Plant Cell Physiol. 47(7): 995–1003 (2006)
doi:10.1093/pcp/pcj072, available online at www.pcp.oxfordjournals.org

Dynamic and Reversible Changes in Histone H3-Lys4 Methylation and H3 Acetylation Occurring at Submergence-inducible Genes in Rice

Hiroyuki Tsuji [1, 3], Hiroaki Saika [1, 4], Nobuhiro Tsutsumi [1], Atsushi Hirai [2] and Mikio Nakazono [1, *]

[1] *Laboratory of Plant Molecular Genetics, Graduate School of Agricultural and Life Sciences, The University of Tokyo, 1-1-1 Yayoi, Bunkyo-ku, Tokyo, 113-8657 Japan*
[2] *School of Agriculture, Meijo University, 1-501 Shiogamaguchi, Tenpaku-ku, Nagoya, Aichi, 468-8502, Japan*

Histone modifications such as methylation and acetylation in the chromatin surrounding a gene are thought to regulate transcriptional activity. In this study, to determine whether dynamic changes occur in histone modification on the loci of stress-responsive genes in plants, we chose rice submergence-inducible *ADH1* and *PDC1* genes. When submerged, the rice *ADH1* and *PDC1* genes were activated in a biphasic manner: the first and second inductions occurred after approximately 2 and 12 h of submergence, respectively. Their expression was transcriptionally induced as shown by increased binding of RNA polymerase II to the *ADH1* and *PDC1* loci during submergence. The Lys4 residues of the histone H3 proteins (H3-K4s) at both the 5′- and 3′-coding regions of *ADH1* and *PDC1* were found to change from a di-methylated state to a tri-methylated state at the first induction period. On the other hand, acetylation of H3 increased throughout *ADH1* and *PDC1* genes at the later induction period. The methylation and acetylation levels recovered to the initial levels during re-aeration. Treatment of seedlings with a histone deacetylase (HDAC) inhibitor, trichostatin A, increased acetylation of histones H3 and association of RNA polymerase II on the *ADH1* and *PDC1* loci, thereby increasing transcript levels of *ADH1* and *PDC1*. Together, these results showed dynamic and reversible changes of histone H3-K4 methylation and H3 acetylation in stress-responsive genes in a higher plant in response to the appearance or disappearance of an environmental stress.

Keywords: Environmental stress — Gene expression — Histone modification — Rice (*Oryza sativa*).

Abbreviations: ADH1, alcohol dehydrogenase 1; ChIP, chromatin immunoprecipitation; HAT, histone acetyltransferase; HDAC, histone deacetylase; PDC1, pyruvate decarboxylase 1; RNA pol II, RNA polymerase II; TSA, trichostatin A; qPCR, quantitative PCR.

Introduction

Chromatin, a highly structured complex of DNA and nuclear proteins, is dynamically modified during several physiological processes including transcription. Modifications mainly occur on the nucleosome, the basic repeated unit of chromatin, which is formed by wrapping approximately 146 bp of DNA around a histone octamer (comprised of two of each of the histones H2A, H2B, H3 and H4) (Khorasanizadeh 2004). The N-termini of the histones undergo several covalent modifications, such as methylation, acetylation, phosphorylation, ADP-ribosylation and ubiquitination. Combinations of these modifications are believed to generate 'histone codes' that provide docking sites for proteins that are needed to regulate chromatin-related processes (Strahl and Allis 2000).

Histone methylation and acetylation play important roles in gene expression and chromatin states (Kurdistani and Grunstein 2003, Sims et al. 2003). Methylation of Lys4 of histone H3 (H3-K4) is generally associated with transcriptionally active chromatin, and methylation of Lys9 of histone H3 (H3-K9) generally correlates with transcriptionally repressed states and is often observed in heterochromatin (Sims et al. 2003). In particular, it has been suggested that di-methylation of H3-K4 correlates with the 'permissive' state of chromatin, in which genes are either active or potentially active, and tri-methylation of H3-K4 is linked to 'ongoing' transcription (Santos-Rosa et al. 2002, Ng et al. 2003, Schneider et al. 2004). Euchromatic regions, which are actively transcribed regions, contain highly acetylated nucleosome histones, and heterochromatic regions, which are transcriptionally repressed, contain less acetylated nucleosome histones (Chua et al. 2001, Kurdistani and Grunstein 2003). Acetylation states are maintained

[3] Present address: Nara Institute of Science and Technology, Ikoma, Nara 630-0101 Japan.
[4] Present address: National Institute of Agrobiological Sciences, 2-1-2 Kannondai, Tsukuba, Ibaraki, 305-8602 Japan.
* Corresponding author: E-mail, anakazo@mail.ecc.u-tokyo.ac.jp; Fax, +81-3-5841-5183.

by the opposing actions of histone acetyltransferase (HAT) and histone deacetylase (HDAC), and thus are reversible (Kurdistani and Grunstein 2003). Unlike histone acetylation, histone methylation has long been considered a stable and irreversible modification. However, recent identification of histone demethylases such as LSD1 (Shi et al. 2004, Metzger et al. 2005) and the JmjC domain-containing proteins (Tsukada et al. 2006, Whetstine et al. 2006, Yamane et al. 2006) suggests that histone methylation is not a permanent modification but a reversible mark. Histone methylation is also removed by the exchange of methylated histones with unmodified histones (Sims et al. 2003, Janicki et al. 2004). To our knowledge, however, there have been no reports of dynamic changes of histone methylation in plants.

Because plants are sessile, their ability to respond quickly to stressful environmental conditions, such as submergence/waterlogging, cold and drought, is crucial for adaptation and survival. Thus, it is important that gene expression is dynamically controlled in response to environmental changes. The gene expression may be partly regulated by dynamic changes in histone modification (Reyes et al. 2002, Loidl 2004). If histone modifications play a direct role in gene expression in response to a stress, the modifications should be reversible, i.e. they should depend on the presence or absence of the stress. In higher plants, there is increasing evidence of regulation of gene expression by histone acetylation and methylation (Chua et al. 2003, Ausin et al. 2004, Bastow 2004, Sung and Amasino 2004, Zhou et al. 2005). However, it is not known whether histone modifications in plants are dynamically and reversibly controlled in response to stress. Although cold exposure in *Arabidopsis* was found to alter the levels of histone H3 acetylation and H3-K9 and K27 methylation in a flowering repressor, *FLOWERING LOCUS C* (*FLC*) (Bastow 2004, Sung and Amasino 2004), the changes were not reversible, i.e. they resulted in a stable and irreversible gene silencing of *FLC* gene expression. Here we report dynamic and reversible changes in histone H3-K4 methylation and H3 acetylation of rice submergence-inducible alcohol dehydrogenase 1 (*ADH1*) and pyruvate decarboxylase 1 (*PDC1*) genes in response to the presence or absence of stress. We chose the two genes as a model system because their expression is reversibly activated or repressed in response to changes of oxygen status (Tsuji et al. 2000). Using chromatin immunoprecipitation (ChIP) and quantitative real-time PCR (qPCR), we demonstrate the occurrence of dynamic and reversible changes of histone H3-K4 methylation and H3 acetylation on chromatin containing the submergence-responsive genes.

Results

Effect of submergence and re-aeration on gene expression

We used reverse transcription–quantitative PCR (RT–qPCR) to monitor *ADH1* and *PDC1* mRNA levels in rice roots during submergence. Signal intensity was normalized by dividing it by the signal intensity of 17S rRNA. Two hours after rice seedlings were completely submerged, transcripts of *ADH1* and *PDC1* began to increase (Fig. 1). The *ADH1* and *PDC1* transcript levels decreased from 6 to 12 h, and then increased again. When the submerged plants were re-aerated, *ADH1* and *PDC1* transcripts decreased to the initial levels (Fig. 1). The transient decrease of *ADH1* and *PDC1* mRNA levels at 12 h after submergence was reproducible ($n = 5$) under our conditions, suggesting that expression of the *ADH1* and *PDC1* genes in rice roots was activated in a biphasic manner.

Changes of RNA polymerase II association

To determine whether the increase of *ADH1* and *PDC1* mRNA levels during submergence is due to an increase of transcription rather than to a decrease of mRNA

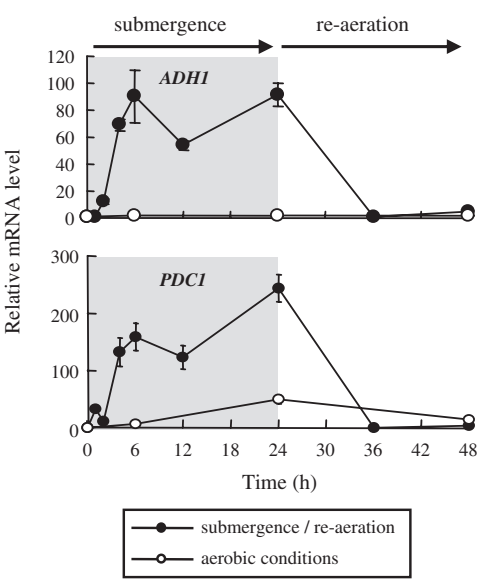

Fig. 1 Representative graphs showing the effects of submergence on transcription of submergence-responsive genes. Real-time RT–PCR analyses of *ADH1* and *PDC1* were performed using total RNA extracted from rice roots. Nine-day-old rice seedlings were completely submerged for 24 h and then re-aerated. Seedling roots were harvested at the indicated times during submergence and following re-aeration (filled circles), and during aerobic control conditions (open circles). The real-time RT–PCR was performed three times for each RNA template. Data show mean values ± SD of three separate PCR analyses. The RT–PCR experiments were done three times or more using different RNA samples for the template. Similar results were obtained for each experiment.

degradation, we carried out ChIP experiments using an antibody against RNA polymerase II (RNA pol II). The RNA pol II-ChIP, like the conventional run-on transcription assay, is a method for estimating transcriptional activity in vivo. However, unlike the run-on transcription assay, it does not require the laborious isolation of highly purified nuclei (Sandoval et al. 2004). The binding of RNA pol II to all three regions (promoter, 5′-coding region and 3′-coding region) of *ADH1* and *PDC1* loci increased during submergence and reverted to the initial levels following re-aeration (Fig. 2), suggesting that the in vivo transcription was enhanced under submergence. The promoter regions for *ADH1* and *PDC1* (–206 to –83 nucleotides and –894 to –699 nucleotides from the transcription initiation sites, respectively) contain predicted anaerobic responsive elements (AREs), which are *cis*-elements required for induction of gene expression under anaerobic conditions (Olive et al. 1990, Kyozuka et al. 1994). Because genomic DNA was cut into 0.5–1.0 kb fragments in our ChIP assay, the PCR amplification of the *ADH1* or *PDC1* promoter regions might detect RNA pol II association or histone modification downstream of the transcription start site (i.e. the 5′ untranslated region) as well as the promoter regions.

The RNA pol II association levels (Fig. 2), unlike the mRNA levels (Fig. 1), did not decrease at 12 h after submergence. These data suggest that the increases of *ADH1* and *PDC1* mRNA are due to induction of transcription, but the biphasic pattern of their mRNA accumulations may be due to some type of post-transcriptional regulation, which decreases the number of transcripts at around 12 h after submergence.

Changes of histone H3-K4 di-methylation and tri-methylation

To examine whether the histone H3-K4 methylation states on the *ADH1* and *PDC1* chromatin dynamically change under submerged conditions and following re-aeration, we monitored the di-methylation and tri-methylation states of histone H3-K4 of *ADH1* and *PDC1* loci at various time points (Figs. 3 and 4).

In all three regions (promoter, 5′-coding region and 3′-coding region) of *ADH1*, the relative abundance of di-methyl H3-K4 decreased after submergence and then increased during re-aeration (Fig. 3). Similar results were obtained for the *PDC1* gene (Fig. 3).

The relative abundance of tri-methyl H3-K4 in the promoter region remained unchanged or slightly decreased during submergence (Fig. 4). However, in the 5′- and 3′-coding regions, tri-methylation of H3-K4 started to increase at about 2 h after the start of submergence, continued to increase until the submerged plants were re-aerated (24 h after submergence), and then dropped to the initial state after re-aeration (Fig. 4). The increase during submergence started at almost the same time that the association of RNA pol II with these genes started to increase (Fig. 2).

We failed to detect any di-methylation of H3-K9 on any of the genes we examined in this study (data not shown). This may have been because H3 histones with di-methylated K9 residues are mainly localized at heterochromatic regions or because the reactivity of the antibody used in our study was too low for our procedure.

Changes of histone H3 acetylation

The kinetic ChIP analyses showed that H3 histones were gradually acetylated at all three regions of the genes under submerged conditions, and the acetylation state of histone H3 reverted to the initial levels following re-aeration (Fig. 5). In this case, the start of the increase of H3 acetylation (Fig. 5) occurred after the start of the increase of *ADH1* and *PDC1* transcripts (Fig. 1) and the RNA pol II association (Fig. 2). This suggests that histone acetylation

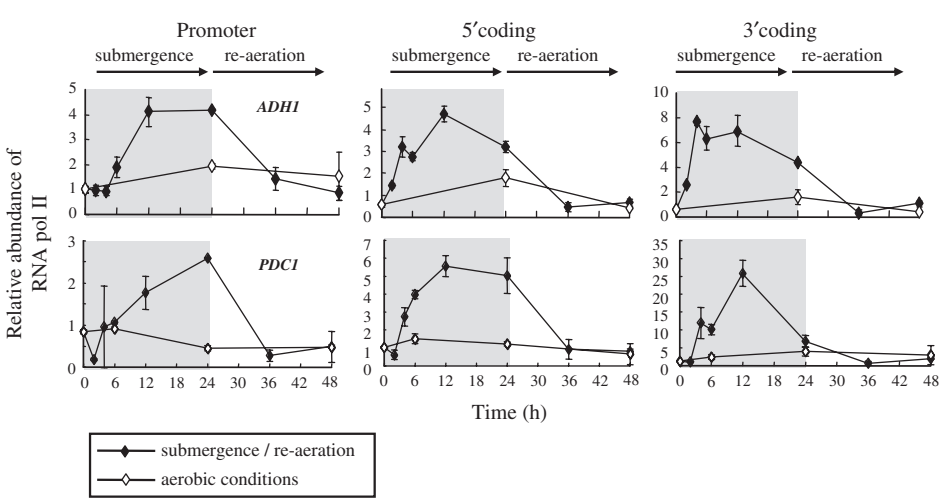

Fig. 2 Representative graphs showing the effects of submergence on association of RNA pol II with submergence-responsive genes. Nine-day-old rice seedlings were completely submerged for 24 h and then re-aerated. Seedling roots were harvested at the indicated times during submergence and following re-aeration (filled diamonds) and during aerobic control conditions (open diamonds). ChIP assays were performed using an antibody against RNA pol II. Values are the mean ± SD of three separate real-time PCR runs in a ChIP experiment. The experiments were done two or three times with similar results.

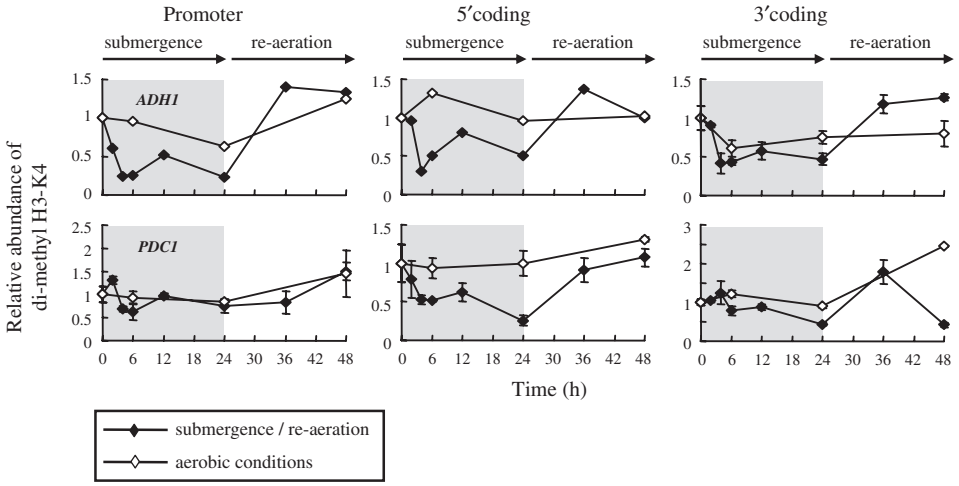

Fig. 3 Representative graphs showing the effects of submergence on di-methylation of histone H3-Lys4 at submergence-responsive genes. Plants were grown and treated as in Fig. 2. ChIP assays were performed using an antibody against di-methylated histone H3 at Lys4. Values are the mean ± SD of three separate real-time PCR runs in a ChIP experiment. The experiments were done two or three times with similar results.

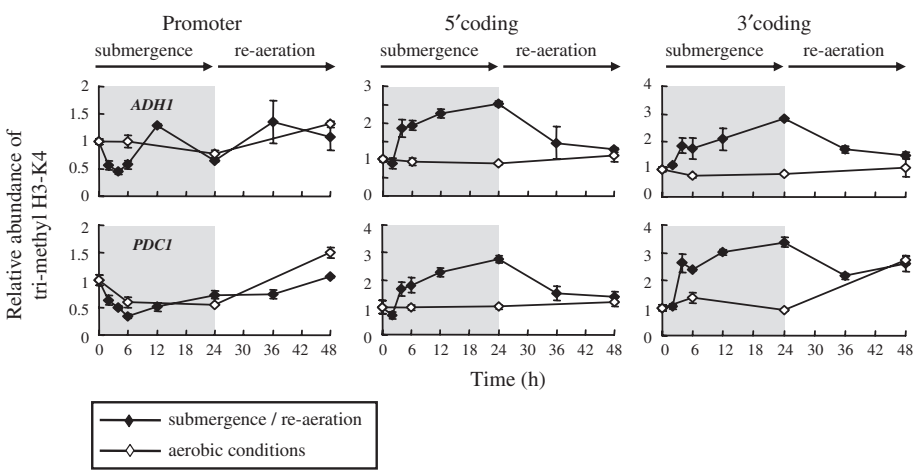

Fig 4 Representative graphs showing the effects of submergence on tri-methylation of histone H3-Lys4 at submergence-responsive genes. Plants were grown and treated as in Fig. 2. ChIP assays were performed using an antibody against tri-methylated histone H3 at Lys4. Values are the mean ± SD of three separate real-time PCR runs in a ChIP experiment. The experiments were done two or three times with similar results.

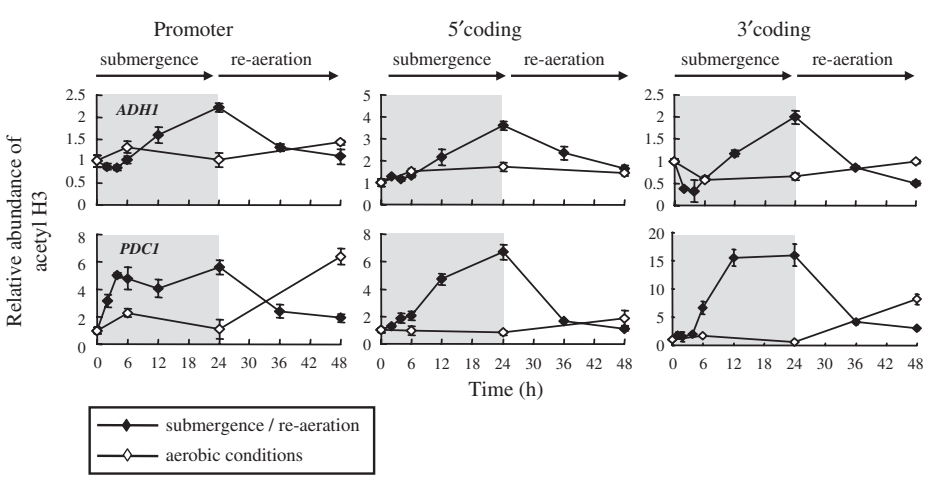

Fig. 5 Representative graphs showing the effects of submergence on acetylation of histone H3 at submergence-responsive genes. Plants were grown and treated as in Fig. 2. ChIP assays were performed using an antibody against acetylated histone H3. Values are the mean ± SD of three separate real-time PCR runs in a ChIP experiment. The experiments were done two or three times with similar results.

does not function at the first induction step of *ADH1* and *PDC1* expression. If this is the case, then what is the function of H3 acetylation? One possibility is that H3 acetylation enhances the expression of *ADH1* and *PDC1* at the late stage, which starts at about 12 h after submergence. Treatment of seedlings with trichostatin A (TSA), which hyperacetylates histones H3 and H4, for 6 h significantly induced H3 acetylation in the 5′-coding region (Fig. 6A) as well as in the promoter and 3′-coding regions (data not shown) of *ADH1* and *PDC1*, even under aerobic conditions. Transcript levels increased with increasing concentration of TSA (Fig. 6B). To examine whether increased acetylation leads to enhanced transcription, we investigated the binding of RNA pol II to the *ADH1* and *PDC1* loci during TSA treatment. As shown in Fig. 6C, RNA pol II was increasingly associated with these loci. These results suggest that increased histone H3 acetylation can enhance *ADH1* and *PDC1* expression via increased association of RNA pol II. Hence, the increase of histone H3 acetylation observed during submergence may enhance activation of *ADH1* and *PDC1* gene expression.

Discussion

The histone H3-K4 methylation state of the chromatin at the *ADH1* and *PDC1* genes (Figs. 3 and 4) changed dynamically in accordance with the change in transcription (Figs. 1 and 2). This suggests that tri-methylation of H3-K4 is associated with active transcription in plants as well as in mammals and yeast. The changes in the tri-methyl H3-K4 states (Fig. 4) at the 5′- and 3′-coding regions of *ADH1* and *PDC1* were in inverse proportion to the changes in the di-methyl H3-K4 states (Fig. 3). This suggests that di-methyl H3-K4 was converted to tri-methyl H3-K4 by histone methyltransferase activity when the *ADH1* and *PDC1* genes were activated under submerged conditions. This is shown schematically in Fig. 7. When rice plants were re-aerated, H3-K4 methylation changed from a tri-methylated state to a di-methylated state at the 5′- and 3′-coding regions of *ADH1* and *PDC1* (Figs. 3 and 4). This result suggests that histone methylation is dynamically controlled. If this is the case, how is the methyl turnover of the histone tail regulated? One possible mechanism is that demethylation of tri-methyl H3-K4 to di-methyl H3-K4 increases methyl turnover of the histone tail. Several recent studies have found evidence for proteins with demethylase activity, including a histone H3-K4-specific or H3-K9-specific histone demethylase (LSD1) (Shi et al. 2004, Metzger et al. 2005), and three JmjC domain-containing proteins with specific demethylase activities. The latter include JHDM1 (Tsukada et al. 2006) and JHDM2A (Yamane et al. 2006), which demethylate only di- or mono-methylated histone H3 at K36 and K9, respectively, and JMJD2 that reverses tri-methylated histone H3-K9 or H3-K36 to di- but not mono- or unmethylated histone H3 (Whetstine et al. 2006). Although a demethylase specific to tri-methyl H3-K4 has not yet been identified, it is possible that such a demethylase contributes to the reversion of H3-K4 methylation states in rice. Another possible mechanism involves histone exchange (Sims et al. 2003, Janicki et al. 2004), in which histone H3 tri-methylated at K4 is first replaced by an unmodified histone H3, and then the unmodified H3 is di-methylated by histone methyltransferase.

Tri-methylation of H3-K4, which is catalyzed by a histone methyltransferase (e.g. Set1), has been shown to be coupled to transcription by RNA pol II (Sims et al. 2004). Consistent with this evidence, the increase of RNA pol II association (Fig. 2) occurred at almost the same time as the increase of histone tri-methylation (Fig. 4) when the submergence-inducible genes were activated. These data suggest that the transcription-coupled H3-K4 tri-methylation is also used in stress-responsive gene expression in plants. In yeast and chicken, tri-methylation of H3-K4 accumulates near the 5′-coding region of genes (Santos-Rosa et al. 2002, Ng et al. 2003, Schneider et al. 2004). Because the tri-methyl H3-K4 states at the 5′- and 3′-coding regions of *ADH1* and *PDC1* increased under submergence (Fig. 4), tri-methylation of H3-K4 in plants may not be limited to the 5′-coding region and may extend to the 3′ portions of the genes.

Changes in gene expression through histone modifications as a result of environmental change have also been observed in winter-annual *Arabidopsis* lines. In these lines, exposure to prolonged cold attenuates expression of the flowering-repressor *FLC* through methylation of histone H3-K9 and H3-K27 at the promoter and first intron of the *FLC* gene, thereby promoting their flowering when spring comes (Sung and Amasino 2004). However, unlike the histone modifications at the *ADH1* and *PDC1* loci, those at the *FLC* locus are irreversible.

The acetylation states of histone H3 at all three regions of the *ADH1* and *PDC1* genes increased under submergence (Fig. 5). This was also the case for the acetylation state of histone H4 (data not shown). We also found that the times at which *ADH1* and *PDC1* transcription started to increase occurred before the start of the increase of histone acetylation at the corresponding genes (Figs. 1, 2, 5). Together, these results suggest that histone acetylation is not required for the initial step of induction of *ADH1* and *PDC1* gene expression by submergence. However, the finding that histone acetylation can induce *ADH1* and *PDC1* gene expression even under aerobic conditions (Fig. 6) suggests that it is involved in enhancing the late phase of *ADH1* and *PDC1* expression during submergence. In fact, increased acetylation leads

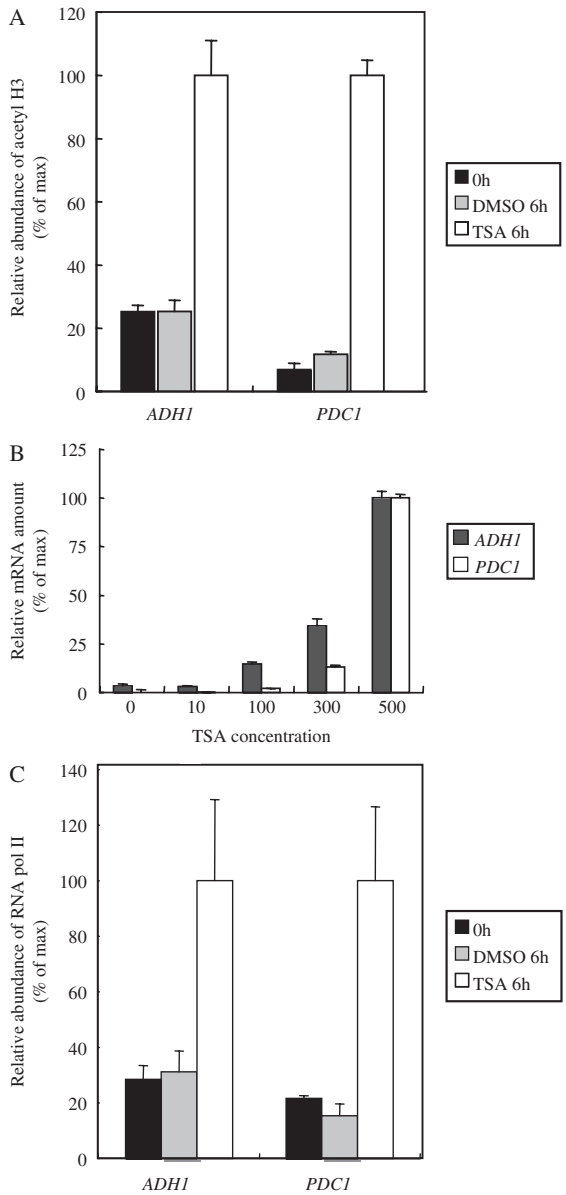

Fig. 6 Effects of trichostatin A (TSA) on *ADH1* and *PDC1* transcription, nucleosome histone acetylation and RNA pol II association in these genes. (A) ChIP analyses using acetylated H3 antibodies in the 5′-coding regions of *ADH1* and *PDC1*. Nine-day-old rice seedlings were treated by 500 μM TSA for 6 h, and roots were harvested and analyzed. (B) Nine-day-old rice seedlings were treated with the indicated concentrations of TSA for 6 h. Relative amounts of *ADH1* and *PDC1* mRNA in rice roots, as determined by quantitative real-time RT–PCR. (C) ChIP analyses using RNA pol II antibodies in the 5′-coding regions of *ADH1* and *PDC1*. Nine-day-old rice seedlings were treated by 500 μM TSA for 6 h, and roots were harvested and analyzed.

to increased RNA pol II association with the *ADH1* and *PDC1* loci (Fig. 6C).

In conclusion, we observed dynamic and reversible changes of the histone methylation and acetylation

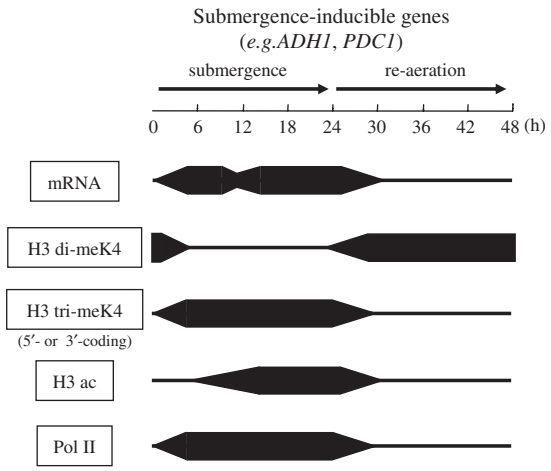

Fig. 7 Dynamic changes of mRNA levels, histone H3-K4 di-methylation (H3 di-meK4), histone H3-K4 tri-methylation (H3 tri-meK4), histone H3 acetylation (H3 ac) and RNA pol II association (pol II) at submergence-inducible genes (e.g. *ADH1* and *PDC1*) during submergence and following re-aeration.

states occurring in chromatin at the submergence-responsive genes in response to changes of oxygen availability as a result of submergence and re-aeration. Many questions remain about how histone modifications, such as methylation and acetylation, are involved in the regulation of gene expression in plants in response to environmental stresses. Submergence-responsive genes in rice may be a good model for answering these questions.

Materials and Methods

Plant materials and treatments

Rice (*Oryza sativa* L., cv. Nipponbare) seedlings were grown in the light at 28°C for 9 d. For submergence treatment, aerobically grown seedlings were completely submerged in water in the dark at 28°C. After 24 h of submergence, seedlings were returned to aerobic conditions in the dark at 28°C. Aerobic control plants were kept for 48 h in the dark. For TSA treatments, seedlings were placed in 1.55% dimethylsulfoxide (DMSO) solution containing various concentrations of TSA (Wako Chemical, Tokyo, Japan) for 6 h.

RNA extraction and quantitative real-time RT–PCR

For RNA extraction, roots of seedlings were harvested and immediately frozen in liquid nitrogen. RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA). qPCR was carried out with an ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) using Quantitect SYBR Green RT–PCR master mix (Qiagen) according to the manufacturer's protocol. Primers used for the qPCR are listed in Table 1. A cDNA clone or a cDNA fragment of each gene was used to make standard curves for quantification. Experiments were repeated two or more times and similar

Table 1 List of primers used in this study

Name	Sequence	Experiment	Amplicon
ADH1-RT-fwd	TCATCCGCATGGAGAACTA	RT–PCR	*ADH1*
ADH1-RT-rev	CCGTATATCATCATTCACCC	RT–PCR	*ADH1*
PDC1-RT-fwd	GCAACTGCTGGACAAAGAAG	RT–PCR	*PDC1*
PDC1-RT-rev	TGGCAGCCCAGCCAATTTCG	RT–PCR	*PDC1*
17S-RT-fwd	TCCTACCGATTGAATGGTCC	RT–PCR	*17S rRNA*
17S-RT-rev	CTTGTTACGACTTCTCCTTCCTC	RT–PCR	*17S rRNA*
ADH1-pro-fwd	AAACAGCGGCTGCAATTC	ChIP	Promoter region of *ADH1*
ADH1-pro-rev	GAGGTTTCGCCACTTCCTTC	ChIP	Promoter region of *ADH1*
ADH1-5′-fwd	TCAAGTGCAAAGGTCAGTGC	ChIP	5′ portion of *ADH1* coding region
ADH1-5′-rev	CGCCGCTCCAGTAATAAAAT	ChIP	5′ portion of *ADH1* coding region
ADH1-3′-fwd	GCTGGAGGTGGAGAAGTTCA	ChIP	3′ portion of *ADH1* coding region
ADH1-3′-rev	CCAACACCATAATCCCCTGA	ChIP	3′ portion of *ADH1* coding region
PDC1-pro-fwd	GCTAGGCGTTACAGCGTAGC	ChIP	Promoter region of *PDC1*
PDC1-pro-rev	CATTCAGTCCAGCCGACAAG	ChIP	Promoter region of *PDC1*
PDC1-5′-fwd	CTCCAACGCCGTCATCAAC	ChIP	5′ portion of *PDC1* coding region
PDC1-5′-rev	TAGTCGAGCAGGGTGAGGTT	ChIP	5′ portion of *PDC1* coding region
PDC1-3′-fwd	CCAAGAAAGACTGCCTCTGC	ChIP	3′ portion of *PDC1* coding region
PDC1-3′-rev	TGAGAAGACGAGCAGCAAGA	ChIP	3′ portion of *PDC1* coding region
ScACT1-fwd	TTTTTCACGCTTACTGCTTTTT	ChIP	5′ portion of yeast *ACTIN1* coding region
ScACT1-rev	GGGACCGTGCAATTCTTCT	ChIP	5′ portion of yeast *ACTIN1* coding region

results were obtained. In each experiment, qPCR was performed three times.

Chromatin immunoprecipitation (ChIP)

This method determines the relative abundance of histones in particular states that are attached to different regions of particular genes. For example, it might show that di-methylation of H3-K4s in the 3′ fragments of a particular gene decreases during submergence. In brief, chromatin was fixed and broken up into fragments corresponding to 500–1,000 bp of DNA. Fragments associated with RNA pol II or fragments containing di-methyl H3-K4, tri-methyl H3-K4 or acetyl H3 were immunoprecipitated, and the numbers of copies of fragments that contain portions (promoter, 5′ and 3′ regions) of the *ADH1* and *PDC1* genes were then quantified with real-time PCR. For *ADH1*, the promoter, 5′ region and 3′ region were defined as nucleotides –206 to –83, 137 to 239, and 2,891 to 3,031, respectively, where nucleotide 1 is the transcription initiation site. The corresponding regions of *PDC1* were nucleotides –894 to –699, 206 to 346, and 2,329 to 2,470. The detailed procedure is given in the following paragraphs.

Unless stated otherwise, all procedures were done at 4°C. Rice seedlings were harvested and immediately immersed in cross-linking buffer [0.4 M sucrose, 10 mM Tris–HCl (pH 8.0), 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride (PMSF), 1% formaldehyde] under vacuum at room temperature for 30 min. Cross-linking was stopped by adding glycine to a final concentration of 82 mM, and incubation was continued for another 5 min. After washing the plants in water, the roots were cut off and frozen in liquid nitrogen.

The frozen roots were powdered using a Multi-Beads Shocker (Yasui Kikai, Osaka, Japan). The powder was suspended in lysis buffer [50 mM HEPES-NaOH (pH 7.4), 150 mM NaCl, 1 mM EDTA, 0.1% (w/v) sodium deoxycholate, 10 mM sodium butyrate, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, Complete Tablet (protease inhibitor cocktail; Roche Diagnostics, Germany)] and sonicated so that DNA was broken into approximately 500–1,000 bp fragments. The mixture was centrifuged at 3,000 r.p.m. for 5 min. The final supernatant was used for immunoprecipitation.

For internal standards of ChIP experiments, we extracted cross-linked solutions from yeast (*Saccharomyces cerevisiae*). Yeast strain AH109 (BD Biosciences, San Jose, CA, USA) was cultured in YPDA medium until the OD_{600} reached 0.8. Cellular proteins and DNA were cross-linked by addition of formaldehyde [final concentration: 1% (v/v)] for 15 min and then the cross-linking was stopped by adding glycine to a final concentration of 82 mM. The yeast cells were centrifuged and suspended in lysis buffer, vortexed with glass beads and centrifuged. The supernatant was sonicated.

Extracts from rice and yeast were pre-cleaned by rotating with 1/100 volume of protein A–agarose (50% slurry) (TOYOBO, Tokyo, Japan) in salmon sperm DNA (Roche Diagnostics) for 2 h. After centrifugation at 3,000 r.p.m., extracts from the yeast and rice were mixed at a ratio of 1 : 5 (v/v). A 4 µl aliquot of antibodies against di-methylated histone H3 at Lys4 (#07-030; Upstate Biotechnology, Lake Placid, NY, USA) or acetylated histone H3 at Lys9 and Lys14 (#06-599; Upstate Biotechnology), 8 µl of antibodies against tri-methylated histone H3 at Lys4 (#ab8580; Abcam, Cambridge, UK) or di-methylated histone H3 at Lys9 (#07-212; Upstate Biotechnology), or 45 µl of an antibody against RNA pol II (#sc-900; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to 600 µl of mixed extract. Extracts were incubated overnight with rotation, and 1/10 volume of protein

A–agarose (50% slurry) in the salmon sperm DNA was added and incubated with rotation for 2 h. Input DNA controls were not immunoprecipitated, and no antibody controls were immunoprecipitated without antibody. The agarose beads were collected by centrifugation, washed twice with lysis buffer, once in LNDET [0.25 M LiCl, 1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl (pH 8.0)] and three times in TE [10 mM Tris–HCl (pH 8.0), 1 mM EDTA]. The immuno-complexes were eluted twice from the beads in elution buffer [1% (w/v) SDS, 0.1 M $NaHCO_3$] at 65°C, and mixed with NaCl to a final concentration of 0.3 M. To reverse the cross-linking, the eluted solutions were incubated at 65°C overnight, treated with RNase A at 37°C for 1 h, and treated with proteinase K (TAKARA SHUZO CO., LTD, Kyoto, Japan) at 45°C for 1 h. Immunoprecipitated DNAs were purified using a QIAquick PCR purification kit (Qiagen).

The number of copies of different regions of the *ADH1* and *PDC1* genes in the purified DNA was quantified by qPCR using a Quantitect SYBR Green PCR Kit (Qiagen). Genomic DNA was extracted from rice and yeast, and these were used for standard curves for real-time PCR. Primer sequences for the qPCR are shown in Table 1. For each sample, the promoter region of the yeast *ACT1* gene, which encodes actin 1, was amplified, and these values were used to normalize the efficiencies of the immunoprecipitation and DNA purification. Input DNA controls were diluted 1 : 4 and quantified by real-time PCR. The values obtained were used to normalize the levels of DNA before immunoprecipitation. The signal intensities in the figures are presented as ChIP PCR signals normalized to the signals of the input DNA controls. Experiments were repeated two or more times and similar results were obtained. In each experiment, PCR was performed three times.

Acknowledgments

We thank Drs H.-Y. Hirano, K. Fukui, S. Matsunaga and S. Arimura for their stimulating discussions. We thank Dr K. Nagaki for the ChIP experiment. This work was partly supported by grants-in-aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan, and by a grant from Ministry of Agriculture, Forestry and Fisheries of Japan to M.N. H.T. acknowledges the support of JSPS Research Fellowships for Young Scientists.

References

Ausin, I., Alonso-Blanco, C., Jarillo, J.A., Ruiz-Garcia, L. and Martinez-Zapater, J.M. (2004) Regulation of flowering time by FVE, a retinoblastoma-associated protein. *Nat. Genet*. 36: 162–166.

Bastow, R., Mylne, J.S., Lister, C., Lippman, Z., Martienssen, R.A. and Dean, C. (2004) Vernalization requires epigenetic silencing of *FLC* by histone methylation. *Nature* 427: 164–167.

Chua, Y.L., Brown, A.P.C. and Gray, J.C. (2001) Targeted histone acetylation and altered nuclease accessibility over short regions of the pea plastocyanin gene. *Plant Cell* 13: 599–612.

Chua, Y.L., Watson, L.A. and Gray, J.C. (2003) The transcriptional enhancer of the pea plastocyanin gene associates with the nuclear matrix and regulates gene expression through histone acetylation. *Plant Cell* 15: 1468–1479.

Janicki, S.M., Tsukamoto, T., Salghetti, S.E., Tansey, W.P., Sachidanandam, R., Prasanth, K.V., Ried, T., Shav-Tal, Y., Bertrand, E., Singer, R.H. and Spector, D.L (2004) From silencing to gene expression: real-time analysis in single cells. *Cell* 116: 633–634.

Khorasanizadeh, S. (2004) The nucleosome: from genomic organization to genomic regulation. *Cell* 116: 259–272.

Kurdistani, S.K. and Grunstein, M. (2003) Histone acetylation and deacetylation in yeast. *Nat. Rev. Mol. Cell Biol*. 4: 276–284.

Kyozuka, J., Olive, M., Peacock, W.J., Dennis, E.S. and Shimamoto, K. (1994) Promoter elements required for developmental expression of the maize *Adh1* gene in transgenic rice. *Plant Cell* 6: 799–810.

Loidl, P. (2004) A plant dialect of the histone language. *Trends Plant Sci*. 9: 84–90.

Metzger, E., Wissmann, M., Yin, N., Muller, J.M., Schneider, R., Peters, A.H., Gunther, T., Buettner, R. and Schule, R. (2005) LSD1 demethylates repressive histone marks to promote androgen-receptor-dependent transcription. *Nature* 437: 436–439.

Ng, H.H., Robert, F., Young, R.A. and Struhl, K. (2003) Targeted recruitment of Set1 histone methylase by elongating Pol II provides a localized mark and memory of recent transcriptional activity. *Mol. Cell* 11: 709–719.

Olive, M.R., Walker, J.C., Singh, K., Dennis, E.S. and Peacock, W.J. (1990) Functional properties of the anaerobic responsive element of the maize *Adh1* gene. *Plant Mol. Biol*. 15: 593–604.

Reyes, J.C., Hennig, L. and Gruissem, W. (2002) Chromatin-remodeling and memory factors. New regulators of plant development. *Plant Physiol*. 130: 1090–1101.

Sandoval, J., Rodriguez, J.L., Tur, G., Serviddio, G., Pereda, J., Boukaba, A., Sastre, J., Torres, L., Franco, L. and Lopez-Rodas, G. (2004) RNAPol-ChIP: a novel application of chromatin immunoprecipitation to the analysis of real-time gene transcription. *Nucleic Acids Res*. 32: e88.

Santos-Rosa, H., Schneider, R., Bannister, A.J., Sherriff, J., Bernstein, B.E., Emre, N.C., Schreiber, S.L., Mellor, J. and Kouzarides, T. (2002) Active genes are tri-methylated at K4 of histone H3. *Nature* 419: 407–411.

Schneider, R., Bannister, A.J., Myers, F.A., Thorne, A.W., Crane-Robinson, C. and Kouzarides, T. (2004) Histone H3 lysine 4 methylation patterns in higher eukaryotic genes. *Nat. Cell Biol*. 6: 73–77.

Shi, Y.J., Lan, F., Matson, C., Mulligan, P., Whetstine, J.R., Cole, P.A., Casero, R.A. and Shi, Y. (2004) Histone demethylation mediated by the nuclear amine oxidase homolog LSD1. *Cell* 119: 941–953.

Sims, R.J.III, Mandal, S.S. and Reinberg, D. (2004) Recent highlights of RNA-polymerase-II-mediated transcription. *Curr. Opin. Cell Biol*. 16: 263–271.

Sims, R.J.III, Nishioka, K and Reinberg, D. (2003) Histone lysine methylation: a signature for chromatin function. *Trends Genet*. 19: 629–639.

Strahl, B.D. and Allis, C.D. (2000) The language of covalent histone modifications. *Nature* 403: 41–45.

Sung, S. and Amasino, R.M. (2004) Vernalization in *Arabidopsis thaliana* is mediated by the PHD finger protein VIN3. *Nature* 427: 159–164.

Tsuji, H., Nakazono, M., Saisho, D., Tsutsumi, N. and Hirai, A. (2000) Transcript levels of the nuclear-encoded respiratory genes

in rice decrease by oxygen deprivation: evidence for involvement of calcium in expression of the alternative oxidase 1a gene. *FEBS Lett*. 471: 201–204.

Tsukada, Y., Fang, J., Erdjument-Bromage, H., Warren, M.E., Borchers, C.H., Tempst, P. and Zhang, Y. (2006) Histone demethylation by a family of JmjC domain-containing proteins. *Nature* 439: 811–816.

Whetstine, J.R., Nottke, A., Lan, F., Huarte, M., Smolikov, S., Chen, Z., Spooner, E., Li, E., Zhang, G., Colaiacovo, M. and Shi, Y. (2006) Reversal of histone lysine trimethylation by the JMJD2 family of histone demethylases. *Cell* 125: 467–481.

Yamane, K., Toumazou, C., Tsukada, Y., Erdjument-Bromage, H., Tempst, P., Wong, J. and Zhang, Y. (2006) JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. *Cell* 125: 483–495.

Zhou, C.H., Zhang, L., Duan, J., Miki, B. and Wu, K.Q. (2005) *HISTONE DEACETYLASE19* is involved in jasmonic acid and ethylene signaling of pathogen response in Arabidopsis. *Plant Cell* 17: 1196–1204.

(Received March 18, 2006; Accepted May 31, 2006)