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Protein Homologous to Human CHD1, Which Interacts with Active Chromatin (HMTase) from Onion Plants

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1. Introduction

Onions are grown as an annual plant for commercial purposes; although since they are biennial it takes two seasons to grow from seed to seed. Bolting (flowering) of onion plants is determined by two factors, the size of the plant and cold temperatures. The critical size for bolting occurs when the onion reaches the five-leaf stage of growth. If onions are seeded in early fall, warm temperatures will result in sufficient size for bolting in the subsequent winter. Early transplants and some onion varieties are especially susceptible to bolting during cold temperatures. However, cold temperatures are not the sole prerequisite for bolting. If onions are not at the critical size in their development, they do not recognize cold as a signal to initiate bolting. Thus, sowing and transplanting at the correct time of year is the most important factor to avoid premature bolting.

Genetic and molecular studies of *Arabidopsis* have revealed a complicated network of signaling pathways involved in flowering time (Boss et al., 2004; Macknight et al., 2002; Putterill et al., 2004). Four genetic pathways, which are known as the photoperiod, autonomous, vernalization, and gibberellin (GA) pathway, have been identified based on the phenotypes of flowering time mutants (Koornneef et al., 1998). The photoperiod pathway includes genes whose mutants show a late flowering phenotype under long day (LD) conditions that is not responsive to vernalization treatments. This pathway contains genes encoding photoreceptors such as *PHYTOCHROME* (*PHY*), components of the circadian clock, clock associated genes such as *GIGANTEA* (*GI*) (Fowler et al., 1999; Park et al., 1999), and the transcriptional regulator *CONSTANS* (*CO*) (Putterill et al., 1995). *FLOWERING LOCUS T* (*FT*) (Kardailsky et al., 1999; Kobayashi et al., 1999) and *SUPPRESSOR OF OVEREXPRESSION OF CO 1* (*SOC1*) (Lee et al., 2000) are targets of *CO* (Samach et al., 2000). The autonomous pathway includes genes whose mutants show a late flowering independently of day length that can be rescued by vernalization. Genes included in this pathway are *FCA*, *FY*, *FVE*, *FLOWERING LOCUS D* (*FLD*), *FPA*, *FLOWERING LOCUS K* (*FLK*), and *LUMINIDEPENDENS* (*LD*) (Ausin et al., 2004; He et al., 2003; Kim et al., 2004; Lee et al., 1994; Lim et al., 2004; Macknight et al., 1997; Schomburg et al., 2001; Simpson et al., 2003). They regulate *FLOWERING LOCUS C* (*FLC*) (Michaels and Amasino, 1999), a floral repressor, through several different mechanisms

such as histone modification and RNA binding (Simpson, 2004). Some genes of this pathway are also involved in ambient temperature signaling (Blazquez et al., 2003; Lee et al., 2007). The vernalization pathway includes genes whose mutations inhibit the promotion of flowering by vernalization. Genes included in this pathway are *VERNALIZATION INSENSITIVE3* (*VIN3*), *VERNALIZATION1* (*VRN1*), and *VERNALIZATION2* (*VRN2*) (Gendall et al., 2001; Levy et al., 2002; Sung and Amasino, 2004). The GA pathway includes genes whose mutations show a late flowering especially under short day (SD) conditions. This pathway has GA biosynthesis genes, *FLOWERING PROMOTIVE FACTOR1* (*FPF1*), and genes involved in GA signal transduction (Huang et al., 1998; Kania et al., 1997). GAs have been known to positively regulate the expression of floral integrator genes such as *SOC1* and *LEAFY* (*LFY*) (Blazquez et al., 1998; Moon et al., 2003).

We report here, genetic and molecular evidences for regulation of bolting time in onion plants using a late bolting-type cultivar (MOS8) and an very early bolting-type cultivar (Guikum). We screened the proteins extracted from onion plants with different bolting times by using a proteomic approach and identified a protein with significant similarities to chromodomains of mammalian chromo-ATPase/helicase-DNA-binding 1 (CHD1) or heterochromatin protein 1 (HP1). Furthermore, we examined *in vitro* histone methyltransferase (HMTase) activity using purified protein isolated from onion plants. Our results suggest that a floral genetic pathway in controlling bolting time may be involved in onion plant.

2. Methodology

2.1 Plant growth and cutivars

Two onion cultivars, MOS8 (Eul-Tai Lee et al. 2009) with a late bolting phenotype and Guikum (provided by Kaneko seed Co., Japan) with a very early bolting phenotype, were used in this study. F_1 plants produced from crosses between MOS8 and Guikum were self-pollinated to produce F_2 populations. Based on the segregation ratio of bolting, inheritances of F_2 generations were evaluated. Bolting was assayed from the time of transplantation into the field to the first open flower.

2.2 Northern

Total RNA was extracted from leaves using an RNeasy plant Mini Kit (Qiagen, USA) according to the manufacturer's instructions. About 15 μg of total RNA was separated via electrophoresis on a 1.2% formaldehyde-agarose gel and then transferred onto a Hybond-N^+ membrane (Amersham, USA) by capillary action (Sambrook et al. 1989). The full-length open reading frame (ORF) regions of *Arabidopsis FRIGIDA* (*FRI*) and *FLC* were amplified from cDNAs prepared from *Arabidopsis* seedlings. These fragments were labeled with [α-^{32}P] and used as Northern blot probes. Hybridization was performed for 20 h at 68°C, and the filters were washed with 2 ×SSC, 0.1% SDS at 68°C for 20 min and 1×SSC, 0.1% SDS at 37°C for 30 min. The filters were exposed to X-ray film at -70°C for 3-7 days.

2.3 2-DE

The meristematically active parts (200 mg) isolated from onion plants were homogenized with lysis buffer containing 8 M urea, 2% NP-40, 5% β-mercaptoethanol, and 5% polyvinyl

pyrrolidene, and then assayed by 2-DE (Yang et al. 2005). Extracted protein samples (100 μg) were separated in the first dimension by isoelectric focusing (IEF) tube gel and in the second dimension by SDS-PAGE. Electrophoresis was carried out at 500 V for 30 min, followed by 1000 V for 30 min and 5000 V for 1 h 40 min. The focusing strips were immediately used for SDS-PAGE or stored at −80°C. After electrophoresis of the first dimension, the focusing strips were incubated for 15 min in equilibration buffer I (6M urea, 2% SDS, 50mM Tris-HCl [pH 8.8], 30% glycerol, 1% DTT, and 0.002% bromophenol blue) and equilibration buffer II (6 M urea, 2% SDS, 50mM Tris–HCl [pH 8.8], 30% glycerol, 2.5% iodoacetamide, and bromophenol blue). Equilibrated strips were then run on an SDS-PAGE gel as the second dimension. The gels were stained with Silver Stain Plus and the image analysis was performed with a FluorS MAX multimager (Bio-Rad, Hercules, CA).

2.4 N-terminal sequencing analysis

Proteins were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Pall, Port Washington, NY) using a semidry transfer blotter (Nippon Eido) and visualized by Coomassie brilliant blue (CBB) staining. The stained protein spots were excised from the PVDF membrane and applied to the reaction chamber of a Procise protein sequencer (Applied Biosystems, Foster city, CA). Edman degradation was performed in accordance with the standard program supplied by Applied Biosystems. The amino acid sequences were compared to known proteins deposited in NCBIBLAST databases.

2.5 Mass spectrometry

Protein spots were excised, destained from 2-DE gels, dehydrated, reduced with DTT, alkylated with iodoacetamide, and digested with trypsin in accordance with the recommended procedures. Samples were then analyzed by Matrix-assisted laser desorption-ionization time-of-flight mass spectrometry (MALDI-TOF) MS on a Voyager-DE STR machine (Applied Biosystems, Framingham MA). Parent ion masses were measured in the reflectron/delayed extraction mode with an accelerating voltage of 20 kV, a grid voltage of 76.000%, a guide wire voltage of 0.01%, and a delay time of 150 ns. A two-point internal standard for calibration was used with des-Arg1-Bradykinin (m/z 904.4681) and angiotensin 1 (m/z 1296.6853). Peptides were selected in the mass range of 700 - 3000 Da. For data processing, the MoverZ software program was used. Peak annotations were checked manually to prevent non-monoisotopic peak labeling. Monoisotopic peptide masses were used to search the databases, allowing a peptide mass accuracy of 100 ppm and one partial cleavage. To determine the confidence of the identification results, the following criteria were used: minimum of four must be matched, and the sequence coverage must be greater than 15%. Database searches were performed using Protein Prospector (http:// prospector.ucsf.edu), ProFound (http:// www.unb.br/cbsp/paginiciais/profound.htm), and MASCOT (www.matrixscience.com).

2.6 Enzyme assays

HMTase assays were carried out at 30°C for 1 h in 20 μl volumes containing 50mM Tris-HCl (pH 8.5), 20mM KCl, 10mM $MgCl_2$, 10mM β-mercaptoethanol, 250mM sucrose, 8 μg/μl histone from calf thymus (Roche, USA), 220 nCi of *S*-adenosyl-$_L$-[methyl-^{14}C]methionine ([^{14}C]SAM), and protein extracts prepared from onion plants. Methylation reactions were stopped by the addition of SDS-PAGE sample buffer, separated on a 16% polyacrylamide gel, and analyzed by autofluorography.

3. Results

3.1 Genetic inheritance of bolting in onion plants

In order to understand to the genetic control of bolting in onion plants, we crossed late bolting-type cultivar (MOS8, days to bolting=165-170 days) with very early bolting-type cultivar (Guikum, days to bolting=130-135 days). The bolting phenotypes of F_1 generations were similar to those of late bolting-type cultivars (data not shown). This suggests that genetic loci affecting bolting may be present in onion plants. Subsequent analysis of the inheritance distribution in F_2 generations is shown in Figure 1. Table 1 shows the distribution pattern and segregation ratio (late bolting:early bolting = about 3:1) indicating that bolting time depends on the segregation of any gene where the dominant allele confers lateness. Furthermore, bolting phenotypes of onion cultivars were reduced by long exposure to cold (E.T. Lee, personal communication). Given the crosses between late and very early bolting onion varieties, and effects of low temperature in onion plants, it appears likely that the genetic basis involved in the regulation of bolting time in onion is similar to that of vernalization requirement in plant species (Sung and Amasino, 2005). Genetic and molecular studies in various winter-annual and summer-annual accessions of *Arabidopsis* as a model plant have shown that *FRIGIDA* (*FRI*) and *FLC* have important functions in distinguishing winter-annual habits and summer-annual habits in *Arabidopsis* accessions (Clarke and Dean, 1994; Gazzani et al., 2003; Shindo et al., 2005). We assessed the expression patterns of these two genes in MOS8, Guikum, and F_1 plants (derived from crosses between MOS8 and Guikum) by northern hybridization (data not shown). The ORF regions of *FRI* and *FLC* amplified from *Arabidopsis* seedlings were used as probes. The mRNA levels of the *FRI* and *FLC* were strongly increased in the late-bolting–type cultivar, MOS8 ; however, the levels of *FRI* and *FLC* expression were significantly decreased in the very early-bolting–type cultivar, Guikum. These results suggest that the bolting time observed in onion plants may be affected by changes in *FRI* and *FLC* expression. However, we cannot dismiss the possibility that loci other than *FRI* and *FLC* may affect the bolting time of onion plants. Consistent with this idea, flowering in cereals is principally controlled by *VERNALIZATION 1* (*VRN1*) and *VERNALIZATION 2* (*VRN2*), which encode *APETALA1* (*AP1*)-like MADS box transcription factor and *CONSTANS* (*CO*)-like transcription factor, respectively (Trevaskis et al. 2003; Yan et al. 2004). Bolting time in other plant species are also determined by a relatively small number of loci, either dominant or recessive locus. With *Hyocyamus niger* (henbane), the biennial habit is governed by a single dominant locus, whereas this habit is governed by a single recessive locus in *Beta vulagris* (sugar beet) (Abegg, 1936; Lang, 1986).

3.2 2-DE analysis in onion plants

In order to examine the components involved in the control of bolting time in onion, we checked protein profiles of MOS8 and Guikum by using a 2-DE proteomics approach. The inner basal tissues of onion bulbs grown for 96 days after transplanting were used for proteomics analysis, because bolting is initiated in this region after cold treatment (Fig. 2a). Initial 2-DE analysis of soluble proteins from onion plants was performed using an IEF range of pH 3 to 6 (data not shown). Because the use of appropriate pH gradients is an effective way to reduce overlapping spots, additional analysis with pH 4 to 6 immobiline pH gradient (IPG) strips was performed (Fig. 2b). After CBB staining, several differences in protein accumulation profiles were detected in onion plants with different bolting times. Although many spots were differentially accumulated in onion plants, we failed to obtain

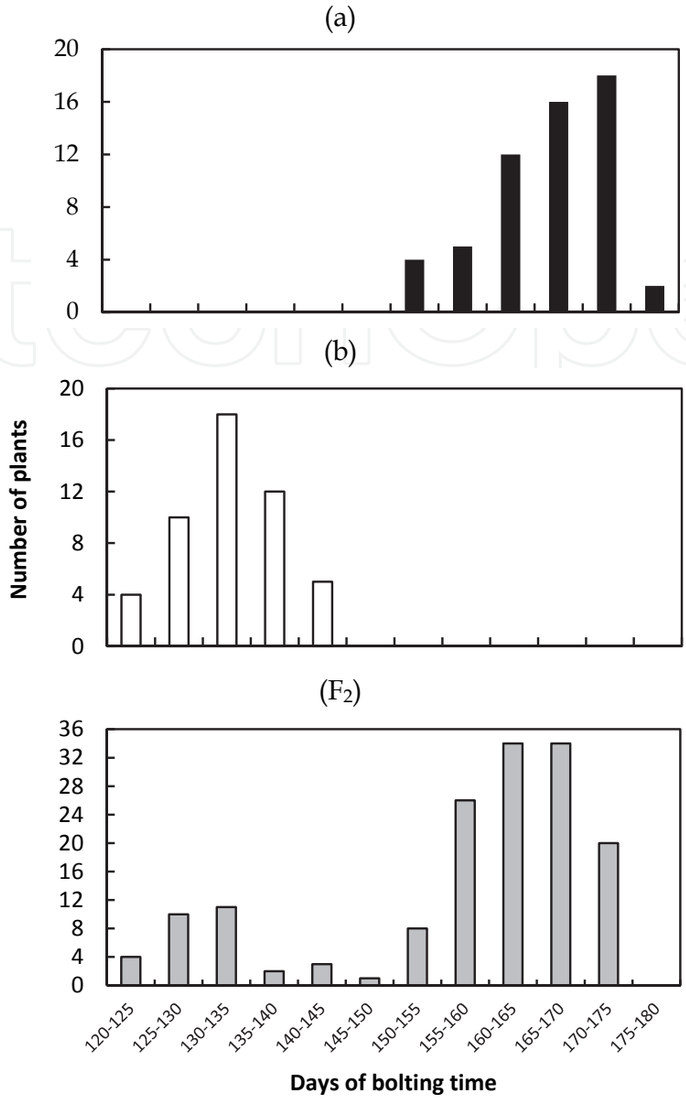
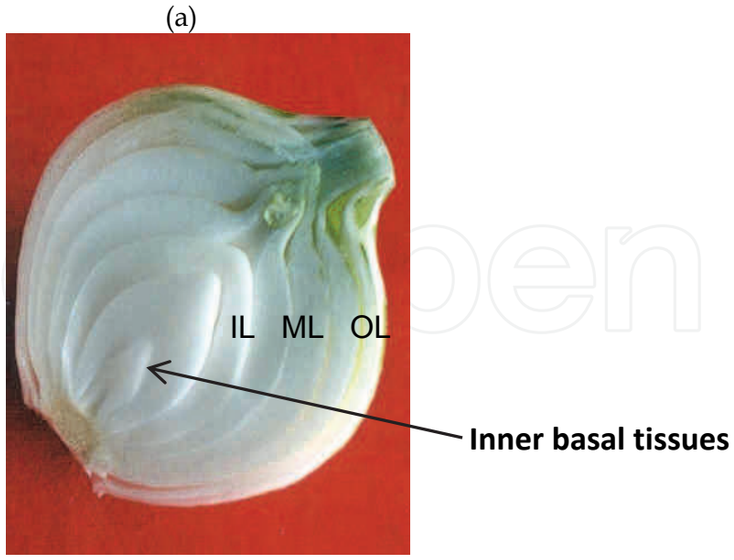


Fig. 1. Distribution patterns of bolting time in F_2 populations derived from crosses between (a) MOS8 (late bolting type) and (b) Guikum (very early bolting type) onion cultivars. These onion cultivars used in this study were inbred lines. The 'days to bolting' time were calculated when 80% of the total population of onion plants had bolted.

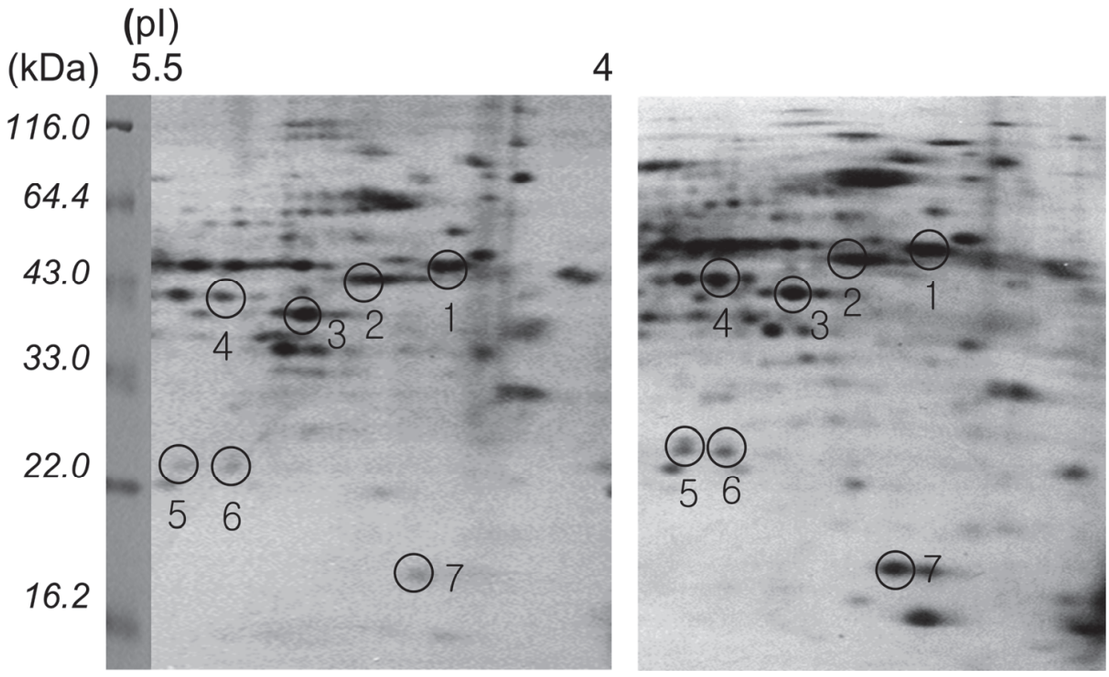
Variety	Total	Very early flower bolting	Late flower bolting	Ratio	Test ratio	x^2	P
MOS8	56	0	56			-	
Guikum	48	48	0			-	
MOS8 × Guikum	152	31	121	1:3.9	1 : 3	1.719	0.001

Table 1. Genetics of crossing MOS8 (Late bolting type) with Guikum (Very early bolting type) to identify genes that confer a vernalization response.

(a)



(b)



(a) The inner basal tissues of onion bulb used for proteomics analysis. *IL* inner layers, *ML* middle layer, *OL* outer layer.
(b) Protein analysis was performed using medium-range IPG strips with pH range from 4 to 6. The protein spots were identified by protein sequencing and MALDI-TOF MS analysis. Molecular masses (kilodalton) are shown on the left and pI ranges at the top comers of each figure.

Fig. 2. Two-dimensional gel electrophoresis of proteins isolated from onion plants (MOS8 and Guikum).

sufficient amounts from many of these spots for successful protein sequencing. Thus, we chose seven protein spots significantly changed in accordance with the degree of bolting time. The amino acid sequences of the differentially regulated proteins were analyzed by protein sequencing (Table 2). Homology searches were performed using the BLAST search tool. N-terminal sequences were successfully obtained for only one protein (spot 7). The remaining proteins were analyzed by MALDI-TOF MS. Among the other six proteins, three proteins (spots 1, 5 and 6) were not identified, whereas three proteins (spots 2, 3 and 4) were identified as actin, tubulin and keratin.

Spot No.[a]	pI/kDa[b]	Sequences[c]	Homologous protein (%)	Accession No.
1	4.8/46	N-blocked/MS[d]	Not hit	-
2	5.0/43	N-blocked/MS	Actin 1 (96)	P53504
3	5.1/39	N-blocked/MS	Tubulin alpha 2 chain (89)	Q96460
4	5.2/40	N-blocked/MS	Keratin, type II cytoskeletal 1 (90)	P04264
5	5.4/23	N-blocked/MS	Not hit	-
6	5.2/23	N-blocked/MS	Not hit	-
7	4.9/17	N-ARTLQTARRSTGGKAP	Chromodomains of mammalian CHD1 or HP1 proteins (93)	2B2W_D 3FDT_T 1GUW_B 1KNE_P

[a]Spot numbers are shown in Fig. 2.
[b]pI and molecular mass (kDa) are from the gel in Fig. 2.
[c]N-terminal amino acid sequences are determined by Edman degradation.
[d]MALDI-TOF MS.

Table 2. Identification of onion proteins whose abundance varied significantly among onion plants with different bolting time

Interestingly, the amino acid sequence of spot 7 showed significant similarities to several chromodomain regions of mammalian CHD1 or HP1 proteins, though we could not confidently identify an onion protein homologous to this spot in the database because of the short amino acid sequence and poorly characterized onion genome (Fig. 3). The chromodomain appears to be a well conserved motif, because it can be found in wide range of organisms such as protists, plants, amphibians, and mammals (Eissenberg, 2001). Furthermore, proteins with this chromodomain are known as both a positive and negative regulator of gene expression in various developmental processes (Hall and Georgel, 2007). For instance, two tandem chromodomains of CHD1 protein are known to interact with methylated lysines on histones, which include H3K4me, H3K36me and H3K79me, associated with active chromatin, thereby inducing active transcription (Flanagan et al., 2005; Sims et al., 2005). However, the chromodomain of the HP1 protein recognizes and binds to H3K9me for promotion of heterochromatin formation (Jacobs and Khorasanizadeh, 2002; Nielsen et al., 2002). Therefore, chromatin remodeling factors with chromodomains may play an important role in regulating gene expression. Because there is a dramatic change in the chromatin in meristematic regions such as inner basal tissues used in this study.

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Onion      ARTLQTARRSTGGKAP_ _ _ _
2B2W_D     ARTXQTARKSTGGKAPRKQY
3FDT_T     ARTKQTARXSTGGKA_ _ _ _ _
1GUW_B     ARTXQTARXSTGGKAPGG
1KNE_P     ARTKQTARXSTGGKAY_ _ _ _
           *** **** *****
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Fig. 3. Multiple alignments of amino acid sequences between onion protein spot 7 with other homologous proteins. Identical amino acid residues are denoted by asterisks. 2B2W_D chain D-tandem chromodomains of human CHD1 complexes with histone H3 tail containing trimethyllysine 4, 3FDT_T chain T-crystal structure of the complex of human chromobox homology 5 with H3K9(Me)3 peptide, 1GUW_B chain B-structure of the chromodomain from mouse HP1 beta in complex with the lysine 9-methyl histone H3 N-terminal peptide, 1KNE_P chain P-chromodomain of HP1 complexes with histone H3 tail containing trimethyllysine 9

Consistent with this, lesions in *Arabidopsis PHOTOPERIOD-INDEPENDENT EARLY FLOWERING 1* (*PIE1*), which encodes an ISW1 family ATP-dependent chromatin remodeling protein, result in a large reduction in *FLC* expression, thereby causing the conversion from winter-annual to summer-annual habits (Noh and Amasino 2003). Given that yeast Isw1p, an *Arabidopsis* PIE1 homolog, can bind H3K4me (Santos-Rosa et al. 2003), it might be assumed that PIE1 will bind H3K4me, which is generated by EARLY FLOWERING IN SHORT DAYS (EFS) (He et al. 2004; Kim et al. 2005), and remodel FLC chromatin to allow active transcription. However, an *in silico* search revealed that an onion protein homologous to human CHD1 was not related to the *Arabidopsis* PIE1 gene. This observation raises the possibility that various ATP-dependent chromatin remodeling factors may interact with various methylation states of lysine on H3 to induce transcriptional activation of target genes. Although there is no evidence that this protein spot is relevant to the regulation of bolting time by vernalization, this observation raises the possibility that chromatin remodeling factors may play roles in regulating this process in onion plants.

3.3 *In vitro* HMTase activity assays in onion plants

In order to assess whether histone methylation correlated with bolting time of onion plants, we performed *in vitro* HMTase activity assays using purified protein spots with significant similarities to chromodomains of mammalian CHD1 or HP1 isolated from two onion cultivars (MOS8 and Guikum) with calf thymus histones as substrates (Fig. 4a). Amino acid sequences of the purified spots used in this assay were confirmed (data not shown). The purified protein spots were able to methylate histone proteins in examined onion plants, indicating that the spots are associated with HMTase activity. Furthermore, differences in HMTase activity were observed in onion plants, though equal amounts of calf thymus histones were used in this assay (Fig. 4a, b). However, chromodomains of chromatin remodeling factors like mammalian CHD1 or HP1 generally act as binding modules for methylated lysines on histones. This could be explained by the SET-domain containing histone methyltransferase (Yeates, 2002) being present in extracts from onion cultivars. We cannot exclude the possibility that the purified protein spot is a histone methyltransferase with a chromodomain-like protein SUV39H1 (Brehm et al., 2004; Koonin et al., 1995).

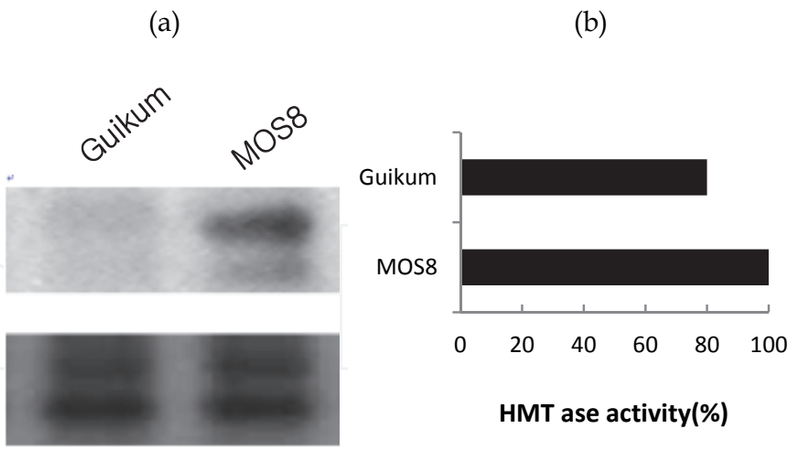

Fig. 4. In vitro HMTase activity in onion plants (MOS8 and Guikum). (a) Fluorography of ^{3}H-methyl calf thymus histone. 200 μg of purified spots containing protein homologous to human CHD1 isolated from two onion cultivars grown for 96 days after transplanting were used in this assay(upper panel). Reaction mixtures were analyzed by 16% SDS-PAGE and autoradiography. Equal amounts of each reaction were confirmed by Coomassie blue stain profiles of calf thymus histones (lower panel). (b) Quantitation of HMTase activity in MOS8 and Guikum

4. Conclusions

Our results suggest that a genetic pathway may be involved in the control of bolting time in onion plants by genetic inheritance, though the regulation of bolting in onion plants may be more complexly governed by several loci. Although it is very difficult to identify confident proteins in onion plants with uncharacterized genome, it appears likely that chromatin remodeling factors involved in histone modification may be conserved in onion plant. Although molecular and genetic analyses of flowering time in *Arabidopsis* have identified several floral promotion and repression pathways, our knowledge of the floral pathways in other economically important crops is limited. Thus, the quantitative trait locus (QTL) mapping and the use of high-throughput experiments such as genomics will provide a better understanding of the regulation of bolting time in onion.

5. Acknowledgment

We thank the Korea Basic Science Institute for their generous technical support of our research.

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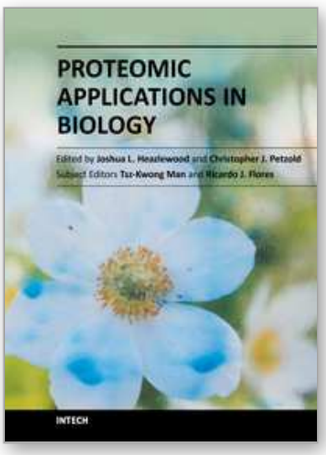

Proteomic Applications in Biology

Edited by Dr. Joshua Heazlewood

ISBN 978-953-307-613-3
Hard cover, 264 pages
Publisher InTech
Published online 18, January, 2012
Published in print edition January, 2012

The past decade has seen the field of proteomics expand from a highly technical endeavor to a widely utilized technique. The objective of this book is to highlight the ways in which proteomics is currently being employed to address issues in the biological sciences. Although there have been significant advances in techniques involving the utilization of proteomics in biology, fundamental approaches involving basic sample visualization and protein identification still represent the principle techniques used by the vast majority of researchers to solve problems in biology. The work presented in this book extends from overviews of proteomics in specific biological subject areas to novel studies that have employed a proteomics-based approach. Collectively they demonstrate the power of established and developing proteomic techniques to characterize complex biological systems.

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