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RESEARCH PAPER

Regulation of *oleosin* expression in developing peanut (*Arachis hypogaea* L.) embryos through nucleosome loss and histone modifications

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Abstract

Nucleosome loss and histone modifications are important mechanisms for transcriptional regulation. Concomitant changes in chromatin structures of two peanut (*Arachis hypogaea* L.) oleosin genes, *AhOleo17.8* and *AhOleo18.5*, were examined in relation to transcriptional activity. Spatial and temporal expression analyses showed that both *AhOleo17.8* and *AhOleo18.5* promoters can adopt three conformational states, an inactive state (in vegetative tissues), a basal activated state (in early maturation embryos), and a fully activated state (in late maturation embryos). Chromatin immunoprecipitation assays revealed an increase of histone H3 acetylation levels at the proximal promoters and coding regions of *AhOleo17.8* and *AhOleo18.5* associated with basal transcription in early maturation embryos. Meanwhile, a decrease of histone H3K9 dimethylation levels at coding regions of *oleosins* was observed in early maturation embryos. However, a dramatic decrease in the histone acetylation signal was observed at the core promoters and the coding regions of the two *oleosins* in the fully activated condition in late maturation embryos. Although a small decrease of histone H3 levels of *oleosins* chromatin was detected in early maturation embryos, a significant loss of histone H3 levels occurred in late maturation embryos. These analyses indicate that the histone eviction from the proximal promoters and coding regions is associated with the high expression of *oleosin* genes during late embryos maturation. Moreover, the basal expression of *oleosins* in early maturation embryos is accompanied by the increase of histone H3 acetylation and decrease of histone H3K9me2.

Key words: Histone acetylation, histone H3K9 dimethylation, histone loss, oleosin, peanut seed.

Introduction

Oleosins are small molecular mass proteins and are located on the surface of oil bodies to form a barrier to prevent the phospholipid (PL) layer from contacting and coalescing with the PL layers of adjacent oil bodies (Huang, 1996). The maintenance of the oil bodies as small droplets provides a large surface area per unit triacylglycerol (TAG) and thus would facilitate lipase binding and lipolysis during seed germination (Kim *et al.*, 2002). More recently, it was found that the ablation of a major oleosin in *Arabidopsis thaliana* resulted in an aberrant phenotype of embryo cells that contain large oil bodies not observed in normal seeds. These results suggest that oleosins are important proteins for modulating the size of oil bodies (Siloto *et al.*, 2006). Peanut seeds are widely used in the food industry mostly because they contain 45–53% of oils and about 30% of proteins. Although pioneer works on oil bodies and their associated proteins were reported from peanut seeds (Jacks *et al.*, 1990), only one cDNA sequence encoding peanut oleosin

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was isolated (Pons *et al.*, 2005). Furthermore, it was reported that this peanut oleosin protein can react with serum IgE of patients who suffer an allergic reaction to peanuts (Pons *et al.*, 2002). Tissue-specific expression of *oleosins* during embryogenesis and seed development requires high transcription activity. The promoters of *oleosins* are absolutely inactive in vegetative tissues, such as roots, stems, and leaves (Plant *et al.*, 1994). However, the molecular mechanisms controlling seed-specific expression of *oleosins* are not well understood.

In eukaryotic cells, it is known that chromatin structure changes play a fundamental role in the establishment and maintenance of transcriptional programmes (Mellor, 2006; Exner and Hennig, 2008). This mainly occurs at three levels, including histone modifications, ATP-dependent chromatin remodelling, and chromatin disassembly (Mellor, 2006; Workman, 2006). The post-synthetic modification of histone, such as acetylation, methylation, phosphorylation, ubiquitinylation, and ADP ribosylation, plays an important role in the epigenetic regulation of transcription. These covalently modified histones can increase or decrease interactions between histones and DNA, or produce a molecular platform for the binding of other factors (Strahl and Allis 2000; Turner, 2000; Jenuwein and Allis, 2001). On the other hand, ATP-dependent chromatin remodelling may result in a sliding of nucleosome along the DNA of histone octamers from the DNA (Längst *et al.*, 1999; Xiao *et al.*, 2001; Boeger *et al.*, 2004). Finally, chromatin disassembly has recently been found to be a profound manner in which the cell can amend the chromatin structure (Boeger *et al.*, 2003; Schwabish and Struhl, 2004; Chen *et al.*, 2005; Zhao *et al.*, 2005). Global analyses of histone occupancy in *Saccharomyces cerevisiae*, *Drosophila*, and human demonstrate that transcribing promoters tend to be depleted of nucleosomes compared to inactive promoters (Bernstein *et al.*, 2004; Lee *et al.*, 2004; Barrera and Ren, 2006). In the case of the yeast *S. cerevisiae*, it has been observed that histones are removed from the *PHO5*, *GAL*, and *HSP82* promoters upon gene activation and that nucleosomes are reassembled as genes turn off (Boeger *et al.*, 2003; Reinke and Horz, 2003; Kristjuhan and Svejstrup, 2004; Zhao *et al.*, 2005).

Although plenty of information about histone modifications impacting on gene expression exists, direct evidence supporting histone displacement taking part in the regulation of plant gene expression is still lacking (Pfluger and Wagner, 2007). Previously, Ng *et al.* (2006; Ng and Hall, 2008) analysed the chromatin structures of the *phaseolin* (*phas*) promoter, which is silent in all vegetative tissues of the bean (*Phaseolus vulgaris*) plant but becomes transcriptionally active during the development of the seed embryo. Although expression from *phas* is normally seed-specific, high levels of expression in leaves can be obtained by ectopic expression of the transcription factors ABI3-like factor (ALF) or Arabidopsis FUS3 as well as application of abscisic acid (ABA). Using the ectopic expression system, a decrease in histone H3 and H4 occupancy levels was detected at the *phas* chromatin during PvALF-mediated *phas* activation in the presence of ABA in leaves (Ng *et al.*, 2006), but it was not observed under FUS3- and ABA-mediated *phas* activation (Ng and Hall, 2008). However, it is unknown whether high *phas* expression during the development of the seed embryo is associated with histone displacement. In this study, the mechanisms of epigenetic regulation of two peanut *oleosin* genes, *AhOleo17.8* and *AhOleo18.5*, were investigated in developing peanut embryos. Our results suggest that the loss of histone H3 from the proximal promoters and coding regions of *oleosins* is associated with their high expression during late embryo maturation. To our knowledge, this is the first report about the involvement of histone displacement in the activation of gene expression during seed development. In addition, the histone modification status of *oleosins* undergoes targeted changes including the increase of histone H3 acetylation and the decrease of histone H3K9me2 in early maturation embryos.

Materials and methods

Plant materials

Mature and developing peanut (*Arachis hypogaea* L. Shanyou 523) plants were grown and provided by Professor YX Zheng (Zhongkai University of Agriculture and Engineering, China). Peanut embryos were harvested separately on the 20th, 30th, 40th, 50th, and 70th day after peg penetrating to the soil (DAPPS). Peanut roots, stems, and leaves were harvested from 12-d-old seedlings.

Arabidopsis used are in the var. Columbia-0 background. Seeds were sown on Petri dishes with Murashige and Skoog (MS) agar medium, vertically oriented at 22 °C under a 16/8 h light/dark photoperiod regime. Seedlings were transferred into soil after 2 weeks.

cDNA library construction and cloning of complete cDNAs

Total RNA was isolated from late-maturation embryos of peanut using the TRIZOL Reagent (Gibco BRL) according to the manufacturer's instructions. cDNA library construction was performed using a SMART cDNA library construction kit (Clontech). Complete cDNAs encoding oleosins were cloned by contig or RACE (rapid amplification of cDNA ends). The 5′-RACE and 3′-RACE gene-specific primers were constructed based on the EST (Expressed Sequence Tags). The PCR products were directly subcloned into the pGEM T-Easy vector (Promega) and then sequenced.

Semi-quantitative and quantitative RT-PCR

Total RNA was isolated from peanut developing embryos, roots, stems, and leaves by using TRIZOL reagent (Invitrogen) according to the manufacturer's instructions. To eliminate contamination with genomic DNA, RNA was treated with 2 μl (5 units μl^{-1}) DNase I (Takara) for 20 min.

First-strand cDNA was synthesized from the total RNA with the Takara RNA PCR kit (Takara). Semi-quantitative RT-PCR was performed using *18S rRNA* as an internal control. Specific primers were as follows: *18S rRNA* forward primer, 5′-GAAACGGCTACCACATCCAAG-3′; *18S rRNA* reverse primer, 5′-CCAACCCAAGGTCCAACTACG-3′; *AhOleo17.8* forward primer, 5′-CCCCGCTTTTCATCCTCTTCAG-3′; *AhOleo17.8* reverse primer, 5′-CTTTGAATCCTGAGCCTTGGTCTGT-3′; *AhOleo18.5* forward primer, 5′-CCCCCACCACACAACGCGTCGA-3′; *AhOleo18.5* reverse primer, 5′-CCGGCACCGTCGTCCCATGTACCT-3′. The PCR was carried out with *Taq* DNA polymerase (Takara) and programmed for 27 cycles with a primer annealing temperature of 61 °C for 18S and for 30 cycles with a temperature of 64 °C for *AhOleo17.8* and 68 °C for *AhOleo18.5*. The PCR fragments were resolved by electrophoresis on a 2% agarose gel and stained with ethidium bromide. Each reaction was performed at least three times.

Quantitative RT-PCR was performed in the IQ5 Multicolor Real-time PCR Detection System following the manufacturer's instructions using the SYBR Green real-time PCR Master Mix (Toyobo). Specific primers were as follows: *AhOleo17.8*, 5′-CGACACCGCTGCTGGAACTAA-3′ (forward), 5′-AACTATGACAGGGCTGAAGAGGA-3′ (reverse); *AhOleo18.5*, 5′-AGTTCACACCCCCACCACACA-3′ (forward); 5′-ACGAGGACGGCGATGATTTG-3′ (reverse); *AhRb7*, 5′-TCATTGCCACTCTTATCTTCGTCT-3′ (forward), 5′-CAGCGATGGACACTCCTACAAAC-3′ (reverse).

The amount of cDNA was calculated using Bio-Rad iQ5 2.0 Standard Edition Optical System Software (Bio-Rad). The number of transcripts was normalized to the constitutively expressed *18S rRNA*, which was amplified using the primers 5′-TTTCTGCCCTATCAACTTTCG-3′ and 5′-TTATTTATTGTCACTACCTCCCCGT-3′.

Cloning of peanut oleosins *promoters*

The promoters of *oleosins* were cloned following the method described in the GenomeWalker universal kit user manual (Clontech). Total genomic DNA was extracted from the leaf of peanut according to the protocol described by Pich and Schubert (1993). Genomic DNA was digested with blunt-ended enzymes (*Dra*I or *Eco*RV) and then ligated to specific adaptors to obtain GenomeWalker DNA libraries. GenomeWalker DNA libraries were subjected to an initial round of polymerase chain reaction amplification with the outer adaptor primer AP1 (5′-GTAATACGACTCACTATAGGGC-3′) and an outer gene-specific primer *AhOleo17.8*-GSP1 (5′-CGTGCCGCCGATAGGGAGTCCAGTGAT-3′), while an inner adaptor primer AP2 (5′-ACTATAGGGCACGCGTGGTC-3′) and inner gene-specific primer *AhOleo17.8*-GSP2 (5′-TAGCGGTTAGTTCCAGCAGCGGTGTCG-3′) were used for the second round of PCR amplification. Finally, the PCR products were purified by using the QIAquick Gel Extraction Kit (Qiagen), and ligated into the pGEM-Teasy vector (Promega), and sequenced. Similar processes were used in cloning of the *AhOleo18.5* promoter. Two gene-specific primers were: *AhOleo18.5*-GSP1 (5′-TCGTCCCATGTACCTGTCGGATGAAGT-3′) and *AhOleo18.5*-GSP2 (5′-CAGCCCGATTATGGTTCCGAGAAGTGA-3′).

Construction of transformation vectors and plant transformation

To make the promoter–GUS fusion construct, a 1072 bp *AhOleo17.8* promoter fragment was amplified using the primer pair, 5′-CGCGGATCCTAAGCGAAATGCTACATTGC-3′ and 5′-TCCCCCGGGTCCCTTTTCTTCCTTAT-3′. The PCR product was digested by *Bam*HI and *Sma*I and subcloned into the corresponding sites of the binary plasmid PBI101 (Clontech) upstream of the *uid*A reporter gene. Transformation vectors were electroporated into *Agrobacterium tumefaciens* strain EHA105. The *Agrobacterium*-mediated transformation of *A. thaliana* was performed as described as Clough and Bent (1998). T_1 seeds were harvested, and germinated on a sterile medium containing 50 μg ml^{-1} kanamycin to select the transformants. Surviving T_1 plantlets were transferred to soil to get T_2 seeds.

Histochemical β*-glucuronidase (GUS) assays*

Histochemical localization of GUS activity in transgenic plants was carried out essentially as described by Jefferson *et al.* (1987). Plant tissue was incubated in 50 mM sodium phosphate (pH 7.0), 0.5 mM potassium ferricyanide, 0.5 mM potassium ferrocyanide, 10 mM EDTA, 0.05% Triton X-100, and 0.35 mg ml^{-1} 5-bromo-4-chloro-3-indolyl-β-D-glucuronide (X-Gluc) at 37 °C. After staining, the samples were washed in 70% ethanol and photographs were taken under a dissecting microscope.

Chromatin immunoprecipitation assay (ChIP)

ChIP assay was performed as described by Bowler *et al.* (2004). Chromatin extracts were prepared from peanut tissues treated by formaldehyde. The chromatin was sheared to an average length of 200–1000 bp by sonication and immunoprecipitated with antibody. The immunocomplexes were harvested with Protein A agarose and heated at 65 °C for 5 h to release DNA cross-linked to the immunoprecipitated proteins. The DNA cross-linked to the immunoprecipitated proteins was analysed by PCR. To assess non-specific binding, the immunoprecipitation reaction was also performed in the absence of antibody. The antibodies used in this study were anti-acetyl-histone H3K9/14 (Upstate, Cat. no. 06-599), anti-acetyl-histone H4K5/8/12/16 (Upstate, Cat. no. 06-866), anti-trimethyl-histone H3K4 (Abcam, Cat. no. ab8580), anti-dimethyl-histone H3K9 (Upstate, Cat. no. 07-441), and anti-histone H3 (Abcam, Cat. no. ab1791).

Purified DNA from the ChIP samples was used for the Quantitative PCR IQ5 Multicolor real-time PCR Detection System (Bio-Rad) using the SYBR Green Realtime PCR Master Mix (Toyobo) with the following cycling conditions: 1 min initial denaturation at 94 °C; 40 cycles of 94 °C for 15 s,

55 °C for 20 s, 72 °C for 20 s; The primer pairs used were: *AhOleo17.8*-P3, 5′-TATTGAGATTGGGAAGCGCATTA-3′ (forward), 5′-ATAAAAACGGGCTTGCTATACATC-3′ (reverse); *AhOleo17.8*-P2, 5′-TTTTTCATACCTTTGGTA-GTCTTTG-3′ (forward), 5′-AGAAACCACTACTAGAAA-GGAATCAG-3′ (reverse); *AhOleo17.8*-P1, 5′-CTTACTC-AACAACTCGCACCTACG-3′ (forward), 5′-GAAGCTC-CCTTTTCTTCCTTATG-3′ (reverse); *AhOleo17.8*-C1, 5′-CGACACCGCTGCTGGAACTAA-3′ (forward), 5′-AAC-TATGACAGGGCTGAAGAGGA-3′ (reverse); *AhOleo18.5*-P1, 5′-CCACCTCATCAAACTCATCCAC-3′ (forward), 5′-TTGGGATTCCTTTTGTTCTCTTT-3′ (reverse); *AhOleo18.5*-C1, 5′-TCACTGGTATTCTCACGGCGG-3′ (forward), 5′-TCCTTTGTCTTCTGCCCCACG-3′ (reverse); *AhRb7*, 5′-TCATTGCCACTCTTATCTTCGTCT-3′ (forward), 5′-CAGCGATGGACACTCCTACAAAC-3′ (reverse).

The precipitated DNA was normalized to an unrelated DNA sequence from the constitutively expressed *18S rRNA*, which was amplified using the primer pair: 5′-TTTCTGCCCTAT-CAACTTTCG-3′ and 5′-TTATTTATTGTCACTACCTCC-CCGT-3′.

The results of C terminus histone H3 of 18S rRNA and Histone H3K9 dimethylation were nomalized for the amount of chromatin used for immunoprecipitation. For the other ChIP assays, the PCR signal of the target genes was quantified and normalized against the *18S rRNA* signals to yield an enrichment value. Relative enrichment values for stem (S), leaf (L), early maturation embryo (EME) and late maturation embryo (LME) were calculated relative to the enrichment value for the root control (R). The size of the amplified regions was confirmed by gel electrophoresis and products were sequenced to ensure specificity of the assay.

Results

Molecular cloning of AhOleo17.8 *and* AhOleo18.5

A cDNA library was constructed using mRNA isolated from embryos of peanut (Yan *et al.*, 2005). Eighty-four cDNA clones were classified into four different *oleosins* (see Supplementary Table S1 at *JXB* online). All peanut oleosins have a central hydrophobic region of 72 amino acid residues including a highly conserved proline knot of 12 residues (see Supplementary Fig. S1 at *JXB* online), which is similar to previously published profiles of seed-specific oleosins from *A. thaliana* (Kim *et al.*, 2002), *sesame* (Tai *et al.*, 2002), and *Coffea canephora* (Simkin *et al.*, 2006).

The *AhOleo17.8* and *AhOleo18.5* (GenBank accession no. EF695400 and EF695401, respectively) promoters were obtained using the PCR-based 'genome walking' technique (see Supplementary Figs S2 and S3, respectively, at *JXB* online). A number of putative *cis*-acting elements, including RY element and G-box that are important for seed-specific expression, were observed within the promoter sequences. The importance of these elements for seed-specificity and high expression has been demonstrated previously (Bobb *et al.*, 1997; Rowley and Herman, 1997; Wu *et al.*, 2000). It was not possible to obtain the promoters of the other three *oleosins* using this method. Further research was therefore focused on *AhOleo17.8* and *AhOleo18.5*. Although most *oleosins* were reported to have no introns, introns do exist in *Arabidopsis oleosins* (Kim *et al.*, 2002; Siloto *et al.*, 2006). Our results showed that *AhOleo17.8* and *AhOleo18.5* have no introns (see Supplementary Fig. S4 at *JXB* online).

AhOleo17.8 *and* AhOleo18.5 *transcripts were highly abundant in peanut embryos but absent in vegetative tissues*

Seed-specific expression of the *oleosin* is believed to be developmentally and spatially regulated primarily at the level of transcription. However, the expression pattern of peanut *oleosins* has not been investigated previously. Semi-quantitative RT-PCR analyses showed that *AhOleo17.8* and *AhOleo18.5* mRNA accumulated specifically in developing seeds but not in roots, stems and leaves (Fig. 1A). Quantitative RT-PCR was further performed to assay the transcription levels of *AhOleo17.8* and *AhOleo18.5* at different seed developing stages. The results indicated that the transcription of *AhOleo17.8* was low at 20 d after peg penetration to the soil (DAPPS), and it reached the peak at 50 DAPPS (Fig. 1B). Similar expression patterns were also found for *AhOleo18.5* (Fig. 1C). In this study, peanut vegetative tissues, early maturation embryos (20 DAPPS) and late maturation embryos (50 DAPPS) represent three different transcriptional states of *oleosin*s, namely, an inactive state, a basal state, and a fully activated state.

Activity of AhOleo17.8 *promoter in transgenic* Arabidopsis *plants*

An *AhOleo17.8:GUS* vector was constructed by fusing a 1072 bp *AhOleo17.8* promoter (from –1072 to –1) to a *GUS* reporter gene and transformed into wild-type *Arabidopsis* plants. Histochemical analyses were performed in three independent transgenic lines and similar results were obtained. Since the 1072 bp *AhOleo17.8* promoter was able to drive strong *GUS* expression in developing seeds, it was therefore chosen for further analysis. The *AhOleo17.8* promoter was activated as early as at 7 days after flowering (DAF) embryos and its activity persisted throughout subsequent embryo development (Fig. 2A–C). GUS staining was also observed in the cotyledons and embryonic roots of young seedlings, but not in the hypocotyls, young roots, and young leaves (Fig. 2D–G). No GUS staining was observed in the flowers, stems, and mature leaves of transgenic *Arabidopsis* (Fig. 2H–J). Ten lines of transgenic plants were stained and all plants showed similar *GUS* expression patterns.

Histones at AhOleo17.8 *and* AhOleo18.5 *were acetylated in early maturation embryos*

Generally speaking, acetylation of Lys residues in histones associated with promoters and coding regions shows

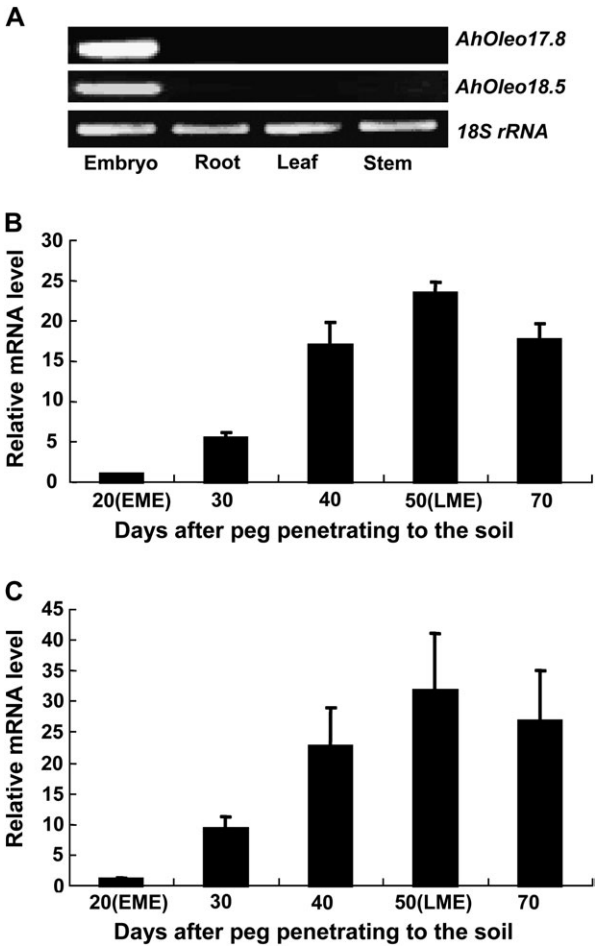

Fig. 1. Expression analysis of *AhOleo17.8* and *AhOleo18.5* in developing peanut embryos. (A) Transcript levels of *AhOleo17.8* and *AhOleo18.5* analysed by RT-PCR. RNA was extracted from roots, stems, leaves, and early maturation embryos. (B) Quantitative real-time RT-PCR analysis of the *AhOleo17.8* transcript during peanut embryo development. (C) Quantitative real-time RT-PCR analysis of the *AhOleo18.5* transcript during peanut embryo development. *18S rRNA* was used as an internal control. Data are the average of three biological replicates. Error bars represent standard errors. EME, early maturation embryo; LEM, late maturation embryo.

a strong positive correlation with gene transcription (Lusser *et al.*, 2001; Pfluger and Wagner, 2007). Chromatin immunoprecipitation (ChIP) assay was used to analyse whether embryo-specific expression of *AhOleo17.8* and *AhOleo18.5* was accompanied by an increase in the acetylation state of histones. DNA and proteins were cross-linked with formaldehyde and chromatin was immunoprecipitated with antibodies against acetylated histones. The coprecipitated DNA was isolated and quantified by real-time PCR. Previous studies indicated that histone acetylation and methylation can occur in both the proximal promoters and/or exons (Benhamed *et al.*, 2006; Wu *et al.*, 2008). Therefore histone modifications of the DNA sequences corresponding to the promoters and the coding regions of *AhOleo17.8* and *AhOleo18.5* were investigated (Fig. 3A; see Supplementary Table S2 at *JXB* online). The PCR signal of the target genes was quantified and normalized against the *18S rRNA* signals to yield an enrichment value. Relative enrichment values for stem (S), leaf (L), early maturation embryo (EME), and late maturation embryo (LME) were calculated relative to the enrichment value for the root control (R).

As shown in Fig. 3B, acetylation levels of histone H3 at the *AhOleo17.8* proximal promoter (P1) and coding region (C1) increased by 2-fold in early maturation embryos. An obvious increase in the acetylation of H3 at the *AhOleo18.5* proximal promoter (P1) and coding region (C1) in early maturation embryos was also observed (Fig. 3C). Similarly, acetylation levels of histone H4 at the *AhOleo18.5* proximal promoter (P1) and coding region (C1) were also increased (see Supplementary Fig. S5 at *JXB* online). Taken together, it was demonstrated that histone H3 and H4 at the *AhOleo17.8* and *AhOleo18.5* proximal promoter (P1) and coding region (C1) became increasingly acetylated in basal transcription in early maturation embryos.

It was further analysed whether the decrease of histone H3 occupancy occurred in early maturation embryos. As shown in Fig. 4A, the histone H3 occupancy from the *AhOleo17.8* proximal promoter (P1) and coding region (C1) was reduced in the early maturation embryo. On the other hand, H3 occupancy in the *AhOleo18.5* proximal promoter (P1) and coding region (C1) was only slightly decreased in the early maturation embryo (Fig. 4B).

Dimethylation histone H3-Lys9 levels of AhOleo17.8 *and* AhOleo18.5 *were higher in vegetative tissues*

Lysine residues in histone tails can also be monomethylated, dimethylated, or trimethylated (Lachner and Jenuwein, 2002; Tariq and Paszkowski, 2004), and dimethylation of histone H3K9 is usually associated with gene silencing and heterochromatin formation through the recruitment of heterochromatin protein 1 (Bannister *et al.*, 2001; Mutskov and Felsenfeld, 2004). To determine whether the silence of *AhOle17.8* and *AhOle18.5* in vegetative tissues is related to dimethylation of histone H3K9, ChIP analysis was performed using the antibody against histone H3K9me2 in early maturation embryos and roots. The levels of histone H3K9me2 in the *AhOleo17.8* proximal promoter (P1) and coding region (C1) were lower in early maturation embryos than those in vegetative tissues (Fig. 5A). Decreased H3K9me2 was also observed in the *AhOleo18.5* proximal promoter (P1) but not the coding region (C1) (Fig. 5B). These data indicate that H3K9me2 levels of *AhOleo17.8* and *AhOleo18.5* proximal promoters (P1) were lower in early maturation embryos than those in vegetative tissues.

Histone modification status of AhOleo17.8 *and* AhOleo18.5 *in late maturation embryos*

Histone H3 and H4 acetylation levels of *AhOleo17.8* and *AhOleo18.5* chromatin in late maturation embryos were also examined. *AhOleo17.8* and *AhOleo18.5* were not expressed in roots, stems, and leaves (Fig. 1A), whereas in

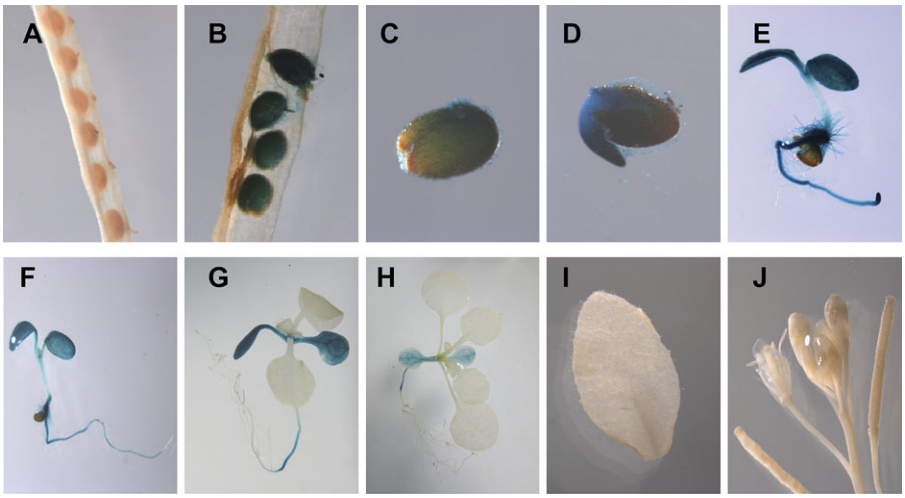

Fig. 2. GUS activity in transgenic *Arabidopsis* plants containing *AhOleo17.8*: *GUS*. GUS staining patterns of 5-d-old (A) and 7-d-old (B) siliques, the mature seed (C), seedling germinated after 2, 4, 6, 12, and 18 d (D–H), mature leaf (I), and stems and flowers (J), in *AhOleo17.8:GUS Arabidopsis* plants.

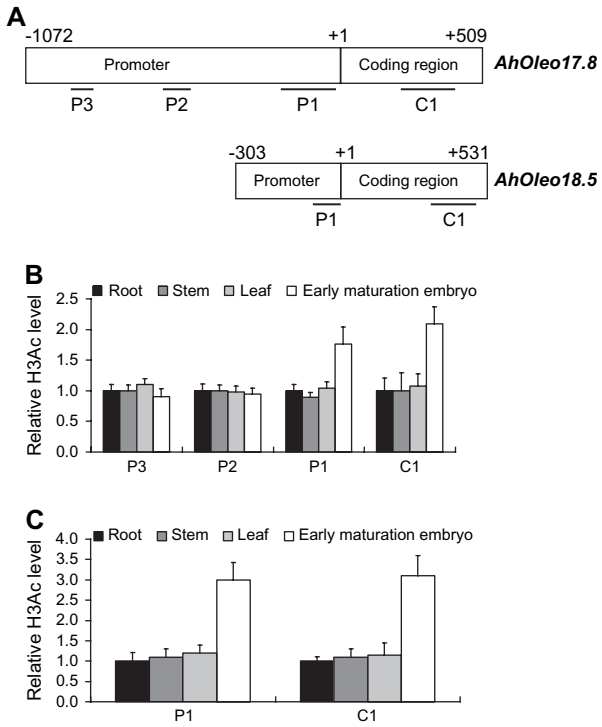

Fig. 3. Histones were hyperacetylated at the *AhOleo17.8* and *AhOleo18.5* proximal promoters (P1) and coding regions (C1) in early maturation embryos. (A) *AhOleo17.8* and *AhOleo18.5* genomic structures and locations of specific primers used in ChIP assay. (B) Acetylated histone H3 levels of *AhOleo17.8* in early maturation embryos compared with vegetative tissues. (C) Acetylated histone H3 levels of *AhOleo18.5* in early maturation embryos compared with vegetative tissues. *18S rRNA* was used as an internal control. The value of roots was arbitrarily given as 1. Data are the average of three biological replicates. Error bars represent standard errors.

late maturation embryos, the transcriptional activity of *AhOleo17.8* and *AhOleo18.5* reached to the highest level (Fig. 1B). Compared with roots, the ChIP signal for acetylated histone H3 at the proximal promoter (P1) decreased dramatically in late maturation embryos (Fig. 6A). Similarly, a decrease in acetylated histone H4 was also observed at the proximal promoter (P1) of *AhOleo17.8* (Fig. 6B). At the distal promoter (P3), middle promoter (P2), and coding region (C1) of *AhOleo17.8*, no apparent changes in histone H3 and H4 acetylation levels were found (Fig. 6A, B). Similar results were also observed for *AhOleo18.5*. Although the acetylated histone H3 level at the coding region (C1) of *AhOleo18.5* increased in late maturation embryos, it does not occur at the proximal promoter (P1) (Fig. 6C). The acetylated histone H4 level at the proximal promoter (P1) and the coding region (C1) of *AhOleo18.5* decreased in late maturation embryos (Fig. 6D).

Next, it was determined if the observed drop in histone H3 and H4 acetylation was restricted to acetylation levels or if there was a general loss due to covalent modifications from the promoter nucleosomes. Our analysis was therefore extended to the trimethylation of histone H3K4 levels of *AhOleo17.8* and *AhOleo18.5*. Again, a decrease of the ChIP signal for histone H3K4me3 at the *AhOleo17.8* and *AhOleo18.5* proximal promoter (P1) and coding region (C1) was observed (Fig. 6E, F). It was not possible to obtain reproducible results for H3K4me3 on early embryogenic extracts. It remains to be determined whether H3K4me3 is associated with basal-expressed oleosin chromatin.

Histones was lost from the fully activated AhOleo17.8 *and* AhOleo18.5

In yeast, Reinke and Horz, (2003) found that an apparent decrease in the histone acetylation level at the activated *PHO5* promoter was attributed to a progressive loss of total

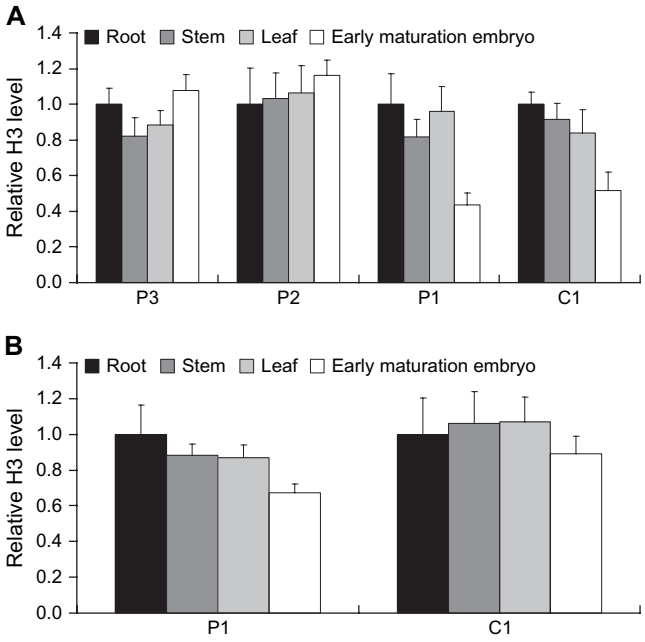

Fig. 4. Histone H3 status of *AhOleo17.8* and *AhOleo18.5* chromatin in roots, stems, leaves, and early maturation embryos. (A) Histone H3 levels of *AhOleo17.8* in early maturation embryos compared with vegetative tissues. (B) Histone H3 levels of *AhOleo18.5* in early maturation embryos compared with vegetative tissues. *18S rRNA* was used as an internal control. The value of roots was arbitrarily given as 1. Data are the average of three biological replicates. Error bars represent standard errors.

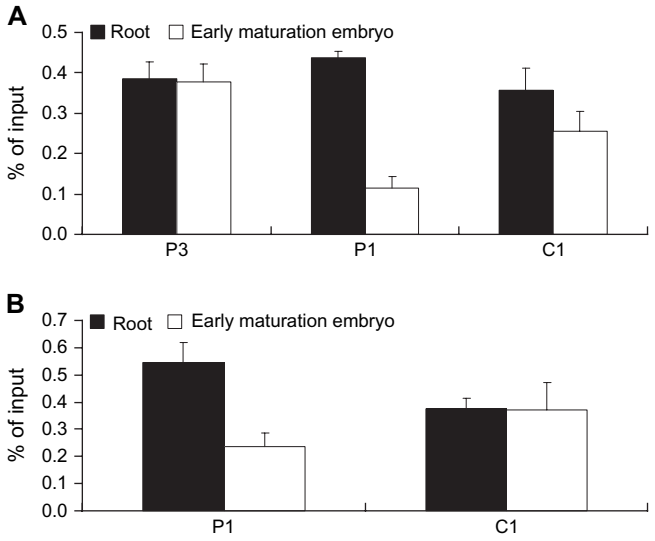

Fig. 5. Histone H3K9me2 status of *AhOleo17.8* and *AhOleo18.5* chromatin in roots and early maturation embryos. (A) H3K9me2 levels of the *AhOleo17.8* promoter and coding region were determined by ChIP in roots and early maturation embryos. (B) H3K9me2 levels of *AhOleo18.5* promoter and coding region were determined by ChIP in roots and early maturation embryos. The results were normalized for the amount of chromatin used for immunoprecipitation. Data are the average of three biological replicates. Error bars represent standard errors.

histones. It was therefore analysed whether the apparent decrease in the acetylation level at the proximal promoters (P1) of activated *AhOleo17.8* and *AhOleo18.5* was because of the same reason. To test this possibility, ChIP assays were performed with antibody against the C terminus of histone H3 that is not influenced by histone modifications. The proximal promoter (P1) of *AhOleo17.8* was enriched in the H3 antibody immunoprecipitates from roots but was largely depleted in the immunoprecipitates from the late maturation embryos (Fig. 7A). This finding suggests that histones H3 of this region might be displaced in late maturation embryos but were reassembled in vegetative tissues. In addition, an obvious increase of H3 occupancy at the coding region (C1) was also observed in roots compared with the late maturation embryos. By contrast, no apparent difference was observed in histone H3 occupancy in the distal (P3) and middle (P2) promoters. Similarly, at the proximal promoter (P1) and coding region (C1) of *AhOleo18.5*, a strong decrease of histone H3 occupancy was also detected in late maturation embryos (Fig. 7B). As a control, the histone H3 occupancy of a root-specific gene, *AhRb7* (Fig. 8A) and *18S rRNA*, was also analysed. No significant difference was observed in histone H3 occupancy of *AhRb7* and *18S rRNA* in roots, stems, leaves, early maturation embryos, and/or late maturation embryos (Fig. 8B, C). Therefore, the decrease in the histone H3 signal from the proximal promoter (P1) and coding region (C1) of *oleosins* in the late maturation embryos is specific for *oleosins*.

Discussion

Loss of histone H3 is associated with the high expression of oleosin *in late maturation embryos*

In this paper, evidence is provided indicating that histone modifications and removal are associated with the specific and high expression of two *oleosins* in peanut embryos. When the nucleosomal state of the *oleosins* was analysed, the nucleosome density of the proximal promoters (P1) of *AhOleo17.8* and *AhOleo18.5* largely decreased in late maturation embryos compared with vegetative tissues. The decrease of histone signals at the core promoters and coding regions of *oleosins* may be due to the loss in histone–DNA interaction. This explanation is in accordance with the study in yeast that nucleosomes were disassembled from the *PHO5* promoter during activation (Reinke and Horz, 2003; Adkins *et al.*, 2004). Although transcriptional process usually causes nucleosome disruption rather than the release of nucleosome in the coding regions, a partial loss of histone H3 from the coding regions of the heavily transcribed genes has been reported (Lee *et al.*, 2004). It was also observed that an obvious loss of histone H3 from the coding region (C1) in late maturation embryos (Fig. 7A, B), which indicated that the removal of histone from the coding region (C1) might be necessary to activate expression of

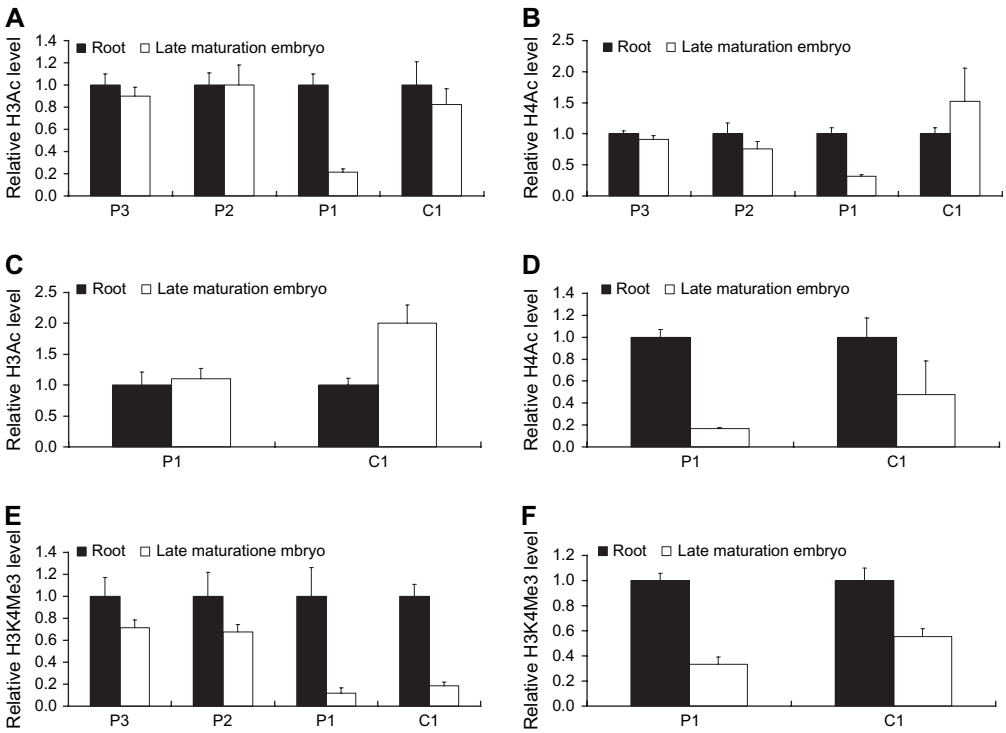

Fig. 6. Histone modification status of *AhOleo17.8* and *AhOleo18.5* chromatin in roots and late maturation embryos. Relative H3ac, H4ac, and H3K4me3 levels were determined by ChIP assays using anti-H3ac, anti-H4ac, and anti-H3K4me3 antibodies, respectively, and normalized to an internal control (*18S rRNA*). The value of roots was arbitrarily given as 1. Data are the average of three biological replicates. Error bars represent standard errors. (A) Histone H3 acetylation status of *AhOleo17.8*. (B) Histone H4 acetylation status of *AhOleo17.8*. (C) Histone H3 acetylation status of *AhOleo18.5*. (D) Histone H4 acetylation status of *AhOleo18.5*. (E) Histone H3K4me3 status of *AhOleo17.8*. (F) Histone H3K4me3 status of *AhOleo18.5*.

oleosins. Taken together, these data suggest that full activitation of *oleosins* in late maturation embryos is associated with histone H3 loss at both the proximal promoter (P1) and coding region (C1). Interestingly, it was found that histone loss at the proximal promoter (P1) of *AhOleo17.8* was more significant than that at *AhOleo18.5*. One possible reason is that the promoter activity of *AhOleo17.8* is much higher than that of *AhOleo18.5* at this stage (see Supplementary Table S1 at *JXB* online). It has also been reported that nucleosome occupancy is inversely correlated with promoter strength (Bernstein *et al.*, 2004).

Chromatin evinction and assembly are performed by histone chaperones and ATP-dependent chromatin remodelling activities *in vitro*. Accordingly, the histone H3–H4 chaperone Asf1 (anti-silencing function 1) was found to mediate chromatin eviction from the yeast *PHO5* and *PHO8* promoters during transcriptional activation (Adkins *et al.*, 2004). Moreover, Asf1 contributes to chromatin disassembly and reassembly during elongation of the yeast *GAL1* gene (Schwabish and Struhl, 2006). It remains to be determined whether similar molecular mechanisms exist in plants.

Histone acetylation is associated with basal oleosin expression

Histone acetylation plays an important role in specific changes in gene expression (Chen and Tian, 2007; Pfluger and Wagner, 2007). The deacetylation of lysine residues on histone tails is strongly correlated with transcriptional silence (Narlikar *et al.*, 2002). Several studies have also found that the expression of seed development and maturation genes was accompanied by an increase in histone acetylation. Overexpression of the plant histone deacetylase HD2A in *Arabidopsis* resulted in the repression of a number of genes involved in seed development and maturation including *oleosins* (Zhou *et al.*, 2004). The observation that histone H3 underwent a substantial increase in histone acetylation upon activation suggested that histone acetylation is associated with the expression of *phasolin* (Ng *et al.*, 2006). A more recent study found that *Arabidopsis* plants treated with histone deacetylase inhibitors trichostatin A displayed the expression of embryo development-related genes and the formation of embryo-like structures on the true leaves of 4-week-old plants (Tanaka *et al.*, 2008). These data suggested that histone acetylation participated in the regulation of seed-specific gene expression. Our results suggested that although histone H3 hyperacetylated from the *oleosin* proximal promoter and coding region accompanied by basal transcriptional activity in early maturation embryo, histone H3 hyperacetylation was not associated with the full transcriptional activity in late maturation embryo. Similarly, no increase in histone acetylation was observed during the transition from almost complete inactivation to an intermediate activity of the maize C4-PEPC

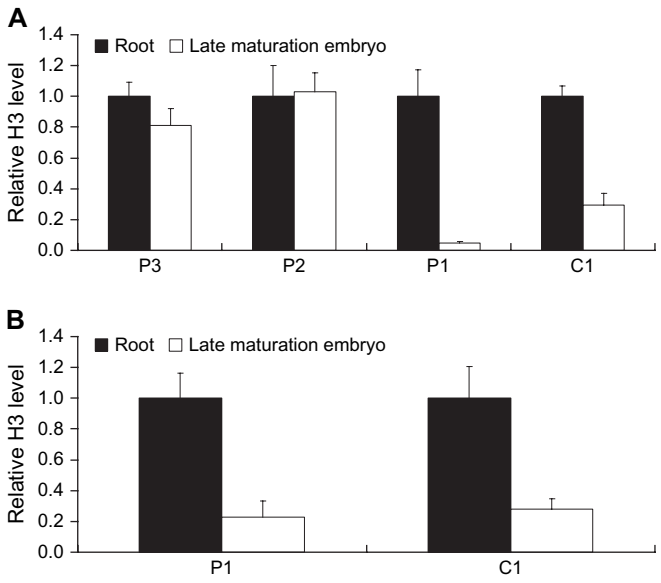

Fig. 7. Histone loss from the *AhOleo17.8* and *AhOleo18.5* proximal promoters and coding regions in late maturation embryos. Histone was lost from the *AhOleo17.8* (A) and *AhOleo18.5* (B) proximal promoters (P1) and coding regions (C1) in late maturation embryos compared with roots. Chromatin was precipitated with an antibody specific for C terminus histone H3. *18S rRNA* was used as an internal control. The value of roots was arbitrarily given as 1. Data are the average of three biological replicates. Error bars represent standard errors.

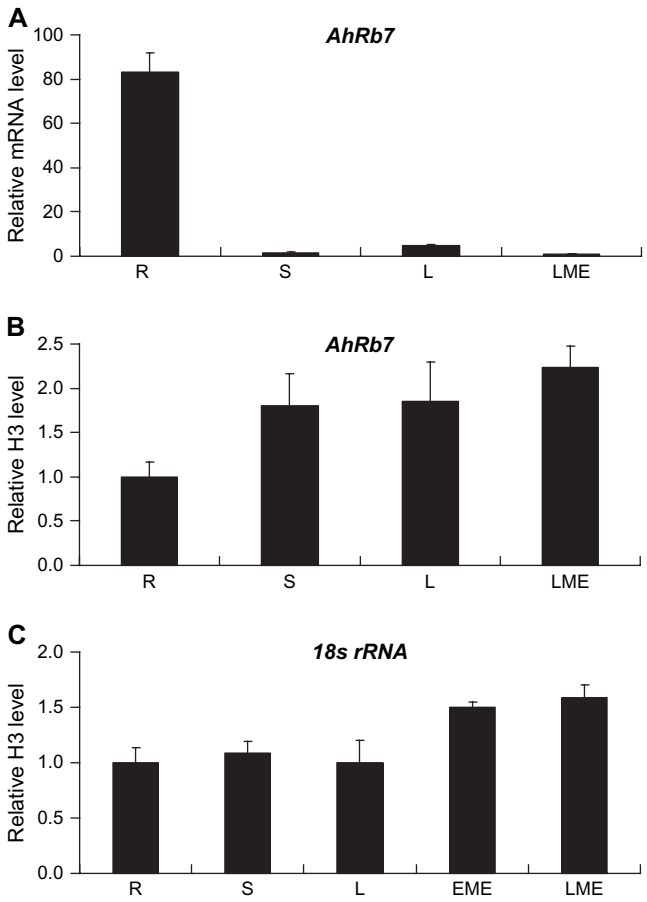

Fig. 8. Analysis of peanut *18S rRNA* and root-specific *AhRb7* nucleosome density in different tissues. *AhRb7* transcript was analysed by quantitative real-time RT-PCR. *18S rRNA* was used as an internal control. (B, C). Analysis of *AhRb7* (B) and *18S rRNA* (C) nucleosome density in different tissues. Chromatins were precipitated with an antibody specific for C terminus histone H3. The results were normalized for the amount of chromatin used for immunoprecipitation. The value of roots was arbitrarily given as 1. Data are the average of three biological replicates. Error bars represent standard errors. R, root; S, stem; L, leaf; EME, early maturation embryo; LME, late maturation embryo.

promoter (Offermann *et al.*, 2006). In yeast, it was found that an apparent decrease in histone acetylation level at the fully activated *PHO5* promoter was attributed to a progressive loss of total histones (Reinke and Horz, 2003). Our analysis indicated that there was a decrease of histone H3 signals from the proximal promoters and coding regions of *olesoins*, suggesting that the apparent decrease in the acetylation levels of fully activated *AhOleo17.8* and *AhOleo18.5* was because of decreased histone occupancy.

Recent studies in yeast and animal systems imply that histone acetylation may also contribute to nucleosome eviction during gene activation. Ito *et al.* (2000) reported that histone acetylation by P300 facilitates the displacement of H2A/H2B dimers onto the histone chaperone NAP-1 in an *in vitro* system. The fact that histones are first acetylated and then lost from the *PHO5* promoter upon induction suggests that modification of histones by acetylation may play a role in nucleosome loss (Reinke and Horz, 2003). Promoter-associated histones are also transiently hyperacetylated at the *HSP82* gene before histones eviction following heat shock (Zhao *et al.*, 2005). The observation that the SWI/SNF complex binds to acetylated histones and that histone loss is dependent on SWI/SNF (Hassan *et al.*, 2001; Reinke and Horz, 2003) suggests a direct connection between histone acetylation and nucleosome displacement. More recently, Govind *et al.* (2007) reported that histone acetyltransferase Gcn5 enhanced nucleosome eviction from the highly transcribed *GAL1* gene and promoted Pol II (RNA polymerase II) elongation. These studies provide evidence that histone acetylation is associated with the subsequent loss of histone. It will be interesting to reveal the role of acetylation in subsequent histone loss during gene activation in plants.

Histone H3K9me2 is associated with oleosin *silence in vegetative tissues*

Histone H3K9me2 is known as a critical mark for DNA methylation and long-term gene silencing (Jackson *et al.*, 2004). In *Arabidopsis*, mutations of histone methyltransferases caused a reduction of methylated histone H3 lysine 9, and reduced gene silencing (Jackson *et al.*, 2004). Alvarez-Venegas and Avramova (2005) found that when transcriptionally silent, *SUP*, a flower-specific gene, carried dimethylation H3K9 on both promoter and coding region.

Furthermore, nickel ions can induce transgene silencing by increasing histone H3K9me2 (Chen *et al.*, 2006). A more recent study showed that silenced genes were strongly enriched for targets of H3K9me2 (Bernatavichute *et al.*, 2008). Our results suggested that chromatin of *oleosin* proximal promoters became dimethylated at histone H3K9 in vegetative tissues when *oleosins* were not expressed, suggesting that dimethylation at histone H3 Lys-9 is associated with *oleosin* repression. H3K9me2 levels were also analysed in late embryogenic extracts. About 30-fold and 6-fold decreases at the AhOleo 17.8 core promoter and coding region was found on late maturation embryos, respectively(data not shown). However, histone occupancy decreased largely at this stage. It remains to be determined whether a decrease of H3K9me2 is because of decreased histone occupancy in late embryogenic extracts.

In conclusion, our analyses indicate that the chromatin structure of *oleosins* undergoes targeted changes during development. During vegetative growth, positioned nucleosomes are present over the *oleosin* core promoter and coding region. Meanwhile, histone H3 at Lys-9 is dimethylated at the *oleosin* core promoter. During embryogenesis, histone H3 at the proximal promoter and coding region of *oleosin* become hyperacetylated in early maturation embryos, and then is lost from the *oleosin* core promoter region in late maturation embryos. The data in this article demonstrate that the chromatin structure of peanut *oleosin*, as assessed by histone acetylation and occupancy, is distinct in different transcriptional states during development. Histone acetylation appears to associate with seed-specific expression of *oleosin*, while high expression of *oleosin* is accompanied by histone displacement. Further research is required to study the molecular mechanism of histone loss and modifications to understand gene regulation in seed development.

Supplementary data

Supplementary data are available at *JXB* online.

Supplementary Table S1. ESTs of *oleosins* derived from late maturation stage peanut embryos.

Supplementary Table S2. Relative positions of *oleosin* primers used in ChIP-QPCR analysis.

Supplementary Table S3. Original data of *Aholeo17.8* histone H3Ac levels of roots, stems, leaves, and early maturation embryos.

Supplementary Table S4. Original data of *Aholeo17.8* histone H3K9me2 levels of roots and early maturation embryos.

Supplementary Fig. S1. Optimal alignment of the peanut oleosin protein sequences.

Supplementary Fig. S2. Nucleotide sequence of the promoter and transcribed sequence of *AhOleo17.8*.

Supplementary Fig. S3. Nucleotide sequence of the promoter and transcribed sequence of *AhOleo18.5*.

Supplementary Fig. S4. Intron analysis of peanut *oleosins*.

Supplementary Fig. S5. Analysis of histone H4 acetylation levels of *AhOleo17.8* and *AhOleo18.5* in early maturation embryos.

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