

Epigenetic regulation of peanut allergen gene *Ara h 3* in developing embryos

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Abstract Peanut (*Arachis hypogaea*) allergy is one of the most serious food allergies. Peanut seed protein, *Ara h 3*, is considered to be one of the most important peanut allergens. Little is known about the temporal and spatial regulation mechanism of *Ara h 3* during seed development. In this study, chromatin structure of the *Ara h 3* promoter was analyzed to examine its transcriptional regulation. Analysis of transgenic plants of *Arabidopsis thaliana* expressing *Arah3: GUS* showed that the *Ara h 3* promoter could efficiently direct the seed-specific expression of the GUS reporter gene. Chromatin immunoprecipitation revealed that nucleosomes were depleted at the core promoter of the *Ara h 3* upon full activation in the late stage of embryo maturation, which was accompanied by a dramatic decrease of

histone acetylation. However, in the early stage of embryo maturation, histone H3 hyperacetylation at the core promoter of *Ara h 3* was detected. A decrease of histone H3-K9 dimethylation levels at core promoter of *Ara h 3* was also observed with concomitant repression of *Ara h 3* in the vegetative tissues. Our results suggest that the histone modification status of *Ara h 3* undergoes targeted changes including the increase of histone H3 acetylation and decrease of histone H3-K9 dimethylation in early maturation embryos. In addition, the loss of histone H3 from the proximal promoter of *Ara h 3* is associated with its high expression during late embryo maturation.

Keywords Peanut · Allergen · *Ara h 3* · Chromatin immunoprecipitation · Epigenetic regulation · Embryo development

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Abbreviations

ABA	Abscisic acid
ALF	ABI3-like factor the bean homolog of ABI3
AP1	Adaptor primer 1
AP2	Adaptor primer 2
ChIP	Chromatin immunoprecipitation
DAF	Days after flowering
DAPPS	Day after peg penetration to the soil
EME	Early maturation embryos
GSP1	Gene specific primer 1
GSP2	Gene specific primer 2
GUS	β -Glucuronidase
LME	Late maturation embryos
MS	Murashige and Skoog medium
ORF	Open reading frame
PCR	Polymerase chain reaction
RT-PCR	Reverse transcriptase-polymerase chain reaction

Introduction

Cultivated peanut (*Arachis hypogaea* L.) is a valuable oil-seed and food crop. However, allergy caused by ingesting peanut seeds is one of the most serious and life-threatening food sensitivities, particularly among children (Sampson 1996; Burks 2003). The major peanut allergens are designated Ara h 1–3, which belong to the peanut seed storage protein conarachin, conglutin and arachin, respectively. To date, 11 peanut allergens (Ara h 1–11) have been officially identified (<http://www.allergen.org/Allergen.aspx>). Among them, Ara h 1–3 are classified as the major peanut allergens which can be recognized by more than 50% of peanut-allergic patients (Burks et al. 1992; Rabjohn et al. 1999; Annick et al. 2005). The peanut major allergen Ara h 3 is a legumin-like seed storage protein that has high sequence similarity to glycinin, which is the major 11S globulin seed storage protein family in soybean (Rabjohn et al. 1999). It is known that the 11S protein has multiple isoforms in many plant species. Each isoform of the 11S globulin is encoded by a single gene (Wang et al. 2002) that produces a precursor which is post-translationally cleaved by an asparaginyl endopeptidase after the formation of the inter-chain disulfide bond between the N-terminal and the C-terminal subunits (Albillos et al. 2008). The 11S storage protein from soybean is known to be a hexameric globulin composed of various mixtures of five different isoforms (Badley et al. 1975). Southern-blot analysis indicated the existence of multiple *Ara h 3*-im genes in peanut (Kang and Gallo 2007). Our previous report also showed that there were at least five isoforms of *Ara h 3* (Yan et al. 2005). Recently, the crystal structure of Ara h 3 has been identified. Mapping of the linear epitopes on the crystal structure of Ara h 3 indicated that linear epitopes with exposure to solvent were more reactive with patient sera (Jin et al. 2009).

Synthesis and accumulation of seed storage protein occurs specifically in seed maturation and is not observed in vegetative organs of the plant (Laux and Jurgens 1997; Vicente-Carbajosa and Carbonero 2005). Although a wealth of information exists concerning the regulation of transcription from seed-specific promoters, much remains to be learned regarding how these promoters are selectively activated. Recent work on the *phaseolin* (*phas*) promoter provided evidence that dynamic histone modifications occur at the chromatin associated with the seed-specific gene expression (Ng et al. 2006). Although the *phaseolin* gene is silent in all vegetative tissues of the bean (*Phaseolus vulgaris*) (Van der Geest et al. 1995), it becomes transcriptionally active in the seed during embryo development (Li et al. 2001). Repressive *phaseolin* chromatin becomes remodeled (potentiated) in the leaf tissue misexpressing an ABI3 homolog (Ng et al. 2006), which is accompanied by

an increased level of acetylation of H3-K9 and H4-K12. A different array of changes, including acetylation of H3-K14 and methylation of H3-K4, was found to be associated with abscisic acid (ABA)-mediated activation of *phaseolin*. More recently, our study in peanut suggests that the histone eviction from the proximal promoters and coding regions is associated with high expression of *oleosin* genes during late embryo-maturation, while the basal expression of *oleosins* in early maturation of embryos is accompanied by the increase of histone H3 acetylation (Li et al. 2009).

Transcriptional regulation of the major peanut allergen genes *Ara h 3* was investigated recently. The transcript of *Ara h 3* was observed in the seeds, but not in leaves, flowers, or roots, and was also undetectable during seed germination (Kang et al. 2007). However, information on the regulation of the promoter activity of peanut allergen genes is limited. Moreover, the relationship between chromatin dynamics and peanut allergen gene activity has not been investigated. In this study we isolated and characterized one isoform of *Ara h 3* (GenBank accession number DQ519079), and demonstrated that the *Ara h 3* promoter can efficiently direct the seed-specific expression of the GUS reporter gene. Moreover, we investigated epigenetic regulation mechanisms of *Ara h 3* in developing peanut embryos. Our results demonstrate that the histone H3 loss from the proximal promoter is associated with high expression of *Ara h 3* during peanut embryo maturation. In addition, the histone modification status of *Ara h 3* undergoes targeted changes including an increase of histone acetylation and decrease of H3-K9 dimethylation at the core promoter in early maturation embryos.

Materials and methods

Plant materials

Mature and immature peanut (*A. hypogaea* L. Shanyou 523) seeds were provided by Dr. Xuanqiang Liang (Guangdong Agricultural Academy, Guangdong, China). Peanut plants were grown in sandy soil in the field for 3 months. The soil was fertilized once before semination with N, P, K, and other microelements. Developing peanut embryos were harvested at 20, 30, 40, 50, and 70 days after peg penetration into the soil (DAPPS) and then stored at -80°C for total RNA and salt-soluble protein extractions. The roots, stems, and leaves were harvested from 14-day-old seedlings and stored at -80°C for total RNA and DNA isolation. *Arabidopsis thaliana* ecotype Columbia was used for generating transgenic plants. Sterilized seeds were grown on Murashige and Skoog medium (MS) supplemented with 1% sucrose and 0.8% agar. Planted seeds were incubated at 4°C for 48 h and then maintained under a 16-h light/8-h

dark photoperiod at 24°C. Seedlings were transferred into soil after 2 weeks.

Isolation of full-length gene and promoter of *Ara h 3*

Genomic DNA was extracted from peanut leaves as described by Pich and Schubert (1993). The primer pair: 5' GTGCCAGTTCCAGCGCTCAAT 3' (forward) and 5' AGGCCATATGAATTTGCAACCAC 3' (reverse) were used to amplify the *Ara h 3* gene. Polymerase chain reaction (PCR) consisted of an initial denaturing step at 95°C for 2 min, followed by 30 cycles of denaturing temperature at 95°C for 30 s, an annealing temperature of 56°C for 30 s, and an elongation temperature of 72°C for 2 min. The final PCR product was cloned into the pGEM-T vector (Promega, Madison, WI, USA) and sequenced.

The promoter of *Ara h 3* was cloned according to the method described in the GenomeWalker Kit (Clontech, Palo Alto, CA, USA). Briefly, genomic DNA was extracted from peanut leaf tissue according to the protocol described by Pich and Schubert (1993). DNA was digested with *Dra*I or *Eco*RV and then blunt-end 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 1 (AP1), 5' GTAATACGACTCACTATAGGGC 3' and an outer gene-specific primer gene specific primer 1 (GSP1), 5' ACCGCCCTCTGATTCAATGC 3', while an inner adaptor primer 2 (AP2), 5' ACTATAGGGCAGCGTGGTC 3' and inner gene-specific primer 2 (GSP2), 5' GC TGCTGCCTGAAGGAGATGC 3' were used for the second round of PCR amplification. PCR products were purified by using the QIAquick Gel Extraction Kit (Qiagen, Hilden, Germany), cloned into the pGEM-T vector (Promega) and sequenced.

Semi-quantitative and quantitative real-time PCR

Total RNA was isolated from peanut cotyledons at approximately 20, 30, 40, 50, and 70 DAPPS, and other tissues including roots, stems and leaves using TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. First-strand cDNA was synthesized from the total RNA with the Takara RNA PCR kit (Takara, Otsu, Japan). Semi-quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) was performed using 18s rRNA as an internal control. The primers for 18s rRNA were 5' GAAACGGCTACCACATCCAAG 3' (forward) and 5' CCAACCCAAGGTCCAACACTACG 3' (reverse). The primers for *Ara h 3* were 5' CGACCAGTATCTTCTGCAAGC 3' (forward) and 5' TTTTCTCCATATCTGCTCATCG 3' (reverse). The PCR program consisted of an initial denaturing step at 95°C for 2 min, followed by 27

cycles at 95°C for 30 s, 61°C for 30 s and 72°C for 30 s for 18s rRNA and by 30 cycles at 95°C for 30 s, 58°C for 30 s, and 72°C for 30 s for *Ara h 3*.

Quantitative real-time PCR was performed in IQ5 Multi-color Real-time PCR Detection System (Bio-Rad, Hercules, CA, USA) following the manufacturer's instruction using the SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan). The amounts of cDNA were 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 5' TTTCTGCCCTATCAACTTTTCG 3' (forward) and 5' TTATTTATTGTCACTACCTCCCCGT 3' (reverse). The primers for *Ara h 3* were 5' TTAGGTGGCTTGGACTTAGTGCTGA 3' (forward) and 5' GTGCGTTCGTGTTGTAGTGAGGGAC 3' (reverse). The amplification was performed at an initial denaturing step at 95°C for 1 min, followed by 40 cycles at 95°C for 15 s, 55°C for 20 s, and 72°C for 20 s.

Protein preparation, electrophoresis, and immunoblotting

The storage protein was extracted by homogenizing developing peanut cotyledons in 10 mM phosphoresce buffer, pH 7.9, containing 10% NaCl, 1 mM PMSF and 10 mM β -ME, and centrifugation at 15,000 $\times g$ for 20 min at 4°C (Yamada et al. 1979). Protein concentration was determined by the method according to Bradford (1976) using Coomassie Blue G250. Salt-soluble protein fractionated by SDS-PAGE was either stained with Coomassie R250 or electroblotted onto Immobilon-NC (0.45 μ M, Millipore, Billerica, MA, USA). Polyclonal anti-*Ara h 3* acidic subunit (38.5 kDa) was prepared according to Liao et al. (2004). Western blot was performed according to Burnette (1981). Results were visualized with ECL Plus detection reagent (Amersham Pharmacia Biotech, Uppsala, Sweden) and by exposing blots to Kodak X-ray Film (Kodak, Rochester, NY, USA).

Construction of *Ara h 3* promoter/*GUS* fusion vector and plant transformation

A DNA fragment of 643 bp containing the putative promoter of *Ara h 3* was amplified by PCR with a pair of primers, 5' CGCGGATCCGGGCAGGTAAAAATAATT 3' (forward) and 5' TCCCCCGGGTTGTGATGAAGGAGAT 3' (reverse). The fragment was ligated into *Bam*HI-*Sma*I digested binary vector pBI101.1 (Clontech) to create the construct *Ara h 3*: *GUS*. The binary vectors, *Ara h 3*: *GUS* containing *nptII* as the plant selectable marker gene, were then transferred to *Agrobacterium tumefaciens* strain EHA105 via the freeze-thaw method (Holsters et al. 1978). Empty pBI121 vectors were used as controls.

Arabidopsis thaliana plants were transformed by the vacuum infiltration method (Clough and Bent 1998). T₁ seeds were harvested and germinated on a sterile medium containing 50 µg/ml kanamycin to select for putative transformants. Surviving T₁ plantlets were transferred to soil for generation of T₂ seeds.

GUS fluorometric and histochemical analyses

Fluorometric analysis of GUS activity was carried out as described by Jefferson et al. (1987). Five independent lines harboring the *Ara h 3* promoter::gusA expression and 35S promoter::gusA were generated and assayed, separately. Three samples of each tissue type were collected from two replicate plants per line. Total protein was extracted from 100 mg of T₃ transgenic *Arabidopsis* seeds and quantified according to Bradford (1976). GUS activity was normalized to the total protein and the results were expressed as GUS-specific activity (nanomole of 4-MU released per milligram protein per minute).

Histochemical staining of GUS activity in plant tissues and seed was performed as described by Jefferson et al. (1987). After GUS staining, the tissues and seedlings were treated with ethanol to clear chlorophyll and photographed under a dissecting microscope.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as described previously (Bowler et al. 2004) with the following modifications. In brief, peanut tissues were cut into pieces and cross-linked in 1% (v/v) formaldehyde by vacuum infiltration for 15 min. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M. Tissues were ground into powder in liquid nitrogen. Nuclei were extracted with 300 µl of nuclear lysis buffer containing 50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS, and 1 protease inhibitor tablet (Roche, Basel, Switzerland). The resulting chromatin was subjected to pulse sonication using a SonicsVC 130PB (Sonics & Materials, Newtown, CT, USA) to obtain DNA fragments with sizes ranging from 200 to 1,000 bp. After sonication, a 10-µl aliquot was removed for the total input DNA control and the rest of the chromatin solution was diluted tenfold with ChIP dilution buffer (1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris-HCl, 167 mM NaCl). The diluted chromatin solution was then subjected to 1 h of preclearing treatment at 4°C with 40 µl of salmon sperm DNA/protein A agarose (Upstate, New York, NY, USA). Overnight immunoprecipitation was performed at 4°C using 500 µl of chromatin solution with or without 5 µl antibodies. Immunoprecipitates were collected with 40 µl of salmon sperm DNA/protein A agarose at 4°C for 1 h. The protein A agarose beads bearing

immunoprecipitates were subjected to sequential washes and immunoprecipitates were eluted twice with 250 µl of elution buffer (1% SDS and 0.1 M NaHCO₃). Samples were then reverse cross-linked overnight at 65°C under high-salt (0.2 M NaCl). The input DNA control (10 µl) was mixed with 20 µl of 5 M NaCl and 500 µl of elution buffer and reverse cross-linked. Protein was removed by proteinase K digestion and DNA was purified by phenol-chloroform extraction and ethanol precipitation. Purified DNA was resuspended in 80 µl of water and stored in -80°C. The antibodies used in this study are anti-acetyl-histone H3K9/14 (Upstate), anti-acetyl-histone H4K5/8/12/16 (Upstate), anti-trimethyl-histone H3K4 (Abcam, Cambridge, UK), anti-dimethyl-histone H3K9 (Upstate), and anti-histone H3 (Abcam).

Purified DNA from the ChIP samples was used for quantitative PCR with the following cycling conditions: 1 min initial denaturation at 94°C, followed by 40 cycles at 95°C for 15 s, 55°C for 20 s and 72°C for 20 s. The primers used to detect *Ara h 3* were 5' CAAGGCTGCTCTCTTTGTA TGCT 3' (forward) and 5' GCTGCTATTATTGTTATT GCTGTGGT 3' (reverse). The primers used for 18s rRNA were 5' TTTCTGCCCTATCAACTTTTCG 3' (forward) and 5' TTATTTATTGTCACTACCTCCCCGT 3' (reverse). The results of histone H3-K9 dimethylation were normalized 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. The size of the amplified regions was confirmed by gel electrophoresis and the specificity by sequencing.

Results

Genomic structure of *Ara h 3*

Previously, we identified five *Ara h 3* gene isoforms in a peanut seed cDNA library (Yan et al. 2005). One of the five isolated *Ara h 3* cDNA (GenBank accession number AY848698) has an open reading frame coding for 531 amino acids. The deduced amino acid sequence of *Ara h 3* displays 97% sequence identity to that of the officially recognized peanut allergen *Ara h 3.01* (GenBank Accession number AAC63045), and contains all four IgE epitopes (Epitope 1–Epitope 4) that have been previously described by Rabjohn et al. (1999), confirming that this gene encodes an *Ara h 3* isoform. Subsequent research was therefore focused on this isoform. The genomic DNA sequence of *Ara h 3* was PCR amplified using the sequence information from the cDNA. The 5' end upstream sequence of *Ara h 3* was obtained by DNA walker method. This resulted in an assembled *Ara h 3* genomic sequence of 2,773 bp (GenBank accession number DQ519079), with four exons

Fig. 1 Nucleotide sequence and deduced amino acid sequence of the allergen gene *Ara h 3* in peanut (GenBank accession no. DQ519079). The start codon (ATG), stop codon (TAA), and polyadenylation signal (AATAAA) are *underlined*. The putative signal peptide is *italicized* and *underlined*. Four IgE-binding epitopes are *boxed*

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-90  tgteecttctctaccctctctctgcccccaaatcaactcgetttcttcttctcacaaccacagcaataacaataatagcagcc
  1  ATGGCTAAGCTTCTTGAGCTTTCTTTTGTCTTTGCTTTCTAGTCTGGGAGCTAGCAGCATCTCTTCAGGCAGCAGCCGAGGAGAAT
  1  M A K L L E L S F C F C F L V L G A S S I S F R Q Q P E E N
  91  GCGTGCCAGTTCAGCGGCTCAATGCGCAGAGACCTGACAATCGCATTGAATCAGAGGGCGGTACATTGAGACTTGGAAACCCCAACAAC
 31  A C Q F Q R L N A Q R P D N R I E S E G G Y I E T W N P N N
                                           Epitope 1
181  CAGGAGTTCGAATGCGCGGCGTGCCTCTCTCGCTTAGTCTCCGCCGCAACGCCCTTCGTAGGCTTTCTACTCCAATGCTCCCCAG
 61  Q E F E C A G V A L S R L V L R R N A L R R P F Y S N A P Q
271  GAGATCTTCATCCAGCAAGgttattacttattactacttttcatatttgagaattttatttttttccatgatctttgtttgttaa
 91  E I F I Q Q
361  ttccattttgaatgaacacatacagGAAGGGGACTTTGGGTGATATCCCGGTTGTCTAGCACATATGAAGAGCTGCACAACAA
 97  G R G Y F G L I F P G C P S T Y E E P A Q Q
451  GGACGTCGATCTCAGTCCCAAGACCAAGACGCTTCAAGGAGAAGACCAAGCAACGAAGAAGTAGTACCAGAAAGGTGCAC
119  G R R S Q S Q R P P R R L Q G E D Q S Q Q Q Q D S H Q K V H
541  CGTTTCGATGAGGGTATCTCATTGCAGTTCACCGGTTGCTTTCTGGCTCTACAACGACCAGCAGACTGATGTTGTTGCTGTTTCT
149  R F D E G D L I A V P T G V A F W L Y N D H D T D V V A V S
631  CTACTGACCAACAACAACGACAACGAGCTTGATCAGTTCACCGGgtatatatttcttatttttgccatctgcataatatacatg
169  L T D T N N N D N Q L D Q F P R
721  aatattgagtcattttcttagaacttgatcactactaaagcaatcaacattttaataatacacaaggAGATTCAATTTGGCTGGGAACCAC
185  R F N L A G N H
811  GAGCAAGAGTTCTTAAGGTACCAGCAACAAGCAGACAAGCAGACGAAGAAGCTTACCATATAGCCCATACAGCCGCAAGTCAGCCT
193  E Q E F L R Y Q Q Q S R Q S R R R S L P Y S P Y S P Q S Q P
901  AGACAAGAAGCGTGAATTTAGTCTCGAGGACGAGCCGAGAGAAGCAGGACAGAAGAAGAAACGAGGTTGGAACATC
223  R Q E E R E F S P R G Q H S R R E R A G Q E E N E G G N I
991  TTCAGCGGCTTACGCGGAGTTCCTGGAACAAGCCTTCAGGTTGACGACAGACAGATAGTGAACCACTAAGAGCGGAGAACGAGAGT
253  F S G F T P E F L E Q A F Q V D D R Q I V Q N L R G E N E S
                                           Epitope 2
1081  GAAGAAGAGGGGCAATTGTGACAGTGAAGGGAGGCCTCAGAATCTTGAGCCAGATAGAAAGAGAGGTGCCGACGAAGAAGAGGAATAC
283  E E E G A I V T V R G G L R I L S P D R K R G A D E E E E Y
                                           Epitope 3
1171  GATGAAGATGAATATGAATACGATGAAGAGGATAGAAGCGTGGCAGGGAAGCAGAGGCAGGGGGAATGGTATTGAAGAGACGATCTGC
313  D E D E Y E Y D E E D R R R G R G S R G R G N G I E E T I C
                                           Epitope 4
1261  ACCGCAAGTGTTAAAAAGAACATTGGTAGAAACAGATCCCTGACATCTACAATCTCAAGCTGGTTCACCTAAAACGCCAACGATCTC
343  T A S V K K N I G R N R S P D I Y N P Q A G S L K T A N D L
1351  AACCTTCTAATCTTAGTGGCTTGACTTAGTGCTGAATATGGAATCTCTACAGGgtttgtactttctctttttctatctcttttttt
373  N L L I L R W L G L S A E Y G N L Y R
1441  accaatatatactagacatttattacatattgaaatgtgattgcagATGCATTGTTGTCCTCACTACAACCAACGCACACAGCA
392  N A L F V P H Y N T N A H S
1531  TCATATATGCATTGAGGGGACGGGCTCAGTGCAAGTCGTGGACGACCAAGCAGAGTGTACGACGAGGAGCTTCAAGAGGGTCAGG
406  I I Y A L R G R A H V Q V V D S N G N R V Y D E E L Q E G H
1621  TGCTTGTTGGTCCACAGAACTTCGCGTCGTGGAAAGTCCAGAGCGACAATTCGAATACGTGGCATTCAAGACAGACTCAAGGCCCA
436  V L V V P Q N F A V A G K S Q S D N F E Y V A F K T D S R P
1711  GCATAGCCAACCTCGCGGTGAAACTCCGTCATAGATAACCTGCGGAGGAGGTGGTTCGAATTCATATGGCTCCAAAGGGAGCAGG
466  S I A N L A G E N S V I D N L P E E V V A N S Y G L Q R E Q
1801  CAAGGCAGCAGCTTAAGAACAACAACCCCTTCAAGTCTCTCGTCCACCGTCTCAGCAGTCTCCGAGGGGTGTGGCTTAAaaacgaccag
496  A R Q Q L K N N N P F K F F V P P S Q Q S P R A V A *
1891  tatcttctgaagcgtgttatccactacatacttttgcacaaatgaataataataataagaagaataatgtagttttaatttttag
1971  tatgaataagaatacaaggggcattgatgccctttttgtttaagatcggaatgtaacatatgtgcaatgagcagatatggagaaacct
tttgcgggaaaaacatgaataataaaagaagttaggtctcagccc

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and three introns (Fig. 1). The three introns in the *Ara h 3* belong to the Class III intron containing the typical AT-rich regions (Grierson and Covey 1988). The first, second, and third intron are 79, 73, and 75% A + T rich, respectively (Table 1). All splice junctions between exons and introns follow the GT-AG consensus sequence rule (Grierson and Covey 1988; Lorković et al. 2000) (Table 1). About 240 bp of the 3' UTR were sequenced and a putative polyadenylation signal (AATAAA) was located 211 nucleotides 3' downstream from the stop codon (TAA) (Fig. 1).

The 657-bp promoter sequence of *Ara h 3* contains several conserved sequence motifs which could be important for gene expression (Fig. 2). A putative TATA box is found at position -57. A CAAT box (AGGA) is located at position -163. These two elements are important for the positioning of RNA polymerase II for transcription initiation. A legumin-box (GCCATAGCCACGCATGCTGAAG AGTGTC) at position -130 is the conserved sequence of legumin-like proteins in legume seeds (Morton et al. 1995). A G-box (TCCGACGTGTCC), which is present in 5'

Table 1 Sequence characteristics of the three introns of the peanut *Ara h 3* gene

Intron	Exon/intron boundaries	Size (bp)	A + T (%)	Nucleotide position
1	GCAAG/GTTATTAC...ACATACAG/GAAGG	96	79.1	250–385
2	CCAGG/GTATATAT...CATAACAG/AGATT	108	73.1	679–786
3	ACAGG/GTTTGTAC...GATTGCAG/AATGC	80	75.0	1,408–1,487

The splice-sites and conserved sequences are in *bold*

regions of many seed specific genes, is located at position –89 (Williams et al. 1992). A sequence highly homologous to a RY element is located at position –275 (CATGCGTG). RY elements are related to the expression of seed storage protein genes (Bäumlein et al. 1992; Chamberland et al. 1992; Fujiwara and Beachy 1994). An ACACA motif (AAC ACA), found in 5' regions of the genes encoding 11S proteins of legumes for conferring seed specificity, is located at –485 (Morton et al. 1995). Several AT-rich sequences were also identified (–380 to –390; –442 to –462 and –657 to –634). AT-rich regions have been reported to enhance gene expression (Strömvik et al. 1999). Thus, the presence of legumin-box, G-box, RY element, and AT-rich regions suggests that the *Ara h 3* promoter is a potentially strong seed-specific promoter. Although the upstream promoter region of *Ara h 3* isolated in this study and that of *Ara h 3* reported previously by Viquez et al. (2004) shows only 69% sequence homology, the two promoters share many identical *cis*-elements, including legumin-box, RY element, and G-box. The region that spans the nucleotide sequence from –657 to –2 bp is defined as the core promoter.

The expression of *Ara h 3* during peanut seed development

Ara h 3 expression patterns were variable depending on the cultivars (Kang et al. 2007). The expression pattern of *Ara h 3* during peanut seed development was investigated by RT-PCR. The result revealed exclusive expression of *Ara h 3* in developing peanut seeds, but not in roots, stems, and leaves (Fig. 3a). Quantitative RT-PCR was further performed to assay the transcription levels of *Ara h 3* at different stages of seed development. The results indicated that transcription of *Ara h 3* was low in seeds harvested on at 20 DAPPS but high in those harvested between 30 and 70 DAPPS (Fig. 3b). Peanut vegetative tissues, early maturation embryos (20 DAPPS), and late maturation embryos (50 DAPPS) thereby represent three different transcriptional states of *Ara h 3*: an inactive state, a basal state, and a fully activated state.

Western blots were performed to correlate the transcript abundance and *Ara h 3* protein expression levels, which confirmed that the relative content of the *Ara h 3* acidic subunit in peanut cotyledons gradually increased from 20 to 70 DAPPS (Fig. 3c).

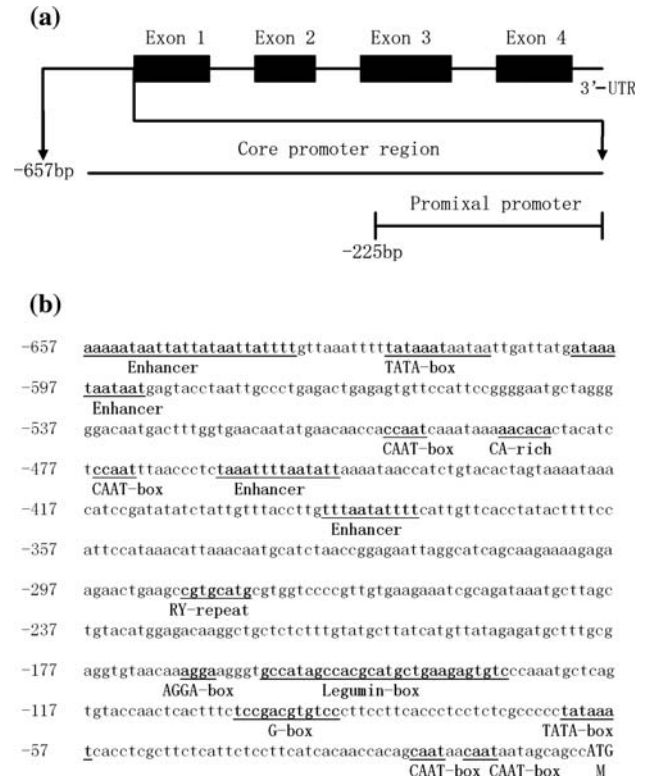


Fig. 2 Schematic diagram and promoter sequence of the *Ara h 3* gene. **a** The *Ara h 3* gene contains four exons and three introns. The profile of core promoter region and proximal promoter are shown. **b** Nucleotide sequence of the *Ara h 3* promoter region. The putative enhancers, transcription factor binding sites, TATA-box, CAAT-box, G-box, CA-rich sequence, legumin-box, and RY-element are labeled, indicated by *bold fonts* and *underlined*. The translation start site (ATG) for the *Ara h 3* is in *upper case*

Expression of GUS reporter gene under control of *Ara h 3* promoter in transgenic *A. thaliana* plants

To further study the expression pattern of *Ara h 3*, a fragment containing the 643-bp promoter region was placed in front of the *uidA* (GUS) reporter gene in a binary transformation vector and transformed into *A. thaliana* plants. When the transgenic *A. thaliana* T₂ homozygous plants were analyzed histochemically, no GUS activity was detected in 5 days after flowering (DAF) siliques and vegetative tissues (stem, leaves), and floral buds (Fig. 4a, i, j). However, the GUS activity was detected at seven DAF embryos and its activity persisted throughout subsequent embryo development (Fig. 4b, c). GUS staining was also

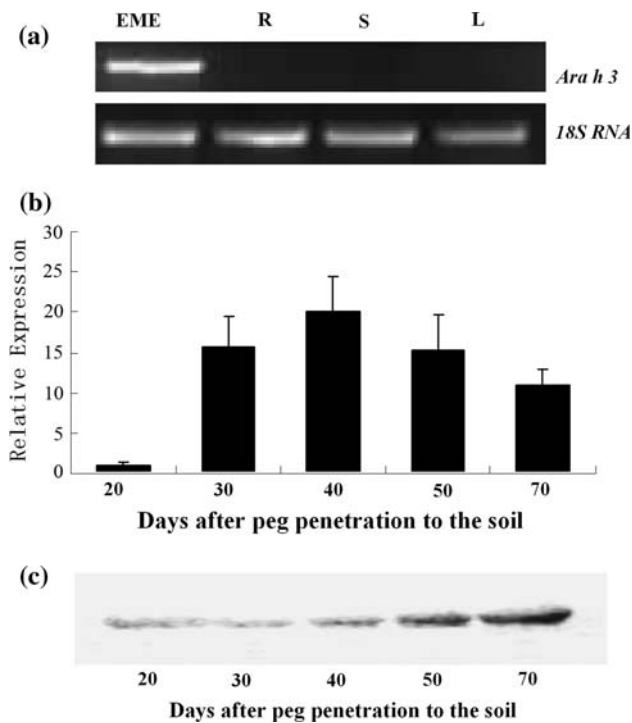


Fig. 3 Expression analysis of *Ara h 3* in developing peanut seeds and vegetative tissues. **a** Semi-quantitative PCR analysis of *Ara h 3* expression in 20 DAPPS seeds (*DS*), roots (*R*), stems (*S*), and leaves (*L*). **b** Real-time PCR analysis of the *Ara h 3* expression in developing peanut seeds at 20, 30, 40, 50, and 70 DAPPS. **c** Western blot analysis of the *Ara h 3* protein. Salt-soluble protein (60 μ g protein) was extracted from developing peanut cotyledons, fractionated by SDS-PAGE, electroblotted onto a nitrocellulose membrane and immunochemically detected using anti-*Ara h 3* acidic subunit antibodies

observed in cotyledons and hypocotyls of young seedlings, but not in young roots (Fig. 4d–h). Five independent lines of transgenic plants were stained and all plants showed similar GUS expression patterns.

Transgenic *Arabidopsis* plants containing the GUS reporter gene under the control of *CaMV35S* promoter (*35S: GUS*) or *Ara h 3* promoter (*Ara h 3: GUS*) were analyzed quantitatively by fluorescence assay (Fig. 5). The T_3 homozygous mature dry seeds of five independent transgenic lines for each construct were used in this experiment. In comparison with those expressing GUS driven by 35S promoter, transgenic *Arabidopsis* plants expressing GUS driven by *Ara h 3* promoter showed considerably higher GUS activity in the mature seeds. The mean GUS activity of *Ara h 3: GUS* is approximately sixfold that of *35S: GUS*, indicating that the *Ara h 3* promoter is a strong promoter.

Histone modification status of *Ara h 3* in peanut late maturation embryos

ChIP was used to analyze whether embryo-specific expression of *Ara h 3* is accompanied by an increase in the acety-

lation state of histones at the promoter. DNA and proteins were cross-linked with formaldehyde and chromatin were immunoprecipitated with antibodies against acetylated histones. The co-precipitated DNA was isolated and quantified by real-time PCR. A pair of *Ara h 3* promoter-specific primers was used to analyze *Ara h 3* promoter histone modifications (Fig. 6a). The targeted sequence for amplification (from –225 to –2 bp) includes the TATA box, the legumin-box, and the G-box, which are essential for seed-specific expression of storage protein (see Fig. 2b). The region that spans the nucleotide sequence from –225 to –2 bp is defined as the proximal promoter.

Histone H3 and H4 acetylation levels of *Ara h 3* chromatin in different organs were examined. *Ara h 3* was not expressed in roots, stems, or leaves (Fig. 3a), while the transcriptional activity of *Ara h 3* reached a very high level in the late maturation embryos (LME) harvested at 50 DAPPS (Fig. 3b). Compared with roots, stems, and leaves, the ChIP signal for acetylated histone H3 decreased dramatically in late maturation embryos (Fig. 6b). Similarly, a decrease in acetylated histone H4 was also observed (Fig. 6c). To test whether the observed decrease in histone H3 and H4 acetylation levels was associated with a general loss of covalent modifications from the promoter nucleosomes, we extended our analysis to trimethylation of histone H3K4 of *Ara h 3*. A decrease of the ChIP signals for histone H3K4me3 at *Ara h 3* core promoter was observed (Fig. 6d), suggesting a loss in histone covalent modifications from the remodeled promoter nucleosomes (Fig. 6).

Histones were lost from the fully activated *Ara h 3* core promoter

Based on the results shown in Fig. 6, we subsequently employed ChIP, using antibodies against the C terminus of histone H3, to test whether the apparent decrease in histone modification of activated *Ara h 3* was due to histone loss. The core promoter of *Ara h 3* is enriched in the H3 antibody immunoprecipitates from vegetative tissues but is largely depleted in the immunoprecipitates from the late maturation embryos (Fig. 7). This finding suggests that histone H3 in this region has decreased interaction with DNA in late maturation embryos but are reassembled in vegetative tissues. As a control, the histone H3 occupancy of 18S rRNA and a root-specific gene, *AhRb7*, was also analyzed. No significant difference was observed in histone H3 occupancy of 18S rRNA and *AhRb7* in roots, stems, leaves, early maturation embryos, and/or late maturation embryos (data not shown). These observations suggest that *Ara h 3* chromatin was devoid of nucleosomes when *Ara h 3* was highly expressed in embryos.

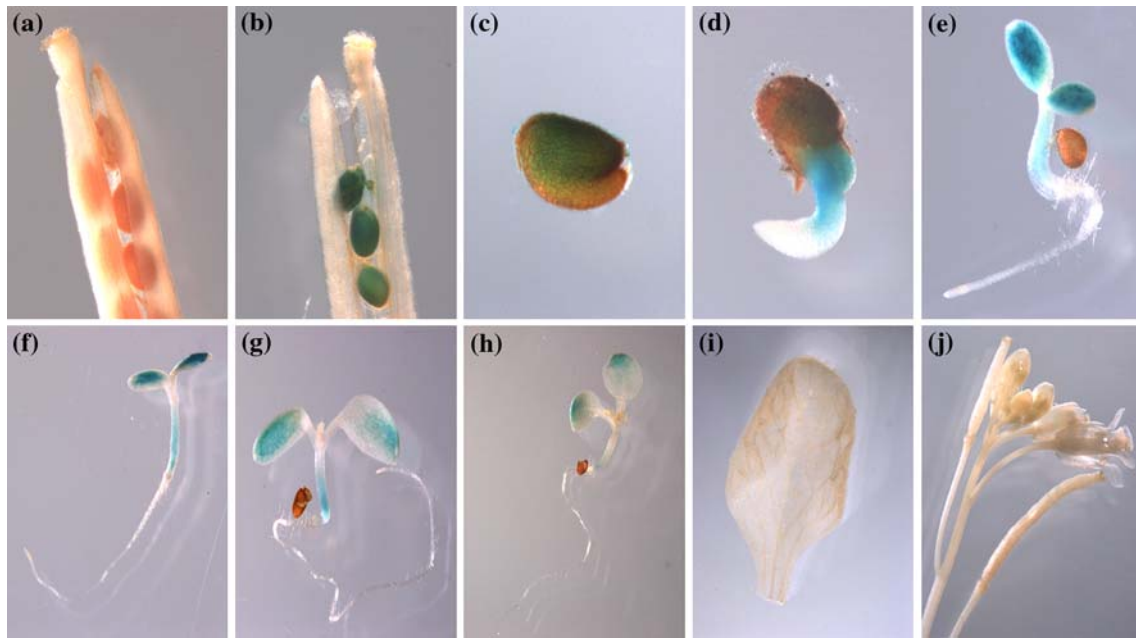


Fig. 4 Activity of the *Ara h 3* promoter in *Ara h 3::GUS Arabidopsis* plants. **a** Five-day-old siliques. **b** Seven-day-old siliques. **c** The mature seed. **d–h** Seedling germinated after 2, 4, 6, 12 and 18 days. **i** Mature leaf. **j** Stems and flowers

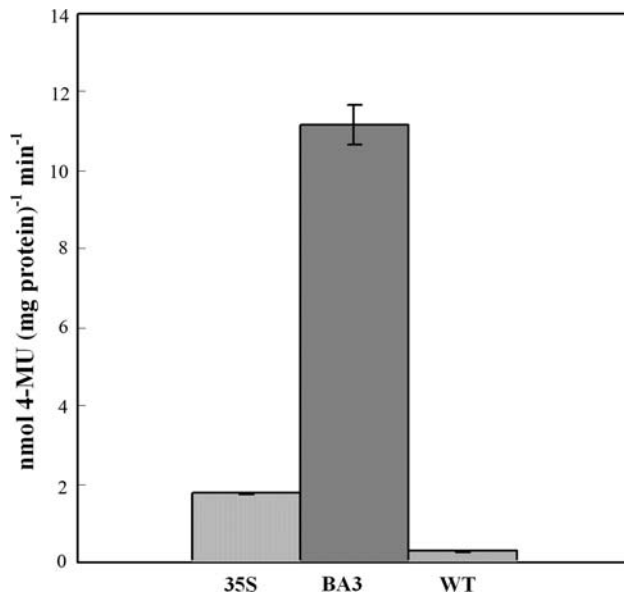


Fig. 5 GUS activity in seeds from *CaMV 35S::GUS*, *Ara h 3::GUS* and wild-type plants. Average GUS activity was measured by fluorometric assay. 35S, *CaMV 35S::GUS*; BA3, *Ara h 3::GUS*; WT, wild type. Five independent transgenic lines were tested for each construct. Error bars are standard deviations representing mean GUS specific activities ($\pm$ SE) of three replicate samples

Histones at *Ara h 3* were acetylated in the early maturation embryos

The loss of the histone H3 ChIP signal from the *Ara h 3* proximal promoter in the late maturation embryos made

it difficult to analyze possible changes in histone acetylation during *Ara h 3* activation. Compared with the late-maturation embryos (see Fig. 3b), we found that the expression of *Ara h 3* was very low in the early maturation embryos (EME) harvested at 20 DAPPS. We therefore examined whether histone H3 occupancy of embryos at the early stage of maturation was lower than that of the late stage. As shown in Fig. 8a, histone H3 occupancy in the *Ara h 3* proximal promoter was only reduced slightly in the embryos in the early stage of maturation. In contrast, there was an eightfold decrease of H3 occupancy of *Ara h 3* core promoter in embryos of the late maturation stage (see Fig. 7). Therefore, loss of histone H3 from *Ara h 3* core promoter in the embryos at early maturation stage was significantly lower than that of the late maturation stage. Measuring the histone acetylation level in embryos at early maturation stage could be useful to analyze acetylation levels without the ChIP signal being dominated by simultaneous histones loss.

As shown in Fig. 8b, acetylation levels of histone H3 at the *Ara h 3* proximal promoter increased about two-fold in the early maturation embryos. Acetylation levels normalized with respect to histone occupancy show unambiguously that acetylation of histone H3 at *Ara h 3* core promoter have increased more than twofold in the embryos at the early maturation stage (Fig. 8c). Therefore, histone H3 at the *Ara h 3* proximal promoter became increasingly acetylated in embryos at the early maturation stage before histone loss.

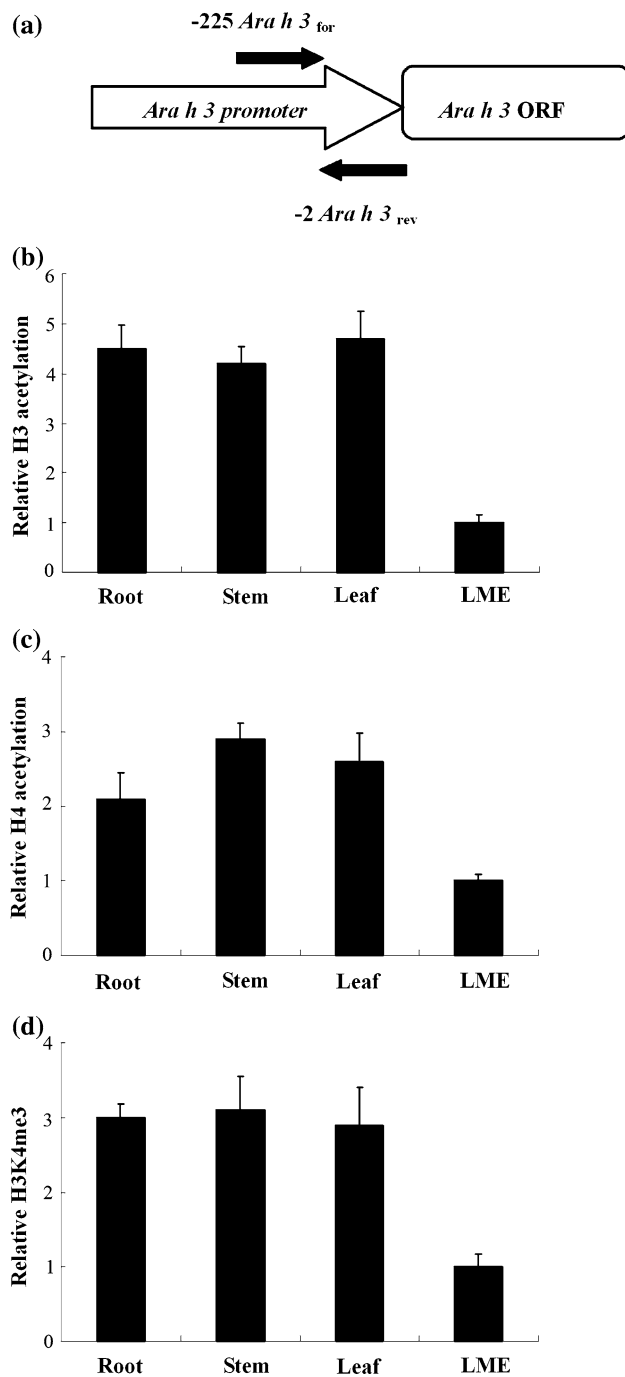


Fig. 6 Histone modification status of *Ara h 3* core promoter in roots, stems, leaves and late maturation embryos (LME). **a** Positions of the primer pairs used for ChIP analysis of the *Ara h 3* promoter are shown. **b–d** Histone modification status at the core promoter of *Ara h 3* promoter. Chromatins were precipitated with an antibody specific for acetylated histone H3, hyperacetylated histone H4 and trimethylation histone H3K4. The amounts of co-immunoprecipitated DNA were determined by quantitative PCR. 18s rRNA was used as an internal control. The value of the late maturation embryos was arbitrarily given as 1. Data are the average of three biological replicates ($\pm$ SE)

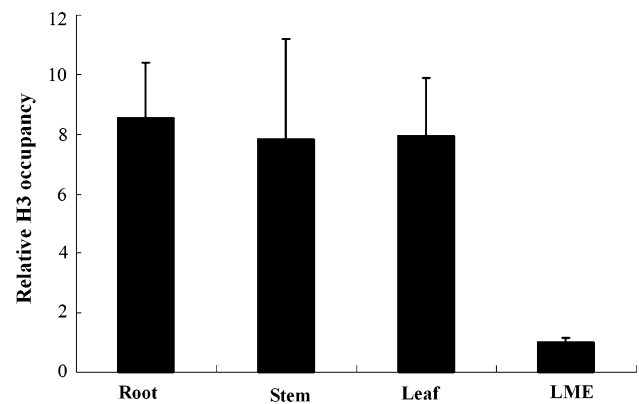


Fig. 7 Histone occupancy in the *Ara h 3* core promoter in peanut late maturation embryos. Chromatins were precipitated with an antibody specific for C terminus histone H3. The amounts of co-immunoprecipitated DNA was determined by quantitative PCR. 18s rRNA was used as an internal control. The value of the late maturation embryos (LME) was arbitrarily given as 1. Data are the average of three biological replicates ($\pm$ SE)

Dimethylation histone H3-K9 levels of *Ara h 3* are higher in vegetative tissues

H3-K9 dimethylation is a known critical mark for DNA methylation and long-term gene silencing (Jackson et al. 2004). Dimethylation of histone H3-K9 is 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 silenced expression of *Ara h 3* in vegetative tissues is related to dimethylation of histone H3-K9, ChIP analysis was performed using the antibody against dimethylated H3-K9 in the early maturation embryos and vegetative tissues. The levels of dimethylated H3-K9 in the *Ara h 3* proximal promoter were lower in the early maturation embryos than those in vegetative tissues (Fig. 9a, b). These data indicated that dimethylation histone H3-K9 levels of *Ara h 3* were lower in the early maturation embryos than those in the vegetative tissues.

Discussion

The expression patterns of the peanut allergen genes during seed development are cultivar-dependent. In particular, *Ara h 3* expression patterns among the cultivars are more variable than those of *Ara h 1* and *Ara h 2* (Kang et al. 2007). These variations may be due to multiple *Ara h 3* isoforms that are differentially expressed. The first genomic sequence of *Ara h 3* was identified by Viquez et al. (2004). Here we described the cloning and characterization of a new *Ara h 3*

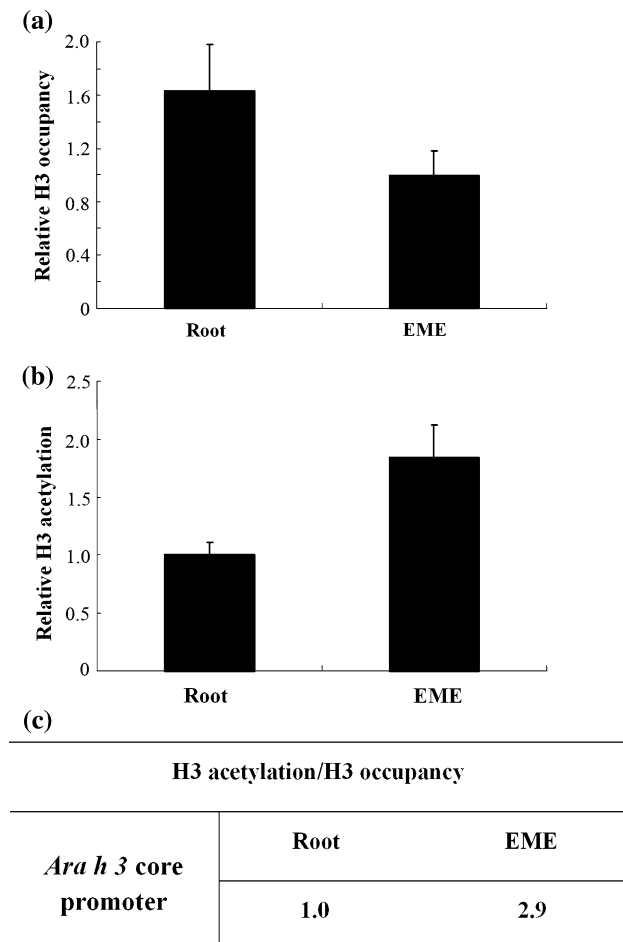


Fig. 8 Histone H3 occupancy and acetylation level at the *Ara h 3* core promoter in the early maturation embryos (EME). **a** The levels of histone H3 were analyzed by ChIP in roots, stems, leaves, and the early maturation embryos. **b** The levels of histone H3 acetylation were analyzed by ChIP in roots, stems, leaves, and the early maturation embryos. **c** H3 acetylation levels normalized with histone occupancy were listed. They were calculated by dividing the values for acetylated H3 by the H3 occupancy values. 18s rRNA was used as an internal control. Data are the average of three biological replicates ($\pm$ SE)

isoform. RT-PCR and promoter activity analyses revealed exclusive expression of *Ara h 3* in seed tissues. Protein accumulation of *Ara h 3* during seed development was similar to transcript accumulation, indicating that this gene was most likely under transcriptional control. In transgenic *Ara-bidopsis*, a 643-bp *Ara h 3* promoter fragment could drive high levels of GUS expression in an embryo-specific manner. Strong GUS activities were detected at 7 DAF in the embryos and subsequent embryo development stages. In addition, we demonstrated that the strength of *Ara h 3* promoter exceeded that of the CaMV 35S promoter with respect to GUS activity (about sixfold) in seeds. Strong seed-specific activity of the *Ara h 3* promoter could be useful for transgene-mediated gene expression during seed development, especially for knocking out peanut allergen in

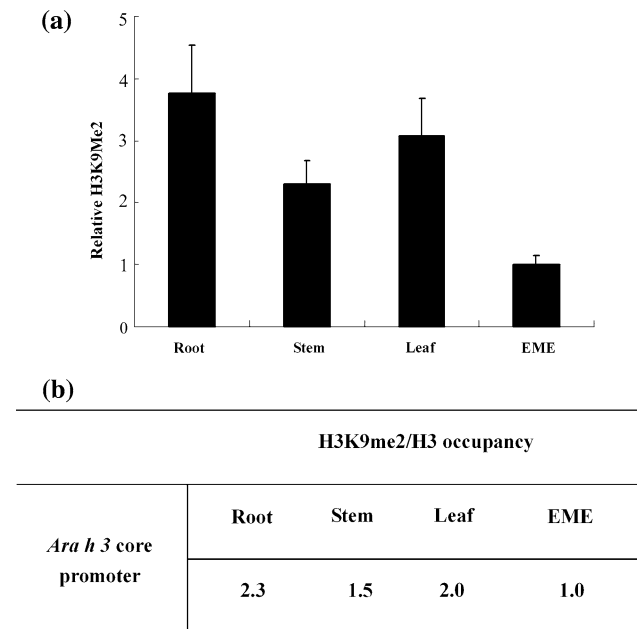


Fig. 9 Histone H3K9 is dimethylated at the *Ara h 3* core promoter in the early maturation embryos (EME). **a** Histone H3K9 dimethylation levels were determined by ChIP in roots, stems, leaves, and the early maturation embryos. **b** H3K9 dimethylation levels normalized with respect to histone occupancy were listed. They were calculated through dividing the values for dimethylated H3K9 by the H3 occupancy values. The value of the early maturation embryos was arbitrarily given as 1. Data are the average of three biological replicates ($\pm$ SE)

the seeds. For example, *Ara h 3* promoter-induced expression of a hypoallergenic ortholog, such as a hypoallergenic ortholog of *Ara h 3-im* (Kang and Gallo 2007) or *Ara h 2.01* (Ramos et al. 2009), could be used to produce hypoallergenic or reduced allergenicity peanut.

Chromatin disassembly is involved in eukaryotic gene transcriptional activation (Bernstein et al. 2004; Lee et al. 2004; Barrera and Ren 2006; Williams and Tyler 2007). In yeast (*Saccharomyces cerevisiae*), it has been observed that in several genes, such as *PHO5*, *GAL*, and *HSP82*, histones are removed upon gene activation and nucleosomes are reassembled when genes are turned off (Boeger et al. 2003; Reinke and Horz 2003; Kristjuhan and Svejstrup 2004; Zhao et al. 2005). Ng et al. (2006) demonstrated that the ABI3-like factor (ALF) mediated *phaseolin* gene expression is associated with nucleosome remodeling. However, direct evidence supporting the involvement of histone loss in the regulation of plant gene expression is still lacking. In this study, we found that *Ara h 3* promoter underwent significant changes associated with the eviction of nucleosomes upon seed development. This eviction is accompanied with transcriptional activation, suggesting that full activation of *Ara h 3* in the late maturation embryos is associated with histone H3 loss at the proximal promoter and histone H3 occupancy is related to the repression of *Ara h 3* in vegetative tissues.

Previous work in yeast has shown that there is a mild transient histone acetylation prior to the major nucleosome displacement at *PHO5* (Reinke and Horz 2003). However, at the *HSP82* promoter, transient increases of histone acetylation do not appear to be required for histone displacement (Erkina and Erkinen 2006). Within the *Ara h 3* promoter, we observed an approximately twofold increase in the abundance of acetylated forms of histones preceding major nucleosome displacement (Fig. 8b), suggesting that histone H3 acetylation may be a required step for chromatin remodeling for *Ara h 3*. Unlike histone acetylation which in general correlates with transcriptional activation, histone methylation can result in either transcription activation or repression, depending on the modified residue within histone proteins (Lachner and Jenuwein 2002; Tariq and Paszkowski 2004). Our results suggested that *Ara h 3* chromatin became dimethylated at histone H3-K9 in vegetative tissues in which *Ara h 3* was not expressed, suggesting that dimethylation at histone H3-K9 is associated with *Ara h 3* repression. In ALF-mediated *phaseolin* expression system, activation of the *phaseolin* promoter was associated with an increase of histone H3 acetylation (Ng et al. 2006). However, in *Ara h 3* promoter, histone H3 acetylation level is highest in early developing seed and decreased as the seed matured. In addition, we observed that the *Ara h 3* chromatin became dimethylated at histone H3-K9 in vegetative tissues.

In conclusion, we have characterized the genomic sequence and expression of an *Ara h 3* isoform during seed development. We have demonstrated that the *Ara h 3* promoter can efficiently direct the expression of the GUS reporter gene in developing *Arabidopsis* seeds. In addition, the seed-specific expression of *Ara h 3* is associated with targeted epigenetic changes, including histone modifications and occupancy.

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