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# Histone H4 acetylation patterns during seed germination and early plant development

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## Abstract

Here we studied whether early development of *Silene latifolia* is accompanied with changes in acetylation of nucleosomal histones H4. Using acid-urea-triton polyacrylamide gel and immunoblotting with specific antisera the histone acetylation in relationship with transcriptional activity, measured by [$^{14}$C]-uridine incorporation, was analysed in dry and germinating seeds, seedlings, and adult leaves. We show that quiescent and germinating seeds, until the root tip is released from testa, are characterised by an absence of transcriptional activity and by a low H4 acetylation level: only mono-acetylated isoforms were present. During the postgermination period and early plantlet development, a high increase of transcriptional activity started and a sharp H4 hyperacetylation, up to the penta-acetylated isoform, was detected. We conclude that epigenetic modification by nucleosomal histone deacetylation plays a role in maintenance of global genome silencing in quiescent seeds.

*Additional key words*: epigenetic modification, *Silene latifolia*, transcription, white campion.

## Introduction

Chemical modifications of DNA and histones influence chromatin properties, including its transcription competence (Vermaak and Wolffe 1998). The role of cytosine methylation in the control of gene expression has been established (Adams 1996). Histones undergo numerous post-translational modifications: acetylation, phosphorylation, methylation, poly-ADP ribosylation, and ubiquitinilation. The subject for acetylation are ε-amino groups of lysines in the N-terminal domain of all core histones. In total there are at least 26 possibly acetylated lysines in histone octamer with H2A, H2B, H3, and H4. A possible link between acetylation and transcriptional activity of genes was first mentioned by Allfrey *et al.* (1964) and later supported by numerous experiments (reviewed by Mizzen and Allis 1998). The introduction of chromatin immunoprecipitation technique allowed to study this relationship on the level of individual genes and their promoters (Chua *et al.* 2001). Acetylation is regulated by two groups of enzymes – histone acetyltransferases (HATs) and histone deacetylases (HDACs). Many transcriptional activators carry HAT activity while corepressors possess HDAC activity, confirming the importance of acetylation in transcription (Spencer and Davie 1999).

In higher plants histones H3, followed by H4, are acetylated most frequently. Quantitative analysis showed an inverse relationship between the plant genome size and the relative abundance of acetylation of these two kinds of histones. While in animal species only four N-terminal lysines in histone H4 (5, 8, 12, and 16) can undergo acetylation, in plants also lysine 20 is acetylatable (Waterborg 1992). Several types of HATs and HDACs have been isolated and characterised in maize (Lusser *et al.* 2001). Various developmental abnormalities appeared when histone deacetylation had been blocked in *Arabidopsis* (Tian and Chen 2001). Immunostaining of metaphase chromosomes has shown hyperacetylation of H4 in a nucleolar organizing region,

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*Abbreviations*: AUT gel - acid-urea-triton polyacrylamide gel; H4 - histone H4; K - lysine; PMSF - phenylmethylsulfonyl fluoride.

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while late replicating heterochromatin was under-acetylated (Houben *et al.* 1996). The colocalisation of histone H4 hypoacetylation with cytological hetero-chromatin in interphase nuclei of *Gagea* (Buzek *et al.* 1998) and *Rumex* (Lengerová and Vyskot 2001), and hyperacetylation of subtelomeric gene rich regions in metaphase chromosomes of *Silene* (Vyskot *et al.* 1999) have been also demonstrated. The quiescent vegetative nucleus in mature pollen compared to its activated status in pollen tubes of *Lilium* displayed a strong H4 underacetylation (Janoušek *et al.* 2000).

Plants survive and spread in space using alternative ways, such as reproductive particles – seed and pollen grains, independent of the mother plant. Shortly after fertilisation and embryo formation, the development is interrupted and the embryo within the seed enters a quiescent stage, characterised by desiccation and restriction of metabolic activity. After imbibition, the germination begins and metabolic activity is rapidly restored. As overall changes in transcriptional activity during early plant development are supported by changes in chromatin structure – from largely condensed chromatin in dry embryo to dispersed chromatin structure in germinating seed (Sargent and Osborne 1980), involvement of modifications like histone acetylation can be expected. Here we present Western blot analyses demonstrating H4 acetylation dynamics during seed germination in relationship with transcriptional activity.

## Materials and methods

**Plants and cultivation:** Seeds of *Silene latifolia* Poiret from the plant collection of the Institute of Biophysics, Brno, were sterilised in 1 % (m/v) $Na_3ClO$ and germinated in water under dim light and constant temperature (25 °C). Seedlings of equal developmental stages after 0.5, 1, 1.5, 2, 2.5, 3, and 6 d of imbibition were analysed. Seed germination, defined as the period till the radicle protrudes from the seed testa (Bewley 1997), lasts about one day in *S. latifolia*. Cotyledons become visible at the end of the second day, seedlings are green and completely released from the testa after three days of imbibition.

**Transcriptional activity:** To estimate transcriptional activity according to Ferrari and Widholm (1973), seeds (100 per sample) were germinated in Petri dishes in water. Twelve hours before the harvest, uridine (10 μM) and immediately afterwards [$^{14}$C]-uridine (37 kBq) were added. During harvesting, water with unincorporated isotope was removed and seedlings were washed twice with 5 $cm^3$ of non-radioactive uridine solution (1 mM). After gentle drying with filter paper, the seedlings were homogenised in liquid nitrogen, transferred to glass funnel with 0.45 μm membrane filter (*Schleicher & Schuell*, Dassel, Germany) and three times washed with 5 $cm^3$ of methanol:chloroform:water (12:5:3, v/v/v) under vacuum. The washed filters with homogenates were placed into scintillation vials and air dried. 5 $cm^3$ of scintillation liquid was added before measuring radioactivity (*Wallac 1410, Pharmacia*, Uppsala, Sweden). Each measurement was done three times. To determinate the non-specific isotope binding to the plant surface, control samples to which radioactive uridine was added directly before harvesting were included.

**Histone extraction:** To extract histones, plant material was homogenised in liquid nitrogen, transferred to extraction buffer (1 M sucrose, 25 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 7 mM β-mercaptoethanol, 0.5 mM PMSF, 10 mM sodium butyrate) and filtered twice through a mesh (50 μm). After centrifugation (5 000 *g* for 10 min) the pellet was twice washed and centrifugated (5000 *g* for 10 min) in extraction buffer plus 0.2 % (v/v) Triton X-100 to lyse mitochondria and chloroplasts. The nuclei were separated by centrifugation (7 000 *g* for 5 min) on a Percoll (*Sigma*, Steinheim, Germany) gradient (mixture of Percoll:extraction buffer with Triton, 9:1.2 m/m), washed (0.25 M sucrose, 10 mM Tris-HCl, pH 7.3, 3 mM $CaCl_2$, 1 mM $MgCl_2$, 1 mM PMSF, 10 mM sodium butyrate) and centrifugated (5 000 *g* for 5 min). Histones were extracted for 2 h in 0.4 M HCl, then 20 times concentrated by lyophilisation, and stored at -70 °C until use. As a marker, histones of HL-60 cells were extracted in buffer containing 10 mM Tris HCl, 0.5 mM EDTA, 0.1 mM PMSF and 0.4 % (v/v) Triton X-100, and in 0.4 M HCl similarly as the plant histones.

**Acid-urea-triton polyacrylamide gels and Western blotting:** Histones were separated on AUT gels (Bonner *et al.* 1980), composed of 8 M urea, 6 % (v/v) glacial acetic acid, 29 mM ammonia, 12 % (m/v) acrylamide, 0.32 % (m/v) bis-acrylamide, and 1.6 % (v/v) Triton X-100. Histone concentration was measured by Bradford (1976) assay and histones were loaded in a buffer (8 M urea, 6 % v/v glacial acetic acid, 35 mM β-mercapto-ethanol, 0.04 % m/v methylene blue) and run for 30 min under 100 V and afterwards for about 5 h under 250 V. To ensure the visualisation of acetylated isoforms, the three times higher amount of proteins (60 μg) were loaded per lane on AUT gel as is usual for SDS-PAGE, because each histone variant is resolved to particular isoforms and usually more than 50 % of the total plant histone H4 occurs in the non-acetylated isoform (Waterborg 1992). AUT gels were stained with Coomassie brilliant blue R-250 or, after equilibration in

0.7 % (v/v) acetic acid (for 1 h), transferred to a *Hybond-C Extra* membrane (*Amersham*, Little Chalfont, England) using *Trans-Blot Cell* apparatus (*Bio-Rad* Laboratories, Hercules, CA) and 0.7 % (v/v) acetic acid as a running buffer. The transfer efficiency was checked with *Ponceau S* (*Sigma*) staining. The acetylated isoforms were detected using rabbit polyclonal antisera R41, R232, and R252, raised against H4 N-terminal synthetic oligopeptides acetylated at lysines 5, 8, and 16, respectively (Turner and Fellows 1989). The specificity of antisera for acetylated isoforms was checked by comparison with the commercial antibody recognising non-acetylated H4 (*Upstate Biotechnology*, Lake Placid, USA). Immunocomplexes were visualised using peroxidase-conjugated goat anti-rabbit antibody (*Sigma*) and enhanced chemiluminiscence (ECL kit; *Amersham*) on X-ray film (*Eastman Kodak*, Rochester, USA).

## Results

**Transcriptional activity during germination and postgermination:** RNA synthesis during germination (0.5 and 1 day of imbibition) and postgermination (seedlings 1.5, 2, 2.5 and 3 d of imbibition) was estimated from incorporation of radioactive uridine. The radioactivity within control samples was negligible for all the investigated stages. During germination, the levels of incorporated uridine were nearly as low as in controls. However, during postgermination period the incorporation of radioactivity increased rapidly and reached the maximum plateau in 2.5-d-old seedlings (Fig. 1).

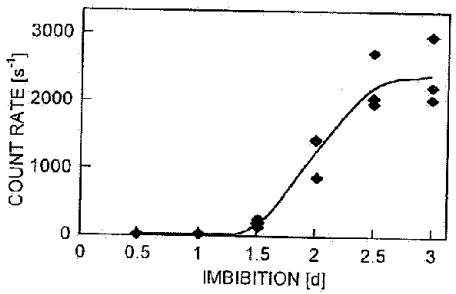


Fig. 1. [$^{14}$C]-uridine incorporation into germinating seeds and seedlings during water imbibition. The scintillation counting (counts per second) was done three times for each stage (*rhombs*), the values for each stage were averaged and are represented by the curve. The seed germination itself lasted approximately 1 day; afterwards the radicle and later cotyledons appeared (postgermination).

**Western analysis of *S. latifolia* histones:** As there are no antisera available to detect histone H4 regardless of its acetylation status, we gained information about a distribution of particular non-acetylated and acetylated isoforms only from Coomassie brilliant blue R-250 staining of AUT gels. In all *S. latifolia* samples we were able to visualise only one isoform (Fig. 2, *lane* 6), which was shifted in comparison with the three isoforms visualised in the marker histones of HL-60 (Fig. 2, *lane* 5). The shift between non-acetylated isoforms reveals a different mobility of mammalian and *S. latifolia* H4 histones in AUT gels. To identify particular H4 isoforms we used the antibody recognising only the non-acetylated histone H4 in both plant (Fig. 2, *lane* 7) and HL-60 samples (Fig. 2, *lane* 4). The identification of these non-acetylated isoforms gave the baseline from which the other isoforms were detected. After Coomassie brilliant blue R-250 staining we visualised abundant non-acetylated and mono-acetylated isoforms in HL-60, but only the non-acetylated isoform in *S. latifolia*. As this staining appeared to be insufficient for analysis of H4 acetylation in the studied plant samples, acetylated isoforms were detected by immunoreactions with antisera against particular acetylated lysines. Specificities of antisera R41 (K5), R232 (K8) and R252 (K16) were checked first for HL-60 samples (Fig. 2, *lanes* 1 - 3). The antisera R41 and R252 recognised all possible (from mono- to tetra-) acetylated H4 isoforms, while the antiserum R232 visualised only mono-, di- and tri-acetylated isoforms. A sensitivity of R232 is affected by its inability to react with H4 already acetylated at lysine 5 (Turner *et al.* 1992). The specificity of antisera was checked also for leaf samples (Fig. 2, *lane* 8, shown for the antiserum against acetylated lysine 5).

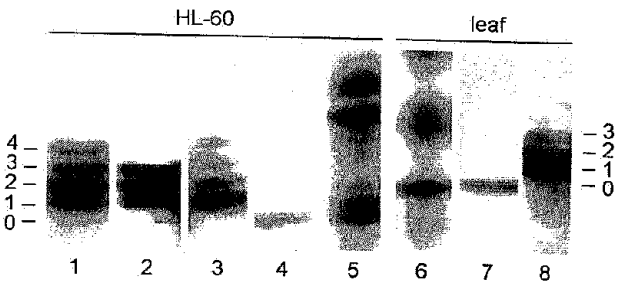


Fig. 2. Specificity of antisera against acetylated K5 (R41), K8 (R232), and K16 (R252) H4 isoforms was checked for histones extracted from HL-60 cells (*lanes* 1 - 3, respectively) and from leaves of mature *S. latifolia* plant (*lane* 8, only R41 shown). Different electrophoretic mobility of HL-60 and plant histones was revealed after Coomassie brilliant blue R-250 staining (*lanes* 5, 6) and after immunodetection with the antiserum which detects only the non-acetylated H4 isoform (*lanes* 4, 7). The histone isoforms are indicated along the side.

**Dynamics of H4 acetylation:** Acetylated H4 isoforms were visualised by immunodetection in dry seeds, 1-d,

3-d and 6-d-old seedlings and in leaves from mature plants. Similar patterns of acetylation were detected in each stage with all antisera used. Only mono-acetylated isoforms of H4 were found in the dry seeds. The acetylation occurred at all lysines studied – K5, 8, and 16 (Fig. 3*A*). Taken this result together with Coomassie brilliant blue R-250 staining, we conclude that in dry seeds H4 histones are non-acetylated (highly prevalent) or mono-acetylated at lysine 5, 8 or 16 (the least frequent). The same distribution of H4 isoforms also appeared in 1-d-old seedlings (Fig. 3*B*). This indicates that during the 24 h of germination no changes in the level of H4 acetylation occur. However, the level of acetylation increased sharply during the following developmental stages. In 3-d-old seedlings all possible acetylated isoforms were found and, moreover, the acetylation was shifted towards tetra- and penta-acetylated isoforms (Fig. 3*C*). From the detection of penta-acetylated isoform it is obvious that besides lysines 5, 8, 12 and 16, also lysine 20 is acetylated. The hyperacetylated isoforms became rather rare in the later stages. Abundant signals of mono-, di- and tri-acetylated isoforms, but faint signals of tetra- and penta-acetylated isoforms were detected in 6-d-old seedlings (Fig. 3*D*). Histones H4 from mature leaves exhibited acetylation up to three terminal lysines (Fig. 3*E*).

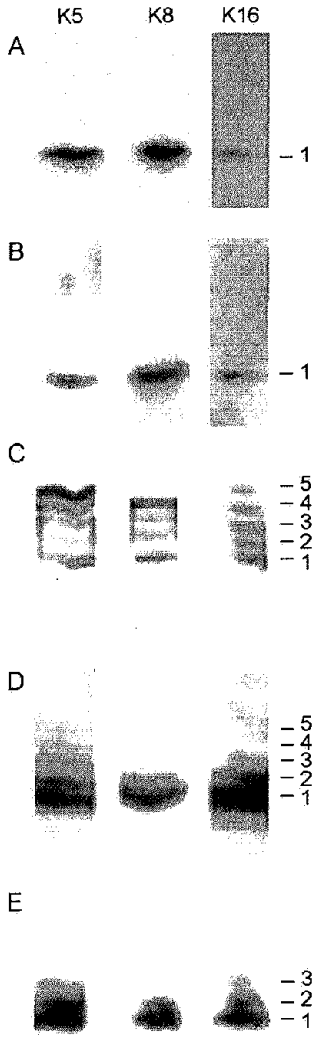


Fig. 3. Acetylated isoforms of histone H4, extracted from dry seeds (*A*), 1-d-old (*B*), 3-d-old (*C*), and 6-d-old (*D*) seedlings and leaves from mature plant *S. latifolia* (*E*) were detected by the specific antisera against acetylated K5, 8 and 16, and visualised by enhanced chemiluminiscence.

## Discussion

The data presented show that during the seed germination in *S. latifolia* transcriptional activity does not proceed. The same is true for cell division and DNA replication (Žlůvová *et al.* 2001). During the subsequent postgerminative growth a sharp increase of both transcriptional and DNA replication activities occurs. These facts strongly support the conclusion that the process of seed germination, which is characterised by uptake of water, respiration activity, and protein synthesis, exclusively relies on mRNA and rRNA already present in the quiescent seed. Similar data have been reported by Arcila and Mohapatra (1992) for tobacco. They demonstrated that the radicle emergence was regulated by translation but independent of replication and transcription, and the translation was mediated by preformed mRNAs.

The fact that neither replication nor transcription are involved in the germination gains further support by DNA methylation analysis in *S. latifolia*. Žlůvová *et al.* (2001) have observed a rapid decrease in global DNA methylation during seed germination, first in endosperm tissue and subsequently in hypocotyl. These demetylation events were observed before cell division indicating that they take place in a non-replicative way. The acetylation of nucleosomal histones represents another epigenetic mechanism with a role in transcriptional control, maintenance of gene expression patterns, and cell memory (for a review, see Turner 2000). Our experimental data demonstrating that only a minor part of histones H4 is subject of acetylation corresponds to the results obtained by Waterborg (1992). In plants with a small genome size, such as *Arabidopsis thaliana*

(0.7 pg/4C), about 53 % of total histones H4 are acetylated, while in species with large genomes, such as *Nicotiana tabacum* (23.4 pg/4C), only 12 %. In *Medicago sativa* (7.0 pg/4C), with a genome size comparable to *S. latifolia* (10.8 pg/4C), 34 % of H4 were acetylated (Waterborg 1992). Our western blotting analysis revealed that in the postgerminative stage the level of acetylation rapidly increased as compared to dry and germinating seeds where only mono-acetylated isoforms were detected. Histone H4 isoforms with all N-terminal lysines acetylated were detected in 3-d-old seedlings. This acetylation also included lysine 20 which undergoes acetylation only in plant species including algae (Waterborg 1992). In 6-d-old seedlings the acetylation pattern decreased almost to the level of mature leaf tissues.

The presence of hypoacetylated H4 in inactive seeds and of hyperacetylated H4 in young seedlings indicates an association between transcription (and probably also DNA replication) and histone acetylation. However, we cannot exclude that the altered H4 acetylation is also influenced by other processes such as acetylation of newly synthesised histones. Dynamic activities of histone acetyltransferases and deacetylases were described during seed germination in maize where a correlation between histone acetyltransferase B (HAT B) and DNA replication was also found. HAT B specifically acetylates lysines 5 and 12 of histone H4 before assembling into new nucleosomes, while other acetyltransferase specific for H3 is connected rather with transcriptional activity. The total histone acetyltransferase activity increased with two maxima at 40 and 72 h after the start of germination (Georgieva *et al.* 1991). Taken together, we conclude that the genomes in dry seeds, similarly as quiescent vegetative nucleus in pollen grains (Janoušek *et al.* 2000), are subject to global epigenetic modifications (histone H4 underacetylation and DNA hypermethylation) to maintain their inactive status and to prevent their premature transcription.

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