Plant Mol Biol (2006) 62:503–512
DOI 10.1007/s11103-006-9036-8

Histone H3 variants in male gametic cells of lily and H3 methylation in mature pollen

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Received: 22 December 2005 / Accepted: 21 June 2006 / Published online: 17 August 2006

Abstract Histones are vital structural proteins of chromatin that influence its dynamics and function. The tissue-specific expression of histone variants has been shown to regulate the expression of specific genes and genomic stability in animal systems. Here we report on the characterization of five histone H3 variants expressed in *Lilium* generative cell. The gcH3 and leH3 variants show unique sequence diversity by lacking a conserved lysine residue at position 9 (H3K9). The gH3 shares conserved structural features with centromeric H3 of *Arabidopsis.* The *gH3* variant gene is strongly expressed in generative cells and gH3 histone is incorporated in to generative cell chromatin. The lysine residue of H3 at position 4 (H3K4) is highly methylated in the nuclei of generative cells of mature pollen, while methylation of H3K4 is low in vegetative cell nuclei. Taken together, these results suggest that male gametic cells of *Lilium* have unique chromatin state and histone H3 variants and their methylation might be involved in gene regulation of male gametic cells.

Keywords Generative cell · Histone H3 · *Lilium longiflorum* · Male gamete · Histone variants · Centromeric histone H3

Abbreviations

CenH3	centromeric histone H3
EST	expressed sequence tag
GC	generative cell
gcH3	a generative cell histone variant reported by Xu et al. (1999a)
gH3	a generative cell histone variant reported by Ueda et al. (2000)
H3K4	Lys 4 residue of histone H3
$H3K4^{Me2}$	H3K4 dimethylation
H3K9	Lys 9 residue of histone H3
$H3K9^{Me2}$	H3K9 dimethylation
DAPI	4',6-diamidino-2-phenylindole

Accession numbers for the sequence dataThe sequences reported in this paper have been deposited in the DDBJ database gcH3 GC1174 (accession no. AB195644), gH3 GC1008 (accession no. AB195646), leH3 GC1126 (accession no. AB195648), soH3-1 GC0075 (accession no. AB195650), soH3-2 GC1661 (accession no. AB195652), genomic sequence of gcH3 (accession no. AB195645), genomic sequence of gH3 (accession no. AB195647), genomic sequence of leH3 (accession no. AB195649), genomic sequence of soH3-2 (accession no. AB195651), genomic sequence of soH3-2 (accession no. AB195653).

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Introduction

The basic repeating unit of chromatin is the nucleosome, which consists of 146 bp of DNA wrapped around an octameric unit of the core histones H2A, H2B, H3, and H4. In addition, a fifth histone (H1) or

linker histone connects nucleosomal cores, and directs the assembly of higher-order structure in the nucleosomal array. Diversity in the nucleosomal assembly as a result of incorporation of specialized histone variants and differential post-translational modifications of the N-terminal tails of the specific histones allows modification of the chromatin structure at various levels (Jin et al. 2005; Pusarla and Bhargava 2005). There is increasing interest in the mechanism of chromatin modification involving the incorporation of histone variants into nucleosomes (reviewed by Jin et al. 2005). Histone variants are non-allelic isoforms of the conventional histones that show sequence variations (Malik and Henikoff 2003). In contrast to conventional histones, the variant forms of histones are synthesized and assembled into nucleosomes in a DNA replication-independent manner, thus enabling the encoding of epigenetic information through the generation of specialized nucleosome arrays (Hake et al. 2004). The variations in chromatin structure resulting from the incorporation of variants can potentially influence various nuclear functions such as gene regulation, DNA repair and meiotic events (Pusarla and Bhargava 2005). Histone variants are mostly encoded by genes remote from the canonical histone gene cluster, and usually occur in single copies and with introns (Albig et al. 1996; Pusarla and Bhargava 2005). In evolutionary terms, the variant histone genes diverged quite early from the canonical histones, which resulted in the development of differential expression patterns (Bramlage et al. 1997; Frank et al. 2003). Accordingly, the variant histone genes are controlled by regulatory mechanisms that are quite distinct from those controlling replication-dependent histones.

Distinct biological functions have been described for certain replication-independent variants. For example, the H2A variant in mouse, H2AX, is critical for facilitating the assembly of specific DNA-repair complexes on damaged DNA (Celeste et al. 2002), and is also required for the sex-body formation and associated silencing in male meiosis (Fernandez-Capetillo et al. 2003). Centromeric histone H3 (CenH3) is one of the well-studied H3 variants in many eukaryotic cells. This molecule is exclusively localized at the centromere, and forms part of the centromere complex (Henikoff et al. 2000; Talbert et al. 2002). Furthermore, another H3 variant H3.3 is very similar to canonical H3, differing only at four or five amino acid positions in animal and plant counterparts (Malik and Henikoff 2003). Ahmad and Henikoff (2002) demonstrated that the *Drosophila* H3 variant H3.3 is deposited at particular loci, including active rDNA arrays and it shows replication-independent deposition, suggesting a mechanism for the activation of genes by replication-independent substitution of H3.3. Therefore, the different histone variants are likely to have distinct biological functions.

In addition to replication-independent variants, tissue-specific variants have been reported in both plant and animal systems. One example is the preferential expression exhibited by testis-specific histone variants in spermatogonia, spermatid or sperm during spermatogenesis (Grimes et al. 1990; Lim and Chae 1992; Witt et al. 1996; Zalensky et al. 2002). Another example is our previous report of male-gamete-specific histone variants *gcH2A* and *gcH3* in *Lilium longiflorum* (lily) generative cell (Xu et al. 1999a). Other histone variants (*gH2A*, *gH2B* and *gH3*) have also been reported to show cell-specific expression in the male gametic cells of lily (Ueda and Tanaka 1995; Ueda et al. 2000). We have recently reported on the H3 gene family in *Arabidopsis*, and identified that AtMGH3 is a male-gamete-specific H3 variant in *Arabidopsis* (Okada et al. 2005) suggesting that the expression of unique histone H3 variants in male gametic cells is a general feature of flowering plants

In this paper, we report five H3 variants (gcH3, gH3, leH3, soH3-1 and soH3-2) that are preferentially expressed in lily generative cell, including three novel H3 variant genes. gcH3 and leH3 lack H3K9 substituted by methionine. gH3 shares conserved structural features with CenH3, but gH3 appears to be distributed throughout generative cell chromatin. Methylation of H3K4 and H3K9 was also investigated. The H3K4 is highly methylated in the generative cell nucleus of mature pollen, while this methylation is low in vegetative cell nuclei. H3 molecules with H3K4 dimethylation are localized not only in GC chromatin but also in the matrix of the generative cell nucleus, suggesting that these free H3 molecules are used to replace H3 in chromatin and activate gene expression in generative cells. H3K9 dimethylation is thoroughly and weakly distributed in generative cell chromatin with distinct spot signals. Possible roles of H3 variants and H3 methylation in male gametic cell are also discussed.

Materials and methods

Plant materials

Leaves of lily (*Lilium longiflorum* cv white fox) were used for genomic DNA isolation and root, shoot, ovary, generative cell, uninucleate microspore and mature pollen were used for RNA isolation.

Genomic Southern blot analysis

Genomic DNA of lily was isolated and digested with *Eco*RI and *Hin*dIII, and Southern blot analysis was performed as described (Okada et al. 2000). The entire cDNAs labelled by digoxigenin was used as probe to hybridize DNA blot. The blots were washed at high stringency (0.1 × SSC, 0.1% SDS at 65°C) and the signal was detected by chemiluminescence using CDP-star (Roche Diagnostics, Basel, Switzerland).

RT-PCR analysis

Total RNA was isolated from different tissues of lily (Sambrook et al. 2001) followed by poly(A) + RNA isolation using Microfast track kit (Invitrogen, Carlsbad, California, USA). The cDNA was synthesized by First-strand cDNA synthesis kit (Amersham Biosciences, Buckinghamshire, England) and RT-PCR was performed using gene specific primers. Gene-specific primers were designed from coding region or 5′- and 3′-UTR of cDNA to amplify specific gene products. Initial denaturation step at 95°C for 2 min was followed by 25–33 cycles of 94°C for 30 s, 58°C 30 s, 72°C for 1 min, and a 2 min final elongation step at 72°C. Amplified DNAs were extracted and sequences were confirmed by standard sequencing protocol (Sambrook et al. 2001). PCR products amplified from genomic DNA were also sequenced to determine the intron location. Primer sequence used for RT-PCR analysis is listed in Table S1 (supplemental data online).

In situ hybridization

Lily mature pollen was collected and fixed with 4% (w/v) paraformaldehyde in PBS buffer. The samples were dehydrated and embedded in paraplast (Structure Probe, West Chester, PA, USA) using standard methods. Sections, 8-μm-thick, were placed on 3-aminopropylethoxysilane-coated slides, deparaffinized with Histoclear (National Diagnostics, Atlanta, GA, USA) and rehydrated through a graded ethanol series.

Digoxigenin-labelled sense and antisense RNA probes were transcribed from a T7 or SP6 promoter in the pGEM-T Easy vector (Promega, Madison, WI, USA) by using a DIG RNA-labelling kit (Roche Diagnostics, Basel, Switzerland). Hybridization and washing were carried out as described (Okada et al. 2005). Prior to colour development, slides were stained with 4′,6-diamidino-2-phenylindole (DAPI) solution to visualize the pollen nuclei. Hybridization signals were detected by treatment with anti-digoxigenin antibodies conjugated with alkaline phosphatase and visualized by overnight incubation with 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt and nitroblue tetrazolium chloride solution. The slides were mounted in Fluorescent Mounting Medium (DakoCytomation, Botany, New South Wales, Australia). Observations and photography were conducted with the aid of a fluorescent microscope BX60 and digital camera DP70 (Olympus, Hamburg, Germany).

Production of anti-gH3 peptide antibody and immunoblot analysis

The peptide corresponding to the amino acid 4–19 of gH3 (PRKEAPQRNLDRDENC, Cys residue was added for KLH conjugation) was synthesized and KLH conjugated (ANU Biomolecular Resource Facility, Canberra ACT, Australia). Rabbit polyclonal antibody raised against gH3 peptide was made by IMVS Veterinary Services (Adelaide, South Australia, Australia).

To assess specificity of anti-gH3 peptide antibody, histone fraction was purified from protein extract of lily mature pollen as described (Ueda and Tanaka 1995). Purified histone proteins were separated by electrophoresis on 15% SDS-polyacrylamide gels, and electroblotted onto PVDF membranes (PVDF-Plus; Micron Separations, Westborough, MA, USA). The blots were probed with anti-gH3 antiserum (1:500 dilution) or preimmune serum, followed by wash with PBS containing 0.05% Tween-20. The blots were then incubated with secondary antibody, alkaline phosphatase (AP)-conjugated anti-rabbit IgG (Promega, Madison, WI, USA), and the signal was visualized by incubation with 5-bromo-4-chloro-3-indolyl-phosphate *p*-toluidine salt and nitroblue tetrazolium chloride solution.

Immunocytochemical staining

Sections of lily mature pollen were prepared as mentioned above. Anti-dimethyl-histone H3 (Lys4) (Cat# 07-030) and anti-dimethyl-histone H3 (Lys9) (Cat# 07-441) were purchased from Upstate (MA, USA). Slides were incubated with anti-gH3 peptide antiserum (1:500 dilution), anti-dimethyl-histone H3K4 (1:200 dilution) or anti-dimethyl-histone H3K9 (1:200 dilution) for over night at 4°C. Following PBS containing 0.1% Tween-20 washes, the slides were incubated with 1:500 dilution of secondary antibody (Alexa 594, Molecular Probes Inc, OR, USA). Following three times wash with PBS with 0.1% Tween-20, the slides were stained by DAPI solution and mounted in Fluorescent Mounting Medium. Observations and photography were conducted as described above.

Phylogenetic analysis of histone H3 variants

Multiple sequence alignment was generated by BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html) and the neighbour-joining method was adopted to make a phylogenetic tree with the aid of GENETYX-MAC 10.0 software (Genetyx, Tokyo, Japan).

Results and discussion

Identification of five H3 variants in lily GC ESTs

We generated a lily generative cell cDNA library (Xu et al. 1999b) and determined ESTs for 886 clones (Okada et al. 2006), of which 12 clones showed significant similarity to H3 histones. Determination of the nucleotide sequences identified two of these as *gcH3* (Xu et al. 1999a) and another five as *gH3* (Ueda et al. 2000). The remaining five clones were novel H3 variant genes, with two clones showing the highest similarity to late embryonic H3 in sea urchin (Kaumeyer and Weinberg 1986), and thus were termed *leH3*. The other three ESTs were grouped into two classes (*soH3-1* and *soH3-2)* because of their similarity to canonical somatic H3. Thus, in addition to already identified variants, we have identified five H3 variants (*gcH3*, *gH3*, *leH3*, *soH3-1* and *soH3-2*) in lily generative cell ESTs.

The deduced amino acid sequences of the five generative cell H3 variants are aligned in Fig. 1. *gcH3* clone GC1174 encodes a putative 84-amino-acid protein and an N-terminal histone tail domain showing significant identity to the conserved canonical H3 sequence (Fig. 1A). In contrast, the histone core domain of gcH3 is not conserved and is much shorter than that of canonical H3, suggesting that gcH3 is unlikely to have a functional H3 core domain. As reported in Ueda et al. (2000), *gH3* clone GC1008 encodes a 149-amino-acid protein and shows a highly diverse sequence in the amino terminal tail, whilst the core domain is relatively conserved. For *leH3*, two clones (GC1126 and GC0584) were shown to be similar to late embryonic H3 of sea urchin; however, the DNA sequences of these two clones differ in the 5′ and 3′ UTR regions of cDNA. GC0584 also has deletions in the coding region resulting in frame shift, and thus encodes a truncated histone protein. The *leH3* GC1126 encodes a 155-amino-acid protein, and significant similarity (64%) was found throughout the protein with a 23-amino-acid insertion in the N-terminal tail domain. As mentioned above, two somatic-type H3 variants were found in GC ESTs: *soH3-1* clone GC0075, which encodes a 137-amino-acid protein with 92% similarity to the *Arabidopsis* H3.3 variant; and *soH3-2* clone GC1661, which encodes a 136-amino-acid protein with 98% similarity to the *Arabidopsis* H3.3 variant. Both soH3-1 and soH3-2 appear to have amino acid substitutions commonly found at positions 31, 87 and 90 in plant histone H3.3 (Malik and Henikoff 2003; Okada et al. 2005), and thus these are likely to be the H3.3 or H3.3-like variants in lily.

Conserved lysine residues of H3 and H4 are the target of acetylation and methylation (Strahl and Allis 2000; Richards and Elgin 2002; Grewal and Moazed 2003). The alignment of GC H3 variants indicates that most of conserved lysine residues are found in GC H3 variants, except for *gH3*, which has a highly diverse sequence in its N-terminal tail. Intriguingly, H3K9 of gcH3 and leH3 is substituted by methionine, although the remaining lysine residues are conserved in these variants. No functional histone H3 genes lacking H3K9 have been reported so far in plants and we did not find such amino acid substitution in H3 of other organisms deposited in the NCBI database.

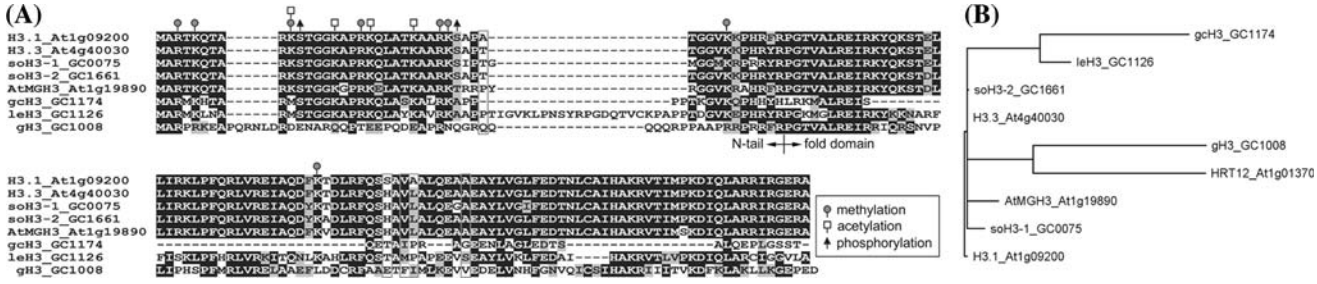

Fig. 1 Phylogenetic analysis and alignment of the protein sequence of lily GC H3 variants and *Arabidopsis* H3 variants. (**A**) Multiple alignment of GC H3 variants with *Arabidopsis* H3.1, H3.3 and AtMGH3, a male gamete-specific histone H3. The known post-translational covalent modification sites are labelled (Sims et al. 2003). Five amino acid positions (31, 87, 89, 90 and 96) that differ between vertebrate H3.1 and H3.3 are indicated by grey open boxes. The At1g09200 and At4g40030 sequences were used as representative sequences for *Arabidopsis* H3.1 and H3.3, respectively. (**B**) A neighbour-joining tree of five GC H3 variants and *Arabidopsis* histone H3 variants (Okada et al. 2005)

Genomic Southern blot analysis revealed a few intense bands corresponding to *gcH3* GC1174, *gH3* GC1008 and *leH3* GC1126, pointing towards a low copy number for these genes in the lily genome (Fig. 2) and suggesting these are unique H3 variant genes. In contrast, *soH3-1* GC0075 and *soH3-2* GC1661 showed a similar pattern of multiple bands, indicating the presence of multiple genes (eight) of somatic type H3 in the lily genome. We have previously reported high redundancy of H3.3 and H3.3-like genes in *Arabidopsis* (Okada et al. 2005). Thus, multiple copies of H3.3 and H3.3-like genes seem to be a common feature in higher plants.

Tissue-specific plant histone gene expression has only been observed in reproductive cells, especially the male gametic cells of pollen (Xu et al. 1999a; Ueda et al. 2000; Okada et al. 2005). The number of ESTs of H3 genes found in lily generative cells ESTs (in total of 886 ESTs) is more than twice that of other histone components such as *H2A*, *H2B* and *H4* (Okada et al. 2006). Interestingly, maize sperm cell EST analysis produced similar results. Twenty ESTs of H3 were found in 1100 ESTs, with less than five for the other histone genes (Engel et al. 2003). Among 20 ESTs of H3 in maize sperm cells, we identified six non-redundant H3 genes (data not shown). We have shown that at least three H3-variant genes are expressed in *Arabidopsis* generative cells, including the male-gamete-specific variant *AtMGH3* as well as variant H3.3 (Okada et al. 2005). Therefore, it appears that the expression of several H3 variants in male gametic cells is common in flowering plants.

Phylogenetic analysis of lily GC H3 variants

H3 is a highly conserved protein in evolutionary terms, with the canonical H3.1 and the H3.3 variant exhibiting only four amino acid differences (Malik and Henikoff 2003). Analysis of the H3 gene family in *Arabidopsis* revealed that although H3 genes are highly conserved, there are several H3.3-like genes with a larger number of amino acid substitutions. Among them, we identified a male-gamete-specific H3 variant, AtMGH3, which contains 10 amino acid substitutions that are additional to the 4 common differences between H3.1 and H3.3, and *AtMGH3* is preferentially expressed in *Arabidopsis* GCs and sperm cells as are other male-gamete-specific histone genes in lily (Xu et al. 1999a; Okada et al. 2005). The promoter regions of both lily gcH3 and Arabidopsis AtMGH3 contain silencer domains that are recognized by an evolutionarily conserved transcriptional repressor that maintains these genes in a silent state in non male gametic cells (Haerizadeh et al. 2006).

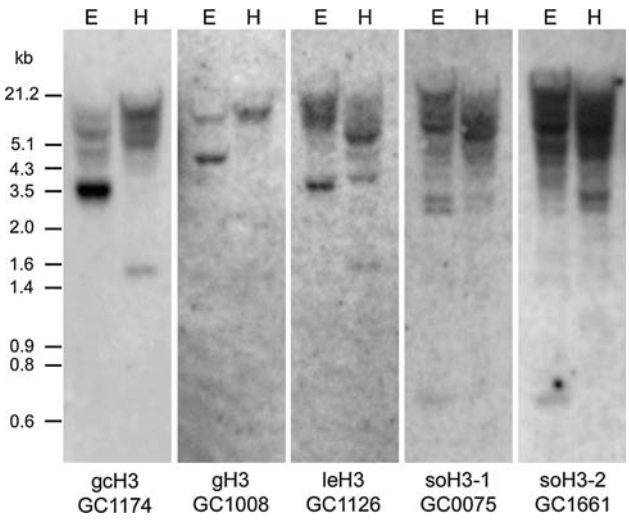

Fig. 2 DNA gel blot analysis of GC H3 variants in lily genome. Genomic DNA of lily was digested with *Eco*RI or *Hin*dIII. The blots were hybridized with digoxigenin-labelled cDNA probe, and washed thoroughly. Molecular markers are indicated in kilobases on the left. E, *Eco*RI digest; H, *Hin*dIII digest

Lily gcH3 and leH3 sequences are highly diverged from the majority of *Arabidopsis* H3 variants (Fig. 1B). soH3-1 and soH3-2 are clustered into the same group as H3.3 variants, supporting the idea that these are H3.3-like variants. The existence of intron is one of the indicators used to assess the type of H3 gene, since the major histone H3.1 which has no intron exhibits replication-dependent expression, whereas the H3.3 variant which has introns is replication-independent (Zweidler 1984; Schumperli 1986; Chaubet et al. 1992; Okada et al. 2005). We have found four conserved intron positions among the *Arabidopsis* H3 genes: one in the 5′ UTR and three in the coding region (Okada et al. 2005). All lily generative cell H3 variants except gH3 have the first intron conserved in the coding region, whereas the second and third conserved introns are not found (Fig. 3). Intriguing features of intron positions and phylogenetic tree were that gH3 does not contain any conserved introns, like CenH3 of *Arabidopsis* (HRT12/At1g01370), and that gH3 is also grouped with HRT12 in the phylogenetic tree (Figs. 1B and 3). These results indicate that quite different types of H3 variant genes are expressed in lily generative cells.

Expression of generative cell H3 variants in male gametic cells

RT-PCR analysis showed that all five H3 variants identified in the GC cDNA library (Okada et al. 2006) are significantly expressed in these cells (Fig 4, lane 4). The corresponding signals were also detectable in

mature pollen (Fig. 4, lane 6), since it contains contributions from both vegetative cells and generative cells. The expression of *gcH3* GC1174, *leH3* GC1126 and *soH3-2* GC1661 in uninucleate microspores was also observed. We confirmed the identity by sequencing RT-PCR products. RT-PCR detected two bands in *leH3* GC1126: an upper band corresponding to the *leH3* GC0584 that encodes truncated protein, as mentioned above; and a lower band corresponding to the *leH3* GC1126. Primers designed using the conserved coding region of *soH3-1* and *soH3-2* revealed the expression of other somatic H3 genes in different tissues (root, shoot and ovary). On the contrary, no bands corresponding to *soH3-1* GC0075 and *soH3-2* GC1661 were observed in these tissues. These results indicate that the expression of all five H3 variants is up-regulated in lily generative cells.

In situ hybridization experiments provided further evidence that all H3-variant genes are transcribed in the generative cells of lily mature pollen (Fig. 5). The results for *gcH3* and *gH3* obtained in this experiment are consistent with previous reports (Xu et al. 1999a; Ueda et al. 2000).

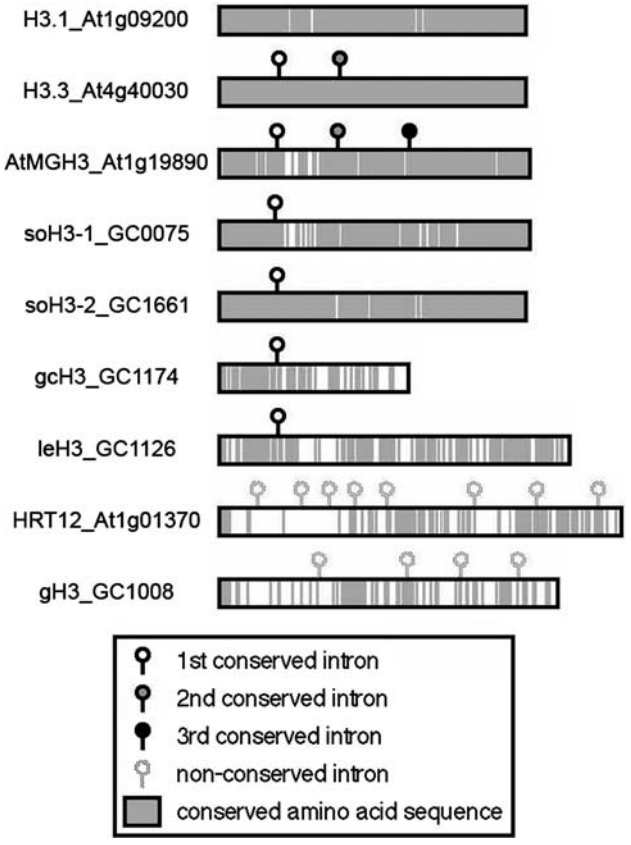

Fig. 3 Comparison of intron positions among H3 variant genes in lily and *Arabidopsis*. Three conserved intron positions in the coding region have been reported in the *Arabidopsis* H3 gene family (Okada et al. 2005) and the intron positions are shown relative to H3 protein. Conserved amino acid residues among H3 variants are also indicated as gray shaded box

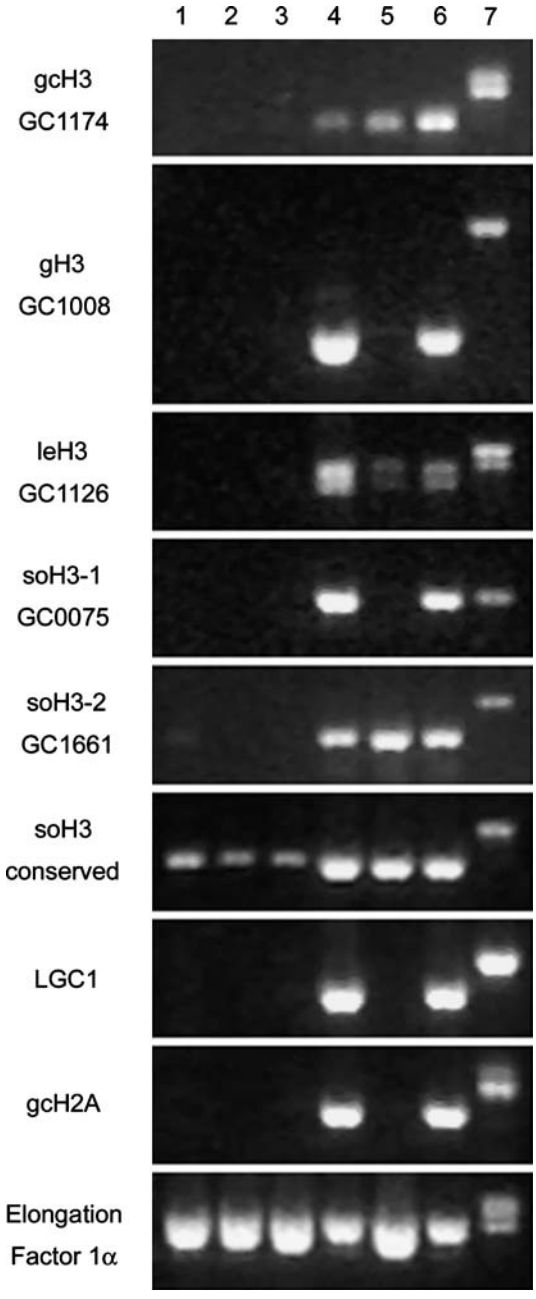

Fig. 4 Expression of GC H3 variants in different tissues of lily. RT-PCR was performed using gene-specific primers in root (lane 1), 2-week-old shoot germinated from bulb (lane 2), ovary (lane 3), GC (lane 4), uninucleate microspore (lane 5), mature pollen (lane 6), and genomic DNA (lane 7). The higher band observed in most genomic DNA samples indicates the existence of intron. *LGC1* and *gcH2A* genes (Xu et al. 1999a, b) were used as control genes for GC-specific expression and elongation factor 1α gene was a control gene for constitutive type expression. The absence of genomic DNA bands in cDNA templates indicates no contamination of genomic DNA. The forward primer for soH3-1 was designed in the coding region after the intron, and thus genomic DNA fragment of soH3-1 does not contain the intron

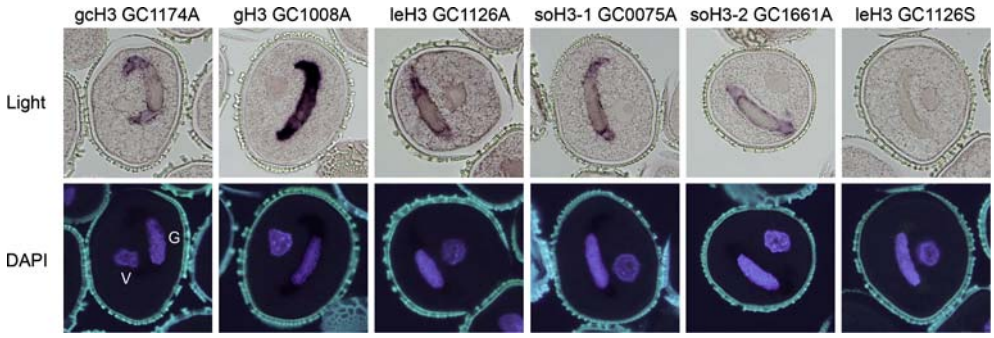

Fig. 5 Spatial expression of lily GC H3 variants in mature pollen examined by *in situ* hybridization. Digoxigenin-labelled antisense (gcH3 GC1174A to soH3-2 GC1661A) or sense (leH3 GC1126S) RNA probes were hybridized with lily pollen sections (top row). Sections were stained by DAPI to observe pollen vegetative nucleus and GC nucleus before colour detection (bottom row). Labelled RNA/RNA hybrids appear as purple staining. V, vegetative nucleus; G, generative nucleus

Histone H3 methylation in lily generative cells

To elucidate histone modification—especially methylations—in male gametic cells, we used anti-dimethyl H3 antibodies for immunocytochemical localization. Lily mature pollen sections were probed with anti-dimethyl H3K4 ($H3K4^{Me2}$) or H3K9 ($H3K9^{Me2}$), and bound antibodies were visualized using a secondary antibody labeled with a fluorescent dye (Fig. 8). The fluorescence signal of $H3K4^{Me2}$ was distributed throughout GC nuclei signals, whereas only a very weak $H3K4^{Me2}$ signal was detected in vegetative cell nuclei. Despite the highly condensed chromatin state of male gametic cell, $H3K9^{Me2}$ is dispersed throughout GC nucleus and also localized in particular regions of GC chromatin detected as intense spot signals (Fig. 8). These intense spot signals might correspond to large heterochromatin domains or centromeric regions.

Histone H3 methylation analysis showed that GC nucleus significantly accumulates replacement H3 molecules with K4 dimethylation, while $H3K4^{Me2}$ in vegetative nucleus was rarely found (Fig. 8). H3-K4 methylation is associated with transcriptionally active genes (Sims et al. 2003). Recent studies have shown high levels of transcriptional activity in lily GCs (Okada et al. 2006). Furthermore, the replication-independent H3.3 is relatively enriched in modifications, including H3K4 dimethylation which is associated with transcriptional activity, in comparison to the canonical H3 (McKittrick et al. 2004). This suggests that histone H3K4 dimethylation and H3 variants might be involved in gene activation in male gametic cells.

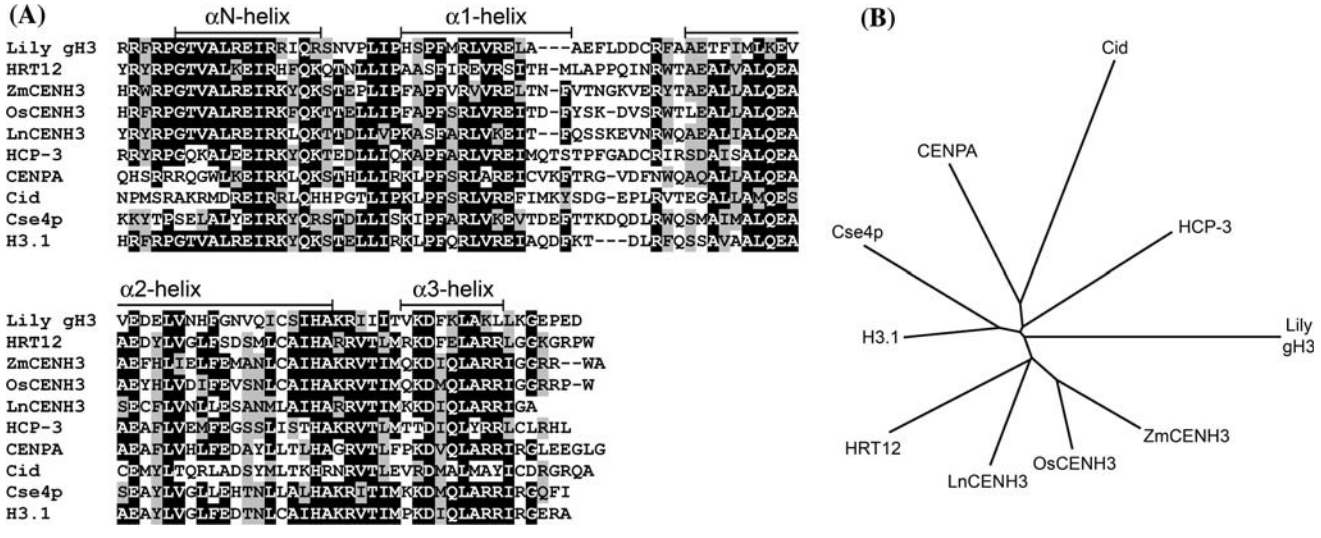

Fig. 6 Comparison of lily gH3 with centromeric histone H3 from plant and other eukaryotes. (**A**) The histone core domain of gH3 is compared to *Arabidopsis* canonical H3.1 and CenH3s from *Arabidopsis* (HRT12), maize (ZmCENH3), rice (OsCENH3), woodrush (LnCENH3), nematode (HCP-3), human (CENPA), *Drosophila* (Cid), Yeast (Cse4P). The α-helix regions in canonical histone H3 are indicated on top. (**B**) Phylogenetic tree of the core domain of lily gH3 and CenH3s

Overall, these observations imply that male gametic cells of lily have unique mechanism for chromatin assembly and gene regulation contributed by unique histone H3 variants and histone H3 methylation.

Assembly of histone gH3 in GC chromatin

The *gH3* in the present study was classified into the same cluster of *HRT12*/At1g01370, and no conserved introns were found (Figs. 1 and 3). The gH3 has a highly diverse sequence in the N-terminal tail, with a long portion that is similar to CenH3. CenH3 histones are exclusively localized in the centromere of eukaryotic cells (Henikoff et al. 2000; Talbert et al. 2002; Henikoff and Ahmad 2005). HRT12/At1g01370 encodes centromeric H3 in *Arabidopsis* (Talbert et al. 2002). Highly diverse sequence in N-terminal tail with

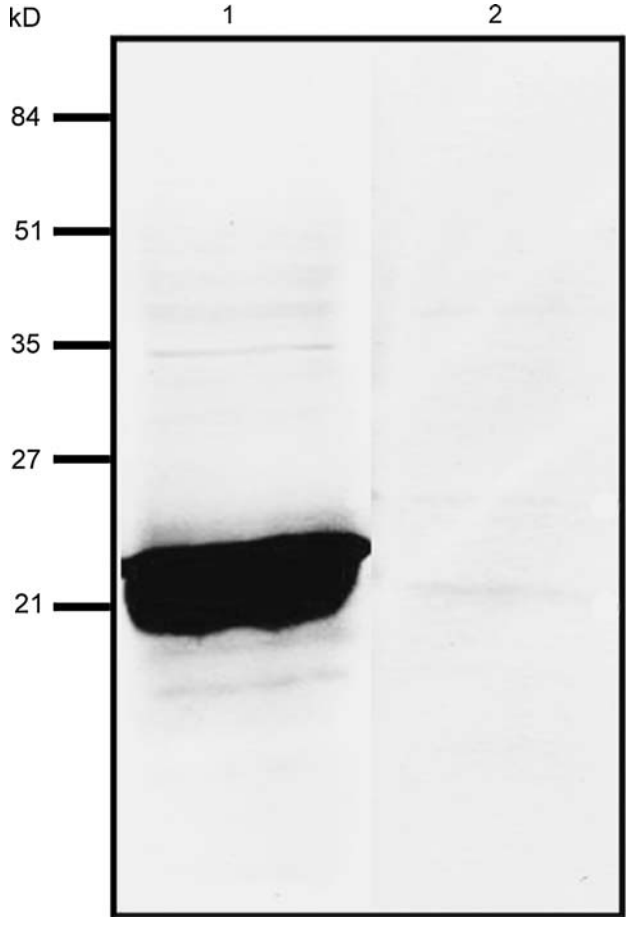

Fig. 7 Immunoblot analysis of gH3 in histone fraction of lily mature pollen. Purified histone protein from mature pollen was blotted and reacted with anti-gH3 peptide antibody (lane 1) or preimmune serum (lane 2). Molecular weight marker is indicated on the left

various length and sequence, and a relatively conserved core domain are characteristics of CenH3s. The region of core domain conserved among CenH3s is also conserved in gH3 but gH3 accumulates more amino acid substitutions in α2-helix compared to the other plant CenH3s (Fig. 6A). Phylogenetic tree of CenH3s indicates that gH3 is a close relative of plant CenH3 but clearly distinct from them (Fig. 6B).

We performed immunological detection of gH3 in lily pollen to verify incorporation of gH3 in male gamete chromatin. At first, we verified specificity of gH3 peptide antibody by immunoblot analysis of histone fraction of lily mature pollen (Fig. 7). The antibody recognized a single intense band at 21 kD which is consistent with previous report that gH3 protein is a 21 kD histone protein in lily generative cell (Ueda and Tanaka 1995; Ueda et al. 2000). No cross-reactive proteins were detected in the blot indicating the anti-gH3 antibody does not react with canonical H3 and other H3 variants. Immunocytochemical staining of gH3 provides unambiguous evidence for the specificity of antibody. Fluorescent signals corresponding to gH3 were only detected in generative cells, consistent with the localization of gH3 transcripts in generative cells only. However, no signal was detected in vegetative cell nuclei (Fig. 8, top row), which is consistent with the absence of gH3 transcripts (Fig. 5) in vegetative cells. Typically, CenH3s appear in nuclei as spot-like signals, which are co-localized with centromeric repeat sequence, during different stages of the mitotic and meiotic divisions of many eukaryotic cells (Henikoff et al. 2000; Talbert et al. 2002). The partial chromosome structure was recognizable in generative cell nuclei by DAPI staining, and gH3 appear to be distributed throughout the generative cell chromatin (Fig. 8). The absence of spot-like signals in the nuclei of generative cells pointed towards gH3 being widely distributed throughout generative cells chromatin. However, the possibility of centromeric localization could not be ruled out and further investigations are required to obtain an conclusive evidence. We have shown that gH3 shares several common features with CenH3s and appears to be an integral component of generative cell chromatin (Figs. 1, 3 and 6–8). We consider that histone variant gH3 due to its male gamete specifity and apparent structural similarity to CenH3 may be involved in male gametic cell specific chromatin organization. This might be achieved in concert with other generative cells-specific histone variants (e.g., gH2A and gH2B) that also have unique sequence and are highly expressed in generative cells (Ueda et al. 2000).

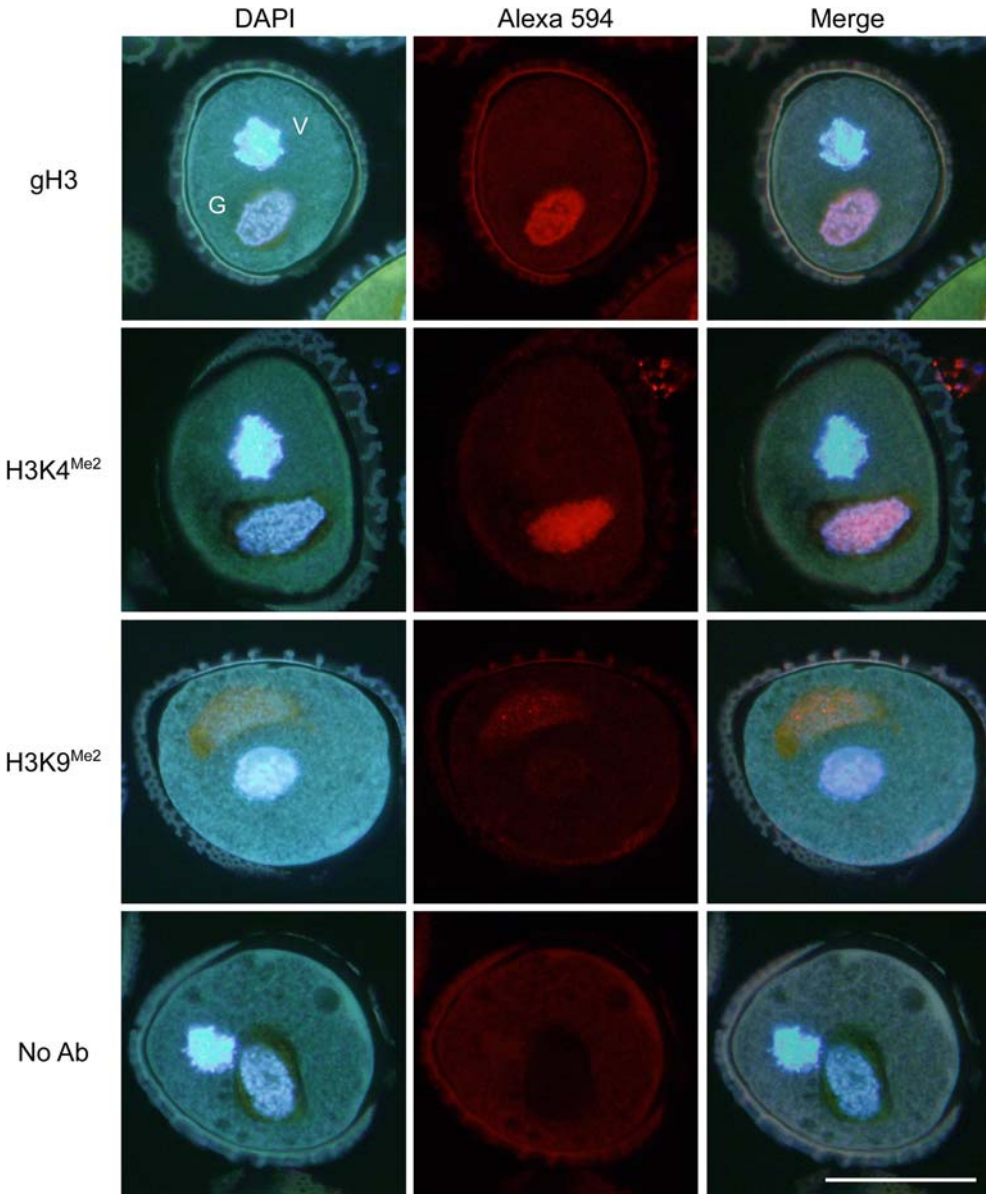

Fig. 8 Immunocytochemical staining of gH3, and dimethylation of H3K4 and H3K9 in lily mature pollen. Pollen sections were reacted with anti-gH3 peptide, anti-dimethyl H3K4 ($H3K4^{Me2}$) , anti-dimethyl H3K9 ($H3K9^{Me2}$) antibody or preimmune serum (No Ab) followed by a secondary antibody labelled with a fluorescent dye (Alexa594, centre lane). Sections were stained by DAPI to observe pollen vegetative cell nuclei and GC nuclei (left lane). A merged image is also shown (right lane). G, GC nucleus; V, vegetative cell nucleus. Bar = 50 μm

The results presented here suggest that lily is a useful plant model for investigating the distinct biological functions of each H3 variant with more diverse sequences as compared to *Arabidopsis* H3 variants, and may enable the generation of specific antibodies as exemplified here for gH3. Lily generative cells offer model system for further investigations to address the relationship between the recruitment of specific H3 variants to chromatin sites and the transcriptional activation or repression of associated genes in the male gametic cells.

Acknowledgement We gratefully acknowledge financial support provided by the Australian Research Council (ARC) for this project.

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