Analysis of the histone H3 gene family in Arabidopsis and identification of the male-gamete-specific variant \textit{AtMGH3}

Takashi Okada\textsuperscript{†}, Makoto Endo, Mohan B. Singh and Prem L. Bhalla*  
Plant Molecular Biology and Biotechnology laboratory, Australian Research Council Centre of Excellence for Integrative Legume Research, Institute of Land and Food Resources, The University of Melbourne, Parkville, Victoria 3010, Australia

*For correspondence (fax +61 3 8344 9651; email premlb@unimelb.edu.au).
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\textsuperscript{†}Present address: Takashi Okada, CSIRO Plant Industry, PO Box 350, Glen Osmond, SA 5064, Australia.

Summary
Histones are major components of chromatin, the protein–DNA complex involved in DNA packaging and transcriptional regulation. Histone genes have been extensively investigated at the genome level in animal systems and have been classified as replication dependent, replication independent or tissue specific. However, no such study is available in a plant system. In this paper we report that there are 15 histone H3 genes in the Arabidopsis genome, including five H3.1 genes, three H3.3 genes and five H3.3-like genes. A gene structure analysis revealed that gene duplication causes redundancy of the histone H3 genes. The expression of one of the H3 genes, termed \textit{AtMGH3}/\textit{At1g19890}, is cell-specific, being restricted to the generative and sperm cells of Arabidopsis pollen as shown by \textit{in situ} hybridisation and reporter gene analysis. Thus, we conclude that in Arabidopsis, \textit{AtMGH3} is a male-gamete-specific histone H3 gene. A T-DNA insertion line for \textit{AtMGH3} revealed decreased expression and ectopic RNA splicing. The T-DNA insertion lines for \textit{AtMGH3}/\textit{At1g19890} and other H3 genes revealed a normal growth phenotype and reproductive fertility. These findings suggest that other H3 genes are likely to compensate for the T-DNA-insertion-induced loss of a single H3 gene because of the high redundancy of these genes in the Arabidopsis genome. These T-DNA mutant lines should be useful for accumulating different H3 gene mutations in a single plant and for studying replication-dependent and replication-independent H3 genes and the specific role of \textit{AtMGH3} in chromatin remodelling and transcriptional regulation during development of male gametes.

Keywords: Arabidopsis, generative cell, histone H3, male gamete variant.

Introduction
The basic structural unit of chromatin is the nucleosome, which consists of a 146-bp fragment of DNA wrapped around a protein octamer (OCT) that contains two molecules of each of the core histones H2A, H2B, H3 and H4. In addition, histone H1 proteins are associated with the linker DNA that connects the nucleosomal cores, termed linker histones. With the exception of H4, these histones exhibit heterogeneity, and thus histone variants are non-allelic forms of conventional histones (Franklin and Zweidler, 1977; Malik and Henikoff, 2003). Recent studies have demonstrated that some of the histone variants have clearly specialised functions, including gene silencing (Jedrusik and Schulze, 2001), sex chromosome inactivation (Fernandez-Capetillo \textit{et al.}, 2003), genomic instability (Celeste \textit{et al.}, 2002) and gene activation (Ahmad and Henikoff, 2002). Thus, each histone variant appears to have a distinct biological function.

From the viewpoint of gene expression, histone genes can be subdivided into three major groups: replication dependent, replication independent and tissue specific (Schumperli, 1986). The replication-dependent variants are the major class of histones and their expression is coupled to the S phase of the cell cycle, when histones assemble with the newly replicated DNA to form a duplicate set of chromatin. The replication-independent histones are synthesised outside the S phase, throughout the cell cycle; they are also known as the replacement histones. In vertebrates, histones H3.1 and H3.3 have been identified as being replication-dependent and replication-independent subtypes, respectively, and they differ at five
amino acid positions (Zweidler, 1984). A recent study has demonstrated clearly that complexes of histones H3.1 and H3.3 contain distinct histone chaperones, CAF-1 and HIRA, and are thereby deposited into the nucleosome via a DNA-synthesis-dependent and a DNA-synthesis-independent nucleosome assembly pathway, respectively (Tagami et al., 2004). Tissue-specific histone variants appear to be expressed during specific developmental stages and their synthesis is not linked with cell cycle progression. The testis-specific histone variants show preferential expression in spermatogonia, spermatids or sperm during spermatogenic development (Grimes et al., 1996; Lim and Chae, 1992; Witt et al., 1996; Zalensky et al., 2002). In the case of a flowering plant, male-gamete-specific histone variants have been previously identified in Lilium longiflorum (Ueda and Tanaka, 1995; Ueda et al., 2000; Xu et al., 1999). The function of these tissue-specific histone H3 variants remains largely unknown.

Covalent modification of histone tails by acetylation, phosphorylation, methylation or ubiquitination makes them into markers or binding sites, leading to the formation of chromatin domains with various functions (Strahl and Allis, 2000). Among the four core histones, histone H3 appears to display more modification sites, and these modifications are involved in gene regulation and chromatin assembly (Sims et al., 2003). Histone H3 variants have been reported to exist in several plant species (Chaubet et al., 1992; Robertson et al., 1996; Waterborg, 1992); however, the expression patterns of these variants have not been elucidated and the precise number of histone variants that exist remains unclear, even in the model plant Arabidopsis. This is probably because many of the histone variants were originally characterised as proteins that show sequence diversity based on variable electrophoretic mobilities. It is still unclear how many of the histone variants represent gene diversity at the genome level, and their expression profile has yet to be elucidated.

In the study described here, we performed bioinformatics-based identification of histone H3 genes and found that there are 15 of them in the Arabidopsis genome. These H3 genes were classified as follows: major H3.1, variant H3.3, H3.3-like and centromeric histone H3. We show that 13 out of the 15 Arabidopsis H3 histone genes are expressed, and we describe their expression patterns. Furthermore, we have identified a tissue-specific histone H3 variant, termed AtMGH3, which shows cell-specific expression in the male gametes of Arabidopsis plants. The T-DNA mutant for AtMGH3 exhibited down-regulated expression and ectopic RNA splicing. The absence of an altered phenotype could be explained by compensation for the loss of AtMGH3 by other members of the H3 gene family that are expressed in male gametic cells of this plant.

Results

Fifteen histone H3 genes are present in the Arabidopsis genome

Although histone proteins have highly conserved sequences, histone variants with minor sequence variations have been demonstrated to play distinct roles in chromatin remodelling, gene inactivation and DNA replication (Ahmad and Henikoff, 2002; Jedrusik and Schulze, 2001; Meneghini et al., 2003; Talbert et al., 2002). A BLASTX search against the Arabidopsis Information Resource (TAIR) database (http://arabidopsis.org/) revealed the existence of 15 histone H3 genes in the Arabidopsis genome (Table 1).

Unlike the histone H3 variants reported to exist in Lilium (Ueda et al., 2000; Xu et al., 1999) 13 out of the 15 Arabidopsis H3 genes have a highly conserved sequence [Figure 1(b)]. Five of them are major histone H3s (H3.1) and three are histone H3 variants (H3.3) which have been previously reported [Chaubet et al., 1992; Figure 1(a)]. HRT12/At1g01370 is a centromeric H3 variant that has a highly diverse sequence (Talbert et al., 2002). The remaining six H3 genes are novel H3 variants. Five of these six novel H3 genes appear to be clustered into H3.3 groups due to the amino acid substitution commonly found in variant H3.3 at positions 31, 87 and 90 (Malik and Henikoff, 2003) but they accumulate more sequence variations, and some variants have sequence deletions or insertions [Figure 1(b)].

Duplication of histone H3 genes in the Arabidopsis genome

Four loci in the Arabidopsis genome were found to display duplication of histone H3 genes [Figure 2(a)], and genes at two loci, H3.1 and H3.3, each encoded identical proteins: At5g10390 and At5g10400, and At4g40030 and At4g40040, respectively. Two of the H3.3 variant genes, which were found at a single locus identified by Chaubet et al. (1992) correspond to At4g40030 and At4g40040. All four H3 genes in these two loci are expressed in the Arabidopsis plant, as shown by reverse transcriptase-polymerase chain reaction (RT-PCR), expressed sequence tagging (EST) and Affymetrix chip data (Table 1 and Figure 3), while another two loci have genes encoding proteins with sequence variations [Figure 1(b)]. At1g75610 has a deletion in the N-terminus of the coding region [Figure 1(b)] and we were unable to detect its expression by RT-PCR analysis, whereas we were able to detect a low level of expression of At1g75600 (Table 1). At5g65360 (H3.1) and At5g65350 (H3.1-like) are closely located on the genome, with only 273 nucleotides separating their coding regions, suggesting that At5g65350 is unlikely to have its own promoter region for transcription. Although there are a few ESTs for At5g65350 deposited in the TAIR data, we were unable to detect transcripts by RT-PCR using 5′ untranslated region (UTR) and 3′ UTR primers.
Table 1 Summary of database search and expression analysis for 15 Arabidopsis histone H3 genes

<table>
<thead>
<tr>
<th>Gene ID</th>
<th>H3 type</th>
<th>E-valuea</th>
<th>EST + cDNAb</th>
<th>RT-PCRc</th>
<th>Open flower</th>
<th>Length (aa)</th>
<th>No. of introns</th>
<th>K4/K9d</th>
<th>T-DNA mutante</th>
<th>Histone motif sequencef</th>
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<td>At1g01370</td>
<td>Centromeric</td>
<td>1.9E-25</td>
<td>0 + 2</td>
<td>+++</td>
<td>+</td>
<td>178</td>
<td>8 Yes/no</td>
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<td>None</td>
<td>OCETYPEINTHISTONE, OCTAMERMOTIFTAH3H4</td>
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<td>2.6E-67</td>
<td>24 + 1</td>
<td>+++</td>
<td>++</td>
<td>136</td>
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<tr>
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<td>H3.3-like</td>
<td>1.5E-62</td>
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<td>+</td>
<td>+++</td>
<td>136</td>
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<td>–</td>
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<td>+++</td>
<td>+++</td>
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<td>160 + 2</td>
<td>+++</td>
<td>+++</td>
<td>136</td>
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<td>–</td>
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<td>20 + 1</td>
<td>+++</td>
<td>+++</td>
<td>136</td>
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<td>56 + 2</td>
<td>+++</td>
<td>+++</td>
<td>136</td>
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<td>+</td>
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<td>H3.1</td>
<td>2.6E-67</td>
<td>55 + 3</td>
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</tbody>
</table>

aE-value against Atg09200 are shown in this table as determined by a TAIR BLAST search (http://arabidopsis.org/Blast/).
bNumber of ESTs and cDNA deposited in the TAIR database (http://arabidopsis.org/).
cExpression of H3 variants in Arabidopsis determined by RT-PCR analysis. Expression level is indicated by – (no expression), + (weak) to ++++ (strong).
dConserved Lys residues of histone H3 at positions 4 and 9 for the target of methylation.
eNumber of T-DNA mutant lines, which have a T-DNA insertion in exon or intron, found in the A. thaliana Insertion Database (http://atidb.cshl.org/index.html).
fHistone-related cis-element was searched by the PLACE database (http://www.dna.affrc.go.jp/PLACE/).
for this gene. However, we could amplify the cDNA of At5g65350 as part of the At5g65360 transcript by using an At5g65360 5' UTR primer and an At5g65350 3' UTR primer (data not shown). The fragment size of this read-through transcript was smaller than that of the genomic DNA, suggesting the presence of an unpredicted intron in this transcript. Indeed, the sequence of this fragment confirms the presence of a 249-bp intron overlapping from the 3' UTR of At5g65360 to the N-terminal coding region of At5g65350 [Figure 2(b)]. Therefore, At5g65350 is unlikely to produce a functional histone H3 protein.

Replication-dependent and -independent expression of Arabidopsis H3 genes

Reverse transcriptase-polymerase chain reaction analysis was used for all 15 Arabidopsis H3 genes so that they could be classified according to their gene expression pattern (Table 1). In addition, Affymetrix chip data obtained from the Nottingham Arabidopsis Stock Centre (NASC; Table S2, supplemental data available online) were also used to elucidate the tissue specificity of gene expression. The expression of 13 out of the 15 Arabidopsis histone H3 genes was confirmed either by RT-PCR (Table 1) or by Affymetrix chip data (Figure 3). Five major H3.1 genes appeared to show significant expression in tissues containing rapidly dividing cells, such as the bud, inflorescence, seedling and cell suspensions (Figure 3). By contrast, three of the H3.3 genes exhibited an extremely high level of expression in most of the tissues examined. It has been reported that major histone H3.1 displays replication-dependent expression, while expression of the H3.3 variant is replication independent (Chaubet et al., 1992; Zweidler, 1984). The expression of H3 genes during cell cycle progression was evaluated using expression data for an Arabidopsis synchronous cell suspension, as reported by Menges et al. (2003). Four out of five of the H3.1 genes appeared to exhibit S-phase-specific expression that peaked in the S phase with a level of expression that was at least a two-fold higher than the lowest level observed (Menges et al., 2003; Figure 4).

The centromeric H3 (At1g01370/HRT12) and two H3.3-like histones (At1g09200 and At1g40030) sequences were used as representative sequences for Arabidopsis H3.1 and H3.3.

Figure 1. Phylogenetic analysis and alignment of the protein sequence of Arabidopsis histone H3 and H3 from other organisms. (a) A neighbour-joining tree of Arabidopsis histone H3s with major H3.1 and variant H3.3 from animals and plants. The centromeric H3 (At1g01370/HRT12) was excluded from the tree. (b) Multiple alignment of Arabidopsis H3s. 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 At1g40030 sequences were used as representative sequences for Arabidopsis H3.1 and H3.3.
Identification of male-gamete-specific histone H3 in Arabidopsis

Affymetrix chip data showed that transcripts corresponding to histone H3 encoded by At1g19890 could only be detected in mature bud, mature bicellular and tricellular pollen (Figure 3). Spatial expression of At1g19890 was analysed by in situ hybridisation to evaluate whether this gene encodes the male-gamete-specific histone H3 of Arabidopsis. In situ hybridisation could not detect transcripts of At1g19890 in uninucleate microspores or in early bicellular pollen, but clear expression was evident in late bicellular and tricellular pollen. Significant staining was confined to generative-cell and sperm-cell cytoplasm, which surrounds the male gametic nuclei, and no signal was detectable in pollen vegetative cells (Figure 5). No At1g19890 transcript was detected in the floral meristem, ovule or any other floral tissues studied in these experiments (data not shown). We conclude that At1g19890, termed AtMGH3, is a male-gamete-specific histone H3 variant in Arabidopsis.

We also tested two H3.3 genes (At4g40040 and At5g10980) that are expressed strongly in pollen (Figure 3). Both genes exhibited similar expression patterns: weak expression in uninucleate microspores, strong expression in the generative cells of early bicellular pollen and no detectable signal in late bicellular and tricellular pollen (Figure 5). These genes are also expressed in the floral meristem, ovule and pollen mother cells (data not shown). Although these two H3.3 genes exhibited replication-independent expression in suspension cells (Figure 4), they appeared to show a replication-dependent expression pattern during pollen development. The first mitotic division is experienced by uninucleate microspores, where weak expression of these genes was confirmed. Moreover, at the early bicellular stage, generative cells in the S phase exhibited the strongest expression of H3.3 genes, while it was barely detectable in late bicellular generative cells where cell cycle is at the G2–M phase (Figure 5).

Expression of AtMGH3 was further confirmed by a β-glucuronidase (GUS) reporter gene assay. GUS staining was evident in the generative cell of the late bicellular stage of pollen and sperm cells of mature pollen [Figure 6(c) and (d)]. AtMGH3 was not expressed in uninucleate microspores, early bicellular pollen [Figure 6(a) and (b)] or any other tissues (data not shown). These results provide additional evidence for male-gamete-specific expression of AtMGH3.

The AtMGH3 promoter sequence was compared with those of other male-gamete-specific genes to establish whether there are any shared cis-regulatory elements. Two motif sequences in the promoters of LGC1 and gcH3 have been proposed as putative cis-regulatory elements that might be common in male gametic genes and that function to suppress gene expression in sporophytic tissues (Okada et al., 2005). AtMGH3 has a 9 bp Box-1-related motif (CCAAAATTCA), which is slightly shifted from the original Box 1 sequence in the lily gene promoter, as a complement-ary sequence in two locations of its promoter [Figure 6(e)]. Intriguingly, this motif is also found in the Duo1 promoter region, which was recently identified as a male-gamete-
specific MYB factor in Arabidopsis. This motif appears to be conserved in four male-gamete-specific gene promoters in two different plant species, which suggests that it is a common silencer element in plants.

**T-DNA insertion lines for H3 genes reveal normal growth and fertilisation**

The function of each of the H3 genes was studied by collecting available T-DNA insertion lines from the NASC. The T-DNA line N110393 has an insertion near the end of the coding region of *AtMGH3*/*At1g19890* [Figure 7(a)]. Insertion of T-DNA in this gene was confirmed by sequencing of the T-DNA flanking region and by genomic Southern blot analysis, which showed restriction fragment length polymorphism (RFLP) in a T-DNA homozygous line [Figure 7(b)]. Expression of *AtMGH3*/*At1g19890* in T-DNA homozygous plants was analysed by RT-PCR. No transcript was detectable in T-DNA homozygous plants when primers P1 and P2, which are located at both sides of the T-DNA, were used, and reduced transcript levels and unspliced transcripts were detected by using primers P1 and P3 [Figure 7(c)]. Therefore, T-DNA insertion near the end of the coding region of *AtMGH3* resulted in decreased expression and a change in the RNA splicing state.

We also analysed the T-DNA insertion lines for the other H3 genes (Table 2), none of which exhibited any developmental abnormality in the T₃ generation, despite reduced or abolished gene expression induced by the T-DNA insertion. This may be explained by the high redundancy and highly conserved sequences of histone H3 multigene in Arabidopsis, which may be able to compensate for the function of other H3-encoding genes.

**Discussion**

The histone H3 gene family in Arabidopsis

We report in this paper that there are 15 histone H3 genes in the model plant Arabidopsis, and that they comprise five H3.1 genes, one H3.1-like gene, three H3.3 genes, five H3.3-like genes and one centromeric H3 gene (Table 1 and Figure 1). A BLAST search of the genomic sequences of other model organisms revealed that the number of histone...
Five H3.3-like genes appeared to show variation in their amino acid sequence, but with the exception of At5g12910, most of the target residues for histone H3 modification were well conserved [Figure 1(b)]. Four of the five H3.3-like histone genes were expressed, thus they might have a distinct function in the nucleosome assembly as novel H3 variants.

**A combination of OCT motif and introns may regulate levels of replication-dependent and replication-independent expression**

It has been reported that the major histone H3.1 exhibits replication-dependent expression, whereas variant H3.3 is replication independent (Chaubet et al., 1992; Schumperli, 1986; Zweidler, 1984). Histones H3.1 and H3.3 are assembled into the nucleosome via DNA-synthesis-dependent and DNA-synthesis-independent nucleosome assembly pathways, respectively (Tagami et al., 2004). Four of the five H3.1 genes were expressed in a replication-dependent manner and all H3.3 genes were replication independent (Figure 4). However, At5g65360 (H3.1) did not exhibit replication-dependent expression and two of the H3.3-like genes (At1g13370 and At1g75600) were expressed in a replication-dependent manner (Figure 4). Two important factors regulate replication-dependent and replication-independent histone gene expression – the OCT motif in the histone promoter, as mentioned earlier, and the intron in histone genes.

It has been reported that the major mouse histone H3.1 has no intron, while variant H3.3 does possess an intron and exhibits replication-independent histone gene expression (Seiler-Tuyns and Paterson, 1986; Wells and Kedes, 1985). Seiler-Tuyns and Paterson (1986) demonstrated the conversion of replication-dependent histone H4 genes into replication-independent genes by inserting introns in the coding regions. All five Arabidopsis H3.1 genes lack an intron and all H3.3 and H3.3-like genes except At5g12910 possess an intron. There are four conserved intron positions among the Arabidopsis H3 genes, one in the 5' UTR and three in the coding region [see Figure 2(a), At4g40040]. Among the three H3.3 genes, At4g40040 has an OCT motif in its promoter, and its expression profile during cell progression is different from that of the other two H3.3 genes but is similar to that of H3.1 (Figure 4). At1g13370 and At1g75600 have imperfect OCT motifs and apparently show S-phase-specific expression despite the presence of introns (Figure 4). Moreover, At5g65360 does have an OCT motif but also has an unpredicted intron at the 3' UTR [Figure 2(b)]. Therefore, both the OCT motif in the proximal promoter region and the introns of H3 genes are likely to be involved in regulation of histone H3 genes, and a balance of both factors may determine the strength of the replication-dependent and replication-independent expression profiles.
AtMGH3/At1g19890 is a male-gamete-specific H3 variant in Arabidopsis

We analysed the expression profile of all Arabidopsis H3 genes by performing RT-PCR (Table 1) and searching Affymetrix chip data (Figure 3) and found that AtMGH3/At1g19890 exhibits pollen-specific expression. Our in situ hybridisation experiments provided evidence that this gene is transcribed specifically in the generative and sperm cells of Arabidopsis pollen (Figure 5). Thus, we conclude that AtMGH3/At1g19890 is a male-gamete-specific H3 histone in Arabidopsis; however, the amino acid sequence of AtMGH3/At1g19890 is very different from those of other male-gamete-specific H3 variants in lily, exhibiting only a 56.4% similarity to gcH3 (Xu et al., 1999) and a 39.4% similarity to gH3 (Ueda et al., 2000).

AtMGH3/At1g19890 has an H3.3-type amino acid substitution at positions 31, 87 and 90 [Figure 1(b)] and exhibits replication-independent expression in male gametic cells (Figure 5). Thus, this H3 variant might be deposited in transcriptionally active loci by a replication-independent nucleosome assembly pathway. (Ahmad and Henikoff, 2002; Tagami et al., 2004) and may contribute to male-gamete-specific gene expression.

Disruption of the AtMGH3 gene might be compensated by other H3 genes

We analysed T-DNA insertion lines for AtMGH3 to assess its function in male gametogenesis and fertilisation. The T-DNA insertion that occurred near the C-terminal end of the AtMGH3 coding region would result in a truncated histone

Figure 5. In situ hybridisation analysis of Arabidopsis H3 variants in microspores and pollen.

Digoxigenin-labelled antisense (At1g19890, At4g40040, At5g10980) or sense (At1g19890) RNA probes were hybridised with microspores and pollen at different developmental stages, as indicated at the top. Sections were stained with DAPI to visualise the nuclei. Slides were observed with the aid of incident light (top column), UV light for DAPI staining (middle column), and both incident and UV light to superimpose the two signals (bottom column). DAPI-stained nuclei of male gametic, generative and sperm cells are indicated by arrowheads.

core whereby the C-terminus, including the α3 helix region, lacked 14 amino acids. The α3 helix of the histone H3 core domain is involved in H3–H4 tetramer formation (Luger et al., 1997), thus loss of the C-terminal tail of H3 might cause a defect in nucleosome function. In addition, T-DNA insertion causes a decrease in the level of gene expression and a change in the RNA splicing status [Figure 7(c)]. However, our T-DNA homozygous lines did not exhibit detectable developmental abnormalities. There are two possible explanations for the absence of an altered phenotype in the T-DNA homozygous lines of AtMGH3. The truncated AtMGH3, with its decreased expression, might be still sufficient for nucleosome function in male gametic cells and the α3 helix region of the histone core domain may not be essential for AtMGH3 function. An alternative explanation for the absence of an altered phenotype involves a significant degree of functional redundancy among H3 variants that can compensate for the deficiency created in these plants.

Disruption of many histone genes (including H3) in chicken DT40 cells has no effect on mutant cell growth, and the remaining members of each of the histone gene families are expressed at higher levels in the mutants than in normal DT40 cells, suggesting the ability to compensate for the genetic disruption (Takami et al., 1995, 1997).

Furthermore, it has been shown that single knock-out mutations for several somatic histone H1 variants and testis-specific H1t in mice exert no detectable change in phenotype in tissues that would normally be abundant in H1 variants, and these animals exhibit normal development, including spermatogenesis (Fan et al., 2001; Lin et al., 2000; Sirotkin et al., 1995). Quantitative measurements of levels of H1 variants in mutant mice showed that the levels of each of the H1 subtypes increased proportionally to compensate for the loss of a particular H1 subtype (Fan et al., 2001; Lin et al., 2000; Sirotkin et al., 1995). As mentioned above, histone H3 genes in Arabidopsis are part of a multigene family, the sequences of which are highly conserved (Figure 1). Although AtMGH3/At1g19890 has a relatively diverse protein sequence as compared with other members of the gene family (90% identity to conventional H3), this diversity is not as high as in mouse H1(0) and testis-specific H1t; other H1 variants compensate for the null mutation. Furthermore, we demonstrated that at least two other histone H3 genes (At4g40040 and At5g10890) are expressed in the generative cells of Arabidopsis (Figure 5). Thus, the high redundancy of histone H3 genes might compensate for

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**Figure 6.** Expression of AtMGH3::GUS during pollen development and putative cis-regulatory elements conserved in male-gamete-specific genes. (a) Uninucleate microspore, (b) early bicellular pollen, (c) late bicellular pollen, (d) mature tricellular pollen. The DAPI-stained nuclei from pollen are shown in the bottom panel of corresponding light-microscope photographs (top panel). Nuclei of generative and sperm cells are indicated by arrowheads. (e) A Box-1-related sequence, a possible silencer element, conserved in a lily (top panel). Nuclei of generative and sperm cells are indicated by arrowheads. Figures 6(a)–(d) are shown in the bottom panel of corresponding light-microscope photographs (top panel). Nuclei of generative and sperm cells are indicated by arrowheads. (e) A Box-1-related sequence, a possible silencer element, conserved in a lily (top panel). Nuclei of generative and sperm cells are indicated by arrowheads.

**Figure 7.** Characterisation of the T-DNA insertion line for AtMGH3/At1g19890. (a) Schematic representation of the AtMGH3/At1g19890 gene and T-DNA insertion site. Boxes and lines indicate the exons and introns, respectively. The coding region and the 3′ UTR are shown as an open box and a shaded box, respectively. Primers used for RT-PCR analysis of T-DNA insertion line are shown below. (b) Confirmation of the T-DNA insertion by Southern blot analysis. Genomic DNA from a wild-type plant and T-DNA homozygous lines were digested by HindIII and hybridised with an AtMGH3 DNA probe. (c) RT-PCR analysis of T-DNA homozygous lines by using two different sets of primers, as shown in (a). RNA was isolated from a flower bud of a wild-type plant and T-DNA homozygous lines were digested by HindIII and hybridised with an AtMGH3 DNA probe.
Table 2 Summary of the analysis of Arabidopsis T-DNA insertion lines for histone H3 genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutant line</th>
<th>Insertion position</th>
<th>Expression in T-DNA homozygous line</th>
<th>Phenotype of T-DNA homozygous line</th>
</tr>
</thead>
<tbody>
<tr>
<td>At1g09200</td>
<td>N522668</td>
<td>105 bp upstream from ATG</td>
<td>Confirmed</td>
<td>Reduced</td>
</tr>
<tr>
<td>At1g19890</td>
<td>N110393</td>
<td>Fourth exon (CDS)</td>
<td>Confirmed</td>
<td>Reduced</td>
</tr>
<tr>
<td>At3g27360</td>
<td>N578768</td>
<td>Exon (CDS)</td>
<td>Confirmed</td>
<td>No expression</td>
</tr>
<tr>
<td>At4g40030</td>
<td>N582765</td>
<td>Second exon (CDS)</td>
<td>Confirmed</td>
<td>ND</td>
</tr>
<tr>
<td>At4g40040</td>
<td>N510583</td>
<td>First exon (CDS)</td>
<td>Confirmed</td>
<td>No expression</td>
</tr>
<tr>
<td>At5g10390</td>
<td>N574285</td>
<td>80 bp upstream from ATG</td>
<td>ND</td>
<td>Normal</td>
</tr>
<tr>
<td>At5g10980</td>
<td>N587850</td>
<td>Second exon (CDS)</td>
<td>Confirmed</td>
<td>No expression</td>
</tr>
<tr>
<td>At5g65360</td>
<td>N696666</td>
<td>50 bp upstream from ATG</td>
<td>Confirmed</td>
<td>Reduced</td>
</tr>
</tbody>
</table>

a The ID number of the T-DNA insertion line obtained from NASC (http://arabidopsis.info/).

b The T-DNA insertion position was confirmed by sequencing of the T-DNA flanking region. CDS, coding sequence.

c T-DNA insertion was also confirmed by genomic Southern blot analysis. ND, not determined.

d Expression of the H3 gene was investigated by RT-PCR analysis as shown in Figure 7. ND, not determined.

the mutation in AtMGH3. Regardless of whether the α3 helix region of H3 is dispensable or of compensation by other H3 variants, null mutants or knock-out mutants of AtMGH3 are required to confirm the hypotheses presented in this paper.

We analysed seven other H3 genes with the aid of T-DNA insertion lines and confirmed the reduced or abolished expression of each gene (Table 2). However, none of the T-DNA homozygous lines exhibited any visible developmentally abnormal phenotype. For example, the T-DNA line N587850 for At5g10980 (H3.3) has an insertion at the second exon and did not produce any histone core domain. Absence of expression of At5g10980 was confirmed by RT-PCR analysis, and yet there was no detectable alteration in the phenotype. At5g10980 encodes a protein that is identical to that encoded by At4g40030 and At4g40040 [Figure 1(a)], and the expression profile of At5g10980 overlaps that of At4g40030 and At4g40040 (Figure 3). Thus, At4g40030 and At4g40040 probably compensate for the loss of At5g10980. Although we did not observe a change in phenotype, the T-DNA insertion lines described here should prove invaluable for future studies aimed at pyramiding two or more mutations of H3 genes in a single plant. Indeed, a triple null mutation of somatic H1 variants in mouse embryos causes a 50% reduction in the normal ratio of H1 to nucleosomes, resulting in embryonic death (Fan et al., 2003). A double-null mutation for H1a and testis-specific H1t led to a 25% decrease in the ratio of H1 to nucleosome cores without perturbation of spermatogenesis or detectable defects in the meiotic processes, but it did cause changes in the expression of specific genes (Lin et al., 2004). By combining the H3 T-DNA insertion alleles, it will be possible to create two or more mutations in H3 genes in male gametic cells. The line with accumulated H3 gene mutations line should be useful for studying the general role of H3 core histones and the specific roles of AtMGH3 in the development of male gametes.

Experimental procedures

Plant materials

Arabidopsis thaliana (ecotype Columbia) plants were grown in a growth chamber and leaves, 2-week-old seedlings, roots, young buds and open flowers were collected for RNA isolation.

Database search and DNA analysis

To investigate how many histone H3 genes exist in the genome of a model organism, a BLASTX search was carried out with a representative Arabidopsis histone H3 gene (At1g09200) using a public database: TAIR (http://arabidopsis.info/) for Arabidopsis, MIPS (http://mips.gsf.de/genome/proj/yeast/index.jsp) for Saccharomyces cerevisiae, BDGP (http://m.fruitfly.org/) for Drosophila melanogaster, WormBase (http://www.wormbase.org/) for C. elegans and NCBI (http://www.ncbi.nlm.nih.gov/blast/) for Mus musculus.

Fifteen Arabidopsis genes showing significant similarity (E-value < 1 x 10^-50) were chosen for further database search. The EST, genomic DNA and protein data of H3 genes were obtained from the TAIR database. The T-DNA mutants of these H3 genes were found in the A. thaliana Insertion Database (http://attdb.cbi.pku.edu.cn/). A search for histone promoter-related cis-elements was carried out using the PLACE database (http://www.dna.afric.cn.pl/PLACE). The results of this database search are summarised in Table 1. Multiple sequence alignment was generated by BioEdit software (http://www.ncbi.nlm.nih.gov/blast/) and the neighbour-joining method was adopted to make a phylogenetic tree with the aid of GENETYX-MAC 10.0 software (Genetyx, Tokyo, Japan).

RT-PCR analysis

Poly(A) + RNAs were extracted from roots, 2-week-old seedlings, leaves, young buds and open flowers using a Microfast track kit (Invitrogen, Mount Waverley, Victoria, Australia) according to the manufacturer’s instructions. The cDNA was synthesised using a first-strand cDNA synthesis kit (Amersham Biosciences, Castle Hill, New South Wales, Australia) and RT-PCR was performed using gene-specific primers. Gene-specific primers were designed from the 5' UTR and 3' UTR of each histone H3 gene to amplify specific gene products. The initial denaturation step of 95°C for 2 min was followed by 25–33 cycles of 94°C for 30 sec, 58°C 30 sec and 72°C for 30 sec.
were determined according to Park above. The developmental stages of the microspores and pollen scope observations and photography were carried out as described sected from flower buds and stained with DAPI solution. Micro-

method of Imaizumi flower buds were stained with GUS assay buffer according to the Bent, 1998). Various tissues and different developmental stages of obtained by floral dip methods, as confirmed by PCR (Clough and

was introduced into

AtMGH3

PCR using primers attached to restriction enzyme sites. A 1.7 kb

1708 (the first ATG of

AtMGH3

þ

1) was amplified by

The Arabidopsis genomic sequence between positions –27 and –1708 (the first ATG of

AtMGH3

marked as –1) was amplified by PCR using primers attached to restriction enzyme sites. A 1.7 kb

AtMGH3

5′ UTR fragment was inserted into a pBl121 plasmid by replacing the CaMV 35S promoter to generate pAtMGH2:GUS. This was introduced into Agrobacterium tumefaciens strain LBA4404 to be used for Arabidopsis transformation. Stable transformants were obtained by floral dip methods, as confirmed by PCR (Clough and Bent, 1998). Various tissues and different developmental stages of flower buds were stained with GUS assay buffer according to the method of Imazumia et al. (2002). After staining, anthers were disected from flower buds and stained with DAPI solution. Microscope observations and photography were carried out as described above. The developmental stages of the microspores and pollen were determined according to Park et al. (1998).

Expression profile of Arabidopsis H3 genes

The expression profile of Arabidopsis H3 genes in different tissues was obtained from NASC Arrays (http://affymetrix.arabidopsis.info); Craigon et al., 2004). The Affymetrix chip data used for this profile are listed in Table S2 (supplemental data available online).

Expression data at different stages during the cell cycle in an Arabidopsis cell suspension were obtained from Menges et al. (2003) and the graphs were drawn using Excel software (Microsoft, Redmond, WA, USA).

Analysis of the T-DNA tagging line for H3 genes

Seeds of T-DNA insertion lines were obtained from NASC (http:// arabidopsis.info; Scholl et al., 2000). The flanking region of T-DNA was amplified by a combination of the H3 gene-specific primer (Table S1, supplemental data available online) and the LBA1-pROK2 primer (TGGTCACGTAGTGGCCATCG), as described on the NASC web site. The dspm1 primer (CTTTAATCGTAA- GAGTGTGGGTGTTGGG) was used instead of the LBA1-pROK2 primer for T-DNA line N110393. The flanking region sequence was determined to confirm the location of the T-DNA insertion in the Arabidopsis genome.

Genomic Southern blot analysis was carried out to determine whether the T-DNA insertion was heterozygous or homozygous from RFLP. Genomic DNAs from Arabidopsis leaves were isolated by using a DNeasy Plant Kit (Qiagen, Doncaster, Victoria, Australia) and digested with appropriate restriction enzymes. The genomic DNA fragments of H3 genes amplified by PCR were labelled with digoxigenin (Roche Diagnostics) and hybridised with a DNA blot, as described previously (Okada et al., 2000). The blots were washed at high stringency (0.1 x SSC, 0.1% SDS at 65°C) and the signal detected by chemiluminescence using a CDP-star (Roche Diagnostics). The T-DNA homozygous lines were grown in normal condi-
tions, as mentioned earlier, and any phenotypic differences noted. The level of expression of H3 genes from the T-DNA insertion line was analysed by RT-PCR using mRNA isolated from buds and gene-specific primers, as mentioned earlier.

Acknowledgements

We are grateful for the financial support provided by the Australian Research Council (ARC) for this project. M.E. is a recipient of a Research Fellowship from the Japan Society for the Promotion of Science for Young Scientists.

Supplementary Materials

The following supplementary material is available for this article online.

Table S1 Primers used for RT-PCR analysis of Arabidopsis histone H3 genes

Table S2 Experimental details of the Affymetrix histone chip used for analysis (NASC Array)

This material is available as part of the online article from http:// www.blackwell-synergy.com

References


