

# Generative cell-specific activation of the histone *gH2A* gene promoter of *Lilium longiflorum* in tobacco

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Received: 3 January 2012 / Accepted: 25 June 2012 / Published online: 21 July 2012  
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**Abstract** The *Lilium longiflorum* *gH2A* promoter is active exclusively in the generative cells of mature pollen in transgenic tobacco expressing the *gH2A* promoter::*GUS* ( $\beta$ -glucuronidase) construct as a reporter gene. Temporal and spatial aspects of *gH2A* promoter activity examined during pollen development in transgenic tobacco reveal that GUS reporter activity was not detected until developing pollen entered the early bicellular developmental stage. Activity was first detected in generative cells at early-mid stages and gradually increased to maximum levels at mid-bicellular stages. The patterns of appearance and longevity of GUS activity in tobacco were very similar to those of *gH2A* mRNA during pollen development in *Lilium*. Exogenous treatment with colchicine, a well-known microtubule depolymerize, blocked microspore mitosis and inhibited generative cell differentiation. No GUS signal was detected in the resulting anomalous pollen, which lacked generative cell differentiation. These data strongly suggest that normal generative cell development is essential for activation of the *gH2A* promoter. Furthermore, these results indicate that common transcriptional activator(s) of the *gH2A* promoter may be present in both *Lilium* and *Nicotiana*, and that such putative factor(s) activates the *gH2A* promoter only when generative cells undergo normal development.

**Keywords** Cell fate marker · Generative cell · Male gamete · Pollen development · Promoter activity

## Introduction

Differentiation of male and female gametes (sperm and egg cells, respectively) is essential to sexual reproduction. Male gametogenesis in angiosperms starts inside the anther locule. Single microsporocytes derived from undifferentiated archesporial cells undergo meiosis to generate four haploid microspores within the anther. The microspore nucleus migrates toward the cell membrane, and then the polarized microspore undergoes haploid mitosis. The mitosis is a typical asymmetric cell division that gives rise to a large vegetative cell and a small generative cell. The generative cell migrates into the cytoplasm of the vegetative cells and then divides to form two mature male gametes (McCormick 2004, Boavida et al. 2005; Honys et al. 2006; Singh et al. 2008; Dickinson and Grant-Downton 2009; Twell 2010).

One of the most important events during pollen development is microspore mitosis. The generative and vegetative cells resulting from microspore mitosis have different cell fates and acquire distinct functions; the vegetative cell produces the pollen tube, while the generative cell is the progenitor of the male gametes (Tanaka 1997; Twell et al. 1998; Scheres and Benfey 1999; Metzinger and Bergmann 2010). A number of vegetative cell-specific genes have been isolated, and some have been assigned biological function via analyses of the corresponding pollen defective mutants (Twell 1994; McCormick 2004; Twell 2010; Reňák et al. 2011). In contrast, only a few genes have been identified in association with generative cells. For example, several genes expressed in the generative and/or sperm cells have been isolated from *Lilium*, *Nicotiana* and

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Communicated by David Twell.

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*Arabidopsis* (Xu et al. 1999; Xu et al. 2002; Singh et al. 2003; Rotman et al. 2005; Okada et al. 2005a, b; Engel et al. 2005; Mori et al. 2005). However, the mechanism of gene regulation of male gametic cells has been largely unclear.

We previously isolated the coding regions of male gamete-specific histone genes *gH2A*, *gH2B*, and *gH3* from *Lilium longiflorum* (Ueda and Tanaka 1995; Ueda et al. 2000). We further successfully isolated the genomic clone of *gH2A* and showed that the 2.8 kb upstream of the coding region possessed promoter activity only in the generative cell upon introduction to *Nicotiana tabacum* (Ueda et al. 2005). In this study, we employed colchicine treatment to interfere with pollen development and showed that the promoter activity of *gH2A* is closely associated with generative cell development. Our data suggest that similar transcriptional activators associated with male gametic cell-specific gene regulation are common in both monocotyledonous and dicotyledonous plants.

## Materials and methods

### Plant materials

T<sub>1</sub> transgenic tobacco plants (*N. tabacum* cv. Samsun NN) were generated from the seeds of T<sub>0</sub> transgenic plants carrying the  $\beta$ -glucuronidase (*GUS*) gene under the control of the male gametic cell-specific 2.8 kb promoter region of the histone *gH2A* gene (defined as *gH2Apro::GUS*, Ueda et al. 2005). The 1–6 transgenic line of T<sub>0</sub> lines contained a single copy of the transgene. Seeds of transgenic plants were sown onto half-strength MS medium containing 50 mg l<sup>-1</sup> kanamycin and allowed to grow for 1 month. The seedlings were transferred to soil and grown to maturity in a growth chamber (CU-225; Tomy Seiko Co., Tokyo, Japan) under greenhouse conditions (16-h light, 8-h dark).

To identify pollen-stage-specific *GUS* activity, pollen of various developmental stages, ranging from 10 to 50  $\mu$ m in length, was collected from developing flowers. Mature pollen grains were collected from the flowers after anthesis.

### Induction of anomalous pollen

Colchicine was used to inhibit asymmetric cell division, or microspore mitosis, according to the methods described for lily by Tanaka et al. (1998), with minor modifications. The terminal ends of tobacco plantlets with flower buds approximately 10–15 mm in length were cut and placed in a solution of 0.05 % (w/v) colchicine and 3 % (w/v) sucrose for 24 h at 25 °C under 16-h light and 8-h dark growth conditions, and then the plantlets were placed in a 3 % sucrose solution for 4–8 days until flowering. The

mature pollen grains from these open flowers were subjected to the *GUS* assay.

### Assays of *GUS* activity

For histochemical staining, anthers containing microspores and developing pollen grains were squeezed with a sterilized toothpick in a solution containing 0.3 M sucrose and 80 mM sodium phosphate buffer (pH 6.8). The cells were fixed with 90 % (v/v) acetone for 1–5 h at –20 °C and then incubated in a reaction solution containing 1.9 mM 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronic acid (X-gluc; Wako Pure Chemical Industries, Osaka, Japan) for 2–12 h at 37 °C. After the *GUS* staining of microspore and pollen grains, the preparations were counter-stained with 2  $\mu$ g ml<sup>-1</sup> 4',6-diamidino-2-phenylindole (DAPI; Sigma Chemical Co.) for DNA staining. *GUS* activity was examined for 150–250 pollen grains.

### RT-PCR analysis

Microspores and developing pollen grains were released from anthers of a 1–6.4 transformant by squeezing with a sterilized toothpick in a solution containing 0.3 M sucrose and 80 mM sodium phosphate buffer (pH 6.8), and then were collected by centrifugation at 13,400g for 5 min at 25 °C. Total RNA was extracted from each cell fraction using the RNeasy Plant Mini Kit (Qiagen, Tokyo, Japan). To avoid amplification of genomic DNA, the RNA fractions were subjected to RNase-free DNase treatment. The DNase reaction solution contained total RNA (~10  $\mu$ g), 1 $\times$  DNase reaction buffer, 10 units of RNase-free DNase (RQ1; Promega KK, Tokyo, Japan), and 1 unit of RNasein (Promega KK) and was incubated for 30 min at 37 °C. Purified total RNA (approximately 0.5  $\mu$ g) was reverse-transcribed with oligo (dT)<sub>18</sub> primer for the first-strand cDNA synthesis (Transcriptor First Strand cDNA Synthesis Kit; Roche Diagnostics KK, Tokyo, Japan) as recommended by the manufacturer. The PCR mixture (20  $\mu$ l) consisted of 1 $\times$  PCR buffer, 0.5  $\mu$ l cDNA product, 0.2 mM dNTP mixture, 0.4  $\mu$ M gene-specific primers, and 1 $\times$  Advantage 2 polymerase mix (Takara Bio, Shiga, Japan). The RT-PCR was performed for the following generative cell-specific genes: *NtS1* (encoding a polygalacturonase) and *NtS2* (unknown function) identified by Xu et al. (2002), and *Ntsp0018* and *Ntsp0412*, both of which encode unknown proteins identified by Xin et al. (2011). In addition, we examined R2R3 MYB-like *B25* gene in close association of dedifferentiation of immature pollen grains (Kyo et al. 2003) and a vegetative cell-specific polygalacturonase gene, *Npg1* (Tebbutt et al. 1994). Primers used to amplify cDNA are listed in Table 1. Amplification was basically performed by 35 cycles of 94 °C/30 s, 60 °C/30 s

**Table 1** PCR primers used in this study

Oligo name	Sequence
gus-L2	TATCAGCGCGAAGTCTTTATACC
gus-R2	CAGTTGCAACCACCTGTTGAT
NtS1-L1	AACAGGTGACGATTGCATCA
NtS1-R1	TGACAAGTTGCAGGCAAAAAG
NtS2-L1	AATACTGCCGTGGTTTCTCC
NtS2-R1	AGCATTGGCTTCAACCTGTC
Ntsp0018-L1	ATTCTTCTTTTTTCTCAATCTTCA
Ntsp0018-R1	CAAATCCATAGTAAACTTTCCCAT
Ntsp0412-L1	CTCAACGGCACCAACAGATTA
Ntsp0412-R1	CGACCTATCGCTTCATTCCAG
B25-L1	CCTCCAATAGAGGCACCAAAA
B25-R1	ATGGCAATCGTTCACAAAT
npg1-L1	TTGTGAGGGTATTGAAGTTGGA
npg1-R1	CCATTTGTAAAGCCCTCTCG
tub-L1	TGAGGAGGGAGATTACTATGAG
tub-R1	CACAACCAACAGAAACAAGTAC

and 72 °C/1 min except for the below case. The annealing temperature of *Ntsp0018* PCR was 57 °C instead of 60 °C, and the number of PCR cycles for the  $\beta$ -*tubulin* gene was 30 instead of 35.

## Results

### Isolation of transgenic tobacco plants homozygous for the *gH2Apro::GUS* construct

We previously reported 11 transgenic tobacco lines ( $T_0$ ) showing GUS activity driven by the *gH2A* promoter (Ueda et al. 2005). In all  $T_0$  plants, GUS activity was detected only in generative cells within pollen grains, and not in vegetative cells of pollen, nor in somatic cells (Ueda et al. 2005). These lines showed a variety of GUS activities. For example, line 2–14 displayed barely detectable activity even after 24 h, whereas 2–1 showed strong generative cell-specific activity immediately after the reaction was started; by 24 h, the indigo blue products had diffused. In contrast, line 1–6 clearly developed clear blue color 2 h after initiation of the reaction and maintained color for 24 h; this line was chosen for further study. Line 1–6 carried a single copy of the transgene, as estimated from segregation data (Ueda et al. 2005). To obtain a homozygous line, seeds of the 1–6  $T_0$  plant were grown on a selective medium containing kanamycin, transferred to soil, and grown until pollen fully developed. Then, GUS activity of the pollen was determined. One of the kanamycin-resistant plants, 1–6.4, produced mature pollen grains that all accumulated indigo blue products only around the generative cell nucleus and hence

was determined to be homozygous for the transgene (Fig. 1a, b). In contrast, only half of the pollen grains after flowering of the 1–6.1 plant displayed GUS activity, indicating that 1–6.1 had a hemizygous constitution (Fig. 1c, d).

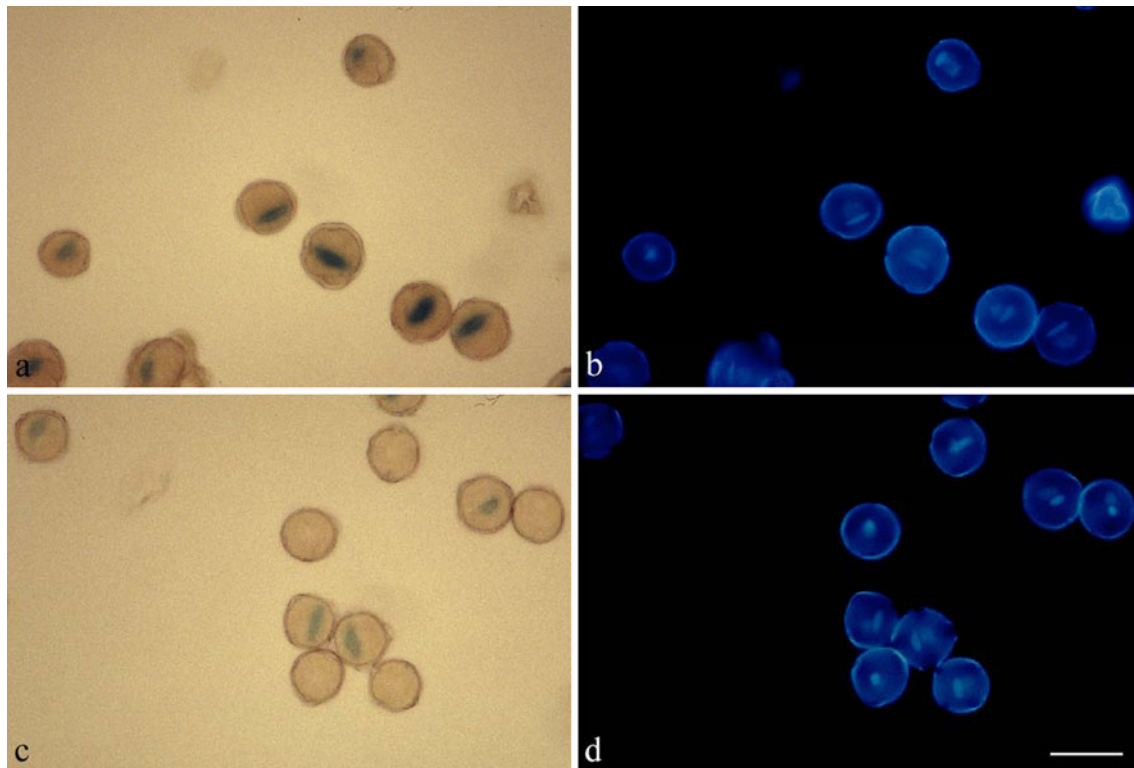
As we reported previously (Ueda et al. 2005), GUS staining was exclusively localized around the generative nuclei (Fig. 1a–d). Generative and sperm cells within pollen grains of many plants have been reported to be spindle-shaped (Russell and Cass 1981; Heslop-Harrison et al. 1988; Tanaka et al. 1989; Xu et al. 1999); the shape of the GUS-stained blue color appeared to be ellipsoidal or spherical, depending on the angle at which we viewed the sample grains (Fig. 1a–d).

### GUS activity during pollen development

We next examined GUS activity in different pollen developmental stages, from microspore stage to maturity. The developmental stages of pollen in tobacco are approximately related to the length of the flower buds (Twell et al. 1993; Twell 1994); we found similar relationships for the 1–6.4 plants under our growth conditions (Fig. 3). GUS activity was not detected when buds were 8–19 mm in length, sizes that corresponded to the microspore, microspore mitosis, and early bicellular pollen stages (Figs. 2a–d, 3). GUS activity was first detected in several pollen grains when buds reached about 20 mm in length (Fig. 3). Thereafter, the percentage of GUS-positive pollen grains increased until almost of all showed GUS activity by the time the buds reached 30 mm in length, corresponding to the early-mid bicellular pollen stage. Furthermore, the intensity of the blue color also varied depending on developmental stages. For example, strong GUS activity was observed after 2 h of incubation in 40-mm bicellular pollen (Figs. 2e–f, 3), whereas 20-mm bicellular pollen displayed only pale blue staining after 24 h (Fig. 3). We concluded that the *gH2A* promoter activity was initiated soon after microspore mitosis, gradually increased by the mid-bicellular pollen stage, and was maintained until pollen maturity.

### GUS activity in anomalous pollen

To further determine the importance of microspore mitosis that precedes the activation of the *gH2A* promoter, we inhibited asymmetric cell division by treating with colchicine. Colchicine is well known to inhibit microtubule assembly and consequently to inhibit the normal asymmetric cell division of microspores (Eady et al. 1995; Twell et al. 1998; Tanaka et al. 1998). The cells in buds of 10–15 mm in length, just before microspore mitosis, were treated with a moderate amount of colchicine (0.05 %) for 24 h, and GUS activity was determined upon flowering.



**Fig. 1** GUS activity of mature pollen in  $T_1$  plants expressing the *gH2Apro::GUS* gene. The  $T_1$  generation of the primary transformant 1–6 line grown to flowering stage. Pollen grains were harvested and

GUS activity was histochemically determined. **a, b** Homozygous line 1–6.4. **c, d** Hemizygous line 1–6.1. **a, c** GUS staining. **b, d** DAPI staining. Bar = 50  $\mu$ m

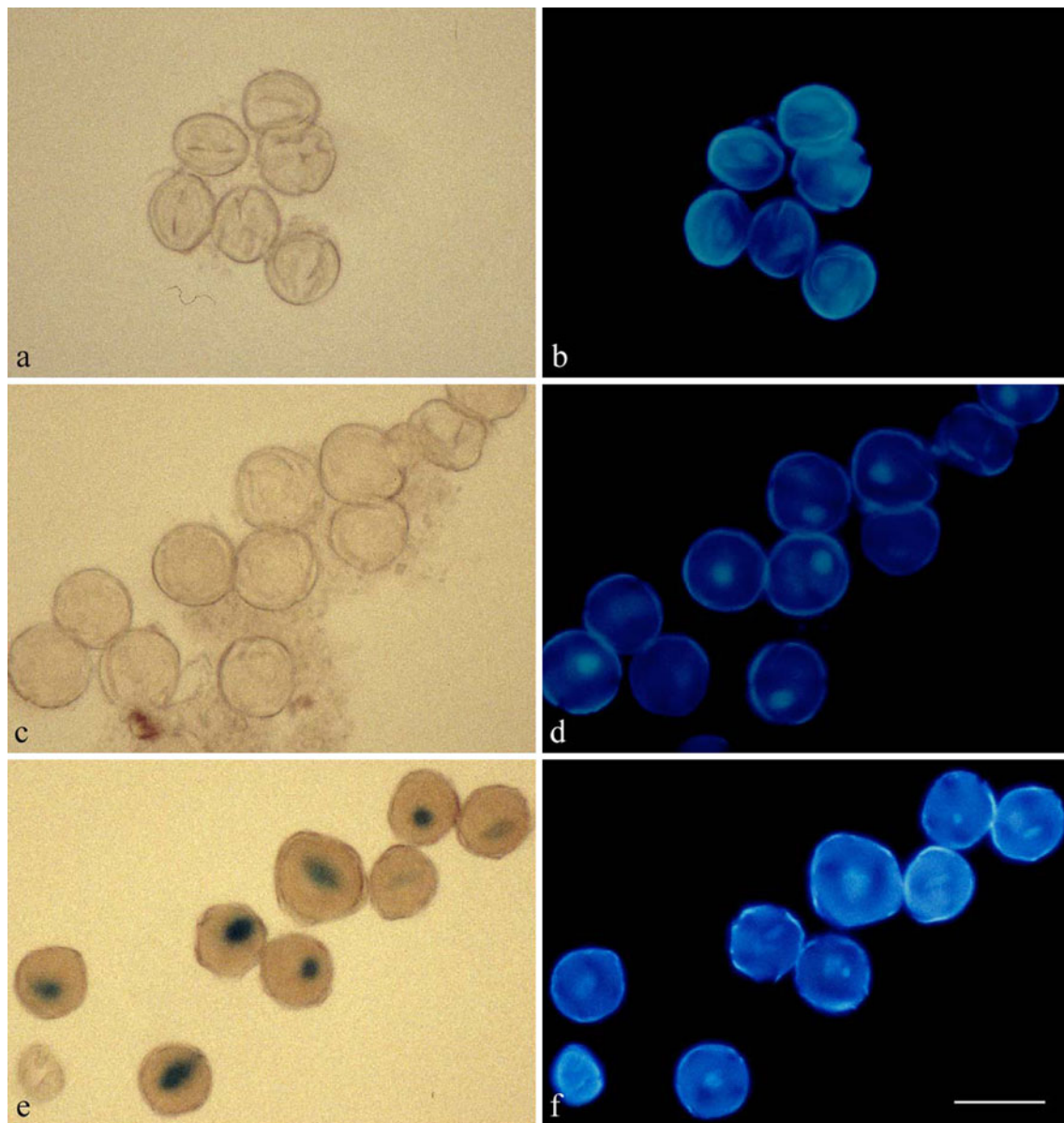
Approximately 46, 56, and 94 % of total mature pollen originating from three independent buds that were treated at the 12, 11 and 10-mm microspore stage, respectively, did not show GUS activity (Fig. 4). When the pollen nuclei were stained with DAPI (4',6-diamidino-2-phenylindole), the GUS-positive pollen displayed both typical ellipsoidal generative and spherical vegetative nuclei (Fig. 4a, b, e–g). In contrast, GUS-negative pollen had only one or two spherical vegetative cell-like nuclei (Fig. 4a, b, h–m). Thus, GUS activity was exclusively observed in normally developed pollen. When mature pollen originating from 13-mm microspore stage or later stages (for example 15 mm) were examined, almost all the generative cells in individual pollen grains were normally formed when viewed with DAPI staining, and these pollen grains displayed positive GUS activity (Fig. 4c, d). Therefore, inhibition of asymmetric cell division led to the loss of GUS activity, and the *gH2A* promoter was activated only when developing pollen produced generative cells.

#### Gene expression analysis for cell fate markers in the *gH2Apro::GUS* transgenic plants

To further study the *gH2Apro::GUS* expression profile and the relatedness to developmental stages, we employed

known cell fate marker genes and determined their transcriptional activation by RT-PCR analysis (Fig. 5). We amplified four generative cell-specific genes: *NtS1* (encoding a polygalacturonase), *NtS2*, *Ntsp0018*, and *Ntsp0412* whose functions are unknown, and a putative R2R3 MYB transcription factor *B25* gene that is associated with the tobacco pollen dedifferentiation (Xu et al. 2002; Xin et al. 2011; Kyo et al. 2003). In addition, *Npg1* gene encoding a polygalacturonase was used as a vegetative cell fate marker gene (Tebbutt et al. 1994). Under our greenhouse conditions, microspore mitosis occurs within the buds of around 15 mm in length (Fig. 3). No PCR signal of *gH2Apro::GUS* was detectable in microspores of buds of 8, 10 and 12 mm in length, and the faint signal was detectable in cells of 15- and 20-mm buds (Fig. 5). Subsequently, prominent signals were detected in bicellular pollen grains of 25–45-mm buds in addition to mature pollen grains (Fig. 5). Thus, temporal appearance and amplitude of *GUS* activity agreed well with the accumulation pattern of *gH2Apro::GUS* transcripts (compare Figs. 3, 5). Transcripts of *NtS1* and *NtS2* genes were detected in both nearly mature and mature pollen grains (Fig. 5). In addition, the signal of *NtS1* gene was uniquely detectable in cells at the microspore mitosis stage (12- and 15-mm buds), suggesting that it is possibly involved in cell division (Fig. 5).





**Fig. 2** Histochemical detection of the *gH2Apro::GUS* activity of the developing pollen. Pollen was harvested at different flower bud lengths from the homozygous plant 1–6.4, and GUS activity was

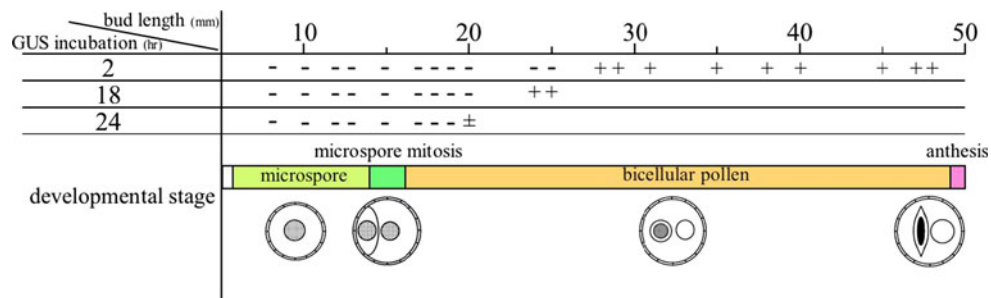
determined. Bud lengths were 8 mm (a, b), 17 mm (c, d), and 40 mm (e, f). a, c, e GUS staining. b, d, f DAPI staining. g, Generative nucleus; v, vegetative nucleus. Bar = 50  $\mu$ m

Transcripts of *Ntsp0018* were only detected in cells within over 25-mm buds, and *Ntsp0412* was detectable initially in cells of 15- and 20-mm buds with low intensity and then gradually accumulated in the developing pollen until maturity (Fig. 5). These expression patterns, especially those of *Ntsp0412* gene, were very similar to the pattern of *GUS* gene control by the *gH2A* promoter (Fig. 5). Transcript of the *B25* gene was detected prominently in the cells of late microspore stage (buds of 12 mm in length) and continued to be detected until the mature pollen stage (Fig. 5). A vegetative cell-specific gene, *Npg1*, was first detected in cells of 15-mm buds and continued to be

detected until mature pollen (Fig. 5). A PCR product of a  *$\beta$ -tubulin* gene was detected prominently in all samples investigated (Fig. 5). No signal was detectable in all samples when a RT-PCR was performed without reverse transcriptase (data not shown).

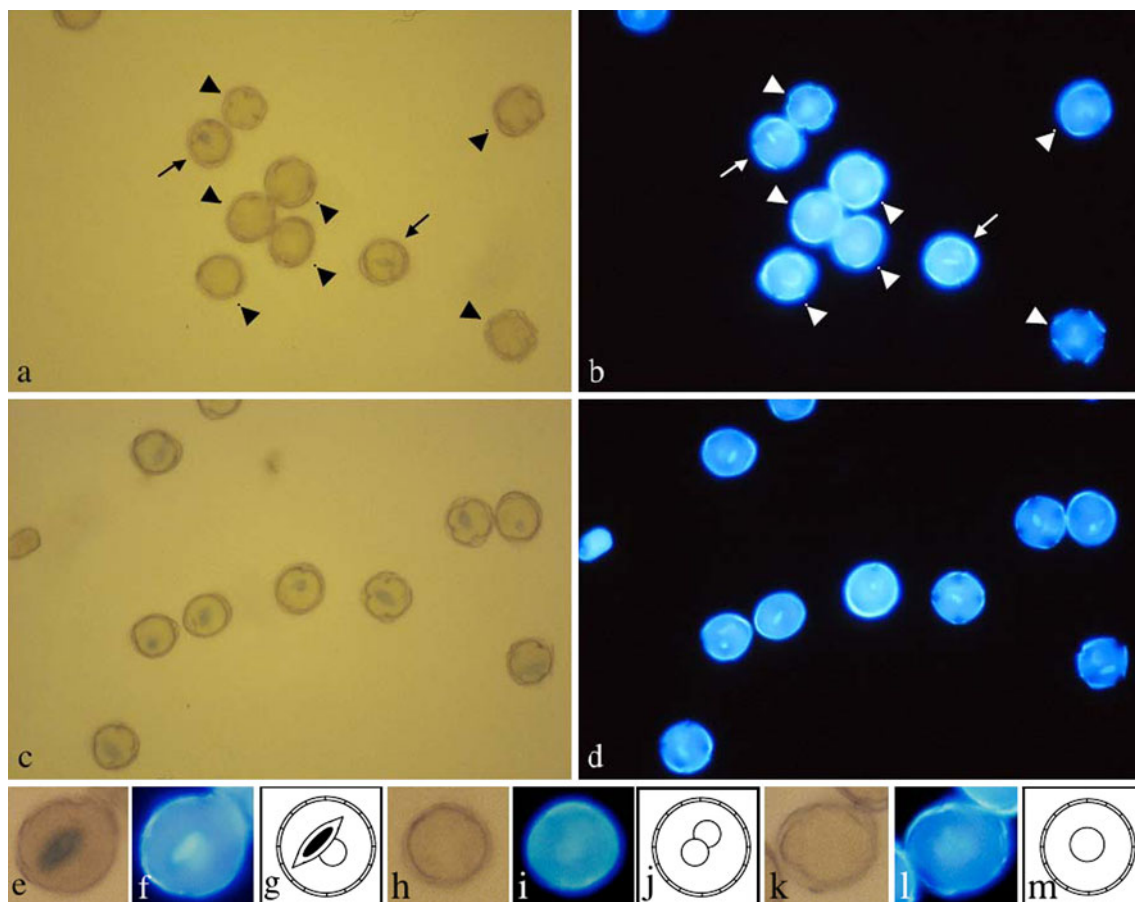
## Discussion

As previously shown, transgenic tobacco plants containing the *gH2Apro::GUS* construct display generative cell-specific expression of the *GUS* gene (Ueda et al. 2005). In the



**Fig. 3** Developmental stage-dependent *gH2Apro::GUS* expression. In tobacco, approximate stages of pollen development correlate with flower bud length. Twenty flower buds of different lengths (8–48 mm) were excised, and developing pollen grains within the buds were subjected to GUS assay. Aliquots containing hundreds of

pollen grains were withdrawn from the reaction mixture after 2, 18, and 24 h and examined for color development under a microscope. Diagram of approximate stages of pollen development according to bud size is depicted below. – no GUS activity; ± and + relative intensity of staining

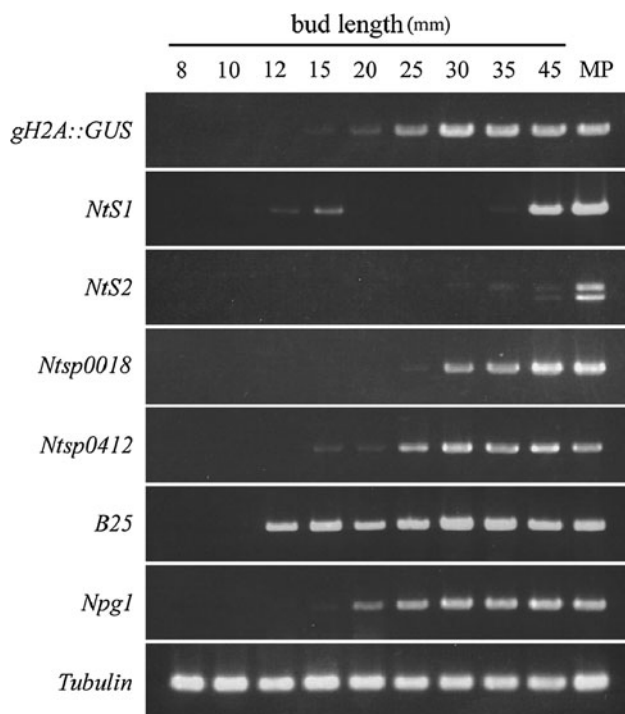


**Fig. 4** Effects of colchicine treatment on *gH2Apro::GUS* activity and normal pollen development. Plantlets with 10 mm (a, b) and 13 mm (c, d) flower buds were treated with colchicine for 24 h and grown without colchicine until anthesis, when GUS activity was determined. Colchicine-treated 10-mm pollen developed normal pollen (e, f, g), anomalous pollen with two vegetative cell-like nuclei (h, i, j), and

anomalous pollen with one large vegetative cell-like nucleus (k, l, m). Treated 13-mm pollen developed normally, similar to pollen shown in e, f, g. a, c, e, h, k GUS staining. b, d, f, i, l DAPI staining. g, j, m illustration based on DAPI staining. Arrows show normally developing pollen. Arrowheads show anomalous pollen. Bars = 50 μm

current study, we refine this observation and demonstrated that the *GUS* gene was activated soon after microspore mitosis. Exogenous application of colchicine interfered simultaneously with generative cell formation and *GUS*

gene expression, suggesting that microspore mitosis is required for the *gH2A* gene to be activated. Colchicine treatment of microspores in tobacco induced the formation of pollen with one or two vegetative cell-like nuclei



**Fig. 5** Expression analysis of *gH2A::GUS* gene with generative cell fate marker genes. Semi-quantitative RT-PCR was performed using 0.5  $\mu$ g total RNA that was extracted from cells of each bud of 8–45 mm in length and from mature pollen grains in *gH2Apro::GUS* transformants. Genes amplified were the *GUS* gene, four tobacco generative cell-specific genes (*NtS1*, *NtS2*, *Ntsp0018* and *Ntsp0412*), a R2R3 MYB-like *B25* gene, a vegetative cell-specific polygalacturonase *Npg1* gene, and a  $\beta$ -*tubulin* gene. Upper numbers showed length of buds (mm) and MP indicates mature pollen

(Fig. 4a, b, h–m, Eady et al. 1995; Twell et al. 1998). In contrast to the case of *gH2A*, these anomalous pollen grains have been shown to express the *LAT52* gene promoter::*GUS* construct specifically in the vegetative cell (Eady et al. 1995; Twell et al. 1998), and thus, this construct has been used as a vegetative cell fate marker (Chen and McCormick 1996; Matsushima et al. 2008; Berger et al. 2008; Chen et al. 2009; Kim et al. 2011). We further suggest that the *gH2A* promoter may be a cell fate marker specific to generative cells.

Several *Arabidopsis* mutants terminate pollen development at specific stages, from microspore through mature pollen stages, depending on the mutant (Borg et al. 2009; Oh et al. 2011; Twell 2011). For example, the *GEMINI POLLEN 1* (*GEMI*) gene encodes a microtubule-associated family protein (Park et al. 1998; Twell et al. 2002). The *Gem1* mutation eliminates asymmetric microspore cell division and thereby produces defective pollen with vegetative-like cells that express a *LAT52* cell fate marker gene. The mutants have reduced genetic transmission (Park et al. 1998; Twell et al. 2002). Another example, *SIDECAR POLLEN* (*SCP*), encodes a novel LOB/AS2 domain

nuclear protein. An *scp* mutation caused symmetric cell division prior to asymmetric cell division and thus produced “four-celled” pollen grains with an extra vegetative cell (Chen and McCormick 1996; Oh et al. 2010). The mutation also reduced male genetic transmission (Chen and McCormick 1996; Oh et al. 2010). These results show the importance of normal asymmetric cell division during pollen development, including the differentiation of generative cells. The *gH2A* gene appears to be activated soon after asymmetric cell division, suggesting that the *gH2A* protein plays an important role in pollen development in *L. longiflorum*.

The lily *gH2A* gene is expressed soon after microspore mitosis (Ueda et al. 2000), which coincides with its expression profile in tobacco (Fig. 3). Similar mechanisms underlying the activation of the *gH2A* gene may exist in both lily and tobacco. In *Arabidopsis*, the male gamete-specific histone H3 gene, *AtMGH3*, seems to be activated soon after microspore mitosis (Okada et al. 2005b), suggesting that a common regulatory mechanism may be operating for histone gene activation in lily, tobacco and *Arabidopsis*. Recently, the *Arabidopsis* R2R3 MYB transcription factor, *AtDUO1*, was reported to be involved in the activation of a number of male germline cell-specific genes or male germline cell-enriched genes, including the *AtMGH3* gene (Brownfield et al. 2009; Borg et al. 2011). The DNA binding sites of the *AtDUO1* protein were identified to be TAACCG, and similar sequences occurred at least four times in the promoter region of the *AtMGH3* gene in both forward and reverse orientations (Borg et al. 2011). MYB transcription factors from both plant and animal cells are well known to recognize the specific consensus sequence AACNG, which is referred to as the “MYB Binding Site” (Biedenkapp et al. 1988; Ogata et al. 1994; Borg et al. 2011). We found in this study an identical *AtDUO1* binding sequence, TAACCG, 141 bp upstream of the initiation codon of *gH2A* gene. In addition, we found three canonical MYB binding sites AACTG (–583, –135 and –117) and four AACCG binding sites (–1262, –207, –142, –55; Ueda et al. 2005). These sequences may provide the target sites for putative MYB-like transcription factors of lily and tobacco. In contrast to the MYB-like activators, the negative regulator, GRSF (germline-restrictive silencing factor), has been reported to bind to the promoter region of the generative cell-specific *LGCI* gene of lily, and it suppresses that gene’s expression in microspores, pollen vegetative cells, and vegetative tissues (Haerizadeh et al. 2006). The GRSF protein recognizes the 8-bp core binding site CTGAATTT, and we found a similar sequence, CTGAATTAAA, about 600 bp upstream of the initiation codon in the *gH2A* promoter described previously (Ueda et al. 2005). In addition to the GRSF binding motif, Okada et al. (2005a, b) reported that a 9-bp motif,



CCAAATTCa, a putative *cis*-regulatory element, was conserved between *AtMGH3* and lily *gcH3* promoters. We also found exactly the same sequence –556 bp upstream of the initiation codon in the *gH2A* promoter. These positive and negative regulators are likely to regulate *gH2A* gene expression, and the developmental switch appears to take place soon after microspore mitosis (Fig. 3).

The results of the GUS enzymatic activity by the *gH2A* promoter during pollen development agreed with GUS gene expression pattern of that by RT-PCR (Figs. 2, 3, 5). We further examined co-expression of *gH2A* with four generative cell fate markers, *NtSI*, *NtS2*, *Ntsp0018*, and *Ntsp0412* genes (Fig. 5). Among these, the expression patterns of the latter two genes, especially *Ntsp0412*, were coincided with those of GUS mRNA by *gH2A* promoter (Fig. 5). This suggests that both *gH2A* promoter and expression of these protein genes are controlled by the same putative regulatory factor or that one of these two unknown proteins can activate transcription of the *gH2A* promoter. On the other hand, the *B25* gene was first identified as a transcript associated with immature pollen dedifferentiation in tobacco (Kyo et al. 2003). Hafidh et al. (2012) reported that *B25* expressed in pollen was a putative tobacco ortholog of a R2R3 MYB *Arabidopsis DUO1* gene, because of its structural similarity (approximately 48 % amino acid sequence identity between them). However, our detailed expression analysis showed that *B25* was abundantly expressed at a late microspore stage (buds of 12 mm in length). In contrast, *DUO1* is not expressed in the late microspore in *Arabidopsis* (Rotman et al. 2005; Brownfield et al. 2009). Therefore, *B25* gene does not seem to behave as an ortholog of *AtDUO1* gene. Future work should determine whether the developmental regulatory promoter regions described here provide the target sites for putative activators and/or suppressors.

**Acknowledgments** We would like to thank K. Toyosawa for excellent technical assistance.

**Conflict of interest** The authors declare that they have no conflicts of interest.

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