# Analysis of the promoter activity of anther-specific genes in *Arabidopsis thaliana*

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**Summary**

Anther-specific genes and their promoters have been extensively studied to identify genes necessary for anther development or genetic engineering of male-sterile plants. In this study, we obtained promoter sequences of six genes in *Arabidopsis thaliana*, and these promoters fused to the β-glucuronidase (*GUS*) gene were transformed into *A. thaliana* to monitor their expression patterns throughout anther development. The GUS expression patterns confirmed the expression in several stages of anther development. Sequence analysis revealed that the promoters contain *cis*-acting elements known to confer anther/pollen-specific gene expression, supporting their anther specific expression pattern.

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**Key words** : *Arabidopsis thaliana*, Anther-specific promoter, GUS staining, *cis*-acting elements

Stamen is the male reproductive organ of the flower, which consists of a filament with anthers at the tip. Stamen produces the pollen to ensure successful reproduction in plants: male sterility (failure of plants to produce functional anthers, pollen and male gametes) cannot produce offspring. Male sterility is generally categorized into two groups, nuclear genic male sterility (caused by defects of nuclear genes) and cytoplasmic male sterility (caused by incompatibility between nuclear and cytoplasmic genomes). Cytoplasmic male sterility is widely used as the $F_1$ hybrid seed production system in plants. However cytoplasmic male sterility has limitations because incompatibility between nuclear and cytoplasmic genomes is not easily generated. Nuclear genic male sterility, especially day-length or temperature dependent, has a potential for $F_1$ hybrid seed production, but requires further development.

Anther development initiates with appearance of the anther primordia at the third whorl of the floral meristem (Sanders *et al*., 1999). Anther primordial cell-specification and differentiation establish pollen mother cells and four-lobed cell layer consisting the epidermis, endothecium, middle layer and tapetum. Pollen mother cells undergo meiosis, and tetrads of haploid microspores are generated. The microspores released from the tetrad subsequently differentiate into tricellular pollen grains. The tapetum is known to provide nutrition and pollen wall components for developing microspores and pollen grains, which degenerate at the late stages of anther development. At the end of anther development, the stamen filament elongates and the anther dehisces to release mature pollen grains.

In *Arabidopsis thaliana*, several male-sterile mutants caused by defects in anther-specific genes have been isolated by forward genetic approaches (*dad1, tpd1, qrt, ms1, ms2, ms5/pollenless3*) (Preuss *et al*., 1994; Aarts *et al*., 1997; Glover *et al*., 1998; Ishiguro *et al*., 2001; Wilson *et al*., 2001; Yang *et al*., 2003). In addition, as anther-specific genes may play important roles in stamen development, characterization of expression pattern is useful for identifying the genes involved in stamen development, and reverse genetic approaches (knock-out or knock-down of candidate genes) can confirm the involvement of these genes during stamen development (Millar and Gubler 2005; Mandaokar *et al*., 2006; de Azevedo Souza *et al*., 2009). Identifications of causative genes of male sterility can contribute to understand the molecular mechanism of stamen development. In addition, anther-specific promoters are useful for directing antisense cDNA/genes or RNA interference of interest in order to perturb the expression of the endogenous target genes, which allows assessing gene function or produce male-sterile plants.

In this study, we isolated the promoter regions of six genes, which are abundantly expressed in anthers at the uninucleate microspore, bicellular pollen, or tricellular pollen stages (Amagai *et al*., 2003). We examined the promoter activity using transgenic *A. thaliana* with these promoter regions fused to β-glucuronidase (GUS) gene during anther development.

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## MATERIALS ANS METHODS

### Construction of promoter-GUS fusion

The promoter regions of #49, #225, #445, #502, #711, and #2272 were identified from a DNA database of *A. thaliana* genomic sequence (Table 1). A 1.02-kb DNA fragment from position -20 to -1,042 bp (the first methionine was marked as +1) was amplified by PCR using the #49-F primer (5'-TCTGCAAACCTAATTTTGCGGC-3') and the #49-R primer (5'- GTTAAATAGAACAGAGCAAGAC-3'). A 0.47-kb DNA fragment from position -28 to -499 bp was amplified by PCR using the #225-F primer (5'-GAGATACCTTTTGCAAGTCCG-3') and the #225-R primer (5'- CTTCCTTACTCACTTTCTACAACC-3'). A 1.96-kb DNA fragment from position -32 to -1999 bp was amplified by PCR using the #445-F primer (5'-AATCGGAGAAGAAAGCGAC - 3') and the #445-R primer (5'- GGAAACATTCGTTTCCGAG- 3'). A 0.84-kb DNA fragment from position -2 to -844 was amplified by PCR using the #502-F primer (5'- GATTCACATGGTCACTTTACTG-3') and the #502-R primer (5'-TCAGGAAGAAGGTTTTGAGC-3'). A 0.72-kb DNA fragment from position -2 to -722 bp was amplified by PCR using the #711-F primer (5'- CGCAAAATGGCGCCGTTTCG - 3') and the #711-R primer (5'-GCTACGGTTGTCGTATACTG - 3'). A 0.5-kb DNA fragment from position -31 to -531 bp was amplified by PCR using the #2272-F primer (5'- CATAATCAGGAGATGTGTGC -3') and the #2272-R primer (5'- GAGTGCCATGTCTAAAAACG - 3'). All amplified products were cloned into the pBlueBac4.5/V5-His-TOPO vector (Invitrogen). The sequences of these promoter fragments were confirmed by sequencing of the plasmid using ABI 310 Genetic analyzer (Applied Biosystems).

The promoter sequence was taken as a *Hin*dIII-X*ba*I fragment from each plasmid and inserted into the *Hin*dIII-X*ba*I site of pSMABuba-GUS, and a *Hin*dIII-S*ac*I fragment of pSMABuba (Toki, 1997) was replaced by a *Hin*dIII-S*ac*I fragment of pBI101 so that the promoter fragment was placed in front of the GUS gene. The constructs, each formed by a promoter-GUS fusion, were named *#49-GUS, #225-GUS, #445-GUS, #502-GUS, #711-GUS* and *#2272-GUS*.

### Transformation into *A. thaliana*

*#49-GUS, #225-GUS, #445-GUS, #502-GUS, #711-GUS* and *#2272-GUS* were transferred to *Agrobacterium tumefaciens* strain EHA105 (Hood *et al*., 1993) using the freeze-thaw method (An *et al*., 1988). Transformation of *A. thaliana* accession Columbia-0 was carried out by the floral dip method (Clough and Bent 1998). Transgenic plants were selected based on hygromycin resistance (10 mg/l).

### Histochemical GUS assay

Flowers, anthers, and pollen (microspores) of different developmental stages were obtained from primary transgenic plants and wild-type plants. The developmental stage was defined based on the number of nuclei in the microspore and pollen using DAPI staining (Okada *et al*., 2000), namely the uninucleate microspore stage, the bicellular pollen stage, and the tricellular pollen stage. The flower of the tetrad stage and one day before anthesis were examined for comparison. The GUS assay was carried out for 12 h at 37 ℃ in a GUS assay solution containing 100 m*M* sodium phosphate (pH 7.0), 20m*M* EDTA, 0.1% TritonX-100, 1m*M* potassium ferricyanide, 1m*M* potassium ferricyanide, 20% methanol, and 1m*M* X-gluc (5-bromo-4-chloro-3-inndoryl-D-glucuronide) (de Block and Debrouwer, 1992). Methanol was added to suppress endogenous GUS-like activities (Kosugi *et al*., 1990). Samples were then washed with 70% ethanol, mounted on slide, and photographed using a light microscope.

## RESULTS AND DISCUSSION

### The assay of promoter activity

GUS activities were compared among transgenic *A. thaliana* with *#49-GUS, #225-GUS, #445-GUS, #502-GUS, #711-GUS, #2272-GUS* and the wild-type (WT). The results of the histochemical GUS assay in flowers at different developmental stages are shown in Fig. 1.

In flowers of transgenic *A. thaliana* with *#502-GUS*, the GUS activity (blue staining) was detected in anther at the tetrad stage (Fig. 1A). Similar intensities of blue staining were observed in the anther at the uninucleate microspore stage (Fig. 1B) and at the bicellular pollen stage (Fig. 1C). GUS expression was detected in the tapetum and not detected in the pollen (data not shown). No GUS activity was detected in the anther at the tricellular pollen stage, but GUS activity was observed in the ovule at the tricellular pollen stage (Fig. 1D). These results were consistent with the microarray

**Table 1**. Description of six anther-specific genes

| Name | Gene locus | Symbol | Description |
|---|---|---|---|
| #49 | At3g28790 | | Protein of unknown function |
| #225 | At3g17980 | ATC2 | Calcium-dependent lipid-binding (CaLB domain) family protein |
| #445 | At3g28780 | | Protein of unknown function |
| #502 | At5g64620 | ACT/VIF2 | Cell wall/Vacuolar inhibitor of fructosidase 2 |
| #711 | At5g59870 | HTA6 | A histone H2A protein |
| #2272 | At1g47980 | | Protein of unknown function |

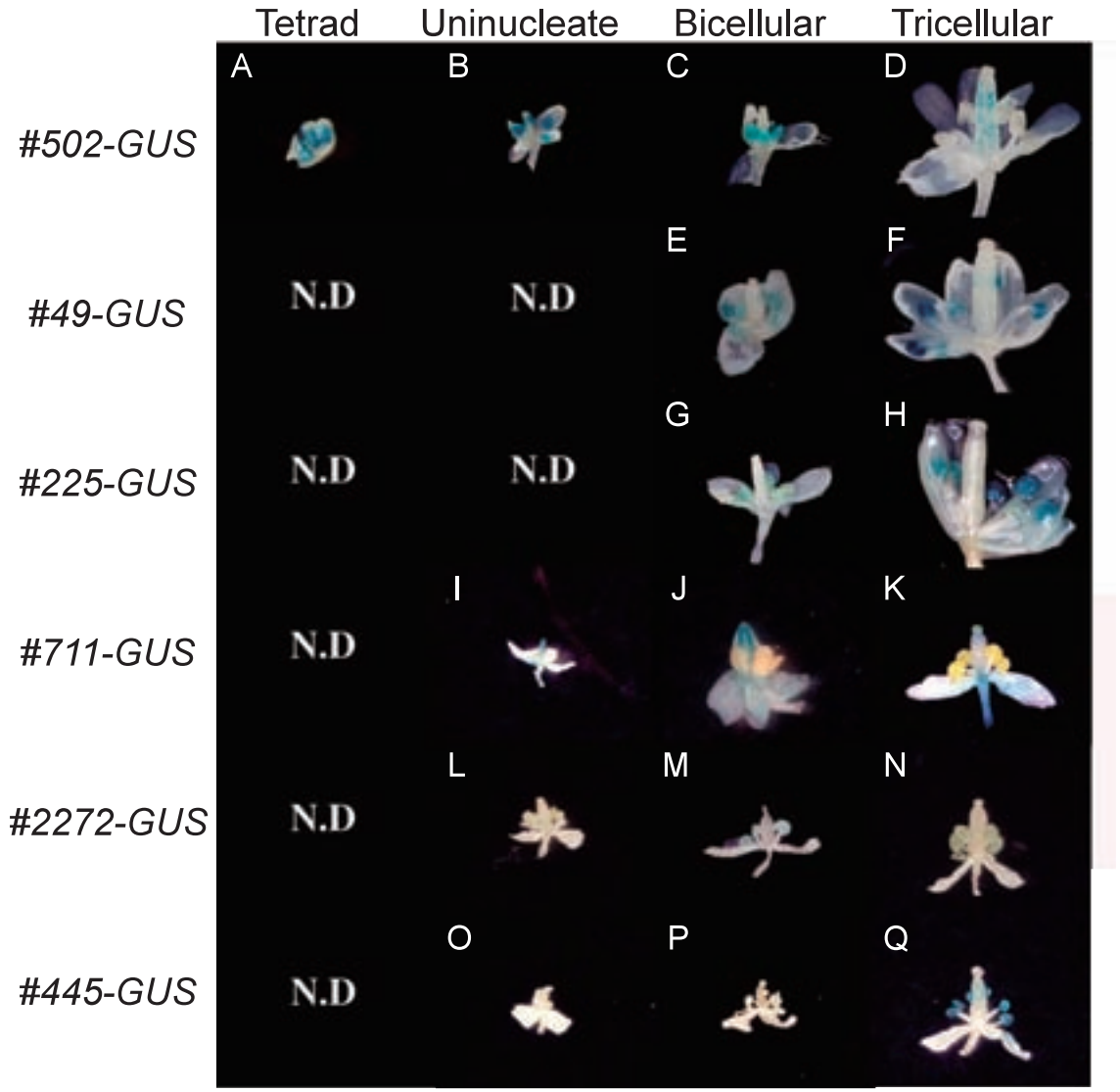


**Figure 1**. GUS expression in flowers at different developmental stages of transgenic *A. thaliana* plants with *#502-GUS* (A-D), *#49-GUS* (E, F), *#225-GUS* (G, H), *#711-GUS* (I-K), *#2272-GUS* (L-N), and *#445-GUS* (O-Q). Blue staining indicates GUS activity.

analysis using *Brassica oleracea* cDNA hybridized with custom cDNA array based on *A. thaliana* (Amagai *et al.*, 2003), suggesting that expression pattern is conserved between two species. Similar expression pattern was observed in *BnSKP1γ1* (*Brassica napus SKP1-like gene*) (Drouaud *et al.*, 2000). #502 encodes cell wall/vacuolar inhibitor of fructosidase (C/VIF). It has been shown that extracellular invertases encode small gene families that show specific spatial and temporal expression pattern (Godt and Roitsch, 1997). The identification of extracellular invertase isoenzymes from tomato, potato, and tobacco (Godt and Roitsch, 1997; Maddison *et al.*, 1999, Goetz *et al.*, 2001) that are expressed in anther tissues and play a critical role in anthers and pollen development, suggesting that #502 also plays a role in anther development of *A. thaliana* or *B. oleracea*.

In flowers of transgenic *A. thaliana* with *#49-GUS*, GUS activity was not detected until the uninucleate microspore stage, and a weak signal was first detected in the anther at the bicellular pollen stage (Fig. 1E). Strong GUS staining was observed in the anther at the tricellular pollen stage (Fig. 1F). These results were also similar to the microarray analysis (Amagai *et al.*, 2003), but the function of #49 is unknown.

In flowers of transgenic *A. thaliana* with *#225-GUS*, GUS activity was not detected until the uninucleate microspore stage, and a weak signal was first detected in the pollen at the bicellular pollen stage (Fig. 1G), but not observed in the tapetum at this stage (data not shown). Higher GUS activity was observed at the tricellular pollen stage (Fig. 1H). No GUS activity was detected in other floral organs, siliques, stems, leaves, and roots, indicating that #225 shows anther-

specific expression. However, the microarray data showed that the expression level is highest at the uninucleate stages and low-level expression was detected at the tricellular pollen stage (Amagai *et al*., 2003), indicating that the expression pattern is different between *A. thaliana* and *B. oleracea*. #225 encodes calcium-dependent lipid-binding (CaLB domain) family protein. Though its function has not yet been well understood, member of protein families including #225 negatively regulates responses to abiotic stress in *A. thaliana* (de Silva *et al*., 2011).

In flowers of transgenic *A. thaliana* with *#711-GUS*, GUS activity was detected in stigma and style at the uninucleate microspore stage (Fig. 1I). GUS activity was also detected stems, leaves, and roots. No activity was detected in the anther, though the expression was detected in anther by microarray data analysis (Amagai *et al*., 2003). #711 encodes for a histone H2A gene, *HTA6*. Histones are highly conserved components of eukaryotic chromatin, and histone H2A encoded by 13-members gene family in *A. thaliana* (Yi *et al*., 2006). Histone modifications regulate gene expression, growth, and development in plants.

In flowers of transgenic *A. thaliana* with *#2272-GUS*, GUS activity was not detected until the uninucleate microspore stage (Fig. 1L), and a weak signal was first detected in anthers at the bicellular pollen stage (Fig. 1M). GUS activity increased during pollen development and showed highest expression one day before anthesis (data not shown). A cross section of anthers at the bicellular pollen stage showed a blue staining of tapetum and pollen (data not shown). However GUS activity was not detected in the isolated pollen grains, indicating that GUS was expressed only in the tapetum in the anther at the bicellular pollen stage (data not shown). GUS activity was detected in the isolated pollen grain at the tricellular pollen stage (Fig. 1N) and one day before anthesis. No GUS activity was detected in other floral organs, siliques, stems, leaves and roots. These indicate that #2272 drives anther specific expression pattern though the protein of #2272 is not known. An anther specific promoter that is active both gametophytically and sporophytically has also been reported in *Polygalacturonase 4* (*PGA4*) (Ariizumi *et al*., 2003) and *S-locus protein 11* (*SP11*) (Shiba *et al*., 2001).

In the flowers of transgenic *A. thaliana* with *#445-GUS*, GUS activity was not detected until the bicellular microspore stage (Fig. 1O), and was first detected in anthers at the tricellular pollen stage (Fig. 1Q). GUS activity was observed in the isolated pollen grains at the tricellular pollen stage (data not shown). #445 shows anther specific expression pattern, but its protein function is unknown.

In wild-type plants, no GUS expression was detected in flowers at the tetrad stage, uninucleate microspore, bicellular and tricellular pollen stages (data not shown).

**The regulatory element of promoters**

To identify regulatory element, these six promoter sequences were analyzed. The putative TATA box sequence and several pollen-specific *cis*-acting regulatory element motifs such as AGAAA and GTGA, which are known to be involved in pollen/anther-specific expression (Twell *et al*., 1991; Rogers *et al*., 2001), were detected in the six promoters (Table 2). Multiple copies of *cis*-acting regulatory element motifs of AGAAA and GTGA in all promoters except for #2272 imply that these promoters have a potential to express at a pollen/anther-specific manner. However the *#711* promoter was not active in anther, although several pollen-specific *cis*-acting regulatory elements are present. As these promoters have redundancy in distribution of anther/pollen-specific *cis*-acting regulatory element, dissection of each *cis*-element will require identifying the role of individual *cis* element.

**Conclusion**

We report the anther/pollen-specific promoter activity of 5 genes, and these pollen/anther-specific promoters can drive gene expression in several stages of pollen development. The promoter sequences analyzed in this study will be useful for the characterization of genes differentially expressed in anthers. Therefore, these promoters, which can drive anther-specific expression, have potential applications in agriculture, especially in nuclear genic male-sterility.

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**Table 2**. *cis*-acting elements of the promoter region in six anther-specific genes

| | TATA box | AGAAA | GTGA |
|---|---|---|---|
| #49 | -76 | -53, -64-, -117, -377, -879 | -200 -227, -349, -889, -935 |
| #225 | -85 | -25, -41, -175, -240, -256 | -37, -142 |
| #445 | -249 | -61, -134, -584, -1058, -1067, -1468, -1761, -1806 | -484, -713 |
| #502 | -69 | -83, -484, -494, -688 | -221, -477, -514, -583, -809 |
| #711 | -96 | -301, -649, -698 | -499 |
| #2272 | -110 | - | -174 |

The first methionine was marked as +1

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# シロイヌナズナにおける葯特異的遺伝子のプロモーター解析

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要　約

葯特異的遺伝子やそれらのプロモーター配列の解析は、葯発達に関わる遺伝子の同定や、遺伝子組換えによる雄性不稔植物の作出を目的として、これまで多くなされてきている。本研究では、６つのプロモーター配列の単離を行い、GUS 遺伝子に連結してシロイヌナズナに導入することで、葯の発達段階における発現誘導様式を調べた。プロモーター GUS の発現は、様々な花粉発達ステージにおいて観察された。また、塩基配列の解析では葯 / 花粉特異的遺伝子の発現に必要とされるシス配列が多く含まれていたことから、これらのシス配列が葯特異的に発現誘導していることが示唆された。

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