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Functional analysis of a rice late pollen-abundant UDP-glucose pyrophosphorylase (*OsUgp2*) promoter

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Abstract *OsUgp2*, a rice UDP-glucose pyrophosphorylase gene, has previously been shown to preferentially express in maturing pollens and plays an important role in pollen starch accumulation. Here, a 1943 bp promoter fragment (P1943) of *OsUgp2* was characterized by 5′ deletion and gain-of-function experiments. P1943 and its 5′ deletion derivatives (P1495, P1005, P665 and P159) were fused to *GUS* reporter gene and stably introduced into rice plants. Histochemical analyses of different tissues and pollens at different developmental stages of the transgenic plants showed that P1943 could only direct *GUS* expression in binucleate pollens. P1495 and P1005 could still drive *GUS* expression in binucleate pollens but at a lower level. On the other hand, neither P665 nor P159 transformant exhibited any GUS activity in pollens. Gain-of-function analyses showed that the region (−1005 to −665 relative to translation start site) combined with a minimal CaMV 35S promoter could direct *GUS* expression in pollens. Further analysis of 5′ deletion truncated at −952, −847 and −740 delimited a 53 bp region (−1005 to −952) essential for pollen-specific expression. The 53 bp sequence contains two motifs of TTTCT and TTTC, which were known to be pollen-specific *cis*-elements. In addition, the same P1943-GUS fusion construct was introduced into tobacco to analyze its specificity in dicotyledon. Interestingly, the GUS expression pattern in transgenic tobacco was quite different from that in rice. High level of GUS expression was detected in mature pollens as well as leaves, roots, sepals and stigmas. These findings suggested a complicated transcriptional regulation of *OsUgp2*.

Keywords Promoter · Pollen · Rice · UDP-glucose pyrophosphorylase · *OsUgp2*

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Introduction

Pollen development is an important male reproductive process in flowering plants. It takes place within a sporophytic anther tissue in a precise chronological order [1, 2]. Pollen development consists of two distinct sequential phases: one is microsporogenesis representing the processes of pollen mother cell to unicellular microspore, and the other is microgametogenesis denoting the stages of microspore to mature pollen [3]. The development of pollen involves participation of a large number of genes coordinately expressed in the gametophytic and surrounding sporophytic cells. Genes related to pollen development are broadly classified into groups of "early" and "late" genes, which are termed to correlate with the formation of young microspore and associated with pollen maturation and pollen function, respectively [4, 5]. Comparative analyses on sporophytic and gametophytic transcriptome datasets indicated that approximately 7200 genes specifically or preferentially expressed in mature pollen grains and usually express with a characteristic of high expression levels [3, 6–8]. A number of "late" pollen genes have been isolated and were found to be expressed in a similar pattern of which transcripts appear around the first mitosis of microspore and increase abundantly with pollen maturation

[9–15]. The similar expression profile of these "late" pollen genes suggested that their expressions are possibly modulated by a common regulatory mechanism. However, the knowledge underlying the regulatory mechanism during late pollen development is still limited.

The spatio-temporal expression of gene is mostly co-determined by the types, numbers and positions of *cis*-regulatory elements within the promoter region. The expression regulation of some pollen-specific genes has been investigated by promoter function analysis based on 5′-deletions, site-directed mutagenesis and gain-of-function experiments in transient and stable transformants. A number of *cis*-regulatory elements including LAT52/56 box, LAT56/59 box, PB core motif, AGAAA and TCCACCATA were identified by dissecting proximal promoter regions of tomato pollen-specific *Lat52*, *Lat56* and *Lat59* genes [16–19]; Functional analyses of the regulatory regions of the pollen-specific tobacco *NTP303*, g10 genes resulted in the identification of sequence motif AAATGA and GTGA [10, 13]. The pollen-specific quantitative element AGGTCA was also found exist in the promoter region of a maize pollen-specific *ZM13* gene [20]. Further identification of such pollen-specific regulatory *cis*-elements should not only provide more insight into the molecular basis of pollen development, but also be of great use in finding its *trans*-acting factors, producing chimeric promoter for applications such as gene function analysis directed to pollen development and producing male sterility for breeding.

We have previously isolated and characterized *OsUgp2*, a rice UDP-glucose pyrophosphorylase gene, which belongs to the "late" pollen gene as it preferentially expressed in maturing pollens. Its expression began at early binucleate stage and reached the highest level in mature pollens [21]. In the present study, we further characterized the promoter function of *OsUgp2* by employing deletions and gain-of-function experiments in stable transformants. The minimal sequence required for pollen-specific expression was defined to a novel 53 bp sequence. The organization of putative regulatory *cis*-elements for late-pollen expression of the *OsUgp2* gene was also discussed.

Materials and methods

Cloning of *OsUgp2* promoter and its sequence analysis

According to the cDNA sequence of *OsUgp2* (AF249880), its corresponding genomic sequence (AP004121) was found in the GenBank database. The predicted promoter region of *OsUgp2* was cloned by PCR amplification and the specific primers P1943/Pr used are listed in Table 1. The amplified promoter fragment (−1943/−5, relative to the translation start site) was designated as P1943. The promoter fragment P1943 was sequenced and analyzed by the PLACE database [22] and PlantCARE database [23] to find putative functional promoter elements.

Generation of promoter deletion-GUS constructs

The promoter fragment P1943 was digested with *Pst*I and *Bam*HI, and fused to GUS gene that was inserted in the primary vector pCAMBRIA1300-G (provided by Linjian Huang, Institute of Plant physiology and Ecology,

Table 1 Oligonucleotides primers

Oligo names	Primers sequence	Underlined endonucleases
P1943	5′ CATCTGCAGGTACATATCAAGCACTCTGAC 3′	*Pst*I
P1495	5′ CATCTGCAGTCTGGTTAGGCAGGGTTCC 3′	*Pst*I
P1005	5′ CATCTGCAGGGTGCAGTATCAGCAGTGC 3′	*Pst*I
P952	5′ CATCTGCAGTACATCACCCTGGAACATGC	*Pst*I
P847	5′ CTTCTGCAGTCAGCCCCTAATGATGATTC	*Pst*I
P740	5′ CATCTGCAGAGTGAAGCTAGTAAGGGTGC	*Pst*I
P665	5′ CATCTGCAGGAACCCTGAATGTTACCTCC 3′	*Pst*I
P159	5′ CATCTGCAGATTGTCCGCCATTGCTTTCG 3′	*Pst*I
Pr	5′ GCTGGATCCGTGTTCTTGCCGCTGCTTC 3′	*Bam*HI
P_{A35S} P_{AMCS}	5′ GGATCTGCAGAGTCAGATCTGATATCTCCACTGACGTAAGG	*Pst*I, *Bgl*II
P1445r	5′ CAGGACGTAACATAAGGGAC	*Bgl*II
P1005r	5′ GATAGATCTGAGAATAACTGATAAGAGC	*Bgl*II
P665r	5′ CCTAGATCTCGTAAAACATTCTGAATCAT	*Bgl*II
GUSf	5′ GTCAGATCTGGTTCTTCAGGTTGTTGGC	
GUSr	5′ CTGTGGGCATTCAGTCTGGATCG 3′	
	5′ GTTACCGCCAACGCGCAATATGC 3′	

Shanghai, China). The derived construct was designated as p1943-G. The 5′ deletions of *OsUgp2* promoter were generated by PCR and plasmid p1943-G was used as template. All oligonucleotides used are given in Table 1. Sense oligonucleotides encompassing 5′-*Pst*I restriction sites were designed at −1495, −1005, −952, −847, −740, −665, and −159 (relative to start site of translation). All sense oligonucleotides were pair-used with the antisense primer Pr, including a *Bam*HI site at the 5′ end, to generate 5′ deletions. The PCR products of 5′-truncated promoter fragments were subcloned to primary vector pCAMBRIA1300-G individually and produced plasmids p1495-G, p1005-G, p952-G, p847-G, p740-G, p665-G, and p159-G.

Gain-of-function promoter-GUS fusion constructs

A minimal 35S promoter (−90/+8) was amplified by primers $P_{\Delta 35S}/P_{\Delta MCS}$ and substituted for the 35S promoter in the primary vector pCAMBRIA1300-G. The derived vector named as Δ35S::*GUS*. Fragments F1–F3 (F1: −1943/−1495; F2:−1945/−1005; F3:−1005/−665) were generated by PCR using primers P1943/P1495r, P1495/P1005r and P1005/P665r. The cloned F1–F3 fragments were subcloned into Δ35S::*GUS* individually by cutting with *Pst*I and *Bgl*II. The generated constructs were named F1Δ35S::*GUS*, F2Δ35S::*GUS* and F3Δ35S::*GUS*.

Plant transformation and growth conditions

Rice Zhonghua 11 (*Oryza sativa* L. *var. japonica*) was used for transformation. A series of 5′ deletion-GUS and gain of function promoter-GUS fusion constructs were stably introduced into rice using the *Agrobacterium*-mediated transformation according to the procedures [24]. Tobacco Chinese cultivar Zhongyan 90 (*Nicotiana tabacum* L. *cv. Zhongyan 90*) was transformed by *Agrobacterium*-mediated leaf disc transformation. All transformants were confirmed for presence of the foreign GUS gene by PCR and Southern Blotting. Transgenic rice and tobacco were potted in greenhouse under routine management. The developmental stages of rice pollens were examined by light microscopy according to Feng et al. [25].

GUS activity assay

Histochemical staining for GUS activity was performed according to the method of Svab et al. [26]. Tissues were obtained from primary transgenic plants and wild-type plants as a negative control. Anthers at five different pollen developmental stages were sectioned with the cryomicrotome (Leica) and then detected by GUS histochemical analysis. Tissues or sections on slides were incubated in fresh prepared GUS staining solution in dark at 37°C for overnight. Every milliliter staining solution contained *N*,*N*-dimethylformamide 20 μl, X-Glu 50 mg, 100 mM sodium phosphate buffer 980 μl (pH 7.0), 5 mM $K_3Fe(CN)_6$ 5 μl, 5 mM $K_4Fe(CN)_6$ 5 μl, and 0.1% Triton X-100 1 μl). After staining, chlorophyll was cleared from the sample by 70% ethanol treatment. The stained samples were examined using a Leica DMRXA microscope. For each construct vector, at least three independent transformants were subjected to histochemical staining.

Fluorometric assay for GUS activity was carried out according to the method of Jefferson et al. [27] with some modifications. Leave or panicles were homogenized in GUS extraction buffer (50 mM PBS, pH 7.0, 1 mM Na_2EDTA, 0.1% Sarkosyl, 0.1% Triton X-100, and 10 mM b-mercaptoethanol). The homogenate was then centrifuged for 10 min at 12,000×*g* at 4°C, and the supernatant was used to assess the GUS activity. Briefly, aliquots of the extracts (100 μl) were added to 900 μl of pre-warmed assay buffer [extraction buffer containing 2 mM 4-methylumbelliferone (MU)], and then incubated at 37°C for 10 min. After incubation, 200 μl samples were removed and placed in 800 μl stop buffer (200 mM sodium carbonate). Fluorescence was determined using a fluorescence spectrophotometer (HITACHI). Protein concentration in plant extracts was measured by the procedure of Bradford [28]. GUS activity was expressed as picomole 4-MU formed per minute per milligram total protein. For each construct vector, at least five independent transgenic plants were detected, and three replicates were performed for each sample.

Results

Sequence analysis of 5′ upstream promoter region of *OsUgp2* gene

According to the *OsUgp2* cDNA (AF249880), its corresponding gene was found in the genomic sequence (AP004121/BAC clone OJ1442_E05) of chromosome 2. The 2 kb upstream promoter region of *OsUgp2* was predicted in Rice Genome Automated Annotation System (http://RiceGAAS.dna.affrc.go.jp/). The putative transcription start site was predicted at −60 relative to the translation start point by software of Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/promoter.html). Two putative TATA-box (one in reverse orientation) are located at positions −87 to −95, and three CAAT-boxes (two in reverse orientation) are located at −146 to −149, −156 to−159 and −399 to −396, respectively (Fig. 1). To look for putative *cis*-acting elements related to pollen-specific expression, the PLACE database [22] and

Fig. 1 Nucleotide sequence of *OsUgp2* promoter. The first base of translation start site A was designated as +1. The predicted transcription initiation site is denoted by an *asterisk*. The putative TATA-box and CAAT-box are *boxed*. The predicted pollen-specific *cis*-acting elements "GTGA" and "AGAAA" are *underlined*. Those *cis*-elements that exist in reverse orientation are in *italics*. PB core, lat56/59 elements and GAAA motif are *underlined with double lines*. All putative enhancer "TGTGG/TGGTTA/TGTGA" are indicated with *waveline*. The nucleotide positions of the 5′-upstream −1943, −1495, −1005, −952, −847, −740, −665 and −159 are numbered. The sequence marked by dotted line matches the 5′ UTR of *OsUgp2* cDNA (AF249880)

PlantCARE database [23] were utilized to analyze the 5′-flanking promoter region of the 2 kb length. Fifteen tobacco g10 late pollen regulatory elements "GTGA" [13] and eight lat52 pollen-specific activation elements "AGAAA" [19] were found in this promoter region (Fig. 1). Additionally, a PB core element "TGTGGTT", a lat56/59-box "GAAWTTGTGA", a GAAA motif (in reverse orientation) and a total of six core motifs of enhancer "TGTGG", "TGTGA" and "TGGTTA" [16–19] were identified (Fig. 1). These *cis*-acting elements are mostly clustered on the regions of −700 to −1200 and −1500 to −1700 (Fig. 6a). The presence of these pollen-specific related motifs implicated some mechanisms of *OsUgp2* preferentially expressed in late pollen development stage.

Spatial and temporal expression of GUS gene under the control of *OsUgp2* promoter in transgenic rice and transgenic tobacco

To investigate the spatial and temporal regulation of *OsUgp2* promoter, the isolated promoter fragment (P1943) was fused with GUS reporter gene. The derived construct p1943-G was stably introduced into rice. The primary construct pCAMBIA1300-G, in which GUS gene was driven by 35S promoter, was transformed as positive control, and wild type rice was used as a negative control. Transformants were determined by PCR and Southern blot (data not shown). More than three independent transformants were obtained for each construct. Histochemical staining showed that the 35S promoter directed efficient expression of GUS gene in all types of rice tissues examined, including callus, roots, stems, seeds, ovaries as well as anthers and pollens at binucleate stage (Fig. 2). However, the promoter of *OsUgp2* only drove GUS gene expression in binucleate pollen but not in any other tissues. No GUS activity was detected in the corresponding tissues from wild type rice (Fig. 2). The results demonstrated that the promoter of the *OsUgp2* gene was pollen-specific.

The expression of GUS gene under the control of *OsUgp2* promoter during different pollen developmental stages was also examined. Anthers at the five different pollen developmental stages of pollen mother cell, meiosis, uninucleate microspore, binucleate pollen and mature pollen were sectioned with cryomicrotome and detected by GUS histochemical staining. The results revealed that high level of GUS activity was observed in pollens at the binucleate stage. On the other hand, only a weak GUS activity was detected in mature pollens (Fig. 3). In positive control, the 35S promoter could also direct GUS gene expressed in pollens at the binucleate stage (Fig. 3) as well as in vascular bundle of anther at the meiosis developmental stage (data not shown).

The activity of *OsUgp2* promoter was also tested in stably transformed tobacco plant. Histochemical staining of the primary transformants with stable integration of the P1943-GUS gene fusion exhibited high level of GUS

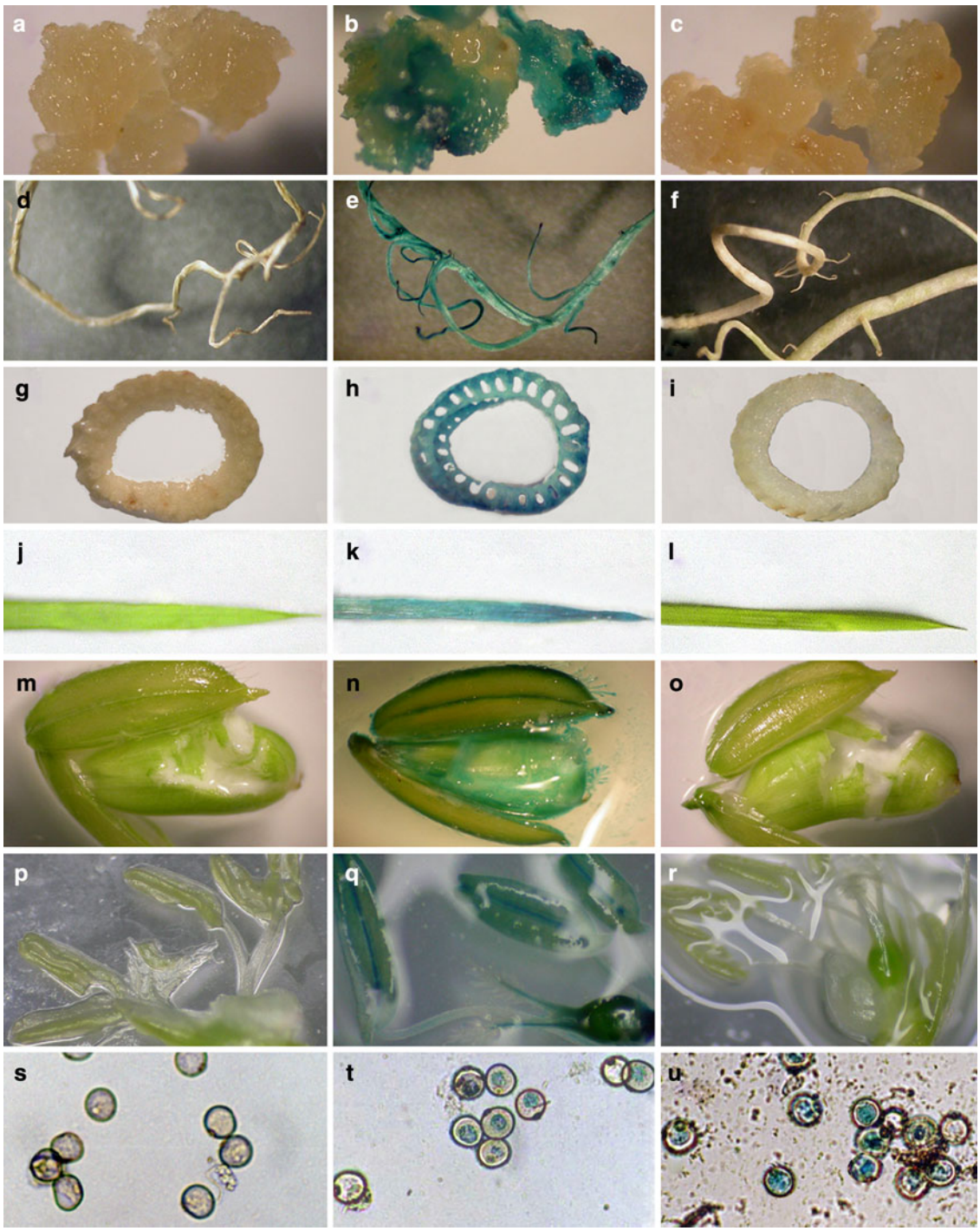
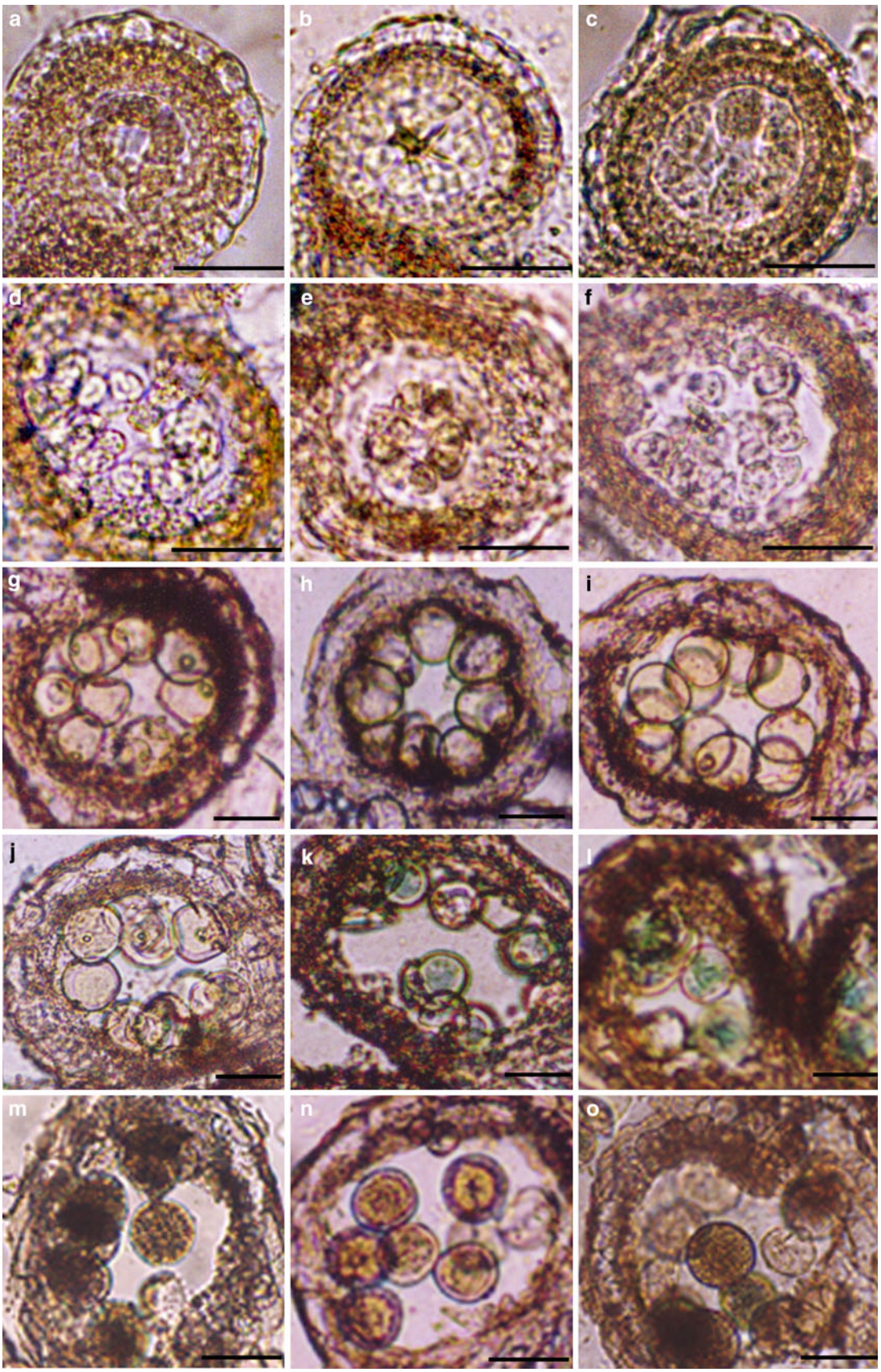


Fig. 2 Histochemical GUS staining of different tissues from rice. *Left panel* (**a**, **d**, **g**, **j**, **m**, **p**, **s**) wild type rice (negative control), *middle panel* (**b**, **e**, **h**, **k**, **n**, **q**, **t**) transgenic rice transformed with pCAMBIA1300-G (positive control), and *right panel* (**c**, **f**, **i**, **l**, **o**, **r**, **u**) transgenic rice transformed with p1943-G, **a–c** callus, **d–f** root, **g–i** stem, **j–l** leave, **m–o** seed, **p–r** anther and ovary **s–u** pollens at binucleate development stage

activity in leave and some old roots but not in young roots. GUS activity was also found in sepals and stigmas (Fig. 4). Florets at the five developmental stages were collected for detection of GUS activity in pollens. No GUS activity was detected in pollens at the early developmental stages I-III. A minor GUS activity was found in maturing pollens (Stage IV); while high level of GUS activity was observed in mature pollens (Stage V) (Fig. 4).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◀ **Fig. 3** Histochemical GUS staining of pollens at five different developmental stages. *Left panel* (**a**, **d**, **g**, **j**, **m**) wild type rice (negative control), *middle panel* (**b**, **e**, **h**, **k**, **n**) transgenic rice transformed with pCAMBIA1300-G (positive control), and *right panel* (**c**, **f**, **i**, **l**, **o**) transgenic rice transformed with p1943-G. Sections of anther in **a–c, d–f, g–i, j–l** and **m–o** represent anther at the pollen mother cell, meiosis, uninucleate microspore, binucleate and mature pollen developmental stages, respectively. All *scale bars* = 50 μm

The 53 bp region from −1005 to −952 is involved in pollen-specific expression of *OsUgp2*

In order to characterize the function of the *OsUgp2* promoter, four promoter derivatives of 5′-progressive deletions were fused to the GUS reporter gene (Fig. 5) and

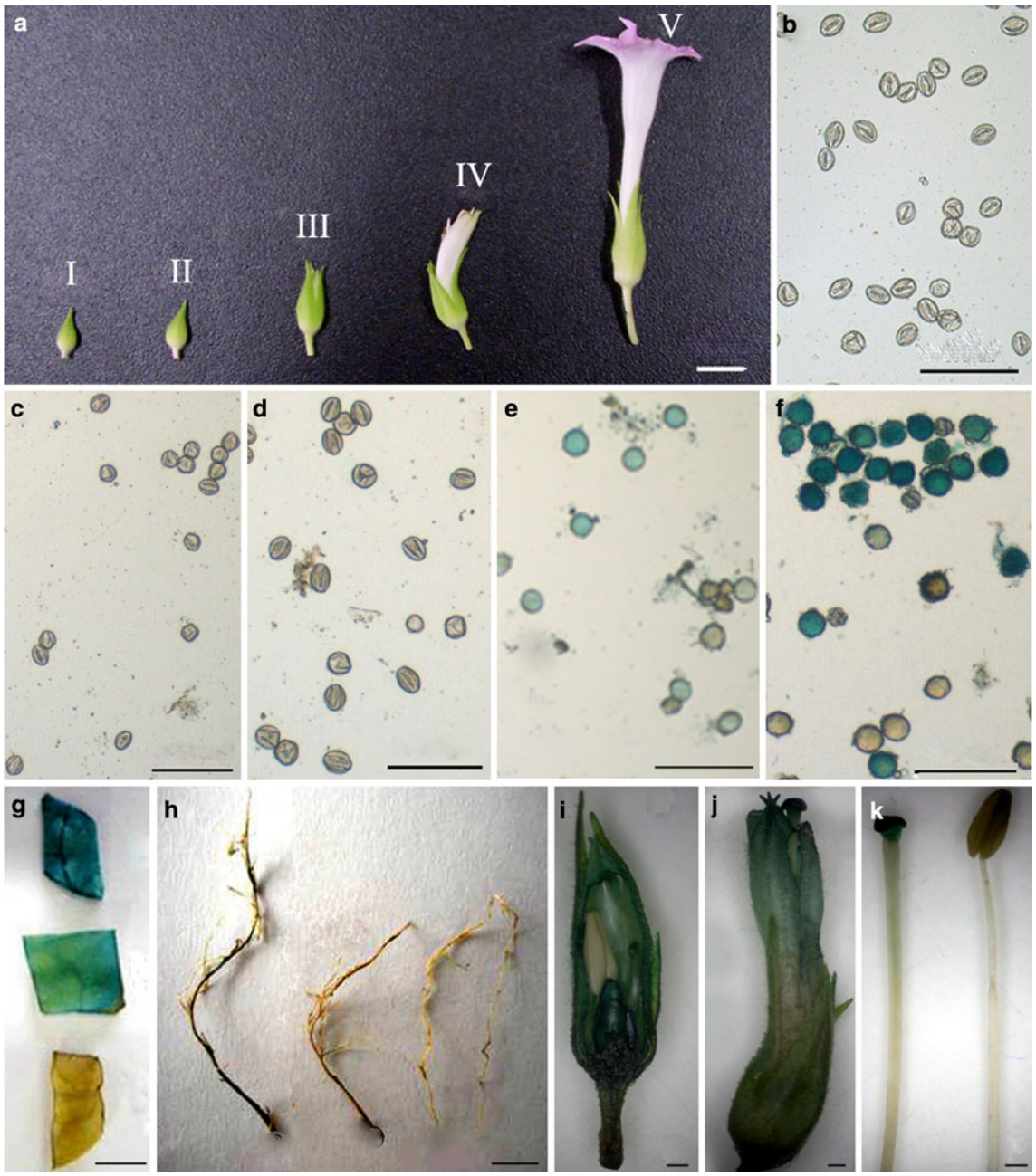

Fig. 4 Histochemical GUS staining of different tissues from transgenic tobacco. **a** Florets developmental stages I–V; **b–f** pollens squeezed from the anthers corresponding to floret stages I–V; **g** leave slice, the lowest *yellow* one is negative control; **h** root, old root (*left*), young root (*right*); **i–j** florets at stage IV and V; **k** stigma and anther from florets at stage V. *Bars* equals to 1 cm in **a**, 100 μm in **b–f**, 0.5 cm in **g–h** and 1 mm in **i–k**

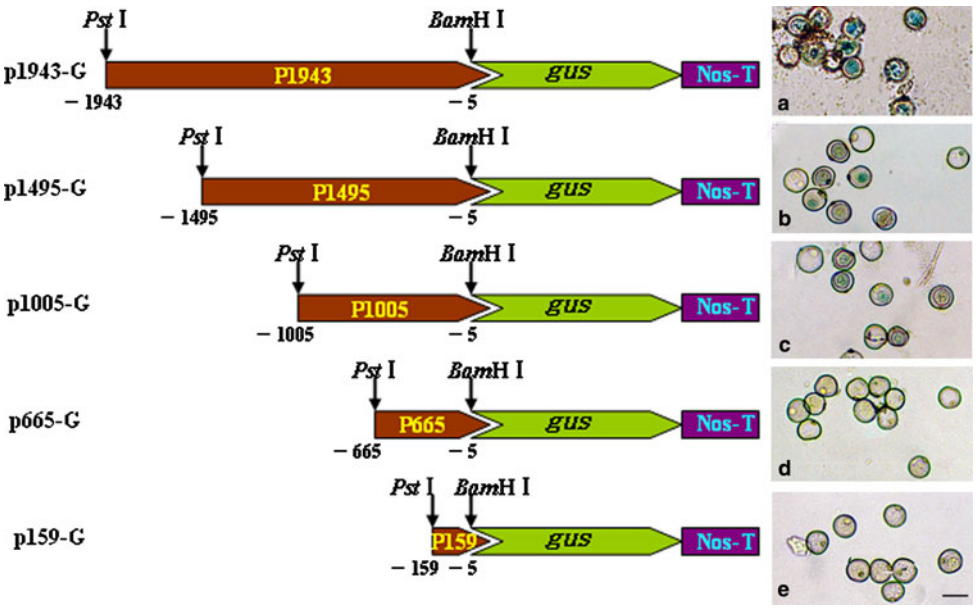

Fig. 5 Schematic illustration of 5′ deletion constructs and GUS activity in pollens from their corresponding transgenic rice. **a–e** Binucleate pollen from transgenic rice harboring construct p1943-G, p1495-G, p1005-G, p665-G and p159-G, respectively. *Scale bar* 50 μm

transformed into rice. As previous study (Fig. 3) showed that high GUS activity in pollen of P1943-G transgenic rice was mainly observed in binucleate pollen grain, the GUS activity in binucleate pollens of transformants was histochemically stained. The results demonstrated that P1495 and P1005 could still drive *gus* gene expression in pollen but at a lower level compared with full sequence promoter. On the other hand, none of the other two deleted promoter fragments (P665 and P159) exhibited pollen-specific GUS activity (Fig. 5). These results suggested that the promoter region −1005 to −665 is critical for GUS expression in pollen.

In an attempt to characterize the function of three promoter regions −1943 to −1495, −1495 to −1005, −1005 to −665 (F1, F2 and F3) deleted above, "Gain of function" analysis was performed. Three fragments F1–F3 were cloned and fused with the minimal 35S promoter. The generated constructs F1Δ35S::*GUS*, F2Δ35S::*GUS* and F3Δ35S::*GUS* were stably introduced into rice, and the minimal Δ35S::*GUS* were used as a control. GUS activity was observed in palea/lemma and ovary of all transformed rice as well as the control (data not shown). However, accumulation of GUS activity in pollens was only detected in the transformants with F3Δ35S::*GUS* (Fig. 6b). Moreover, quantitative GUS assay showed that the ability of enhancement of F3 was stronger than that of F1 and F2 in spikelets, but weaker in leaves (Fig. 6d). These findings of gain-of-function experiments further indicated that promoter region −1005 to −665 probably contain *cis*-elements related to pollen-specificity, and also suggested the presence of enhancers in both promoter regions F1 and F2.

On the basis of 5′-deletions and "gain of function" analysis, a further deletion analysis was carried out within promoter region −1005 to −665 to locate the *cis*-elements related to pollen-specific expression. Deletion from −1005 to −952 resulted in losing almost all activity of *OsUgp2* promoter in pollens (Fig. 6c), suggesting the 53 bp (−1005 to −952) region plays a crucial role in conferring the pollen expression pattern of this promoter.

Discussion

Previous studies have revealed the pollen-abundant expression of a rice native UDP-glucose pyrophosphorylase (*OsUgp2*) and its rather low transcription level in leaves [21, 29]. In an attempt to locate the potential *cis*-regulatory elements which are important for pollen expression, a 1943 bp sequence upstream of the ATG translation start site of *OsUgp2* was cloned and analyzed in this study. The spatial and temporal expression activities of this promoter were firstly clarified by dissecting GUS activity in transgenic plants containing the *OsUgp2* promoter-GUS fusion construct. The construct was stably transformed into both rice and tobacco in order to examine if the function of this promoter is conserved between different species of monocot (rice) and dicot (tobacco).

In transgenic rice, histochemical GUS staining indicated GUS activity was restricted to pollens and not detectable in any other vegetative or flora tissues examined, which correlated well with the previous studies on expression pattern of *OsUgp2* [21]. In contrast to endogenous *OsUgp2,* strong GUS activity was only found in binucleate pollens but not observed in rice mature pollen grains. The discrepancy suggested that the 1943 bp 5′ flanking DNA of *OsUgp2* we used in GUS fusion construct was sufficient to

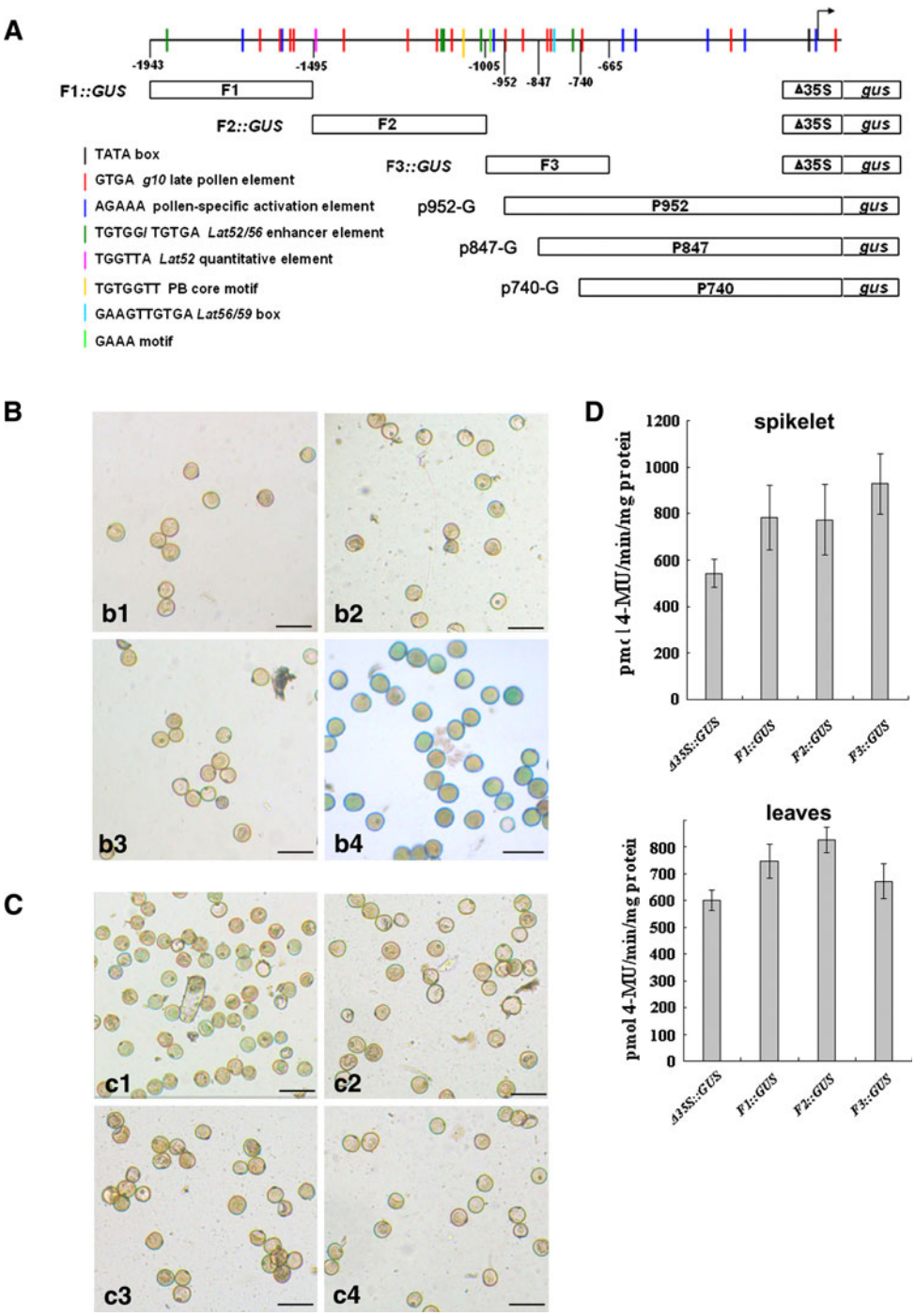

Fig. 6 Histochemical and quantitative analyses of GUS activity changes in transgenic rice containing gain-of-function and promoter deletion constructs. **A** physical map of *OsUgp2* promoter and promoter structures in F1Δ35S::*GUS*, F2Δ35S::*GUS,* F3Δ35S::*GUS*, p952-G, p847-G and p740-G; **B**, **C** histochemical analysis of GUS activity in binucleate pollens of transgenic rice. *b1–b4* pollens in rice plants transformed with Δ35S::*GUS*, F1Δ35S::*GUS*, F2Δ35S::*GUS,* and F3Δ35S::*GUS* respectively, *c1–c4* pollens in transformed rice containing constructs p1005-G, p952-G, p847-G and p740-G respectively; **D** quantification of GUS activity in the spikelets and leaves of transgenic rice transformed with Δ35S::*GUS*, F1Δ35S::*GUS*, F2Δ35S::*GUS,* and F3Δ35S::*GUS* respectively. The *error bars* represent standard deviations of the means of five samples. *Scale bars* 100 μm

direct gene expression in an essentially pollen-specific manner, but deficient for high level gene expression in mature pollens. The expression profile lacking correlation between the endogenous gene and the introduced promoter-GUS fusion construct was also found in tomato *lat 52* and *lat 59*. In their report, absent transcriptional repressor sequence and differences such as mRNA stability, processing or translation between native transcripts and chimeric GUS activity was proposed to account for the observed discrepancies [16]. We thus postulated some *cis*-elements probably reside in the upper region and had tried a longer 5′ upstrem sequence (3000 bp) instead of 1943 bp in GUS fusion construct. However, a similar result was obtained for this longer construct (data not shown).

To date, accumulating evidences have showed that introns can affect both gene expression level and expression profile [30–32]. Introns located in 5′ UTR, coding region or 3′ UTR were found to regulate gene expression

not only in the amount but also the tissue-specificity [33–41]. Furthermore, sequences close to the translation start codon were also reported to enhance gene expression as much as exons [36, 42, 43]. The 1943 bp *OsUgp2* promoter ranging from −1943 to −5 relative to translation start point included the 5′ UTR region but excluded the initial AUG flanking region, introns and 3′ UTR. Therefore, it is possible that the *OsUgp2* upstream sequences used as the promoter for GUS fusions do not contain all necessary elements for full expression. Some *cis*-elements important for regulation of GUS expression in mature pollen of transgenic rice probably existed in the downstream intron or 3′ UTR regions.

In contrast to the expression pattern in rice, the same 1943 bp promoter drove GUS expression not exclusively in pollen but also in leave, root, sepal, petal and stigma in transgenic tobacco. Similar expression pattern was also observed in transgenic *Arabidopsis* (data not shown). Although the tissue-specific pattern changed in transgenic tobacco and *Arabidopsis*, the temporal expression pattern in pollen correlated well with that of the native *OsUgp2* gene. High GUS activity accumulated in pollen at late developmental stages, especially in mature pollens; and no GUS activity was detected in pollen at early developmental stages. Spatiotemporal promoter-GUS analysis in different plant species was widely used to demonstrate the functional conservation of promoter sequence, and results showing promoter activity conservative or changed across plant species had been documented [12, 44–47]. Our result here suggested that the mechanisms regulating *OsUgp2* promoter in rice and tobacco were not evolutionally conserved as the same promoter resulted in two different GUS expression patterns between transgenic rice and tobacco. The finding also indicated that it is important to test the promoter activity in various plant species before the application of the anther/pollen-specific promoter in producing male sterility or targeting gene function analysis.

5′ deletions and gain-of-function experiments resulted in the identification of a 53 bp region vital for pollen-specific expression and two regions F1 (−1943 to −1495) and F2 (−1495 to −1005) that are involved in expression enhancement. Within the defined 53 bp region, only two potential pollen-specific *cis*-elements "TTTCT" and "TTTC" were found, and both are present in reverse orientation. Pollen-specific activation element "AGAAA" and "GAAA" motif had been identified in tomato pollen-specific gene *lat52* [18, 19] and were found to be conservative within the region essential for some late-pollen promoters [40, 48–51]. Regulatory element TTTCT, which is the reverse complementary sequence of the AGAAA motif, was defined to serve a vital role in pollen-specific expression of maize *Zm13* [20]. Moreover, one to five copies of the potential enhancers TGTGG,TGGTTA and TGTGA [16] were found in region F1 and F2. The reverse orientated TTTCT and TTTC motifs and the putative enhancers mentioned above would be mutated to elucidate their functions in directing pollen-abundant expression of *OsUgp2* in our following studies.

In summary, our present study identified a specific 53 bp region of the *OsUgp2* promoter that confers pollen-specific expression at binucleate pollen stage. Two sequences located upstream of −1005 were shown to modulate *OsUgp2* expression level in pollens, though not essential for its expression *per se*. The actual function(s) of the two potential pollen-specific regulatory elements "TTTCT" and "TTTC" in the 53 bp region and other potential enhancers in the two 5′-upstream regions await verification by mutagenesis analysis. Moreover, the mechanism(s) underlying the discrepancy of expression pattern between native and promoter-GUS chimeric gene need further studies so as to identify more regulatory elements that work collaboratively with the identified elements here to regulate the preferentially high level expression of *OsUgp2* in mature pollens.

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