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Isolation and characterization of an anther-specific gene, RA8, from rice (*Oryza sativa* L.)

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Abstract

An anther-specific cDNA clone of rice, RA8, was isolated from an anther cDNA library by differential screening. RNA blot analysis indicated that the RA8 transcript is present specifically in anthers and the transcript level increased as flowers matured, reaching the highest level in mature flowers. The RA8 clone contains an open reading frame of 264 amino acid residues with a hydrophobic N-terminal region. The deduced amino acid sequences did not show significant homology to any known sequences. Genomic DNA blot analysis showed that RA8 is a single-copy gene. A genomic clone corresponding to the RA8 cDNA was isolated and its promoter region was fused to the β-glucuronidase (*GUS*) gene. Transgenic rice plants exhibited anther-specific expression of the *GUS* reporter gene. Histochemical GUS analysis showed that the RA8 promoter was active in the tapetum, endothecium, and connective tissues of anthers. Experiments showed that expression of the gene starts when microspores are released from tetrads, and it reaches to the maximum level at the late vacuolated-pollen stage. The RA8 promoter may be useful for controlling gene expression in anthers of cereal plants and for generating male-sterile plants.

Introduction

Male reproductive processes in flowering plants occur in the anther [8]. This organ is composed of several tissues and cell types and is responsible for producing pollen grains that contain the sperm cells. The study of anther development is immensely important because not only for the understanding of gene regulation in the sexual reproduction of flowering plants, but also because of its potential application in agriculture such as male-sterility.

Anther development can be divided into two general phases [11]. During phase 1, the morphology of the anther is established and microspore mother cells undergo meiosis to generate tetrads of microspores. During phase 2, pollen grains and anthers differentiate and tissue degeneration, dehiscence, and pollen grain release occur. These processes are accomplished by many diverse genes expressed in various tissues of the anther [18]. Among these genes, only a small fraction (10–20%) is anther-specific, while the large majority is also expressed in other tissues [43, 44]. The former are useful for producing male sterility by antisense expression of the gene or use of anther cell-specific promoters that are fused to cytotoxic genes, such as the diphtheria toxin gene or RNase genes [21, 23, 28, 46].

In recent years, several laboratories have identified cDNA clones that are specific or preferential to the anther. Some anther-specific clones, such as TA29 and TA32 of tobacco [21], SF2 and SF18 of sunflower [7], Osc4, Osc6, YY1, and YY2 of rice [15, 40], 108 of tomato [37], and BA112 and A9 of *Brassica napus*

The nucleotide sequence data reported will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number AF042275.

[34, 36] are found exclusively in sporophytic tissues of the anthers. Others are pollen grain-specific or are present in both sporophytic and gametophytic tissues of the anthers. NTM19 of tobacco [29], BA42 of *B. napus* [36], LAT52, 56, and 59 of tomato [42], Zmc13 of maize [13], LMP131A of lily [19], and α_1-tubulin of *Arabidopsis thaliana* [22] belong to this category.

It is believed that temporal and spatial regulation of anther-specific gene expression is primarily controlled at the transcriptional level [11]. This was confirmed by transgenic approaches using chimeric gene fusions between the 5′ sequence of an anther-specific gene and either the β-glucuronidase, diphtheria toxin, or barnase gene. With these approaches, several genes, including TA29 and NTM19 of tobacco [5, 21], LAT52 of tomato [41], α_1-tubulin and MS2 of *A. thaliana* [1, 20], Zmg13 of maize [12], Bgp1 of *Brassica campestris* [45], and Osg6 of rice [47], were shown to be expressed in specific cell types of anthers.

Genes expressed in anthers for male gametogenesis can be divided into two groups based on their expression timing [24]. The genes of the first group begin to express soon after meiosis, reaching maximum expression level by the late pollen interphase and decreasing thereafter. The I3 and E2 clones of *B. napus*, which fall into this 'early' gene category, have been isolated [10, 30]. The genes in the second group start to express after microspore mitosis and the expression increases until maturity. These 'late' genes have been isolated from a number of plant species including the tobacco, tomato, *A. thaliana*, *B. napus*, *Oenothera organensis*, sunflower, petunia, ragweed, ryegrass, maize, and lily [26, 27]. Most of these gene products are accumulated abundantly in pollen grains and are involved in pollen maturation or germination.

In this report, we describe the isolation of an anther-specific cDNA clone, RA8, from rice that is specifically expressed in the tapetum, endothecium, and connective tissues. We show that the 5′ sequence of the RA8 gene confers sporophytic cell-specific expression in anthers of transgenic plants during anther development.

Materials and methods

Plant materials and bacterial strains

Rice (*Oryza sativa* L. cv. Nackdong) flowers at the late-vacuolated stage were cross-sectioned into three parts. The middle portion, which contains intact anthers and parts of the palea and lemma, was used as an anther-enriched sample. Leaves were harvested from six-day-old seedlings. These materials were used for the construction of cDNA libraries. *Escherichia coli* strains MC1000 and JM83 were used as hosts for molecular cloning. The f1 helper phage R408 and *E. coli* strain XL-1Blue were used for *in vivo* excision of the pBluescript plasmid vector from the λZapII phage (Stratagene, California).

Screening of cDNA and genomic libraries

cDNA libraries were constructed from mRNA prepared from the anther-enriched sample or leaves as described previously [4]. After *in vivo* excision of the anther library, cDNA inserts ranging from 0.6 to 1.5 kb were recovered and used as hybridization probes. 1×10^5 plaques from the anther library and leaf library were lifted onto nitrocellulose membranes and hybridized with probes that were radioactively labeled using [α-^{32}P]dCTP by the random priming method [32]. A genomic library constructed in the λDASH vector with IR36 DNA (kindly provided by Steve Kay) was used for isolation of a genomic clone as described by Sambrook *et al.* [32]. Phage DNA was prepared by as described [3].

Genomic DNA blot analysis

Genomic DNA was isolated from young leaves of two-week-old rice seedlings using the CTAB (Cetyltrimethylammonium bromide) method [31]. Five μg of genomic DNA were digested with *Eco*RI, *Hin*dIII, or *Pst*I, separated on a 0.7% agarose gel, blotted onto a nylon membrane, and hybridized with a ^{32}P-labeled probe [32].

RNA gel blot analysis

Flowers were harvested from rice flowers at four different developmental stages; floral primordia (less than 1 cm panicles), young flowers (1–5 cm panicles), early vacuolated-pollen-stage flowers, and late vacuolated-pollen-stage flowers. Mature flowers were separated into anthers, carpels, and paleas/lemmas under a dissecting microscope. Leaves and roots were collected from two-week-old seedlings. A 25 μg portion of total RNA, which was isolated by the guanidium thiocyanate-CsCl method, was used for the blot analysis as described previously [32].

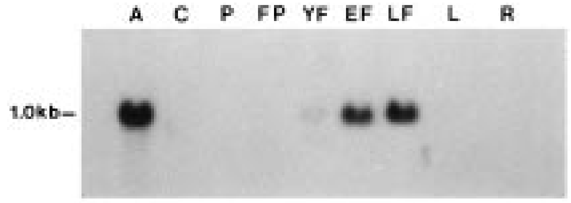

Figure 1. RNA blot analysis of the RA8 gene. A 25 μg portion of total RNAs isolated from leaves (L), roots (R), anthers (A), carpels (C), paleas/lemmas (P), flower primodia (less than 1 cm panicle) (FP), young flowers (1–5 cm panicle) (YF), immature flowers at early vacuolated-pollen stage (EF), and flowers at late vacuolated-pollen stage (LF) was blotted onto a nylon membrane and hybridized to the ^{32}P-labeled RA8 probe. Equal amounts of total RNA loading were identified with ethidium bromide staining of rRNAs.

Sequence analysis

DNA sequencing was performed by the dideoxynucleotide chain termination method [33]. Both strands of the cDNA and genomic clones were sequenced using double-stranded plasmid DNA templates.

Construction of RA8-GUS fusion plasmid

A *Bam*HI site was generated in the exon II of the RA8 gene using a synthetic oligomer (5′-GCGGATCCAGGTTGAACCAC-3′). In the resulting plasmid, pGA1639, the 2.7 kb *Sac*I-*Bam*HI fragment contains the 5′-flanking region, first exon, first intron, and a part of the second exon of the RA8 gene. This fragment was connected to the β-glucuronidase coding sequence (derived from pBI 101.1) in the binary vector, pGA1633, which contains the *hph* (hygromycin phosphotransferase) gene as a selection marker. This plasmid, pGA1647, was transferred to the *Agrobacterium tumefaciens* strain LBA4404 using the freeze-thaw method [2].

Rice transformation

A japonica rice variety, Nackdong, was used for transformation by the *Agrobacterium* cocultivation method as described previously with the following modifications [14]. Calli were induced from the scutellum of mature seeds on an N6 medium containing 2 mg/l 2,4-D. An *A. tumefaciens* strain LBA4404 carrying the pGA1647 plasmid was grown for 3 days in an AB liquid medium supplemented with 30 mg/l hygromycin B and 3 mg/l tetracycline. Three-week-old calli were cocultivated with *Agrobacterium* on a 2N6-As medium supplemented with 1 mM betaine for 2–3 days in darkness at 25 °C. The cocultivated calli were washed with sterile water containing 100 mg/l cefotaxime, and incubated on an N6 medium containing 40 mg/l hygromycin B and 250 mg/l cefotaxime for 3 weeks. Actively growing calli were transferred onto a regeneration medium, MS media supplemented with 0.1 mg/l NAA, 2 mg/l kinetin, 2% sorbitol, 1.6% phytagar (Gibco), 50 mg/l hygromycin B, and 250 mg/l cefotaxime. After 2–3 weeks under continuous light (40 μmol m^{-2} s^{-1}), plantlets were potted and grown in a growth chamber with 10 h light per day.

Histochemical GUS assay

Histochemical GUS staining was performed according to Dai *et al.* [6] except for addition of 20% methanol in the staining solution. After staining, tissues were fixed in a solution containing 50% ethanol, 5% acetic acid, and 3.7% formaldehyde and embedded in a Paraplast (Sigma). The samples were sectioned to 10 μm thickness and observed under a microscope using dark-field illumination.

Results

Isolation of an anther-specific cDNA clone

A rice flower cDNA library that was prepared from anther-enriched samples and another cDNA library from leaves were differentially screened to obtain anther-specific clones. Of the 33 cDNA clones tested, six were present only in the anther-enriched library and another six were more abundant in the anther-enriched library. The remaining 21 clones were present at almost equal levels in both libraries. Among the six clones that were present only in the anther-enriched cDNA library, the RA8 clone, which was the most abundantly present in the library, was selected for further study.

To determine whether the RA8 transcript is present in other organs, the expression pattern of this clone was further studied by RNA blot analysis. Figure 1 shows that the RA8 transcript is detectable in the flowers at various developmental stages but not in leaves, roots, and during young inflorescence (panicle size $<$1 cm). It was weakly detectable in young flowers (panicle size 1–5 cm) which are at pre- and post-meiosis stages, and the amount increased as flowers matured, reaching the highest level in mature flowers. In order to determine the organ specificity within a flower, an RNA blot experiment was conducted with

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-1196                                                                         cattcagaatca
-1184   tctccagcctacaatgtactctctcccataatacaagtgtctctatgattcaaaatttgtcctacaatataaac
-1110   atttccagcatgaaatccatacattaattttcagctaatcagatgcttggagggaaaaatctaagcgattcaat
-1036   atgcaaaaattgatcactgaagtaactgaaagagaatatctcgttttaacattagtgctagtatttattaaaca
 -962   actaaaaaattgtttatatttagtacaaatcgagtagtagcagtagcagagctagcgtaagatcgtgttccga
 -888   tcacctgagaaaccgtcaggtggtttgtctgtgccgtccagccgatcagaattcggagatccgccgtcgtttct
 -814   ttcctgaaatctgcaagtcccagcagcagcagcagcagagcaagagcaatggcgtgcagggagtttgatacttt
 -740   gatgcactagctagctactaggcgttcgttccatgtcgctctcacgccgtgcgaatgtgccatgatcctgcatg
 -666   catcatcgccaagattatattcctcacatttttcttcctatcgctcctagtcgtctgtttgggagcttaaaat
 -592   tatgaaaagcagctgctgagaagctagctggtgagaatctgaagaatttgagttctacgttcattctccagatt
 -518   ctacaattacagattcttataatttaggtaaaaagctggactgtttgggagcttctgtcagccggagattctgt
 -444   gagaagctgcagctgctagaagcttccccaaacagacccctagttgtactctagctgatcgattcactctattt
 -370   tatatacaccttgctctctagcttatcaaacgtagccaagacttgaattttaaagcttaaattgattttgatgt
 -296   tcttttcatcgtaattcacttaccgaccttagtcggcatttgaattttaaaaataattttagagctgatttt
 -222   gatttttttttcagcggaatttattttcacgtatgtaaaagttttacctataaattattaattttcagcggagt
 -148   aagcattagtgttatgggttataatcatctggtatgcttaaatctctttacttggacttagttgggacaattcg
  -74   taatgcattctcgtgccatctctataatacggcctgctagctttgctcttgtatctgcacacaagaactagctg
    1   gcaaagtcctcaaggcgaaccgcctccatcttctccttccagctcctcccATGGCGTCCCTCGTCGCCATCGCC
                                                           M  A  S  L  V  A  I  A     8
   75   ATCGCCATGGCTCTCATGgtgcagagatatccagcatgctgaaattaacttgacgcatatcatctcatcatcac
         I  A  M  A  L  M                                                             14
  149   ttgcatttcaacttctggattgtgcagacatgtttagctgatcagtaaacgttgccgtgttgaattggtcctta
  223   tcagGTGGTTCAACCTGGCCGGCAGATGACTGCCTTTGCTGCTCGGACGTCGCCGGCGGCGGCGGCGGAGGCGT
             V  V  Q  P  G▲ R  Q  M  T  A  F  A  A  R  T  S  P  A  A  A  A  E  A  F   38
  297   TCTGGCGCGCCGCCATGCCCGGCGCTCCCATGCCGGACGCCATCGTCGAGCTCCTCCACCATGgtgaatccgtg
           W  R  A  A  M  P  G  A  P  M  P  D  A  I  V  E  L  L  H  H  E              59
  371   aacttaattgtgtacactactttaaatgatactagtattagtgatggtcactactagaaaacaagttttgcag
  445   gcagctagtaaatatttcgccggtaggctaaggcctgcgaaagtcgttgattttgcaggcggagctctatccg
  519   cttgcgcagacggcatccttgtatcacccacttgcaaaaagttttggggcaaaaagaaaagattaaaaa
  593   aaagcaatttccactacatgcaaagcatcctattcaaatcaaatcagattcattcaacaagaaacacatcgaac
  667   agcagctacaatcagatgcactagaataaacactataagctgacatgtaagatcctcacatatatgaatgcttt
  741   tgctgttatatatctatttttttaacgacggttatatatctaattaactaactatactataaacttatctttt
  815   gtttttgcttttatcttttataatatccgttgcaacgcacgaatctttagctactgatcataaaatgaacaga
  889   aataagagattatgttttatacgtggagcctctcttacatgcgtagctgcatgcgtgcatgcagAGCACGGCG
                                                                          H  G  V     62
  963   TTGCTAGCGCCGGCGGCAAGGCCAACGGCGGCGGCGACGGTCCACCGCCGCCGATGAACTTCAACTACGACGAC
           A  S  A  G  G  K  A  N  G  G  G  D  G  P  P  P  P  M  N  F  N  Y  D  D     86
 1037   TACAGGGCCCTGCCTCGGAGCGACGCCCCTTCCCCGGATGCATTGAACCGCGTCGCCGCCGTACAGAACGCCGA
         Y  R  A  L  P  R  S  D  A  P  S  P  D  A  L  N  R  V  A  A  V  Q  N  A  D    111
 1111   CGAGAACGGCGTGTCCTCGCCGCCGCCGCCGCCGCCGACGGTGTTCTTCCTCGAGGACGCCGTGCGCGTCGGCG
          E  N  G  V  S  S  P  P  P  P  P  P  T  V  F  F  L  E  D  A  V  R  V  G  E   136
 1185   AGAGCCTGCCCCTACCGCGACCGGCGGCCGACGCAACCGCCGCCGGCGCAGCGGCGGCCACGGCGTTGCCGCCG
           S  L  P  L  P  R  P  A  A  D  A  T  A  A  G  A  A  A  A  T  A  L  P  P     160
 1259   CTGCGGCTGTACACCGTCCGGTCGGTGAGGGCAATCGAGGGGTCCAGCTTCGTCGTGTGCCGCGGCGAGACCAC
         L  R  L  Y  T  V  R  S  V  R  A  I  E  G  S  S  F  V  V  C  R  G  E  T  T    185
 1333   GGCGGGCGCCGGCGTGTACGGGTGCCGCCATGCCGCCACCGGCCCGGCGAGGGCGTACGCGGTGGACGCGGCCG
          A  G  A  G  V  Y  G  C  R  H  A  A  T  G  P  A  R  A  Y  A  V  D  A  A  G   210
 1407   GCGGCGGCGGCGGGGACGCGGTGATCGCCGCCGTCGTGTGCCACGCCGACACGTCCCGCTGGGACCCGGACCAC
           G  G  G  G  D  A  V  I  A  A  V  V  C  H  A  D  T  S  R  W  D  P  D  H     234
 1481   GCCGCGTTCCGGCTGCTCGGCGTCAGGCCCGGCGGCGCCGCCGTCTGCCGCGCCGTCGCCGACGCGCACATCCT
         A  A  F  R  L  L  G  V  R  P  G  G  A  A  V  C  R  A  V  A  D  A  H  I  L    259
 1555   CCCGACGAACAAGGATtgagtacttctcgccatgatcgaggactcctcgatcgattagtgctcgatcgatctta
          P  T  N  K  D  *                                                            264
 1629   atcttattgccatgcataattagtgtgttaattacgtgcttaattgtatgtgtgaagtgtgcattagtcacagt
 1703   ttgtaatgtaataatgtttcgcttgttcgactgtatgatctcgatttgggtgatatatacgttgataaaataaa
 1777   ggaaaaagtttgtgttggttcatatgcacga
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Figure 2. Nucleotide and deduced amino acid sequences of the RA8 gene. The non-coding sequence is in lower case and the coding sequence is in upper case. The encoded amino acids are designated by single-letter codes. The asterisk indicates the translation stop codon. The putative CAAT and TATA boxes in the 5′-flanking region and the putative poly(A) addition signals in the 3′-flanking region are underlined. The double-underlined T residue indicates the last sequence of the cDNA clone where the poly(A) tail is added. The position +1 corresponds to the start site of the cDNA clone. The hydrophobic amino-terminal region, SPPPPPP motif, and glycine repeat region are shown in bold letters. The arrowhead indicates the position of the translation fusion between RA8 and the *GUS* gene.

the total RNAs isolated from different floral organs. Each of the floral organs was dissected under a dissecting microscope to minimize cross-contamination. The result revealed that the RA8 mRNA was present in anthers, but not in the carpel or the palea/lemma (Figure 1).

Sequence analysis of the RA8 cDNA clone

The DNA sequence of the RA8 cDNA clone revealed that it is 1008 bp long and contains an open reading frame of 264 amino acid residues with a calculated molecular mass of 26.4 kDa and a pI of 6.1 (Figure 2). The poly(A) tail is located 164 nucleotides downstream from the TGA translation termination codon. In the 3′ non-coding region, the AATAA consensus polyadenylation signal sequence was found. The sequence surrounding the first ATG fits well with the translational start consensus sequence of monocots [17]. Taken together, the size of the cloned cDNA agrees with that of the RNA blot data (Figure 1), indicating that RA8 is close to full-length. The predicted amino acid sequence is rich in alanine (21.9%), glycine (9.9%), and proline (10.2%). A hydropathy plot analysis of the translated protein sequence showed that it contains a hydrophobic N-terminal region which may be involved in targeting the protein into a membrane fraction or extra cellular space. In addition, an extensin-like SPPPPPP motif and a glycine-rich region also exist in the coding region (Figure 2). However, the protein did not show a significant homology to any known proteins deposited to protein databases.

Isolation and characterization of the RA8 genomic clone

To study the genomic complexity of the RA8 clone, genomic DNA blot hybridization was performed. The RA8 probe hybridized to a single band, indicating that the RA8 gene exists as a single copy in the rice genome (Figure 3).

A genomic clone containing the RA8 gene was isolated from a rice genomic library by plaque hybridization experiments. A ca. 13.7 kb genomic fragment was isolated and a restriction map of the clone was established (Figure 4). It was found that the 2.9 kb *Sac*I fragment contains the 5′-flanking sequence and the 5′ end of the coding region, whereas the adjacent 1.5 kb *Sac*I-*Eco*RI fragment contains the remaining coding region as well as the 3′-flanking sequence. Sequencing these DNA fragments revealed the presence of two introns in the coding region. The first intron is 134 bp

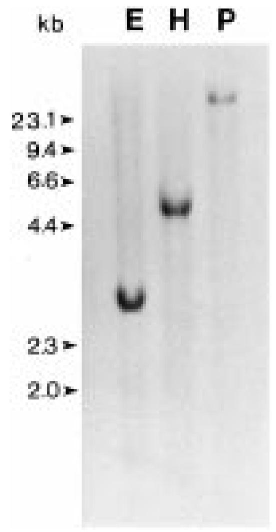

Figure 3. Genomic DNA blot analysis of the RA8 gene. Genomic DNA was isolated from the leaves of two-week-old seedlings and digested with *Eco*RI (E), *Hin*dIII (H), or *Pst*I (P) restriction endonucleases. The probe was prepared using the entire RA8 cDNA.

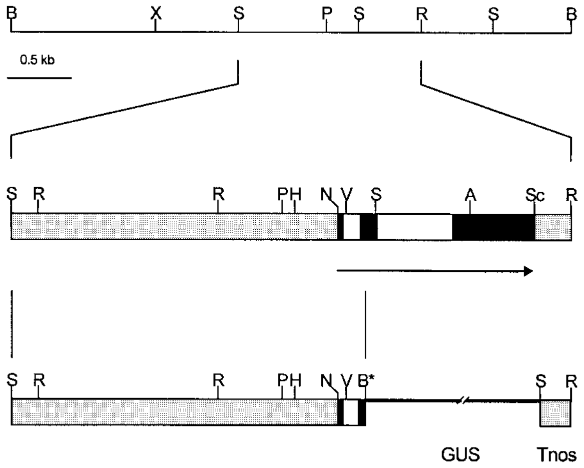

Figure 4. Restriction map of the RA8 gene and construction of the chimeric plasmid between the RA8 promoter and *GUS* gene. Introns are shown as white bars. The coding regions are shown in black bars. The promoter and terminator regions are shown in gray. The arrow indicates direction of transcription. Tnos means the nopaline synthase terminator. Letters represent restriction enzyme sites: B, *Bam*HI; H, *Hin*dIII; P, *Pst*I; R, *Eco*RI; V, *Eco*RV; S, *Sac*I; X, *Xho*I. B* indicates a *Bam*HI site generated for construction of the chimeric fusion with the *GUS* gene.

long and located between codons 14 and 15 (Figure 2). The 594 bp second intron is located at codon 59. Both introns contain the consensus GT and AG sequences at the 5′ and 3′ ends, respectively. The 5′-flanking sequence of the RA8 coding region contains the putative CAAT box sequence, CAAT, and TATA box sequence, TATAATA, at −82/−79 and −53/−47, respectively.

Expression analysis of the RA8 promoter in transgenic rice

To study spatial and temporal regulation of the RA8 gene expression, a translational fusion between the RA8 and β-glucuronidase genes was constructed. To retain the first intron that may be needed for specificity of expression in the fusion construct, a *Bam*HI site was generated within exon II (see Materials and methods) and the site was used for connecting the RA8 and β-glucuronidase coding sequences in the same reading frame (Figure 4). This fusion was introduced into a binary vector, making pGA1647.

The fusion gene was introduced into rice chromosomes by the *Agrobacterium*-mediated transformation method. Twenty-two independent transgenic rice plants were regenerated and expression of the fusion gene in anthers was analyzed by a histochemical GUS assay. The experiment showed that 21 plants exhibited GUS expression in anthers and the level was highest in the transgenic line A11279.

The expression patterns of the gene in various vegetative and reproductive organs were studied with the transgenic line A11279 (Figure 5). The GUS staining was observed only in the anthers of spikelets, and the intensity increased as flowers matured (Figure 5B and C). The GUS staining was hardly detectable in the anthers at the early pre-meiosis stage (Figure 5A). There was also no detectable GUS activity in other floral organs, leaves (Figure 5E), or roots (Figure 5F). Although the intensity was lower, anther-specific expression of the gene was also observed from other transgenic plants (data not shown). Flowers of untransformed control plants did not exhibit any detectable GUS activity (Figure 5D).

The tissue-specific expression pattern of the gene was analyzed under a microscope using sections of anthers at four different developmental stages (Figure 6). At the pre-meiosis stage, GUS activity was not detected in any parts of the anther (Figure 6A). The GUS expression was first detectable at the time when microspores were released from tetrads (Figure 6B). The highest amount of GUS activity was observed in anthers at the vacuolated-pollen stage (Figure 6C). In anthers at mature pollen stage just before or after dehiscence, however, GUS activity suddenly decreased (Figure 6D). It was observed that GUS staining was restricted to tapetum, connective, and endothecium tissues.

No GUS staining was observed in the vascular tissue. Microspores or pollen grains exhibited occasional color spots, but this was probably due to an artifact caused by the diffusion of dye, because the pollen grains squeezed out of the anthers in various stages showed no GUS activity.

Discussion

In this study, we have isolated an anther-specific gene, RA8, from rice. RNA blot analysis and transgenic experiments showed that the expression of RA8 is restricted to the anther, and expression of this gene is developmentally regulated. Expression of the gene starts in anthers at the tetrad stage and reaches a maximum level at the late vacuolated-pollen stage. This result coincides with that of histochemical analysis of transgenic rice plants expressing the RA8-*GUS* fusion. Expression of the *GUS* reporter gene was first detectable in anthers at the time when microspores were released from tetrads, and the expression level increased as the anthers matured. These results indicate that RA8 can be classified as a gene that is expressed at the late stage of anther development because of its strong expression in the anthers at the mature stage. The Osc4, Osc6, YY1, and YY2 clones which were isolated from anthers of rice plants are early genes that become active soon after meiosis, and their mRNAs are present at reduced levels in mature anthers [15, 40].

Within anthers, the RA8 gene was expressed specifically in the tapetum, connective, and endothecium tissues. This expression pattern is similar to that of TA55, which is also expressed preferentially in tapetum, connective, and endothecium tissues of tobacco anthers [11]. However, the expression times of the two genes were different from each other. The TA55 transcript disappeared when the tapetum was completely degenerated, indicating that the TA55 gene belongs to the early type gene group, whereas RA8 was still expressed at the same stage of anther development. Most anther-specific genes, which are classified as late genes, are expressed in pollen grains [26, 27]. Recently, it was reported that the promoter of the

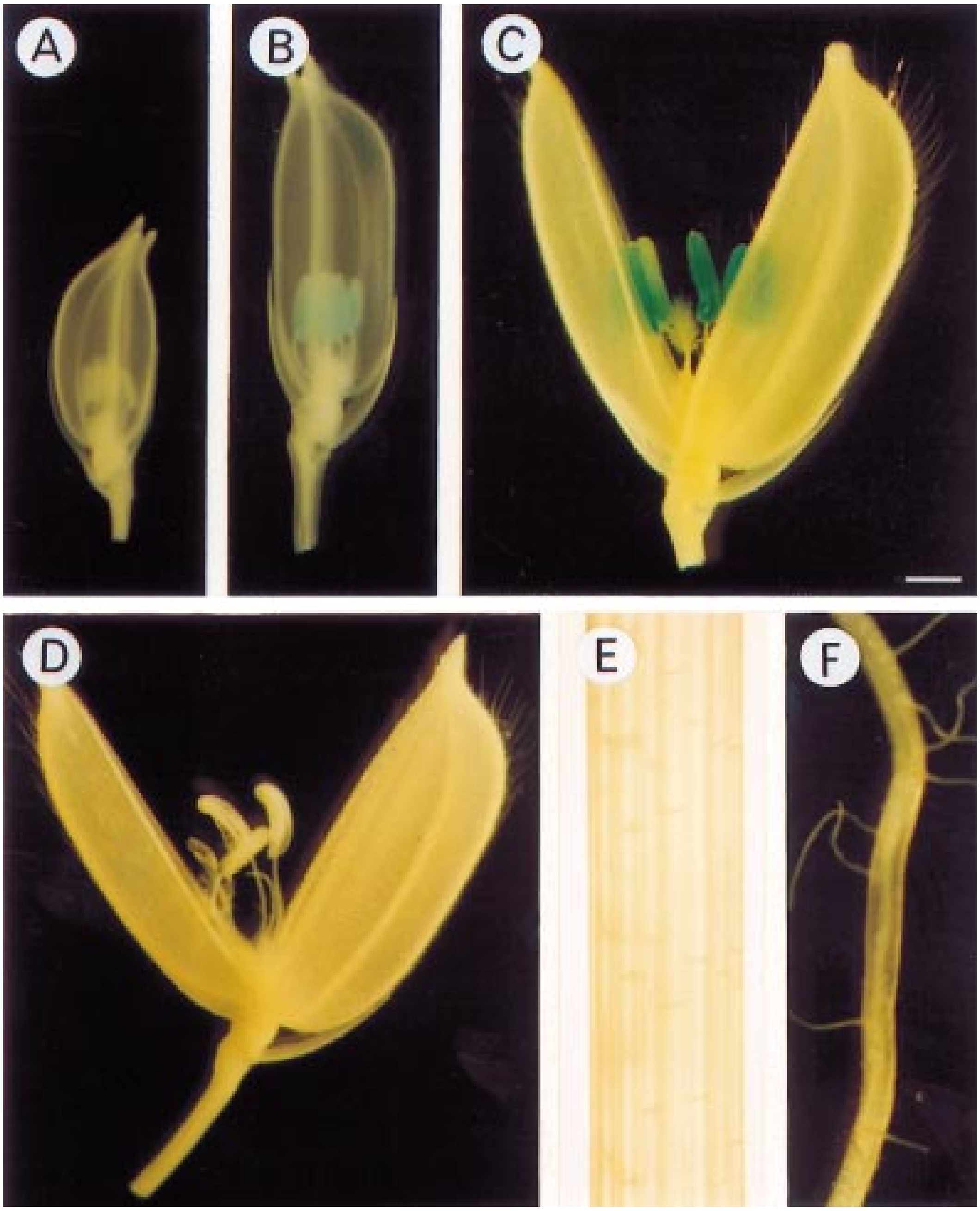

Figure 5. The RA8 promoter-driven GUS expression pattern. A spikelet at the pre-meiosis stage (A), a spikelet at the early vacuolated-pollen stage (B), and a mature spikelet (C) of the transgenic rice flower; a mature flower of wild type (D). The stages were determined on the basis of panicle size; a leaf (E) and a root (F) of the transgenic rice plant. Scale: 1 mm.

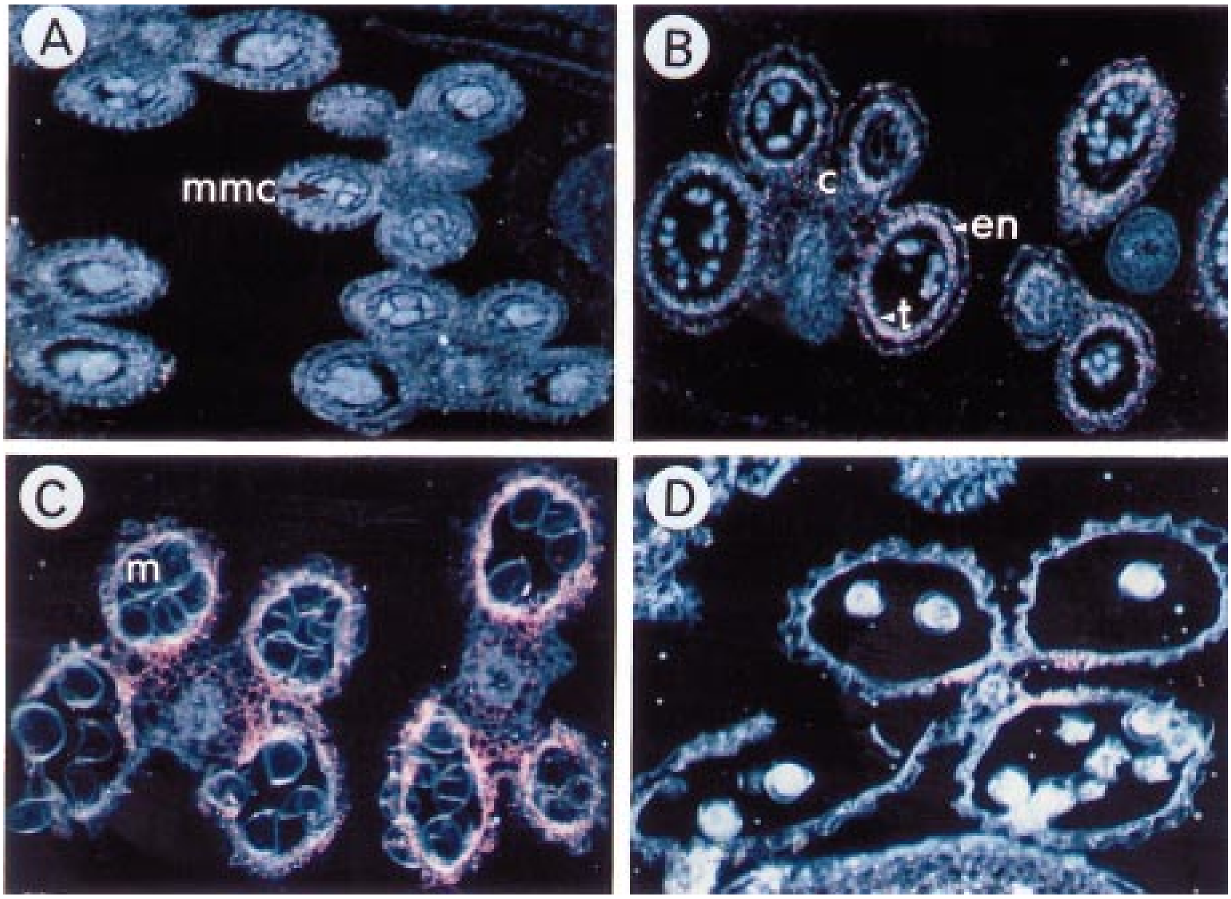

Figure 6. Histochemical analysis of temporal and spatial expression patterns of the RA8-*GUS* fusion during anther development in the transgenic rice. Cross-section of anthers at the pre-meiosis stage (A), microspore-release stage (B), vacuolated-pollen stage (C), and mature pollen stage immediately prior to anthesis (D). Red staining indicates GUS activity. c, connective tissue; en, endothecium; m, microspore; mmc, microspore mother cell; t, tapetum.

Osg6 gene from rice exhibits a tapetum-specific expression [47]. It appears that the temporal and spatial expression patterns of the RA8 gene are unique in the expression of the anther-specific genes.

Database searches of the RA8 amino acid sequences did not show a significant homology to any known proteins. Sequence analysis showed that the RA8 protein includes an extensin-like SPPPPPP motif. A proline-rich sequence motif such as this is also found in maize BET1, that is a cell wall protein in transfer cells [16]. Transcripts of the sunflower plant, SF2, SF18, and SF19, which encode proline-rich proteins, were found only in the epidermis of mature anthers [9]. Thus, the RA8 protein may function in the cell wall matrix in the anther. The RA8 protein also contains a glycine repeat region near the carboxyl terminus. Some anther-specific proteins, such as TA29 of tobacco [35] and MROS2 of campion [25], encode glycine-rich proteins. The glycine-rich sequences form so-called glycine loops, which are expected to be highly flexible in proteins [38].

It is worthy to note that 21.9% of the RA8 protein consists of alanine. An anther-specific clone, Bcp1of *Brassica campestris,* encodes a 12 kDa alanine-rich protein of unknown function [39]. This gene was expressed in both pollen grains and tapetum tissues after microspore mitosis, and the level was elevated at the time of pollen maturation and during pollen germination. However, the RA8 gene was not expressed in pollen grains but rather in tapetum, endothecium, and connective tissues.

The endothecium and connective tissues play critical roles during the dehiscence of the anther. Dehiscence probably requires expression of many genes, including those encoding proteins for thickening of fibrous bands on the endothecial cell walls, breakdown of the connective tissues, and the rupture of anthers at the stomium [11]. Expression pattern of the RA8 gene suggests that the gene product may function in the dehiscence process. Antisense expression of the RA8 cDNA in rice may help clarify the function of the gene.

The present study shows that the 2.5 kb 5′ region of the RA8 gene contains all the regulatory elements required for anther-specific expression in transgenic rice. Therefore, the promoter identified in this study will be useful for controlling gene expression in anthers of rice and for generating male-sterile rice plants. It appears that this region did not contain any obvious sequence elements that are found in other anther-specific promoters. Further studies of the RA8 promoter are needed to define the precise *cis*-regulatory sequences.

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