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Isolation and characterization of two cDNA clones for mRNAs that are abundantly expressed in immature anthers of rice (*Oryza sativa* L.)

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Abstract

The relationship between the length of anthers and the stage of development of microspores was examined in rice (*Oryza sativa* L. cv. Hayayuki). Anthers of ≤2 mm and 2.1–2.2 mm in length and those ready to dehiscence were determined to be at the uninucleate, binucleate and trinucleate microspore stage, respectively.

Two cDNAs (YY1 and YY2), representing genes that are specifically expressed in anthers at the uninucleate microspore stage, were isolated and characterized. YY1 cDNA encoded an open reading frame of 95 amino acids. Eight cysteine residues with the potential to form disulfide bridges were present in the amino acid sequence. There was a hydrophobic region at the N-terminus of the putative protein, suggesting that the YY1 protein might be secreted. This cysteine motif and the hydrophobic N-terminus are conserved among products of several anther-specific genes or cDNAs isolated from various plant species. These proteins are thought to form a superfamily of proteins that are confined to anthers. The YY1 transcript was localized in the tapetal cells and the peripheral cells of the vascular bundle. YY2 cDNA encoded an open reading frame of 389 amino acids and the deduced amino acid sequence exhibited substantial homology to that of chalcone synthase. Expression of YY2 mRNA was confined to the tapetal cells. The genes correspond to YY1 and YY2 cDNAs were shown to exist as single copies in the rice genome.

Introduction

In higher plants, the formation of pollen is a unique developmental process, involving cytological and biochemical changes in several types of cells [14]. Underlying these processes is a complex program of gene expression in both sporophytic and gametophytic tissues [15]. Genes that are preferentially expressed in anthers during the development of microspores might be expected to

The nucleotide sequence data reported will appear in the GenBank, EMBL, DDBJ and NCBI Nucleotide Sequence Databases under the accession number D50575 (YY1) and D50576 (YY2).

play important roles in anther-specific developmental pathways. To isolate these genes, differential screening of cDNA libraries has been carried out by several groups [16, 17, 32]. Mascarenhas [15] classified these genes into two classes according to their patterns of expression. The 'early' genes become active soon after meiosis and the levels of their transcripts are reduced or they are undetectable in mature pollen. The 'late' genes become active after microspore mitosis and their transcripts continue to accumulate until the pollen reaches maturity. Based on their nucleotide sequences and the localization of their transcripts, the two classes of genes are likely to have very different roles. The 'early' genes are probably involved in pollen development such as wall synthesis. For example, the *Sa*tap35 and *Sa*tap44 proteins from *Sinapis alva* are thought, from their localization, to be involved in sporopollenin formation and/or deposition to the pollen wall [31]. The 'late' genes are likely to be required during pollen maturation or growth of pollen tube. LAT56 from tomato [37] and P2 from *Oenothera organensis* [1] have amino acid sequences that exhibit homology to those of pectate lyase and polygalacturonase, respectively. They may be required during growth of pollen tubes. Recently, the LAT52 gene, which is expressed in a pollen-specific manner, was shown to play a role in pollen hydration and/or germination from results of antisense inhibition experiment [18]. However, with the exception of a few examples, such as LAT52, the functions of these anther-specific genes are still a matter of conjecture. In order to dissect the molecular processes that occur during anther development, we must isolate many more anther-specific genes and determine their functions.

In this report, we describe the isolation and characterization of two cDNAs for mRNAs that are specifically expressed in anthers at the uninucleate microspore stage in rice plants. These two cDNAs can be classified as cDNAs of 'early' genes, and the corresponding genes may participate in microspore development. One of the cDNAs, YY1, encodes an amino acid sequence with eight cysteine residues, as do several other anther-specific genes. It is suggested that the YY1 protein is a member of a superfamily of proteins that are specifically localized in anthers. The protein encoded by another cDNA, YY2, exhibited significant homology to chalcone synthase (CHS) and it might be involved in the synthesis of flavonoids, which play important roles in anthers.

Materials and methods

Plant materials

Rice plants (*Oryza sativa* L. cv. Hayayuki) were grown in a greenhouse. For construction of a cDNA library and northern blot analysis, anthers were collected and immediately frozen in liquid nitrogen. Samples of shoots and roots were obtained from 1-week-old plants. Frozen tissues were stored at −80 °C until use.

Determination of anther length and the stage of microspore development

Lengths of florets and anthers were determined under the light microscope. The stage of microspore development was assessed by light-microscopic examination of microspores (or pollen grains) that had been extruded from anthers squashed on a glass slide. For observations of nuclei, anthers dissected from florets were fixed in a mixture of 99% ethanol and acetic acid (3:1, v/v) for 1 h. They were rinsed first in 70% ethanol and then in water. Microspores in a solution of 4′-6-diamidino-2-phenylindole (DAPI) (1 μg/ml DAPI, 0.05 M Tris-HCl pH 7.0, and 0.5% Triton X-100) were observed under UV light.

Construction of a cDNA library

Poly(A)$^+$ RNA was extracted from anthers at the uninucleate microspore stage with a Fast Track mRNA isolation kit (Invitrogen). cDNAs were synthesized with a cDNA synthesis kit (Pharmacia) and ligated with *Eco*RI adapters for bidirec-

tional introduction into the *Eco*RI site of the λZAPII vector (STRATAGENE). *In vivo* excision of pBluescript plasmids was performed in the *Escherichia coli* K-12 strain XL1-Blue.

Preparation of total RNA

Total RNA was prepared from anthers at different stages, from shoots and from roots by the phenol-SDS method [21]. Tissues were homogenized in a solution of 0.2 M Tris-HCl pH 8.0, 0.1 M NaCl, 10 mM EDTA, 0.5% SDS, 0.1% 2-mercaptoethanol and an equal volume of TE-saturated phenol, with the subsequent addition of an equal volume of chloroform. After centrifugation (3000 × *g*, 5 min), the supernatant was shaken with a mixture of phenol and chloroform (1:1, v/v). The mixture was centrifuged (3000 × *g*, 5 min) and chloroform was added to the supernatant. After centrifugation (3000 × g, 5 min), nucleic acids were pelleted by ethanol precipitation and dissolved in diethylpyrocarbonate (DEPC)-treated water. Then LiCl (10 M) was added to the solution of nucleic acids to give a final concentration of 2.5 M, and the mixture was kept on ice for more than 1 h. RNA was pelleted by centrifugation (15000 × *g*, 20 min) and dissolved in DEPC-treated water. The yield was determined by monitoring absorbance at 260 nm.

Analysis of mRNA levels by dot blot hybridization

Five hundred cDNA clones were spotted onto a nylon membrane (0.5 μg/clone; Hybond-N+; Amersham). After denaturation by 1.5 M NaCl and 0.5 M NaOH and neutralization by 0.5 M Tris-HCl pH 7.2, 1.5 M NaCl and 1 mM EDTA, membranes were first incubated at 65 °C for 1 h in hybridization solution that contained 5 × SSC, 5 × Denhardt's solution, 0.5% SDS and 0.1 mg/ml heat-denatured salmon sperm DNA. Then they were incubated for 15 h with cDNA probes that had been synthesized from poly(A)$^+$ RNA extracted from anthers or shoots. Double-stranded cDNAs were synthesized as described above and labelled with [α-^{32}P] dCTP by the random primer labelling method. After hybridization, membranes were washed in 2 × SSC that contained 0.1% SDS for 10 min at 65 °C with shaking, and then twice in 1 × SSC that contained 0.1% SDS for 30 min at 65 °C. They were dried and exposed to X-ray film (RX-50; Fuji). Plasmid clones that gave strong signals with anther cDNA probes but weak signals with shoot probes were selected. For the second screening, slot blot hybridization was performed. RNAs from anthers, shoots and roots (3 μg/slot) were blotted on a nylon membrane (Hybond-H+; Amersham) with a BIO-DOT SF system (Bio-Rad). After denaturation by 0.05 M NaOH, membranes were first incubated at 65 °C for 1 h in hybridization solution that contained 1% SDS, 1 M NaCl, 10% dextran sulfate and 0.1 mg/ml heat-denatured salmon sperm DNA. Then they were incubated with cDNA probes for 15 h. After hybridization, membranes were washed in 2 × SSC for 10 min at 65 °C with shaking, and then twice in 2 × SSC that contained 1% SDS for 30 min at 65 °C. Membranes were dried and exposed to X-ray film.

Plaque hybridization

A small aliquot of a diluted plate lysate was plated with *E. coli* XL1-blue as host bacterium, which supports the rapid growth of the λZAPII phage. After incubation at 37 °C, plaques were transferred to a nylon membrane. cDNA clones used as probes were amplified by the polymerase chain reaction. They were labelled with a DIG DNA Labelling Kit (Boehringer-Mannheim) in accordance with the manufacturer's instructions. Hybridization was performed in a solution that contained 5 × SSC, 2% blocking reagent, 0.1% sodium lauroylsarcosine, 0.02% SDS, 50% formamide and 10 ng/ml DIG-labelled probe at 42 °C for 12 h. Each membrane was washed twice with 2 × SSC that contained 0.1% SDS for 5 min at room temperature and twice with 0.1 × SSC that contained 0.1% SDS for 15 min at 42 °C. Chemiluminescence was detected with a

DIG Luminescent Detection Kit (Boehringer-Mannheim) in accordance with the manufacturer's instructions. Plaques that hybridized to cDNA probes were selected and phage particles in each plaque were eluted in phage-dilution buffer SM. The screening was repeated twice.

Northern blot analysis

Total RNA from anthers, shoots and roots (5 μg/lane) was subjected to electrophoresis in a 1.2% formaldehyde agarose gel in 1 × MOPS buffer (20 mM MOPS, 5 mM CH_3COONa, 1 mM EDTA). After electrophoresis, RNA was transferred to a nylon membrane (Hybond-N+; Amersham) as described by the manufacturer (Amersham). Procedures for preparation of probes, hybridization and washing were the same as those for slot blot analysis.

Southern blot analysis

Total DNA was isolated from 2-month-old rice plants. Tissues were ground to a fine powder in liquid nitrogen and incubated at 60 °C in an extraction buffer that contained 200 mM Tris-HCl pH 8.0, 100 mM EDTA, 1% sodium laurylsarcosine and 100 μg/ml proteinase K. After addition of an equal volume of a mixture of phenol and chloroform (1:1, v/v), the entire mixture was centrifuged (10000 × *g*, 10 min) at 4 °C. The supernatant was collected and nucleic acids were pelleted by ethanol precipitation. Pellets of DNA were rinsed with 70% ethanol and dissolved in TE buffer. Then 1.54 ml of the solution in TE buffer, 1.52 g of CsCl and 60 μl of a solution of ethidium bromide (10 mg/ml) were mixed and genomic DNA was separated by ultracentrifugation (100000 × *g* for more than 6 h) at 20 °C. Genomic DNA was collected, washed with CsCl-saturated isopropanol and pelleted by ethanol precipitation. After it had been dissolved in TE buffer, the DNA was digested with restriction enzymes (*Eco*RI, *Hin*dIII, *Xba*I or *Bam*HI) and subjected to electrophoresis on a 0.8% agarose gel (15 μg/lane). The gel was treated with 0.25 M HCl for 30 min. Then DNA was transferred to a nylon membrane in 0.4 M NaOH. The membrane was incubated at 55 °C for 1 h in a solution that contained 5 × SSC, 50% formamide, 5 × Denhardt's solution, 0.5% SDS and 0.1 mg/ml heat-denatured salmon sperm DNA (for the YY1 probe) or incubated at 65 °C for 1 h in a solution that contained 5 × SSC, 5 × Denhardt's solution, 0.5% SDS and 0.1 mg/ml heat-denatured salmon sperm DNA (for the YY2 probe). Then the membrane was incubated for 15 h with a cDNA probe in the appropriate solution. After hybridization, the membrane was washed in 2 × SSC that contained 0.1% SDS for 10 min at 65 °C with shaking, and then in 1 × SSC that contained 0.1% SDS for 30 min at 65 °C and in 0.25 × SSC that contained 0.1% SDS for 30 min at 65 °C. Membranes were dried and exposed to X-ray film. Full-length cDNAs labelled with [α-^{32}P] dCTP by the random primer labelling method were used as probes.

Nucleotide sequencing of YY1 and YY2 cDNAs

DNA sequencing was performed by the dideoxy chain-termination method using *Bca* BEST dideoxy sequencing kit (TAKARA). Both strands of the cDNA clone were sequenced using double-stranded plasmid DNA templates, with synthetic oligonucleotides as primers.

In situ *hybridization*

Anthers at different stages were fixed in a solution containing 4% paraformaldehyde in 20 mM cacodylate buffer (pH 7.0), followed by dehydration in graded ethanol series, and cleaning in ethanol/xylene series. After soaking in xylene and paraffin, they were embedded in paraffin (Paraplast Plus; Oxford). The paraffin blocks were sliced into 10 μM sections, which were then mounted onto poly-L-lysine (Sigma) treated glass slides. Samples were kept over night at 45 °C. Prehybridization was carried out as described by Drews *et al.* [4].

Digoxigenin-labelled sense and antisense RNA probes were generated with T7 and T3 promoter of pBluescript SK$^-$ (Stratagene), in which the cDNA of either YY1 or YY2 was cloned. *In vitro* transcription reactions were performed with the TransProbe T Kit (Pharmacia). The probes hydrolyzed by alkaline to an average length of 150 bases [2] were mixed with hybridization solution that contained 50% formamide, 300 mM NaCl, 10 mM Tris pH 7.5, 1 mM EDTA, 1 × Denhardt's solution, 10% dextran sulfate, 70 mM DTT and 150 μg/ml tRNA for 16 h at 50 °C. After hybridization, the samples were washed three times in 4 × SSPE solution that contained 5 mM DTT, and treated with 25 μg/ml RNase in 500 mM NaCl, 10 mM Tris pH 7.5 and 1 mM EDTA for 30 min at 37 °C. The samples were washed again in 2 × SSPE solution that contained 5 mM DTT for 1.5 h at room temperature, and then in 0.1 × SSPE solution that contained 1 mM DTT for 1 h at 57 °C. Hybridization signal was detected with DIG Nucleic Acid Detection Kit (Boehringer Mannheim) in accordance with the manufacturer's instruction.

Results and discussion

Determination of the relationship between the length of anthers and the stage of microspore development

In several plants including rice, there is a strong relationship between bud length and/or anther length and the stage of microspore development [10, 23, 26, 28]. Since such a relationship facilitates collection of anthers at a specific stage of development, the floret or anther length at each stage of development of microspores (uninucleate, binucleate or trinucleate) was measured. In the case of rice pollen, nuclei can hardly be seen after the binucleate stage because of the accumulation of starch. Therefore, the developmental stages of microspores were classified as follows by light microscopy [25, 35]: (1) microspore stage, at which only one nucleus was seen; (2) vacuolated pollen stage, at which vegetative cell and generative cell were observed; (3) early engorged pollen stage, when starch began to accumulate; and (4) engorged pollen stage, at which pollen was filled with starch and maturation was completed (Fig. 1). Furthermore, staining with DAPI revealed that early engorged pollen and engorged pollen corresponded to the binucleate and the trinucleate stage, respectively (Fig. 1).

Anther length, rather than bud length (data not shown), was closely related to the stage of microspore development. Figure 2 shows the frequency distribution of anther lengths at each of the four stages of microspore development. We defined anthers of ≤ 2 mm in length as 'uninucleate', those of 2.1 to 2.2 mm as 'binucleate' and anthers that were ready to dehiscence as 'trinucleate'.

Isolation of cDNA clones of transcripts that were abundantly expressed in anthers at the uninucleate microspore stage

To obtain cDNA clones of transcripts that are specifically expressed in immature anthers, we screened a cDNA library of rice anthers at the uninucleate microspore stage by dot blot analysis. Sixteen clones that gave a strong signal with a ^{32}P-labelled mixture of anther cDNAs but a weak signal with that of shoot cDNAs were tentatively identified among 500 cDNA clones. Further analysis by slot blot hybridization using each of the 16 cDNAs as a probe revealed that three clones, with inserts of 0.2 kb, 0.25 kb and 1.4 kb, respectively, corresponded to mRNAs that were specifically expressed in anthers.

Using the 0.2 kb fragment as probe, we screened the cDNA library of rice anthers to obtain full-length cDNA clones. Sixteen positive clones were obtained from 4.2×10^5 plaques and inserts were subcloned into pBluescript by the *in vivo* excision method. A cDNA clone with the longest inserts was named YY1. It included the nucleotide sequence of the 0.25-kbp fragment, suggesting that both were derived from the same gene. Using the 1.4-kbp fragment as probe, we obtained eight positive clones from 2.1×10^5 plaques and the longest one was named YY2. Lengths of YY1 and YY2 cDNAs coincided with the sizes of transcripts that were estimated by

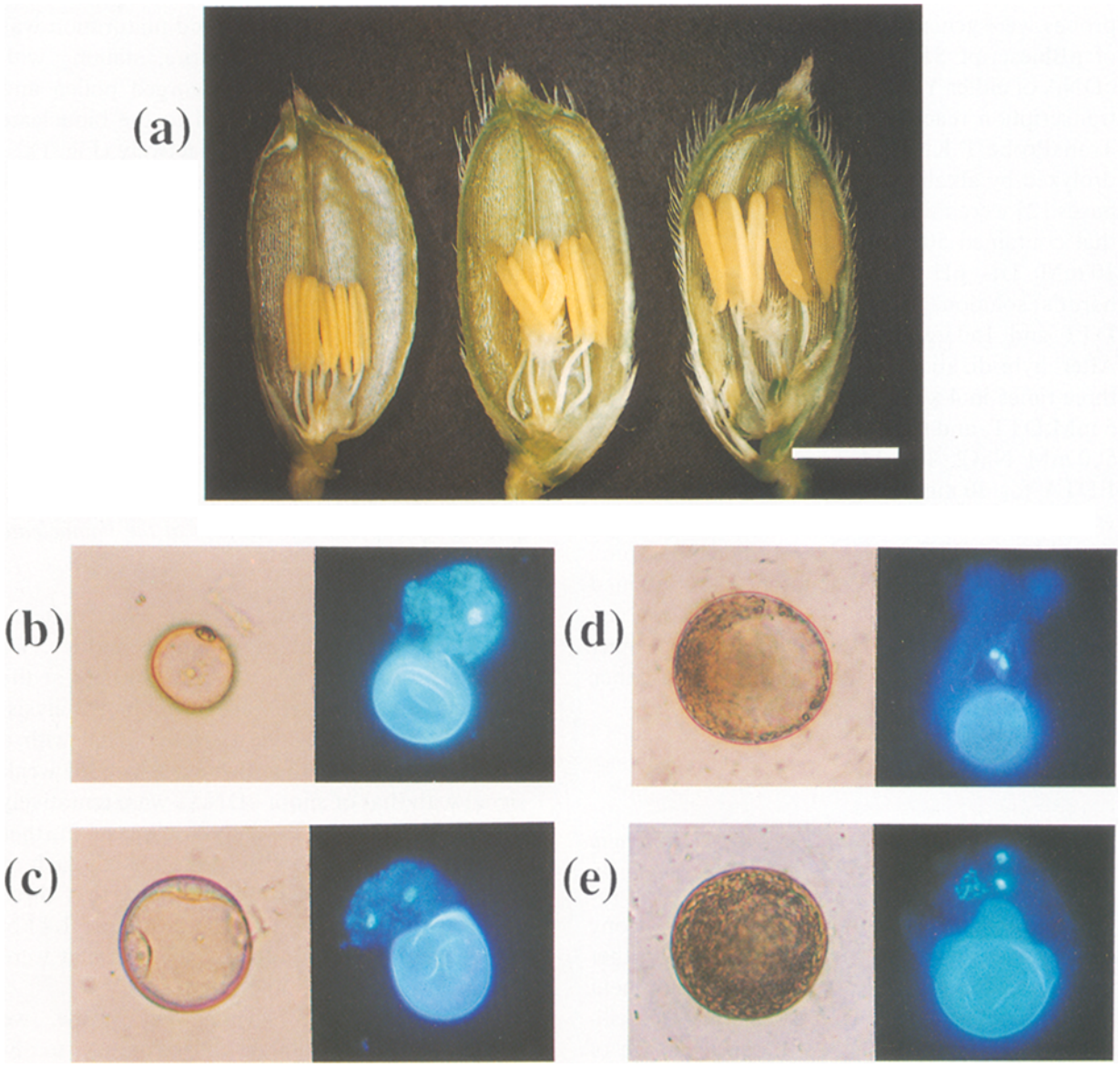

Fig. 1. Development of anthers and microspores in rice plants. (a) Anthers at different stages; (b) microspore stage (uninucleate); (c) vacuolate pollen stage (binucleate); (d) early engorged pollen stage (binucleate); (e) engorged pollen stage (trinucleate). Pictures on the right in (b)–(e) are samples stained with DAPI. Scale in (a) indicates 2 mm.

Northern blot hybridization (Fig. 3), suggesting that these cDNA clones contained full-length inserts. YY1 mRNA was expressed exclusively in anthers at the uninucleate microspore stage. YY2 mRNA was strongly expressed in anthers at the uninucleate microspore stage, with low-level expression in shoots. Southern blot analysis showed that both the YY1 and the YY2 gene existed as single copies in the rice genome (Fig. 4).

Characterization of YY1 cDNA

The complete nucleotide sequence (605 bp) of YY1 cDNA was determined (Fig. 5). An open

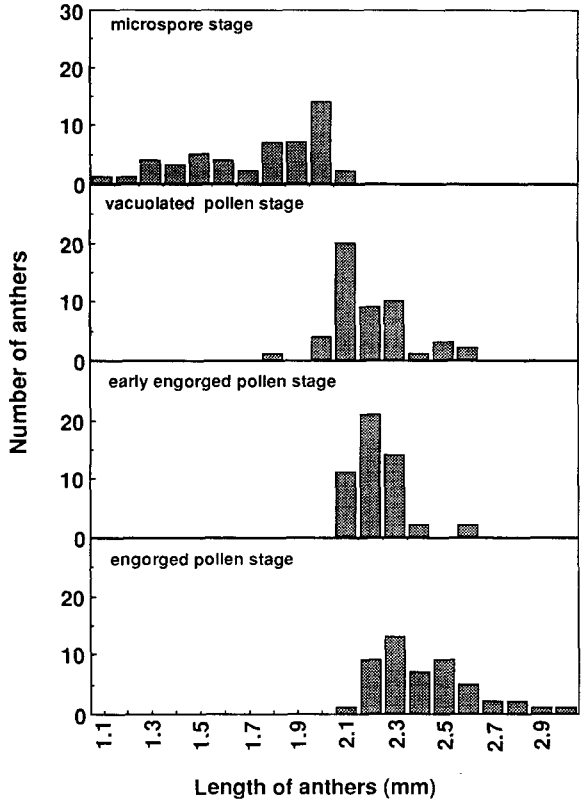

Fig. 2. Frequency distribution of length of anthers at various developmental stages. For each stage, lengths of anthers from 50 florets were measured.

reading frame (288 bp), a 5′ non-coding region (65 nucleotides) and a 3′ non-coding region (252 nucleotides) were identified. The predicted protein contained 95 amino acid residues and had a molecular mass of 9.5 kDa.

YY1 cDNA exhibited no homology to nucleotide sequences in DNA databases. However, the deduced amino acid sequence exhibited some homology to LIM1 (33.7%) and LIM3 (37.2%) which are products of meiosis-specific genes from trumpet lily [8], to the product of Fil1 (30.9%) whose mRNA is expressed in the filament of the

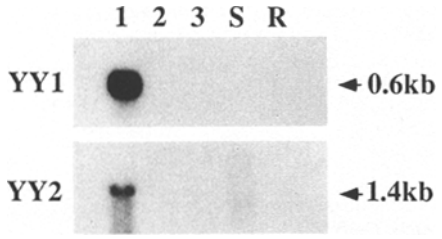

Fig. 3. Northern blot analysis of YY1 and YY2 mRNAs. Total RNA (5 μg/lane) was subjected to electrophoresis and allowed to hybridize with ^{32}P-labelled YY1 or YY2 cDNA probes. Arrows indicate the sizes of mRNAs. 1, anthers at the uninucleate microspore stage; 2, anthers at the binucleate microspore stage; 3, anthers at the trinucleate microspore stage; S, shoots of 1-week-old plants; R, roots of 1-week-old plants.

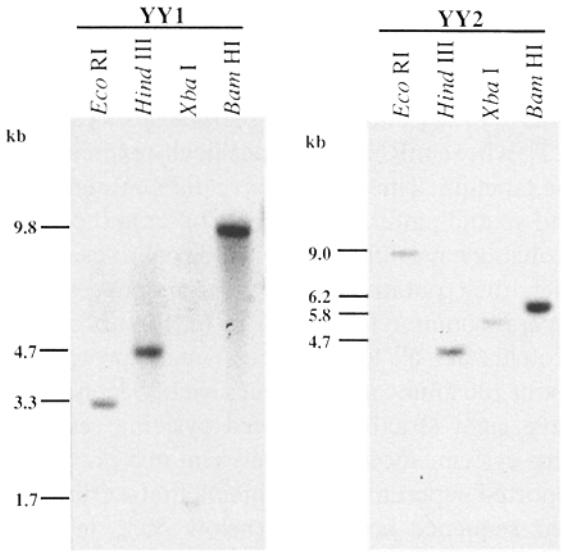

Fig. 4. Southern blot analysis of genes that correspond to YY1 and YY2 cDNAs. Total DNA (15 μg) from 2-month-old rice plants was digested with *Eco*RI, *Hin*dIII, *Xba*I or *Bam*HI, subjected to electrophoresis and allowed to hybridize with ^{32}P-labelled YY1 or YY2.

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-65                                                        CGCAC
-60 GCACCGTGCAGGCATCGACGACGAGCGAGAGCCCCTCGGCGGCAGAAGACACTCACGGCG
  1 ATGGCGGTGACGAGGACGGCGCTGCTGGTGGTGTTGGTAGCGGGGGCGATGACGATGACG
    M  A  V  V  R  T  A  L  L  V  V  L  V  A  G  A  M  T  M  T
 61 ATGCGCGGGGCGGAGGCGCAGCAGCCGAGCTGCGCGGCGCAGCTCACGCAGCTGGCGCCG
    M  R  G  A  E  A  Q  Q  P  S  C  A  A  Q  L  T  Q  L  A  P
121 TGCGCGCGAGTCGGCGTGGCGCCGGCGCCGGGGCAGCCGCTGCCGGCGCCCCCGGCGGAG
    C  A  R  V  G  V  A  P  A  P  G  Q  P  L  P  A  P  P  A  E
181 TGCTGCTCGGCGCTGGGCGCCGTGTCGCACGACTGCGCCTGCGGCACGCTCGACATCATC
    C  C  S  A  L  G  A  V  S  H  D  C  A  C  G  T  L  D  I  I
241 AACAGCCTCCCCGCCAAGTGCGGCCTCCCGCGCGTCACCTGCCAGTGATGGAGATGGTGT
    N  S  L  P  A  K  C  G  L  P  R  V  T  C  Q  *
301 GCCAAGGTAATTGCGTTTGCTCGTGCGAGGATGAGAAGAGAAGATTGAATAAGATGTTTG
361 ATGGCAACAAGTCATCAGGCGATCCGATCCCTGCAGCTATGAATGGGAGTATACGTAGTA
421 GTGGTCTCGTTAGCATCTGTGTGTCGCATATGCACGCCGTGCGTGCCGTGTCTGTCCTGC
481 TTGCTCTGCTGATCGTTCAATGAACGACAAATTAATCTAACTCTGGAGTGACAAGTCGTT
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Fig. 5. The nucleotide sequence of YY1 cDNA and the deduced amino acid sequence. YY1 cDNA has 605 nucleotides and encodes 95 amino acid residues.

stamen and at the bases of petals in the garden snapdragon [19], and to the product of clone 108 from tomato (36.8%) [30], A9 from *Brassica* (30.3%) [22] and A9 from *Arabidopsis* (29.9%) [22], whose mRNAs are specifically expressed in the tapetum. These genes have different temporal and spatial patterns of expression in anthers and probably have different physiological roles. However, their putative amino acid sequences have several common features (Fig. 6). First of all, the proteins are all low-molecular-mass proteins of about 100 amino acid residues each. Second, they have eight strictly conserved cysteine residues. This cysteine motif is also present in a previously reported superfamily of proteins that exhibit limited sequence homology (below 50% in some cases). This superfamily consists of seed proteins, such as prolamins from the Triticeae, the 2S globulins from castor bean and rape, and some inhibitors of proteases and α-amylase [11]. Resembling these proteins, which are confined to seeds, products of YY1, Fil1, LIM1, LIM3, clone108 and A9 seem to constitute a superfamily of proteins that are confined to anthers. In a few members of the superfamily of seed proteins, the cysteine residues are known to be involved in intramolecular disulfide bridges [12, 13]. The YY1 protein might also stabilize its tertiary structure by intramolecular cross-linking. Of particular interest is the presence of a hydrophobic region at the N-terminus of the YY1 protein. Some products of anther-specific genes have been reported to have hydrophobic N-termini (e.g., TA29 [10], MFS14 [38], *Sa*tap35 [31]), and these regions are thought to be signal sequences for secretion. In the YY1 protein, a potential signal for cleavage, which follows the −3, −1 rule [5] is located at 18 amino acid residues downstream of the initiator methionine. *In situ* hybridization was used to determine the location of YY1 mRNA. The pattern of antisense hybridization to rice anthers at the uninucleate microspore stage is shown in Fig. 9a. Signals were detected in the tapetal cells and in the peripheral cells of vascular bundles. As expected from the results of Northern hybridization, anthers at the trinucleate microspore stage did not generate hybridization signals above the background level (Fig. 9d).

Characterization of YY2 cDNA

The complete nucleotide sequence (1405 bp) of YY2 cDNA is presented in Fig. 7. An open reading frame (1170 bp), a 5′ non-coding region (25 nucleotides) and a 3′ non-coding region (210 nucleotides), followed by an eight-nucleotide poly(A) tract were all identified. There was no obvious polyadenylation signal upstream of the poly(A) tract. The predicted protein has 389

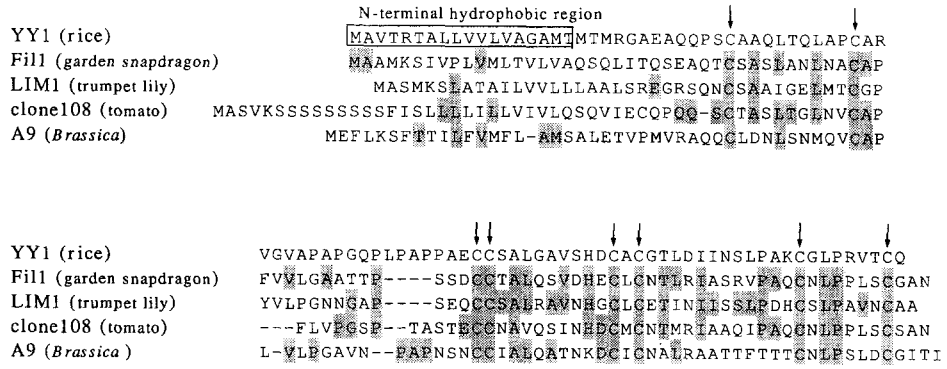

Fig. 6. Comparison of amino acid sequences deduced from rice YY1 cDNA [this study], garden snapdragon Fil1 [19], trumpet lily LIM1 [8], tomato clone 108 [30] and *Brassica* A9 [22]. Identical amino acid residues are indicated by shadowing. Gaps have been introduced to obtain maximum homology, and they are shown by hyphens. Conserved cysteine residues are indicated by arrows. The N-terminal hydrophobic region in the YY1 protein is enclosed in a box.

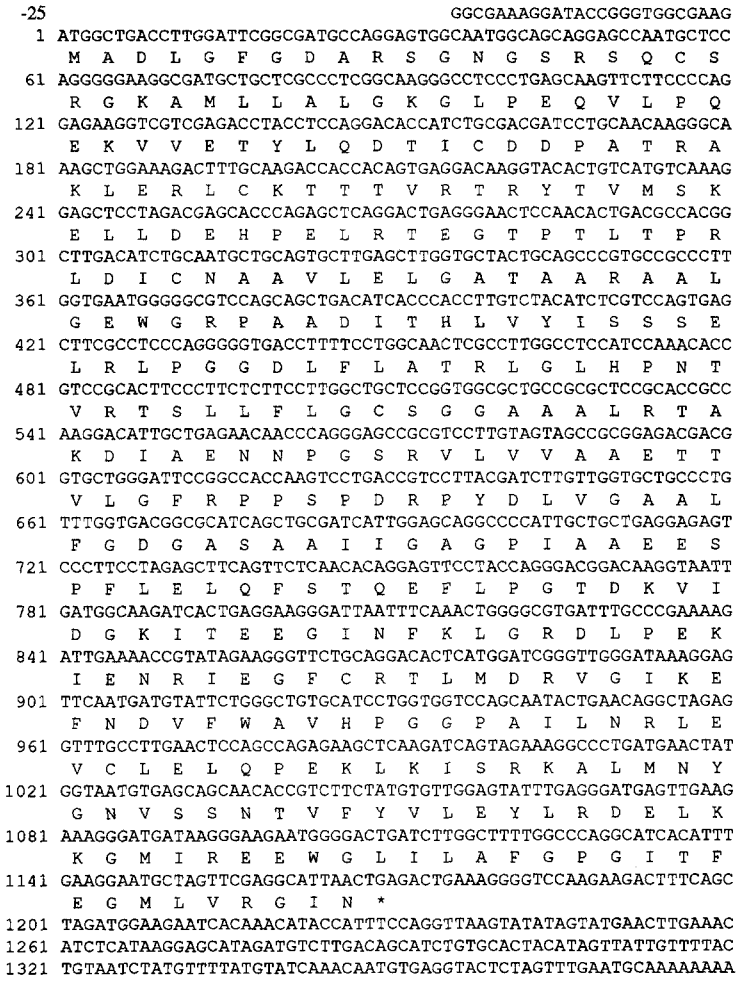

Fig. 7. The nucleotide sequence of YY2 cDNA and the deduced amino acid sequence. The YY2 cDNA has 1405 nucleotides and encodes 389 amino acid residues.

amino acid residues and a molecular mass of 42 kDa.

A search in nucleotide and protein databases with the YY2 sequence revealed the similarity of YY2 cDNA to A1 and BA42, partial cDNAs of transcripts that are specifically expressed in immature anthers of *Brassica napus* [28, 29], and to genes for chalcone synthase from various plant species. The nucleotide sequence of YY2 was 62.2% identical to that of A1 and about 50% to those of genes for chalcone synthase from various plant species. The predicted protein was 65.5% identical to the protein encoded by BA42 and about 40% identical to chalcone synthases. Figure 8 shows an alignment of amino acid sequences encoded by YY2, A1 and BA42 with that of chalcone synthase from parsley [24]. Considerable differences exist in amino acid sequences between products of BA42 and A1, though both cDNAs were isolated from *B. napus*. Shen and Hsu [29] observed that several fragments of genomic DNA hybridized to the BA42 probe. By contrast, in rice, the YY2 gene exists as a single copy, as shown by Southern blot hybridization (Fig. 4). YY2 cDNA also exhibits homology to genes for chalcone synthase (CHS). The predicted sizes of CHS mRNAs of *Hordeum vulgare*, *Petunia hybrida* and *Zea mays* are 1477 nt, 1335 nt and

Fig. 8. Comparison of amino acid sequences deduced from rice YY2 cDNA [this study], *Brassica* A1 [28], and *Brassica* BA42 [29] with that of chalcone synthase (CHS) from parsley [24]. Identical amino acid residues are indicated by asterisks. Gaps have been introduced to obtain maximum homology, and they are shown by hyphens. Amino acid residues conserved among CHSs from various plant species [20] are indicated by shadowing. The amino acid sequences of A1 and BA42 were deduced from reported partial cDNA sequences.

1461 nt, respectively [20]. YY2 mRNA is 1402 nt long, corresponding to the expected size of CHS mRNA. Though BA42 and A1 also exhibit a high degree of homology to genes for CHS, they are not full-length cDNA clones and lack a consensus site for initiation of translation. It will, therefore, be of interest to compare the full-length sequence of YY2 cDNA with those of genes for CHS. Little homology is observed between the N-termini of the YY2 protein and parsley CHS. There are some conserved domains in the central part of the YY2, A1, BA42 and CHS proteins, which correspond roughly to a consensus sequence for CHSs [20], shown by shaded amino acid residues in Fig. 8. We have previously isolated a partial cDNA clone that exhibited 89% homology to CHS gene from *Hordeum vulgare*. However, YY2 cDNA did not show any similarity to this sequence (data not shown). These observations imply that the YY2, A1 and BA42 proteins are not CHS itself, but are divergent members of the CHS gene family. From the similarities in their protein sequences, we consider that YY2 cDNA is a rice homologue of A1 and BA42 cDNAs. In contrast to transcripts for CHS and BA42, which are expressed in several types of cell in the anther (CHS: tapetal cells, outer parenchyma cells of the connectivum [9]; BA42: tapetal cells, peripheral cells of the vascular bundle, developing microspores [29]), expression of YY2 mRNA was confined to the tapetum (Fig. 9b).

Possible roles of YY1 and YY2 in the development of anther

Both YY1 and YY2 mRNAs are specifically expressed in the tapetum at the uninucleate microspore stage (Figs. 3 and 9). Osc4 and Osc6, which were isolated from rice anther by Tsuchiya *et al.* [32], show similar expression pattern [33], but both YY1 and YY2 cDNAs did not exhibit any homology with them. According to the classification of anther-specific genes by Mascarenhas [15], the YY1 and YY2 genes may be 'early' genes that become active soon after meiosis and whose mRNAs are present at reduced levels or

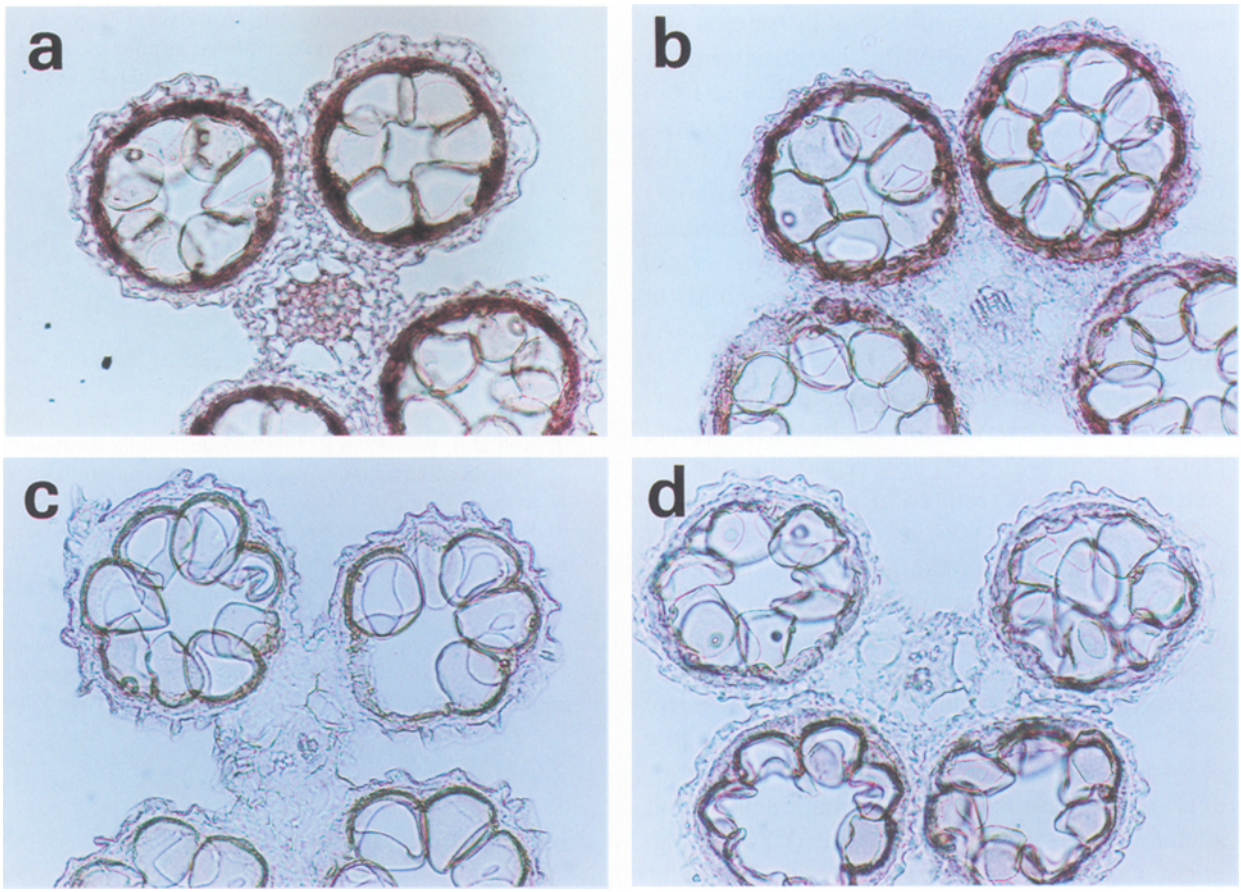

Fig. 9. In situ localization of YY1 and YY2 transcripts in developing anthers. Digoxigenin-labelled sense and antisense RNA probes were used. YY1 (a) or YY2 (b) antisense probe was used for the uninucleate microspore stage of anther, respectively. (c) Sense probe of YY1 shows no hybridization signal. (d) No signal was detected in the trinucleate microspore stage of anther with YY1 antisense probe.

are undetectable in mature anthers. At the uninucleate microspore stage, microspores are released from tetrads and rapidly increase in volume at the anther locule. During the growth of the microspores, tapetal cells, which surround the anther locule, provide nourishment and structural components, such as cell-wall materials to microspores. The 'early' genes are expected to encode materials secreted to the microspores or enzymes that function in the tapetum.

Among the 'early' genes, there are some genes for proteins with putative signal sequences at their N-termini. For example, glycine-rich protein and lipid-transfer protein are abundantly expressed in the tapetum of tobacco, and they are thought to be secreted to developing microspores in view of their putative signal sequences [10]. In the case of *Sa*tap35 and *Sa*tap44 from *Sinapis alva*, processing the translated products actually occurs and mature proteins are localized in the exine cell-wall layer of the developing microspore, suggests evidence that the proteins are indeed secreted from the tapetum into the locule [31]. The YY1 polypeptide with a putative signal sequence might be secreted and act as an extracellular structural component, which is stabilized by intramolecular disulfide bridges.

The YY2 cDNA exhibits considerable homology to genes for CHS, which is known to play an important role in anther development. CHS is the

key enzyme in the biosynthesis of flavonoids and catalyzes the formation of the central intermediate in this pathway, naringenin chalcone, from 4-coumaroyl-CoA and three molecules of malonyl-CoA [6]. In anthers, CHS is located in the tapetal cells [7], and newly synthesized flavonoids are transported into the locule and deposited on the outer surface of the pollen grains (the exine) [36]. The significance of CHS and flavonoids in anthers was demonstrated in antisense mRNA-inhibition experiments. Van der Meer *et al.* [34] report that aberrant pollen development or germination was observed in *chs*-antisense *Petunia* plants. Ylstra *et al.* [39] also report that the absence of flavonols resulted in structural damage to the pollen apex in *Petunia* plants. The strong homology in terms of the amino acid sequence between YY2 protein and CHS suggests the possibility of a similar catalytic function for the YY2 protein. In fact, a number of enzymes, such as 6-deoxychalcone synthase [3] and resveratrol synthase [27], are capable of utilizing the same substrates as those of CHS, but their final products are different. From the information presented so far, it seems possible that the YY2 protein might catalyze one step in the biosynthesis of flavonoids that are required for anther development. By introduction of *yy2*-antisense gene into rice and analysis of the composition of flavonoids, as well as the development of anthers in the transgenic plants, we should be able to obtain more information about the function of the YY2 protein.

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References

1. Brown SM, Crouch ML: Characterization of a gene family abundantly expressed in *Oenothera organensis* pollen that shows sequence similarity to polygalacturonase. Plant Cell 2: 263–274 (1990).
2. Cox KH, Goldberg RB: Analysis of plant gene expression. In: Shaw CH (ed) Plant Molecular Biology: A Practical Approach, pp. 1–34. IRL Press, Oxford (1988).
3. Dixon RA: The phytoalexin response: eliciting, signalling and control of host gene expression. Biol Rev 61: 239–291 (1986).
4. Drews GN, Bowman JL, Meyerowits EM: Negative regulation of the Arabidopsis homeotic gene AGAMOUS by the APETALA2 product. Cell 65: 991–1002 (1991).
5. von Heijne G: A new method for predicting signal sequence cleavage sites. Nucl Acids Res 14: 4683–4690 (1986).
6. Heller W, Hahlbrock K: Highly purified 'flavanone synthase' from parsley catalyzes the formation of naringenin chalcone. Arch Biochem Biophys 200: 617–619 (1980).
7. Kehrel B, Wiermann R: Immunochemical localization of phenylalanine ammonia-lyase and chalcone synthase in anthers. Planta 163: 183–190 (1985).
8. Kobayashi T, Kobayashi E, Sato S, Hotta Y, Miyajima N, Tanaka A, Tabata S: Characterization of cDNAs induced in meiotic prophase in lily microsporocytes. DNA Res 1: 15–26 (1994).
9. Koes RE, van Blokland R, Quattrocchio F, van Tunen AJ, Mol JNM: Chalcone synthase promoters in petunia are active in pigmented and unpigmented cell types. Plant Cell 2: 379–392 (1990).
10. Koltunow AW, Truettner J, Cox KH, Wallroth M, Goldberg RB: Different temporal and spatial gene expression patterns occur during anther development. Plant Cell 2: 1201–1224 (1990).
11. Kreis M, Forde BG, Rahman S, Miflin BJ, Shewry PR: Molecular evolution of the seed storage proteins of barley, rye and wheat. J Mol Biol 183: 499–502 (1985).
12. Lilley GG, Inglis A: Amino acid sequence of conglutin δ, a sulphur-rich seed protein of *Lupinus angustifolius* L. FEBS Lett 195: 235–241 (1986).
13. Maeda K, Wakabayashi S, Matsubara H: Disulphide bridges in an α-amylase inhibitor from wheat kernel. J Biochem 94: 865–870 (1983).
14. Mascarenhas JP: The biochemistry of angiosperm pollen development. Bot Rev 41: 259–311 (1975).
15. Mascarenhas JP: Gene activity during pollen development. Annu Rev Plant Physiol Plant Mol Biol 41: 317–338 (1990).
16. Mascarenhas JP: Pollen gene expression: molecular evidence. Int Rev Cytol 140: 3–18 (1992).
17. McCormick SM: Molecular analysis of male gametogenesis in plants. Trends Genet 7: 298–303 (1991).
18. Muschietti J, Dircks L, Vancanneyt G, McCormick S: LAT52 protein is essential for tomato pollen development: pollen expressing antisense *LAT52* RNA hydrates and germinates abnormally and cannot achieve fertilization. Plant J 6: 321–338 (1994).
19. Nacken WKF, Huijser P, Beltran JP, Saedler H, Sommer H: Molecular characterization of two stamen-specific genes, *tap1* and *fil1*, that are expressed in the wild type,

but not in the deficiens mutant of *Antirrhinum majus*. Mol Gen Genet 229: 129–136 (1991).
20. Niesbach-Klösgen U, Barzen E, Bernhardt J, Rohde W: Chalcone synthase genes in plants: A tool to study evolutionary relationships. J Mol Evol 26: 213–225 (1987).
21. Palmiter RD: Magnesium precipitation of ribonucleoprotein complexes: Expedient techniques for the isolation of undergraded polysomes and messenger ribonucleic acid. Biochemistry 13: 3606–3615 (1974).
22. Paul W, Hodge R, Smartt S, Draper J, Scott R: The isolation and characterization of the tapetum-specific *Arabidopsis thaliana* A9 gene. Plant Mol Biol 19: 611–622 (1992).
23. Raghavan V: Anther and pollen development in rice (*Oryza sativa*). Am J Bot 75: 183–196 (1988).
24. Reimold U, Kröger M, Kreuzaler F, Hahlbrock K: Coding and 3′ non-coding nucleotide sequence of chalcone synthase mRNA and assignment of amino acid sequence of the enzyme. EMBO J 2: 1801–1805 (1983).
25. Satake T: Male sterility caused by cooling treatment at the young microspore stage in rice plants. IX. Revision of the classification and terminology of pollen developmental stages. Proc Crop Sci Soc Japan 43: 31–35 (1974).
26. Satake T, Hayase H: Male sterility caused by cooling treatment at the young microspore stage in rice plants. V. Estimations of pollen developmental stage and the most sensitive stage to coolness. Japan J Crop Sci 50: 495–501 (1981).
27. Schröder G, Brown JWS, Schröder J: Molecular analysis of resveratrol synthase: cDNA genemic clones and relationship with chalcone synthase. Eur J Biochem 172: 161–169 (1988).
28. Scott R, Dagless E, Hodge R, Paul W, Soufleri I, Draper J: Patterns of gene expression in developing anthers of *Brassica napus*. Plant Mol Biol 17: 195–207 (1991).
29. Shen JB, Hsu FC: *Brassica* anther-specific genes: characterization and *in situ* localization of expression. Mol Gen Genet 234: 379–389 (1992).
30. Smith AG, Gasser CS, Budelier KA, Fraley RT: Identification and characterization of stamen- and tapetum-specific genes from tomato. Mol Gen Genet 222: 9–16 (1990).
31. Staiger D, Kappeler S, Müller M, Apel K: The proteins encoded by two tapetum-specific transcripts, *Sa* tap 35 and *Sa* tap 44, from *Sinapis alba* L. are localized in the exine cell wall layer of developing microspores. Planta 192: 221–231 (1994).
32. Tsuchiya T, Toriyama K, Nasrallah ME, Ejiri S: Isolation of genes abundantly expressed in rice anthers at the microspore stage. Plant Mol Biol 20: 1189–1193 (1992).
33. Tsuchiya T, Toriyama K, Ejiri S, Hinata K: Molecular characterization of rice genes specifically expressed in the anther tapetum. Plant Mol Biol 26: 1737–1746 (1994).
34. van der Meer IM, Stam ME, van Tunen AJ, Mol JNM: Antisense inhibition of flavonoid biosynthesis in petunia anthers results in male sterility. Plant Cell 4: 253–262 (1992).
35. Wada T, Ito T, Ito M, Takeoka Y: Light microscopic observation on pollen and anther in rice (*Oryza sativa* L.). II. Stages from early microspore to mature pollen. Jpn J Crop Sci 61: 136–144 (1992).
36. Wiermann R, Vieth K: Outer pollen wall, an important accumulation site for flavonoids. Protoplasma 118: 230–233 (1983).
37. Wing RA, Yamaguchi J, Larabell SK, Ursin VM, McCormick S: Molecular and genetic characterization of two pollen-expressed genes that gave sequence similarity to pectate lyases of the plant pathogen *Erwinia*. Plant Mol Biol 14: 17–28 (1990).
38. Wright SY, Suner MM, Bell PJ, Vaudin M, Greenland AJ: Isolation and characterization of male flower cDNAs from maize. Plant J 3: 41–49 (1993).
39. Ylstra B, Busscher J, Franken J, Hollmen PCH, Mol JNM, van Tunen AJ: Flavonols and fertilization in *Petunia hybrida*: localization and mode of action during pollen tube growth. Plant J 6: 201–212 (1994).