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Isolation of a gene preferentially expressed in mature anthers of rice (*Oryza sativa* L.)

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Summary. Using monoclonal antibodies raised against pollen-specific proteins, we have isolated a cDNA clone, designated *Ory-C1* from a rice anther cDNA expression library. A transcript corresponding to the *Ory-C1* gene showed preferential expression in anthers. This transcript was not detected in any vegetative tissues analysed. RNA gel blot analysis of different developmental stages of anthers showed that the *Ory-C1* gene is expressed at later stages of pollen development. In situ hybridisation showed that the *Ory-C1* transcript is only present in mature pollen.

Keywords: Anther-specific gene; In situ hybridisation; Pollen; *Oryza sativa*.

Introduction

The development of the pollen grain is of central importance in sexual reproduction of flowering plants. The role of the pollen grain is to produce and deliver male gametes, sperm cells, to the embryo sac (Knox 1984). This results in successful fertilisation and ultimately seed set. Pollen development occurs within the diploid sporophytic anther tissue and encompasses a series of sequential developmental events. These include the differentiation of sporogenous cells, meiotic divisions, postmeiotic development of the microspores and eventually pollen maturation, including formation of male gametes. Studies in various plants indicate that each stage of pollen development is tightly controlled by a specific set of nuclear genes (Chaudhury 1993). These genes are likely to be expressed exclusively in anthers. A number of anther-specific genes has been isolated from a wide variety of plants including some agronomically important crops such as tomato (Twell et al. 1989), oilseed rape (Theerakulpisut et al. 1991, Albani et al. 1990), and maize (Hanson et al. 1989). The isolation and characterisation of such genes is essential for a better understanding of pollen development and function. Furthermore, study of these genes may have potential applications in creating nuclear male sterility, an important tool for facilitating production of hybrid varieties in plants of agronomic importance (Goldberg et al. 1993).

Most of the anther-specific genes obtained to date are from dicotyledonous plants (McCormick 1991, 1993). Relatively few anther-specific genes have been identified from monocots, particularly from crops such as rice, where only two early-acting microspore-specific genes, *Osc4* and *Osc6*, have been characterised (Tsuchiya et al. 1992). We are interested in identifying genes involved in development of rice anthers, particularly those preferentiall expressed in tapetum and pollen. We report here the isolation of a cDNA clone corresponding to an mRNA specifically expressed in mature rice anthers.

Materials and methods

Plant material

Rice (*Oryza sativa* L. var. japonica YRM42) plants were grown in a glasshouse under 12–14 h daylight and 30/20 °C day/night temperatures.

Construction and screening of cDNA library

Poly(A)$^+$ RNA was extracted from mature rice anthers using a Quick-Prep mRNA purification kit (Pharmacia). Approximately

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5 µg of poly(A)$^+$ RNA was obtained from 200 mg of fresh anthers. A cDNA library, containing 150,000 independent clones, was considered in λgt11 expression vector. The library was screened with monoclonal antibodies 3A2 and 4D2 (Smith 1993) raised against Bermuda grass (*Cynodon dactylon*) pollen-specific proteins, using the method described by Singh et al. (1991). Positive clones were purified and cDNA inserts subcloned into plasmid vectors pBluescript SK (Stratagene) for further analysis.

RNA and DNA gel blot analysis

Total RNA was extracted from various tissues as described (Chomocynski and Sacchi 1987). 20 µg of total RNA was electrophoresed and transferred onto Hybond N$^+$ nylon membrane (Amersham) under vacuum for 4 h using Vacugene gel blotting system (Pharmacia) following manufacturer's instructions. Hybridisation and washing was carried out as described by Singh et al. (1991).

Genomic DNA was extracted from young leaves (Murray and Thompson 1980) and digested with restriction enzymes. DNA (10 µg) was then separated on 0.7% agarose gel and transferred onto Hybond N$^+$ nylon membrane (Amersham) under alkaline conditions according to the manufacturer's specification. Hybridisation and washing were conducted as described by Sambrook et al. (1989).

DNA sequencing and sequence analysis

DNA sequence was determined by the dideoxy chain termination method using a T7 sequencing kit (Pharmacia). Specific oligonucleotide primers were used to obtain the complete sequence of both strands. Sequence analysis was performed using the Melbot/Angis database which incorporates sequence data from the following major sources: GeneBank; EMBL and NBRF nucleic acid libraries; NBRF PIR protein and Swiss-Prot libraries.

In situ hybridisation

Anthers were fixed in glutaraldehyde and embedded in LR gold resin as described by Chaudhury et al. (1994). Biotin-UTP labelled sense and antisense RNA riboprobes were generated by in vitro transcription. The labelled probes were then purified through Nick Spin column (Pharmacia) and degraded to a mean length of 75–100 bp by alkaline hydrolysis (Meyerowitz 1987). Hybridisation and washing were conducted on 2 µm sections as described by McFadden (1991). The hybridisation signal was detected using colloidal gold-antibiotin antibodies (BioCell) followed by silver enhancement (Amersham). Sections were then observed with a bright field microscope. The signal was analysed using IMAGE-1/AT version 4.0 computer software (Universal Image Corp. Media). The processed image was displayed on a Sony color video monitor and recorded off the screen using a Nikon FE2 SLR camera.

Results and discussion

In order to isolate genes specifically expressed in rice anthers, a cDNA expression library was constructed and probed with monoclonal antibodies to Bermuda grass pollen-specific proteins (Smith 1993). Immunoblotting analysis revealed that these antibodies cross-react with pollen-specific proteins of other grasses, including rice (Smith 1993). Several cDNA clones were identified, purified and subcloned into pBluescript SK plasmid vectors. To determine the tissue specificities of the cDNA clones obtained, RNA gel blot analyses were performed on total RNA isolated from rice leaf, seedling, lemma and palea, and mature anther. One of the cDNA clones hybridised to a ~1kb transcript, which is abundantly expressed in mature anthers (Fig. 1 A). This clone was designated *Ory-C1*. No corresponding transcript was detected in any of vegetative tissues analysed, suggesting that the *Ory-C1* gene is expressed preferentially in mature anthers.

To establish at which stage anther development the *Ory-C1* gene expression is switched on, RNA gel blot analysis was performed using total RNA extracted from rice anthers of five developmental stages. The size of spikelets can be used as an efficient index for pollen developmental stages. Anthers were dissected from florets of lengths varying from 6 mm to mature spikelets and developmental stages determined by acetocarmine staining of squashed anthers. Anthers

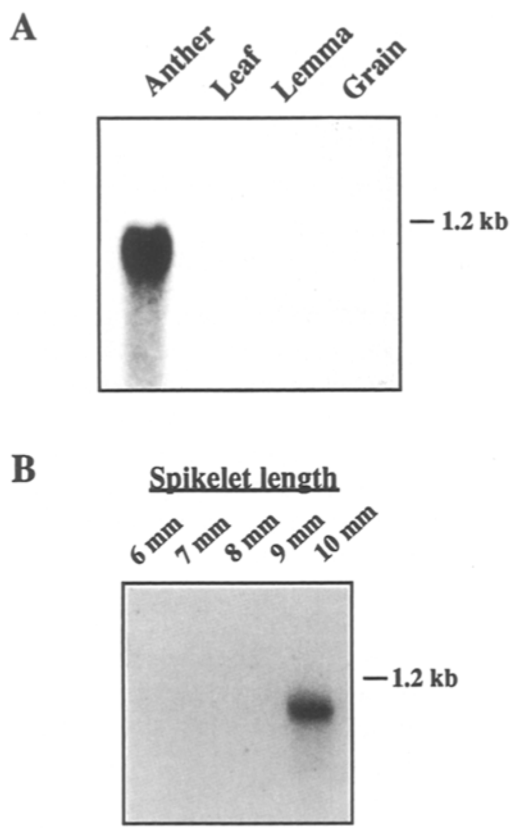

Fig. 1. RNA gel blot analyses showing the spatial and temporal expression pattern of *Ory-C1* gene. 20 µg of total RNA per lane was used. **A** Expression of *Ory-C1* gene is detected in anther tissue, but not in leaf, seedling, lemma, and palea. **B** Expression of the *Ory-C1* gene during anther development

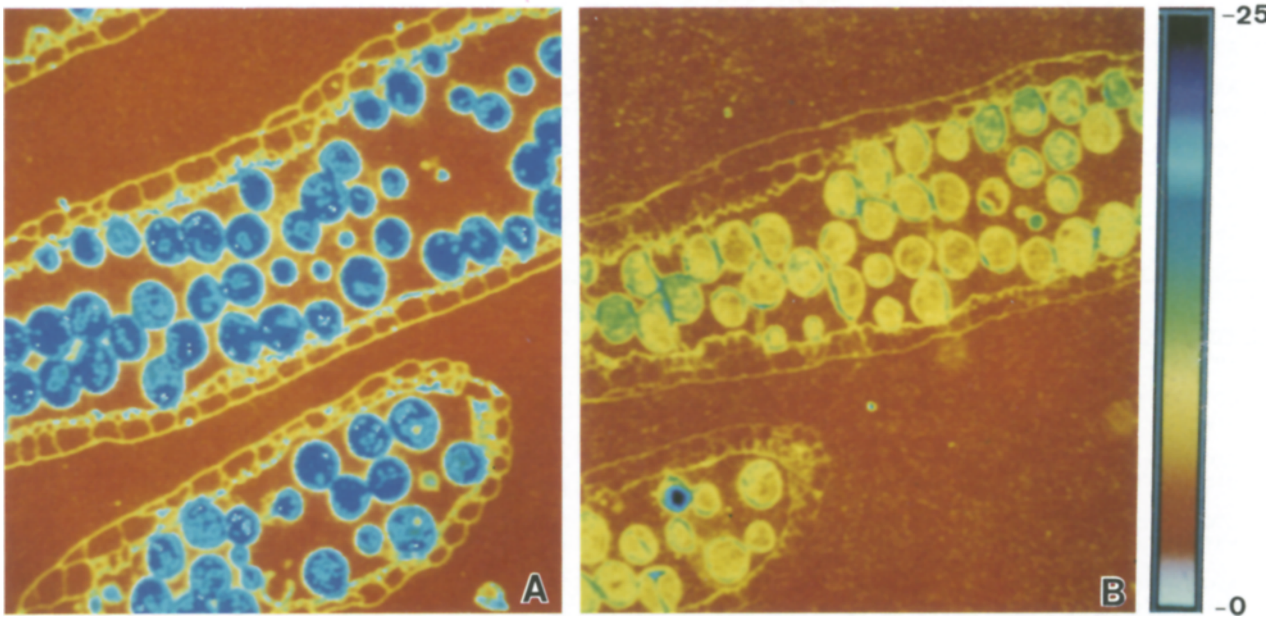

Fig. 2. In situ hybridisation of rice anthers showing that the *Ory-C1* gene transcript is only present in mature pollen grains. Mature anther sections were probed with biotin-labelled antisense (**A**) and sense (**B**, control) riboprobe generated from the *Ory-C1* cDNA clone. The hybridisation signal was detected using gold-conjugated anti-biotin antibodies followed by silver enhancement. Sections were viewed by bright field microscopy and signal was analysed using IMAGE-1/AT computer software. Strong hybridisation signal was detected in pollen grains as indicated by the intensity to the blue colour. The wedge represents the colour pixel intensity profile from 0 to 255 units

from 6 mm spikelets contained microsporocytes undergoing meiotic division. Newly released uninucleate microspores were found in anthers dissected from 7 mm spikelets. Anthers from 8 mm spikelets contained predominantly vacuolate uninucleate microspores, whereas those from 9 mm spikelets contained late uninucleate microspores and bicellular pollen. Anthers taken from open spikelets (10 mm) contained starch-filled mature pollen grains. RNA gel blot analysis (Fig. 1 B) shows that the *Ory-C1* gene transcript is not expressed in anthers from 6–9 mm spikelets in which most of the microspores are at uninucleate stage. High levels of expression of the *Ory-C1* gene transcript is found only in mature anthers.

In order to determine in which anther tissue the transcript corresponding to the anther-specific *Ory-C1* cDNA clone was expressed, in situ hybridisation was performed on sections of anthers at two developmental stages, uninucleate microspore and mature pollen stages. Semi-thin sections (2 µm) were hybridised with the antisense strand of biotin-labelled riboprobes synthesized from the *Ory-C1* cDNA clone. Parallel sections were probed with the biotin-labelled sense strand of the *Ory-C1* riboprobe as a control. As shown in Fig. 2, a very strong hybridisation signal was detected in mature pollen grains (Fig. 2 A), whereas no hybridisation was observed in the anther wall cells, the epidermis and endothecium (Fig. 2 B). At this stage of pollen development, the tapetum had degenerated. No hybridisation signal was detected at the uninucleate microspore stage in any anther tissues

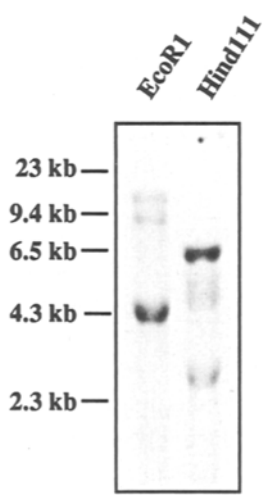

Fig. 3. DNA gel blot analysis of rice genomic DNA. Genomic DNA was digested separately with Eco RI and Hin dIII. Digested DNA (10 µg per lane) was subjected to DNA gel blot analysis using ^{32}P-labelled *Ory-C1* cDNA insert as a probe

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                                                      GA ATT CAA CCT TCC AAA   17

GGT ACA GCA CAT TTA TGT TCA GTG TTT TGA GTG GAT AAT ATC TTT CTC GGA GCA ATA ACC   77

ATG AAT CAT TTA GCC TTG ACA CTC TCC GTT GTA GCT TGC TCC TTG ACA GGC GGA CTC TCC  137
Met asn his leu ala leu thr leu ser val val ala cys ser leu thr gly gly leu ser

GGT GTA CCT TCC TTG TTG ACA GGC CGA ATC CGT TCA TAC CGC TTC CTC GTC AGT GTC TCC  197
gly val pro ser leu leu thr gly arg ile arg ser tyr arg phe leu val ser val ser

CCT TTG AGG CAG CAA CAT ATC TGT CAT TTG TGT CCT CTT TCC TTT GCA GCT CTC CAA GAA  257
pro leu arg gln gln his ile cys his leu cys pro leu ser phe ala ala leu gln glu

GAC TGG TGG TCC GGG GAC AGG GAG AGC TAC CTC GTC GAC GAG CTG GAG CCG CTG CCG CTG  317
asp trp trp ser gly asp arg glu ser tyr leu val asp glu leu glu pro leu pro leu

CCG TTG ACC GTT CCG ACA CCG AGC CGA TGT CCC GCG AGA GCT CGA CCG CCG CCT CAG CTG  377
pro leu thr val pro thr pro ser arg cys pro ala arg ala arg pro pro pro gln leu

CGA CGT GGA GAT CGA GGA TTG CAA GAC TGT CTC CTA CGA GTG GAC GGG CAA GTG CGG AGT  437
arg arg gly asp arg gly leu gln asp cys leu leu arg val asp gly gln val arg ser

TGC CAG GGG ACA GGG CTG GTG AGC TAT TTC AGG AAG AAG GGG AGG GAG ACC ATC TGC AAA  497
cys gln gly thr gly leu val ser tyr phe arg lys lys gly arg glu thr ile cys lys

TGC GTG CCA TGC GCT GGC ATT GGT TAT GTT CGG AAA ATC ACA TTT CGC CAG GAC ATT GAA  557
cys val pro cys ala gly ile gly tyr val arg lys ile thr phe arg gln asp ile glu

AAC ATG GAT GAG TTA GAC AAT GGG AAA CCA CCA GTT AGA TGG CCC TGT TCG CTT GGC AGG  617
asn met asp glu leu asp asn gly lys pro pro val arg trp pro cys ser leu gly arg

CTG TCA TTT ATT AAC TGC TGC TGG ATA GAT TGT GTT CAC AAC TTG GAC GAT TTA ACC AGT  677
leu ser phe ile asn cys cys trp ile asp cys val his asn leu asp asp leu thr ser

GAT TCT TTC TGT TAC AGT CAG GCA CGG TTA TCT ATG GAT GAG GTG CCA TTT TTA AGT GTT  737
asp ser phe cys tyr ser gln ala arg leu ser met asp glu val pro phe leu ser val

GTA AAC CAT TAT CTG CTA ATA ATT GTA CTC CTT TGT TCT TAT CAT TAT AGT GAT AAA GAA  797
val asn his tyr leu leu ile ile val leu leu cys ser tyr his tyr ser asp lys glu

AAG GAA AGA AAA TTA ACG GCA AGA TCA TGC ACA TTA TCG GCA GGA AGG AGA GGA AGA ATT  857
lys glu arg lys leu thr ala arg ser cys thr leu ser ala gly arg arg gly arg ile

TAC GGT GCT CTA AAG ACC AAA GAA AAG GAA AAT TCT GTC TGA TCT ACT GAT CAA GAC TCG  917
tyr gly ala leu lys thr lys glu lys glu asn ser val OPA

AGG GCG CCG TGT CCT TCC CTT CCT GAT CCT AGC CGT GGC CCC GCC GCC GAC TAC GGC GGC  977

CGG CTC CGG GTG GAT GAG CAG CTT GAA CCT GCC GTC CTC CTT GCA CGG CTG AGC GGC CGC 1037
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Fig. 4. Nucleotide and deduced amino acid sequence of the *Ory-C1* cDNA. The predicted amino acid sequence is shown below the nucleotide sequence. The region showing 60% identity with rice grain cysteine proteinase (Watanabe et al. 1991) is underlined

(data not shown). These in situ hybridisation results strongly suggest that expression of the *Ory-C1* gene is confined to mature pollen. This is consistent with the results obtained from RNA gel blot analyses (Fig. 1 B).

Previous studies have shown that two distinct sets of genes are involved in the development of pollen (Mascarenhas 1992). The genes belonging to the first set are expressed at an early stage of pollen development. Expression of these early genes commences shortly after meiosis and reaches a maximum around microspore mitosis, then declines during pollen maturation. The two previously isolated rice anther-specific genes, *Osc4* and *Osc6*, belong to this class (Tsuchiya et al. 1992). The second set of genes are expressed in maturing pollen and expression of these late genes begins after microspore mitosis. Our RNA gel blot analyses showed a high level of *Ory-C1* gene expression in mature pollen, suggesting that this gene belongs to the late set of genes.

DNA gel blot analysis was conducted to determine the number of genes in the *Ory-C1* gene family. Rice genomic DNA was digested and probed with the *Ory-C1* cDNA insert. As shown in Fig. 3, one genomic DNA fragment hybridised strongly to the probe while an additional fragment hybridised weakly, representing related sequences. These data indicate that the *Ory-C1* gene belongs to a small gene family.

Both strands of the *Ory-C1* cDNA were sequenced. The nucleotide sequence of *Ory-C1* cDNA and its derived amino acid sequence are shown in Fig. 4. The *Ory-C1* cDNA is 1037 bp long, which is close to the size of *Ory-C1* transcript (approximately 1 kb) revealed by RNA gel blot analysis (Fig. 1 A). No putative polyadenylation signal is present in the sequence, suggesting that a short length of nucleotides in the cDNA clone obtained may be missing at the 3′ end. The translation start of *Ory-C1* is considered to be the first ATG codon present in the sequence at position 78. This putative start codon gives the longest possible open reading frame and encodes a predicted polypeptide of 276 amino acid residues. The *Ory-C1* polypeptide is rich in leucine (13%), with a calculated molecular mass of approximately 31 kDa. A hydropathy plot showed a hydrophobic region at the N-terminus, which represents a putative signal peptide sequence. A search of existing data bases showed that a region of 120 nucleotides from the *Ory-C1* sequence (nucleotide position 230 to 350; Fig. 4) displays 60% sequence identity with cysteine proteinases of rice grains (Watanabe et al. 1991). However, the active site of cysteine proteinases is not conserved in the *Ory-C1* polypeptide, suggesting that the relationship between the two genes may be of evolutionary origin rather than due to a conserved function. Function of the *Ory-C1* gene product in pollen development is not known. The temporal expression pattern of this gene suggests that it may play a role during pollen maturation as a storage protein or during germination and pollen tube growth. Studies are in progress to investigate the functional aspect of the *Ory-C1* gene product using various approaches including antisense RNA.

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