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Expressed Sequence Tags in Developing Anthers of Rice (*Oryza sativa* L.)

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In the early stages of microspore development, dramatic changes in nuclear phase and cell morphology occur [1]. The pollen mother cells undergo meiosis, giving rise to a tetrad of haploid cells. After microspores are released to the anther locule, they grow rapidly and cell walls are actively synthesized. These successive changes are not caused by the male gametophyte alone but are consequences of the cooperative works of gametophyte and surrounding sporophytic tissues such as the tapetum. For example, tapetal cells secrete β-1, 3-glucanase (callase) at the time of microspore release to dissolve the callose wall which surrounds the tetrads, and provide nourishment and structural components to the microspores at the appropriate time of development.

A large number of proteins may participate in the intricate process of anther development showing a strictly controlled expression pattern both temporally and spatially. Further, it is remarkable that their gene expression seems to be controlled differentially or in a coordinated manner between sporophytic and gametophytic tissues. To gain insight into the molecular processes underlying anther development, several anther-specific genes have been obtained by differential screening. The isolation of anther-or pollen-specific genes have been reported for some plant species (reviewed by [2, 3]). The roles of their gene products have been elucidated by sequence similarity, immunolocalization and the introduction of antisense constructs using transgenic techniques. However, for the systematic analysis of gene expression, it is necessary to isolate many homologous probes from one plant species and to characterize their expression patterns in terms of developmental stages and cell types.

Random cDNA sequencing is a powerful approach for the isolation of many functional genes which are expressed in a particular tissue or under specific environmental conditions [4]. Recently, a large number of cDNA clones were identified by random sequencing from human [5, 6], *Caenorhabditis elegans* [7, 8], mouse [9], maize [10], *Arabidopsis thaliana* [11], *Brassica napus* [12] and rice [13-15]. Now it is clear that these studies (the expressed sequence tags, ESTs) have greatly contributed to increased gene resources in each organism. Moreover, the cDNA clones thus identified can be used for expression analysis of genes engaged in specific metabolic pathways such as glycolysis, alcohol fermentation and the TCA cycle [15, 16]. These results showed that ESTs provide us the opportunity to analyze the regulation of gene expression under specific conditions.

In order to survey the ESTs of early stages of anther development, we identified a number of putative genes from cDNA clones of rice anthers at the uninucleate microspore stage. Further, by using the isolated cDNA clones as probes, the transcript levels during anther development were investigated.

Oryza sativa L. var. Hayayuki plants were grown in a greenhouse. Poly(A)$^+$ RNA was extracted from anthers at the uninucleate microspore stage ($\leq$2 mm in length) using the Fast Track mRNA isolation kit (Invitrogen). cDNAs synthesized using cDNA synthesis kit (Pharmacia) were ligated with *Eco*RI adapters to introduce them 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. Partial nucleotide sequences of cDNA inserts were determined by the fluorescence detection method [17] using either dye-labeled T3 or T7 primer. A Model 373A sequencer (Applied Biosystems) was used for automated sequence analysis. The homology of each cDNA sequence averaging 300 bases was searched in the recorded nucleic acid sequences of GenBank and EMBL databases using the FASTA program. Homology scores (>160) were searched for amino acids [13, 15].

From this analysis, partial nucleotide sequences of 653 cDNA clones were determined. Seventy-seven cDNA clones, which occupy 11.8% of the total cDNAs, had significant similarity to nucleotide sequences registered in the databases (**Table 1**). The

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Table 1.

Summary of ESTs isolated from cDNA library of rice anthers at uninucleate microspore stage.

Accession No.	Gene	Length compared (bp)	% matched (bp)	Length compared (aa)	% matched (aa)	Source of comparison
D21130	Acidic ribosomal protein P0	135	69.6	45	57.8	HUMPPARP1
D29689	Acidic ribosomal protein P2	113	77.9	37	78.4	TCP2BMRNA
D29690	Acyl carrier protein II	178	73.6	58	75.9	BLYACL2
D29691	ADP-ribosylation factor*	137	82.5	47	93.6	ATHADPRFA
D29692	ATP-dependent protease	348	80.5	115	94.8	TOMCD4B
D29693	Calmodulin*	178	97.8	59	100	OSCALM
D29695	CARSR12	132	62.1	44	68.2	DINCARSR12
D29694	Casein kinase 1-γ	342	56.7	111	44.0	BOVCKIG
D29696	Casein kinase II-α	177	81.4	56	87.5	ZMACK2
D29697	Chalcone synthase	179	88.8	59	84.7	X58339
D29698	Chaperonin 10	132	73.5	43	72.1	SPICPN10X
D29699	Chloroplast genome (ORF109)	306	99.7	91	100	RICCPOSXX
D29700	Chloroplast genome (psbB)	174	99.4	55	98.2	CHOSXX
D29701	Cyclophilin*	269	81.4	89	79.8	MZECYP
D29702	Cytochrome b-5*	150	67.3	51	71.0	BNACYTB5A
D29703	Elongation factor 1-α*	264	93.9	86	100	S116489
D29704	G-protein β-subunit-like polypeptide	141	60.3	25	80.0	CREGPLP
D29705	GAmRNA (cloneF)	140	70.0	47	77.0	PEAGAMRF
D29706	Glutamate 1-semialdehyde-aminotransferase	129	75.2	43	90.7	BLYGSA
D29707	Glycine-rich protein*	122	84.4	34	79.4	ZMGLYR
D29708	Heat shock protein 70*	293	69.3	97	78.4	PSPHSP1
D29709	Heat shock protein 82*	330	63.0	66	79.0	TOBHSP82
D29710	Heat shock protein 90*	293	75.1	95	84.2	THEHSP90
D29711	High mobility group protein*	163	72.4	50	68.0	ZMHMGPMR
D29712	Histone H2B*	195	87.7	65	90.8	TAHISTH2B
D29713	Histone H3*	204	81.4	65	97.0	SPHISH34
D29714	Hydroxyproline-rich glycoprotein	133	100	40	100	S85024
D29715	Initiation factor (eIF-4A)*	293	98.0	7	100	RICEIF4A
D50570	Initiation factor (eIF-4C)	163	65.6	46	63.0	2006333A
D29716	NADPH HC-toxin reductase	226	80.5	71	78.0	MZETOXR
D17443	Nodulin 26	210	64.8	69	80.0	SOYNOD26R
D29717	Oryzacystatin	204	100	68	100	RICOCS
D29739	Osc6	315	85.1	—	—	RICANT2
D21109	33kDa oxygen evolving protein	80	76.3	26	62.0	WHTPSB0
D21113	Polypeptide chain-binding protein	431	87.2	141	83	MZEB70A
D50572	Polyubiquitin	101	86.1	—	—	OSPORUBI
D29718	pPLB07	248	68.5	—	—	LUPPLB07M
D50569	Protein kinase-Alfalfa (MSK-3)	253	66.0	82	57.3	AMMSK3A
D29719	Ras-related GTP binding protein*	264	97.0	7	100	RICSS230
D21114	Ribosomal protein A2	334	58.7	93	63.0	YSPRPA2
D29720	Ribosomal protein L7	139	72.7	46	78.0	HUMRPL7Y
D29721	Ribosomal protein L19*	86	89.5	20	90.0	RICSS504
D50571	Ribosomal protein L26	142	62.0	44	59.1	HUMRPL26X
D29722	Ribosomal protein L31	204	61.8	63	64.0	CRERIBPL31
D29723	Ribosomal protein L37a	217	65.0	72	70.8	MMRP37A
D29724	Ribosomal protein L38	200	69.5	62	74.0	ENRIPRL38
D29725	Ribosomal protein L39	161	63.4	49	69.4	HUMRIBPROG
D50573	Ribosomal protein L41*	78	92.3	26	92.3	GHRP60S
D29740	Ribosomal protein rp21c	154	72.7	43	62.8	DRORP21C
D29726	Ribosomal protein S5	121	84.3	38	35.0	RNRPS5
D29727	Ribosomal protein S11	239	92.9	79	100	ZMRPS11C
D29728	Ribosomal protein S12*	125	62.4	28	60.7	TTRPS12

Accession No.	Gene	Length compared (bp)	% matched (bp)	Length compared (aa)	% matched (aa)	Source of comparison
D29729	Ribosomal protein S15*	121	76.9	33	93.9	CHKRIGA
D29730	Ribosomal protein S19	286	66.4	85	66.0	HUMS19RP
D21117	Signal recognition particle 7SRNA	208	72.6	—	—	WHTSRP7S2
D29731	smD small nuclear ribonucleoprotein	130	73.1	—	—	MUSSMDAUTA
D29732	Sucrose synthase (RSs1)*	358	98.3	54	94.4	OSSUPHSY
D29733	Sucrose synthase (RSs2)*	137	95.6	33	94.0	RICRSS2
D29734	Superoxide dismutase*	330	99.1	109	99.1	RICSODAOA
D25534	Tonoplast intrinsic protein	188	71.8	60	86.7	ATHGTIP
D17766	Triosephosphate isomerase*	223	96.0	62	97.0	RICRIC
D29735	α-tubulin*	143	87.4	49	93.9	PRUATUB
D50574	Ubiquitin-conjugating enzyme*	163	75.5	46	97.8	ATHUBCB-1

Accession numbers registered in DDBJ, EMBL NCBI and GenBank databases are indicated. Homologies are given as percent identity (% matched) and length compared in base pairs (bp) for nucleotide matches and in amino acid residues (aa) for peptide matches. Sources of comparison were based on the recorded sequences in the GenBank database. Genes with asterisks had been identified in other cDNA libraries previously (Uchimiya *et al.* 1992, Umeda *et al.* 1994).

expected functions of the identified genes were distributed in a broad spectrum. Putative genes encoding ribosomal proteins accounted for most of the identified genes as in previous studies [11, 13-15]. In plants, ribosomes are classified into three types, namely chloroplastic, mitochondrial and cytosolic. Though many chloroplastic ribosomal protein genes which are homologous to those of bacteria have been identified [18], there are relatively few reports about isolation of cytosolic ones. In this study, we isolated 17 cytosolic ribosomal protein genes. For instance, genes encoding cytosolic ribosomal proteins L7, L26, L31, L39, rp21c, S5 and S19 (**Table 1**) are not well understood in higher plants.

We isolated several genes associated with signal transduction pathways: ADP-ribosylation factor, Calmodulin, Casein kinase I-γ, Casein kinase II-α, G-protein β-subunit-like polypeptide, protein kinase isolated from Alfalfa, and Ras-related GTP-binding protein (**Table 1**). We identified a cDNA clone similar to the gene (Osc6) which is specifically expressed in rice anther tissue [19]. We also isolated a homologue to CARSR12 which is a senescence-related gene strictly regulated by ethylene, and specific to floral organs, primarily to petals [20].

Concerning the redundancy of cDNA clones of rice anther, about 80% of the clones appeared once. We did not see much redundancy in other clones. Nevertheless, cDNA encoding sucrose synthase appeared six times, and those encoding glycine-rich protein were detected three times, suggesting their important role in the early stage of anther development. Glycine-rich protein works as a cell-wall component. Since pollen wall is rapidly synthesized immediately after meiosis, proteins such as glycine-rich protein are expected to be transcribed and translated actively in tapetal cells and be secreted to microspores.

To analyze gene expression pattern during anther development, we conducted RNA blot analysis using cDNA clones identified in this study. We have chosen two cDNAs encoding sucrose synthase isozymes which were repeatedly identified from the anther library, and several cDNAs encoding ribosomal proteins. cDNAs were amplified by PCR method from plasmid clones, and labeled with [α-^{32}P] dCTP (110 TBq/mmol, ICN) using a random primer DNA labeling kit (TAKARA). Total RNA was isolated from anthers at the uninucleate microspore stage, at the binucleate pollen stage (2.1~2.2 mm in length) and at the trinucleate pollen stage (ready to dehiscence), and also from shoots and roots of 1-week-old seedlings by the phenol-SDS method [21]. RNAs (3 μg/slot) were blotted on the nylon membrane (Hybond-N+: Amersham) using a BIO-DOT SF (BioRad). Membranes were prehybridized at 65°C for 1 h in hybridization solution containing 1% SDS, 1 M NaCl, 10% dextran sulfate and 0.1 mg/m*l* heat-denatured salmon sperm DNA. They were then hybridized with cDNA probes for 15 h. After hybridization, they were washed in 2 × SSC for 10 min. at 65°C with shaking, and then twice in 2 × SSC containing 1% SDS for 30 min. at 65°C. Membranes were dried and exposed to X-ray films (Fuji RX-50). Quantities of slot blots were measured using a Imaging Plate Scanner BAS2000 (Fuji Film Co.).

As expected from the redundancy in the cDNA library, sucrose synthase gene was highly expressed in anthers compared with shoots and roots (**Fig. 1a**). We repeated the same experiments with different clones, and the result was reproduced. Sucrose synthase catalyzes the reversible reaction, namely, sucrose + UDP ↔ UDP-glucose + fructose. Its main physiological

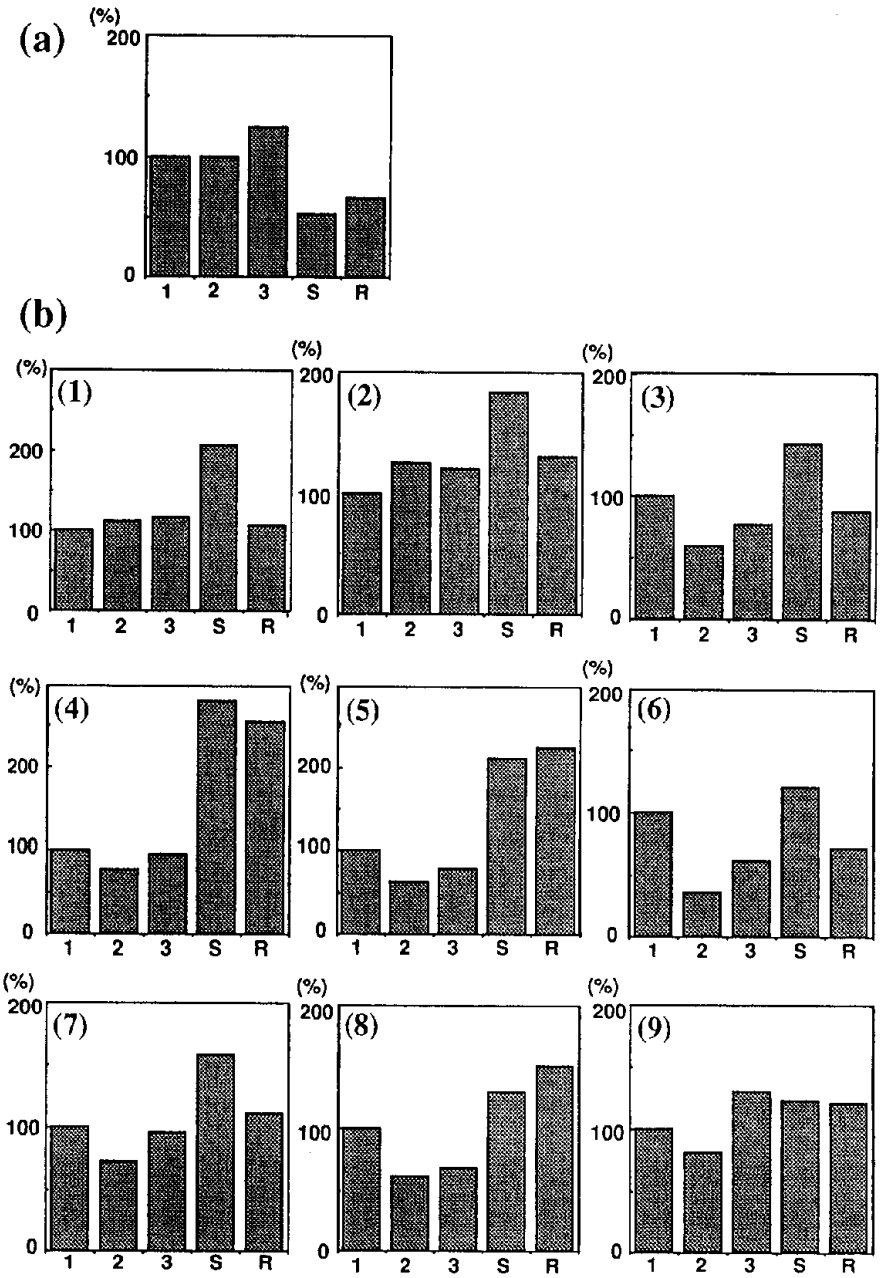

Fig. 1 Expression analysis of various genes during anther development.
(a) Sucrose synthase gene and (b) ribosomal protein gene for (1) S12, (2) S19, (3) S22, (4) L3, (5) L7a, (6) L31, (7) L37a, (8) L38 and (9) P0. Results of RNA blot analysis were quantitated using a Imaging Plate Scanner BAS2000 (Fuji Film). Amounts of transcript in anther at uninucleate microspore stage were taken as 100%. 1: anthers at uninucleate microspore stage. 2: anthers at binucleate microspore stage. 3: anthers at trinucleate microspore stage. S: shoots. R: roots.

role is to cleave sucrose, providing UDP-glucose for the metabolism such as starch, cellulose and hemicellulose synthesis, and respiration [22, 23]. During microspore development, the accompanying cell enlargement, cell division, starch accumulation and formation of intine, the innermost wall of the microspore containing cellulose and hemicellulose [24], may require a constant supply of UDP-glucose. Throughout such metabolic pathway, sucrose synthase as well as glycine-rich protein may take an important role in microspore development.

In addition to six clones encoding ribosomal protein (S12, S19, L31, L37a, L38, P0) from the anther library, we used three cDNA clones (S22, L3, L7a) previously isolated in our laboratory [13]. As shown in **Fig. 1b**, several clones (S19, L3, L7a, L37a, L38 and P0) showed relatively lower transcript levels throughout anther development than those observed in shoots and roots of rice seedlings. With respect to the transcript levels in anthers at different developmental stages, coordinated decline of transcript accumulation from uninucleate stage to bi- and/or tri-nucleate stage was noted with the cDNA probes such as S22, L3, L7a, L31, L37a, L38 and P0, suggesting the ribosomal proteins may be

involved in active translation in young anthers. In order to supply some 60 ribosomal proteins at equimolar concentration, it is known that both transcriptional and post-transcriptional regulation is important [25]. Although there is little information on the structure and regulation of cytoplasmic ribosomal protein in plants, reported instances suggest that their gene expression is regulated in a coordinated manner. For example, two ribosomal protein genes encoding S19 and L7 showed coordinated expression patterns in parallel to cell growth in potato [26]. Namely, they are strongly expressed in the stolon tip during the early stage of tuberization, and transcript levels declined as the tuber increased in size. It is also demonstrated here that ribosomal protein gene expression seems to be coordinately regulated during anther development.

The expression analysis described here could be applicable to other genes. This enables us to make a transcriptional map of genes associated with anther development. Further work may be needed to explore the temporal and spatial gene expression events in rice anther.

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