Molecular cloning of rice cDNAs related to pollen development using the subtraction hybridization technique

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Abstract The meiotic stage of pollen mother cell is a very important stage in controlling the development and formation of pollen. In order to clone the rice cDNA(s) of this stage, a normal rice, Annong N and its thermosensitive mutant, Annong S-1 were used as the plant material. The mRNA has been extracted from the young panicle at the meiotic stage. By using the cDNA subtraction hybridization technique, three cDNA fragments, *RP-1*, *RP-2* and *RP-3* have been successfully cloned from Annong N. Northern blot analysis reveals that the mRNA of these three clones are expressed only in anthers, and not leaves. The mRNA levels of these clones are lower in anthers of Annong S-1 than in Annong N. Furthermore, the amount of mRNA extracted from anthers of Annong S-1 growing under high temperature (28℃) is lower than plants growing at lower temperature (25℃). Sequence analysis and homology search indicate that these three clones display no similarity to the current database. It is concluded that the three novel cDNA cloned are related to pollen development in rice.

Keywords: cDNA, subtraction hybridization, pollen development, rice.

POLLEN development and its formation is a very unique process in higher plants. It involves systematic cell

differentiation and complicated changes in its morphology and biochemistry. These changes are controlled by genes differentially expressed at different stages of pollen development until the completion of the whole developmental process. Many researchers have expressed wishes to identify the genes that will express specifically during pollen development. And more importantly, they also wish to find out the functions of those genes. In order to solve those problems, researchers have used different molecular biology tools to clone the genes that express specifically during pollen development[1—3].

Based on the expression of pollen genes, Mascarenhas[1] have classified the specifically expressed genes in pollen into two groups: one group is referred to as the "early expressed genes". The expression of these genes can be detected after pollen meiosis and the expression level then decreases gradually until it can no longer be detected at about pollen maturation. The other group is referred to as the "late expressed genes". These genes begin to express starting from the stage of microspore mitosis until pollen maturation. In rice, several pollen specific genes have already been cloned. Some of these genes express at the early stage[4,5], and some at the late stage[6]. The meiotic stage is the most important stage during pollen development. But, as far as we are aware, seldom reports have been published on genes expressed at this stage, especially for the case of rice. Furthermore, in the literature we can see that researchers have used different techniques and strategies to clone pollen specific genes. Some used the differential screening technique and some the DDRT-PCR technique which involved the combination of RT-PCR and differential screening[7]. However, all these techniques have limitations. To overcome those limitations, we have in this study used the cDNA subtraction hybridization technique and the young panicle of *Indica* rice, Annong N and its thermosensitive male sterile mutant, Annong S-1, to extract mRNA and clone genes. We have successfully isolated three novel cDNA clones of low expression levels and showed that these clones were related to pollen development in rice. The results are described in detail below.

1 Materials and methods

(ⅰ) Plant materials. Fertile Annong N, its thermosensitive sterile mutant Annong S-1 (critical temperature, 24.2℃[8]) and IR36 seeds were first grown at 37℃ to obtain seedlings. They were then transferred to growth chamber or netted chamber at the stage of 5—6-leaf stage. The growth chamber was set at day length of 14 h with the highest day temperature at 30℃ and the lowest at 25℃. Annong S-1 grown under these temperatures are referred to as Annong S-1 (25℃). The growing condition of the netted chamber maintained at the lowest night temperature of 26℃ and the highest day temperature of 32℃ (at the developmental stage of the pollen mother cell the lowest temperature was kept at 28℃). Annong S-1 grown under these temperatures are referred to as Annong S-1 (28℃). Different parts of the plant, e.g. tiller, young panicle and leaves were excised from both Annong N and Annong S-1 at the meiotic stage of pollen mother cell development. Anthers, hull and leaf sheath were excised from IR36. All tissues were frozen in liquid nitrogen and kept at −80℃ for storage. After the plant parts were excised for analysis the rest of the tiller of Annong N and Annong S-1 was left to mature. Fertility of the mature plants were examined and checked.

(ⅱ) Extraction and isolation of total RNA. After extraction with the one-step method and TRIzo reagent (GIBCOL/BRL), total RNA was dissolved in DEPC-treated water. LiCl was added to precipitate the RNA twice (to a final concentration of 2 mol/L). After vortex mixing, it was kept at 4℃ for at least 2 h. Then it was centrifuged and resuspended in DEPC-treated water. Ethanol was added to precipitate the total RNA and it was finally resuspended in DEPC-treated water and stored at −80℃. Isolation of mRNA was performed using the magnetic beads kit (Promega, USA) following the instructions supplied by manufacturers.

(ⅲ) Construction of subtraction hybridization library. cDNA subtraction hybridization was performed using the PCR-Select cDNA subtracion kit (Clontech). Procedures were followed according to the methods described by Diatchenko[9] except that the ratio of the tester and driver was modified. The cDNA synthesized from the young panicle of Annong N was taken as the tester while that from the young panicle of Annong S-1 as the driver. The subtraction hybridization products were subjected to two rounds of suppression PCR. The PCR products were then ligated to pGEM-T vector (Promega). Ligation products were

transformed into DH10B by electroporation.

(ⅳ) Differential screening of the cDNA subtraction library. The identification of the recombinants was carried out using a modified protocol[10]. The insert sequences were amplified by PCR. 500 ng of each insert was loaded and electrophoresed in a 0.8% agarose gel. Then the DNA was transferred to a nylon membrane (Hybond-N$^+$, Amersham). Two copies were made for each membrane. One of the membranes was hybridized with labeled Annong N cDNA (the same as that used in the subtraction hybridization). The other membrane was hybridized with labeled Annong S-1 cDNA (same as above). The labeling and hybridization conditions were performed according to standard procedures[9].

(ⅴ) Northern blot. 2 μg of mRNA was extracted from the young panicles of Annong N, Annong S-1(25℃) and Annong S-1(28℃), and also from the leaf blades of Annong N (sampling time was the same as that for harvesting the young panicle). Formaldehyde was added to agarose gel and the mRNA was separated by electrophoresis. After electrophoresis, the gel was stained with EB. mRNA was then transferred onto Hybond-N membrane. Another Hybond-N membrane containing 2 g of mRNA of each of the anthers, hull and leaf sheath of IR36 was prepared following the same procedures described above. The specific cDNA obtained after screening was labeled. The labeling reaction of the probe was the same as above. Hybridization was carried out at 50℃ in a high concentration solution of SDS (7% SDS, 50% formaldehyde, 5 × SSC, 1% Blocking Reagent (Boehringe Mannhein), 50 mmol/L phosphate buffer (pH 7.0), 0.1% Sarcosine). After hybridization, the membrane was washed with high stringency. Then the hybridization signal of the membrane was first analyzed with PhosphorImager (Molecular Dynamics) so that the relative expression levels of the corresponding mRNA could be confirmed. Then the membrane was exposed to X-ray film at −70℃.

(ⅵ) DNA sequencing and homology analysis. The pGEM-T plamsid that contained the insert was used as template to perform Cy5 automatic sequencing using the ALF DNA automatic sequencer (Pharmacia). The sequences obtained from each plasmid were analyzed using a computer. Database searching and analysis was performed through Internet to the four databases of Gene Bank, EMBL, DDBJ and PDB. Comparison of homology was performed using the Blast program.

2 Results and discussions

(ⅰ) Construction and screening of cDNA subtraction library. The subtracted products were amplified by PCR and then ligated with pGEM-T vector. All the ligation products were transformed into *E. coli* DH10B, of which the transformation efficiency was about $1 \times 10^{10}/\mu g$. A small quantity of bacterial culture was spread onto LB plates. The recombinant efficiency reached 30% after being checked by the quick method and PCR amplification. 120 recombinants were randomly picked and their insert sequences amplified by PCR. Then they were electrophoresed and transferred to nylon membrane. The blot was hybridized correspondingly with the cDNA synthesized from the young panicles of both Annong N and Annong S-1. Results showed that there were altogether 20 cDNA clones hybridized strongly with Annong N cDNA. If hybridized with Annong S-1 cDNA, the hybridization signal was lower in comparison with that hybridized with Annong N cDNA. But all clones were found to be expressed in Annong S-1. These results indicated that the 20 clones are related to pollen development. The DDRT-PCR was the commonly used protocol for cloning pollen specific genes. This involved the use of a number of primer-groups for screening gene clones. By using this method some of the messages could be lost due to the limitations of the primer-groups. However, the current strategy we used in the present study did not involve the use of any primer-groups and therefore the subtraction hybridization technique has some advantages over the DDRT-PCR method. Moreover, this method required the construction of cDNA subtraction library that could easily be stored for a long period of time. Hence the cloning efficiency of using cDNA subtraction hybridization is much higher.

(ⅱ) cDNA cloning and the characterization of its interaction during pollen development. Probes were made by PCR amplification using the 20 cDNAs clones obtained above. Northern blot analysis was performed using the mRNA isolated from leaves and young panicles of both Annong N and Annong S-1. Among the 20 cDNAs clones, only 6 clones showed expression specificity differences between Annong N

and Annong S-1. However, 3 out of the 6 clones hybridized with the same mRNA with molecular weight of about 1 kb. Two of them hybridized with the same mRNA of about 2 kb. One clone hybridized with a 1.5-kb mRNA. Therefore only 3 clones were obtained. These 3 clones were designated *RP-1*, *RP-2* and *RP-3* respectively (*RP* stands for Rice Pollen while *1*, *2* and *3* represent different isolation dates of the clones). Northern blot analysis was carried out for these 3 clones. Results showed that the expression specificity of these 3 transcripts was the same in Annong N and Annong S-1 (fig. 1). There was no signal when they were hybridized with leaf mRNA and the hybridization signal was much lower in sterile Annong S-1 in comparison with the fertile Annong N. Furthermore, the level of transcripts in Annong S-1 grown under high temperature was much lower than Annong N grown at lower temperature. These results showed that temperature was an important factor in affecting the expression levels of the 3 cDNA clones. Sequential analysis of pollen fertility further suggested that the level of transcripts of the 3 cDNA clones were related to pollen fertility rate. For example, the average pollen fertility rate of Annong N was 99% (the lowest was not less than 96%) and in it the expression level of the 3 clones was normal. Pollen sterility rate of Annong S-1 (25℃) was 100% and the expression level of the 3 clones in it showed a much lower expression level.

In order to investigate whether the expression of the 3 clones was tissue-specific, mRNA isolated from pollen, leaf sheath and hull of IR36 was hybridized with the 3 cDNAs (fig. 2). In fig. 2, we can see that the 3 cDNA clones hybridized only with the pollen mRNA, but not the mRNA from the hull and the leaf sheath. These results clearly indicated that the 3 clones expressed only specifically in pollen. And the genes corresponding to the 3 cDNA clones were related to pollen development.

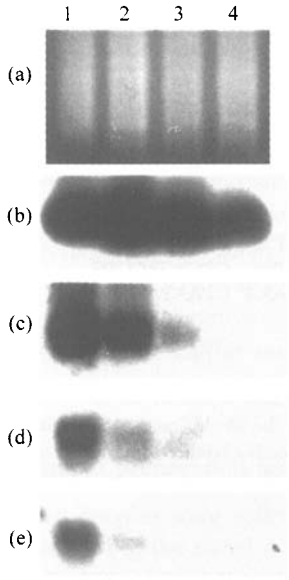

Fig. 1. Northern blot analysis using mRNA isolated from panicle at the meiotic stage of pollen mother cells of Annong N, Annong S-1 (25℃), Annong S-1 (28℃) and Annong N leaves at the same developmental stage. (a) EB staining of mRNA; (b) rRNA as probe; (c) *RP-1* as probe; (d) *RP-2* as probe; (e) *RP-3* as probe. Lane 1, Panicle of Annong N; lane 2, panicle of Annong S-1 (25℃); lane 3, panicle of Annong S-1 (28℃); lane 4, Annong leaves.

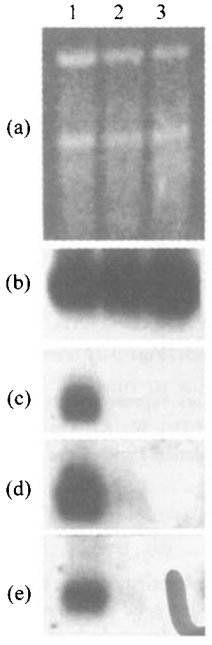

Fig. 2. Northern blot analysis of mRNA isolated from anthers of IR36, hull and leaf sheath at the meiotic stage of pollen mother cells. (a) EB staining of mRNA; (b) rRNA as probe; (c) *RP-1* as probe; (d) *RP-2* as probe; (e) *RP-3* as probe. Lane 1, anther; lane 2, hull and lane 3, leaf sheath.

We have taken the cDNA from the young panicle of Annong S-1 at the same developmental stage as the tester and the cDNA from the young panicle of Annong N as driver. Another subtraction hybridization was carried out using these two cDNAs pools. We have not found any cDNA fragments not expressed in Annong N and yet expressed in Annong S-1, although we have found some abnormalities in the developing pollen of Annong S-1 at the cellular level. Nor have we found any cDNA fragments expressed at low level in Annong N but at high level in Annong S-1. Moreover, we have not found any cDNA clone that expressed in Annong N and not in Annong S-1 either. The 3 cDNA fragments obtained only exhibited differences on the level of expression in Annong N and Annong S-1. All these results indicated that the mechanism in controlling thermosensitive male genic sterility was different from that of cytoplasmic sterility. The mecha-

nism controlling thermosensitive sterility may be the result of DNA mutation in certain genes at the early stage of pollen development which then led to a lowering in the production level of transcripts from the meiotic stage to the formation stage of microspore. The meiotic stage of pollen mother cell development thus marked the beginning of the initiation of thermosensitive sterility. The cDNA fragments that we have cloned all began to express at this stage. Thus by analyzing the structure, function and expression characteristics of these genes, we should be able to understand more about the control mechanism of thermosensitive sterility in rice.

(iii) Analysis of the partial cDNA sequences. With the use of Cy5 DNA automatic sequencer, the insert fragment which ligated to pGEM-T vector of the *RP-1*, *RP-2* and *RP-3* clones was sequenced. The insert size of *RP-1*, *RP-2* and *RP-3* was 237, 230 and 218 bp respectively (fig. 3). Each sequence was analyzed using a computer. Sequence analysis and database searching were performed through Internet to the four databases of GeneBank, EMBL, DDBJ and PBD. Results showed that these 3 clones bear no resemblance to those in the database. It is thus highly plausible that *RP-1*, *RP-2* and *RP-3* may represent 3 novel genes that begin to express specifically at the early stages of development of the pollen of rice. Full-length cDNA sequencing of each clone was still in progress and the function of these clones also has been investigated and the results will be reported later.

RP-1 (237bp)

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ACACTTACCA AACCAGACAT GAGACTGCCA GGAACACACC TCCGAGTCTC TCCCTAACTA
ATTCTTCACT CAAGAACAAA GCAATTGCCG AGGAAGCGAG ATACTCTCGA AGTCTCAAGC
CATACGCTAC GGGGCGGAAA GGGGGGAAAA CTACGCAGCA CCAATTTTCT GAATCGTATC
GACAATGCCA GCAAGGAAGT CGCCTTCGTA TTAGTTTGAC GACACAAACA TGCAAAT
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RP-2 (230bp)

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ACACAAACTT CCATAGCTAG CCCAAGATCA GACCAGCAAA CACAAGAACA TACACACCAG
TATTACAATA TGTATATATC TCTCTATATA TACTATAAAA CGAAGTGTTA AGCGACTGGT
GGTAATTAAT TAACACACAG CAAAATACCA TAATACTGCT CTTCTTAATC ACACTTACCA
GTGCTAATCC TGAAGAAAAC ACGCAGCTTA AGCTCAGTAA TGCATGGTAC
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RP-3 (218bp)

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GAGGTCCAAC TAAAGATGGA TCAATGCTGT TACAGTATAT TTCGGTGTTA GGTGTGGTCC
AACTAAATAA GATGCCCTGA GCTGTGTCAC AGTATATTTT CATGTTTTCG CCCTGAGCTG
TCACAGTATA TTTGCAGATT GAGGGAATTT GTGGTTTAGT TAACCACAAA CTAATATTGT
ATGTGGTTCA TCGTGATATA ATTAATTACA AAAAAAAA
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Fig. 3. Partial cDNA sequences of *RP-1*, *RP-2* and *RP-3*.

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