Isolation and analysis of differentially expressed genes in dominant genic male sterility (DGMS) *Brassica napus* L.

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Abstract

Dominant genic male sterility (DGMS) is an important approach to utilize the heterosis of *Brassica napus*. It can be presumed that male sterility is caused by the abnormity of the male gametogenesis. This study was undertaken to compare the genes that were differentially expressed between the fertile and sterile plants of the homozygous DGMS two-type line Rs1046AB, as an initial step towards understanding the molecular mechanism of DGMS and male gametogenesis. In this study, SSH was used to construct two subtracted libraries for enriching genes that were up-regulated in the fertile plants or sterile ones, and then a customed cDNA microarray with the clones of two subtracted libraries on it, was used to profile global gene expression patterns. cDNA clones showing a ±2.0-fold change in expression signal were regarded as up- or down- regulated. A total of 1200 significantly differentially expressed clones were isolated between the fertile and sterile plants, Northern blot further demonstrated the credibility of the microarray data. Subsequently, about 400 clones were selected for sequencing, and they represented 216 unigenes. Of these genes, 181 homologous sequences could be divided into 17 groups excluding those that encode the unclassified proteins, genes involved in metabolism; cell rescue, defense and virulence; biogenesis of cellular components and protein fate were the four largest groups, possessed 11.11%, 7.69%, 6.55% and 5.70% respectively; in contrast, genes related to protein activity regulation occupied the smallest proportion (0.9%). And the other 35 genes had no homology in the databases at the National Center for Biotechnical Information (NCBI), so including the 81 genes belonged to the unclassified proteins, there were totally 115 (54.2%) genes function unknown or novel genes. Furthermore, some important pathways related to male gametogenesis were found by using the program of KOBAS, such as nitrogen metabolism, nitrobenzene degradation, and starch and sucrose metabolism. Further analyses of the results are still in progress.

Introduction

Dominant genic male sterility (DGMS) is an important approach to utilize the heterosis of *Brassica napus*, the remarkable advantage of which lies in its complete sterility. Li et al. (1988) and Dong et al. (2000) revealed the genetic rule of DGMS. Based on this, they constructed a three lines system by using the homozygous two-type line, temporarily maintainer line and restorer line which could produce 100% sterile population and was not necessary to remove 50% fertile plants during F_1 seed production. Using this system, Zhou et al. (2003) had bred a DGMS hybrid variety Heza No.3 with low erucic acid and low glucosinolates in *Brassica napus*. However, there are still some difficulties in applying the DGMS for hybrid seeds production, e.g. developing new resources of homozygous two-type lines and restorer lines. Using the same material as ours, Lu et al. (2004) had found two molecular markers linked to the *Ms* gene, and this made the molecular marker-assisted selection (MAS) possible. Nevertheless the molecular mechanism of DGMS is still not well understood until now.

Male gametogenesis is a complicated process which is involved in the strict spatial-temporal expression of a mass of genes. During this process, mutation of any gene related to gametogenesis will induce the abortion of the male gamete's development and result in male sterility (McCormick 1993; McCormick 2004). Homozygous DGMS two-type line Rs1046AB was derived from the natural mutation material Yi-3A. The anatomical observations on the anther's development of the dominant genic male sterile line Yi-3A by light microscope and transmission electron microscope showed that the abortion of the anther's development of Yi-3A occurred at the pollen mother cell (PMC) stage (Yang et al. 1999b; Yu and Fu 1990). Nevertheless, we still know little about the gene expression during male gametogenesis.

In the present study, SSH was used to construct two subtracted libraries for enriching genes that were up-regulated in the fertile plants or sterile ones. The emergence of cDNA microarray made the rapid and high throughput screening of differentially genes in SSH subtracted libraries feasible (Yang et al. 1999a). Combining these two technologies, many differentially expressed genes between the male sterile plants and the male fertile ones in Rs1046AB were identified. Thus, our study will provide a platform for further investigation of the mechanism of DGMS and the gene expression related to male gametogenesis.

Materials and methods

Plant materials

The homozygous DGMS two-type line, Rs1046AB, was kindly provided by the National Center of Rapeseed Improvement in Wuhan. Plants were prepared by growing the seeds in the rapeseed research field of Huazhong Agricultural University. At the pollen mother cell (PMC) stage, the anthers, i.e. the buds that are about 2 mm in length, were used in the present research. Harvested samples were snap-frozen in liquid nitrogen and stored at −80°C before use.

RNA preparation and construction of subtracted cDNA libraries

Total RNA was extracted from the harvested samples by using TRIzol reagent (Gibco BRL) and following the manufacturer's instructions. 2 μg total RNA was reverse-transcribed and amplified using SMART PCR cDNA Synthesis Kit (Clontech, USA), and the LD PCR was performed for 17 cycles on MJ Research PTC-200 Peltier Thermal Cycler. Subsequently, SSH was performed as described in the PCR-Select cDNA Subtraction Kit (Clontech, USA) according to the manufacturer's protocols. In the forward subtracted library the cDNA from the fertile plants of Rs1046AB was used as the "tester"; and it was the cDNA from the sterile ones used as the "tester" in the reverse subtracted one.

Two rounds of suppression subtractive hybridization and PCR amplification were carried out to normalize and enrich the differentially expressed cDNAs. Products of the secondary round PCR from the forward and reverse subtraction were directly inserted into p-GEMT vector (Promega, USA). The ligation mixture was then transformed into Escherichia coli DH5α cells and cultured on a LB media plate containing ampicillin and X-Gal/IPTG. The white clones were picked out to construct the subtracted cDNA library, each of which contained more than 2000 clones.

Amplification of cDNA inserts

The cDNA inserts were amplified using Nested PCR primer 1 and 2R provided in the PCR-selected cDNA subtraction kit. The 50 μl reaction mixture contained 1×reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 μM of each primer, 1 U Tag DNA polymerase (Takara, Japan), and 2 μL bacterial culture template. PCR products were purified by ethanol precipitation, and dissolved in 30 μl distilled water. Excluding the two-banded and short-fragment clones, the rest were used to construct cDNA microarray.

Construction of cDNA microarray

Besides the above purified clones, the housekeeping genes from *Brassica napus*, *actin*, *β-tubulin* and *BNACBP* (Hills et al. 1994; Mandrup et al. 1992), were used as internal controls. In order to equalize hybridization signals generated from different samples, a PCR-amplified fragment from the λ control template DNA fragment (DTX803; Takara, Dalian, China) was used as the external control. And the human transferrin receptor (TFR) gene (DTX806; Takara, Dalian, China) and pUC19 (D3219; Takara, Dalian, China) were used as negative controls. Subsequently, all these purified clones and control cDNA were resuspended into 50% DMSO and 50% MiliQ water to a final concentration of 0.1 mg/ml, and then spotted to the glass slides (DTX704; Takara, Dalian, China) by the Array Spotter Generation III (Amersham Pharmacia Biotech, Uppsala, Sweden). The purified cDNA inserts were printed in duplicate while the control cDNAs were spotted 48 times randomly in different locations on each slide. After spotting, cDNA was crosslinked to the slides by UV light (60 mJ/cm^2).

Microarray hybridization and scanning

Total RNA was extracted from the pooled anthers of fertile and sterile plants of Rs1046AB as described above, before labeling, equal amount of the external control was spiked into these two RNA, and then the mixture was applied to synthesize Cy3- or Cy5- conjugated dUTP-labeled cDNA probe using RNA Fluorescence Labeling Core Kit (MMLV Version) Ver.2.0 (Takara, Dalian, China). Two independent biological replicates were applied in each sample, and each replicate was used for independent RNA extraction and labeling reaction. The successfully labeled probes (one with Cy3 and the other with Cy5) were combined, precipitated and dissolved in 25 μl hybridization buffer (6×SSC, 0.2% SDS, 5×Denhardt solution, and 0.1 mg/ml denatured salmon sperm DNA). This probe solution was denatured at 95°C for 2 min, dropped onto the center of array surface, and then covered with a coverslip without any bubbles. The slides were placed into a sealed cassette to hybridize in a 65°C water bath for 12-16 h. To increase the reliability of microarray analysis, dye-swapping experiments were performed.

After hybridization, slides were washed at 55°C in 2×SSC and 0.2% SDS, in 0.1×SSC and 0.2% SDS, and in 0.05×SSC for 5 min, respectively. The slides were immediately dried by centrifuging at 2500×g for 2 min. Subsequently, hybridized slides were scanned for Cy3 at 532 nm and Cy5 at 635 nm using the Affymetrix 428™ Array Scanner (Affymetrix, USA). Thus, two separate TIFF images were generated.

Data analysis

Data analysis was performed with ImaGene™ 4.2 software (BioDiscovery, USA). Grid placement and manipulation was performed automatically with the specified parameters, and spot segmentation was then performed using a histogram segmentation method with slight manual adjustment for the precise localization of the spots. Quantification of the intensity values for each of the spots prior to being segmented was done using the ImaGene fixed circle method. Briefly, raw intensity for each spot was measured with the mean of pixels in the spot contour, and the area outside the spot contour but inside the cell was used to calculate local background. The product of background per pixel (a median of the pixels in this area) and spot area was given an estimated spot background value. Finally, this background value was subtracted from the raw spot intensity to produce the background-subtracted intensities used for further analysis. All the poor-quality spots and those with signal intensity lower than the background plus 2 SDs (standard deviations) and the spots with dust were excluded from the analysis.

In order to equalize hybridization signals generated from different samples and reduce the systematic error, two different methods (housekeeping genes and external control) were used to normalize the microarray data. At last, the average ratio of each spot was calculated for at least three replicates, and a cutoff of twofold up- or down-regulation was chosen to define differential expression (Schena et al. 1995) in this report.

Sequence analysis

DNA sequencing was performed by using ABI 3730 Genetic Analyzer in Shanghai Sangon Biological Engineering Technology & Services CO. Ltd. After about 200 clones were sequenced, these fragments were pooled and used as probes for dot hybridization to eliminate duplicated clones. Homology searches were performed by BLAST at web servers of the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). These sequences were classified according to the biological processes they participated in and MIPS *A. thaliana* Genome Database (http://mips.gsf.de/proj/funcatDB/search_main_frame.html). KEGG Orthology (KO)-Based Annotation System (KOBAS) was used to automatically annotate a set of sequences with KO terms and identify both the most frequent and the statistically significantly enriched pathways (Mao et al. 2005).

Northern analysis

Nine clones that were identified to be differentially expressed by cDNA microarray were further confirmed using RNA gel blot. Total RNA (20 μg) was fractionated in 1% agarose gels containing 6% formaldehyde, transferred to nylon membrane following the standard procedure (Sambrook and W.Russell 2001), and then fixed to the membrane by baking at 80°C for 2 h. The selected cDNAs were amplified using Nested PCR Primer 1 and 2R. About 50 ng of each PCR product was labeled using TaKaRa Random Primer DNA Labeling Kit (Takara, Japan). The membrane was pre-hybridized at 65°C for 4 h and hybridized at 65°C for 12-16 h in hybridization buffer (0.5 M NaH_2PO_4 buffer, 1 mM EDTA (PH8.0), 7% SDS, 0.6% BSA). The hybridized membrane was washed at 42°C in 2×SSC and 0.1% SDS for 5 min, then in 0.5×SSC and 0.1% SDS up to reaching the desired value. The results were visualized by exposing to X-ray films at -80°C for 3 to 7 days.

Results

Construction of subtracted cDNA libraries

Using SSH, two subtracted cDNA libraries were constructed. The tester cDNA was from fertile plants in the forward-subtracted library and it was from sterile plants in the reverse-subtracted one. In this study, the subtraction efficiency was evaluated by PCR amplification of the housekeeping gene, *BNACBP*. If subtraction is efficient, transcripts of housekeeping genes should be reduced. Fig.1 shows that the *BNACBP* fragment is detectable only after 33 cycles of amplification in the subtracted samples, whereas it is clearly detectable in the unsubtracted samples after 18 cycles, indicating high subtraction efficiency.

Differential screening of subtracted cDNA libraries

In the present study, the custom-designed cDNA microarrays were used to identify the differentially expressed genes. The results for the four hybridizations were averaged for each clone, and those with a normalized expression ratio more than 2-fold were selected. Thus, 1200 up-regulated clones were identified in the forward-subtracted library. By contrast, only 11 up-regulated clones were selected in the reverse-subtracted library (Figure not shown).

Northern blot analysis

To validate the results of cDNA microarray, nine cDNA clones were selected randomly for Northern blotting in the forward-subtracted library, each clone was repeated in at least three times using different membranes prepared from different pooled samples. On the whole, the results of RNA blots for eight clones were consistent with the expression data obtained by cDNA microarray analysis (Fig.2-A), demonstrating the credibility of microarray data. But as to the four genes in reverse subtracted library there was only one result was positive (Fig.2-B).

From Fig.2 we could see some cDNA clones only expressed in the fertile plants, for example, 1-P09, 2-C22, 2-J09 and 3-I16; and some others expressed in differential levels in the two materials (2-D01, 2-K06, 3-D22, 3-P11 and 6-H17).

Sequence analysis of selected cDNA clones

In the forward-subtracted library, 1200 significantly differentially expressed genes were isolated. To reduce the redundant sequencing of clones, about 200 clones were sequenced and analyzed firstly. Our results indicated that only 41 unigenes were identified. Subsequently, these genes were pooled and used as probes to hybridize with the left 1000 clones. Thus, about 200 un-hybridized clones were picked out for sequencing. So, totally about 400 clones were sequenced. Then these sequences were analyzed by BLAST, after the redundant sequences were removed; only 216 different genes were identified. 181 clones could find homologous sequences in NCBI, the other 35 clones without any homology (The data is available as additional data files with the online version of this paper, and their sequences are available from the corresponding author upon request).

With the information in the MIPS *A. thaliana* Genome Database, these 181 genes were divided into 18 groups. Fig.3 shows the detailed distribution of 17 groups (the unclassified proteins were not included), it demonstrated that genes involved in metabolism; cell rescue, defense and virulence; biogenesis of cellular components and protein fate were the four largest groups, possessed 11.11%, 7.69%, 6.55% and 5.70% respectively. In contrast, genes related to protein activity regulation occupied the smallest proportion (0.9%). Moreover, there were 84 genes belonged to the unclassified proteins, so including the 35 no homology genes, there were totally 119 (55.1%) genes function unknown or novel genes. In the reverse-subtracted library, the 11 selected clones represented four unigenes, including three genes of known function and one novel gene (Table 1).

KOBAS analysis

In this study, KOBAS was used to identify the most frequent and statistically significantly enriched pathways of genes from

the forward-subtracted library. Of all the sequences, 41 (19.07%) were assigned to 47 KO terms. Table 2 displays partial results of the KOBAS analysis. Based on the *p*-values, all these pathways were important in our results, especially the pathways of nitrogen metabolism and nitrobenzene degradation (False Discovery Rate (FDR) <0.05).

Discussion

In this study, many differentially expressed genes were isolated and identified between the fertile plants and sterile ones of the homozygous DGMS two-type line Rs1046AB. Up to now, although the key genes controlling fertility were unknown, these genes may relate to male gametogenesis. Therefore, our results may offer some useful information for the research of male gametogenesis.

Differentially expressed genes in two subtracted libraries

In the present study, 220 differentially expressed unigenes were identified, including 216 genes from the forward-subtracted library and 4 genes from the reverse-subtracted library. This is because the fertile and sterile plants of the homozygous DGMS two-type line Rs1046AB have a similar genetic background (Li et al., 1988). Consequently, the fertile plants and sterile ones should have a similar gene expression pattern before the pollen mother cell stage, thereafter; the male gametes of sterile plants stop its development, so the differentially expressed genes reduced greatly. However, its development of the fertile plants is normal, and after pollen mother cell stage, there will be the expression of huge amount of genes. On the other hand, we can only decide the male gamete's stage by the bud's length. Therefore, besides the key genes that related to the fertility control of this homozygous two-type line, a mass of genes may be included after the pollen mother cell stage. Now it is still difficult to distinguish the key genes from so many genes.

Distribution of the differentially expressed genes in forward-subtracted library

As shown in Fig. 3, metabolism; cell rescue, defense and virulence; biogenesis of cellular components and protein fate were the four largest groups for all the differentially expressed genes. Metabolism is a fundamental process for plant development, and male gametogenesis is a complicated program that needs all kinds of nutrition. So we think that metabolism is the most important process for male gametogenesis according to our results. Also some genes that related to cell rescue, defense and virulence were identified, the possible explanation about it is that the buds were needed to cut from the inflorescence to get the anther samples, and this may induce some plant defense reactions, leading to the up-regulation of some genes. Cell wall formation (and dissolution) during and after meiotic divisions is a unique feature of male meiosis in plants, and after male meiotic cytokinesis, the individual microspores of the tetrad initiating development of the pollen wall etc (Scott et al. 2004). These processes were all involved in the biogenesis of cellular components. As for protein fate, it includes protein folding, modification, and destination. In previous study, it had been reported that the UPR (unfolded protein response; reviewed by Schroder and Kaufman 2005) represses both nitrogen starvation induced developmental programs, pseudohyphal growth, and meiosis and, thus, contributes to nitrogen sensing in budding yeast (Schroder et al. 2000). This indicated that protein folding was very important for normal development under some conditions.

Genes related to male gametogenesis

In our study, totally 216 genes were identified in the forward-subtracted library, more than 50% of which were function-unknown or novel. Among the known genes, some are related to male gametogenesis, such as *AGL18, AtMYB103*, *Ms2* and *QRT3*. *AGL18* is a member of MADS-box genes family that plays central roles in flower and fruit development of plants (Weigel 1995). Moreover, *AGL18* is expressed in the endosperm and in developing male and female gametophytes (Alvarez Buylla et al. 2000). *AtMYB103* belonged to the R2R3 *MYB* gene family, and it is required for tapetal development and microsporogenesis, and negatively regulates trichome endoreduplication linked to the trichome branching (Higginson et al. 2003). As for *Ms2* and *QRT3*, they play specific roles during microspores released from tetrad (Aarts et al. 1997; Rhee et al. 2003). In addition, some genes that related to meiosis, such as those involved in cell cycle, cell type differentiation, were also identified in this study. As we known, microsporogenesis involved several fundamental cellular processes, including cell division, cell differentiation, cell-cell communication, and cell death (Ma 2005). Accordingly, all these results indicated that we identified some important genes that related to male gametogenesis. On the other hand, these results implicated that the 34 function-unknown genes were maybe novel genes involved in male gametogenesis.

In the reverse-subtracted library, the *RAN3* gene was identified. It is known that *Ran* GTPase belongs to the Ras superfamily of monomeric G proteins. Ran is a small, evolutionarily conserved, eukaryotic GTPase that is essential for viability in every organism tested to date (Sazer and Dasso 2000). In addition to regulating nucleocytoplasmic transport, Ran also controls mitotic spindle assembly (Quimby and Dasso 2003), mitotic checkpoint (Arnaoutov and Dasso 2003), DNA replication (Yamaguchi and Newport 2003), and nuclear envelope assembly (Quimby and Dasso, 2003). *RAN3* is a member of the Ran GTPase subfamily, and it was found that *RAN3* was highly up-regulated in the sterile plants in our experiment (Fig.2-b). Previous study has reported that PMC does not conduct meiosis and no dyad or tetrad was formed of our material (Yu and Fu, 1990; Yang et al., 1999b), and this may be caused by the abnormal assembly of the spindle. On the other hand, the higher expression level of this gene in the sterile plants may cause the abnormal nuclear trafficking. These may be the reasons of male sterility.

What is more, another gene, *importin* α, that is essential for both spindle assembly and nuclear formation (Gruss et al. 2001; Riddick and Macara 2005) was also expressed in different level (Fig.4). Previous research had demonstrated that the effect of Ran on spindle assembly via TPX2, potential downstream targets for Ran in spindle formation, was mediated by

regulating its interaction with importin α, (Gruss et al. 2001). In our research, *importin α* was only expressed in early buds of normal anthers, but in sterile ones it could not be detected. So, it gave us further evidence that male sterility was maybe caused by the abnormality during Ran GTPase cycle.

Important pathways during male gametogenesis

Although many differentially expressed genes were isolated in our study, it is difficult to identify the key genes that related to the fertility control, and a lot of genes may be related to male gametogenesis as described above. Therefore, it is possible to get some information about male gametogenesis based on our results. In order to know how these genes work in the gene networks, the software of KOBAS was used to find out the important pathways related to male gametogenesis. Nonetheless, no information of *Brassica napus* exsits in the KEGG database, so we had to use the *Arabidopsis*'s complete genome sequence for the background distribution. As a result, only 41 sequences were assigned to KO terms, but it still supplied us some interesting information.

Just as discussed above, diploid budding yeast exhibited two developmental programs in response to nitrogen starvation, pseudohyphal growth, and sporulation (Schroder et al. 2000), suggesting that some creatures were nitrogen sensitive. The result of KOBAS analysis indicated that nitrogen metabolism and nitrobenzene degradation were the most important two pathways in our research, and they were both related to nitrogen, but the connection between nitrogen and male gametogenesis in plants was still unknown. Another important pathway was starch and sucrose metabolism. Previously, Dorion et al. (1996) found that water deficit induced the male sterility which affected the pollen mother cells during meiosis of wheat, and the starch and sucrose synthesis was quite different between the sterile and normal plants. Moreover, the starch and sugar were also accumulated at the different level during pollen maturation of maize, and this suggested that combined effects of both reduced sugars and their reduced flux in starch biosynthesis along with a strong possibility for altered redox passage may lead to the temporal changes in gene expression, and ultimate pollen sterility (Datta et al. 2002). Therefore, this pathway was also an important factor for male gametogenesis.

For further investigation the genes involved in these pathways, four genes, 1-B14 (involved in starch and sucrose metabolism), 3-I16 (involved in nitrogen metabolism), 5-K08 and 5-L09 (both involved in nitrobenzene degradation), were picked out to get its temporal expression pattern during male gametogenesis (Fig.4). It showed us that all these genes were only expressed in normal plants at the early stage of anther development. So, it could be concluded that in the sterile plants these important pathways were disrupted in early stage. Especially for the nitrobenzene degradation pathway, two of four genes in it were induced significantly.

In summary, understanding of molecular mechanism of DGMS is of great importance and interests. This paper shown us some very interesting data and would constitute the start of an interesting research in male gametogenesis.

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References

Aarts MG, Hodge R, Kalantidis K, Florack D, Wilson ZA, Mulligan BJ, Stiekema WJ, Scott R, Pereira A (1997) The *Arabidopsis* MALE STERILITY 2 protein shares similarity with reductases in elongation/condensation complexes. Plant J 12: 615-623.

Alvarez-Buylla ER, Liljegren SJ, Pelaz S, Gold SE, Burgeff C, Ditta GS, Vergara-Silva F, Yanofsky MF (2000) MADS-box gene evolution beyond flowers: expression in pollen, endosperm, guard cells, roots and trichomes. Plant J 24: 457-466.

Arnaoutov A, Dasso M (2003) The Ran GTPase regulates kinetochore function. Dev Cell 5: 99-111.

Datta R, Chamusco KC, Chourey PS (2002) Starch biosynthesis during pollen maturation is associated with altered patterns of gene expression in maize. Plant Physiol 130: 1645-56.

Dong ZS, Zhao ZH (2000) Discusses on dominant nuclear male sterile gene interaction in rape and Li's viewpoint. Acta Agriculturae Boreali-occidentalis Sinica 9: 91-95.

Dorion S, Lalonde S, Saini HS (1996) Induction of male sterility in wheat by meiotic-stage water deficit is preceded by a decline in invertase activity and changes in carbohydrate metabolism in anthers. Plant Physiol 111: 137-145.

Gruss OJ, Carazo-Salas RE, Schatz CA, Guarguaglini G, Kast J, Wilm M, Le Bot N, Vernos I, Karsenti E, Mattaj IW (2001) Ran induces spindle assembly by reversing the inhibitory effect of importin alpha on TPX2 activity. Cell 104: 83-93.

Higginson T, Li SF, Parish RW (2003) AtMYB103 regulates tapetum and trichome development in *Arabidopsis thaliana*. Plant J 35: 177-192.

Hills MJ, Dann R, Lydiate D, Sharpe A (1994) Molecular cloning of a cDNA from *Brassica napus* L. for a homologue of acyl-CoA-binding protein. Plant Mol Biol 25: 917-920.

Li SL, Qian YX, Wu ZH (1988) Genetic male sterility in rape (*B.napus* L.) conditioned by interaction of genes at two loci. Can. J. Plant Sci 68: 1115-1118.

Lu GY, Yang GS, Fu TD (2004) Molecular mapping of a dominant genic male sterility gene Ms in rapeseed (*Brassica napus*). Plant Breeding 123: 262-265.

Ma H (2005) Molecular genetic analyses of microsporogenesis and microgametogenesis in flowering plants. Annu Rev Plant Biol 56: 393-434.

Mandrup S, Hummel R, Ravn S, Jensen G, Andreasen PH, Gregersen N, Knudsen J, Kristiansen K (1992) Acyl-CoA-binding protein/diazepam-binding inhibitor gene and pseudogenes. A typical housekeeping gene family. J Mol Biol 228: 1011-1022.

Mao X, Cai T, Olyarchuk JG, Wei L (2005) Automated genome annotation and pathway identification using the KEGG Orthology (KO) as a controlled vocabulary. Bioinformatics 2005 19:3787-3793.

McCormick S (1993) Male gametophyte development. Plant Cell 5: 1265-1275.

McCormick S (2004) Control of male gametophyte development. Plant Cell 16 Suppl: S142-S153.

Quimby BB, Dasso M (2003) The small GTPase Ran: interpreting the signs. Curr Opin Cell Biol 15: 338-344.

Rhee SY, Osborne E, Poindexter PD, Somerville CR (2003) Microspore separation in the quartet 3 mutants of Arabidopsis is impaired by a defect in a

developmentally regulated polygalacturonase required for pollen mother cell wall degradation. Plant Physiol 133: 1170-1180.
Riddick G, Macara IG (2005) A systems analysis of importin-{alpha}-{beta} mediated nuclear protein import. J Cell Biol 168: 1027-1038.
Sambrook J, W.Russell D (2001) Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory Press.
Sazer S, Dasso M (2000) The ran decathlon: multiple roles of Ran. J Cell Sci 113: 1111-1118.
Schena M, Shalon D, Davis RW, Brown PO (1995) Quantitative monitoring of gene expression patterns with a complementary DNA microarray. Science 270: 467-470.
Schroder M, Chang JS, Kaufman RJ (2000) The unfolded protein response represses nitrogen-starvation induced developmental differentiation in yeast. Genes Dev 14: 2962-2975.
Schroder M, Kaufman RJ (2005) The mammalian unfolded protein response. Annu Rev Biochem 74: 739-789.
Scott RJ, Spielman M, Dickinson HG (2004) Stamen structure and function. Plant Cell 16: S46-S60.
Weigel D (1995) The genetics of flower development: from floral induction to ovule morphogenesis. Annu Rev Genet 29: 19-39.
Yamaguchi R, Newport J (2003) A role for Ran-GTP and Crm1 in blocking re-replication. Cell 113: 115-125.
Yang GP, Ross DT, Kuang WW, Brown PO, Weigel RJ (1999a) Combining SSH and cDNA microarrays for rapid identification of differentially expressed genes. Nucleic Acids Res 27: 1517-1523.
Yang GS, Qu B, Fu TD (1999b) Anatomical studies on the anther's development of the dominant genic male sterile line Yi-3A in *Brassica napus* L. Journal of Huazhong Agricultural University: 405-408..
Yu FQ, Fu TD (1990) Cytomorphological research on anther development of several male—sterile lines in *Brassica Napus* L. Journal of Wuhan Botanical Research 8: 209-216.
Zhou XR, Li SL, Zhou ZJ, Zhuang J, Gu LD (2003) Breeding of dominant genic male sterility (GMS) hybrid variety Heza No.3 with low erucic acid and low glucosinolates in *Brassica napus* L. Journal of Shanghai Jiaotong University (Agricultural Science) 21: 304-308.

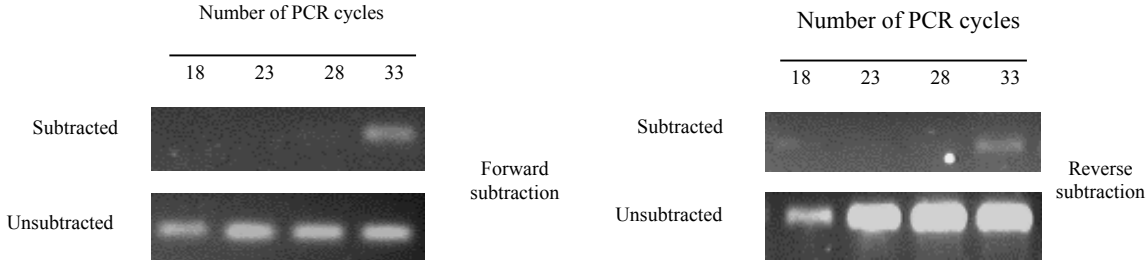

Fig.1. Analysis of subtraction efficiency by PCR. In forward and reverse subtraction, the unsubtracted and subtracted pools of cDNA were amplified using primers for the constitutively expressed *BNACBP* gene. 5 μl of the products were taken after 18, 23, 28 and 33 cycles of PCR amplification and analyzed on 1.2% agarose gel.

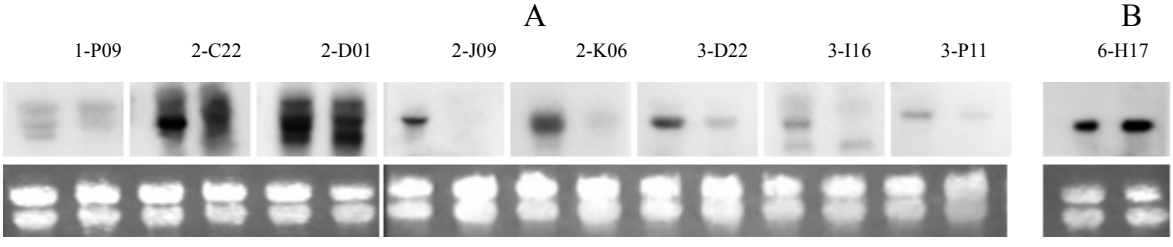

Fig.2. Northern blot analysis of nine clones and their corresponding microarray data. Nine ESTs selected from the microarray experiments were applied to probes in Northern blot. Anthers of the fertile and sterile plants were taken at PMC stage. (a) RNA blots of the cDNA clones from the forward-subtracted library. (b) RNA blot of *RAN3* from the reverse-subtracted library. The left line in every figure represented the fertile plants; the right line represented the sterile ones. And the bottom figure was the result of total RNA transferred to nylon membranes.

Clone no.	Gene description	Clone no.	Gene description	Clone no.	Gene description
1-P09	LOB domain family protein	2-J09	leucine-rich repeat family protein	3-I16	carbonic anhydrase family protein
2-C22	auxin-induced protein IAA17	2-K06	cytochrome P450 family protein	3-P11	strictosidine synthase family protein
2-D01	TAZ zinc finger family protein	3-D22	hexose transporter, putativeb	6-H17	Ras-related GTP-binding protein

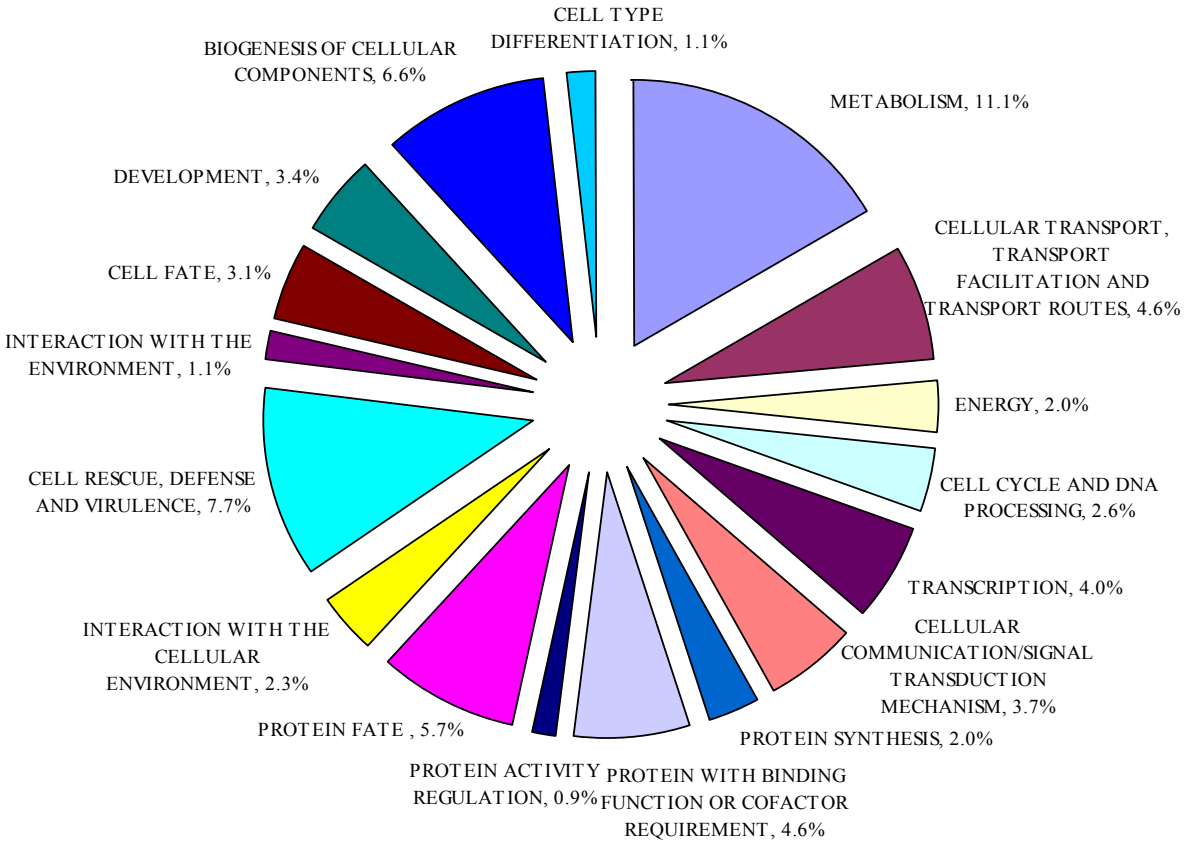

Fig.3. Pie chart shows the proportion of differentially expressed genes from the forward-subtracted library in each of the functional categories described in MIPS database and Gene Ontology website. Genes encoding unclassified proteins were not shown.

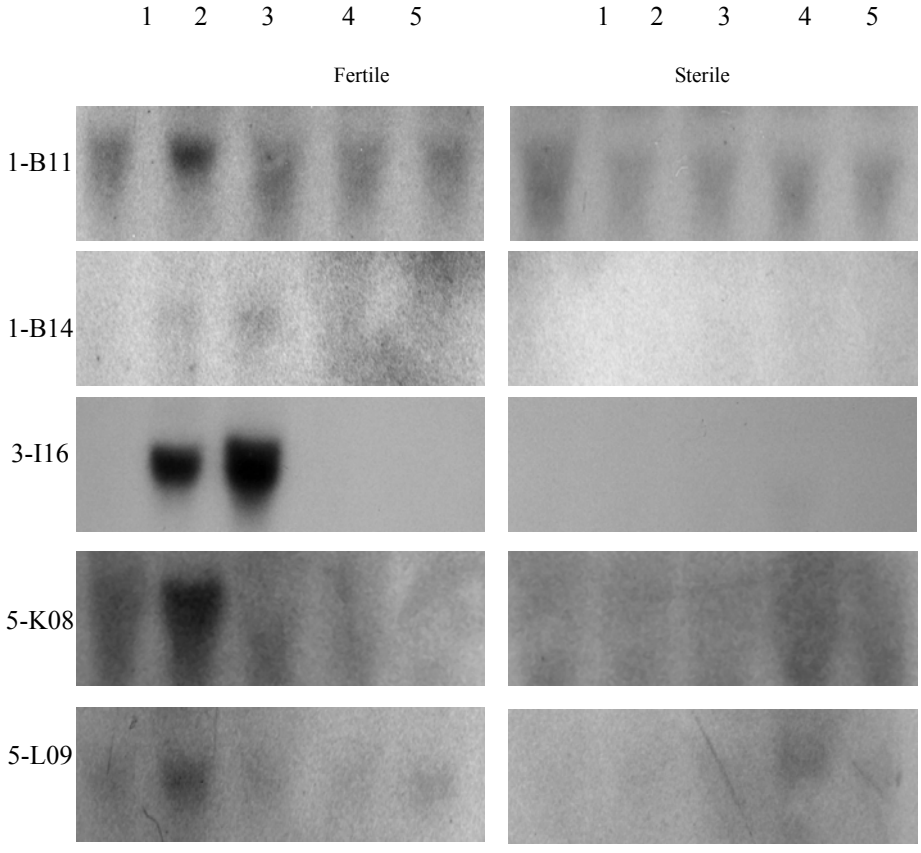

Fig.4 Tempral expression pattern of selected clones. The left four figures reprsented the fertile plants, the right figures represented the sterile ones. The number 1 to 5 above represented the bud's length.

Table 1 Significantly up-regulated genes in the reverse- subtracted library

Clone	Accession no.	Gene description
1-D16	AF093751	lipid transfer protein (wax9E)
7-I14	AAM63628	putative small heat shock protein 17.6 kDa class I small heat shock protein (HSP17.6B-CI)
6-H17	NM_124901	Ras-related GTP-binding protein (RAN3)
5-H11		No homology

Table 2: Partial results of KOBAS analysis

Pathway	Sample Count	Background Count	*p*-value	FDR
Nitrogen metabolism	4	31	0.00157	0.03737
Nitrobenzene degradation	2	4	0.00162	0.03737
Starch and sucrose metabolism	8	158	0.00399	0.06111
Stilbene,coumarine and lignin biosynthesis	8	174	0.00718	0.08254
Fluorene degradation	5	86	0.01345	0.11319
gamma-Hexachlorocyclohexane degradation	5	88	0.01476	0.11319
Ascorbate and aldaratemetabolism	5	95	0.02003	0.13161
Ethylbenzene degradation	1	2	0.0334	0.19204
D-Glutamine and D-glutamate metabolism	1	3	0.04969	0.2239