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

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Abstract

Dominant genic male sterility (DGMS) is an important approach to utilize the heterosis of *Brassica napus*, but the molecular mechanism of DGMS is not well understood. As an initial step towards understanding this event, some pilot studies were performed. Using suppression subtractive hybridization (SSH) and cDNA microarray, about 1200 significantly differentially expressed clones were isolated between the fertile and sterile plants of the homozygous DGMS two-type line, Rs1046AB. Northern blot further demonstrated the credibility of the microarray data. Subsequently, about 400 clones were selected for sequencing and they represented 216 unigenes (212 were up-regulated in fertile buds, and the other 4 were up-regulated in sterile ones). Of the 212 up-regulated genes in forward-subtracted library, 178 homologous sequences could be divided into 17 groups excluding those that encode the unclassified proteins; and the other 34 genes had no homology in the databases at the National Center for Biotechnical Information (NCBI). Furthermore, some important pathways related to male gametogenesis were identified using the program of KEGG Orthology (KO)-Based Annotation System (KOBAS), such as nitrogen metabolism, nitrobenzene degradation and starch and sucrose metabolism.

Keywords: Suppression subtractive hybridization; Male gemetogenesis; KOBAS

1. 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. [1] and Dong and Zhao [2] revealed the genetic rule of DGMS and constructed a ''three-line'' system, using the homozygous two-type line, temporarily maintainer line and restorer line, which could produce 100% sterile population and not need to remove 50% fertile plants during F_1 seeds production. Using this system, Zhou et al. [3] had bred a DGMS hybrid variety ''Heza No. 3'' with low erucic acid and low glucosinolates in *B. napus*. However, there are still some difficulties in applying the DGMS for hybrid seeds production, such as developing new resources of homozygous two-type lines and restorer lines. Using the same material as ours, Lu et al. [4] had found two molecular markers linked to the *Ms* gene, and this made the molecular marker-assisted selection (MAS) become possible. Nevertheless, the molecular mechanism of DGMS is still not well understood.

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 [5,6]. Homozygous DGMS two-type line Rs1046AB was derived from the natural mutation material Yi-3A. The anatomical observations on the anther's development of Yi-3A by light microscope and transmission electron microscope showed that the abortion of

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the anther's development of Yi-3A occurred at the pollen mother cell (PMC) stage [7,8]. Nevertheless, little was known about the transcriptome profiling 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 [9]. 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 investigating the mechanism of DGMS and the transcriptome profiling related to male gametogenesis.

2. Materials and methods

2.1. 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 stage, the anthers, i.e. the buds about 2 mm in length, were used in the present research. To investigate the temporal expression pattern of interested genes, the buds that are <1, 1–2, 2–3, 3–4 and >4 mm which numbered 1–5 were also harvested. All harvested samples were snap-frozen in liquid nitrogen and stored at -80 °C before use.

2.2. 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. Two micrograms 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 the cDNA from the sterile ones was used as the ''tester'' in the reverse-subtracted one.

Two rounds of suppression subtractive hybridization (SSH) 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 selected to construct the subtracted cDNA library, each of which contained more than 2000 clones.

2.3. 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 deioned water. Excluding the two-banded and short-fragment clones, the rest were used to construct cDNA microarray.

2.4. Construction of cDNA microarray

Besides the above-purified clones, the housekeeping genes from *B. napus*, *actin*, *β-tubulin* and *BNACBP* [10,11], 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 cDNAs 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).

2.5. Microarray hybridization and scanning

Total RNA was extracted from the pooled anthers of fertile and sterile plants of Rs1046AB, respectively, 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 with same amount of total RNA from different buds 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 with ethanol and then 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/0.2% SDS, in 0.1× SSC/0.2% SDS and in 0.05× SSC for