African Journal of Biotechnology Vol. 10(27), pp. 5296-5303, 15 June, 2011
Available online at http://www.academicjournals.org/AJB
DOI: 10.5897/AJB10.2048
ISSN 1684–5315 © 2011 Academic Journals

Full Length Research Paper

Construction and analysis of a suppression subtractive hybridization (SSH) library of genic multiple-allele inherited male-sterility in Chinese cabbage (*Brassica campestris* L. ssp. *pekinensis*)

Ji Rui-Qin[1], Xin Xi-Feng[1], Li Zi-Qin[2] and Feng Hui[1]*

[1]Department of Horticulture, Shenyang Agricultural University, Shenyang 110866, China.
[2]Inner Mongolia Academy of Agricultural and Animal Husbandry Sciences, Hohhot 010031, China.

Accepted 4 March, 2011

Utilization of male sterility is a key method for producing crossbred Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis* (Lour.) Olsson. In this study, suppression subtractive hybridization (SSH) was used to construct sterility and fertility cDNA libraries, which included differentially, expressed clones between fertile and sterile buds of the A/B line 'AB01'. The positive clones were randomly selected by polymerase chain reaction amplification (PCR) and 25 high quality sequences (22 from the fertile-tester library and three from the sterile-tester libraries) were generated. The fragment lengths varied from 77 to 469 bp. Differential expression patterns between fertile and sterile buds were selected and verified using five expressed sequence tags (ESTs). Results indicated that, three ESTs were expressed only in fertile buds and two ESTs were down-regulated in sterile buds. According to the Basic Local Alignment Search Tool (BLAST) screening and functional annotation, the 25 ESTs were homologous to known sequences deposited in National Center for Biotechnology Information (NCBI). These genes had homology to known proteins such as flowers/buds development proteins, metabolic-related proteins, cell structure proteins, cell growth/division proteins and secondary metabolic-related proteins. The results suggested that, these proteins played a critical role in nuclear male sterility progression of genic multiple-allele inherited male-sterility in Chinese cabbage.

Key words: Key words: Chinese cabbage, male sterility, suppression subtractive hybridization (SSH), expressed sequence tags (ESTs).

INTRODUCTION

Chinese cabbage (*Brassica rapa* L. ssp. *pekinensis*) (Lour.) Olsson is an important vegetable crop worldwide. Male sterility is a much sought-after trait in many crop plants as it avoidsthe need for hand emasculation in the production of hybrid seed (Bino, 1985). The utilization of male-sterile lines in cross-breeding programs is an economical and stable approach to breed new varieties of Chinese cabbage. The male-sterile materials in Chinese cabbage can be divided into genic male sterility (GMS) and cytoplasmic male sterility (CMS) (Van Der Meer, 1987). GMS has obvious advantages, including stable and complete sterility performance, extensive restorer distribution and the absence of negative cytoplasmic effects. However, manual removal of the fertile plants from the parental line is necessary in the utilization of GMS resources (Van Der Meer, 1987). The

*Corresponding author. E-mail: fenghuiaaa@263.net. Tel: 86-24-884871436. Fax: 86-024-88487144.

Abbreviations: GMS, Genic male sterility; **CMS,** cytoplasmic male sterility; **AFLP,** amplified fragment length polymorphism; **DDRT-PCR,** differential display reverse transcriptase polymerase chain reaction; **SSH,** suppression subtractive hybridization; **RDA,** representational difference analysis; **DGMS,** dominant genic male sterility; **BLAST,** Basic Local Alignment Search Tool; **ESTs,** expressed sequence tags; **RT,** reverse transcription; **PCD,** programmed cell death; **ROS,** reaction oxygen species.

percentage of male-sterile plants in an ideal male-sterile line should be 100%. 'AB01' is a formerly bred GMS A/B line of Chinese cabbage. Four stable hereditary lines comprising of 100% male-sterile plants were obtained by crossing male sterile plants with male fertile plants between A/B lines, ('AB01') (Feng et al., 1995). Feng et al. (1996) reported the first genetic hypothesis of genic multiple allele male sterile genes in Chinese cabbage. Most male sterile lines had been obtained following this genetic hypothesis (Yue and Feng, 2005; Li and Feng, 2006; Feng et al., 2007) and some molecular makers have subsequently been identified (Feng et al., 2009; Wei et al., 2009; Liu et al., 2010; Wang et al., 2010). However, the molecular mechanisms of genic multiple-allele inherited male-sterility in Chinese cabbage remains elusive.

Pollen preferentially expressed genes, such as CYP86MF, BcMF2, BcMF3 through BcMF15 have been identified by differential display reverse transcriptase polymerase chain reaction (DDRT-PCR) and cDNA-amplified fragment length polymorphism (AFLP) using 'ZUBajh97-01 A/B', a Chinese cabbage GMS A/B line (Cao et al., 2006; Wang et al., 2005; Liu et al., 2006; Tian et al., 2009). In order to resolve the molecular mechanisms of genic multiple-allele inherited male-sterility, differentially expressed genes between fertile and sterile of genic multiple-allele inherited male-sterility in Chinese cabbage must be uncovered.

Suppression subtractive hybridization (SSH) is a simple yet powerful technique that isolates highly abundant differentially expressed genes and amplifies signals of low-abundance genes. Compared with DDRT-PCR and representational difference analysis (RDA), SSH is quick, highly efficient and exhibits a low number of false positives. SSH has been widely applied in isolation of differentially expressed genes of fertile and sterile in various plant species. For example, Wu et al. (2007) isolated approximately 1200 significantly differentially expressed clones between fertile and sterile homozygous dominant genic male sterility (DGMS) of *Brassica napus*. Following identification of differentially expressed genes during anther abortion of Taigu genetic sterile wheat, Li et al. (2008) demonstrated that, 87.5% of the clones were expressed differentially between sterile and fertile anthers. However, despite the research completed to gain insights into male sterility, a cDNA subtractive library of genic multiple-allele inherited male-sterility in Chinese cabbage has not been generated.

In this study, SSH was used to construct two subtracted libraries for enriching genes that were up-regulated in fertile or sterile Chinese cabbage plants. Many differentially expressed genes between male sterile and male fertile 'AB01' plants were identified using this technology. Therefore, our study provided a platform to further investigate the mechanisms and transcriptome profiling of genic multiple-allele inherited male-sterility in Chinese cabbage

MATERIALS AND METHODS

Plant materials

'AB01' , a previously bred AB line of Chinese cabbage (Brassica rapa L. ssp. pekinensis (Lour.) Olsson) was used as the study materials. The GMS A/B 'AB01' line generates a 1:1 proportion of fertile (AB01-2, Ms^fMs) and sterile (AB01-1, MsMs) progeny and is therefore, considered a stable system. In this study, the GMS A/B 'AB01' line was reproduced continuously by a sister-line cross (A×B, nA×B) for more than 10 years. The seeds were sown in the field station of Shenyang Agriculture University. During the flowering stage, fertile and sterile flower buds were respectively divided into different grades according to filament length, anther color and presence or absence of pollen and then, harvested quickly on ice. All harvested samples were snap-frozen in liquid nitrogen and stored at -80℃ prior to use.

Total RNA extraction and mRNA isolation

Total RNA was extracted from each individual harvested sample at each stage using RNA simple total RNA kit (TianGen, Beijing, China) according to the manufacturer's protocol. Fertile and sterile bulks were separately prepared by pooling an equal quantity of RNA from each of the six stages of fertile and sterile buds for mRNA isolation. Micro-fast track 2.0 (Invitrogen) was used to isolate mRNA according to the manufacturer's instructions.

Total RNA, mRNA yield and quality were determined spectrophotometrically at wavelengths of 260 and 280 nm; mRNA was adjusted to a final concentration of 1 µg/µl. The integrity and quality of total RNA and mRNA were verified by electrophoresis on 1.0% agarose/EB gels using GeneSnap from SynGene (Cambridge, UK).

SSH cDNA library construction and amplification of cDNA inserts

The SSH library was constructed using the PCR amplification cDNA subtraction kit (Clontech, USA) following the manufacturer's instructions. The fertile mRNA were used as "testers" in the forward-subtracted cDNA library; and the sterile mRNA were "testers" in the reverse-subtracted cDNA library.

Briefly, after double-stranded cDNA synthesis by reverse transcription from poly (A+) mRNA and a digestion with *Rsa*I, the tester cDNA was subdivided into two portions and a different adaptor ligated to each portion followed by two successive hybridizations. PCR amplification was performed with specific primers to adaptors allowing exponential amplification of only the tester cDNA molecules carrying both types of adaptors, representing differentially-expressed transcripts present in the tester. The other hybrids were not amplified or linearly amplified. A second round of PCR amplification was performed to reduce the background noise and to enrich differentially-expressed cDNA sequences.

The efficiency of subtraction was analyzed by comparing cDNA abundance before and after subtraction by PCR using specific primers for the constitutively expressed gene *β-actin* (sense: 5´-ATCTACGAGGGTTATGCT-3´, antisense: 5´- CCACTGAGGACG ATGTTT -3´). PCR amplification was performed using *Taq* DNA polymerase (TaKaRa, Dalian, China) and 5 µl aliquots were removed following determined numbers of PCR cycles. The amplified products were examined in 2% agarose gel. The differences in the number of cycles, which were needed to generate an approximately equal amount of the corresponding PCR product in subtracted and unsubtracted samples, served to indicate the subtraction efficiency.

The final PCR products were purified with the QIAquick PCR

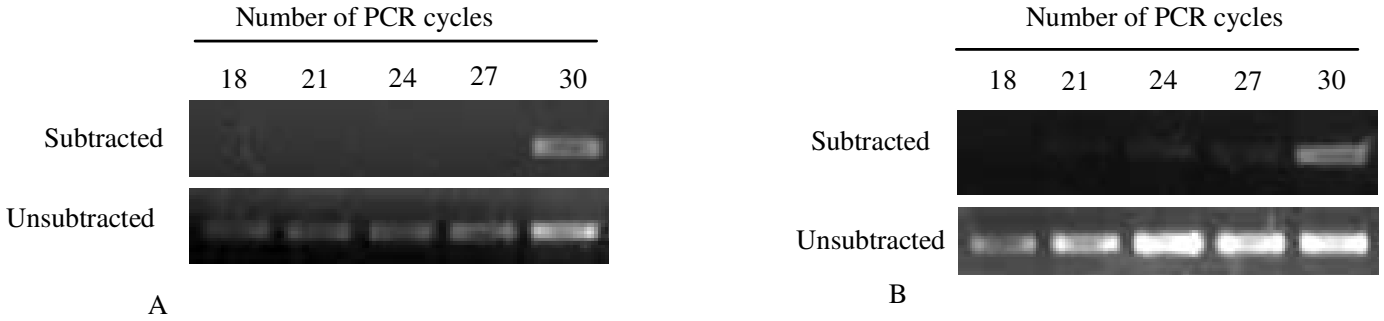

Figure 1. (A) Analysis of subtraction efficiency in forward unsubtracted and subtracted pools of cDNA; (B) analysis of subtraction efficiency in Reversed unsubtracted and subtracted pools of cDNA.

purification kit (Qiagen, Holland). The fragments obtained were cloned into the PGEM-T vector (Promega, USA). The ligation mixture was then, transformed into *Escherichia coli* DH5a cells and cultured on LB media containing ampicillin and X-Gal/IPTG. The white clones were selected to construct the subtracted cDNA library. The cDNA inserts were amplified using nested PCR primers 1 and 2R provided in the PCR-selected cDNA subtraction kit.

Sequencing and Basic Local Alignment Search Tool (BLAST) analysis

Fifty white positive clones from each subtractive cDNA library were selected for sequencing. The vector and adaptor sequences were subsequently removed to obtain the expressed sequence tags (ESTs). ESTs were compared with database nucleotide collection (nr/nt) using Tblastx (Search translated nucleotide databases using a translated nucleotide query) (http://www.ncbi.nih.gov).

Analysis of gene expression by semi-quantitative RT-PCR

Total RNA was extracted from each individual harvested sample at each stage using the method as described previously. Fertile and sterile bulks were separately prepared by pooling an equal quantity of RNA from each of the six stages of fertile and sterile buds for 1st stand cDNA synthesis. One-step reverse transcription-PCR (RT-PCR) was performed using total RNA as the template and *M-MLV* reverse transcriptase (Promega, USA) according to the manufacturer's instructions. *β-actin* was chosen as the reference gene. The EST primers were determined following sequence results. PCR reactions were performed in a total volume of 25 µl, comprising 170 ng cDNA, 1.25 mmol L^{-1} dNTPs, 10 µmol L^{-1} of each primer, 37.5 mmol L^{-1} $MgCl_2$, and 0.2 U of Taq DNA polymerase (Henan Sino-American Biotechnology Co., Ltd., China). The PCR cycling parameters included an initial denaturation at 94°C for 5 min; 25 to 27 cycles of 94°C for 1 min, 54°C for 30 s and 72°C for 50 s and a final 72°C for 10 min.

RESULTS

Fertility investigation of 'AB01' and classification of flower buds

Investigation of plant fertility levels showed that there was a 1:1 ratio for fertility:sterility in these A/B line 'AB01' Chinese cabbage. Following filament length, anther color and presence or absence of pollen, the flower buds were divided into six size stages: Stage I (length: < 1.5 mm); stage II (length: 1.5 to 2 mm); stage III (length: 2 to 2.5 mm); stage IV (length: 2.5 to 3 mm), stage V (length: 3 to 3.5 mm); and stage VI (length > 3.5 mm).

Library construction

A sterile-tester library was obtained, which was enriched in the cDNA of genes preferentially expressed in sterile flower materials. A fertile-tester library was obtained, which should be enriched in the cDNA of genes preferentially expressed in fertile flower materials. The subtraction efficiency was evaluated by PCR amplification of the *β-actin* housekeeping gene. If subtraction is efficient, a reduction in housekeeping gene transcripts should be observed. Figure 1 shows the *β-actin* fragment is detectable only following 30 cycles of amplification in the subtracted samples, whereas, it is clearly detectable in the unsubtracted samples after 18 cycles, indicating high subtraction efficiency.

PCR product examination of differentially expressed cDNAs

Random differentially expressed cDNA clones were screened by PCR amplification from the two subtraction libraries. Twenty-five clones were amplified and the cDNA insert size ranged from 77 to 639 bp after removing the primer and adapter sequences (Figure 2).

EST sequence analysis

Functional analysis of ESTs from fertile-tester library

Twenty-two ESTs were obtained from fertile-tester library after low-quality and repeated sequences were

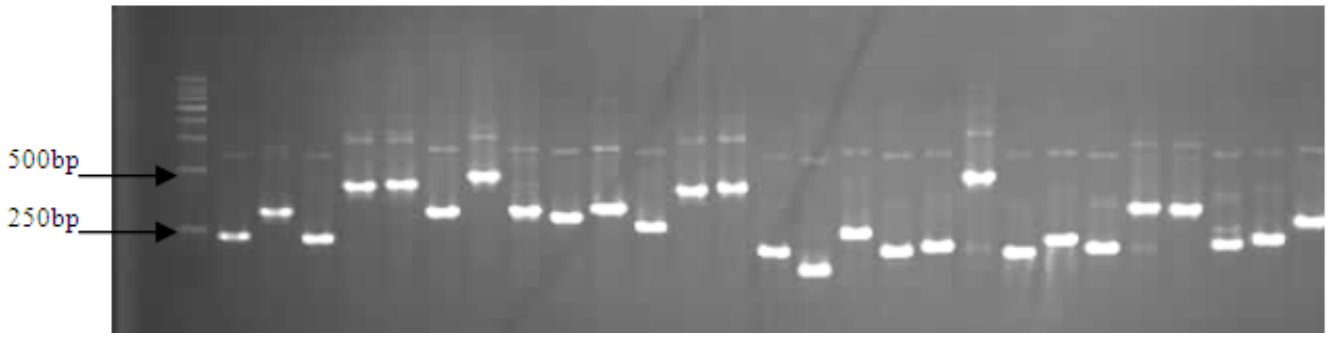

Figure 2. Electrophoresis patterns of PCR products amplified from the inserted fragments. M: DL2000; 1 to 12: PCR products of a single clone.

eliminated. TBLASTx was used to analyze ESTs with database nucleotide collection (nr/nt). The results showed that all the 22 ESTs were homologous to known sequences deposited in NCBI. Of the 22 ESTs, 20 were homologous to ESTs from *Arabidopsis thaliana* or *B. rapa*. These genes with homology to known proteins could be divided into several functional categories: Seven were categorized in flowers/ buds development proteins; eight were meta-bolize proteins (five lipid metabolize proteins and two energy metabolize proteins) and others included three cell structure proteins, three cell growth/division proteins and two secondary metabolic-related proteins (Table 1). Among these seven flowers/buds development proteins, there were two anther development proteins, one pollen-specific protein and four unknown proteins (two clones from silique and two clones from flowers and buds).

Functional analysis of ESTs from sterile-tester library

Only three EST sequences were obtained after removing the low-quality and repeated sequences in the sterile-tester library. The length of ESTs ranged from 77 to 173 bp, TBLASTx analysis showed that all the three ESTs were homologous to known sequences deposited in NCBI. Two of these ESTs were homologous to ESTs from *A. thaliana* or *B. rapa*. Functional analysis showed there was one flowers/buds development protein and two cell structure proteins (Table 2).

RT-PCR assay

Five ESTs (length > 400 bp) were selected to verify different expression patterns between fertile and sterile buds via RT-PCR. Results indicated that, three of these ESTs were expressed only in fertile buds and others were down-regulated in sterile buds (Figure 3). Function analysis showed that among these ESTs expressed only in fertile buds, there were two anther development proteins and one lipase.

DISCUSSION

In this study, sterile and fertile buds of a two-type line Chinese cabbage 'AB01' were used to construct a SSH library and to seek fertility-related genes. Although, high-throughput is one characteristic of SSH, we only selected 25 differentially expressed genes, including 22 up-regulated genes in the fertile buds (from the forward-subtracted library) and three up-regulated genes in sterile buds (from the reverse-subtracted library). This result confirmed that, there is high similarity between the two sets of materials and two-type line was ideal material to research the molecular mechanisms of genic multiple-allele inherited male-sterility. This is because the fertile and sterile plants of two-type line Chinese cabbage 'AB01' have similar genetic backgrounds (Feng et al., 2009). Consequently, the fertile plants and sterile ones should have a similar gene expression pattern before the key stage for fertility control. Thereafter, the male gametes of sterile plants stopped its development and the differentially expressed genes reduced greatly. However, the development of the fertile plants is normal and after this stage, huge amount of genes will be expressed. Two-type line had been widely used to isolate differentially expressed clones between the fertile and sterile plants. Cao et al. (2006) had used GMS A/B line 'ZUBajh97-01A/B' to find pollen preferentially expressed genes in Chinese cabbage (*B. campestris L. ssp. chinensis Makino*). The homozygous DGMS two-type line 'Rs1046AB' was used to isolate differentially expressed clones between the fertile and sterile plants of *B. napus* L. (Wu et al., 2007).

SSH is a powerful tool to study gene expression at the transcriptional level in specific states tissues. In this study, a total of 25 differentially expressed genes in the nuclear genetic male-sterile and male-fertile Chinese cabbage were ultimately obtained by differential screening and sequencing in two constructed SSH cDNA libraries.

Table 1. TBLASTx analysis of fertile-tester library EST with function identified genes in GenBank.

Classification	Sample no.	Length (bp)	Putative identification	Organism	Max. score	E-value
Flowers/buds Development protein	3F-5	469	Anther development protein	*Brassica juncea*	179	1.00E-52
	1F80	100	Anther development protein	*B. juncea*	62.5	2.00E-08
	3F-95	432	Clone GSLTFB5ZE09 of Flowers and buds of strain col-0	*A. thaliana*	219	6.00E-67
	1F89	176	Clone GSLTSIL87ZH09 of silique of strain col-0	*A.thaliana*	52.2	3.00E-05
	1F203	447	Putative pollen-specific protein	*A.thaliana*	321	4.00E-85
	3FD5	433	Flowers and buds of strain col-0	*A.thaliana*	278	2.00E-72
	1F228	348	clone GSLTSIL76ZG06 of silique	*A.thaliana pekinensis*	148	8.00E-34
Lipid metabolize	3F-22	253	Phospholipid transfer proteins	*B.napus*	177	2.00E-42
	IIF11	308	Lipid transfer protein	*B. rapa*	169	7.00E-40
	1F57	307	Phospholipase activator/ ARFA1D	*A.thaliana*	53.8	1.00E-06
	F15	355	Calcium ion binding / DGK1	*A.thaliana*	458	7.00E-126
	3F-37	603	Acyltransferase/ carboxylesterase/ lipase (EXL6)	*A.thaliana*	225	5.00E-77
Energy metabolize	1F86	209	ATP binding (HSP60-2)	*A.thaliana*	129	3.00E-28
	1F195	144	Chlorophyll a-binding protein (psbC)	*Euglena mutabilis*	90	1.00E-16
Cell structure protein	3F-16	208	Chloroplast sequence	*Brassica rapa* subsp. *pekinensis*	132	3.00E-29
	3F-94	334	18S ribosomal RNA	*Brassica oleracea*	239	9.00E-61
	3F-43	238	BAC clone T16L24; putative membrane protein	*A.thaliana; Pseudomonas aeruginosa*	57.4	3.00E-07
Cell growth/division Protein	3FA10	162	Xyloglucan 6-xylosyltransfera (XXT5) mRNA	*Brassica rapa* subsp. *pekinensis*	102	2.00E-20
	3FC10	178	Ubiquitin protein ligase binding	*Brassica rapa* subsp. *pekinensis*	51.9	8.00E-10
	3FC4	125	Ubiquitin protein ligase binding	*Brassica rapa subsp. pekinensis*	53.3	3.00E-10
Secondary Metabolic-related protein	3F-56	284	Type 2 peroxiredoxin (PrxII)	*Brassica rapa* subsp. *pekinensis*	205	1.00E-50
	1F209	112	Peptidylprolyl isomerase B	*Homo sapiens*	35.4	3.4

Table 2. TBLASTx analysis of sterile-tester library EST with function identified genes in GenBank.

Classification	No.	Size of fragment (bp)	Best homolog score	Category of homology	Max score	E-value
Flowers /buds development protein	1S86	118	cDNA clone from total adult females.	*Anopheles gambiae*	26.7	0.62
Cell structure protein	1S333	173	Chloroplast sequence	*B. rapa subsp. pekinensis*	109	2.00E-22
Cell structure protein	1S406	77	Protein binding mRNA	*A. thaliana*	65.7	3.00E-09

Of the 22 ESTs from the fertile-tester library, 20 (90.9%) are homologous ESTs in *A. thaliana* or *B. rapa* and function analysis showed 31.8% were flowers/buds development proteins (Table 1). These results indicated that, our libraries are representative to research the molecular mechanisms of genic multiple-allele inherited male-sterility. Results of five ESTs (length > 400 bp) validated by RT-PCR indicated that, the

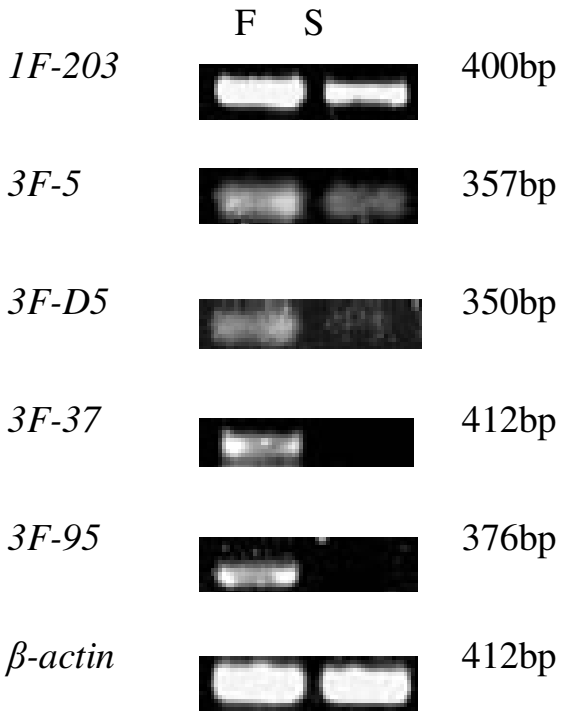

Figure 3. RT-PCR expression patterns of 5 ESTs in the SSH library between fertile and sterile buds.1-5: detected EST; Actin: the reference gene; F: fertile buds; S: sterile buds.

genes were either specially expressed in fertile buds or differentially expressed between fertile and sterile buds (Figure 3), which indicated a very low false positive rate. Therefore, the results support that a high quality library had been constructed.

A number of new and 100% sterile plants from the male-sterile lines have been bred using a hypothesis of multiple-allele nuclear genetic male sterility in Chinese cabbage. However, the molecular mechanisms of genetic multiple-allele inherited male-sterility have not yet been reported. As shown in Table 1, special protein of flowers or bud proteins and metabolism were the two largest groups for all the differentially expressed genes. So we think the development of anther and pollen and metabolism are two important processes in multiple-allele nuclear genetic male sterility in Chinese cabbage.

The abnormal development of anther and pollen is a common phenomenon in male sterility of most plants. This is, either because the male organs fail to develop normally, microsporogenesis is abnormal, the pollen is not able to fully mature or because the anthers fail to dehisce. Male sterility is a common phenomenon across the whole plant kingdom (Chen et al., 2006) and has been widely proven such as in *B. rapa* L.ssp. *pekinensis* flowers/buds development proteins (Guo et al., 2001), *Zinnia elegans* (Ye et al., 2008), *B. napus* L. (Dong et al., 2004) and *Capsicum annum* L. (He et al., 2008). In this study, eight (32%, seven from the fertile-tester library and one from the sterile-tester library) flowers/buds development proteins were detected among the 25 EST homologous sequences, where two were anther development proteins, one pollen-specific protein and five unknown proteins from silique or flowers and buds. Therefore, it can be concluded that the development of anther and pollen is an important process in multiple-allele nuclear genetic male sterility in Chinese cabbage. This was supported by the results of RT-PCR, of the three specially-expressed genes in fertile buds; two were anther development protein genes (Figure 3).

Metabolism also was one of the largest groups for all the differentially expressed genes in this study. Five lipid metabolism protein genes and two energy metabolism protein genes were detected among the 22 EST homologous sequences in the fertile-tester library in the study, which accounted for 31.8% of the EST sequences. Metabolism is a fundamental process for plant development and male gametogenesis is a complicated process that needs all kinds of nutrition. Therefore, it can be concluded that metabolism is the most important process for male gametogenesis from our results. Lipid metabolism is an important process for male gametogenesis and has been proven by the study of Shi et al. (2007), whose study showed that, regulating rice lipid metabolism-related gene *OsMS2* expression can lead to sterility.

In combination with previous research, it was inferred that male-sterility cytoplasm was likely a programmed cell death (PCD) of pollen mother cell damaged by reaction oxygen species (ROS) accumulation in virtue of the mutation of mitochondrial genes, which was a hypersensitive reaction of male cells to excess ROS accumulation (Jiang et al., 2007). Excessive accumulation of ROS was reported in the male-sterility anthers of rice (Chen and Liang, 1991; Li et al., 2004) and wheat (Zhao et al., 1996). Miao et al. (2009) confirmed that male-sterility was related to abnormal metabolism of active oxygen in onion CMS. In this research, one peroxiredoxin EST was isolated in the fertile-tester library. We imagined that, it may be the possible action mechanism of peroxiredoxin in male sterility that more peroxiredoxin avoids ROS accumulation to damaged pollen mother cells in fertile plants, but less peroxiredoxin leads to the production of abnormal pollen in sterile plants. Research on the relationship between active oxygen metabolism and sterility have been reported. For example, Miao et al. (2009) confirmed that male-sterility was related to abnormal metabolism of active oxygen in onion CMS. In addition, a relationship was suggested between *Lycium* sterility (Mi et al., 2008) and the formation of a type of wheat physiological sterility caused by a shortage of energy supply in cells. Moreover, studies have shown that an ATP decrease may directly affect maize male sterility (Xia and Liu, 1994) and beet CMS (He and Tian, 2008).

Also, some genes related to cell structure protein, cell

growth/division protein and secondary metabolic-related protein were identified. The possible explanation was that, these processes were all involved in the biogenesis of cellular components. As for protein fate, it includes protein folding, modification and destination. It had been reported that the unfolded protein response (Schroder and Kaufman, 2005), represses both nitrogen starvation induced developmental programs, pseudohyphal growth and meiosis, thus, contributing to nitrogen sensing in budding yeast (Schroder, 2000). This indicated that, protein folding was very important for normal development under some conditions. Former studies confirmed a relationship between photosynthesis and male sterility in wheat (Zhao et al., 1997; Jiao et al., 2007) and other male sterility structures (Su et al.,1999). Shi et al. (2004) confirmed that, cabbage CMS was related to leaf pigment content and its ultrastructure.

The utilization of genic multiple-allele inherited male-sterility in cross-breeding programs is an economical and stable approach to breed new varieties of Chinese cabbage. Therefore, to understand the mechanism of genic multiple-allele inherited male-sterility and identity fertility-related genes by using SSH method is one of the important strategy to breed more new Chinese cabbage cultivation. However, the molecular mechanisms in Chinese cabbage remains elusive. It is more important to deeply understand the mechanism of these genes expressed and gene regulated and apply it in practice exactly. Further study is needed to obtain full-length genes and determine their definitive function in genic multiple-allele inherited male-sterility in Chinese cabbage.

REFERENCES

Bino RJ (1985). Histological aspects of microsporogenesis in fertile, cytoplasmic stetile and restored fertile Petunia hybrida, Theor. Appl. Genet. 69: 423-428.

Cao JS, Yu XL, Ye WZ, Lu G, Xiang X (2006). Functional analysis of a novel male fertility CYP86MF gene in Chinese cabbage (*Brassica campestris* L. ssp. *chinensis Makino*). Plant Cell Rep. 24(12): 715-723.

Chen SH, Chung NJ, Wang YN, Lee CL, Lee YL, Tsai PF (2006). Study of male sterility in Taiwania cryptomerioides Hayata (*Taxodiaceae*), Protoplasma, 228: 137-144.

Chen XF, Liang CY (1991). Accumulation of hydrogen peroxide and lipid peroxidation in male-sterile anthers of *Orya sativa*. Acta Phytophysiologic Sin. 17: 44-48.

Dong JG, Dong ZS, Liu XX, Liu CS, Li HB (2004). Cytological studies on anther development of ecological male sterile line 533S in *Brassica napus* L. J Northwest Sci-Tech. Univ. Agric. For. 32(7): 61-66.

Feng H, Wei YT, Zhang SN (1995). Inheritance of and utilization model for genic male sterility in Chinese cabbage (*Brassica pekinensis Rupr.*). Acta Horticult. 402: 133-140.

Feng H, Wei YT, Ji SJ (1996). Multiple allele model for genic male sterility in Chinese cabbage. Acta Horticult. 467: 133-142.

Feng H, Xu W, Wang YG (2007). Directive transfer of the genetic male sterile line of milk Chinese cabbage AI023. Acta Horticult. Sin. 34(3): 659-664.

Feng H, Wei P, Piao ZY, Liu ZY, Li CY, Wang YG, Ji RQ, Ji SJ, Zou T, Choi SR, Lim YP (2009). SSR and SCAR mapping of a multiple-allele male-sterile gene in Chinese cabbage (*Brassica rapa* L.). Theor. Appl. Genet. 119: 333-339.

Guo JX, Sun RF, Song JX, Zhang SJ (2001). Microsporogenesis of several male sterile lines in *Brassica rapa* L. ssp. *Pekinensis*. Acta Horticult. Sin. 28(5): 409-414.

He CZ, Liu ZM, Xiong XY, Zou XX, Xiao LT (2008). Cytologic observa tions on anther development of 9704a, a cytopla smic male sterile line in *capsicum annum* l. Acta Horticulturae Sinica, 35 (4): 521-528.

He XQ, Tian ZH (2008). Analysis on terminal enzyme and atpase activities between cms lines and their maintainers during vegetative period of sugarbeet. Acta Bot. Boreali-Occidentalia Sin. 28(8): 1614-1618.

Jiang PD, Zhu YG, Wang XL, Zhu W, Zhang XG, Xie HY, Wang XD (2007). Metabolism of reactive oxygen species in the cytoplasmic male-sterile cotton anther. Agric. Sci. Chin. 6(3): 275-280.

Jiao J, Gao QR, Zhang AM, Hao YY, Wang DW, Wang L, Qiu XM (2007). Diurnal changes of photosynthetic and physiological parameters in different male sterile lines of wheat. Acta Agron. Sin. 33(8): 1267-1271.

Li CY, Feng H (2006). Transfer of the multiple allele male sterile line of Chinese cabbage. Chin. Agricul. Sci. Bull. 22(7): 377-379.

Li HX, Zhang LY, Zhang GS, NIiu N, Zhu ZW (2008). Construction of cdna library from fertility related genes of male sterile wheat with aegilops kotschyi cytoplasm by ssh. Acta Agron. Sin. 34(6): 965-971.

Li SQ, Wan CX, Kong J, Zhang ZJ, Li YS, Zhu YG (2004). Programmed cell death during microgenesis in a Honglian CMS line of rice is correlated with oxidative stress in mitochondria. Funct. Plant Biol. 31: 369-376.

Liu LC, Cao JS, Yu XL, Xiang X, Pei YJ (2006). Expression of an antisense BcMF3 affects microsporogenesis and pollen tube growth in Arabidopsis. Agric. Sci. Chin. 5(5): 339-345.

Liu ZY, Feng H, Li CY, Wei P, Wang LL (2010). Scar markers linked to ms-a genetic male sterile gene in chinese cabbage. Acta Horticult. Sin. 37(5): 757-762.

Miao JS, Yang WC, Li MQ, Shen HL (2009). Studies on the relationships between cytoplasmic male sterility and reactive oxygen species metablisms during development of welsh onion (*a. Fistulosum*) buds. Acta Agric. Boreali-Sin. 24(4): 92-95.

Mi HL, Zhang XY, Fan YF, Li YK, Cao YL (2008). A study on activated oxygen metabolism in the male sterile plants of lycium barbarum. Acta Agricul. Univer. Jiangxiensis, 30(5): 796-798.

Schroder M, Chang JS, Kaufman RJ (2000). The unfolded protein response represses nitrogen-starvation induced developmental differentiation in yeast, Genes. 14: 2962-2975.

Schroder M, Kaufman RJ (2005). The mammalian unfolded protein response, Annu. Rev. Biochem. 74: 739-789.

Shi GJ, Hou XL, Yuan JY (2004). Pigment contents and ultrastructure of leaf on cytoplasmic male sterile lines and their maintainer of non-heading Chinese cabbage. J. Nanjing Agricul. Univer. 27(4): 31-35.

Shi J (2007). Mapping of a Rice (*Oryza Sativa* L.) Fatty Acyl Reductase OsMS2 Gene and Its Function in Pollen Wall Development. Shanghai: [Master's dissertation]. Shanghai Jiaotong Univer.

Su N, Meng K, Shen GF (1999). The regulation of chloroplast gene expression. Biotech. Inform. (1): 14-17.

Tian AM, Cao JS, Huang L, Yu XL, Ye WZ (2009). Characterization of a male sterile related gene BcMF15 from *Brassica campestris* ssp. chinensis. Mol. Boil. Rep. 36(2): 307-314.

Van Der Meer QP (1987). Chromosomal monogenic dominant male sterility in Chinese cabbage *(Brassica rapa* subsp. Pekinensis (Lour.) Hanelt). Euphytica, 36: 927-931

Wang LL, Wei P, Liu ZY, Li CY, Wang YG, Ji RQ, Feng H (2010). SSR Mapping of the Msf-a Multiple-allele Male-fertility Restorer Gene in Chinese Cabbage-*Brassica campestris* L. ssp. Pekinensis. Acta Horticult. Sin. 37(6): 923-930.

Wang YQ, Ye WZ, Cao JS (2005). Cloning and characterization of the microspore development-related gene BcMF2 in Chinese cabbage pak-choi (*Brassica campestris* L. ssp. chinensis Makino) Aseanbiotechnology. Info. J. Integrative Plant Biol. 47(7): 863-872.

Wei P, Feng H, Piao ZY, Li CY, Liu ZY, Wang YG, Ji RQ, Zou T, Ji SJ(2009). Identification of AFLP markers linked to Ms, a genic multiple allele inherited male-sterile gene in Chinese cabbage. Breeding Sci. 59: 333-339.

Wu JY, Shen JR, Mao XZ, Liu KD, Wei LP, Liu PW, Yang GS (2007).

Isolation and analysis of differentially expressed genes in dominant genic male sterility (DGMS) *Brassica napus* L. using subtractive PCR and cDNA microarray. Plant Sci. 72: 204-211.
Xia T, Liu JL (1994). Cytochrome oxidase activity and atp content of male-sterile cytoplasm in maize (zea mays l.). Acta Agricult. Boreali-Sin. 9(4): 33-37.
Ye YM, Huqs Chen TH, Bao MZ (2008). Male Sterile Lines of Zinnia elegans and Their Cytological Observations, Agricul. Sci. Chin. 7(4): 423-431.
Yue YL, Feng H (2005). Transfer of the nuclear gene male sterile lines in various ecotype of Chinese cabbage. China Vegetables. (7): 22-23.
Zhao HJ, Liu HS, Lin XW, Wu YT (1996). Study on relationship between anther abortion of cytoplasmic male-sterile wheat lines and metabolism of active oxygen. Acta. Agron. Sin. 22: 365-367.
Zhao M, Zhang JX, Wang MY (1997). Changes of leaves photosynthesis when Male Sterility is induced by short time high temperature in wheat. Acta Agron. Sin. 23(4): 496-500.