Isolation of an ascorbate peroxidase in *Brassica napus* and analysis of its specific interaction with ATP6

Zhi-bin Liu, Yu-fei Yue, Jun-bei Xiang, Jian-mei Wang, Jun Wu, Xu-feng Li and Yi Yang*

Key Laboratory of Bio-resources and Eco-environment, Ministry of Education, College of Life Science, Sichuan University, Chengdu, Sichuan 610064, P.R. China.

Accepted 16 March, 2010

In *Brassica napus*, ATP6 is related to *Pol* cytoplasmic male sterility. To understand the mechanism of *Pol* CMS, proteins which interact with ATP6 were screened in a yeast two-hybrid system. A partial sequence of a putative and ascorbate peroxidase (*Bn-APX*) was isolated from *Brassica napus*. By use of rapid amplification of cDNA ends method, the full length of *Bn-APX* coding sequence was cloned. The deduced amino acid sequence contained 438 amino acid residues with a conserved ascorbate peroxidase domain and shared 77% identity with that of *APX* from *Arabidopsis thaliana*. Further analyses revealed that the region of *Bn-APX* interacting with ATP6 was at its c-terminal. It was also observed that the expressions of ATP6 and *Bn-APX* were strongly similar in the floral tissue of CMS line and the restoring line. Furthermore, in restoring line, the expression of *Bn-APX* is higher in the flower than that in other tissues.

**Key words:** ATP6, *Pol* CMS, yeast two-hybrid system, ascorbate peroxidase.

**INTRODUCTION**

Cytoplasmic male sterility (CMS), a maternally inherited condition in which a plant is unable to produce functional pollen, has been observed in numerous species (Laser and Lersten, 1972). Detailed molecular analyses of a number of CMS systems in various plant species have revealed that male sterility is associated with some essential mitochondrial genes, accompanied by either appearance of novel, often chimerical transcripts or altered expression usually in a tissue-specific manner (Schnable and Wise, 1998; Hanson and Bentolila, 2004). Such changes in mitochondrial gene expression usually result from rare mitochondrial genome mutations, alien mitochondrial substitutions subsequent to wide hybridization or mitochondrial recombination in somatic hybrids. Although the regions of the mitochondrial genome relative to certain forms of CMS have been identified, the molecular basis of the trait is not precisely understood in any system.

*Brassica napus*, which is widely grown as the oilseed crop of rape or canola, offers several advantages as a system for the molecular analysis of CMS. The relatively simple organization of its mitochondrial genome in the *Brassica* and allied genera (Palmer and Herbon, 1986) facilitates detailed analysis of structural differences between sterile and fertile mitochondrial DNA (Makaroff and Palmer, 1988). In addition, the capability of producing *Brassica* somatic hybrids with recombinant mitochondrial genomes potentially allows for direct genetic analysis of the cytoplasmic determinants of CMS. There is also considerable interest in applying

---

*Corresponding author. E-mail: yangyi528@scu.edu.cn. Tel: 86-28-85412281. Fax: 86-28-85410957.

**Abbreviations:** CMS, Cytoplasmic male sterility; RT-PCR, reverse transcriptase PCR; SMART, switching mechanism at 5’ end of RNA transcript; SD, synthetic dropout; GAL, galactose; GAL4BD, GAL4 binding domain; GAL4AD, GAL4 activation domain; APX, ascorbate peroxidase; Bn-APX, *Brassica napus* ascorbate peroxidase; HMG, high mobility group; ROS, reactive oxygen species; RACE, rapid amplification of cDNA ends; ONPG, Ortho nitro phenyl β-D-galactopyranoside.
Brassica CMS in the production of hybrid rapeseed, because seed yield in Brassica napus hybrids may be enhanced by as much as 60% above that of parental lines.

*Polima* cytoplasm confers a relatively temperature sensitive male sterility (Polowick and Sawhney, 1988; Young et al., 2004) and because of the availability of restorer genotypes, this system appears to be advantageous for hybrid rapeseed production. The ATP6 mitochondrial gene region has also been found to be associated with the *Polima* or Pol CMS system of B. napus (Singh and Brown, 1991; Witt et al., 1991; Handa and Nakajima, 1992). In this case, the ATP6 gene is situated downstream of and co-transcribed with a chimerical mitochondrial gene open reading frame named *orf224* (Singh and Brown, 1991). The N-terminal coding and 5' non-coding regions of this chimerical gene are derived from a normal mitochondrial gene, *orfB*, while the origin of the remaining portion is unknown.

In order to study the reason that an alternation of ATP6 leads to Pol CMS, we screened ATP6 interacting proteins in a yeast two-hybrid system and identified *Bn-APX*, a putative ascorbate peroxidase. The interaction proteins in a yeast two-hybrid system and identified *Bn-ATP6* of the remaining portion is unknown. (Singh and Brown, 1991). The N-terminal coding and 5' non-coding regions of this chimerical gene are derived from a normal mitochondrial gene, *orfB*, while the origin of the remaining portion is unknown.

Yeast two-hybrid screening

The *B. napus* cDNA library was constructed using RNA from floral tissue of fertility restorer lines messenger RNA transcripts are efficiently copied into ds cDNA using BD SMART (Switching Mechanism at 5' end of RNA Transcript) technology and cDNA library construction was performed as described (Zhu et al., 2001). The pGBK7-ATP6 and library plasmids were co-transformed into yeast host strain AH109. A total of $1 \times 10^6$ transformants were plated onto synthetic dropout (SD) selection medium that lacked tryptophan, leucine and histidine. Yeast transformants that appeared on the selection medium within 5d were streaked on a nylon membrane soaked with SD medium lacking tryptophan, leucine and histidine and then filter-lift assays were performed as described in the protocol supplied by the manufacturer. The pGADT7 plasmids isolated from both his3- and lacZ-positive yeast transformants were co-transformed with the empty pGBK7 into the AH109 yeast strain to determine the specificity of growth.

5'RACE and full-length cDNA cloning of *Bn-APX*

The 5'RACE was performed using the SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA, USA), according to the instructions of the manufacturer. By comparing and aligning the sequence of yeast two-hybrid and 5'RACE products, the full-length cDNA sequence of *Bn-APX* was obtained, which then was amplified via PCR using a pair of primers (apx1: 5'AGATGGGCTCTATCATGCTCTCCGCC-3'/apx2: 5'TTAGAAACCAGAGTAGATTATAGCATCATTC-3') and cloned into the *EcoR*I and *BamH*I sites of pGADT7 by PCR amplification. All plasmids were sequenced for correct construction.

β-Galactosidase activity assay

Various pairs of the pGBK7 and pGADT7 plasmids were co-transformed into yeast reporter strain AH109. Transformants were plated onto SD medium lacking tryptophan and leucine and incubated for 5 days at 30°C. The colonies were collected and assayed for β-galactosidase activity. β-Galactosidase assays with ONPG and X-gal as substrates, respectively, were performed, as described previously (Matchmaker two-hybrid system, Clontech).

Expression analysis of *ATP6* and *Bn-APX* by quantitative RT-PCR

Real-time PCR was performed using the LightCycler Quick System 350S (Roche Diagnostics, Mannheim, Germany) with SYBR Premix Ex Taq (Takara, China). Each PCR reaction contained 1X SYBR Premix Ex Taq, 0.2 µM of each primer and 2 µl of a 1:5 dilution of the cDNA in a final volume of 20 µl. The following PCR program was used: initial denaturation, 95°C, 10 s; PCR, 40 cycles of 95°C, 10 s, 57°C, 15s, and 72°C, 15s. In melting curve analysis, PCR reactions were denatured at 95°C, reannealed at 55°C, then a monitored release of intercalator from PCR products or primer dimmers by an increase to 95°C with a temperature transition rate of 0.1°C s$^{-1}$. To create a standard curve, homologous standards for each gene were used as external standards in all experiments. cDNA quantities were calculated by the second derivative maximum methods of Light-Cycler Software Ver.3.5 (Roche Diagnostics, Mannheim, Germany) and all quantifications were normalized using β-actin mRNA as an internal control.
RESULTS

Isolation of proteins that interact with ATP6

To isolate proteins that interact with ATP6, the entire ATP6 protein that fused with GAL4 binding domain (GAL4BD) was first used as bait and screened in a yeast two-hybrid system. In the yeast strain AH109, ATP6 did not activate the transcription of reporter genes. pGBKKT7-ATP6 and pGADT7 inserted by cDNAs were co-transformed and screened by growth selection on medium lacking histidine and by the activity of β-galactosidase. A clone designated 653 was isolated as a clone that activated transcription of the two reporter genes in the presence of ATP6. The specific interaction was confirmed by the reintroduction of plasmid extracted into yeast AH109 (Figure 1).

Characterization of specific interaction of ATP6 and Bn-APX

Since clone designated 653 encoded a partial sequence of Bn-APX, the region of Bn-APX that is required for interaction with the ATP6 protein was further characterized. The entire and truncated versions of Bn-APX (1-140, 1-250, 140-250, 250-439 and 140-250) were cloned into pGADT7 to fuse to the GAL4 activation domain (GAL4AD) and used for the determination of interaction. Moreover, the clones containing both pGBKKT7-ATP6 and pGADT7 ligated entire or truncated versions of Bn-APX, grew on the SD-Leu-Trp-His-Ade medium and were verified by PCR. The results showed that Bn-APX 140-439aa, Bn-APX 250-439aa and Bn-APX 1-439aa could grow on SD medium lacking tryptophan, leucine and histidine. Furthermore, they became blue using X-gal as a substrate (Figure 3). Moreover, β-galactosidase activity was assayed in liquid using ONPG as a substrate. The whole region of Bn-APX contributed to the interaction and the Bn-APX 250-439 showed a higher reporter activity.
Figure 2. Alignment of amino acid sequences of putative Brassica napus ascorbate peroxidase with Brassica oleracea (GenBank accession BAD14931) and Arabidopsis thaliana (GenBank accession CAA67426). HMG (High Mobility Group) domain is from 312 to 423.

Figure 3. a: The yeast cotransformants containing both GAL4BD and GAL4AD fusion proteins grew on medium without histidine. b: β-galactosidase activity of each yeast cotransformant was monitored by blue staining (+: positive control, -: negative control, 1:GAL4AD-APX1-140, 2:GAL4AD-APX1-250, 3:GAL4AD-APX1-439, 4:GAL4AD-APX1-50-439, 5:GAL4AD-APX1-140, and 6:GAL4AD-APX).
Expression of **ATP6** and **Bn-APX** in the floral tissue of CMS line and the restoring line

To investigate whether **Bn-APX** and **ATP6** were related to CMS, their expression patterns were analyzed in the floral tissue of CMS line and the restoring line using the quantitative RT-PCR method. The **ATP6** expression level was significantly reduced in the CMS and the **Bn-APX** transcripts in restoring were 2.96 folds than in CMS. We also analyzed their expression in the flower, leaf and stem. The result showed that the transcripts of **ATP6** and **Bn-APX** were highest in the leaf and flower, respectively (Figure 5).

**DISSCUSSION**

In this work, our results indicated that **ATP6** could interact with **Bn-APX** and **APX** was necessary for the interaction through the yeast two-hybrid system. Protein sequence analysis revealed that **APX** contain a HMG (high mobility group) domain (http://smart.embl-heidelberg.de/), which appears to play important archi-tectural roles in the assembly of protein complexes in a variety of biological processes (Thomas, 2001).

Quantitative RT-PCR data indicated that the expressions of **ATP6** and **Bn-APX** were strongly similar in flowers of both CMS and restoring line. In addition, the transcripts of **ATP6** and **Bn-APX** were found in all tissues of *B. napus* examined, including leaf, stem and flower, which suggested they are constitutively expressed. Furthermore, in restoring line, the expression of **Bn-APX** was the highest in the flower, suggesting that **Bn-APX** plays an important role in plant development and physiology, especially flower development.

Ascorbate peroxidases, a protein family which use ascorbate as the electron donor for H$_2$O$_2$ reduction (Asada, 1992), involved in ascorbate-glutathione cycle, utilize reduced glutathione to regenerate ascorbate (Foyer and Halliwell, 1976), while glutathione is regenerated by glutathione reductase. The ascorbate/glutathione cycle is the most important H$_2$O$_2$- detoxifying system in the chloro-plast, but it has also been identified in the cytosol, peroxisomes and mitochondria (Jimenez et al., 1997).

It is now widely accepted that most environmental stresses such as high temperature and salt, lead to the accumulation of H$_2$O$_2$ (Dat et al., 2000; Mittler, 2002). This accumulation has a number of implications for biological processes within the plant as a whole. H$_2$O$_2$, maybe closely related to abscission and pollination, has been proposed as signals for programmed cell death during petal senescence (Robson and Vanlerbergh, 2002). Likewise, APX is considered to play a role as a defense line against ROS-induced programmed cell death in petals and other organs of the plant (Bartoli et al., 1995; Rubinstein, 2000; De Pinto, 2006; Rogers, 2006).

*Pol* cytoplasm confers a relatively temperature sensitive male sterility (fertile in low temperature and sterile in high temperature). The mechanism is unclear so far. It would suggest a hypothesis based on our work: H$_2$O$_2$ accumulates while temperature is increasing. Because the **Bn-APX** interacts with chimerical protein (orf224/atp6 co-transcriptions translated), the ascorbate peroxidases activity are depressed. Then excessive H$_2$O$_2$ is not scavenged, as signals for programmed cell death during
petal senescence leading to sterility.

ACKNOWLEDGEMENTS

This work was supported by the National Natural Science Foundation of China (NSFC projects: 30570968, 30671165) and the Key Project of Ministry of Education (105140).

REFERENCES