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cDNA cloning, sequence characterization and expression analyses of a novel gene-TPI from pepper cytoplasmic male sterility (CMS)

Ming-hua DENG¹,², Jin-fen WEN³, Jin-long HUO⁴, Hai-shan ZHU², Xiong-ze DAI¹, Zhu-qing ZHANG¹, Hui ZHOU² and Xue-xiao ZOU¹*

¹Institute of Vegetable Crops, Hunan Academy of Agricultural Science, Changsha 410125, China.
²College of Horticulture and Landscape, Yunnan Agricultural University, Kunming 650201, China.
³Faculty of Modern Agricultural Engineering, Kunming University of Science and Technology, Kunming 650224, China.
⁴Faculty of Animal Science and Technology, Yunnan Agricultural University, Kunming 650201, China.

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The complete coding sequence (CDS) of the gene triose phosphate isomerase (TPI) was amplified using the reverse transcriptase polymerase chain reaction based on the conserved sequence information of some Solanaceae plants and known highly homologous pepper ESTs. The results showed the pepper TPI gene encodes a protein of 254 amino acids that has high homology with the proteins of six other species, Solanum tuberosum (95%), Arabidopsis thaliana (87%), Ricinus communis, Vitis vinifera and Oryza sativa (87%) and Zea mays (85%). The pepper TPI gene was expressed in root, stem, leaf, flower, pericarp, placenta and seed. At the abortion stages, the ATP and NADH pool in male-sterile line were distinctly lower than those in the maintainer; The activities and expression levels of TPI in anthers of sterile line were obviously reduced, while levels in F₁ hybrid and maintainers kept normal, which indicated that stable transcripts of TPI are beneficial to keep the energy metabolism with a normal level. But when the restorer gene was transferred into CMS line, activities and expression level of TPI could be regulated by the restorer gene and kept stably. The restorer gene likely plays an important role in keeping the balance of the energy metabolism with a normal level during the microspore development.

Key words: Pepper (Capsicum annuum L.), triose phosphate isomerase, cloning, characterization, cytoplasmic male sterility.

INTRODUCTION

Cytoplasmic male sterility (CMS), a maternally inherited trait resulting in the inability to produce or release functional pollen, has been identified and characterized in many crop species (Wise and Pring, 2002). Numerous studies have shown that CMS is related to abnormal mitochondrial gene reorganization (Touzet and Budar, 2004; Linke and Börner, 2005). The reorganization of mitochondria often leads to abnormality of CMS energy metabolism (Connetttm and Hanson, 1990). In aerobic tissues, mitochondria provide energy for cell activities (Balk and Leaver, 2001; Sweetlove et al., 2002). Numerous studies have shown that inadequacy of energy metabolism is related to CMS (Sun et al., 2009; Carlsson et al., 2007; Fujii et al., 2007; Teixeira et al., 2005).

Triose phosphate isomerase (TPI) (Lolis et al., 1990) is the glycolytic enzyme (EC:5.3.1.1) that catalyses the reversible interconversion of glyceraldehyde 3-phosphate and dihydroxyacetone phosphate. TPI plays an important role in several metabolic pathways and is essential for efficient energy production. It is present in eukaryotes as well as in prokaryotes. TPI is generally considered as an extremely efficient enzyme, and is even referred to as a perfect catalyst (Albery and Knowles, 1976).

There is an abundance of literature on the role, structure and regulation of animal TPIs (Oláh et al., 2002; Ahmed et al., 2003). This situation is in sharp contrast to the current research on plant TPIs. In fact, TPI catalyzes a very important step in carbohydrate metabolism, and
TPI activity could play a role in the balance of metabolic fluxes in plant primary metabolism. In vascular plants, a small number of studies have been conducted on TPI expression (Xu and Hall, 1993; Zhang and Chinnappa, 1994; Dorion et al., 2005), but until today the pepper TPI gene has not been reported yet.

In the present experiment, we will isolate the complete coding sequences of the pepper (Capsicum annum L.) TPI gene; we will also study the tissue expression distribution of the gene, and finally we will also study the activity and expression level of TPI gene in anthers of pepper CMS-9704A, its maintainer line 9704B and F1, in order to establish the primary foundation of further understanding of the pepper gene TPI and the relationship with the CMS.

**MATERIALS AND METHODS**

**Samples collection, RNA extraction and first-strand cDNA synthesis**

A typical CMS line, 9704A, its near-isogenic fertile maintainer line, 9704B and a F1 hybrid line were grown in experimental fields of Yunnan Agricultural University on campus in the summer of 2011. Flowers at four developmental stages were picked out from the corresponding young flowers and collected for experiments. The whole flower development was divided into four stages according to the microsporogenesis progress by checking under the microscope, that is (Stage 1) sporogenous cell division stage, (Stage 2) pollen mother cell meiosis stage, (stage 3) uninucleate microspore stage and (Stage 4) mature pollen stage. Fresh tissues (root, stem, leaf, flower, pericarp, placenta and seed) in the full bloom stage for RNA isolations were frozen in liquid nitrogen and stored at -80°C until further use.

Total RNA and first-strand cDNA synthesis for those samples were performed as the methods described by previously (Deng et al., 2011).

**Isolation of the pepper TPI gene**

The reverse transcription PCR (RT-PCR) was performed to isolate the pepper gene using the cDNAs of CMS line and maintainer line from different tissues aforementioned. The primers for pepper TPI gene isolation were designed based on the conserved coding sequences information from tomato, tobacco, potato TPI genes and their highly homologous pepper ESTs. The primers for pepper TPI were: 5'- TGCAGGAATCCACGAGACTAC -3' and 5'- TACCCACCTGAGCACAATGT -3'. The pepper primers which were used to perform the semi-quantitative RT-PCR for tissue expression profile analysis were the same as the primers for isolation RT-PCR aforementioned.

**Bioinformatics analysis**

Sequence analysis of pepper TPI gene was performed using software in NCBI (http://www.ncbi.nlm.nih.gov) and ExPaSy (http://www.expasy.org). The cDNA sequence was predicted using the GenScan software (http://genes.mit.edu/GENSCAN.html). Putative protein theoretical molecular weight (Mw) and isoelectric point (pI) prediction, signal peptide prediction, subcellular localization prediction and transmembrane topology prediction were performed using the Compute pi/Mw Tool (http://us.expasy.org/tools/pi_tool.html), SignalP 3.0 server (http://www.cbs.dtu.dk/services/SignalP/), PSort II (http://psort.hgc.jp/), TMHMM-2.0 server (http://www.cbs.dtu.dk/services/TMHMM-2.0/), respectively. The Blast program and Conserved Domain Architecture Retrieval Tool were used to search for similar proteins and conserved domain, respectively (http://www.ncbi.nlm.nih.gov/Blast). The alignment of the nucleotide sequences and deduced amino acid sequences were computed using ClusterX, and the phylogenetic trees were computed using the ClustaIX and Mega 4.0 software with standard parameters. Secondary structures of deduced amino acid sequences were predicted with SOPMA (http://npsa-pbil.ibcp.fr/). The 3D structure was predicted based on the existed 3D structures by the amino acids homology modeling on Swiss server (http://swissmodel.expasy.org/).

**RESULTS**

**Cloning and identification of pepper TPI cDNA**

Through RT-PCR with different tissue cDNAs, for pepper TPI gene, the resulting PCR product was 818 bp (Figure 1). These cDNA nucleotide sequence analysis revealed that pepper TPI gene was not homologous to any of the
known pepper genes and they were then deposited into the GenBank database (Accession No. JN885195). The sequence prediction showed that the 765 bp cDNA sequences represent a single gene which encoded 254 amino acids. The cDNA sequences in 9704A and 9704B are exactly the same. It indicated that the TPI gene CMS line was not reorganized. The complete CDS and the encoded amino acids were presented in Figure 2.

Bioinformatics analysis of pepper TPI

The pI and Mw of pepper TPI were 5.72 and 27094, respectively. The signal peptide prediction revealed that pepper TPI did not contain a potential signal peptide (Bendtsen et al., 2004). Transmembrane topology prediction showed that pepper TPI was not potential membrane protein. Subcellular localization analysis showed pepper TPI was probably located in the cytoplasm (Nakai and Horton, 1999).

Examined indicated that pepper TPI contains one separated conserved domain—TIM-phosphate_binding. The prediction of secondary structure indicates that the deduced pepper TPI contains 126 alpha helices, 43 extended strands, 12 beta turn and 73 random coils.

The homology modeling of TPI was performed to estimate their 3D structure (Benkert et al., 2011). The 3D structure of the TPI (3-249AA) was similar to that of the rabbit TPI (1r2r: C).

The deduced protein sequence of pepper TPI was submitted to generate BLAST reciprocal best hits, and similarity comparison revealed that pepper TPI protein has high homology with the TPI proteins of six other species, Solanum tuberosum (95%), Arabidopsis thaliana (87%), Ricinus communis (87%), Vitis vinifera (87%), Oryza sativa (87%) and Zea mays (85%) (Figure 3).

The phylogenetic tree analysis revealed that the pepper TPI gene has a closer genetic relationship with the TPI genes of S. tuberosum than with those of A. thaliana, R. communis, V. vinifera, O. sativa and Z. mays (Figure 4).

mRNA tissue-specific expression profile

To check the relative expression levels of pepper TPI mRNA in various pepper tissues, semi-quantitative RT-PCR was performed in seven pepper tissues aforementioned. Results revealed that pepper TPI gene was expressed in root, stem, leaf, flower, pericarp, placenta and seed (Figure 5).

ATP and NAD⁺-NADH content in isolated mitochondria

The ATP levels in 9704A, 9704B and a F₁ hybrid line preserved a durative uptrend, but the ATP levels in 9704A were significantly lower than that in 9704B and F₁ at each of the developmental stage (Figure 6a). The NAD⁺-NADH levels in the extracted mitochondria were also investigated, and a gradual rise was found in 9704B and a F₁ hybrid line. However, except a notable decline at the Stage 2, no distinct change was observed in other three stages in 9704A (Figure 6b). At the same time, the ATP and NAD⁺-NADH levels were no more different in 9704B and a F₁ hybrid line at every stage (Figure 6).

Activities and expression level

The activity and expression level of TPI was investigated in this paper (Figure 7). Results showed the activity of TPI had no significant difference in 9704B and F₁ in the all stages. But the activities of TPI were obviously reduced, relative to the anthers of 9704B and F₁ especially in the Stage 2.

Transcriptional analysis of TPI gene by semi-quantitative RT-PCR was used to test the gene expression difference in the process of flower development among the anthers of 9704A and 9704B. The amplification results showed that the expressions of TPI were very similar to the activities of TPI. As could be seen in Figure 7, the levels of actin mRNA were relatively constant in all of the RNA samples analyzed.

DISCUSSION

It had been reported that there are very highly energy demands during pollen development in higher plants (Tedege and Kuhlemeier, 1997). A widely accepted hypothesis on the mechanism of CMS is that the increased demand for respiratory function and cellular energy in the form of ATP during anther development may be compromised by expression of the aberrant mitochondria genes.
Figure 2. The complete cDNA sequence and amino acid sequence of the protein encoded by *TP1* (GenBank accession number: JN885195).

<table>
<thead>
<tr>
<th>Capsicum annum</th>
<th>Solanum tuberosum</th>
<th>Arabidopsis thaliana</th>
<th>Ricinus communis</th>
<th>Vitis vinifera</th>
<th>Oryza sativa</th>
<th>Zea mays</th>
</tr>
</thead>
</table>
| ATGCCAGAATTCTTCTCAGCGCCACACTGAAAATGCGGACCGGACTCAGCATGACAGAGATAATACCAACTCCTATGCTCTG
| MGRKKFFVGGHNCSTSLNAGTPSPSVDVEVUSPPFVFLPKVKEKLPIH-VAAQNWVVR 70 | MGRKKFFVGGHNCSTSLNAGTPSPSVDVEVUSPPFVFLPKVKEKLPIH-VAAQNWVVR 70 | MGRKKFFVGGHNCSTSLNAGTPSPSVDVEVUSPPFVFLPKVKEKLPIH-VAAQNWVVR 70 | MGRKKFFVGGHNCSTSLNAGTPSPSVDVEVUSPPFVFLPKVKEKLPIH-VAAQNWVVR 70 | MGRKKFFVGGHNCSTSLNAGTPSPSVDVEVUSPPFVFLPKVKEKLPIH-VAAQNWVVR 70 | MGRKKFFVGGHNCSTSLNAGTPSPSVDVEVUSPPFVFLPKVKEKLPIH-VAAQNWVVR 70 | MGRKKFFVGGHNCSTSLNAGTPSPSVDVEVUSPPFVFLPKVKEKLPIH-VAAQNWVVR 70 |

Figure 3. Alignment of the protein encoded by the pepper *TP1* and six other types of *TP1* from *Solanum tuberosum* (ABB02628), *Arabidopsis thaliana* (NP_191104), *Ricinus communis* (XP_002533147), *Vitis vinifera* (XP_002283693), *Oryza sativa* (AAB63603) and *Zea mays* (ACG24648).

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The citric acid cycle is a series of enzyme-catalysed chemical reactions, which is of central importance in all living cells, especially those that use oxygen as part of cellular respiration. In euukaryotic cells, the citric acid...
cycle occurs in the matrix of the mitochondrion. TPI is an enzyme in the Embden-Meyerhof pathway which catalyzes the reversible conversion of dihydroxyacetone phosphate to glyceraldehyde-3-phosphate and is essential for efficient energy production (Lolis et al., 1990). TPI has been found in nearly every organism searched for the enzyme, including animals such as mammals and insects as well as in fungi, plants, and bacteria (Dorion et al., 2005).

Our results (Figure 6) showed that the ATP and NADH pool in male-sterile line were distinctly lower than that in the male fertile (maintainer and F1). This indicates that the CMS plants are in a sub-hungriness state and the reduced availability of energy. At the same time, the low the transcripts and protein activities of TPI were checked in the in male-sterile line (Figure 7). So we can speculate that the transcripts and protein activities of TPI were too low to keep balance of the energy metabolism in sterile line, but they were significantly higher in the anthers of maintainer and hybrid F1, which indicated that stable

Figure 4. Phylogenetic tree for pepper TPI gene.

Figure 5. Tissue expression profile of pepper TPI gene. 1, root; 2, stem; 3, leaf; 4, flower; 5, npericarp; 6, placenta; 7, seed. The Actin expression level is used for the internal control. M: DL2000 DNA mark.

Figure 6. ATP and NAD+ - NADH content in isolated mitochondria during flowers development. A, Generation of ATP in isolated mitochondria from the anthers. B, Content of NAD+ - NADH in the mitochondria.
ACKNOWLEDGEMENTS

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REFERENCES


Figure 7. Activities and expression level of pepper TPI gene.