

Molecular cloning, sequence characterization of a novel pepper gene *NADP-ICDH* and its effect on cytoplasmic male sterility

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ABSTRACT. NADP-dependent isocitrate dehydrogenase (*NADP-ICDH*) is an important enzyme involved in energy metabolism. The complete coding sequence of the pepper (*Capsicum annuum*) *NADP-ICDH* gene was amplified using a reverse transcriptase PCR based on the conserved sequence information of the tomato and other Solanaceae plants and known highly homologous pepper ESTs. Nucleotide sequence analysis revealed that the pepper *NADP-ICDH* gene encodes a protein of 415 amino acids that has high homology with the proteins of seven species, *Solanum tuberosum* (100%), *Citrus limon* (98%), *Daucus carota* (98%), *Nicotiana tabacum* (98%), *Vitis vinifera*

(99%), *Arabidopsis thaliana* (97%), and *Oryza sativa* (98%). Tissue expression analysis demonstrated that the pepper *NADP-ICDH* gene is over expressed in flower, pericarp and seed, moderately in placenta, weakly in stem and leaf, hardly expressed in root. At the abortion stages, activities and expression levels of *NADP-ICDH* in anthers of a sterile line were strongly reduced, while those in an F_1 hybrid remained normal. Activities and expression levels of *NADP-ICDH* were too low to maintain balanced energy metabolism in the sterile line, which indicated that stable transcripts of *NADP-ICDH* are necessary to maintain energy metabolism at a normal level. When the restorer gene was transferred to the cytoplasmic male sterile line, activities and expression level of *NADP-ICDH* were regulated by the restorer gene and became stable. The restorer gene likely plays an important role in keeping the balance of the energy metabolism within normal levels during microspore development.

Key words: *Capsicum annuum* L.; NADP-isocitrate dehydrogenase; Cloning; Sequence characterization; Cytoplasmic male sterility

INTRODUCTION

Cytoplasmic male sterility (CMS) is a genetic phenomenon characterized by the absence of the production or release of viable pollen; it has been reported in more than 150 species of plants and plays an important role in the utilization of hybrid vigor (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 respiratory metabolism in CMS lines (Connettm and Hanson, 1990).

In aerobic tissues, mitochondria provide energy for cell activities (Bartoli et al., 2000; Gueguen et al., 2000; Balk and Leaver, 2001; Sweetlove et al., 2002). Numerous studies have shown that the inadequacy of energy metabolism is related to CMS (Bergman et al., 2000; Ducos et al., 2001; Sabar et al., 2003; Yui et al., 2003; Teixeira et al., 2005; Carlsson et al., 2007; Fujii et al., 2007; Sun et al., 2009).

The tricarboxylic acid (TCA) cycle is the central metabolic pathway for all aerobic processes in living organisms (Corpas et al., 1999). NADP-isocitrate dehydrogenase (*NADP-ICDH*) catalyses the first oxidative decarboxylation reaction of the TCA cycle, yielding 2-oxoglutarate, CO_2 , and NADPH from isocitrate via a two-step reaction (Sienkiewicz-Porzućka et al., 2010). NADPH can be used for reductive biosynthetic reactions, and 2-oxoglutarate can be further oxidised within the cycle or reductively aminated to glutamate. *NADP-ICDH* thus supplies the cell with a very important intermediate of energy metabolism, as well as precursors and reducing power for anabolic pathways (Sassi et al., 2008). The gene encoding isocitrate dehydrogenase has been cloned and sequenced from several living organisms (Fieuw et al., 1995; Gálvez et al., 1998; Camacho et al., 2002; Kirimura et al., 2002).

In the present experiment, we isolated the complete coding sequence of the pepper *NADP-ICDH* gene on the basis of the conserved gene sequences of tomato or other Solanaceae plant species, and subsequently performed sequence analysis. We also studied the tissue expres-

sion of the gene *NADP-ICDH* and the activity and expression levels of the gene in the anthers of pepper CMS-9704A and its maintainer lines 9704B and F₁, in order to establish a foundation for further understanding the action of the pepper gene *NADP-ICDH* and its relationship with CMS.

MATERIAL AND METHODS

Sample collection, RNA extraction, and first-strand cDNA synthesis

A typical CMS line, 9704A, its 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 4 developmental stages were selected and used for the experiments. Whole flower development was divided into 4 stages according to the progression of microsporogenesis, which was assessed by microscopic examination: stage 1, sporogenous cell-division stage; stage 2, pollen mother cell (PMC) meiosis stage; stage 3, uninucleate microspore stage; and stage 4 mature pollen stage. Fresh tissues (root, stem, leaf, flower, pericarp, placenta, and seed) were obtained for RNA isolation, frozen in liquid nitrogen, and stored at -80°C until further use.

Total RNA was extracted using the RNAiso Plus (TaKaRa, Dalian, China) according to manufacturer instructions. To remove genomic DNA contamination, total RNA was digested with RNase-free DNase I (TaKaRa). Three microgram of RNA was reverse transcribed with oligo (dT)₁₈ primer and M-MLV reverse transcriptase (Invitrogen, USA). The efficiency of reverse transcription was verified on 2% agarose gels stained with ethidium bromide.

Isolation of the pepper *NADP-ICDH* gene

RT-PCR was performed to isolate the pepper gene using the cDNAs of the CMS line and maintainer line from the different tissues mentioned above. The 20-μL reaction system contained the following: 1.5 μL 25 ng/μL cDNA, 1 μL 2.5 mM mixed dNTPs, 2 μL 10X Taq DNA polymerase buffer (MgCl₂ plus), 0.4 μL 10 μM forward and reverse primer, 0.3 μL 5 U/μL Taq DNA polymerase, and 13.4 μL sterile water. The PCR program was initially started by incubation at 94°C for 4 min, followed by 35 cycles of 94°C for 1 min, 57°C for 45 s, 72°C for 90 s, and then 72°C extension for 10 min, and finally 4°C to terminate the reaction. The primers for isolation of the pepper *NADP-ICDH* gene were designed on the basis of the conserved coding sequence data for tomato, tobacco, and potato *SCS* genes and their highly homologous pepper ESTs. The primers for pepper *NADP-ICDH* were as follows: 5'-TCTTCCTCCCACTTCTCAT-3' and 5'-ACTGTCCTCTTGCTACCG-3'. After the PCR, the gene product was cloned into a pMD18-T vector (TaKaRa) and sequenced bidirectionally with the commercial fluorometric method. At least 5 independent clones were sequenced.

Bioinformatics analysis

Sequence analysis of pepper *NADP-ICDH* gene was performed using the software in NCBI (<http://www.ncbi.nlm.nih.gov>) and ExPaSy (<http://www.expasy.org>). The cDNA sequences were predicted using the GenScan software (<http://genes.mit.edu/GENSCAN.html>). Predictions of the theoretical molecular weight (Mw) and isoelectric point (pI) of the putative

protein, the signal peptide, the subcellular localization, and transmembrane topology 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/>), and TMHMM-2.0 server (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), respectively. The Blastp 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 ClustalX 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 on the basis of the amino acid homology modelled on a Swiss server (<http://swissmodel.expasy.org/>).

Semi-quantitative RT-PCR

RT-PCR was conducted to determine the expression levels of the *NADP-ICDH* gene in 7 pepper tissues to characterize this gene further. RT-PCR was also conducted using cDNA from the flowers in various developmental stages of CMS-9704A, maintainer lines and F₁ as templates to study the relationship between the expression of the *NADP-ICDH* gene and CMS. To eliminate the effect of cDNA concentration, we repeated the RT-PCR 5 times using 1, 2, 3, 4, and 5 μ L cDNA as templates. We selected the housekeeping gene *actin* as the positive control. The control primers used were as follows: 5'-TGCAGGAATCCACGAGACTAC-3' and 5'-TACCACCACTGAGCACAATGTT-3'. The pepper primers used to perform the semi-quantitative RT-PCR for tissue expression profile analysis were the same as those mentioned above for isolation RT-PCR. The PCRs were optimized for a number of cycles to ensure product intensity within the linear phase of amplification.

Enzyme activity determinations

NADP-ICDH activity was assayed as described in Gibon et al. (2004).

Statistical analyses

The experiments were carried out in 3 independent series with 3 replicates. Values are the mean \pm SD.

RESULTS

Cloning and identification of the pepper *NADP-ICDH* cDNA

Through RT-PCR of different tissue cDNAs for evaluating the expression of the pepper *NADP-ICDH* gene, the resulting PCR product was 1298 bp (Figure 1). cDNA nucleotide sequence analysis using the BLAST software at the NCBI server revealed that pepper *NADP-ICDH* gene was not homologous to any of the known pepper genes, and the sequences were then deposited into the GenBank database (accession No. JN885192). The sequence prediction was performed using the GenScan software and results showed that the 1248 bp cDNA

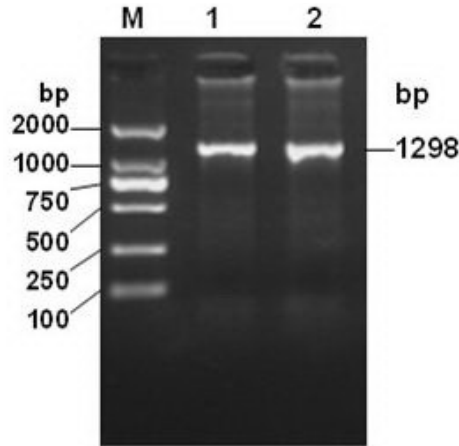


Figure 1. RT-PCR result for pepper *NADP-ICDH* gene. Lane M = DL2000 DNA marker; lane 1 = PCR product for pepper 9704A; lane 2 = PCR product for pepper 9704B.

sequences represent a single gene, which encoded 415 amino acids. The cDNA sequences in the CMS line and maintainer line were exactly the same, thereby indicating that the *NADP-ICDH* gene in the CMS line was not reorganized. The complete CDS and the encoded amino acids are presented in Figure 2.

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ATGGCGTTTCAGAAATCAACGTTCAAAATCCCATCGTTGAGATGGACGGAGATGAATGACGCGAGTTATTTGGAATCAATCAAGGAT
M A F Q K I N U Q N P I U E M D G D E M T R U I W K S I K D
AAGCTCATCCTTCCCTTTCTGGAGTTGGACATCAATACCTTTGACCTTGGACTTCCCACCGTGATTTGACAGACGATAAGGTTACAGTT
K L I L P F L E L D I K Y F D L G L P H R D L T D D K U T U
GAAAGTCCGGAGCCCACTCTAAAGTACAACGTAGCTATTAAAGTGTCCGACCATTACTCCAGATGAGGCCCGTAAAGGAGTTAAAGTTG
E S A E A T L K Y N U A I K C A T I T P D E A R U K E F K L
AAATCCATGTGGAGGAGCCCAATGGGACAATCAGAAACATCCTTAATGGAACGGTCTTTAGAGAACCAATATGTGCAAAAATATTCCT
K S M W R S P N G T I R N I L N G T U F R E P I M C K N I P
CGACTTGTCCCGAGTGGACCAACCAATATGCATAGGGAGACATGCTTTTGGAGATCAATACCGAGCTACTGATGCAGTTATTCAGGA
R L U P G W T K P I C I G R H A F G D Q Y R A T D A U I Q G
GCTGGAANAATCAATTTGGTGTTTGTCGAGAGGATCAGACGAGAGACTGAATACGAAGTTTACAATTTTACTGGTGTGTTGGAGTA
A G K L K L U F U P E G S D E K T E Y E U Y N F T G A G G U
GCTTTATCCATGTACAACACAGACGAGTCTATTGCGCATTTGCTGATGCTTCAATGAACATGGCATCCAAAAGAAATGGCCACTCTAT
A L S M Y N T D E S I R A F A D A S M N M A Y Q K K W P L Y
CTTAGTACAAGAATACCATTCTTAAGAATATGATGGGAGGTTCAAGGACATCTTCCAAGAAGTTTATGAAGCAAGTTGGAAGTCCAAG
L S T K N T I L K K Y D G R F K D I F Q E U Y E A S W K S K
TACGAGGAGGCTGGAATCTGGTATGAACACCGTCTTATCGATGATATGTTGCTTATGCTTTGAAGAGTGAAGGTGTTATGATGGGCC
Y E E A G I W Y E H R L I D D M U A Y A L K S E G G Y U W A
TGCAAGAATATGATGGGGATGACAGAGTGATTTCTTAGCACAGGTTTTGGATCCCTTGGTTGATGACATCTGCTGCTGCTGCTCCT
C K N Y D G D U Q S D F L A Q G F G S L G L M T S U L U C P
GATGGCAAGACCATTGAGGCTGAAGCTGCCCATGGAATGTTACCCGCCACTACAGGTTTACCAGAAAGGAGGTGAGACCAGCACAAAC
D G K T I E A E A A H G T U T R H Y R U H Q K G G E T S T N
AGCATTGCCCTCGATCTTGCCTGGACTCGTGGACTTGCACACAGGGCAACATTGGACAAGAATGAAGGCTCTTGGATTTCACTGAGAAA
S I A S I F A W T R G L A H R A T L D K N E R L L D F T E K
CTAGAGGCCGCATGTCATTTGTCAGTGGAAATCTGGAAGATGACCAAGATCTTGCATCATCATCCATGGATCCAGGCTGTCAAGAGAG
L E A A C I G A U E S G K M T K D L A L I I H G S K L S R E
CATTATCTGAACACAGAGAGTTTATTGACGCTGTGGCCGATGAGCTCAAGCAAACTTCTGAAGCAAGGCTTAA
H Y L N T E E F I D A U A D E L K A K L L K A K A .
    
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Figure 2. Complete cDNA sequence and amino acid sequence of the protein encoded by *NADP-ICDH* (GenBank accession No. JN885192).

Physical and chemical characteristics of pepper *NADP-ICDH*

The theoretical pI and Mw were computed using the Compute pI/Mw Tool. The pI and Mw of pepper *NADP-ICDH* were 6.54 and 46705, respectively. The signal peptide was predicted by SignalP 3.0 on the basis of a combination of several artificial neural networks and hidden Markov models revealed that pepper *NADP-ICDH* did not contain a potential signal peptide (Bendtsen et al., 2004). Using a hidden Markov model algorithm, transmembrane topology prediction made by the TMHMM program (Moller et al., 2001), showed that pepper *NADP-ICDH* was not a potential membrane protein. For subcellular localization analysis, the amino acid sequence was submitted to the PSORT program, and Reinhardt's method showed pepper *NADP-ICDH* was probably located in the peroxisome, with up to 52.4% probability (Nakai and Horton, 1999).

Prediction and analysis of structures and conserved domains of pepper *NADP-ICDH*

Proteins often contained several domains, each of which had their own evolutionary origins and functions. Examined using the Conserved Domain Architecture Retrieval Tool of BLAST at the NCBI server (<http://www.ncbi.nlm.nih.gov/BLAST>) indicated that pepper *NADP-ICDH* contains one separated conserved domain-Iso-dh superfamily (Figure 3).



Figure 3. Putative domains of the protein encoded by pepper *NADP-ICDH*.

Predictions of the secondary structure by SOPMA indicated that the deduced pepper *NADP-ICDH* contained 172 alpha helices, 84 extended strands, 32 beta turn, and 127 random coils (Figure 4).

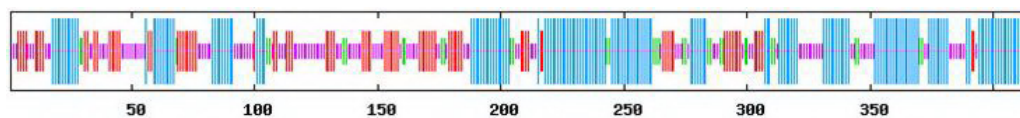


Figure 4. Secondary structure of the pepper *NADP-ICDH* protein predicted by S OPMA. Helices, strands and coils are indicated, respectively, with long, middle and short vertical lines.

Homology modeling

In order to better understand the detailed structure of pepper *NADP-ICDH*, homology modeling of *NADP-ICDH* was performed to estimate their 3D structure (Wang et al., 2007; Benkert et al., 2011). The 3D structure of the *NADP-ICDH* (2-410AA) by homology modeling was similar to that of the *NADP-ICDH* (1euc Chain: A). The 3D structure analysis may provide a basis for further studying the relationship between structure and function of *NADP-ICDH*.

Analysis of sequence identity and evolutionary relationships of pepper *NADP-ICDH*

The deduced protein sequence of pepper *NADP-ICDH* was submitted to generate BLAST reciprocal best hits, and similarity comparison revealed that pepper *NADP-ICDH* protein has high homology with the *NADP-ICDH* proteins of 7 other species: *Solanum tuberosum* (100%), *Vitis vinifera* (99%), *Nicotiana tabacum* (98%), *Citrus limon* (98%), *Daucus carota* (98%), *Oryza sativa* (98%), and *Arabidopsis thaliana* (97%) (Figure 5).

Capsicum annuum	-----	0
Citrus limon	-----	0
Daucus carota	-----	0
Solanum tuberosum	-----FFHQLFFIRLRFLC	14
Oryza sativa	-----	0
Vitis vinifera	-----	0
Arabidopsis thaliana	MLMKLTHGVFTYRASLTANLSSSTSAAGLSSSFVSRFLSSGIFS SGASRNRVTFP VQPHRAS	62
Nicotiana tabacum	-----NASVASFISSSSASTSSAVTKNLPFS IISNRQLFKNRVYL LHRIPNA	47
Capsicum annuum	-----NAFQKIKVAMP IVEHDGENTRVFHKSIKDKLILPFLIED IKYFDLGLPHRDLT	54
Citrus limon	-----NAFQKIKVAMP IVEHDGENTRVFHKSIKDKLILPFLIED IKYFDLGLPHRDLT	54
Daucus carota	-----NAFQKIKVAMP IVEHDGENTRVFHKSIKDKLILPFLIED IKYFDLGLPHRDLT	54
Solanum tuberosum	LIFLSICKNAFQKIKVAMP IVEHDGENTRVFHKSIKDKLILPFLIED IKYFDLGLPHRDLT	76
Oryza sativa	-----NASTKIKVAMP IVEHDGENTRVFHKSIKDKLILPFLIED IKYFDLGLPHRDLT	54
Vitis vinifera	-----NALEKIKVAMP IVEHDGENTRVFHKSIKDKLILPFLIED IKYFDLGLPHRDLT	54
Arabidopsis thaliana	AVRCFASSGSDRIQVQNP IVEHDGENTRVFHSIKDKLILPFLIED IKYFDLGLPHRDLT	124
Nicotiana tabacum	SIRSFASTTASSKIRVENP IVEHDGENTRVITHTIKEKLIYVYLELD IKYFDLGLPHRDLT	109
Capsicum annuum	DDKVTVESAATLKYNVAIKCATITPDEARVKEFNLKSHWRSPNGTIRNINLNGTVFREPIMC	116
Citrus limon	DDKVTVESAATLKYNVAIKCATITPDEARVKEFNLKSHWRSPNGTIRNINLNGTVFREPIMC	116
Daucus carota	DDKVTVESAATLKYNVAIKCATITPDEARVKEFNLKSHWRSPNGTIRNINLNGTVFREPIMC	116
Solanum tuberosum	DDKVTVESAATLKYNVAIKCATITPDEARVKEFNLKSHWRSPNGTIRNINLNGTVFREPIMC	138
Oryza sativa	DDKVTVESAATLKYNVAIKCATITPDEARVKEFNLKSHWRSPNGTIRNINLNGTVFREPIMC	116
Vitis vinifera	RDQVTVESAATLKYNVAIKCATITPDEARVKEFNLKSHWRSPNGTIRNINLNGTVFREPIMC	106
Arabidopsis thaliana	DDKVTVESAATLKYNVAIKCATITPDEARVKEFNLKSHWRSPNGTIRNINLNGTVFREPIMC	186
Nicotiana tabacum	DDQVTVESAATLKYNVAIKCATITPDEARVKEFNLKSHWRSPNGTIRNINLNGTVFREPIMC	171
Capsicum annuum	KNIPRLVPGMTKPIGGRHAFDQYRATDAVIQGGAKLKLIVFVPEGDEKTELEVYNTGAG	178
Citrus limon	KNIPRLVPGMTKPIGGRHAFDQYRATDAVIQGGAKLKLIVFVPEGDEKTELEVYNTGAG	178
Daucus carota	KNIPRLVPGMTKPIGGRHAFDQYRATDAVIQGGAKLKLIVFVPEGDEKTELEVYNTGAG	178
Solanum tuberosum	KNIPRLVPGMTKPIGGRHAFDQYRATDAVIQGGAKLKLIVFVPEGDEKTELEVYNTGAG	200
Oryza sativa	KNIPRLVPGMTKPIGGRHAFDQYRATDAVIQGGAKLKLIVFVPEGDEKTELEVYNTGAG	176
Vitis vinifera	-----GSKPPIGGRHAFDQYRATDAVIQGGAKLKLIVFVPEGDEKTELEVYNTGAG	160
Arabidopsis thaliana	SNIPRLVPGMEKPIGGRHAFDQYRATDAVIQGGAKLKLIVFVPEGDEKTELEVYNTGAG	248
Nicotiana tabacum	KNIPRLVPGMEKPIGGRHAFDQYRATDAVIQGGAKLKLIVFVPEGDEKTELEVYNTGAG	233
Capsicum annuum	GVALSHYNTDESIRAFADA SSMHAYQKKWPLYLSTKNTILKKYDGRFKDIFQEVYEAQWESK	240
Citrus limon	GVALSHYNTDESIRAFADA SSMHAYQKKWPLYLSTKNTILKKYDGRFKDIFQEVYEAQWESK	240
Daucus carota	GVALSHYNTDESIRAFADA SSMHAYQKKWPLYLSTKNTILKKYDGRFKDIFQEVYEAQWESK	240
Solanum tuberosum	GVALSHYNTDESIRAFADA SSMHAYQKKWPLYLSTKNTILKKYDGRFKDIFQEVYEAQWESK	262
Oryza sativa	GVAQSHYNTDESIRAFADA SSMHAYQKKWPLYLSTKNTILKKYDGRFKDIFQEVYEAQWESK	238
Vitis vinifera	GVALSHYNTDESIRAFADA SSMHAYQKKWPLYLSTKNTILKKYDGRFKDIFQEVYEAQWESK	222
Arabidopsis thaliana	-VALAHYNTDESIRAFADA SSMHAYQKKWPLYLSTKNTILKKYDGRFKDIFQEVYEAQWESK	309
Nicotiana tabacum	-VALAHYNTDESIRAFADA SSMHAYQKKWPLYLSTKNTILKKYDGRFKDIFQEVYEAQWESK	294
Capsicum annuum	YEEAGIMYEHLRDDHVAVALKSEGGYVMACKNYDGDVQSDFLAQQFGSLGLMTSLVLCPDG	302
Citrus limon	YEEAGIMYEHLRDDHVAVALKSEGGYVMACKNYDGDVQSDFLAQQFGSLGLMTSLVLCPDG	302
Daucus carota	YEEAGIMYEHLRDDHVAVALKSEGGYVMACKNYDGDVQSDFLAQQFGSLGLMTSLVLCPDG	302
Solanum tuberosum	YEEAGIMYEHLRDDHVAVALKSEGGYVMACKNYDGDVQSDFLAQQFGSLGLMTSLVLCPDG	324
Oryza sativa	YEEAGIMYEHLRDDHVAVALKSEGGYVMACKNYDGDVQSDFLAQQFGSLGLMTSLVLCPDG	300
Vitis vinifera	FEAVGIMYEHLRDDHVAVALKSEGGYVMACKNYDGDVQSDFLAQQFGSLGLMTSLVLCPDG	284
Arabidopsis thaliana	FEHSIMYEHLRDDHVAVALKSEGGYVMACKNYDGDVQSDFLAQQFGSLGLMTSLVLSADG	371
Nicotiana tabacum	FEHSIMYEHLRDDHVAVALKSEGGYVMACKNYDGDVQSDFLAQQFGSLGLMTSLVLSADG	356
Capsicum annuum	KTIEAEAAHGTVTRHYRVHQGGGETSTNSIASIFAMTRGLAHRATLDKNERLLDFTEKLEAA	364
Citrus limon	KTIEAEAAHGTVTRHYRVHQGGGETSTNSIASIFAMTRGLAHRATLDKNERLLDFTEKLEAA	364
Daucus carota	KTIEAEAAHGTVTRHYRVHQGGGETSTNSIASIFAMTRGLAHRATLDKNERLLDFTEKLEAA	364
Solanum tuberosum	KTIEAEAAHGTVTRHYRVHQGGGETSTNSIASIFAMTRGLAHRATLDKNERLLDFTEKLEAA	386
Oryza sativa	KTIEAEAAHGTVTRHYRVHQGGGETSTNSIASIFAMTRGLAHRATLDKNERLLDFTEKLEAA	362
Vitis vinifera	KTIEAEAAHGTVTRHYRVHQGGGETSTNSIASIFAMTRGLAHRATLDKNERLLDFTEKLEAA	346
Arabidopsis thaliana	KTIEAEAAHGTVTRHYRVHQGGGETSTNSIASIFAMTRGLAHRATLDKNERLLDFTEKLEAA	433
Nicotiana tabacum	KTIEAEAAHGTVTRHYRVHQGGGETSTNSIASIFAMTRGLAHRATLDKNERLLDFTEKLEAA	418
Capsicum annuum	CIGAYESGKHKRDLAL-IIHGSKLSREHYLNTEE FIDAVADELRARLAKA	415
Citrus limon	CVGTYESGKHKRDLAL-IIHGSKMTRREHYLNTEE FIDAVADELRARLAKA	414
Daucus carota	CVGTYESGKHKRDLAL-IIHGSKLSREHYLNTEE FIDAVADELRARLAN---	412
Solanum tuberosum	CIGAYESGKHKRDLAL-IIHGSKLSREHYLNTEE FIDAVADELRARLAKA	438
Oryza sativa	CIGAYESGKHKRDLAL-LVHGSSNVTRSHYLNTEE FIDAVADELRARLAN---	412
Vitis vinifera	CIATYESGKHKRDLAL-LIHGPKVTRAQYLNTEE FIDAVADELRARLAKA	397
Arabidopsis thaliana	CVMTYESGKHKRDLAL-LIHGPKVSRDLFVNTEE FIDAVADELRARLAKA	484
Nicotiana tabacum	CVGTYESGKHKRDLAL-LVHGPKVSRREHYLNTEE FIDAVADELRARLAKA	469

Figure 5. Alignment of the protein encoded by the pepper *NADP-ICDH* and seven other types of *NADP-ICDH* from *Solanum tuberosum* (CAA53300), *Citrus limon* (AF176669_1), *Daucus carota* (BAA34112), *Nicotiana tabacum* (CAA65504), *Vitis vinifera* (CAN83081), *Arabidopsis thaliana* (NP_196963), and *Oryza sativa* (NP_001056381).

To evaluate the evolutionary relationships of pepper *NADP-ICDH* with other species, we then constructed a phylogenetic tree using DNASTar, Cluster, Mega, and the DNAMAN software on the basis of the *NADP-ICDH* amino acid sequences. The phylogenetic tree analysis revealed that the pepper *NADP-ICDH* gene has a closer genetic relationship with the *NADP-ICDH* genes of *S. tuberosum* than with those of *C. limon*, *D. carota*, *N. tabacum*, *V. vinifera*, *A. thaliana*, and *O. sativa* (Figure 6).

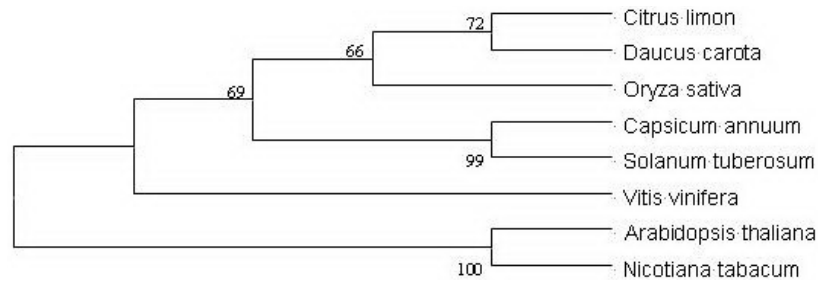


Figure 6. Phylogenetic tree for the pepper *NADP-ICDH* gene.

mRNA tissue-specific expression profile

To check the relative expression levels of pepper *NADP-ICDH* mRNA in various pepper tissues, semi-quantitative RT-PCR was performed in the 7 pepper tissues mentioned above. The continuously expressed gene, *actin*, was used and served as an endogenous reference for determination of targeted mRNA profiles. Results revealed that pepper *NADP-ICDH* gene was overexpressed in the flower, pericarp, and seed; moderately, in the placenta; weakly, in the stem and leaf; and hardly expressed, in the root (Figure 7).

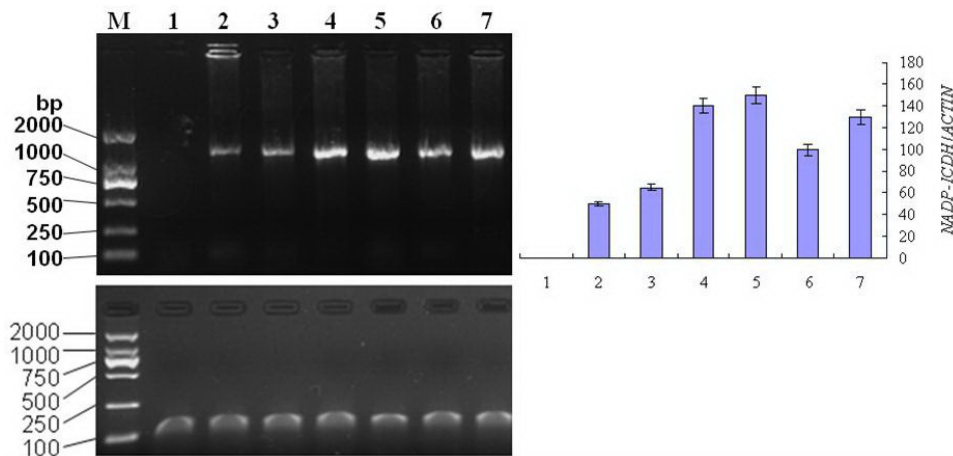


Figure 7. Tissue expression profile of the pepper *NADP-ICDH* gene. Lane 1 = root; lane 2 = stem; lane 3 = leaf; lane 4 = flower; lane 5 = pericarp; lane 6 = placenta; lane 7 = seed. The *Actin* expression level is used for the internal control. Lane M = DL2000 DNA marker.

Activities and expression level

The activity and expression levels of *NADP-ICDH* were analyzed in this study (Figure 8). Assay of *NADP-ICDH* activity showed that there were no more changes in the sterile line, maintainer, and hybrid F₁ at stage 1. In contrast, at stages 2, 3, and 4, the *NADP-ICDH* activity in the sterile line was 35.3, 58.1, and 75.0% that of the maintainer, respectively, and was 30.0, 55.3, and 70.8% that of the hybrid F₁, respectively.

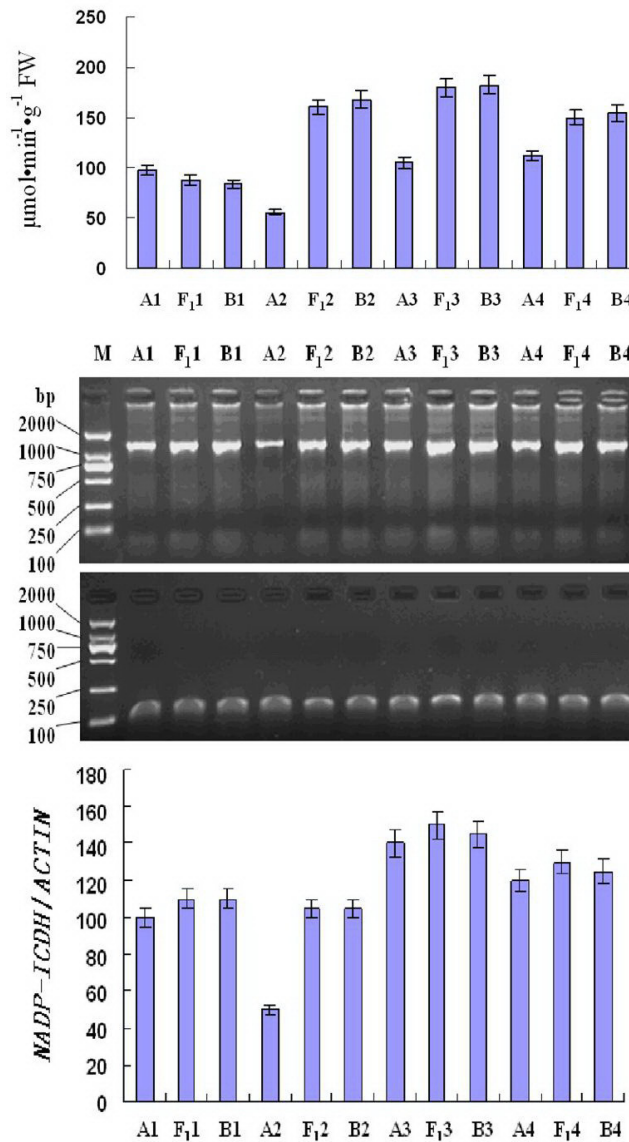


Figure 8. Activities and expression level of the pepper *NADP-ICDH* gene.

Transcriptional analysis of the *NADP-ICDH* gene by RT-PCR was used to test the differences in the gene expression levels during the process of flower development among the sterile and fertile anthers. The results showed that there was no significant difference in the expression of *NADP-ICDH* in the anthers of the sterile line, maintainer, and hybrid F₁ at stage 1. At stages 2, 3, and 4, expression of *NADP-ICDH* mRNA in the anthers of the sterile line reduced markedly, relative to the anthers of the maintainer and hybrid F₁. As seen in Figure 4, the levels of *actin* mRNA were relatively constant in all of the RNA samples analyzed.

DISCUSSION

Reports indicate that energy demands during pollen development are very high in higher plants (Tadege and Kuhlemeier, 1997). A widely accepted hypothesis regarding 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 the expression of the aberrant mitochondria genes.

In aerobic tissues, mitochondria provide energy for cell activities. Numerous studies have shown that CMS is related to abnormal mitochondrial gene reorganization (Touzet and Budar, 2004; Linke and Börner, 2005).

The citric acid cycles involves a series of enzyme-catalyzed chemical reactions, which is of central importance in all living cells, especially those that use oxygen for cellular respiration. In eukaryotic cells, the citric acid cycle occurs in the matrix of the mitochondrion.

NADP-ICDH catalyzes the oxidative decarboxylation of isocitrate to 2-oxoglutarate and produces the reduced coenzyme NADPH (Gálvez and Gadal, 1995). It is the main cellular source of NADPH (Gálvez and Gadal, 1995)

Our results showed that during the abortion stage (stages 2, 3, and 4), the levels of transcripts and protein activities of *NADP-ICDH* in the sterile line were too low to maintain a balance of energy metabolism, but they were significantly higher in the anthers of the maintainer and hybrid F₁, which indicated that stable transcripts of *NADP-ICDH* are beneficial to maintain energy metabolism at a normal level.

Considerable evidence has revealed that nuclear gene expression can regulate mitochondrial gene expression through mitochondria-to-nucleus signaling (Karpova et al., 2002; Maxwell et al., 2002; Dutilleul et al., 2003; Rhoads et al., 2006). In our study, there was an interesting finding that during the anther abortion, unlike the sterile line, the hybrid F₁ always exhibited transcripts and protein activities of *NADP-ICDH* that were maintained at levels consistent with those in the maintainer. This phenomenon has scarcely been reported to date. We presume that this phenomenon is probably the result of the regulation by the nuclear restorer gene during the stage of anther abortion.

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