Full Length Research Paper

Sequence analysis and expression of the calcium-dependent protein kinase gene, *MCDPK* in mulberry (*Morus* L.)

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A full-length cDNA sequence coding for calcium-dependent protein kinase of mulberry tree, which we designated was *MCDPK* and this was cloned based on mulberry expressed sequence tags (ESTs). Sequence analysis showed that the *MCDPK* is 2249 base pairs in length, encoding 532 amino acids with a predicted molecular weight of 59.96 kDa and an isoelectric point of 6.84. The mRNA expression level of *MCDPK* at different developmental stages in mulberry leaves and flowers and in different tissues was investigated. The results showed that *MCDPK* transcripts were most abundantly expressed in the terminal bud, and the expression level of the mRNA was reduced significantly under the conditions of low temperature and salt stress when compared to the normal growth environment. The findings of the study laid the foundation for understanding the resistance mechanism of functional genes in mulberry in the future.

Key words: Mulberry, calcium-dependent protein kinase gene (CDPK), gene cloning, abiotic stress, gene expression.

INTRODUCTION

The past two decades revealed a plethora of Ca^{2+} responsive proteins and downstream targets in plants, of which several of them are unique to plants. More recent high-throughput 'omics' approaches and bioinformatics are exposing Ca^{2+} responsive cis-elements and the corresponding Ca^{2+} responsive genes (Lanteri et al., 2006). Here, we reviewed the current knowledge on Ca^{2+} signaling pathways that regulate gene expression in plants, and linked these to mechanisms by which plants respond to biotic and abiotic stresses (Galon et al., 2010). In higher plants, calcium ion (Ca^{2+}) plays crucial roles as a second messenger mediating the actions of many hormone and environmental factors, including biotic and abiotic stresses (Shao et al., 2008). CDPKs as cytoplasmic Ca^{2+} receptor protein kinases play a very important role in plant cells activated through Ca^{2+} signal transduction (Chung et al*.,* 2004). Drought, low temperature, high salinity, disease, insects and other environmental stresses can stimulate plant cell Ca^{2+} signaling (Szczegielniak et al., 2005), CDPK recognition of Ca^{2+} signaling in the form of phosphorylation signaling cascade and then impart to downstream stress response gene and regulate expression and production activity of this gene to reduce or avoid stress or plant damage (Patharkar et al., 2000; Vitart et al., 2000). Plants possess many unique, putative Ca^{2+} sensors, including a large family (50 in *Arabidopsis*) of calmodulin-like CDPKs

interact with target proteins (that is, protein kinases, metabolic enzymes, cytoskeleton-associated proteins) and regulate their activity (Vanderbeld and Snedden, 2007).

Abiotic stresses, including salinity, drought and cold, have been the important causes for crop loss worldwide (Mahajan and Tuteja, 2005) including mulberry. Higher plants always adapt to the environmental stresses by the activation of cascades of molecular networks involved in stress perception, signal transduction, and expression of specific stress-related genes (Kumar et al., 2004). Stress perception and signaling pathways are critical components of adaptive response that is vital for the plant species to survive under extreme environmental constraints (Cao et al., 2006). Under these conditions, a number of signal pathway, such as the unique Ca^{2+} signal networks will be activated, which results in the transcriptional reprogramming to bestow plants with the resistance to these stresses (Gut et al., 2009).

CDPKs control plant development and respond to various stress environments through their key roles in the regulation of Ca^{2+} signaling (Ye et al., 2009). In plants, CDPKs constitute a unique family of enzymes consisting of a protein kinase catalytic domain fused to carboxy-terminal autoregulatory and calmodulin-like domains (Syam and Jayabaskaran, 2006). Calcium-dependent protein kinases (CDPKs) are proposed to play an essential role in plant defense responses (Kiselev et al., 2010).

In this study, we cloned the MCDPK gene based on the expressed sequence tags (ESTs) from mulberry cDNA library constructed previously (Fang et al., 2008; Zhao, 2008), and got the full-length sequence by RT-PCR (Gao and Zhao, 2010; Zhang and Liu, 2011) and RACE method (Guo, 2011) and analyzed the expression of the gene in different tissues and under different kinds of stresses in mulberry. The purpose of this study is to lay a good foundation for understanding the signal transduction mechanism underlying the stress response and design new strategies for improving mulberry production through the study of transgenic resistance in the future.

MATERIALS AND METHODS

Plant materials and reagents

To analyze the gene expression under different conditions, mulberry variety Yu71-1 (*Morus multicaulis*) was grown under standard conditions in the National Mulberry Gene Bank of the Sericultural Research Institute, Chinese Academy of Agricultural Sciences, Zhenjiang, Jiangsu Province, China was utilized. The mulberry shoots were grafted, and then the grafted plants were transferred into an incubator, and maintained at 25°C and 12 h photoperiod to induce burgeoning until the winter buds grew to about 30cm in length (50_d).

To study and analyze the expression level of *MCDPK* at different developmental stages and in different tissues, samples were collected from a 10 years old mulberry tree of the same variety of Yu71-1 grown in the same field. RNAiso Plus, Reverse

Transcriptase M-MLV (RNaseH⁻), RNase Inhibitor, dNTP, rTaq polymerase, T4 DNA ligase, and Agarose Gel DNA Purification Kit,pMD18-T vectors were products of Takara Company. All PCR primers were synthesized by Shanghai Sangong Biological Engineering Technology and Services Co. Ltd., and all chemicals used were analytical grade reagents. Forward and reverse primers were designed according to the EST library of mulberry leaves.

cDNA cloning

Total RNA was isolated from browses (net weight about 100 mg) of grafted mulberry seedlings using RNAiso plus reagent following the manufacturer's protocol, resuspended in DEPC-treated water and stored at -80°C. The quality of total RNA was determined using UV spectrophotometer combined with electrophoresis. The first strand cDNA was synthesized from total RNA by Reverse Transcriptase M-MLV (RNaseH) at 42°C for 60 min with oligo-dT-adaptor primer following the manufacturer's protocol. The first-strand cDNA was used as the template for PCR in gene cloning. The RT-PCR reactions were performed in a total volume of 50 µL including 1µL first-strand cDNA, 41 µL ddH₂O, 1 µL each of the gene-specific primers, 0.5 µL dNTP, 5 µL buffer, and 0.5 µL rTaq DNA polymerase (5 U/ml). The RT-PCR amplifications were performed using the following parameters: DNA was denatured at 94°C for 5 min followed by 28 amplification cycles (94°C for 30 s, 58°C for 1 min, 72°C for 1 min), with a final extension step of 7 min at 72°C. The RT-PCR products were analyzed in 1% agarose gels and purified using Takara Agarose Gel DNA Purification Kit (TAKARA Bio Co., Ltd.) following the manufacturer's protocol. The purified fragment, which was confirmed to have the predicted length, was then cloned into pMD18-T vector and sequenced to confirm the presence of an open reading frame (ORF) related to the tentative consensus sequence.

5' cDNA end amplification

The homology comparison showed that cDNA sequence of the MCDPK gene fragment obtained contained only the complete 3'-UTR and complete 5'-UTR. Reverse primers were designed according to the two sequences obtained and 5'RACE amplification was performed. Total RNA from browses was used as template to generate first strand total cDNA using Clontech SMART™ RACE Kit in terms of manual instruction. Based on the amplified product of MCDPK, antisense primers F-MCDPK (5'-AAGCAGTGGTATCAACGCAGAGT-3'), and R-MCDPK (5'-CCATCTCCATCAACATCAGCAGAATCCA-3') were synthesized for 5' rapid amplification of cDNA ends (RACE) of the MCDPK gene. R-MCDPK was paired with Clontech SMART™ RACE Kit 5'-Primer to carry out the primary amplification of 5' RACE in a standard 50 µl Taq PCR system. Amplification conditions were as follows: predenaturation at 94°C for 2 min, followed by 25 cycles of amplification (94°C for 1 min, 50°C for 1 min, 72°C for 1 min 30 s) and by 72°C for 10 min. The size of the 5' RACE product is about 1700bp. Sequence encoding MCDPK was determined by homology searches in the NCBI (http://www.ncbi.nlm.gov/) databases using the BLAST program, and the homology sequences were downloaded from the database. Alignment of the MCDPK protein with other structurally related CDPK proteins was performed using the Clustal X program. Some other basic properties were analyzed by tools at the website (http://www.expasy.org) and DNAStar software, such as the searching of the ORF and translation of nucleotide sequence, as well as, isoelectric point prediction and molecular weight of MCDPK. To determine the relationship between MCDPK and other MCDPK proteins, phylogenetic analysis was carried out by the MEGA 4.1 program (Tamura et al., 2007). All the
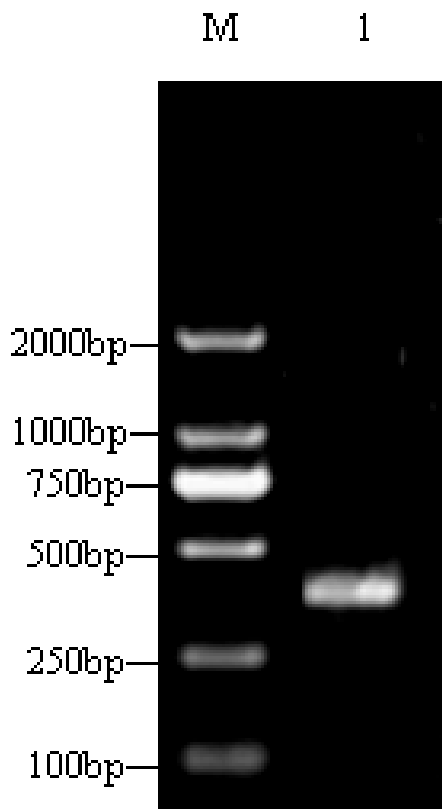
Figure 1. RT-PCR result of mulberry gene MCDPK. M: DL2000 DNA molecular marker; 1: Product of RT-PCR.

Sense: 5'-CAGTGCTTCTCACTGAGGCTC-3'
Anti-sense: GGAAGAGGACTTCTGGGCATC-3'.

The primers for *MCDPK* and the parameters of semi-quantitative RT-PCR amplification were the same as for the RT-PCR analysis. Quantitative analysis of the gel was performed using LabImage software v. 2.7.1. (Kapelan GmbH Co, Germany). The target band density (gray level) was used to represent the relative expression level of the target gene, *MCDPK*. All experiments were repeated at least six times.

Expression patterns of *MCDPK* under different abiotic stresses

To reveal the putative biological function of the *MCDPK* protein, semi-quantitative RT-PCR was performed to detect the expression level of *MCDPK* mRNA under various abiotic stress-induced conditions in mulberry. When the winter buds grew to about 30 cm in length (50_d), these grafted seedlings were transferred into a series of stress treatments, including low temperature (15, 8, 3, 0, -1, 1 + 28°C) and salt (treating time: 0, 6, 12 h, 1, 2, 3, 4, 11, 16 and 18 d). 0.3 mol/L salt was sprayed over plants when the winter buds grew to about 30 cm in length (50_d). Stresses were administered starting from the beginning of the photo-period without changing light intensity, humidity or photoperiod. Control plants were maintained at 25°C and a 12 h photoperiod in the incubator without changing light intensity and humidity before RNA extraction. The browses subjected to different stress conditions were collected for RNA extraction until the appearance of symptoms. All tissues harvested for nucleic acid extraction were weighed and immediately frozen in liquid nitrogen, and stored at -70°C until use.

Total RNA isolated from browses of mulberry under different stress conditions were converted into 1st strand cDNAs with reverse transcriptase to a constant volume of 20 μL. Semi-quantitative RT-PCR amplification and the methods of quantitative analysis of the gel were the same as indicated earlier.

RESULTS AND DISCUSSION

Amplification of full-length cDNAs and sequence analysis of the *MCDPK* gene

To clone the full-length cDNA of the mulberry *MCDPK* gene, a cloning strategy combining bioinformatics analysis and the mulberry cDNA library screening technique was used. RT-PCR products were detected by 1% agarose gel, and obtained the expected size (Figure 1). Sequencing result showed that it was basically the same sequence with related EST. Nested amplification yielded a specific bright band of about 500 bp, which was also in consensus with homology-based length prediction (Figure 2).

Sequence analysis showed that the isolated cDNA and designated *MCDPK* is 2249 base pairs (bp) in length and contains a 381 bp 5' untranslated region (5'-UTR) and a 269 bp 3'-UTR. Its open reading frame (ORF) is 1599 bp, encoding 532 amino acids with a predicted molecular weight of 59.96 kDa and an isoelectric point of 6.84 (Figure 3). To understand the structure and predict the function of MCDPK protein, we carried out the molecular modeling using SWISS-MODEL (http://swissmodel.expasy.org/) and established the

sequences of other species obtained from Genbank were subjected to multiple sequences alignment using ClustalW program (Thompson et al., 2002), and a phylogenetic tree was constructed of the amino acid sequences according to the neighbor-joining method. The reliability of the tree was accessed by a bootstrap analysis with 1,000 replicates. The prediction of protein domains were carried out using SMART (http://smart.embl-heidelberg.de/) and searching literature references. SWISS-MODEL (http://swissmodel.expasy.org/) was used to analyze the molecular modeling of MCDPK protein.

Semi-quantitative analysis of *MCDPK* transcript levels of *MCDPK* at different developmental stages of leaves and flowers as well as in different tissues

To investigate *MCDPK* mRNA content in leaves and flowers at different developmental stages and in different tissues of mulberry, semi-quantitative RT-PCR were performed. Total RNAs were isolated from spires, tender leaves (3 days old), climax leaves (10 days old), phloem, xylem, roots, flourishing female flowers (stigma unpollinated), withered female flowers (stigma pollinated) and maturated female flowers (black mulberry fruits). Total RNAs were converted into cDNAs with reverse transcriptase to constant volume of 20 μl. Semi-quantitative PCR was carried out with 1μL cDNA as template, and mulberry *Maactin* gene (GenBank accession No. DQ785808), a house-keeping gene, was used as an internal control to allow for normalization by visual inspection of mRNA levels. The following primers were used for the *Maactin* gene:
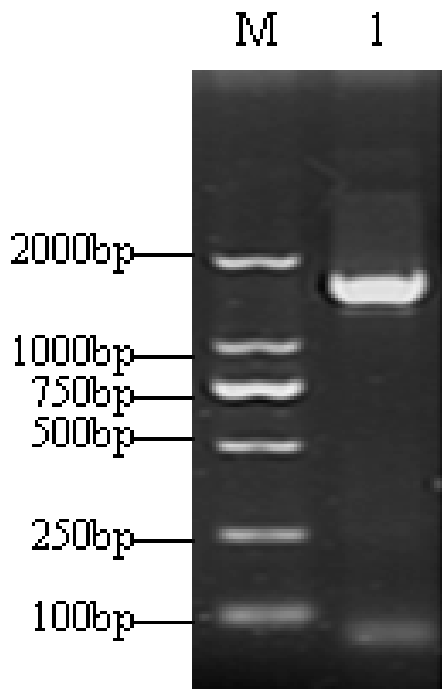
Figure 2. The amplification result of 5' RACE. M: marker DL-2000; 1: the PCR amplification products.
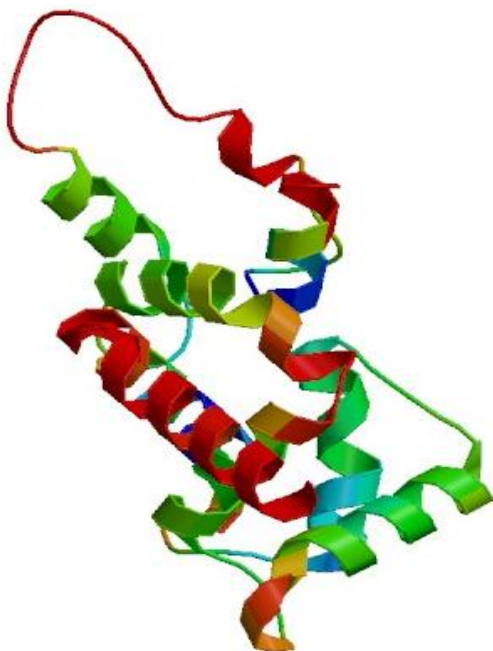

Figure 4. Predicted three dimensional model of *MCDPK*.

three-dimensional structure of the MCDPK protein (Figure 4).

Homology search and phylogenetic analysis of the *MCDPK* gene

To understand the function and evolutionary relationshipof the deduced amino acid sequence encoded by mulberry *MCDPK* gene, multiple sequence alignment was carried out by Clustal X program using the amino acid sequences of MCDPK and other nine species (Figure 5). Sequence alignment revealed that all the proteins have conserved domains additionally, the MCDPK sequences shared very high identity with those of *Nicotiana tabacum* (ADO79931), *Gossypium hirsutum* (ACX37460), *Jatropha curcas* (BAJ53260) with 100% and *Triticum aestivum* (ABY59017) and *Hevea brasiliensis* (AEI70328) with 99%. In order to determine the evolutionary relationship between MCDPK and other proteins, phylogenetic analysis was further carried out by Clustal X program with default parameters using the amino acid sequences of CDPK from mulberry and other 19 species. The phylogenetic relationship among 20 CDPK from different species (Figure 6) shows that the mulberry (*Morus multicaulis*), *Arabidopsis lyrata subsp. Lyrata* and *Arabidopsis thaliana* have a relatively closer relationship. Nonetheless, the MCDPK showed the close relationships with all the CDPK compared earlier which indicates that the amino acid residues of CDPK are highly conserved, suggesting the possibility of structural and functional conservation of CDPK during the course of evolution.

Expression levels of *M-CDPK* at different developmental stages of mulberry leaves and flowers and in different tissues

To elucidate the mechanisms underlying *CDPK* gene expression in mulberry, the expression of *MCDPK* was further analyzed in roots, flowers and leaves of mulberry at different developmental stages using semi-quantitative RT-PCR (Figure 7). We investigated the relative levels of *MCDPK* transcripts from nine samples, comparing five different tissues: leaf, phloem, xylem, root, and flower. *MCDPK* transcripts were detected in all samples and seemed to be most abundant on spire matured leaf with the lowest expression level in mature fruit.

During the three different leaf developmental stages, the expression level of *MCDPK* mRNA was highest in the mature leaf, lower in the spire, and even lower in the young leaf. During the three different developmental stages of female flower, the expression level of *MCDPK* mRNA was highest in the full bloom. The *MCDPK* gene expression exhibits a tissue-specific and developmental stage-specific pattern even in the same tissues in mulberry.

These results suggest that *MCDPK* expression may be predominantly associated with tissue aging or with particular sites.

1
ATTAAGCAGTGGTATCAACGCAGAGTACGCGGGGAGAGAAGAGAGAGTGAAAGAAAAAAGGGGGTGAAGAGAGA
GA
76
AAGAAGCGTCTGCAAAAGAGCTTTGACTCTTCTCTCTCGAAATCCATGGCTCATTCATACCTACGTTTATACGTT
151 TTCAAATTCTTTACCTTCTCGAAACCTTTCATTTCTTGCATTTAAGTCAAACCTCATCCTCTACTTCTTCCACAC
226
ACAAAAAAACCTTCTAGGTTTTCCACGGGATTCCTTCTCAGCTCCTGAGAAATTCACCACGAGATTCCCAGATTT
301
GATTCCCGTCCAAATCTATAGAATCAAAATCTATAGATAGAAAGTAGGGAAATTTTGTAAAAAGATTTGAGGGTT
376 TGCTTA
382
[ATG]GGAAATTGTTGTGCATCGCCGAGCACTCCTTCCCGGAACAACCGGAAGAACAAGAAGAAACCTAATCCCTT
T
1 M G N C C A S P S T P S R N N R K N K K K P N P F
457
GCGGGCGATTATGGTGTTGGTAATGAAAATGGAAGTGGGAACAAGCTCTGGGTCTTGAAAGACCCGACGGGTC
GA
26 A G D Y G V G N E N G S G N K L W V L K D P T G R
532
GACATTTTGGAGCGGTACGATCTGGGTCGGGAGCTCGGGAGAGGCGAATTTGGGATCACGTATCTGTGCATAG
AT
51 D I L E R Y D L G R E L G R G E F G I T Y L C I D
607
GTTTCCACGGGCGAAAAGTTTGCTTGCAAATCGATATCGAAGAAGAAGCTGAGGACTTCGGTGGATGTCGATGA
T
76 V S T G E K F A C K S I S K K K L R T S V D V D D
682
GTAAGGCGAGAAGTTGAGATTATGAAACACTTGCGAAATCACCAGAACATTGTGTCATTGAAGGACACATTCGAG
101 V R R E V E I M K H L R N H Q N I V S L K D T F E
757
GACGAAAGTGCGGTTCACATTGTTATGGAATTGTGTGAGGGAGGCGAGTTGTTCGATCGCATTGTCGCGAGAGG
G
126 D E S A V H I V M E L C E G G E L F D R I V A R G
832
CATTACACGGAAAGAGCGGCTGCAGCTGTTATGAAGACAATTGTTGAAGTTGTTCAGAATTGTCATGAACATGGA
151 H Y T E R A A A A V M K T I V E V V Q N C H E H G
907
GTGATGCATCGTGATCTGAAACCTGAGAACTTTCTGTTTGCAAATAAGAAGGAAAATTCCCCATTAAAGGCAATT
176 V M H R D L K P E N F L F A N K K E N S P L K A I
982
GATTTTGGGTTGTCGGTGTTCTTTAAACCCGGTGAACAATTTAATGAGATAGTGGGAAGTCCTTACTACATGGCT
201 D F G L S V F F K P G E Q F N E I V G S P Y Y M A
1057
CCAGAGGTTCTGAAACGGAACTATGGTCCTGAGGTGGACGTTTGGAGTGCTGGAGTTGTCCTGTATATTTTGCTT
226 P E V L K P N Y G P E V D V W S A G V V L Y I L L
1132
TGTGGTGTTCCTCCTTTTTGGGCAGAAACTGAACAAGGGGTAGCACAGGCAATTATTCGCTCAGTCGTCGATTTT
251 C G V P P F W A E T E Q G V A Q A I I R S V V D F
1207
AAGAGGGACCCATGGCCTAGAGTCTCTGATAATGCGAAAGATCTTGTTAAGAAAATGCTTGATCCAGATCCAAAA
276 K R D P W P R V S D N A K D L V K K M L D P D P K
1282
CGGCGGCTTACAGCTCAGAAAGTACTTGAGCATCCATGGTTACAAAATGCCAAAAAAGCTCCAAATGTCTCACTG
301 R R L T A Q K V L E H P W L Q N A K K A P N V S L
1357
GGCGAGACAGTGAGAGCAAGGCTCAAACAATTTTCTGTCATGAACAAGCTCAAAAAACGAGCTTTAGGGGTCAT
A
326 G E T V R A R L K Q F S V M N K L K K R A L G V I
1432
GCTGAACATTTGTCAGTTGAGGAAGTGGCTGGCATAAAGGAGGCATTTGATACGATAGACATCGCTAAGAAAGG
C

Figure 3. The full length cDNA sequence and deduced amino acids of mulberry gene *MCDPK*; [ATG] indicates the start codon; [TAA] indicates the stop codon.
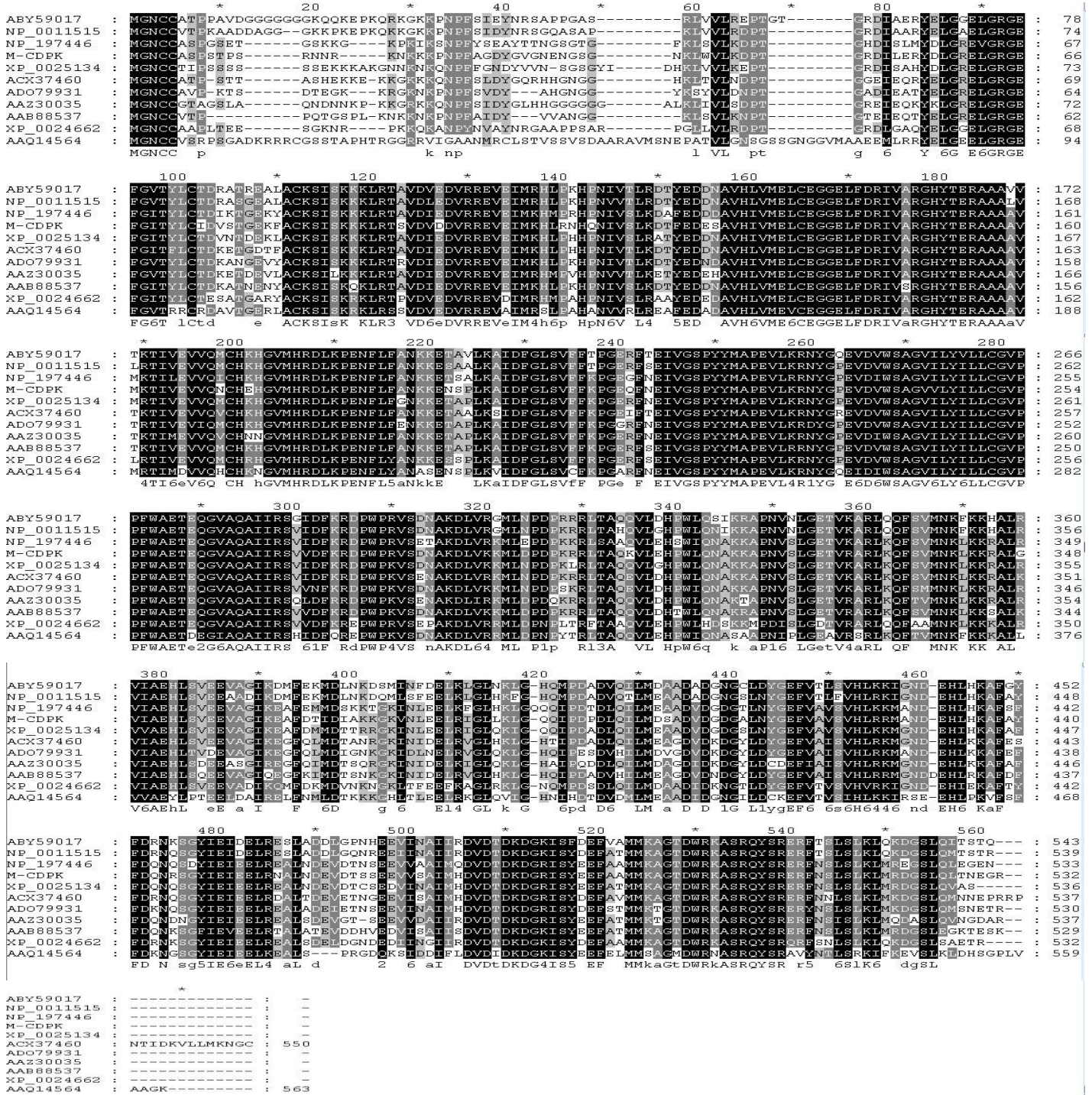
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                *          20           *          40           *          60           *          80           *
ABY59017    : MGNCCATEPAVDGGGGGGGKQQKEPKQRKGKPNPFSIEYNRSAPPGAS---------RIVVLREPTGT-------ERDIAERYELGGELGRGE : 78
NP_0011515  : MGNCCVTEKAADDAGG--GKKPKEPKQRKGKPNPFSIDYNRSGQASAP---------KIVVLRDPT---------ERDIAARYELGAELGRGE : 74
NP_197446   : MGNCCASPGSET------GSKKG----KPKIKSNPFYSEAYTTNGSGTG--------FKLSVLKDPT---------EHDISLMYDLGFEVGRGE : 67
M-CDPK      : MGNCCASPSTPS------RNNR-----KNRKKPNPFAGDYGVGNENGSG--------NKIWVLKDPT---------ERDILERYDLGRELGRGE : 66
XP_0025134  : MGNCCTIPSSSS------SSEKKKAKGNNKNKQNPFFGNDYVVN-SGSGYI------DHKIVVLKEPT---------ERDISAHYDLGGELGRGE : 73
ACX37460    : MGNCCATP-STT------ASHEKKE-KKGKKQNPFSLDYGQRHHGNGG--------HKLTVLNDPT---------GGEIEQRYELGGELGRGE : 69
ADO79931    : MGNCCAVE-KTS------DTEGK---KRGKNKPNPFSVDY---AHGNG--------YKSYVLDNPT---------CADIEATYELGGELGRGE : 64
AAZ30035    : MGNCCGTAGSLA------QNDNNKP-KKGRKQNPFSIDYGLHHGGGGGG-------ALKIIVLSDPT---------EREIEQKYKLGRELGRGE : 72
AAB88537    : MGNCCVTE--------PQTGSPL-KNKRNKPNPFAIDY---VVANG--------KISVLKNPT---------TEIEQTYELGGRELGRGE : 62
XP_0024662  : MGNCCAAELTEE------SGKNR----PKKQRANPYNVAYNRGAAPPSAR-------PGLLVLRDPT---------ERDIGAQYELGGELGRGE : 68
AAQ14564    : MGNCCVSRPSGADKRRRCGSSTAPHTRGGERVIGAANMRCLSTVSSVSDAARAVMSNEPATVLGNSGSSGNGGVMAAEEMLRRYEIGEELGRGE : 94
              MGNCC     p                 k np                         l VL pt                g 6  Y  6G E6GRGE
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Figure 5. Multiple sequence alignment of amino acids encoded by mulberry gene *MCDPK*.

Stress-induced expression patterns of *MCDPK*

To further investigate whether the expression of *MCDPK* was induced by abiotic stresses, the mRNA transcript an isoelectric point of 6.84. Homology analysis revealed that *MCDPK* gene is highly conserved in mulberry and other species.

Earlier studies carried out by semi-quantitative RT-PCR analysis showed that *MCDPK* transcripts changed significantly in different tissues (Syam and Jayabaskaran, 2007) and these were most abundant in the bud and leaf with lowest expression level in the mature fruit
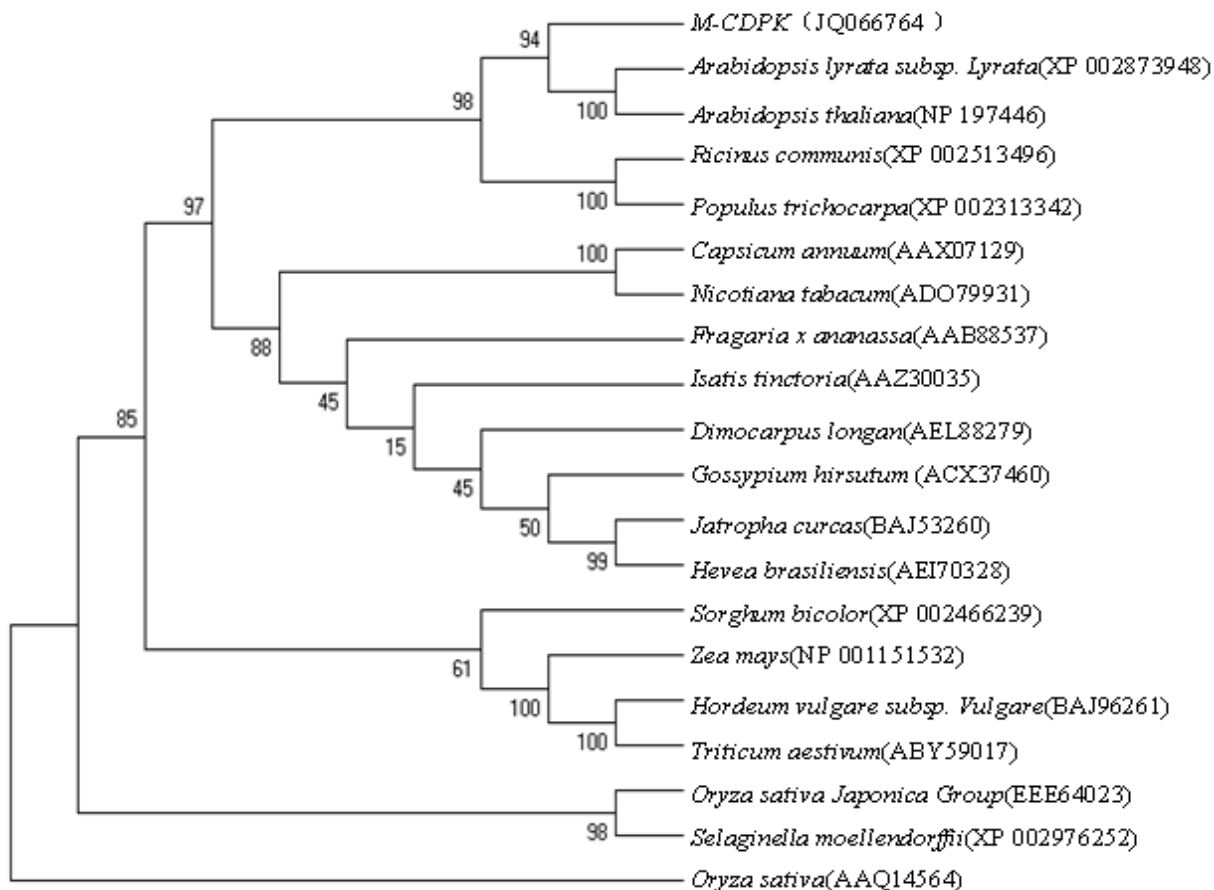
Figure 6. The phylogenetic tree based on amino acid sequence of *MCDPK* and other homologous sequences.
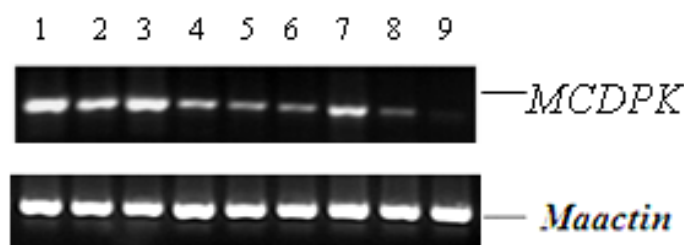

Figure 7. Expression profiles of the *MCDPK* gene in mulberry. 1: top bud; 2: young leaf; 3: mature leaf; 4: phloem; 5: xylem; 6: root; 7: full bloom; 8: wilting flower; 9: mature fruit. Maactin: mulberry actin gene (GenBank accession No. DQ785808), a house-keeping gene, was used as an internal control to allow for normalization by visual inspection of mRNA levels.

(Martin et al., 2000 and Harmon et al., 1987). These results are in agreement with the previous reports that the more mature the tissue, the lower the expression (Ivashuta et al., 2005).

The results of semi-quantitative RT-PCR analysis taken up in the present investigation also showed that the transcriptional level of *MCDPK* mRNA significantly decreased under the conditions of salt and low temperature stresses respectively as compared to the normal growth environment which was consistent with previous studies (Dammann et al., 2003, Wang et al., 2005).

In summary, based on the results presented in this paper, it is reasonable to expect that *MCDPK* most likely acts as an important common component responsive to abiotic stresses in mulberry. Further studies are underway for a more detailed elucidation of the biological function of *MCDPK*, especially, in the context of the Ca^{2+} signal transduction between biotic and abiotic stress signaling.
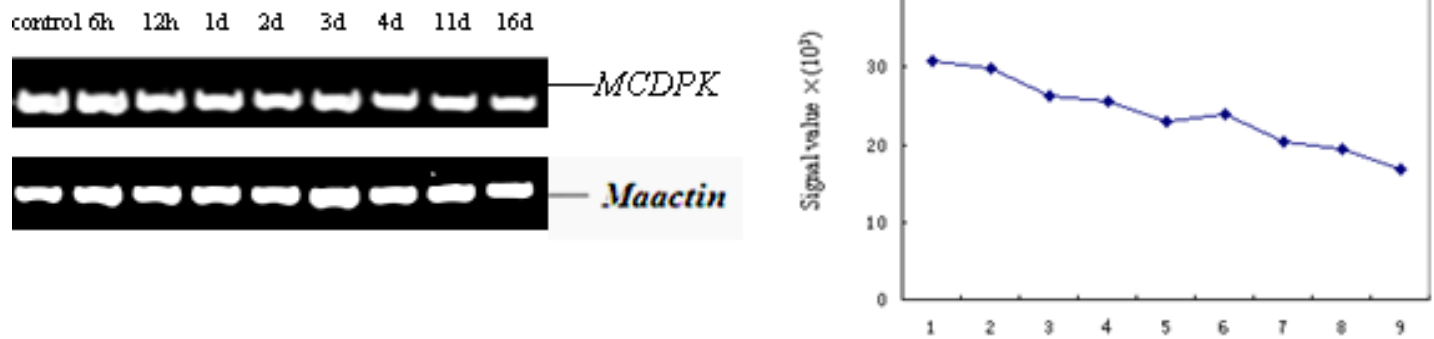
ACKNOWLEDGMENTS

Figure 9. *MCDPK* expression profile after treatment with NaCl. Maactin: mulberry actin gene (GenBank accession No. DQ785808), a house-keeping gene, was used as an internal control to allow for normalization by visual inspection of mRNA levels.

Program (nyhyzx07-020).

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