Genome-wide investigation of malate dehydrogenase gene family in poplar (Populus trichocarpa) and their expression analysis under salt stress

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Malate dehydrogenase (MDH) is widely distributed in plants and animals, and plays an important role in many metabolic processes. However, there have been few studies on MDH genes in poplar. In this study, 16 MDH gene sequences were identified from the Populus trichocarpa genome and renamed according to their chromosomal locations. Based on phylogenetic analysis, the PtMDH genes were divided into five groups, and genes that grouped together all shared the same subcellular location and had similar sequence lengths, gene structures, and conserved motifs. Two pairs of tandem duplication events and three segmental duplication events involving five genes were identified from the 15 PtMDH genes located on the chromosomes. Each pair of genes had a Ka/Ks ratios <1, indicating that the MDH gene family of P. trichocarpa was purified during evolution. Based on the transcriptome data of P. trichocarpa under salt stress and qRT-PCR verification, the expression patterns of PtMDH genes under salt stress were analyzed. The results showed that most of the genes were upregulated under salt stress, indicating that they play a role in the response of poplar to salt stress. The PtmMDH1 gene can be used as an important salt-tolerant candidate gene for further investigations of molecular mechanisms. This study lays the foundation for functional analysis of MDH genes and genetic improvement in poplar.
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**Abstract**

Malate dehydrogenase (MDH) is widely distributed in plants and animals, and plays an important role in many metabolic processes. However, there have been few studies on *MDH* genes in poplar. In this study, 16 *MDH* gene sequences were identified from the *Populus trichocarpa* genome and renamed according to their chromosomal locations. Based on phylogenetic analysis, the *PtMDH* genes were divided into five groups, and genes that grouped together all shared the same subcellular location and had similar sequence lengths, gene structures, and conserved motifs. Two pairs of tandem duplication events and three segmental
duplication events involving five genes were identified from the 15 PtMDH genes located on the chromosomes. Each pair of genes had a Ka/Ks ratios <1, indicating that the MDH gene family of P. trichocarpa was purified during evolution. Based on the transcriptome data of P. trichocarpa under salt stress and qRT-PCR verification, the expression patterns of PtMDH genes under salt stress were analyzed. The results showed that most of the genes were upregulated under salt stress, indicating that they play a role in the response of poplar to salt stress. The PtmMDH1 gene can be used as an important salt-tolerant candidate gene for further investigations of molecular mechanisms. This study lays the foundation for functional analysis of MDH genes and genetic improvement in poplar.

Introduction

Malate dehydrogenase (MDH, EC1.1.1.37), which is widely distributed in animals and plants, catalyzes the interconversion of malate and oxaloacetate (OAA) and plays important roles in several metabolic pathways including the tricarboxylic acid cycle, glyoxylate bypass, photosynthesis, and the C4-dicarboxylic acid cycle (Selinski et al., 2014; Gietl, 1992). MDH is a multimeric enzyme that usually occurs as dimers or tetramers consisting of the same or similar subunits; an exception is the MDH from Nitzschia alba, which is an octamer (Yueh, Chung & Lai, 1989). Each subunit of MDH contains functional and structural domains: the nicotinamide adenine dinucleotide (NAD)-binding domain occupies the N-terminus, and the C-terminal domain is the substrate-binding site; the necessary amino acid residues for catalysis, as well as the active site of the enzyme, exist in the spaces between these two domains (Musrati et al., 1998). Higher plants contain multiple forms of MDH, which differ in coenzyme specificity, subcellular localization, and physiological functions (Gietl, 1992). According to the coenzyme specificity and subcellular location, MDH is divided into NAD-dependent MDH (NAD-MDH) and NADP-dependent MDH (NADP-MDH). NAD-MDH, with the coenzyme NAD+, is distributed in mitochondria, cytoplasm, microbodies (peroxisomes and glyoxysomes), and chloroplasts, and the molecular weight of each subunit ranges from 32–37 kDa. NADP-MDH, with the coenzyme NADP+, is distributed in chloroplasts, and the molecular weight of each subunit ranges from 42–43 kDa (Ding & Ma, 2004; Tomaz et al., 2010).

Many studies have shown that MDHs in plants play crucial roles in seed germination, plant growth, pollen and fruit development, and the response to abiotic stress (Beeler et al., 2014; Rudrappa, 2008). The expression of the cytosolic NAD-dependent MDH gene in apple is positively correlated with growth viability and metabolic activity, and plays an important role in plant growth and response to low temperatures and salt stress (Yao et al., 2011). Overexpression of the NADP-dependent MDH gene in chloroplasts significantly affects the redox state of ferredoxin in transgenic potato plants (Backhausen et al., 1998). The activity of the mitochondrial NAD-dependent MDH gene in strawberry increases significantly with the maturity of the fruit, indicating that the gene is closely related to the growth and development of the fruit.
The NAD-dependent \(MDH\) gene isolated from cucumber was shown to play roles in the peroxisome and glyoxysome, and its expression increased significantly during seed germination (Kim & Smith, 1994). At present, studies on MDH are becoming more and more extensive, but most focus on crops and horticultural plants, and studies that focus on poplar are relatively rare.

The poplar has a long history of cultivation and is widely used as an important industrial material as well as in ecological protection as an economical forest tree species (Tun et al., 2018). With the increase of soil salinization, salt stress has become an important factor restricting the growth of forest trees (Zhou et al., 2018); therefore, research on the functions of genes related to salt tolerance has received increasing attention. In this study, we applied bioinformatics methods to identify \(MDH\) gene family members from the genome of \(Populus trichocarpa\), and analyzed the phylogeny, gene structure, chromosomal location, and duplication of each member. Using quantitative real-time reverse-transcription PCR (qRT-PCR) and transcriptome data, the expression patterns of \(MDH\) gene family members under salt stress were analyzed. The results lay the foundation for further analysis of the function of \(MDH\) genes in poplar.

**Materials & Methods**

**Identification of MDH family members in the \(P. trichocarpa\) genome**

The whole genome and protein sequences of \(P. trichocarpa\) were downloaded from the Ensembl Plants database (http://plants.ensembl.org/index.html). First, the identified MDH protein sequences from \(Arabidopsis thaliana\) (downloaded from the Arabidopsis Information Resource (TAIR); https://www.arabidopsis.org/) were used as queries in BLASTP searches against the \(P. trichocarpa\) genome databases with an e-value cutoff of 1.0 (Imran, Tang & Liu, 2016). The redundant sequences were manually removed, and all candidate genes were analyzed using InterProScan (http://www.ebi.ac.uk/interpro/search/sequence-search) and SMART (http://smart.embl-heidelberg.de/) to confirm the presence of the MDH domain (PF00056, PF02866, and PS00068) (Quevillon et al., 2005; Letunic & Bork, 2018). Finally, the sequence lengths, isoelectric points, and molecular weights of the MDH protein sequences from \(P. trichocarpa\) were calculated using ExPASy (http://cn.expasy.org/tools), and the online software WoLF PSORT (http://www.genscript.com/wolf-psort.html) and EuLoc (http://euloc.mbc.nctu.edu.tw/) were used to predict the subcellular localization of MDH proteins in \(P. trichocarpa\).

**Phylogenetic analysis and classification of the \(MDH\) gene family in \(P. trichocarpa\)**

The MDH protein sequences from \(P. trichocarpa\) and \(A. thaliana\) were used for phylogenetic analysis. First, a multiple sequence alignment of MDH protein sequences was generated using
ClustalW in MEGA 7 with the default parameters. A neighbor-joining phylogenetic tree was constructed based on the alignment results with the Poisson model, pairwise deletion, and 1,000 bootstrap replications (Xie et al., 2018). Finally, the MDH proteins were divided into groups according to the topology of the phylogenetic tree.

**Structure and motif analysis of the MDH gene family in P. trichocarpa**

Structural information on the MDH gene family members was extracted from the genome annotation file of *P. trichocarpa*. The online Gene Structure Display Server (GSDS; http://gsds.cbi.pku.edu.cn) was used to map the structures of the *PtMDH* genes (Guo, Zhu & Chen, 2007). MEME software was used to analyze the motifs of PtMDH protein sequences, and the parameters were set as follows: amino acid length, 6–50; number of repeats of the motif, arbitrary; threshold number of motif discovery, 10 (Bailey et al., 2006).

**Chromosomal localization and duplication analysis of MDH genes in P. trichocarpa**

Based on the genome annotation file of *P. trichocarpa*, positional information on *PtMDH* genes was obtained, and the chromosomal localizations of *PtMDH* genes were mapped using MapChart software. *PtMDH* gene duplication events were analyzed using the Multiple Collinearity Scan toolkit (MCScanX). The definition criteria for segmental gene duplication were as follows: 1) the length of the shorter aligned sequence covered >70% of the longer sequence, and 2) the similarity of the two aligned sequences was >70% (Wang et al., 2012; Gu et al., 2002). In addition, homologous genes located on the same chromosome with no more than one gene between them were identified as tandem duplication genes (Wu et al., 2014). Circos was used to map the results of MDH gene duplication events as images (Krzywinski et al., 2009). KaKs_Calculator 1.2 was used to calculate non-synonymous (Ka) and synonymous (Ks) substitutions of each duplicated MDH gene (Zhang et al., 2006).

**Transcriptomic analysis of PtMDH genes under salt stress**

The expression levels of *MDH* gene family members were extracted from the transcriptome data of *Populus × euramericana* cv. ‘74/76’ treated with different salt concentrations (0, 3‰, and 6‰) obtained by high-throughput sequencing in a previous study (Chen et al., 2018). After the data were log$_2$(1+RPKM) transformed, a gene expression heat map was generating using MEV 4.0 (Saeed et al., 2003). According to differences in gene expression under salt stress, the 16 *PtMDH* genes were classified and their expression patterns were explored.

**Plant material and salt stress treatment**

Tissue culture seedlings of *Populus × euramericana* cv. ‘74/76’ were used as experimental material. Stem segments with two leaves were inoculated into 1/2 MS medium containing 0 mmol/L or 100 mmol/L NaCl (1.5% sucrose + 0.65% agar + 0.3 mg/L IBA). The tissues were cultured at 25°C under a light intensity of 2,000 lux with a 14-h light/10-h dark photoperiod.
Root, stem, and leaf tissues were collected at 15, 20, 25, and 30 days, and immediately frozen in liquid nitrogen and stored at −80°C for subsequent analysis.

RNA extraction and qRT-PCR analysis

The EASYspin plant RNA rapid extraction kit was used to extract RNA from tissue samples collected at different time points. The concentration and quality of RNA were determined using a NanoDrop One Microvolume UV-Vis spectrophotometer and an Agilent 2100 Bioanalyzer. cDNA was synthesized via reverse transcription using the First Strand cDNA Synthesis Kit with the ReverTra Ace qPCR RT Master Mix. Primer Premier 5.0 was used to design primers for the PtMDH genes (Table 1). qRT-PCR was performed on an Agilent Mx3005P using SYBR Green chemistry. The PtRG5 gene encoding a poplar ribosomal L29e family protein was used as an internal control. The reaction was carried out as follows: 95°C for 30 s, followed by 50 cycles of 95°C for 10 s, 56°C for 30 s, and 72°C for 34 s. Each reaction was performed with three biological replicates and the expression levels of the genes were calculated using the $2^{-\Delta\Delta C_t}$ method (Livak & Schmittgen, 2001).

Results

Identification and analysis of MDH genes in P. trichocarpa

After removal of redundant sequences and database validation, a total of 16 MDH genes were identified in the genome of P. trichocarpa. The 16 PtMDH genes were renamed according to their chromosomal positions and the results of subcellular localization (Table 2). Except for PtMDH5, the remaining 15 PtMDH genes were mapped to chromosomes. The lengths of the PtMDH genes varied; PtMDH4 was the shortest at 1,491 bp, whereas PtchMDH2 was the longest at 4,762 bp. In contrast, the lengths of the coding sequences and protein sequences were less varied, with averages of 1,099 bp and 365 amino acids, respectively. The molecular weights of the PtMDH proteins ranged from 27.68 KDa to 49.76 KDa, with an average of 38.87 KDa. The isoelectric points ranged from 5.87 to 8.93, with an average of 7.96. Of the 16 PtMDH genes, five were localized to the mitochondrion, three to the peroxisome, four to the cytoplasm, and four to the chloroplast.

Phylogenetic analysis and classification of MDH genes in P. trichocarpa

To identify similar regions and conserved sites among PtMDH genes and to clarify their evolutionary relationships with different species, we performed multiple sequence alignment with the 16 PtMDH protein sequences and nine AtMDH protein sequences, and built a phylogenetic tree based on the alignment results. Based on the analysis, the 25 MDH sequences were divided into five groups, and the PtMDH genes in each group shared the same subcellular location, indicating that the subcellular localization of MDH proteins is closely related to
information contained in the sequence. It is worth noting that although Group I and Group II share the same subcellular location, they did not cluster together. According to the National Center for Biotechnology Information database, AtchMDH1 (in Group I) is a NAD-dependent MDH, whereas AtchMDH2 (in Group II) is a NADP-dependent MDH, indicating that among the four PtchMDH genes, PtchMDH1, PtchMDH3, and PtchMDH4, whose coenzyme is NAD+, are closely related to MDH genes that localize to the mitochondrion and peroxisome, whereas PtchMDH2, whose coenzyme is NADP+, is closely related to MDH genes that localize to the cytoplasm (Fig. 1).

**Gene structure and motif analysis of MDH members in *P. trichocarpa***

We analyzed the structures of the PtMDH genes to further understand their structural evolution. The number of introns in the PtMDH2 genes ranged from 0 to 12, with the greatest number of introns in the NADP-dependent PtchMDH2 gene, and no introns in the NAD-dependent PtchMDH1, PtchMDH3, and PtchMDH4 genes. Although all four of their proteins localized to the chloroplast, the number of introns in the genes varied greatly, and may be related to their different coenzymes and functions. PtMDH genes in the same group had similar numbers of exons and introns, whereas the difference between groups was significant (Fig. 2).

Ten conserved motifs were found in the PtMDH proteins by MEME analysis. The conserved motifs ranged from 21 to 100 amino acids in length. Each PtMDH gene contained 4–5 conserved motifs, and all 16 contained motif 1. Based on analysis using the Pfam and SMART databases, motif 1 corresponds to the C-terminal domain. The NAD-binding domains of the PtMDH protein sequences consist entirely of motifs 2 and 4 or motif 8. In the same group, the types and quantities of motifs were generally the same. Some conserved motifs were exclusive to a certain group, such as motif 8 (Group I) and motif 5 (Group III). The motifs are structural components of proteins with specific spatial conformations and functions, indicating differences in evolution and function between MDH members with different motifs.

**Chromosomal localization and duplication analysis of MDH genes in *P. trichocarpa***

Figure 3 shows that except for the PtmMDH5 gene, which was not located on a chromosome, the remaining 15 PtMDH genes are unevenly distributed on nine chromosomes of *P. trichocarpa*. Chromosome 17 contained the most PtMDH genes (three), whereas chromosomes 7, 9, 10, and 11 each contain only one.

As tandem duplication and segmental duplication affect the formation of the gene family to a certain extent and are the driving force of genome evolution, we analyzed the duplication events of PtMDH genes. Four PtMDH genes, PtcyMDH1/PtcyMDH2 and PtchMDH3/ PtchMDH4, were identified as tandem duplication genes on chromosomes 2 and 17, respectively. In addition, three segmental duplication events involving five MDH genes were also identified (Fig. 4). To better understand the evolutionary pressure on the PtMDH genes, we calculated the Ka/Ks ratios...
of the duplicated gene pairs. All duplicated gene pairs had Ka/Ks ratios <1, indicating that the
MDH gene family of P. trichocarpa has experienced purifying selection.

Expression pattern analysis of the MDH gene family of P. trichocarpa under salt stress

Based on transcriptome data from Populus × euramericana cv. '74/76' treated with different salt concentrations, we analyzed the expression changes of P. trichocarpa MDH genes. Fourteen genes showed significant changes in their expression levels, indicating that these genes are involved in the response of poplar to salt stress. The expression patterns of the PtMDH gene family members were roughly divided into five categories: class I showed decreased gene expression in response to salt stress; class II showed increased expression levels under low salt stress, and decreased expression under high salt stress; class III showed decreased expression under low salt stress, and increased expression under high salt stress; class IV showed no changes in expression under low salt stress, but increased expression under high salt stress; and class V genes were not expressed under salt stress, and may be pseudogenes that formed during the evolutionary process of the MDH gene family but lost function due to sequence changes, or they may exhibit spatiotemporal expression specificity (Fig. 5).

To examine temporal and tissue-specific expression, we analyzed the expression levels of six PtMDH genes in the roots, stems and, leaves of class IV genes under salt stress by qRT-PCR. Most of the genes were significantly upregulated in various tissues on the 25th and 30th day after salt stress. At the four time points, in various tissues, the expression levels of PtmMDH1 were significantly higher than those in the control, indicating that salt treatment had a significant induction effect on the PtmMDH1 gene. Therefore, the PtmMDH1 gene can be used as an important salt tolerant candidate gene for functional verification in subsequent experiments (Fig. 6).

Discussion

MDH is a highly active enzyme in plants and plays an indispensable role in many metabolic processes (Yao et al., 2011). At present, most studies on MDH genes are focused in crops and horticultural plants, such as cotton (Imran, Tang & Liu, 2016; Imran & Liu, 2016), corn (Metzler et al., 1989), wheat (Ding & Ma, 2004), apple (Yao et al., 2011), grape (Sweetman et al., 2009), etc. By contrast, there is very little research on the function of MDH genes in poplar, a model plant for forest genomics. We identified 16 MDH gene sequences in the genome of P. trichocarpa using bioinformatics methods, which is more than the number identified in Gossypium arboreum (Imran & Liu, 2016), but less than in Gossypium hirsutum (Imran, Tang & Liu, 2016). Based on phylogenetic analysis with A. thaliana, the 16 PtMDH genes were divided into five groups, with the genes in each group sharing the same subcellular location. The four PtcMDH genes, which localize to the chloroplast, were divided into two groups, likely because they have different coenzymes, resulting in differences in function.
A total of 10 motifs were identified in the MDH gene family of *P. trichocarpa*. Although the sequences in different groups have different types and numbers of motifs, they all have motifs corresponding to the NAD-binding domain and C-terminal domain of MDH proteins. For example, in groups I, IV, and V, motifs 2 and 4 correspond to the NAD-binding domain, whereas motifs 1 and 3 correspond to the C-terminal domain; in group III, motif 6 corresponds to the NAD-binding domain, whereas motifs 1 and 5 correspond to the C-terminal domain. Motif 1 is present in every MDH sequence because it contains an active site that is an indispensable component of MDH sequences and plays a catalytic role when the substrate binds to MDH. A previous study showed that the critical residue of the active site distinguishes between malate dehydrogenase and lactate dehydrogenase, two enzymes that share high sequence similarity (Hannenhalli & Russell, 2000). The five groups of *PtMDH* genes have different intron-exon structures and different numbers of introns. Unlike the other groups, the genes in group I have no introns, consistent with the results of a study on the cotton MDH gene family by Imran et al. (Imran, Tang & Liu, 2016). Ren et al. reported that highly expressed genes have more and longer introns than genes expressed at low levels, suggesting that the *PtMDH* genes in group I are expressed at relatively low levels in response to biotic or abiotic stresses (Ren et al., 2006).

Gene duplication events, mainly including tandem duplication, segmental duplication, and transposition events, are important ways to expand gene families (Kong et al., 2007). Among the 15 *PtMDH* genes distributed unevenly on nine chromosomes, two pairs of tandem duplication events and three segmental duplication events involving five genes were identified. Based on analysis of transcriptome data, the expression patterns of different *PtMDH* genes under salt stress vary, indicating that the functions of some *PtMDH* genes may have changed during the evolution of the gene family. The *PtcyMDH2* gene may have lost its function and become a pseudogene after being formed from the *PtcyMDH1* gene, which often occurs in multigene families in eukaryotes (Maestre et al., 1995).

According to the expression levels of *PtMDH* genes in transcriptome data, the genes were divided into five categories, each of which exhibits a certain expression pattern. The expression levels of most *PtMDH* genes increased significantly in response to salt stress, but at different salt concentrations, indicating the existence of multiple molecular mechanisms through which *PtMDH* genes respond to salt stress. qRT-PCR was used to detect the transcription levels of several *PtMDH* genes in various tissues under salt stress. The expression of most *PtMDH* genes increased significantly compared to the control during the late stage of treatment, especially the *PtmMDH1* gene, the expression of which was higher than that of the control at multiple time points in multiple tissues, indicating that this gene plays an important role in the response of poplar to salt stress, and serves as an important salt tolerance candidate gene for further research on molecular mechanisms.

**Conclusions**
A total of 16 MDH gene sequences were identified in the *P. trichocarpa* genome. Based on the phylogenetic tree constructed with *A. thaliana* sequences, the 16 PtMDH genes were divided into five groups; genes in the same group shared the same subcellular location and had similar gene structures. Analysis of gene duplication events and evolutionary selection revealed the formation and evolutionary processes of the MDH gene family in *P. trichocarpa*. The expression patterns and changes in PtMDH genes under salt stress were further analyzed based on published transcriptome data and qRT-PCR. The *PtmMDH1* gene can serve as a candidate gene for studying salt tolerance mechanisms. This study lays the foundation for further analysis of the function of MDH genes in poplar.

**References**


Table 1 (on next page)

Primer sequences for quantitative real-time reverse-transcription PCR (qRT-PCR).
<table>
<thead>
<tr>
<th>Gene name</th>
<th>Forward primer</th>
<th>Reverse primer</th>
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</thead>
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<tr>
<td>PtpMDH2</td>
<td>CTTCTGCTGTGGTTCGTGGTCTT</td>
<td>TCATCCCTCGTCATTCCTGTTTTTC</td>
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<td>GACAAGACTGGACCATAACAGGGCCT</td>
<td>TTGACATCAGGGTACTGGGAAGACGA</td>
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<td>PtmMDH1</td>
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<td>PtRG5</td>
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Table 1. Primer sequences for quantitative real-time reverse-transcription PCR (qRT-PCR).
Table 2 (on next page)

Characteristics of the malate dehydrogenase (MDH) gene family in *Populus trichocarpa*.

Chr: chromosome number; Un: unknown; CDS: coding sequence length (nucleotides); aa: amino acid length; MW: molecular weight; pI: isoelectric point.
Table 2. Characteristics of the malate dehydrogenase (MDH) gene family in *Populus trichocarpa*.

<table>
<thead>
<tr>
<th>Name</th>
<th>Gene ID</th>
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<td>1065</td>
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1. Table 2. Characteristics of the malate dehydrogenase (MDH) gene family in *Populus trichocarpa*.

2. Chr: chromosome number; Un: unknown; CDS: coding sequence length (nucleotides); aa: amino acid length;

3. MW: molecular weight; pI: isoelectric point.
Figure 1

Phylogenetic analysis of MDH members in *P. trichocarpa* and *A. thaliana*.

Phylogenetic tree was constructed by MEGA7 with Neighbour-Joining method and bootstrap of 1000 replications. The different colored arcs indicate different groups of MDH proteins.
Figure 2

Gene structure and conserved motif analysis of MDH genes in *P. trichocarpa*.

(A) Phylogenetic tree of 16 PtMDH proteins. (B) Distributions of conserved motifs in PtMDH genes. (C) Exon/intron organization of PtMDH genes.
Figure 3

Chromosomal mapping of *MDH* genes in *P. trichocarpa*.

The chromosome numbers and size (bp) are indicated at the top and bottom of each chromosome, respectively. Tandemly duplicated gene pairs are highlighted in yellow.
Figure 4

Synteny analysis of the MDH gene family in *P. trichocarpa*

Chromosomes were drawn in different colours. Red curves linking *PtMDH* genes represent the segmental duplication events.
Figure 5

Expression analysis of *PtMDH* genes in leaf tissues under salt stress.

FPKM values of *PtMDH* genes were transformed by log2. The colour scale represents relative expression levels.
Figure 6

Expression analysis of six *PtMDH* genes in roots under salt stress by qRT-PCR.

Data were normalized to *PtRG5* gene and vertical bars indicate standard error.
Figure 7

Expression analysis of six PtMDH genes in stems under salt stress by qRT-PCR.

Data were normalized to PtRG5 gene and vertical bars indicate standard error.
Figure 8

Expression analysis of six *PtMDH* genes in leaves under salt stress by qRT-PCR.

Data were normalized to *PtRG5* gene and vertical bars indicate standard error.