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Research article

Molecular cloning and expression analysis of mulberry MAPK gene family

Congjin Wei [a,b], Xueqin Liu [a,b], Dingpei Long [a,b], Qing Guo [a,b], Yuan Fang [a,b], Chenkai Bian [a,b], Dayan Zhang [a,b], Qiwei Zeng [a,b], Zhonghuai Xiang [a,b], Aichun Zhao [a,b,*]

[a] State Key Laboratory of Silkworm Genome Biology, Southwest University, Chongqing 400716, China
[b] Key Laboratory for Sericulture Functional Genomics and Biotechnology of Agricultural Ministry, Southwest University, Chongqing 400716, China

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ABSTRACT

Mitogen-activated protein kinase (MAPK) cascades play an important role in regulating various biotic and abiotic stresses in plants. Although MAPKs have been identified and characterized in a few model plants, there is little information available for mulberry *Morus* sp. L., one of the most ecologically and economically important perennial trees. This study identified 47 mulberry *Morus notabilis* MAPK (MnMAPK) family genes: 32 MnMAPKKK, five MnMAPKK and ten MnMAPK genes, and cloned ten MnMAPK cDNA genes based on a genome-wide analysis of the morus genome database. Comparative analysis with MAPK gene families from other plants suggested that MnMAPKs could be divided into five subfamilies (groups A, B, C, D and E) and they could have similar functions in response to abiotic and biotic stresses. MnMAPK gene expression analysis of different stresses (high/low temperature, salt and drought) and signal molecules (ABA, SA, H_2O_2 and methyl jasmonate (MeJA)) revealed that all ten MnMAPK genes responded to high/low temperature, salt and drought stresses, and that nine of the ten MnMAPKs (*MnMAPK7* excepted) could be induced by ABA, SA, H_2O_2 and MeJA, which suggested that MnMAPKs may play pivotal roles in signal transduction pathways. Our results indicated that almost all of the MnMAPKs may be involved in environmental stress and defense responses, which provides the basis for further characterization of the physiological functions of MnMAPKs.

1. Introduction

Plants often encounter a variety of adverse conditions, including: drought, high and low temperatures and high salt stress, during their growth and development (Meng and Zhang, 2013; Mizoguchi et al., 1997). Mitogen-activated protein kinase (MAPK) cascades play an important role in regulating various biotic and abiotic stresses during the plant life cycle. Those cascades are highly conserved signaling components in all eukaryotic cell signal transduction. They can be linked to different cell-membrane receptors and cell responses and play an important role in endogenous and exogenous signaling (Zhang et al., 2006). Plant MAPKs are similar to animal and yeast MAPKs (Nishihama et al., 1995). Their common features are that they have similar molecular weights (38–55 kD) and have 11 conserved subdomains (Hanks et al., 1988). MAPK cascades consist of three kinds of kinases: MAPKKK, MAPKK and MAPK, which are progressively phosphorylated by upstream stimulatory signals. TXY, an ATP phosphorylation site, is an essential factor present in the catalytic domain between the seventh and eighth sub-domains (Ichimura et al., 2002). Plant cells respond to environmental stresses (such as wounding, drought, temperature, etc.) and growth signals (such as auxin, ethylene, abscisic acid, etc.) via the receptor protein kinase (Jonak et al., 1999; Mishra et al., 2006). MAPKKK, MAPKK and MAPK are activated consecutively by the receptor protein kinase or by an upstream activator. MAPKKK is the most upstream in the cascade system and phosphorylates itself directly via stimulation of receptors, signal molecules or extracellular stimuli. The phosphorylated MAPKKK is able to activate the phosphorylation of its downstream factor, MAPKK. The MAPKK, which is phosphorylated at the Thr and Tyr residues of the activation loop for MAPKs, can activate MAPK phosphorylation (Kültz, 1998). When organisms are

* Corresponding author. State Key Laboratory of Silkworm Genome Biology, Southwest University, BeiBei, Chongqing 400716, PR China. Tel.: +86 23 68251803; fax: +86 23 68251128.
E-mail addresses: zhaoaichun@hotmail.com, zhaoaichun@swu.edu.cn (A. Zhao).

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confronted by stresses, such as: UV radiation, osmotic stress, heat stress, wounding and other hormones, MAPK is activated via a number of upstream signal molecules, which leads to the phosphorylation of downstream molecules by MAPK. MAPK cascades eventually transfer the external signal to the nucleus, which regulates the expression of the specific genes that cause physiological responses to occur (Pitzschke et al., 2009).

To date, the responses of many MAPK genes to abiotic stress and molecular signaling pathways have been widely studied in several model plants (Jonak et al., 2002; Singh et al., 2012). Jammes et al. proved that two MAP kinases: *MPK9* and *MPK12*, were preferentially expressed in guard cells and positively regulated ROS-mediated ABA signaling (Jammes et al., 2009). Arabidopsis *MPK6* has been shown to be involved in a stress responses mechanism that modulated the biosynthesis of ethylene (Liu and Zhang, 2004). In rice, mitogen-activated protein kinase, *OsMAPK5*, was activated by ABA, various abiotic stresses and pathogen infection (Xiong and Yang, 2003). *OsBIMK1* was rapidly activated after exposure to jasmonic acid and *Pseudomonas syringae pv. syringae*, and after wounding. These results suggest that *OsBIMK1* plays an important role in rice disease resistance (Song and Goodman, 2002). More recently, transgenic Arabidopsis overexpressing *GhMPK16* has been shown to have significant resistance to fungi, bacterial pathogens, drought and H_2O_2 (Shi et al., 2011) and activity at the transcript of *CrMPK3* was induced by wounding, UV treatment and MeJA (Raina et al., 2012). To date, many studies have shown that the MAPK gene family is not only closely related to biotic and abiotic stresses, but is also involved in various important biological processes, such as growth and development.

Genome-wide analysis of several plant genomes has identified MAPK families in a number of plant species. For example, Arabidopsis encodes 20 MAPKs compared with 26 MAPKs in apple and 21 MAPKs in poplar (Ichimura et al., 2002; Nicole et al., 2006; Zhang et al., 2013a). However, as far as can be ascertained, there have been no studies into the mulberry MAPK gene family at the whole genome level. Mulberry (*Moraceae morus*) is a perennial woody plant and is ecologically and economically important (Ramachandra Reddy et al., 2004). Mulberry leaves are the main source of food for the silkworm, *Bombyx mori*, and its fruit is very popular and nutritious (Singhal et al., 2010). In addition, mulberry can adapt to many different environments, including cold, waterlogged, drought and saline environments (Checker and Khurana, 2013), but there has been little research into its stress physiology, biochemistry and molecular biology. There is also very little mulberry MAPK information in the public databases (such as the NCBI, EMBL, etc.). After complete analysis of the *Morus notabilis* genome had been undertaken (He et al., 2013), it became possible to analyze the defense genes, such as MAPKs, involved in abiotic and biotic responses in mulberry at the genome wide level.

This study provides a list of MAPK family members (32 MAPKKK, five MAPKK and ten MAPK genes), and cloned and sequenced all ten MAPK genes from *M. notabilis*. We classified these ten MAPKs into five groups according to their phylogenetic analysis. In addition, we investigated the conserved domains among the ten MAPKs by multi-alignment of protein sequences. Further expression profile analysis using the quantitative real time-polymerase chain reaction technique (qRT-PCR) showed that most of the MAPK genes from mulberry were induced by various stresses (high/low temperature, salt and drought) and signal molecules (ABA, SA, H_2O_2 and MeJA). Our data provide some insights into new potential functions of mulberry MAPKs and the research can be used as a basis for further characterization of the physiological functions of mulberry MAPKs.

2. Materials and methods

2.1. Plant materials and stress treatments

The *M. notabilis* leaves used for total RNA extraction were collected in Yingjing county of Ya'An city, Sichuan Province, China during May 2011. *Morus multicaulis cv. Husang No. 32* mulberry seedlings were planted in a PQX type plant incubator with artificial intelligence capability (Ningbo Southeast Instrument Corporation, China) with a 22 °C/26 °C (night/day) and 8 h/16 h (night/day) temperature and light cycle. After about two months, mulberry seedlings that were about 25 cm tall were subjected to the abiotic stresses of: high temperature (40 °C for 12 h), low temperature (4 °C for 12 h), salt (200 mM NaCl for 2 d) and dehydration (drought for 10 d). There were also signal substance treatments, consisting of: abscisic acid (ABA) (400 μM ABA for 24 h), salicylic acid (SA) (2 mM SA for 24 h), hydrogen peroxide (H_2O_2) (10 mM H_2O_2 for 24 h) and methyl jasmonate (MeJA) (2 mM MeJA for 24 h), as described in previous papers (Caitriona Dowd et al., 2004; Zong et al., 2009). The leaves of the treated seedlings were preserved at −80 °C for total RNA extraction.

2.2. Cloning and sequence identification of MAPKs from mulberry

All of the logged MAPK amino acid sequences for several sequenced species were downloaded from the NCBI (http://www.ncbi.nlm.nih.gov/). The protein sequences were modeled in order to find mulberry MAPKs genes based on the *M. notabilis* database (http://morus.swu.edu.cn/morusdb/). Ten pairs of primers were designed by Primer Premier 5.0 (Premier Biosoft International, CA, USA) (Supplementary Table 1) to amplify the full-length coding sequence (CDS) of ten mulberry MAPK genes from the total cDNA in the *M. notabilis* leaves. The PCR conditions included an initial denaturation for 4 min at 94 °C, followed by 30 cycles of: 94 °C denaturation for 30 s, 45 s annealing at 60 °C, 72 °C elongation for 2 min and a final 72 °C extension for 10 min. The PCR products were purified and cloned into the PMD®19-T Vector (TaKaRa, Dalian of China). The positive clones were sequenced by Invitrogen™ from Life Technologies (Shanghai, China). The exon-intron structures of the mulberry MAPK genes were confirmed by aligning their coding sequences to their corresponding genomic sequences. The exon-intron structure diagram was generated using the online Gene Structure Display Server (GSDS: http://gsds.cbi.pku.edu.cn) and the exon position and gene length method.

2.3. Conserved domain and phylogenetic analysis

We used the GeneDoc program, a full featured multiple sequence alignment editor, to investigate the amino acid sequences and kinase domains of MnMAPKs. The MAPK family protein sequence alignments and the phylogenetic tree were created using the MEGA5 program (Tamura et al., 2011). The phylogenetic trees for MAPKs from *M. notabilis*, *Arabidopsis thaliana*, *Oryza sativa*, *Populus trichocarpa*, *Nicotiana tabacum* and *Fragaria vesca* were constructed using the Neighbor-Joining (NJ) method and assessed by bootstrap analysis with 1000 resampling replicates.

2.4. Analysis of quantitative real-time PCR

Total RNA was extracted from *M. multicaulis cv. Husang No. 32* seedling leaves and preserved as outlined above. The first strand cDNA were synthesized using the Perfect Real Time version of the PrimerScript™ RT reagent Kit with gDNA Eraser (Perfect Real Time) (TaKaRa, Dalian, China). The primer pairs for the qRT-PCR analysis of ten mulberry MAPK genes were designed using Primer Premier