Cloning and evolutionary analysis of tobacco MAPK gene family

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Abstract The mitogen-activated protein (MAP) kinase
cascade is an important signaling module which is involved
in biotic and abiotic stress responses as well as plant
growth and development. In this study, we identified 17
tobacco MAPKs including 11 novel tobacco MAPK genes
that have not been identified before. Comparative analysis
with MAPK gene families from other plants, such as
Athaliana thaliana, rice and poplar, suggested that tobacco
MAPKs (such as NtMPK1, NtMPK3 and NtMPK8) might
play similar functions in response to abiotic and biotic
stresses. QRT-PCR analysis revealed that a total of 14
NtMPKs were regulated by SA and/or MeJA, suggesting
their potential roles involved in plant defense response. In
addition, 6 *NtMPKs* were induced by drought treatment,
implying their roles in response to drought stress. Our
results indicated that most of tobacco MAPK might be
involved in plant defense response, which provides the
basis for further analysis on physiological functions of
tobacco MAPKs.

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Introduction

The mitogen-activated protein kinase cascade is an
important signaling module that transduces exogenesis or
endogenesis signaling molecules to intracellular responses
in eukaryotic organisms. Typically, the conserved signal-
ing module consists of three kinds of protein kinases:
MAPK kinase kinase (MAP3 K), MAPK kinase (MKK)
and MAPK [1]. As the terminal component of this cascade,
MAPKs are activated through phosphorylation on their
threonine and tyrosine residues by their upstream MAP2 K
and MAP3 K. At the downstream of this cascade, some
transcription factors and other signaling components [2, 3],
which have important roles in plant immunity through
activation of downstream genes, serve as the physiological
substrates of MAP kinases.

For recent decades years, mitogen-activated protein
kinase cascade drew much attention not only because its
crucial roles in responding to various extracellular stimuli
[4–8] but also its roles in severing as a universal signaling
pathway which regulates various cellular activities, such as
cell division, differentiation and programmed cell death
[6, 9, 10]. In *Arabidopsis*, the flagellin *flg22* may activate a
MAPK cascade including MEKK1, MKK4/MKK5 and
MPK3/MPK6, which, in turn, increase the expression of
WRKY29 and confer resistance to *P. syringae* (Ps) as well
as *B. cinerea* [2]. Interestingly, isolation of another MAPK
from *Arabidopsis*, AtMPK4, showed its role to negatively
regulate systemic acquired resistance [11]. Alfalfa MAP
kinase 3 (MMK3) could be found during all stages of the
cell cycle, but its protein kinase activity was only detected

in mitosis and correlated with the timing of phragmoplast formation, suggesting its roles in the regulation of plant cytokinesis [10]. Besides, increasing evidence reveals that MAP kinases are involved in plant priming mechanism and induced-resistance (IR) phenomena, which suggests plants have the capacity of "remembering" and responding to environment stimuli in a more rapid and robust manner [12, 13]. A typical research on *Arabidopsis* revealed that prestress deposition of the signaling components MPK3 and MPK6 was a critical step in priming plants for full induction of defense responses during IR [14].

Based on the genome scan of several plant genomes, MAPK families have been identified in various plants. For example, *A. thaliana* genome possesses genes encoding 23 MAPKs [15, 16] compared with 17 MAPKs in rice [8] and 21 MAPK homologs in poplar [17]. Interestingly, as a dicot plant, grapevine possesses only 12 MAPK genes [18], which is very similar with human [19] and zebrafish [20] (both has 10 MAPKs respectively). However, there is no such study on analysis of tobacco MAPK gene family on the whole genome. According to our knowledge, only six tobacco MAPKs (SIPK [21], WIPK [22, 23], Ntf4 [24], NRK1 [25], Ntf3 [26] and MPK4 [27]) were analyzed. The salicylic acid-inducible protein kinase (SIPK) and the wound-inducible protein kinase (WIPK), which are the first two isolated MAPKs from tobacco, were proved to be activated in response to various environmental stresses [21, 28–31]. Then, another study showed that suppression of *NtSIPK* and *NtWIPK* attenuated the *N* gene-mediated resistance against tobacco mosaic virus (TMV), which suggests that these two MAPK were required for TMV defense [32]. Another two MAPKs, Ntf4 and NRK1, were reported to be involved in regulating pathogen-induced HR cell death in tobacco [24, 25, 29]. In addition to the function of plant resistance, other two MAPKs (Ntf4 and NRK1) were considered as positive regulators of plant development and cell cytokinesis [33–35]. Loss-of-function experiment of NtMPK4 revealed that NtMPK4 had played important roles in JA signaling and stomatal movement [27].

In this study, we firstly provided a list of MAPK members from tobacco. Based on cloning and sequencing, we got 17 MAPKs and 11 of 17 MAPK genes were novel. We classified these 17 MAPKs into six groups according to the phylogenetic analysis. Besides, we investigated the conserved domains and divergent sequences among the 17 MAPK by multi alignment of protein sequences. Further investigation revealed that some tobacco MAPK genes could be regulated by methyl jasmonate acid (MeJA), salicylic acid (SA) and drought using quantitative reverse transcription-polymerase chain reaction (qRT-PCR) analysis. Our data provide some new insights into potential functions of the tobacco MAPKs.

Materials and methods

Plant materials and preparation of RNA and first strand cDNA synthesis

Common tobacco (*Nicotiana tabacum* cv HHDJY) seeds sterilized by 10 % NaClO were germinated in sterilized planets with MS solid culture medium in a green house programmed for 22 °C and a 14-h light cycle. After 8 weeks, tobacco plants were treated by phytohormone as followed: for SA, and MeJA treatments, tobacco seedlings were cultured in solutions with 2 mM SA in water, and 100 μM JA in 0.01 % ethanol, respectively [36]. For drought treatment, tobacco seedlings were placed sterilized filter paper without any water.

TRIpure reagent (Roche, China) was used to extract total RNA from various treated tobacco seedlings according to the manufacturer's protocol. RT-PCR was completed to synthesize the first-strand cDNA with 2 μg of purified total RNA (pre-treated with DNase I) according to the manufacturer's protocol from Promega. The oligo $(dT)_{18}$ was used as a primer and the reverse transcription reaction was incubated at 42 °C for 65 min in a total volume of 26 μl. The reverse transcription products were diluted to 100 μl with sterile ddH$_2$O.

Cloning and identification of MAPKs from tobacco

Several sources, such as ESTs, TGI (Tobacco Genome Initiative, http://www.pngg.org/tgi/) and *N. benthamiana* genome (http://solgenomics.net/organism/Nicotiana_bentha miana/genome) are available to provide bioinformatics analysis of tobacco MAPKs. Using these released data, 13 primer pairs were designed using PrimerPremier 5.0 (Supplementary Table 1) to amplify the full-length open reading frame of tobacco MAPK genes. Each polymerase chain reaction (PCR) contained a total volume of 50 μl, containing 10 ng cDNA template, 0.2 μM of each primer and 25 μl GoTaq Green mix (Promega, USA). The PCR conditions included 4 min initial denaturation at 94 °C, followed by 33 cycles of the following: 30 s denaturation at 94 °C, 45 s annealing at appropriate temperature, 2 min of extension at 72 °C and a final 10 min extension at 72 °C. The amplified products were purified with agarose gel purification kit (Bioteck, China) and then cloned into pEASY-T1 vector (Transgen, China). All positive clones were sequenced by BGI Company. The raw reads were assembled using BioEdit software.

To identify novel tobacco MAPKs, we followed the criteria suggested by Nathan [8]: (i) their amino acid sequences are significantly homologous with other known MAPKs; (ii) there are 11 conserved subdomains in the

MAPKs kinase domain; (iii) the TDY or TEY motif is essential in the activation site.

Conserved domain detection

Protein secondary structure and domain provide much information for researchers to understand the function of these proteins. Therefore, the multiple sequence alignment of the amino acid sequences and kinase domains of MAPKs was performed using the MultAlin program [37].

Phylogenetic analysis

The amino acids sequences of MAPKs from four plants (Arabidopsis, tobacco, rice, poplar) were initially aligned using Clustal W (v.1.83) [38]. The phylogenetic tree for the plants MAPKs was reconstructed by Neighbor-Joining method in which distance was estimated by JTT amino acid matrix implemented in the program MEGA 4.0 [39]. The pairwise deletion option was set in the NJ tree reconstruction and the accuracy of the tree topology was assessed by bootstrap analysis with 1,000 resampling replicates.

Quantitative real-time PCR

A pair of gene-specific primers was designed by PrimerPremier 5.0 according to tobacco MAPK gene sequences (Supplementary Table 2). In order to avoid the effect of potential genomic DNA contamination, these primers were designed to span intron-splicing sites if possible. Tobacco *EF-1α* gene (Genbank: D63396.1) was used as a control. Tobacco *PR1a* and *ODC* were analyzed to verify the accuracy of SA and MeJA treatment, respectively. The real-time PCR analysis were performed using BIO-RAD CFX96 real-time PCR system (Bio-Rad, USA 96 well) formats with programs as following: an initial 95 °C denaturation step for 3 min, followed by 40 cycles of denaturation at 95 °C for 10 s and annealing/extension at 60 °C for 1 min. The disassociation curve was used to detect the effect of amplification. Each PCR reaction was mixed with 7.5 μl of SYBR Green (Takara, China), 4.5 μl of ddH2O, 1 μl of each reverse-transcribed and digested cDNA product, and 1 μl of each primer (10 μM).

Relative gene expression level was analyzed according to $2^{-\Delta\Delta C}T$ method. The ΔCT and $\Delta\Delta CT$ were calculated by the formulas $\Delta CT = CT$ target$-CT$ control and $\Delta\Delta CT = \Delta CT$ treated sample$-\Delta CT$ untreated sample (0 h treatment), respectively. In our study, genes with only threefold change compared with 0 h control were considered induced.

Results

Identification and nomenclature of the tobacco MAPK genes

As we have mentioned above, six tobacco MAPK genes were reported previously and most of them were involved in environmental stimuli as well as plant development and cell cytokinesis. However, other members of tobacco MAPK gene family have not been identified and characterized. After extensive in silico analysis of the released data from tobacco EST and TGI, we cloned 13 novel MAPK homologs. However, according to the criteria proposed by Nathan [8], two of them were not considered as MAPK gene family members since the first two conserved subdomains did not exist in their protein kinase domains (data not shown), despite of their significant homology with other tobacco MAPKs at the amino acid level. Thus, in total, we got 17 tobacco MAPKs and 11 of 17 were novel which were not identified before. To avoid confusing names, these 17 tobacco MAPKs (Table 1) were named according to the nomenclature followed by previous study in *Arabidopsis* [16].

Phylogenetic analysis of tobacco MAPK genes

In order to evaluate the evolutionary relationship of MAPKs between tobacco and other plants, and predict the potential orthologous and paralogous relations among MAPKs in the four model plants, a combined phylogenetic tree (Fig. 1) was constructed using software MEGA 4.0 with NJ method. The results showed that MAPK proteins could be divided into six major groups (Group A–F), and each group contains at least two tobacco MAPKs. According to the difference of TEY or TDY motif in their activation site, all the six groups can be divided into two subtypes. Groups A, B, C and F contains TEY motif, whereas TDY motif exists in group D and E. We investigated the numbers of MAPK belonging to the TEY type as well as TDY type from the four species. The results showed that the members belonging to TEY were more than members belonging to TDY in dicot plants, whereas in monocots plants, this situation would be different. For example, we found 15 AtMPKs, 12 PtMPKs and 10 NtMPKs belonged to TEY motif whereas only 8 AtMPKs, 9 PtMPKs and 6 NtMPKs belonged to TDY. However, in rice, 5 OsMPKs contained the TEY motif whereas 11 OsMPKs contained TDY motif. Those results suggested that MAPKs containing the TEY motif might play more important roles than MAPKs belonging to the TDY motif in dicot plants. Interestingly, not all of the MAPK family members contain the TEY or TDY motif in their kinase activation loop. A MEY motif was also found in both

Table 1 The mitogen-activated protein kinase (MAPK) genes in tobacco

New name	Also known as	Accession No.	T-loop	Group	Induced by	Amino acids
NtMPK1	NtMPK4	AB212070.1	TEY	B	MeJA/drought	373
NtMPK2		JX076820	MEY	B		374
NtMPK3		JX076821	TEY	B	SA/drought	380
NtMPK4	NRK1	AB055515.1	TEY	B	MeJA	371
NtMPK5	WIPK	D61377.1	TEY	A	MeJA	375
NtMPK6	SIPK	U94192.1	TEY	A	MeJA	393
NtMPK7	Ntf4	DQ229077	TEY	A	SA/MeJA	394
NtMPK8		JX076822	TDY	D	SA/MeJA	372
NtMPK9		JX076823	TDY	D	Drought	370
NtMPK10		JX076813	TDY	E	SA/MeJA/drought	509
NtMPK11		JX076814	TDY	E	SA	594
NtMPK12		JX076815	TDY	E		590
NtMPK13		JX076816	TDY	E	SA	566
NtMPK14	Ntf3	X69971	TEY	C	MeJA/drought	610
NtMPK15		JX076817	TEY	C	SA/MeJA/drought	573
NtMPK16		JX076818	TEY	F	SA/MeJA	425
NtMPK17		JX076819	TEY	F	SA/MeJA	470

OsMPK2 [40] and NtMPK2 whereas none was found in *Arabidopsis* and poplar MAPK genes.

Group A contains the most extensively studied plant MAPKs, such as AtMPK3/6, OsMPK1/5 and NtMPK5/6 (known as WIPK/SIPK). Most of MAPKs in this group have been reported to be associated with a variety of biotic and abiotic stress responses. For example, NtMPK7 (known as Ntf4) has been illustrated to be involved in plant defense response [24] and a possible relationship to the gametophytic function during plant development [35]. Group B contains four NtMPKs (NtMPK1, NtMPK2, NtMPK3 and NtMPK4). All of the four NtMAPKs share high similarity with OsMPK6 and AtMPK4, both of which are reported to regulate negatively systemic acquired resistance [11, 41]. Group C includes NtMPK14 and NtMPK15. NtMPK14 share higher similarity with OsMPK4, a gene proved to be induced by wounding, JA, SA, ABA, ET, H_2O_2, salt in rice [7]. While NtMPK15 has high identities with AtMPK7 whose circadian-rhythm-regulated expression was detected by microarray analysis.

Group D and E, which are notable for their TDY motif in the activation site and the extended C-terminal sequences, include a total of six NtMPKs (for Group D: NtMPK8/9; for Group E: NtMPK10-13). However, only one of them, NtMPK8 (known as Ntf3) [26], was isolated and studied.

Group F contained the least members of plant MAPKs. Only two NtMPKs (NtMPK16 and NtMPK17) were found in this branch. To date, few of work have been done to analyze the function of MAPKs in this group.

Multiple sequence alignment and structure analysis

To explore the conserved and diverse function of tobacco MAPKs, multiple sequence alignment was performed with the deduced kinase domains (Fig. 2). As reported previously, all tobacco MAPKs proteins contained 11 highly conserved kinase subdomains as well as TXY motif (except NtMAPK2), which was considered as the typical characteristic of MAPK family. It was suggested that these MAPK domains should play important roles in plants.

In addition to the conserved domains, divergent sequences were also detected in the regions between subdomain VII and VIII (Fig. 2), although we are not familiar with their functions. Previous research demonstrated that SIPK (NtMPK6) contained a common docking (CD) domain (LNSLHDISDEPICM) at the C-teminus, which is involved in the interaction with their upstream kinases, substrates and negative regulator including MAPK phosphatases [5]. In our analysis, CD domains were only observed in members of Group A, B and C (Supplementary Fig. 1), indicating these genes may have similar functions. Group D, E and F have quite divergent C-terminal extension (Supplementary Fig. 1) suggesting that they might have diverse functions in plant cellular activities.

Expression profiles of NtMAPKs in response to various signals

Increasing evidence showed that phytohormone SA and MeJA play diverse and important roles in plant defense

Fig. 1 Phylogenetic relationships of MAPK gene families from tobacco, *A. thaliana*, rice and poplar. Amino acid sequences were aligned using ClustalX and subjected to phylogenetic analysis using the NJ method with 1,000 resampling replicates. The *red asterisks* in the branches indicate potential orthologous/paralogous relationship
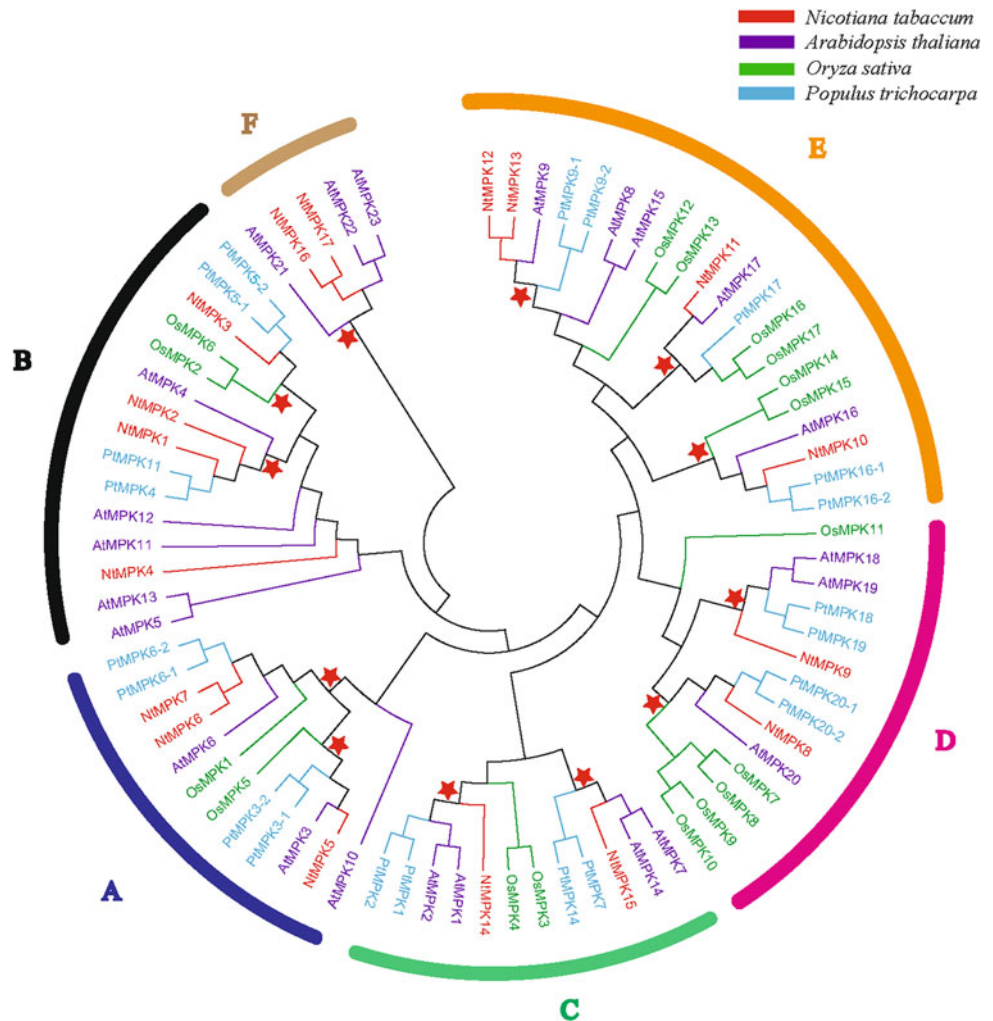
response [42]. To explore the function of tobacco MAPKs in response to these defense signaling molecules, we analyzed the expression patterns of tobacco MAPKs under treatment of SA and MeJA (Fig. 3a, b). Nine tobacco MAPK genes have been regulated by SA (Fig. 3a). Among them, the expression of five NtMPKs (*NtMPK7/8/10/11/13*) was up-regulated under SA treatment, with *NtMPK11* and *NtMPK13* to be considered highly induced at 10 h treatment by SA. The remaining four MAPKs (*NtMPK3/15/16/17*) showed down-regulated expression under SA treatment, exceptionally *NtMPK15*. In our MeJA treatment experiment, 11 MAPKs (*NtMPK1/4/5/6/7/8/10/14/15/16/17*) had inducible expression. Among them, 10 genes showed up-regulated expression, especially *NtMPK5* and *NtMPK16*, which were up-regulated 40-folds higher than control at 10 h of treatment. And only one gene, *NtMPK4*, was slightly down-regulated by MeJA. Our results indicated most of tobacco MAPKs were involved in response to SA and MeJA signaling pathway and might have important functions in plant defense response.

Many studies demonstrated that MAPKs involved in response to drought stress [5, 43, 44]. For example, the transcript levels of an alfalfa MAPK gene *MMK4* [43] and *GhMPK16* [44] accumulated after drought treatment. In our qRT-PCR analysis, the mRNA levels of six tobacco MAPKs (*NtMPK1/3/9/10/14/15*) were induced by drought treatment. *NtMPK1* and *NtMPK9* were significantly induced by drought treatment (Fig. 3c). The results suggested several tobacco MAPKs might be involve in response to drought stress.

Discussion

A list of 17 tobacco MAPKs were firstly analyzed in our study. And among them, we cloned 13 novel MAPK homologs in common tobacco, among which 2 genes were excluded according to the criteria proposed by Nathan [8]. Although *N. tabacum* has a very large genome size with 4.5 billion base pairs compared with other plants, mobile DNAs may make up the majority of the nuclear genome [45]. Therefore, considering the conserved numbers of
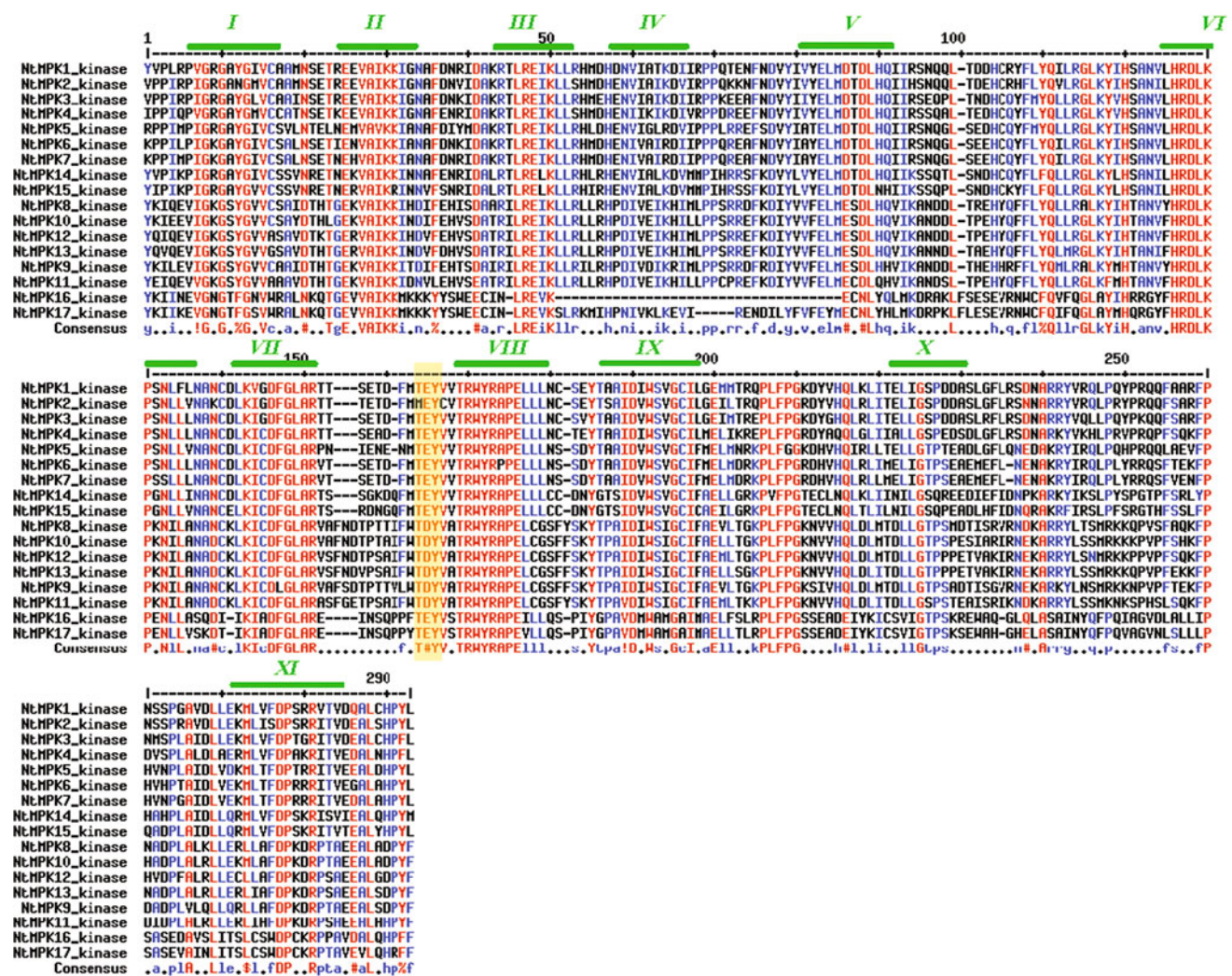
Fig. 2 Protein sequence alignment of tobacco MAPKs kinase domain. Alignment was performed using the MultAlin 5.4.1 program. The high-, low-, and neutral- consensus amino acid (aa) residues are depicted in *red, blue* and *black colors*, respectively. Highly conserved aa residues appear in *high-consensus color* and as an *uppercase letter* *in the consensus line*. The PK subdomains are in *italicized roman numerals* (I–XI) on the top of each row and aa positions are marked by *green lines*. The phosphorylation-activation motif (TEY and TDY) is indicated by *yellow box*

MAPKs from other plants (Arabidopsis: 23, rice: 17, poplar: 21), it is likely that we have identified most, if not all, of the MAPKs in *N. tabacum*.

Previous study in *A. thaliana* indicated that MAPK were clustered into four groups [16]. However, the phylogenetic tree in this study reveals that tobacco MAPKs appear to be clustered into six groups (Group A–F). Base on the phylogenetic analysis, several potential orthologous/paralogous subclusters were identified (Fig. 1) in our research. These potential orthologous relationships help us to understand possible functions of tobacco MAPKs. Previous research revealed that AtMPK4 was required for jasmonic acid-responsive gene expression and negatively regulated systemic acquired resistance in *Arabidopsis* [11]. We speculate its orthologs, NtMPK1 and NtMPK2, may share a similar

function in plant defense response. Actually, Kenji has reported NtMPK1 (old name NtMPK4) is required for jasmonic acid signaling and involved in ozone tolerance via stomatal movement in tobacco [27]. Our qRT-PCR analysis (Fig. 3b) reveals *NtMPK1* is induced by MeJA treatment, which is similar to Kenji's results. Similarly, it has been investigated that OsMPK6 functioned both as an activator and a repressor to regulate rice defense responses [46]. As its ortholog, *NtMPK3* was down-regulated by SA in our qRT-PCR analysis (Fig. 3a), which suggested NtMPK3 had a similar function with OsMPK6 in negatively regulating systemic acquired resistance (SAR). In addition, it is likely that NtMPK3 is also involved in response to drought stress since its transcript level is induced by drought treatment (Fig. 3c). Although no more information about NtMPKs in

Fig. 3 Expression pattern of tobacco MAPKs in response to SA, MeJA and drought treatments
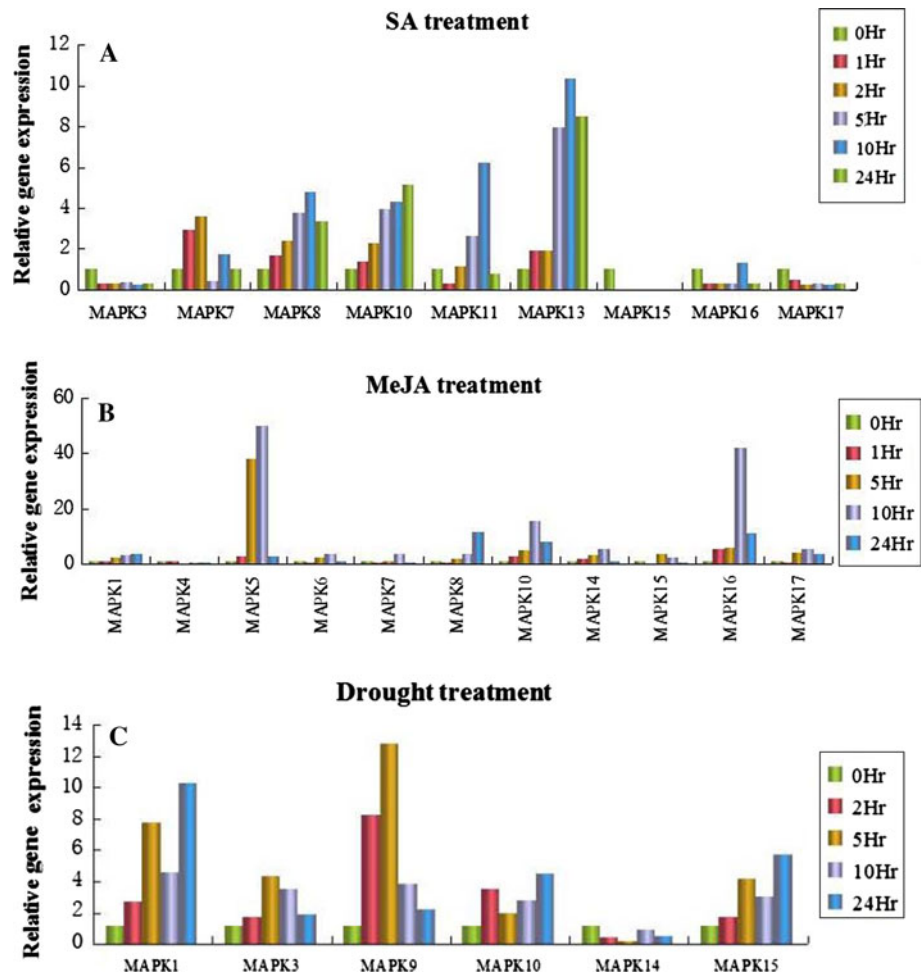
Group D is known, rice MAPKs in the group support more useful details to analyze NtMPKs. Two OsMPKs (OsMPK7 and OsMPK8) were found to be involved in biotic and abiotic stress responses in group D. Over-expression and suppression of *OsMPK7* revealed that the MAPK participate in JA signaling and defense response [40]. While *OsMPK8* could be regulated by ABA, H_2O_2, drought, temperature changing, heavy metals and UV–C irradiation, and was also associated with host cell death [8]. These studies suggest that NtMPK8 (also known as Ntf3), the ortholog of OsMPK7/8, may play an important role in response to various environmental stimuli and phytohormone signaling. Our qRT-PCR analysis partly verified the speculation since we detected up-regulated expression of *NtMPK8* by SA and MeJA treatment (Fig. 3a, b); however, no obvious change was observed under drought treatment.

Salicylic acid (SA) and jasmonic acid (JA) are two important signaling molecules involved in plant defense against pathogen infection. Accumulation of SA is required for the induction of SAR [47]; while, JA is involved in induced systemic resistance (ISR) against pathogens [48].

MAPK induced by SA and/or JA may be involved in plant defense response against pathogens. *OsMPK12* (also known as BWMK1) was induced by both SA and JA treatments in Nathan's qRT-PCR analysis [8]. Constitutive expression of *OsBWMK1* also enhanced resistance against pathogen infections [49]. We speculate tobacco MAPKs (*NtMPK1/3/4/5/6/7/8/10/11/13/14/15/16/17*) induced by SA and/or MeJA in our qRT-PCR analysis may play important roles in plant SAR and ISR. It has been reported that kinase activity of SIPK (NtMPK6) was activated by SA [21]. However, mRNA level of SIPK was not detected obviously induced by SA in our qRT-PCR analysis. To validate the accuracy and effect of our treatments with SA and MeJA, expression of *PR1a* [50] and *ODC* [51] in tobacco was analyzed as marker genes through qRT-PCR (Supplementary Fig. 2a, b), respectively. Our results showed that the treatments in our research were effective. This phenomenon suggests that those MAPKs, which are not induced by SA at mRNA level, may play important roles in plant defense response. Interestingly, both WIPK and SIPK were induced by MeJA at mRNA levels in qRT-PCR analysis (Fig. 3b). Actually, our

analysis verified the fact that WIPK and SIPK were involved in JA production [52].

Our results will provide important information of tobacco MAPKs for further research. Detection of mRNA level change could not return real information sometimes. We expect transgenic analysis and kinase activity assay, which are effective ways to understand the functions of MAPKs, will be established in the future.

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