Validation by isolation and expression analyses of the mitogen-activated protein kinase gene family in the grapevine (*Vitis vinifera* L.)

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Abstract

Background and Aims: Mitogen-activated protein kinases have been found to play essential roles in mediating biotic and abiotic stress responses in plants. Investigation of their possible involvement in grapevine resistance to biotic or abiotic stresses and of their development will be possible only through future functional genomics experiments of gain/loss of function in the grapevine.

Methods and Results: We identified and re-annotated all 12 mitogen-activated protein kinases genes from the 12X V1 sequenced grapevine genome and re-nominated them according to international standards as VvMPK. All were validated by cloning their cDNA sequences through polymerase chain reaction amplification. Expression analysis of VvMPK genes using microarray analysis and quantitative real-time polymerase chain reaction demonstrated that all VvMPK genes are expressed during grapevine growth and development. Based on expression analysis of grapevine tissues and organs at several developmental stages, and of leaf tissues treated with *Erysiphe necator* (powdery mildew), salicylic acid, ethylene, hydrogen peroxide and drought, we identified for further functional characterisation several VvMPK candidate genes which might be involved in grapevine growth and development and in biotic and abiotic responses.

Conclusions: We identified several grapevine MPK genes modulated at the transcriptional level in several stages of grapevine growth and development and during the response to development and environmental stresses.

Significance of the Study: This is the first comprehensive experimental survey of the grapevine MPK gene family, which provides insights into their potential roles in regulating responses to biotic and abiotic stresses. Ongoing functional characterisation of important candidate VvMPK genes will assist unravelling their biological roles in grapevine.

Keywords: *environmental stress, expression analysis, grapevine, growth and development, mitogen-activated protein kinase (MAPK), powdery mildew*

Introduction

Plants are constantly exposed to a variety of biotic and abiotic stresses throughout their life. To meet these challenges, plants have developed sophisticated mechanisms to perceive external signals and respond to them with proper physiological and morphological alterations (Bohnert et al. 1995). Protein phosphorylation via protein kinases is one of the major mechanisms controlling intracellular response to extracellular signals (Xiong et al. 2002, Zhu 2002, Li et al. 2008).

In this context, mitogen-activated protein kinases (MAPK) form one of the largest and most important categories of protein kinases, and transduce extracellular signals into intracellular responses through phosphorylation (Shi et al. 2009, Rodriguez et al. 2010). The MAPK signalling cascade in plants typically consists of functionally interlinked protein kinases: a MAP kinase kinase kinase (MAPKKK/ MAP3K), a MAP kinase kinase (MAPKK/MKK) and a MAP kinase (MAPK/MPK) (Pitzschke et al. 2009, Popescu et al. 2009). Activation of a MAP3K phosphorylates a MKK at conserved serine and/or threonine residues in the activation loop (T-loop), which then phosphorylates and consequently activates the downstream MPKs (Tena et al. 2001, Hamel et al. 2006). MPK proteins have 11 domains with the active site containing either a Thr-Glu-Tyr (TEY) or a Thr-Asp-Tyr (TDY) phosphorylation motif. The T-loop is located between subdomains VII and VIII with conserved threonine and tyrosine residues in the motif TXY (Lewis et al. 1998, Widmann et al. 1999, Mishra et al. 2006). In contrast to TEY type of MAPKs, all TDY types of MAPK have a long C-terminal extension. The phosphorylated MAPKs then target various proteins in the cytoplasm or nucleus, thereby reprogramming gene expression (Fiil et al. 2009, Andreasson and Ellis 2010, Rodriguez et al. 2010).

The MPK family is a multi-gene family. For example, *Arabidopsis thaliana* genome contains 20 (Ichimura et al. 2002), rice contains 17 (Agrawal et al. 2003, Reyna and Yang 2006) and *Populus* has 21 genes (Nicole et al. 2006). Molecular and

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biochemical characterisation of individual MPK genes has revealed that they are involved in growth, cell differentiation and response to biotic and abiotic stresses (Asai et al. 2002, Rodriguez et al. 2010), as well as in the production of their related hormonal signals, such as abscisic acid, salicylic acid (SA), jasmonic acid (JA), and ethylene (ETH), and in the accumulation of reactive oxygen species, such as H_2O_2 (Jonak et al. 1996, Bogre et al. 1997, Zhang and Klessig 1997, Ichimura et al. 2000, Asai et al. 2002, Ren et al. 2002, Xiong and Yang 2003, Xu et al. 2008, Beckers et al. 2009).

Grapevine (*Vitis vinifera* L.) is one of the most widely grown fruit crops in the world. All cultivated, high-quality wine grapes are susceptible to diseases and subject to multiple environmental stresses. Understanding the resistance mechanisms to biotic and abiotic stresses will greatly assist developing resistant cultivars and reducing pesticide use and yield losses. Towards this major goal, a high-quality whole genome sequence has been generated from the highly homozygous grapevine PN40024 genotype (Jaillon et al. 2007). This has greatly facilitated the grapevine comparative and functional genomics studies at the whole genome-, gene-family- or individual gene level, similarly to those of Arabidopsis and rice (Jaillon et al. 2007, Nonis et al. 2008). Recent preliminary data indicated that the grapevine possesses 12 MPK genes based on the genome scan of the 8× sequence coverage, but without experimental verification (Hyun et al. 2010). In addition, no gene-family expression analysis and few functional analyses of each MPK gene have been undertaken in terms of their roles in biotic and abiotic stress responses in grapevine.

In the present study, we validated 12 earlier identified (Hyun et al. 2010) non-redundant MPK genes using the 12× sequence coverage of the grapevine genome by polymerase chain reaction (PCR)-based cloning. We then examined the expression pattern of VvMPK genes in tissues/organs at several developmental stages and in response to biotic and abiotic stresses, and related chemical signals. Based on all these data, we identified several MPK genes which might be involved in the regulation of biotic and abiotic stress responses.

Materials and methods

Identification of the MAPK genes in the grapevine genome

The sequences of 20 Arabidopsis MPK proteins retrieved from The Arabidopsis Information Resource 10 Genome Release (http://www.arabidopsis.org) were used as queries to search the *V. vinifera* Proteome (12X genome coverage, release V1) at the Grape Genome (http://genomes.cribi.unipd.it/grape/) and Vitis-URGI (http://urgi.versailles.inra.fr/Species/Vitis) databases by using the profile Hidden Markov Model-based search (HMMER:http://hmmer.wustl.edu/). The proteins with e values >1E-5 were additionally queried against the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). The sequences with conserved kinase domains were considered as grapevine MPK (VvMPK).

Isolation of the MAPK genes in the grapevine

The open reading frames (ORFs) of the 12 VvMPK genes were amplified by PCR using specific primers (listed in Supporting Information Table S1) for each gene. Total RNA was extracted from grapevine (*V. vinifera*, PN40024) young leaves using the TRIZOL reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instruction. The first-strand cDNA was generated following the manufacturer's protocol using the Improm reverse transcription system (Promega, Madison, WI, USA). The PCR cycling conditions consisted of an initial denaturation at 95°C for 4 min, 35 cycles of 94°C for 30 s, 51–58°C for 30 s and 72°C for 1 min, followed by extension at 72°C for an additional 10 min. The total volume of the PCR was 25 μL including 1 μL of first strand cDNA as template, 0.5 μL of each primer and a PCR mix [0.2 μL Prime STAR HS DNA polymerase (TaKaRa, Dalian, China), 2.5 μL 10 × PCR buffer, 2 μL 2.5 mmol dNTP, 2.0 μL 25 mmol $MgCl_2$ and 16.3 μL double distilled H_2O]. The 3′ untranslated region (UTR) sequences were obtained by genome walking using the SMART rapid amplification of cDNA ends (RACE) cDNA amplification kit (Clontech, Mountain View, CA, USA). The primary PCR to amplify the 3′ region was performed with cassette primer 3W and each of the gene-specific primer VvMPK-W. The primary PCR products were diluted 1:10 and then amplified using cassette primer 3N and nested primer, VvMPK-N. The 3′ RACE primer pairs are listed in Supporting Information Table S2. The PCR amplifications were performed as described above. The PCR and RACE products were cloned into pMD19-T vector (TaKaRa) and sequenced (Invitrogen Biotechnology Co. Ltd, Shanghai, China). Cloning of PCR products and sequencing were repeated three times.

Sequence alignments and conserved domain analysis

Multiple-sequence alignments of predicted VvMPK sequences with cloned VvMPK coding sequences were performed using the ClustalX program (version 1.83; Centre National de la Recherche, Paris, France) both at the protein and nucleotide level. The domain architectures of VvMAPK amino acid (AA) sequences were predicted by searching against the SMART database (http://smart.embl-heidelberg.de/). The MEME and MAST software (http://meme.sdsc.edu/meme/website/intro.html) were employed to identify conserved motifs in the VvMPK protein sequences.

Microarray data analysis of tissue-specific expression of VvMPK genes

To understand the potential functions of VvMPK genes during the grapevine life cycle, the spatial and temporal expression profiles of the VvMPK genes were analysed based on published high-throughput microarray data (Fasoli et al. 2012). In the data sets, 54 grapevine samples – node, inflorescence, carpel, petal, pollen, berry, withering berry, leaf, root, seed, seedling, rachis, stem and tendril – covering most organs at several developmental stages, were analysed. The expression data were transformed into $\log_2$ values. The heat maps were made by MeV4.8 software (http://www.tm4.org/mev/) (Dana-Farber Cancer Institute, Boston, MA, USA) (Mar et al. 2011) and VvMPKs were clustered according to their expression profiles.

Plant materials and treatments for expression analysis

In vitro grapevine plants (*V. vinifera* genotype PN40024, the sequenced genotype) were kindly provided by Dr Anne-Françoise Adam-Blondon, National Institute for Agricultural Research (INRA), Evry, France, and maintained in vitro on half strength Murashige and Skoog medium supplemented with 0.3 mg/L indole-3-butyric acid (IBA, Sigma-Aldrich Co., St Louis, MO, USA), under a 16/8 h photoperiod [100 μmol/(m^2 • s)] at 25°C in the growth chamber.

For treatments involving SA, ETH and hydrogen peroxide (H_2O_2), plants that were subcultured for 5 weeks were sprayed with 5 mmol SA (Sigma-Aldrich Co.), 5 mmol ethephon (as an ETH donor) (Sigma-Aldrich Co.) and 10 mmol H_2O_2 (Sigma-Aldrich Co.). In these experiments, each treatment consisted of three clonal plants; the samples were harvested at 4, 8, 12, 24, 48 and 72 h post-treatment (hpi). Mock-treated plants served as

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control. For powdery mildew [*Erysiphe necator* (Schw.) Burr] infection, a local strain of *E. necator* was maintained in Pinot Noir plants in a greenhouse. The inoculation was conducted by laying the same size *E. necator*-infected leaves on detached leaves of PN40024 grown in the growing chamber. Samples were collected at 4, 8, 12, 24, 48 and 72 hpi. Untreated leaves served as a mock control.

For drought treatments, in vitro PN40024 plants were acclimated to potting mix and grown in the greenhouse until about 40 cm tall with 14 leaves. The plants were first watered thoroughly and then not watered. The sixth leaves were collected at 4, 8 and 12 days after watering interruption (dpi), then immediately frozen in liquid nitrogen and stored at −70°C. Regularly watered plants served as a control. Each treatment consisted of three independent biological replications (leaves from three independent plants).

Total RNA isolation and cDNA synthesis

Total RNA was extracted from the frozen samples following the method proposed by Gonzalez-Mendoza et al. (2008), and then the RNA was treated with an RNase-free DNase I (TaKaRa) according to the manufacturer's instruction. The concentration of RNA was measured with a One-Drop OD-1000 spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA); purity was determined by the optical density (OD) absorption ratio OD 260 nm/OD 280 nm. The RNA integrity was monitored by electrophoresis on 1.0% agarose gels by staining with ethidium bromide. About 1 μg total RNA was used to generate the first-strand cDNA for quantitative real-time PCR (qRT-PCR) analysis in a 20 μL reaction mixture. The PrimeScript RT reagent Kit (TaKaRa) was used according to the manufacturer's instructions. The first strand cDNA samples were diluted 1:10 with sterile double distilled water (ddH_2O) and stored at −20°C before being used as templates.

Gene expression analysis using qRT-PCR

The specific expression of VvMPKs was examined by qRT-PCR using a SYBR Green method on an ABI 7300 real-time PCR system (Perkin-Elmer Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The oligonucleotide primers of the VvMPKs were designed based on the identified 3′ UTR and the 3′ terminal sequences of the coding region using Primer Express software (Perkin-Elmer Applied Biosystems). The primers' specificity for each gene was set so that at least one 3′ end, and three other nucleotides at the 5′ end were unique compared with sequences of other genes in the gene family. The housekeeping gene (actin-101-like, VIT_12s0178g00200) was used as an internal control for qRT-PCR. The primer pairs are listed in Supporting Information Table S3. The total volume of each real-time RT-PCR reaction was 20 μL which included 1 μL cDNA from each sample as a template, 10 μL SYBR Premix Ex Taq, 0.2 μL of each primer and 8.6 μL ddH_2O. The PCR conditions consisted of denaturation for 4 min at 95°C, followed by 40 cycles of 95°C for 20 s, 60°C for 20 s and 72°C for 43 s. A dissociation curve for each amplification was prepared immediately after completion of the qRT-PCR to verify the specificity of each amplification reaction from 60 to 95°C. Each sample had three replicates to ensure the accuracy of results and no-template controls were included. Real-time PCR data of VvMPKs were analysed according to $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta Ct = (Ct_{target\ gene} - Ct_{actin})_{treatment} - (Ct_{target\ gene} - Ct_{actin})_{ck}$ (Livak and Schmittgen 2001). To visualise the relative fold difference, all data were represented by setting the relative expression level of mock samples to 1: 'above 1' and 'below 1' are considered as up or down regulation during the stress treatment. Mean values and standard deviations (SDs) were obtained from three biological replicates.

Statistical analysis

The software in SPSS version 13.0 (SPSS, Chicago, IL, USA) and EXCEL (Microsoft Corporation, Redmond, WA, USA) were used for statistical analysis. All results were expressed as means ± SDs based on Duncan's multiple range test, and $P < 0.05$ and $P < 0.01$ were taken as statistically significant and highly significant, respectively.

Results and discussion

Validation and isolation of VvMPK family genes

By scanning the V1 genome sequence (http://genomes.cribi.unipd.it/grape/), we confirmed the 12 VvMPK genes previously reported in grapevine based on 8× coverage (Hyun et al. 2010). These genes were renamed following the new international naming convention based on the gene order of their chromosomal locations: from VvMPK1 to VvMPK12. Twelve VvMPK genes locate in ten different chromosomes (Table 1). The predicted ORF length ranged from 1107 bp (VvMPK3) to 1842 bp (VvMPK1), with an average length of 1409 bp. The ORFs encoded polypeptides from 368 AA to 613 AA long, with a predicted molecular mass in the range of 42.54–70.48 kDa and an isoelectric point in the range of 4.69–9.57 (Table 1).

The nucleic acid sequences of the cloned genes are reported in Supporting Information Table S4. The cloned full-length cDNA sequences of all 12 VvMPK genes from PN40024 were largely the same as the predicted cDNA sequences (Supporting Information Figure S1), with almost 100% identity. The alignment between the predicted AA sequences of *each* cloned cDNAs and the corresponding sequence predicted by the genome (Supporting Information Figure S2) was almost identical.

The deduced protein sequences of the cloned VvMPKs genes also contained the 11 domains (I-XI) typical of MPK proteins in other species (Supporting Information Figure S3a), confirming that all 12 VvMPKs belong to the MPK family. The threonine and tyrosine residues are located in domain VIII, namely the T-X-Y motif that has to be phosphorylated for the activation of VvMPKs. The TEY or TDY motif of VvMPKs are located in the activation loop between kinase subdomain VII and VIII (Supporting Information Figure S3b). The VvMPK2, 5, 6, 7 and 9 possess a common docking domain at the C-terminus (Supporting Information Figure S3c) with two invariable AA residues, which is involved in the interaction with their upstream kinases, substrates and negative regulators (Tanoue et al. 2000), whereas other VvMPKs contain a domain with hydrophobic residues [LAXX (D/E)]. The grapevine contained fewer MPKs than Arabidopsis (20 MPKs) (Ichimura et al. 2002) and rice (17 MPKs) (Liu and Xue 2007).

Tissue-specific expression of VvMPK genes at several developmental stages in grapevine organs

To draw hypotheses about the possible biological functions of VvMPK genes in specific developmental processes in the grapevine, we examined the expression profiles of VvMPK genes through a survey of a wide published data set of 54 different grapevine tissues/organs (Fasoli et al. 2012). Figure 1 represents the relative abundance of each transcript in each sample, compared with the median value of the whole data set. The transcript abundance of most VvMPKs was relatively similar in all examined tissues and stages, with some notable exceptions. A much higher variability of gene expression was detected for

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Table 1. The mitogen-activated protein kinase genes predicted in grapevine genome and their characteristics.

Name	Gene model name	Identical genes in 8X	Chromosomal localisation	AA length	MM (kDa)	pI	Gene length (kbp)
VvMPK1	VIT_01s0011g04920	GSVIVT00030495001	chr1: 04565523-04574755	613	70.48	8.62	9.233
VvMPK2	VIT_02s0025g00270	GSVIVT00002553001	chr2: 00380307-00386869	375	42.69	6.24	6.563
VvMPK3	VIT_04s0023g02420	GSVIVT00036900001	chr4: 18972891-18976842	368	42.72	7.95	3.952
VvMPK4	VIT_05s0020g02530	GSVIVT00019886001	chr5: 04205645-04215886	563	63.91	8.44	10.24
VvMPK5	VIT_05s0094g00900	GSVIVT00034702001	chr5: 24220367-24241098	398	45.77	5.67	20.73
VvMPK6	VIT_06s0004g03540	GSVIVT00024457001	chr6: 04432888-04436315	375	43.12	5.95	3.428
VvMPK7	VIT_06s0004g03620	GSVIVT00024447001	chr6: 04580727-04584943	371	42.54	4.69	4.217
VvMPK8	VIT_12s0142g00130	GSVIVT00018914001	chr12: 00126317-00133214	610	68.96	9.33	6.898
VvMPK9	VIT_15s0046g02010	GSVIVT00026919001	chr15: 18821588-18826743	375	43.28	6.9	5.156
VvMPK10	VIT_17s0000g02570	GSVIVT00017805001	chr17: 02368197-02377721	601	68	7.36	9.525
VvMPK11	VIT_18s0001g13010	GSVIVT00014559001	chr18: 11126023-11129236	376	43.23	7.56	3.214
VvMPK12	VIT_19s0014g00220	GSVIVT00027929001	chr19: 00224334-00234190	598	67.61	9.57	9.857

AA, amino acids; MM, molecular mass; pI, isoelectric point.

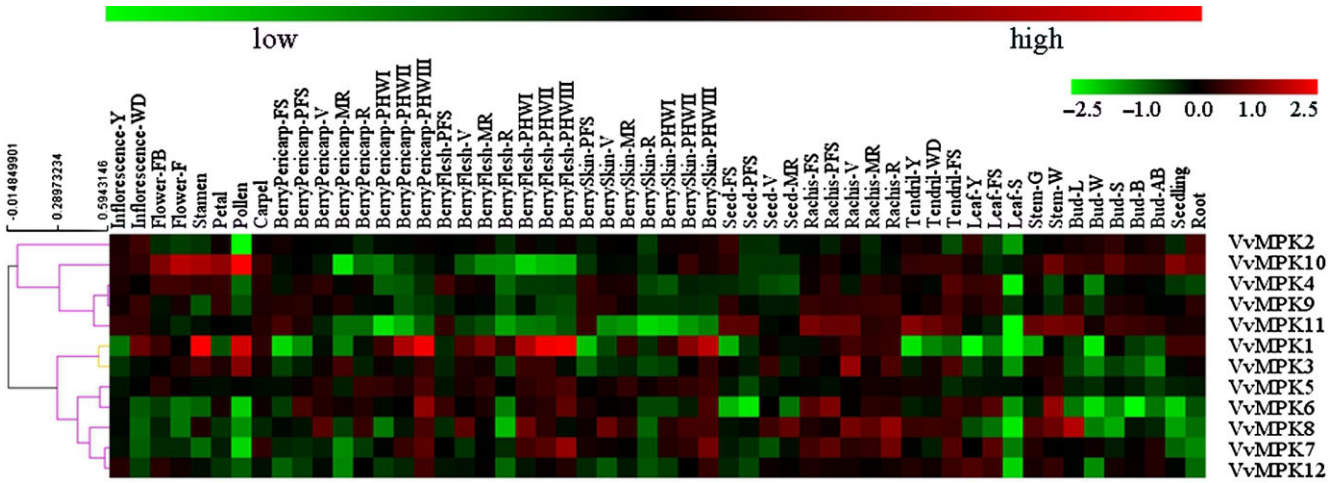

Figure 1. Expression profiles of VvMPK genes in grapevine development. Results are based on a published high-throughput microarray data set that analysed 54 grapevine samples (node, inflorescence, carpel, petal, pollen, berry, leaf, root, seed, seedling, rachis, stem and tendril) at several developmental stages. Expression values were the average of three biological replicates.

VvMPK1 and 10. VvMPK1 was highly expressed in stamen and pollen, and its transcript abundance progressively increased in berries during postharvest withering. The expression of VvMPK10 was high in all flower organs and especially in pollen, but declined in the late stages of berry development and postharvest. The Arabidopsis homologues closest to VvMPK1 and 10 also show strong pollen-specific expression, as revealed by the AtMPK gene expression atlas (Menges et al. 2008). These data support a possible involvement of these MAP kinases in pollen development. A few other VvMPKs (VvMPK4, 6 and 11) were expressed at a much lower level in certain organs or stages: VvMPK4 was poorly expressed in senescing leaves and winter nodes; VvMPK6 was strongly expressed in stems but much less in flower organs, nodes and seeds; while VvMPK11 expression decreased during berry maturation and postharvest and in senescing leaves. The genes VvMPK5 and VvMPK6 cluster together in the expression analysis as well as the corresponding genes in the previously reported phylogenetic analysis (Hyun et al. 2010), although VvMPK5 expression level is mostly higher than that of VvMPK6 in all examined organs. A parallelism with this observation was also reported in poplar – another perennial crop – for the two closest homologues PtMPK6 and 3 respectively, with a relatively higher abundance of the first over the second gene, especially in nodes (Nicole et al. 2006). The same was observed in Arabidopsis, in which the two paralogous genes AtMPK6 and 3, corresponding to VvMPK5 and 6, both considered positive regulators of the stress response, show an expression pattern in different organs similar to that in the grapevine (Menges et al. 2008). The genes VvMPK4 and VvMPK11 are highly similar to AtMPK16 and AtMPK1 and 2, respectively, but functional information on their possible role is lacking.

Expression profiles of VvMPK genes in response to powdery mildew infection, SA, ETH, hydrogen peroxide and drought treatments

To the best of our knowledge, there are no reports on the expression profile of defence-related MPK genes in infected or stressed plants. The VvMPK genes exhibited different expression patterns in response to powdery mildew infection (Figure 2a). VvMPK5 and VvMPK6 (AtMPK6 and AtMPK3 orthologues) showed predominantly a reduced expression level in response

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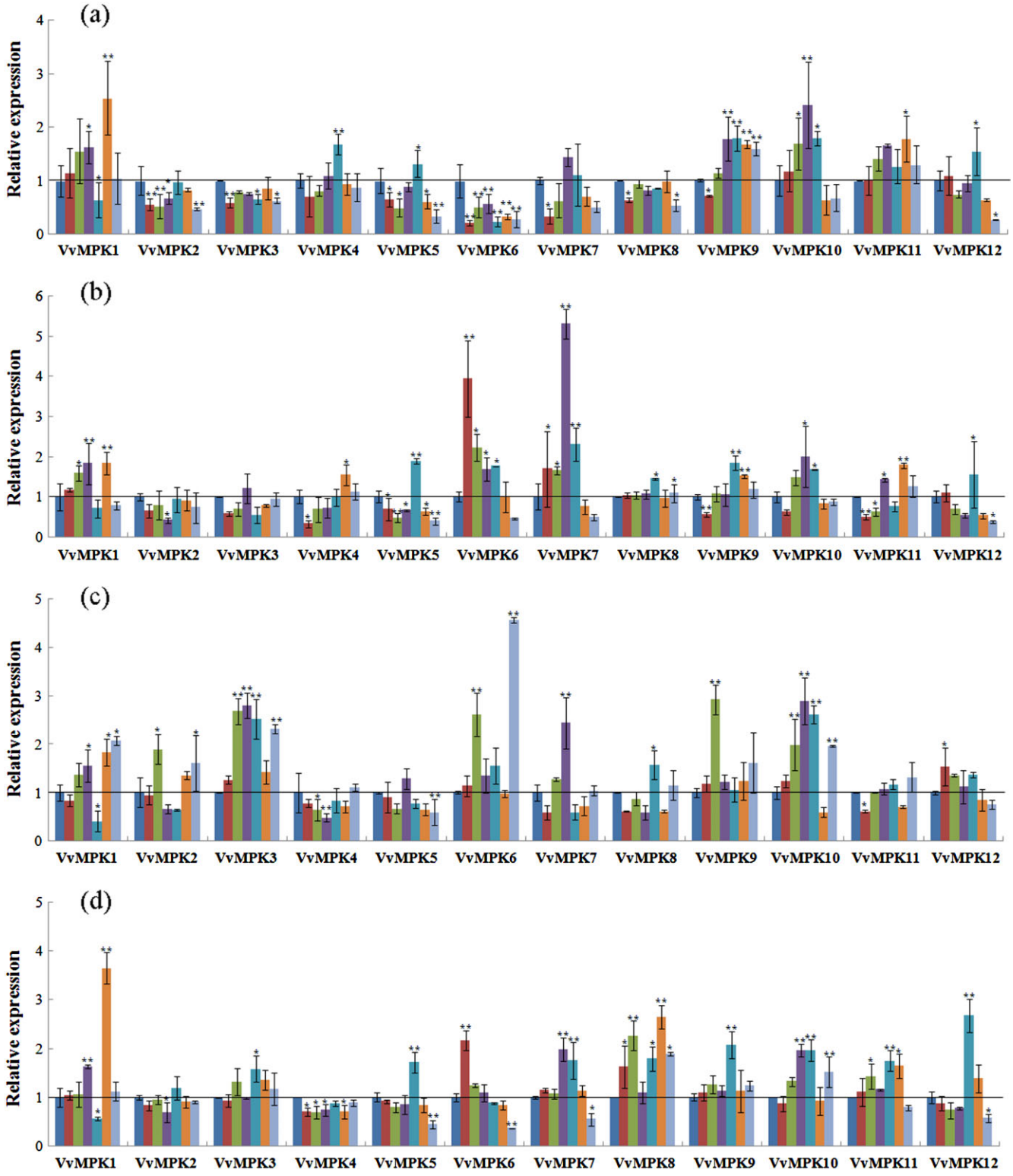

Figure 2. Expression profiles of VvMPK genes in grapevine leaves in response to (a) *Erysiphe necator* treatment and after exogenous application of defence-signalling compounds, (b) salicylic acid, (c) ethylene and (d) H_2O_2. The leaves were harvested at 4 (■), 8 (■), 12 (■), 24 (■), 48 (■) and 72 h (■) post-treatment. Mock-treated plants served as control (■). To visualise the relative fold difference, the normalised relative expression level in mock samples was set up as '1'. Data are shown as the means ± standard deviation (SD) of the relative level of each gene expression from nine data sets (three biological replicates in each experiment and three repeated experiments), and bars represented SDs. ** and * indicate a significant difference in comparison with the control at $P < 0.01$ and $P < 0.05$, respectively.

to powdery mildew inoculation. It is well documented that the signalling role of MPKs depends on upstream activation of the kinase cascade, and transcript abundance could be unrelated to the final activity. Considering, however, the already mentioned involvement in the resistance response of their corresponding orthologues in Arabidopsis (Pitzschke et al. 2009), such a reproducible decrease in the mRNA levels for both VvMPK5 and VvMPK6, at several time points after infection,

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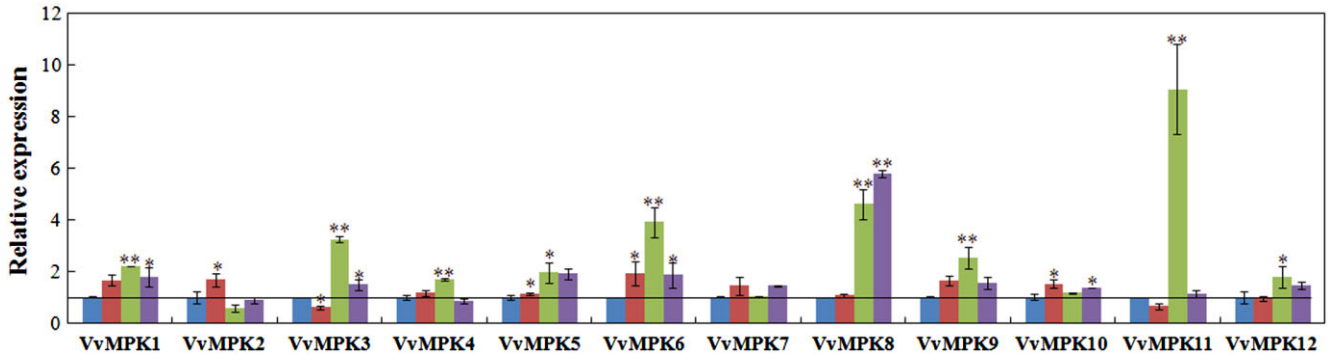

Figure 3. Expression profiles of VvMPK genes in grapevine leaves in response to drought stress. Leaves were collected at 4 (■), 8 (■) and 12 (■) days post-drought. To visualise the relative fold difference, the normalised relative expression level of the control (■) was set up as '1'. Data are shown as the mean ± standard deviation (SD) of the relative level of each gene expression from nine data sets (three biological replicates in each experiment and three repeated experiments), and bars represented SDs. ** and * indicate a significant difference in comparison with the control at $P < 0.01$ and $P < 0.05$, respectively.

probably deserves further investigation. Genes VvMPK1 and 10 showed the most prominent induction following *E. necator* infection, being expressed twofold more than that in the control, at 48 and 12 h post-inoculation, respectively (Figure 2a), while VvMPK9 showed a lower but sustained increase over time during the experiment, consistent with its counterpart AtMPK4 in powdery mildew-infected Arabidopsis plants (Michel et al. 2006).

To further investigate the roles of VvMPK genes in response to abiotic responses, PN40024 leaves were treated with the defence-signalling compounds SA, ETH and H_2O_2, and the expression level of VvMPK genes was measured. Within 72 h after the SA treatment (Figure 2b), most genes were expressed at about the same level as that in controls, while several genes showed a slight but significant increase of the expression level. Two genes that increased threefold or more than in the control were VvMPK6 and VvMPK7 at 4 and 12 h after treatment, respectively. Several other VvMPKs showed a significant although sometimes low increase in response to SA.

After treatment with ETH, the majority of VvMPKs genes exhibited significant changes compared with those in the mock control (Figure 2c). Some genes, VvMPK1, 3, 6, 7, 9 and 10 (Figure 2c), responded to treatment with a highly significant increase in expression level of more than twofold, in comparison to that of the controls at least at one time point. In previous phylogenetic analysis (Hyun et al. 2010), VvMPK genes corresponding to VvMPK6 and 9 show a great similarity with genes AtMPK3 and AtMPK4, two well-known stress-responsive MAPK in Arabidopsis. Genetic analysis indicate that Arabidopsis MPK4 acts as a negative regulator of SA-mediated defence against biotrophic pathogens but is required for ETH- and JA-mediated defence against necrotrophic pathogens (Petersen et al. 2000, Brodersen et al. 2006). Gene AtMPK3 was also shown to be involved in ETH signalling (Hahn and Harter 2009) and both respond to different abiotic stresses (Droillard et al. 2000, Ichimura et al. 2000, Ahlfors et al. 2004).

After treatment with H_2O_2, nearly all VvMPK genes were expressed at a level higher than that in the mock controls, especially VvMPK1 which was expressed about 3.5-fold at 48 hpi and VvMPK8 and 12 which were expressed about 2.5-fold at least at one time point (Figure 2d).

When grapevine plants were subjected to drought, all VvMPK genes were expressed at a level higher than that in mock controls. Among them, VvMPK6, 11 and 8 genes were expressed fourfold more than the control at 8 days after drought treatment (Figure 3).

In plants, increasing evidence supports that MAPKs play important roles in signalling to biotic and abiotic stresses (Nicole et al. 2006, Reyna and Yang 2006, Zhang et al. 2006), but no systematic investigation has been applied to this gene family in the grapevine. Further, there are few reports on the biological functions of MAPKs in the grapevine. Survey of the expression profile of the whole gene family has been one of the effective strategies to identify candidate genes or specific promoters that may be involved in a particular biological process, such as the identification of the cambium region, highly expressed YUCCA gene in *Populus* (Ye et al. 2009) or the multiple hormone-inducible XTH gene promoter (Ye et al. 2012). Disease-resistance-related candidate genes, such as EDS in Arabidopsis, were also identified through a gene-family-wide expression survey (Gao et al. 2010).

We surveyed the grapevine MAPK gene family and found that VvMPK6 increased its expression level in response to all the stress-related treatments but was downregulated by *E. necator* infection, suggesting that in the grapevine this gene might be involved in response to abiotic stresses. Genes VvMPK1 and 10 and possibly VvMPK9 are likely responders to infection by powdery mildew, one of the most serious fungal diseases of grapevine worldwide (Donald et al. 2002).

Several observations suggest that many MAPKs, such as Arabidopsis AtMPK3, 4 and 6 (Droillard et al. 2002, Colcombet and Hirt 2008, Pitzschke et al. 2009), rice OsMSRMK2 and OsMSRMK3 (Agrawal et al. 2003), respond to several environmental stresses. The plant defence-related compounds SA, ETH and H_2O_2 are also important signalling compounds involved in overall disease resistance and defence responses (Hamel et al. 2005). In this study, we found that in the grapevine, several VvMPKs showed various gene expression patterns following each tested stress-related treatment. A possible involvement of each VvMPK in the response to different stimuli is difficult to envisage; MAP kinases are indeed known to participate in a highly interconnected and finely tuned network of signals transduction cascades in the plant cell, regulating different processes. Their fast responsiveness has been proven by the many experiments in which MAPKs are activated by a wide range of different perturbations, especially in Arabidopsis (Colcombet and Hirt 2008). According to our experiments, however, a few VvMPKs deserve further investigation, such as VvMPK1, 10 and 9, which showed a recurrent significant increase in expression level in response to several stress-related treatments and more specifically during powdery mildew infection. In contrast, induction of VvMPK8 and 11 appeared to be more related to the

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drought response, although the timing and dynamic of the experiment was different from that of the other treatments. For these MAPKs and their homologues in other species, the available information is still scarce. Investigations of their possible involvement in grapevine resistance to biotic or abiotic stresses will be possible only through future functional genomics experiments of gain/loss of function in grapevine.

Conclusion

In this paper, we validated 12 VvMPKs genes in the grapevine by cloning, and we revised their nomenclature according to international standards. We also investigated their expression in various tissues/organs at several developmental stages and in response to several biotic and abiotic stresses. Based on these treatments, we identified several grapevine MPK genes that are worthy of, and currently are under, further characterisation of their roles in grapevine growth and development and in their response to biotic and abiotic stresses.

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Supporting information

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Figure S1. Alignment between the nucleic sequences of each cloned VvMPK gene and the corresponding sequence predicted in the grapevine genome database. Sequence homologues were highlighted in black by the sequence alignment software while differences were highlighted in grey.

Figure S2. Alignment between the predicted amino acid sequences of each cloned VvMPK coding region and the corresponding protein sequence predicted in the grapevine genome database. Similar amino acid sequences were highlighted in black by the sequence alignment software, while differences were highlighted in grey. GS, genome sequence; C, cloned sequence.

Figure S3. Alignment of amino acid sequences from experimentally identified VvMPK coding regions. (a) Schematic representation of the domain organisation. The shaded boxes represent the 11 domains found in all serine/threonine protein kinases, whereas open areas indicate less conserved regions. (b) Multiple protein sequence alignment of 12 VvMPKs. Roman numerals indicate the 11 conserved domains (I–XI). The red box shows the Thr and Tyr residues in the TxY phosphorylation motif and CD domains. (c) Amino acid sequences of the conserved domain (CD) of the VvMPKs. Letters in bold red indicate the negatively charged amino acids in the common docking (CD) domain.

Table S1. Primer sequences used for polymerase chain reaction amplification of cDNAs.

Table S2. Primer sequences used for 3′ rapid amplification of cDNA ends.

Table S3. Primer sequences used in quantitative real-time polymerase chain reaction.

Table S4. The nucleic acid sequences of the cloned genes. The black colour represents 5′ and 3′ untranslated regions; red colour denotes the open reading frames of VvMPK genes.

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