Identification and Characterization of 12 Mitogen-activated Protein Kinase Genes Implicated in Stress Responses in Cherry Rootstocks

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ABSTRACT. Improving the poor resistance to environmental stress and the weak development of roots system in the cherry (*Prunus*) rootstock ‘Gisela 6’ (*Prunus cerasus* × *Prunus canescens*) is of great importance for sustainable sweet cherry (*Prunus avium*) production. Although a stable genetic transformation system has been developed for ‘Gisela 6’ rootstock, there is little information on the identification of genes involved in stress resistance. Using the cherry rootstock cultivar Gisela 6, we identified a total of 12 novel mitogen-activated protein kinase (MAPK) genes, designated *PcMPKs*. Phylogenetic analysis revealed that the *PcMPKs* could be divided into four groups, designated A, B, C, and D. In addition, an intron–exon structure analysis for the *PcMPKs* was conducted to help further understand the structure–function relationships within the cherry family. The expression profiles of *PcMPKs* in response to abiotic and biotic stresses were characterized using quantitative reverse transcription-polymerase chain reaction (qRT-PCR). Five *PcMPKs* (i.e., *PcMPK4-1*, *PcMPK4-2*, *PcMPK3*, *PcMPK6*, and *PcMPK18*) exhibited differential expression, and suggested their potential roles in plant responding to various stresses. This study provides the basis for further analysis on the physiological functions of *PcMPKs* in environmental tolerance in cherry rootstocks.

‘Gisela’ rootstock, a triploid hybrid of *P. cerasus* and *P. canescens*, is currently the most popular dwarf rootstock for sweet cherry (Koc et al., 2013; Usenik et al., 2010). However, the main disadvantages in this cultivar are the weak development of root systems and poor resistance to environmental stresses such as cold, drought, salinity, or pathogen infection. Conventional approaches for cherry rootstock improvement are generally difficult and occur over long time frames as a result of the heterozygosity and polyploidy of the cross. Genetic transformation with a single gene or multiple genes offers an attractive approach to efficiently improve the problems existing in this cultivar. Although a stable genetic transformation system has been developed for ‘Gisela 6’ (Song, 2014; Song and Sink, 2006; Song et al., 2013; Zhao and Song, 2014), little has been reported on gene identification for this cultivar.

MAPK cascades are highly conserved signal transduction modules that play crucial roles in regulating many biological processes in plants, such as growth, development, and responses to biotic and abiotic stresses (Ligterink, 2000; Ligterink and Hirt, 2001; MAPK Group, 2002; Mizoguchi et al., 2000; Nakagami et al., 2005; Zhang et al., 2006; Zwerger and Hirt, 2001). MAPKs are dual-specificity protein kinases that are activated by mitogen-activated protein kinase kinases through double phosphorylation of the Thr-x-Tyr (T-x-Y) motif in the activation loop (T-loop). Four subgroups, designated A, B, C, and D, have been identified based on phylogenetic analysis of MAPKs, and distinct functions in signal transduction within

cells in plants have been reported for each group (MAPK Group, 2002). Groups A–C, which possess a Thr-Glu-Tyr (T-E-Y) motif, have been studied in many plant species, including arabidopsis (*Arabidopsis thaliana*), tobacco (*Nicotiana tabacum*), and maize (*Zea mays*), and were reported to cause positive effects on stress tolerance during plant transformation with single genes under controlled conditions. For example, in arabidopsis, AtMPK3 and AtMPK6 from group A (Conroy et al., 2013; Droillard et al., 2002; Moon et al., 2003; Raghuram et al., 2015; Schikora et al., 2011; Wan et al., 2004) and AtMPK4 from group B (Ichimura et al., 2000, 2006; Roux et al., 2015) have been shown to play important roles in response to salt and cold stress and pathogen attacks. Ectopic expression of *ZmSIMK1*, a member of group B from maize, was reported to be involved in an improved tolerance to both biotic (e.g., *Pseudomonas syringae*) and abiotic stresses (e.g., salt and drought) in transgenic tobacco plants (Gu et al., 2010; Wang et al., 2014). Overexpression of another maize group A MAPK gene, *ZmMPK5*, in tobacco induced defense responses and conferred tolerance to salt and low-temperature stress in transgenic plants (Berberich et al., 1999; Zhang et al., 2014). These findings demonstrated the various roles of MAPK cascades in environmental stress responses and highlighted the potential application of MAPK genes for improving plant tolerance through biotechnology methods.

Because of the importance of MAPK cascades in plant resistance to biotic and abiotic stress, our goal was to identify and characterize MAPK genes in ‘Gisela 6’ to allow further work that would improve stress resistance in cherry rootstock through genetic engineering.

Materials and Methods

PLANT MATERIAL AND TREATMENT. In vitro plantlets of ‘Gisela 6’ were selected for qRT-PCR analysis. The plantlets

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were subcultured on Murashige and Skoog (MS) medium in the culture room held at 25 °C with a 16/8-h (light/dark) cycle for 6 weeks, and were then selected for different stress treatments. For osmotic and salinity stress challenges, *in vitro* plantlets of ‘Gisela 6’ were cultured in solid MS medium complemented with 200 mmol mannitol or 200 mmol NaCl, respectively. For the low-temperature treatment, the plantlets were cultured in solid MS medium in an artificial climate chest in which the temperature was held at 4 °C with a light/dark cycle of 16/8 h. For the salicylic acid (SA) and methyl jasmonate (MeJA) treatments, the plantlets were cultured in solid MS medium complemented with 150 µmol SA and 200 µmol MeJA, respectively. For *P. syringae* pv. *syringae* infection, single bacterial isolates were cultured on King’s B medium at 25 °C for 48 h. Before inoculation, bacterial cells were suspended in sterile tap water to produce a suspension containing 10⁸–10⁹ colony-forming units/mL. The whole plantlets were inoculated by dipping in the bacterial suspension and the cocultivation occurred at 25 °C with a 16/8-h (light/dark) photoperiod. For all stress challenges, the whole *in vitro* plantlets were collected at 2, 4, 6, 12, 24, 48, and 72 h, respectively, after treatment, immediately frozen in liquid nitrogen, and stored at –80 °C for RNA extraction. Plantlets cultured in the normal MS medium at 25 °C with a 16/8-h photoperiod served as the control for the qRT-PCR analysis. All samples included at least three independent biological repeats that were randomly selected from the vessels.

RNA EXTRACTION AND REVERSE TRANSCRIPTION. For total RNA extraction, frozen plantlets were crushed in liquid nitrogen with a mortar and pestle. RNA was extracted using the RN38-EASYspin Plus Plant RNA Kit (Aidlab, Beijing, China), in accordance with the manufacturer’s instructions. The first strand complementary DNA (cDNA) was obtained using HiFiScript Quick gDNA Removal cDNA Kit (CWBIO, Beijing, China), according to the manufacturer’s instructions.

CLONING AND IDENTIFICATION OF MAPKS FROM THE CHERRY ROOTSTOCK ‘GISELA 6’. A genome-wide identification of MAPK gene families in ‘Gisela 6’ rootstock was difficult due to the lack of genomic information for this cultivar. Thus, we searched and identified the MAPK candidate genes from a cDNA library of the ‘Gisela 6’ leaf samples based on the transcriptome sequencing, which was provided by a colleague in our group. Total RNA of the leaf samples taken from six

different branches of two independent 5-year-old ‘Gisela 6’ trees was pooled before library preparation. The library preparations were sequenced by Novogene Bioinformatics Technology Co. (Beijing, China) on an Illumina HiSeq 2000 platform and 100–base pair (bp) paired end reads were generated. A total of 50,919 transcripts were assembled and obtained using the Trinity software (Grabherr et al., 2011). A number of MAPK candidate genes were identified from the de novo transcriptome assembly database by BLASTx search. Target sequences that were more than 1000 bp in length, contained intact open reading frames (ORFs), and had greater than 60% homology to MAPK genes in other species were used as the templates for cDNA cloning. A total of 12 primer pairs were designed using Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA) to amplify the full-length open reading frame of MAPK genes from cDNA and genomic DNA of ‘Gisela 6’ (Supplemental Table 1). All the amplified products were cloned into pEASY-Blunt Simple Cloning Vectors (Transgen, Beijing, China) and sequenced (Sangon Biotech, Beijing, China). The sequences were designated as *PcMPKs* and submitted to GenBank.

SEQUENCE ANALYSIS OF THE MAPKS. Multiple alignments of the deduced MAPK amino acid sequences were performed using the Clustal W program (Larkin et al., 2007). A phylogenetic tree was constructed using the MEGA 6 software (Tamura et al., 2013). Because no reference genome was available for cherry, the gene structure analyses of the *PcMPKs* were generated from the alignments of the cDNA and genomic DNA amplicon sequences.

REAL-TIME qRT-PCR ANALYSIS. Primers for real-time qRT-PCR analysis were designed using Beacon Designer 8 software (Premier Biosoft) (Supplemental Table 1). Reactions were carried out on an ABI 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA) using the UltraSYBR mixture with Rox (CWBIO) following the manufacturer’s instructions. The sweet cherry house-keeping gene *β-actin* (accession no. FJ560908) was selected as the internal control to calibrate the cDNA template of the corresponding samples. PCR conditions were as follows: an initial 95 °C denaturation step for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 55 °C for 1 min. The specificity of the PCR amplification procedure was determined with a heat-dissociation protocol after the final PCR cycle. Each

Table 1. The information of MAPK family genes in ‘Gisela 6’ cherry rootstock.

Name	Accession no.	ORF size (bp)	MAPK group	Homologs	Regulated by					
					Mannitol	NaCl	Low temp	SA	MeJA	Bacterium infection
<i>PcMPK3</i>	KT357359	1113	A	AtMPK3	Up	Up	Up	Up	Up	Up
<i>PcMPK6</i>	KT357360	1224	A	AtMPK6	Up	Up	—	Up	—	Up
<i>PcMPK4-1</i>	KT357356	1131	B	AtMPK4/11	—	Up	Up	Up	—	Up
<i>PcMPK4-2</i>	KT357357	1122	B	AtMPK4/11	Up	Up	Up	Up	—	Up
<i>PcMPK13</i>	KT357358	1113	B	AtMPK13	—	—	Up	—	—	—
<i>PcMPK1</i>	KT357361	1119	C	AtMPK1/2	Down	Down	—	Up	—	—
<i>PcMPK7</i>	KT357362	1107	C	AtMPK7/14	—	Up	Up	—	—	—
<i>PcMPK9-1</i>	KT357363	1281	D	AtMPK9	Up	Up	—	—	—	—
<i>PcMPK9-2</i>	KT357364	1881	D	AtMPK9	—	—	—	—	—	—
<i>PcMPK16</i>	KT357365	1683	D	AtMPK16	—	Up	Up	—	—	—
<i>PcMPK20</i>	KT357366	1869	D	AtMPK20	Up	Up	Up	—	—	—
<i>PcMPK18</i>	KT357367	1830	D	AtMPK18/19	Down	—	Down	—	Down	Down

MAPK = mitogen-activated protein kinase; MeJA = jasmonic acid methyl ester; ORF = open reading frame; SA = salicylic acid.

measurement was carried out with at least three independent biological replicates, with PCR in triplicate to determine Ct values. Relative gene expression was analyzed according to the $2^{-\Delta\Delta Ct}$ method. A two-sample F test was performed to compare two population variances. $P \leq 0.05$ indicated that significant difference of the variation of the gene expression could be deduced.

Results

IDENTIFICATION AND NOMENCLATURE OF MAPKs GENES IN 'GISELA 6'. A total of 12 putative *MAPK* sequences with intact ORFs have been identified from the de novo transcriptome assembly of 'Gisela 6'. The sequence data were verified by PCR amplification from cDNA of 'Gisela 6'. The 12 target sequences met the following criteria: 1) significant homology with known MAPKs, 2) possessing 11 conserved subdomains in the amino acid sequence, and 3) possessing the activation loop with T-x-Y motif. Therefore, the target sequences were considered to be 'Gisela 6' MAPK genes. The nomenclature

and the molecular characterization of the 12 novel MAPK genes from 'Gisela 6' are detailed in Table 1.

MULTIPLE SEQUENCE ALIGNMENT, PHYLOGENETIC RELATIONSHIPS, AND GENE STRUCTURE ANALYSIS OF MAPK GENES IN 'GISELA 6'. To explore the conserved and divergent amino acid sequences of 'Gisela 6' MAPKs, multiple sequence alignment was performed with the deduced amino acids sequences using Clustal W. The 12 novel PcMAPKs possessed the 11 conserved domains that are known to be characteristic of plant MAPKs. The activation loop containing a T-x-Y motif, which was considered the phosphorylation site for MAPK activity, was found between domains VII and VIII in all of the 12 PcMPKs. Seven PcMPKs carried a Thr-Glu-Tyr (T-E-Y) motif, whereas five PcMPKs carried a Thr-Asp-Tyr (T-D-Y) motif. The TDY domain-containing MPKs also had an extended C-terminal region, which is similar to MPKs from other plant species (Fig. 1).

To evaluate the evolutionary relationship of PcMPKs and other plant MAPKs, a phylogenetic tree was constructed using the MEGA 6 software with the neighbor-joining method. The

Fig. 1. Multiple alignment of PcMPKs amino acid sequences. The alignment was performed using the Clustal W program (Larkin et al., 2007). The characteristic regions containing 11 domains (I–XI), the activation loop, and Thr-x-Tyr (TXY) motif are presented in serine/threonine protein kinase and marked on the top of each row. The 12 novel PcMAPKs possessed the characteristic regions of plant mitogen-activated protein kinase (MAPKs). Seven PcMPKs carried a Thr-Glu-Tyr (T-E-Y) motif, whereas five PcMPKs carried a Thr-Asp-Tyr (TDY) motif.

12 novel *PcMPKs* could be divided into four major groups (groups A–D). Among them, two *PcMPKs* were classified into each of group A and group C, and three were classified into group B. Group D, which possessed a TDY motif, contained the most MAPKs homologs (five members) (Fig. 2); a similar distribution of MAPKs homologs has been observed in other plant species including arabidopsis and tobacco (MAPK Group, 2002; Zhang et al., 2013b). For example, eight of 20 *AtMPKs* were classified as group D in arabidopsis, and six of 17 *NtMPKs* were classified as group D in tobacco. As mentioned above, *PcMPKs* in group D were notable for their TDY motif in the activation loop and an extended C-terminal region.

To understand duplication events within the MAPK gene family, full-length cDNA sequences were compared with the corresponding genomic DNA sequences to reveal the exon/intron structures of each *PcMPK* gene. The number of introns varied from one (group C members) to nine (*PcMPK16* from group D) (Fig. 3). Previous studies showed that most genes within the same subfamily shared a similar exon/intron structure (Cakir and Kilickaya, 2015; MAPK Group, 2002; Zhang et al., 2013a, 2013b). Similar results were observed here. For example, *PcMPK1* and *PcMPK7* from group C possessed only one intron, whereas *PcMPK4-1*, *PcMPK4-2*, and *PcMPK13* from group B, each contained five introns. However, the length of the introns and exons were quite different even between members of the same group. Some modest differences in organization were observed between *PcMPK3* and *PcMPK6*, both of which were classified into group A. For example, *PcMPK3* contained five introns, whereas *PcMPK6* contained four introns. *PcMPKs* from group D included the most complex gene structures, containing eight or nine introns and possessing large differences in the length of genomic sequences between group members.

EXPRESSION PROFILES OF *PcMPKs* IN RESPONSE TO STRESS STIMULI, SIGNAL MOLECULES, AND PATHOGEN INFECTION. To identify the candidate genes potentially involved in responses to abiotic or biotic stresses, we analyzed the expression profiles of *PcMPKs* following exposure to environmental stress (osmotic, salinity, or low temperature), exposure to defense signaling molecules (SA and MeJA), and pathogen infection (*P. syringae* pv. *syringae*).

A total of eight *PcMPK* genes exhibited changes in expression following mannitol treatment (Fig. 4A). Among them, five *PcMPKs* (*PcMPK4-2/3/6/9-1/20*) were upregulated, with *PcMPK4-2*, *PcMPK3*, and *PcMPK6* being especially highly induced at 6 to 12 h after treatment. The remaining three genes (*PcMPK1/7/18*) showed downregulated expression at different levels under mannitol treatment.

Following exposure to NaCl treatment, eight *PcMPKs* (*PcMPK4-1/4-2/3/6/7/9-1/16/20*) showed significant changes in expression (Fig. 4B). Inductive effects caused by NaCl showed different patterns across genes, with either an initial increase followed by a slight decrease (e.g., *PcMPK7* and *PcMPK20*) or increased levels that were maintained throughout the treatment (e.g., *PcMPK4-1/4-2/3/6*). *PcMPK1* displayed an obvious downregulation following NaCl treatment; similar suppression of gene expression was also observed during treatment with mannitol (Fig. 4A).

Seven *PcMPKs* (*PcMPK 4-1/4-2/13/3/7/16/20*) were induced by low temperature (Fig. 4C). The expressions of *PcMPK13*, *PcMPK3*, *PcMPK16*, and *PcMPK20* were increased significantly and maintained at a relatively high level

Fig. 2. Phylogenetic analysis of the *PcMPKs*. A phylogenetic tree was constructed based on the complete protein sequence alignment of mitogen-activated protein kinase (MAPKs) in arabidopsis, tobacco, and ‘Gisela 6’ cherry rootstock using the neighbor-joining method with bootstrapping analysis. Different gene clusters are denoted as A–D. The subgroups are marked by the black lines. Among the 12 novel *PcMPKs*, two were classified into each of group A and group C, and three were classified into group B. Group D contained the most MAPKs homologs (five members).

during the entire temperature experiment. *PcMPK4-1*, *PcMPK4-2*, and *PcMPK7* were moderately induced. The expression of *PcMPK18* was suppressed immediately after treatment and then recovered slowly to the control levels.

To explore whether MAPK cascades were responsive to *P. syringae* pv. *syringae* infection, we inoculated ‘Gisela 6’ plantlets with *P. syringae* pv. *syringae* and analyzed the expression profiles of *PcMPKs*. *PcMPK4-1* was greatly induced when plants were infected with the pathogen (Fig. 4D). The expression of *PcMPK4-2*, *PcMPK3*, and *PcMPK6* were also stimulated substantially after pathogen exposure. In

Fig. 3. The exon/intron structure of 'Gisela 6' cherry rootstock *PcMPKs* genes. Exons and introns of the genes are indicated by gray boxes and black lines, respectively. The size of exons and introns is given inside the boxes and on top of the lines. Among the 12 novel *PcMPKs*, the number of introns varied from one (group C members) to nine (*PcMPK16* from group D). Most members within the same subgroup shared a similar exon/intron structure.

contrast, the expression of *PcMPK18* was sharply suppressed after pathogen infection, and the expression of *PcMPK13* was also slightly decreased.

SA and MeJA are two important signaling molecules involved in plant defense against pathogen infection. To explore more candidate loci implicated in response to potential biotic stress, we analyzed the expression profiles of *PcMPKs* treated with SA and MeJA (Fig. 4E–F). *PcMPK4-1*, *PcMPK4-2*, *PcMPK3*, *PcMPK6*, and *PcMPK1* were upregulated 2- to 4-fold over the controls under SA treatment. *PcMPK3* was also induced significantly by MeJA. However, the expression of *PcMPK13* and *PcMPK18* were downregulated when treated with SA, with a mild recovery 24 h after treatment. These two genes were also downregulated slightly by MeJA. Notably, both of them were suppressed by *P. syringae* pv. *syringae* infection, as shown in Fig. 4D. These results indicate that *PcMPK13* and *PcMPK18* may respond to pathogen infection in a different way from those candidates that were upregulated, such as *PcMPK3* and *PcMPK6*.

Discussion

The engineered MAPK cascades were proposed to be the potential application for improving plant stress tolerance (Moustafa, 2014a, 2014b). To date, a conserved number of MAPK families have been identified and functionally characterized in arabidopsis, tobacco, grape (*Vitis vinifera*), apple (*Malus domestica*), etc. (Cakir and Kilickaya, 2015; MAPK Group, 2002; Zhang et al., 2013a, 2013b). However, no data on MAPK were available for either sweet cherry or close-related species. A genome-wide identification of MAPK genes family in 'Gisela 6' rootstock was difficult due to the lack of genomic information for this cultivar. Herein, a total of 12 novel MAPKs were isolated and analyzed from cherry rootstock 'Gisela 6'. Although we could not identify MAPK families at a genome-wide scale in this study, the whole genome assembly for peach (*Prunus persica*) may offer some hints for analysis. A total of

15 MAPK candidates were identified from the released phytozome, which included the gene annotation of 225.7 Mb arranged in eight chromosomes (Verde et al., 2013). Therefore, due to the conserved number of MAPKs in other plants, it is likely that we have identified most, if not all, of the MAPKs in 'Gisela 6'.

Phylogenetic relationship analysis showed that *PcMPK3* and *PcMPK6* were homologs of *AtMPK3* and *AtMPK6*, which are the most extensively studied MAPKs in arabidopsis, and are involved in plant resistance (Conroy et al., 2013; Droillard et al., 2002; Moon et al., 2003; Raghuram et al., 2015; Schikora et al., 2011; Wan et al., 2004). Their homologs in tobacco, WIPK and SIPK, have also been shown in many studies to regulate response to abiotic and biotic stresses (Kallenbach et al., 2010; Katou et al., 2005; Kim et al., 2011; Kobayashi et al., 2010; Sharma et al., 2003; Zhang and Klessig, 1998). *AtMPK4*, another well-studied MAPK in arabidopsis, was associated with response to low temperature, drought, wounding injury, and pathogen infection (Ichimura et al., 2006; Roux et al., 2015). As shown in Fig. 2, *PcMPK4-1* and *PcMPK4-2* were clustered into the same subgroup as *AtMPK4* in the phylogenetic tree. Because MAPK genes with the same function typically cluster into the same subgroup, the phylogenetic analysis may provide a basis for exploring the functions of *PcMPKs*. It is reasonable to speculate that *PcMPK3*, *PcMPK6*, *PcMPK4-1*, and *PcMPK4-2* may be involved in stress responses in 'Gisela 6'.

In addition, this study reported the intron–exon structures of *PcMPK* genes. Because no genomic sequences are currently available, we identified and sequenced each *PcMPK* gene from plant total cDNA and genomic DNA separately and aligned these sequences to determine the intron–exon structure for each gene. The purpose of the structure analysis was not only to compare the intron–exon splicing with that in other plant species, but also to provide sequence references for the identification of the regulatory regions of genes with important functions in stress resistance.

Fig. 4. Expression profiles of *PcMPKs* in response to (A) mannitol, (B) salinity, (C) low-temperature treatments, (D) *Pseudomonas syringae* pv. *syringae* infection, (E) salicylic acid, and (F) jasmonic acid methyl ester stimuli. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was used to detect the mRNA levels of *PcMPKs* under the experimental treatments. The constitutive gene β -actin was used as a reference. Each measurement was carried out with at least three independent biological replicates, with PCR in triplicate to determine Ct values. Relative gene expression was analyzed according to the $2^{-\Delta\Delta C_t}$ method. Error bars represent the SE of at least three independent biological replicates.

Because gene expression patterns can provide important clues to gene function, we conducted real-time qRT-PCR analysis to reveal the expression profiles of the 12 *PcMPK* genes in response to several environmental stresses, signaling molecules, and potential pathogen infection. In our study, *PcMPK3* and *PcMPK6* were significantly induced by most of the treatments, including exposure to mannitol, NaCl, a bacterial pathogen, SA and MeJA (Fig. 4). Similar expression profiles were found in their orthologs *AtMPK3/AtMPK6* in arabidopsis. *AtMPK3* has been shown to be particularly strongly associated with numerous abiotic and biotic stress responses in arabidopsis. For example, *AtMPK3/AtMPK6* and their upstream kinase *AtMKK4/AtMKK5* were activated by various biotic and abiotic stimuli (Colcombet and Hirt, 2008; Pitzschke et al., 2009; Rodriguez et al., 2010; Samajova et al., 2013; Sinha et al., 2011). Both *AtMPK3/6* and *AtMKK4/5* have overlapping functions. Mutant *AtMKK4s* that were defective in the *AtMPK3/6*-upstream regulatory MAPK kinase were less tolerant to osmotic stress. In contrast, *AtMPK3/6* hyperactivation via *AtMKK4* overexpression enhanced stress tolerance (Kim et al., 2011). Given the expression profiles of *PcMPK3/6* in response to stress and the previous reports of orthologs in other plant species, it was reasonable to speculate that these two MAPK members may be involved in the cross-talk node of different signaling transduction pathways. *PcMPK3* and

PcMPK6 could act as a potential “regulating switch” during rewiring of MAPK transgenic circuits to improve plant stress tolerance.

In addition, *PcMPK4-1* and *PcMPK4-2* were also induced by NaCl, low temperature, or bacterial infection. Similar characteristics were found for *AtMPK4*, the ortholog of *PcMPK4-1/4-2* in arabidopsis. *AtMPK4* was reported to be activated by low temperature, low humidity, hyperosmolarity, and wounding (Ichimura et al., 2000); *AtMPK4* also appears to play a role in negatively regulating immune responses (Berriri et al., 2012; Gao et al., 2008; Kong et al., 2012) and in hyperosmolarity resistance (Droillard et al., 2004) in arabidopsis. These studies suggest that *PcMPK4-1* and *PcMPK4-2* may play a potential role in plant defensive responses and resistance to osmotic stress or other environmental stresses.

Finally, *PcMPK18* was especially interesting due to its significant negative expression profile in response to stress treatments. As shown in Fig. 4, the expression of *PcMPK18* was sharply suppressed throughout the mannitol treatment. When treated with low temperature, the gene expression level decreased rapidly, but recovered slowly over the 24 h after treatment. *Pseudomonas syringae* pv. *syringae* was reported to induce a number of important diseases in *Prunus* species. It caused a variety of symptoms in host plants, such as flower blast, dead dormant buds, shoot-tip dieback, and stem cankers

(Qiu et al., 2008; Spotts and Cervantes, 1995). Infection with *P. syringae* pv. *syringae* also caused significant suppression of *PcMPK18*. The transcripts of *PcMPK18* were rapidly reduced to $\approx 1/10$ of that in control samples 2 h after treatment. Exogenous SA molecules may also suppress the expression of *PcMPK18* at the beginning of treatment. However, expression increased slightly 24 h after the signaling molecule application. Although group D MAPKs were notable for their TDY motif and extended C-terminal sequences, no additional details about gene function could be obtained in the present study. Thus, it will be interesting to explore the potential roles that *PcMPK18* plays in stress resistance.

Herein, we presented a comprehensive analysis of 12 novel MAPK genes in the cherry rootstock 'Gisela 6', with an emphasis on the potential functional characterization of stress responses. *PcMPK4-1*, *PcMPK4-2*, *PcMPK3*, *PcMPK6*, and *PcMPK18* were revealed to be potentially involved in plant resistance to environmental stress. This study may provide the basis for further analysis on the physiological functions of *PcMPKs* in environmental tolerance in cherry rootstocks.

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Supplemental Table 1. Sequences of primers used for PCR amplification and qRT-PCR analysis of *PcMPKs*.

Gene name	Primer sequences
Primers for gene amplification of <i>PcMPKs</i>	
<i>PcMPK4-1</i>	Fwd: TCGAGAATCACGGGCACCAT Rev: ATCTACAGCCCCAAACAAACG
<i>PcMPK4-2</i>	Fwd: TATTCTCCTCCATCATTAGCG Rev: AATCCAATCACTCCAGCACA
<i>PcMPK13</i>	Fwd: TCCACCAGCTTTTCTAAATT Rev: GAAATTAGATCGCTGCTGAC
<i>PcMPK3</i>	Fwd: ACGGACTTGCACAGTGTATT Rev: CATAGGTATAGAAACCGAGTA
<i>PcMPK6</i>	Fwd: TTCTCACTGCCATTACTACAA Rev: ACCAAACAAACGGAACATAG
<i>PcMPK1</i>	Fwd: TAGGGCTTGCGTCTGTGAAT Rev: GCTGGCACCATAACAAAGAG
<i>PcMPK7</i>	Fwd: GCCTCTTACTTGTCTCCACG Rev: TGTTGAGGAACTGGAAAGC
<i>PcMPK9-1</i>	Fwd: TCCTTCGGCTGCTACATCAC Rev: AAATGAAGCCATGCCCTTGT
<i>PcMPK9-2</i>	Fwd: ACAGGAGCGACCATTGAACC Rev: GGACTGTTACAGAGCACCTT
<i>PcMPK16</i>	Fwd: ACCAAACGCTCTTCAGGTAG Rev: CTATTGCTAAACTTCCTCC
<i>PcMPK20</i>	Fwd: TGTGGGCTTGTGATAAGGAG Rev: ACCCGACTCTTTGAATGCTC
<i>PcMPK18</i>	Fwd: CTTGTCATTTCGGAGTGGGTCT Rev: TACAATCACTCCACCCTCCC
Primers for qRT-PCR analysis of <i>PcMPKs</i>	
<i>PcMPK4-1</i>	Fwd: GGCTTATCACAGAGTTAA Rev: AACACTAATGCGTCTATG
<i>PcMPK4-2</i>	Fwd: GTTGCCATTAAAGAAGATTG Rev: TGTAGACATCATTGAAGTT
<i>PcMPK13</i>	Fwd: CGTATAACTGTTGAGGAA Rev: GTTGAAGTGTAGAGACTC
<i>PcMPK3</i>	Fwd: GAGTATGCTTGATTGAAA Rev: CCACCTAAGTAATGATGTA
<i>PcMPK6</i>	Fwd: ATATTCAGCAACTTCCTC Rev: ATCTTCAACTGTAATTCTCT
<i>PcMPK1</i>	Fwd: AAGTTGCGATTAAGAAGA Rev: CTCATACACCAAGTAGAC
<i>PcMPK7</i>	Fwd: GGATGTTATGATGCCTAT Rev: TGAGTGAAGATACTTGAG
<i>PcMPK9-1</i>	Fwd: TTGAGTTGATGGAATCTG Rev: AGTCTGAATTAGCAAGTATA
<i>PcMPK9-2</i>	Fwd: AAACAATGATGTGGAAAG Rev: TCTTACACCTATACACTTC
<i>PcMPK16</i>	Fwd: AACCAGAAAGATGTAAGA Rev: ATAGTGCTCCTCAAGATA
<i>PcMPK20</i>	Fwd: CCAGTTATCCACTTGAA Rev: ATTCTTGTATGCCTCTTC
<i>PcMPK18</i>	Fwd: TCTAAGACATCCTGATATTG Rev: CCTTGATAACTTGGTGAA
β -actin	Fwd: CCTGGACTATGAGCAAGAAC Rev: AATGAGTGATGGTTGGAAGAG

Fwd = forward primer; PCR = polymerase chain reaction; qRT-PCR = quantitative reverse transcription polymerase chain reaction; Rev = reverse primer.