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CLONING, CHARACTERIZATION, AND EXPRESSION ANALYSIS OF TWO *MAPKKK* GENES IN *CHRYSANTHEMUM*

REN LIPING[1,2#], YIN DANDAN[1#], WAN WENYANG[1], FANG TINGTING[1], SU XIAOHUI[1] AND CAO XIAOHAN[1*]

[1]*Key Laboratory of Horticultural Plant Biology of Biological and Food Engineering School, Fuyang Normal University, Fuyang, Anhui 236037, China*
[2]*Fuyang Academy of Agricultural Sciences, Fuyang, Anhui 236000, China*
[#] *Contributed equally to this work*
[*]*Corresponding author's email: caoxiaohan@163.com*

Abstract

MAPKKK (mitogen-activated protein kinase kinase kinase) genes are involved in plant growth and stress responses.The expression profiles of *MAPKKK* genes in response to various stimuli, including high temperature, drought, and salt, as well as different hormones, were investigated in this study using a *Chrysanthemum morifolium* variety called 'Jinba' (such as abscisic acid, methyl jasmonate, salicylic acid and ethylene). According to our previous transcriptome data, we identified and cloned two *MAPKKK* genes with complete open reading frames. These two MAPKKK genes were studied in bioinformatics and expression models in response to various stimuli. Both of these genes belonged to the *MEKK* subfamily, according to cluster analysis. They were most abundantly expressed in the leaves, according to expression analysis, and their levels of expression were subjected to treatments. Our findings suggest that *CmMAPKKK* genes may be involved in multiple stress responses and hormone responses, which will facilitate our future researches on their functions.

Key words: *Chrysanthemum morifolium*; *MAPKKK*; Stress; Hormone treatment; Gene expression.

Introduction

MAPK signal transduction pathway is a complex and highly conserved cellular signal transduction pathway that mainly consists of three protein kinases, *MAPKKK*, *MAPKK* and *MAPK*, all three of which are sequentially phosphorylated and each kinase has numerous members. Plants can react quickly and effectively to a variety of external stimuli because of this sequential phosphorylation (Sun *et al*., 2014; Song *et al*., 2015). The MAPK protein family is a complex family of serine/threonine kinases that can phosphorylate a variety of substrates, including transcription factors, protein kinases, and cytoskeleton-associated proteins (Zhang *et al*., 2014). Many cellular processes, including growth, proliferation, development, differentiation, programmed cell death, stress responses, and signal transduction, rely on this cascade reaction (Kumar *et al*., 2020b). *MAPKKK* (also known as *MEKK* or *MKKK*) is the most upstream of the MAPK cascade pathway and is responsible for receiving external signals and transmitting them to downstream MAPKK, thus regulating plant defence responses, such as saline, drought and low temperature, which consists of the defence signalling pathway in plants (Nakagami *et al*., 2004; Pedley & Martin, 2005; Song *et al*., 2015; Li *et al*., 2016; Jiao *et al.*, 2017). In *Arabidopsis thaliana*, the *MAPKKK* family is divided into three subfamilies, *MEKK*, Raf, and ZIK (Wang *et al*., 2020). *MAPKKK* has been found to be involved in the response to stress and hormonal treatments in plants such as *Oryza sativa* (Na *et al*., 2019), *Malvaceae Gossypium* (Yin *et al*., 2021), *Triticum aestivum* (Kumar *et al*., 2020a), *Nicotiana tabacum* (Shou *et al*., 2004), *Medicago sativa* (Nakagami *et al*., 2004), *Musa nana* (Hu *et al*., 2016), implying that it may be a key gene involved in the stress tolerance phase in plants. At present, bioinformatics analysis of *MAPKKK* genes in *Chrysanthemum morifolium* and their response to adversity stresses and hormones have not been reported.

Chrysanthemum is one of the top ten traditional famous flowers in China and the four major cut flowers in the world. There is a high demand in the market, but *Chrysanthemum* s are susceptible to a variety of biotic and abiotic stresses during their growth, which affects their ornamental and economic values, impeding the industry's safe and long-term production (An *et al*., 2014). The material used in this study was *C. morifolium* variety 'Jinba', and two *CmMAPKKK* genes were obtained through gene cloning. Bioinformatics analysis of these two genes were performed, and then the expression pattern of *CmMAPKKK* genes in response to stress and hormone treatments were analyzed by RT qPCR. The results may help to investigate the functions of *CmMAPKKK* genes, which will facilitate the understanding of the key point that causes growth retardation and quality reduction in *Chrysanthemum* exposed to environmental stresses such as low temperature and drought stresses as well as hormone treatment.

Materials and Methods

Plant materials: Cuttings of the cut flower *Chrysanthemum* cultivar 'Jinba', provided by the Main Laboratory of Horticultural Plant Biology of School of Biology and Food Engineering, Fuyang Normal University, were chosen with consistent growth for rooting. After 14 days, the cuttings were transplanted into plastic bottles containing vermiculite, perlite, and nutrient soil (1:1:1). The seedlings were placed in a chamber under the following environmental conditions: temperature of 22°C, the light intensity of 100 $\mu mol \cdot m^{-2} \cdot s^{-1}$, and light/ dark time of 16h/8h. Plants at 6-8 leaf age with uniform growth were picked for use.

Plant treatments: For high and low-temperature stress treatments, both experimental and control groups were incubated in an incubator with a photoperiod of 16h/8h and light intensity of 50μmol·m^{-2}·s^{-1}. The high temperature was set at 40°C, while the low temperature was set at 4°C, and the control group (CK) was set at 22°C (Song *et al*., 2014a). For hormone treatment, the experimental group was sprayed with 50 μmol/L abscisic acid (ABA) (Felipe *et al*., 2010), 1 mmol/L methyl jasmonate (MeJA) (War *et al*., 2013), 200 μmol/L salicylic acid (SA) (Kazemi *et al*., 2017), 50 mg/L ethylene (ethephon, ETH) (Miki *et al*., 2006). The control group sprayed with equal volume sterile water containing 8 ml absolute ethanol. After incubated in pure water for 3 days, both experimental and control groups were subject to drought and salt stress treatments. The drought stress group was treated with 20% (W/V) polythene glycol 6000(PEG6000) (Song *et al*., 2012) and the salt stress group was treated with 200 mmol/L sodium chloride (NaCl) (Song *et al*., 2014b). Three biological replicates were set to minimize the errors. Samples were picked at 0, 1, 2, 4, and 8 h after treatments, the second true leaves were collected at various time points and frozen in liquid nitrogen, and then storied at -80℃ for test.

Cloning of *CmMAPKKK* genes: Total RNA extraction was performed according to manufactures instructions (RNA Extraction Kit 3.0, Huayueyang Biotech, Beijing). The cDNA was synthesized by using RNA as a template according to the spark script Ⅱ RT plus Kit (with gDNA eraser) (sparkjade). The *MAPKKK* orthologs were identified based on the results of our previous transcriptome, and the predicted *CmMAPKKK* ORF sequence was used to design primers (Table 1). The PCR reaction system was set as follows: 1 μL cDNA as template, 2 μL of each sense and anti-sense primers (10 μmol·L^{-1}), 25 μL of 2×One Step Mix, 2.5 μL of One Step Enzyme Mix, ddH_2O was added to make final volume as 50 μL. The PCR reaction program was set as follows: pre-denaturation at 94°C for 3 min, 30 cycles of denaturation at 94°C for the 30 s , annealing at 55°C for 30 s, and extension at 72°C for 1 min, with a final extension at 72°C for 5 min. After PCR, DNA products were collected using a kit and then ligated into the pMD19-T vector (Takara Co, Ltd. Japan). The recombinant was transferred into DH5α competent cells, and the positive clones were picked for sequencing.

Bioinformatic analysis of *CmMAPKKK* genes: Sequences were aligned using the online BLSAT program in GenBank. Homology analysis of sequences was performed using DNAMAN 6.0 and MUSCLE software (Edgar, 2004). Phylogenesis analysis was built using Mega 7.0, with a bootstrap of 500 (Sudhir *et al*., *2004*). ProtParam Tool and ProtScale were used to predict the physicochemical and hydrophilic properties of the proteins, respectively. NetPhos3.1 was used to predict the potential phosphorylation site of the proteins. SWISS-MODEL and PyMOL were used to predict the tertiary structure of the proteins (Schiffrin *et al*., 2020).

qRT-PCR analysis: Primers for RT-qPCR were developed using primer premier 5.0 based on the *CmMAPKKK* gene sequence (Table 1). qRT-PCR was performed using a Roche (LightCycler®480II system), according to the SYBR Green method in a 25μL reaction system. The procedure was set as follows: 94°C for 3 min, 94℃ for 20 s, 55°C for 20 s, 72°C for 30s, 40 cycles. To reduce the error, three biological replicates were made. *CmEF1α* was used as an internal reference gene. The relative gene expression level was calculated according to a $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001).

Results and analysis

Cloning of *CmMAPKKK* gene and its protein structure analysis: Based on our previous transcriptome data, CmMEKK3 and CmMEKK21 were cloned with ORFs of 1941 BP and 1008 BP, respectively (Fig. 1). The *CmMEKK3* gene encodes a putative protein containing 647 amino acids. Its isoelectric point (PI) is 9.16, and its molecular weight is 70275.44KD. It has an average hydrophilicity coefficient of -0.61795, representing a hydrophobic protein (Fig. 2A). There were 147 potential phosphorylation sites, of which 89 were Ser phosphorylation sites, 39 were Thr phosphorylation sites, and 19 were Tyr phosphorylation sites (Fig. 3A). The *CmMEKK21* gene encodes a protein containing 336 amino acids, with an isoelectric point (PI) of 5.35, and a molecular weight of 36884.96KD. The protein has an average hydrophilicity coefficient of -0.26602, representing a hydrophobic protein (Fig. 2B). There were 57 potential phosphorylation sites, of which 38 were Ser phosphorylation sites, 12 were Thr phosphorylation sites, and 7 were Tyr phosphorylation sites (Fig. 3B). SWISS-MODEL was used to predict the tertiary structure of both proteins, and the results are shown in Fig. 4. Both proteins were predicted to be extracellular based on their subcellular localization.

Table 1. Sequences of primers in this study.

Gene	Primer F (5'-3')	Primer R (5'-3')	Usage
CmMEKK3	ATGCCTGCTTGGTTTGGTAAAAATCATCA	TTAAATGAGCCGTGACCTTGGGGATCTGAT	Amplification of full-length primer
CmMEKK21	ATGGAGTGGGTACGAGGTAAAAAAATTGGT	TCATCTTACTTTTAACCAACTGCTGTTAAC	Amplification of full-length primer
CmMEKK3	TTTACGACCCCCAGCCTC	ACTCGCAGTTGGGGACAC	qRT-PCR primer
CmMEKK21	GGCGGTTGGATGTTTGG	CGATTGCTCCGATTCCCA	qRT-PCR primer
EF1α	TTTTGGTATCTGGTCCTGGAG	CCATTCAAGCGACAGACTCA	reference gene primer

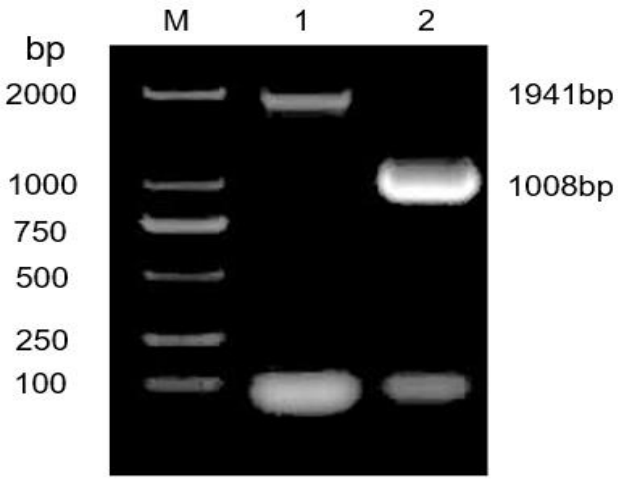

Fig. 1. The PCR product of *CmMAPKKK* genes. (DL2000 marker; 1. CmMEKK3; 2. CmMEKK21).

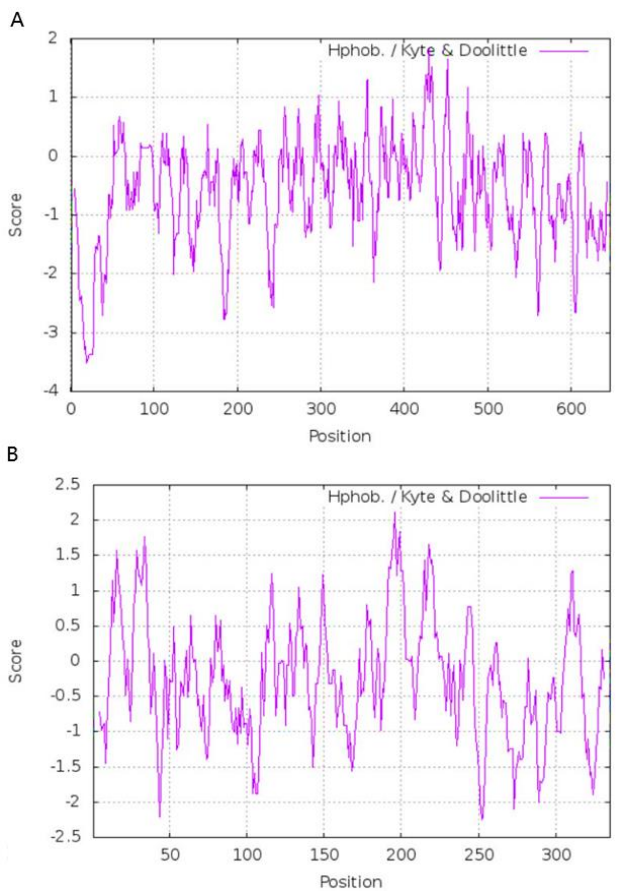

Fig. 2. The hydrophilicity and hydrophobicity of CmMEKK3 and CmMEKK21 proteins.

Homology analysis of CmMAPKKK proteins: The results showed that both CmMEKK3 and CmMEKK21 were clustered into the MEKK subfamily in Arabidopsis, based on multiple sequence alignment and phylogenetic analysis of the screened two CmMAPKKK genes with MAPKKK genes from other organisms. CmMEKK3 is similar to LsMAPKKK3 (*Lactuca sativa*), and CmMEKK21 is similar to HaMAPKKK1 (*Helianthus annuus*), as shown in Fig. 5, implying that they have higher homology.

Differential expression patterns of *CmMAPKKK* genes in different organs: As shown in Fig. 6A, *CmMEKK3* was less expressed in all organs of the *Chrysanthemum* than *CmMEKK21*. *CmMEKK3* was least expressed in flowers, while its expression in roots, stems, and leaves were 1.67-, 1.23-, and 2.48-fold higher than that in flower, respectively. CmMEKK21, on the other hand, had the lowest expression in stems and the highest expression in leaves. There was 32.65 times as many in leaves as there was in stems. There is no discernible difference in language between roots and flowers (Fig. 6B).

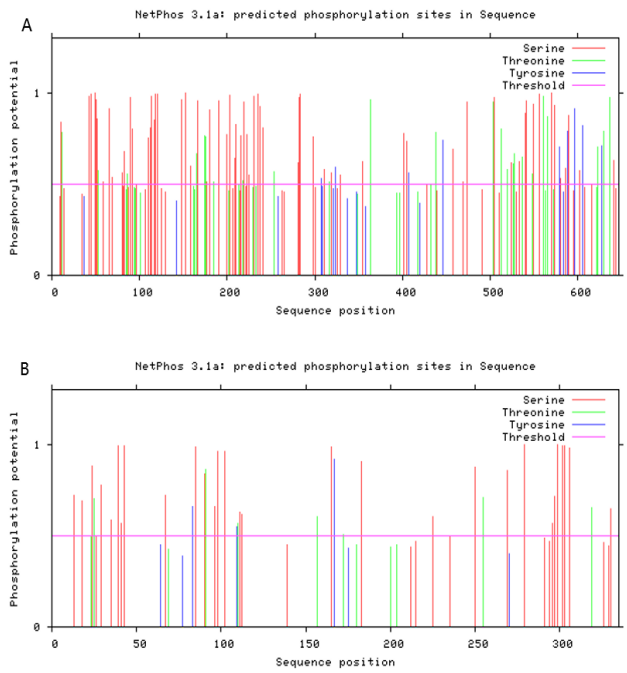

Fig. 3. The predicted phosphorylation sites of CmMEKK3 and CmMEKK21 proteins.

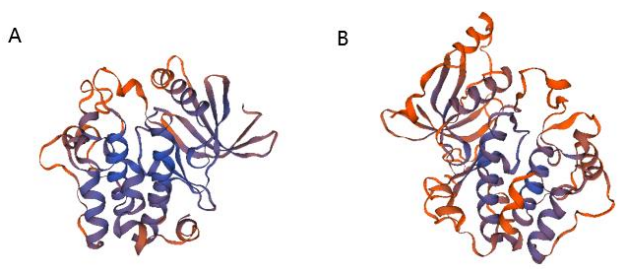

Fig. 4. The prediction of the tertiary structure of CmMEKK3 (A) and CmMEKK21 (B) proteins.

Expression profiles of *CmMAPKKK* genes under abiotic stresses: As shown in Fig. 7A, under 40°C treatment, the expression of *CmMEKK3* was not changed at 1h. It slightly increased at 2 h, and decreased after 4 h, while it remained almost unchanged subsequently. *CmMEKK21* expression, on the other hand, began at 1 h, decreased from 2-4 h, and then increased at 8 h after treatment. CmMEKK21 was 8.69 times more abundant than in the control group. As shown in Fig. 7B, after 2 hours of 4°C treatment, *CmMEKK3* expression continued to grow, reaching 3.23-fold that of the control group after 8 hours; however, expression showed an initial increase,

followed by a decrease. It was induced for 1 hour, then down-regulated for 2-4 hours before increasing for 8 hours. The abundance was 1.82-fold higher than that of the control at 8h. The expression level of *CmMEKK3* under prolonged high-temperature treatment was lower than that under low-temperature treatment, whereas *CmMEKK21* was apposite. As shown in Fig. 7C, CmMEKK3 was slightly expressed at 4h after drought treatment, which was 1.04-fold that of the control group. It was down-regulated at 8h. However, CmMEKK21 was not expressed under drought stress. As shown in Fig. 7D, *CmMEKK3* expressed 4 hours after salt treatment, and the level of expression at 8 hours was 1.71 times higher than in the control group, while *CmMEKK21* did not. *CmMEKK3* expression was significantly higher in the salt treatment than in the drought treatment. The expression level of *CmMEKK21* in salt treatment was significantly higher than that in drought treatment.

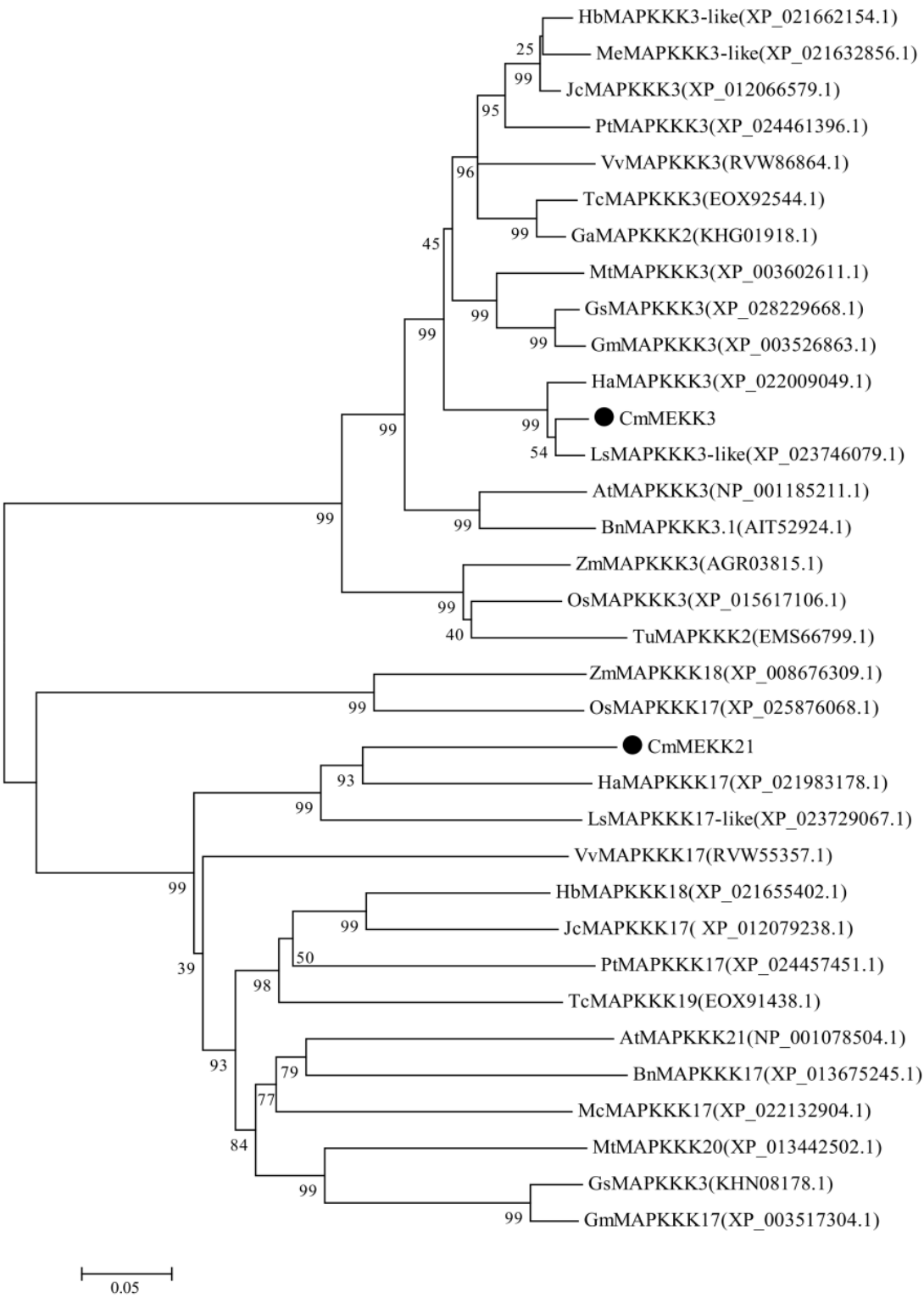

Fig. 5. Phylogenetic analysis of of CmMEKK3, CmMEKK21 and other MAPKKKs.

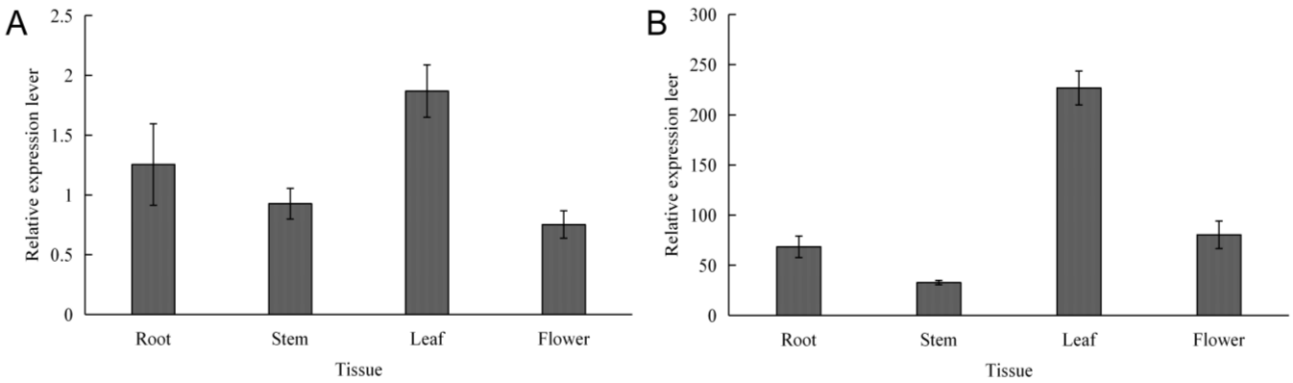

Fig. 6. Expression patterns of CmMEKK3 (A) and CmMEKK21 (B) in different organs/tissues obtained by qRT-PCR analysis.

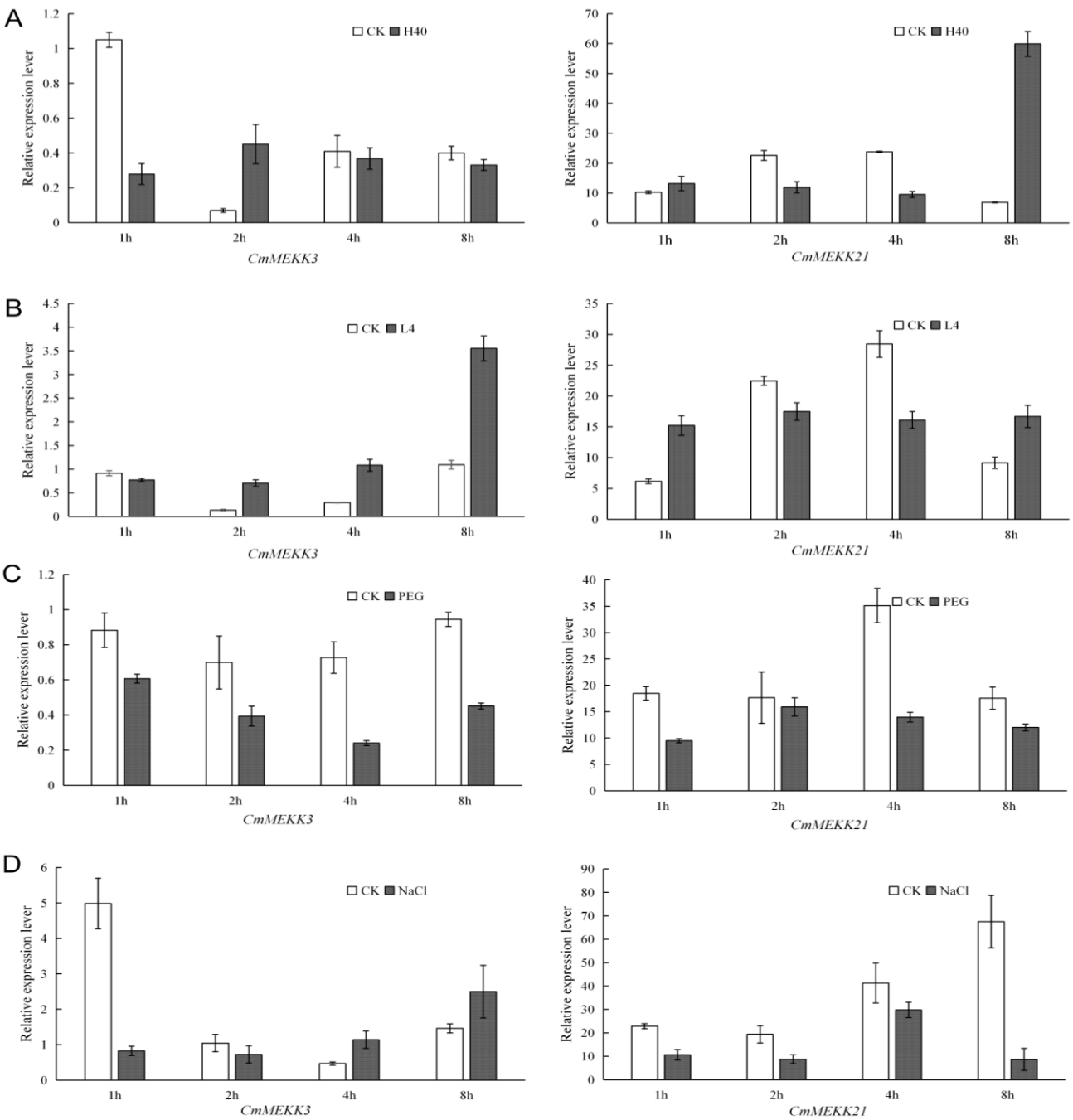

Fig. 7. Differential expression patterns of the *CmMAPKKK* genes in leaves to abiotic stress.
(A) high temperature treatment with 40℃; (B) low-temprature treatment with 4℃; (C) drought treatment by PEG; (D) salt treatment by NaCL

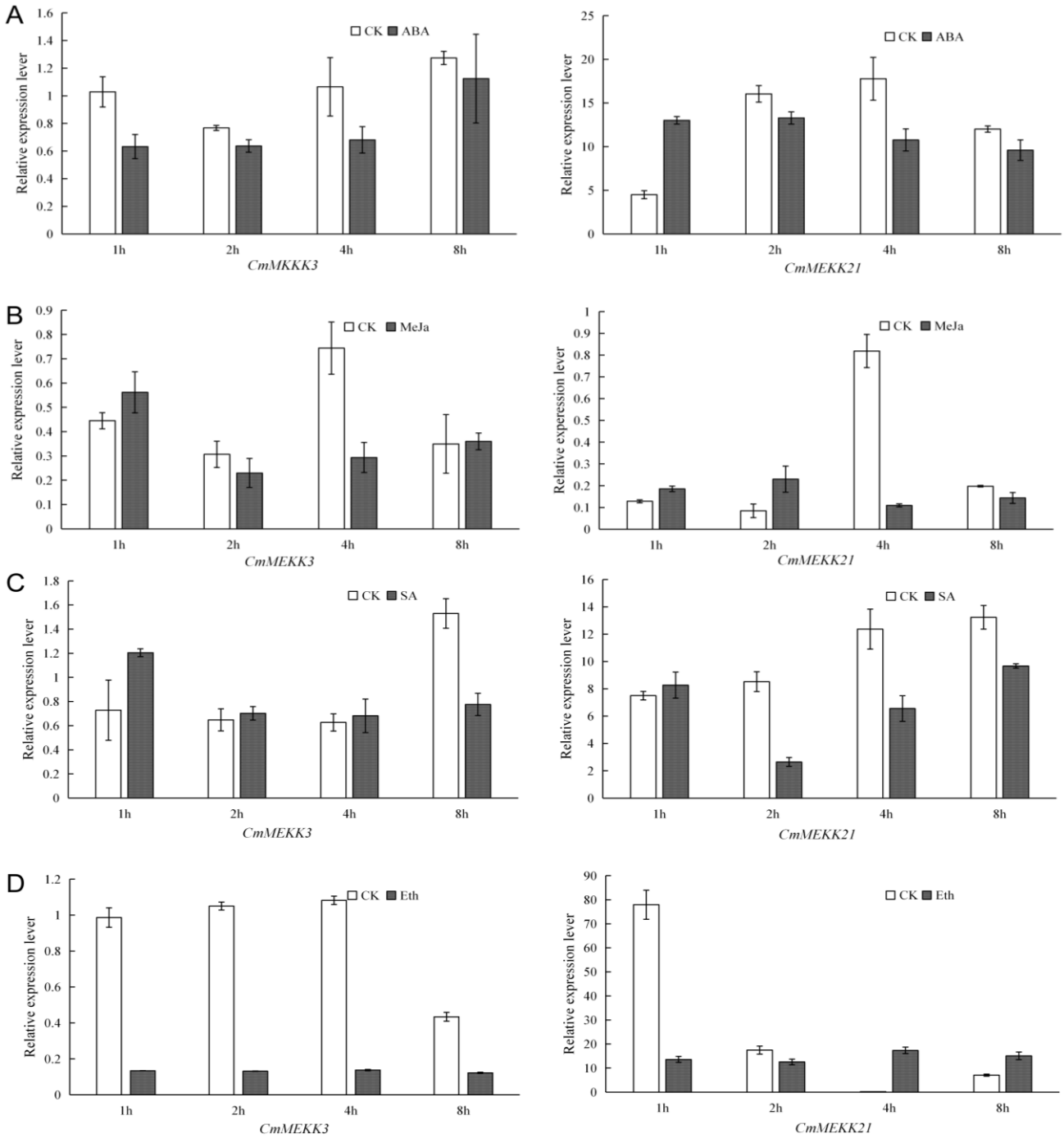

Fig. 8. Differential expression patterns of the *CmMAPKKK* genes in leaves to hormone treatments. (A) ABA; (B) MeJA; (C) SA; (D) Eth.

Differential responses of the *CmMAPKKK* genes to hormone treatments: Under ABA treatment, the expression of was similar in the control and experimental groups, as shown in Fig. 8A. The expression of *CmMEKK21* increased rapidly at 1h after ABA treatment, nearly 2.89-fold than that of the control group, and was down-regulated after 2h. From Fig. 8B, it can be seen that the expression of *CmMEKK3* raised first and then decreased and then raised again under MeJA treatment. At 2h, the expression of *CmMEKK3* increased slowly and then decreased sharply, then slightly increased again; *CmMEKK21* was initiated at 1h after MeJA treatment, and continued to increase till 2h after treatment. After 2 hours, its expression began to decline. *CmMEKK3* expression was significantly increased at 1 h after SA procedure, as shown in Fig. 8C., but the abundance was not changed at 2-4 h compared with the control group; the expression of *CmMEKK21* was slightly increased at 1h after SA treatment, but it was almost not changed subsequently.*CmMEKK21* expression was higher than *CmMEKK3*. *CmMEKK3* expression was not different in the ETH treatment community than in the control group, as shown in Fig. 8D. *CmMEKK21* expression was up-regulated 4 hours after ETH treatment and was 90.94 times higher than in the control group. *CmMEKK21* expression was 6.68-fold higher at 8 hours than in the control group.

Discussion

The cascades in which *MAPKKK* genes participate are closely related to plant resistance to biotic and abiotic stresses. In *Arabidopsis*, Raf5 mutants are more salt tolerant than the wild type. But some *MAPKKK* genes were found to negatively regulate salt-tolerance in Arabidopsis (Gao & Xia, 2008). One *Arabidopsis* Raf *MAPKKK* gene, HT1, positively regulates its response to CO_2 (Mimi *et al*., 2016). The *MAPKKK* gene *GhRaf19*, a Raf gene positively regulates its cold resistance, while negatively regulates its drought and salt resistance, through virus-induced gene silencing (Jia *et al*., 2016). In *Gossypium hirsutum*, the *GhMAPKKK49* gene can respond significantly to abiotic stresses such as high salt, drought and low temperature, as well as some biotic stresses (Liu *et al*., 2016). It's reported that in Maize some members of the Raf family were associated with disease resistance, and some members of the *ZIK* family involved in drought resistance (Yang *et al*., 2010). In wheat, *Tamekk14*, *TaRaf10*, *TaRaf34* and *TaRaf53* genes were responsive to salt stress; The *TaRaf87* and *TaRaf105* genes could be induced by drought stress; While the *TaRaf36*, *TaRaf49* and *TaRaf112* genes could be induced by cold and heat stress (Liu *et al*., 2016). In watermelon, multiple members of *MAPKKK* were also responsive to abiotic stresses (such as drought, salt, cold, and heat treatments) and biotic stresses (such as *Fusarium oxysporum*) (Song Liu *et al*., 2015). Genes of the *CsZIK* family in cucumber showed a certain response to abiotic stress (Wang *et al*., 2015). The expression of *SlMAPKKK51*, *SlMAPKKK53* and *SlMAPKKK55* genes in tomato increased at least 100-fold under abiotic stresses (such as drought, cold, salt, etc.), whereas the expression of *SlMAPKKK45*, *SlMAPKKK48* and *SlMAPKKK49* genes increased more than 10-fold under *Pseudomonas syringae* stress (Wu *et al*., 2014). In cassava, multiple *MeMAPKKK* genes are responsive to drought and hormonal treatments (Ye *et al*., 2017).

In addition, we discovered two *MAPKKK* genes in *Chrysanthemum* that were caused by salt stress. We came to the conclusion that *MAPKKK* genes were significant in plant stress resistance. In some plants, the *MAPKKK* gene family has been extensively studied (Xu & Zhang, 2014), but there have been few studies in *Chrysanthemum*. Here, we screened two *Chrysanthemum MAPKKK* genes, *CmMEKK3* and *CmMEKK21* based on our previous transcriptome data. To further understand their roles in stress responses, we performed a series of experiments, including high and low-temperature treatments, drought and salt treatments as well as hormone treatments such as ABA, SA, MeJA and Eth. We found that both *CmMEKK3* and *CmMEKK21* responded to various stresses. *CmMEKK21* could respond fast to high and low-temperature, MeJA and SA, but respond slowly to Eth until 8h after treatment. *CmMEKK3* could respond rapidly to low temperature, MeJA and SA, as well as low temperature, drought and salinity treatments. These findings provide further insights into their functions and their practical use in horticulture to improve its resistance and quality; what's more, it will facilitate our knowledge on their roles in the signal pathway of ABA, SA, MeJA and Eth.

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