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Research Article

Expression Analysis of Mitogen-Activated Protein Kinases (*MAPKs*) Gene Family in Grapevine Berries

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Abstract

Plants, as sessile living organisms, are dependent on signalling mechanisms. Mitogen-activated protein kinases (*MAPKs*) are a highly conserved gene family that take a role in switching an extracellular signal into an intercellular signal. Ripening-related processes in non-climacteric fruits are not as well understood as in climacteric fruits. In this regard, studying *MAPKs* in grape berries during developmental stages may lead to a better understanding of physiological interactions during commercially relevant stages, such as pigmentation, ripening, and phenolics accumulation in the berries. Each MAPK cascade involves three or four MAPK proteins that facilitate signal transduction by phosphorylation of downstream targets. We examined the relative expression of *VvMAP2Ks* and *VvMAP4Ks* in berries at two-weekly intervals, from flowering to over-ripening. Expression analysis of 5 *MAP2Ks* and 7 *MAP4Ks* suggested that both gene families may play an active role in development of berries. Expression of *VvMAP2K1* showed a correlation with abscisic acid (ABA) and ethylene accumulation. Moreover, the expression pattern of *VvMAP2K2* and *VvMAP2K3* shows a correlation with auxin, and ABA accumulation respectively. Furthermore, *VvMAP2K4* may have a role in berry size increment and halting stomatal development. In addition, *VvMAP2K5* may play a role in floral organ development. *VvMAP4Ks* expression pattern moves them forward to be excellent markers for monitoring the effect of for instance climate change-related stress on berry development.

Keywords: Ripening; Veraison; Flowering; Fruit-set; Viticulture; Transcriptomics

Abbreviations

MAPK: MPK: Mitogen Activated Protein Kinase; MAP2K: MAPKK: MKK: Mitogen Activated Protein Kinase Kinase; MAP3K: MAPKKK: MKKK: Mitogen Activated Protein Kinase Kinase Kinase; MAP4K: MAPKKKK: MKKKK: Mitogen Activated Protein Kinase Kinase Kinase Kinase; SDS: Sodium Dodecyl Sulfate; PEG: Polyethylene Glycol; PVPP: Polyvinylpolypyrrolidone; ABA: Abscisic Acid; JA: Jasmonic Acid; BRs: Brassinosteroids; SIMK: Stress-Induced Mitogen-Activated Protein Kinase; SAMK: Stress-Activated Mitogen-Activated Protein Kinase; WIPK: Wound-Induced Mitogen-Activated Protein Kinase; HR: Hypersensitive Response; CTR: Constitutive Triple Response; NAA: Naphthaleneacetic Acid; DEPC: Diethyl Pyrocarbonate

Introduction

Grapevine is the most widely grown fruit crop in the world, covering approximately 7.454 million hectares in 2016 and producing more than 270 million hectoliters of wine [1]. The development of the grape berry, from fruit setting to overripening, is a complex process that requires a large number of events. Although the changes that occur as the berry begins to ripen have received considerable attention, their overall control and coordination remain poorly understood. Signalling regarding coordination is a crucial issue in berry development, as the changes that occur at both the physical and biochemical levels are considerable and rapid, occurring over only a few weeks.

According to Coombe [2], grape berry development is a dynamic process divided into three major phases. During Phase I, starting at fruit set, the diameter of the grape berry may double in size due to cell division and subsequent cell expansion, and organic acids, tannins, and hydroxycinnamates accumulate to peak levels. The second major phase (Phase II) is defined as a lag phase in which cell expansion ceases and berries remain firm. Sugars begin to accumulate and berries lose chlorophyll. The beginning of the third major phase (Phase III) is marked by 'veraison' as the onset of ripening in which berries undergo a second period of growth due to additional mesocarp cell expansion, accumulation of sugars, pigments, volatile compounds and a decline in organic acid accumulation.

The complexity of the molecular control during berry ripening has been exemplified by recent development in transcriptomics. Moreover, differential screening, cDNA, and oligonucleotide microarray analysis have shown that the expression of thousands of genes, including large numbers of transcription factors, do actually change during grape berry ripening [3-9].

Mitogen-activated protein kinases (*MAPKs*) represent a large group of proteins taking role in signal transduction within a cell. They consist of three or four MAPK proteins: MAPK, MAPK kinase (MAPKK = MAP2K), MAPK kinase kinase (MAPKKK = MAP3K), and MAPK kinase kinase kinase (MAPKKKK = MAP4K). The MAPK

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pathway plays an important role in the conversion of an extracellular signal into an intercellular one through protein phosphorylation. MAPK proteins sequentially activate each other by phosphorylation. In other words, *MAPKs* form signalling modules where MAPK kinase kinases (MAPKKKs) activate MAPK kinases (MAPKKs) which in turn activate *MAPKs*. Activated MAPK can trigger a pathway or activate a transcription factor [10,11]. In addition to this, MAPK proteins have the potential to crosstalk with other pathways or can act as a negative regulator [12]. For instance, CTR1, which is a MAPKKK, is known as a negative regulator of ethylene signalling [13]. They are highly conserved in eukaryotes including yeast, animals, and plants where they are involved in cytokinesis, differentiation, proliferation, hormonal responses; abiotic and biotic stress signalling and developmental programs [10,14-16].

Expression analysis of MAPK genes during berry developmental stages can create an understanding of both primary and secondary metabolisms during berry ripening. To our knowledge, this is the first report about the expression of *VvMAP2Ks* and *VvMAP4Ks* during grape berry development. These markers may lead us to a better understanding of signalling processes specifically, and grape berry development physiology in general.

Materials and Methods

Plant tissue

Seedless grapes, *Vitis vinifera* 'Sultana' berries were used for analysis. Berries were collected every two weeks from fruit set (26.05) until full ripening (17.09) from an 8-years-old vineyard located at the Ege University Agricultural Experiment Station, Izmir, Ege, Turkey. Berries were sampled from different blocks, immediately frozen and ground in liquid nitrogen to be stored at -80˚C.

RNA extraction

Total RNA from grape berries of the variety Sultana was extracted according to Davies and Robinson [17], with an additional step of selective precipitation with 2 M LiCl. The extraction buffer contained 0.3 M Tris, 5 M Sodium perchlorate monohydrate (ClH_2NaO_5), 1% SDS, 2% PEG, 8.5% PVPP, and just prior to use, 1% β-mercaptoethanol. Two grams of ground powder was added to a pre-warmed (30-35°C) extraction buffer at 5ml/g of tissue and shaken at 37°C for 90 minutes at 180 × rpm. Vacuum filtration was done before adding 96% alcohol and was kept overnight at -20°C.

On the second day, samples were centrifuged at 4°C for 30 minutes at 5000 × rpm. 2ml of 70% alcohol was added prior to centrifugation at 4°C for 5 minutes at 5000 × rpm. The precipitate was dissolved in DEPC water, and mixtures were extracted twice with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) then centrifuged at 10,000 × rpm for 7 minutes at 4°C. Chloroform:isoamyl alcohol (24:1, v/v) was added to the supernatant before a centrifuge at 10,000 × rpm for 7 minutes at 4°C. Ten % (V/V) of 3 M NaOAc (pH 5.2) was added to the supernatant, mixed, and then stored at -80°C for 90 minutes. Total RNA pellets were collected by centrifugation at 10,000 × rpm for 20 minutes at 4°C. Each pellet was dissolved in 200µl of DEPC water prior to adding 100µl of 10 M LiCl as the last step of the second day and this was stored at 4°C for 16-20 hours. On the third and the last day, pellets were collected by centrifugation at 10,000 × rpm for 40 minutes at 4°C, 500µl of 70% alcohol was added to the pellet prior to centrifugation at 10,000 × rpm for 5 minutes at 4°C. The pellet was dissolved in 30µl of DEPC water and was stored at -20°C.

Quantitative real-time PCR (qRT-PCR)

The expression of MAPK genes in grape berries were confirmed by qRT-PCR analysis. Total RNA was extracted from samples as above and treated with DNase I (Fermentas, USA). First-strand cDNA was performed by using the Transcriptor First Strand cDNA Synthesis Kit (Roche, Switzerland) according to the manufacturer's protocol.

Çakır and Kılıçkaya [18] revealed the existence of 14 *MAPKs*, 5 *MAP2Ks*, 62 MAP3Ks, and 7 *MAP4Ks* in *Vitis vinifera*, which were used as primer data information. *VvActin* was used as a housekeeping gene in regard to normalization and elimination of pipetting errors [19].

Primers used are listed in Table 1. LightCycler® FastStart DNA Master SYBR Green I Kit (Roche, Switzerland) was utilized for preparation of qRT-PCR reactions and reactions were run by using Roche LightCycler 480. Three replicates were conducted to analyze the expression of each gene under each condition. The $2^{-\Delta\Delta CT}$ method was used for calculation of relative expression levels [20]. Transcript abundance was normalized to that of *Vvactin*.

Statistical data analyses

A T-test was performed. The P-value for all primers was less than 0.01, indicating that the results were statistically significant.

Results

In order to examine expression levels of various *MAPKs* in *Vitis vinifera* at different stages of berry development, we performed qRT-PCR. Details about *VvMAP2Ks* expression are presented in Figure 1. There was a significant increase in transcript levels of *VvMAP2K1* at the flowering stage, one month before veraison, during veraison and a month after veraison. However, the expression of *VvMAP2K1* was strongly suppressed during the ripening period and suddenly increased in the over-ripening period. Furthermore, an increase was observed in the expression of *VvMAP2K2* during flowering, as well as in week six and explicit at veraison. When the expression profile of the *VvMAP2K3* gene was examined, it was clear that its expression also increases during the veraison period. The expression level was quite low before and after veraison. Relative expression of *VvMAP2K4* showed increments at flowering and veraison and a month after veraison. The *VvMAP2K5* gene showed high expression in the period of flowering, veraison, and just before ripening. Expression of this gene was suppressed one month after the fruit set and during ripening.

Details about the expression *VvMAP4Ks* were shown in Figure 2. Expression of the *VvMAP4K1* gene gradually increased from veraison at two weeks before ripening and was suddenly suppressed during the ripening period; expression of *VvMAP4K2* increased one month after the fruit set. In other periods, the expression of the *VvMAP4K2* gene was low. It was observed that the expression of the *VvMAP4K3* gene increased considerably during the veraison period, in contrast with the slight expression of the gene after the flowering period and one month after veraison. In other periods, the expression of the gene remained at deficient levels and was even almost non-existent in the ripening period. The *VvMAP4K4* suddenly showed a high level

Table 1: *Vitis vinifera* MAP2K and MAP4K subfamilies and related primers.

Gene Locus ID	Gene Name	Forward Primers (5'-3')	Reverse Primers (5'-3')
GSVIVT01008476001	*VvMAP2K1*	AACTCCTACGTGGGCACCTG	AATTGGAGCCGTGGGAGTCG
GSVIVT01015155001	*VvMAP2K2*	ACCAGTTGAGCTTGGCTGACA	GCTTGAGAGCAGCCTCCTGA
GSVIVT01015283001	*VvMAP2K3*	TGCTCAAGAAACCCCTATCAC	TCAGAGATAAGCCGCAAACC
GSVIVT01016115001	*VvMAP2K4*	AGCCGGATCTTGGCTCAGAC	CACACCGAGGCTCCAGATGT
GSVIVT01032414001	*VvMAP2K5*	TGCTCATTTATTGATGCTTGCCTTCA	TGCTTCGGACAAATGCCGTT
GSVIVT01012233001	*VvMAP4K1*	GCGGTGCTAGTGCATCGTC	GCCTGAAATCGGGCTTCGTC
GSVIVT01013739001	*VvMAP4K2*	AGGCTGGGAATATCTTGGTTG	TGCATAACTTCAGGAGCCATC
GSVIVT01014297001	*VvMAP4K3*	GCAATTGGTCATGCAGTGGCA	AGTTGCCAGCTCCTGAAGGT
GSVIVT01016074001	*VvMAP4K4*	TCATCATCCTCATGCCCTTCCC	GAGCAGCAGACACGGAGGAA
GSVIVT01019643001	*VvMAP4K5*	GCTCCTCGAAGAGGTCGGTT	CCAAACACTTAACGGCCACCA
GSVIVT01027718001	*VvMAP4K6*	TTCTCAGAGCCCACTGTTCGT	TGGCAATGCAGGGTTCTGGT
GSVIVT01032461001	*VvMAP4K7*	CCCGAGCGGAACTATAGTGGT	TGGGACTGAGCTCTGCTGAAG
XM_010652725	*VvActin*	GGAATGGTTAAGGCTGGATTTG	GGTTGAGAGGAGCTTCAGTTAG

Primer information for *VvMAP2Ks* and *VvMAP4Ks*. The first column shows the putative gene IDs in the *Vitis vinifera* genome sequencing project Genoscope (Genoscope website: http://www.genoscope.cns.fr/externe/ GenomeBrowser/vitis). Actin gene's ID is according to NCBI (National Centre for Biotechnology Information). The second column contains gene's preferred name according to Cakir and Kilickaya [18].

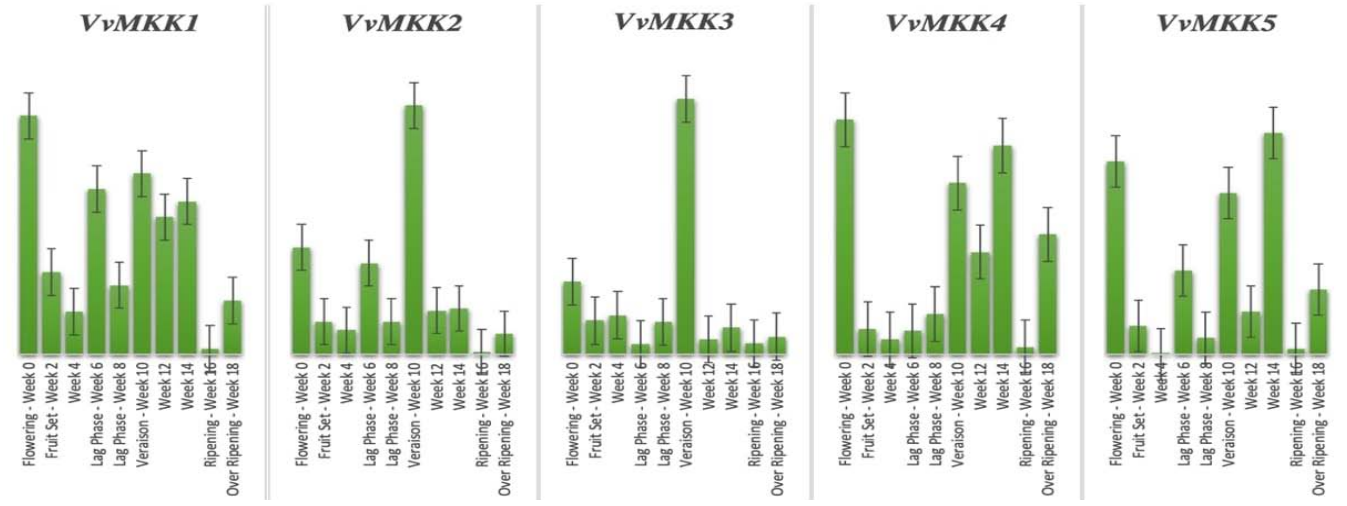

Figure 1: Relative expression of *VvMAP2K* genes from flowering (Week 0) to over-ripening (week 18). Week 2 represents the fruit set that marks the beginning of Phase I of berry development. The period from week 6 until the end of week 8 is considered as Lag phase (Phase II). Phase III (veraison) starts at week 10. Week 16 represents the ripening point.

of expression one month after fruit set but was then suppressed two weeks later. However, it had a low expression level from veraison prior to the ripening period. It was also observed that the expression of the *VvMAP4K5* increased during the period from veraison until the period before ripening, and was again suddenly cut out during the ripening period. Expression of the *VvMAP4K6* increased from flowering to fruit set and then dropped down, abruptly increasing one month after the fruit set, and becoming suppressed before veraison. It increased gradually from the period of veraison to ripening and was totally dropped in the ripening period. The expression of the *VvMAP4K7* again increased suddenly in the veraison period and was abruptly suppressed afterward, showing a similar expression until the period of overripening.

Discussion

When the expression profiles of the *VvMAP2K*, and *VvMAP4K* gene families were examined, a decrease in the expression levels of all genes was observed during week 16. Besides, it is known that a vast number of physiological processes are regulated in week 16 including but not limited to sugar accumulation, flavour compound and anthocyanin production, suggesting the role of *VvMAP2Ks* and *VvMAP4Ks* in these ranges of processes [21]. Since MAPK genes are mainly known to play a role in abiotic and biotic stress, most MAPK-related breeding programs are aimed at creating resistant vines. Our data suggest that any change in MAPK genes will affect not only resistance but also berry ripening.

Several studies showed a relation between the expression of MAP2K1 and ABA and its effect on defence responses, especially abiotic stresses, including salt stress [22-26]. Although the sampled vines were not in apparent stress, our data show a positive correlation between the expression of *VvMAP2K1* and the presence of ABA in

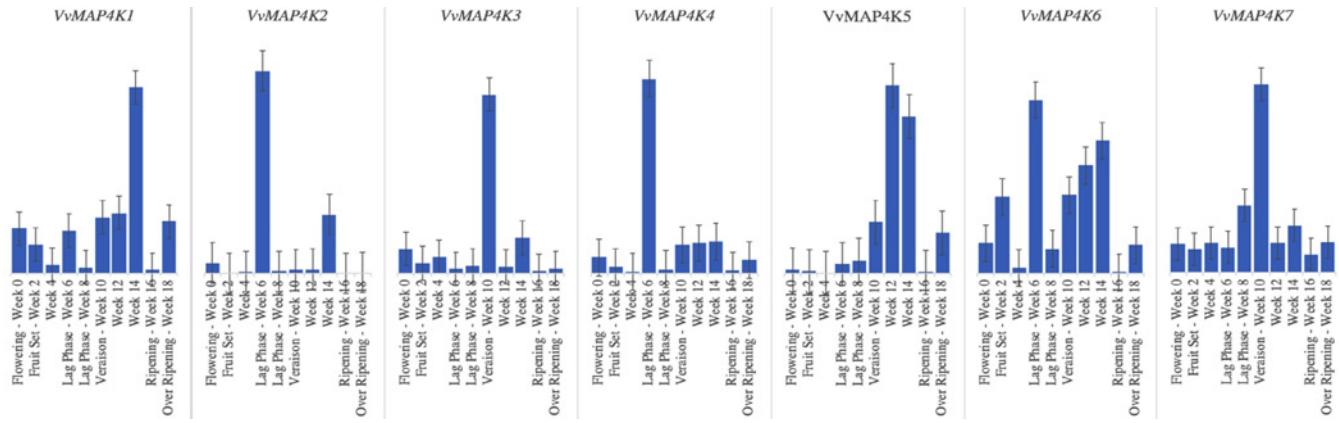

Figure 2: Relative expression of *VvMAP4K* genes from flowering (week 0) to over ripening (week 18). Week 2 represents the fruit set that marks the beginning of Phase I of berry development. The period from week 6 until the end of week 8 is considered as Lag phase (Phase II). Phase III (veraison) starts at week 10. Week 16 represents the ripening point.

berries [27], which remains relatively high from weeks 10 to 14 after flowering.

Stanko et al. [28] showed a correlation between the AtMKK2-AtMPK10 pathway and auxin accumulation. Plus, Ziliotto et al. [29] showed that Naphthalene Acetic Acid (NAA) inhibits ripening in berries. Although *VvMAP2K2*'s expression indicates a correlation with IAA in berries from flowering to veraison, it shows a peak in expression during veraison when IAA level is low [30]. Since this cannot be related to auxin accumulation, our data suggests that *VvMAP2K2* is not only involved in auxin related pathways and it may play a role in other pathways too.

De Zelicourt et al. [31] showed that the MAP3K17/18-MKK3-MPK1/2/7/14 cascade is under the control of ABA core signalling pathway. Similarity in ABA accumulation pattern [27] and *VvMAP2K3* expression during berry development supports the thesis of ABA's effect on above-mentioned MAPK pathway.

MAP2KK4 genes are known to play a role in controlling cell division, organ shape, and size [32,33]. Accordingly, it is assumed that the *VvMAP2K4* gene may similarly play a role in the berry size increment along a sigmoidal growth curve that drops abruptly, due to its increased expression level during berry growth. It has been reported in previous studies that the MAP2K4 gene is responsible for suppressing stomatal development [34,35]. In addition, it is known that the stomata of the fruit become dysfunctional in the veraison period [36]. An increase in the level of *VvMAP2K4* expression in this period suggests that it may be associated with halting stomatal development.

Further, MAP2K5 is part of the MKK4/MKK5-MPK6 cascade control in maternal embryogenesis and floral organ development [37, 38]. Relatively high expression of *VvMAP2K5* during the flowering period supports this idea. Khan et al. [35], showed a correlation between brassinosteroids and YODA-MKK4/5-MPK3/6 cascade responsible for cellular patterning, and cell's fate in A. thaliana. Since the BRs accumulation in grape berries is high after veraison until ripening ([39], our data suggest a relation between BRs and *VvMAP2K4/5*.

There are two main models about the accumulation of ethylene during grape berry development. The first one states its level starts high at flowering time and then drops quickly to stay at a low level during subsequent berry development ([40, 41]). The second model indicates that there is a peak not only during flowering, but also at week 8 [42]. In addition to this, Novikova et al. [43], and Ouaked et al. [44] showed a correlation between the ethylene and MAPK cascade. Our data only supports the first model: *VvMAP2K1* is strongly present during flowering period and likewise stays relatively low until the end of ripening. However, our data do not constitute evidence for the second model. In other words, there is no candidates *VvMAP2K* or *VvMAP4K* that show a peak in expression during week 8.

The only GA, which is detected in grape berries, is GA3, which concentration is relatively high during flowering, decreases dramatically and remains low and unchanged through the end of berry development [39]. To our knowledge, there is no research showing a correlation between GAs and *MAPKs*. Our data also do not show any direct correlation between GA3's accumulation and *VvMAPKs* expression.

It is clear that *MAP4Ks* play a role in cascade initiation by sensing start signals in the MAPK pathway. A sudden increase and decrease of their expression in specific periods may relate to this. In addition to this, the characteristic, relatively high expression in only a specific period for *MAP4Ks* suggests their ability to be utilized as markers. For instance, *VvMAP4K2* could be used as a marker to show the initiation of the lag phase. In another example, *VvMAP4K3* could be used as a marker to show the veraison. The lack of published findings on *MAP4Ks* in plants makes it impossible to come to any more conclusions. Additional supportive data are required.

Conclusion

Each expression pattern of the 12 genes belonging to the *Vitis vinifera VvMAP2K* and *VvMAP4K* subfamilies was examined by real-time PCR in different developmental periods of grape berries. Although all-over, their expression decreased in the ripening period, their expression during other developmental periods widely differed. This confirms their role in this important phase of the grape life cycle. In addition to this, it is noteworthy to mention that *MAP4Ks* subfamily in plants is not so well studied as it is in human and animal

biology. Our results suggested that the MAP4K gene subfamily should receive more attention. Their high expression (or lack of expression) at specific developmental phases moves them forward to be excellent markers for monitoring the effect of for instance climate change related stress on berry development. It is noteworthy that MAPK proteins can also behave as negative regulators. In other words, not only the expression of MAPK genes but their suppression can trigger specific pathways. In addition to this, they can crosstalk with other pathways. Therefore, the decisive conclusion of their role requires more supportive data.

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