

Quantification of Stress-Induced Mitogen-Activated Protein Kinase Expressional Dynamic Using Reverse Transcription Quantitative Real-Time PCR

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Abstract

Although it is generally accepted that signal transduction in plant mitogen-activated protein kinase signaling cascades is regulated via rapid posttranslational modifications, there are also several compelling examples of swift stress induced transcriptional activation of plant MAP kinase genes. A possible function of these fast and transient events is to compensate for protein losses caused by degradation of phosphorylated MAP kinases within stimulated pathways. Nevertheless, there is still need for additional evidence to precisely describe the regulatory role of plant MAP kinase transcriptional dynamics, especially in the context of whole stress stimulated pathways including also other signaling molecules and transcription factors. During the last two decades a reverse transcription quantitative real-time PCR became a golden choice for the accurate and fast quantification of the gene expression and gene expression dynamic. In here, we provide a robust, cost-effective SYBR Green-based RT-qPCR protocol that is suitable for the quantification of stress induced plant MAP kinase transcriptional dynamics in various plant species.

Key words Plant mitogen-activated protein kinase, Abiotic stress, Biotic stress, Tri reagent, Reverse transcription quantitative real-time PCR, SYBR green

1 Introduction

Plant mitogen-activated protein kinase (MAPK) cascades are complex signaling pathways that transduce myriads of extracellular environmental and developmental signals into cellular responses. Signal transduction in a MAP kinase pathway is mediated via consecutive phosphorylation of key pathway components in which the most upstream MAPKKK phosphorylates MAPKK which in turn phosphorylates downstream MAPK. Once activated MAPK transmits phosphorylation signal into variety of substrates like transcription factors, protein kinases, and cytoskeleton-associated proteins [1]. In many well documented cases, especially those addressing abiotic and biotic stress stimulation, the phosphorylation of MAP

kinase pathway components is extremely fast, occurring within minutes post-stress. Such a fast signal transduction enables plants to quickly reprogram cellular metabolism resulting into a timely adaptation to stress. Nevertheless, not only are plant MAP kinases swiftly activated, but also the gene expression of MAP kinases might be induced by stress in short time periods.

There are several compelling examples of rapid stress induced transcriptional activation of plant MAP kinase genes. We summarize here and compare the expression data mainly for the well-studied *Arabidopsis* MAP kinase genes *AtMPK3* and *AtMPK6* together with their orthologues in other plant species. In one of the pilot studies, the expression levels of *AtMPK3* and *AtMEKK1* (*Arabidopsis* MAPKKK) were shown to be markedly increased within 1 h following cold and salt exposure [2]. The same work shows in parallel rapid increase in mRNA levels of these two MAP kinase genes occurring within 10 min after touch stimulation of rosette leaves. Interestingly, at least transcriptional activation of *AtMPK3* appears to be of transient nature as evidenced by the resumption of gene expression basal levels within 40 min only. The transient nature of the rapid transcriptional activation of *AtMPK3* was also documented in hydroponically grown *Arabidopsis* seedlings elicited with chitin and in soil grown *Arabidopsis* plants exposed to ozone [3, 4]. On the other hand, the expression of *AtMPK6* was indifferent to chitin treatment for up to 4 h while ozone induced weakly only but more persistently the expression of *AtMPK6* in the same experimental frameworks. Other stimuli including low temperature, wounding, touch and low humidity induced not any or rather weak transcriptional activation of *AtMPK6* and *AtMPK4* in the 6–8 weeks old *Arabidopsis* plants [5]. Interestingly, these contrasting expression patterns of *AtMPK3* and *AtMPK6* induced by various stress factors were also observed in the case of *Nicotiana* *WIPK* orthologue of *AtMPK3* and *Nicotiana* *SIPK* orthologue of *AtMPK6*. While *WIPK* gene is rapidly and transiently transcriptionally activated within minutes post wounding and this activation is even more pronounced post wounding when oral secretions of *Manduca sexta* are co-supplied, the expression of *SIPK* is rather weakly but more persistently induced under the same experimental regime [6, 7]. *WIPK* gene is also transiently induced within 20–30 min after infiltration with water, cutting and abrasion, conditions against which *SIPK* expression is non-responsive for as long as 6 h [8]. In addition to this, no changes in *SIPK* mRNA levels were detected for nearly 1 day after salicylic acid treatment or fungal elicitation of tobacco cell suspension culture [9]. Another example concerns the transcriptional activation of an *AtMPK3* orthologue from alfalfa, namely, *MMK4*, which was shown to be rapidly induced by cold, drought, and wounding [10, 11].

A possible explanation for the rapid and transient transcriptional activation of plant MAP kinases was exemplified in the case of wound induced alfalfa MAP kinase pathway (*reviewed in* [12]). In this work, it is mentioned that wound induced transcriptional upregulation of the MAPK gene is not correlated with an increase in the amounts of the MAPK protein. To explain this discrepancy, evidence is stated there for the degradation of wound-activated MAP kinase, followed by the compensation of MAPK protein lost by the rapid transient activation of MAPK gene. Thus, the rapid transcriptional activations of MAPK gene are to reset the MAP kinase pathway. The MAP kinase pathway is switched off by both rapid degradation of activated MAPK protein and the transcriptional induction of *MP2C* encoding a protein phosphatase type 2C, a protein that degrades wound induced MAP kinase cascade (*reviewed in* ref. 12). In many cases described in the previous paragraph, the rapid transient increase in the activity is provided as an evidence for the involvement of MPK6 kinase or its functional protein homologue SIPK in a particular stress signaling [3–5, 7–9]. However, in those occasions, the expression of *AtMPK6* or *SIPK* is either not induced or induced rather weakly as already mentioned. Conversely, the rapid transient increase in the activity of *AtMPK3*, *WIPK*, or *MMK4* is accompanied by the rapid, pronounced transient transcriptional induction of *AtMPK3* or its orthologues [3–7, 10, 11]. Hypothetically the differential transcriptional regulation of *AtMPK3* and orthologues (*WIPK* or *MMK4*) and *MPK6* or *SIPK*, may relate to the need for compensation at the protein level.

The occurrence, the mechanisms and the rationale behind plant MAP kinase transcriptional regulation and its role in MAP kinase signaling needs to be further substantiated. Future experiments intended for the characterization of stress induced expression of MAP kinase gene pathways should be designed carefully by combining short and long term stress treatments. Wherever possible, protein abundance and kinase activity data should be comparatively studied together with transcriptional dynamics. Moreover, the experiments should be performed in an organ or tissue specific manner and should involve not only proved or putative MAP kinase gene pathways but also other signaling components and transcription factors. An excellent framework for such analyses was recently provided in *Arabidopsis* [13].

Since its appearance in mid-1990s [14–16], quantitative real time PCR (qPCR) was quickly commercialized and became an essential tool for the quantification of nucleic acids. The basic principle of the method relies on the usage of fluorescent dyes for the labeling of PCR products that arise during PCR cycling. The reaction is performed in real-time PCR devices that in addition to the rapid thermal cycling simultaneously measure the accumulation of

fluorescent signal during the PCR exponential phase. The online measurement of fluorescence accumulation enables the accurate quantification of PCR product and further objective data analysis. The reverse transcription quantitative real-time PCR (RT-qPCR) based quantification of the gene expression offers several advantages [17] with its high sensitivity being the most important one. It was demonstrated that the detection limit of qPCR could be as low as two copies of gene [18]. The SYBR Green or TaqMan assays were shown to provide 4- to 5-log dynamic range of amplification, while being much more sensitive than semiquantitative RT-PCR with subsequent densitometric analysis or ribonuclease protection assay [19, 20]. On the other hand, the high sensitivity of RT-qPCR is also its major disadvantage. Therefore, good laboratory practice precautions must be taken to prevent cross-contamination of samples, chemicals, consumables, and devices with plasmid DNA, PCR products, or genomic DNA.

Herein we describe a robust RT-qPCR method which is routinely used in our laboratory for the quantification of stress induced plant MAP kinase transcriptional dynamics. Our approach relies on TRI reagent RNA extraction which proved to be efficient in various plant organs and tissues from different plant species such *Arabidopsis thaliana*, *Medicago* sp., and *Hordeum vulgare*, using of M-MLV reverse transcriptase for cDNA synthesis and SYBR Green based amplicon detection. As an example of the method application, quantification of MAP kinase transcriptional dynamics in the roots of *Arabidopsis* exposed to osmotic stress (NaCl) is presented.

2 Materials

All standard chemicals should be of analytical grade. Except for sterilization of seeds, growing and treatment of *Arabidopsis* plants, solutions should be prepared in RNase and DNase-free ultrapure water (18.2 M Ω ×cm specific resistivity). Toxic compounds should be handled appropriately under fume hood and according to institutional safety regulations. Always wear gloves and protective clothing.

2.1 Growth Media, Sterilization of Plants and Their Treatment

1. ½ Murashige–Skoog medium (1/2 MS; solid/1 L): 2.2 g MS medium (Duchefa) without vitamins, 10 g sucrose, 0.8 % (v/v) Phytagel, pH 5.8 (KOH).
2. ½ Murashige–Skoog medium (liquid/1 L): 2.2 g MS medium without vitamins, 10 g sucrose, pH 5.8 (KOH).
3. 70 % (v/v) ethanol, 96 % (v/v) ethanol, 150 mM NaCl.

2.2 RNA Isolation

1. TRI Reagent solution (Sigma-Aldrich).
2. 1-Bromo-3-chloropropane (BCP) (Molecular Research Center).
3. Micropestle (Eppendorf).
4. Isopropanol.
5. 75 % (v/v) ethanol.
6. Filtered tips.

**2.3 DNase
I Treatment**

1. DNase I (Thermo Scientific).
2. 10× DNase I reaction buffer (Thermo Scientific).
3. EDTA (Thermo Scientific).

2.4 cDNA Synthesis

1. 100 μM PAGE purified 18 bp oligo dT primers.
2. M-MLV Reverse Transcriptase 5× reaction buffer (Promega).
3. Deoxynucleotide mix (dNTPs) (Fermentas).
4. RNasin® Plus RNase inhibitor (40 U/μL) (Promega).
5. M-MLV Reverse Transcriptase (100 U/μL) (Promega).

**2.5 Quantitative
Real-Time PCR**

1. Power SYBR® Green PCR Master Mix (Life Technologies).
2. 96-well microtiter plates (Life Technologies).
3. MicroAmp™ Optical Adhesive Film (Life Technologies).

3 Methods**3.1 General Working
Precautions**

1. Designate a clean working area, where only RNA and RT-qPCR work is conducted. Ideally, this area should be isolated from laboratory spaces dedicated to cloning, transformation, and gel electrophoresis in order to avoid contamination with plasmids, PCR products, or genomic DNA.
2. Designate pipettes, pipette tips, tubes, and other tools and consumables for RNA and RT-qPCR work only (*see Note 1*). Such equipment and consumables should stay only in the RNA and RT-qPCR working area.
3. To avoid RNase contaminations coming from hands always use gloves when working with RNA.
4. Clean the working area with diluted household bleach and let it dry for a while immediately before starting work. Afterwards clean the working area with 70 % (v/v) ethanol. These steps should decontaminate the working area from microorganisms and RNase contamination. Also clean the fume hood, where TRI reagent based RNA isolation will be carried out.

3.2 Growing of *Arabidopsis* Seedling and Their Treatment

1. Sterilize seeds of *Arabidopsis thaliana* ecotype Col-0 by 70 % (v/v) ethanol for 5 min, then follow with 96 % (v/v) ethanol sterilization for 1 min and finally wash the seeds by sterilized water two times for 5 min. During each step shake gently the tube with the seeds (*see* **Note 2**).
2. Keep the seeds on the sterilized filter paper to dry.
3. Plate the seeds on square petri dishes containing ½ MS medium and place them for germination and growth in environmental chamber for 2 weeks under following conditions: 21 °C, 70 % humidity, 16 h light–8 h dark.
4. Prepare 150 mM NaCl solution, pour it to the dish in horizontal position and incubate plants for 3 h at the root temperature. The dish should be very gently shaken. Collect roots of *Arabidopsis* plants at 15 min, 30 min, 1 h, 2 h and 3 h post stress exposure. Be careful that all plants are well submerged in the solution (*see* **Note 3**).

3.3 RNA Isolation

Unless indicated otherwise, work at the room temperature.

1. Using sterile scalpel and forceps collect approximately 50 mg fresh weight of *Arabidopsis* roots into a 2 mL round bottom microfuge tube and immediately freeze the samples by immersion into liquid nitrogen (*see* **Note 4**).
2. Disrupt root samples in a 2 mL round bottom tubes using micropestle and liquid nitrogen to a fine powder (*see* **Note 5**).
3. Add 0.7 mL of TRI Reagent solution into liquid nitrogen or dry ice prechilled sample tube, homogenize sample via pipetting up and down several times, and incubate the homogenate for 7 min at room temperature (*see* **Note 6**).
4. Centrifuge sample tubes at 12,000×*g* for 10 min at 4 °C to get rid of insoluble debris and transfer the resulting supernatant into a clean tube.
5. Add 70 µL of 1-bromo-3-chloropropane, cap sample tubes tightly, and mix by vigorous shaking for at least 15 s. Incubate for 5 min at room temperature.
6. Centrifuge sample tubes at 12,000×*g* for 15 min at 4 °C. Following centrifugation transfer upper aqueous phase containing RNA into to a clean tube (*see* **Note 7**).
7. Add isopropanol (70 % (v/v) of aqueous phase volume), mix by inverting the tubes several times, and incubate 8 min at room temperature (*see* **Note 8**).
8. Centrifuge at 12,000×*g* for 8 min at 4 °C and decant the supernatant (*see* **Note 9**).
9. Wash samples with 0.7 mL of 75 % (v/v) ethanol. Do not vortex (*see* **Note 10**).

10. Centrifuge sample tubes at $7,500 \times g$ for 5 min, remove ethanol, and briefly dry RNA pellet on laboratory bench (*see Note 11*).
11. Dissolve RNA pellet in water by pipetting several times up and down and determine the RNA concentration using NanoDrop spectrophotometer. Aliquot each RNA sample into two to three tubes, and store at -80°C .

3.4 DNase I Treatment (See Note 12)

Unless indicated otherwise, work on ice.

1. Use 2 μg of RNA sample per reaction. Based on the RNA concentration calculate for each RNA sample the volume needed to provide 2 μg of RNA per reaction and transfer this volume into a new tube. Fill up each sample tube containing 2 μg of RNA with water to a final volume of 10 μL (*see Note 13*).
2. According to the number of samples processed prepare master mix containing 6 μL of water, 2 μL of $10\times$ DNase I reaction buffer, and 2 μL (2 units) of DNase I per each sample. Mix by gently flicking the tube (*see Note 14*). Briefly spin down.
3. Add 10 μL of master mix prepared in **step 2** into each sample tube containing 2 μg of RNA in 12 μL of water. Mix by gently flicking the tubes. Briefly spin down.
4. Incubate sample tubes for 40 min at 37°C and following incubation briefly spin down.
5. Add 2 μL of EDTA to the sample tube, mix by flicking the tube and briefly spin down. Incubate the reaction mixture for 10 min at 70°C to inactivate DNase I. Briefly spin down and let cool in a room temperature for a while. Store DNase I treated samples on ice for further processing.

3.5 cDNA Synthesis

Unless indicated otherwise, work on ice.

1. Transfer 5.5 μL of each DNase I treated RNA sample (500 ng of RNA) into a new tube.
2. According to the number of samples processed prepare master mix containing 4.5 μL of water and 0.5 μL of oligo-dT primer (0.25 μg of oligo-dT primer per reaction) per sample. Mix by shortly vortexing the tube, briefly spin down and add 4.5 μL of master mix into each sample tube containing 5.5 μL of DNase I treated RNA. Incubate the samples for 5 min at 70°C to denature secondary structures in mRNA and immediately chill on ice. Briefly spin down and keep on ice up to further processing.
3. According to the number of processed samples prepare cDNA synthesis master premix containing 1.2 μL of water, 4 μL of M-MLV Reverse Transcriptase $5\times$ reaction buffer (*see Note 15*) 4 μL of dNTPs (2.5 mM), 0.4 μL (40 units) of M-MLV Reverse Transcriptase, and 0.4 μL (16 units) of RNasin[®] Plus

RNase inhibitor. Mix by gently but thoroughly flicking the tube and briefly spin down.

4. Add 10 μL of cDNA synthesis master mix prepared in **step 3** into each sample tube processed according **step 2**. Mix by gently but thoroughly flicking the tubes, briefly spin down and incubate at 42 °C for 120 min.
5. Inactivate M-MLV Reverse Transcriptase by heating the sample tubes at 70 °C for 10 min. Briefly spin down. Add 60 μL of water into each sample containing synthesized cDNA in 20 μL volume. Mix by flicking the tubes and briefly spin down. Aliquot each cDNA sample into two to three tubes and store at -80 °C (*see Note 16*).

3.6 Primer Design

When designing primers for RT-qPCR one should follow certain guidelines in order to obtain reliable results. We present here primer design strategy relaying on the use of Primer-BLAST software [21] and outline primer designing rules as user defined program settings.

1. Use Primer-BLAST web software to design primers (*see Note 17*).
2. Insert target mRNA sequence in FASTA format.
3. Set primer melting temperature (T_m) from 58 to 65 °C; however, follow the rule that maximal T_m difference between two primers within a primer pair should be 1 °C. In addition, whenever possible, this rule should be followed also for the T_m differences among different primer pairs.
4. Set PCR product size from 50 bp to 150 bp and size of the primers from 18 bp to 24 bp.
5. If possible primers should be designed to avoid amplification of unintended targets from contaminating genomic contaminating DNA. For this purpose a program option “primer must span an exon-exon junction” might be used. Alternatively primers might be designed to span introns using “intron inclusion” option. In the example provided, primers are designed using “primer must span an exon-exon junction” option (Table 1).
6. Select “nr” database and define “organism” in “Primer Pair Specificity Checking Parameters”.
7. Otherwise use program default settings and run program.
8. On the program output select suitable primer pairs primarily based on specificity; further primer selection should be guided by lowest “any” and “3’” complementarity.
9. Check the template secondary structure using mFold server (<http://www.bioinfo.rpi.edu/applications/mfold/>) to avoid secondary structures that might interfere with the RT-qPCR efficiency.

Table 1
Specific primer properties

Gene and accession number	Primer name	Sequence (5' → 3')	Length (bp)	T _m (°C)	Amplicon length (bp)	PCR efficiency (%)
<i>AtMPK4</i>	qMPK4F	TGTCGGCTGGTGCAGTCGATT	22	65	85	90.4
At4g01370	qMPK4R	TGGCACAACGCCTCATCAACTGT	23	65.29		
<i>AtMPK6</i>	qMPK6F	ACAGCTTCCACCTTATCCTCGCCA	24	65.79	85	92.3
At2g43790	qMPK6R	TGGGCCAATGCGTCTAAACTGTG	24	64.12		

10. In the Table 1 an example is provided of the primer pairs designed for the RT-qPCR quantification of *Arabidopsis thaliana* MPK4 and MPK6 using Primer-BLAST.

3.7 Set up, Run and Analysis of Quantitative Real-Time PCR

Unless indicated otherwise, work on ice.

- Using 10 μ M primer stock solutions prepare primer master mix for each tested target that should contain forward primer, reverse primer, and water in 3:3:19 ratio. Vortex briefly twice and spin down.
- According to the number of samples analyzed prepare for each tested target a RT-qPCR master mix containing 2.5 μ L of primer mix (**step 1**, running concentration of each primer = 0.3 μ M) and 5 μ L of Power SYBR[®] Green PCR Master Mix per single reaction. Mix reaction by gently but thoroughly flicking the tube and briefly spin down.
- Design 96-well plate running schedule and pipet 7.5 μ L of each RT-qPCR master mix according to this schedule into the bottom of 96-plate wells.
- Follow 96-well plate running schedule and pipet 2.5 μ L of each cDNA sample or control sample to the walls of the wells.
- Cover plate with optical adhesive film and briefly centrifuge.
- Run the plate in the StepOne[™] Thermal cycler under the following conditions: 95 °C for 10 min, 40 cycles of 95 °C for 15 s, and 60 °C for 1 min.
- Run the melt curve analysis under the following conditions: 95 °C for 15 s, 60 °C for 1 min and heating from 60 °C to 95 °C with 0.3 °C increments.
- Analyze obtained data using the StepOne[™] software (Applied Biosystem[®]). Check the negative controls for the amplification

of unintended targets (*see* **Note 18**) and perform melting curve analysis for the targets amplified from cDNA samples (*see* **Note 19**). Calculate relative gene expression using $2^{(-\Delta\Delta C_q)}$ method [25] implemented in StepOne software and verify the calculation in Microsoft Excel spreadsheet (*see* **Note 20**).

3.8 PCR Efficiency Test

1. Determine the efficiency of target amplification for each primer pair that is going to be used in RT-qPCR analyses.
2. Prepare serial dilutions of the appropriate template at ratios of 1, 1/4, 1/16, 1/64 and 1/256 (*see* **Note 21**).
3. Run at least in duplicate RT-qPCR on the dilution series samples using conditions described in Subheading 3.7. Determine PCR efficiency from the slope of the calibration curve. In particular, $\text{PCR efficiency} = [10^{(-1/\text{slope})} - 1]$, when logarithm of template concentrations is on the x axis and C_q values are on the y axis. Standard curve analysis can be also performed using StepOne™ software.
4. Good PCR efficiency ranges within 85–115 %; otherwise design new primers (Table 1).

4 Notes

1. We generally find plastic ware to be RNase free and routinely use relatively cheap tubes and tips without any special designations. For RNA isolations (starting from the pipetting of water phase) and cDNA preparations we use filtered tips.
2. Always work in sterile laminar flow box.
3. Dissolve NaCl in liquid ½ MS medium and use liquid ½ MS medium only for time parallel controls.
4. Following freezing in liquid nitrogen, samples should be stored in –80 °C freezer prior to further processing.
5. During the sample disruption procedure 2 mL round bottom tubes bearing samples are maintained on dry ice or floating on liquid nitrogen in a polystyrene box. Samples are disrupted individually in the tubes prechilled in liquid nitrogen by hand using micropestle (also prechilled in liquid nitrogen). During disruption step, samples should be occasionally chilled in liquid nitrogen to avoid thawing of the samples. In general, it is critical to maintain the samples deep frozen during storage and disruption step. After processing of each sample, wash the pestle in 70 % (v/v) ethanol and demi water. Finally, wipe the pestle dry with lint-free tissue.
6. Samples homogenized in TRI reagent solution can be safely kept on ice or in a fridge from several hours up to 1 day.

7. Aqueous phase should be colorless and not viscous, otherwise it might be contaminated with high molecular genomic DNA, proteins, polysaccharides, and phenol. Such contaminants in the aqueous phase might interfere with RT-qPCR, and therefore, they should be avoided. Most often, contaminants in water phase occur when the <1:10 ratio of wet plant material weight to TRI reagent volume is not followed. Aqueous phase could be additionally purified using chlorophorm extraction. Chlorophorm extraction is usually indispensable when working with *H. vulgare* leaves.
8. When working with *Medicago* roots, mix isopropanol in 1:1 ratio with high salt RNA precipitation solution containing 0.8 M sodium citrate and 1.2 M NaCl. Add this mixture (70 % (v/v) of aqueous phase volume) into a tube containing collected aqueous phase.
9. White pellet should be visible at the bottom of 2 mL tube at the end of centrifugation. Remove supernatant using pipet in order to minimize the risk of sample contamination that might occur if supernatant is simply poured out of the tube.
10. It is important to keep the RNA pellet compact. Pellet should be released from the bottom of tube into 75 % (v/v) ethanol simply by carefully inverting the tube.
11. This is the most challenging step. RNA pellet should not be overdried as this might lead to poor RNA solubility and impurity. Initially, ethanol should be removed using pipet and then the pellet is dried out for a few minutes until it becomes clear. No ethanol drops should be visible in the tube before RNA pellet is dissolved.
12. Ideally, especially when processing large number of samples, DNase I treatment and cDNA synthesis should be done in PCR 8-tube strips and thermocycler dedicated to RNA work only.
13. If the RNA concentration is lower than 200 ng/ μ L, final volume can be increased up to 16 μ L. The volume of water added per single sample should be decreased accordingly in the following DNase I master mix preparation.
14. DNaseI is sensitive to physical denaturation.
15. M-MLV Reverse Transcriptase 5 \times reaction buffer contains high amounts of salt that may precipitate upon freezing. Therefore, it should be vigorously vortexed to dissolve all salts when thawing.
16. Repeated freeze–thaw cycles might have tremendous effect on cDNA quality and in turn on performance of RT-qPCR. Even two freeze–thaw cycles alone can significantly decrease RT-qPCR sensitivity. When analyzing different batches of cDNA samples, one should consider analyzing batches of similar age only that in addition underwent the same low number of freeze–thaw cycles.

17. Primer3 software [22, 23] is also well suitable for the RT-qPCR primer design. Nevertheless, it bears no primer specificity check option; therefore, prior to primer design, a sequence region specific to the target mRNA should be identified using BLAST [24] searches.
18. Positive but also negative controls should be included into the every plate run. The simplest negative control is the “water”, so-called non template control (NTC) control that is run with water template supplied instead of cDNA template. No PCR products of correct size are tolerable in NTC controls. Occasionally, primer dimer products might be detected in NTC control runs. We find those to be usually acceptable when giving late Cq values. However, the identity of amplicons should be always checked using melting curve analysis or in the case of uncertainty, PCR products should be resolved using 2 % (w/v) agarose gel electrophoresis. Additionally, in order to check for genomic DNA contamination, especially when using primer pairs that amplify targets also from genomic DNA templates, no-RT control should be included. This control employs DNase I treated RNA template that is diluted with water to the exactly same concentration as is putative RNA concentration in standard cDNA sample. To give a numerical example according to our protocol, 5.5 μL of DNase I treated RNA sample (500 ng of RNA) should be diluted with 74.5 μL of water to get RNA concentration of 34.37 ng/ μL . No-RT control should be run for each respective cDNA sample; however, it can be omitted in further runs once it has been validated as free of DNA contamination.
19. Only single “clear” melting curve should be visible. RT-qPCR products can be also resolved in 2 % (w/v) agarose gel electrophoresis in order to verify the specificity of amplification.
20. A reference gene should be always checked for the invariant expression in the running experimental conditions.
21. When working with genes exhibiting sufficient expression (C_q =at least 25–26), cDNA template (undiluted cDNA template corresponds to dilution 1) can be used directly for the preparation of standard curve dilution series. Otherwise, when the gene expression is low (based on initial runs) and cannot be easily induced, plasmid templates should be used.

Acknowledgements

This work was supported by Czech Science Foundation (GACR) grant GA CR, P501/12/P455.

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Plant MAP Kinases

Methods and Protocols

Komis, G.; Šamaj, J. (Eds.)

2014, XI, 266 p. 33 illus., 17 illus. in color., Hardcover

ISBN: 978-1-4939-0921-6

A product of Humana Press