

Full Length Research Paper

# Role of calcium in the expression of MAP kinase kinases (MKKs) and MAP kinases in tomato flower abscission

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The mitogen activated protein kinase (MAPK) cascade is an important intracellular signaling module that functions as a convergent point for crosstalk during stress signaling. In this study, we constructed a phylogenetic tree for MAP kinase kinases (MKKs) and MAP kinases (MPKs) in *Arabidopsis* and *Lycopersicon esculentum* (tomato) and found that abscission-related AtMKK4 and AtMKK5, and AtMPK3 and AtMPK6 clustered with LeMKK2, and LeMPK1, and LeMPK2 and LeMPK3, respectively. To investigate whether there was a link between homology and abscission, we studied all the LeMKKs at the messenger ribonucleic acid (mRNA) transcript level. LeMKK2 and its homologue AtMKK4/5 both have roles in regulating abscission. Quantitative real time polymerase chain reaction (qRT-PCR) of LeMPK1 and LeMPK2, which are highly homologous to AtMPK3 and AtMPK6, most probably mediate abscission downstream through LeMKK2. Calcium depressed the expression of these genes in the early stages of abscission. Treatment with W7 (a calmodulin inhibitor) indicated that the expression of LeMKKs and LeMPKs depended on calmodulin during tomato flower explant abscission.

**Key word:** Abscission, calcium, qRT-PCR, MAPK, tomato.

## INTRODUCTION

Abscission, the developmental process regulating the detachment of an organ from the main body of a plant, can be initiated in response to environmental factors, such as to disease or pathogen challenge and for shedding organs that no longer provide essential functions to the plant for example when the floral organs abscise following pollination and fruit set (Abeles 1968; Bleecker et al., 1997). Among the model plant used in abscission studies, tomato is the most widely studied due to its specialized flower pedicel joint-like abscission zone (AZ), which is recognized by its distinct indentation (Roberts et al., 2000).

At the cellular level, abscission is the hydrolysis of the

middle lamella of an anatomically specialized cell layer, the AZ, by cell wall-modifying and hydrolyzing enzymes. Thus, abscission requires both the formation of the AZ early in the development of a plant organ and the subsequent activation of the cell separation response (Bleecker et al., 1997; Patterson et al., 2001; Roberts et al., 1997, 2002; Lewis et al., 2006; Rose et al., 2003; Wang et al., 2007).

All eukaryotes utilize MAPK cascades to convey signals that are generated from the perception of extracellular and intracellular stimuli (Widmann et al., 2007). These cascades generally involve three functionally linked protein kinases: a MAP kinase kinase kinase (MKKK) that activates a MAP kinase kinase (MKK), which in turn activates a MAP kinase (MPK). MAPK cascades play important roles in plant responses to pathogens, hormones and development (Wang et al., 2007). In a recent study, MAPK cascades were shown to regulate floral organ abscission in *Arabidopsis thaliana*. AtMKK4 and AtMKK5 are not required for formation of the AZ, but rather function subsequently during the cell separation phase of abscission. AtMPK6 is a positive regulator

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**Abbreviations:** MKKs, MAP kinase kinases; MPKs, MAP kinases; AZ, abscission zone; RNA, ribonucleic acid; qRT-PCR, quantitative real time polymerase chain reaction.

of abscission and AtMPK3 can substitute for AtMPK6 in plants lacking a functional AtMPK6 (Sung et al., 2007).

Calcium (Ca) is an important secondary messenger that regulates the expression of genes that are important for abscission in tomato pedicel explants (Xu et al., 2009). Ca influences the abscission process by contributing to the maintenance of cellular integrity in cell walls and membranes. Bean explants incubated in 1-100 nM Ca solution showed a marked decline in abscission rate (Poovaiah and Rasmitssen, 1973). Similarly, when a Ca solution is applied to tomato flower pedicel explants, it results in an increase in abscission time (Xu et al., 2008).

In order to illuminate whether MAPK cascade signals are important during tomato flower abscission, and if so which members are involved, we used MAPK cascades that regulate floral organ abscission in *A. thaliana* as a template to construct phylogenetic trees. This also permitted detailed analysis of the LeMKKs and LeMPKs expressed during tomato flower abscission. Moreover, we aimed to investigate how Ca inhibits abscission and mediates MAPK cascade signals, as this remains elusive. In this study, we used treatments of Ca, W7 [a calmodulin (CaM) inhibitor] and W7 combined with Ca to study Ca function on abscission and MAPK cascade signals in *Lycopersicon esculentum* (tomato).

## MATERIALS AND METHODS

### Plant materials

Tomato liaoyuanduoli (*L. esculentum* Mill) plants were grown in a glass greenhouse under standard greenhouse condition at Shenyang Agricultural University, China. The culture chamber room was set as follow: 16/8 h photoperiod, 25/20°C day/night and 80% relative humidity. Yellow flowers with all petals opened to an angle of almost 90° were selected to study petiole abscission. The flowers were excised from the inflorescence and immediately placed under water to reduce the risk of xylem embolism and dehydration during explanation. The flowers were removed and pedicel explants were trimmed to 20 mm in length.

### Chemical treatment and abscission rate investigation

This study consisted of three experimental treatments (80 mM CaCl<sub>2</sub>, 100 μM W<sub>7</sub>, and 80 mM CaCl<sub>2</sub> combined with 100 μM W<sub>7</sub>) and a non-treated control. The proximal ends of 50 explants were inserted into 1% agar medium in one dish. The three dishes for each treatment were placed into a container with a glass cover (40 × 25 × 20 cm<sup>3</sup>) and the explants were incubated at 25°C. The pedicel abscission rate was estimated according to Wang et al. (2005), and recorded for each treatment.

### Phylogenetic tree

A multiple protein sequence alignment was generated by the Clustal W 1.83 program (Thompson et al., 1994) using putative amino acid sequences from putative MKKs and MPKs from *A. thaliana* and tomato. Phylogenetic analysis was performed using MEGA4.0 (Tamura et al., 2007) and the neighbor-joining method was employed for phylogenetic tree searching and inference.

Predictions of the structures of MKK and MPK genes were inferred using information available from the genome sequences of *A. thaliana* and *L. esculentum*.

### RNA extraction and RT-PCR

Total ribonucleic acid (RNA) for qRT-PCR was isolated from a 2-mm wide piece of tissue containing the flower AZ. Approximately 0.05 g of frozen AZ tissue was used to extract total RNA using the RNA Prep Pure Plant Kit (Tiangen, BeiJing, China) according to the manufacturer's instructions. Total RNA concentration was determined spectrophotometrically. First strand c-deoxyribonucleic acid (cDNA) synthesis with Superscript II reverse transcriptase (Invitrogen, Carlsbad, CA) was performed in a total volume of 20 μl using 1350 ng of total RNA as template.

### qRT-PCR

Specific primer pairs were designed for each of the four LeMKKs and three LeMPKs (Table 1), and their specificities were confirmed by running the RT-PCR product in agarose gel before using these for qRT-PCR analysis. The amplicon product of each primer pair was between 60 and 200 bp. Each PCR reaction consisted of 9 μl SYBR Green PCR Master Mix (TianGen, BeiJing, China), 2 μl of each primer, 7 μl of ddH<sub>2</sub>O, and 2 μl of each reverse transcribed cDNA product. qRT-PCR was carried out using an ABI7500 thermocycler running the following program: initial step of 95°C for 3 min, followed by 40 cycles of 30 s denaturation at 95°C, 30 s annealing at 55 to 60°C and a 1 min extension step at 68°C. The fluorescence signal was collected for each cycle during elongation at 68°C. At the end of each reaction, a dissociation curve was created to detect the presence of primer dimers or other unwanted amplified products that would produce a detectable CT value, and therefore negatively skew the results.

## RESULTS

### Chemical treatment and abscission rate

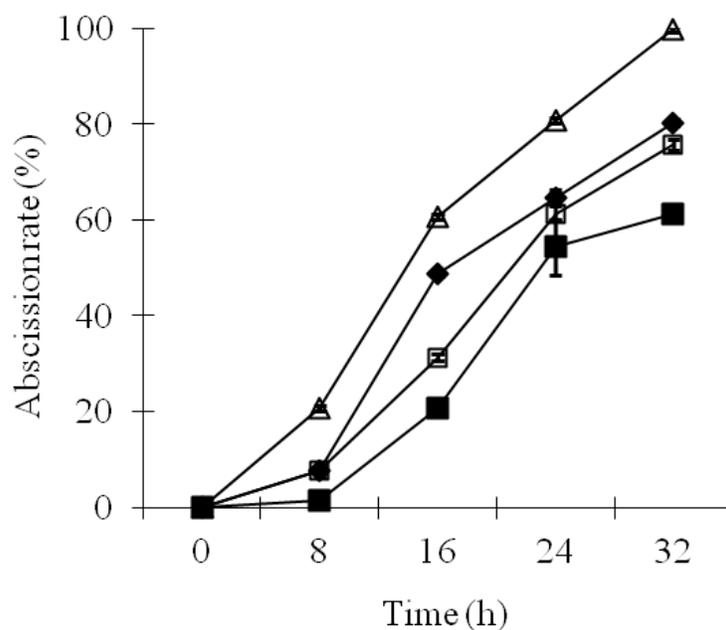
Approximately 50% abscission was obtained after 16 h in the control treatment, and the abscission rate was up to 80% at 32 h (Figure 1). Treatment with W7 accelerated the rate of abscission and these explants had shed completely at 32 h; however, Ca postponed the time course of abscission of the tomato flower pedicel explants, and abscission rate was almost zero before 8 h. Compared with the Ca treatment, abscission rate was elevated at each time point in the Ca combined with W7 treatment group. The relative abscission rate for the control peaked at about 42% after 16 h (Figure 2). W7 promoted abscission before 16 h, while the Ca and Ca plus W7 treatments inhibited relative abscission before 16 h.

### Generation of a MKK phylogenetic tree

To generate the phylogenetic tree, protein sequences were blasted from NCBI (<http://www.ncbi.nlm.nih.gov/>) and SGN (<http://solgenomics.net/>) using AtMKK4 and AtMKK5 sequences that mediate abscission in *A.*

**Table 1.** Real-time PCR primers for *LeMKKs* and *LeMPKs*.

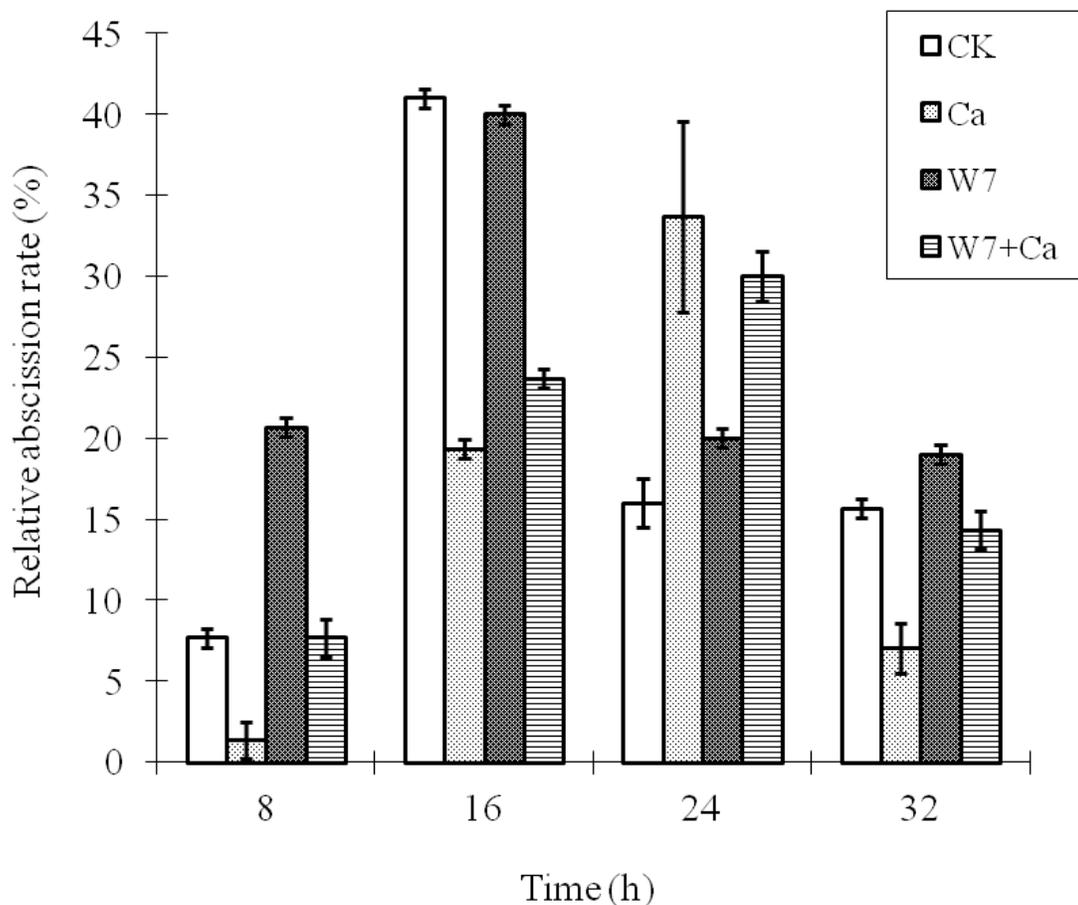
Gene	Primer sequence (5'-3')
<i>LeMKK1</i>	F:TCCAGA AGCACCAGA AGG R:CCTGCGGAAGTGAAGTAA G
<i>LeMKK2</i>	F:TACTGATTTGACCCTTCC TCT TC R:GGACGATGTAGAACCTTG TAAAC
<i>LeMKK3</i>	F:TACAGTCAGAGGACCAGCAAGC R:TGG AGC AGA AGG TGG TGG AC
<i>LeMKK4</i>	F:CCT TACCTCTCCCTCCATCTTC R:TTATACACAGTTCCACCGTTTCC
<i>LeMPK1</i>	F:CTGCGTCTTATTATGGAGTTGATTG R:TAAAGAGGAAGTTGTCCGATATAGC
<i>LeMPK2</i>	F:CGTCTGCTTATGGAGTTGATTGG R:CGTCTGCTTATAGAGTTGATTGG
<i>LeMPK3</i>	F:TCTTCTTCTCATCTTCTCCTTCC R:CAGCACCCATATTAGCATCAACC
<i>ACTIN</i>	F:TGTCCCTATTTACGAGGGTTATGC R:AGTTAAATCACGACCAGCAAGAT



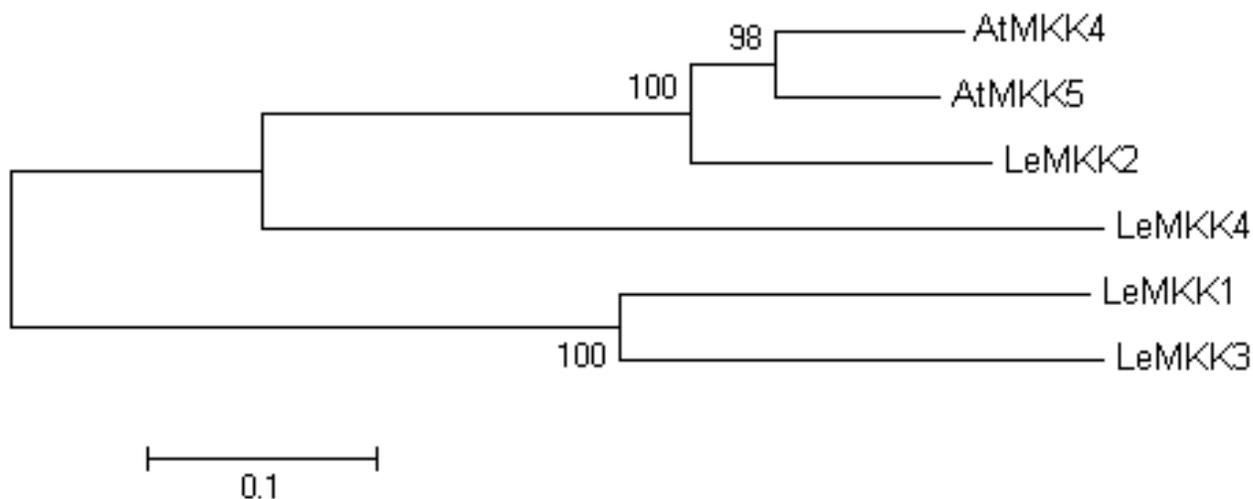
**Figure 1.** Abscission of tomato flower pedicel explants imbedded in different treatments. ◆ control; Δ, W7; ◻, Ca combined with W7; ■, Ca. Percentage abscission was calculated as the number of plants that had undergone floral pedicel shedding expressed as a percentage of the total explants within a treatment.

*thaliana*. The obtained tomato sequences were aligned using the Clustal W 1.83 program. The phylogenetic tree groups formed a number of clusters based on amino acid homology, and sequence divergence concurred with that

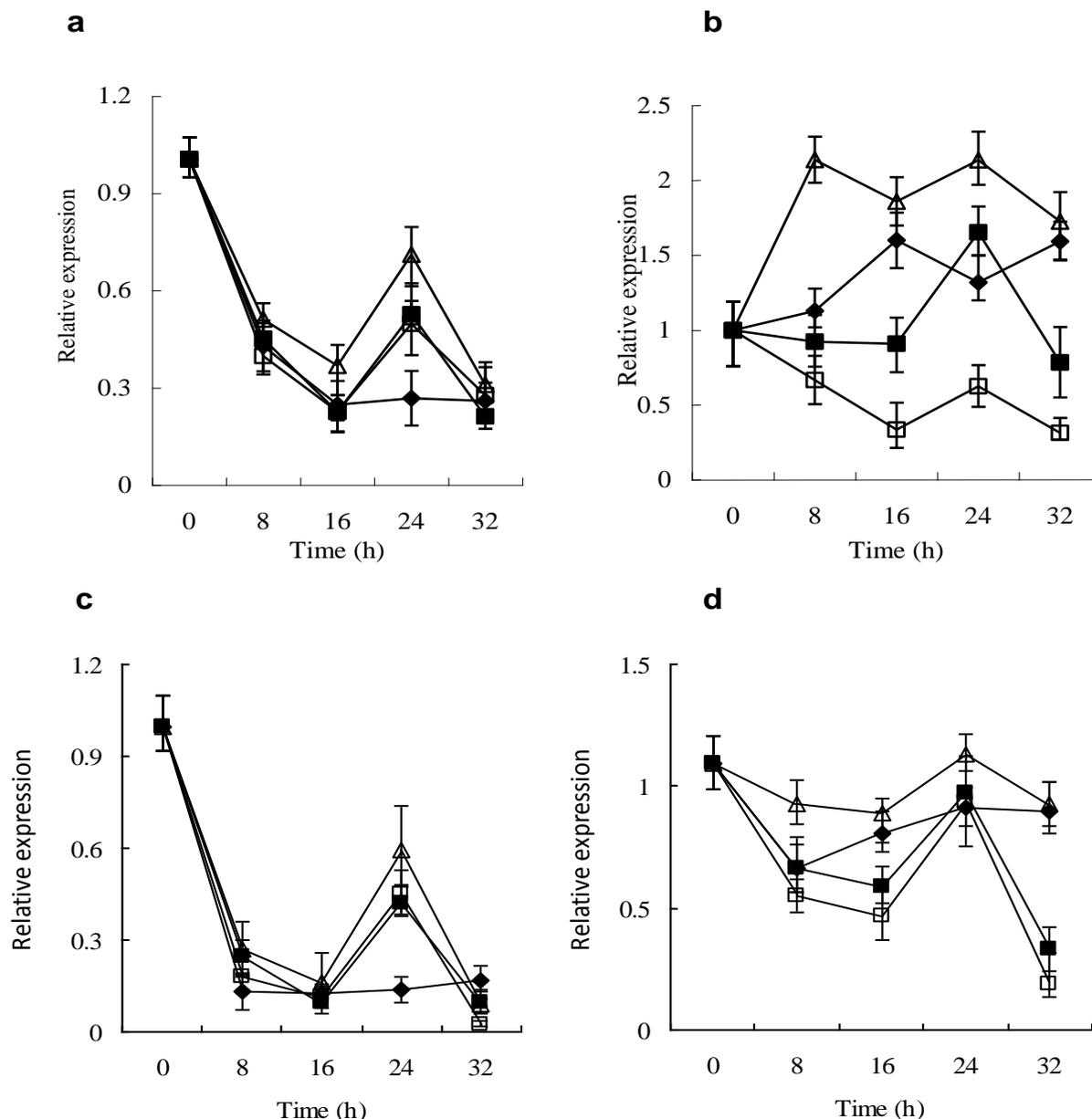
generated by the MEGA4.0 software. Phylogenetic analysis of amino acid sequences of different LeMAPKK members suggested that LeMKK2 shared high homology with abscission-related AtMKK4 and AtMKK5, while



**Figure 2.** Effects of different treatments on the cumulative abscission rate of tomato pedicel explants. CK, control.



**Figure 3.** Phylogenetic tree generated using amino acid sequences of putative MKKs from *A. thaliana* and *L. esculentum*. The branch lengths are proportional to the amount of inferred evolutionary change, and boot strap values are included. The scale bar indicates a distance of 0.1 from the root of the tree. The accessions used for generating the predicted protein sequences were AtMKK4 (NM\_104044), AtMKK5 (NM\_113017), LeMKK1 (AY691330), LeMKK2 (AY691331), LeMKK3 (AY691332) and LeMKK4 (AY691333).



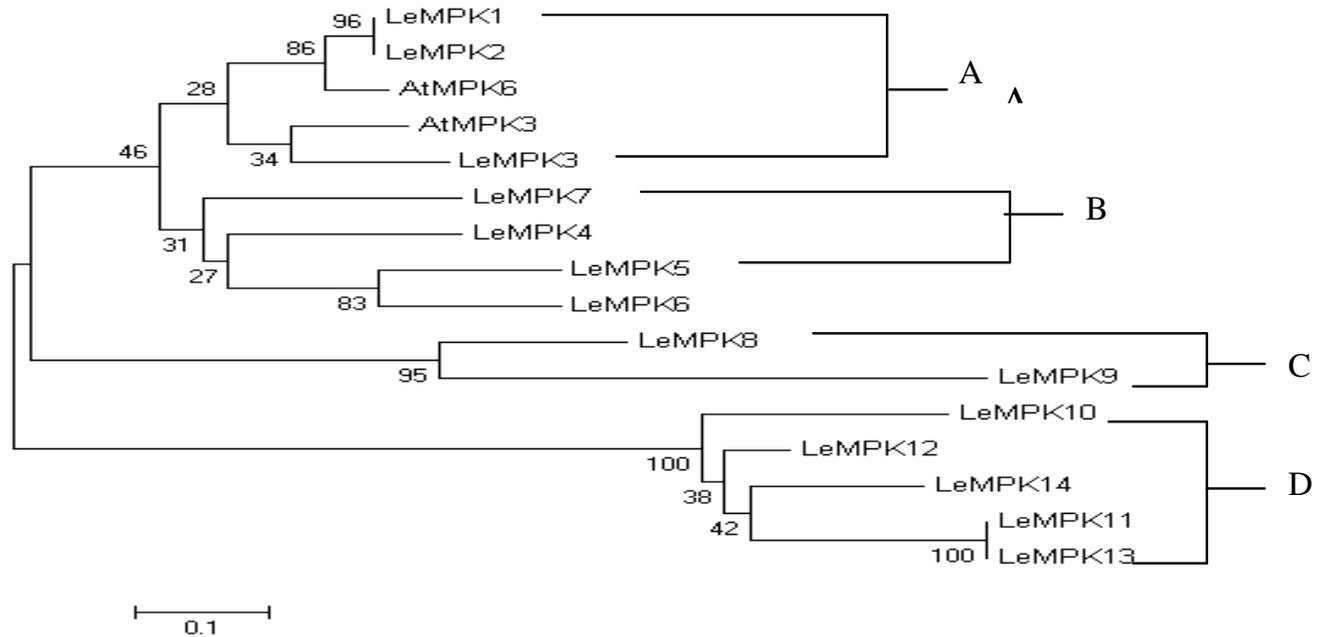
**Figure 4.** Relative gene expression assessed by qRT-PCR in the AZ of tomato pedicel explants. (a) LeMCK1; (b) LeMCK2; (c) LeMCK3; (d) LeMCK4.  $\blacklozenge$ , Control;  $\triangle$ , W7;  $\blacksquare$ , Ca combined with W7 treatment;  $\blacksquare$ , Ca. Vertical bars indicate standard error (n=9). Percentage abscission was calculated as the number of plants that had undergone floral pedicel shedding expressed as a percentage of the total explants within a treatment.

LeMCK1 is closely genetically related with LeMCK3 (Figure 3).

#### Expression of LeMCKs in the AZ

To further identify which LeMCKs are related to abscission, the expression patterns of LeMCK1, LeMCK2, LeMCK3 and LeMCK4 were analyzed by qRT-PCR during tomato abscission. Furthermore, possible effects of Ca on

expression were assessed by treatment with Ca, W7, and Ca combined with W7 (Figures 4a to d). In the control, LeMCK1 and LeMCK3 expression levels both quickly reduced to low levels even after 8 h, and expression remained low throughout abscission. LeMCK2 expression was up-regulated at 16 h and 32 h (1.5-fold greater transcript levels), and this corresponded with the timing of high relative abscission rate. LeMCK4 was down-regulated at 8 h, before showing a trend of increasing expression until complete abscission. For the explants



**Figure 5.** Phylogenetic tree generated using amino acid sequences of putative MPK from *A. thaliana* and *L. esculentum*. Branch lengths are proportional to the amount of inferred evolutionary change, and boot strap values are included. The scale bar indicates a distance of 0.1 from the root of the tree. The accessions used for generating the predicted protein sequences were AtMPK3 (At3G45640), AtMPK6 (At2G43790), LeMPK1 (SGN-U316697), LeMPK2 (SGN-U316695), LeMPK3 (SGN-U313928), LeMPK4 (SGN-U323634), LeMPK5 (SGN-U313996), LeMPK6 (SGN-U313995), LeMPK7 (SGN-U323219), LeMPK8 (SGN-U318773), LeMPK9 (SGN-U316113), LeMPK10 (SGN-U317229), LeMPK11 (SGN-U322516), LeMPK12 (SGN-U318438), LeMPK13 (SGN-U316366), and LeMPK14 (SGN-U318361).

treated with W7, the transcript level of each LeMCK was induced significantly. LeMCK1 and LeMCK3 expression levels peaked at 24 h, while the expression of LeMCK4 was induced to a relatively high level. However, the most notable finding was the up-regulation of LeMCK2 expression, which occurred at 8 h and 24 h during abscission. Ca treatment mainly depressed the expression of the LeMCKs in the early stages (before 16 h), with enhanced expression at 24 h, but reduced levels at 32 h. In the Ca combined with W7 treatment, except for LeMCK2, the mRNA expression levels of the other LeMCKs were very similar to the Ca treatment only. These data indicate that LeMCK2, which shares high homology with AtMCK5 and AtMCK5, plays a role in regulating abscission.

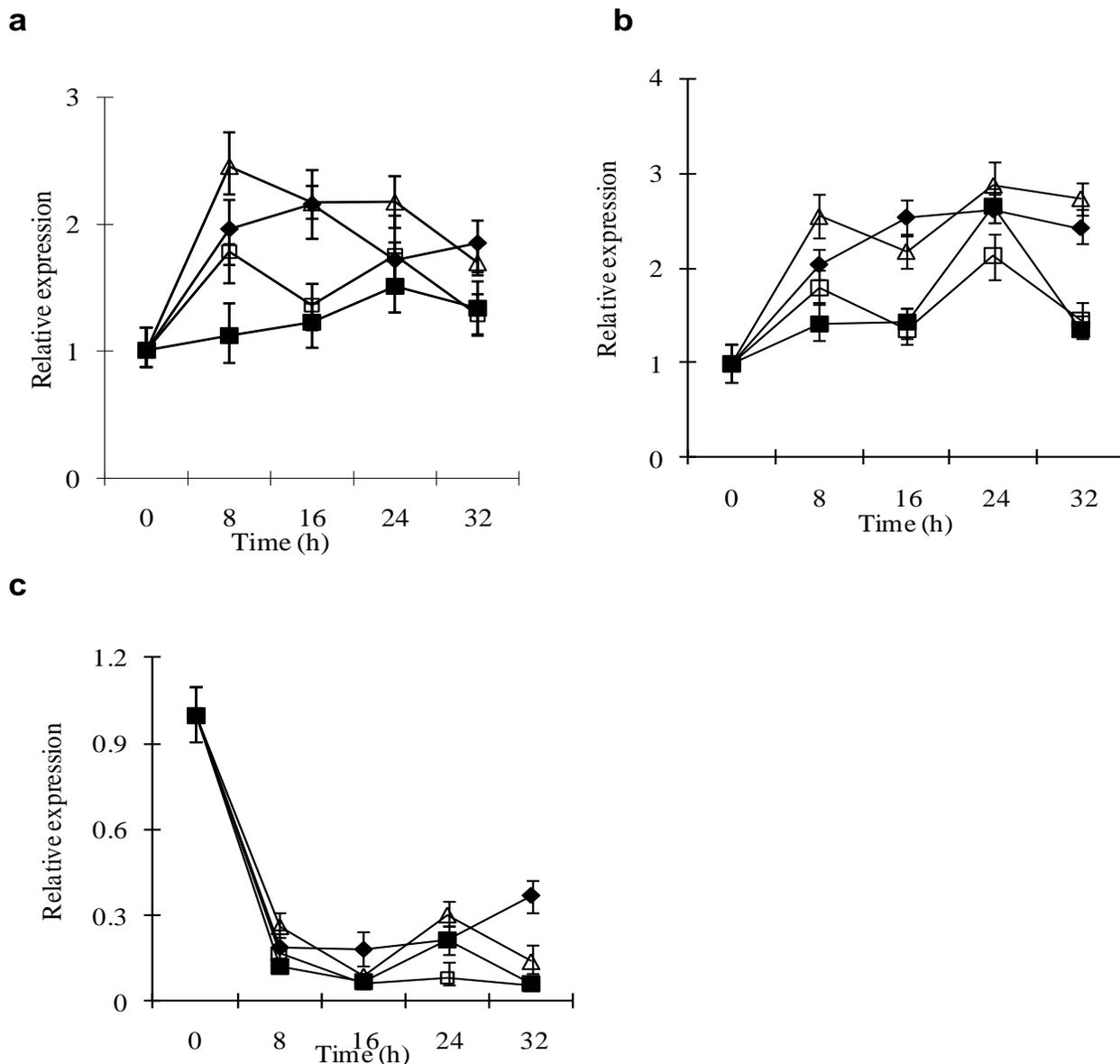
#### Generation of a LeMPK phylogenetic tree

For Arabidopsis, the 20 AtMPKs clustered into four groups, namely Groups A to D (Ichimura et al., 2002). MAPKs that cluster in Group A are thought to be positive regulators of defense signaling (Mishra et al., 2006). AtMPK3 and AtMPK6 belong to this group and these play a role in regulating floral organ abscission in Arabidopsis. In tomato, members of the LeMPKs also cluster into Groups A to D. Phylogenetic analysis showed that

LeMPK1 and LeMPK2 clustered with AtMPK6, while LeMPK3 clustered with AtMPK3, which plays a role in abscission. Based on their clustering in the phylogenetic tree, LeMPK1, LeMPK2 and LeMPK3 might play similar roles in the AZ in tomato as their Arabidopsis counterparts.

#### Expression of LeMPKs in the AZ

To identify the functions of the Group A LeMPKs, LeMPK1, LeMPK2 and LeMPK3 in abscission, the transcript levels of these genes were investigated (Figure 5). In the control, LeMPK1 transcript levels increased after incubation and peaked at 16 h, before reducing to a low level. Differing from LeMPK1, LeMPK2 transcript levels were up-regulated before 16 h and remained high throughout abscission. Transcript levels of LeMPK3 reduced rapidly after 8 h and remained low until 24 h, but then increased slightly at 32 h (Figures 6a, b, c). Compared to the control, Ca treatment mainly depressed LeMPK1 and LeMPK2 expression during abscission, especially before 24 h. Treatment with W7 increased the transcript levels of LeMPK1 and LeMPK2, especially at 8 h (early stages of abscission). The Ca combined with W7 treatment resulted in complex expression patterns for the LeMPKs. To some extent, mRNA expression of the



**Figure 6.** Relative gene expression assessed by qRT-PCR in the AZ of tomato pedicel explants. (a) LeMPK1; (b) LeMPK2; (c) LeMPK3. ◆, Control; △, W7; ∩, Ca combined with W7 treatment; ■, Ca. Vertical bars indicate standard error ( $n = 9$ ). Percentage abscission was calculated as the number of plants that had undergone floral pedicel shedding expressed as a percentage of the total explants within a treatment.

LeMPKs was similar to the Ca treatment, but notable differences were observed for the expression of LeMPK1 and LeMPK2 at 8 h and 24 h, where peak relative abscission rate occurred. These data indicate that LeMPK1 and LeMPK2 play a significant role in abscission, and Ca also participates in regulating the expression of LeMPKs.

## DISCUSSION

AtMKK4 and AtMKK5 have been shown to act through

AtMPK3 and AtMPK6 in the Arabidopsis defense response (Wang et al., 2007). In a recent study, the expression of AtMKK4 and AtMKK5 was demonstrated to play a role in floral organ abscission. Indeed, AtMKK4 and AtMKK5 are functional during the cell separation phase of abscission, rather than in the formation of the AZ. AtMPK6 is a positive regulator of abscission, but AtMPK3 can substitute for AtMPK6 in plants lacking a functional AtMPK6 (Sung et al., 2008). Here, we report that components of a MAPK signaling cascade also play roles in regulating abscission in tomato.

In this study, Clustal W 1.83 and MEGA4.0 software

were used to cluster the tomato MAPKKs with related kinases from Arabidopsis. The results indicate that LeMKK2 belongs to Group C, and it clusters with AtMKK4 and AtMKK5, which act upstream of AtMPK3 and AtMPK6 (Ichimura et al., 2002). Therefore, it was hypothesized that LeMKK2 could function similarly in abscission as these Arabidopsis homologues.

To determine whether any of the other three tomato MAPKK genes had similar functions, each one was analyzed in pedicel explants. Transcript levels of LeMKK2 (also Group C) were up-regulated in the AZ, while expression of the Group A MAPKKs, LeMKK1 and LeMKK3, were down-regulated. These results show that these genes play different roles in abscission in tomato pedicel explants.

Ca is an essential nutrient for plant growth and it can influence the abscission process by contributing to the maintenance of cellular integrity in terms of cell membranes and cell walls. Application of Ca solutions results in cell wall hardening in coleoptile tissues (Tagawa and Bonner, 1957), and bean explants incubated in 1-100 mM Ca showed a marked decline in abscission (Poovaiah and Leopold, 1973). Tomato pedicel explant abscission is also delayed when they are treated with 80 mM CaCl<sub>2</sub>. A CaM-sensitive Ca-dependent protein kinase inhibitor (TPF) has been used as a means to eliminate Ca, which subsequently hastens tomato pedicel explant abscission (Xu et al., 2009).

In this study, during abscission, pedicels treated with Ca or W7 (a CaM inhibitor) had different abscission rates and there was differential expression of LeMKKs in these two treatment groups. Ca mainly depressed the expression of LeMKKs in the early stages, and this may explain the lower abscission rate in this treatment group at 8 to 24 h. To investigate whether CaM directly mediates the expression of LeMKKs, changes in their expression during treatment with W7 were also analyzed. Compared to the control, the W7 treatment induced the expression of LeMKKs throughout abscission, but there was a notable depression of their expression in early stages. These results also indicate that expression of LeMKKs is dependant on CaM during abscission.

In this study, LeMPK1 and LeMPK2 expression was up-regulated during abscission. On the contrary, LeMPK3 expression was down-regulated. Different LeMPK mRNA expression responses were found in the AZ of tomato pedicels treated with Ca, W7 or Ca combined with W7. Ca mainly depressed LeMPK2 and LeMPK3 expression during the early stages of abscission. As W7 enhanced the expression of different LeMPKs, these data indicate that their expression is dependant on CaM during abscission. Furthermore, LeMPK1 and LeMPK2 belong to the A2 subgroup of plant MAPK and share 95% identity at the amino acid level (Holley et al., 2003). LeMPK1 and LeMPK2 are activated by the same upstream MAPKKs, namely LeMKK2 and LeMKK4 (Pedley and Martin, 2004).

In addition, AtMPK3 and AtMPK6 are coordinately activated by wounding, systemic oligosaccharide elicitors and UV-B (Higgins et al., 2007; Holley et al., 2003) in Arabidopsis. Finally, LeMPK1 and LeMPK2 are presumed to be functionally redundant and activated by LeMKK2 during abscission of tomato pedicel explants. Nevertheless, more experiments are required for comprehensively defining the role of LeMKK2 in pedicel abscission

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