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Expression of *MaMAPK* Gene in Seedlings of *Malus* L. under Water Stress

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Abstract Seedlings of three species of *Malus* were used to study the expression of mitogen-activated protein kinase (MAPK) in response to water stress: *Malus hupehensis*, a drought-sensitive species; *Malus sieversii*, a drought-tolerant species; and *Malus micromalus*, a middle type. Results showed that *Malus MAPK* (*MaMAPK*, GenBank accession No. AF435805) was expressed in both roots and leaves of seedlings of the three *Malus* species treated with 20% polyethylene glycol for different time periods. Expression levels peaked at 1.5 h after treatment with polyethylene glycol, then decreased to their lowest levels. Liquid kinase assays indicated that the dynamic changes of MAPK activity were very similar to those of the relative expression of *MaMAPK* mRNA. However, the peak of the former occurred slightly behind the latter. It was noticed that, although the kinase activity decreased after the peak, it was still higher than that of the control during the whole time period. These results suggested that *MaMAPK* was regulated not only by water stress at the transcription level, but also by phosphorylation and dephosphorylation at the protein level. In addition, of these three apple species, the highest MAPK activity and *MaMAPK* expression level was found in *M. sieversii*, followed by *M. micromalus* and *M. hupehensis*, suggesting that MAPK might be correlated with drought tolerance in these three species. The different expression levels might be one of the molecular mechanisms of the different drought tolerances in *Malus*.

Key words expression characteristics; *Malus* L.; *MaMAPK* gene; water stress

Many signal pathways and similar models of gene expression and regulation can be induced in plant cells by drought, high salinity and low temperature stress [1]. The phosphorylation and dephosphorylation of protein are the important biochemical reactions of energy metabolism and signal transduction in an organism in response to extracellular signals [2]. Mizoguchi *et al.* [3] proved that there was a mitogen-activated protein kinase (MAPK) cascade pathway composed of mitogen-activated protein kinase kinase kinase (MAPKKK/AtMEKK1)→mitogen-activated protein kinase kinase (MAPKK/MEK1)→MAPK (AtMPK4) in plant cells. MAPK is located downstream of the cascade pathway and is a conserved serine/threonine protein kinase in eukaryotes. MAPKs were activated by the upstream components through phosphorylation on both threonine and tyrosine residues in the conserved threonine-X-tyrosine sequence in kinase subdomain VIII [4] and can be de-activated by both tyrosine and serine/threonine-specific phosphatase [5], therefore many stress signals were transmitted.

Apples are an important economical crop. Some apple species are used as stock. Their roots are flourishing and greatly affected by soil water, so their growth and products are seriously affected by water stress. Research showed that there were phosphorylation and dephosphorylation in apple seedlings in response to water stress [6], which suggested that there were protein kinase genes regulating the plants' adaptability to water stress. In order to find out the expression characteristics of the protein kinase gene and its regulation mechanism in apple species under water stress, *Malus MAPK* (*MaMAPK*, GenBank accession No. AF435805), the homologous gene of *MAPK*, has been cloned from *Malus micromalus* [7]. In the present study, we demonstrated that the transcription levels of the *MaMAPK* gene gradually increased in response to water

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stress. The dynamic change in kinase activity was basically similar to that of the relative expression of *MaMAPK* mRNA. The regulation of the *MaMAPK* gene at both transcription and protein levels, in relation to water stress, is also discussed.

Materials and Methods

Plant materials, growth conditions and stress treatment

The plant materials were *Malus hupehensis* (Pamp.) Rehd., a type sensitive to drought, *Malus sieversii* (Ledeb.) Roem., a drought-tolerant type, and *M. micromalus* Makine, a middle type. Apple (*Malus* L.) seeds were disposed at 4 ºC for 30–40 d and sown in a culture medium composed of vermiculite, peat soil and sand (2:2:1, *V*/*V*/*V*). Seedlings grew at 25 ºC and received a photoperiod of 12 h every day. After the sixth true leaf emerged they were transferred into Hoagland nutrition solution containing 20% polyethylene glycol (PEG) 6000 for water stress treatment. The young leaflets and roots were harvested after being treated for different durations, quickly immersed in liquid nitrogen, then stored at –80 ºC for RNA extraction and at –20 ºC for protein extraction.

RNA extraction

Plant materials were ground in liquid nitrogen, suspended in cetyl trimethyl ammonium bromide immediately for 30 s, and preheated in water at 65 ºC for 2 min. Samples were extracted with equal volumes of chloroform:isoamyl alcohol (1:1, *V*/*V*), repeated three times. RNA was precipitated overnight with one-third volume of 8 M LiCl at 4 ºC, washed with 70% ethanol, reprecipitated with double volumes of 100% ethanol at –20 ºC for 2 h, then dissolved in water treated with diethylpyrocarbonate.

Northern blot analysis

RNA samples (20 μg), after electrophoresis in 1% (*W*/*V*) formaldehyde-denatured agarose gels, were blotted onto a Hybond-N nylon membrane by capillary transfer using paper towels. The membrane was dried and fixed at 80 ºC for 2 h. The reverse transcription-polymerase chain reaction product at the 3′ end of *MaMAPK* acted as the probe. The cDNA probes were ^{32}P-labeled with random primers (Sigma-Aldrich, St. Louis, USA) and used for hybridization with RNA blots in 50% (*V*/*V*) formamide, 5×standard saline citrate, 5×Denhartd's solution, 0.5% (*W*/*V*) sodium dodecyl sulfate, and 0.1 mg/ml denatured herring sperm DNA at 42 ºC for 20 h. The blots were washed in 1×standard saline citrate, 0.1% (*W*/*V*) sodium dodecyl sulfate at 50 ºC two or three times, and X-ray films were exposed for various time periods with an intensifying screen at –80 ºC.

Protein extraction

Plant sample (2 g) was taken and kept on ice in a two-fold volume of extraction buffer which contained 250 mM sucrose, 1 mM dithiothretol, 1 mM EDTA, 50 mM Tris-Mes (pH 7.6), and 0.5% polyvinylpolypyrrolidone. The homogenate was filtered through a four-layer nylon cloth and centrifugated (14,000–15,000 *g*) at 4 ºC for 15 min. The supernatant was taken out and centrifugated (42,000–45,000 *g*) at the same temperature for 90 min. The protein concentration of supernatant, the component of soluble protein, was determined by Coomassie Brilliant Blue G250 (Sigma-Aldrich) [6].

Determination of protein kinase activity

In order to start the reaction, the following materials were added to 100 μl of reaction system: 50–100 μg/ml undetermined protein, 50 mM Tris-Mes (pH 7.0), 10 mM $MgCl_2$, 1 mM $CaCl_2$ or 2 mM EGTA, 0.3 mg/ml myelin basic protein or histone III, and 10 μCi/ml [γ-^{32}P]ATP (final concentration of 186 TBq mM^{-1}). After treatment for 6 min at room temperature, 20 μl of reaction mixture was immediately taken out and dropped on Whatman P-81 filter paper (Whatman, Maidstone, UK) which was treated with 20% trichloroacetic acid (TCA) and 1% NaPPi. After it was dried slightly, the filter paper was washed in 5% TCA solution containing 1% NaPPi four times, 15 min for each time. After the filter paper was dried, it was put into the glimmer solution to determine the radioactivity intensity of phosphorylated protein by the XH-6925 solution glimmer counter. The cpm/μg/min was taken as the unit of protein kinase activity, and 20% TCA of final concentration was added to the control before ^{32}P was added.

Results

Expression of *MaMAPK* in roots of three apple species under water stress

The expression of *MaMAPK* was analyzed at the mRNA level by Northern hybridization. The results showed that the same expression dynamics of *MaMAPK* occurred in roots of the three apple seedlings after treatment with 20% PEG for different time periods (**Fig. 1**). Hybridization signals strengthened as the time of PEG treatment

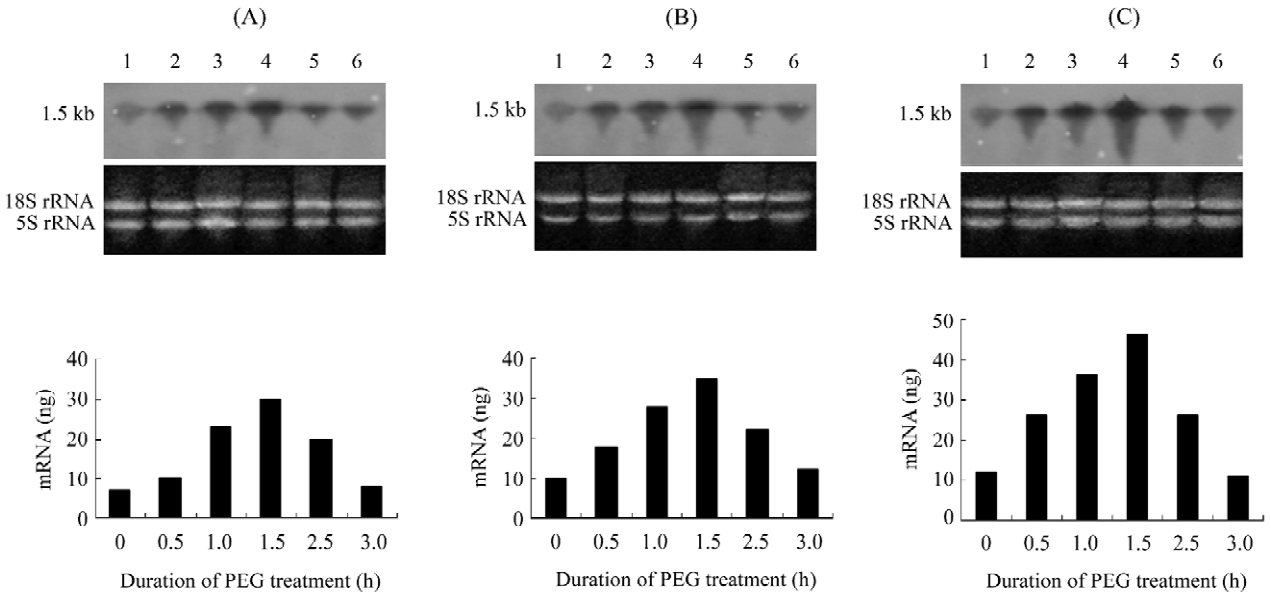

Fig. 1 Northern blotting and levels of *Malus* mitogen-activated protein kinase mRNA in roots of three apple species treated with 20% polyethylene glycol (PEG) at different time points

(A) *Malus hupehensis*. (B) *Malus micromalus*. (C) *Malus sieversii*. 1–6, treated with PEG for 0, 0.5, 1.0, 1.5, 2.5 and 3.0 h respectively.

lengthened, and peaked at 1.5 h after treatment, which implied that *MaMAPK* was regulated by water stress at the transcription level. Furthermore, we found that the highest expression level of *MaMAPK* was in *M. sieversii*, followed by *M. micromalus* and *M. hupehensis*. This pattern of expression was consistent with that of antioxidant enzyme activity in three apple species under water stress [8]. In addition, there was slight expression in the roots of the control. This might be due to one of two reasons: *MaMAPK* was constitutively expressed; or there was a small *MaMAPK* family in the *Malus* L. gene group [7].

Expression of *MaMAPK* in leaves of three apple species under water stress

As shown in **Fig. 2**, *MaMAPK* was also expressed in leaves of the three apple seedlings after treatment with 20% PEG for different time periods. The pattern of expression was the same as that in the roots. The highest level of *MaMAPK* mRNA was found in *M. sieversii*, and the lowest was in *M. hupehensis* with the same treatment. However, the difference in *MaMAPK* expression between roots and leaves was that *MaMAPK* did not express in the leaves of *Malus* in control seedlings. This result implied that the expression of the *MaMAPK* gene was inducible, but not constitutive, in leaves.

The relative expression amount of *MaMAPK* mRNA in roots and leaves of the three apple seedlings under water stress was analyzed by means of UVP Lab Work software (**Figs. 1** and **2**). The results showed that the relative expression of mRNA in roots was higher than that in the leaves (data not shown). The comparison of the relative expression of *MaMAPK* mRNA among the three apple species indicated that, in both roots and leaves, *M. sieversii* was the highest, followed by *M. micromalus*, and *M. hupehensis* was the lowest (**Figs. 1** and **2**). These reflected the differences in the adaptability of each of the three apple species to drought stress.

Relationship between relative expression of *MaMAPK* mRNA and activity of MAPK under water stress

This research showed that *MaMAPK* can express at different levels under water stress, and be regulated at the transcription level. Earlier research proved that MAPK played important roles in signal transduction through phosphorylation-dephosphorylation of protein kinase [1]. Did MAPK, the expression product of *MaMAPK*, transmit the signal of water stress through reversible phosphorylation? What was the relation between the activity of MAPK and the relative expression amount of *MaMAPK* mRNA? Taking *M. sieversii* as an example, the liquid kinase assay manifested that the dynamic changes of MAPK activity were basically consistent with the mRNA level (**Fig. 3**). This

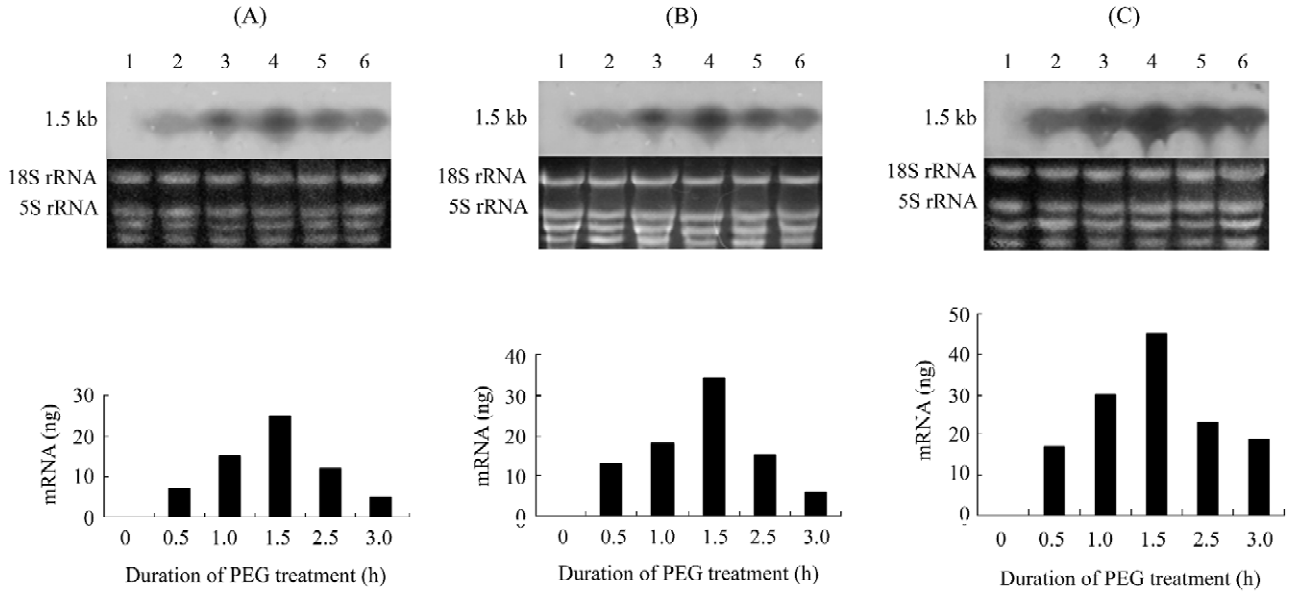

Fig. 2 Northern blotting and levels of *Malus* mitogen-activated protein kinase mRNA in leaves of three apple species treated with 20% polyethylene glycol (PEG) at different time points

(A) *Malus hupehensis*. (B) *Malus micromalus*. (C) *Malus sieversii*. 1–6, treated with PEG for 0, 0.5, 1.0, 1.5, 2.5 and 3.0 h, respectively.

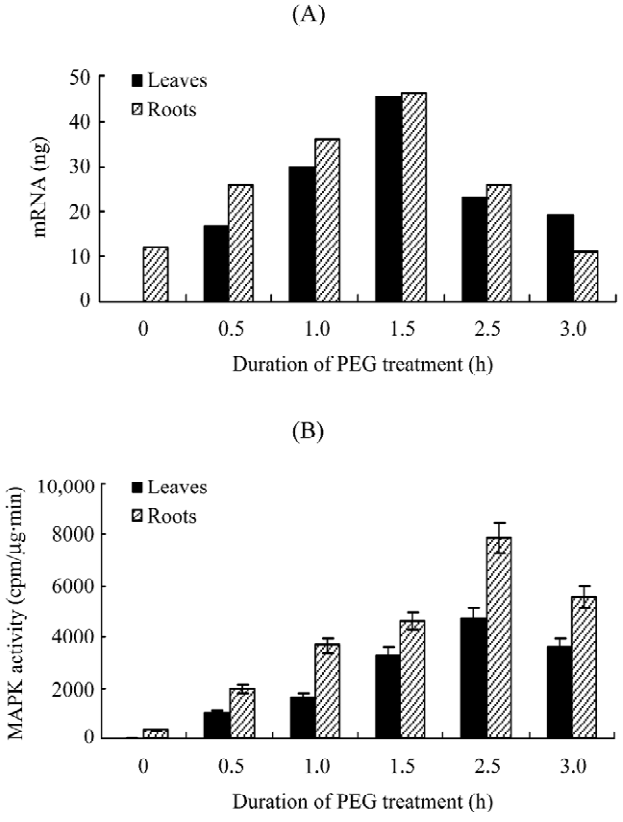

Fig. 3 Relative expression of *Malus* mitogen-activated protein kinase (MAPK) mRNA (A) and activity (B) of MAPK in *Malus sieversii* under water stress

suggested that *MaMAPK* was regulated not only by water stress at the transcription level, but also by phosphorylation of MAPK at the protein level.

Discussion

MAPKs are types of protein kinases that are important in cell signal transduction. They participate in many signal transduction processes through the cascade pathway and play important roles in mediating cell differentiation [9, 10], cell development [11], hormone action [12], the transduction of extracellular environmental stress signals and the regulation of intracellular stress responses [1,13–15]. MAPKs regulate the expression of many genes through the phosphorylation and dephosphorylation of protein, especially the phosphorylation of transcription factors. Research in *Arabidopsis* found that the expression of *AtMEKK1* (coding MAPKK) and *AtMPK3* (coding MAPK) genes [16] could be induced by drought, high salinity and low temperature. *AtMEKK1* and *AtMPK3* could be induced and expressed in 5 min under these conditions, their expression could be markedly increased within 1 h, then continued to increase, and peaked after 24 h. These results certified that *AtMEKK1* and *AtMPK3* were regulated not only by environmental signals at the transcription level, but also by phosphorylation and dephosphorylation at the

protein level.

Our Northern blotting results indicated that there was expression of *MaMAPK* mRNA in roots and leaves (except for the control) of the three apple species (**Figs. 1** and **2**) under water stress. These results suggested that *MaMAPK* could be induced by water stress at the transcription level. But differences in the levels of *MaMAPK* mRNA were produced by different durations of stress treatment. The highest levels of *MaMAPK* mRNA were induced after water stress for 1.5 h. This implied that the expression of this gene could also be regulated by water stress. But what is the reason for the difference in the maximal expression times between *Arabidopsis* MAPK (24 h) and *Malus* MAPK (1.5 h)? There may be two reasons. The *MAPK* expression in *Malus* was transient, because, after maximal expression at 1.5 h, the expression decreased again (**Figs. 1** and **2**). In contrast, it was continuous in *Arabidopsis*. Another reason might be the use of different pathways in different plant species, because many MAPK cascade pathways have been found in plants [3].

The results showed that the relative expression of mRNA in roots was higher than in leaves (**Figs. 1** and **2**), suggesting that the transcription amount of *MaMAPK* mRNA in roots was different from that in leaves. Roots are more sensitive to water stress, and respond rapidly; therefore, the increase in levels of *MaMAPK* mRNA could be induced by slight water stress.

Liquid kinase assays indicated that the dynamic change of kinase activity was basically similar to that of the expression of *MaMAPK* mRNA. The difference was that the peak of kinase activity was later than that of *MaMAPK* mRNA. These results were identical with those of the alfalfa *MMK4* gene [17]. It might be possible that the *MaMAPK* gene was transcribed and accumulated, but not translated to protein or only partly translated. Another possibility is that, after transcription, the *MaMAPK* mRNA was translated into protein; different pools of protein might have different rates of turnover, but steady state levels of protein might stay constant. Little is known about evaluating the significance of these observations, so further research is needed to investigate different possibilities. However, although the kinase activity decreased after a peak, the increased extent was still higher than that of the control during the whole time period, which suggested that the *MaMAPK* gene was regulated not only by water stress at the transcription level, but also by phosphorylation and dephosphorylation at the protein level.

The dynamic changes of MAPK activity of three apple species were basically similar to that of their relative expression of *MaMAPK* mRNA, which was consistent with the different drought tolerances of the three apple species [7]. But the differences between gene expression and enzymatic activity among the three species were slight. Could they cause the differences in drought-stress tolerance of these species? Cowan and Storey [18] reported that the phosphorylation of the MAPK results in a conformational change and a >1000-fold increase in specific activity, so that, in effect, MAPKs are inactive unless phosphorylated by their respective upstream kinases [18]. Therefore, the difference between the levels of *MaMAPK* mRNA and the activity of MAPK might be one of the molecular mechanisms of different drought tolerance in *Malus*.

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