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...
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. microsmaus Makine, a middle type. Apple (Malus L.) seeds were disposed at 4 °C for 30–40 d and sown in a culture medium composed of vermiculite, peat soil and sand (2:2:1, V/V/V). The young leaflets and roots were harvested after being treated for different durations, quickly immersed in liquid nitrogen, then stored at −80 °C. The supernatant was taken out and centrifugated (14,000 × g) at the same temperature 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 MgCl2, 1 mM CaCl2, or 2 mM EGTA, 0.3 mg/ml myelin basic protein or histone III, and 10 μCi/ml [γ-32P]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 32P 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
Expression of MaMAPK Gene in Seedlings of Malus L. under Water Stress

Li-Xin PENG et al.: Expression of MaMAPK Gene in Seedlings of Malus L. under Water Stress 283

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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
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.
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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