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BRIEF COMMUNICATION

# The effects of abiotic stresses on the NADP-dependent malic enzyme in the leaves of the hexaploid wheat

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## Abstract

The objective of this study was to examine the effects of different abiotic stresses on the activity of an NADP-dependent malic enzyme (NADP-ME) and the corresponding gene transcription in the leaves of the hexaploid wheat (*Triticum aestivum* L.) The activity of the NADP-ME enzyme was increased by water stress (20 % polyethylene glycol 6000), low temperature (4 °C), darkness, salinity (200 mM NaCl), abscisic acid and salicylic acid. The transcription of the *TaNADP-ME1* gene decreased in response to all of the stresses except darkness and NaCl. In addition, the transcription of *TaNADP-ME2* was down-regulated by all of the tested treatments and could not be detected under dark stress.

*Additional key words:* abscisic acid, enzyme activity, gene transcription, low temperature, salicylic acid, salinity, water stress.

The NADP-dependent malic enzyme (NADP-ME) catalyzes the following reaction: (*L*)-malate + NADP → pyruvate + $CO_2$ + NADPH (Edwards and Andreo 1992, Taub and Lerdau 2000, Cheng and Long 2007). In plants, NADP-ME is divided into photosynthetic and non-photosynthetic forms according to various physiological roles held by the enzyme (Drincovich *et al.* 1998, Laporte *et al*. 2002). The photosynthetic forms of the NADP-ME supply $CO_2$ for carbon fixation in the bundle sheath chloroplasts of $C_4$ plants as well as in the cytosol of CAM plants. The non-photosynthetic forms of the NADP-ME enzyme are involved in regulating the cellular pH, ionic balance, fatty acid biosynthesis and the synthesis of other organic acids (Famiani *et al.* 2000, Shearer *et al.* 2004, Gerrard Wheeler *et al.* 2008). Recently, some of the non-photosynthetic forms of the NADP-ME enzymes have also been associated with the plant defence (Fushimi *et al.* 1994, Casati *et al*. 1999, Pinto *et al.* 1999, Maurino *et al.* 2001, Chi *et al.* 2004).

The NADP-ME has been well studied in $C_4$ and CAM plants (Honda *et al*. 2000, Drincovich *et al*. 2001, Falcone-Ferreyra *et al*. 2003, Saigo *et al.* 2004), however, few studies have been conducted on $C_3$ plants such as rice, tobacco, *Arabidopsis thaliana* and wheat (Casati *et al.* 1997, Maurino *et al.* 1997, Liu *et al.* 2007, Müller *et al.* 2008, Wheeler *et al.* 2008). In this study, we measured the effects of various stressors including polyethylene glycol (PEG), NaCl, low temperature (4 ºC), salicylic acid (SA), abscisic acid (ABA) and darkness on the NADP-ME enzyme activity and the gene transcription in the leaves of wheat plants.

Wheat (*Triticum aestivum* L.) cv. Jinmai 47 was grown in *Vermiculite* at 25 °C, relative humidity 60 - 70 % and irradiance of 400 μmol $m^{-2}$ $s^{-1}$ with a 12-h photoperiod. The selected treatments were applied by transferring 2-week-old seedlings into a Hoagland solution containing either 200 mM NaCl, 20 % (m/v) PEG 6000, 200 μM ABA, or 3 mM SA. The low

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*Abbreviations*: ABA - abscisic acid; CAM - Crassulacean acid metabolism; ME - malic enzyme; PEG - polyethylene glycol; SA - salicylic acid.
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temperature and dark treatments were administered by transferring the plants into the 4 °C or into dark. The untreated seedlings were used as a control. The leaves were harvested at 0, 3, 6, 12, 24 and 48 h after treatments and used for the enzyme assay and the RNA extraction.

Approximately 0.5 g of wheat leaves were homogenized in an extraction buffer containing 100 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 2 mM EDTA, 10 % (v/v) glycerol, 10 mM 2-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF) and then centrifuged at 9 000 *g* for 10 min. The supernatants were used for the measurement of the NADP-ME activity according to Liu *et al.* (2007), by monitoring on the spectrophotometer (type, producer, location) the increase in absorbance at 340 nm as the NADPH was produced. The standard reaction mixture contained 0.95 $cm^3$ of the reaction buffer (50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 0.5 mM NADP, 4 mM *L*-malate) and 0.05 $cm^3$ of the supernatants of the enzyme into a final volume of 1 $cm^3$ of solution. The reaction was then started by adding 0.05 $cm^3$ of 4 mM *L*-malate into the mixture. One unit of enzyme activity is defined by an increase of 0.01 in the absorbance of the mixture at 340 nm per minute.

The plastidic *TaNADP-ME1* (EU170134) and the cytosolic *TaNADP-ME2* (EU082065) were cloned from the leaf tissue of the hexaploid wheat before and after above mentioned treatments. A semi-quantitative RT-PCR was performed using the total RNA that was extracted from the wheat leaves. The DNA-free RNA was then reverse transcribed using the *PrimeScript* reverse transcriptase at 42 °C for 1 h. The gene-specific primers were designed in the 3' untranslated region. The specific primers are as follows:
*TaNADP-ME1:* Be1-5 (5-GAAGCATACAAATGGA CCAAGG-3) and Be1-3 (5-CAAGAACAG CGACAG ACAACAA-3); *TaNADP-ME2:* Be2-5 (5-GTGGAG TACGAGGGGAAAAC-3) and Be2-3 (5-GCATAT GGGGGAAGGAGATT-3). The primers for the wheat actin gene (WAC-F:5-GTTCCAATCTATGAGGGA TACACG-3, WAC-R: 5-GAACCTCCACTGAGAACA ACATTACC-3) were used as the internal standard in this study.

The statistical analysis was performed by a one-way *ANOVA* using the *SPSS* software package. The data are the average of a minimum of three replicates. The smallest significant differences (LSD) between the means were estimated at the 95 % confidence level.

The activity of the NADP-ME was mostly enhanced under various stresses (Table 1). Under the PEG, SA and NaCl stresses, the NADP-ME activity initially increased being highest after 6-h (NaCl) or 12-h (PEG, SA) treatments and then decreased. Both the cold and dark stresses caused a continuous rise in the NADP-ME enzyme activity, with activity peaks after 48-h treatment that were 4.3 and 4.7 times higher than the control, respectively. The ABA treatment resulted in an increase in the enzyme activity with time, except in the 24-h treatment. Likewise, the enzyme activity in the 48-h ABA treated plants was more than four times higher than that of the control. Based on the enhanced enzyme activity under all of the tested treatments, we conclude that the wheat NADP-ME responds to various abiotic stresses. Previous researches have also reported that NADP-ME activity can be induced by wounding, pathogen attacks, by cellulase or UV-B irradiation (Maurino *et al.* 1997, 2001, Casati *et al.* 1997, 1999, Pinto *et al.* 1999). We believe that some inactive isozymes may turn into active isoforms through phosphorylation during stress. Under stress the catalyzing efficiency of the NADP-ME enzyme might be further enhanced with the consumption of NADPH. Furthermore, plants have many pathways for adaptation to abiotic stresses (Ferreira *et al.* 2008).

The semi-quantitative RT-PCR showed that the transcript accumulation of *NADP-ME* in the leaves is distinctly affected by various stresses (Fig. 1). The expression levels of *TaNADP-ME1* in the leaves gradually decreased until 24 h following the application of PEG. When the leaves were treated with NaCl, the *TaNADP-ME1* was down-regulated in the first 6 h of the treatment and then began to rise until 24 h of treatment. At a low temperature (4 °C), the production of the *TaNADP-ME1* transcripts was depressed until 3 h of treatment, at which point they began to ascend until 6 h of treatment, and finally they diminished at 24 h of treatment. After the SA treatment, the mRNA amounts were reduced in the first 6 h, they then began to increase until 24 h of treatment. Under the ABA treatment, the *TaNADP-ME1* transcript levels declined through the

Table 1. The activity of the wheat NADP-ME [U $mg^{-1}$(protein)] under different treatments (PEG, NaCl, cold, SA, ABA and darkness) during the experimental period. Means ± SE of at least 3 replicates. Different letters marked significantly different observations at $P < 0.05$.

| Treatment | 0 h | 3 h | 6 h | 12 h | 24 h | 48 h |
|---|---|---|---|---|---|---|
| Control | 0.47 ± 0.04c | 0.68 ± 0.04c | 1.95 ± 0.26a | 1.69 ± 0.39ab | 1.07 ± 0.05bc | 0.92 ± 0.05c |
| PEG | 0.47 ± 0.04c | 1.40 ± 0.08b | 2.58 ± 0.19a | 1.72 ± 0.19a | 2.53 ± 0.09a | 1.87 ± 0.17b |
| NaCl | 0.47 ± 0.04d | 2.28 ± 0.48b | 3.46 ± 0.28a | 2.48 ± 0.27b | 2.28 ± 0.28b | 1.40 ± 0.06c |
| Dark | 0.47 ± 0.04c | 1.27 ± 0.18c | 2.68 ± 0.35b | 2.98 ± 0.17b | 3.57 ± 0.38ab | 4.28 ± 0.29a |
| cold | 0.47 ± 0.04e | 1.93 ± 0.15d | 2.63 ± 0.10c | 2.86 ± 0.12c | 3.46 ± 0.13b | 3.98 ± 0.28a |
| SA | 0.47 ± 0.04d | 2.46 ± 0.46bc | 2.83 ± 0.32bc | 5.01 ± 0.42a | 3.62 ± 0.24ab | 1.63 ± 0.10cd |
| ABA | 0.47 ± 0.04e | 0.93 ± 0.07d | 3.13 ± 0.19c | 3.90 ± 0.10b | 3.38 ± 0.19c | 4.79 ± 0.21a |

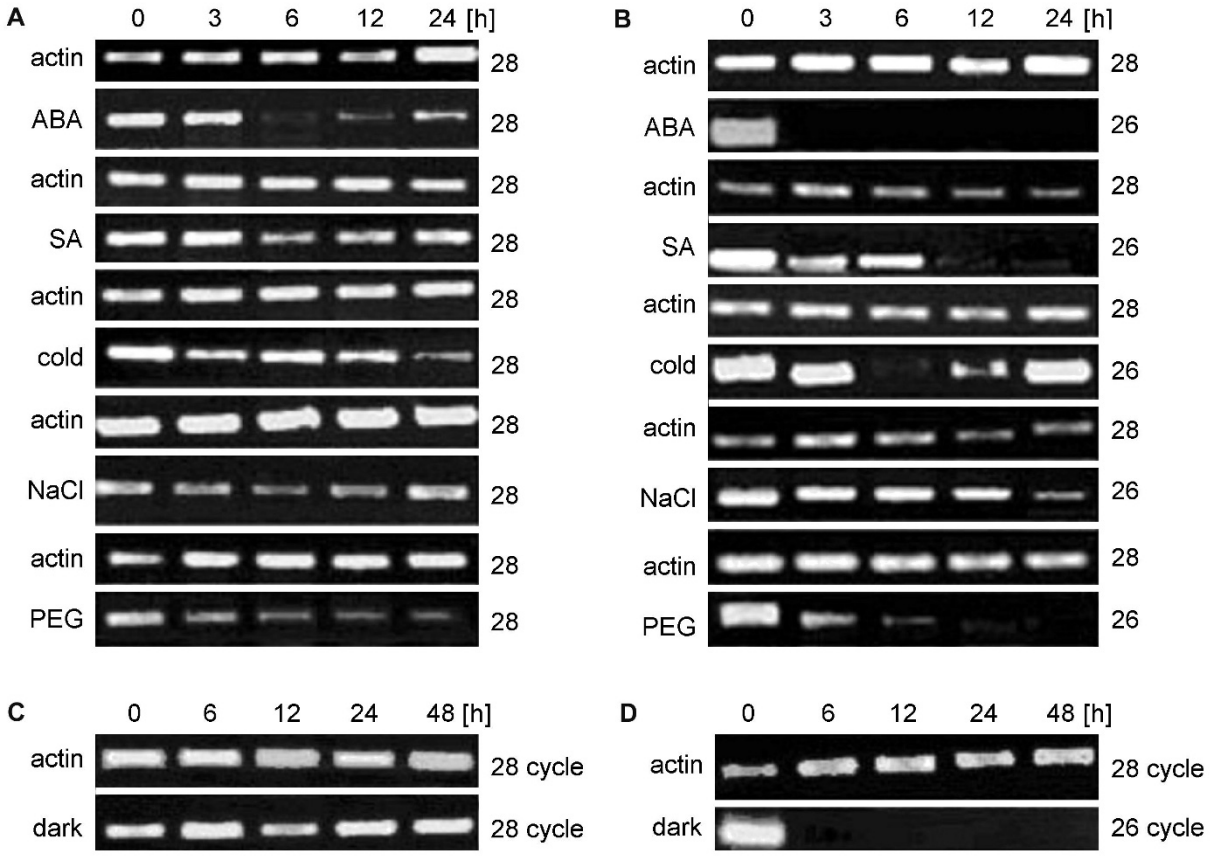


Fig. 1. The expression of the *TaNADP-ME* genes in the leaves of Jinmai47 under the various treatments administered. The DNA-free total RNA was analyzed using a semi-quantitative RT-PCR. The actin (accession no. AB181991) transcript was used as an internal control. The experiments were repeated at least four times with similar results. *A* - The expression of *TaNADP-ME1* in the leaves under various treatments. *B* - The expression of *TaNADP-ME2* in the leaves under various treatments. *C* - The expression of *TaNADP-ME1* in the leaves under darkness. *D* - The expression of *TaNADP-ME2* in the leaves under darkness.

first 6 h of treatment and then rose slightly at the 24 h treatment point (Fig. 1*A*).

Compared with the expression of the *TaNADP-ME1* gene, the expression of *TaNADP-ME2* gene was mostly down-regulated (Fig. 1*B*). In the PEG and NaCl treatments, the *TaNADP-ME2* expression levels continuously decreased. The expression levels were hardly detected in the 6 h treatment with PEG. When the leaves were exposed to the cold, the *TaNADP-ME2* transcript levels dropped to a minimum value in the first 6 h, and then recovered over the next 18 h. Under the SA treatment, the expression of the *TaNADP-ME2* transcripts decreased in the first 3 h and then began to rise slightly at 6 h, after which it decreased until 24 h. We could not detect any transcripts in the ABA treatment.

Previous studies have reported that several enzymes, such as Rubisco, glyceraldehyde 3-phosphate dehydrogenase, ribulose 5-phosphate kinase, fructose-1,6-bisphosphatase, pyruvate orthophosphate dikinase (PPDK) and maize chloroplastic NADP-ME are activated by irradiance (Buchanan 1980, Faske *et al.* 1995, Sage and Seemann 1993, Scheibe 1987, Edwards *et al.* 1985, Tausta *et al.* 2002, Murmu *et al.* 2003). To determine whether the two *NADP-ME* genes were responsive to irradiance, we examined their expression patterns under dark. The expression of *TaNADP-ME1* decreased slightly in the first 12 h after dark treatment, and then began to recover through 48 h (Fig. 1*C*). The expression of *TaNADP-ME2* through transcripts was not detected during the dark treatment (Fig. 1*D*). In addition, the putative molecular mass of the *TaNADP-ME2* gene transcript is 63 kDa according to *ChloroP1.1* server (http://www.cbs.dtu.dk/services/chloroP/). The photosynthetic isoforms of the NADP-ME enzyme have been reported to range from 62 to 67 kDa (Casati *et al.* 1999, Honda *et al,* 2000). We therefore postulate that although it reacts to different abiotic stresses, the *TaNADP-ME2* gene may be a light-activated gene and may have a photosynthetic role in $C_3$ wheat. Since abiotic stresses often limit the photosynthetic rate and the subsequent crop production (Pandey and Yeo 2008, Santos *et al.* 2009, Wang *et al.* 2009, Yu *et al.* 2009), the breeding of wheat with TaNADP-ME2 activity might improve yields under conditions of abiotic stress, especially drought stress. This effect is particularly interesting as non-cytosolic NADP-ME has not been previously reported to

have a photosynthetic role in $C_3$ plants. It should be mentioned, however, that in addition to the mode of light-regulation and subunit size, the photosynthetic MEs can also be distinguished from non-photosynthetic isoforms by using optimum pH, subcellular localization and higher affinity for malate (Maurino *et al.* 1997, Drincovich *et al.* 2001, Tausta *et al.* 2002). Further biochemical experiments are needed to verify the photosynthetic function of *TaNADP-ME2* gene.

Under various stress treatments, the enzymatic activity was enhanced while the transcript accumulation for the two *TaNADP-ME* genes was decreased. We speculate that hexaploid wheat, which possesses a large genome, may have some additional isozymes and more than two *TaNADP-ME* genes. Three or four *NADP-ME* genes have been identified in tobacco, maize, rice and *Arabidopsis thaliana* (Tausta *et al.* 2002, Chi *et al.* 2004, Saigo *et al.* 2004, Detarsio *et al.* 2008, Müller *et al.* 2008, Wheeler *et al.* 2008). These potential additional NADP-ME genes in the hexaploid wheat that have yet to be isolated could perhaps lead to the observed increase in enzyme activity that was seen in our study. In addition, the two *TaNADP-ME* genes responded differently under various stresses, and this may be due to their promoter characteristics and their *cis*-responsive elements.

In conclusion, our results suggest that the NADP-ME enzyme plays an important role in the response of the wheat plant to various stresses. Stress may stimulate a certain signal system in the plant and subsequently regulate plant defence genes, including *NADP-ME,* by signal transduction.

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