

## The activity and isoforms of NADP-malic enzyme in *Nicotiana benthamiana* plants under biotic stress

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**Abstract.** The activity and presence of isoforms of NADP-dependent malic enzyme (NADP-ME, EC 1.1.1.40) were studied in non-transgenic and transgenic *Nicotiana benthamiana* plants containing potyviral gene for helper component protease (HC-pro) and in plants infected by *Potato virus Y* strain NTN (PVY<sup>NTN</sup>). No significant changes in enzyme activities and isoenzyme pattern were observed due to foreign gene introduction and PVY<sup>NTN</sup> infection. However, the activity and isoenzyme composition of NADP-ME measured in extracts from different parts of the plants showed significant differences. Non-denaturing electrophoresis followed by specific detection of NADP-ME activity in polyacrylamide gel detected the presence of only one isoform in roots and younger leaves. Two isoforms of NADP-ME were detected in older leaves and stem (relative molecular mass ~248,000 and ~280,000) and three isoforms corresponding to tetramer, dimer and monomer were found in flowers. The activity of NADP-ME and the isoenzyme pattern was discussed in relation to its role in plant metabolism within distinct plant parts.

**Key words:** NADP-malic enzyme isoforms — *Nicotiana benthamiana* — HC-pro — *Potato virus Y*

**Abbreviations:** EDTA, ethylenediaminetetraacetate; NADP-ME, NADP-dependent malic enzyme; Tris, tris(hydroxymethyl)aminomethane; PVY, *Potato virus Y*

### Introduction

NADP-dependent malic enzyme (NADP-ME, L-malate: NADP<sup>+</sup> oxidoreductase (oxaloacetate-decarboxylating), EC 1.1.1.40) catalyzes the oxidative decarboxylation of L-malate and NADP<sup>+</sup> to produce pyruvate, CO<sub>2</sub> and NADPH (Edwards and Andreo 1992; Maurino et al. 2001). To decarboxylate malate enzyme requires a divalent cation. The most effective cations are Mg<sup>2+</sup>, Mn<sup>2+</sup> and Co<sup>2+</sup> (Wedding 1989). The NADP-ME was found in animal and plant tissues as well as in prokaryotic and eukaryotic microorganisms (Edwards and Andreo 1992).

In mammals, malic enzyme is a tetrameric protein with double dimer structure and enzymes assigned as EC 1.1.1.40 are either cytosolic or mitochondrial enzymes (Chang and Tong 2003). In mammalian liver, NADP-ME is major NADPH-generating enzyme for lipogenesis (Chang and Tong 2003).

The NADP-ME is widely distributed among all types of plants, but the isoform present in bundle sheath chloroplasts of several C<sub>4</sub> plants is the best characterized. In this type of plants, NADP-ME plays an important role in photosynthetic metabolism as it generates CO<sub>2</sub> and reducing power (NADPH) in the chloroplasts, where Calvin cycle operates (Maurino et al. 2001). Three isoforms of NADP-ME have been characterized in maize: 62,000 form, which is implicated in C<sub>4</sub> metabolism and predominated in green leaves, 66,000 non-photosynthetic isoform assembles as a dimer and 72,000 non-photosynthetic form of the protein, present

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mainly in etiolated leaves (Maurino et al. 1996; Saigo et al. 2004). However, the 72,000 protein, which was also purified from wheat stems (Casati et al. 1997), *Egeria densa* leaves (Casati et al. 2000), *Aptenia cordifolia* leaves (Falcone et al. 2003), *Ricinus communis* cotyledons (Colombo et al. 1997), and immunochemically identified, was later defined as a heat shock protein (Lara et al. 2005). The photosynthetic enzyme isolated from maize leaves exists as a tetramer (at pH 7.5) or a dimer (at pH 7.0) depending on pH and a buffer used. The enzyme purified from sugar cane ( $C_4$ ) leaves can readily undergo changes in oligomerization between monomer, dimer and tetramer with the tetramer form being favoured at high pH (8.0). All three forms of the enzyme are enzymatically active (Edwards and Andreo 1992). In CAM (crassulacean acid metabolism) plants, a cytosolic isoform (e.g. ice plant 64,000 form) also functions as a decarboxylase of malate to donate  $CO_2$  for Calvin cycle (Drincovich et al. 2001).

Also in  $C_3$  plants, the NADP-MEs are encoded by small gene families. The best studied family of malic enzymes is from *Arabidopsis thaliana* ( $C_3$  dicot plant), where the whole genome sequence is known (Wheeler et al. 2005). The *Arabidopsis* genome contains four genes encoding NADP-MEs. One isoform is localized in plastids and exists in equilibrium of active dimers and tetramers, whereas the other three isoforms are cytosolic and are present as hexamers or octamers (Wheeler et al. 2005). The rice (*Oryza sativa*,  $C_3$  monocot plant) NADP-ME family is composed of four members, one plastidic and three cytosolic versions. Each rice NADP-ME gene has presumably a different tissue-specific and developmental profile (Chi et al. 2004).

Non-photosynthetic forms of NADP-ME seem to be present in all plants ( $C_4$ , CAM,  $C_3$ ) and they play diverse functions. These roles may be anaplerotic, providing pyruvate and NADPH, which could be used in assimilatory processes requiring NADPH, e.g. lipid biosynthesis (Edwards and Andreo 1992). NADP-ME has also been suggested to serve as a pH stat in a combination with phosphoenolpyruvate carboxylase. In addition, non-photosynthetic NADP-ME has been proposed to function in plant defence response. For example, a direct evidence of the induction of bean NADP-ME by UV-B radiation was obtained (Drincovich et al. 2001). The increase in NADP-ME activity was also observed, when a fungal elicitor preparation, cellulase or jasmonate were applied directly to roots of intact maize seedlings (Maurino et al. 2001). In our previous work, the influence of viral infection caused by *Potato virus Y* strain NTN (PVY<sup>NTN</sup>) on plant metabolism of *Nicotiana tabacum* L. cv. Petit Havana SR1 was studied. PVY<sup>NTN</sup>-infected tobacco plants responded with the enhanced activity of NADP-ME (Ryšlavá et al. 2003). Gels after non-denaturing electrophoresis stained for NADP-ME activity showed the presence of one protein band with the NADP-ME activity in leaves of *N. tabacum* L.

In this study, we used control, non-transgenic *Nicotiana benthamiana* and transgenic *N. benthamiana* plants transformed with the gene for helper component protease (HC-pro) of *Potato virus A* (PVA). Plants expressing HC-pro could change susceptibility to infection with homologous viruses (Savenkov and Valkonen 2002). The aim of this study was to test the influence of viral infection caused by PVY<sup>NTN</sup> on the activity of NADP-ME and on the presence of NADP-ME isoforms in different parts of transgenic and non-transgenic *N. benthamiana* plants. The approximately relative molecular masses of corresponding NADP-ME isoforms were determined after non-denaturing polyacrylamide gel electrophoresis.

## Materials and Methods

### Plant material

*N. benthamiana* plants and transgenic *N. benthamiana* plants transformed with HC-pro of PVA were kindly provided by Dr. Savenkov (Uppsala, Sweden). The plants were grown in a greenhouse under temperatures 22/18°C day/night. Seeds were sown in pots with sand and plantlets were transferred to soil after 3 weeks. Leaves of 7-weeks old plants were mechanically inoculated with PVY<sup>NTN</sup>, kindly provided by Dr. Dědič (Potato Research Institute, Havlíčkův Brod, Czech Republic). The samples were collected from control and infected leaves within 26 days and 26<sup>th</sup> day also from flowers, stems and roots. As a control the plants without viral infection were used. Samples were immediately frozen in liquid  $N_2$  and stored at  $-75^\circ C$ . The extent of viral infection was determined by double antibody sandwich-ELISA (Clark and Adams 1977) in homogenates of the leaves of infected plants using polyclonal antibodies raised against PVY<sup>NTN</sup> (Čeřovská 1998). Sample of maize leaves were collected from plants *Zea mays* var. 2013 Čejč.

### The enzyme activity assays

For assay of NADP-ME activity, 0.5 g of plant tissue was homogenized in 1.5 ml 100 mmol/l Tris-HCl (pH 7.8) containing 1 mmol/l dithiothreitol, 1 mmol/l EDTA and 5 mmol/l  $MgCl_2$ ; then 0.02 g of polyvinylpyrrolidone was added and the homogenate was centrifuged at  $23,000 \times g$  for 15 min at  $4^\circ C$ . The supernatant was used as plant extract for measuring NADP-ME activity. The NADP-ME activity was determined spectrophotometrically at  $25^\circ C$  by monitoring NADPH production at 340 nm. The NADP-ME assay mixture contained 100 mmol/l Tris-HCl buffer (pH 7.4), 10 mmol/l malate, 2 mmol/l  $MgCl_2$  and 0.2 mmol/l  $NADP^+$  in total volume of  $1\text{ cm}^3$ . The reaction was started by addition of 50  $\mu l$  of the enzyme extract.

### Electrophoretic separations

Native gel electrophoresis was performed according to Lee and Lee (2000). The same volume (25  $\mu$ l) of all plant extracts with 20% of sucrose was applied to the gel. The gels (6%) were assayed for the NADP-ME activity by incubating in a solution of 100 mmol/l Tris-HCl (pH 7.4) containing 10 mmol/l L-malate, 10 mmol/l  $MgCl_2$ , 2 mmol/l  $NADP^+$ , 0.1 mg·ml<sup>-1</sup> nitroblue tetrazolium and 5  $\mu$ g·ml<sup>-1</sup> phenazine methosulfate at room temperature (Maurino et al. 2001).

### Native molecular mass estimation

The molecular mass of the protein NADP-ME was evaluated by native gel electrophoresis using a Biometra multigel apparatus and commercial markers for native electrophoresis.  $100[\log(R_f \times 100)]$  values were plotted against the acrylamide concentrations in gels, where  $R_f$  is relative mobility of standard proteins. Consequently, the logarithms of negative values of slopes were plotted against the logarithm of relative molecular masses (248,000; 132,000; 66,000; 45,000; 29,000; 14,200). The size of isoforms of NADP-ME was calculated from the regression line thus obtained (Ferguson 1964; Lottspeich and Zorbas 1998).

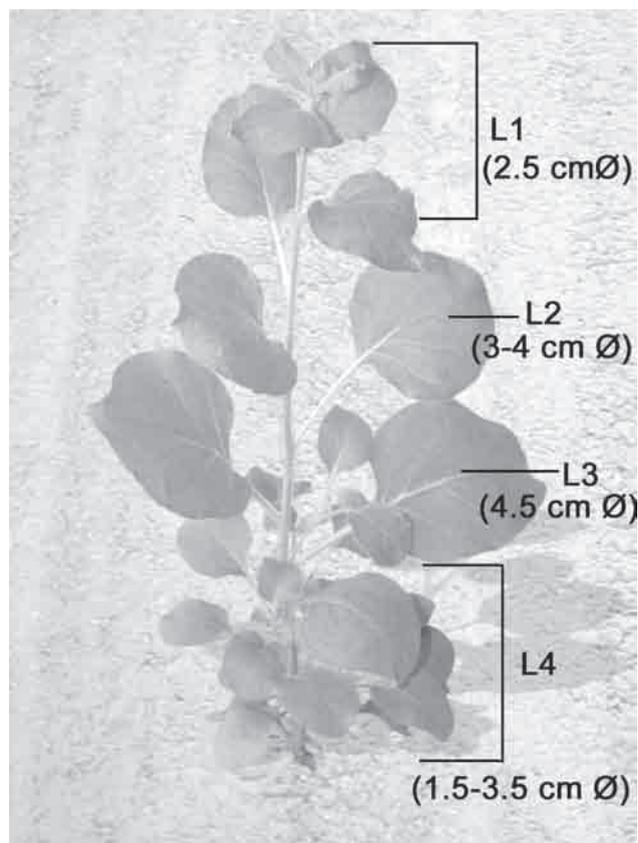
### In vivo localization of NADP-ME

Malic enzyme was localized by staining for its activity on fresh hand-cut sections of the leaf midribs and the stem. The sections were incubated immediately in 10 ml of staining solution consisting of 100 mmol/l Tris-HCl buffer (pH 7.4), 10 mmol/l L-malate, 10 mmol/l  $MgCl_2$ , 7.6 mg  $NADP^+$ , 12  $\mu$ mol/l nitroblue tetrazolium chloride (NBT), and 0.16  $\mu$ mol/l phenazine methosulfate for 30 min in the dark at the room temperature. The control staining was done without malate or  $NADP^+$ . The blue dye indicated NADP-ME activity, which was not present in the control staining. The sections were examined in the light microscope Nikon Eclipse E600 equipped with CCD camera.

## Results

### Activity and isoforms of NADP-ME in different parts of *N. benthamiana*

Plant extracts from different parts of *N. benthamiana* (leaves, stems, roots) were assayed for activity of NADP-ME and the presence of enzyme isoforms was studied. The whole plant was divided into several sections, labelled as L1–L4, indicating the position of leaves from top of the plant (Fig. 1). The stem was divided in two parts, upper (S1) and lower (S2). Leaves at top of the plant L1 exhibited the highest activity of NADP-ME calculated *per* fresh weight. The activity of



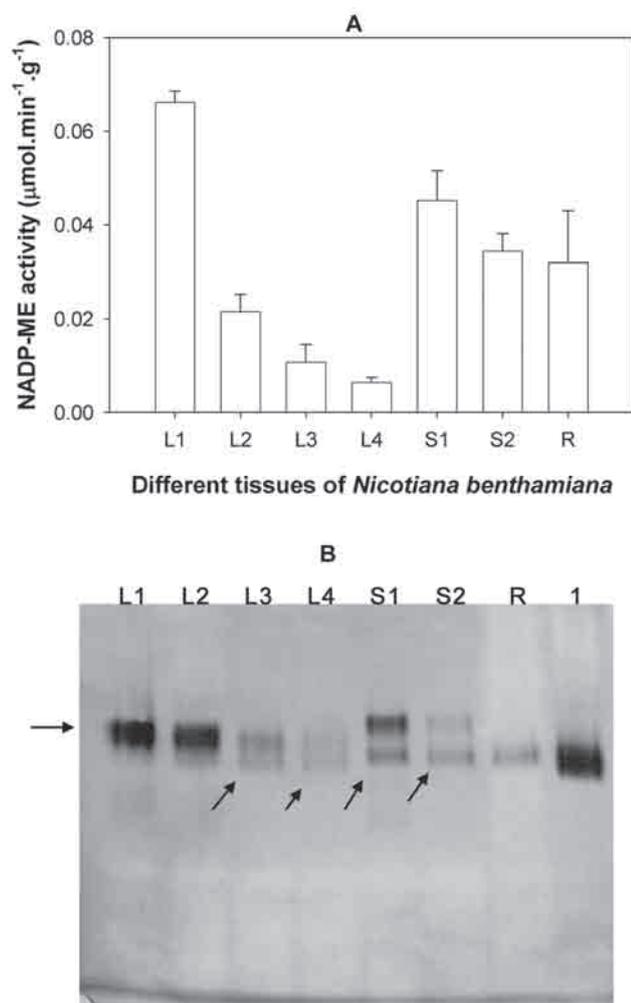
**Figure 1.** Leaves from 7-weeks old plants *N. benthamiana* were divided into 4 groups (L1, L2, L3 and L4) according their localization and size.

NADP-ME decreased gradually from the upper parts of the plant to the older basal leaves (Fig. 2).

Native gel electrophoresis and staining for NADP-ME activity detected one band in young (L1) leaves and in the roots (R), but with different electrophoretic mobility (Fig. 2). The occurrence of one band probably indicates the presence of solely isoform of NADP-ME in young leaves and roots. Two isoforms were observed in older leaves (L2, L3, L4) and in two different sections of the stem (S1, S2; see Fig. 2). Moreover, three isoforms of NADP-ME were apparent in flowers of *N. benthamiana* (Fig. 3).

### Influence of PVY<sup>NTN</sup> infection on NADP-ME

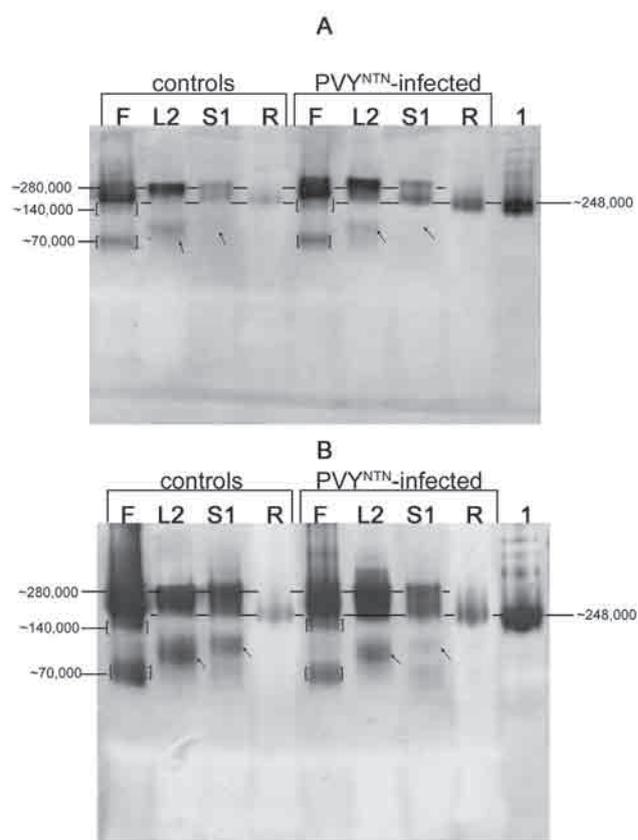
The relative content of virus PVY<sup>NTN</sup> and activity of NADP-ME in leaves of control *N. benthamiana* and transgenic plants of HC-pro *N. benthamiana* were measured in samples collected three times a week during 26 days of infection. The time course of activity of NADP-ME in leaves was expressed as percentage of the activity of control healthy plants (calculated *per* fresh leaf matter). No statistically significant changes were



**Figure 2. A.** Activities of NADP-ME calculated *per* fresh mass from different parts of *N. benthamiana* (from different leaves L1, L2, L3 and L4; from upper stems S1, lower stems S2, and from roots R). The activity was measured in triplicate, S.E. are shown. **B.** Detection of activity of NADP-ME in 6% polyacrylamide gel after non-denaturing electrophoresis. The line with label 1 contains plant extract from green maize leaves, 7-times diluted compared to others samples. The straight arrow indicates a position of NADP-ME isoforms with lower electrophoretic mobility, whereas isoforms with higher electrophoretic mobility are marked with oblique arrows.

found in activities of leaves from *N. benthamiana* and transgenic HC-pro *N. benthamiana* plants infected by PVY<sup>NTN</sup> (Fig. 4A). Only in PVY<sup>NTN</sup>-infected roots from both groups of plants were found slight increase (180 and 215%) in activities of NADP-ME in comparison to healthy controls representing 100% (Fig. 4B) in the maximum of infection.

The maximal content of PVY<sup>NTN</sup> virus was found in the end of the infection (26<sup>th</sup> day) in both transgenic (HC-pro) and non-transgenic plants of *N. benthamiana* (Table 1).

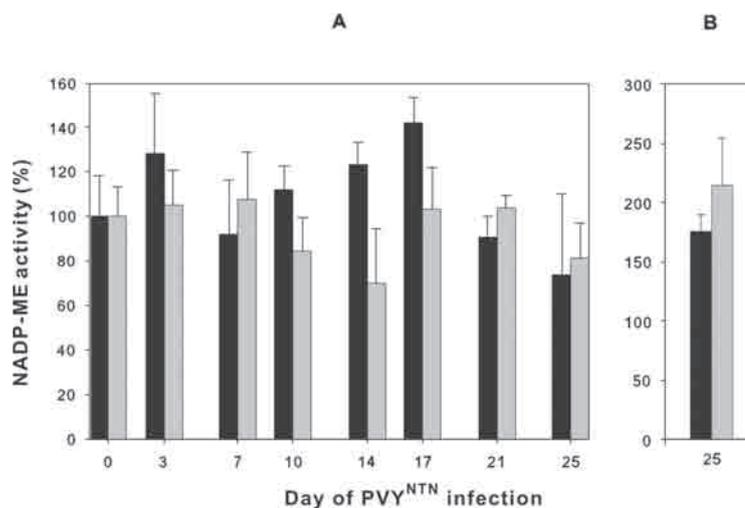


**Figure 3.** Detection of isoforms of NADP-ME from control and PVY<sup>NTN</sup>-infected flowers (F), leaves (L2), stems (S1) and roots (R) of *N. benthamiana* plants (**A**) and from transgenic *N. benthamiana* plants transformed with HC-pro (**B**) in 7% polyacrylamide gel after non-denaturing electrophoresis. The line with label 1 contains plant extract from green maize leaves, 7-times diluted compared to others samples. The same results were obtained from two independent courses of PVY<sup>NTN</sup> infection. The oblique arrows indicate position of bands corresponding to NADP-ME activity, which were visible only when 8 and 7% polyacrylamide gel was used.

**Table 1.** The relative content of virus PVY<sup>NTN</sup> was determined by DAS-ELISA with *p*-nitrophenylphosphate as enzyme substrate. Absorbance at 405 nm is proportional to the virus content

Day of PVY <sup>NTN</sup> infection	Absorbance (405 nm)	
	<i>N. benthamiana</i>	<i>N. benthamiana</i> - HC-pro
3	0.03	0.02
7	0.06	0.05
10	0.06	0.08
14	0.78	0.67
17	0.85	0.92
21	1.19	1.28
25	1.74	1.31

**Figure 4.** Activities of NADP-ME in leaves L2 (A) and roots (B) of *N. benthamiana* (■) and in transgenic plants *N. benthamiana* with HC-pro (□) 0–26<sup>th</sup> day after inoculation by PVY<sup>NTN</sup>. The activity of each sample is calculated as percentage of the non-infected control, where 100% of NADP-ME activity corresponds to  $0.022 \pm 0.004 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  in *N. benthamiana* leaves and  $0.032 \pm 0.011 \mu\text{mol}\cdot\text{min}^{-1}\cdot\text{g}^{-1}$  in *N. benthamiana* roots. In each repetition of the experiment, the activity was measured triplicate. S.E. are shown.



NADP-ME isoform analysis of crude extracts from flowers, leaves, stems, and roots from healthy and infected non-transgenic and transgenic *N. benthamiana* plants was done on 26<sup>th</sup> day of the PVY<sup>NTN</sup> infection, when the virus content was very high (Fig. 3). No significant differences in the isoform contents were observed among transgenic and non-transgenic plants.

#### Estimation of relative molecular masses of NADP-ME isoforms in *N. benthamiana*

Native electrophoresis carried out in various concentrations of polyacrylamide gel, which simultaneously enables to stain the gels for enzyme activity and distinguish isoform of NADP-ME and its oligomerization states, was applied. Non-denaturing electrophoresis with native standard proteins in various concentrations of polyacrylamide gel was used for molecular mass estimation of corresponding isoforms of NADP-ME. The presence of the isoforms of NADP-ME in flowers, leaves, stems and roots is shown in Table 2. When the native electrophoresis is carried out in a set of gels of various polyacrylamide concentrations, the enzyme isoforms differing both in molecular mass and/or charge could be determined (Lottspeich and Zorbas 1998). In 5, 6, 7 and 8% polyacrylamide gels we measured relative mobility of three bands presented in *N. benthamiana* flowers samples, two bands in leaves and stems samples and one band in roots and maize leaves sample (only 7% gel is shown in Fig. 3). No differences in isoenzyme content were observed between healthy and PVY<sup>NTN</sup>-infected parts of *N. benthamiana* plants. Another (third) band was also visible in leaves and stems (Fig. 3), but only in 7 and 8% polyacrylamide gel. Therefore, this form couldn't be exactly analyzed with Ferguson's method (Ferguson 1964; Lottspeich and Zorbas 1998). Probably it is a partially degraded NADP-ME, but with retained enzyme activity. Also the presence of another

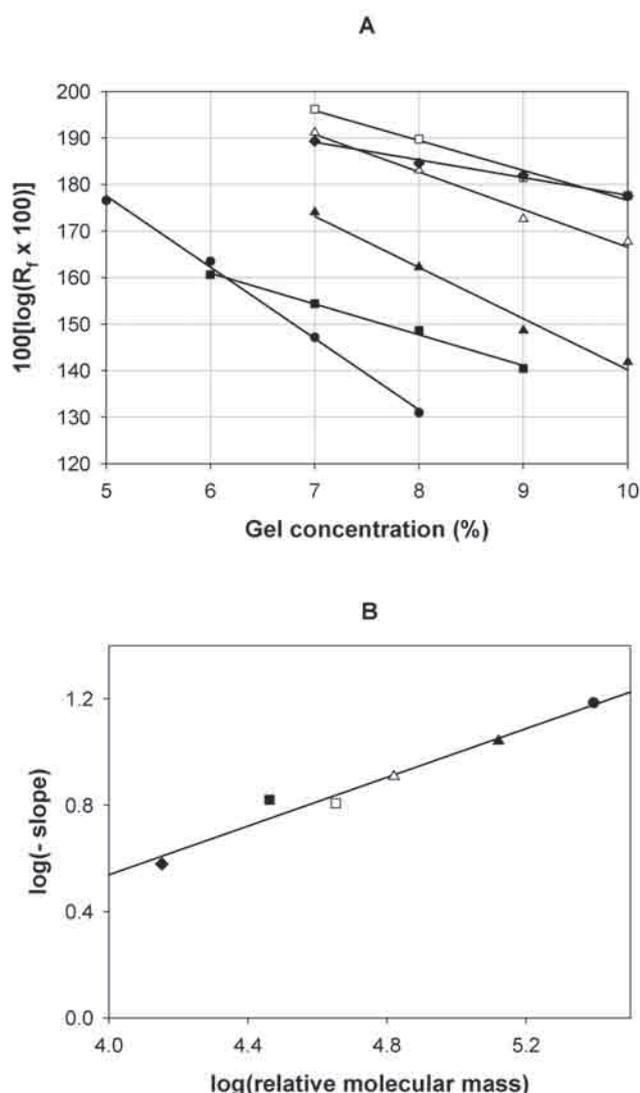
**Table 2.** The presence of isoforms of NADP-ME in flowers (F), leaves (L2), stems (S1) and roots (R) from both control and PVY<sup>NTN</sup>-infected plants *Nicotiana benthamiana* and transgenic plants *N. benthamiana* transformed with HC-pro

	~280,000 (~70,000)	~248,000 (~62,000)	~140,000 (~70,000)	~70,000	Fragment NADP-ME
F	+	-	+	+	-
L2	+	+	-	-	(+)
S1	+	+	-	-	(+)
R	-	+	-	-	-

The numbers indicate apparent relative molecular mass of whole native protein NADP-ME, the number in brackets corresponds to relative molecular mass of a monomer in case of the native NADP-ME is a tetramer or dimer. +, the presence of the isoform; -, the absence of the isoform; (+), facultative presence of the isoform.

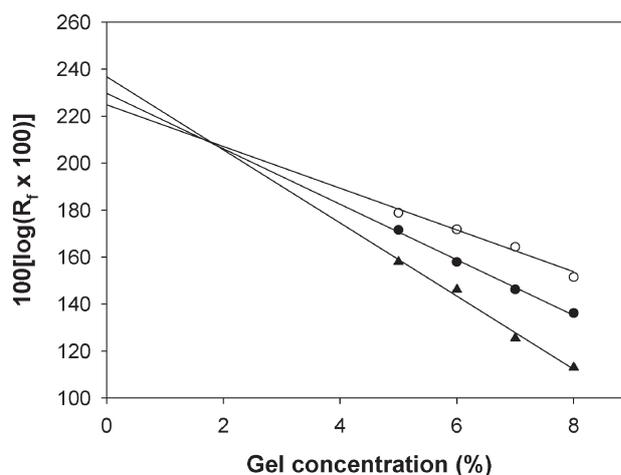
isoform is possible as well. Ferguson's plots (dependence of  $100[\log(R_f \times 100)]$  on polyacrylamide gel concentration) allowed determine, which isoform is larger in size or charge, alternatively found how given isoforms are related to each other. For example, when in Ferguson's graph two plots are connected in one point, which is positioned below value 2% of polyacrylamide gel on x-axis, then two relevant proteins have different molecular weight, but the same charge. Generally it could be two oligomerization forms of one protein (Lottspeich and Zorbas 1998). It was the case of three forms presented in flower of *N. benthamiana*. All three Ferguson's plots were intersect in one point ( $T < 2\%$  of polyacrylamide gel concentration) (Fig. 6). Each curve had different minus slope value (retardation coefficient) and calculated relative molecular masses from calibration curve (Fig. 5B) correspond to tetramer, dimer and monomer, when monomer was about 70,000 (Table 2).

Tetramer NADP-ME with molecular mass of one subunit about 70,000 found in flowers (~280,000 isoform) had ana-



**Figure 5.** A. Ferguson plots for determination of standards slopes.  $R_f$  corresponds to relative mobility of native protein in various concentrations of polyacrylamide gel (5–10%).  $\alpha$ -lactalbumin (14,200; ◆), carbonic anhydrase (29,000; ■), albumin from chicken egg (45,000; □), albumin from bovine serum (66,000 monomer  $\Delta$ ; 132,000 dimer  $\blacktriangle$ ) and NADP-ME from maize green leaves (248,000; ●) were used as the standard proteins. B. Secondary regression line obtained from plot A, from which relative molecular masses of isoforms of NADP-ME from different parts of *N. benthamiana* were calculated.

logues in leaves and stems of *N. benthamiana*, because we established for appropriate forms identical relative mobility in gels. Second isoform of NADP-ME in leaves and stems and one isoform present in roots of *N. benthamiana* have very similar relative mobility in gels with NADP-ME from maize leaves. Ferguson's plots were near each other; retardation coefficients were almost the same. Calculated relative

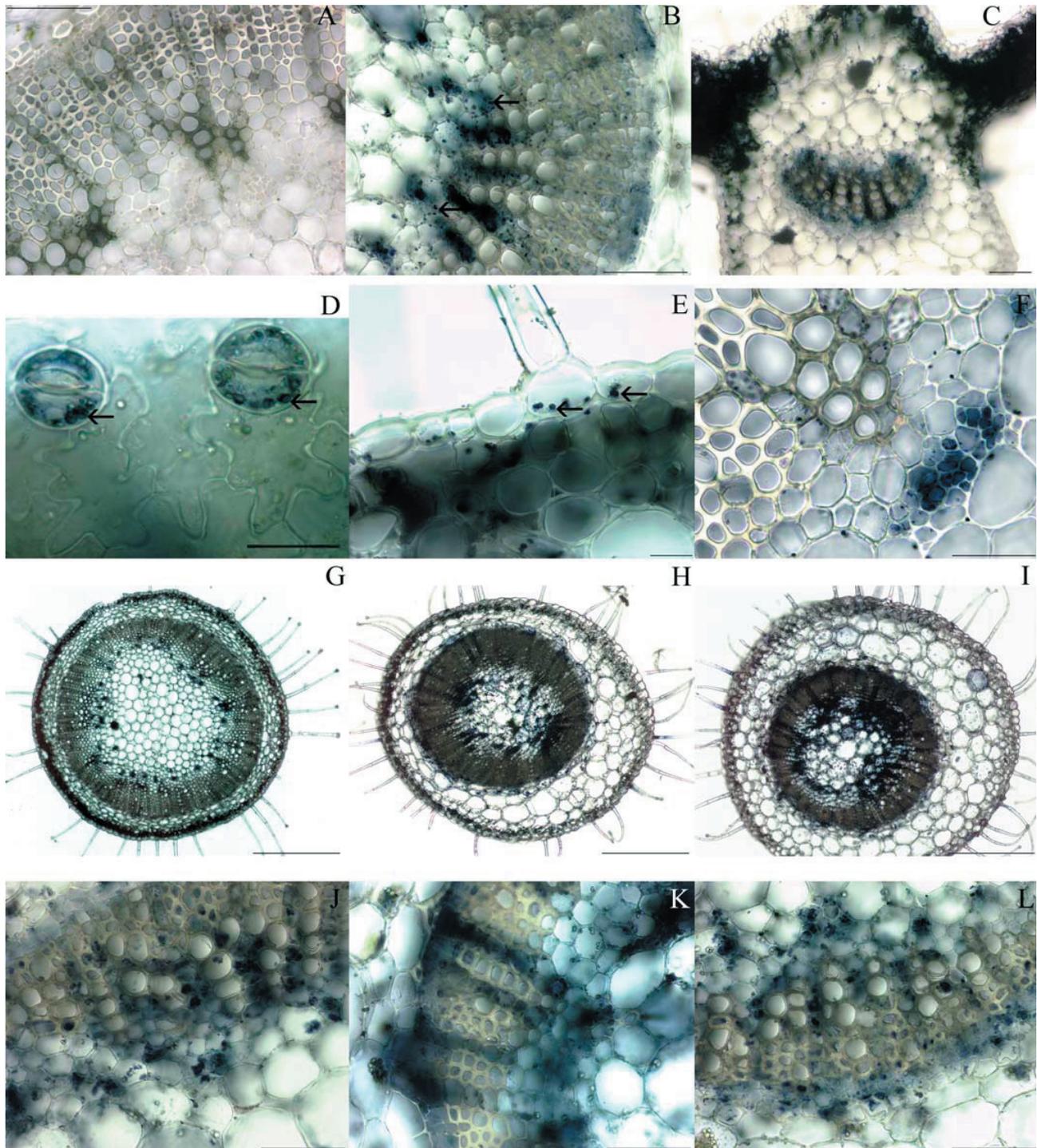


**Figure 6.** Ferguson's plots for determination of slopes for 3 isoforms NADP-ME present in *N. benthamiana* flowers.  $R_f$  corresponds to relative mobility of native protein in various concentrations of polyacrylamide gel (5–8%). The point of intersection indicates that there is one protein in three different oligomerization states. From the slopes relative molecular masses of 70,000; 140,000 and 280,000 can be estimated.

molecular masses from calibration curve (Fig. 5B) agree with tetramer of NADP-ME from maize leaves. Therefore, it could indicate, that NADP-ME isoform present in *N. benthamiana* leaves, stems and roots has relative molecular mass about 248,000, because it is known, that NADP-ME from maize leaves is tetramer with molecular mass 248,000 (Edwards and Andreo 1992; Detarsio et al. 2007). Ferguson's plot belonging to NADP-ME from maize leaves (248,000 isoform) and plot belonging to  $\sim$ 280,000 isoform weren't intersect, with 248,000 isoform plot above  $\sim$ 280,000 isoform (data not shown). It correlates with fact, that upper protein according to Ferguson's method is smaller and with larger charge (Lottspeich and Zorbas 1998).

#### Localization of NADP-ME in *N. benthamiana* plants

Localization of NADP-ME activity was carried out on fresh hand cut sections from various parts of *N. benthamiana* plant using NBT staining method (Fig. 7). NADP-ME activity was indicated by blue colour. Fig. 7A represents control staining without the presence of malate. In leaves, the enzyme was localized particularly in cells around the veins in the midrib (Fig. 7C). Blue stains were also apparent in chloroplasts of stomatal, epidermal, and mesophyll cells (small arrows, see Fig. 7D and E). In stem sections taken from upper and lower part of the stem (S1, S2), NADP-ME activity was localized in xylem parenchyma cells vessels and in phloem cells (Fig. 7B,E,G,H). Contrary to small changes in activities of crude extracts found in PVY<sup>NTN</sup>-infected leaves, the stem sections of infected *N. benthamiana*



**Figure 7.** Localization of NADP-ME activities in fresh hand cut sections from different parts of *N. benthamiana* plant by the method of NBT staining. The blue coloration indicates NADP-ME activity. **A.** Control staining without the presence of malate. **B.** The section of the stem (S2). **C.** The section of the leaf midrib. **D.** Stomatal cells. **E.** Epidermal cells. **F.** Detailed view of the xylem and phloem cell in the stem section. **G.** Cross-section of the stem (S1). **H.** Cross-section of the stem (S2) of the healthy *N. benthamiana*. **I.** Cross-section of the stem (S2) of the PVY<sup>NTN</sup>-infected *N. benthamiana*. **J., K., L.** Detailed view of S2 section from the PVY<sup>NTN</sup>-infected *N. benthamiana*. Small arrows mark stained chloroplasts. Scale bars represent: A, B, C, J, K, L = 100  $\mu\text{m}$ ; D = 35  $\mu\text{m}$ ; E = 25  $\mu\text{m}$ ; F = 50  $\mu\text{m}$ ; G, H, I = 500  $\mu\text{m}$ .

plants showed stronger coloration, particularly in phloem and along the xylem vessels (Fig. 7I,J,K,L).

## Discussion

### Occurrence of NADP-ME isoforms

Our results showed that NADP-ME in *N. benthamiana* plants is present at least in two isoforms differing in their electrophoretic properties (Figs. 2, 3). Plant NADP-MEs are coded by a small gene family (Wheeler et al. 2005). In  $C_3$  plants *Arabidopsis thaliana* and *Oryza sativa* with known genome, the sequences of three cytosolic and one chloroplastic isoforms were found (Chi et al. 2004; Wheeler et al. 2005). However, it is not sure until now if such a composition of the gene family is common for all plants. Differences in the expression of particular NADP-MEs may occur in various tissues, in different developmental stages, and among plant species (Chi et al. 2004; Wheeler et al. 2005).

### Localization of NADP-ME

We found NADP-ME activity in various parts of the plant *N. benthamiana*. It was present in epidermal cells, particularly in stomatal cells (Fig. 7D,E). The function of NADP-ME in these cells is in close relation to pH changes and stomatal movements (Latzko and Kelly 1983; Schnabl 1983).

We localized NADP-ME activity in cells surrounding main veins in stems and leaves (Fig. 7B,C,F,G,H). In these cells NADP-ME could contribute to photosynthetic  $CO_2$  fixation via decarboxylation of malate transported through floem (Hibberd and Quick 2002). This possible function maintains the presence of NADP-ME in chloroplasts (Fig. 7). Probably, the cytosolic isoform is present but its contribution is not so easy to distinguish as that of plastids.

### Molecular mass of subunits of NADP-ME

The molecular weights of monomers of isoforms NADP-ME found in *N. benthamiana* were estimated at ~62,000 and ~70,000. Even though molecular weights couldn't be determined with high accuracy, we used native polyacrylamide gel electrophoresis, because this method enables to detect enzyme isoforms and simultaneously estimate relative molecular masses of native proteins from Ferguson's calibration graph. Furthermore, mutual position of curves belonging to appropriate isoenzymes in Ferguson's graph could confirm which protein is smaller or which proteins are identical (Lottspeich and Zorbas 1998). Detected sizes of monomers are common for plant NADP-MEs (Drincovich et al. 2001). Monomer with relative molecular mass of 66,000–67,000 is typical for non-photosynthetic forms ( $C_3$  plants, non-pho-

tosynthetic parts of  $C_4$  plants) (Saigo et al. 2004; Lara et al. 2005). In *N. benthamiana* this isoform is mainly present in young leaves and together with other isoform in stems and in older leaves (Fig. 2). The molecular weight estimated for the other isoform was surprisingly 62,000, nevertheless, this lower molecular weight is typical for photosynthetic forms in  $C_4$  plants (Edwards and Andreo 1992). Moreover, this isoform was the main one in roots of *N. benthamiana*.

### Oligomerization of NADP-ME

NADP-ME occurs in plant tissues in several oligomeric stages (monomer, dimer, tetramer, hexamer, octamer) (Edwards and Andreo 1992; Wheeler et al. 2005). In some plants NADP-ME is in the form of larger oligomers, due to the absence of a sequence near the C terminal, which is responsible for tetrameric structure of animal NADP-ME (Chang and Tong 2003). Hexamers or octamers are supposed in some NADP-ME isoforms in *A. thaliana* (Wheeler et al. 2005) and *Zea mays* (Fig. 3, upper bands in sample 1) but such aggregates were not detected in our experiments. In leaves, stems, and roots of *N. benthamiana* isoforms with molecular weight of ~248,000 and ~280,000 were found, therefore NADP-ME build tetrameric molecules. The pattern of isoforms of NADP-ME in flowers is more complicated, because also molecules of molecular weight of ~140,000 and ~70,000 were found. It means the presence of dimers and monomers (Fig. 3).

### Function of gene for HC-pro protein in plants

HC-pro is a multifunctional potyviral protein, enhancer of genome amplification and suppressor of PTGS (post-transcriptional gene silencing). It affects symptom development, cell-to-cell movement and long distance movement and has cystein-type proteinase activity (Waterhouse et al. 2001; Savenkov and Valkonen 2002). In our experiments we have not found any differences in susceptibility to PVY<sup>NTN</sup> infection in *N. benthamiana* plants (Table 1). The activity and isoform composition of NADP-ME in leaves and roots of both types of *N. benthamiana* plants during PVY<sup>NTN</sup> infection were very similar.

### Stress response

The expression and activity of NADP-ME is associated with plant responses to biotic or abiotic stresses (Maurino et al. 2001; Sun et al. 2003; Chi et al. 2004; Smeets et al. 2005; Liu et al. 2007). Recently, we have reported, that the activity of NADP-ME of *N. tabacum* L. in PVY<sup>NTN</sup> infected leaves increased 5 to 6 times (Ryšlavá et al. 2003). However, in analogous experiments performed with *N. benthamiana* plants, any significant change in NADP-ME activity in leaves was not found (Fig. 4). The reason for such differences between two

species of genus *Nicotiana* is not clear. However, in roots of *N. benthamiana* PVY<sup>NTN</sup>-infected plants enhanced activity of NADP-ME was found (Fig. 4). Similar results were reported by Maurino et al. (2001) in maize roots after jasmonate, cellulase and fungal elicitor treatment.

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## References

- Casati P., Spampinato C. P., Andreo C. S. (1997): Characterization and physiological function of NADP-malic enzyme from wheat. *Plant Cell Physiol.* **38**, 928–934
- Casati P., Lara M. V., Andreo C. S. (2000): Induction of C<sub>4</sub>-like mechanism of CO<sub>2</sub> fixation in *Egeria densa*, a submersed aquatic species. *Plant Physiol.* **123**, 1611–1621
- Čeřovská N. (1998): Production of monoclonal antibodies to potato virus Y<sup>NTN</sup> strain and their use for strain differentiation. *Plant Pathol.* **47**, 505–509
- Chang G. G., Tong L. (2003): Structure and function of malic enzymes, a new class of oxidative decarboxylases. *Biochemistry* **42**, 12721–12731
- Chi W., Yang J., Wu N., Zhang F. (2004): Four rice genes encoding NADP malic enzyme exhibit distinct expression profiles. *Biosci. Biotechnol. Biochem.* **68**, 1865–1874
- Clark M. F., Adams A. N. (1977): Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. *J. Gen. Virol.* **34**, 475–483
- Colombo S. L., Andreo C. S., Podestá F. E. (1997): Carbon metabolism in germinating *Ricinus communis* cotyledons. Purification, characterization and developmental profile of NADP-dependent malic enzyme. *Physiol. Plant.* **101**, 821–826
- Detarsio E., Alvarez C. E., Saigo M., Andreo C. S., Drincovich M. F. (2007): Identification of domains involved in tetramerization and malate inhibition of maize C<sub>4</sub> NADP-malic enzyme. *J. Biol. Chem.* **282**, 6053–6060
- Drincovich M. F., Casati P., Andreo C. S. (2001): NADP-malic enzyme from plants: a ubiquitous enzyme involved in different metabolic pathways. *FEBS Lett.* **490**, 1–6
- Edwards G. E., Andreo C. S. (1992): NADP-malic enzyme from plants. *Phytochemistry* **31**, 1845–1857
- Falcone Ferreyra M. L., Andreo C. S., Podestá F. E. (2003): Purification and physical and kinetic characterization of a photosynthetic NADP-dependent malic enzyme from CAM plant *Aptenia cordifolia*. *Plant Sci.* **164**, 95–102
- Ferguson K. A. (1964): Starch-gel electrophoresis: application to the classification of pituitary proteins and polypeptides. *Metabolism* **13**, 985–1002
- Hibberd J. M., Quick W. P. (2002): Characteristics of C<sub>4</sub> photosynthesis in stems and petioles of C<sub>3</sub> flowering plants. *Nature* **415**, 451–454
- Lara M. V., Drincovich M. F., Müller G. L., Maurino V. G., Andreo C. S. (2005): NADP-malic enzyme and Hsp70: co-purification of both proteins and modification of NADP-malic enzyme properties by association with Hsp70. *Plant Cell Physiol.* **46**, 997–1006
- Latzko E., Kelly G. J. (1983): The many-faceted function of phosphoenolpyruvate carboxylase in C<sub>3</sub> plants. *Physiol. Veg.* **21**, 805–815
- Lee D. H., Lee C. B. (2000): Chilling stress-induced changes of antioxidant enzymes in the leaves of cucumber: in gel enzyme activity assays. *Plant Sci.* **159**, 75–85
- Liu S., Cheng Y., Zhang X., Guang Q., Nishiuchi S., Hase K., Takano T. (2007): Expression of an NADP-malic enzyme gene in rice (*Oryza sativa* L.) is induced by environmental stresses; over-expression of that gene in Arabidopsis confers salt and osmotic stress tolerance. *Plant Mol. Biol.* **64**, 49–58
- Lottspeich F., Zorbas H. (1998): Bioanalytics. Spektrum Akademischer Verlag, Heidelberg-Berlin (in German)
- Maurino V. G., Drincovich M. F., Andreo C. S. (1996): NADP-malic enzyme isoforms in maize leaves. *Biochem. Mol. Biol. Int.* **38**, 239–250
- Maurino V. G., Saigo M., Andreo C. S., Drincovich M. F. (2001): Non-photosynthetic malic enzyme from maize: a constitutively expressed enzyme that responds to plant defence inducers. *Plant Mol. Biol.* **45**, 409–420
- Ryšlavá H., Müller K., Semorádová Š., Synková H., Čeřovská N. (2003): Photosynthesis and activity of phosphoenolpyruvate carboxylase in *Nicotiana tabacum* L. leaves infected by *Potato virus A* and *Potato virus Y*. *Photosynthetica* **41**, 357–363
- Saigo M., Bologna F. P., Maurino V. G., Detarsio E., Andreo C. S., Drincovich M. (2004): Maize recombinant non-C<sub>4</sub> NADP-malic enzyme: A novel dimeric malic enzyme with high specific activity. *Plant Mol. Biol.* **55**, 97–107
- Savenkov E. I., Valkonen J. P. T. (2002): Silencing of a viral RNA silencing suppressor in transgenic plants. *J. Gen. Virol.* **83**, 2325–2335
- Smeets K., Cuypers A., Lambrechts A., Semane B., Hoet P., Van Laere A., Vangronsveld J. (2005): Induction of oxidative stress and antioxidative mechanisms in *Phaseolus vulgaris* after Cd application. *Plant Physiol. Biochem.* **43**, 437–444
- Schnabl H. (1983): The key role of phosphoenolpyruvate carboxylase during the volume changes of guard cell protoplast. *Physiol. Veg.* **21**, 955–962
- Sun S.-B., Shen Q.-R., Wan J.-M., Liu Z.-P. (2003): Induced expression of the gene for NADP-malic enzyme in leaves of *Aloe vera* L. under salt stress. *Acta Biochim. Biophys. Sin. (Shanghai)* **35**, 423–429
- Waterhouse P. M., Wang M.-B., Lough T. (2001): Gene silencing as an adaptive defense against viruses. *Nature* **411**, 834–842
- Wedding R. T. (1989): Malic enzymes of higher plants. Characteristics, regulation and physiological function. *Plant Physiol.* **90**, 367–371
- Wheeler M. C. G., Tronconi M. A., Drincovich M. F., Andreo C. S., Flüge U. I., Maurino V. G. (2005): A comprehensive analysis of the NADP-malic enzyme gene family of Arabidopsis. *Plant Physiol.* **139**, 39–51