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Fumarate and cytosolic pH as modulators of the synthesis or consumption of C_4 organic acids through NADP-malic enzyme in *Arabidopsis thaliana*

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Abstract *Arabidopsis thaliana* is a plant species that accumulates high levels of organic acids and uses them as carbon, energy and reducing power sources. Among the enzymes that metabolize these compounds, one of the most important ones is malic enzyme (ME). *A. thaliana* contains four malic enzymes (NADP-ME 1–4) to catalyze the reversible oxidative decarboxylation of malate in the presence of NADP. NADP-ME2 is the only one located in the cell cytosol of all *Arabidopsis* organs providing most of the total NADP-ME activity. In the present work, the regulation of this key enzyme by fumarate was investigated by kinetic assays, structural analysis and a site-directed mutagenesis approach. The final effect of this metabolite on NADP-ME2 forward activity not only depends on fumarate and substrate concentrations but also on the pH of the reaction medium. Fumarate produced an increase in NADP-ME2 activity by binding to an allosteric site. However at higher concentrations, fumarate caused a competitive inhibition, excluding the substrate malate from binding to the active site. The characterization of ME2-R115A mutant, which is not activated by fumarate, confirms this hypothesis. In addition, the reverse reaction (reductive carboxylation of pyruvate) is also modulated by fumarate, but in a different way. The results indicate pH-dependence of the fumarate modulation with opposite behavior on the two activities analyzed. Thereby, the coordinated action of fumarate over the direct and reverse reactions would allow a precise and specific modulation of the metabolic flux through this enzyme, leading to the synthesis or degradation of C_4 compounds under certain conditions. Thus, the physiological context might be exerting an accurate control of ME activity *in planta*, through changes in metabolite and substrate concentrations and cytosolic pH.

Keywords *Arabidopsis thaliana* · NADP-malic enzyme · Regulation · Fumarate

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Introduction

NADP-dependent malic enzymes (NADP-ME; EC 1.1.1.40) catalyze the reversible oxidative decarboxylation of malate to pyruvate, CO_2 and NADPH in the presence of a divalent cation (Chang and Tong 2003). This enzyme is widely distributed in nature. In plants, multiple isoforms have been identified in diverse organs and with varied subcellular localizations (Drincovich et al. 2010). Apart from the photosynthetic role of NADP-ME in some C_4 and CAM species, several functions have been proposed for the non-photosynthetic isoforms, some of which are still speculative. Thus, NADP-ME would play a key role in regulating the levels of C_4, C_3–C_2 and C_1 compounds, which are involved in vital processes such as respiration, biosynthesis of proteins and lipids, cellular pH regulation, stomatal movement and defense responses (Famiani et al. 2000; Lai et al. 2002; Shearer et al. 2004; Hurth et al. 2005; Laporte et al. 2002; Liu et al. 2007; Parker et al. 2009).

Arabidopsis thaliana, a C_3 dicot species, contains four NADP-ME isoforms. Three of them are cytosolic (NADP-ME1–3) and the fourth one is plastidic (NADP-ME4). These isoforms all have different kinetic, regulatory and structural properties (Gerrard Wheeler et al. 2005, 2008, 2009; Maurino et al. 2009). NADP-ME2 is constitutively expressed and is

responsible for most of the activity measured in mature plant tissues (Gerrard Wheeler et al. 2005). This isoform, a homotetramer constituted by monomers of 65 kDa (Maurino et al. 2009), is able to catalyze both direct (oxidative decarboxylation of malate) and reverse (reductive carboxylation of pyruvate) malic enzyme reactions at high rates (Gerrard Wheeler et al. 2008). Moreover, the $K_{0.5}$ values for the substrates in both directions are in the range of the physiological concentrations found in the plant (Fahnenstich et al. 2007), indicating that both reactions could be relevant in vivo (Maurino et al. 2009). In addition, NADP-ME2 was the most regulated isoform when the activity of *Arabidopsis* NADP-ME family members was assayed in the presence of various metabolites (Gerrard Wheeler et al. 2008). Particularly, fumarate stimulated both forward and reverse reactions of NADP-ME2 in studies conducted with fixed metabolite and substrates concentrations (Gerrard Wheeler et al. 2008). However, it is unclear which activity is prevailing in vivo and whether this organic acid may influence the direction of the reaction in any condition. It was also seen that Arg115 is part of an allosteric site that controls NADP-ME2 activation by fumarate (Gerrard Wheeler et al. 2008). The homolog to this residue has been implicated in fumarate allosteric activation of human NAD(P)-ME (Yang et al. 2002) and *Ascaris suum* NAD-ME (Karsten et al. 2003). Nevertheless, the lack of conservation in other residues suggested that binding capacity and fumarate response could be different among the three isoenzymes (Hung et al. 2005; Hsieh et al. 2009; Gerrard Wheeler et al. 2009).

The growing recognition of the importance of organic acids, especially malate and fumarate, in *A. thaliana* metabolism (Fernie and Martinoia 2009; Araújo et al. 2011) makes necessary a complete characterization of the regulation of NADP-ME2 by these compounds. Since this enzyme catalyzes the degradation and/or synthesis of malate in vitro, it could provide a link between the biosynthetic precursors' generation and the respiratory metabolism. In this way, recent studies have shown a significant alteration in the metabolic profiles of NADP-ME2 knockout lines, both under normal growth conditions and stress treatments (Brown et al. 2010; Voll et al. 2012). Several regulatory metabolites have been described for different ME isoforms, but their effects are variable depending on the tissue and the plant species where they are localized (Shearer et al. 2004; Tronconi et al. 2010). Moreover, little is known about their mode of action, probably due to the absence of crystallographic data for these enzymes in the plant kingdom (Chang and Tong 2003). Thus, the study of NADP-ME2 fumarate modulation is crucial for the understanding of organic acid metabolism in C_3 plants. Especially considering that other NADP-ME with different fumarate regulations are found in *Arabidopsis* (Gerrard Wheeler et al. 2008, 2009), which suggests a differential physiological contribution of each of the isoforms. Overall, the results obtained in the present work indicate that fumarate would be able to exert a dual role in the in vivo regulation of NADP-ME2 forward activity depending on its concentration, the levels of NADP and malate and cytosolic pH. Besides, fumarate would also affects the direction of the carbon flux through ME, leading to the synthesis or consumption of C_4 organic acids. The cellular conditions are a reflection of the highly varying environments to which plants are exposed. In this regard, a possible mechanism involving diurnal-nocturnal pH fluctuations, NADP-ME2 and cytosolic fumarase isoform (that converts the ME substrate into a ME modulator) is discussed.

Materials and methods

Heterologous expression and purification of the recombinant enzymes

pET32 expression vectors containing the full length cDNA sequences of NADP-ME2 and the mutant version ME2-R115A (Gerrard Wheeler et al. 2005, 2008) were used to express each NADP-ME fused in frame to a His-tag, to allow purification by a nickel-containing His-binding column (Novagen). Induction and isolation of the proteins were performed as previously described (Gerrard Wheeler et al. 2005, 2008). The fusion proteins were digested with 0.05 units of enterokinase (EK-Max; Invitrogen) per milligram of protein at 16 °C for 2 h to remove the N-terminus encoded by the expression vector. The protease was eliminated and the proteins were further purified using an affinity Affi-Gel Blue column (BioRad) The purified enzymes were concentrated on Centricon YM-50 (Millipores), analyzed by SDS-PAGE to verify integrity and purity and stored at −80 °C in 50 mM Tris–HCl pH 8.0, 10 mM $MgCl_2$ and 10 % (v/v) glycerol for further studies.

NADP-ME activity assays and protein concentration measurement

Oxidative decarboxylation of malate (forward reaction) was assayed spectrophotometrically using a standard reaction mixture containing 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 1 mM NADP and 30 mM malate in a final volume of 0.5 ml. Reductive carboxylation of pyruvate (reverse reaction) was measured in an assay medium containing 50 mM MOPS-KOH pH 7.0, 10 mM $MgCl_2$, 0.2 mM NADPH, 10 mM $NaHCO_3$ and 50 mM pyruvate in a final volume of 0.5 ml. In both cases, the reaction was started by the addition of the enzyme. One unit (U) is defined as the amount of enzyme that catalyzes the formation or consumption of 1 μmol of NADPH min^{-1} under the specified conditions

($\varepsilon_{340nm} = 6.22\ mM^{-1}\ cm^{-1}$). In assessing the effects of fumarate, succinate, maleate, tartrate, malonate and 2-ketoglutarate on enzyme activity, each metabolite was used in concentrations between 0 and 50 mM. All organic acid solutions were prepared with Mg^{2+} according to their dissociation constants (Dawson et al. 1986), in order to avoid the chelation of this cofactor from the reaction medium. Besides, to avoid modifying pH, the solutions were brought to pH 7.5. Fumarate modulation was determined using different buffers as follows: 50 mM MES-NaOH (pH 6.5), 50 mM MOPS-KOH (pH 7.0), 50 mM Tris–HCl (pH 7.5 and 8.0) and 50 mM Tricine (pH 8.5).

Initial velocity studies were performed by varying the concentration of one of the substrates around its $K_{0.5}$ value while keeping the other substrate concentrations at saturating or sub-saturating fixed levels. Previous reported $K_{0.5}$ values were considered for NADP-ME2: 72 μM for NADP and 3.3 mM for malate (direct reaction) and 0.54 mM for pyruvate (reverse reaction); and for ME2-R115A: 50 μM and 2.4 mM for NADP and malate, respectively (Gerrard Wheeler et al. 2005, 2008). As the true substrates of malic enzymes are the free forms, uncomplexed by metal ions, the data were analyzed based on free concentrations of NADP and malate. The following values for the dissociation constants (K_d) were used in the corrections: K_dMg-NADP = 19.1 mM and K_dMg-malate = 28.2 mM (Detarsio et al. 2003). All kinetic parameters were calculated at least by triplicate determinations. The substrate dependent rates were fitted alternatively to the Michaelis–Menten equation or the Hill equation by non-lineal regression (Sigma Plot). Significant changes in the kinetic parameters were decided using one way analysis of variance (ANOVA). Minimum significance differences were calculated by the Bonferroni, Holm-Sidak, Dunett and Duncan tests ($\alpha = 0.05$) using the Sigma Stat Package.

All activity assays were carried out at 30 °C in a Helios β spectrophotometer (Unicam). Protein concentration was determined by the BioRad protein assay using total serum protein as standard.

Circular dichroism (CD) measurements

CD spectra were obtained on a Jasco J-810 spectropolarimeter using a 0.1 cm-path length cell and averaging ten repetitive scans between 250 and 200 nm. Typically, 30 μg of each protein in 20 mM NaPi pH 7.5 were used for the assay. Mean amino acid residue ellipticity was calculated as described in Detarsio et al. (2003).

Urea denaturation experiments

The proteins (3 μg) were incubated in 50 mM Tris–HCl pH 7.5 for 30 min at 25 °C at different urea concentrations (until 3 M) in the absence or presence of 20 mM fumarate in a final volume of 200 μl. When appropriate, pre-incubation of the proteins with fumarate for 30 min at 0 °C was performed prior to urea addition. Ten microliters of incubation media were used for enzymatic activity determination, as was described above.

Gel electrophoresis

SDS-PAGE was performed in 10 % (w/v) polyacrylamide gels according to Laemmli (1970). Proteins were visualized with Coomassie blue.

Native PAGE was performed using 6 % (w/v) polyacrylamide gels. Electrophoresis was run at 150 V at 10 °C using running buffer (25 mM Tris–HCl; 192 mM glycine, pH 8.0) with or without 10 mM fumarate. Gels were assayed for NADP-ME activity by incubation in a solution containing 50 mM Tris–HCl pH 7.5, 10 mM $MgCl_2$, 30 mM malate, 1 mM NADP, 35 μg ml^{-1} nitroblue tetrazolium and 0.85 μg ml^{-1} phenazine methosulfate at 30 °C.

Gel filtration chromatography

Recombinant NADP-ME2 molecular mass was evaluated in the absence and presence of 10 mM fumarate by gel filtration chromatography using an ÄKTA purifier system (GE Healthcare) and a Tricorn Superdex 200 10/300 GL column (GE Healthcare). The column was equilibrated with 20 mM Tris–HCl at pH 7.5 and calibrated using the following molecular mass standards: carbonic anhidrase, 29 kDa; bovine serum albumin, 66 kDa; alcohol dehydrogenase, 150 kDa; β-amylase, 200 kDa; apoferritin, 443 kDa; and thyroglobulin, 669 kDa (Sigma-Aldrich). The sample and the standards were applied separately in a final volume of 100 μl at a constant flow rate of 0.5 ml min^{-1}.

Results

Regulatory properties of fumarate over NADP-ME2 forward reaction

Arabidopsis NADP-ME2 isoform was expressed as a recombinant protein in *E. coli* and purified to homogeneity. Fumarate modulation on NADP-ME2 forward activity was examined in the presence of sub-saturating concentrations of substrates NADP (72 μM) and malate (3.3 mM). Fumarate acted as an activator on NADP-ME2 in a wide range of concentrations, although the highest activation was achieved at 20 mM, reaching activities 18 times greater than those determined in the absence of the

metabolite (Fig. 1a). Above this concentration, the percentage of activation started to decrease (Fig. 1a).

Effect of fumarate over the NADP saturation curve of NADP-ME2 forward reaction

Using a malate concentration equivalent to the $K_{0.5}$ value (3.3 mM), an activating effect of fumarate was observed, which became more evident at low concentrations of NADP (Fig. 1b). Thus, the presence of 10 mM fumarate activated the NADP-ME2 forward reaction by about 15-fold at 0.05 mM NADP, but only at about 1.5-fold at 0.35 mM NADP. The increase of fumarate levels resulted in a decrease in the n_H values, which was accompanied with an increase in the affinity for NADP (Fig. 1b; Table 1) of the enzyme. In the absence of the modulator, a sigmoid response was observed ($n_H = 2.1$) and the apparent $K_{0.5}$ for NADP was equal to 0.4 mM. However, at 10 mM fumarate, the saturation curve presented a lower n_H value ($n_H = 1.4$) with an apparent $K_{0.5}$ value of 0.2 mM. In addition, no significant modifications in the apparent k_{cat} values were observed (Fig. 1b; Table 1).

At saturating malate concentrations, saturation curves of NADP at different fixed levels of fumarate were not significantly different, indicating the absence of fumarate modulation in these conditions in particular (data not shown).

Effect of fumarate over the malate saturation curve of NADP-ME2 forward reaction

When varying malate at a sub-saturating NADP concentration, the response of increasing the fumarate concentration resulted in a decrease in the n_H value, with a slight decrease in the apparent $K_{0.5}$ for malate (Fig. 1c; Table 1). The presence of fumarate did not produce significant modifications in k_{cat} values (Table 1). The activation effect of

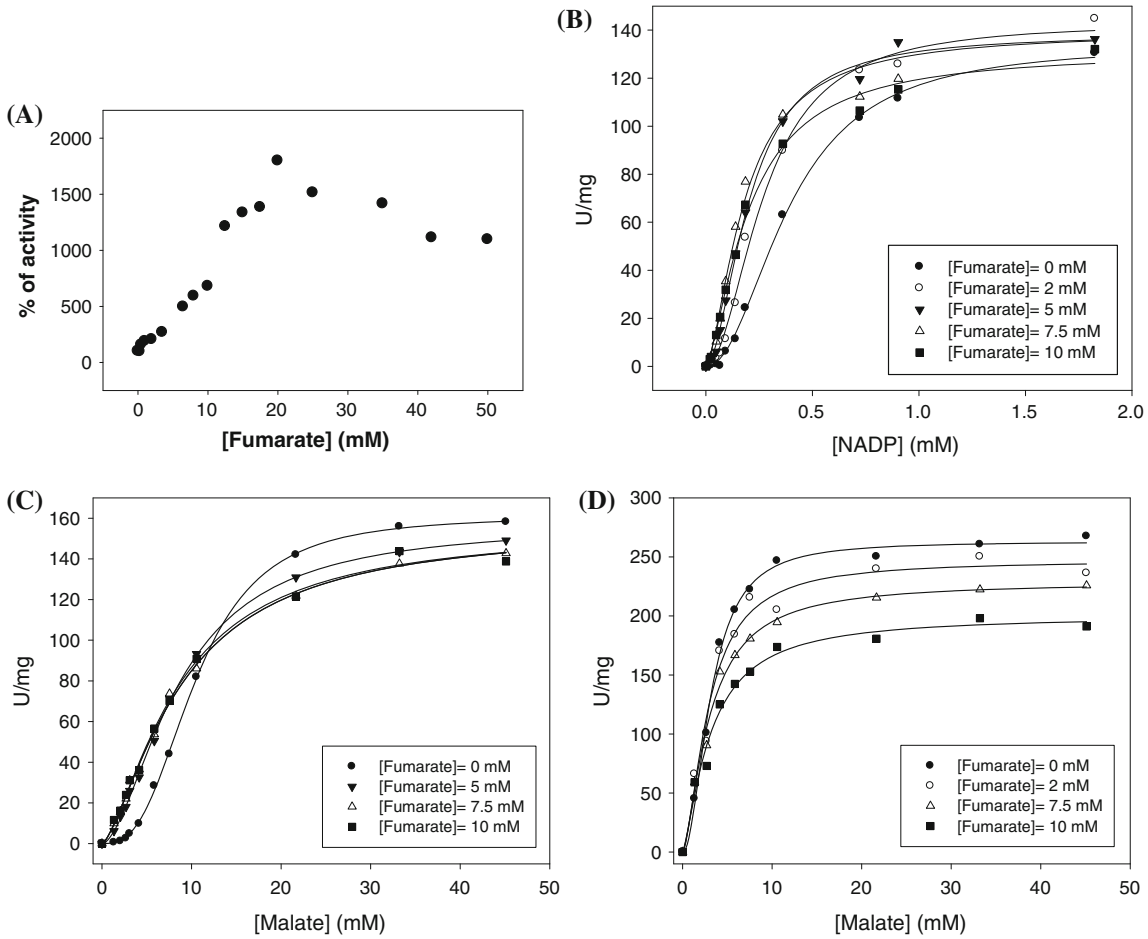

Fig. 1 Fumarate regulation of recombinant *A. thaliana* NADP-ME2 NADP-ME2 forward enzymatic activity was determined at pH 7.5 in the absence or presence of different fixed level of fumarate. Typical results are shown from at least three independent determinations. **a** The substrate concentrations used were 72 μM NADP and 3.3 mM malate and the results are presented as the % of activity in the presence of the effector in relation to the activity measured in the absence of it (100 % of activity). **b** NADP as variable substrate at 3.3 mM malate. **c** Malate as variable substrate at 72 μM NADP. **d** Malate as variable substrate at 1 mM NADP. The values of k_{cat}, n_H and $K_{0.5}$ obtained are shown in Table 1

Table 1 Kinetic parameters obtained for NADP-ME2 using NADP or malate as variable substrate

Malate = $K_{0.5}$	Fum = 0 mM	Fum = 2 mM	Fum = 5 mM	Fum = 7.5 mM	Fum = 10 mM
NADP as variable substrate at 3.3 mM malate					
$k_{cat\ apparent}$ (s^{-1})	145 ± 4	147 ± 7	146 ± 5	140 ± 13	141 ± 8
n_H	2.1 ± 0.1	2.0 ± 0.2	1.8 ± 0.1	1.6 ± 0.2	1.4 ± 0.1
$K_{0.5\ apparent}$ (mM)	0.39 ± 0.01	0.27 ± 0.02	0.20 ± 0.09	0.18 ± 0.03	0.20 ± 0.02
NADP = $K_{0.5}$	Fum = 0 mM		Fum = 5 mM	Fum = 7.5 mM	Fum = 10 mM
Malate as variable substrate at 72 μM NADP					
$k_{cat\ apparent}$ (s^{-1})	174 ± 4		171 ± 8	168 ± 15	168 ± 20
n_H	2.8 ± 0.1		1.7 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
$K_{0.5\ apparent}$ (mM)	11.0 ± 0.1		8.7 ± 0.2	9.0 ± 0.6	8.7 ± 0.6
NADP = 1 mM	Fum = 0 mM		Fum = 2 mM	Fum = 7.5 mM	Fum = 10 mM
Malate as variable substrate at 1 mM NADP					
k_{cat} (s^{-1})	285 ± 2		261 ± 4	238 ± 3	214 ± 4
n_H	2.1 ± 0.2		1.6 ± 0.3	1.5 ± 0.2	1.4 ± 0.2
$K_{0.5}$ (mM)	3.3 ± 0.1		3.0 ± 0.3	3.0 ± 0.2	3.0 ± 0.3

The other substrate is maintained at fixed level, as indicated. The values are the average of at least three different measurements ± SD

fumarate was maximal at low malate concentrations, with 10 mM fumarate generating a 24-fold activation at 1.5 mM malate (Fig. 1c). As malate concentration increases, the effect of fumarate decreases, resulting in an activation of 1.6 times at 7.5 mM malate. Above this concentration of malate, fumarate lost its activating effect at all fumarate concentrations tested (Fig. 1c).

On the other hand, saturation curves of malate at higher NADP levels (1 mM) in the presence of different fixed fumarate levels (Fig. 1d) showed that the modulator caused a decrease in NADP-ME2 maximum specific activity without altering its affinity for malate (Table 1). In turn, the curves lost the sigmoid behavior at increasing fumarate concentrations. Under these conditions, the activating effect of fumarate was observed only at low fumarate and malate concentrations (2 mM and 1.5 mM, respectively). However, at 10 mM fumarate this metabolite behaved as an inhibitor, producing velocity values between 69 and 76 % compared to those measured in the absence of the metabolite (Fig. 1d).

Regulatory properties of fumarate over the forward reaction of ME2-R115A

The replacement of Arg115 by Ala was enough to turn NADP-ME2 into a fumarate non-activated isoform (Gerrard Wheeler et al. 2008). In this work, the effect of fumarate on ME2-R115A was thoroughly analyzed in the presence of sub-saturating levels of NADP and malate at pH 7.5 (Fig. 2a). The results indicated that, for all concentrations tested, the enzyme activity value obtained was always lower than the one determined in the absence of fumarate, reaching a value of 19 % at 25 mM fumarate.

In addition, initial velocities were determined when malate was varied at fixed concentrations of fumarate and at a saturating NADP concentration. In this case, a hyperbolic response was obtained for all fumarate concentrations. The representation of data according to the Lineweaver–Burk equation suggests that fumarate acted as a competitive inhibitor with respect to malate (Fig. 2b). The generated secondary plot (slope versus inhibitor concentration) is representative of a non-linear parabolic inhibition mechanism (Fig. 2b).

Urea denaturation of NADP-ME2 and its mutant form

Fumarate binding to NADP-ME2 and ME2-R115A was evaluated by urea-induced denaturation assays. The proteins were incubated with increasing urea concentrations in the absence or presence of 20 mM fumarate. Then, overall protein integrity was analyzed by measuring residual enzymatic activity at saturating substrates concentrations.

The denaturation process induced by urea was different for each enzyme analyzed (Fig. 3). In this regard, a significant loss of NADP-ME2 activity in the absence of fumarate started at concentrations greater than 1.5 M urea, leading to complete inactivation at 2.5 M urea (Fig. 3a). However, only about 40 % of ME2-R115A activity was lost at the highest concentration tested (3 M urea; Fig. 3b).

In addition, protein denaturation patterns were differentially modified in both proteins by the presence of

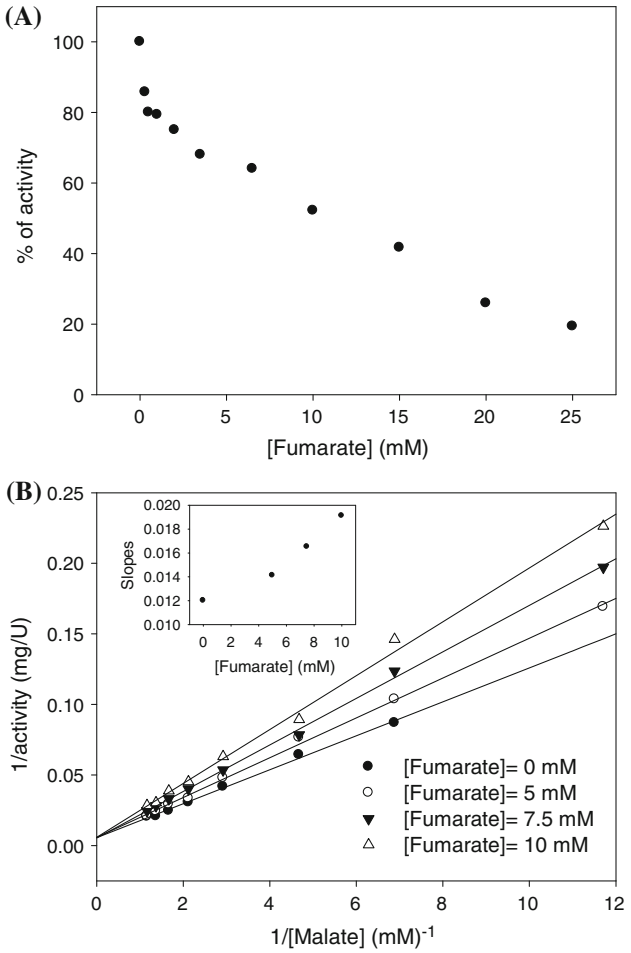

Fig. 2 Effect of fumarate over ME2-R115A mutant protein **a** % of ME2-R115A forward activity in the presence of the different fumarate concentrations in relation to the activity measured in the absence of it (100 % of activity). Typical results are shown from at least three independent determinations. The substrate concentrations used were 50 μM NADP and 2.4 mM malate. **b** Double reciprocal graph of ME2-R115A forward activity determined using malate as variable substrate at 1 mM NADP in the absence or presence of different fixed levels of fumarate. Typical results are shown from at least three independent determinations. In the inset is shown the secondary graph obtained by plotting the slopes versus the different inhibitor concentrations used

fumarate (Fig. 3). Interestingly, in the case of parental NADP-ME2, fumarate produced a decreased sensibility to urea denaturation, especially at high concentrations of the denaturing agent. The residual activity slightly fell in the presence of fumarate after incubation with 0.25 M urea and, above this concentration, this parameter remained more or less stable (Fig. 3a). On the other hand, when fumarate was added to the ME2-R115A incubation media, the opposite effect was observed, exhibiting a greater loss of activity (between 12 and 30 %) at urea concentrations greater than 1 M (Fig. 3b).

Effects of fumarate structural analogues

In addition to fumarate (a four-carbon *trans* dicarboxylic acid), a number of related acids were tested as modulators of NADP-ME2 forward activity. The R115A mutant, which is completely insensitive to fumarate activation, was used as a negative control. As shown in Fig. 3c, succinate (a four-carbon dicarboxylic acid with a carbon–carbon single bond) could also activate NADP-ME2. However, in this case, the activating effect was less pronounced than the one observed with fumarate (Figs. 1a, 3c). The *cis* isomer of fumarate, maleate, significantly inhibited NADP-ME2 activity, suggesting that the *trans* configuration of fumarate is crucial for the activating effect to take place. Other structural analogues, including malonate (a three-carbon dicarboxylic acid) and 2-ketoglutarate (a five carbon α-ketodicarboxylic acid), also acted as inhibitors (Fig. 3c). On the other hand, tartrate (a four carbon dicarboxylic acid with two hydroxyl groups) produced no significant effects on enzyme activity. ME2-R115A displayed a marked inhibition in the presence of all the organic acids tested (Fig. 3d), as was previously observed with fumarate (Fig. 2a).

Modulation by fumarate and its dependence on the pH

Fumarate also stimulated NADP-ME2 pyruvate reductive carboxylation in assays measured using 0.2 mM NADPH, 30 mM HCO_3^- and 0.54 mM pyruvate (Fig. 4). Thus, in order to clarify whether this organic acid influences the prevalence of one of the two activities, an analysis of the forward and reverse reactions catalyzed by NADP-ME2 at pH 7 and 7.5 was performed. The results show dependence on pH of fumarate activation with opposite effects on the two activities analyzed (Fig. 4). The forward reaction is no longer activated by fumarate when pH is shifted 0.5 units, to pH 7 (Fig. 4a). Instead, in these conditions, fumarate acted as an inhibitor of NADP-ME2 enzyme activity, reaching values below 80 % of the activity measured in the absence of metabolite (Fig. 4a). For the reverse reaction, an opposite behavior was observed. At pH 7, the degree of activation is close to 10 times higher that the activation observed at pH 7.5 at 15 mM fumarate (Fig. 4b).

The direct reaction was also evaluated at more acidic and basic pHs (data not shown). At more alkaline values (greater than pH 7.5) the percentages of activation were even higher, reaching a maximum activation of 37-fold at pH 8.5 in the presence of 10 mM fumarate. This represented a 5-fold higher activation than the one observed at pH 7.5. At pH 6.5 fumarate activation was not observed, coincident with the result obtained at pH 7.

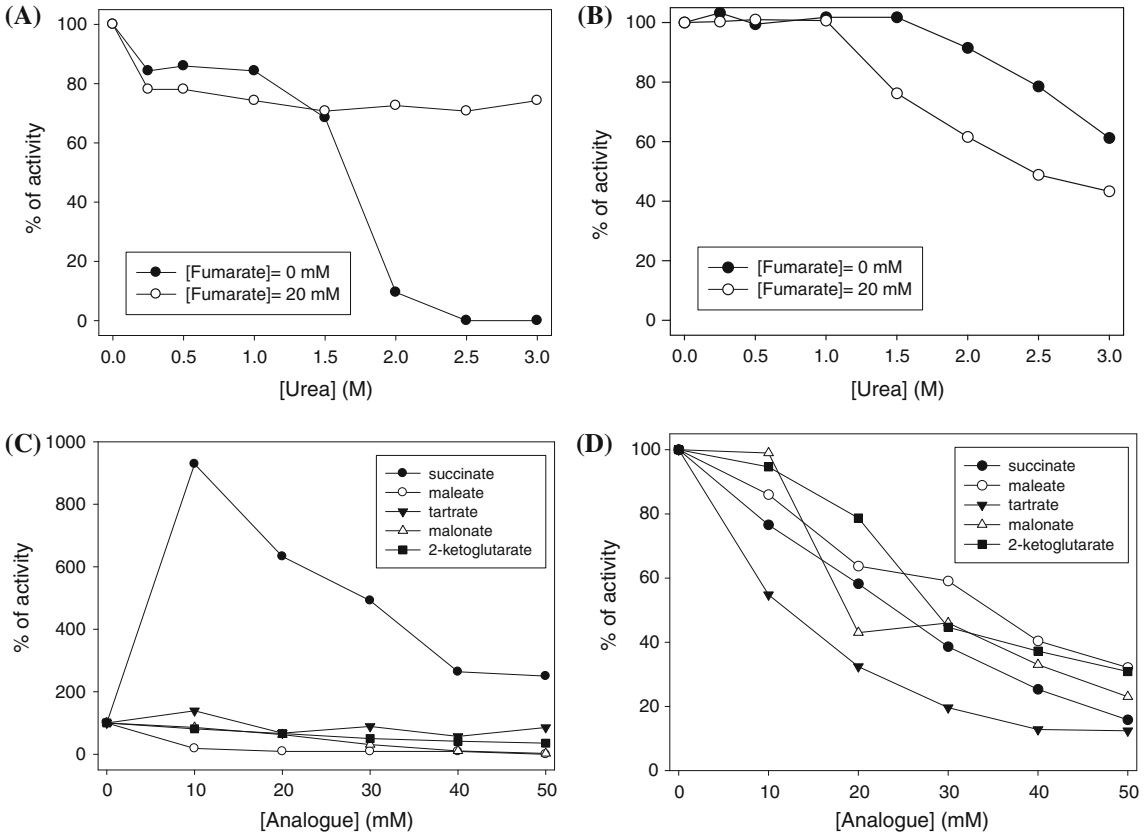

Fig. 3 Residual forward activity (as % of activity) of NADP-ME2 (**a**) or ME2-R115A (**b**) after incubation with different urea concentrations in the absence or presence of fumarate. 100 % of activity corresponded to the activity without urea in the presence or absence of fumarate according to the case. Enzymatic forward activity of NADP-ME2 (**c**) or ME2-R115A (**d**) determined at pH 7.5 in the absence or presence of different concentration of fumarate analogues. Results are presented as the % of activity in the presence of the effector in relation to the activity measured in the absence of it (100 % of activity). The substrate concentrations used were 72 μM NADP and 3.3 mM malate in (**c**) or 50 μM NADP and 2.4 mM malate (**d**). Typical results are shown from at least three independent determinations

Oligomerization state of NADP-ME2 in the presence of fumarate

NADP-ME2 oligomerization state in the presence of fumarate was evaluated by native electrophoresis and gel filtration chromatography and compared with the results determined in the absence of metabolite (Gerrard Wheeler et al. 2005; Maurino et al. 2009). The results indicated that fumarate did not alter the tetramer structure of recombinant *Arabidopsis* NADP-ME2 (data not shown).

Discussion

The highly changing environments to which plants are exposed demand similar extensions of veering responses in order for these organisms to survive (Jump et al. 2009). Modulation of enzyme content through synthesis and/or degradation is a common long term acclimation strategy (Kilian et al. 2007). However, fast enzyme activity changes are critical and can be achieved by allosteric regulation (Laskowski et al. 2009). In this work, we present the multiple factors that control *A. thaliana* NADP-ME2 allosteric regulation by fumarate. This complex system integrates several environmental stimuli in the forms of malate, NADP and fumarate levels and pH, which together reflect the cell metabolic status.

Substrate concentrations play a main role in the regulation of NADP-ME2 forward activity by fumarate. This metabolite can act as a strong activator or a partial inhibitor depending on the NADP and malate levels (Fig. 5). The ability of fumarate to enhance NADP-ME2 activity is seen only at sub-saturating concentrations of the substrates and is maximal at concentrations equal to or lower than their $K_{0.5}$ values (Fig. 1). This organic acid produces an increase in the NADP-ME2 affinity for both substrates, expressed by a decrease in the apparent $K_{0.5}$ values, causing greater active site occupancy and therefore an increase in the forward reaction rate (Table 1). However, if at least one of the substrates reaches a saturating level, the activation is not

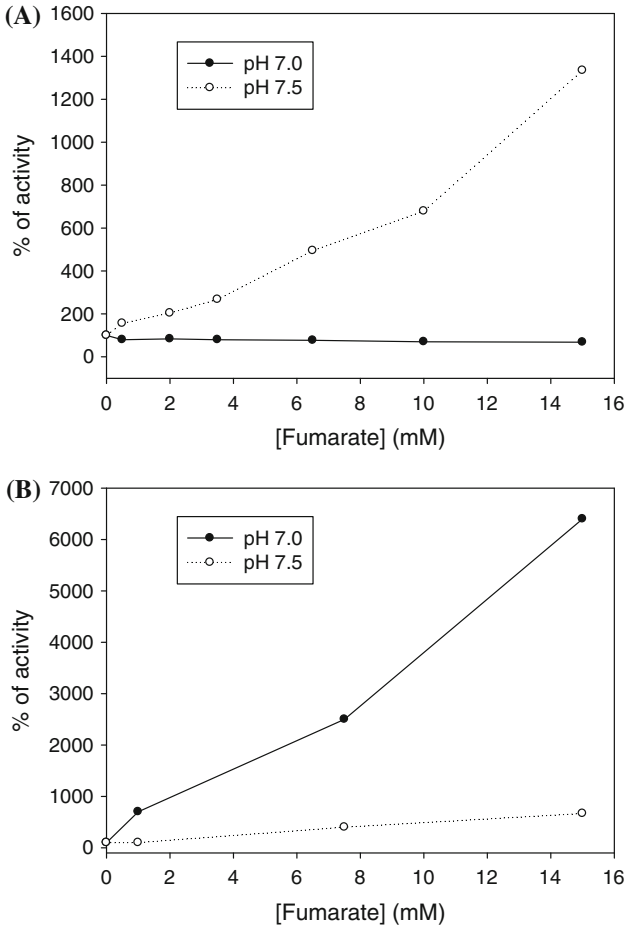

Fig. 4 pH-dependent fumarate modulation Fumarate regulation of NADP-ME2 forward (**a**) and reverse (**b**) activity was determined at pH 7 and pH 7.5. Results are presented as the % of activity in the presence of different concentrations of fumarate in relation to the activity measured in the absence of it (100 % of activity). Typical results are shown from at least three independent determinations. The substrates concentrations used for the forward reaction were 72 μM NADP and 3.3 mM malate and for the reverse reaction were 0.2 mM NADPH, 30 mM HCO_3^- and 0.54 mM pyruvate

observed. Instead, over a certain L-malate concentration, fumarate begins to show an inhibitory effect (Fig. 1d).

ME2-R115A is a useful tool for studying the molecular mechanism of fumarate inhibition, as this point mutant enzyme is not activated by fumarate but maintains the inhibitory effect even at sub-saturating substrate concentrations (Fig. 2). The results are consistent with parabolic competitive inhibition of fumarate against malate (Fig. 2b); this means that the binding of a molecule of fumarate allows the binding of a second molecule, so that two effector molecules contribute to the exclusion of the substrate from the active site (Roverts 1975). Furthermore, fumarate prevented the loss of NADP-ME2 activity in urea denaturation experiments (Fig. 3a). This behavior was not observed in ME2-R115A (Fig. 3b), reflecting differential fumarate binding properties for both enzymes.

Fumarate concentration itself is another determinant of its action (Fig. 5). NADP-ME2 forward reaction activation is maximal at 20 mM fumarate and, above that level, activation starts to decrease (Fig. 1a). This biphasic effect suggests that fumarate can bind at two distinct sites with different affinities and causing opposite effects. At low fumarate concentrations, the effector can only bind to the activation site, the allosteric site, that presents a high affinity for fumarate. But once fumarate concentration is high enough, it could also bind to the active site, causing competitive inhibition, which is reflected in the gradual loss of activation seen over 20 mM of fumarate. It is worth mentioning that all the changes in activity determined in the presence of different levels of fumarate are not accompanied by changes in the oligomeric state of the enzyme.

In this context, flexible regulation of NADP-ME2 by fumarate would allow the cell to easily vary the activity of this key metabolic enzyme in a range of a thousand times simply by the adjustment of metabolite concentrations. In

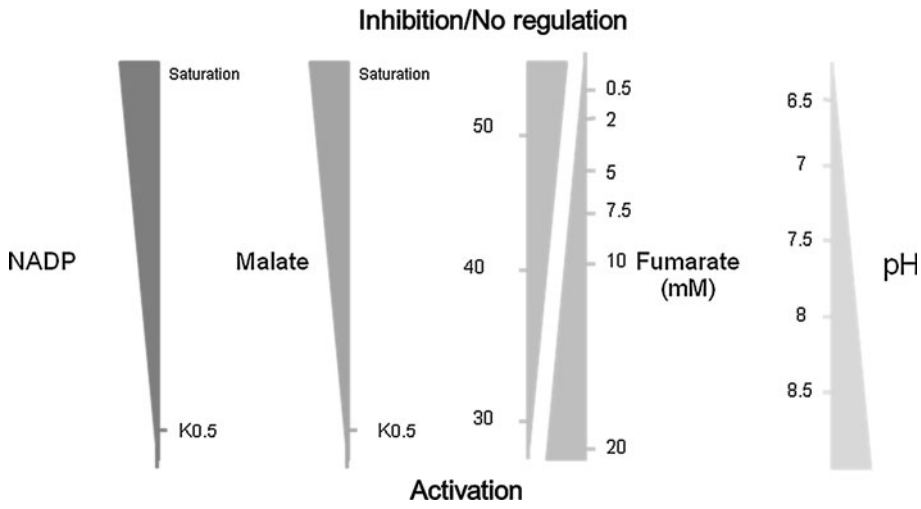

Fig. 5 Schematic representation of the factors that affect the regulation by fumarate in *Arabidopsis* NADP-ME2 forward activity The *bar* width increases with the concentration of NADP, malate or fumarate or a pH increase. Depending on the different factors, NADP-ME2 may be in a state of activation or not regulation or inhibition

cellular conditions, in which substrate levels are low (associated with low enzyme activity), the activator action of fumarate should be determinant in order to allow a fast response to environmental changes. On the other hand, the inhibitor role of fumarate should be important when enzyme activity is high (high levels of substrates), giving the cell the possibility to decrease its activity as quickly as the changes in concentrations take place.

The regulation of NADP-ME2 by fumarate can be relevant in vivo, as *A. thaliana* can accumulate great amounts of fumarate and malate in the cell cytosol (Hurth et al. 2005; Gout et al. 1993) and use them as carbon, energy and reducing power sources (Fernie and Martinoia 2009; Araújo et al. 2011). Their levels are extremely variable depending on tissue, plant age and growth conditions, such as light and nutrient availability, and can reach concentrations of up to 50 mM (Chia et al. 2000; Fahnenstich et al. 2007). Also, organic acid levels increase during the day due to photosynthetic activity and decrease at night (Zell et al. 2010). Thus, the regulatory action of fumarate in NADP-ME2 activity in vivo will ultimately depend on their changing levels in the different contexts. The recent identification of a specific cytosolic fumarase isoform in *Arabidopsis*, reflects the critical role that fumarate plays in the cytosolic metabolism of this species (Pracharoenwattana et al. 2010). The regulation of this reversible enzyme should be tightly coordinated with the NADP-ME2 regulation, as fumarase converts the ME modulator into a ME substrate (Fig. 6).

NADP-ME2 forward activity is also modulated distinctively by fumarate at different pHs (Fig. 4a). The degree of activation decreases with decreasing pH values between 8.5 and 7.5, with no activation at pH values equal to or lower than 7 (Fig. 5). Thus, the presence of amino acid side chains in the allosteric site that should be in a proper protonation state to produce activation could be postulated. At more acidic pH values, the protonation of some ionizable residue(s) of the enzyme causes a decrease in fumarate binding and/or the transmission of the positive signal to the active site.

Furthermore, fumarate also affects NADP-ME2 reverse reaction (Fig. 4b). At pH 7, pyruvate carboxylation catalyzed by NADP-ME2 is activated by fumarate (Fig. 4b), while malate decarboxylation is inhibited by this metabolite (Fig. 4a). These results reflect a strong preference of the reverse direction over the forward one at pH 7.0 and in the presence of fumarate (Fig. 6). Interestingly, at pH 7.5 the opposite statement is true: fumarate activation is higher for the direct reaction (Fig. 4), favoring the decarboxylation of malate (Fig. 6). It is worth mentioning that both NADP-ME2 activities present similar catalytic efficiencies (Gerrard Wheeler et al. 2008). Thereby, the net flux through this key metabolic point would result from concerted fumarate regulation of both reactions by changes in the cytosolic pH. Thus, a change of 0.5 units of pH would be enough to change the direction of the metabolic flux.

Intracellular pH must be kept close to neutrality to be compatible with cellular functions. However, cytosolic pH fluctuations occur in certain conditions, with variations of up to two pH units having been reported (Rienmüller et al. 2012). In this way, changes in light intensity represent a signal that can define pH differences (Felle 2001). It has been reported that in some species, dark-adapted cells respond to light with an acidification of the cytosol (Okazaki et al. 1994). Considering this fact together with the regulation of NADP-ME2 activity by fumarate, it is possible to postulate a mechanism to understand the role of this enzyme in organic acid metabolism. Thus, during the day, when cytosolic pH would be around 7, NADP-ME2 would catalyze mainly the reverse reaction, allowing the synthesis of malate (Fig. 6). Part of this malate would be converted into fumarate through the action of the cytosolic fumarase, which could auto-stimulate its own synthesis, generating the high levels of C_4 organic acids found in *Arabidopsis* during the light period (Pracharoenwattana et al. 2010). On the other hand, at nocturnal pH (possibly around 7.5) the NADP-ME2 direct reaction would be favored and could lead to the consumption of malate and, due to its equilibrium with fumarate, also to the consumption of this acid (Fig. 6). At the beginning of the dark period, while the concentration of fumarate is still high, it could auto-stimulate its own consumption due to its activator effect on the direct reaction at that pH (Fig. 4a). This is consistent with the consumption of organic acids and

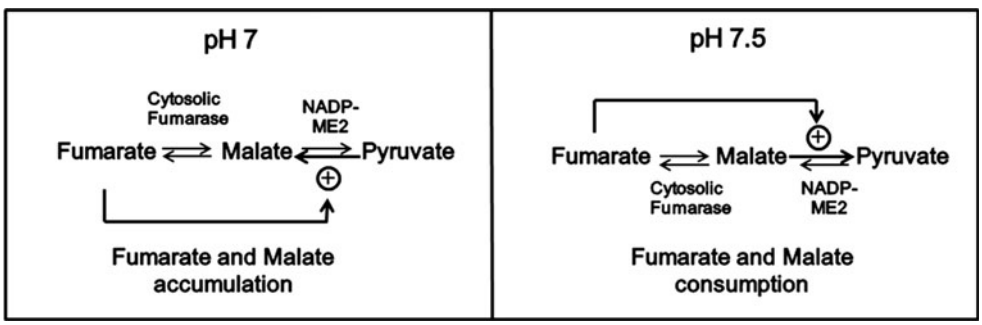

Fig. 6 Schematic representation of a possible mechanism that allow the accumulation of organic acids and their consumption during the dark heterotrophic metabolism, according to the fumarate regulation of NADP-ME2

sugars during night heterotrophic metabolism, in order to fulfill the energetic demand (Tronconi et al. 2008). This hypothesis should, however, be proved in vivo and the exact concentration of the substrates in both directions should be determined before any final conclusions are made.

Finally, it is important to consider that, besides fumarate, NADP-ME2 is also regulated by other metabolites such as succinate, aspartate and glucose-6P (Gerrard Wheeler et al. 2008). The possibility for regulations as complex and sophisticated as in the case of fumarate exists for all these other compounds. Succinate, for example, also behaves as a positive regulator of NADP-ME2 activity, showing a similar, although more attenuated, biphasic behavior (Fig. 3c). This means that the final effect on ME activity should come from the integrated action of all the modulators present in a particular set of conditions.

Concluding remarks

The studies here reported reveal a complex behavior between substrates, metabolites and pH in *Arabidopsis* NADP-ME2 regulation. This enzyme would use a very sophisticated binding mechanism to regulate its own activity. Thus, minimal changes in the cellular environment could adjust malic enzyme activity and thus flux direction, to respond optimally to the needs of plant metabolism. This work highlights the fact that only monitoring total enzyme contents and activities in order to analyze metabolic fluxes could lead to erroneous conclusions. Thus, a complete view of in vivo enzyme activities should include knowledge of the concentrations of all regulators present.

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