Specific Arabidopsis thaliana malic enzyme isoforms can provide anaplerotic pyruvate carboxylation function in Saccharomyces cerevisiae

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In vivo pyruvate carboxylation activity for specific *Arabidopsis thaliana* malic enzyme isoforms

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Running title: Carboxylation activity of plant malic enzymes

Abstract

NAD(P)-malic enzyme (NAD(P)-ME) catalyzes the reversible oxidative decarboxylation of malate to pyruvate, CO_2 and NAD(P)H and is present as a multigene family in Arabidopsis. The carboxylation reaction catalyzed by purified recombinant *A. thaliana* NADP-ME proteins is faster than those reported for other animal or plant isoforms. In contrast, no carboxylation activity could be detected *in vitro* for the NAD-dependent counterparts. In order to further investigate their putative carboxylating role *in vivo*, Arabidopsis NAD(P)-ME isoforms, as well as the NADP-ME2del2 (with a decreased ability to carboxylate pyruvate) and NADP-ME2R115A (lacking fumarate activation) versions, were functionally expressed in the cytosol of pyruvate carboxylase negative (Pyc^-) *Saccharomyces cerevisiae* strains. The heterologous expression of NADP-ME1, NADP-ME2 (and its mutant proteins), and NADP-ME3 restored the growth of Pyc^- *S. cerevisiae* on glucose, and this capacity was dependent on the availability of CO_2. On the other hand, NADP-ME4, NAD-ME1 and NAD-ME2 could not rescue the Pyc^- strains from C_4 auxotrophy. NADP-ME carboxylation activity could be measured in leaf crude extracts of knockout and over-expressing Arabidopsis lines with modified levels of NADP-ME, where this activity was correlated with the amount of *NADP-ME2* transcript. These results indicate that specific *A. thaliana* NADP-ME isoforms are able to play an anaplerotic role *in vivo* and provide a basis for the study on the carboxylating activity of NADP-ME, which may contribute to the synthesis of C_4 compounds and redox shuttling in plant cells.

Keywords: anaplerotic role, C_4 organic acids, *Saccharomyces cerevisiae*, malate synthesis, .plant metabolism

Abbreviations: ME, malic enzyme; Pyc^-, pyruvate carboxylase negative strain.

1. Introduction

Malic enzyme (ME; **EC 1.1.1.38-40**) is a widely distributed protein [1, 2] which catalyzes the oxidative decarboxylation of malate generating pyruvate, CO_2 and reducing power in the form of NADPH or NADH [3]. The standard free energy change (ca. -8 kJ/mol) categorizes the ME reaction as potentially reversible *in vivo* [4]. Although its participation in the C_4- or CAM photosynthesis has been extensively studied [5], all plant species present several isoforms whose individual biological roles are still unknown [6]. The proposed physiological roles for the different isoforms of ME are related to its capacity to control the availability of its substrates and products for other metabolic pathways. Although the most characterized reaction is the one in the decarboxylation direction, some carboxylating roles for ME have been previously suggested [7-11].

The first characterization of a complete plant ME family has been conducted in the C_3 dicot *Arabidopsis thaliana*. This family is composed of three cytosolic proteins (NADP-ME1 to 3), a plastidic isoform (NADP-ME4) and two enzymes with mitochondrial localization (NAD-ME 1 and 2) [12, 13] (Table 1). These proteins do not only differ in subcellular localization and cofactor requirement, but also in abundance, developmental and tissue-dependent expression patterns, and biochemical properties [14-21] (Table 1). NADP-ME2 (**TAIR: AT5G11670**) is responsible for the major decarboxylating activity measured in all mature plant organs. This protein has been involved in sugar metabolism in veins [22] and in the oxidative burst triggered by hemibiotrophic fungal pathogen infection [23]. Recent studies showed that the over-expression of NADP-ME2 in *A. thaliana* resulted in altered metabolic profiles, which reflect the importance of this enzyme in maintaining redox and carbon cellular balances in plants [24]. Different amino acid residues and sequence segments are involved in its catalytic and regulatory features and through mutagenesis studies it was found that Arg115 participates in allosteric activation by fumarate, while the amino terminal part is crucial for the carboxylation reaction [14]. The isoforms NADP-ME4 (**AT1G79750**), NAD-ME1 (**AT2G13560**) and NAD-ME2 (**AT4G00570**) are also ubiquitously expressed [12, 13] (Table 1). It has been suggested that NADP-ME4 provides precursors for plastidic fatty acid biosynthesis [25], while NAD-ME1 and NAD-ME2 function as homo and/or

mixed heterodimer participating in malate respiration [17]. By contrast, the expression of NADP-ME1 (**AT2G19900**) and NADP-ME3 (**AT5G25880**) is subject to cell or plant growth-specific signals: NADP-ME1 is localized in embryonic tissues and roots of adult plants, while NADP-ME3 is found mainly in trichomes and pollen [12] (Table 1).

Recombinantly expressed *A. thaliana* NAD-ME1 and NAD-ME2 isolated from *Escherichia coli* do not perform the reductive carboxylation of pyruvate, supporting the notion that they act only in malate oxidation in plant mitochondria [18, 26]. In contrast, the carboxylation reaction catalyzed by recombinant purified Arabidopsis NADP-ME1-4 is faster than previously reported for other ME isoforms, presenting higher k_{cat} values than the "average enzyme" [14, 27]. Moreover, considering the high amount of pyruvate found in *A. thaliana* leaves (0.4 and 0.6 µmol per gram of fresh weight at the end of the night and the day period, respectively) [24] and the relatively low K_m for pyruvate obtained for NADP-ME1-4 [14], we postulate that the carboxylation activity possibly takes place *in planta*. In this sense, carboxylating and decarboxylating activities of each enzyme were reciprocally regulated by several cellular metabolites [14, 21], which could enable a specific modulation of the metabolic flux through NADP-ME, leading to the synthesis or degradation of C_4 compounds according to cellular needs.

The aim of this work was to investigate the ability of the Arabidopsis ME family to carboxylate pyruvate *in vivo*. For this purpose, each isoform was expressed in pyruvate carboxylase negative (Pyc^-) *Saccharomyces cerevisiae* strains - which are not able to grow on glucose as the sole carbon source due to C_4 organic acid auxotrophy - and its ability to rescue growth on glucose was investigated. In addition, the carboxylation reaction was evaluated for recombinant ME isoforms in the presence of cellular metabolites and in wild type and transgenic Arabidopsis plants with modified levels of NADP-ME (mutant and over-expressing lines). Together, the results indicated that specific *A. thaliana* NADP-ME isoforms are able to play an anaplerotic role *in vivo* and provide a basis for future studies on the carboxylation activity of ME, which may contribute to the synthesis of C_4 compounds and redox shuttling in plant cells.

2. Results

2.1 Testing pyruvate carboxylation activity of Arabidopsis NAD(P)-ME within a cellular context

In *S. cerevisiae* cells grown on glucose, the glyoxylate cycle is suppressed and anaplerosis is solely carried out by the two isoforms of pyruvate carboxylase (Pyc1 and Pyc2), which convert pyruvate to oxaloacetate at the expense of ATP and CO_2 in the cytosol [28]. Strains with deletions in both *PYC1* and *PYC2* genes (Pyc^-) cannot grow on glucose as sole carbon source and provide a simple eukaryotic system to test the carboxylation activity of the Arabidopsis NAD(P)-ME isoforms [11, 29]. *S. cerevisiae* possess a unique ME localized to the mitochondria that can use NAD and NADP [30]. This ME acts exclusively as a decarboxylase since its presence does not allow the growth of Pyc^- strains [31].

cDNAs encoding the mature proteins - without localization signals - of the different *A. thaliana* NAD(P)-ME isoforms (Table 1) were cloned into the single-copy p416GPD vector. Two mutant versions of NADP-ME2, NADP-ME2del2 (with reduced pyruvate carboxylation performance *in vitro*) and NADP-ME2R115A (lacking the allosteric regulation by fumarate) [14], were also included in the analysis. The Pyc^- mutant IMK299 [11] was transformed with the different plasmids and the functional expression of the plant proteins in yeast was checked by measuring the ME decarboxylating activities in cell-free extracts. All strains showed significantly higher levels of NADP-ME (0.070-0.997 µmol min^{-1} mg $protein^{-1}$) or NAD-ME (0.251-0.252 µmol min^{-1} mg $protein^{-1}$) activity than the control strain transformed with an empty vector without NAD(P)-ME insert (0.028 and 0.174 µmol min^{-1} mg $protein^{-1}$ for NADP and NAD as cofactor, respectively; Figure 1), indicating that all plant NAD(P)-ME proteins were functional in *S. cerevisiae*.

After confirmation of the functional expression, the ability of the different NAD(P)-ME variants to restore growth on synthetic medium with glucose as the sole carbon source was investigated. The growth of each strain was characterized on plates under anaerobic conditions consisting of either 100% N_2 or a mixture of 10% CO_2 and 90% N_2 to thermodynamically favor pyruvate carboxylation. As expected, the reference yeast strain CEN.PK113-7D grew well and the negative control, IMK299 (*pyc1,2*Δ*pdc2*Δ) transformed with an empty p416GDP vector, did not show growth in medium with ammonium as nitrogen source in thermodynamically favorable conditions (10% CO_2) (Figure

2A). Strains expressing NADP-ME2, NADP-ME3 and to a lesser extent NADP-ME2R115A and NADP-ME2del2 were able to complement the C_4 auxotrophy of IMK299. However, no growth was observed for strains expressing NADP-ME1, NADP-ME4, NAD-ME1 and NAD-ME2 (Figure 2A). In addition, no growth was observed for any of the complemented strains incubated on synthetic medium with ammonium as nitrogen source in 100% N_2 atmosphere (data not shown), indicating that the presence of CO_2 is essential for growth. As a positive control, all strains were grown on plates supplemented with the C_4 acid aspartate under ambient conditions (Figure 2B).

Since the rate of malate synthesis from pyruvate carboxylation is most likely the rate limiting step for cell growth in the constructed mutants, a comparison was made between the ability of each isoform to complement the C_4 auxotrophy based on cell growth rate in shake-flask cultures with favorable thermodynamic conditions (6% CO_2) (Figure 3). Growth was observed for the strains expressing NADP-ME1, NADP-ME2, NADP-ME2del2, NADP-ME2R115A and NADP-ME3, with maximum specific growth rates ranging between 0.010 and 0.022 h^{-1} (Figure 3B). In accordance with the spot plate results (Figure 2A), no growth was observed for strains expressing NADP-ME4 and the NAD-ME isoforms after 350 hours of incubation (Figure 3A). IMK299 expressing NADP-ME1 grew well in shake-flask cultures, presenting a maximum specific growth rate of 0.022 h^{-1} (Figure 3B). However, this strain exhibited a long lag phase of 200 h (Figure 3A), which could explain the absence of colonies on solid media (Figure 2A). Thus, some NADP-ME isoforms were able to complement growth of the Pyc^- *S. cerevisiae* strains, whilst NADP-ME4, NAD-ME1 and NAD-ME2 failed to fulfill the anaplerotic role under these conditions.

2.2 In vitro activity of recombinant NAD(P)-ME proteins under conditions that mimic the cellular environment

The performance of some of the ME isoforms in the *in vivo* complementation experiments seemed to contradict previously determined *in vitro* pyruvate carboxylating capacities. Therefore, the possible modulation of this activity by metabolites present in the cellular environment was assayed using purified ME isoenzymes, recombinantly obtained from *E. coli*.

The mutant protein NADP-ME2del2 lacks the first 44 amino acid residues [14] and compared to the parental NADP-ME2, it had a much lower pyruvate carboxylation capacity *in vitro* (9% of the NADP-ME2 activity; Figure 4A). However, NADP-ME2del2 was able to complement the C_4 auxotrophy of the Pyc$^-$ *S. cerevisiae* strain on solid media and in liquid cultures (Figures 2 and 3). Therefore, the NADP-ME2 and NADP-ME2del2 carboxylating reactions were evaluated in the absence or presence of 1 or 7.5 mM of fumarate and succinate. NADP-ME2 was activated by fumarate and succinate at both concentrations, as previously reported [14]. Interestingly, NADP-ME2del2 also exhibited an increased activity in presence of both organic acids and reached activity levels similar to those of NADP-ME2 with 7.5 mM succinate (Figure 4A).

On the other hand, the *in vitro* activity of NADP-ME4, which failed to complement the auxotrophy of Pyc$^-$ mutants although having a high value of the catalytic constant for the carboxylation reaction, was not increased by the addition of either C_4 acid [14] (Figure 4A), presenting activity levels between 50 and 75% depending on the concentration and nature of the modulator.

To test whether modulation of the enzymatic activity could also take place *in planta*, the ability of the recombinant enzymes to carboxylate pyruvate was evaluated in the presence of total polar metabolite extracts of *A. thaliana* leaves. The results showed increased activities of NADP-ME2 and to a lesser degree NADP-MEdel2 in the environments containing the plant compounds (Figure 4B). Furthermore, NADP-ME4 activity decreased compared to the control without plant metabolites (Figure 4B), in a similar way to the results obtained with either fumarate or succinate (Figure 4A).

Consistent with previous work [18], the carboxylating activity of NAD-ME recombinant proteins was below the detection limit in the conditions tested (<0.02 nmol NAD(P)H consumed per min), even if the assays were repeated with different batches of the enzymes and at both low and high enzyme and substrate concentrations. Moreover, the addition of fumarate, succinate or the plant metabolite extracts to the reaction medium did not result in detectable activity.

2.3 Carboxylating activity of cell extracts of A. thaliana lines with modified levels of specific NADP-ME isoforms

In order to evaluate the physiological contribution of the ME carboxylation reaction *in planta*, the total NADP-ME carboxylating activity was examined in leaf protein extracts of *A. thaliana* loss-of-function and over-expressing plants of the different NADP-ME isoforms. The carboxylating activity was measured in wild type leaf extracts (Figure 4C), although with lower rates than those determined for the decarboxylation reaction [24]. It should be noted that the plant protein extracts showed no detectable carboxylase activity with NADH instead of NADPH. Only the NADP-ME carboxylating activity measured in the extracts from single, double or triple mutant lines affecting the *NADP-ME2* gene was significantly lower compared to wild type (Figure 4C). The lines deficient in the other *NADP-ME* genes presented similar levels to those of the wild type plant. In addition, two NADP-ME2 over-expressing lines (7.11 and 4.20) showed an increase of ~4 times in the pyruvate carboxylation activity (Figure 4C). These results indicate a direct correlation between carboxylating activity and NADP-ME2 level in each line. Also, they suggest that this isoform is the largest contributor to the total NADPH-dependent pyruvate carboxylation activity in Arabidopsis, at least in leaves.

3. Discussion

In this work, all *A. thaliana* NAD(P)-ME proteins were tested as pyruvate reductive carboxylases in a cellular context. The results indicated that the expression of some NADP-ME isoforms was sufficient to rescue the C_4 auxotrophy of Pyc^- strains of *S. cerevisae*. In liquid medium, NADP-ME1, NADP-ME2 and, to a lesser extent, NADP-ME3 supplied the anaplerotic role normally carried out by pyruvate carboxylase in non-mutant yeast cells (Figure 3). Malate synthesized by NADP-ME1-3 acting in the carboxylating direction is used to replenish the intracellular pool of C_4 acids in order to produce biomass, resulting in a measurable growth rate (Figure 3). On the other hand, NADP-ME4 did not allow the growth of mutant *S. cerevisiae* on glucose (Figures 2 and 3). This result was unexpected, considering the high value of the catalytic constant of this isoform for the carboxylating reaction *in vitro* [14], but could be explained on the basis of its inhibition by metabolites present in the cellular environment (Figures 4A and 4B). Several reports indicate that NADP-ME plastidic isoforms, such as NADP-ME4, would be involved in anabolic functions, working as

decarboxylases and providing pyruvate and NADPH for the synthesis of amino and fatty acids [25, 32, 33]. Nevertheless, due to the relatively low activity of NADP-ME4 measured in yeast extracts (Figure 1), it cannot be ruled out that the lack of complementation observed is due to a low expression of this plant protein in *Saccharomyces*, caused by different codon usage or folding machinery. Yeast transformed with NAD-ME1 and NAD-ME2 did not show any growth on solely glucose (Figures 2 and 3). This is consistent with the absence of carboxylating activity *in vitro* [18] and suggests that NAD-ME1 and NAD-ME2 only participate in malate catabolism, resulting in mitochondrial pyruvate for the tricarboxylic acid cycle.

NADP-ME2R115A carries a point mutation at the Arg115 residue that does not substantially alter the kinetic parameters of the enzyme, but abolishes its activation by fumarate [14]. Our results indicate that this change in regulatory features did not affect the ability of NADP-ME2R115A of complementing the C_4 auxotrophy of Pyc$^-$ system (Figures 2 and 3). In addition, the truncated NADP-ME2del2 was also able to support growth of the mutant strains (Figures 2 and 3). This could be attributed again to the fact that *in vitro* tests do not always reflect the conditions of a cellular context. An activation of the NADP-ME2del2 carboxylating reaction mediated by specific metabolites present in *S. cerevisiae* cells probably occurred, as happened in the assays with the purified recombinant proteins in presence of fumarate, succinate or the plant metabolite mix (Figures 4A and B). In this sense, the intracellular levels of succinate found in yeast are within the range of concentrations tested in this work [34].

So far, the strongest indications for *in vivo* ME activity in the carboxylation direction came from experiments with engineered *E. coli*, in which the over-expression of endogenous NAD(P)-ME isoform SfcA improved the succinate titers [35, 36]. However, the interpretation of these results is complicated by the fact that these strains still expressed phosphoenolpyruvate carboxylase, the main carboxylating enzyme in glucose-grown wild type *E. coli* [37]. SfcA was recombinantly obtained and characterized, and although it could catalyze the carboxylating reaction, the decarboxylating activity was over 28 times faster [38]. It is worth mentioning that no complementation of Pyc$^-$ *S. cerevisiae* strains was achieved by over-expressing this enzyme, despite reaching relatively high levels of activity (1.03 µmol NADH min^{-1} mg protein^{-1}) [11]. Only after several laboratory evolution cycles, SfcA allowed growth on

glucose of yeast mutants and the value of the maximum specific growth rate (0.015 h^{-1}) was comparable to those obtained with the plant enzymes (Figure 3). The evolution accomplished a cofactor switch from NADH to NADPH, so it was postulated that the NADPH specificity would be a key factor in fulfilling anaplerosis, probably due to the higher NADPH/NADP ratio compared to NADH/NAD present in yeast cells [11]. The results obtained in this work with Arabidopsis enzymes with different cofactor specificity support this hypothesis (Figures 2 and 3).

Previous characterization of Arabidopsis lines with modified levels of NADP-ME isoforms showed that NADP-ME2 over-expressing plants have higher malate and lower pyruvate levels than the wild type throughout day and night periods [24]. The pyridine nucleotide pool sizes also suggested that NADP-ME2 may be preferentially catalyzing the reductive carboxylation of pyruvate over the oxidative decarboxylation of malate *in vivo* in the transgenic plants [24]. In a contrasting way, *A. thaliana* plants over-expressing the photosynthetic NADP-ME from maize, which is basically a decarboxylating protein [39] presented lower levels of C_4 metabolites [40, 41]. In this work, the carboxylating NADP-ME activity was measured in leaf crude extracts from *A. thaliana* wild type plants (Figure 4C). Although this activity was at least one order of magnitude lower than the decarboxylating activity [24], it suggests that both directions of the ME reaction may occur *in vivo*. NADP-ME2 is the isoform that contributes the most to the carboxylating activity, and this activity correlates well with NADP-ME2 levels in the loss-of-function mutants or in over-expressing Arabidopsis lines (Figure 4C).

This work provides evidence that sustain the hypothesis that the pyruvate carboxylation activity of cytosolic NADP-ME1-3 would take place in plant cells, probably participating in C_4 organic acid biosynthesis and CO_2 fixation. This proposed role would not be redundant, since each isoform could act in specific tissues or metabolic situations according to their differential expression patterns (Table 1) [12]. Malate and its derivative fumarate accumulate in large levels in *A. thaliana*, accomplishing many different and essential roles for plant development and survival [42, 43]. The study of CO_2 fixing pathways is of fundamental importance for approaches aiming to improve plant productivity and sustainability of food and energy production [44]. In this regard, NADP-ME may participate in the recycling of CO_2 produced during photorespiration in C_3

plants, thereby increasing the efficiency of the photosynthetic process. Finally, the reversibility of the reaction would also allow NADP-ME to participate in the transport of reducing equivalents between compartments, jointly to malate dehydrogenase [45]. In order to draw conclusions with regards to the operation of Arabidopsis ME proteins *in planta*, an exhaustive analysis of enzyme affinities by the substrates should be accomplished. Future investigations must be addressed to establish the physiological concentrations of substrates, products and regulatory metabolites in the plant compartments, as well as the pathways that generate them and their potential changes in response to the changing environment.

These findings are also a promising basis for improvement of yeast strain for industrial production of C_4 dicarboxylic acids from renewable resources. Metabolic engineering for optimization of *A. thaliana* ME carboxylating performance or organic acid export and/or restriction of branching pathways from glucose [46, 47] would be useful for this purpose. These C_4 acids are key building blocks for chemical industry and *S. cerevisiae* is a very convenient host for their synthesis [47, 48]. Moreover, ME does not require ATP to complete carboxylation, improving the energy balance of the production process, an advantage over pyruvate carboxylase [11].

4. Material and Methods

4.1 Cloning of the ME genes into the centromeric yeast expression vector

The cDNA encoding the mature proteins (without signal peptides) for the different NAD(P)-ME isoforms from *A. thaliana* contained in pET32 vectors [12, 13] were introduced in p416GPD yeast vector (*URA3* marker; *TDH3* promoter; *CYC1* terminator) [49] via Gibson assembly or restriction and ligation procedure (New England Biolabs, Ipswich, Massachusetts, United States). Previously, the ATG codon was added to NADP-ME2del2, NADP-ME4, NAD-ME1 and NAD-ME2 through PCR and cloning into the pBluescript vector (Table 2; Stratagene, San Diego, California, United States). Each of the isoforms, with the exception of NADP-ME2del2 and NAD-ME1, were first cloned into p426GPD [49] using *Eco*RV (*Sma*I) or *Bam*HI (5´-end) and *Xho*I or *Sal*I (3´-end) restriction enzymes. Then, the plasmid backbone from MB5573 [11] was linearized using primers CYC1f and TDH3r (Table 2), excluding the open reading frame encoding for *E.*

coli sfcA. The NAD(P)-ME cassettes were obtained by PCR using primers TDH3f and CYC1r (Table 2) with the p426GPD plasmids containing the various NAD(P)-ME isoforms as templates. The PCR products were subject to gel purification (Zymoclean, Zymo Research, Irvine, California, United States) and the Gibson assembly reaction was performed following the manufacturer's recommendations. The open reading frames of NADP-ME2del2 and NAD-ME1 were isolated from pBluescript plasmids using *Bam*HI and *Xho*I or *Sal*HI. The fragments were isolated by gel purification and subsequently combined using T4 ligase (Thermo Fisher Scientific, Waltham, Massachusetts, United States). Then, the reaction mixtures were used to chemically transform *E. coli* DH5α (Life Technologies, Carlsbad, California, United States). The structure of the resulting plasmids was verified by analysis with restriction enzymes (Thermo Fisher Scientific). The NAD(P)-ME sequences were confirmed by barcode sequencing using generic (seqTDH3fw and seqCYC1rv) and specific primers for each construct (Table 2). Only one (synonymous) mutation (C1296T) was detected in the cDNA of NADP-ME2.

4.2 Transformation into Pyc⁻ Saccharomyces cerevisiae strain

Saccharomyces cerevisiae strain IMK299 (*pyc1,2*Δ*pdc2*Δ) [11, 29] was transformed according to [50] with 1-2 µg of each NAD(P)-ME construct. Selection of successful transformants was done on 2% (w/v) agar plates containing synthetic medium consisting of 3 $g \cdot l^{-1}$ KH_2PO_4, 0.5 $g \cdot l^{-1}$ $MgSO_4$, 0.5 $g \cdot l^{-1}$ L-aspartic acid, 20 $g \cdot l^{-1}$ glucose, trace elements and vitamins (pH 6.0) [51] and confirmed by colony PCR using diagnostic primers (Table 2). IMK299 has the CEN.PK genetic background [52] and presents targeted deletions of the pyruvate carboxylase genes *pyc1* and *pyc2* and a disrupted *ura3* locus. An additional deletion in *pdc2*, encoding a transcriptional activator of pyruvate decarboxylase genes *pdc1* and *pdc5*, results in reduced activity of these enzymes and allows the potential accumulation of higher levels of intracellular pyruvate [53, 54].

4.3 Growth studies in shake-flasks and on plates

Yeast frozen stock cultures were prepared by addition of 30% (w/v) glycerol to exponentially growing cultures and subsequent storage in 2 ml screw-cap tubes at -80 °C. Pre-cultures were prepared in 50 ml round, flat

bottom flasks containing 20 ml of synthetic medium, inoculated with yeast frozen stock cultures and incubated in an orbital shaker set at 200 rpm at 30°C. Cells from exponentially growing pre-cultures were collected, washed with demineralized water and transferred to a 50 ml flask containing 30 ml of new medium containing 5 $g \cdot l^{-1}$ $(NH_4)_2SO_4$ instead of L-aspartic acid and the anaerobic growth factors Tween 80 (0.42 $g \cdot l^{-1}$) and ergosterol (10 $mg \cdot l^{-1}$) [51]. The flasks were incubated on an orbital shaker set at 150 rpm under anaerobic conditions (5% H_2, 6% CO_2 and 89% N_2) in a Bactron anaerobic chamber (Sheldon MFG Inc., Cornelius, North Carolina, United States) at 30°C. Growth was monitored via optical density measurements at 660 nm using a Libra S11 spectrophotometer (Biochrom, Cambourne, Cambridge, United Kingdom). Maximum specific growth rates were determined for duplicate cultures.

For plate cultivation, solid medium was prepared by addition of 2% (w/v) agar to synthetic medium. Pre-culture flasks were prepared as above. Prior to plating, the cells were washed and diluted in demineralized water to a cell density of 200,000 $cells \cdot ml^{-1}$. A serial dilution was made by diluting the cells ten times with each progressive step and 5 µl from each dilution was plated. The plates were incubated at 30°C, under ambient condition, in a jacketed vessel continuously flushed with 100% N_2 or in a gas-tight jar with 0.3 bar overpressure and a gas mixture consisting of 10% CO_2 and 90% N_2.

4.4 Plant lines and growing conditions

Arabidopsis lines analysed in this work include two non-segregating T_3 independent NADP-ME2 over-expressing lines (7.11 and 4.20) [24], knockout lines with T-DNA inserted into each of the genes encoding NADP-ME (SALK_036898, SALK_073818, SALK_139336 and SALK_064163; Nottingham Arabidopsis Stock Center) and double and triple mutants obtained by crosses [12]. The plants were grown in soil pots in a chamber with a 16:8 h light:dark regimen at 25°C using a photosynthetic photon flux density of 100 µE m^{-2} s^{-1}.

4.5 Heterologous expression and purification of the recombinant enzymes

pET32 vectors containing the cDNA sequences of NADP-ME2, NADP-ME2del2, NADP-ME4, NAD-ME1 and NAD-ME2 [12-14] were used to express each NAD(P)-ME fused in frame to a His-tag in *E. coli*, in order to allow purification by a nickel containing His-binding column (Novagen, Madison,

Wisconsin, United States). The induction in BL21 (DE3) cells and the isolation and digestion of the enzymes were performed as previously described [12-14]. The purified proteins were concentrated using Centricon YM-50 filters (Millipore, Billerica, Massachusetts, United States) and analysed by SDS-PAGE to verify integrity and purity.

4.6 Preparation of extracts and enzyme activity assays

Yeast cells from exponentially growing aerobic shake-flask cultures in media supplemented with aspartate were collected via centrifugation, washed with demineralized water and resuspended in 10 mM potassium phosphate buffer, 2 mM EDTA (pH 7.5) and stored at -20 °C. Cell-free extracts were prepared as described earlier [11] without the dialysis step and protein concentrations were determined as in [55]. Enzymatic activities were measured with 0.4 mM NADP or 2 mM NAD as redox cofactor, following the production of NAD(P)H at 340 nm as in [30].

Samples of rosette leaves from *A. thaliana* lines were collected 32 days after sowing in the middle of the light period, frozen in liquid N_2 and stored at -80°C. Enzyme extraction and protein quantification were carried out according to [24]. ME pyruvate carboxylation activity assays in the extracts were performed at 30ºC in cuvettes (0.5 ml) containing 50 mM MOPS-KOH, 10 mM $MgCl_2$, 0.2 mM NADPH, 10 mM $NaHCO_3$ and 50 mM pyruvate (pH 7.0), following the consumption of NADPH at 340 nm.

Protein concentration and carboxylating activity of the recombinant purified enzymes was performed as indicated in the previous paragraph, but using a subsaturating concentration of pyruvate (0.5 mM). For NAD-ME isoforms, the reaction mixture contained 0.2 mM NADH instead of NADPH. The proteins were assayed in the presence of 1 or 7.5 mM fumarate and succinate, and different quantities of extracts containing total polar metabolites present in *Arabidopsis* leaves obtained according to [24].

Significance was determined by one way analysis of variance in all pairwise comparison type using the Holm-Sidak test ($p < 0.05$) with the SigmaStat software.

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Author Contributions

MBB, RM, AVL, MAT, CLA performed the experiments. VGM contributed with the plant lines and the edition of the manuscript. MCGW, AJAvM, MBB, RM, MFD and CSA planned the experiments, analysed the data and wrote the paper.

References

1. Vorapreeda T, Thammarongtham C, Cheevadhanarak S & Laoteng K (2013) Repertoire of malic enzymes in yeast and fungi: insight into their evolutionary functional and structural significance. *Microbiology* 159, 2548-2557.

2. Jiang P, Du W, Mancuso A, Wellen KE & Yang X (2013) Reciprocal regulation of p53 and malic enzymes modulates metabolism and senescence. *Nature* 493, 89-693.

3. Chang GG & Tong L (2003) Structure and function of malic enzymes, a new class of oxidative decarboxylases. *Biochemistry* 42, 12721-12733.

4. Kunkee RE (1968) Malo-lactic fermentation. *Adv Appl Microbiol* 9, 235-279.

5. Drincovich MF, Lara MV, Maurino VG & Andreo CS (2011) C_4 decarboxylases: different solutions for the same biochemical problem, the provision of CO_2 to Rubisco in the bundle sheath cells. In *C_4 photosynthesis and related CO_2 concentrating mechanisms* (Raghavendra AS & Sage RF, eds), pp. 277-300. Springer Netherlands, Heidelberg.

6. Maurino VG & Engqvist MKM (2015) 2-hydroxy acids in plant metabolism. In *The Arabidopsis book* (The American Society of Plant Biologists, eds), 13e0182.

7. Sundqvist KE, Heikkila J, Hassinen IE & Hiltunen JK (1987) Role of NAD^+-linked malic enzymes as regulators of the pool size of tricarboxylic acid-cycle intermediates in the perfused rat heart. *Biochem J* 243, 853-857.

8. Pound KM, Sorokina N, Ballal K, Berkich DA, Fasano M, LaNoue KF,

Taegtmeyer H, O'Donnell JM & Lewandowski ED (2009) Substrate-enzyme competition attenuates upregulated anaplerotic flux through malic enzyme in hypertrophied rat heart and restores triacylglyceride content: attenuating upregulated anaplerosis in hypertrophy. *Circ Res* 104, 805-812.

9. Tang KH, Feng X, Tang YJ & Blankenship RE (2009) Carbohydrate metabolism and carbon fixation in *Roseobacter denitrificans* OCh114. *PLoS One* 4, e7233.

10. Sun L, Liang C, Chen Z, Liu P, Tian J, Liu G & Liao H (2014) Superior aluminium (Al) tolerance of *Stylosanthes* is achieved mainly by malate synthesis through an Al-enhanced malic enzyme, SgME1. *New Phytol* 202, 209-219.

11. Zelle RM, Harrison JC, Pronk JT & van Maris AJA (2011) Anaplerotic role for cytosolic malic enzyme in engineered *Saccharomyces cerevisiae* strains. *Appl Environ Microbiol* 77, 732-738.

12. Gerrard Wheeler MC, Tronconi MA, Drincovich MF, Andreo CS, Flügge UI & Maurino VG (2005) A comprehensive analysis of the NADP-malic enzyme gene family of *Arabidopsis thaliana*. *Plant Physiol* 139, 39-51.

13. Tronconi MA, Fahnenstich H, Gerrard Wheeler MC, Andreo CS, Flügge UI, Drincovich MF & Maurino VG (2008) Arabidopsis NAD-malic enzyme functions as a homodimer and heterodimer and has a major impact during nocturnal metabolism. *Plant Physiol* 146, 1540-1552.

14. Gerrard Wheeler MC, Arias CL, Tronconi MA, Maurino VG, Andreo CS & Drincovich MF (2008) *Arabidopsis thaliana* NADP-malic enzyme isoforms: high degree of identity but clearly distinct properties. *Plant Mol Biol* 67, 231-242.

15. Gerrard Wheeler MC, Arias CL, Maurino VG, Andreo CS & Drincovich MF (2009) Identification of domains involved in the allosteric regulation of cytosolic *Arabidopsis thaliana* NADP-malic enzymes. *FEBS J* 276, 5665-5677.

16. Maurino VG, Gerrard Wheeler MC, Andreo CS & Drincovich MF (2009) Redundancy is sometimes seen only by the uncritical: does Arabidopsis need six malic enzyme isoforms? *Plant Sci* 176, 715-721.

17. Tronconi MA, Maurino VG, Andreo CS & Drincovich MF (2010) Three different and tissue-specific NAD-malic enzyme generated by alternative subunit association in *Arabidopsis thaliana*. *J Biol Chem* 285, 11870-11879.

18. Tronconi MA, Gerrard Wheeler MC, Maurino VG, Drincovich MF & Andreo CS (2010) NAD-malic enzymes of *Arabidopsis thaliana* display distinct kinetic mechanisms that support differences in physiological control. *Biochem J* 430,

295-303.
19. Tronconi MA, Gerrard Wheeler MC, Drincovich MF & Andreo CS (2012) Differential fumarate binding to Arabidopsis NAD^+-malic enzymes 1 and -2 produces an opposite activity modulation. *Biochimie* 94, 1421-1430.
20. Tronconi MA, Gerrard Wheeler MC, Martinatto A, Zubimendi JP, Andreo CS & Drincovich MF (2015) Allosteric substrate inhibition of Arabidopsis NAD-dependent malic enzyme 1 is released by fumarate. *Phytochemistry* 111, 37-47.
21. Arias CL, Andreo CS, Drincovich MF & Gerrard Wheeler MC (2013) Fumarate and cytosolic pH as modulators of the synthesis or consumption of C_4 organic acids through NADP-malic enzyme in *Arabidopsis thaliana*. *Plant Mol Biol* 81, 297-307.
22. Brown NJ, Palmer BG, Stanley S, Hajaji H, Janacek SH, Astley HM, Parsley K, Kajala K, Quick WP, Trenkamp S, Fernie AR, Maurino VG & Hibberd JM (2010) C_4 acid decarboxylases required for C_4 photosynthesis are active in the mid-vein of the C_3 species *Arabidopsis thaliana*, and are important in sugar and amino acid metabolism. *Plant J* 61, 122-133.
23. Voll LM, Zell MB, Engelsdorf T, Saur A, Gerrard Wheeler MC, Drincovich MF, Weber APM & Maurino VG (2012) Loss of cytosolic NADP-malic enzyme 2 in Arabidopsis is associated with enhanced susceptibility towards *Colletotrichum higginsianum*. *New Phytol* 195, 189-202.
24. Badia MB, Arias CL, Tronconi MA, Maurino VG, Andreo CS, Drincovich MF & Gerrard Wheeler MC (2015) Enhanced cytosolic NADP-ME2 activity in *A. thaliana* affects plant development, stress tolerance and specific diurnal and nocturnal cellular processes. *Plant Sci* 240, 193-203.
25. Gerrard Wheeler MC, Arias CL, Righini S, Badia MB, Andreo CS, Drincovich MF & Saigo M (2016) Differential contribution of malic enzymes during soybean and castor seeds maturation. *PLoS ONE* 11, e0158040.
26. Artus NN & Edwards GE (1985) Properties of leaf NAD-malic enzyme from the inducible crassulacean acid metabolism species *Mesembryanthemum crystallinum*. *Plant Cell Physiol* 26, 341-350.
27. Bar-Even A, Noor E, Savir Y, Liebermeister W, Davidi D, Tawfik DS & Milo R (2011) The moderately efficient enzyme: evolutionary and physicochemical trends shaping enzyme parameters. *Biochemistry* 50, 4402-4410.
28. Pronk JT, Yde Steensma H & Van Dijken JP (1966) Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* 12, 1607-1633.

29. Zelle RM, Trueheart J, Harrison JC, Pronk JT & van Maris AJ (2010) Phosphoenolpyruvate carboxykinase as the sole anaplerotic enzyme in *Saccharomyces cerevisiae*. *Appl Environ Microbiol* 76, 5383-5389.

30. Boles E, de Jong-Gubbels P & Pronk JT (1998) Identification and characterization of *MAE1*, the *Saccharomyces cerevisiae* structural gene encoding mitochondrial malic enzyme. *J Bacteriol* 180, 2875-2882.

31. Stucka R, Dequin S, Salmon JM & Gancedo C (1991) DNA sequences in chromosomes II and VII code for pyruvate carboxylase isoenzymes in *Saccharomyces cerevisiae*: analysis of pyruvate carboxylase-deficient strains. *Mol Gen Genet* 229, 307-315.

32. El-Shora HM & ap Rees T (1991) Intracellular location of $NADP^+$-linked malic enzyme in C_3 plants. *Planta* 185, 362-367.

33. Lai LB, Wang L & Nelson TM (2002) Distinct but conserved functions for two chloroplastic NADP-malic enzyme isoforms in C_3 and C_4 *Flaveria* species. *Plant Physiol* 128, 125-139.

34. Canelas AB, ten Pierick A, Ras C, Seifar RM, van Dam JC, van Gulik WM & Heijnen JJ (2009) Quantitative evaluation of intracellular metabolite extraction techniques for yeast metabolomics. *Anal Chem* 81, 7379-7389.

35. Stols L & Donnelly MI (1997) Production of succinic acid through overexpression of NAD-dependent malic enzyme in an *Escherichia coli* mutant. *Appl Environ Microbiol* 63, 2695-2701.

36. Hong SH & Lee SY (2001) Metabolic flux analysis for succinic acid production by recombinant *Escherichia coli* with amplified malic enzyme activity. *Biotechnol Bioeng* 74, 89-95.

37. Ashworth JM & Kornberg HL (1966) The anaplerotic fixation of carbon dioxide by *Escherichia coli*. *Proc Biol Sci* 165, 179-188.

38. Bologna FP, Andreo CS & Drincovich MF (2007) *Escherichia coli* malic enzymes: two isoforms with substantial differences in kinetic properties, metabolic regulation, and structure. *J Bacteriol* 189, 5937-5946.

39. Saigo M, Alvarez CE, Andreo CS & Drincovich MF (2013) Plastidial NADP-malic enzymes from grasses: Unraveling the way to the C_4 specific isoforms. *Plant Physiol Biochem* 63, 39-48.

40. Fahnenstich H, Saigo M, Niessen M, Drincovich MF, Zanor MI, Fernie A, Andreo CS, Flügge UI & Maurino VG (2007) Low levels of malate and fumarate cause accelerated senescence during extended darkness in *Arabidopsis*

thaliana overexpressing maize C_4 NADP-malic enzyme. *Plant Physiol* 145, 640-655.

41. Zell MB, Fahnenstich H, Maier A, Saigo M, Voznesenskaya EV, Edwards GE, Andreo CS, Schleifenbaum F, Zell C, Drincovich MF & Maurino VG (2010) Analysis of *A. thaliana* with highly reduced levels of malate and fumarate sheds light on the role of these organic acids as storage carbon molecules. *Plant Physiol* 152, 1251-1262.

42 Fernie AR & Martinoia E (2009) Malate. Jack of all trades or master of a few? *Phytochemistry* 70, 828-832.

43. Araújo WL, Nunes-Nesi A & Fernie AR (2011) Fumarate: multiple functions of a simple metabolite. *Phytochemistry* 72, 838-843.

44. Bar-Even A, Noor E, Lewis NE & Milo R (2010) Design and analysis of synthetic carbon fixation pathways. *Proc Natl Acad Sci* 107, 8889-8894.

45. Hebbelmann I, Selinski J, Wehmeyer C, Goss T, Voss I, Mulo P, Kangasjärvi S, Aro EM, Oelze ML, Dietz KJ, Nunes-Nesi A, Do PT, Fernie AR, Talla ST, Raghavendra AS, Linke V & Scheibe R (2012) Multiple strategies to prevent oxidative stress in Arabidopsis plants lacking the malate valve enzyme NADP-malate dehydrogenase. *J Exp Bot* 63, 1445-1459.

46. Abbott DA, Zelle RM, Pronk JT & van Maris AJ (2009) Metabolic engineering of *Saccharomyces cerevisiae* for production of carboxylic acids: current status and challenges. *FEMS Yeast Res* 9, 1123-1136.

47. Raab AM & Lang C (2011) Oxidative versus reductive succinic acid production in the yeast *Saccharomyces cerevisiae*. *Bioeng Bugs* 2, 120-123.

48. Nielsen J, Larsson C, van Maris A & Pronk J (2013) Metabolic engineering of yeast for production of fuels and chemicals. *Curr Opin Biotechnol* 24, 398-404.

49. Gueldener U, Heinisch J, Koehler GJ, Voss D & Hegemann JH (2002) A second set of loxP marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. *Nucleic Acids Res* 30, e23.

50. Gietz RD & Woods RA (2002) Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. *Methods Enzymol* 350, 87-96.

51. Verduyn C, Postma E, Scheffers WA & Van Dijken JP (1992) Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. *Yeast* 8, 501-517.

52. Nijkamp JF, van den Broek M, Datema E, de Kok S, Bosman L, Luttik MA, Daran-Lapujade P, Vongsangnak W, Nielsen J, Heijne WH, Klaassen P, Paddon CJ, Platt D, Kötter P, van Ham RC, Reinders MJ, Pronk JT, de Ridder D & Daran JM (2012) De novo sequencing, assembly and analysis of the genome of the laboratory strain *Saccharomyces cerevisiae* CEN.PK113-7D, a model for modern industrial biotechnology. *Microb Cell Fact* 11, 36.

53. Hohmann S (1993) Characterisation of PDC2, a gene necessary for high level expression of pyruvate decarboxylase structural genes in *Saccharomyces cerevisiae*. *Mol Gen Genet* 241, 657-666.

54. Flikweert MT, Kuyper M, van Maris AJ, Kötter P, van Dijken JP & Pronk JT (1999) Steady-state and transient-state analysis of growth and metabolite production in a *Saccharomyces cerevisiae* strain with reduced pyruvate-decarboxylase activity. *Biotechnol Bioeng* 66, 42-50.

55. Lowry OH & Rosebrough NJ (1951) Protein measurement with the Folin phenol reagent. *J Biol Chem* 193, 265-275.

Tables

Table 1 Overview of *A. thaliana* ME isoforms Nomenclature, cellular and tissue localization, and preferential cofactor of the different Arabidopsis ME proteins used in this work are shown [12, 13].

Name	TAIR code	Subcellular localization	Principal cofactor	Expression
NADP-ME1	AT2G19900	Cytosol	NADP	Embryonic tissues and roots of adult plants
NADP-ME2	AT5G11670	Cytosol	NADP	Ubiquitously expressed
NADP-ME3	AT5G25880	Cytosol	NADP	Trichomes and pollen
NADP-ME4	AT1G79750	Plastids	NADP	Ubiquitously expressed
NAD-ME1	AT2G13560	Mitochondria	NAD	Ubiquitously expressed
NAD-ME2	AT4G00570	Mitochondria	NAD	Ubiquitously expressed

Table 2 Oligonucleotide primers used in this work

Name	Sequence
Addition of ATG codon	
NADP-ME2del2ATG	GGATCCATGGTCGCTAGTGGCTACCACATC
NADP-ME2del2r	CTCGAGTTAACGGTAGTTTCTGTACAC

NADP-ME4ATG	GATATCATGAAATCCACCGTATCTGGTGG
NADP-ME4r	CTCGAGCTTATCCTCAGCGGTAG
NAD-ME1ATG	GGATCCATGCCCACCATCGTTCATAAA
NAD-ME1r	GTCGACTTAGTCATCCTTGTAGAC
NAD-ME2ATG	GGATCCATGTGCATCGTCCACAAGCGT
NAD-ME2r	GTCGACCTATTTCTCGTGAAC
Amplification of MB5573 backbone	
CYC1f	CGAGTCATGTAATTAGTTATGTCACG
TDH3r	ATCCGTCGAAACTAAGTTCTGG
Amplification of NAD(P)-ME cassettes	
TDH3f	ACCAGTTCCCTGAAATTATTCC
CYC1r	TTTCGGTTAGAGCGGATGTGG
Barcode sequencing	
seqTDH3fw	GACCCACGCATGTATCTATCTC
seqCYC1rv	GCCGCAAATTAAAGCCTTCG
seqNADP-ME1fw	CGTCCTTCAGCGTGTTTACC
seqNADP-ME1rv	TTCTTGCGGCTTTCCTCTAC
seqNADP-ME2fw	CGTCCATCAGCTTGTCTTCC
seqNADP-ME3fw	TCGGCTTGCCTTCCAATAAC
seqNADP-ME2-3rv	TGGAGTCCACAAGCCAAATC
seqNADP-ME4fw	CCCGTAACAATCGATGTTGG
seqNADP-ME4rv	ATTGCCTTGACGGCATCAAC
seqNAD-ME1fw	TCCAGAGCAAGTGGGCTTTC
seqNAD-ME1rv	CCGGCCTTCGGTGATAAGAC
seqNAD-ME2fw	AAAGGCCGTCGTACAGTTCG
seqNAD-ME2rv	CTTGCTCCCTCGCGTATCTC
Diagnostic colony PCR	
dgfw	TGCTCTGATGCCGCATAG
dgrv	GGGATCGCCAACAAATACTACC

Figure legends

Fig. 1 NAD(P)-ME malate decarboxylating activity in Pyc$^-$ strain IMK299 transformed with different Arabidopsis NAD(P)-ME isoforms (A) NADP-ME activity. (B) NAD-ME activity. Determinations were performed in duplicate or triplicate. The average and mean deviation (duplicate determinations) or standard deviation (triplicate determinations) are shown. The asterisk (*) indicates significantly increased activity ($p < 0.05$) comparing to the control strain transformed with the empty vector.

Fig. 2 Growth of *S. cerevisiae* Pyc$^-$ strain IMK299 transformed with each of the *A. thaliana* NAD(P)-ME isoforms in aerobic or anaerobic conditions with increased CO_2 pressure (A) Synthetic media with glucose under anaerobic conditions with 10% CO_2. (B) Synthetic media with glucose and aspartate under ambient atmosphere. Plates were incubated for 12 (A) or 3 (B) days. The numbers indicate the targeted number of cells plated.

Fig. 3 Growth on synthetic liquid media with glucose of Pyc⁻ strain IMK299 transformed with each of the Arabidopsis NAD(P)-ME isoforms (A) Growth curves of one representative culture for each strain. (B) Maximum biomass specific growth rates. Experiments were performed in duplicate, under anaerobic conditions with 6% CO_2. The average and mean deviation are shown. n. g.: no growth was detected. Values of maximum specific growth rate which share at least one letter are not significantly different ($p < 0.05$).

Fig. 4 NADP-ME carboxylating activity of recombinant purified NADP-ME2, NADP-ME2del2 and NADP-ME4 enzymes (A and B) or leaf protein extract of Arabidopsis lines with modified levels of NADP-ME (C) The activity of recombinant enzymes was evaluated in the presence of 1 or 7.5 mM fumarate or succinate (A) or in the presence of 5 or 10 µl of polar metabolite extracts of *A. thaliana* (B). These amounts are equivalent to the addition of the metabolites contained in 0.5 or 1 mg of leaf, respectively. They also correspond to a final concentration of 10 or 20 µM fumarate, respectively, in the enzymatic reaction medium [24], together with the whole pool of polar metabolites extracted. In (B) the ratio between enzymatic activity in the presence and absence of plant extract metabolites is indicated. Each mutant line is named after the mutated gene (*nadp-me1-4* or their combinations in double or triple mutants). 7.11 and 4.20 are two NADP-ME2 independent over-expressing lines. Determinations were performed in duplicate in two independent batches of enzyme or plant protein extracts. The average and standard deviation are shown. The asterisk (*) indicates significant difference ($p < 0.05$) compared to wild type (wt).