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Expression of novel cytosolic malate dehydrogenases (cMDH) in *Lupinus angustifolius* nodules during phosphorus starvation

Marcellous Le Roux[a], Ethel Phiri[d], Wesaal Khan[b], Muhammet Şakiroğlu[c], Alex Valentine[d,*], Sehaam Khan[e]

[a] Department of Agronomy, Faculty of Agriculture, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa
[b] Department of Microbiology, Faculty of Science, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa
[c] Department of Bioengineering Kafkas University, 36100 Kars, Turkey
[d] Department of Botany and Zoology, Faculty of Science, University of Stellenbosch, Private Bag X1, Matieland 7602, South Africa
[e] Department of Biomedical Sciences, Faculty of Health and Wellness Science, Cape Peninsula University of Technology, P.O. Box 1906, Bellville 7535, South Africa

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ABSTRACT

During P deficiency, the increased activity of malate dehydrogenase (MDH, EC 1.1.1.37) can lead to malate accumulation. Cytosolic- and nodule-enhanced MDH (cMDH and neMDH, respectively) are known isoforms, which contribute to MDH activity in root nodules. The aim of this study was to investigate the role of the cMDH isoforms in nodule malate supply under P deficiency. Nodulated lupins (*Lupinus angustifolius* var. Tanjil) were hydroponically grown at adequate P (+P) or low P (−P). Total P concentration in nodules decreased under P deficiency, which coincided with an increase in total MDH activity. A consequence of higher MDH activity was the enhanced accumulation of malate derived from dark CO_2 fixation via PEPC and not from pyruvate. Although no measurable neMDH presence could be detected via PCR, gene-specific primers detected two 1 kb amplicons of cMDH, designated *Lang*MDH1 (corresponding to +P, HQ690186) and *Lang*MDH2 (corresponding to −P, HQ690187), respectively. Sequencing analyses of these cMDH amplicons showed them to be 96% identical on an amino acid level. There was a high degree of diversification between proteins detected in this study and other known MDH proteins, particularly those from other leguminous plants. Enhanced malate synthesis in P-deficient nodules was achieved via increased anaplerotic CO_2 fixation and subsequent higher MDH activities. Novel isoforms of cytosolic MDH may be involved, as shown by gene expression of specific genes under P deficiency.

Introduction

There is widespread consensus that malate is the preferred carbon (C) source for bacteroids in almost all legume–*Rhizobium* symbioses studied to date (Rosendahl et al., 1990; Driscoll and Finan, 1993; Schulze et al., 2002). The apparent high flux of malate in root nodules is the result of active synthesis via malate dehydrogenase (MDH). This enzyme catalyzes the reversible reduction of oxaloacetate (OAA) to malate. In root nodules, though, the kinetics of the reaction suggests that this reaction is favored in the forward direction toward malate synthesis (Miller et al., 1998). This may further depend on the physiological state of the cell and the subcellular location of the enzyme within the cell (Schulze et al., 2002). Nodule MDH has to maintain a fine balance between malate as respiratory source and optimal functioning of bacteroids and malate for conversion to OAA and subsequent use in N assimilation.

Numerous earlier immunological and biochemical studies have detected novel forms of the MDH enzyme (Appels and Haaker, 1988; Ratajczak et al., 1989), but it was only much later that several of these forms were isolated and characterized (Miller et al., 1998). This is highly indicative of the importance of the enzyme in several metabolic pathways (Schulze et al., 2002). Of special interest in root nodules is the isolation of a unique nodular enhanced MDH (neMDH) isoform, which was shown to account for up to 50% of the total MDH activity in root nodules of alfalfa (Miller et al., 1998). Conversely, approximately 20% of the MDH activity is found in the cytosolic fraction of root nodules (Miller et al., 1998). The lower

* Corresponding author. Tel.: +27 21 808 3067; fax: +27 21 808 2405.
E-mail addresses: mrl@sun.ac.za (M. Le Roux), ephiri@sun.ac.za (E. Phiri), wesaal@sun.ac.za (W. Khan), msakiroglu@gmail.com (M. Şakiroğlu), alexvalentine@mac.com, ajv@sun.ac.za (A. Valentine), khans@cput.ac.za (S. Khan).

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cMDH activity in root nodules may be as a result of the microaerobic conditions, which impede complete oxidative phosphorylation of more complex C substrates as one progress to the inner core of the nodule (Kaur and Singh, 1999; Cabrerizo et al., 2001).

Recently, it was proposed that C provision to bacteroids in root nodules is acquired through eloquent manipulation of the surrounding host cell cytoplasmic P_i concentration (Colebatch et al., 2004). This model (Colebatch et al., 2004) notes the tendency of bacteroids in root nodules to actively scavenge P_i, probably through the excretion of acid phosphatases (APs), from the surrounding plant cell fraction (Al-Niemi et al., 1997, 1998). High levels of APs have been shown even under conditions of apparent P sufficiency, which suggests that bacteroids, in addition to functioning optimally at low O_2 levels, also operate within narrow limits of P_i concentration (Sa and Israel, 1991; Al-Niemi et al., 1997, 1998). Nevertheless, because of this, bacteroids induce the alternative pathway via the concerted action of the non-phosphorylating enzyme reactions phosphoenolpyruvate carboxylase (PEPc) and MDH to metabolize PEP in the plant cell fraction (Colebatch et al., 2004). Implicit to this model would be the extent to which nodules acquire such high malate levels. Furthermore, the sink strength of nodules for P can also be inferred from this, which would explain why nodules are almost always unaffected by even very acute P deficiencies of host root Le Roux et al., 2006, 2009). Nodules consistently maintain higher P levels than either root or shoots under P deficiency (Israel, 1993; Sa and Israel, 1991; Tang et al., 2001). MDH activity has been assayed under P deficient conditions in the nodules of lupins and soybean and its role in nodule malate supply is pivotal under various conditions, including P deficiency (Le Roux et al., 2006, 2009; Schulze et al., 2006). However, in spite of this vital role of MDH in P-starved nodules, it is not known how many nodular isoforms are involved in the P-deficient response and how they affect C supply to nodules during N_2 fixation and assimilation.

The objective of this study was therefore to assess how many cMDH isoforms could be detected in *Lupinus angustifolius*, and to determine if these isoforms have different expression patterns under N_2 fixing nodules in P stressed conditions.

Materials and methods

Plant material, growth conditions and harvesting

Seeds of *Lupinus angustifolius* (L. cv. Tanjil) were germinated in vermiculite. When planted, seeds were coated with a genus-specific rhizobial inoculum containing *Bradyrhizobium* sp. (*Lupinus*) bacteria (Agricol, Western Cape, South Africa). Seedlings were transferred 7–10 days after planting to an aerated nutrient solution (4 mM $CaCl_2$, 1.5 mM $MgSO_4$, 2 mM K_2SO_4, 2 mM NaH_2PO_4/Na_2HPO_4, 139 μM H_3BO_3, 21 μM $MnSO_4$, 2 μM $ZnSO_4$, 3 μM $CuSO_4$, 0.2 μM Na_2MoO_4, 89 μM FeEDTA, and no N). The nutrient solution was changed once a week and the pH adjusted to 5.8 daily by addition of either 1 M HCl or 1 M NaOH. Plants were initially grown in a non-limiting P supply (2 mM P, designated +P) for approximately three weeks, after which half of the plants were switched to a limiting P supply (2 μM P, designated −P). Phosphorus starvation was induced for 14 days after which nodule tissues from the +P and −P plants were harvested. Nodules were excised from adjacent roots, frozen in liquid nitrogen, and stored at −80 °C until further analysis was completed. Roots were oven-dried to a constant weight at 72 °C for 48 h. The dry weights were recorded and the dried root material was sent to a commercial laboratory (Bemlab, De Beers Road, Somerset West, South Africa) where P concentrations were quantified using inductively coupled mass spectrometry (ICP-MS) with suitable standards. Total P concentration was expressed as per root dry weight.

Calculations of $\delta^{15}N$

The δ^{15}nitrogen (N) and carbon to nitrogen ratio (C:N) analyses were carried out at the Archeometry Department, University of Cape Town. The isotopic ratio of $\delta^{15}N$ was calculated as $\delta = 1000‰ [R_{sample}/R_{standard}]$, where R is the molar ratio of the heavier to the lighter isotope of the sample and standards as defined by Farquhar et al. (1989). The oven-dried plant components were milled in a Wiley mill using a 0.5 mm mesh (Arthur H Thomas, CA, USA). Between 2.1 mg and 2.2 mg of each sample was weighed into 8 mm by 5 mm tin capsules (Elemental Microanalysis Ltd., Devon, UK) on a Sartorius microbalance (Goettingen, Germany). The samples were then combusted in a Fisons NA 1500 (Series 2) CHN analyzer (Fisons Instruments SpA, Milan, Italy). The $\delta^{15}N$ values for the nitrogen gases released were determined on a Finnigan Matt 252 mass spectrometer (Finnigan MAT GmbH, Bremen, Germany), which was connected to a CHN analyzer by a Finnigan MAT Conflo control unit. Three standards were used to correct the samples for machine drift: two in-house standards (Merck Gel and Nasturtium) and one IAEA (International Atomic Energy Agency) standard $(NH_4)_2SO_4$. Percentage NDFA was calculated according to Shearer and Kohl (1986):

$$\%\text{NDFA} = 100 \times \frac{\delta^{15}\text{N}_{\text{reference plant}} - \delta^{15}\text{N}_{\text{legume}}}{\delta^{15}\text{N}_{\text{reference plant}} - \text{B value}}$$

where the reference plant was wheat (*Triticum aestivum*) grown under the same glasshouse conditions, the B-value is the $\delta^{15}N$ natural abundance of the N derived from biological N-fixation of the above-ground tissue of *Lupinus luteus*, grown in an N-free solution.

Malate concentration and MDH activity

Malate concentrations and NADH–MDH activities, which equate to cytosolic MDH, were estimated in accordance with methods from Le Roux et al. (2006). Roots of plants with nodules still attached were used in feeding experiments. These attached root nodule pieces were submerged in the same nutrient solutions (15 ml) in which the plants were grown, but modified to contain 1% sucrose, to compensate for the loss of photosynthate supply via the shoot. The system was aerated with ambient air (360 ppm CO_2) for the duration of the experiment. Root nodule pieces were equilibrated for 5 min, prior to feeding, with 10 μl of ^{14}C label in the form of $NaHCO_3$. The experiment was stopped after 5 min by discarding the incubation solution. The various segments were bagged, quenched in liquid N_2 and stored at −80 °C. Components were homogenized with 80% (v/v) ethanol and separated into soluble and insoluble components. The soluble component was subsequently separated into chloroform-soluble and water-soluble components, of which the latter was fractionated into amino acid, organic acid, and carbohydrate fractions, as described by Atkins and Canvin (1971).

High performance liquid chromatography (HPLC) separations were made isocratically on a 30 × 0.78 cm Bio-Rad Aminex Ion Exclusion HPX-87H organic acid column. HPLC analysis was carried out on an Alliance 2690 Separations Module equipped with a 996 Photodiode array detector (Waters). The mobile phase consisted of 30 mM H_2SO_4 at a flow rate of 0.6 ml min^{-1}. Eluting peaks were detected by ultraviolet absorption at 247 nm and at a column temperature of 50 °C. The system was calibrated with known standards (0–100 mM of malate) for quantification and estimation of retention time, and was co-chromatographed with the sample for identification. Data analysis was conducted using Millenium32 Chromatography software (Waters). In addition, the organic acid fractions of interest were manually collected to assess radioactivity in the specific organic acid, i.e. malate. Radioactivity was measured on a liquid scintillation counter.