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Glutamate dehydrogenase is essential in the acclimation of *Virgilia divaricata*, a legume indigenous to the nutrient-poor Mediterranean-type ecosystems of the Cape Fynbos

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ABSTRACT

Glutamate dehydrogenase (NAD(H)- GDH, EC 1.4.1.2) is an important enzyme in nitrogen (N) metabolism. It serves as a link between C and N metabolism, in its role of assimilating ammonia into glutamine or deaminating glutamate into 2-oxoglutarate and ammonia. GDH may also have a key in the N assimilation of legumes growing in P-poor soils. *Virgilia divaricata* is such a legume, growing in the nutrient limited soils of the mediterranean-type Cape fynbos ecosystem. In order to understand the role of GDH in the nitrogen nutrition of *V. divaricata*, the aim of this study was to identify the GDH gene transcripts, their relative expressions and enzyme activity in P-stressed roots and nodules during N metabolism. During P deficiency there was a reduction in total plant biomass as well as total plant P concentration. The analysis of the GDH cDNA sequences in *V. divaricata* revealed the presence of *GHD1* and *GHD2* subunits, these corresponding to the *GDH1*, *GDH-B* and *GDH3* genes of legumes and non-legume plants. The relative expression of *GDH1* and *GDH2* genes in the roots and nodules, indicates that two the subunits were differently regulated depending on the organ type, rather than P supply. Although both transcripts appeared to be ubiquitously expressed in the roots and nodules, the *GDH2* transcript evidently predominated over those of *GDH1*. Furthermore, the higher expression of both GDH transcripts in the roots than nodules, suggests that roots are more reliant on on GDH in P-poor soils, than nodules. With regards to GHD activity, both aminating and deaminating GDH activities were differently affected by P deficiency in roots and nodules. This may function to assimilate N and regulate internal C and N in the roots and nodules. The variation in *GDH1* and *GDH2* transcript expression and GDH enzyme activities, indicate that the enzyme may be regulated by post-translational modification, instead of by gene expression during P deficiency in *V. divaricata*.

1. Introduction

Carbon (C) and nitrogen (N) metabolism is fundamental to plant development and growth (Miyashita and Good, 2008). The enzyme, glutamate dehydrogenase (NAD(H)- GDH, EC 1.4.1.2) seems to play an essential role during the complex co-ordination of C and N metabolism in plants (Miyashita and Good, 2008). GDH serves as a link between C and N metabolism as it is capable of assimilating ammonia into glutamine or deaminating glutamate into 2-oxoglutarate and ammonia (Melo-Oliveira et al., 1996; Miyashita and Good, 2008). A primary function of GDH for assimilating ammonium has been less supported, owing to a a more efficient route (GS-GOGAT pathway) that been described by Lea and Miflin (1974). However, several authors still propose that this enzyme may play an alternative role to the GS-GOGAT pathway in the ammonium assimilation under specific physiological conditions that make ammonium concentration increase (Melo-Oliveira et al., 1996; Masclaux-Daubresse et al., 2002). Even more, there is evidence in favor of the catabolic function of GDH in glutamate metabolism, to help fuel the tricarboxylic acid (TCA) cycle under carbon insufficiency and help the cellular carbon: nitrogen ratios during stress conditions (Melo-Oliveira et al., 1996; Ficarelli et al., 1999; Dubois et al., 2003; Purnell et al., 2005; Miyashita and Good, 2008). Therefore

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GDH may play an essential role in the assimilation of N and the integration of C and N metabolism in *Virgilia divaricata* growing in the nutrient limited soils of the Mediterranean-type Cape fynbos ecosystem.

V. divaricata (Adamson) like other temperate legumes is an amide exporting indigenous tree legume (Magadlela et al., 2017), growing in nutrient rich soils of forest-margins, but is reported to invade the mature fynbos (Coetsee and Wigley, 2013) The mature fynbos is characterised by soils that are acidic, highly leached and nutrient-poor, specifically in regards to phosphorus (P) (Coetsee and Wigley, 2013; Maseko and Dakora, 2013). Even though the fynbos vegetation has evolved with regular fires, the, post-fire alterations in the dynamics of soil nutrient availability, have been proposed as major factors in limiting the growth of legumes in mature fynbos (Manders et al., 1992; Coetsee and Wigley, 2013). However the legume plant, *V. divaricata*, invades the mature fynbos even in the absence of fire and is described as a forest precursor. Furthermore, it enhances the fertility of fynbos soils (Coetsee and Wigley, 2013).

Fynbos soils can contain organic P levels of between 58–77% (Straker, 1996), with most of the organic P not available for plant assimilation and use due to complexion with cations (calcium and iron) (Dakora and Phillips, 2002). In acidic soil conditions, the P ions can readily precipitate with cations (Dakora and Phillips, 2002), but may bind to organic compounds via microbial action (Vance et al., 2003; Uhde-Stone et al., 2003). Therefore P concentration in fynbos soils, is generally available in micromolar or lower concentrations, and these P concentrations are extremely low to drive the P requiring metabolic processes, including N assimilation and metabolism (Maseko and Dakora, 2013). As such, low P availability is a critical limitation for plants, especially fynbos legume plants, as P is essential during N_2 fixation, N assimilation and metabolism during plant growth (Maseko and Dakora, 2013; Sulieman et al., 2013). P deficiency affects N nutrition consequently the C costs of plants during growth (Magadlela et al., 2014). Therefore GDH may play a role during N metabolism and C provision, as it is capable of assimilating ammonia into glutamine or deaminating glutamate into 2-oxoglutarate and ammonia (Melo-Oliveira et al., 1996).

The GDH protein is a hexamer comprised of two subunit polypeptides that differ slightly in mass and charge. The largest subunit, (43.0 kDa) designated α, is encoded by the gene *GDH2* while the second subunit, (42.5 kDa) designated β, is encoded by the gene *GDH1* (Loulakakis and Roubelakis-Angelakis, 1991, 1996; Loulakakis et al., 1994; Purnell et al., 2005; Lehmann et al., 2011). GDH gene families coding for both α and β subunits of the protein have been characterised in *Arabidopsis thaliana* and *Nicotiana plumbaginifolia* (Turano et al., 1997; Ficarelli et al., 1999). Regulation of GDH gene expression may occur at various levels, and it has been established that a substantial constituent in this regulation is not only the metabolic environment, but also the varied expression of the subunits of GDH (Tercé-Larforgue et al., 2004a; Fontaine et al., 2006; Labboun et al., 2009; Lehmann et al., 2011). Considering this pivotal role of varied GDH subunit expression of *GHD1* and *GDH2* (Lehmann et al., 2011), it is an intriguing possibility that this may also play a role in GDH regulation in the nodulated root systems of a legume from a nutrient-poor ecosystem.

Given the importance of GDH in linking C and N metabolism during P stress, the aim of this study was to identify for the first time the *GHD1* and *GDH2* transcripts and their regulatory role in the activity of the enzyme GDH in a legume from a P-poor ecosystem. This was achieved via the transcript identification, quantitative expression levels and enzyme activities in P-stressed *V. divaricata* roots and nodules.

2. Materials and methods

2.1. Plant material and growth conditions

V. divaricata seeds were acquired from Silverhill Seeds (Kenilworth, South Africa). Seed acid scarification method was used, this method involved soaking the seeds for 30 min in 95–99% Sulphuric acid (H_2SO_4), and followed by rinsing 10 times in distilled H_2O (Magadlela et al., 2014). Following scarification, the seeds were soaked overnight, at room temperature in diluted smoke water, which was obtained from Kirstenbosch National Botanical Gardens (Claremont, Cape Town, South Africa) (Magadlela et al., 2014). Seed germination and growth was in sterile sand under ambient conditions in the north facing glasshouse at the Botany and Zoology Department, University of Stellenbosch. The glasshouse midday irradiances was between 600 to 800 μmol m^{-2} s^{-1} and the night and day temperatures averaged between 15 and 25 °C. Seedlings were inoculated with effective *Burkholderia* sp. N362. The seeddlings were supplied with a 25% strength Long Ashton nutrient solution, which was modified to contain 500 μM $NaH_2PO_4{\cdot}2H_2O$ as High P and 5 μM $NaH_2PO_4{\cdot}2H_2O$ as Low P, 500 μM NH_4NO_3 as the N source and the pH was adjusted to 5.8 (Hewitt, 1966). The use of NH_4NO_3 as the N source, is to simulate the natural conditions of native soils, where both NH_4^+ and NO_3- occur, and it is known that *V. divaricata* roots can assimilate both forms of N (Magadlela et al., 2016).

At 80 days after seedlings were inoculated with *Burkholderia* sp. N362 the plants were harvested and kept at chilled condition, so that plants could be separated into nodules, roots, stems and leaves. The roots and nodules were flash frozen with liquid N thereafter store at −80 °C for biochemical analysis.

2.2. Plant harvest

After 80 days of growth, following inoculation, the plant material was harvested. The leaves, stems, roots and nodules were oven dried in brown paper bags in a 50 °C drying oven for 5–7 days, or until a constant dry weight was reached. The dried material was ground with a tissue-lyser (Central Analytical Facilities, Stellenbosch University, South Africa).

2.3. Tissue phosphorus assay

Total phosphorus (P) analysis was performed in the oven dried ground plant material, where approximately 0.25 g of the ground plant material was digested in 7 ml HNO_3 using a Mars CEM microwave digester (CEM Coporation, USA). The digested material was then diluted in 50 ml of deionised H_2O. Total plant P was determined using a Thermo ICAP 6300 ICP-AES (Thermo Scientific, South Africa), following calibration of the instrument with NIST-traceable standards at Central Analytical Facilities, Stellenbosch University, South Africa.

2.4. Enzymatic assay

Enzymes were assayed according to El-Shora and Ali (2011), where a crude enzyme extraction from the −80 °C stored fresh plant samples (roots and nodules), were ground in liquid N using a pre-chilled mortar and pestle. The extraction buffer included 50 mM KH_2PO_4 buffer, pH 7.5, consisting of 2 mM EDTA, soluble casein at 1.5% (w/v), 2 mM dithiothreitol (DTT) and polyvinylpyrrolidine (PVP insoluble) at 1% (w/v). In addition, a tablet of Complete Protease Inhibitor Cocktail (Roche) was added to the buffer. The resulting homogenate was centrifuged for 5 min at 3,000 g

(4 °C), and thereafter the supernatant was further centrifuged for 20 min at 30,000 g (4 °C). To measure enzyme activities of aminating and deaminating glutamate dehydrogenase (GDH) the resulting supernatant was used. The protein concentration was determined using the Bradford (1976) method, using protein assay reagent (Bio-Rad) and bovine serum albumin (BSA) as a standard. Aminating-GDH activities were assayed according to Glevarec et al. (2004), following the oxidation of NADH at 340 nm. Glevarec et al. (2004) reaction mixture contained 100 mM Tris–HCl (pH 8), 1 mM $CaCl_2$, 13 mM 2-oxoglutarate, 50 mM $(NH_4)_2SO_4$ and 0.25 mM NADH. The deaminating-

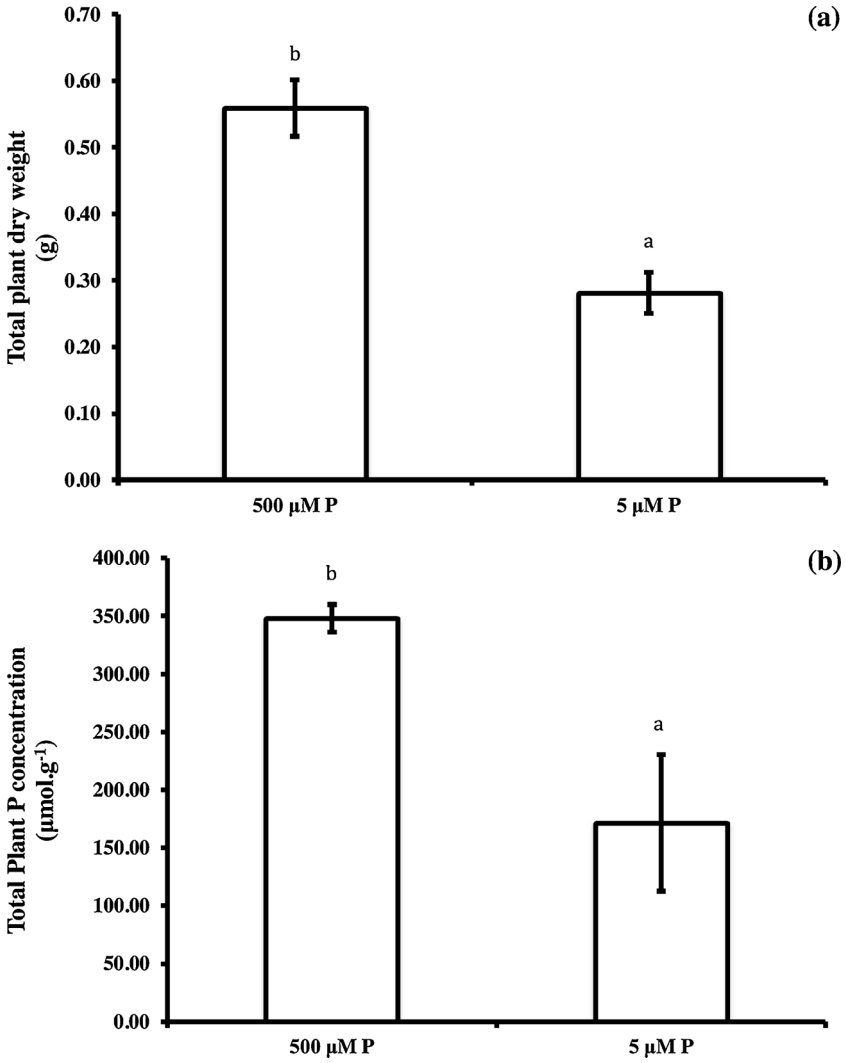

Fig. 1. (a) Biomass and (b) total plant P concentration of *Virgilia divaricata* grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4{\cdot}2H_2O$. Both the high and Low P plants were supplied with 500 µM NH_4NO_3 as soil nitrogen (N) source. The different letters indicate significant differences among the treatments (*P < 0.05).

GDH enzyme activity was assayed based on the reduction of NAD to NADH. This reaction mixture consisted of 100 mM Tris–HCl buffer (pH 9) which was supplemented with 1 mM $CaCl_2$ (pH 9), 33 mM Glu and 0.25 mM NAD or NADP.

2.5. PCR, cloning and sequencing

A pair of primers was used for the reverse transcriptase-polymerase chain reaction (RT-PCR) for gene subunits. The primers used for *GHD1* were as follows: RTfgdh1 5′-GAGTATATCAGGACAACGGTTTGTCA-3′ and RTrgdh1 5′-GGATCACCACCATGGAATCCTT-3′ (Lehman et al., 2011) and for *GDH2* were as follows: forward *GDH2*, 5-GGATTCATGT GGGAAGAGGA-3′ and reverse *GDH2* 5′-GCGACTCGGTTAACTCC AAG-3′ (Miyashita and Good, 2008). Total RNA (Supplementary Fig. 1a) was isolated using the RNeasy Mini Kit obtained from QIAGEN, Qiagen Strasse 1, 40724 Hilden, Germany. Genomic DNA was removed using the DNase 1, RNase-free (Thermo Scientific, Johannesburg, South Africa) according to the manufacturers protocol. Subsequently, first-strand cDNA was synthesized using AMV reverse transcriptase (RevertAid TMH Minus First Strand cDNA Synthesis kit, Fermentas, Vilnius, Lithuania) and oligo(dT)18 anchor. PCR was carried out on BioRad Mini Opticon thermal cycler (BioRad, South Africa) in a volume of 50 µL, containing 5 µL of the first-strand cDNA, 1x GoTaq PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 mM of each primer and 1.25U of 5U/µL GoTaq G2 Flexi DNA polymerase (Promega, USA). The PCR program was as follows: 94 °C for 1 min, 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s, for 35 cycles and a final extension for 5 min at 72 °C and viewed in a 1,2% agarose gel electrophoresis (Supplementary Fig. 1b).

Amplification products were cloned (Supplementary Fig. 1c) using the pGEM-T easy plasmid vector (Promega) according to the manufacturer's protocol, thereafter sequenced at the Central Analytical Facilities (CAF), at the University of Stellenbosch, Stellenbosch, South Africa. The raw sequence files were viewed, edited where necessary, and aligned with the aid of the BioEdit and Geneious (ver 8.0.2, Biomatters) software programmes. The aligned GDH sequences were all subjected to a BLAST analysis on the GenBank database and matching hits were selected.

With regards to the maximum-likelihood *GDH1* and *GDH2* cluster analysis, a range of Fabaceae sequences and out-group sequences and *GDH 1* (177 base pairs) and *GDH2* (141 basepairs) sequences were aligned using BioEdit software programme and Geneious version 8.0.2 created by Biomatters (http://www.geneious.com/). The aligned sequences for both *GDH 1* and *GDH 2* subunits were subjected through MrBayes 3.2 (Ronquist et al., 2012), for a 1,000000 generations using the NST mixed command to model over model space and a gamma correction for amongst site variation. A burn-in of 25% was used and all

parameters converged at an estimated sample sizes greater than 500.

2.6. q-RT PCR analyses

Quantitative real-time PCR was performed using cDNA normalized to 20 ng/reaction (1 μL), using KAPA SYBR FAST Universal One-Step qRT-PCR kit (KAPABIOSYSTEMS, Cape Town, South Africa) according to manufactures protocol. The primers used for *GHD1* were as follows: RTfgdh1 5′-GAGTATATCAGGACAACGGTTTGTCA-3′ and RTrgdh1 5′-GGATCACCACCATGGAATCCTT-3′ (Lehman et al., 2011) and for *GDH2* were as follows: forward *GDH2*, 5-GGATTCATGTGGGAAGA GGA-3′ and reverse *GDH2* 5′-GCGACTCGGTTAACTCCAAG-3′ (Miyashita and Good, 2008). The analysis was carried out in an Applied Biosystems Step-One Plus RT-PCR (Thermo Fisher Scientific) using the following program: 30 s at 95 °C, followed by 40 cycles 3 s at 95 °C and 20 s at 50 °C. In each assay for one of the two targets, triplicate standard curve were prepared for the appropriate primer set using the diluted plasmid samples. Furthermore, standards, cDNA samples and negative control were analyzed in three repeats in each assay. To quantify the transcript of each gene, the copy numbers of each target were determined by the standard curve constructed using the diluted plasmid samples. The determined copy numbers of *GDH1* and *GDH2* were then normalized the copy numbers of *Actin2* transcripts.

3. Results

3.1. Biomass and P nutrition

Plant biomass increased with increasing supply of P, this is because plants grown under high P conditions accumulated more biomass than did plants under low P conditions (Fig. 1a). Similarly, total plant P concentration decreased in P deficient plants compared to plants supplied with high P (Fig. 1b).

3.2. GDH activity assay

During N assimilation, aminating GDH show an increased activity in the legume roots during low P supply compared to roots supplied sufficient P (Fig. 2a). The contrary was observed in the nodules, where low P nodules showed reduced activity compared to high P nodules. Deaminating GDH showed the same trend of activity in the roots and nodules as the aminating GDH. The enzyme activity of deaminating GDH shows an increase in the roots under P deficiency, while the opposite was found in the nodules (Fig. 2b).

3.3. Virgilia divaricata GDH gene sequencing

In the current study only two previously known genes, *GDH1* and *GDH2*, were investigated. The cDNA encoding GDH in *V. divaricata* was identified. The aligned subjected to GenBank BLAST, *GDH1* nucleotide sequence in *V. divaricata* showed approximately 92%, 90%, 90%, 88%, 88%, identities with *Lupinus luteus GDH1* (AY681352.1), predicted *Glycine max* GDH1-like (LOC100789509) (XM_006603650.1), *Glycine max GDH1* (NM_001249475.1) and *Medicago truncatula* NADP-specific *GDH* (XM_003618924.2), *Lupinus luteus* clone Ylgdh-L.07 GDH (AY871065.1) respectively. The *GDH2* nucleotide sequence in *V. divaricata* showed approximately 97%, 94%, 92%, 89%, 87% identity to predicted *Glycine max GDH1*-like (LOC100789509) (XM_006603650.1), predicted *Elaeis guineensis GDH* (XR_830269.1), predicted *Vitis vinifera GDH-B* (XM_010655427.1), predicted < *Beta* > *vulgaris* subsp. *vulgaris GDH-B* (XM_010673749.1) and *Cicer arietinum* probable *GDH3* (XM_004489651.2) respectively.

With regards to the maximum-likelihood cluster, isolates of *V. divaricata GDH1* (Fig. 3a) formed a monophyletic cluster with a legume, *Lupinus luteus*, which forms a clade with other legumes such as *Cicer arietinum* and *Glycine max*. Where *GDH2* (Fig. 3b) isolates formed a monophyletic cluster with a legume *Lotus japonicum*, which forms a clade with *Cicer arietinum, Glycine max* and a non-legume species, *Prunus persica*.

3.4. Target GDH transcript organ-dependent expression

In this study, the relative expression of *GDH1* and *GDH2* results indicated that *GDH1* is highly expressed in both roots supplied with sufficient and low P supplied plants compared to the nodules of these plants (Fig. 4). Regarding *GDH2*, in the roots there was no significant difference between the P treatments, this was the same in nodules. However the roots showed higher *GDH2* expression than nodules (Fig. 4). Both relative expression of both GDH transcripts was higher in the roots compared to nodules.

4. Discussion

The success of the legume *V. divaricata* in P-poor soils, may in part be influenced by the interaction C and N metabolism in roots and nodules. In this regard, the aminating and deaminating roles of the enzyme GDH is central in regulating C and N metabolism under P limitation. The contrinution of the enzyme's activities, the identification and quantification of gene transcripts for the GDH subunits α and β, are discussed.

The reduced plant growth during limited P supply is in agreement previous studies on model and endemic fynbos legumes, in which P limitation reduced the plant growth and biological N_2 fixation (Muofhe and Dakora, 1999; Nielsen et al., 2001; Olivera et al., 2004; Dakora and Phillips, 2002; Power et al., 2010; Magadlela et al., 2014; Vardien et al., 2014). Furthermore, the lower plant P levels during P deficiency, is congruent with previous work in legumes, which were also cultivated under conditions of low P supply (Hernández et al., 2007; Le Roux et al., 2006). P deficiency in *V. divaricata* affects N nutrition, C costs and allocation during plant growth (Magadlela et al., 2014). Given the importance of GDH in linking C and N metabolism, it is surprising that very few studies have been carried out on legumes, moreover on legumes that have evolved to grow in both P richer and poorer soils, like *V. divaricata* (Coetsee and Wigley, 2013; Maseko and Dakora, 2013). In the current study, low P roots showed an enhanced aminating GDH activity, due to GDH contributing to NH_4^+ assimilating capacity, along with the GS-GOGAT system. This may occur when GS-GOGAT is not be able to achieve its function, and then the mitochondrial NAD(H) dependent GDH may become prominent, by synthesising glutamate from NH_4^+ and 2-oxoglutarate (Melo-Oliveira et al., 1996; Miflin and Habash, 2002). The up-regulation of GDH in response to elevated ammonium levels suggest that GDH is important in the detoxification of ammonium by assimilating some of the ammonium ions (Tercé-Laforgue et al., 2004a, 2004b). This concurs with findings by Sarasketa et al. (2014) in *Arabidopsis thaliana,* where supply with both NH_4^+ and NO_3^- caused aminating GDH activities to be induced.

The assimilation of ammonium via GDH may also confers a saving in energy (ATP and NADPH) compared with GS-GOGAT cycle (Helling, 1998; Qui et al., 2009). In particular, the contribution of GDH to NH_4^+ assimilation during P deficiency, might be an energy conservation strategy as recently found for the same species (Magadlela et al., 2015). However, in addition to GDH assimilating NH_4^+, the activities of deaminating GDH in roots of P stressed plants, may contribute to the recycling of amino acids, in order to generate organic C for the TCA cycle, under conditions of P limitation. GDH is one of the few enzymes capable of releasing amino nitrogen from amino acids to yield a keto-acid and ammonium, of which the 2-oxoglutarate can be separately recycled for utilisation in TCA cycle respiration and further amide formation (Miflin and Habash, 2002). The increased deaminating GDH activity in the P stressed roots indicates that glutamate is broken down to form 2-oxoglutarate and NH_4^+. Although this has not been established in P stressed plants roots and nodules before, studies have

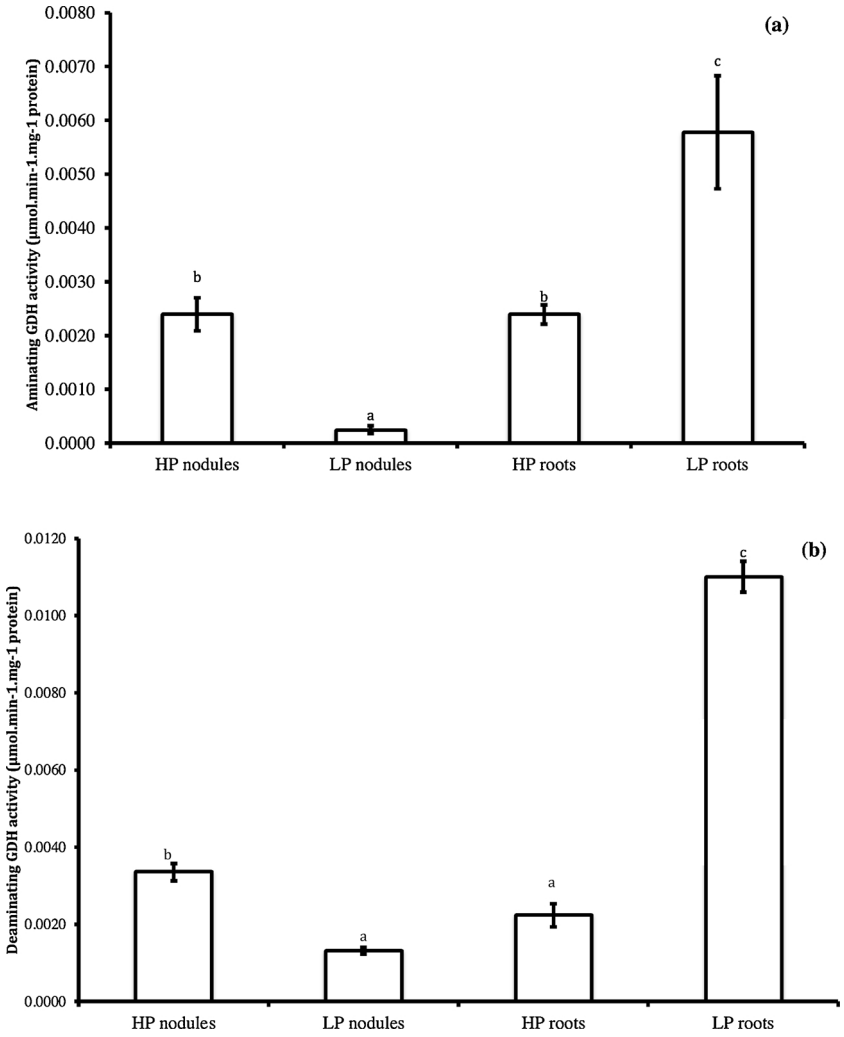

Fig. 2. (a) Aminating and (b) Deaminating GDH activities in the roots and nodules of *Virgilia divaricata* grown in sand culture under high P (500 µM) or low P (5 µM) as $NaH_2PO_4{\cdot}2H_2O$. Both the high and Low P plants were supplied with 500 µM NH_4NO_3 as soil nitrogen (N) source. The different letters indicate significant differences among the treatments (*P < 0.05).

implied that in times of stress, particularly carbon starvation, there maybe a strong demand to obtain carbon from amino acids to feed into the TCA cycle, this achieved by deaminating GDH (Lea and Miflin, 1980; Miflin and Habash, 2002; Miyashita and Good, 2008). Evenmore, there was a 243 max fold increase of GDH peptides in the roots during low P. This might be the case in *V. divaricata* plants during P stress, as it has been reported that P stress not only affects biomass and N nutrition in *V. divaricata* but also carbon (C) costs and allocation during plant growth (Magadlela et al., 2014, 2015). The control of GDH enzyme activities may be subject to regulation at the gene or protein level.

GDH transcripts have been extensively studied using biochemical, genetic and physiological approaches in model non-legume plants (Miyashita and Good, 2008; Lehmann et al., 2011). Studies on GDH have focused on carbon starvation (Miyashita and Good, 2008) and ammonium tolerance (Sarasketa et al., 2014). In contrast, a few studies have addressed the role of GDH in P stress, for example Qui et al. (2009) investigated the GDH responses of rice to N and P deprivation. Based on recent literature on GDH genes, there are four genes described as GDH have been identified in *Arabidopsis*, including two previously described by Turano et al. (1997) *GHD1* (At5g18170) and *GDH2* (At5g07440) as well as two assumed ones, *GDH3* by Purnell et al. (2005) and Miyashita and Good (2008), (Atg03g0390, encoding the β-subunit and GDH gene encoding NADP(H)-dependent GDH, At1g551720), four GDH genes in rice by Qui et al. (2009) and by Lehmann et al. (2011) in yellow Lupine. In this study we have found that at least two GDH transcripts are present in *V. divaricata*. Our analysis of cDNA sequences coding for GDH revealed that both *GDH1* and *GDH2* are present in the roots and nodules and expressed differently between the organs (roots and nodules) but not largely due to varying P supply. The phylogenetic comparison of these *GDH1* and *GDH2* sequences, indicate that these genes are conserved within the legume group. It is interesting to note that the *GDH1* largely clustered with the group of amino acid exporting legumes, while the *GDH2* largely clustered with the ureide exporting legumes. Recent work from our group indicated that under P limitation, *V. divaricata* nodules were less stressed than roots, synthesised mostly amino acids compared to the ureide synthesis of the severely stressed roots (Magadlela et al., 2015). In the current study, it may therefore be that the α-subunit of the enzyme GDH as encoded for by *GDH2*, possibly plays a role in the ureide synthesis, by means of the GDH provision of the substrate glutamate.

The variability of the quantitative expression of GDH transcripts in this study, concurs with findings where GDH coding cDNA sequences in yellow lupine were expressed differently in various organs during the developmental stages (Lehmann et al., 2011). Furthermore, the comparison of the yellow lupine sequences with *Arabidopsis,* confirmed that they correspond to the products of *GDH1* and *GDH2* genes (Lehmann et al., 2011). Additional findings indicated that the expression of GDH genes was differently regulated, depending on the organ or tissue types

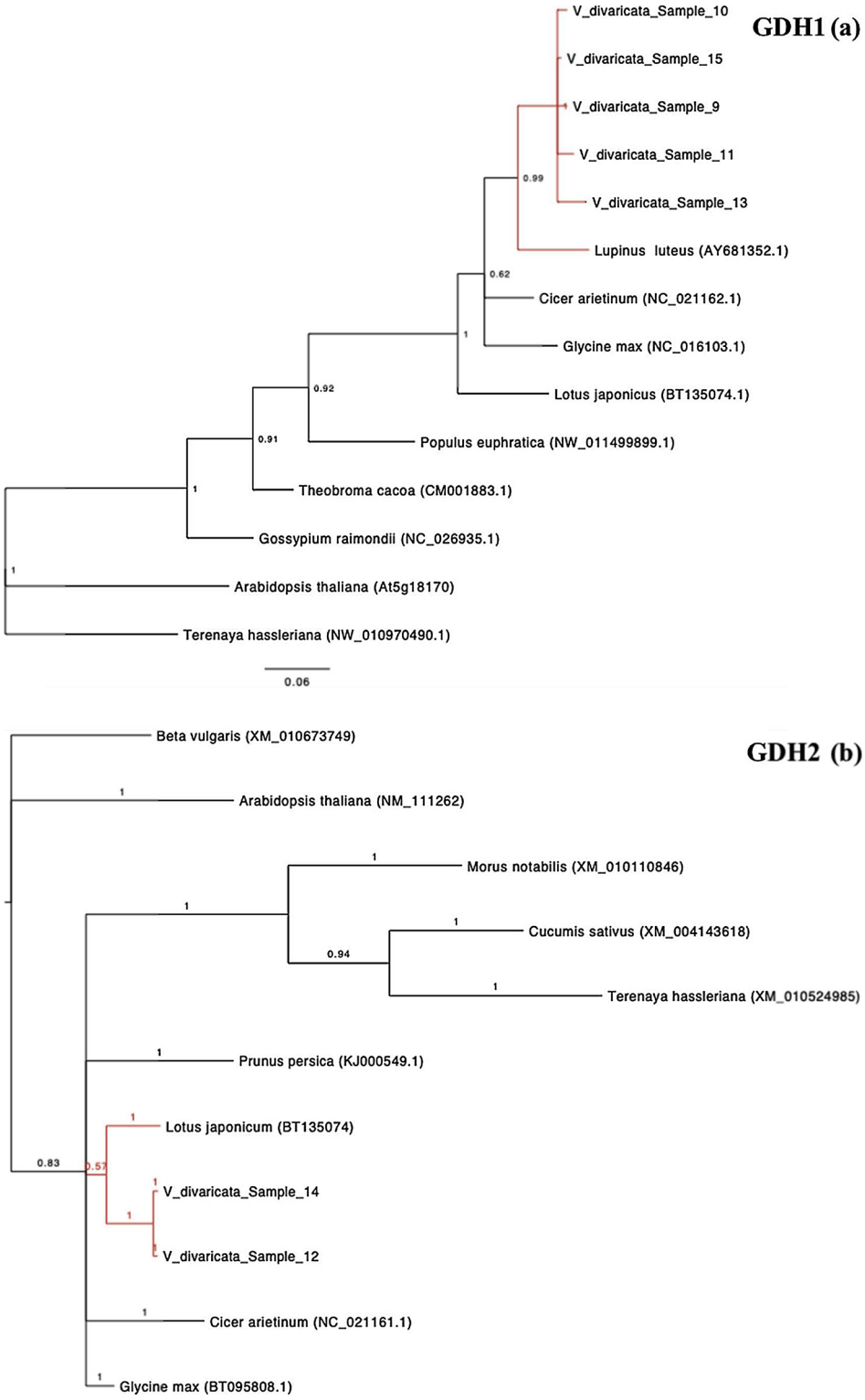

Fig. 3. Evolutionary relationships of (a) GDH1 and (b) GDH2 subunits encoded by *Virgilia divaricata* cDNAs. The aligned sequences of GDH 1 subunits were subjected through MrBayes 3.2 (Ronquist et al., 2012), for a 1,000000 generations using the NST mixed command to model over model space and a gamma correction for amongst site variation. A burn-in of 25% was used and all parameters converged at an estimated sample sizes greater than 500. Accession number is shown in parentheses, according to the sequences deposited in GenBank (NCBI, EMBL/GenBank/DDBJdatabase).

and cellular C availability in *Arabidopsis thaliana* (Miyashita and Good (2008). This concurs with our findings, where the differences in expression of the GDH subunits α and β, were largely between the roots and nodules. In contrast, the large differences in GDH enzyme activities, as influenced by P supply, suggests that the enzyme may not be regulated by gene expression of the subunits, but by post-translational modifications of the enzyme.

In a recent study by Magadlela et al. (2015) on *V. divaricata* grown under the same P conditions as the current study, nodules were found to have a lower percentage of decline in their ATP and ADP levels,

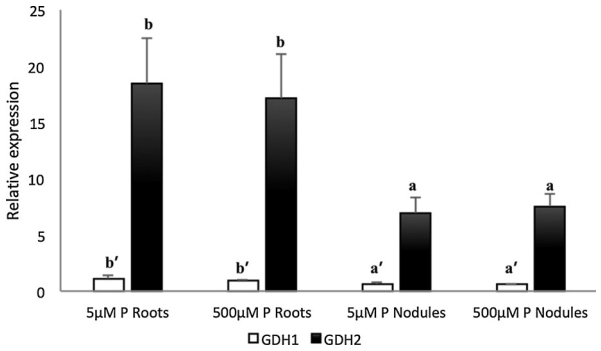

Fig. 4. Gene analysis expression of GDH1 and GDH2 (Glutamate dehydrogenase) in roots and nodules of *Virgilia divaricata*. Gene expression was normalized to that of *ACTIN2*. Error bars indicate standard deviation. *Virgilia divaricata* was grown in sand culture under high P (500 μM) or low P (5 μM) as $NaH_2PO_4{\cdot}2H_2O$. Both the high and low P plants were supplied with 500 μM NH_4NO_3 as soil nitrogen (N) source. The different letters indicate significant differences (*P < 0.05) among the treatments for GDH1 (with ‘) and GDH2 (without ‘).

compared to the roots. This suggests that these nodules may have a strategies to minimize their internal P usage and reduce the effects of P deficiency (Magadlela et al., 2015). In this study the higher relative expression of both GDH transcripts in the roots than nodules may play a role in the ability of roots to regulate their C and N and conserve their internal P during P deficiency as they were reported to be more stressed than the nodules due to the percentage decline in ATP and ADP levels (Magadlela et al., 2015).

5. Conclusion

These findings indicate that the control of C and N metabolism is important for the success of *V. divaricata* in soils ranging from nutrient rich soils in the forest to nutrient poorer soils in the fynbos. The regulation of GDH enzyme activity appear to be more complex than merely by gene expression, and suggests a role for post-translational modification at the protein level.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jplph.2019.153053.

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