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Differential expressions and enzymatic properties of malate dehydrogenases in response to nutrient and metal stresses in *Stylosanthes guianensis*

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Highlights (no more than 85 characters including spaces)

- At least seven *SgMDH* genes existed in stylo.
- *SgMDH* genes were regulated by nutrient and metal stresses in stylo root.
- SgMDH proteins displayed higher catalytic efficiency towards OAA than malate.
- Activities of the recombinant SgMDH proteins were affected by metal ions.

Abstract (no more than 250 words)

Malate dehydrogenase (MDH, EC 1.1.1.37) is a key enzyme that catalyzes a reversible NAD-dependent dehydrogenase reaction from oxaloacetate (OAA) to malate. Although MDH has been documented to participate in cellular metabolism and redox homeostasis in plants, the roles of MDH members in the tropical legume *Stylosanthes guianensis* (stylo) remain less definitive. In this study, except SgMDH1 that had been previously characterized, six novel *MDH* genes were isolated from stylo, which were then designated as *SgMDH2* to *SgMDH7*. All of the SgMDH proteins possessed the common features of NAD binding, dimerization interface and substrate binding sites. Expression analysis showed that three *SgMDHs* exhibited preferential expressions in leaf, and one *SgMDH* was mainly expressed in root. Furthermore, *SgMDHs* were regulated by nutrient deficiencies in stylo roots, especially for phosphorus (-P) and potassium (-K) deficiencies. Differential responses of *SgMDHs* to trace metal stress and heavy metal toxicity were observed in stylo roots, suggesting the involvements of *SgMDHs* in stylo response to metal stresses. The six novel SgMDHs were subsequently expressed and purified from *Escherichia coli* to analyze their biochemical properties. Although SgMDHs exhibited variations in subcellular localizations, each SgMDH protein displayed a high level of catalytic efficiency towards OAA and NADH, but a low level of catalytic efficiency towards malate and NAD^+. In addition, the activities of recombinant SgMDH proteins were pH-dependent and temperature-sensitive, and exhibited differential regulations by various metal ions. These results together suggest the potential roles of *SgMDHs* in stylo coped with nutrient and metal stresses.

Keywords: Malate dehydrogenase, Malate, Gene expression, Enzymatic properties, Nutrient deficiency, Metal stress, *Stylosanthes guianensis*

Abbreviations:

GST, glutathione S-transferase; IPTG, isopropylthio-D-galactoside; MDH, malate dehydrogenase; OAA, oxaloacetate; ORF, open reading frame; PVDF, polyvinylidene difluoride; qRT-PCR, quantitative real-time polymerase chain reaction; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

1. Introduction

In plants, malate dehydrogenase (MDH, EC 1.1.1.37) is a key enzyme of the malate-oxaloacetate (OAA) shuttle and participates in various metabolic pathways, including respiration, photosynthesis and energy generation (Nunes-Nesi et al., 2005; Imran et al., 2016; Ma et al., 2018). MDH catalyzes a reversible reaction from OAA to malate, accompanied by a redox reaction between NADH and NAD^+, which are the essential components in electron transport chain (Gietl, 1992; Hadži-Tašković Šukalović et al., 2011; Imran et al., 2016). The reaction direction catalyzed by MDH is mainly depended on the ratio of substrate and product, redox status and the changing environment of plant growth (Tomaz et al., 2010; Chen et al., 2015). Furthermore, MDH and its catalytic product, malate, have been documented to participate in the regulation of root growth (van der Merwe et al., 2009), leaf respiration (Tomaz et al., 2010) and embryo development (Beeler et al., 2014). In addition, malate also plays central roles in increasing phosphorus (P) acquisition, symbiotic nitrogen (N) fixation and aluminum (Al) toxicity tolerance (Schulze et al., 2002).

To date, a variety of MDH homologues have been identified from different plant species, such as Arabidopsis (Tomaz et al., 2010; Beeler et al., 2014), soybean (*Glycine max*) (Chen et al., 2011; Zhu et al., 2021), alfalfa (*Medicago sativa*) (Miller et al., 1998; Tesfaye et al., 2001), cotton (*Gossypium hirsutum*) (Wang et al., 2015; Imran et al., 2016) and apple (*Malus domestica*) (Yao et al., 2011a; Ma et al., 2018). MDHs exhibit various subcellular localizations, including cytoplasm, mitochondria, glyoxisome and peroxisome, serving various roles in plant metabolisms (Gietl, 1992). For example, nine putative MDH members have been identified in Arabidopsis, including eight NAD-MDHs and one NADP-MDH. Among the eight NAD-MDHs, two members are mitochondrial MDHs (mMDHs), two members are peroxisomal MDHs (pMDHs), one member is plastidial MDH (pdNAD-MDH), and the remaining three members are thought to be cytosolic MDHs (cyMDHs) (Beeler et al., 2014). The mitochondrial MDHs in Arabidopsis are involved in leaf respiration and photorespiration, thereby affecting plant growth (Tomaz et al., 2010). Arabidopsis peroxisomal MDHs are demonstrated to participate in β-oxidation of fatty acids and CO_2 release in the photorespiratory pathway (Pracharoenwattana et al., 2007; Cousins et al., 2008). The plastidial NAD-dependent

MDH is critical for redox homeostasis and is involved in embryo development in Arabidopsis (Beeler et al., 2014). Arabidopsis cytosolic MDH is found to catalyze malate synthesis, which subsequently contributes to Al detoxification and facilitates P acquisition (Wang et al., 2010). In addition, cell wall-localized MDH in maize (*Zea mays*) is proposed to provide NADH for the catalytic reaction of cell wall-bound peroxidases (PODs) as reducing equivalent, which further participate in phenolic metabolism (Hadži-Tašković Šukalović et al., 2011). Recently, it has been documented that MDHs in nodules are probably involved in regulation of symbiotic N fixation and nodule growth in soybean (Zhu et al., 2021).

Nutrient stress, such as nutrient deficiency and trace element excess, together with heavy metal toxicity that inhibits plant growth and yield, has been found to regulate the expressions of *MDH* genes (Wang et al., 2000; Uhde-Stone et al., 2003; Armengaud et al., 2009; Abd El-Moneim et al., 2015). For example, deficiencies of a set of macronutrients, such as N, P and potassium (K), regulate the expressions of various *MDH* homologues in Arabidopsis, soybean, cotton and white lupin (*Lupinus albus*) (Uhde-Stone et al., 2003; Armengaud et al., 2009; Wang et al., 2015; Vengavasi et al., 2016; Zhu et al., 2021). Furthermore, the expressions of *MDH* homologues are also regulated by excess copper (Cu) and manganese (Mn) stresses and Al toxicity (Yang et al., 2012; Abd El-Moneim et al., 2015; Chen et al., 2015). In addition, changes in enzyme activities of MDH under phosphate (Pi) starvation as well as metal stresses are observed in soybean, *Lupinus angustifolius*, maize and rape (*Brassica napus*) (Ligaba et al., 2004; Le Roux et al., 2006, 2008; Hadži-Tašković Šukalović et al., 2011; Vengavasi et al., 2016). Therefore, studying the comprehensive changes in expression profiles and enzyme properties of MDHs during stress conditions will help to provide new insights into the roles of MDHs in plants coped with nutrient and metal stresses.

Stylosanthes guianensis (stylo), an important forage legume with high quality and high yield, is commonly used for livestock nutrition and soil improvement in tropical and subtropical areas (Chandra, 2009; Guo et al., 2019). As it originates from the tropics, stylo exhibits great adaptability to acid soil-based nutrient and metal stresses (e.g., P deficiency, Al and Mn toxicity), which is possibly attributed to the fine modification of root characteristics through involvements of a variety of candidate genes (Sun et al., 2014;

Chen et al., 2015; Liu et al., 2016; Jiang et al., 2018; Chen et al., 2021). Among those genes, a gene encoding MDH (*SgMDH1*) was found to participate in malate synthesis and exudation in stylo roots, alleviating Mn toxicity by decreasing Mn accumulation (Chen et al., 2015). Furthermore, several *MDH* homologues in stylo had also been found to be regulated by low P availability, and Al and Mn toxicity through transcriptomic analyses (Jiang et al., 2018; Jia et al., 2020; Chen et al., 2021). Due to the critical roles of MDHs in plant metabolic processes, it is of considerable importance to investigate the *SgMDHs* expression profiles and biochemical properties in response to nutrient and metal stresses in stylo. Thus, in this study, six novel *SgMDHs* were isolated from stylo, and transcriptional profiles of *SgMDHs* in response to nutrient and metal stresses were investigated by quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Enzymatic properties of the six novel SgMDH proteins were further investigated.

2. Materials and methods

2.1. Isolation of *S. guianensis MDHs*

Except for *SgMDH1* (GenBank accession no. KJ123727), which has been previously characterized from stylo (Chen et al., 2015), six novel genes encoding MDHs were obtained from the sequencing data of transcriptome analyses (Jiang et al., 2018; Jia et al., 2020; Chen et al., 2021). The coding sequence of each *MDH* gene was amplified from a full-length cDNA library constructed using P-deficient roots of stylo (Sun et al., 2013). The six novel *MDHs* were named from *SgMDH2* to *SgMDH7*. The sequence data of the six novel *SgMDHs* were deposited in GenBank under accession numbers OK188912, OK188913, OK188914, OK188915, OK188916 and OK188917 for SgMDH2 to SgMDH7, respectively. All of the SgMDH members contained the conserved Ldh_1_N (PF00056) and Ldh_1_C (PF02866) domains according to the Pfam database (http://pfam.xfam.org/search/sequence). Phylogenetic analysis was performed by MEGA 4.1.

2.2. Plant growth and treatments

In this study, the elite stylo cultivar ‘RY2’ that is commonly cultivated in South China (Tang et al., 2009) was used. After seed germination for 3 d, seedlings were transferred to Hoagland solution containing 3 mM KNO_3, 2 mM $Ca(NO_3)_2$, 0.25 mM KH_2PO_4, 0.5 mM

$MgSO_4$, 5 μM $MnSO_4$, 0.5 μM $ZnSO_4$, 1.5 μM $CuSO_4$, 0.09 μM $(NH_4)_6Mo_7O_{24}$, 23 μM NaB_4O_7 and 80 μM Fe-Na-EDTA as previously described (Chen et al., 2021). The pH of the nutrient solution was adjusted to 5.8 every 2 d. Plants were grown in a greenhouse with a photoperiod of about 13 h and temperatures ranging from 25 to 35 ℃, under natural sunlight condition. After 21 d of growth, leaf and root were harvested for RNA extraction and gene expression analysis.

To analyze the transcripts of *SgMDHs* under nutrient deficient conditions, fourteen-day-old seedlings grown in half strength Hoagland solution were separately transplanted into fresh nutrient solution without N, P and K application according to Qin et al. (2012). For -N treatment, KNO_3, $Ca(NO_3)_2$ and $(NH_4)_6Mo_7O_{24}$ were replaced by K_2SO_4, $CaSO_4$ and Na_2MoO_4, respectively. For -P treatment, KH_2PO_4 was replaced by K_2SO_4. For -K treatment, KNO_3 and KH_2PO_4 was replaced by $Ca(NO_3)_2$ and NaH_2PO_4, respectively. Plants grown in full-strength nutrient solution were used as the control (CON). After 7 d treatments, roots were harvested for RNA extraction and gene expression analysis.

To detect the expressions of *SgMDHs* in stylo in response to excess trace elements, fourteen-day-old seedlings were separately transplanted into fresh solution (pH 5.0) containing 800 μM Fe-Na-EDTA, 400 μM $MnSO_4$, 10 μM $CuSO_4$ or 20 μM $ZnSO_4$ for 7 d, which were set as individual Fe, Mn, Cu or Zn stresses, respectively. To assay the transcripts of *SgMDHs* in stylo exposed to heavy metal treatments, fourteen-day-old seedlings were transplanted into a 0.5 mM $CaCl_2$ solution (pH 4.5) individually supplied with 0, 100 μM $AlCl_3$, 40 μM $CdCl_2$ or 20 μM $LaCl_3$ for 2 d, which were set as the control (CON), Al, Cd or La stresses, respectively. Roots with the above treatments were separately harvested for RNA extraction and gene expression analysis. For all experiments, a hydroponic box containing three seedlings was set as one biological replicate, and three biological replicates were included.

2.3. RNA extraction and qRT-PCR analysis

Total RNA was extracted using Trizol reagent according to the manufacturer's instructions (TIANGEN Biotech, China). First strand cDNA was synthesized using the HiScript III cDNA synthesis kit (Vazyme, China) from 2 μg total RNA after treated with DNase I. qRT-PCR reaction was performed using SYBR Green Master mix (Vazyme,

China) and monitored by QuantStudio™ 6 Flex Real-Time system (Thermo Fisher, USA). The primers for qRT-PCR analysis are listed in Supplementary Table S1. Gene expression was calculated by comparison with a standard curve using cycle threshold value. Relative expression level was calculated by the ratio of expression of each *SgMDH* to that of the housekeeping gene, *SgEF-1a*, according to Chen et al. (2021). Gene expression analysis included three biological replications.

Subcellular Localization of SgMDH proteins

To assay the subcellular localization of SgMDH proteins, the open reading frame (ORF) of each *SgMDH* gene was amplified using *SgMDHs-GFP-F/R* primers (Supplementary Table S1). The amplified product was subcloned into the N-terminal of the green fluorescent protein (GFP) of the binary vector pBWA(V)HS-ccdb-GLosgfp as described by Li et al (2021). Arabidopsis mesophyll protoplasts from 4-week-old seedlings were prepared, and then used to analyze the subcellular localization of each SgMDH protein according to Yoo et al. (2007). Each *35S:SgMDH-GFP* construct was transiently expressed in protoplasts, and empty vector was used as a control. The GFP fluorescence in Arabidopsis mesophyll protoplasts was imaged using a Nikon C2 confocal microscopy (Nikon, Japan) at 488 nm for GFP and at 640 nm for chloroplast autofluorescence.

2.4. Expression, purification and biochemical analyses of SgMDHs

The ORF of each *SgMDH* gene was amplified using *SgMDHs-GST-F/R* primers (Supplementary Table S1). The resulting amplified product was digested with the corresponding restriction endonuclease (Takara, Japan), and then subcloned into the pGEX6P-3 vector containing a glutathione S-transferase (GST) tag (GE Healthcare, USA). Six GST:SgMDH constructs and the empty vector were separately transformed into the *Escherichia coli* strain BL21. The recombinant proteins were induced by supplying 0.5 mM isopropylthio-D-galactoside (IPTG) for 4-6 h, and then purified from the *E. coli* extracts using BeaverBeads^{TM} (Beaver, China) according to the manufacturer's instructions. The resulting recombinant SgMDH proteins were analyzed by the sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (12% acrylamide) combined with Coomassie Blue R-250 staining and western blot analysis. Protein concentrations were measured by the Coomassie Brilliant Blue method (Bradford, 1976).

MDH activity of each recombinant protein was determined according to Chen et al.

(2015) with some modifications. To detect MDH activity in OAA reduction, approximately 0.2 μg recombinant MDH protein was incubated in reaction buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM ethylenediaminetetraacetic acid (EDTA), 0.025-0.3 mM NADH and 0.02-3 mM OAA. The reaction was started by protein addition. MDH activity towards OAA reduction was spectrophotometrically detected at 340 nm by measuring the levels of NADH within 1 min with a spectrophotometer (UV-2010, Hitachi, Japan), and is expressed as the amount of enzyme required to catalyze the oxidation of NADH (extinction coefficient 6.22 mM^{-1} cm^{-1}) per minute. To assay MDH activity in malate oxidation, 0.2 μg recombinant MDH protein was incubated in reaction buffer containing 100 mM Tris-HCl (pH 9.0), 5 mM EDTA, 0.125-3 mM NAD^+ and 0.1-10 mM L-malate. MDH activity towards malate oxidation was detected at 340 nm by measuring the levels of NADH within 1 min and calculated as the amount of enzyme required to catalyze the reduction of NADH per minute.

To detect the activities of SgMDH proteins in catalyzing the reversible reaction using NADPH and $NADP^+$, the recombinant SgMDH proteins were incubated in 100 mM Tris-HCl (pH 8.0) containing 25 mM dithiothreitol (DTT), 10 μM thioredoxin and 2 mM EDTA for 30 min at 30°C according to Ferte et al. (1986) with some modifications. The DTT-thioredoxin-activated enzyme was then used to analyze MDH activity in OAA reduction using NADPH. Approximately 0.4 μg recombinant MDH protein was incubated in reaction buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 0.3 mM NADPH and 3 mM OAA. For MDH activity in malate oxidation using $NADP^+$, 0.4 μg recombinant MDH protein was incubated in reaction buffer containing 100 mM Tris-HCl (pH 8.0), 5 mM EDTA, 1 mM $NADP^+$, 10 mM L-malate, 20 mM glutamate and 1 unit glutamate:oxaloacetate aininotransferase. MDH activity was detected at 340 nm by measuring the levels of NADH within 1 min.

The K_m and V_{max} values were determined by Lineweaver-Burke plot analysis. To determine the optimal reaction pH, 0.2 μg recombinant MDH protein was incubated in 100 mM Tris/HCl buffer (pH 7.0-9.5) using 1 mM OAA as a substrate. For temperature optimization, MDH activity was detected at different temperatures ranging from 30 to 60 °C. To detect the effects of various metal ions on the catalyzed activity, MDH activity was analyzed at the reaction buffer supplied with 1 mM $FeSO_4$, $MnCl_2$, $CuSO_4$, $ZnSO_4$,

$MgCl_2$, or $AlCl_3$. Analyses of MDH activity were conducted in triplicate. As the control, the GST protein from the empty vector displayed no detectable MDH activity.

2.5. Western blot analysis

After the purified SgMDH proteins were resolved by 12% SDS-PAGE, they were further electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane (Merck Millipore, USA) according to Chen et al. (2015). The hybridization of the PVDF membrane was started by addition of the anti-GST antibody (1:5,000 dilution) in blotting buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 0.1% (v/v) Tween 20 and 3% (w/v) nonfat dry milk for 1 h. After that, the PVDF membrane was hybridized with the alkaline phosphatase-tagged secondary antibody (1:2,000 dilution) in the fresh blotting buffer for 1 h. The signal of target protein in the PVDF membrane was observed after alkaline phosphatase reaction according to Chen et al. (2015).

2.6. Statistical analyses

Data analysis was performed by Microsoft Excel 2010 (Microsoft Company, USA). Assays with one-way ANOVA and Student's *t*-test were performed by the SPSS program v13.0 (SPSS Institute, USA).

3. Results

3.1. Identification and characterization of *MDHs* in *S. guianensis*

In this study, six novel *MDH* genes were isolated from stylo, which were designated as *SgMDH2* to *SgMDH7*. Together with SgMDH1 that had been cloned in a previous study (Chen et al., 2015), the ORF of the seven *SgMDHs* ranged from 999 to 1317 nucleotides. The deduced amino acid sequences of SgMDHs contained 330 to 438 amino acid residues, with calculated molecular masses differing from 33.6 to 48.9 kDa (Supplementary Table S2). The common features, including NAD binding, dimerization interface and substrate binding sites, were observed among the amino acid sequences of SgMDHs; the SgMDHs possessed the conserved amino acid sequences of MDH homologues from Arabidopsis, soybean, alfalfa and cotton (Fig. 1 and Supplementary Fig. S1). Furthermore, the deduced amino acid sequences of SgMDH1 to SgMDH7 shared 15.7 to 90.7% homology identities with AtcMDH1 in Arabidopsis, 13.2 to 57.2%

homology identities with MsneMDH in alfalfa, 16.8 to 90.7% homology identities with GmMDH12 in soybean and 16.9 to 83.4% homology identities with GhmMDH1 in cotton (Supplementary Fig. S2). In addition, the seven SgMDHs shared 13.0 to 97.3% homology identities with each other (Supplementary Fig. S2). According to WoLF PSORT program, most SgMDHs were predicted to be localized to the cytoplasm, except SgMDH4 and SgMDH5 that were localized to the chloroplast (Supplementary Table S2).

A phylogenetic tree was constructed using plants MDH homologues, including SgMDHs from stylo and all MDH members from Arabidopsis. Results showed that plant MDH members could be classified into three major groups (Fig. 1B). Among them, two SgMDHs (SgMDH2 and 5) together with three MDH members from Arabidopsis, four MDH members from rice, MsAAB99757 from alfalfa, PsAAC28106 from pea (*Pisum sativum*), SlNM001247072 from tomato (*Solanum lycopersicum*) and ZmNP001148518 from maize were classified into group I. Group II included only SgMDH1 with high similarity with GmNP001341093 from soybean and MsAAB99755 from alfalfa. Group III contained four SgMDHs (SgMDH3, 4, 6 and 7), three MDH members from Arabidopsis, two MDH members from rice, two MDHs from white lupin, MdDQ221207 from apple and other MDH members from alfalfa, cotton, maize, *Prunus persica*, *Plantago major* and *Ananas comosus* (Fig. 1B).

3.2. Expressions of *SgMDHs* under nutrient deficiency

In this study, we first detected the expressions of *SgMDHs* in leaf and root of 21-day-old stylo seedlings by qRT-PCR analysis. Variations in *SgMDHs* expressions were observed in leaf and root of stylo. Among them, three *SgMDHs* (*SgMDH1*, *4* and *5*) showed higher expressions in leaf, while *SgMDH6* were mainly expressed in root (Fig. 2). In addition, the transcripts of *SgMDH2*, *SgMDH3* and *SgMDH7* were constitutively expressed in leaf and root (Fig. 2).

Subsequently, we analyzed the expression profiles of *SgMDHs* in response to nutrient deficiencies, including N, P and K deficiencies. Results showed that *SgMDHs* exhibited differential regulations by nutrient deficiencies in stylo roots, and all *SgMDHs* responded to at least one nutrient deficiency treatment (Fig. 3). Among them, the transcript of *SgMDH3* was increased by -N treatment, while the remaining *SgMDHs* did not respond to N deficiency (Fig. 3). Four *SgMDHs* (*SgMDH1*, *3*, *6* and *7*) and two *SgMDHs*

(*SgMDH4* and *SgMDH5*) were increased and decreased by -P treatment, respectively (Fig. 3). In addition, six out of seven *SgMDHs* responded to K deficiency, including three up-regulated *SgMDHs* (*SgMDH4*, *5* and *7*) and three down-regulated *SgMDHs* (*SgMDH1*, *2* and *3*). Interestingly, *SgMDH2* and *SgMDH6* exhibited specific responses to -K and -P treatments, respectively (Fig. 3).

3.3. Responses of *SgMDHs* to metal stress

To examine the potential regulation of *SgMDHs* by exposure to trace metals, transcript levels of these genes were assessed in roots of stylo grown in nutrient solution supplied with excess Fe, Mn, Cu and Zn for 7 days. Diverse responses of *SgMDHs* were observed: under excess Fe treatment, transcripts of five *SgMDHs* (*SgMDH1*, *2*, *3*, *4* and *7*) were increased in roots compared to the control (CON) (Fig. 4). Similarly, four *SgMDHs* (*SgMDH2*, *3*, *5* and *7*) were also enhanced by excess Zn treatment. In contrast, three *SgMDHs* (*SgMDH3*, *4* and *7*) and two *SgMDHs* (*SgMDH5* and *SgMDH7*) were suppressed by excess Mn and Cu treatments, respectively, while the remaining *SgMDHs* did not respond to these two trace metals. Interestingly, the transcripts of *SgMDH6* were not affected by any of the trace metals (Fig. 4).

We further investigated the responses of *SgMDHs* in stylo roots to heavy metal toxicity, including Al, Cd (cadmium) and La (lanthanum) treatments. As shown in Fig. 5, transcripts of *SgMDHs* were regulated by at least one of the heavy metal treatments. Among them, five *SgMDHs* (*SgMDH1*, *2*, *3*, *4* and *6*) were up-regulated by Al treatment in roots, whereas *SgMDH5* and *SgMDH7* were not affected by Al. In contrast to the regulation by Al toxicity, Cd treatment mainly resulted in inhibiting the transcripts of six *SgMDHs* (*SgMDH1*, *3*, *4*, *5*, *6* and *7*), except *SgMDH2*. Besides, four *SgMDHs* (*SgMDH4*, *5*, 6 and *7*) were suppressed and only *SgMDH3* was enhanced by La treatment. Interestingly, *SgMDH2* exhibited specific up-regulation by Al but not by Cd and La treatments (Fig. 5).

3.4. Subcellular localization of SgMDH proteins

As the subcellular localization of SgMDH1 had been previously studied (Chen et al., 2015), the coding sequence of the six novel *SgMDHs* was separately cloned and fused with the N-terminal of the *GFP* reporter gene and transiently expressed in Arabidopsis mesophyll protoplasts. Results showed that the fluorescence of GFP in cells transformed

with the empty vector was found in many areas in protoplast cells, particularly in the cytoplasm. GPF signals of SgMDH2 and SgMDH 5 were detected in the organelles, probably in the peroxisome. SgMDH3 was mainly localized in the cytosol, while SgMDH4, SgMDH6 and SgMDH7 were observed in both the cytosol and plasma membrane. (Fig. 6).

3.5. Enzymatic properties of SgMDHs

Subsequently, the biochemical properties of six novel SgMDH proteins were detected by fused with a GST tag. After expressed in *E. coli*, the recombinant SgMDH proteins were purified from the lysates. After verification by SDS-PAGE and western-blot analyses (Supplementary Fig. S3), the purified SgMDH proteins were further used to test their biochemical properties in vitro, including catalytic properties and pH-dependent, temperature-sensitive and metal ion-sensitive activities. The catalytic properties were investigated by calculating K_m, V_{max} and catalytic efficiency (K_{cat}/K_m) values. The K_m values of SgMDHs ranged from 0.022 to 0.121 mM for OAA and from 0.168 to 1.144 mM for malate, and the V_{max} values of SgMDHs differed from 455.2 to 939.4 $nmol^{-1}min^{-1}$ for OAA and 65.8 to 135.1 $nmol^{-1}min^{-1}$ for malate (Table 1). Furthermore, the V_{max} values of SgMDHs for OAA and NADH were higher than those for malate and NAD^+, respectively. Similarly, all purified SgMDH proteins showed a higher level of catalytic efficiency (K_{cat}/K_m) towards OAA and NADH, and a lower catalytic efficiency towards malate and NAD^+. For example, the catalytic efficiency (K_{cat}/K_m) of SgMDH2 was 475,000 for OAA and 126,017 for NADH, which was significantly higher than that for malate and NAD^+ (Table 1). In addition, we also detected the activities of SgMDH proteins in catalyzing the reversible reaction using NADPH and $NADP^+$. Results showed that compared to NADH and NAD^+, each SgMDH protein exhibited a very small activity by using NADPH and $NADP^+$ to catalyze OAA reduction and malate oxidation, respectively (Supplementary Fig. S4).

As shown in Figs. 7 and 8, using OAA as the substrate, the enzyme activities of recombinant SgMDH proteins were pH-dependent and temperature-sensitive. Increasing activities of recombinant proteins were observed with increasing pH of the reaction buffer, then the MDH activities decreased with increasing pH value (Fig. 7). The optimum pH for activities of SgMDH2 and SgMDH6 was 7.5, while the optimum pH for SgMDH3,

SgMDH4 and SgMDH7 activities was 8.5. SgMDH5 exhibited the highest level of activity at pH of 8.0 (Fig. 7). Similar to the trends of MDH activities under different pH conditions, MDH activities increased and then decreased with increasing temperature of the reaction buffer (Fig. 8). The optimum temperature for activities of SgMDH2, SgMDH4 and SgMDH6 was 40, 50 and 50 ℃, respectively, while SgMDH3, SgMDH5 and SgMDH7 exhibited the highest level of activity at 45 ℃ (Fig. 8).

In addition, SgMDH activities were differentially influenced by metal ions, including Fe^{2+}, Mn^{2+}, Cu^{2+}, Zn^{2+}, Mg^{2+} and Al^{3+}, when using OAA as the substrate (Table 2). Among them, SgMDH2 activities were increased by Mn^{2+} and Zn^{2+}, but were inhibited by Cu^{2+} and Al^{3+}, while SgMDH3 activities were decreased by more than 20% when Fe^{2+}, Cu^{2+}, Zn^{2+} and Al^{3+} were applied. SgMDH4 activities were inhibited by all tested ions, except Cu^{2+}. SgMDH5 activities were increased by Mn^{2+}, but were inhibited by Fe^{2+} and Cu^{2+}, while SgMDH6 activities were decreased by all tested ions, except Mg^{2+} (Table 2). SgMDH7 activities were more than 20% increased by Mn^{2+} and Mg^{2+}, but more than 40% inhibited by Fe^{2+}, Cu^{2+} and Zn^{2+}. Interestingly, the activities of all SgMDHs were inhibited by Cu^{2+} and Fe^{2+}, except SgMDH2 and SgMDH4, respectively. Furthermore, the activities of most SgMDHs were not affected by Mg^{2+}, except SgMDH7 (Table 2).

4. Discussion

The ability to catalyze a reversible reaction from OAA to malate by the NAD-dependent MDH has been well elucidated in plants, and thus MDH plays a role in various physiological processes (Tomaz et al., 2010; Beeler et al., 2014; Ma et al., 2018). The number of MDH members differs among various plant species, suggesting they may possess diverse roles in cellular metabolic pathways. Although one cytosol-localized MDH member in stylo, SgMDH1, had been previously characterized to function in catalyzing malate synthesis (Chen et al., 2015), data are still limited on the potential roles of different MDH members in this important tropical legume due to lack of genome information. Thus, in this study, six novel *SgMDH* genes were further cloned and functionally characterized. Including SgMDH1, all seven SgMDH members contained the conserved features of plant NAD-MDH proteins (Fig. 1 and Supplementary Fig. S1), suggesting that SgMDH members are putative MDH proteins and possess roles in

399 catalyzing the reversible reaction from OAA to malate.

400 According to the phylogenetic analysis, in group I, SgMDH2 and SgMDH5 exhibited
401 high similarity to Arabidopsis peroxisomal MDHs (AtNP1979863 and AtNP196528),
402 which were demonstrated to be involved in β-oxidation of fatty acids and CO_2 release in
403 the photorespiratory pathway (Pracharoenwattana et al., 2007; Cousins et al., 2008).
404 Furthermore, alfalfa neMDH (MsAAB99757) in group I was suggested to function in
405 malate synthesis, contributing to increase Al tolerance and facilitate Pi acquisition
406 (Tesfaye et al., 2001, 2003). In group II, the cytosol-localized SgMDH1 had been
407 previously characterized to be involved in malate synthesis, improving Mn tolerance by
408 decreasing Mn accumulation in stylo (Chen et al., 2015). The closest homologue of
409 SgMDH1, GmMDH12 (GmNP001341093), was proposed to catalyze malate synthesis; it
410 regulates nodule growth in soybean under P deficient condition (Zhu et al., 2021). In
411 addition, AtNP564625 (mMDH1) and AtNP001078156 (mMDH2) from Arabidopsis in
412 group II were involved in leaf respiration and photorespiration, thereby affecting plant
413 growth (Tomaz et al., 2010). Furthermore, mMDH (SlNM001247072) in tomato was
414 found to participate in altering photosynthetic activity and aeration as well as root growth
415 in tomato (Nunes-Nesi et al., 2005; van der Merwe et al., 2009). Four SgMDHs
416 (SgMDH3, 4, 6 and 7), together with three MDHs from Arabidopsis and two MDHs
417 (LaAF459645 and LaAF459646) from white lupin, were divided into group III, clustering
418 with apple MdcyMDH (MdDQ221207), which participated in plant growth and tolerance
419 to cold and salt stresses (Yao et al., 2011a,b), and OscMDH (OsNP001064860), which
420 was involved in starch synthesis and seed development in rice (Teng et al., 2019).
421 Therefore, stylo SgMDH proteins are probably involved in diverse metabolic processes.

422 Subsequent expression analysis showed that *SgMDHs* exhibited various responses to
423 nutrient deficiencies in stylo roots (Fig. 3). It has been observed that nutrient deficiencies
424 regulate the expressions of many metabolism-related genes, including *MDH* (Wang et al.,
425 2000; Scheible et al., 2004; Armengaud et al., 2009). For example, a variety of *MDH*
426 genes were found to be regulated by P deficiency in Arabidopsis, soybean and white lupin
427 (Hammond et al., 2003; Uhde-Stone et al., 2003; Wu et al., 2003; Vengavasi et al., 2017;
428 Zhu et al., 2021). Enhanced expressions of *LaMDH1* were observed in P-deficient tissues
429 of white lupin, especially in normal and proteoid roots, facilitating acclimation to low-P

stress through regulation of carbon metabolism (e.g., organic acid synthesis) (Uhde-Stone et al., 2003). In addition, five out of 16 *GmMDH* genes were significantly enhanced by low-P stress in soybean nodules, suggesting the involvement of GmMDHs in nodule growth during Pi starvation (Zhu et al., 2021). Furthermore, overexpressions of *MDH* homologues could enhance malate synthesis and exudation, thereby increasing P availability in the rhizosphere (Tesfaye et al., 2003; Wang et al., 2010; Lü et al., 2012). In this study, thus, the four low-P stress-enhanced *SgMDHs* (*SgMDH1*, *3*, *6* and *7*) were probably involved in increasing P utilization of stylo. Besides, we also found that the expressions of *SgMDHs* were regulated by N and K deficiencies in stylo roots (Fig. 3). Similar regulations of *MDH* homologues have been reported in other plants, such as Arabidopsis and cotton (Scheible et al., 2004; Armengaud et al., 2009; Wang et al., 2015). In addition to catalyze malate synthesis, changes in expressions of *SgMDHs* might regulate nitrate and carbon metabolism as well as redox equilibrium in stylo response to N and K deficiencies. The roles of SgMDHs in nutrient deficiencies require further study.

It has been demonstrated that MDH is involved in metal tolerance by catalyzing malate synthesis in roots and subsequent exudation from roots to chelate metal ions, thereby improving metal tolerance in plants (Tesfaye et al., 2001; Wang et al., 2010; Chen et al., 2015). In the present study, transcripts of *SgMDHs* in stylo roots were differentially regulated by exposure to trace and heavy metals. Among them, five *SgMDHs* (*SgMDH1*, *2*, *3*, *4* and *7*) and four *SgMDHs* (*SgMDH2*, *3*, *5* and *7*) were enhanced by excess Fe and Zn, respectively, while four *SgMDHs* (*SgMDH2*, *3*, *4* and *6*) were up-regulated by Al (Figs. 4 and 5). Similarly, *MDH* homologues were also found to respond to various metal treatments, including Cu, Mn and Al (Kumari et al., 2008; Yang et al., 2012; Abd El-Moneim et al., 2015; Chen et al., 2015). For example, the transcript of *mMDH* but not *cyMDH* was increased by Al treatment in *Citrus*, which was associated with the tolerance of *Citrus* to Al toxicity (Yang et al., 2012). In addition, *mMDH1* in roots of Arabidopsis was also found to be induced by Al (Kumari et al., 2008). Furthermore, overexpression of *neMDH* in alfalfa and *amdh* in tobacco led to increased organic acid synthesis and secretion, conferring Al tolerance in transgenic plants (Tesfaye et al., 2001; Wang et al., 2010). In our previous study, we found that both the transcripts of *SgMDH1* and its protein levels were increased under excess Mn in roots of the Mn-tolerant stylo genotype,

461 contributing to increase Mn tolerance (Chen et al., 2015). Therefore, these results suggest
462 the potential roles of *SgMDHs* in metal tolerance.
463 We thus investigated the biochemical properties of the recombinant SgMDH proteins,
464 catalyzed OAA reduction and malate oxidation. Among them, the K_m values of the
465 remaining recombinant SgMDH3 and SgMDH6 for OAA and NADH were lower than
466 those for malate and NAD^+ (Table 1), as the case observed in neMDH/cyMDH from
467 alfalfa, cell wall-associated and plasma membrane-bound MDHs from maize, MDH from
468 pineapple (*Ananas comosus*) and MDH from *Aptenia cordifolia* (Miller et al., 1998;
469 Cuevas and Podesta, 2000; Tripodi and Podesta, 2003; Hadži-Tašković Šukalović et al.,
470 2011). In contrast, the values of V_{max} and K_{cat}/K_m of the six recombinant MDH proteins
471 were higher for the OAA reduction than for malate oxidation (Table 1). Similar results
472 were also found in MDH members from wheat, apple, maize, pineapple and *A. cordifolia*
473 (Cuevas and Podesta, 2000; Tripodi and Podesta, 2003; Ding and Ma, 2004;
474 Hadži-Tašković Šukalović et al., 2011; Yao et al., 2011b). However, alfalfa neMDH
475 exhibited higher V_{max} values for malate and NAD^+ but lower V_{max} values for OAA and
476 NADH, whereas the catalytic efficiency (K_{cat}/K_m) for malate and NAD^+ was lower than
477 that for OAA and NADH (Miller et al., 1998). Although values of kinetic properties
478 differed in SgMDH proteins and other plant MDH members, which were probably due to
479 the recombinant proteins or native proteins used in the analyses, our results revealed that
480 SgMDH proteins displayed higher catalytic efficiency towards OAA than malate by using
481 NADH.
482 The optimum pH for catalyzed activities of the recombinant SgMDH proteins towards
483 OAA reduction ranged from 7.5 to 8.5 (Fig. 7). A wide range of optimum pH values for
484 MDH activities have been observed from 6.5 to 8.5 in maize, *A. cordifolia*, pineapple and
485 spinach (*Spinacia oleracea*), and basic pH values, such as 8 and 8.5, were the common
486 optimum pH for MDH activity (Hadži-Tašković Šukalović et al., 1999, 2011; Cuevas and
487 Podesta, 2000; Tripodi and Podesta, 2003; Cvetić et al., 2008). Furthermore, the optimum
488 temperature for activities of the recombinant SgMDH proteins ranged from 40 to 50 ℃
489 (Fig. 8), which was similar to that for MDH members from soybean, *A. cordifolia* and
490 pineapple (Cuevas and Podesta, 2000; Tripodi and Podesta, 2003; Zhu et al., 2021). In
491 addition, increased or inhibited effects of metal ions on MDH activities were observed in

stylo (Table 2), and in soybean and maize as well. For example, activities of the cell wall-associated MDH from maize were inhibited by Zn^{2+} and Cu^{2+}, while soybean GmMDH12 activities were reduced when exposed to Al^{3+}, Fe^{2+}, Ag^{+}, Cu^{2+}, Mn^{2+}, Mg^{2+} and Zn^{2+} (Hadži-Tašković Šukalović et al., 2011; Zhu et al., 2021). Furthermore, stylo SgMDH1 activities had been previously found to be increased by Mn^{2+} and inhibited by Fe^{2+} (Chen et al., 2015). Interestingly, increases in activities of the SgMDH1 and SgMDH2 proteins exposed to Mn^{2+} and Zn^{2+} seemed to relate to the increasing gene expressions under excess Mn and Zn stresses, respectively (Fig. 4 and Table 2) (Chen et al., 2015). These results together suggest that SgMDH proteins possess different properties due to their potential roles in cellular ion homeostasis and microenvironment adaptation.

Although the seven SgMDH members in stylo exhibited similar structures, variations in the expression patterns, subcellular localization and biochemical properties of SgMDHs were observed in this study, suggesting these SgMDHs may have distinct or redundant biological functions in various physiological processes. Similarly, although mMDH1 and mMDH2 were mitochondrial MDH homologues in Arabidopsis, the transcripts and protein levels of mMDH1 were higher than those of mMDH2 (Millar et al., 2001; Hruz et al., 2008; Lee et al., 2008). Furthermore, the two single T-DNA insertion mutants, *mmdh1* and *mmdh2*, had no detectable phenotypes, but the double mutant *mmdh1mmdh2* displayed small and slow growth; the leaf respiration rate of this double mutant was higher than that of the wild type in both dark and light conditions (Tomaz et al., 2010), suggesting that mMDH1 coordinates with mMDH2 regulated leaf respiration and photorespiration in Arabidopsis. Besides, different enzymatic properties of MDH members in the same plant were also observed, such as nodule-enhanced and cytosolic MDHs from alfalfa, and cell wall-associated and plasma membrane-bound MDHs from maize (Miller et al., 1998; Hadži-Tašković Šukalović et al., 1999, 2011). Therefore, SgMDHs might possess various functions in the response of stylo to nutrient and metal stresses. The biological significance of SgMDHs in these processes merits further investigation.

Conclusion

In this study, a total of seven *SgMDHs* were characterized in stylo. The expressions of *SgMDHs* were differentially regulated by nutrient deficiencies and metal stresses in stylo roots. Despite various subcellular localizations, each recombinant SgMDH protein displayed a higher level of catalytic efficiency towards OAA and NADH compared to that towards malate and NAD^+. The activities of recombinant SgMDH proteins were pH-dependent and temperature-sensitive, and were differentially regulated by various metal ions. This study suggests the involvements of SgMDHs in stylo adapted to nutrient and metal stresses.

Authors contributions

ZC and LL designed the research. JS, XZ and PL performed the experiments and analyzed the data. GL prepared the plant material for this work. JS and ZC wrote the manuscript. JAC, RS and LL discussed and revised the manuscript. All authors have read and approved the manuscript.

Conflicts of interest statement

The authors declare that they have no competing interests.

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Table 1. Kinetic parameters of the recombinant SgMDH proteins.

Protein	Substrate	K_m (mM)	V_{max} ($nmol^{-1}min^{-1}$)	K_{cat}/K_m ($mM^{-1}min^{-1}$)
SgMDH2	OAA	0.065±0.02c	492.10±7.94a	1415734
	NADH	0.182±0.02b	387.20±21.70b	397837
	Malate	0.168±0.03bc	85.60±2.38c	95281
	NAD^+	0.460±0.05a	122.98±1.33c	49994
SgMDH3	OAA	0.032±0.01b	455.17±12.0a	2523349
	NADH	0.098±0.02b	416.67±34.4a	754258
	Malate	0.610±0.05a	65.83±2.43b	15954
	NAD^+	0.600±0.05a	66.87±4.10b	16476
SgMDH4	OAA	0.047±0.00b	939.40±30.30a	3607122
	NADH	0.194±0.01b	944.44±13.10a	1131058
	Malate	1.144±0.24a	135.08±13.10b	24567
	NAD^+	0.256±0.17b	103.87±4.69b	45357
SgMDH5	OAA	0.022±0.01c	626.60±22.70b	5921054
	NADH	0.504±0.02a	803.00±71.60a	331220
	Malate	0.168±0.03b	85.60±2.38c	77822
	NAD^+	0.370±0.07a	75.48±3.40c	31158
SgMDH6	OAA	0.047±0.00c	750.90±18.30b	3607122
	NADH	0.207±0.01b	1037.00±37.00a	1131058
	Malate	0.605±0.05a	65.83±2.43c	24567
	NAD^+	0.481±0.08a	96.63±4.90c	45357
SgMDH7	OAA	0.121±0.03c	939.39±30.30b	1303501
	NADH	0.268±0.03b	1157.40±46.30a	725102
	Malate	0.542±0.07a	77.09±5.21c	23881
	NAD^+	0.216±0.02bc	71.50±1.53c	55578

The K_m and V_{max} of the recombinant MDH proteins were calculated by Lineweaver-Burke plots method. Values indicate means of three replicates with standard error. Values followed with different letters indicate significant differences between various substrates at $P<0.05$ according to least significant difference (LSD) test.

Table 2. Effects of metal ions on the activities of the recombinant SgMDH proteins.

Metal ion	Relative activity (%)					
	SgMDH2	SgMDH3	SgMDH4	SgMDH5	SgMDH6	SgMDH7
Fe^{2+}	97.2±4.3bc	45.6±5.1c	53.0±1.4c	76.3±3.4c	61.9±2.0c	48.3±2.1cd
Mn^{2+}	107.8±1.8a	95.6±2.6a	86.6±1.4b	111.0±5.9a	88.2±4.2ab	122.7±4.7a
Cu^{2+}	88.1±4.2cd	14.2±2.1d	100.3±1.6a	43.1±4.3d	32.1±1.9d	55.8±4.3c
Zn^{2+}	111.6±3.5a	53.8±4.6c	25.1±1.3d	97.5±1.3b	57.9±2.2c	44.7±2.3d
Mg^{2+}	103.4±0.8ab	105.8±1.0a	88.7±1.5b	105.7±5.8ab	98.1±2.6a	128.5±5.1a
Al^{3+}	84.1±3.2d	77.1±3.3b	88.9±3.0b	98.5±2.7ab	85.4±5.0b	102.6±1.5b

Relative activity (%) was calculated by the ratios of MDH activity with metal ion treatment to its activity without application of metal ion. Values indicate means of three replicates with standard error. Values followed with different letters within a column indicate significant differences between various treatments at $P<0.05$ according to LSD test.

Supplementary data

Supplementary Fig. S1. Conserved domains of SgMDHs. Conserved domains were predicted by CDD program (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

Supplementary Fig. S2. Homology identity of MDH proteins from stylo and other plants. (A) Identity of SgMDHs with those closely related MDH proteins from other plants. (B) Homology identity among SgMDH proteins. The first two letters of each protein represent the abbreviated species name. Sg, *Stylosanthes guianensis*; At, *Arabidopsis thaliana*; Gm, *Glycine max*; Ms, *Medicago sativa*; Gh, *Gossypium hirsutum*. The identity (%) was determined by Clustalw (https://www.genome.jp/tools-bin/clustalw).

Supplementary Fig. S3. Purification of the recombinant SgMDH proteins. A, SDS-PAGE analysis of the recombinant SgMDH proteins in *E. coli*. Lane 1, molecular mass marker. Lane 2, total protein of *E. coli* transformed with the empty vector pGEX-6P-3 after IPTG induction. Lane 3, purified GST protein. Lanes 4, 6, 8, 10, 12 and 14, total protein of *E. coli* transformed with SgMDH2, 3, 4, 5, 6 and 7 after IPTG induction, respectively. Lanes 5, 7, 9, 11, 13 and 15, purified SgMDH2, 3, 4, 5, 6 and 7 proteins, respectively. B, Western blot analysis of the purified SgMDH proteins using anti-GST antibody. Lane 1, molecular mass marker. Lane 2, purified GST protein. Lanes 3, 4, 5, 6, 7 and 8, the purified SgMDH2, 3, 4, 5, 6 and 7 proteins, respectively.

Supplementary Fig. S4. Effects of NADH, NADPH, NAD^+ and $NADP^+$ supplements on activities of the recombinant SgMDH proteins. 0.3 mM NADH and NADPH were separately added into the reaction buffer to analyze OAA reduction, while 1 mM NAD^+ and $NADP^+$ were used to analyze malate oxidation. All values represent means of three replicates with standard error. Different letters indicate significant differences among various supplements at $P<0.05$ according to Duncan test.

Supplementary Table S1. Primers used for qRT-PCR and enzymatic properties analyses.

Supplementary Table S2. General information for the *SgMDH* genes in *S. guianensis*.

Figure legends

Fig. 1. Multiple alignment and phylogenetic analysis of SgMDH proteins. (A) SgMDHs with those closely related MDH members from other plants. (B) Phylogenetic analysis of MDH proteins in stylo and other plants. Except for SgMDHs, AtcyMDH1 (AtNP171936), GmMDH12 (GmNP001341093) and MsneMDH (MsAAB99757), the first two letters of each protein represent the abbreviated species name, followed by GenBank number. At, *Arabidopsis thaliana*; Ac, *Ananas comosus*; Cm, *Cucumis melo*; Gh, *Gossypium hirsutum*; Gm, *Glycine max*; La, *Lupinus albus*; Md, *Malus domestica*; Ms, *Medicago sativa*; Os, *Oryza sativa*; Pm, *Plantago major*; Pp, *Prunus persica*; Ps, *Pisum sativum*; Sl, *Solanum lycopersicum*; Zm, *Zea mays*. The red arrows represent SgMDH proteins.

Fig. 2. Expressions of *SgMDHs* in leaf and root of stylo. After seeds germinated for 3 d, seedlings were transplanted into full-strength Hoagland solution. After 21 d of growth, leaf and root were separately harvested for gene expression analysis. Each bar represents means of three independent replicates with standard error. Asterisks indicate significant differences between leaf and root according to Student's *t*-test. **, $0.001<P<0.01$; ***, $P<0.001$.

Fig. 3. Expression patterns of *SgMDH* genes in response to phosphorus (-P), nitrogen (-N) and potassium (-K) deficiency in stylo. Fourteen-day-old seedlings grown in Hoagland solution were transplanted into -P, -N and -K treatments. Plants grown in full-strength nutrient solution were used as the control (CON). After 7 d treatments, roots were harvested for gene expression analysis. Each bar represents means of three independent replicates with standard error. Different letters indicate significant differences among treatments at $P<0.05$ according to Duncan test.

Fig. 4. Effects of exposure to trace metal on *SgMDHs* expression in stylo. Fourteen-day-old seedlings grown in Hoagland solution were exposed to excess Fe, Mn, Cu and Zn treatments for 7 d. Plants grown in full-strength nutrient solution were used as the control (CON). Roots were harvested for gene expression analysis. Each bar represents means of three independent replicates with standard error. Different letters indicate significant differences among treatments at $P<0.05$ according to Duncan test.

Fig. 5. Responses of *SgMDHs* to heavy metal toxicity in stylo. Fourteen-day-old seedlings were subjected to 0 (CON), 100 μM $AlCl_3$, 40 μM $CdCl_2$ or 20 μM $LaCl_3$

814 treatments for 2 d. Roots were harvested for gene expression analysis. Each bar
815 represents means of three independent replicates with standard error. Different letters
816 indicate significant differences among treatments at $P<0.05$ according to Duncan test.
817 **Fig. 6.** Subcellular localization of SgMDH proteins in Arabidopsis mesophyll protoplasts.
818 Signals of GFP fusion protein (GFP), chlorophyll autofluorescence (Auto), bright-field
819 images (DIC) and the merged images (Merge) were examined using confocal microscopy.
820 Scale bar is 20 μm.
821 **Fig. 7.** Effects of pH on activities of the recombinant SgMDH proteins using OAA as the
822 substrate. All values represent means of three replicates with standard error.
823 **Fig. 8.** Effects of temperature on activities of the recombinant SgMDH proteins using
824 OAA as the substrate. All values represent means of three replicates with standard error.
825
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Differential expressions and enzymatic properties of malate dehydrogenases in response to nutrient and metal stresses in Stylosanthes guianensis

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