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Novel glutamate dehydrogenase genes show increased transcript and protein abundances in mature tomato fruits

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ABSTRACT

NAD(P)H-glutamate dehydrogenase (GDH, EC 1.4.1.3) contributes to the control of glutamate homeostasis in all living organisms. In bacteria and animals, GDH is a homohexamer allosterically regulated, whereas in plants NADH-GDH (EC 1.4.1.2) is also found as heterohexamer of α- and β-subunits, but its regulation remains undefined. In tomato (*Solanum lycopersicum*), GDH activity increases during the fruit ripening along with the content of free glutamate, the most abundant amino acid of ripe fruit involved in conferring the genuine tomato flavour. In this work, novel *Slgdh-NAD* genes were identified in the recently deciphered tomato genome: three encoding the α-subunit (*Slgdh-NAD;A1-3*) and one additional gene encoding the β-subunit of GDH (*Slgdh-NAD;B1*) isolated from a genomic library. These genes are located in different chromosomes. *Slgdh-NAD;A1-3* show conserved structures, whereas *Slgdh-NAD;B1* includes a novel 5′-untranslated exon. *Slgdh-NAD;A1-3* transcripts were detected in all tomato tissues examined, showing the highest levels in mature green fruits, contrasting with *Slgdh-NAD;B1* transcripts which were detected mainly in roots or in mature fruits when treated with glutamate, NaCl or salicylic acid. Analyses of GDH activity and protein distribution in different tissues of the Micro-Tom cultivar showed that only the active homohexamer of GDH β-subunits was detected in roots while heterohexamers of GDH α- and β-subunits were found in fruits. These results indicate that GDH β-subunit could modulate the heteromeric isoforms of GDH in response to the environment and physiology of the tomato fruit. This information is relevant to manipulate glutamate contents in tomato fruits genetically.

Introduction

Glutamate dehydrogenase (GDH) catalyses the reductive amination of α-ketoglutarate to glutamate, using either NADH or NADPH as a coenzyme. In plants, NADH-dependent GDH (EC 1.4.1.2) is the most abundant form (Dubois et al., 2003). The GDH protein exists as a homohexamer in bacteria and animals, being the latter highly regulated (Smith and Stanley, 2008). In plants, GDH is also a hexamer, but composed of two distinct subunits (α and β) that have very similar molecular masses and slightly different charges. The two subunits combine at random, in different ratios, to form seven possible NADH-GDH isoenzymes according to the current model (Turano et al., 1997; Purnell et al., 2005). There are homohexameric complexes consisting either solely of β-subunits (GDHB), the most cathodic isoform, or solely of α-subunits (GDHA), the anodic isoenzyme. The five heterohexamers migrate between the cathodic GDHB and anodic GDHA homohexamers in native gel electrophoresis (Loulakakis and Roubelakis-Angelakis, 1991). GDH α- and β-subunit cDNAs have been identified in several plant species. The GDH α-subunit has been found to be encoded by a single gene, whereas the β-subunit seems to be encoded by one gene in monocots and by at least two genes in dicots (Purnell et al., 2005). Recently, it has been reported that the rice *GDH* gene family comprises one gene (*OsGDH1*) encoding the β-subunit and two genes (*OsGDH2* and *OsGDH3*) encoding the α-subunit (Qiu et al., 2009). These three genes show different expression patterns in response to nutrient conditions depending on the rice tissue and time assayed (Qiu et al., 2009).

The role of GDH as an ammonium-fixing enzyme has been questioned by the use of transgenic tobacco plants modified in the expression of the genes encoding for the two GDH polypeptides α and β (Purnell and Botella, 2007; Skopelitis et al., 2007). These

Abbreviations: ESTs, expressed sequence tags; GDH, glutamate dehydrogenase; GDHA, GDH homohexamer of α-subunits; GDHB, GDH homohexamer of β-subunits; qPCR, real time PCR; rGDHA, recombinant fragment of the α-subunit; rGDHB, recombinant fragment of the β-subunit; RTL, relative transcript levels; SA, salicylic acid.

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studies have shown that overexpression of the α-subunit polypeptide from *Vitis vinifera* (GDHA) exhibits strong deaminating and only very low aminating activity (Skopelitis et al., 2007), whereas the overexpression of β-subunit polypeptide from *Solanum lycopersicum* (GDHB) solely deaminates glutamate (Purnell and Botella, 2007). Nevertheless, GDH can operate in the direction of ammonium assimilation under stress conditions favouring ammonium accumulation in grapevine suspension cells and tobacco leaf discs (Skopelitis et al., 2006). More recently, it has been proposed that GDH, acting in concert with GS and GOGAT, two enzymes that are also present in the phloem, is directly involved in the control of glutamate synthesis and trafficking to the vascular tissue (Labboun et al., 2009). Other findings have shown induction of GDH during avocado and tomato fruit ripening (Loulakakis et al., 1994; Boggio et al., 2000), raising the question about the role of GDH in fruit metabolism. Overexpression of a NADP-dependent GDH gene from *Aspergillus nidulans* in tomato leads to an increased level of glutamate in fruits (Kisaka and Kida, 2003). During the ripening of the tomato fruits, there is a sharp increase in the content of free glutamate (Boggio et al., 2000) concomitantly with an increase in the activities of GDH (aminating/deaminating ratio) and γ-amino butyric acid (GABA) transaminase (Sorrequieta et al., 2010). This suggests that these enzymes are involved in glutamate homeostasis. Glutamate plays a central role in a variety of metabolic processes at the interface of the carbon and nitrogen assimilatory pathways, and as a signalling molecule (Forde and Lea, 2007).

To clarify the role of GDH in tomato fruit ripening, additional data about the structure and function of *Slgdh-NAD* genes in tomato are needed. Although a cDNA encoding for a GDH β-subunit has been cloned and roughly characterized (Purnell et al., 1997), no further information has been published about the presence and regulation of *Slgdh-NAD* genes in tomato. In this work, *Slgdh-NAD* genes encoding the α- and β-subunits of tomato GDH were identified and characterized at the level of their genomic organization and their expression pattern in different organs, particularly in ripening fruits of tomato. This was done under normal growing conditions, and in the presence of glutamate and molecules like salicylic acid (SA) and NaCl, which are related to biotic and abiotic stresses, respectively. Additionally, distribution of enzyme activities and polypeptides of the α- and β-subunits of the hexameric GDH in different Micro-Tom organs were analysed. Taken together, these data provide novel information about the environmental and physiological control of GDH in tomato fruits.

Materials and methods

Plant material. Tomato (*Solanum lycopersicum* L.) plants of cv. Micro-Tom were grown in a controlled environment cabinet under a light intensity at the top of a fruit-containing plant of 400 μmol m^{-2} s^{-1}. The temperature ranged from 23 °C during the light period (14 h) to 18 °C during the dark period and the relative humidity was 70%. Plants were grown in soil, maintained under optimal irrigation and supplied with a standard nutrient medium. Fruits were allowed to ripen naturally on the plant and then selected for uniformity to maximise homogeneity between samples. They were harvested at mature green stage (when fruit stopped growing), and red stage (5.5 ± 2.1 days after breaker stage), when fruits began to soften. Pericarp tissue of harvested fruits was obtained by peeling off, removing the locular tissues (placenta) and seeds and immediately processed or frozen in liquid nitrogen and stored at −80 °C until use. Plants were grown for 4–6 weeks for leaf tests, and for 10–12 weeks for fruit analysis. For root tests, plants were grown in soil. Seeds were surface-sterilized in 10% commercial bleach for 15 min, rinsed with sterilized water three times and then put in a plant box with 1X Murashige & Skoog Medium (Sigma Aldrich, Argentina) supplemented with 15 g L^{-1} sucrose and 8 g L^{-1} agar medium. They were maintained at 25 ± 2 °C under 16 h light/8 h dark with fluorescent light (irradiance of 50 μmol m^{-2} s^{-1}) for 4–6 weeks. All the fresh material was harvested between 5 and 8 h after the lights were switched on.

Slgdh-NAD gene isolation. A λEMBL-3 tomato genomic library (Clontech, Palo Alto, CA, USA) was screened by plaque hybridization according to standard procedures (Sambrook et al., 1989). A total of 3.3×10^5 plaques were plated at a density of 3.5×10^4 plaques per 15 cm petri dish, and then transferred to Hybond-N filters (Amersham Biosciences, Argentina) and screened using a tomato *gdh* cDNA fragment (Bortolotti et al., 2003; Scarpeci et al., 2007) as a ^{32}P-labelled probe. The filters were further processed as described previously (Sambrook et al., 1989). The host strain used for library amplification was *E. coli* NM538. Phage DNA was isolated from putative positive clones using DNA lambda prep kit (Qiagen, Tecnolab SA, Argentina) and sequenced. To obtain the full-length *Slgdh-NAD;B1* gene (including the 3′-end) PCR amplification was performed on genomic DNA, which was isolated from tomato young leaves using DNeasy Plant Mini Kit (Qiagen). Approximately 50 ng of genomic DNA was used as template and specific primers corresponding to the sequences 5′-GAGGCTGCTAACCATCCAACTG-3′ on exon 7 of the *Slgdh-NAD;B1* gene and 5′-ACTTTGTCCATGGGCAGAACTTAC-3′ on the complementary strand of *legdh1* cDNA 3′-UTR. PCR reaction (94 °C, 5 min; 30 cycles of: 94 °C, 90 s; 45 °C, 2 min; 72 °C, 90 s; and 10 min of final extension at 72 °C) was performed in 100 μL of final volume with the following additions: 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, 150 pmoles of each primer, 1 X buffer (Promega, USA), 5U of Taq DNA polymerase (Promega). The DNA fragment was isolated from agarose gels and subsequently sequenced.

Primer extension analysis. Total RNA was extracted from leaves, roots and fruit pericarp using an RNeasy Plant Mini kit (Qiagen) according to the manufacturer's protocol. Briefly, 10 μg of each RNA was mixed with 20 pmol of [5′-^{32}P] specific primer (5′-CTAAAGCATTCATGTTTTCTCCACA-3′) and 30–40 U of RNAsine (Promega) in a final volume of 12 μL, and incubated at 70 °C for 10 min, followed by incubation on ice for 1 min. The extension reaction was performed by adding 5 μL of 5 X Superscript II reverse transcriptase buffer (GIBCO-BRL), 2 μL of 0.1 M DTT, 2 μL of dNTPs mix (dCTP, dGTP, dTTP) 10 mM each, 2 μL of 0.1 mM dATP, 1.5 μL of α[^{32}P]dATP (10 μCi/μL), and 1 μL of Superscript II reverse transcriptase (200 U/μL) (GIBCO) followed by 50 min of incubation at 42 °C. The enzyme was inactivated by heating for 15 min at 70 °C. A sequencing ladder was generated with the above primer and the 5′-flanking region of genomic DNA as template.

Sequence analyses. Multiple nucleotide and protein sequence alignments were performed using the CLUSTAL W software (Thompson et al., 1994). Clustering of *gdh* genes was done by UPGMA and Neighbour-joining methods using MEGA 2.1 software (Kumar et al., 2001). Trees were obtained with bootstrap replication of 1000, addition of sequences at random with five replications. Mapping of *Slgdh* genes onto the EXPEN 2000 genetic map (solgenomics.net) was performed exactly as described in Almeida et al. (2011).

Extraction of soluble proteins and enzyme activity analyses. Leaf, fruit and root protein extracts were obtained as previously described (Scarpeci et al., 2007) and immediately used, or stored at −80 °C until analysis. Total soluble protein concentration in each sample was estimated according to Bradford's assay using bovine serum albumin as standard (Bradford, 1976). In-gel GDH activity was determined by substrate incubation and further staining of non-denaturing 8% (w/v) polyacrylamide gel as previously described (Turano et al., 1996).

Expression and purification of recombinant GDHA and GDHB. Partial GDH cDNAs expressed in *E. coli* for GDHA (corresponding to residues 244 and 324) (rGDHA) and GDHB (from residue 61 to 169)

(rGDHB) were obtained by PCR using the following primers: for rGDHA, forward 5′-GCTAGCAAGAACTCCAATGGGATCG-3′ and reverse 5′-AAGCTTTAAGGCCTTGACGTTATCAGC-3′, where the underlined sequences indicate the *Nhe*I and *Hind*III restriction sites, respectively; and for GDHB, forward 5′-GTGGGATCCGCACGAGGGCCTATGAAAG-3′ and reverse 5′-CTCGTCGACTCCAGTTACCACAGCAGG-3′, where the underlined sequences correspond to the *BamH*I and *Sal*I restriction sites, respectively. The PCR products were digested with the corresponding restriction enzymes and cloned into the expression vectors pET-28a(+) (rGDHA) and pET-32a (rGDHB). DNA sequencing was carried out to ensure that no mutations had been introduced. Overexpression was achieved by transforming BL21(DE3) pLysS *E. coli* strain with the recombinant plasmid. Cells were grown in Luria–Bertani broth at 37 °C until OD_{600} 0.6, and GDH expression was induced by addition of isopropyl β-D-thiogalactoside to a final concentration of 0.4 mM.

Antibody production. The antibodies were generated against tomato rGDHA and rGDHB. Anti-rGDHA was expected to be specific for α-subunit. The antisera were raised in white rabbit. rGDHB protein was purified by large-scale SDS-PAGE, from which the GDH band was cut and made a fine powder under liquid nitrogen. Ni–NTA column (Qiagen) was used to purify the rGDHA protein. Bacteria expressing rGDHA were lysed with lysis buffer pH 8 (150 mM NaCl, 50 mM Tris-HCl, 5 mM imidazole). Lysate was sonicated, centrifuged at 10,000 × *g* for 20 min at 4 °C and the supernatant collected and directly loaded onto a Ni–NTA column previously equilibrated with lysis buffer. The column was washed four times with washing buffer (150 mM NaCl, 50 mM Tris–HCl (pH 8), 10 mM imidazole). Bound rGDHA was eluted with elution buffer (300 mM NaCl, 50 mM Tris–HCl (pH 8), 100 mM imidazole). Both recombinant proteins were concentrated using Centriplus-10 concentrators (Millipore, USA). Then, 500 μg of purified protein was injected subcutaneously into the animal. Two booster injections of 250 μg protein were given 3 and 6 weeks later. Six weeks after the initial injection, blood was collected and the serum recovered by centrifugation at 5000 *g* for 5 min. Before using, the collected antiserum, diluted in TBS-T-milk, was incubated with total protein extract from induced *E. coli* cells carrying the empty expression vector.

Immunoblot analyses. Non-denaturing 8% (w/v) polyacrylamide gel or SDS-PAGE on 12% (w/v) polyacrylamide gels was electrophoresed and the gels were either stained for GDH activity or electroblotted to nitrocellulose membranes. Immunodetection was carried out according to the manufacturer's instructions (ECL Amersham Biosciences, Argentina), using antibodies against rGDHA or rGDHB (see above).

SA, NaCl and glutamate treatments of Micro-Tom plants. Groups of plants harbouring fruits at green and breaker stages were soil soaked every day for 5 days with a solution of either 2.5 mM SA, 100 mM NaCl or 20 mM glutamate. For NaCl treatment, plants were subjected to a daily increase of 50 mM NaCl as previously described (Debouba et al., 2006). Each treatment was carried out in triplicate, each consisting of four plants grown in soil, one plant per pot of 500 mL capacity, and soaked with 100 mL solution every day. After the treatment, leaves, roots and fruits were collected for RNA isolation and real-time PCR (qRT-PCR) analysis.

RNA isolation. Total RNA was isolated using TRIzol (Invitrogen Life Technologies, Karlsruhe, Germany) following the manufacturer's instructions. Quality and quantity of RNA were monitored spectrophotometrically at 260 and 280 nm, and RNA integrity was checked by electrophoresis in 1.5% (w/v) agarose gel.

Quantitative real-time PCR. For this experiment total RNA was isolated from roots, leaves or fruits from four different plants randomly selected (three leaves and three fruits from each plant at green or red stage). Experiments were carried out in triplicate and data are shown as the means ± SE or SD of the mean of each independent experiment. Total RNA (0.75 μg) was digested with DNAse (Sigma) and reverse-transcribed with Superscript III reverse transcriptase (Invitrogen) in a reaction volume of 20 μL to generate first-strand cDNA. qPCR analyses of *Slgdh-NAD;A1-3* and *Slgdh-NAD;B1* transcripts were performed using the following primers: for *Slgdh-NAD;B1* forward 5′-AAGGAGTCACCATCCTACCG-3′ and reverse 5′-TGTGAGTCTTGCACATATCCTTG-3′, for *Slgdh-NAD;A1-3* forward 5′- CCAGACATCTATGCCAATGC-3′ and reverse 5′- ATTCACCCCCAATGTGAATG-3′, for *Slgdh-NAD;A1* forward 5′- GAAAGCTCATCGATTTT-GCT-3′ and reverse 5′- TAATGAATTTGGCCTTGACGTT, for *Slgdh-NAD;A2* forward 5′- GGAACCTAATTGATTTCGGC-3′ and reverse 5′- CGATGAACTTGGCATTTACATG-3′, for *Slgdh-NAD;A3* forward 5′- AGCTGATGAGGTACTGTGC-3′ and reverse 5′-CAGGTTATGGAAGGCATTC-3′. *Rpl2* (encoding the ribosomal protein large subunit 2) was chosen as housekeeping gene and was measured using the primers *Rpl2* forward 5′-CGTGGTGTTGCTATGAATCC-3′ and *Rpl2* reverse 5′- GTCAGCTTTGG-CAGCAGTAG -3′. cDNAs were amplified using Mastercycler ep Realplex2 thermocycler (Eppendorf, Westbury, USA). PCR conditions were 1 min at 95 °C and 40 cycles of 15 s at 95 °C, 30 s at 58 °C and 40 s at 72 °C. Following amplification, products were denatured by heating from 60 to 95 °C to check amplification specificity. qPCR was performed using a SYBR Green fluorescence-based assay. Gene-specific cDNA amounts were calculated from threshold cycle (Ct) values, expressed as relative to controls, and normalized with respect to tRpl2 cDNA, used as an internal reference (Ctr), according to the equation ΔCt = Ct − Ctr and quantified as 2-ΔCt. A second normalization by a control (Ctc) ΔΔCt = Ct − Ctc produces a relative quantification: 2ΔΔCt (Livak and Schmittgen, 2001).

Promoter-GUS fusion. To generate the *Slgdh-NAD;B1* promoter-*GUS* (*uidA* from *E. coli*) fusion lines of tomato, a 0.9-kb genomic fragment 5′ upstream of the ATG initiation codon of *Slgdh-NAD;B1* (see GenBank accession no. AY768544) was amplified by PCR using Micro-Tom genomic DNA as template and as reverse primer 5′-AGGTACCATTAACCAACACTTTTTAGAG-3′ (added *Kpn*I restriction site underlined) and forward primer (added *Nco*I restriction sites underlined): 5′-ACCATGGAGTCAATAATCAGAGACTGC-3′. Cotyledon transformation in Micro-Tom was performed according to Meissner et al. (1997).

Treatments of plants for GUS staining and GUS enzymatic assay. Roots, leaves and fruits were excised from tomato plants. Fruits were cut longitudinally. These organs were submerged in either 100 mM NaCl, 2 mM SA or 25 mM glutamate for 6 h. After the treatments, tissues were collected and rinsed in 50 mM sodium phosphate (pH 7.2), 10 mM EDTA, 0.33 mg mL^{-1} potassium ferricyanide, and then transferred to the same solution containing in addition 0.5 mg/mL 5-bromo-4-chloro-3-indolyl-β-D-glucuronic acid (X-Gluc; Gold Biotechnology, St Louis, MO, USA). Leaves and roots were vacuum-infiltrated for 1 min three times and then incubated at 37 °C for 12–24 h, while fruits were cut in the middle and incubated in X-Gluc solution at 37 °C for 18 h. Tissues were destained by soaking in 70% (v/v) ethanol.

Statistical analysis. Significant difference was determined at $p < 0.05$ using an unpaired two-tailed Student's *t* test. Data from SA, NaCl and glutamate treatments of Micro-Tom plants were subjected to ANOVA followed by Holm Sidak test analysis ($p < 0.05$).

Results

Structural analysis of tomato GDH encoding genes

To isolate the tomato GDH encoding genes, a genomic DNA library was screened with a probe designed on a *gdh-NAD* highly conserved region in all plant genes reported to date (Scarpeci

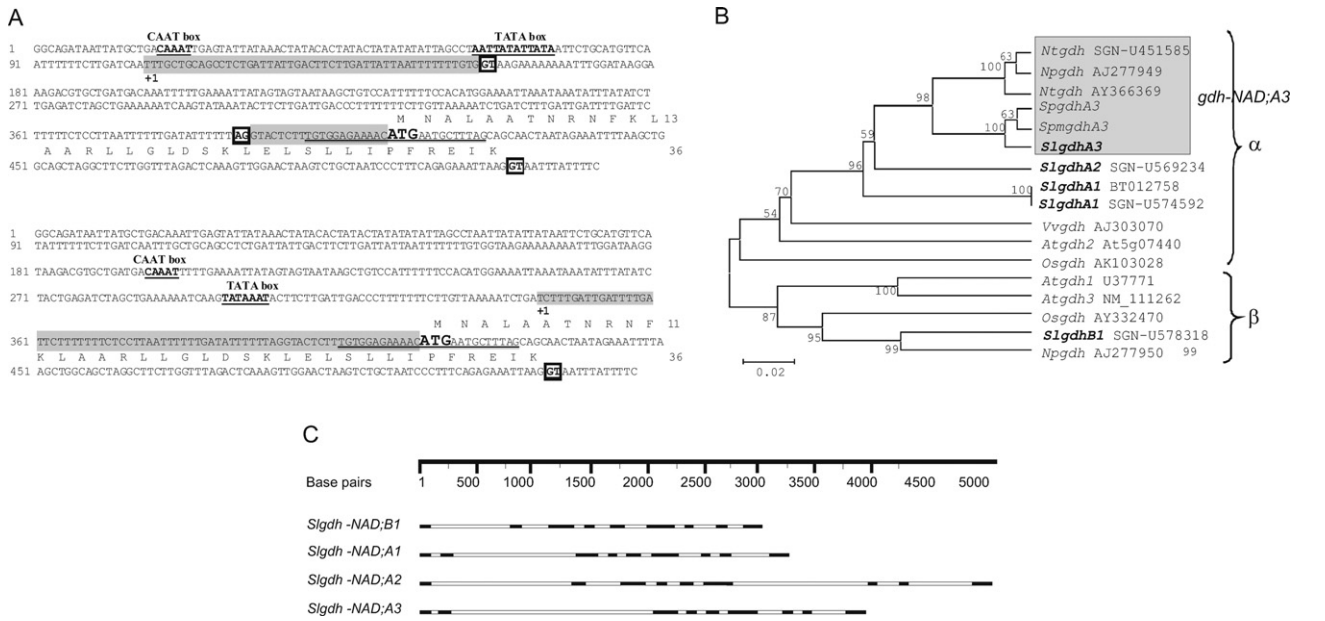

Fig. 1. Structural analysis of *Slgdh-NAD* genes. (A) Sequences of the 5′-UTR of *Slgdh-NAD;B1*: upper panel: 5′-UTR of the cDNA deposited in GenBank (accession no. U48695; Purnell et al., 1997) showing the first untranslated exon in grey; lower panel: 5′-UTR of the predicted Unigene SGN-U578318 (The International Tomato Genome Sequencing Consortium-www.solgenomics.net/tomato-) is shown in grey. Positions of the CAAT- and TATA-boxes upstream 5′-UTR are highlighted in bold and underlined. The splicing sequences GT and AG are boxed. The initiation of transcription is indicated by +1. The oligonucleotide sequence used in the primer extension experiment is underlined. The ATG translation start codon is shown in bold and enlarged. (B) Phylogenetic relationships among plant *gdh-NAD* genomic sequences. The *gdh-NAD* genes encoding the α- and β-subunits of GDH from different plant species are indicated. The grey box indicates the *gdh-NAD;A3* genes from different plant species. Nucleotide coding regions were aligned using Clustal W method. The unrooted tree was constructed using the Neighbour-joining method. The Treeview software was used as a tree-drawing tool. Confidence of the groupings was estimated using 1000 bootstrap replications. Bootstrap values present at each node represent the percentage of times out of 1000 bootstrap re-samplings that branches were grouped together. Branch lengths are to scale, 0.1 representing 10 changes per 100 nucleotides. The aligned sequences and their GenBank or SGN accession numbers are indicated on the right. *At, Arabidopsis thaliana; Np, Nicotiana plumbaginifolia; Nt, Nicotiana tabacum; Os, Oryza sativa; Sl, Solanum lycopersicum; Sp*, *Solanum pennellii*; *Spm*, *Solanum pimpinellifolium; Vv, Vitis vinifera.* (C) Comparison of the structures of the *Slgdh-NAD* genes. The scaled diagrams of exon-intron structure of all genes are represented from the ATG start codon to the stop codon. Exons are represented as black boxes.

et al., 2007). After consecutive screenings, phage purification and sequencing, only one clone was assigned to the *Slgdh-NAD;B1* gene (10 exons and 9 introns) and a 5′-upstream region of 3780 bp (GenBank accession no. AF403178 and AY768544). The intron borders matched the canonical plant intron sequences (GT. . .AG) for all nine introns. This *Slgdh-NAD;B1* gene structure is similar to other plant *gdh* genes except for the presence of an exceptional untranslated first exon (49 bp) followed by an intronic region of 242 bp (Fig. 1A, upper panel). The existence of this untranslated exon was deduced by comparison with the 5′-UTR sequence of the previously isolated cDNA (GenBank accession no. U48695). By querying the Unigene collection deposited at the International Tomato Genome Sequencing Consortium web site (www.solgenomics.net/tomato), we found a single Unigene (SGN-U578318) associated with *Slgdh-NAD;B1*, containing a 73-bp 5′-UTR, but not showing the 49-bp untranslated fragment mentioned above (Fig. 1A, lower panel). It is remarkable to note that the upstream 5′-UTR contained TATA boxes at -32 and -39 relative to the transcription start site and putative CAAT boxes at -85 and -142 (Fig. 1A). In order to map the transcription start sites of the *Slgdh-NAD;B1* gene a primer extension experiment was performed using an antisense oligonucleotide including the ATG start codon (Fig. 1A). Two extension products that differed in size by five nucleotides, mapping 67 to 72 bp upstream of the first ATG, were observed using mRNA from roots (Suppl. Fig. 1). Similar results were obtained using mRNA from green and red fruits (not shown), raising the possibility that both 5′ upstream regions are transcribed *in vivo* (Fig. 1A) regardless of the ripening process or tissue specificity.

To ascertain the existence of other *Slgdh-NAD* genes in the tomato genome two databases were surveyed: the Unigene collection and the Tomato WGS Chromosomes (version SL2.4) deposited at the International Tomato Genome Sequencing Consortium web site (www.solgenomics.net/tomato). For the Unigene search, the sequence of the *Slgdh-NAD;B1* coding region was used. Three Unigenes were found (*E*-values <2e^{-20}), one for *Slgdh-NAD;B1* and two highly similar to *gdh-NAD;A* of *Nicotiana plumbaginifolia* and *GDH2* of *Arabidopsis thaliana* (Table 1), suggesting that they may correspond to the *Slgdh-NAD;A* genes. They were named *Slgdh-NAD;A1* (SGN-U574592) and *Slgdh-NAD;A2* (SGN-U569234) (Table 1). Next, the three Unigene sequences were used as queries in a search onto the complete tomato genome by BLASTN. We found three loci with significant identities to the *Slgdh-NAD;A1*, *Slgdh-NAD;A2* and *Slgdh-NAD;B1* sequences (Table 2), and an additional one (Scaffold SL2.40sc04279) for which no expressed sequence tags (ESTs) were found, suggesting that it could correspond to a very low abundant transcript.

As untranscribed genes could be lost during evolution, the existence of the *gdh-NAD;A3* gene was searched for in the genomes of other Solanaceae plants. The coding region of *Slgdh-NAD;A3* was blasted against the SGN database and sequences analysed according to Kamenetzky et al. (2010). Scaffolds that were highly similar to *Slgdh-NAD;A3* in *Solanum pimpinellifolium* (99% identity), *Solanum pennellii* (97% identity) and the Unigene SGN-U451585 from *Nicotiana tabacum* (90% identity) were found. Using the coding region from these sequences and from all the coding regions of the *gdh-NAD* genes described from other higher plants, the phylogeny of the *gdh-NAD* gene family was constructed (Fig. 1B). The topology of the phylogenetic tree shows two main clusters: one for the α-subunits, including the newly identified *Slgdh-NAD;A1-3* genes, and another one containing all the genes encoding the β-subunit, and including the single *Slgdh-NAD;B1* (Fig. 1B).

To characterize the genomic structure of *Slgdh-NAD;A1-3*, the sequences of the identified cDNA clones corresponding to *Slgdh-NAD;A1* (GenBank accession no. BT012758 and AK325267) were

Table 1
Tomato Unigenes with significant identities with *Slgdh-NAD* genes. The SGN Tomato Combined – WGS, BAC, and Unigene sequences of Tomato 200607#2 was queried with the coding region of *Slgdh-NAD;B1* (1,233 nt from AF403178).

Unigene identifier	ESTs	mRNA sequence length (bp)	Annotation	Identity (%)	Gene name
SGN-U578318	67	2114	GDH1 from *A. thaliana* (At5g18170) and *S. lycopersicum* (AF403178)	100	*Slgdh-NAD;B1*
SGN-U574592	10	1647	GDH2 from *A. thaliana* (At5g07440) and GDHA *N. plumbaginifolia* (Y08292)	80	*Slgdh-NAD;A1*
SGN-U569234	3	619	GDH2 from *A. thaliana* (At5g07440) and GDHA *N. plumbaginifolia* (Y08292)	78	*Slgdh-NAD;A2*

aligned with sequences of *Slgdh-NAD;A2* and *Slgdh-NAD;A3*. This revealed that all *Slgdh-NAD;A1-3* genes have similar genomic organization, with nine exons separated by eight introns of different lengths (Fig. 1C). These *Slgdh-NAD* genes are located in different chromosomes in *Solanum lycopersicum* (Table 2, Suppl. Fig. 2). These data suggest the existence of three newly identified *Slgdh-NAD* genes of the *gdh-NAD;A* type in the tomato genome.

Putative conserved domains in the GDH deduced amino acid sequences

The deduced amino acid sequences of all four tomato GDH encoding genes contained 411 residues, and the estimated molecular masses are summarized in Table 2. The alignment of the three GDH α-subunits showed a high degree of similarity, although they presented a heterogeneity region between residues 254–271 (Fig. 2). The GDH β-subunit showed more dissimilarity in the region between residues 251 and 281, which contained several negatively charge residues in the GDH α-subunits. These data are coincident with those shown in Suppl. Fig. 3, where it can be seen that the sequence conservation in each subunit (α or β) of the protein family is higher than that of both subunits in intra plant species comparisons. Additionally, the GDH α-subunit, but not the GDH β-subunit, contained a Ca^{2+}-binding variant of a helix-loop-helix motif known as EF-hand motif (Grabarek, 2006) reaching from residues Asp_{265} to Glu_{276} (see arrows in Fig. 2). All Cys residues are conserved among the four GDHs, except for Cys_{288}, which is conserved in the GDH α-subunits but not in the β–subunit, and Cys_{324}, which is present only in GDHA3 (Fig. 2). Nevertheless, prediction of disulfide bridges using the web tool DISULFIND (http://disulfind.dsi.unifi.it) was negative for GDH α- and β-subunits with high levels of confidence (Ceroni et al., 2006).

The deduced amino acid sequence of the GDH α-subunit was screened for conserved domains at the Conserved Domain Database (CDD at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml). A Glu/Leu/Phe/Val dehydrogenase dimerization domain, a NAD(P) binding site and a multidomain PLN02477 were detected in several GDH and predicted proteins from plants and *Chlamydomonas reinhardtii* (Suppl. Fig. 4) (Marchler-Bauer et al., 2009). Additionally, several putative phosphorylation and N-myristoylation sites were found in the deduced amino acid sequence of the GDH α-subunit when queried in the Predict Protein web tool (Rost et al., 2004).

As expected for a mitochondrial enzyme, the four GDH deduced amino acid sequences possess the predicted mitochondrial target peptides (18 amino acid residues at the N terminus), which were found by TargetP Web server (http://www.cbs.dtu.dk/services/TargetP/) with scores of 0.398 for GDHB1 and 0.718, 0.713 and 0.647 for GDHA1 to GDHA3.

Slgdh-NAD expression and distribution patterns of GDH α- and β-subunits in different tomato cv. Micro-Tom organs

Expression patterns of the *Slgdh-NAD;A1-3* and *Slgdh-NAD;B1* genes were examined in different organs of Micro-Tom plants by qRT-PCR (Fig. 3A). For this experiment, leaf transcript levels were taken as reference and the *Rpl2* gene was used for normalization (Balbi and Lomax, 2003). The transcripts of *Slgdh-NAD;B1* were much more abundant in roots than in any other organ. For the joint detection of all three *gdh* α-subunit transcripts (*Slgdh-NAD;A1-3*), a primer common for the three genes, which did not anneal to any region of the *Slgdh-NAD;B1* gene, was used. The expression pattern of *Slgdh-NAD;A1-3* in the tissues examined was different from that of *Slgdh-NAD;B1* (Fig. 3A), with green fruit showing the highest transcript levels for *Slgdh-NAD;A1-3*. When primers specific for each *Slgdh-NAD;A1-3* gene were used (Fig. 3A lower panels), predominant expression of *Slgdh-NAD;A1 and Slgdh-NAD;A2* was observed in mature green fruit, while *Slgdh-NAD;A3* show no expression in the tissues examined (data not shown).

To assess the functionality of the tomato GDH in plants, an in-gel assay was used to detect activity of GDH in different tomato organs (cv. Micro-Tom) (Fig. 3B). It is interesting to note that the organs analysed have specific oligomeric isoforms shown by GDH activity. Particularly, both in fruits and leaf seven active isoenzymes of GDH were observed, whereby the activity was differently distributed among the GDH isoforms. Thus, the more cathodic isoform of the enzyme (GDHB) predominated in leaf, while the anodic and the cathodic GDH isoforms seemed to be equally active in fruits. In root, however, the activity was due mainly to a unique oligomeric state of the GDH isoform, which is coincident with a homohexamer composed of β-subunits.

To distinguish between α- and β-subunits, immunoblot analyses were performed in protein extracts from different tomato organs after electrophoresis in native as well as in denaturing polyacrylamide gels. The immunoblots used antisera that reacted with both GDH subunits (anti-α+ β) or specifically with the α-subunit (anti-α). The cross-reactivity of both antibodies against each GDH recombinant protein was checked (Suppl. Fig. 5). The GDH β-subunit was hardly detected using anti-α antiserum (less than 0.5 μg protein), while both GDH subunits were quite visible using anti-α+ β antibody (Suppl. Fig. 5). Immunoblot analyses of protein extracts from leaf, root, mature green and ripe fruits in

Table 2
Structure of tomato *Slgdh-NAD* genes.

Tomato WGS scaffolds (2.40)	Chromosome position (in cM)	Gene	Length (nt)[a]	Predicted protein mass (Da)
SL2.40sc04199	10 (67)	*Slgdh-NAD;B1*	3038	44,689
SL2.40sc03796	3 (75.1)	*Slgd-NAD;A1*	3258	44,712
SL2.40sc03902	5 (101.7)	*Slgdh-NAD;A2*	5100	44,570
SL2.40sc04279	6 (10.9)	*Slgd -NAD;A3*	3992	44,731

[a] The length of each nucleotide sequence is given from the proposed ATG start to the stop codon.

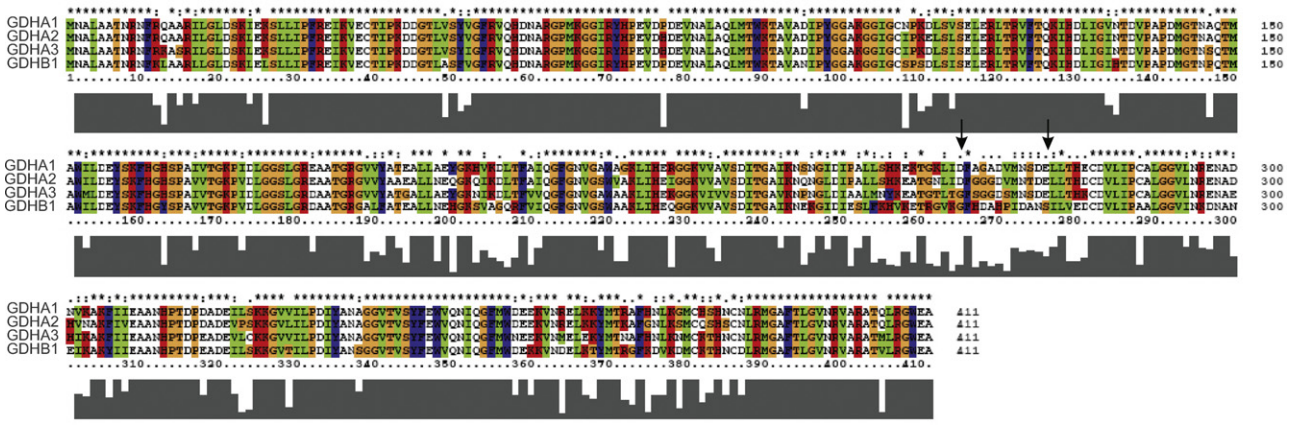

Fig. 2. Alignment of deduced amino acid sequences of GDH proteins from *Slgdh-NAD* genes found in tomato. Alignment of GDHA (GDHA1, GDHA2 and GDHA3 amino acid sequences from *Slgdh-NAD;A1-3*) and GDHB1 (the deduced amino acid sequence of *Slgdh-NAD;B1*; GenBank accession No. AAL36888) (see Table 1). Arrows indicate the EF-hand motif from GDHA expanding from Asp_{265} to Glu_{276}.

native as well as in denaturing gels using both antisera are shown in Fig. 3C and D. It can be observed that the seven isoforms of GDH are active and similarly distributed in mature green and red fruits. In the leaf, several oligomeric GDH isoforms were detected, although the prevalent isoforms were those constituted by β-subunits. The relatively lower transcript level found for *Slgdh-NAD;B1* in fruits in comparison with leaf (Fig. 3A) was not coincident with the protein level found in these tissues (Fig. 3D). In root, GDH seemed to be constituted mainly by the β-subunit, while the α-subunit was barely detected under the experimental conditions assayed (Fig. 3C and D). In this tissue, the relative content of the *Slgdh-NAD;B1* transcript and protein levels with respect to those in leaf was similar.

These results suggest that the level of GDH α- and β-subunits in tomato plants is regulated differently in each tomato organ, considering the specific patterns present in all the organs analysed.

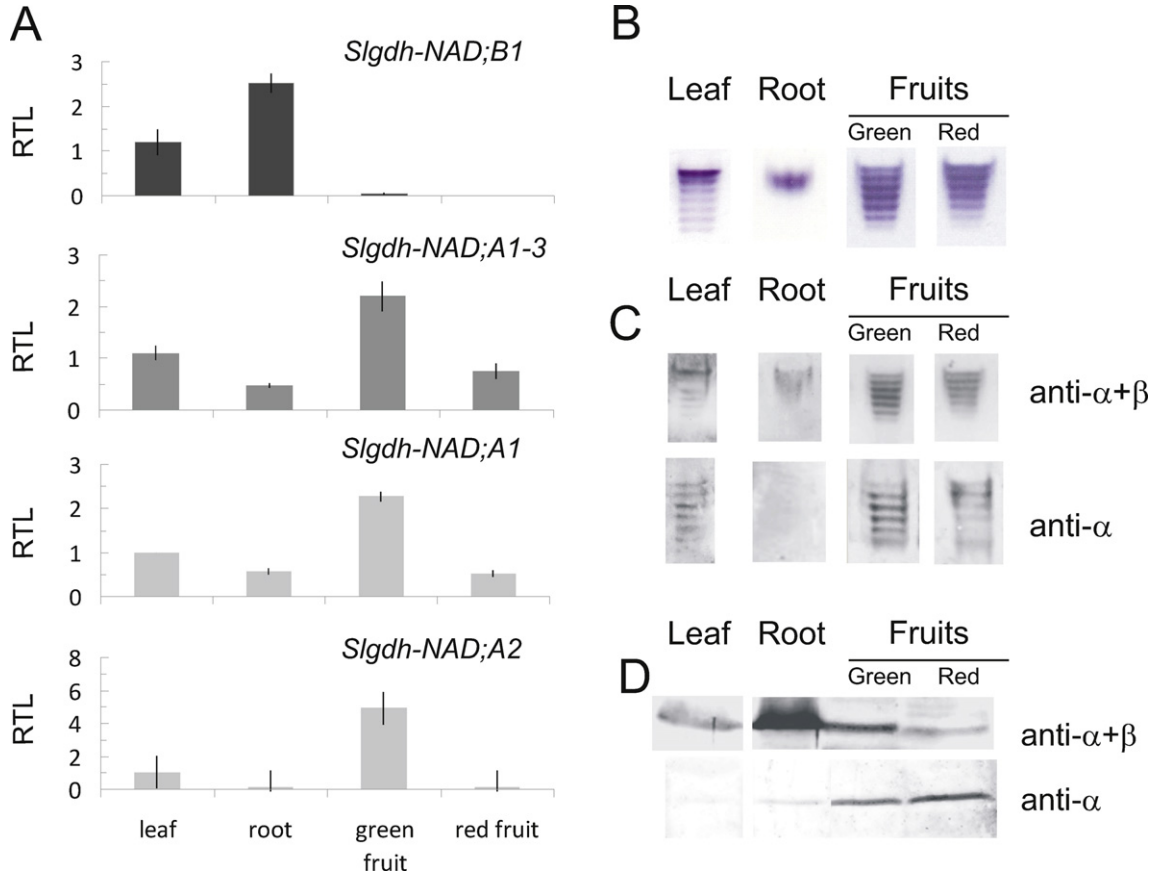

Fig. 3. *Slgdh-NAD* transcript, protein and activity levels of different tomato organs. (A) Relative transcript levels of *Slgdh-NAD;B1*, *Slgdh-NAD;A1-3*, *Slgdh-NAD;A1* and *Slgdh-NAD;A2* genes. Total RNA of fruits were retro-transcribed using a poly-dT primer, and then subjected to real-time PCR analysis using specific oligonucleotides. Leaf transcripts were arbitrarily fixed to 1. Results are the means of at least three independent experiments ± SE. The experimental data were subjected to Student's *t* test analysis ($p < 0.05$). (B) GDH activity in non-denaturing polyacrylamide gels. (C) Immunoblot analysis of GDH in non-denaturing polyacrylamide gels. (D) Immunoblot analysis of GDH protein in SDS-PAGE. Representative images from different parts of the same gel from several immunoblot analyses are shown in (B-D).

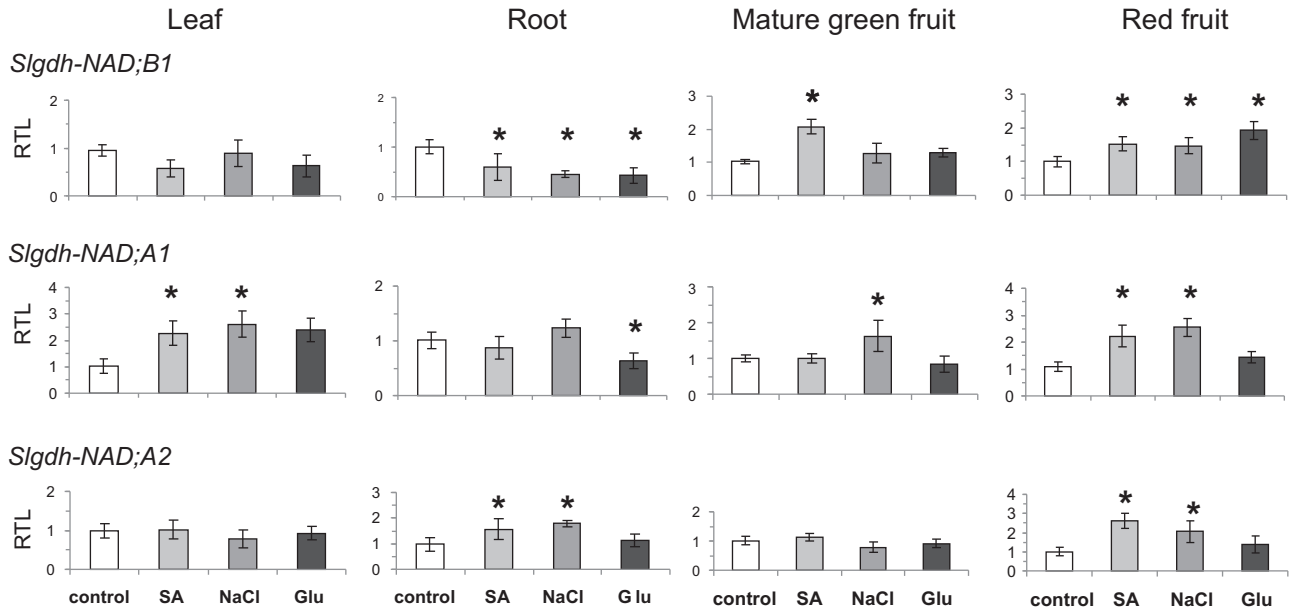

Fig. 4. Relative transcript levels of *Slgdh-NAD* genes in tissues from tomato plants subjected to different treatments. Groups of plants containing green fruits were soaked in water (control), 2.5 mM SA, 100 mM NaCl or 20 mM glutamate for five days. For the salt treatment, plants were previously soaked with 50 mM NaCl for one day. After the treatment, tissues were harvested and RNA isolated. Transcripts from tissues without treatment (control) were arbitrarily fixed to 1. Results are the means of three independent experiments ± SD. The experimental data were subjected to ANOVA followed by Holm Sidak test analysis ($p < 0.05$). Asterisks indicate significant data.

Regulation of Slgdh-NAD expression by glutamate and stress-related molecules

To further analyse the behaviour of these genes in different tomato organs from plants subjected to modified conditions, tomato cv. Micro-Tom plants carrying green and breaker fruits were soaked for five days with different solutions, including glutamate (20 mM). The content of this amino acid shows a large increase in fruits during ripening (Sorrequieta et al., 2010). The other treatments included the addition of SA (2.5 mM) or NaCl (100 mM), using water soaking as control. These molecules are related to biotic and abiotic stresses. At the end of the treatments, organs were collected, total RNA isolated and transcripts quantified by qRT-PCR. Data are presented as means ± SD of three independent experiments. The results show that *Slgdh-NAD;B1* was responsive to glutamate in root and red fruit showing an opposite pattern of transcription, *i.e.*, *Slgdh-NAD;B1* transcript level was significantly increased in red fruit but decreased in root (Fig. 4). A similar opposite effect on *Slgdh-NAD;B1* transcription was caused by NaCl and SA in these two organs. In addition, SA significantly increased *Slgdh-NAD;B1* transcript level in green fruit. Nevertheless, *Slgdh-NAD;B1* transcription was unresponsive to glutamate and NaCl in green fruit and to all these treatments in the leaf. On the other hand, *Slgdh-NAD;A1* transcript level significantly increased in the leaf and in red fruit by SA and NaCl, and in green fruit by NaCl, while glutamate decreased its level in root. The *Slgdh-NAD;A2* transcript level increased by SA and NaCl in root and in red fruit, but it was unaffected by glutamate, SA or NaCl in leaf and green fruit (Fig. 4). *Slgdh-NAD;A3* showed no expression in any of the tissues examined. In summary, these data suggest that glutamate up-regulated *Slgdh-NAD;B1* expression in red fruit and down-regulated *Slgdh-NAD;A1* and *Slgdh-NAD;B1* expression in the root, without causing a detectable effect on *Slgdh-NAD;A2* expression in any of the tissues examined. Stress-related molecules such as SA and NaCl up-regulated *Slgdh-NAD;B1*, *Slgdh-NAD;A1* and *Slgdh-NAD;A2* expression in red fruit, *Slgdh-NAD;A1* expression in the leaf, *Slgdh-NAD;A2* expression in the root, and down-regulated *Slgdh-NAD;B1* expression in the root.

Additional functional characterization of *Slgdh-NAD;B1 in planta* was performed in transgenic tomato plants harbouring the 900 bp of the *Slgdh-NAD;B1* promoter sequence upstream the *β-glucuronidase* reporter gene. Under physiological conditions, no detectable levels of GUS staining were found in root, leaf or fruit of transgenic tomato plants (Fig. 5). *Slgdh-NAD;B1* expression was induced in all these organs by treatment with SA (Fig. 5). Moreover, the *Slgdh-NAD;B1* promoter was activated by glutamate in root (Fig. 5B) and fruit (Fig. 5C), and NaCl enhanced *Slgdh-NAD;B1* expression in fruit only (Fig. 5C).

Discussion

This study reports about the structure of four different tomato *loci* encoding *Slgdh-NAD* genes and an initial functional characterization. The starting point was a genomic clone spanning the full-length sequence encoding the GDH β-subunit and its regulatory regions (GenBank accession no. AF403178 and AY768544). An *in silico* search within the tomato genome allowed the identification of three novel genes encoding the α-subunit of GDH. Transcripts for *Slgdh-NAD;A1* and *Slgdh-NAD;A2* were detected by qPCR and analysis of ESTs, indicating that they are functional genes. In the case of *Slgdh-NAD;A3*, transcripts were not detected under the conditions and tissues investigated in this study, but it cannot be ruled out that it may be functional in other cell types or developmental stages or stress-induced conditions. Remarkably, *Slgdh-NAD;A3* orthologues were found in other Solanaceae species (*S. pennellii*, *S. pimpinellifolium* and *N. tabacum*) suggesting that it is a conserved gene and, by implication, functional. As a whole, in contrast to other plants and, in particular, dicots, and similarly to rice (Qiu et al., 2009), tomato has more than one gene encoding the GDH α-subunit.

The deduced amino acid sequence of the GDH α-subunit (Fig. 2) possessed a consensus EF-hand sequence (Grabarek, 2006) at 265–276 residues. This motif is absent in the GDH β-subunit. Putative Ca^{2+} binding domains have been found in a similar position in the gene products of *Npgdh-NAD;A1* (Ficarelli et al., 1999) and *Atgdh-NAD;A1* (Turano et al., 1997). The key attribute

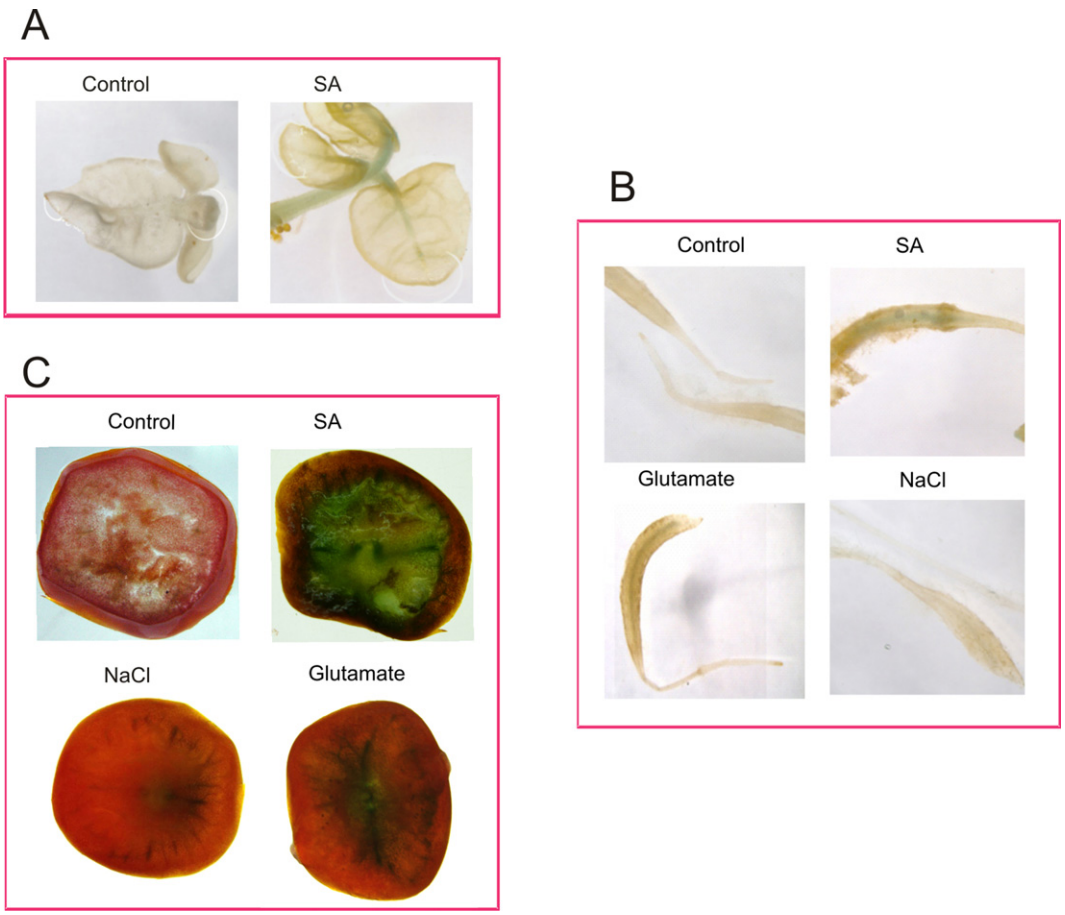

Fig. 5. Expression of *GUS* in transgenic tomato plants carrying a fragment of *Slgdh-NAD;B1* promoter after different treatments. Promoter activity was followed by histochemical localization of GUS activity in leaf (A), root (B) and red fruit (C) of transgenic plants after treatments with water (control), SA, NaCl or glutamate. Representative images are shown.

of the regulatory EF-hand proteins is the ability to change their conformation upon Ca^{2+} binding, thus acquiring different interactive properties (Grabarek, 2006). This might be relevant for the biological responses of GDH arrangements with different proportions of α- and β-subunits.

GDH activity was detected in all the Micro-Tom organs analysed. However, the composition of the GDH protein complex varied. In root, GDH seemed to be constituted solely by a homohexamer of β-subunits (Fig. 3C). This is consistent with the predominance of *Slgdh-NAD;B1* transcripts in this organ (Fig. 3A, upper panel). This may differ from that observe in rice, where the two GDH genes that were predominantly expressed in roots, *OsGDH1* and *OsGDH2*, probably encode GDH β- and α-subunits, respectively (Qiu et al., 2009). In tomato leaf and fruits, the active hexamers were constituted by both subunit types. However, the relative abundance, and hence the composition of the GDH complexes, varied. In leaf, a higher proportion of the β-subunit homohexamer was found, while there was a higher proportion of the α-subunit in fruits (Fig. 3C and D). Although *Slgdh-NAD;A1-3* transcripts were higher in mature green tomato fruits than in leaf (Fig. 3A, lower panels), the *Slgdh-NAD;B1* transcript level in fruits was rather low in relation to the transcript level in the leaf (Fig. 3A, upper panel). Thus, a direct correlation of *Slgdh-NAD;B1* transcript level with GDH activity in fruits did not seem possible.

There is growing evidence that GDH is a stress-responsive protein (Skopelitis et al., 2006). It has been shown that NaCl treatment induces transcription of the *gdh-NAD;A1* genes encoding the GDH α-subunits in tobacco and grapevine (Skopelitis et al., 2006). To analyse the regulation of the expression of these genes, tomato plants were soaked with glutamate and molecules related to biotic (SA) and abiotic stresses (NaCl) and the abundance of *Slgdh-NAD;B1* and *Slgdh-NAD;A1-3* transcripts was evaluated in different tissues by qPCR (Fig. 4). In a previous work, red fruits from salt-stressed Micro-Tom plants (160 mM NaCl) showed a two-fold increase in glutamate content in the pericarp of red fruits compared with those found in control conditions (Yin et al., 2010). In another tomato cultivar NaCl (25–100 mM) increased GDH activity in leaves and roots (Debouba et al., 2006). In the present study, glutamate significantly enhanced *Slgdh-NAD;B1* expression in tomato cv. Micro-Tom red fruit (Fig. 4). Glutamate represented more than 50% of the free amino acid content in tomato cv. Micro-Tom ripe fruit (Sorrequieta et al., 2010). This is consistent with the proposal that GDH might deaminate glutamate when this amino acid is in excess, and thus provide 2-oxoglutarate to the tricarboxylic acid cycle, as previously proposed for Arabidopsis GDH (Miyashita and Good, 2008). On the other hand, *Slgdh-NAD;B1* transcripts and protein levels showed high variation depending on the organ analysed, indicating the existence of tissue-specific post-translational regulatory mechanisms that probably adjust the level of β-subunit to modulate the abundance of every heterohexameric GDH isoform. It is interesting to note that *Slgdh-NAD;B1* expression was oppositely regulated by glutamate and stress-related molecules in root and fruits. In red fruit from tomato plants soaked for five days with SA and NaCl, *Slgdh-NAD;B1*, *Slgdh-NAD;A1* and *Slgdh-NAD;A2* expressions were all up-regulated. Nevertheless, in the leaf and root, SA and NaCl seem to only modulate *Slgdh-NAD;A1* and *Slgdh-NAD;A2* expressions, respectively. *Slgdh-NAD;A1-3* transcripts were undetected by these treatments (data not shown). These results suggest that

Slgdh-NAD;B1 is oppositely regulated by glutamate in root and red fruit and, together with *Slgdh-NAD;A1* and *Slgdh-NAD;A2*, are differently regulated by molecules related to stress, such as SA and NaCl, depending on the tissue examined. In agreement with this, SA and NaCl enhanced GUS expression driven by a −900 bp *Slgdh-NAD;B1* promoter in fruits. SA has important roles related to biotic stresses and has been implicated in both local and systemic responses to disease-causing microorganisms (Loake and Grant, 2007).

In summary, in this study a full-length gene and its promoter region, encoding a tomato GDH β-subunit and three new *Slgdh-NAD;A1-3* genes, two of which are highly expressed in tomato fruits, were identified, isolated and functionally characterized. These four genes are derived from four independent *loci*. We found that distinct GDH subunits predominate in different tissues; *Slgdh-NAD;A1-3* occurs mainly in fruits and *Slgdh-NAD;B1* mainly in roots at the level of expression, protein abundance and enzyme activity. Stress-related molecules enhanced expression of the three genes in fruit, and also *Slgdh-NAD;A1* in leaf and of *Slgdh-NAD;A2* in root. Additionally, glutamate up-regulated the expression of *Slgdh-NAD;B1* in red fruit. These results suggest that the β-subunit modulate the heteromeric isoforms of GDH in response to the environment and the physiology of ripe tomato cv. Micro-Tom fruit.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jplph.2012.02.002.

References

Almeida J, Quadrana L, Asis R, Setta N, de Godoy F, Bermudez L, et al. Genetic dissection of vitamin E biosynthesis in tomato. J Exp Bot 2011;62:3781–98.
Balbi V, Lomax TL. Regulation of early tomato fruit development by the diageotropica gene. Plant Physiol 2003;131:186–97.
Boggio SB, Palatnik JF, Heldt HW, Valle EM. Changes in amino acid composition and nitrogen metabolizing enzymes in ripening fruits of *Lycopersicon esculentum* Mill. Plant Sci 2000;159:125–33.
Bortolotti S, Boggio SB, Delgado L, Orellano EG, Valle EM. Different induction patterns of glutamate metabolising enzymes in ripening fruits of the tomato mutant *green flesh*. Physiol Plant 2003;119:384–91.
Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. Anal Biochem 1976;72:248–54.
Ceroni A, Passerini A, Vullo A, Frasconi P. DISULFIND: a disulfide bonding state and cysteine connectivity prediction server. Nucleic Acids Res 2006;34:W177–81.
Debouba M, Gouia H, Suzuki A, Ghorbel MH. NaCl stress effects on enzymes involved in nitrogen assimilation pathway in tomato "*Lycopersicon esculentum*" seedlings. J Plant Physiol 2006;163:1247–58.
Dubois F, Tercé-Laforgue T, Gonzalez-Moro MB, Estavillo JM, Sangwan R, Gallais A, et al. Glutamate dehydrogenase in plants: is there a new story for an old enzyme? Plant Physiol Biochem 2003;41:565–76.
Ficarelli A, Tassi F, Restivo FM. Isolation and characterization of two cDNA clones encoding for glutamate dehydrogenase in *Nicotiana plumbaginifolia*. Plant Cell Physiol 1999;40:339–42.
Forde BG, Lea PJ. Glutamate in plants: metabolism, regulation, and signalling. J Exp Bot 2007;58:2339–58.
Grabarek Z. Structural basis for diversity of the EF-hand calcium-binding proteins. J Mol Biol 2006;359:509–25.
Kamenetzky L, Asis R, Bassi S, de Godoy F, Bermudez L, Fernie AR, et al. Genomic analysis of wild tomato introgressions determining metabolism- and yield-associated traits. Plant Physiol 2010;152:1772–86.
Kisaka H, Kida T. Transgenic tomato plant carrying a gene for NADP-dependent glutamate dehydrogenase (*gdhA*) from *Aspergillus nidulans*. Plant Sci 2003;164:35–42.
Kumar S, Tamura K, Jakobsen IB, Nei M. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 2001;17:1244–5.
Labboun S, Terce-Laforgue T, Roscher A, Bedu M, Restivo FM, Velanis CN, et al. Resolving the role of plant glutamate dehydrogenase I. In vivo real time nuclear magnetic resonance spectroscopy experiments. Plant Cell Physiol 2009;50:1761–73.
Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001;25:402–8.
Loake G, Grant M. Salicylic acid in plant defence—the players and protagonists. Curr Opin Plant Biol 2007;10:466–72.
Loulakakis KA, Roubelakis-Angelakis KA. Plant NAD(H)-glutamate dehydrogenase consists of two subunit polypeptides and their participation in the seven isoenzymes occurs in an ordered ratio. Plant Physiol 1991;97:104–11.
Loulakakis KA, Roubelakis-Angelakis AK, Kanellis AK. Regulation of glutamate dehydrogenase and glutamine synthetase in avocado fruit during development and ripening. Plant Physiol 1994;106:217–22.
Marchler-Bauer A, Anderson JB, Chitsaz F, Derbyshire MK, Weese-Scott C, Fong JH, et al. CDD: specific functional annotation with the conserved domain database. Nucleic Acids Res 2009;37:D205–10.
Meissner R, Jacobson Y, Melamed S, Levyatuv S, Shalev G, Ashri A, et al. A new model system for tomato genetics. Plant J 1997;12:1465–72.
Miyashita Y, Good AG. NAD(H)-dependent glutamate dehydrogenase is essential for the survival of *Arabidopsis thaliana* during dark-induced carbon starvation. J Exp Bot 2008;59:667–80.
Purnell MP, Botella JR. Tobacco isoenzyme 1 of NAD(H)-dependent glutamate dehydrogenase catabolizes glutamate in vivo. Plant Physiol 2007;143:530–9.
Purnell MP, Stewart GR, Botella JR. Cloning and characterisation of a glutamate dehydrogenase cDNA from tomato (*Lycopersicon esculentum* L.). Gene 1997;186:249–54.
Purnell MP, Skopelitis DS, Roubelakis-Angelakis KA, Botella JR. Modulation of higher-plant NAD(H)-dependent glutamate dehydrogenase activity in transgenic tobacco via alteration of beta subunit levels. Planta 2005;222:167–80.
Qiu X, Xie W, Lian X, Zhang Q. Molecular analyses of the rice glutamate dehydrogenase gene family and their response to nitrogen and phosphorous deprivation. Plant Cell Rep 2009;28:1115–26.
Rost B, Yachdav G, Liu J. The PredictProtein server. Nucleic Acids Res 2004;32:W321–6.
Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: a laboratory manual. Cold Spring Harbor: Cold Spring Harbor Laboratory Press; 1989.
Scarpeci TE, Marro ML, Bortolotti S, Boggio SB, Valle EM. Plant nutritional status modulates glutamine synthetase levels in ripe tomatoes (*Solanum lycopersicum* cv Micro-Tom). J Plant Physiol 2007;164:137–45.
Skopelitis DS, Paranychianakis NV, Paschalidis KA, Pliakonis ED, Delis ID, Yakoumakis DI, et al. Abiotic stress generates ROS that signal expression of anionic glutamate dehydrogenases to form glutamate for proline synthesis in tobacco and grapevine. Plant Cell 2006;18:2767–81.
Skopelitis DS, Paranychianakis NV, Kouvarakis A, Spyros A, Stephanou EG, Roubelakis-Angelakis KA. The isoenzyme 7 of tobacco NAD(H)-dependent glutamate dehydrogenase exhibits high deaminating and low aminating activities in vivo. Plant Physiol 2007;145:1726–34.
Smith TJ, Stanley CA. Untangling the glutamate dehydrogenase allosteric nightmare. Trends Biochem Sci 2008;33:557–64.
Sorrequieta A, Ferraro G, Boggio SB, Valle EM. Free amino acid production during tomato fruit ripening: a focus on L-glutamate. Amino Acids 2010;38:1523–32.
Thompson JD, Higgins DG, Gibson TJ, Clustal W. Improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994;22:4673–80.
Turano FJ, Dashner R, Upadhyaya A, Caldwell CR. Purification of mitochondrial glutamate dehydrogenase from dark-grown soybean seedlings. Plant Physiol 1996;112:1357–64.
Turano FJ, Thakkar SS, Fang T, Weisemann JM. Characterization and expression of NAD(H)-dependent glutamate dehydrogenase genes in Arabidopsis. Plant Physiol 1997;113:1329–41.
Yin YG, Tominaga T, Iijima Y, Aoki K, Shibata D, Ashihara H, et al. Metabolic alterations in organic acids and gamma-aminobutyric acid in developing tomato (*Solanum lycopersicum* L.) fruits. Plant Cell Physiol 2010;51:1300–14.