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PHYSIOLOGY AND BIOCHEMISTRY

Molecular analyses of the rice *glutamate dehydrogenase* gene family and their response to nitrogen and phosphorous deprivation

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Abstract Glutamate dehydrogenases (GDH, EC 1.4.1.2 ~ 4) are ubiquitous enzymes encoded by *GDH* genes. So far, at least two *GDH* members have been characterized in plants, but most members of this family in rice remains to be characterized. Here, we show that four putative *GDH* genes (*OsGDH1-4*) are present in the rice genome. The GDH sequences from rice and other species can be classified into two types (I and II). *OsGDH1-3* belonged to type II genes, whereas *OsGDH4* belonged to type I like gene. Our data implied that the expansion rate of type I genes was much slower than that of type II genes and species-specific expansion contributed to the evolution of type II genes in plants. The expression levels of the different members of *GDH* family in rice were evaluated using quantitative real-time PCR and microarray analysis. Gene expression patterns revealed that *OsGDH1*, *OsGDH2*, and *OsGDH4* are expressed ubiquitously in various tissues, whereas *OsGDH3* expression is glumes and stamens specific. The expression of the *OsGDH* family members responded differentially to nitrogen and phosphorus-deprivation, indicating their roles under such stress conditions. Implications of the expression patterns with respect to the functions of these genes were discussed.

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Abbreviations

GDH	Glutamate dehydrogenase
Glu	Glutamate
GOGAT	Glutamate synthase
GS	Glutamine synthetase
HCA	Hierarchical cluster analysis
N^-	Nitrogen depriving or nitrogen-free solution
P^-	Phosphorus depriving or phosphorus-free solution
qPCR	Quantitative real-time polymerase chain reaction
α-KG	α-Ketoglutarate

Introduction

Glutamate dehydrogenase (GDH, EC 1.4.1.2 ~ 4) is a family of enzymes catalyzing a reversible deamination of L-glutamate to 2-oxoglutarate or α-ketoglutarate (α-KG), directly connected to the Krebs cycle (Fisher 1985) and thereby plays a key role by providing a link between carbon and nitrogen metabolism. The GDHs are ubiquitous and present in bacteria as well as in most sub-cellular compartments of the eukaryotic cell. This enzyme family has drawn more attention because assimilation of ammonia via GDH is more energy-efficient than via glutamate synthase (GOGAT, Fd-GOGAT: EC 1.4.7.1 and NADH-GOGAT: EC 1.4.1.14) (Windass et al. 1980; Helling 1998), which suggested that the potential industrial or agricultural savings could be made by identification of features that incur "energy penalty". Before the discovery of the

glutamine synthetase (GS, EC 6.3.1.2)/GOGAT cycle (Lea and Miflin 1974), GDH was considered to be a key enzyme in ammonium assimilation. Since then, many attempts have been made to redefine the physiological role(s) of GDH, but this has still remained obscure (Dubois et al. 2003; Masclaux-Daubresse et al. 2006; Lehmann and Ratajczak 2007; Purnell and Botella 2007; Skopelitis et al. 2007; Miyashita and Good 2008).

The GDH is abundant in several plant organs, and was shown to be localized in mitochondria, chloroplasts, and cytosol (Dubois et al. 2003). Several reports have shown that GDH in plants are encoded by at least two genes, each encoding α or β subunits which randomly associate to give rise to the various hexameric isoenzymes (Loulakakis and Roubelakis-Angelakis 1991; Melo-Oliveira et al. 1996; Purnell et al. 2005; Miyashita and Good 2008). In Arabidopsis (*Arabidopsis thaliana*), two *GDH* genes have been cloned and their expression was shown to differ with development, organ/tissue type, and nutritional conditions (Melo-Oliveira et al. 1996; Turano et al. 1997; Miyashita and Good 2008). And recently, another two putative *GDH* sequences have been found in public databases (Inokuchi et al. 2002; Purnell et al. 2005; Miyashita and Good 2008). Two unique *GDH* genes were cloned from tobacco (*Nicotiana plumbaginifolia*) and their expression was shown to differ with environmental signals, such as salinity, temperature shifts, and heavy metals (Restivo 2004). In rice (*Oryza sativa*), three *GDH* genes (*OsGDH1*, *OsGDH2*, and *OsGDH3*) were cloned. The expression of *OsGDH1* and *OsGDH2* was induced by NH_4Cl in seedling roots, while *OsGDH3* was unexpressed (Abiko et al. 2005). Based on available evidence, it is tempting to speculate that specific *GDH* shows differential regulation and their expression patterns have functional significance. However, the evolutionary relationship between the presently known *GDH* genes and the degree of possible functional redundancy is still unclear.

An earlier comparison of GDHs from plants and archaebacteria shows that the plant isoforms are evolutionarily more close to the archaebacterial isoforms than to the vertebrate mitochondrial (human and Drosophila) (Syntichaki et al. 1996). Comparison of the GDHs from monocotyledons and dicotyledons shows that plant *GDH* genes may have been derived from common ancestors (Pavesi et al. 2000; Purnell et al. 2005). However, the broader evolutionary histories of the *GDH* genes in the plants remain unclear since few studies have investigated the evolution of the *GDH* genes in a wide range of plants. Recently, Andersson and Roger (2003) proposed that lateral gene transfer may play a significant role in the evolution of *GDH* family, at least among eubacteria. However, the details of the gene duplications and deletions experienced by the *GDH* genes in most groups of the plants remains unclear. Thus, additional studies are needed to understand the evolutionary history of the *GDH* genes in various plant groups, such as unicellular green algae (*Chlamydomonas reinhardtii* and *Chlorella sorokiniana*) and moss *Physcomitrella patens*.

A plausible way to solve the questions posed at present about the plant GDHs could be a systematic functional identification of all of them in a specific plant. Rice is the staple food for almost half of the world population, and it has become a model plant of monocot species for functional genomics and gene function studies. However, limited knowledge of the *GDH* gene family is available in this species (Abiko et al. 2005). Therefore, a molecular approach is needed to characterize GDHs in the rice species by analyzing the structure and expression of this gene family. As part of systematic work aimed at comparing with GDH homolog from other species by sequence and phylogenetic analysis, herein we reported the expression patterns of the *GDH* gene family in the whole life cycle of rice plants and under depriving nitrogen or phosphorus stress conditions in young seedlings. In addition, we assigned genes in this family to different functions and/or pathways by expression analysis. It is expected that such a comprehensive analysis may provide a framework for future functional dissection of the *GDH* gene family in plant growth and development.

Materials and methods

Data search and analyses

To identify the members of the *GDH* gene family in rice (*OsGDH*), the protein family ID PF02812 or PF00208 was queried in the TIGR database (release 5, http://www.tigr.org/tdb/e2k1/osa1/domain_search.shtml). Information about the chromosomal localization, coding sequence (CDS) length, amino acid (aa) length, and full-length cDNA accessions was obtained for each gene from TIGR, NCBI (www.ncbi.nlm.nih.gov), and KOME (http://cdna01.dna.affrc.go.jp/cDNA/). For comparison, we also obtained the information for *GDH* family from Arabidopsis (*AtGDH*) from TAIR (release 8, http://arabidopsis.org/). To study the evolutionary relationships of *GDH* genes in rice and other plant species, GDH protein sequences were obtained from NCBI and confirmed with the Pfam database (Finn et al. 2006). The analyses included GDH sequences from six angiosperm species, three dicots (Arabidopsis, *Nicotiana plumbaginifolia*, and *Nicotiana tabacum*) and three monocots (rice, *Zea mays*, and *Asparagus officinalis*). In this analysis, we also included sequences from two unicellular green algae (*Chlamydomonas reinhardtii* and *Chlorella sorokiniana*) and the moss *Physcomitrella patens* subsp.

patens. In addition, the *Escherichia coli* (*E. coli*) GDH sequence was also included in the analysis for rooting purposes. For sequence and phylogenetic analyses, only the longest frame was used if a *GDH* gene has different splice transcripts.

Multiple sequences alignment of GDH proteins was performed using the CLUSTAL_X 1.83 (Thompson et al. 1997). Gaps in the alignments were removed manually. MEGA 4.0 (Tamura et al. 2007) was used to generate phylogenetic tree by using neighbor-joining algorithms. The reliability of different phylogenetic groupings was evaluated by using the bootstrap test (1,000 bootstrap replications) available in MEGA 4.0 (Tamura et al. 2007). MEME 3.5.7 (Bailey and Elkan 1994) was used to find conserved motifs in GDH group of the proteins. The parameters of this analysis were set with default settings, except that the maximum number of motifs to find was defined as 15. To predict putative functions of identified motifs, all motifs discovered by MEME were searched against the InterPro database using the InterProScan tool (http://www.ebi.ac.uk/Tools/InterProScan/). Gene structure display server (GSDS) program (Guo et al. 2007) was used to illustrate the structure of intron and exon of *OsGDH* genes according to the genome and CDS sequences from TIGR.

Plant materials, treatments, and tissue collection

Rice variety Minghui 63 (*Oryza sativa* ssp. *indica*) was used for detecting transcript levels of rice *GDH* genes under depriving nitrogen or phosphorus stress conditions. Rice seeds were treated and grown as described (Lian et al. 2006). Minghui 63 seedlings at the emergence of four-leaf stage were transferred from control solution (CK, 1.44 mM NH_4NO_3 and 0.3 mM NaH_2PO_4) to nitrogen-deficient (N^-, 0 mM NH_4NO_3) or phosphorus-deficient (P^-, 0 mM NaH_2PO_4) solutions. Roots and shoots were harvested separately at 1 h, 1 day, and 7 days after treatment. The planting and harvesting were conducted three times with an interval of 1 month for three biological replicates.

All materials prepared were immediately frozen in liquid nitrogen and stored at −70°C freezer.

qPCR analysis

For expression analyses of *OsGDH*, total RNA was prepared using Trizol reagent (Invitrogen, CA, USA. http://www.invitrogen.com). For qPCR analysis, 4 μg total RNA (10 μl reaction volume) was first treated with 2 U DNaseI (Invitrogen) and then reverse transcribed in a total volume of 20 μl with 0.5 μg oligo$(dT)_{15}$, 0.75 mM dNTPs, 10 mM dithiothreitol (DTT), and 200 U SuperScript II RNase H-reverse transcriptase (Invitrogen). qPCR was performed using gene-specific primers (Supplemental Table 1) in a total volume of 25 μl with 1.5 μl of the RT reactions, 0.25 μM gene-specific primers, and 12.5 μl SYBR Green Master mix (Takara Biotechnology, Japan) on a 7500 real-time PCR machine (Applied Biosystems, USA) according to the manufacturer's instructions. The rice *actin2* gene was used as control. The relative expression level of each *OsGDH* gene was determined in roots and in shoots under nitrogen and phosphorous deficient conditions separately, and in roots under normal conditions compared to shoots under normal conditions. Moreover, the expression level of each gene in treated plants was considered insignificant with the corresponding control plants if the expression levels were less than 2-fold. For each analysis, qPCR assays were repeated at least twice with each repetition having three replicates; similar results were obtained in repeated experiments.

Microarray analysis

Microarray data of rice *GDH* genes, as well as *OsGS* (genes code for rice glutamine synthetase) and *OsGOGAT* (genes code for rice glutamate synthase), were extracted from CREP database (http://crep.ncpgr.cn; Weibo Xie, unpublished data). In this database, hybridization was conducted using RNA samples obtained with at least two biological repeats from 39 tissues covering the entire life cycle of the plants from three genotypes of cultivated rice, Minghui 63, Zhenshan 97, and their hybrid Shanyou 63, an elite hybrid widely grown in China (Tan et al. 2000). For relevance, only hybridization data for 27 tissues of Minghui 63 were analyzed in this study: *Callus*, calli at 15 days after subculture; *Seed*, seed at 72 h after imbibition; *Radicle*, radicle at 48 h after emergence in the dark; *Plumule*, plumule at 48 h after emergence in the dark; *Seedling 1*, embryo and radicle after germination; *Seedling 2*, leaf and root at three-leaf stage; *Root*, root at seedling with 2 tillers; *Shoot*, shoot at seedling with 2 tillers stage; *Leaf 1*, leaf at young panicle of secondary branch primordium differentiation stage; *Leaf 2*, leaf at 4–5 cm young panicle stage; *FL 5DBH*, flag leaf at 5 days before heading; *FL 14DAH*, flag leaf at 14 days after heading stage; *Sheath 1*, sheath at young panicle of secondary branch primordium differentiation stage; *Sheath 2*, sheath at 4–5 cm young panicle stage; *Stem 5DBH*, stem 5 days before heading stage; *Stem HS*, stem at heading stage; *Panicle 3*, young panicle of secondary branch primordium differentiation stage; *Panicle 4*, young panicle at pistil/stamen primordium differentiation stage; *Panicle 5*, young panicle at pollen-mother cell formation stage; *Panicle 6*, panicle at 4–5 cm young panicle stage; *Panicle 7*, panicle at heading stage; *Stamen 1DBF*, stamen at one day before flowering stage; *Glume 1DBF*, glume at one day before flowering stage; *Spikelet*

3DAP, spikelet at 3 days after pollination stage; *Endo 7DAP*, endosperm at 7 days after pollination stage; *Endo 14DAP*, endosperm at 14 days after pollination stage; *Endo 21DAP*, endosperm at 21 days after pollination stage.

In performing the analysis, we included only genes with 100% identity over the entire length with the entire set of the probes for each gene, which were labeled 'Present' by Affymetrix MAS 5.0 with average signal values of more than 100. Data for only one probe of each gene were used for expression analysis. To get the expression values, average of two biological replicates for each tissue was used, except for five tissues (Seedlings 1, 2; Panicles 3, 4, and 5) which had six biological replicates each. The $\log_2$ ratio of expression values were clustered on the basis of Pearson correlation coefficients using R package (Eisen et al. 1998; http://www.R-project.org). In performing the clustering analysis, we used only those genes with expression values more than 100, in two or more tissues.

Results

Identification of rice *GDH* gene family

To obtain *GDH* gene family in rice, the protein family ID PF02812 was queried in TIGR database. This search identified four GDH-like protein genes, which are named *OsGDH1* to *OsGDH4* (Table 1). Three of the *OsGDH* genes have one or two differently spliced transcripts, with sequences collected in the databases (Table 1 and Supplemental Table 2); the transcripts resulting from alternative splicing of the same gene are named with the suffixes 1, 2 or 3 following the gene name (Table 1).

In addition to the four genes, one sequence (LOC_Os07g13460 or GenBank Accession Number: NM_001065791) encoding 5′–3′ exoribonuclease identical to *XRN4* in Arabidopsis (At1g54490 or AF286718) was found to have high similarity with *OsGDH1* (score: 87, 1.0e-17). But neither of the two feature domains for GDH (see below), PF02812 (ELFV_dehydrog_N, [LIV]XXGG [SAG]KX[GV]XXX[DNST][PL]) and PF00208 (ELFV_dehydrog, GXGXX[GA]) (Baker et al. 1992), was found in the putative amino acid sequence of Os07g13460. Therefore, Os07g13460 was not included in the following analysis.

Gene structure analysis of the *OsGDH* gene family

Comparison of the full-length cDNA sequence with corresponding genomic DNA sequence showed that the coding sequences of all the *OsGDH* genes are disrupted by at least two introns (Fig. 1). Alternative splicing was found in all *OsGDH* genes except *OsGDH3* (Fig. 1). This may reflect a mechanism for regulating the rice *GDH* gene structure. The splice site position of *OsGDH* genes with respect to the ORFs is referred to as the intron phase (Sharp 1981). An excess of phase 0 introns and symmetric exons (with the same phase on both ends) may facilitate exon shuffling, recombination, and exchange of protein domains by avoiding interruptions of the ORFs (Gilbert 1987; Patthy 1987). The *OsGDH* genes had a strong bias toward phase 0 introns relative to *AtGDH* genes (Fig. 1 and Supplemental Table 3), suggesting that the structures of rice *GDH* genes were more variable than those of Arabidopsis *GDH* genes based on the intron-early theory (Patthy 1987).

Alternative splicing is a mechanism invented by evolution to generate a variety of proteins derived from a same chromosomal locus via multiple combinations of individual peptide domains encoded by this locus (Kubo et al. 1999). And alternative splicing, allowing a cell to diversify its limited genetic capacity of the chromosomes, is strictly regulated and is often cell type-dependent or associated with a specific developmental stage of cells. This is very common in plants and significantly contributes to transcript and protein diversity in cells (Kazan 2003). In rice genome, about 21.2% genes display alternative

Table 1 Rice glutamate dehydrogenate (GDH) like protein gene family members

Family member	TIGR locus ID	Chromosome (cM position)	Length (bp)	Predicted peptide length (transcript)	Full-length cDNA GenBank accession no.	Genome	
						japonica	*indica*
OsGDH1	LOC_Os03g58040.1	3 (147.7–148.8)	810	269 (*OsGDH1.1*)	AK071839	AC090871	AAAA02011203.1
	LOC_Os03g58040.2	3 (147.7–148.8)	1236	411 (*OsGDH1.2*)	AY332470	AC090871	AAAA02011203.1
	LOC_Os03g58040.3	3 (147.7–148.8)	1236	411 (*OsGDH1.3*)	EST sequences	AC090871	AAAA02011203.1
OsGDH2	LOC_Os04g45970.1	4 (83.5–84.1)	1236	411 (*OsGDH2.1*)	AK063467	AL606728	AAAA02014474.1
	LOC_Os04g45970.2	4 (83.5–84.1)	510	169 (*OsGDH2.2*)	AK065301	AL606728	AAAA02014474.1
OsGDH3	LOC_Os02g43470.1	2 (107.4)	1236	411	AK103028	AP007203	AAAA02007030.1
OsGDH4	LOC_Os01g37760.1	1 (72.9–73.5)	1923	640 (*OsGDH4.1*)	AK120139	AP003443	AAAA02002655.1
	LOC_Os01g37760.2	1 (72.9–73.5)	1719	572 (*OsGDH4.2*)	EST sequences	AP003443	AAAA02002655.1

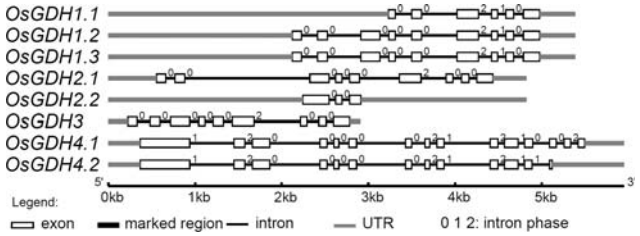

Fig. 1 Scaled diagram of exon–intron structure of the glutamate dehydrogenase genes in rice. 0, 1, 2 intron phase indicate splicing patterns: in phase 0, splicing occurs after the third nucleotide of the first codon; in phase 1, splicing occurs after the first nucleotide of the single codon; and in phase 2, splicing occurs after the second nucleotide

splicing events (Wang and Brendel 2006). The *gdh* in rice also show alternative splicing events with EST/cDNA evidence (Supplemental Table 2).

Phylogenetic and motif analysis of plant GDHs

A subset of plant GDH protein sequences from representative species was used to reconstruct the phylogeny of the gene family. The analysis included GDH sequences from six angiosperm species, three dicots, and three monocots, as well as sequences from the moss *Physcomitrella patens* and two unicellular green algae. In addition, the *E. coli* GDH sequence was included in the analysis for rooting purposes. These sequences exhibited very high degree of similarity by having two highly conserved domains (GenBank Accession Number: PF02812 and PF00208) (Supplemental Table 4). Therefore, the two regions were used for multiple sequence alignment (Fig. 2). Two features were present in PF02812 and PF00208 domains: a Glu/α-KG binding site and an NAD(P)-binding site (Baker et al. 1992). PF02812 domain was more conserved than PF00208 domain (Fig. 2), while within the PF00208 domain, amino acids present in N- and C-terminal regions are more conserved than ones present in the middle (Fig. 2b). A phylogenetic tree was constructed using only the conserved PF02812 domain (Supplemental Fig. 1). Two clusters, GDHI and GDHII cluster, as described by Inokuchi et al. (2002) were identified containing all *GDH* genes of Arabidopsis. All the Arabidopsis GDH proteins were found to lie in clusters similar to those identified previously, except that the more species were included in this study (Inokuchi et al. 2002).

A separate phylogenetic tree was also generated from GDH full-length protein sequences of all the 10 species. These proteins fell broadly into two major clusters (I and II), with well-supported bootstrap values (Fig. 3). Cluster I has only seven members with one member from rice (OsGDH4) and can be divided into subcluster IA with five members and IB with two members. All the members of this cluster have an NADPH-specific motif ($GXGX_2AX_{10}G$), except OsGDH4 whose motif sequence is slightly different ($GXGX_2AX_{10}E$) (Inokuchi et al. 2002). Thus, *OsGDH4* was named a type I like gene. Cluster II contains 17 members that can be further divided into subcluster IIA with eight members, IIB with five members, IIC with two members, and two divergence members (PpGDH1 and PpGDH2, two GDHs from *P. patens*). OsGDH1 belonged to subcluster IIA, while OsGDH2 and OsGDH3 were placed in IIB. PpGDH2 (a GDH from the moss *P. patens*) was located more closely to EcGDH (GDH from *E. coli*) than GDHs from the alga *C. reinhardtii*. Some putative orthologous and paralogous pairs can be identified from the tree (Fig. 3), such as NpGDH1 and NtGDH3, OsGDH1 and ZmGDH1 in subcluster IIA, NpGDH2 and NtGDH2, OsGDH2 and OsGDH3 in subcluster IIB, suggesting that duplication events within the GDH lineage occurred.

To understand the basis of divergence between the phylogenetic groups, the MEME tool for motif identification (Bailey and Elkan 1994) was employed. Fifteen distinct motifs were identified (Supplemental Fig. 2), of which, nine (motifs 5, 2, 3, 4, 14, 8, 7, 1, and 6) matched well with the conserved regions, PF02812 and PF00208, as revealed by multiple sequence alignment analysis (Fig. 2). Differences were also observed between cluster I and II. For example, motifs 13 (40 residues within N-terminal domain) and 14 (41 residues within the PF00208 domain) were specifically found in members of cluster I, and motifs 12 (15 amino acid residues within the middle domain) and 8 (41 amino acid residues within the PF00208 domain) were specifically found in members of cluster II. Moreover, motif 6 (50 amino acid residues within the PF00208 domain) was found in 15 of the 17 members of cluster II. In addition, a putative mitochondrial transit polypeptide profile was hit by motif 10 (29 residues within the N-terminal domain) (Hartl et al. 1989), which was found only in 14 of the 17 members of cluster II.

Expression analysis of *OsGDH* under nitrogen and phosphorus-deprivation

N-deprivation leads to a general repression of genes required for amino acid synthesis and induction of genes required for amino acid degradation. However, the result from a microarray analysis indicated that *GDH* genes showed little response in the early phase of low-N stress (Lian et al. 2006). It was reported that P-deprivation caused an induction of *AtGDH1* (*At3g03910*) and *AtGDH2* (*At5g07440*) (Morcuende et al. 2007). To investigate the response of the *GDH* genes in extended period of low-N and low-P stresses, Hejiang 19 plants were deprived of N and P separately in culture solutions, and samples were

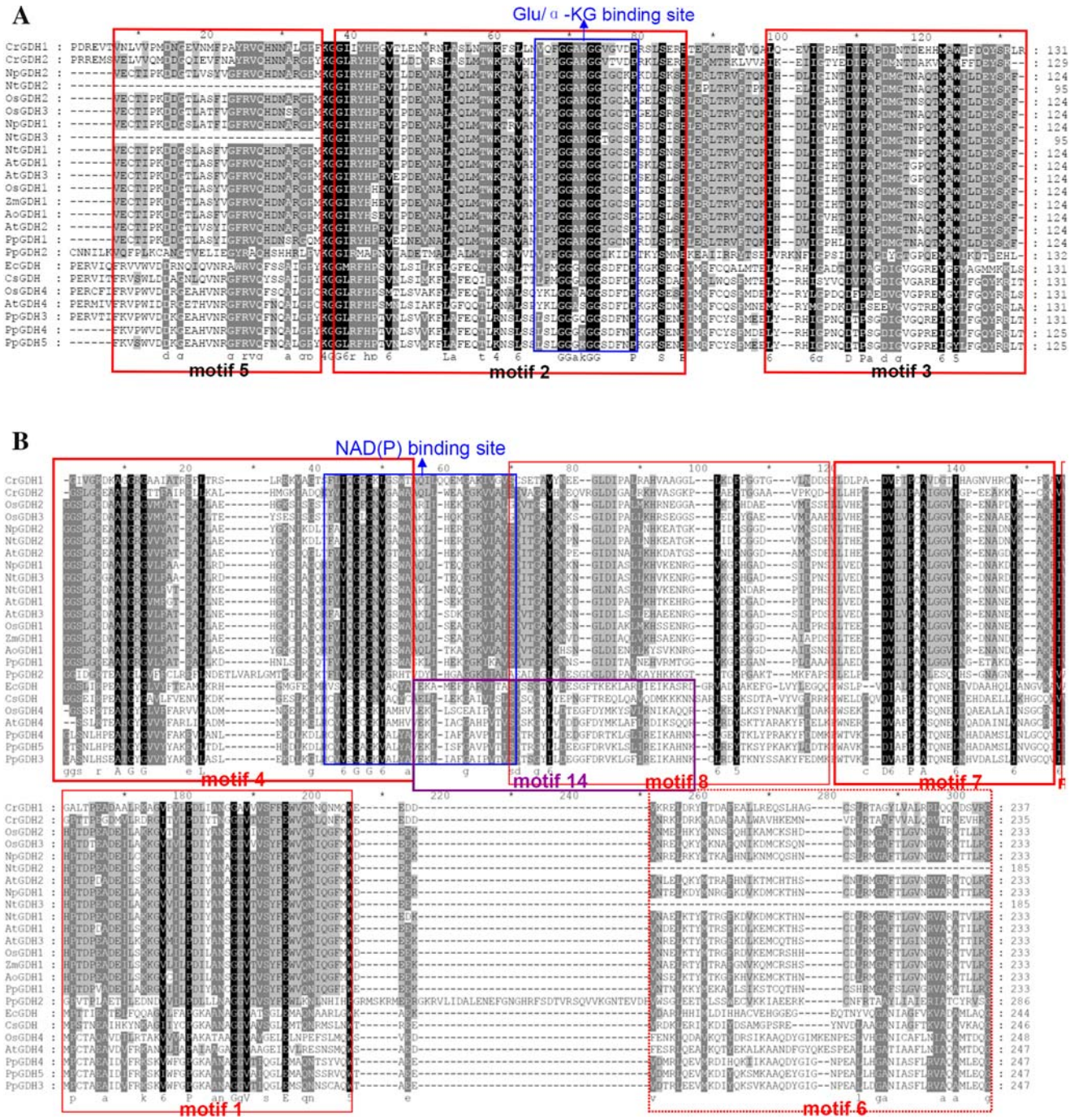

Fig. 2 Amino acid sequence alignment of glutamate dehydrogenase. Sequences were aligned with CLUSTAL_X 1.83 program (Thompson et al. 1997). Only the domain of **a** PF02812 and **b** PF00208 are shown here. Putative motifs are indicated in *boxes*. Cr, *Chlamydomonas reinhardtii*; Np, *Nicotiana plumbaginifolia*; Nt, *Nicotiana tabacum*; Os, *Oryza sativa*; At, *Arabidopsis thaliana*; Zm, *Zea mays*; Ao, *Asparagus officinalis*; Pp, *Physcomitrella patens* subsp. *Patens*; Ec, *Escherichia coli*; Cs, *Chlorella sorokiniana*

collected for qPCR analysis using member-specific primers (Supplemental Table 1).

OsGDH genes gave distinct expression patterns (Fig. 4) under N- and P-deficient conditions. In shoots, *OsGDH2* and *OsGDH3* were dramatically induced after N-deprivation for 1 day, with 5.4- and 9.7-fold increase, respectively. *OsGDH3* expression was also up-regulated by 3.1-fold after P-deprivation for 7 days. In roots, a 2.4-fold increase of the *OsGDH1* transcript was observed after N-deprivation for 1 h. In contrast, *OsGDH2* expression was largely reduced by both N- and P-deprivation after 1 h, whereas its expression increased greatly after 7 days of P-deprivation.

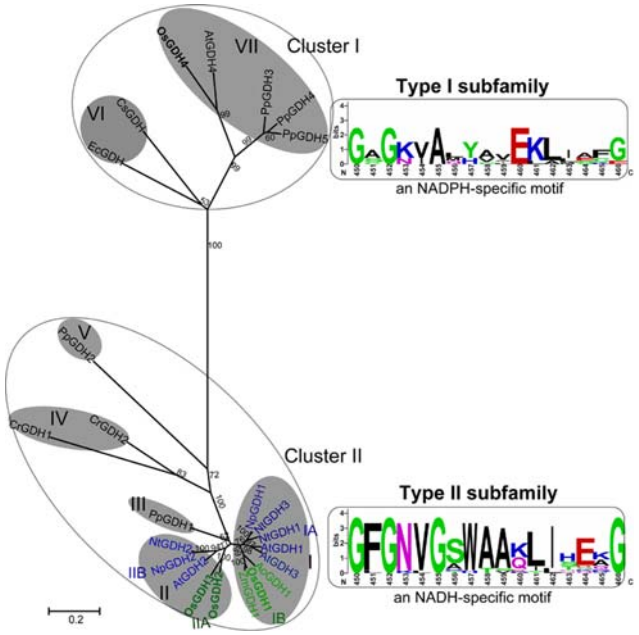

Fig. 3 Phylogenetic tree of the glutamate dehydrogenase (GDH) family. On the base of alignment of full-length GDH protein sequences, a phylogenetic tree was inferred by the neighbor-joining algorithms of MEGA 4.0 (Tamura et al. 2007). The evolutionary distances were calculated by employing the JTT model. Bootstrap analysis was computed with 1,000 replicates. Numbers in branches indicated bootstrap values (percentage). The scale bar indicates the number changes per unit length. Accession numbers are described in Supplemental Table 4. Sequence logos of type I and II subfamilies showed on the right were generated using WEBLOGO (http://weblogo.berkeley.edu/logo.cgi)

OsGDH3 transcript was undetectable under the normal conditions in roots, and increased by 7.6-fold after P-deprivation for 1 day. Overall, it can be seen from Fig. 4 that *OsGDH1* and *OsGDH2* were predominant in roots, *OsGDH3* is expressed mainly in shoots, and *OsGDH4* is expressed both in root and shoot which is relatively constant with or without the treatments.

Global gene expression analysis of *OsGDH*

The CREP database was searched to characterize the expression patterns of the *OsGDH* genes in the entire life cycle of the rice plant. *GS* and *GOGAT* expression patterns were also investigated for comparison. All the genes could be found in the expression database (Supplemental Table 5). The average signal values of at least two biological replicates for these genes were obtained and given in Supplemental Fig. 3A. To assay possible functional redundancy and to formulate hypotheses regarding the function of the *OsGDH* genes, a hierarchical cluster analysis (HCA) (Eisen et al. 1998) based on the mean $\log_2$ transformed intensity value of the *GS*, *GOGAT*, and *GDH* genes was performed. Among these genes, *OsGDH3* was hardly expressed in any tissue except glumes and stamens (one day before flowering, see Supplemental Fig. 3A), and was therefore not included in the analysis.

The expression patterns of the genes could be roughly categorized into four clusters, with each *OsGDH* gene in a different cluster (Fig. 5), suggesting functional non-redundancy among the *OsGDH* genes. None of the *GDH* genes was in cluster 4 (Fig. 5). *OsGDH1*, the sole member in cluster 1, was expressed in all the 27 tissues studied. The highest expression was detected in roots (at seedling with 2 tillers), radicles (48 h after emergence in the dark), and plumules (48 h after emergence in the dark); high expression was also detected in developing tissues, such as calli (15 days after subculture), seedlings (after germination and at three-leaf stage), young stems, developing panicles and spikelets. Low expression was detected in endosperms, stamens (at one day before flowering stage), sheaths, and leaves (Fig. 5 and Supplemental Fig. 3A). *OsGDH2* was placed in cluster 2, together with *OsGS1;1* (a gene encoding cytosolic GS1, LOC_Os02g50240), *OsGLT1* (*OsNADH-GOGAT1*, a gene encoding NADH-GOGAT, LOC_Os01g48960), *OsGS1;2* (a gene encoding cytosolic

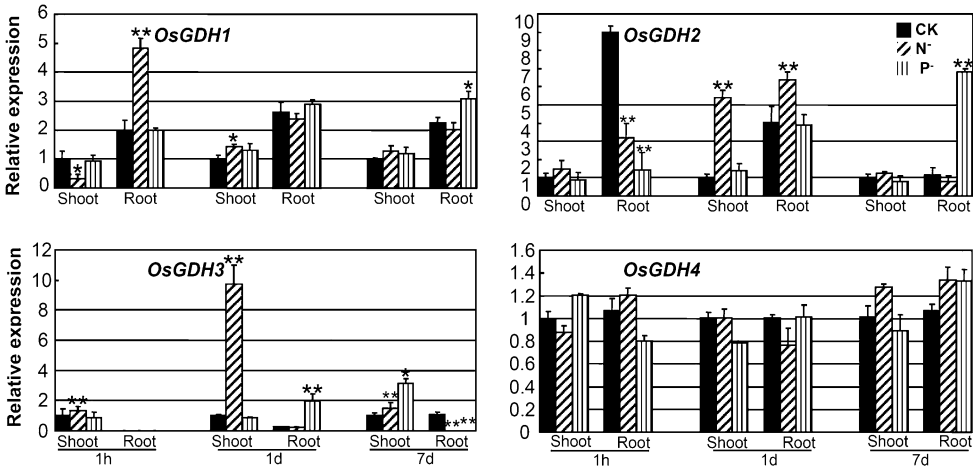

Fig. 4 Quantitative real-time PCR (qPCR) results for *OsGDH* genes under nitrogen-deprivation (N^-) or phosphorus-deprivation (P^-) conditions. Plant tissues were harvested as described in "Materials and methods". The transcript level of each gene is expressed relative to its transcript abundance in the shoot under the normal condition (CK). The error bar indicates one standard error, and asterisks indicate a statistically significant difference ($P < 0.05$) from the treatment

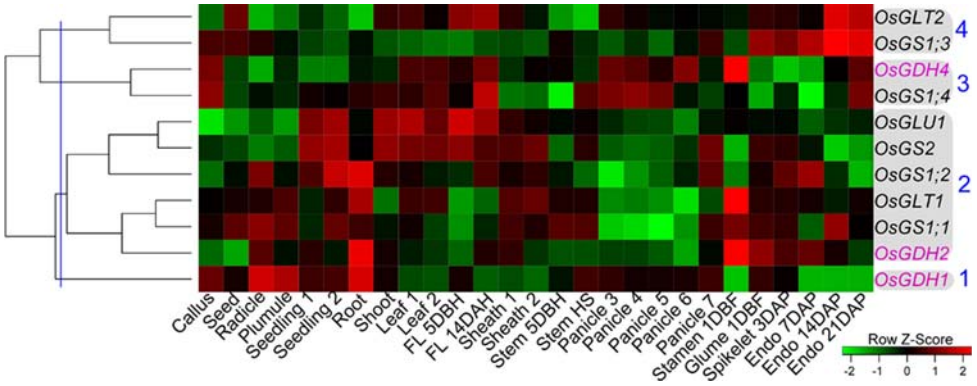

Fig. 5 Hierarchical clustering of the genes encoding glutamine synthetase (GS), glutamate synthase (GOGAT), and glutamate dehydrogenase (GDH) represented on Affymetrix rice genome array in 27 various tissues or organs (described in "Materials and methods") in Minghui 63. *OsGS1*: *OsGS1;1*, *OsGS1;2*, *OsGS1;3,* and *OsGS1;4*. *OsGS1* genes code for rice cytosolic glutamine synthetase, *OsGS2* gene codes for rice chloroplast/plastid glutamine synthetase, *OsGLT1* and *OsGLT2* genes code for rice NADH dependent glutamate synthase, *OsGLU1* codes for rice reduced-ferredoxin (Fd) glutamate synthase, *OsGDH1* and *OsGDH3* genes code for rice NADH dependent glutamate dehydrogenase, and *OsGDH4* code for rice NADPH dependent glutamate dehydrogenase. Genes with similar expression patterns are grouped into four clusters (1–4). The color scale, representing z-score of signal values, is shown down right

GS1, LOC_Os03g12290), *OsGS2* (a gene encoding chloroplast/plastid GS2, LOC_Os04g56400), and *OsGLU1* [*OsFd-GOGAT*, a gene encoding for reduced-ferredoxin (Fd) GOGAT, LOC_Os07g46460]. These genes were expressed in relatively high levels in seedlings and roots, and very low in young panicles (panicles 3–6). *OsGDH2* showed very high expression in roots and stamens (Fig. 5). *OsGDH4* was closely grouped with *OsGS1;4* [a gene encoding putative similar to GS1 (glutamate-ammonia ligase), LOC_Os10g31820] in cluster 3. Both genes showed relatively high expression in callus, shoot, leaves, and young panicles. In addition, *OsGDH4* showed the highest expression in stamens (Fig. 5).

Discussion

The *GDH* gene family in rice

This study reports the identification of four members of the *GDH* gene family in rice. The phylogenetic analysis separated the plant GDH proteins into two types, which is similar to the classification based on coenzymes: NAD(P)H-GDH (EC 1.4.1.3, EC 1.4.1.4) and NADH-GDH (EC 1.4.1.2), each type of GDH containing a motif with binding activity only to a specific coenzyme (Coruzzi and Last 2000; Inokuchi et al. 2002). Most type I genes identified in this study have an NADPH-specific motif (located in motifs 4, 8, and 14), while type II genes have an NADH-specific motif (located in motifs 4, 8, and 14, Figs. 2 and 3). In rice, there are only one type I gene (*OsGDH4*) but three type II genes (*OsGDH1-3*).

The OsGDH1-3 sequences harbor a putative mitochondrial transit polypeptide profile (located in motif 10, Supplemental Fig. 2) (Hartl et al. 1989) and a NADH-specific motif (Inokuchi et al. 2002), suggesting that the translation products of *OsGDH1-3* are mitochondrial NADH-GDH in plants. Plant mitochondrial NADH-GDH is generally believed to be a hexamer consisting of α- and β-subunit polypeptides associated in an ordered ratio to form two homohexamers and five heterohexamers: β_6 (isoenzyme 1), $\beta_5\alpha_1$ to $\beta_1\alpha_5$, α_6 (isoenzyme 7) (Loulakakis and Roubelakis-Angelakis 1991; Melo-Oliveira et al. 1996; Purnell et al. 2005; Miyashita and Good 2008). Previous phylogenetic analysis also revealed that the encoded products of NADH-*GDH* genes in higher plants can be divided into two groups, α or β, according to the nature of the subunit encoded (Purnell et al. 2005). Comparison of the results of Purnell et al. (2005) with the present study suggests that IIB and IIA are equivalent to the α and β groups, respectively. Generally, the genes in the same subcluster might have similar functions. The tobacco proteins NpGDH1, NtGDH3 and the rice protein OsGDH1 were located in the same clade in the phylogenetic tree (Fig. 3). It was shown that the encoded product of *NtGDH3* could be assembled as GDH-isoenzyme 1 (β_6) that deaminates Glu in vivo (Purnell and Botella 2007). Thus, it is highly likely that *OsGDH1* also encodes the β-subunit of GDH, and the assembly of such subunits would produce a positively charged GDH isoenzyme (β_6) that may be involved in deamination of Glu in vivo. *OsGDH2* and *OsGDH3* may have the same function as *NtGDH2* (Skopelitis et al. 2006, 2007), the assembly of which would produce a negatively charged GDH isoenzymes (α_6) primarily acting toward the

deamination of Glu. OsGDH2 and OsGDH3 may also have low-aminating activity as indicated by a putative EF-hand loop motif (located in C-terminal domain of motif 8, DFXXAEVMD[SA][SA]E, Fig. 2b), which may be important for the amination reaction (Abiko et al. 2005). However, the NADPH-specific motif was absent in OsGDH4 thus could only be referred to as a type I like gene, suggesting that rice may lack an NAD(P)H-*GDH* gene.

The existence of type I and type II *GDH* genes in both monocot and dicot plants suggests that the two types of *GDH* genes had already existed before the monocot and dicot plants diverged. The finding that there are only six members of type I genes compared to 17 of type II suggests that the expansion rate of type I genes is much slower than that of type II genes. This is also clearly the case in both Arabidopsis and rice. Two rounds of large-scale genome duplications have been predicted in the rice genome: one occurred before the divergence of cereals and the other before the monocot–dicot separation (Vandepoele et al. 2003). The pattern of clustering of the GDH genes in the subclusters IIA and IIB resolved in this study is consistent with this prediction: GDHs from dicot species are clearly separated from those of monocots, indicating dicot–monocot divergence; within subcluster IIA, GDHs from rice and maize are clearly separated from Asparagus sequence, indicating cereal/non-cereal divergence. In addition, these results also suggest that most GDH genes from angiosperms were in species-specific expansion.

Developmental and stress-inducible expression patterns of *GDH* family in rice

It is well known that the expression pattern of a gene is usually closely related to its function. In the present study, we illustrated developmental and stress-inducible expression patterns of *GDH* gene family in rice,which may be useful for speculating their functions. A high level of expression was observed for *OsGDH1* and *OsGDH4*. In contrast, the expression of *OsGDH2* is low, and *OsGDH3* is hardly expressed in most tissues (Supplemental Fig. 3A), as was previously suggested by RT-PCR analysis (Abiko et al. 2005).

OsGDH1 was the dominant form in most tissues examined, perhaps suggesting its greater functional significance than other *OsGDH* genes. Development affects *OsGDH1* expression: strong accumulation of specific transcripts occur in roots at seedling with 2 tillers (booting stage), radicles, plumules, and calli (at 15 days after subculture). Thus, OsGDH1 may be a major contributor to GDH catabolic activity in transaminations with amino acids from the breakdown of storage proteins, as suggested previously in lupine (Lehmann and Ratajczak 2007). A pair of paralogous protein, OsGDH2 and OsGDH3 was identified by phylogenetic analysis. Paralogs usually display different functions, which can be due to their different expression patterns (Figs. 4 and 5). *OsGDH2* appears to be expressed in most tissues tested (Supplemental Fig. 3A), and its expression also varied with the developmental stages: strong accumulation of specific transcripts occur in inflorescences (stamen at one day before flowering) and a transcriptional induction is observed in senescing leaves, which is in accordance with the results of *AtGDH2* in Arabidopsis (Supplemental Fig. 3B). It is well known that inflorescence is a tissue with elevated energy demands. Assimilation of NH_4^+ via GDH confers a saving in energy compared with the GS/GOGAT cycle (Windass et al. 1980; Helling 1998). It seems that OsGDH2 may be major contributor to GDH anabolic activity in reassimilation of ammonia from sources tissues (Hadzi-Taskovic Sukalovic 1990). However, the oxidation of glutamate to GDH also generates energy (10 molecules of ATP) (Lehmann and Ratajczak 2007). Thus, whether this strong accumulation of specific transcripts of *OsGDH2* in stamens (at one day before flowering) is due to GDH anabolic activity or to GDH catabolic activity is not known. However, OsGDH2 can be considered as a major isoform of GDH in inflorescence organs especially stamens and function in energy-efficiently transporting nitrogen from sources to sinks. Ammonium has been shown to induce expression of the gene encoding the α-subunit of GDH (Turano et al. 1997). Thus, the increase of *OsGDH2* transcripts during leaf senescence is due to the accumulation of ammonium in senescing leaves (Chen and Kao 1996; Hirel et al. 2005). It is well known that expression variation is largely due to the changes of regulatory elements. Consequently, we found absence of the CAAT box (Ha and An 1989) in the promoter region of *OsGDH3* using PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/), which may be the possible reason for the low expression of this gene. *OsGDH4* appears to exhibit a near constitutive expression pattern, which was also influenced by the developmental stages to certain extent, such that increasing transcripts occur in calli, leaves (at reproductive growth stage), and reproductive tissues (Figs. 4 and 5).

Expression of each *GDH* was further analyzed under nitrogen and phosphorus-deprivation. *OsGDH4* transcripts levels remained unchanged after deprivation of N and P (Fig. 4), which may be caused by absence of the NADPH-specific motif in its amino acid sequence. While the other three genes *OsGDH1-3* showed gene-specific behavior with respect to response time, tissue/organ, and nutrient conditions (Fig. 4). Under N-deprivation conditions, the expression of *OsGDH1* was induced in roots, indicating a special role of *OsGDH1* under nitrogen starvation. *OsGDH3* was induced in both roots and shoots after long-term (≥1 day) N and P starvation. In seedlings of Arabidopsis and tomato (*Lycopersicon esculentum*), N- and

P-deprivation caused an increase in flavonols (Stewart et al. 2002). The induced expression of *OsGDH1* and *OsGDH3* can partially contribute to the increasing level of flavonols by N- and P-deprivation. Global transcription pattern in rice revealed that *OsGDH* genes showed little response to low-N stress (1/6 nitrogen or 0.24 mM NH_4NO_3) (Lian et al. 2006). Similar result was obtained in wild type Arabidopsis (Peng et al. 2007), such that only *AtGDH2* among *GDH* family members was highly induced by low nitrogen (1/3 nitrogen or 3 mM nitrate) stress in the nitrogen limitation adaptation (nla) mutant. The expression pattern of *OsGDH2* is also unique, such that its expression was reduced in roots after short-term (1 h) N-and P-deprivation, while increased after long-term P starvation (Fig. 4). This suggests that *OsGDH2* has a special role under N starvation and energy limitation.

Hierarchical cluster analysis

The GDH occupies a biochemically critical position at the junction between carbon (α-KG) and nitrogen (Glu) metabolism and participates in the balancing of the cellular levels of three major components: the ammonium ions, α-KG, and Glu. But its function in ammonia assimilation in plants is still not fully understood because of difficulties in determining the in vivo direction of GDH reaction (Dubois et al. 2003). Therefore, the judgment in the direction of GDH (anabolic or catabolic activity; amination or deamination reaction) in plant metabolic pathways plays an important role.

A HCA, especially the HCA on the base of Pearson correlation coefficients, has been widely used to identify groups of coregulated functionally unknown and annotated genes based on the similarity of their expression patterns across a wide range of tissue and treatment samples (Eisen et al. 1998; Slonim 2002; Horan et al. 2008). In the present study, we performed a HCA to compare the expression of each *GDH* (not including *OsGDH3*) with that of annotated primary ammonium assimilation (including *GS* and *GOGAT*) genes in rice (Tabuchi et al. 2007). The results indicate distinct expression patterns among *OsGDH* family (Fig. 5). In addition, the results also indicate only two GDH genes (*OsGDH2* and *OsGDH4*) clustered with *GS* and *GOGAT* genes. *OsGDH2* is clustered with *OsGS1;1*, *OsGLT1*, *OsGS1;2*, *OsGS2*, and *OsGLU1*, annotated primary ammonium assimilation genes (Lea and Miflin 2003; Tabuchi et al. 2007). They show similar expression mainly in immature panicles (Fig. 5), suggesting that OsGDH2 may catalyze a reductive amination of α-KG to Glu in developing panicle/pollen. But panicle/pollen development is a complex process that requires carbohydrate import, indicating carbon-deficient in immature panicles. This suggests that OsGDH2 in immature panicles may catalyze an oxidative deamination of Glu to α-KG, as suggested by Miyashita and Good (2008). *OsGDH4* is clustered closely with an unknown gene, *OsGS1;4* (Fig. 5). Both genes show similar expression mainly in immature panicles, seed germination, and senescence of leaves (Fig. 5). It has been postulated that both seed germination and senescence of leaves are processes, in which proteins are hydrolyzed and transported again as soluble amino acids (Forde and Lea 2007). This also suggests that during that processes GDH may catalyze a reductive amination of α-KG to Glu or an oxidative deamination of Glu to α-KG. Therefore, we still cannot determine the in vivo direction of individual *GDH* genes based on HCA (Fig. 5). However, results from HCA indicate non-redundant functions among *OsGDH* family members under the normal conditions. Similar results were also obtained when we performed the HCA for the signal values of *GS*, *GOGAT*, and *GDH* genes in Arabidopsis (Supplemental Fig. 4). This suggests non-redundant functions among plant *GDH* family members under the normal conditions.

In conclusion, our data suggest that the *GDH* gene family members in rice are not only diverse in gene sequences, but are so different in the expression patterns under normal and stress conditions. Phylogenetic analysis and protein motif organization analysis revealed that *GDH* family in rice and other plants is obviously divided into two types. Both types of genes expanded in different manners. *OsGDH* genes showed different temporal–spatial expression patterns and responded differently to N- and P-deficient stress treatments. This work will provide a firm platform to answer pending questions about the physiological role(s) of GDH in plants.

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