JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Six Regulatory Elements Lying in the Promoter Region Imply the Functional Diversity of Chloroplast GAPDH in *Duanliella bardawil*

Yong-Min Lao,[†,‡] Yan Lu,[‡] Jian-Guo Jiang,*[,†] and Li-Xin Luo*[,‡]

[†]College of Food Science and Engineering, South China University of Technology, Guangzhou 510640, China

[‡]School of Biological Science and Engineering, South China University of Technology, Guangzhou 510006, China

ABSTRACT: Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a well-known proverbial protein involved in various functions in vivo. The functional diversity of GAPDH from *Dunaliella bardawil* (DbGAPDH) may relate to the regulatory elements lying in the promoter at the transcriptional level. Using RT-PCR and RACE reactions, *gapdh* cDNA was isolated, and the full-length genomic sequence was obtained by LA-PCR-based genome walking. The full-length cDNA sequence was 1645 bp containing an 1128 bp putative open reading frame (ORF), which coded a 375 amino acids-deduced polypeptide whose molecular weight was 40.27 kDa computationally. Protein conserved domain search and structural computation found that DbGAPDH consists of two structural conserved domains highly homologous in most species; multiple sequence alignment discovered two positive charge residues (Lys164 and Arg 233), which play a critical role in the protein−protein interaction between GAPDH, phosphoribulokinase (PRK), and CP12. Phylogenetic analysis demonstrated that DbGAPDH has a closer relationship with analogues from algae and higher plants than with those from other species. In silico analysis of the promoter region revealed six potential regulatory elements might be involved in four hypothesized functions characterized by chloroplast GAPDH: oxygen-, light-, pathogen-, and cold-induced regulation. These results might supply some hints for the functional diversity mechanisms of DbGAPDH, and fresh information for further research to bridge the gap between our knowledge of DNA and protein structure and our understanding of functional biology in GAPDH regulation.

KEYWORDS: *Dunaliella bardawil, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), genome walking, regulatory element, functional diversity*

INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, EC 1.2.1.12) is a proverbial highly conserved protein with little variance between species to catalyze the carbohydrate breakdown in the glycolytic pathway.[1] Because of its apparent high degree of conservation across the phylogenetic scale, conventional dogma indicated only a limited role for this gene and protein.[2] Thus, it is considered to be a "house-keeping" protein and a proved classical model protein frequently utilized for protein structure analysis and enzyme catalytic mechanism investigation.[3,4]

However, recent research results disclosed that the trite and commonplace of GAPDH turned out to be a quite daedal and elaborate story: it plays a multifunctional role in a large number of fundamental cell pathways with crucial activities.[5,6] As Figure 1 indicated, these functions, separately at the DNA, RNA, and protein levels, are involved in intracellular membrane trafficking, receptor-mediated cell signaling, maintenance of DNA integrity, and cell apoptosis, etc. GAPDH is regulated by various factors or environmental stresses, including insulin,[7,8] calcium,[9] and hypoxia.[10,11] It can be expected that the regulatory differentiation of GAPDH, to some extent, accounts for its functional diversity in vivo, respectively, at the DNA, RNA, and protein layers. With respect to the DNA level, promoter area is one of the key points to investigate the regulatory mechanisms of GAPDH. Hitherto several regulatory elements harboring in *gapdh* promoter were found, for instance, the #104 region[12] related to apoptotic stimulus, hypoxia responsive element (HRE) activated by hypoxic stress,[11]

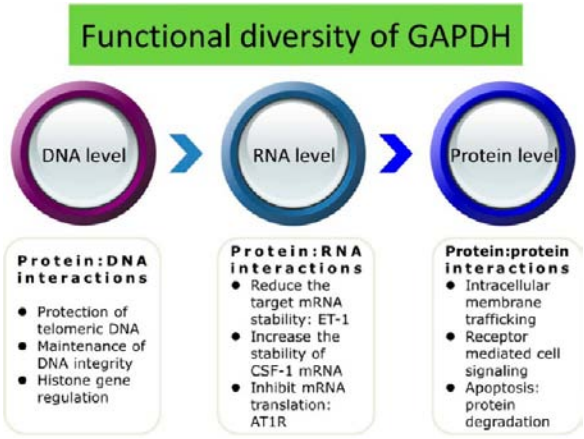

Figure 1. The functional diversity of GAPDH. Regulatory GAPDH participates in various metabolisms at the DNA, RNA, and protein levels. The regulation mechanisms include protein−DNA, protein−RNA, and protein−protein interactions to endow species, tissue, and temporal specificity, accordingly. These functions refer to intracellular membrane trafficking, receptor-mediated cell signaling, maintenance of DNA integrity, and cell apoptosis, etc.

Received: June 20, 2012
Revised: August 16, 2012
Accepted: August 20, 2012
Published: August 20, 2012

Table 1. Primers Used in This Study

procedure	primer	primer sequence (5→3)
EST isolation	DbGAPDHinF	TCSAACGCSTCBTGCACSACCAAC
	DbGAPDHinR	GGGGGTBGGMACRCGSAGVGC
3′ RACE reaction	DbGAPDH3′F1	CAACATCATTCCTTCCTCCAC
	DbGAPDH3′F2	TGCCTGCTCTGAAGGGAAAG
5′ RACE reaction	DbGAPDH5′R	GTGGCAGTGGTGGCGTGGACAGT
	SMARTAO	AAGCAGTGGTATCAACGCAGAGTACGCGGG
	5′-RACE CDS	$(T)_{25}$VN
promoter isolation	ProSP1	CCCTCACCCTCCCTGAAATCTACAA
	ProSP2	AGCATTCCACAGGTCCAAGGCAGA
	ProSP3	ACCTCACATCCATTCCGCTTTCA
terminator isolation	TerSP1	ATGCTGTAATTGCCAGACCTCCG
	TerSP2	AGCTTGTTTTGCGGTTGTTATGGG
	TerSP3	CTTGTTTTGCGGTTGTTATGGGG
genomic DNA analysis	g1SP1	ATTAGCGGGTGCTGTGCCTTCC
	g1SP2	CGGGTGCTGTGCCTTCCACTTTC
	g1SP3	CCCCATAACAACCGCAAAACAAGC
	g2SP1	TACCACCTCATCCTCAGTGAAGCC
	g2SP2	AGAAAAATCTGTGTGCCGTGCTGG
	g2SP3	TGCCCTGGGAATGGAACTGAAAGC

anaerobic response elements in response to anaerobiosis,[13] and the *fa* respective region effected by insulin in adipose tissue,[14] which are genetically regulated at the level of promoter activation or inactivation.

According to the cofactor required, the plant GAPDH can be divided into three types:[15] the cytoplasmic GADPH (EC 1.2.1.12), which absolutely requires NADH as coenzyme; the chloroplast one (EC 1.2.1.13) using either NADH or NADPH as coenzyme; and the third one (EC 1.2.1.9) is specific for NADPH. In photosynthetic tissues, especially in chloroplast, GAPDH executes two major functions using either NADH or NADPH as coenzyme: (1) it has a central role to participate in the unique reductive step of the Calvin cycle to assimilate CO_2; as well as (2) its conventional dephosphorylation in glycolysis.[16] Protein structure analysis found that the chloroplast GAPDH is composed of four identical GapA subunits (A_4) in cyanobacteria, green and red algae, and higher plants.[3] In *Chlamydomonas reinhardtii*, the NADPH-GAPDH, constituted of a single type of subunit (A) arranged in homotetramers, is not autonomously regulated as other heteropolymers; instead, interaction of GAPDH with GapB-like protein CP12 is required to fulfill its full function.[3] CP12 acts like GapB to endow regulatory characteristic by providing necessary C-terminal extension to NADPH−GAPDH in *C. reinhardtii*.[3]

Unlike *C. reinhardtii*, lack of detailed information about GAPDH from the *Duanliella* algae blocks advanced studies on the regulatory mechanisms in these algae. Up to now, merely mRNA sequence was obtained from *Dunaliella salina* by Jia et al.;[17] no information was even available in *D. bardawil*. Consequently, to preliminarily explore the interrelationship between the sequence specificity and the regulatory diversity of GPADH, in the present study, we separately conducted RT-PCR reactions and RACE operations to obtain cDNA of GAPDH from *D. bardawil* (DbGAPDH), and, based on the UTR sequences of GAPDH cDNA, we performed genome walking to achieve the full-length genomic sequence covering from promoter to terminator; eventually, applying some bioinformatics tools, we analyzed and predicted the conserved regulatory elements encompassed in the promoter and protein structure of GAPDH.

MATERIALS AND METHODS

Strains and Culture Conditions. The green algae *D. bardawil* strain 847 was obtained from the Institute of Hydrobiology, Chinese Academy of Science. The cells were cultured at 26 °C under a 14/10 h dark/light cycle in defined medium[18] and were collected at the late log phase. *E. coli* GT116 was used as the host for the multiplication of plasmids.

Isolation of RNA and Genomic DNA. Total RNA was extracted from 6 mL of *D. bardawil* cells grown at the late log phase using E.ZNA Total RNA Kit II (OMEGA) following conditions recommended by the manufacturer. Genomic deoxyribonucleic acid (DNA) extraction from cells in the late log phase was performed according to the method described by Yang et al.[19]

Primer Design. Several species were selected, and protein BLAST was carried out to design degenerate primes; 5′ RACE primers were synthesized according to the SMARTer RACE cDNA Amplifcation Kit User Manual; and gene-specific primers were designed using Primer Premier 5 software. The primer sets used in this study are listed in Table 1.

Characterization of *gapdh* Expression Sequence Tag (EST) from *D. bardawil*. Alignment of GAPDH polypeptides deposited in GenBank revealed the conserved amino acid sequences "SNASCTTN" and "ALRVPTP", and a pair of degenerate primers were designed and synthesized correspondingly. RT-PCR was performed with RNA PCR Kit (AMV) Version 3.0 (TaKaRa) using oligo dT-Adaptor Primer. All manipulations followed the manufacturer's protocol.

Cloning of the 3′ and 5′ Ends of *gapdh* by Rapid Amplification of cDNA Ends (RACE). On the basis of the EST fragment of *gapdh*, two gene-specific primers were designed, and 3′ RACE was conducted with DbGAPDH3′F1 and oligo dT-Adaptor primers using RNA PCR Kit (AMV) Ver.3.0 (TaKaRa). The RT-PCR product was diluted at the ratio of 1:100, and sequential nested PCR using DbGAPDH3′F2 primer was performed by LA Taq (TaKaRa) with the parameters set as follows: 94 °C, 4 min; 30 cycles of 94 °C, 30 s, 46 °C, 30 s, and 72 °C, 1 min with a final extension at 72 °C for 10 min.

The 5′ RACE operation was accomplished with SMART MMLV Reverse Transcriptase (Clontech) and synthesized primers SMARTAO and 5′-RACE CDS. Gene-specific primer design and other handlings including touchdown PCR were employed according to the SMARTer RACE cDNA Amplifcation Kit User Manual, with the

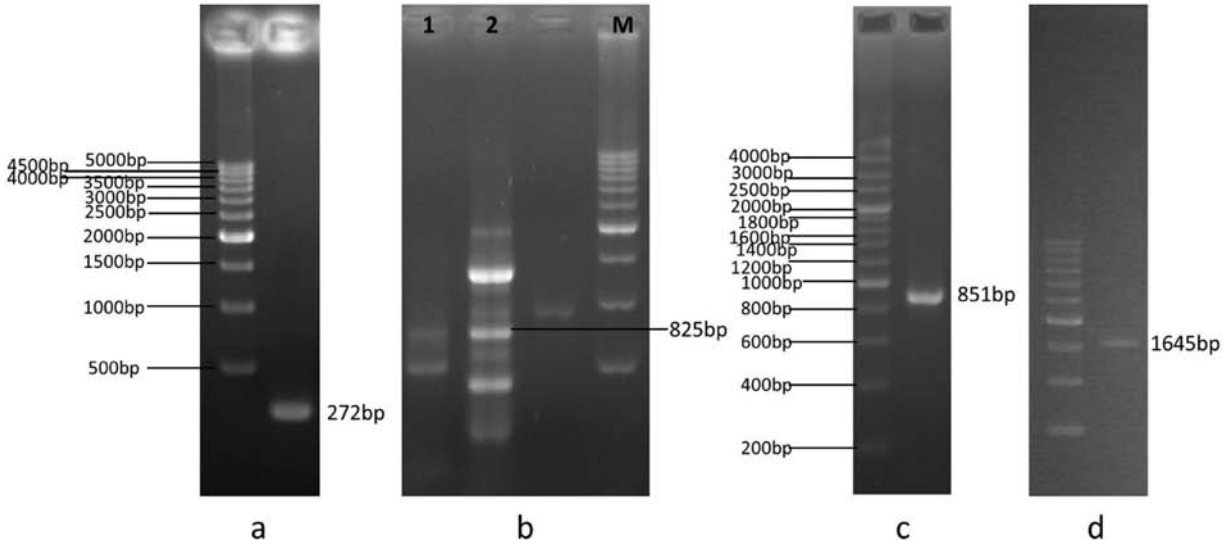

Figure 2. Isolation of *gapdh* cDNA from *D. bardawil*. (a) *gapdh* EST fragment with 272 bp in length; (b) nested PCR was conducted during 3′ RACE manipulation; lane 1, first round PCR products; lane 2, second round PCR products retrieving a 825 bp 3′ end of *gapdh*; M, 500 bp DNA ladder marker; (c) 5′ RACE reaction using SMART technique obtained the *gapdh* 5′ end; and (d) full-length *gapdh* cDNA was isolated with high fidelity PrimeSTAR HS DNA polymerase.

exception of LA Taq DNA polymerase (TaKaRa) for touchdown PCR, rather than Advantage 2 Polymerase Mix.

The full-length GAPDH cDNA was obtained with primers corresponding to the 5′ and 3′ ends of the *gapdh* gene. All amplified fragments were cloned into pCR2.1 vector (Invitrogen) and sequenced.

Identification of *gapdh* Genomic DNA. Genome walking was implemented with gene-specific primers to identify the genomic DNA of *gapdh* (Figure 2). First, the initial PCR was fulfilled using gene-specific primers complemented with 3′ UTR of *gapdh* and genomic DNA as template. Subsequently, additional gene-specific primers were synthesized for genome walking in the light of the initial PCR product. For isolation of *gapdh* promoter and terminator, two sets of gene-specific primers were designed, and genome walking was carried out with genome walking kit (TaKaRa) independently. All manipulations were in accordance with the user manual.

Promoter prediction was operated by PLACE (http://www.dna.affrc.go.jp/PLACE/signalscan.html). Terminator scan program Poly (A) Signal Miner (http://dnafsminer.bic.nus.edu.sg/) and RibEx (http://132.248.32.45/cgi-bin/ribex.cgi) were used to analyze the putative terminator sequence of *gapdh*.

Sequence Analysis and Phylogenetic Construction. Sequence analysis was performed using BLAST Software (http://blast.ncbi.nlm.nih.gov/). Component analysis of *gapdh* was calculated using DNAStar software 7.1.0. Physical and chemical features of DbGAPDH were analyzed by ProtParam tool (http://expasy.org/tools/protparam.html). Subcellular localization presumption was performed using WoLF PSORT (http://wolfpsort.org/). Conserved domains in DbGAPDH were detected using the Conserved Domains Search tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Quaternary structure was constructed using SWISS-MODEL Automatic Modeling (http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1). Multiple alignments among similar enzymes were conducted using Clustal X 1.83. Phylogenetic and molecular evolutionary analyses of the amino acid sequences of different GAPDHs were conducted using the Neighbor Joining method and the molecular evolution genetics analysis (MEGA) software, version 4.0.2.

RESULTS AND DISCUSSION

Isolation and Characterization of *gapdh* cDNA. According to *gapdh* cDNA sequences of *C. reinhardtii* (XM_001689819.1) and *D. viridis* (DQ301766.1), also referring to two conserved regions of the GAPDH protein sequences of several plants and cyanobacteria, a pair of degenerate primers were designed, and a resultant 272 bp *gapdh* EST fragment was obtained (Figure 2a). The nucleotide and putative protein sequence of this EST fragment is highly homologous to those of previously reported *gapdh* demonstrated by BLASTX (data not shown). On the basis of the EST fragment, 3′ and 5′ RACE reactions were performed to clone the 3′ and 5′ regions of *gapdh* cDNA. An 825 bp partial cDNA sequence was amplified by 3′ RACE procedure from *D. bardawil* (Figure 2b). The nucleotide and deduced protein sequences characterize 76% and 70% identities with *Chlamydomonas* counterpart, respectively. Finally, isolation of an 851 bp fragment (Figure 2c) corresponding to the 5′ end of *gapdh* was achieved by 5′ RACE treatment. Homologous searches through the BLAST programs demonstrated that it is the expected 5′ ends of the *D. bardawil gapdh* cDNA.

A 1645 bp full-length cDNA was obtained by assembling the above fragments, and confirmed by PCR amplification using a pair of primers (DbGAPDH For/Rev) corresponding to the 3′ and 5′ ends of *gapdh* gene (Figure 2d). ORF search found that DbGAPDH cDNA consists of an 1128 bp CDS, a 175 bp 5′ UTR, and a 342 bp 3′ UTR.

The deduced 375 aa polypeptide possesses a computed molecular weight of 40.27 kDa, a theoretical isoelectric point (pI) of 9.035, comprises 45 strongly basic (+) amino acids (K, R), 37 strongly acidic (−) amino acids (D, E), 150 hydrophobic amino acids (A, I, L, F, W, V), and 84 polar amino acids (N, C, Q, S, T, Y). BLAST search displayed identities of 83% and 80% with *C. reinhardtii* referring to DNA and protein sequence individually. However, this gene shares only 75% or 72% homology with that of *D. salina* (data not shown), which might imply an isoform differentiated from the known counterpart of *D. salina*. ProtParam analysis characterized a stable protein with formula of $C_{1778}H_{2881}N_{501}O_{530}S_{16}$. SignalP 3.0 predicted no signal peptide in this GAPDH, which indicates a nonsecretory protein. Transmembrance analysis by TMHMM Server v. 2.0 found no transmembrane component, and WoLFP SORT

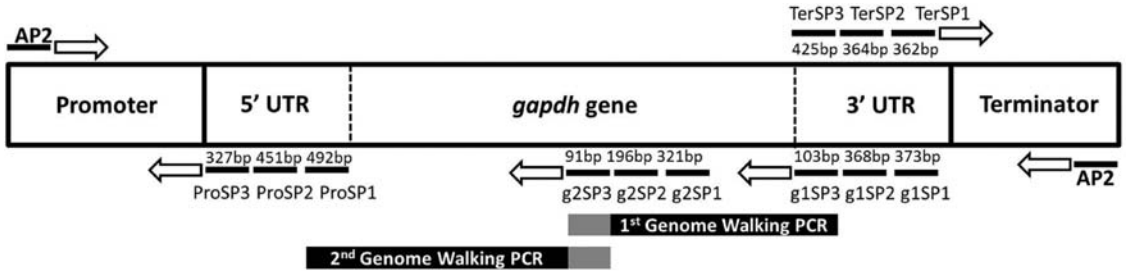

Figure 3. Strategy for cloning of *gapdh* genomic DNA in *D. bardawil* by LA PCR-based genome walking. For isolation of genomic gene of *gapdh*, two consecutive steps of genome walking PCR were conducted. First genome walk PCR: according to the 3′ UTR of *gapdh* cDNA, a set of adjacent gene-specific primers (g1SP1, g1SP2, and g1SP3) was designed for isolation of *gapdh* full-length gene fragment; second genome walking PCR: successively, another genome walking manipulation was conducted to obtain full-length gene of *gapdh*, using another set of gene-specific primers (g2SP1, g2SP2, and g2SP3) designed according to the above obtained fragment. The overlapped region they shared was highlighted as a gray box. Similarly, gene-specific primers (ProSP1, ProSP2, and ProSP3; TerSP1, TerSP2, and TerSP3) for cloning of promoter and terminator regions of *gapdh* were designed to reside in the 5′ and 3′ UTR region of *gapdh*, correspondingly. While cloning the full-length gene and promoter of *gapdh*, degenerate primer AP2 supplied by the Kit was used as a sense primer, g1SP1−3, g2SP1−3, and ProSP1−3 as antisense primers, correspondingly; conversely, AP2 was used as an antisense primer and TerSP1−3 as sense primers when isolating the *gapdh* terminator. The numbers proximal to the SP primers indicate the extending length of *gapdh* gene by LA Taq polymerase with corresponding SP primers when conducting nested PCRs.

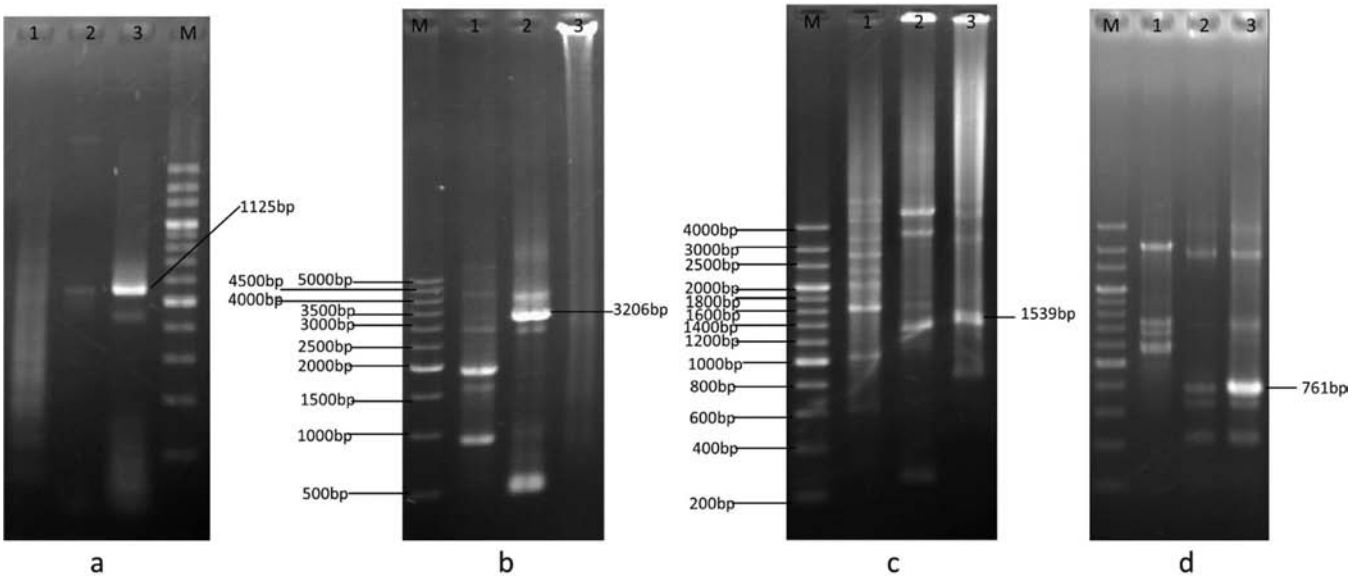

Figure 4. Isolation of *gapdh* genomic DNA by genome walking approaches. (a) First genome walking handling to obtain a 1125 bp partial sequence of *gapdh* genomic DNA; lane 1, first nested PCR products; lane 2, second nested PCR products; lane 3, third nested PCR products (similarly hereinafter); M, 200 bp DNA ladder marker; (b) second genome walking to obtain full-length *gapdh* genomic DNA sequence; M, 500 bp DNA ladder marker; (c) according to the full-length genomic DNA template, gapdh promoter was cloned; M, 200 bp DNA ladder marker; and (d) a 761 bp terminator was recovered; M, 200 bp DNA ladder marker.

suggested a chloroplast isoform as it exhibits 80% identity with G3PA_CHLRE.

Analysis of the Genomic Structure of *gapdh*. For the sake of acquirement of *gapdh* promoter and terminator sequences, as well as further elucidation of *gapdh* genomic structure, we isolated the full-length *gapdh* genomic sequence based on its cDNA information. TaKaRa genome walking technique enables these aims relying on thermal asymmetric interlaced PCR (TAIL-PCR),[20] which requires application of three sequential nested PCRs using a set of gene-specific primers (SPs) in combination with asymmetric primer (AP) of lower Tm as compared to SPs (Figure 3). Through these manipulations, we can obtain upstream and downstream sequences flanking the known sequence, such as promoter and terminator, and other regulatory sequences.

Two successive steps of genome walking were performed successively to assemble *gapdh* gene, with two sets of adjacent gene-specific primers, respectively. An 1125 bp fragment of the third nested PCR was recovered in the first round genome walking using primers g1SP1−3 (Figure 4a), and a 3206 bp sequence of the second nested PCR was obtained in the second round genome walking with g2SP1−3 residing in 805 bp, 930 bp, and 1035 bp of the above fragment, respectively (Figure 4b). Sequence assembly of the two fragments and subsequent alignment with the *gapdh* cDNA revealed that the gene contains six exons interrupted by five introns with a total length of 4219 bp (Figure 5a). The GC content (%) of the six exons and all introns ranges from 49.67% to 60.54% and 47.2% to 54.95%, correspondingly. The locations of these introns in this gene show no similarity with those of plant known up to now.

With regard to the regulatory sequences of *gapdh*, we subsequently cloned the promoter and terminator on the strength of *gapdh* gene information: the brightest fragment of 761 bp was recovered from the third nested PCR when

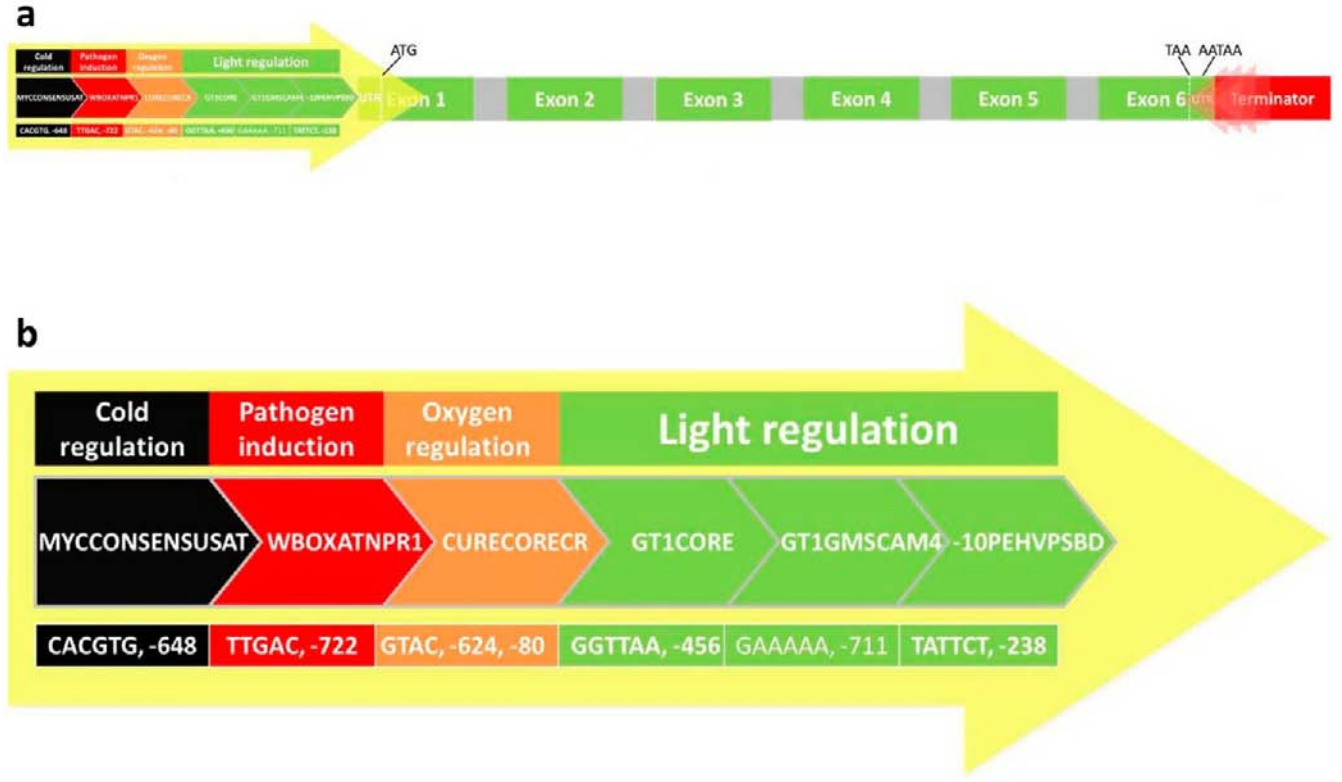

Figure 5. The genomic structure of *gapdh* ranging from promoter to terminator. (a) *gapdh* gene is made up of six exons inserted by five introns, including a 175 bp long 5′ UTR and a 3′ UTR of 342 bp. A poly (A) signal was also found at position 237 downstream of the TAA stop codon. Adjacent to the 3′ UTR is the terminator of 400 bp in length. (b) A 921 bp promoter was isolated and analyzed by PLACE database. Six conserved motifs were found harboring in this promoter. They are predicted to be involved in cold-, pathogen-, oxygen-, and light-induced activation or inactivation, independently or coordinately.

conducting terminator isolation (Figure 4d); and a 1539 bp fragment was retrieved while performing promoter separation, as Figure 4c shows. Sequence analysis indicated that both the 1539 bp and the 761 bp fragments contain overlapping regions predesigned with the 5′ and 3′ UTR of *gapdh*, independently. Therefore, these sequences are the upstream and downstream of the UTR of *gapdh* correspondingly. A poly (A) signal (AAATAA) was found in the hypothetic terminator at position 237 downstream of the terminal codon TAA by Signal Miner and RibEx programs (Figure 5a),[21] indicating a canonical terminator characteristic *gapdh* possessed.

***cis*-Acting Element Analysis of *gapdh* Promoter.** The putative promoter was submitted to the PLACE server[22] to analyze candidate *cis*-acting elements located in this query sequence. Several plant promoter-specific conserved motifs were found in the promoter (Figure 5b), for example, TATA box with consensus sequence TATATAA located at position −29 upstream of the TSS, CAAT box located at −574, and GATA box at −99. Remarkably, six regulatory *cis*-acting elements were also found in this promoter (Figure 5b); they were predicted to be involved in four functions, which imply a central position of DbGAPDH in the complex regulatory network of photosynthetic algae.

Oxygen Response. Two pieces of literature[23,24] reported that human *gapdh* is transcriptionally activated by hypoxia in normal and malignant cells, and the promoter region of which contains two distinct hypoxia responsive element (HREs) coffering such activation. In *C. reinhardtii*, two copper regulatory elements (CuREs) were found to function as a target of transcriptional activator in *cyc6* gene.[25] Further research[26,27] revealed *cyc6* and *cpx1* were activated coordinately in response to copper and oxygen deficiency through a copper-sensing signal transduction pathway, with a CuRE consensus sequence GTAC. The same CuRE (also called HRE) found in chloroplast *gapdh* promoter might imply that similar regulatory mechanisms underline copper and hypoxia response in another anaerobic alga, *D. bardawil*, as most hypoxia-induced genes are involved in sugar metabolism.[27] It is suggested that photosynthetically produced oxygen radicals are involved in triggering massive β-carotene accumulation in *D. bardawil*.[28] Carotenoids can act as protective response against oxidative damage; its overaccumulation is paralleled by an increase in catalase activity.[29] Thus, the upstream GAPDH should be an emphasis to investigate the oxygen role on carotenogenesis.

Light Regulation. Three candidate elements were found in the *gapdh* promoter of *D. bardawil*, which might participate in light response: −10PEHVPSBD, GT1CONSENSUS (also named GT1GMSCAM4), and GT1CORE. In barley, −10PEHVPSBD was found to activate chloroplast *psbD* gene encoding a photosystem II reaction center chlorophyll-binding protein by blue, white, or UV-A light.[30] Mutation of this element reduced transcription to very low levels in all light regimes. Consensus GT-1 binding sites, referred to GT1CONSENSUS and GT1CORE in this paper, are located in many light-induced genes.[31] GT-1 factors can activate downstream host genes through direct interaction between TFIIA and GT-1 to stabilize the TFIIA-TBP-DNA (TATA box) complex.[32] Binding of GT-1-like factors to PR-1a promoter influences the level of salicylic acid (SA) inducible gene expression.[33] In our previous study, we found these regulatory boxes in phytoene

dx.doi.org/10.1021/jf302659z | J. Agric. Food Chem. 2012, 60, 9211−9220

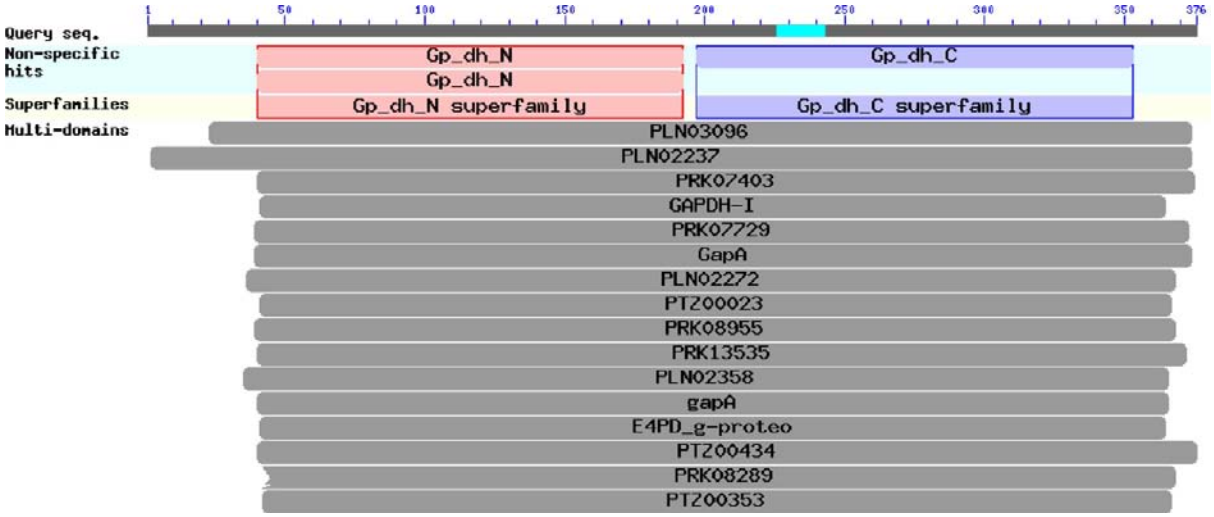

Figure 6. Conserved domains in DbGAPDH. Two superfamilies were found in this protein: Gp_dh_N is a Rossmann-fold cofactor binding domain and is in charge of NAD (P) binding, while Gp_dh_C is responsible for catalytic reaction.

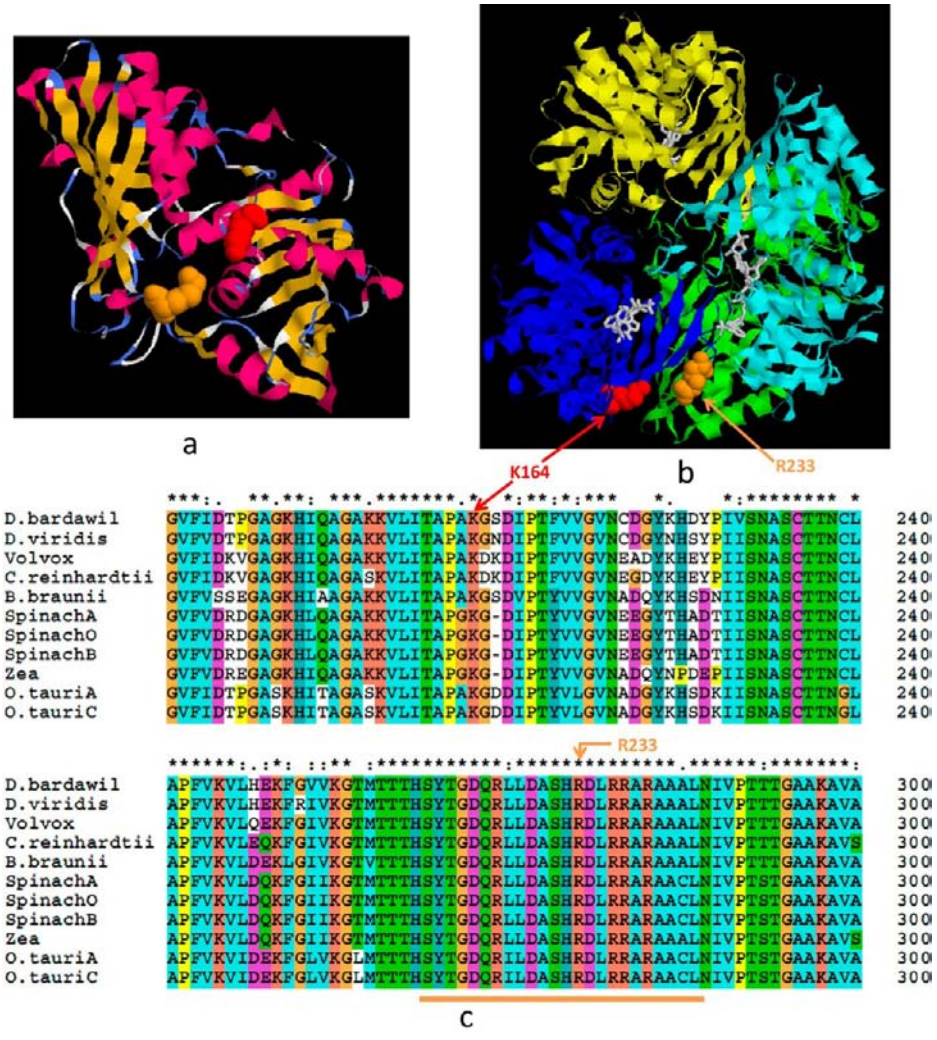

Figure 7. The predicted quaternary structure of DbGAPDH. (a) The ribbon structure of monomer subunit A. (b) DbGAPDH is forecasted as homotetramers formed by 4 subunits A. Different chains are colored in different colors. The predicted Lys164 and Arg233 in the same chain are highlighted by spacefill in red and orange, correspondingly. Four NAD coenzymes are also present as sticks. The α-helix and β-sheet regions of the putative protein are indicated with cylinders and bands, respectively. (c) The Lys164 and Arg233 residues, participating in protein−protein interaction, are conserved between chloroplast GAPDHs. The S loop in the catalytic domain is underlined.

dx.doi.org/10.1021/jf302659z | J. Agric. Food Chem. 2012, 60, 9211−9220

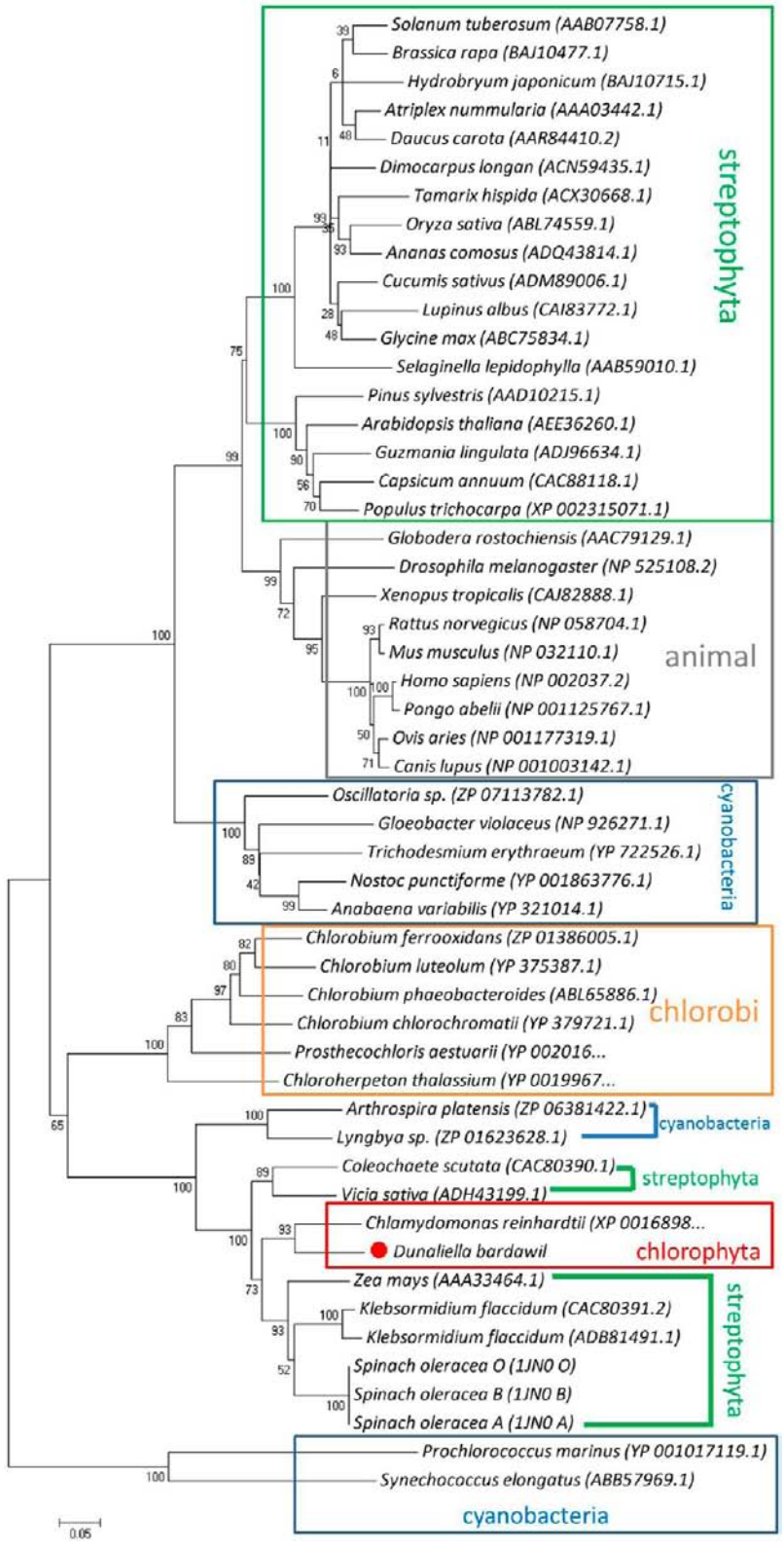

Figure 8. Phylogenetic analysis of 52 GAPDHs. The red circle shows the position of the *D. bardawil* on the phylogenetic tree. GenBank accession numbers of reference protein sequences in parentheses are shown next to the name of each species. Numbers associated with the branches are the neighbor-joining bootstrap values (n = 1000). The length of the branch is proportional to evolutionary distance with its scale being 0.05.

synthase (*psy*) promoter, which might function as salt-induced elements;[34] overexpression of *Pleurotus sajor-caju* GAPDH can confer salt stress resistance in transgenic potato;[35] moreover, a cognate TBOXATGAPB together with GT1CONSENSUS in this gene was also found, respectively, in the chloroplast lycopene β-cyclase (*lycB*) and plastidic glycerol 3-phosphate dehydrogenase (*gpdh*) promoters of *D. bardawil* in our ongoing research (data not shown). It is well-known that *psy* and *lycB*

are two key enzyme genes responsible for carotenoid metabolism, and *gpdh* is of vital importance for glycerol metabolism. GT1CONSENSUS might take charge of light or salt induction of carotenoid accumulation under light and salt stress.[34] Furthermore, PSY and GPDH were simultaneously upregulated by NaCl.[36] Because GAPDH is on the upstream of the both pathways in the metabolic network, we hypothesize some links among GAPDH, carotenogenesis, and osmoregulation in *D. bardawil* under light- and salt-stress. Further investigations into the exact mechanisms below remain to be conducted.

Pathogen-Induced Response. WBOXATNPR1 acts as a negative regulatory element in *Arabidopsis thaliana* to control AtWRKY18, which enhances expression of pathogenesis-induced genes and resistance to bacterial pathogen by specific binding of SA-induced WRKY DNA binding proteins.[37] Suspicious WBOXATNPR1-like element found *gapdh* promoter might also prevent overexpression of DbGAPDH during the activation of defense responses, which implies a distinct role of GAPDH in pathogen-induced response in algae. Interestingly, the found GT1CONSENSUS both in *gapdh* and in *psy* promoter was also predicted to be pathogen inducible.[34] However, the original GT1CONSENSUS of SCaM-4 promoter from *Glycine max* is of positive-controlled property.[36] The actual regulatory role of such *cis*-acting element is still to be confirmed.

Cold Stress Regulation. Cold stress can trigger some transcription factors, especially the C-repeat binding factor (CBF) family, which in turn activates many downstream genes that confer freezing tolerance to plants.[38] MYC recognition site MYCCONSENSUSAT is discovered in the promoter of CBF3, dehydration-responsive gene *rd22*, and many other genes in *Arabidopsis*;[38] it is the specific binding site of ICE1 (inducer of CBF expression 1) that regulates the transcription of CBF3 in cold stress in *Arabidopsis*.[39] Furthermore, MYCCONSENSUSAT also relates to osmotic stress response at the transcriptional level in plant.[40] Osmotic stress can evoke abscisic acid (ABA)-independent transcription of stress responsive genes including MYC/MYB through MYCCONSENSUSAT site in plant.[40] As a halophilic alga, *D. bardawil* can adjust itself to hyperosmotic environment via glycerol synthesis by GPDH; our ongoing research also found such element in GPDH promoter, which implies a deduced common osmoregulation mechanism shared by GAPDH and GPDH in *D. bardawil*.

As a natural rich source of β-carotene, *D. bardawil* can massively accumulate β-carotene under stress conditions such as high light density, high salt concentration, and nutrient starvation.[41] Meanwhile, in response to salt stress and osmotic pressure, osmoregulation is triggered through glycerol synthesis pathway.[42] Clue implied that these adjustments should be attributed to the regulatory elements resided in the promoter region of relevant enzymes.[34] Consequently, further research should surround these elements; foreign reporter driven by mutated promoter, which is deficient with related elements, should be detected in *D. bardawil* under control or stress conditions.

Protein Structure and Phylogenetic Analysis for DbGAPDH. *Conserved Domains and Quaternary Structure of DbGAPDH.* Two conserved domains, NAD binding domain and C terminal domain, are included by DbGAPDH utilizing the NCBI Conserved Domain Search tool[43] (Figure 6). It is well-known that GAPDH is a tetrameric NAD-binding enzyme involved in glycolysis and glyconeogenesis. The N-terminal domain was predicted to present a Rossmann NAD (P) binding fold, and the C-terminal domain was calculated to form a mixed α/antiparallel β fold, which are consistent with experimental data.[44] In addition, a highly similar template 1rm4B (77.68% identity with DbGAPDH, modeled residues range from 39 to 374) was selected and automatically modeled by SWISS-MODEL.[45] The representative homotetramers A_4 of chloroplast GAPDH were also predicted and shown in Figure 7b. In this model, two positive charge residues Lys164 and Arg 233, corresponding to Lys128 and Arg197 in *C. reinhardtii*, or Lys122 and Arg191 in spinach, were as well predicted to be involved in protein−protein interaction[46] (Figure 7a and b). In the chloroplast of *C. reinhardtii*, GAPDH cooperates with phosphoribulokinase (PRK) and CP12, a peptide sharing homology with C-terminal extension of GAPDH subunit B,[47] to form a complex to exert related function; Lys128 and Arg197 can stabilize such complex and facilitate native complex formation.[46] Therefore, these residues are expected to be conserved among other algae chloroplast GAPDHs and function in a similar way (Figure 7c).

Phylogenetic Analysis of DbGAPDH. Five groups of 52 GAPDH proteins, including the deduced DbGAPDH, were chosen to construct the phylogenetic tree using MEGA 4.0.2 software.[48] Universally, animal, chlorobi, chlorophyta, and most of streptophyta and cyanobacteria are clustered definitely into their own group, as Figure 8 shows. Nevertheless, the chlorophyta algae exhibit ambiguous relationship with a few streptophyta (such as *Zea mays*, *Spinach oleracea*, and *Vicia sativa*, etc.) and cyanobacteria (*Arthrospira platensis* and *Lyngbya* sp.), which implies a conserved evolutionary relationship among these species. Furthermore, the phylogenetic tree also revealed a closer relationship between DbGAPDH and streptophyta homologue than those of cyanobacteria, although still a far distance from animal counterparts (Figure 8).

In conclusion, chloroplast GAPDH is a regulatory isoform, which takes part in various metabolism modulation processes in photosynthetic organisms. We believe that the functional diversity of chloroplast GAPDH results from the sequence characteristics emphasizing at the DNA, mRNA, and protein levels, respectively. Consequently, the cDNA of chloroplast GAPDH was isolated, and the deduced protein structure was analyzed by computational tools. The calculated results are in accordance with experimental data on other closer species, implying a regulatory isoform of DbGAPDH. At the DNA level, specifically referring to the promoter region, six notable regulatory elements were found and conjecturally identified to underlie the functional diversity in four aspects using PLACE database. These findings offer new ideas for the functional diversity mechanisms of DbGAPDH; further evidence should be supplied to confirm the discussed findings.

AUTHOR INFORMATION

Corresponding Author

*Tel.: +86-20-87113849 (J.-G.J.); +86-20-39380628 (L.-X.L.). Fax: +86-20-87113849 (J.-G.J.); +86-20-39380628 (L.-X.L.). E-mail: jgjiang@scut.edu.cn (J.-G.J.); btlxluo@scut.edu.cn (L.-X.L.).

Funding

This project was supported by the National Natural Foundation of China (grant 31171631).

Notes

The authors declare no competing financial interest.

REFERENCES

(1) Caputto, R.; Dixon, M. Crystallization and identity of the triose and triosephosphate dehydrogenase of muscle. *Nature* **1945**, *156*, 630−631.

(2) Sirover, M. A. Role of the glycolytic protein, glyceraldehyde-3-phosphate dehydrogenase, in normal cell function and in cell pathology. *J. Cell. Biochem.* **1997**, *66*, 133−140.

(3) Li, A. D.; Anderson, L. E. Expression and characterization of pea chloroplastic glyceraldehyde-3-phosphate dehydrogenase composed of only the B-subunit. *Plant Physiol.* **1997**, *115*, 1201−1209.

(4) Erales, J.; Mekhalfi, M.; Woudstra, M.; Gontero, B. Molecular mechanism of NADPH-glyceraldehyde-3-phosphate dehydrogenase regulation through the C-terminus of CP12 in *Chlamydomonas reinhardtii*. *Biochemistry* **2011**, *50*, 2881−2888.

(5) Sirover, M. A. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. *Biochim. Biophys. Acta* **1999**, *1432*, 159−184.

(6) Sirover, M. A. On the functional diversity of glyceraldehyde-3-phosphate dehydrogenase: Biochemical mechanisms and regulatory control. *Biochim. Biophys. Acta* **2011**, *1810*, 741−751.

(7) Nasrin, N.; Ercolani, L.; Denaro, M.; Kong, X. F.; Kang, I.; Alexander, M. C. An insulin response element in the glyceraldehyde-3-phosphate dehydrogenase gene binds a nuclear protein induced by insulin in cultured cells and by nutritional manipulations in vivo. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 5273−5277.

(8) Alexander-Bridges, M.; Dugast, I.; Ercolani, L.; Kong, X. F.; Giere, L.; Nasrin, N. Multiple insulin-responsive elements regulate transcription of the GAPDH gene. *Adv. Enzyme Regul.* **1992**, *32*, 149−59.

(9) Chao, C. C.; Yam, W. C.; Lin-Chao, S. Coordinated induction of two unrelated glucose-regulated protein genes by a calcium ionophore: human BiP/GRP78 and GAPDH. *Biochem. Biophys. Res. Commun.* **1990**, *171*, 348−431.

(10) Graven, K. K.; Troxler, R. F.; Kornfeld, H.; Panchenko, M. V.; Farber, H. W. Regulation of endothelial cell glyceraldehyde-3-phosphate dehydrogenase expression by hypoxia. *J. Biol. Chem.* **1994**, *269*, 24446−24453.

(11) Lu, S.; Gu, X.; Hoestje, S.; Epner, D. E. Identification of an additional hypoxia responsive element in the glyceraldehyde-3-phosphate dehydrogenase gene promoter. *Biochim. Biophys. Acta* **2002**, *1574*, 152−156.

(12) Tsuchiya, K.; Tajima, H.; Yamada, M.; Takahashi, H.; Kuwae, T.; Sunaga, K.; Katsube, N.; Ishitani, R. Disclosure of a pro-apoptotic glyceraldehyde-3-phosphate dehydrogenase promoter: anti-dementia drugs depressits activation in apoptosis. *Life Sci.* **2004**, *74*, 3245−3258.

(13) Manjunath, S.; Sachs, M. M. Molecular characterization and promoter analysis of the maize cytosolic glyceraldehyde3-phosphate dehydrogenase gene family and its expression during anoxia. *Plant Mol. Biol.* **1997**, *33*, 97−112.

(14) Rolland, V.; Dugail, I.; Liepvre, X. L.; Lavau, M. Evidence of increased glyceraldehyde-3-phosphate dehydrogenase and fatty acid synthetase promoter activities in transiently transfected adipocytes from genetically obese rats. *J. Biol. Chem.* **1995**, *270*, 1102−1106.

(15) O'Brien, M. J.; Powls, R. Algal glyceraldehyde-3-phosphate dehydrogenase. *Eur. J. Biochem.* **1976**, *63*, 155−161.

(16) Falini, G.; Fermani, S.; Ripamonti, A.; Sabatino, P.; Sparla, F.; Pupillo, P.; Trost, P. Dual coenzyme specificity of photosynthetic glyceraldehyde 3-phosphate dehydrogenase interpreted by the crystal structure of A4 isoform complexed with NAD. *Biochemistry* **2003**, *42*, 4631−4639.

(17) Jia, Y.; Xue, L.; Liu, H.; Li, J. Characterization of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene from the halotolerant alga *Dunaliella salina* and inhibition of its expression by RNAi. *Curr. Microbiol.* **2009**, *58*, 426−431.

(18) Zhu, Y. H.; Jiang, J. G. Continuous cultivation of *Dunaliella salina* in photobioreactor for the production of β-carotene. *Eur. Food Res. Technol.* **2008**, *227*, 953−959.

(19) Yang, Z. Y.; Zhang, Q. Q.; Jiao, X. Z. Construction of a genomic DNA library of *Dunaliella salina*. *Acta Phytophysiol. Sin.* **2000**, *26*, 25−28.

(20) Li, Z. T.; Gray, D. J. Isolation and characterization of the 2S albumin gene and promoter from grapevine. *Genome* **2005**, *48*, 312−320.

(21) Abreu-Goodger, C.; Merino, E. RibEx: a web server for locating riboswitches and other conserved bacterial regulatory elements. *Nucleic Acids Res.* **2005**, *33*, W690−W692.

(22) Chang, W. C.; Lee, T. Y.; Huang, H. D.; Huang, H. Y.; Pan, R. L. PlantPAN: Plant Promoter Analysis Navigator, for identifying combinatorial *cis*-regulatory elements with distance constraint in plant gene group. *BMC Genomics* **2008**, *9*, 561−574.

(23) Graven, K. K.; Yu, Q.; Pan, D.; Roncarati, J. S.; Farber, H. W. Identification of an oxygen responsive enhancer element in the glyceraldehyde-3-phosphate dehydrogenase gene. *Biochim. Biophys. Acta* **1999**, *1447*, 208−218.

(24) Lu, S.; Gu, X.; Hoestje, S.; Epner, D. E. Identification of an additional hypoxia responsive element in the glyceraldehyde-3-phosphate dehydrogenase gene promoter. *Biochim. Biophys. Acta* **2002**, *1574*, 152−156.

(25) Quinn, J. M.; Merchant, S. Two copper-responsive elements associated with the *Chlamydomonas* Cyc6 gene function as targets for transcriptional activators. *Plant Cell* **1995**, *7*, 623−638.

(26) Quinn, J. M.; Barraco, P.; Eriksson, M.; Merchant, S. Coordinate copper- and oxygen-responsive *Cyc6* and *Cpx1* expression in *Chlamydomonas* is mediated by the same element. *J. Biol. Chem.* **2000**, *275*, 6080−6089.

(27) Quinn, J. M.; Eriksson, M.; Moseley, J. L.; Merchant, S. Oxygen deficiency responsive gene expressionin *Chlamydomonas reinhardtii* through a copper-sensing signal transduction pathway. *Plant Physiol.* **2002**, *128*, 463−471.

(28) Shaish, A.; Avron, M.; Pick, U.; Ben-Amotz, A. Are active oxygen species involved in induction of β-carotene in *Dunaliella bardawil*? *Planta* **1993**, *190*, 363−368.

(29) Bouvier, F.; Backhaus, R. A.; Camara, B. Induction and control of chromoplast-specific carotenoid genes by oxidative stress. *J. Biol. Chem.* **1998**, *273*, 30651−30659.

(30) Thum, K. E.; Kim, M.; Morishige, D. T.; Eibl, C.; Koop, H. U.; Mullet, J. E. Analysis of barley chloroplast *psbD* light-responsive promoter elements in transplastomic tobacco. *Plant Mol. Biol.* **2001**, *47*, 353−366.

(31) Zhou, D. X. Regulatory mechanism of plant gene transcription by GT-elements and GT-factors. *Trends Plant Sci.* **1999**, *4*, 210−214.

(32) Le Gourrierec, J.; Li, Y. F.; Zhou, D. X. Transcriptional activation by Arabidopsis GT-1 may be through interaction with TFIIA-TBP-TATA complex. *Plant J.* **1999**, *18*, 663−668.

(33) Buchel, A. S.; Brederode, F. T.; Bol, J. F.; Linthorst, H. J. M. Mutation of GT-1 binding sites in the Pr-1A promoter influences the level of inducible gene expression in vivo. *Plant Mol. Biol.* **1999**, *40*, 387−396.

(34) Yong-Min Lao, Y. M.; Xiao, L.; Ye, Z. W.; Jiang, J. G.; Zhou, S. S. *In silico* analysis of phytoene synthase and its promoter reveals hints for regulation mechanisms of carotenogenesis in *Duanliella bardawil*. *Bioinformatics* **2011**, *27*, 2201−2208.

(35) Jeong, M. J.; Park, S. C.; Byun, M. O. Improvement of salt tolerance in transgenic potato plants by glyceraldehyde-3 phosphate dehydrogenase gene transfer. *Mol. Cells* **2001**, *12*, 185−189.

(36) Park, H. C.; Kim, M. L.; Kang, Y. H.; Jeon, J. M.; Yoo, J. H.; Kim, M. C.; Park, C. Y.; Jeong, J. C.; Moon, B. C.; Lee, J. H.; Yoon, H. W.; Lee, S. H.; Chung, W. S.; Lim, C. O.; Lee, S. Y.; Hong, J. C.; Cho, M. J. Pathogen- and NaCl-induced expression of the SCaM-4 promoter is mediated in part by a GT-1 box that interacts with a GT-1-like transcription factor. *Plant Physiol.* **2004**, *135*, 2150−2161.

(37) Chen, C.; Chen, Z. Potentiation of developmentally regulated plant defense response by AtWRKY18, a pathogen-induced arabidopsis transcription factor. *Plant Physiol.* **2002**, *129*, 706−716.

(38) Agarwal, M.; Hao, Y.; Kapoor, A.; Dong, C. H.; Fujii, H.; Zheng, X.; Zhu, J. K. A R2R3 type MYB transcription factor is involved in the

cold regulation of CBF genes and in acquired freezing tolerance. *J. Biol. Chem.* **2006**, *281*, 37636−37645.
(39) Chinnusamy, V.; Ohta, M.; Kanrar, S.; Lee, B.; Hong, X.; Agarwal, M.; Zhu, J. K. ICE1: a regulator of cold-induced transcriptome and freezing tolerance in *Arabidopsis*. *Gene Dev.* **2003**, *17*, 1043−1054.
(40) Chinnusamy, V.; Schumaker, K.; Zhu, J. K. Molecular genetic perspectives on cross-talk and specificity in abiotic stress signalling in plants. *J. Exp. Bot.* **2004**, *55*, 225−236.
(41) Ben-Amotz, A.; Katz, A.; Avron, M. Accumulation of b-carotene in halotolerant algae: purification and characterization of b-carotene-rich globules from *Dunaliella bardawil* (Chlorophyceae). *J. Phycol.* **1982**, *18*, 529−537.
(42) Ben-Amotz, A.; Avron, M. On the mechanism of osmoregulation in *Dunaliella*. In *Energetics and Structure of Halophilic Microorganisms*; Caplan, S. R., Ginzburg, M., Eds.; Elsevier/North Holland Biomedical Press: Amsterdam, 1978; pp 529−541.
(43) Marchler-Bauer, A.; Anderson, J. B.; Chitsaz, F.; Derbyshire, M. K.; DeWeese-Scott, C.; Fong, J. H.; Geer, L. Y.; Geer, R. C.; Gonzales, N. R.; Gwadz, M.; He, S.; Hurwitz, D. I.; Jackson, J. D.; Ke, Z.; Lanczycki, C. J.; Liebert, C. A.; Liu, C.; Lu, F.; Lu, S.; Marchler, G. H.; Mullokandov, M.; Song, J. S.; Tasneem, A.; Thanki, N.; Yamashita, R. A.; Zhang, D.; Zhang, N.; Bryant, S. H. CDD: specific functional annotation with the Conserved Domain Database. *Nucleic Acids Res.* **2009**, *37*, D205−D210.
(44) Kim, H.; Feil, I. K.; Verlinde, C. L.; Petra, P. H.; Hol, W. G. Crystal structure of glycosomal glyceraldehyde-3-phosphate dehydrogenase from *Leishmania mexicana*: implications for structure-based drug design and a new position for the inorganic phosphate binding site. *Biochemistry* **1995**, *34*, 14975−14986.
(45) Schwede, T.; Kopp, J.; Guex, N.; Peitsch, M. C. SWISS-MODEL: an automated protein homology-modeling server. *Nucleic Acids Res.* **2003**, *31*, 3381−3385.
(46) Graciet, E.; Mulliert, G.; Lebreton, S.; Gontero, B. Involvement of two positively charged residues of *Chlamydomonas reinhardtii* glyceraldehyde-3-phosphate dehydrogenase in the assembly process of a bi-enzyme complex involved in CO_2 assimilation. *Eur. J. Biochem.* **2004**, *271*, 4737−4744.
(47) Wedel, N.; Solls, J. Evolutionary conserved light regulation of Calvin cycle activity by NAPDH-mediated reversible phosphoribulokinase/CP12/glyceraldehyde-3-phosphate-dehydrogenase complex dissociation. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 9969−9704.
(48) Tamura, K.; Dudley, J.; Nei, M.; Kumar, S. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. *Mol. Biol. Evol.* **2007**, *24*, 1596−1599.