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A *Phytophthora sojae* gene of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) induced in host infection and its anti-oxidative function in yeast

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Abstract Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a multifunctional protein well defined in eukaryotes, especially in mammalian and *Saccharomyces cerevisiae*. Using the method of suppression subtractive hybridization (SSH), we identified a *Phytophthora sojae* cDNA coding GAPDH, which was up-regulated during the early stage of soybean infection. The termed *PsGapdh* gene possessed three copies in the *P. sojae* genome. Its amino acid sequence harbored overall conserved domain of GADPH, homologous closest to GapC1 of *Achlya bisexualis* (oomycete) and adjoined to GapC2s of *Odontella sinensis* and *Phaeodactylum tricornutum* (diatom), on the C-Ⅱ branch of subfamily GapC in phylogeny tree of GAPDH. The transcriptional level of *PsGapdh* was up-regulated throughout early infection. Heterogenous expression of *PsGapdh* in the yeast *tdh*1-deleted mutant could rescue growth arrest under continuous exposure to H_2O_2. These results indicated active roles of *PsGapdh* in pathogen-host interaction and anti-oxidation.

Keywords: ***Phytophthora sojae*, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), pathogen-host interaction, anti-oxidation.**

Phytophthora sojae (Kaufmann & Gerdemann) is the causative agent of soybean root rot, one of the most devastating diseases of soybean worldwide[1]. The genus *Phytophthora* is cataloged into Oomycetes, which has already been classified as Stramenopiles, a diverse group of protoctists that includes golden-brown algae and diatoms[2,3]. Despite their peculiar phylogenetic affinities and economic importance, oomycetes were chronically understudied at the molecular level.

Researches on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) had established it as an intricate protein with extraordinary abundant functions, especially in mammalian cells. GAPDH was involved in endocytosis, translational control of histone gene expression, nuclear tRNA exporting, DNA replication and DNA repair[4]. Recent studies revealed the roles of GAPDH in neuronal apoptosis, senescence-related neurodegenerative disorders, prostate cancer and viral pathogenesis[5,6]. Its new nuclear roles emerged lately, including its essential function in nuclear membrane fusion, the recognition of fraudulently incorporated nucleotides in DNA and its mandatory participation in the maintenance of telomere structure[7].

Moreover, knowledge about GAPDH functions in microbial cells had been acquired, which was initially triggered in yeast. S-thiolation of GAPDH, actually called TDH in yeast, had been proven directly related to resistance against hydrogen peroxide (H_2O_2) in *Saccharomyces cerevisiae*[8] and regulated by glutaredoxin 5, an active protein in redox homeostasis[9]. Besides, TDHs were interacted with Gts1p, a rhythmically expressed protein that appeared to be involved in regulating oscillations of the glycolytic pathway[10]. Furthermore, one minor isoform, TDH1, was particularly regulated by reductive stress caused by an excess of cytoplasmic NADH[11]. All of these results implicated active roles of GAPDH in diverse processes, which were probably more intimate with pathogen-host interaction because of its affiliation to redox homeostasis.

Nevertheless, GAPDH functions were poorly reported in oomycetes. The coding gene of GAPDH in *Phytophthora infestans*, *gpdA*, was isolated from a phage DASH genomic library using a fragment encompassing the gene of *Aspergillus nidulans*[12]. Then, GAPDH was determined to fuse with triose-phosphate isomerase (TPI) and form a single transcriptional unit (*tigA*) in *Phytophthora* species[13]. This TPI-GAPDH fusion protein was demonstrated to be existing in diatom and further determined to be imported compartment-specifically into mitochondria, in favor of a mitochondrial origin of the eukaryotic glycolytic pathway[14]. Recently, transcript accumulation of GAPDH was testified and found to be significantly increased in germinated cysts of *Phytophthora nicotianae*[15], sug-

gesting a positive role of GAPDH in an important infective structure and enlightening our study on GAPDH in *P. sojae*.

To explore the molecular mechanisms involved in *P. sojae* infection, we utilized the method of suppression subtractive hybridization (SSH) to isolate *P. sojae* genes differentially expressed prior to saprophytic infection. Among the up-regulated genes, a cDNA coding GAPDH, named *PsGapdh* (GenBank Accession No. CX873807), was identified. As the first step of determining physiological function of GAPDH in *P. sojae*, we cloned and analyzed the coding sequence of *PsGapdh*, detected its transcriptional level during early stage of susceptible infection, and performed a complementation experiment in a *tdh*1-deleted mutant to testify whether the *P. sojae* protein has anti-oxidation function similar to yeast TDH.

1 Materials and methods

1.1 P. sojae strain and culture conditions

P. sojae strain used in this study, Pmg2, was isolated from Heilongjiang Province of China, and stored in the collection of Nanjing Agricultural University. For DNA and RNA extraction, culture blocks were transferred into a sterile 250 mL flask containing 100 mL 10% V8 juice medium (10% Campbell's V8 juice and 0.02% $CaCO_3$) and incubated at 25℃ in the dark for 4 d.

1.2 cDNA cloning and homology analysis of PsGapdh

To obtain intact ORF, total RNA of Pmg2 was prepared according to the TRIzol Reagent kit (Invitrogen, Carlsbad, CA) and reverse transcribed into single-strand cDNA using the synthesis system of M-MLV Reverse Transcriptase (Promega Biosciences, Inc.). Based on corresponding sequence in COGEME, a pair of primers was designed as 5′-ATGAGTGAAGTCAAGATCG-3′ and 5′-CTACTTGTCGACAGTGGC-3′, named sojGap2-F/R respectively. A full-length gene was amplified by PCR from Pmg2 cDNA. Cycling conditions for 30 cycles were 94℃ for 30 s, 54℃ for 30 s and 72℃ for 1 min, with pre-denaturing at 94℃ for 5 min and prolongation at 72℃ for 7 min subsequently. PCR product was isolated from 1% agarose gels using Agarose Gel DNA Purification kit (TaKaRa, Japan), linked into pMD18 T-Vector and sequenced by TaKaRa Biotechnology (Dalian) Co., Ltd.

The amino acid sequences of 54 homologues from different species, along with PsGAPDH, were analyzed in Mega3.0Beta[16] to generate a phylogeny tree. Conserved domains of the protein with similar putative functions were revealed by BioEdit (Version 7.0.5.1) (Tom Hall Copyright C 1997-2005) in comparison with some model microorganisms such as *P. infestans*, *S. cerevisiae*, *Neurospora crassa* and *Magnaporthe grisea*.

1.3 Southern hybridization

Genomic DNA of *P. sojae* was extracted from lyophilized mycelium of 4-d-old Pmg2 according to Cenis[17]. Under the guidance of DIG DNA Labeling and Detection kit (Roche Molecular Biochemicals, Germany), a 577 bp fragment of *PsGapdh* DNA was labeled as probe, at a final concentration of 25 $ng \cdot mL^{-1}$ for hybridization. Ten micrograms of chromosomal DNA was restrictively digested with *Eco*R I and *Hin*d III, both of which had none restriction site within the gene. The resulting DNAs were fractioned on 0.8% agarose gels, transferred to positively charged nylon filters (HybondTM-N^+, Amersham, Biosciences UK Limited) and hybridized to DIG-labeled probe in hybridization solution (including 6× SSC, 5× Denhard's reagent, 0.5% SDS, 100 $\mu g \cdot mL^{-1}$ salmon sperm DNA) at 54℃ overnight. The filters were washed in a low stringency solution (2× SSC and 0.1% SDS) and then a high stringency solution (0.5× SSC and 0.1% SDS) at 68℃ for 30 min each, and then washed, immunologically detected according to the kit.

1.4 RT-PCR of PsGapdh during early stage of compatible interaction

For compatible interaction, two-week-old leaves of soybean variety Hefeng35 were detached from the plant and placed in petri plates for inoculation with Pmg2 mycelium, which had been cultured in 10% V8 juice medium for 4 d. Leaves inoculated with mycelium were incubated at 25℃ for 0, 1, 3, 6 and 12 h, respectively. Each sample of Pmg2 mycelium was then separated from the leaves simultaneously and prepared for RNA extraction immediately.

Total RNA was extracted as described above and all RNA used for RT-PCR was treated with DNase I (Qiagen, Valencia, CA) prior to cDNA synthesis to exclude DNA contamination. Ten micrograms of total RNA from each sample were used to create first strand cDNA according to the manufacturer's protocol. Two microliter of diluted first strand cDNA was used as template in 25 μL PCR solution, according to the LA

PCR kit (TaKaRa, Japan). To proliferate shorter fragment adjacent to 3′-PolyA end rather than full-length sequence from cDNA, primers were designed as follows: fGap F 5′-TGCTGAACGGCAAGCTGACG-3′, fGap R 5′-ACTTGTCGACAGTGGCCATG-3′ (for *PsGapdh*); actinRT 5′-GTACTGCAACATCGTGCTGTCG-3′, actinAD 5′-TTAGAAGCACTTGCGGTGCACG-3′ (for *actin*). After equating of template concentration among samples and optimization for cycles within exponential stage, PCRs were performed in an MJ-PTC 200 thermocycler (MJ-Research, Watertown, MA) with the following program: 95℃ for 5min followed by 28 cycles of 95℃ 15 s, 60℃ (for *PsGapdh*) or 64℃ (for *actin*) 30 s, 72℃ 30 s, and a final extension for 7 min at 72℃. PCR products were electrophoresed in 1.2% ethidium bromide-containing agarose gels, visualized with Gel Doc 2000 (BIO-RAD, Segrate Italy) and quantitatively analyzed in Quantity One 4.4.0 (BIO-RAD, Segrate Italy).

1.5 Yeast strains and culture conditions

A series of *tdh*-mutant strains originating from BY4741 were purchased from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/), described as Y00000 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0), Δ*tdh*1 (Mat a; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0;YJL052w::kanM×4). Strains were cultured either in YPD medium (2% glucose, 2% peptone, 1% yeast extract) or SD medium (0.17% yeast nitrogen base without amino acids, 5% ammonium sulfate, 2% glucose) supplemented with appropriate amino acids and bases: 20 mg·L^{-1} methiouine, 20 mg·L^{-1} histidine, 20 mg·L^{-1} uracil and 60 mg·L^{-1} leucine (with or without in SD or SD-leu$^-$). Media were solidified by the addition of 2% agar.

1.6 Plasmid construction and transformation of yeast mutant strain

Expression vector was constructed on the basis of pGAD424, a shuttle plasmid containing a 0.4 kb ADH1 promoter and leucine selection marker. The derivative, pGAD424::*PsGapdh*, was made by fusing *PsGapdh* into multiple cloning site (MCS) with *Eco*R I forward and *Bam*H I reversely. Δ*tdh*1, the strain most sensitive to H_2O_2 at stationary stage, was transformed with both empty and re-constructional vector by the lithium acetate method[18], generating transformants of Δ*tdh*1+pGAD424 or Δ*tdh*1+pGAD424::*PsGapdh*. Transformants were selected on SD-leu$^-$ media for twice at least.

1.7 Yeast viability assays for H_2O_2 tolerance

Sensitivity to H_2O_2 was examined by spot assay[19], in which yeast strains were under constant exposure to oxidant. Cells were grown to stationary phase in SD (for wild-type and original strains) or SD-leu$^-$ (for leu$^+$-derivatives), and diluted to various concentrations (A_{600}=1, 10^{-1}, 10^{-2}, 10^{-3}). An aliquot (5 μL) from each dilution was spotted onto YPD medium containing 0, 4.5 and 6 mmol/L H_2O_2, respectively. To quantify cell viability, equal liquid (5 μL) of each strain at A_{600}=1 was diluted up to 50 μL and spread on the same series of H_2O_2 plates, with three-time repetition. Plates were incubated for 4 d at 30℃. Spot images and cell survival ratios were used for estimating H_2O_2 sensitivity of strains.

2 Results

2.1 Cloning and sequence analysis of PsGapdh

As an up-regulated gene identified during the early stage of compatible interaction between *P. sojae* and soybean, *PsGapdh*, a thoroughly translatable ORF with a length of 1020 bp, was cloned from cDNA of *P. sojae*. The clone from genome was identical with that from cDNA, suggesting none intron in the coding region, as this gene in *P. infestans*[12] and a majority of other genes in oomycete[20]. The DNA sequence of *PsGapdh* was matched to that in the Functional Genomics of Microbial Eukaryotes (COGEME), Phytopathogenic Fungi and EST Database version1.5 (http://cbr-rbc.nrc-cnrc.gc. ca./service/cogeme) and the *P. sojae* Genome Database release version1.0 (http://genome.jgi-psf.org/sojae1/sojae1.home.html), resulting in 100% identity. Southern blot resulted in three strips on each lane of digested genome DNA, in which the gene *PsGapdh* was still intact, indicating that *P. sojae* harbored three copies of *PsGapdh* in the genome (Fig. 1(a)).

As discussed previously, GAPDH was involved in compartment-specification[14] and recently used as a mark for lateral gene transfer[21]. Thus, homologues were divided into two distinct classes among diplonemid, called subfamilies Gap1-GapC (related to the cytosolic *gapC* of eukaryotes) and Gap2-GapA/B (related to cyanobacterial and proteobacterial *gap3*), respectively, showing differential intracellular locations and functions. In order to deduce its putative characteristics in *P. sojae*, amino acid sequence of *PsGapdh* was aligned with 54 other sequences from different species

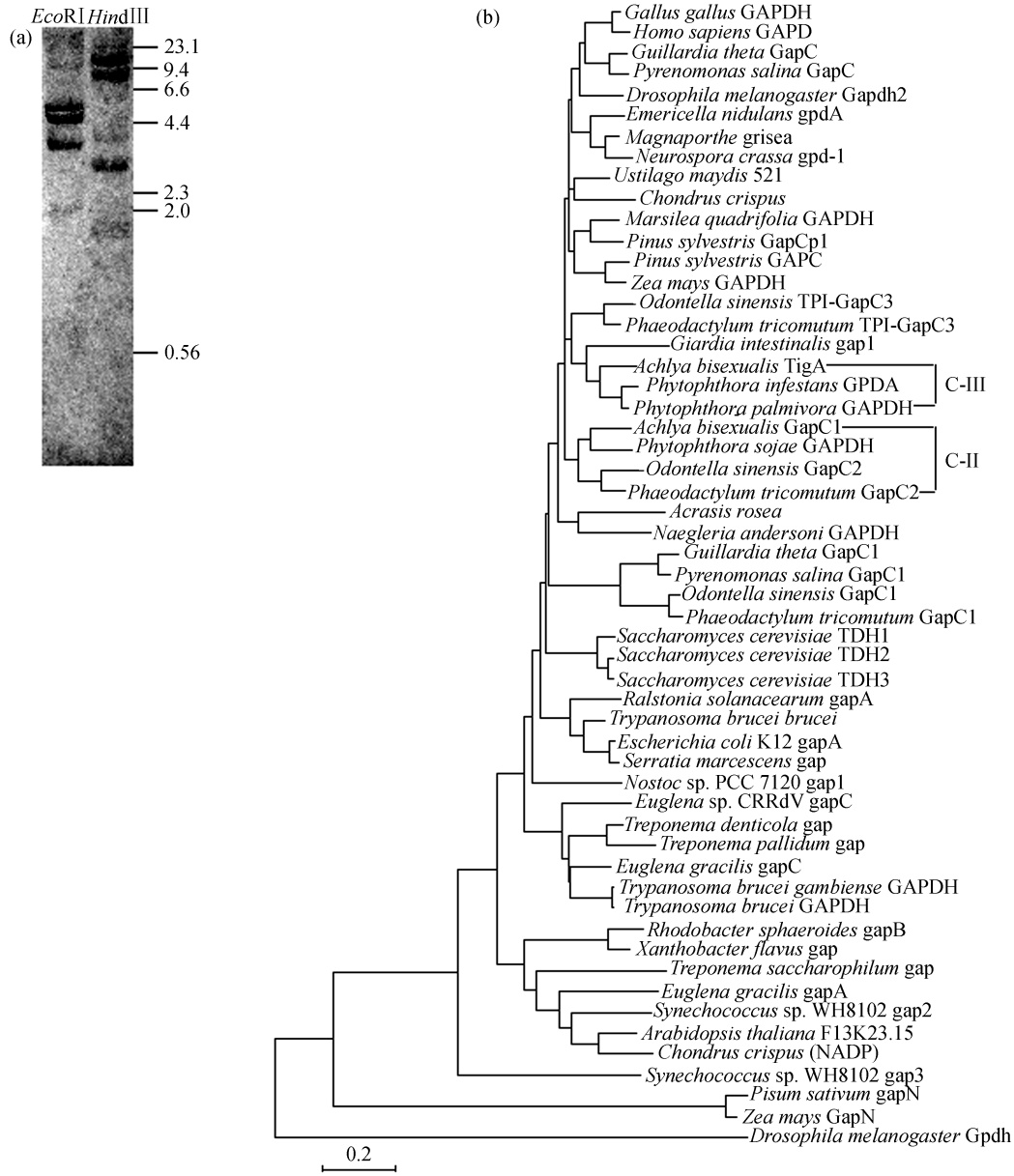

Fig. 1. Characterization of *PsGapdh* both in the genome and protein phylogeny tree. (a) Southern blot analysis of *PsGapdh*. Genome DNA (10 μg/lane) from strain Pmg2 was digested with *Eco*R Ⅰ and *Hin*d III, electrophoresed in 0.8% agrose gel. Southern hybridization was performed according to the kit. The positions of molecular size markers are indicated in kilobases. (b) Protein phylogeny tree of GAPDH. Tree of GAPDH amino acid sequences was conducted by the neighbor-joining (NJ) method using Mega30Beta, with a root at Gpdh of *Drosophila melanogaster*. This clustering was in accordance of that generated by Liaud *et al.*[14] and localized PsGAPDH on C-II branch of subfamily GapC, apart from C-III branch comprising that of other *Phtophthora*, indicating its cytosolic-specific compartmentation.

(Fig. 1(b)). In protein phylogeny tree of GAPDH, PsGAPDH was closest to GapC1 of *Achlya bisexualis* (oomycete) and adjoined to GapC2s of *Odontella sinensis* and *Phaeodactylum tricornutum* (diatom) on the C-II branch of subfamily GapC, indicating its cytosolic location. Comparatively, the reported homologues of other oomycetes (GPDA of *P. infestans*, GAPDH of *P. palmivora* and GapC2 (TigA) of *A. bisexualis*) were clustered on the C-III branch and compartment-specific in mitochondria. Although TPI-GAPDH fusion occurred throughout the genus *Phytophthora*[13], PsGAPDH had a distinctive protein structure catalogued rather in C-II than in C-III.

To estimate the potential function of PsGAPDH,

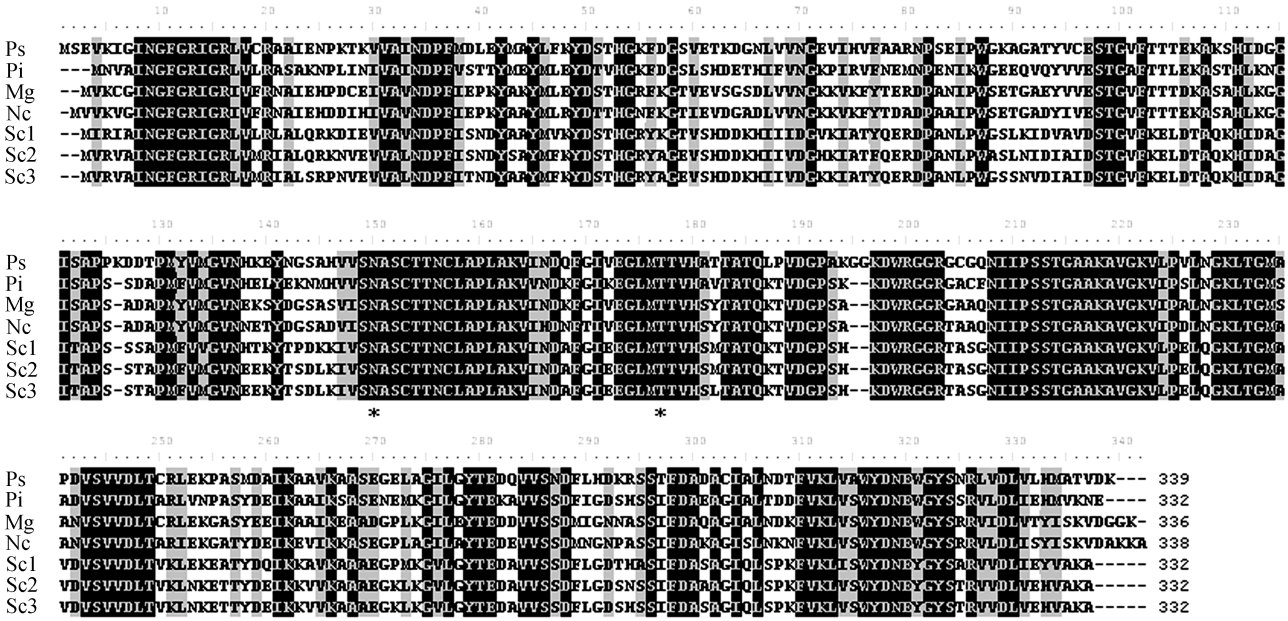

Fig. 2. A multiple-sequence alignment of GAPDH orthologs from both *P. sojae* and other model fungi. Abbreviations of species names are explained as follows: Ps, *P. sojae*; Pi, *P. infestans*; Mg, *M. grisea*; Nc, *N. crassa*; Sc1－3, *S. cerevisiae* (TDH1－3). Identical amino acid residues in all sequences are denoted in black background and that with 70% similarity are shown in grey blocks. The conserved asparagines (N) 150 and threonine (T) 177 residues, which are important for NAD^+ binding and thiol group activity during catalysis respectively, are indicated with asterisks.

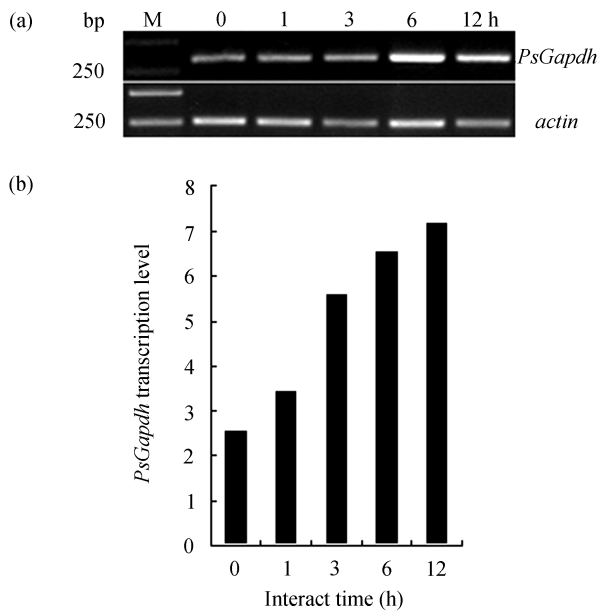

Fig. 3. RT-PCR analysis of *PsGapdh* transcriptional level during early stage of compatible interaction between Pmg2 and Hefeng35. (a) Luminance of PCR products in agrose gel. Because of non-compatibility, *PsGapdh* and *actin* (as internal control) were amplified respectively; nevertheless the PCR products of the same sample were added into agrose gel with equal quantity and electrophoresed to meet the uniformity. RNA was isolated from mycelium inoculated for 0, 1, 3, 6 and 12 h (0, 1, 3, 6, and 12) respectively, and used in RT-PCR. Marker lane (M) shows the 250 bp bands. (b) Quantitative analysis of electrophoretic stripes. In Quantity One 4.4.0, absorption of each stripe was transferred into volume concentration data. Relative transcriptional level was determined by the ratio generated from the corresponding data of *PsGapdh* and *actin* from the same treatment, taking the latter as divisor.

conserved regions were revealed on the basis of comparison with several model fungi (Fig. 2), which were far-off in the phylogeny tree but well researched previously. Apparently, PsGAPDH harbored the putative functional regions for NAD^+ binding (in 150 site) and catalytic activity (in 177 site), and even shares "conflict" regions (in 248, 287 and 329 site) with three TDH isoforms of *S. cerevisiae*.

2.2 PsGapdh transcription was modulated throughout early stage of susceptible infection

RT-PCR was used to give an insight into *PsGapdh* transcription tendency throughout the early stage of soybean infection. Within exponentially amplified cycles, originating from equate quantity of cDNAs, PCR products of different samples showed varied luminance on agrose gel (Fig. 3(a)), which was transformed into volume concentrations and quantitatively analyzed (Fig. 3(b)). In the process of early infection stage, a remarkable increase from 0 to 12 h could be implicated, suggesting that *PsGapdh* was induced throughout the early stage of *P. sojae* colonization on the susceptible host.

2.3 PsGapdh complemented H_2O_2 tolerance of a yeast Δtdh mutant

In the absence of effective and stable transformation system of *P. sojae*, we resorted to yeast genetic com-

plementation to clarify the anti-oxidative function, since *PsGapdh* possessed identical conserved regions in common with its yeast homologues. The *P. sojae* gene appeared functionally equivalent to *tdh*1 of *S. cerevisiae* based on its complementation of the Δ*tdh* mutation. This was shown by expressing the *PsGapdh* open reading frame under a constitutive promoter in the yeast mutant, which would otherwise show a growth arrest under continuous exposure to H_2O_2. In the presence of 4.5 and 6 mmol/L H_2O_2 in YPD plates, *PsGapdh* restored growth to the mutant by one progression in spot assay and up to 60% viability as wild type strain statistically (Fig. 4). It was indirectly proved that *PsGapdh* could be essential for survival in oxidative stress environment.

3 Discussion

On the aspect of genetic phylogeny of GAPDH, there seemed to be some canvasses on the classification of PsGAPDH. Firstly, GAPDH of *P. sojae* was bunched up with GapC1 of *A. bisexualis* (oomycete), GapC2s of *O. sinensis* and *P. tricornutum* (diatom), apart from homologues of other oomycetes (GPDA of *P. infestans*, GAPDH of *P. palmivora* and GapC2 (TigA) of *A. bisexualis*), even far from that of some model fungi (*Ustilago maydis*, *N. crassa* and *M. grisea*). These results are in accordance with the catalogue formed previously[14]. As GAPDH was taken as a label

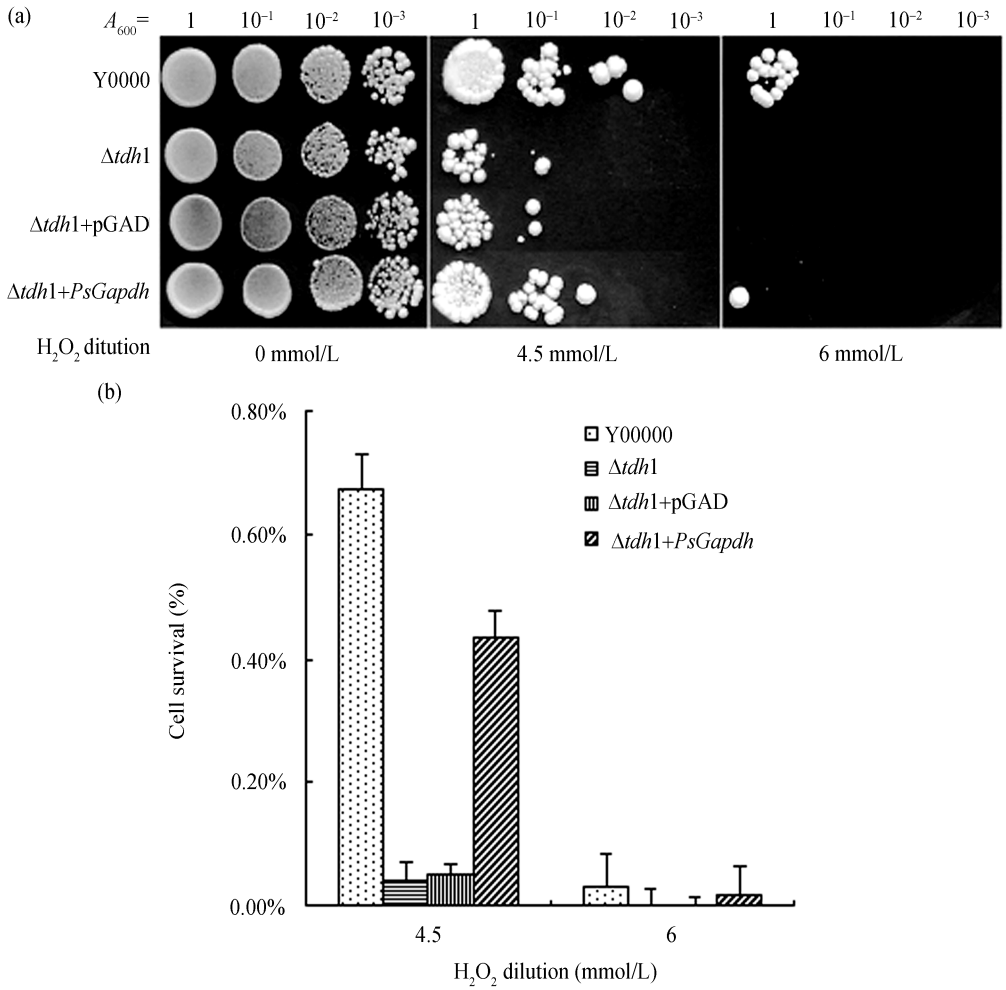

Fig. 4. Complementation of the *tdh*1 allele of *S. cerevisiae* by *PsGapdh*. (a) Cells of Y00000, Δ*tdh*1, Δ*tdh*1+pGAD424 (Δ+pGAD) and Δ*tdh*1+pGAD424::*PsGapdh* (Δ+*PsGapdh*) were grown to stationary phase in SD (for wild-type and original strains) or SD-leu$^-$ (for leu$^+$-derivatives), and serial 10-fold diluted (A_{600}=1, 10^{-1}, 10^{-2}, 10^{-3}). An aliquot (5 μL) from each dilution was spotted onto YPD medium containing 0, 4.5 and 6 mmol/L H_2O_2, respectively. The blotted plates were incubated for 4 d at 30℃. (b) Cell survival ratio. Equal liquid (5 μL) of each strains at A_{600}=1 was diluted up to 50 μL and spread on the same series of H_2O_2 plates. After 4-d incubation at 30℃, colonies on each plate were counted and cell survival ratio was made by dividing the value for H_2O_2-treated from mock-treated samples. Every statistic data was generated from three repetition sample.

in the investigation of lateral gene transfer from prokaryote to eukaryote[21], the location of PsGAPDH in protein phylogeny tree was likely to tell the kindred relation between *P. sojae* and diatom. Secondly, PsGAPDH's entry enlarges the C-II branch of subfamily GapC, implicating its cytosolic orientation. Since gene function was always dependent on its sub-cellular localization, new roles of GAPDH in mammalian and yeast had been found to be relevant to different localizations fulfilling non-glycolytic functions[22−26]. Based on the phylogeny analysis, PsGAPDH probably exerted its effect in cytosol, which would need experimental evidence in both yeast and *P. sojae*.

There could be different interpretations of *PsGapdh*'s prominence through SSH during the early stage susceptible infection, to which particular attention was paid in this study. In a sense, the concerned pathogen, *P. sojae* should promote energy metabolism for struggling with its host to hold an advantageous status, and as a result, GAPDH was up-regulated. Whereas, in our selection of 487 clones from SSH screening, none of other glycolytic enzymes appeared in forward library, and even conversely, pyruvate kinase presented itself in reverse library, leaving this commentation an antinomy. Therefore, we resort to unknown properities of *PsGapdh* in the light of foregone in *S. cerevisiae*, in which some valuable evidences for its involvement in anti-oxidation and redox homeostasis had been achieved[8,9,11]. Oxidative burst, a well-known event in the early stage of pathogen and plant interaction[27], could induce programmed cell death (PCD) and trigger defense responses in plants[28]. However, it remained unknown how phytopathogen struggled in these oxidative stress during plant infection. It was prognosticated that the functional validation on PsGAPDH could reveal its connection with oxidative resistance during early stage of susceptible infection, which had not been represented in previous papers but fulfilled in both *P. sojae* and *S. cerevisiae* as our study indicated.

The transcriptional level of *PsGapdh* was modulated dramatically during the inoculation of *P. sojae* mycelium on host leaves, when and where the host executed ROS burst to challenge the pathogen, implicating its active demeanour. Although it had been believed as a house keeping gene, recent studies supported a different standpoint that GAPDH could be regulated under different abiological stress in maize[29,30] and biological stress in potato[31]. Thus, the continuous increasing trend and maximum elevation of 3-fold were considered of prominence, as this level of variation was also justificative in temporal accumulation of GAPDH mRNA in whole potato plates during an incompatible interaction with *P. infestans*[32] and in quantitative analysis of GAPDH gene expression in germinated cysts of *P. nicotianae*[15].

As a proof of its anti-oxidative competence, *PsGapdh* rescued the Δ*tdh* mutant to revive under continuous exposure to H_2O_2, demonstrating its probable anticipation in defense of oxidative burst during the early stage of pathogen-plant interaction. Although heterogenous complementation was not always consummate, yeast genetic complementation system was used widely to validate genes' putative functions, as previous examples of *picdc14* of *P. infestans*[33], *ypt1* of *Trichoderma reesei*[34] and two genes of *P. tricornutum* and *Crypthecodinium cohnii* related to amino-acid synthesis[35]. Thus, the characterization of *PsGapdh* in yeast was convincing , thus validating our hypothesis.

In this study, we cloned *PsGapdh* of *P. sojae*, testified its involvement in compatible pathogen-host interaction and its competence of anti-oxidation in *S. cerevisiae,* dropping a hint on the novel roles of GAPDH in *P. sojae*. Though we know whether and how *PsGapdh* helped to protect the pathogen from oxidative damage caused by the host, the possibilities could be ascertained and the mechanism should be revealed through further studies on the basis of mutant's phenotype in *P. sojae*.

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