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Molecular cloning, characterization, expression and chromosomal location of *OsGAPDH*, a submergence responsive gene in rice (*Oryza sativa* L.)

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Abstract Differential clones from submergence stress and control treatment from rice seedlings were isolated by the differential screening method. One of the clones, *OsGAPDH*, represented a gene that was expressed at high level during 12-h submergence. A homology search of GenBank databases showed that *OsGAPDH* had significant sequence homology with maize non-reversible glyceraldehyde-3-Phosphate dehydrogenase. The *OsGAPDH* sequence consists of 1,772 bp with the longest open reading frame encoding 499 amino acids with a calculated relative mass of 54.2 kDa. Genomic Southern analysis indicated that one or two copies of the *OsGAPDH* gene occur in the Yukihikari genome. The chromosomal location of the *OsGAPDH* gene was identified by RFLP analysis indicating that *OsGAPDH* was located on chromosome 8. Tissue-specific expression of *OsGAPDH* indicated that the high level of mRNA was detected in the panicle. Plants exposed to drought, submergence and ABA treatment showed an increased accumulation of *OsGAPDH* transcripts. The induction of *Escherichia coli* cells containing the pGST-OsGAPDH plasmid resulted in the accumulation of a large amount of the 83.2-kDa recombinant protein. The purified GAPDH enzyme showed an optimum activity at pH 8.5 and 50 °C, and was strongly inhibited by ATP and ADP.

Keywords Rice · Submergence · Gene expression · Glyceraldehyde-3-phosphate dehydrogenase · Differential screening

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Introduction

Submergence is a common feature in rainfed lowland rice during the monsoon season, resulting in exposure of plants to hypoxia/anoxia. Although rice is well adapted to aquatic environments, its survival is low if complete submergence persists for several days. Survival in submergence largely depends on adequate reserves and maintaining energy production through augmented rates of alcoholic fermentation during oxygen shortage. The inability of crops to withstand low oxygen conditions in the root zone results in substantial yield losses. Submergence tolerance of rice seedling is related to the maintenance of energy supply partly through fast rates of alcoholic fermentation, which require high levels of carbohydrates. Studies on expression of anaerobic protein genes in higher plants under hypoxia and anoxia carried out during the last 15 years have highlighted the importance of energy metabolism in plant tolerance to anaerobic stress; many of these anaerobic proteins (ANPs) have since been the enzymes of glycolysis and fermentation (Vartapetian and Jackson 1997).

In anaerobic environments glycolysis and alcoholic fermentation are important for energy production of plants. The expressions of the genes involved in this process (e.g. glyceraldehyde-3-phosphate dehydrogenase, enolase, alcohol dehydrogenase and pyruvate decarboxylase) are dramatically induced by anaerobiosis (Umeda and Uchimiya 1994; Sachs et al. 1996). This induction is essential for anaerobic tolerance. The causes of cell death in anoxia includes: (1) self poisoning by ethanol formed by alcoholic fermentation, (2) cytoplasmic acidosis, (3) insufficient energy generation (ATP) to sustain cell integrity, and (4) death from metabolic lesions caused by the re-entry of oxygen after a period without oxygen. Slowing down of ethanol fermentation, or its deviation from ethanol formation into less toxic compounds, such as malate, was proposed as the biochemical basis for adaptation and survival (Vartapetian and Jackson 1997).

Anaerobic proteins have since been studied intensively in maize roots. In this tissue, 20 proteins account

for approximately 70% of the total amount of protein formed after 5-h anaerobiosis and the majority catalyze the reaction in glycolysis or sugar phosphate metabolism (Sachs et al. 1996). The production of ANPs continues until the root begins to die after 72 h without oxygen (Sachs et al. 1980) and is a consequence of enhanced gene transcription and selective translation mediated by the phosphorylation of protein synthesis initiating factors (Webster et al. 1991). Pioneering work on the mechanism of anoxic induction led to the conclusion that anoxia induces transcription and the accumulation of novel set of mRNAs, for example the maize adh1 (Rowland and Strommer 1986) gene exhibits increased transcription during anoxia. Recent analysis of crosses between submergence tolerant and intolerant genotypes suggests that tolerance traits can segregate in a manner implying that only a few genes specify tolerance. Their isolation, sequencing and identification is a promising avenue that will benefit basic understanding and facilitate the selection and breeding of crops with greater resilience to flooding (Setter et al. 1997). Although numerous studies of submergence tolerance have been carried out (Mazaredo and Vergara, 1982; Chang et al. 2000; Dominique et al. 2001), the molecular aspects have not been clearly understood. The regulatory mechanism of anaerobic metabolism is complex, involving substrate and product concentration, enzyme synthesis and degradation and enzyme activation or repression.

High nutritional and high yielding lowland cultivars could be adapted by genetic engineering, allowing them to grow in flooded areas. To achieve this objective we isolated a full-length cDNA encoding non-reversible glyceraldehyde-3-phosphate dehydrogenase (*OsGAPDH*), a gene involved in the fermentation process that is induced by submergence in rice. The non-reversible glyceraldehyde-3-phosphate dehydrogenase (EC 1.2.1.9) catalyzes the oxidation of D-glyceraldehyde-3-P (D-G3P) to 3-phosphoglycerate (3-PGA) with the generation of NADPH. Here we report the first cloning of the full structure of the rice glyceraldehyde-3-phosphate dehydrogenase (*OsGAPDH*) gene and the study of the enzymatic characterization and the pattern of expression in different tissues of rice in response to various environmental stimuli.

Materials and methods

Plant materials and growth conditions

A popular high yielding japonica rice variety Yukihikari was used for this experiment. The seeds were de-husked mechanically and surface sterilized by soaking successively in 70% ethanol, 2% sodium hypochlorite and 10 mM HCl, as described elsewhere (Huang et al. 1990). After washing extensively in sterile distilled water, 50 seeds were germinated in a 300-ml Erlenmeyer flask containing 5 ml of sterile distilled water at 28 °C. For tissues of mature rice plants, each tissue was obtained from 6-week-old rice plants grown in a 16-h light and 8-h dark period at 28 °C in a growth chamber. Six-day old rice seedlings grown in the dark at 28 °C were separately exposed to various abiotic stresses and plant hormones for 2 days. Total RNA was extracted from the whole seedlings and used for Northern-blot analysis with following treatments, control (grown at 28 °C), drought (dehydrated until losing 30–40% of the fresh weight), cold (exposed to 5 °C) and salt (irrigated with 0.25 M NaCl). For hormone treatments, seedlings were both irrigated and sprayed with mannitol (0.5 M mannitol), ethephon (10^{-2} M ethephon) and ABA (10^{-5} M abscisic acid) solutions. Under control conditions, only roots were submerged in distilled water; for submergence stress, whole seedlings were submerged in distilled water without aeration at 28 °C in the growth chamber.

Construction and screening of the cDNA library

Poly (A)+RNA was isolated directly from rice seedlings treated for 24-h submergence treatment (whole seedlings were submerged in distilled water without aeration at 28 °C) by using the Fast track 2.0 kit (Invitrogen, Carlsbad, Calif. USA). A cDNA library was constructed using 5 µg of this poly (A)+RNA and a λZAP-cDNA/Gigapack cloning Kit from Stratagene, La Jolla, Calif. USA. The library was screened with single-stranded cDNAs synthesized from both control and 24-h submergence treatment. The phage library was plated out and transferred to duplicate sets of Hybond-N+ Nylon membrane (Amersham). One of the sets of filters was probed with DIG-labeled (Boehringer Mannheim, Version 3.0, 1995) cDNA prepared by reverse transcription of RNA from the seedlings treated with 24-h submergence. The other set of filters was hybridized to a labeled cDNA probe prepared from untreated control seedlings. Clones were identified that hybridized more strongly to the first probe and negligibly to the control probe. These clones were selected for further investigation. In vivo excision of the pBluescript from the Uni-Zap XR vector was carried out and cDNA inserts were separated after digestion with desired restriction enzymes (*Xho*I/*Eco*RI).

DNA extraction and Southern-blot analysis

Genomic DNA was isolated from young seedlings based on the methods described by Murray and Thomson (1980). The purified DNA was digested with *Bam*HI, *Bgl*II, *Eco*RI and *Hin*dIII, and a parallel set of samples was separated by electrophoresis on a 0.8% agarose gel. DNA fragments were transferred to nylon membranes (Hybond N+ Amersham) by the capillary transfer method. Hybridization was carried out at 55 °C overnight. The inserts of cDNA clones were used as probes labeled by the Alkphos labeling system (Amersham). After hybridization the membranes were washed twice in primary wash buffer (2 M-Urea, 0.1%-SDS, 0.5 M-Na phosphate,150 mM-NaCl, 1.0 M-$MgCl_2$, 0.2% Blocking reagent) at 55°C for 15 min and twice in secondary wash buffer (1 M Tris base, 2 M NaCl) for 5 min at room temperature as per the manufacturer's recommendations.

RFLP-mapping

The mapping population consists of 127 di-haploids (DH) derived from a *indica*/*japonica* rice (ZYQ8/JX17) cross. The chromosomal posistion of *OsGAPDH* was found out using molecular linkage maps (He et al. 1999). The genomic DNA of the 127 DH lines was digested with *Sca*I, separated on a 0.8% agarose gel and then transferred to nylon membranes. The hybridization was done by using *OsGAPDH* cDNA as a probe. Mapping analysis was carried out by the software MAPMAKER/EXP version 3.0 (Lander et al. 1987).

RNA extraction and Northern-blot hybridization

For Northern-blot analysis, total RNA was isolated from rice seedlings or 6-week-old mature plants by the SDS–phenol method of Bachem et al. (1996). All samples were frozen in liquid nitrogen and kept at –80 °C until use. Total RNA was separated in a 1.2% (w/v) agarose gel (3 Vcm-1) containing 2.2 M formaldehyde and transferred to a Highbond-N+ nylon membrane (Amersham) using 20 × SSC (Sambrook et al. 1989). Equal loading of RNA was verified by staining with ethidium bromide and comparing the amount

Fig. 1 Nucleotide and deduced amino-acid sequence of cDNA clone *OsGAPDH*

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CGCGCCCCCATCACCACCCACTCCACTCCCTCCTCCCCCGAGCCCGAGTGCCTCACCGCC   60
ACTAGCTAGCAGCAGCGGCGGCGGCGGCGGAGAAGAGGAGGAGATATGGCGGCGGTGGCG  120
                                                  M  A  A  V  A    5
GGGACGGGGGTGTTCGCGGAGATCCTGGAGGGGGAGGTGTACAGGTACTACGCCGACGGG  180
 G  T  G  V  F  A  E  I  L  E  G  E  V  Y  R  Y  Y  A  D  G   25
GAGTGGCGCGTCTCGGCCTCCGGCAAGTCCGTCGCCATCGTCAACCCCACCACCCGCCTC  240
 E  W  R  V  S  A  S  G  K  S  V  A  I  V  N  P  T  T  R  L   45
ACCCAGTACAGGGTGCAAGCATGCACCCAGGAGGAGGTGAACAAGGTGATGGAGACGGCG  300
 T  Q  Y  R  V  Q  A  C  T  Q  E  E  V  N  K  V  M  E  T  A   65
AAGGTGGCGCAGAAGGCGTGGGCGCGCACGCCGCTGTGGAAGCGCGCGGAGCTCCTGCAC  360
 K  V  A  Q  K  A  W  A  R  T  P  L  W  K  R  A  E  L  L  H   85
AAGGCGGCCGCCATCCTCAAGGAGCACAAGACCCCGATTGCAGAGTGCCTCGTCAAGGAG  420
 K  A  A  A  I  L  K  E  H  K  T  P  I  A  E  C  L  V  K  E  105
ATCGCCAAGCCTGCCAAGGACGCGATCTCTGAAGTGGTGAGGTCAGGGGATTTGGTGTCG  480
 I  A  K  P  A  K  D  A  I  S  E  V  V  R  S  G  D  L  V  S  125
TACACAGCTGAGGAGGGTGTTCGGATACTGGGGGAAGGCAAGCTGCTGGTGTCTGATAGC  540
 Y  T  A  E  E  G  V  R  I  L  G  E  G  K  L  L  V  S  D  S  145
TTCCCGGGCAATGAACGGAACAAGTACTGTTTGAGCTCCAAGGTACCTCTTGGAGTAGTT  600
 F  P  G  N  E  R  N  K  Y  C  L  S  S  K  V  P  L  G  V  V  165
TTGGCAATCCCACCATTTAACTATCCTGTCAACCTAGCAGTCTCCAAGATTGGCCCAGCA  660
 L  A  I  P  P  F  N  Y  P  V  N  L  A  V  S  K  I  G  P  A  185
CTAATTGCTGGCAATGCTCTTGTTCTGAAGCCTCCAACTCAGGGAGCGGTGGCTGCACTA  720
 L  I  A  G  N  A  L  V  L  K  P  P  T  Q  G  A  V  A  A  L  205
CATATGGTGCACTGCTTCCACCTTGCTGGTTTCCCCAAAGGCTTGATCAATTGTGTCACC  780
 H  M  V  H  C  F  H  L  A  G  F  P  K  G  L  I  N  C  V  T  225
GGGAAAGGTTCTGAAATTGGTGATTTTCTTACGATGCATCCTGGAGTCAACTGCATAAGT  840
 G  K  G  S  E  I  G  D  F  L  T  M  H  P  G  V  N  C  I  S  245
TTTACGGGAGGTGATACTGGTATAGCCATTTCAAAGAAGGCTGGAATGGTCCCGCTTCAG  900
 F  T  G  G  D  T  G  I  A  I  S  K  K  A  G  M  V  P  L  Q  265
ATGGAACTTGGAGGAAAAGATGCTTGTGTTGTGTTAGAGGATGCAGATCTGGATTTAGTG  960
 M  E  L  G  G  K  D  A  C  V  V  L  E  D  A  D  L  D  L  V  285
GCAGCAAATATAGTAAAAGGAGGCTTCTCTTACAGTGGCCAGAAGTGCACTGCTGTGAAA 1020
 A  A  N  I  V  K  G  G  F  S  Y  S  G  Q  K  C  T  A  V  K  305
GTGGTGCTGATCATGGAATCCGTTGCTGATATCGTGGTAGAGAAGGTGAAGGCCAAGTTG 1080
 V  V  L  I  M  E  S  V  A  D  I  V  V  E  K  V  K  A  K  L  325
GCAAAACTGACAGTTGGGCCACCTGAGGCTGACTCTGATATCACCCCAGTTGTAACAGAA 1140
 A  K  L  T  V  G  P  P  E  A  D  S  D  I  T  P  V  V  T  E  345
TCCTCAGCAAATTTTATTGAGGGTTTGGTCATGGATGCCAAGGAGAAAGGAGCAACCTTT 1200
 S  S  A  N  F  I  E  G  L  V  M  D  A  K  E  K  G  A  T  F  365
TGTCAGGAGTACAGGAGAGAAGGCAACCTTATCTGGCCGTTGCTACTGGATCACGTCCGG 1260
 C  Q  E  Y  R  R  E  G  N  L  I  W  P  L  L  L  D  H  V  R  385
CCTGACATGAGGATTGCTTGGGAGGAGCCATTTGGCCCTGTCTTGCCTGTGATTAGGATC 1320
 P  D  M  R  I  A  W  E  E  P  F  G  P  V  L  P  V  I  R  I  405
AACTCGGTCGAGGAAGGCATCCACCATTGCAATGCCAGCAATTTTGGGCTGCAGGGATGT 1380
 N  S  V  E  E  G  I  H  H  C  N  A  S  N  F  G  L  Q  G  C  425
GTATTCACTAAAGATATCAACAAAGCGATCATGATCAGCGATGCGATGGAGACCGGAACC 1440
 V  F  T  K  D  I  N  K  A  I  M  I  S  D  A  M  E  T  G  T  445
GTTCAGATCAACTCTGCACCGGCTCGAGGACCTGACCATTTCCCCTTCCAGGGCTTGAAG 1500
 V  Q  I  N  S  A  P  A  R  G  P  D  H  F  P  F  Q  G  L  K  465
GACAGTGGGATTGGATCCCAGGGGATAACTAACAGCATAAACATGATGACCAAGGTGAAG 1560
 D  S  G  I  G  S  Q  G  I  T  N  S  I  N  M  M  T  K  V  K  485
AGCACTGTCATAAACCTACCATCTCCATCCTACACCATGGGCTGAGGTGTTCCATATAGA 1620
 S  T  V  I  N  L  P  S  P  S  Y  T  M  G  *                499
TGATCAGATTTCTATAACCACATGAGTACAATGTAGAGAATTCAAATGTTTTGTAACCAC 1680
GTGAGCTTTGTACGCTATTTGCACCACAGTTCTCCCTGTATGTTGTTGGCTCTTTTACAA 1740
TTAAGAATAATGCATGTTGAAGTAAAAAAAAA
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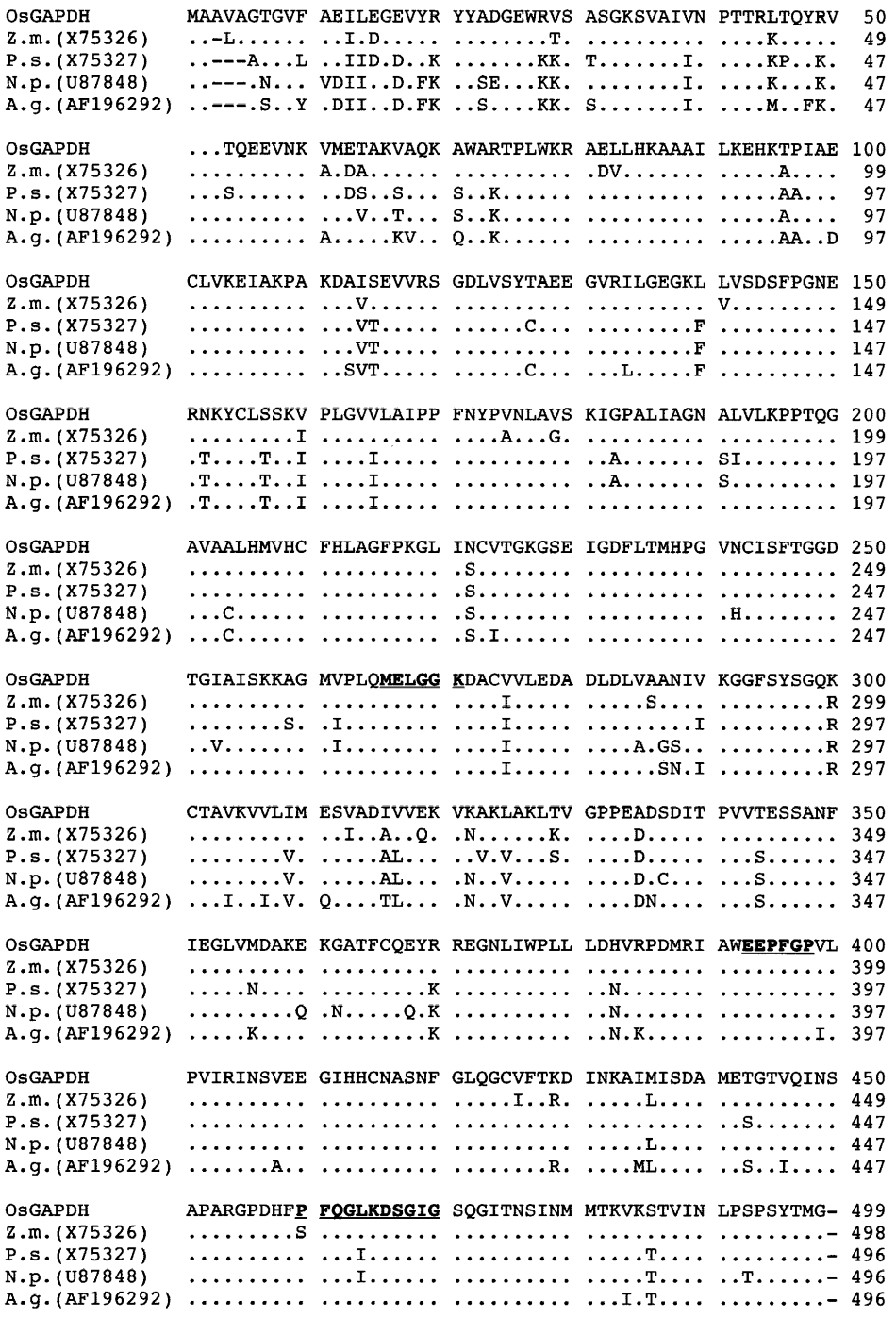
of ribosomal RNAs in each sample before transfer to the membrane. The blots were hybridized with DNA probes labeled by the Alkphos labeling and detection system (Amersham). Hybridization was performed at 55 °C and washings were carried out under high stringency conditions as recommended by the manufacturer.

DNA sequence analysis

Plasmid DNA was prepared by the Alkaline-lysis method, purified by the Quantum Prep Plasmid Miniprep kit (Bio-Rad) and sequenced by the dideoxynucleotide chain-termination method (Sanger et al. 1977) using the T7 DNA polymerase sequencing kit (Pharmacia, Buckinghamshire, England). The nucleotide sequences and deduced amino-acid sequences were analyzed by the gene software package (Intelli Genetics, Mountain View, Calif., USA). Homology searches of sequences in databases were conducted by using the BLAST program (Altschul et al. 1994).

Construction of recombinant protein and expression in *Escherichia coli*

The expression of recombinant OsGAPDH protein was made by the GST gene fusion system (Amersham Pharmacia Biotech, Japan). The coding region of the OsGAPDH mature protein was amplified by a pair of primers, 5′CGGAATTCAGGAGGAGA TAT GGC-3′ and 5′ATA**GTCGAC**TGATCATCTATATGG-3′. Parts underlined indicate the *Eco*RI site, and those bold underlined indicate the *Sal*I site. After PCR amplification, the DNA fragments were digested with *Eco*RI and *Sal*I restriction enzymes and then ligated into the *Eco*RI and *Sal*I sites of pGEX-6P-3 to produce the pGST-OsGAPDH vector. The vector pGST-OsGAPDH was transformed into *E. coli* strain DH5α cells and grown in LBA (Luria broth supplemented with 40 μg ml^{-1} Ampicillin) at 30 °C. The 4 ml of overnight culture was diluted into 400 ml of LBA and grown at 30 °C. To the culture 0.1 mM IPTG was added to induce



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OsGAPDH         MAAVAGTGVF AEILEGEVYR YYADGEWRVS ASGKSVAIVN PTTRLTQYRV  50
Z.m.(X75326)    ..-L...... ..I.D..... ........T. .......... ....K.....  49
P.s.(X75327)    ..---A...L ..IID.D..K .......KK. T.......I. ....KP..K.  47
N.p.(U87848)    ..----.N... VDII..D.FK ..SE...KK. ........I. ....K...K.  47
A.g.(AF196292)  ..----.S..Y .DII..D.FK ..S....KK. S.......I. ....M..FK.  47

OsGAPDH         ...TQEEVNK VMETAKVAQK AWARTPLWKR AELLHKAAAI LKEHKTPIAE 100
Z.m.(X75326)    .......... A.DA...... .......... .DV....... .....A.....  99
P.s.(X75327)    ...S...... ..DS..S... S..K...... .......... .....AA....  97
N.p.(U87848)    .......... ...V..T... S..K...... .......... .....A.....  97
A.g.(AF196292)  .......... A.....KV.. Q..K...... .......... .....AA..D  97

OsGAPDH         CLVKEIAKPA KDAISEVVRS GDLVSYTAEE GVRILGEGKL LVSDSFPGNE 150
Z.m.(X75326)    .......... ...V...... .......... .......... V.........  149
P.s.(X75327)    .......... ...VT..... ......C... .........F ..........  147
N.p.(U87848)    .......... ...VT..... .......... .........F ..........  147
A.g.(AF196292)  .......... ..SVT..... ......C... ...L.....F ..........  147

OsGAPDH         RNKYCLSSKV PLGVVLAIPP FNYPVNLAVS KIGPALIAGN ALVLKPPTQG 200
Z.m.(X75326)    .........I .......... ....A...G. .......... ..........  199
P.s.(X75327)    .T....T..I ....I..... .......... ..A....... SI........  197
N.p.(U87848)    .T....T..I ....I..... .......... ..A....... S.........  197
A.g.(AF196292)  .T....T..I ....I..... .......... .......... ..........  197

OsGAPDH         AVAALHMVHC FHLAGFPKGL INCVTGKGSE IGDFLTMHPG VNCISFTGGD 250
Z.m.(X75326)    .......... .......... .S........ .......... ..........  249
P.s.(X75327)    .......... .......... .S........ .......... ..........  247
N.p.(U87848)    ...C...... .......... .S........ .......... .H........  247
A.g.(AF196292)  ...C...... .......... .S.I...... .......... ..........  247

OsGAPDH         TGIAISKKAG MVPLQMELGG KDACVVLEDA DLDLVAANIV KGGFSYSGQK 300
Z.m.(X75326)    .......... .......... ....I..... .....S.... .........R  299
P.s.(X75327)    ........S. .I........ ....I..... .........I .........R  297
N.p.(U87848)    ..V....... .I........ ....I..... ....A.GS.. .........R  297
A.g.(AF196292)  .......... .......... ....I..... ......SN.I .........R  297

OsGAPDH         CTAVKVVLIM ESVADIVVEK VKAKLAKLTV GPPEADSDIT PVVTESSANF 350
Z.m.(X75326)    .......... ..I..A..Q. .N......K. ....D..... ..........  349
P.s.(X75327)    ........V. .....AL... ..V.V...S. ....D..... ...S......  347
N.p.(U87848)    ........V. .....AL... .N..V..... ....D.C... ...S......  347
A.g.(AF196292)  ...I..I.V. Q....TL... .N..V..... ....DN.... ...S......  347

OsGAPDH         IEGLVMDAKE KGATFCQEYR REGNLIWPLL LDHVRPDMRI AWEEPFGPVL 400
Z.m.(X75326)    .......... .......... .......... .......... ..........  399
P.s.(X75327)    .....N.... .........K .......... ..N....... ..........  397
N.p.(U87848)    .........Q .N.....Q.K .......... ..N....... ..........  397
A.g.(AF196292)  .....K.... .........K .......... ..N.K..... ........I.  397

OsGAPDH         PVIRINSVEE GIHHCNASNF GLQGCVFTKD INKAIMISDA METGTVQINS 450
Z.m.(X75326)    .......... .......... .....I..R. .....L.... ..........  449
P.s.(X75327)    .......... .......... .......... .......... ..S.......  447
N.p.(U87848)    .......... .......... .......... .....L.... ..........  447
A.g.(AF196292)  .......A.. .......... ........R. ....ML.... ..S..I....  447

OsGAPDH         APARGPDHFP FQGLKDSGIG SQGITNSINM MTKVKSTVIN LPSPSYTMG-  499
Z.m.(X75326)    .........S .......... .......... .......... .........-  498
P.s.(X75327)    .......... ...I...... .......... .....T.... .........-  496
N.p.(U87848)    .......... ...I...... .......... .....T.... ..T......-  496
A.g.(AF196292)  .......... .......... .......... ...I.T.... .........-  496
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Fig. 2 Sequence alignment of rice *OsGAPDH* with other sequences. The deduced amino-acid sequence of rice *OsGAPDH* aligned with nr*GAPDH* from *Zea mays* (accession no X75326), *Pisum sativum* (accession no X75327), *Nicotiana plumbaginifolia* (accession no U87848), and *Apium graveolens* (accession no AF196292). Identical amino acids are given as *dots*, *dashes* are introduced to optimize the alignment. The three most-conserved regions of all non-phosphorylating ALDHs are indicated by a bold underline

the GST-OsGAPDH fusion protein. After 2.5-h incubation at same temperature the cells were harvested by centrifugation and sonicated in PBS (phosphate-buffered saline) on ice. Cellular debris was removed by centrifugation and the supernatant was loaded on a glutathione-Sepharose 4B column (Amersham Pharmacia Biotech). After extensive washing the column with PBS, the bound GST-recombinant protein was eluted with 100 mM of Tris-HCl (pH 8.9) containing 10 mM of glutathione according to the manufacturer's instruction. Protein concentrations were determined according to the methods of Bradford (1976) using the Coomassie Protein Assay Reagent (Pierce, Rockford) with bovine serum albumin as a standard.

Assay for non-reversible glyceraldehyde-3-phosphate dehydrogenase

Non-reversible glyceraldehyde-3-phosphate dehydrogenase (nr-GAPDH) was assayed by the method of Kelly and Gibbs (1973) with slight modifications. The standard reaction mixture contained 50 mM of Tris [tris(hydroxymethyl) aminomethane] buffer, pH 8.0, 3.0 mM of reduced glutathione, 1 mM of glyceraldehyde-3-phosphate, 0.1 mM of NADP+ and was incubated at 30 °C. By using a spectrophotometer (SmartSpec 3000, Bio-Rad, Calif.) the assays were monitored at 340 nm absorbance with a different pH, temperature and inhibitors. The kinetic data were collected at periodic intervals for a specified length of time.

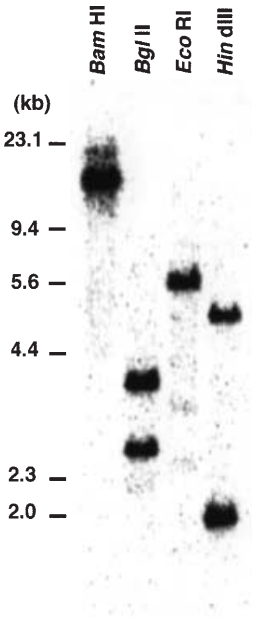

Fig. 3 Southern-blot analysis of *OsGAPDH*. Genomic DNA from rice variety Yukihikari was digested with *Bam*HI, *Bgl*II, *Eco*RI and *Hin*dIII, separated on a 0.8% agarose gel, blotted and probed with the *OsGAPDH* gene. The filter was probed with a full-length *OsGAPDH* clone. The size of the fragments is indicated on the left

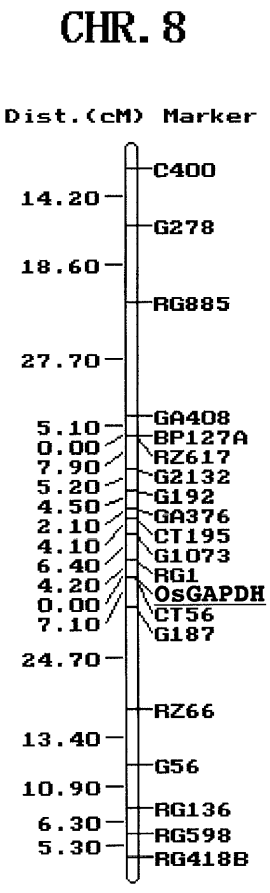

Fig. 4 Genetic map of rice chromosome 8 showing the location of *OsGAPDH*. Molecular markers are indicated to the right of chromosome and the genetic distances in Kosambi centiMorgan (cM) are shown on the left

Results

Molecular cloning of *OsGAPDH* cDNA

A Differential screening approach was employed to identify the genes preferentially expressed during submergence stress in rice seedlings. The submergence stress cDNA library was hybridized with DIG-labeled first-strand cDNA obtained from the mRNA of submergence stress-treated seedlings, followed by a second hybridization against cDNA established from control seedlings. The differentially hybridized purified phage clone was then selected. One of these clones, which showed sequence homology with maize non-reversible glyceraldehyde-3-phosphate dehydrogenase and strong expression in Northern analysis for submergence treatments, was named as *OsGAPDH* and used for further analysis.

Since the isolated *OsGAPDH* cDNA represented a partial transcript, we screened the same cDNA library with the partial *OsGAPDH* as a probe enabling us to obtain its full-length cDNA and deposit it in GenBank (AF 357884). Figure 1 shows the nucleotide sequence of the full-length cDNA of *OsGAPDH*. The full-length cDNA consists of a 1,772 bp-long DNA starting with a ATG codon. The longest open reading frame encodes a 499 amino-acid peptide with a calculated relative mass of 54.2 kDa. The sequence alignment of rice *OsGAPDH* with other sequences is indicated in Fig. 2.

Genomic Southern and RFLP analysis

Southern-blot analysis of genomic DNA was performed to estimate the number of *OsGAPDH* related genes in the rice genome. Total genomic DNA was isolated from seedlings of the Yukihikari variety. Genomic DNA was digested with *Bam*HI, *Bgl*II, *Eco*RI and *Hin*dIII respectively. Digests of genomic DNAs were separated on an agarose gel by electrophoresis and then transferred to a nylon membrane. The blot was hybridized with *OsGAPDH* as a probe. Single bands of 16.0 kb and 5.6 kb were detected in the *Bam*HI and *Eco*RI lanes respectively. The *Bgl*II and *Hind*III lanes showed two bands of different intensity at 2.8 and 3.8 kb, and at 2.0 and 5.0 kb, respectively (Fig. 3). The signals of Southern blots showed that one or two copies of the *OsGAPDH* gene occur in the rice genome. A previously constructed linkage map for rice (He et al. 1999) was used for RFLP mapping. This map includes a total of 243 RFLP and microsatellite markers distributed over all 12-rice chromosomes. After analyzing polymorphism in the parental genotypes the Southern hybridization was carried out in the di-haploid population. The *OsGAPDH* detected in the di-haploid F_2 population was located in between markers RG1 and CT56 on chromosome 8 (Fig. 4).

OsGAPDH is strongly expressed in panicles

We distinguished the pattern of *OsGAPDH* mRNA accumulation in various tissues of rice (Fig. 5). RNAs were extracted from the panicle, leaf, leafsheath, node, inter-

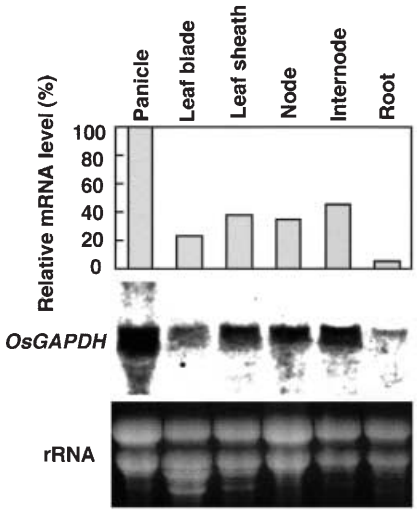

Fig. 5 Northern-blot analysis of *OsGAPDH* mRNA from total RNA (30 µg/lane) extracted from panicle, leaf blade, leaf sheath, node, internode and root of the rice plant. The blots were probed with *OsGAPDH*-PCR fragments. Equal loading of RNA was verified by staining with ethidium bromide and by comparing the amount of ribosomal RNAs in each sample before transfer to the membrane

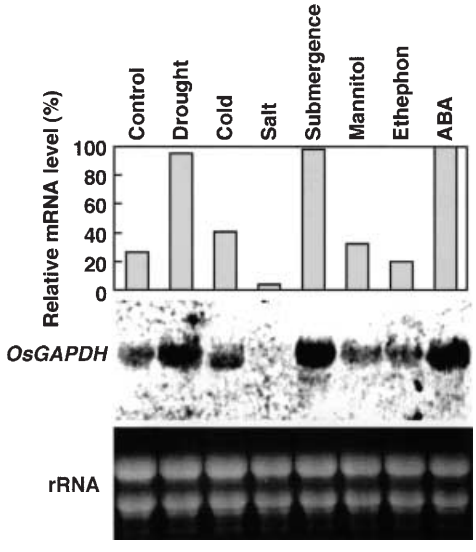

Fig. 6 Effect of various stresses and plant hormones on *OsGAPDH* transcript level. Total RNA (30 µg/lane) extracted from control (grown at 28 °C), drought (dehydrated until losing 30–40% of the fresh weight), cold (exposed to 5 °C), salt (irrigated with 0.25 M NaCl), submergence and mannitol (irrigated with 0.5 M mannitol). For hormone treatments, seedlings were both irrigated and sprayed with ethephon (10^{-2} M ethephon) and ABA (10^{-5} M abscisic acid) solutions

node and root of greenhouse-grown rice plants. Very high *OsGAPDH* mRNA was detected in panicles, indicating strong *OsGAPDH* transcription in flower tissues. Trace amounts were detected from the leaf sheath, node and internode. On the other hand *OsGAPDH* transcript levels were hardly detectable in the mature leaf and root.

Expression of the *OsGAPDH* gene with other stress treatments and plant hormones

To check whether expression of the *OsGAPDH* gene in rice seedlings is influenced by various environmental stresses, total RNA was isolated from the seedlings of different stress treatment and subjected to Northern analysis. Plants exposed to drought, submergence and ABA treatment had increased amounts of mRNA accumulation. On the other hand cold treatment at 5 °C, with mannitol and with ethephon showed relatively less accumulation (Fig. 6).

The time-course expression of the *OsGAPDH* transcript was found out under drought, submergence stress and ABA treatment. For drought treatment the highest rate of *OsGAPDH* transcript accumulation took place at 12-h treatment. However, a decreased transcript level was noticed on the next day and then the accumulation reached a maximum level after 3-days of drought treatment. A stronger submergence response was observed in the 12-h treatment; subsequently the expression declined progressively. The plant hormone ABA showed strong induction within 1 day of treatment and thereafter the transcript level decreased slightly under 2 and 3 days of treatment (Fig. 7).

Glutathione-S transferase (GST) fusion and expression of OsGAPDH

To confirm whether the OsGAPDH protein has glyceraldehyde-3-phosphate dehydrogenase activity, the mature protein of OsGAPDH encoding 54.2 kDa (499 amino acids) was expressed in *E. coli* cells as a GST-OsGAPDH recombinant protein. The accumulation of 29-kDa GST was observed when *E. coli* cells containing pGEX-6P-3 without an insert was induced by IPTG. The induction of *E. coli* cells containing the pGST-OsGAPDH plasmid by IPTG resulted in the accumulation of a large amount of 83.2-kDa recombinant protein in the cell-soluble fraction (Fig. 8, lane 4).

The recombinant protein was further purified from the soluble fraction by affinity chromatography on glutathione-Sepharose 4B. The GST-OsGAPDH recombinant protein was eluted from the affinity column as a single band of 83.2 kDa by 10 mM of glutathione, as judged by SDS–PAGE (Fig. 8, lane 5). In order to remove GST from the recombinant protein, the protein was digested with protease and digests were loaded on the second glutathione-Sepharose 4B column. The OsGAPDH protein was eluted from the column as a single band of 54.2 kDa (Fig. 8, lane 7). The activity of purified rice GAPDH expressed in *E.coli* was measured in terms of a different pH, temperature and inhibitors. The GAPDH showed optimum activity at pH 8.5 and 50 °C (Fig. 9A, B). The inhibitors ATP and ADP inhibit the enzyme activity by 29 and 13% respectively (Fig. 9C).

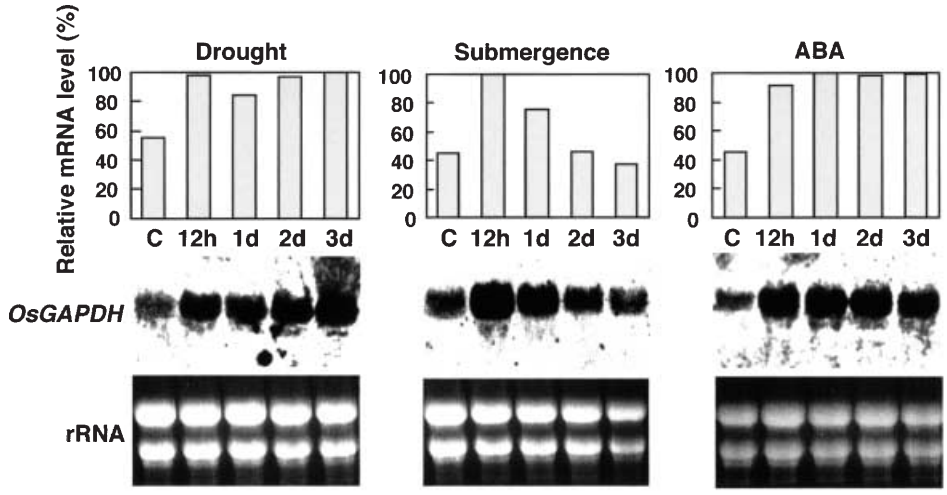

Fig. 7 Northern-blot analysis of the expression of the *OsGAPDH* gene upon drought, submergence and ABA treatment. Total RNA (30 µg/lane) was extracted from rice seedlings treated for drought, submergence and ABA, for control, 12-h, 1-day, 2-day, and 3-day intervals

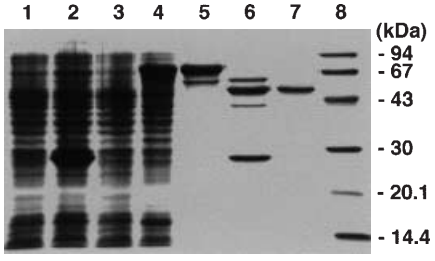

Fig. 8 Expression of the GST-OsGAPDH fusion protein in *E. coli* and its purification to apparent homogeneity. *Lane 1*, total protein fraction of DH5α cells containing pGEX-6P-3 without an insert minus IPTG; *lane 2*, total protein fraction of DH5α cells containing pGEX-6P-3 without an insert plus IPTG; *lane 3*, total protein fraction of DH5α cells containing pGST-OsGAPDH minus IPTG; *lane 4*, total protein fraction of DH5α cells containing pGST-OsGAPDH plus IPTG; *lane 5*, pGST-OsGAPDH fusion protein fraction purified by glutathione-Sepharose 4B chromatography; *lane 6*, purified pGST-OsGAPDH protein fraction digested by PreScission Protease (Amersham Pharmacia Biotech); *lane 7*, purified OsGAPDH protein by passing through a glutathione-Sepharose 4B column; *lane 8*, molecular-mass markers: phosphorylase b, 94 kDa, BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; trypsin inhibitor, 20.1 kDa; and lysozyme, 14.4 kDa. All protein samples were separated on a 12% SDS-polyacrylamide gel and stained with Coomassie Brilliant Blue R250

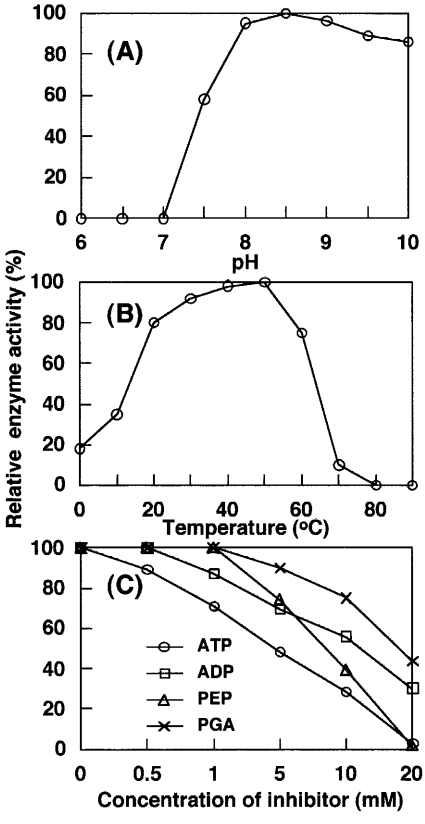

Fig. 9A–C Effect of pH, temperature and metabolites on activity of the purified rice GAPDH expressed in *E. coli*. (**A**) Effect of pH on GAPDH activity: Tris-maleate buffer (pH 6.5–7.0); Tris-HCl buffer (pH 7.0–8.5); Glycine-NaOH buffer (pH 8.5–10). (**B**) Effect of temperature on GAPDH activity at pH 8.5. (**C**) Effect of metabolites on GAPDH activity. *ATP*, adenosine 5′-triphosphate; *ADP*, adenosine 5′-diphosphate; *PEP*, phosphoenol pyruvic acid; *PGA*, 3-phosphoglyceric acid. The values are means of duplicate assays and activity is expressed as a percentage of the control that does not contain any metabolites. Assays were performed by varying concentration of each metabolite from 0.5 mM to 20 mM in the standard reaction mixture

Discussion

We discussed changes in transcript levels, in vivo expression, and the isolation and characterization of cDNA encoding *OsGAPDH*, a submergence tolerant gene, in rice. Submergence of rice seedlings was caused by mRNA accumulation of glycolysis and alcohol fermentation-related genes. The *OsGAPDH* contained an open reading frame coding for a polypeptide of 499 amino acids (Fig. 1). The predicted Mr of the OsGAPDH mature protein with the 499 amino-acid residue was 54.2 kDa and a pI of 6.83. The data base search of the deduced amino acid sequence showed a significant similarity to the sequence of maize and, in other plants, non-reversible glyceraldehyde-3-phosphate dehydrogenase indicated that the enzyme was

highly conserved (Fig. 2). The rice nr-*GAPDH* sequence closely resembles nr-*GAPDH* from *Zea mays* (accession no X75326; 94% identity), *Pisum sativum* (accession no X75327; 89% identity), *Nicotiana plumbaginifolia* (accession no U87848; 88% identity) and *Apium graveolens* (accession no AF196292; 86% identity). The conserved domain database using RPS-BLAST identified the *OsGAPDH* sequence as a member of the aldehyde dehydrogenase superfamily (ALDHs). Enzymes of this superfamily catalyze the irreversible oxidation of a wide variety of aldehydes to their corresponding acids via the formation of a thioacyl intermediate (Lindahl 1992).

Southern-blot analysis of genomic DNA was performed using four different restriction enzymes, that do not cut the *OsGAPDH* gene. When the Southern blot was probed with the full-length probe, followed by a high stringency wash, one or two banding patterns were observed in certain digests where both strong and weak bands were detected. These banding patterns revealed that one or two copies of the *GAPDH* gene occurs in the rice genome. The chromosomal location of the *OsGAPDH* gene was identified by RFLP analysis indicating that *OsGAPDH* was located on chromosome 8 (Fig. 4).

Northern hybridization using total RNA extracted from several organs showed that *OsGAPDH* was expressed at a high level in the panicle. Therefore we suggest that alcoholic fermentation might occur in this tissue. The aerobic alcoholic fermentation is a general phenomenon in pollen and developing microspores in plants, and is controlled not by oxygen availability but by substrate availability (Tadege and Kuhlemeier 1997; Tadege et al. 1999). In rice, Nakazono et al. (2000) found out that Aldh1 and Adh1 transcripts accumulate to high levels in young panicles and panicles after heading. Xie and Wu (1989) reported that the ADH1 isozyme is present in rice pollen. These findings suggests that, even in a aerobic environment, alcoholic fermentation takes place in the floral tissues (the panicles) as reported in rice (Nakazono et al. 2000) and tobacco (Bucher et al. 1995; Tadege and Kuhlemeier 1997). Expression analysis of various environmental stresses and growth hormones indicated a coordinate suppression after cold, salt and exogenous application of mannitol and ethephon treatment. Concomitantly, an increase in mRNA accumulation has been noted on drought, submergence and ABA treatments.

Under complete drought conditions the mRNA reached a maximum after 12 h and then suddenly decreased on the next day. The second and third day drought treatments showed an increased accumulation of the *OsGAPDH* transcript. These results imply that enhanced rates of glycolysis are one of the immediate cellular responses to water deficit. Riccardo et al. (1994) proposed that by this mechanism the plant cells prepare for a demand of ATP and $NADH_2$ during recovery.

Since rice is capable of germinating under anerobic conditions and growing under anoxia, it is likely that the mRNAs of fermentation-related genes are kept at higher levels for several days under submergence stress. In our case the *OsGAPDH* transcript increased by 12-h submergence treatment and then decreased after subsequent treatment. The possible reason for the decrease of the *OsGAPDH* transcript after 1-day submergence is due to the shortage of ATP. Nakazono et al. (2000) proposed that the decrease in the ALDH2 transcript after 24-h submergence is due to the efficiency of respiration and that ATP synthesis in mitochondria is reduced because of a lack of oxygen. Further submergence after 12 h might cause an ATP shortage and a reduction in the membrane potential and reduces the efficiency of importing ALDH2 protein into the mitochondria. Since purified GAPDH activity was inhibited by ATP, ADP, and the metabolites PEP and PGA, the enzyme activity may be regulated by these metabolites under certain physiological conditions.

Activation or repression of different members of a gene family can cause differential expression of an enzyme as reported in the glyceraldehyde-3-phosphate dehydrogenase and pyruvate decarboxylase genes of maize and *Arabidopsis* (Russell and Sachs 1989; Peschke and Sachs 1993; Yang et al. 1993). Since the inheritance system of quantitative traits, like submergence and drought tolerance, might consist both of a few major genes and a number of polygenes, efforts have been undertaken to identify the critical region of *GAPDH* necessary for submergence and drought tolerance.

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