Full Length Research Paper

Identification of *GPD1* gene from yeast via fluorescence differential display-polymerase chain reaction (FDD-PCR)

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The main task of this work was to identify abiotic stress-induced gene(s) from yeast (*Saccharomyces cerevisiae*) and introduce it to a prokaryotic system to detect its effect on conferring tolerance to salt stress. Six isolates of yeast (*S. cerevisiae*) were evaluated under salt and osmotic stresses at concentrations of 2 M NaCl and 2 M sorbitol, respectively, in which one isolate was selected as the most tolerant against both stresses. Fluorescence differential display-polymerase chain reaction (FDD-PCR) was conducted for cDNAs after been exposed to 0 and 2 M NaCl for 0, 20, 40 and 60 min. After a number of DD-PCR runs, 350 fragments were observed, out of which 30 of them (9.14%) showed differential expression versus exposure times. They were classified into 12 patterns of gene expression. Three up-regulated DD fragments above 100 bp in size were chosen and cloned for subsequent molecular analysis and gene construction. DNA sequences were detected and subjected to homology searching via computer software. The results indicate that one DD fragment showed significant homology with a yeast DNA fragment on chromosome 4 expressing glycerol-3-phosphate dehydrogenase (GPD1); a NAD dependent key enzyme of glycerol synthesis essential for growth under osmotic stress. This fragment was chosen to recover full-length gene following the rapid amplification of cDNA ends (RACE) strategy, then gene was cloned and transformed into *Escherichia coli*. Expression of *GPD1* gene was proven in transformed bacteria via northern blotting and glycerol-3-phosphate dehydrogenase enzyme activity. The overall results of stress tolerance for *GPD1*-transformed bacteria indicate the efficacy of utilizing the gene in conferring salt tolerance at the prokaryotic level.

Key words: Salt stress, osmotic stress, cDNA, Northern blotting, rapid amplification of cDNA ends (RACE), GeneRacer.

INTRODUCTION

All organisms ranging from microbes to animals and plants, synthesize compatible solutes in response to osmotic stress (Burg et al., 1996). Compatible solutes are non-toxic molecules such as amino acids, glycine betaine, glycerol, sugars and sugar alcohols. They do not interfere with normal metabolism and accumulate predominantly in the cytoplasm at high concentrations under osmotic stress (Chen and Murata, 2002). Initially, it

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was thought that compatible solutes have their main role in osmotic adjustment, but there is increasing argument over other roles (Serraj and Sinclair, 2002). It is claimed that accumulation of these solutes may be unrelated to osmotic stress tolerance, but their metabolic pathways may have adaptive value (Hasegawa et al., 2000). It is hypothesized that compatible solutes are also involved in scavenging reactive oxygen species (Hong et al., 2000; Akashi et al., 2001; Chen and Murata, 2002).

Differential display (DD), first described by Liang and Pardee (1992), is one of the methods for analyzing gene expression in eukaryotic cells and tissues. DD has been widely applied to study changes in mRNA expression induced by temporal developments, biotic and abiotic factors (Linskens et al., 1995). This powerful technique simultaneously screens for both up- and down-regulated transcripts in multiple cell populations under different developmental and environmental conditions. To recover full-length gene, cDNA is used as a template to obtain the 5′ and 3′ cDNA ends of the gene using the GeneRacer® kit, a RACE-dependent kit with SuperScript III RT enzyme. GeneRacer® promotes the amplification of only full-length transcript ends by eliminating truncated mRNA from the amplification process and products can be cloned and sequenced.

The study aimed to isolate and characterize stress-related cDNA from yeast conferring tolerance to salt and osmotic stresses. Eventually, this gene will be utilized in transformation experiment for a model plant like Arabidopsis to ensure the present findings.

MATERIALS AND METHODS

Yeast material

Six isolates of yeast were kindly provided by Dr. Fatma Badawy, Professor of Genetics, Department of Genetics, Faculty of Agriculture, Ain Shams University, Cairo, Egypt. These isolates (S. cerevisiae) were isolated, among a huge number of isolates, from salty lands around the Mediterranean Sea to be characterized for their tolerance to salt as well as drought stress.

Screening experiment to detect salt-tolerant yeast strains

The six yeast isolates were grown in yeast extract peptone dextrose (YPD) on agar plates medium (10 g yeast extract, 20 g peptone, 20 g dextrose and 15 g agar) with sorbitol (2 M) or NaCl (2 M) for 24 h at 27°C in three replications and the growth rate (level) was determined using optical density at 600 nm.

RNA isolation and DD-PCR for cDNA fragments

Total RNAs were isolated from yeast cells under normal and stress conditions (0 and 2 M NaCl) at different time exposure using an RNeasy kit (Qiagen, Germany). DNA contaminants were removed using RNase-free DNase I. Cells were harvested from different treatments after being exposed to stress for 20, 40 and 60 min using Fluorescence differential display reverse transcriptase (FDDRT)-PCR (RNA Image kit GenHunter Corp., USA). Bands with

differential expression were determined using gradient transparent sheets then excised from the gel, and cDNAs were purified from the gel. The up- regulated cDNA fragments were eluted from polyacrylamide gel. Recovered fragments were re-amplified via polymerase chain reaction (PCR) with the same two primers in order to obtain high amount of cDNAs for subsequent downstream approaches.

Cloning of the cDNA fragments

Cloning was carried out with pGEM-T easy vector system (Promega, USA) according to the manufacturer's manual and then introduced to *Escherichia coli* XL1 blue (Stratagen, USA).

Northern blotting

This technique was done twice; the first was to prove differential expression of the three gene fragments in yeast under different stress conditions (cells treated with 2M NaCl for 0 and 60 min), while the second was done to prove constitutive expression of *GPD1* gene in the three *E. coli* strains DH5α, XL1Blue and TOPO10 after genetic transformation. Labeling of the probes for detecting the three fragments was performed using DIG-High Prime DNA Labeling and Detection Starter kit II (Roche, Germany) following the manufacturer's manual.

Sequencing and analysis of the cDNA fragment

The dideoxyribonucleoside chain termination procedure originally developed by Sanger et al. (1977) was employed for sequencing the double-stranded recombinant DNA plasmids obtained during the cloning procedure. The DNA sequences were determined by automated DNA sequencing method performed using ABI PRISM Big Dye terminator cycle sequencing ready reaction kit (PE applied Biosystems, USA), in conjunction with ABI PRISM (310 Genetic Analyzer).

Computer analysis was done using Blast programs from National Center for Biotechnology Information (NCBI), USA (http://www. ncbi.nlm.nih.gov/BLAST) and *Saccharomyces* genome database (http://www.yeastgenome.org/, Altschul et al., 1997) to detect sequence homologies.

Detection of glycerol-3-phosphate dehydrogenase activity

The procedure for estimating glycerol-3-phosphate dehydrogenase activity following Rawls et al. (2011) was used. Three *E. coli* strains DH5α, XL1Blue and TOPO10 were transformed with pCR4-TOPO containing *GPD1* gene and grown on Luria-Bertani (LB) medium in addition to 100 μg/ml ampicillin and 20 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG) for 1 day at 37°C. Log-phase cells (OD$_{600}$, 0.3 to 0.5) were harvested by centrifugation (20 min, 4,300 $x\,g$, 4°C), washed once with 20 ml buffer A (100 mM Tris-HCl at pH 7.5, 2 M NaCl), resuspended in 1 ml of buffer A containing 1 mM phenylmethylsulfonyl fluoride (PMSF) and lysed by sonication (4 times, for 20 s each time, at 140 W). Debris was removed by centrifugation (10 min, 12,000 $x\,g$, 4°C). The protein concentration was estimated using the Bradford assay with bovine serum albumin as a standard. Enzyme activity was carried out at 42°C in a 96-well microtiter plate. Reaction mixtures (0.1 ml) contained 5 mM dihydroxyacetone phosphate (DHAP), 0.25 mM NADH, 2 M NaCl, and 1 to 4 g cell lysate in buffer A. The change in absorbance at 340 nm (A_{340}) for reaction mixtures containing no substrate (DHAP) was subtracted from that for reaction mixtures in which the substrate was included to yield the overall change in absorbance.
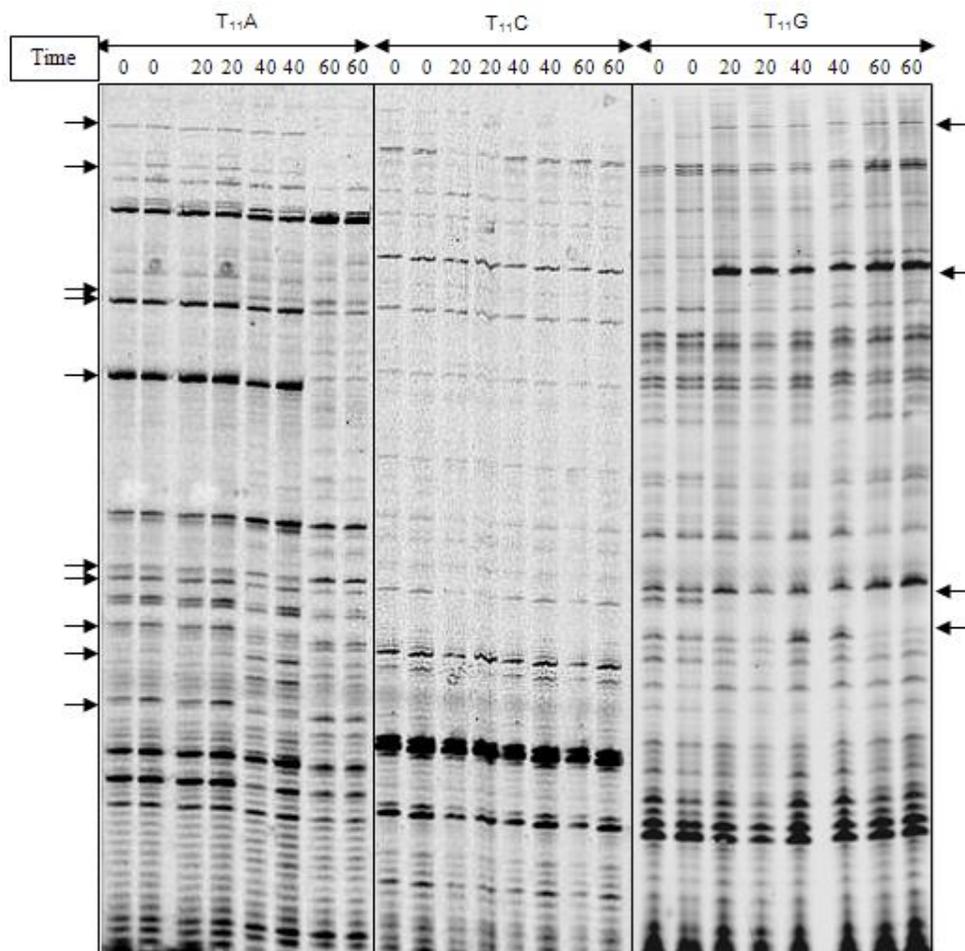
Figure 1. FDD-polyacrylamide gels of yeast (strain no. 1) cDNAs at 0, 20, 40 or 60 min exposure times to NaCl (2 M) stress condition utilizing AP1 primer. Differentially displayed fragments are indicated by the arrows. T11G, T11C and T11A represent the anchor primers.

Reaction mixtures containing boiled enzyme and no NADH were included as negative controls. One unit of enzyme activity is defined as 1 μmol substrate consumed or product formed per min with a molar extinction coefficient of 6,220 $M^{-1} \cdot cm^{-1}$ at 340 nm.

The G3PDH activity was determined for cell lysates grown to log phase. Experiments were performed in biological triplicate, and the means ± standard deviations were calculated. No activity was detected for controls with no substrate or with boiled cell lysates.

Detection of salt tolerance in *GDP1*-transformed *E. coli*

Three *E. coli* strains DH5α, XL1Blue and TOPO10 transformed with pCR4-TOPO containing *GPD1* gene were grown on LB medium containing NaCl (0, 100 or 200 mM) for 1 day at 37°C in three replications and the growth degrees were determined using an optical density of 600 nm.

RESULTS AND DISCUSSION

Screening experiment

Six yeast isolates were used to detect the most salt- and

osmotic-stress tolerant isolate based on the ability to grow on YPD media supplemented with 2 M NaCl or 2 M sorbitol. This isolate (no.1) was chosen for downstream molecular isolation and detection of stress-related genes using FDD-PCR.

FDD-PCR

cDNA was employed in which fluorescence-labeled anchor primers ($T_{11}A$, $T_{11}C$ or $T_{11}G$, Metabion, Germany), to target the poly A tail at the 3′ end of stress-related genes, and the random primers (ARP1 or ARP2, GenHunter RNA Image kit, USA), attempting to hit the open reading frames (ORFs) at the 3′ end of these genes, were used. Hot-start Taq DNA polymerase (Bioron, Germany) was used to detect successive FDD-PCR products involving partial-length cDNA fragments of these ORFs. The gels were visualized with Green laser (532 nm) for fluorescence excitation using Typhoon high-quality confocal optical imaging system scanner (Figures 1 and 2).
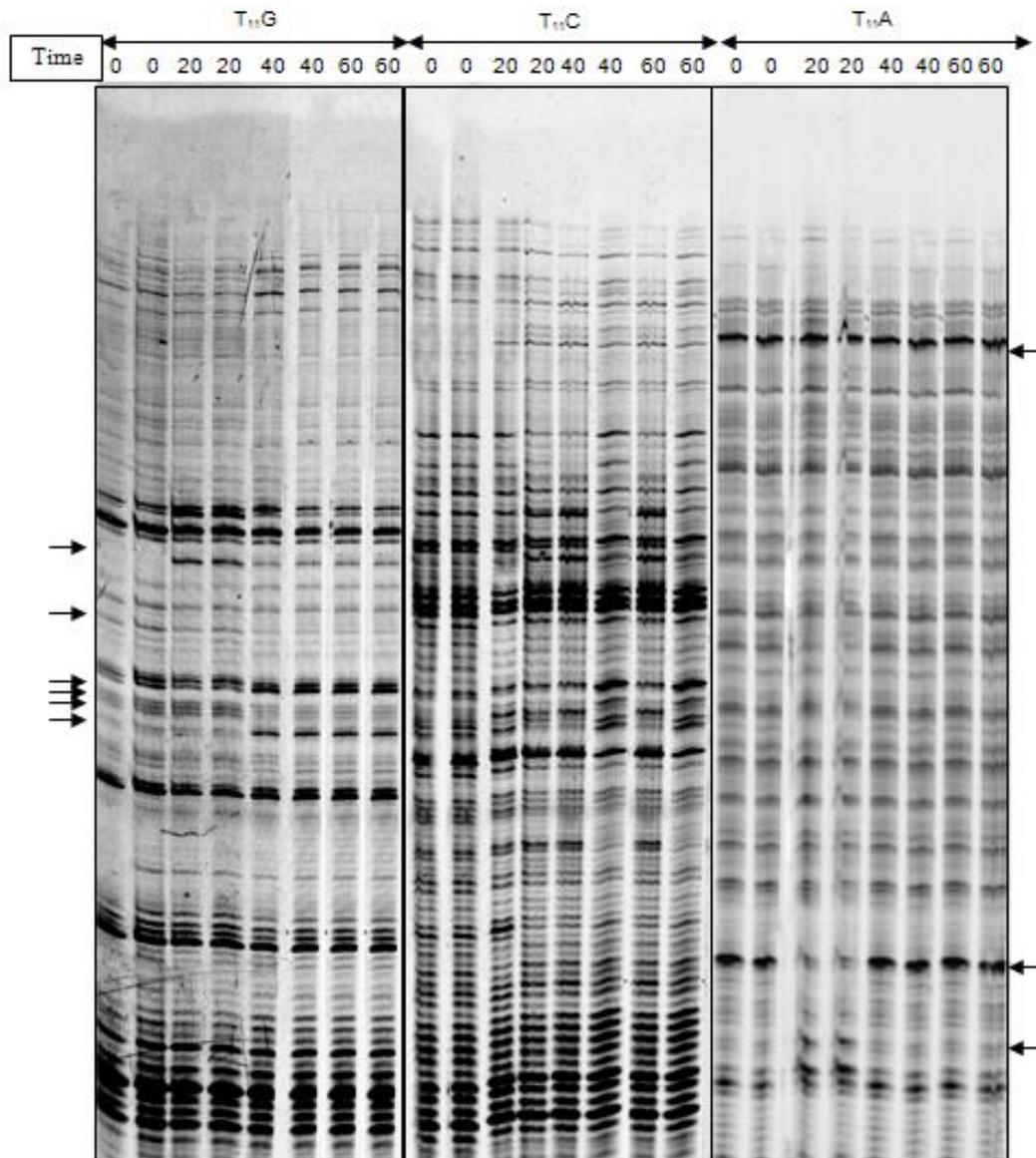
Figure 2. FDD-polyacrylamide gels of yeast (strain no. 1) cDNAs at 0, 20, 40 or 60 min exposure times to NaCl (2 M) stress condition utilizing AP2 primer. Differentially displayed fragments are indicated by the arrows. T11G, T11C and T11A represent the anchor primers.

A total number of 324 bands were observed across the three DD gels, 30 of them (9.26%) showed differential expression against different exposure time. These fragments were categorized into 12 patterns of gene expression as shown in Table 1. Molecular sizes of the three longest cDNA (over 100 bp) DD fragments (nos.7, 19 and 20), as shown in Figure (3), were detected by PCR re-amplification (Figure 4), while differential expression was confirmed by Northern blotting (Figure 5).

DNA sequencing

The automated DNA sequencing reactions were con-

ducted for the three fragments (no.7, 20 and 19) with M13 forward and reverse universal primers.

Data analysis for sequenced fragments

As shown in Table 2 and Figure 6, the three fragments were highly similar to three *S. cerevisiae* identified genes, all of which had a role in the cell's response to osmotic stresses. They are the following: (a) *GPD1* (fragment no. 7): encoding glycerol-3-phosphate dehydrogenase, NAD-dependent key enzyme of glycerol synthesis essential for growth under osmotic stress; expression regulated by high osmolarity glycerol response (HOG) pathway; (b)

Table 1. Expression patterns of the obtained DD fragments across exposure time.

Pattern	0 min	20 min	40 min	60 min	DD fragment
1	Normal	Normal	none	none	nos. 25, 4
2	Normal	Normal	up-regulated	up-regulated	nos. 1, 3, 27
3	Normal	Normal	up-regulated	normal	nos. 14, 16, 22
4	Normal	up-regulated	up-regulated	up-regulated	nos. 7, 20, 28, 29, 30,31, 32
5	Normal	None	None	none	no. 21
6	Normal	Normal	down-regulated	normal	nos. 17, 13
7	Normal	down-regulated	Normal	normal	no. 8
8	Normal	Normal	down-regulated	down-regulated	nos. 5, 6, 15, 23, 24, 26
9	Normal	Normal	Normal	down-regulated	nos. 10, 12
10	Normal	Normal	Normal	none	no. 11
11	None	up-regulated	up-regulated	up-regulated	nos. 18, 19
12	Normal	up-regulated	Normal	normal	nos. 2, 9
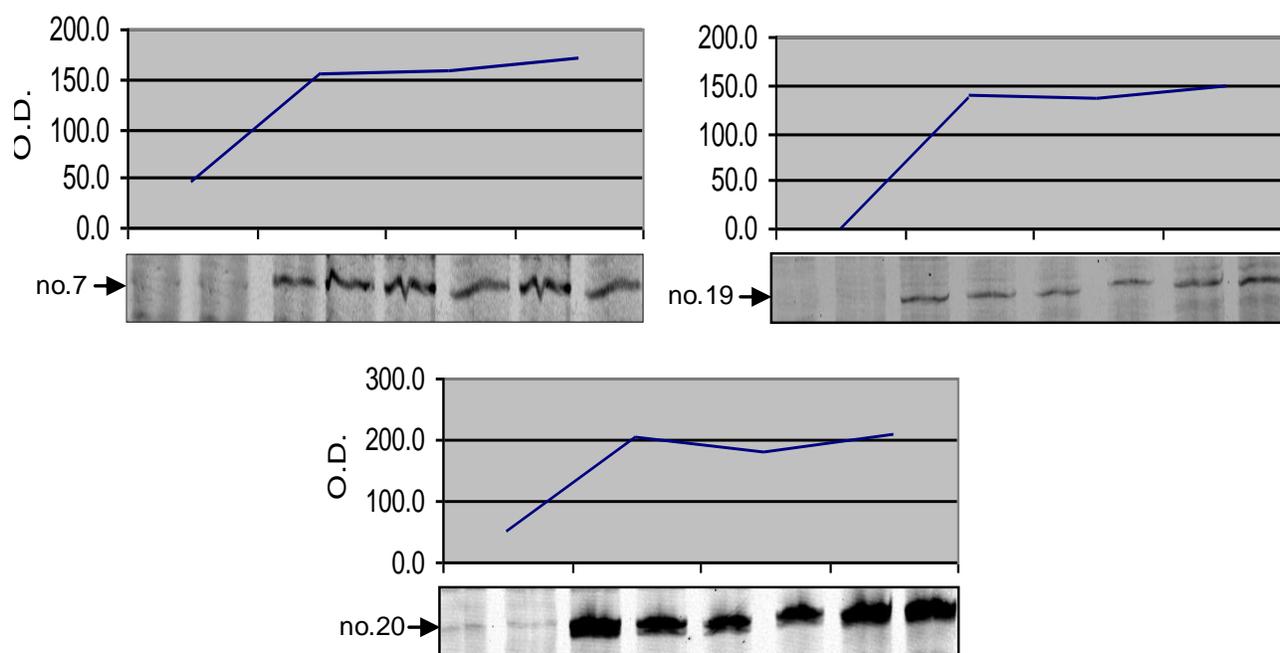
Figure 3. Expression profiles of the three DD fragments. Lanes 1 and 2 = 0 min; lanes 3 and 4 = 20 min; lanes 5 and 6 = 40 min; lanes 7 and 8 = 60 min from exposure to 2 M NaCl. Arrows indicate the positions of the three DD bands.

STL1 (fragment no. 19): encoding glycerol proton symporter of the plasma membrane, subjects to glucose-induced inactivation, strongly (but transiently) induced when cells are subjected to osmotic shock. *STL1* has been described as an osmostress-specific gene with very low basal levels of transcription. Its high induction after acute stress relies exclusively on *HOT1* and *HOG1* (Rep et al., 2000); (c) HOT1 (fragment no. 20): A transcription factor required for the transient induction of glycerol biosynthetic genes *GPD1* and *GPP2* in response to high osmolarity; targets Hog1p to osmostress responsive promoters. *Hot1* seems particularly important for the osmostress-specific induction of genes involved in glycerol production such as *GPD1* and *GPP2* (Alepuz et al. 2001).

E-values, or the expect value (E), is a parameter that describes the number of hits expected to see by chance when searching a database of a particular size (Pearson and Lipman, 1988) and indicated high homology by default. Fragment no. 7, which represents partial *GPD1* ORF, has been chosen for rapid amplification of cDNA ends (RACE) analysis to recover the full-length gene due to its important role in glycerol accumulation within the cell. In addition, the recovered fragment through DD was the longest in size (276 bp), and practically more suitable for the recovery of full-length gene fragment. A gene with
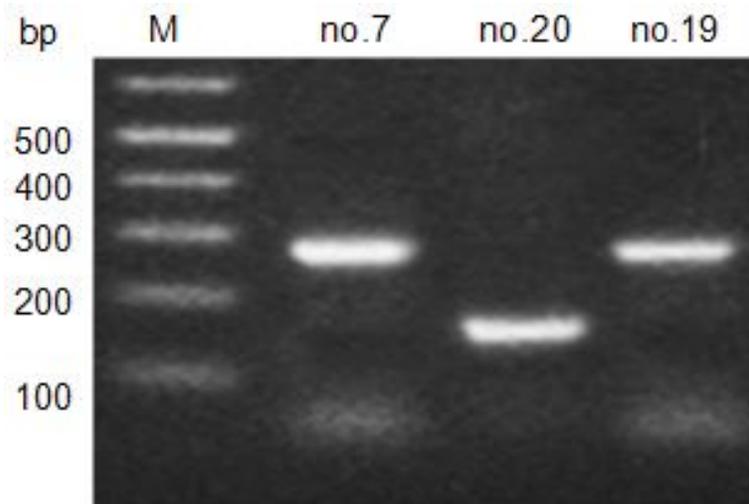
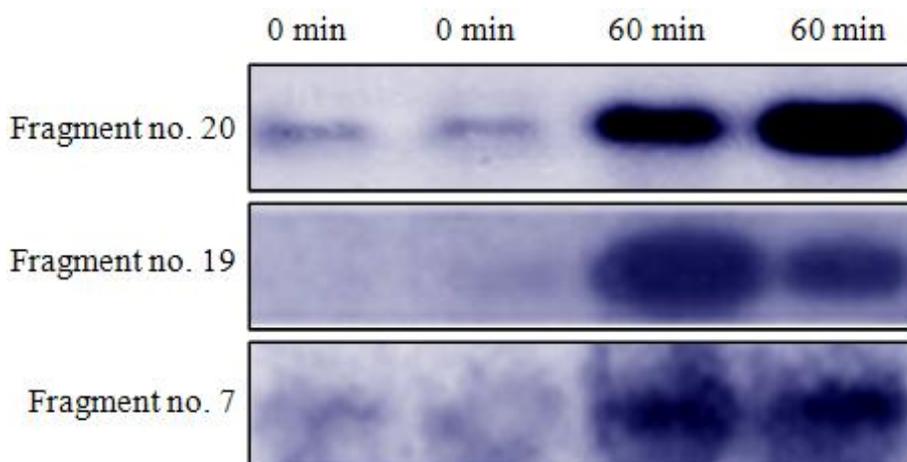**Figure 4.** Re-amplification of cDNA fragments no.7 (~290 bp), 20 (~150 bp), and 19 (~280 bp).

Figure 5. Northern blotting screening of different expressed fragments at 0 and 60 min after being exposed to 2M NaCl.

Table 2. Selected DD fragments and homology results using BLAST (NCBI) database.

Fragment number	Molecular size (bp)	Homology search result (Gene Bank accession number and E-value)
7	276	Gpd1 (Z24454.1; $7e^{-153}$)
19	160	Stl1 (L07492.1; $3e^{-76}$)
20	260	Hot1, (YMP 172W; $3e^{-55}$)

similar function and sequence has been isolated from oyster mushroom (*Pleurotus sajor-caju*) and introduced into potato plants. A bioassay of these transformants revealed that the *P. sajor-caju GPD* gene was able to confer salt stress tolerance in the potato plant cell system (Jeong et al., 2001).

Isolation of full-length *GPD1* gene using RACE

RACE is based on oligo-capping and RNA ligase-mediated (RLM) approach for full-length cDNA recovery (Maruyama and Sugano, 1994; Schaefer, 1995). The GeneRacer® kit (Invitrogen, USA) involves selectively
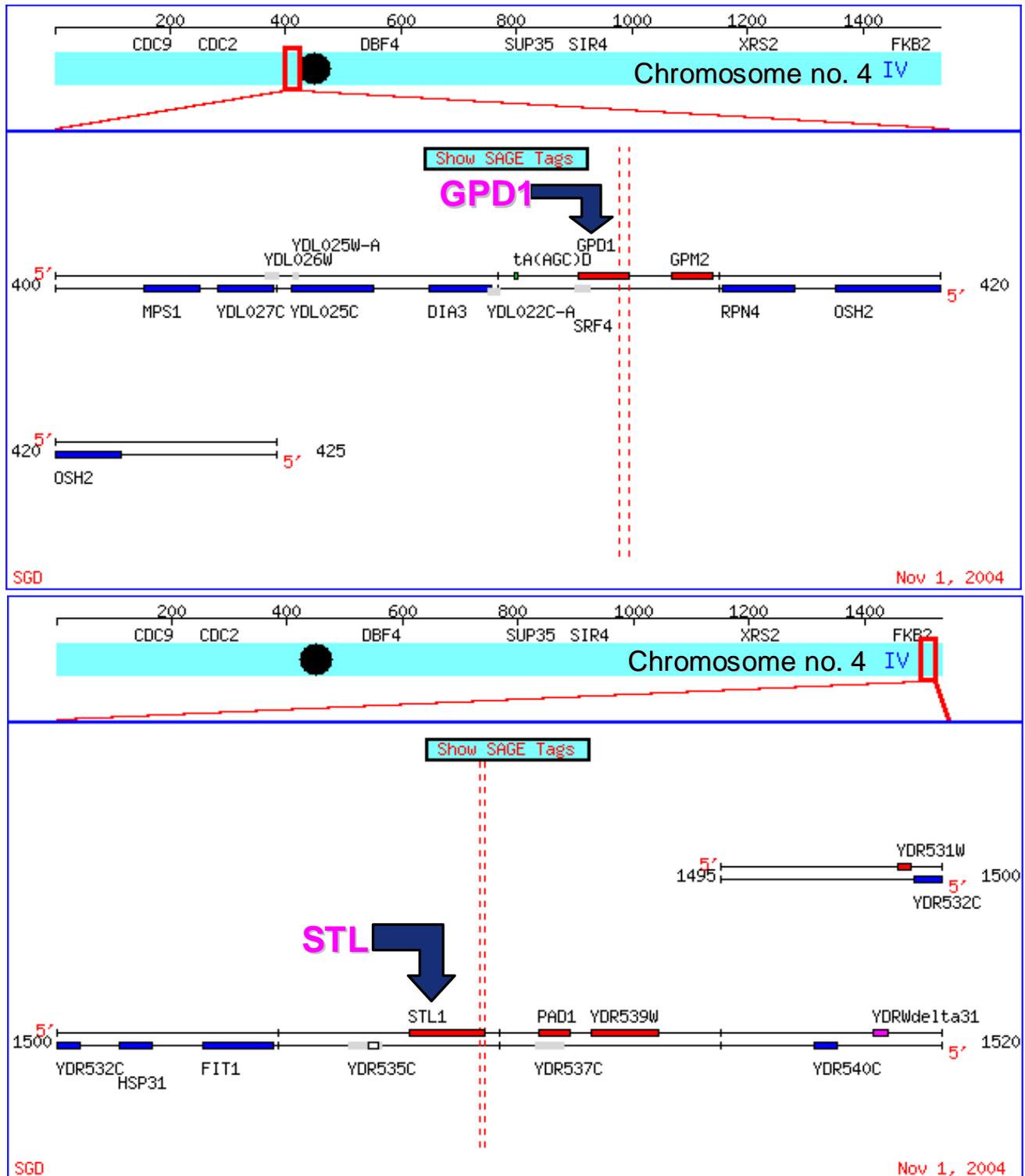
Figure 6. Alignment and positions of the three differentially expressed fragments after sequencing using SGD database.

ligating an RNA oligonucleotide (GeneRacer® RNA Oligo) to the full-length 5′ ends of decapped mRNA using T4 RNA ligase. The system relies on the highly purified mRNA, which is to be dephosphorylated at the 5′ ends to eliminate the 5′ phosphates from partial-length and non-mRNA codes. To select full-length mRNA for further amplification via PCR of their cDNA, tobacco acid pyro-phosphatase (TAP) is used to remove the 5' cap structure
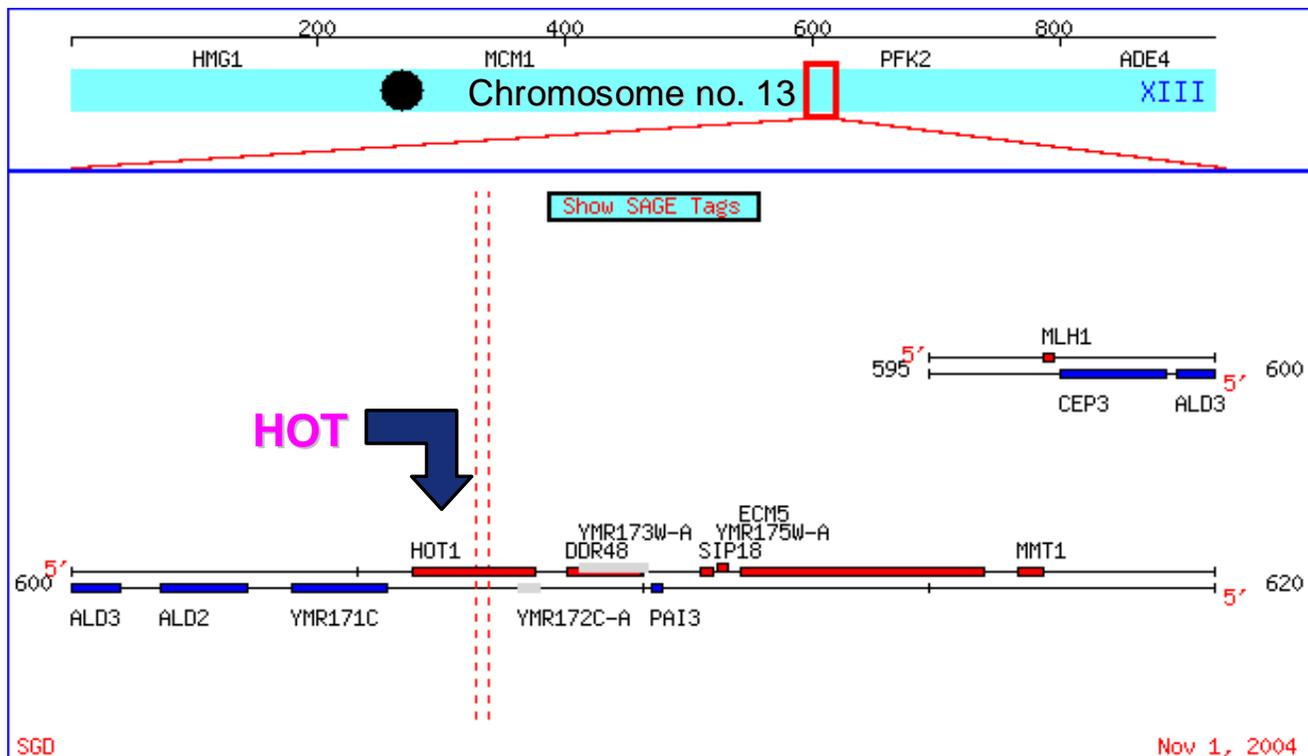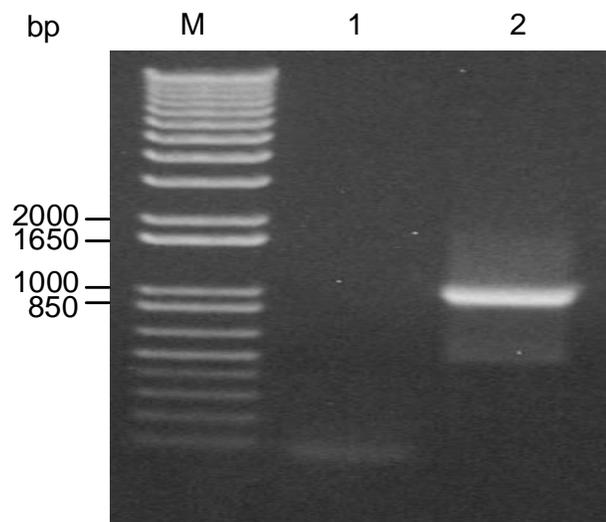
Figure 6. Contd.

Figure 7. GeneRacer® PCR for amplification of *GPD1* full-length gene. Lane 1 = negative control; lane 2 = GeneRacer® 5' Primer + 3' specific GPD1 primer.

from intact, full-length mRNA. This reaction resulted in the recovery of only 5' phosphorylated decaped full-length mRNA. This 5' end is suitable for ligation using T4 RNA ligase. The later was designed to be a template to 5' PCR primer provided by the kit. No PCR product will be

successful for the gene of interest (*GPD1*) unless cDNA contains the sequence of GeneRacer®. RNA Oligo with reverse sequence of the *GPD1* fragment recovered through differential display (5' ATGAATATGATATAG-AAGAGCCTC 3') is shown in Figure (7).

RNase H was used for degradation of mRNA strand of cDNA for more efficient RACE-PCR. To reduce the chance of DNA contamination, PCR was done for the originally purified mRNA with *GPD1* and poly T primers. After successful amplification of *GPD1* gene using touch-down PCR and 3' speciefic GPD1 primer, PCR product was purified from the gel, cloned into pCR4®TOPO® vector, transfromed into *E. coli* TOPO10 strain and tested by EcoRI restriction digestion.

Sequencing of *GPD1* gene

The complete cDNA sequence contained 105 bp of a 5' untranslated region, 645 bp of an open reading frame (ORF), and 111 bp of a 3' untranslated region. The ORF encodes a 215 amino acid polypeptide with a calculated molecular mass of 8.888 kDa. The ORF is likely to represent the complete sequence of the gene. The 5' untranslated region contains no stop codons on this reading frame, and no other ATG codons are present beyond the first ATG codon of the assigned ORF. Comparison of the deduced amino acid sequence of the enzyme G3PDH with the sequences in the databases

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gb|EDV08306.1| glycerol-3-phosphate dehydrogenase [Saccharomyces cerevisiae

Length=392

 Score =  387 bits (994),  Expect(2) = 3e-110
 Identities = 195/206 (94%), Positives = 196/206 (95%), Gaps = 0/206 (0%)
 Frame = +1

Query  1    MSAAADRLNLTSGNLNAGRKRSSSSVSLKAAEKPFKVTVIGSGNWGTTIAKVVAENCKGY  180
            MSAAADRLNLTSG+LNAGRKRSSSSVSLKAAEKPFKVTVIGSGNWGTTIAKVVAENCKGY
Sbjct  1    MSAAADRLNLTSGHLNAGRKRSSSSVSLKAAEKPFKVTVIGSGNWGTTIAKVVAENCKGY   60

Query  181  PEVLAPIVQMWVLEEEINGEKLTEIINTRHQNVKYLPGITLPDNLVANPDLIDSVKDDDI  360
            PEV APIVQMWV EEEINGEKLTEIINTRHQNVKYLPGITLPDNLVANPDLIDSVKD DI
Sbjct  61   PEVFAPIVQMWVFEEEINGEKLTEIINTRHQNVKYLPGITLPDNLVANPDLIDSVKDVDI  120

Query  361  IVFNIPHQFLPRICSQLKGHVDSHVRAISCLKGFEVGAKGVQLLSSYITEELGIQCGDLS  540
            IVFNIPHQFLPRICSQLKGHVDSHVRAISCLKGFEVGAKGVQLLSSYITEELGIQCG LS
Sbjct  121  IVFNIPHQFLPRICSQLKGHVDSHVRAISCLKGFEVGAKGVQLLSSYITEELGIQCGALS  180

Query  541  GANIATEVAQEHWSETTVCLXXSKGF   618
            GANIATEVAQEHWSETTV     K F
Sbjct  181  GANIATEVAQEHWSETTVAYHIPKDF  206
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Figure 8. Alignment of the deduced amino acids sequence of G3PDH with its related sequences in *Saccharomyces cerevisiae*.
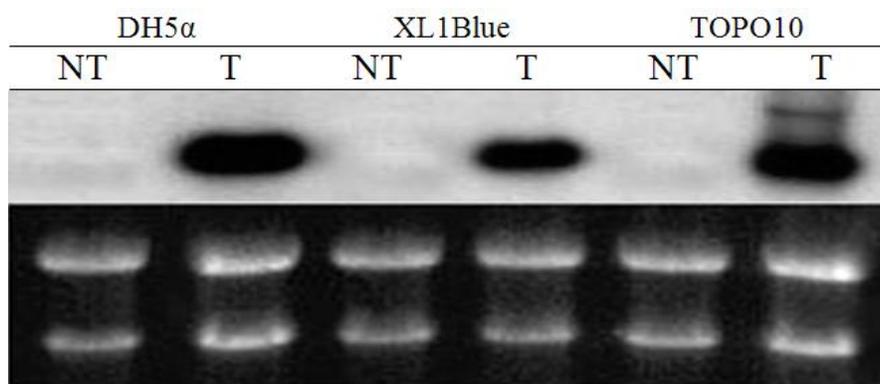

Figure 9. Northern blot with *GPD1* gene as a probe, for the three *E. coli* transformed bacterial strains (T) as compared to the non-transformed bacteria (NT).

revealed that the first is related to other yeast glycerol-3-phosphate dehydrogenase gene (Figure 8), including *S. cerevisiae* NAD-dependent glycerol-3-phosphate dehydrogenase NP_010262.1 (94% identity and 95% similarity) and *S. cerevisiae GPD1* gb_AAT27375.1 (92% identity and 94% similarity).

Expression of *GPD1* gene in transformed bacteria

In this study, we investigated the oxidation of G3P to DHAP by the enzyme G3PDH, a metabolic step likely to be central to glycerol catabolism and subsequent to the phosphorylation of glycerol by glycerol kinase. It was noted that both bacteria and eukaryotes use G3PDH to synthesize G3P for the backbones of their membrane

lipids. The latter process is indicative to G3PDH activity in bacterial cell lysates. The results of Northern blotting to prove transgene constitutive expression for the three *GPD1*-transformed bacteria are shown in Figure 9. It was obvious that no detectable differences in gene expression among the three transformed strains are shown. However, the results of G3PDH indicated that the transformed bacterial strain TOPO10 showed the highest activity level when compared to the other two transformed bacterial strains (Table 3). Experiments were performed in biological triplicate, and the means ± standard deviations were calculated. No activity was detected for controls with no substrate or with boiled cell lysates. In addition, the results of Northern blotting for the three non-transformed bacteria indicated no gene expression and consequently the results of the enzyme activity indicated

Table 3. G3PDH activity measured for the three *E. coli* transformed bacterial strains (T) as compared to the non-transformed bacteria (NT).

Bacterial strain	DH5α		XL1Blue		TOPO10	
	T	NT	T	NT	T	NT
Activity (Um · mg^{-1})	45.4 ± 9.2	-	61.2 ± 12.7	-	73.8 ± 7.0	-

T = transformed; NT = non-transformed.

Table 4. Effects of different NaCl concentrations on the growth of the three *E coli* strains as referred to by the culture turbidity (absorbance at 600 nm) after one day.

Media	DH5α		XL1Blue		TOPO10	
	T	NT	T	NT	T	NT
LB (control)	2.23	2.28	1.9	1.88	2.9	2.86
LB + 50 mM NaCl	2.09	1.19	2.01	1.37	2.89	1.67
LB + 100 mM NaCl	1.99	0.85	1.64	1.07	2.15	1.08
LB + 150 mM NaCl	1.79	0.66	1.67	0.6	1.8	0.95
LB + 200 mM NaCl	1.01	0.27	1.2	0.35	1.6	0.33

T = transformed; NT = non-transformed.
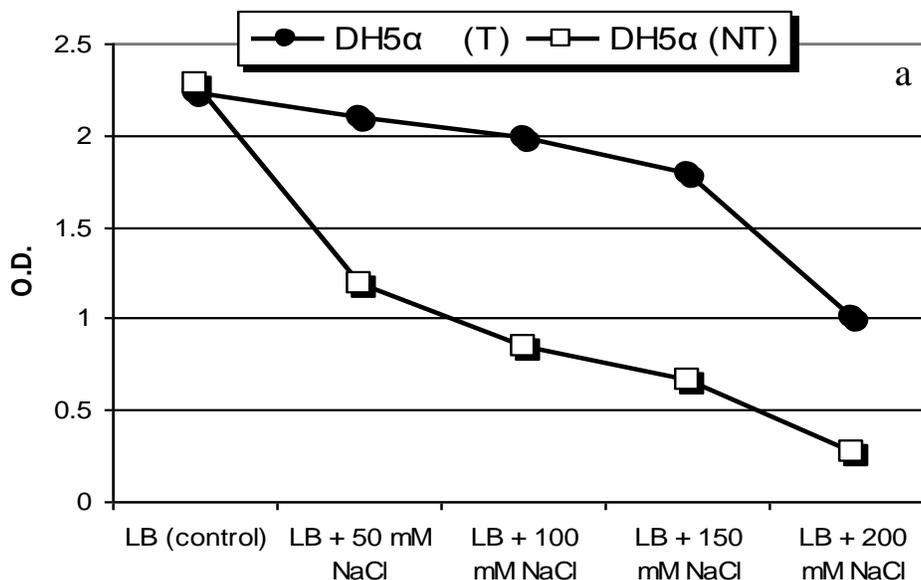
Figure 10. Effects of NaCl on *E coli* strains DH5α (A), XL1Blue (B) and TOPO10 (C) at concentrations of 50 mM NaCl, 100 mM NaCl, 150 mM NaCl and 200 mM NaCl. T= Transformed; NT= non-transformed.

no detectable levels (Figure 9 and Table 3).

Salt tolerance in a bacterial system using the *GPD1* gene

Three bacterial (*E. coli*) strains, DH5α, XL1Blue and TOPO10 were used; each was transformed by pCR4-TOPO-*GPD1* vector and grown in LB medium supple-mented with 50, 100, 150 and 200 mM NaCl in addition to the control. Growth degree was determined after 24 h at OD of 600 nm. As shown in Table 4 and Figure 10, there were significant differences between the transformed and non-trasformed strains under concen-trations of 50, 100, 150 and 200 mM NaCl in each strain, indicating that the *GPD1* gene might enhance the tolerant of these strains to salt stress as compared to non-transformed strains.

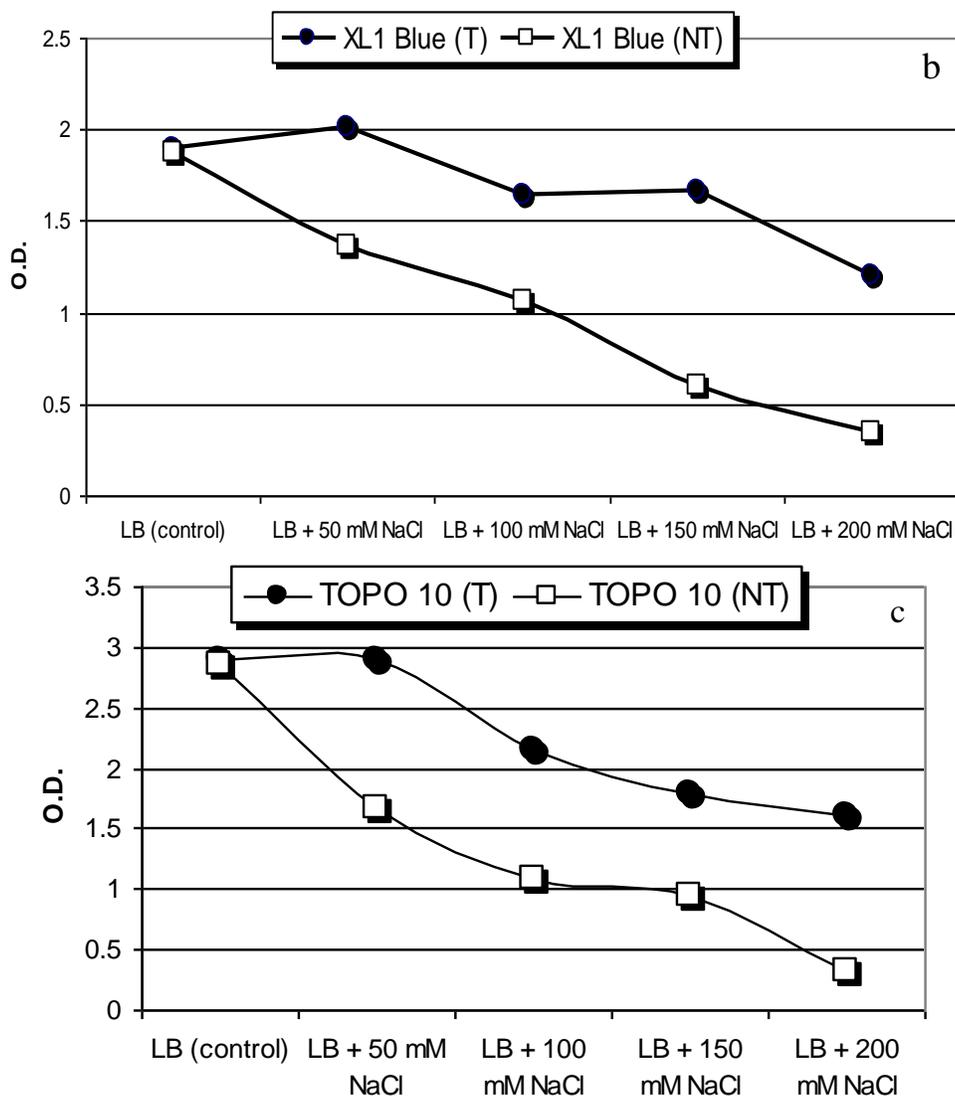In conclusion, the results of this finding indicates the

Figure 10. Contd.

possible utilization of this gene (*GPD1)* to confer tolerance to salt stress upon being introduced in an eukaryotic system, like tobacco or Arabidopsis.

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