Isolation and Molecular Characterization of the Trehalose Gene from Yemen Soil Bacterial Genome

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Abstract: The trehalose gene is found in a wide range of organisms such as bacteria, yeast, fungi, plants and animals. Twelve bacterial isolates were purified from soil samples collected from three governorates (Thamar, Hodidah and Sana'a) in Yemen state. Unique biochemical and physical properties make the nonreducing disaccharide, trehalose a good protectant against various stresses (temperature and pH). Ten random primers were used in the present study to characterize these bacterial isolates. Among those, six primers generated reproducible and easily scorable RAPD profiles. The number of amplified DNA fragments ranged from 8 to 11 amplicons per primer. The results of molecular analysis (RAPD) revealed 85.18% polymorphism and the similarity indices ranged from 44.1 to 97.1%. Two specific oligonucleotide primers were used for the amplification of the two trehalose-6-phosphate synthesis (TPS or *Ost A* gene) and trehalose-6-phosphate phosphate (TPP or *Ost B* gene) genes. The electrophorized band that is equivalent to 1425 bp in length was characterized in two bacterial isolates (s1-A) and (s1-B). Moreover, DNA sequencing was performed to the retrieved band (1425 bp). The reading frame of the 398 bp as a forward and 404 bp as a reverse reading frame was carried out in Germany for the proposed trehalose gene. The DNA sequencing alignment was screened using database of the trehalose gene in the Genbank databases. The 26 strains of *E. coli* showed 100% and another 5 and 25 strains of the same bacterium showed 99 and 98% similarity respectively to the characterized trehalose gene in the present study.
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1. Introduction

Saline soil causes a serious problem to the agricultural production all over 100 countries especially in China, India, Pakistan, United States, Thailand and Egypt. Salinity soil causes results in poor growth and lossing yield of plants. Soluble ions in the saline soil can affect plants by reducing the osmotic potential. Thus, salinity contributes to forces which reduce photosynthesis and transpiration by preventing water from entering plant roots (Waisel, 1972). Halophilic microorganisms are organisms that grow optimally in the presence of at least 0.2 M NaCl. The high potential for biotechnological remediation applications using halophilic bacteria were reported by several authors (Oren, 2002; Ramos- Cormenzana, 1990; Ventosa *et al*., 1998). The applications of halophilic bacteria include recovery of saline soil by directly supporting the growth of vegetation thus indirectly increasing crop yields. Trehalose (α,α-trehalose or α-D-glucopyranosyl α-D-glucopyranoside) is a non-reducing disaccharide of glucose that commonly occurs in a large range of organisms such as bacteria, fungi, nematodes and crustaceans (Elbein, 1974). Trehalose has a capacity to stabilize proteins and membranes under stress conditions, especially desiccation and heat since it prevents the denaturation of proteins and the fusion of membranes (Wingler, 2002).

The protective properties of trehalose during heat, dehydration and oxidative stresses are explained by its ability to stabilize the native conformation of proteins and membranes, but the molecular basis for this phenomenon is not well understood (Singer and Lindquist, 1998). Contradictory models have been proposed to explain the stabilizing effects of trehalose, either by replacement of water molecules from the surface of biomolecules, or by entrapment of residual water molecules close to the biomolecule (Thevelein, *et.al* 1996; Pereira *et al*., 2004; Skibinsky *et al*., 2005).

Since trehalose was first identified in a living system, it has been isolated from at least 80 species including plants, algae, fungi, yeasts, bacteria, insects and other invertebrates (Elbein, 1974). In addition to its proposed roles as carbon source and stress protectant, the 6-phosphate derivative (trehalose-6-phosphate) has been implicated in the regulation of carbon partitioning in yeast. This regulation is achieved through the control of carbon influx into glycolysis, by the inhibition of hexokinases and other yet undefined mechanisms (Gancedo *et al.,* 2004). Trehalose plays an important role in stress tolerance in

plants. Trehalose-producing, transgenic rice (*Oryza sativa*) plants was generated by the introduction of a gene encoding a bifunctional fusion (TPSP) of the trehalose-6-phosphate (T-6-P) synthase (TPS) and T-6-P phosphatase (TPP) of *Escherichia coli,* under the control of the maize (*Zea mays*) ubiquitin promoter (Ubi1) (Ishida *et al.,* 1996). The present study was conducted to with two major objectives, the first is screening soil collected from Yemen state for the isolation of halophilic bacteria. Second is characterizing the trehalose gene through the genetic engineering and the bioinformatics tools.

2. Material and Methods

Bacterial strains

The twelve bacterial isolates used in this study were isolated from soil samples of different Yemen governorates as indicated in Table (1).

Table (1) Soil samples collected from different regions and cities in Yemen

Sample	Region	City
S1	Wosab	Thamar
S2	Jarobah	Hodeidah
S3	Jahran	Thamar
S4	Wadeerima	Thamar
S5	Wa'alan	Sana'a
S6	Otmah	Thamar

Sample were transferred into sterile bottles and stored at 4°C. The *E. coli* strain that provided from microbiological department, faculty of agriculture, Cairo University was used as a positive control. Bacterial isolation was carried out by dissolving 10 g soil in 100 ml sterile distilled water and then 40 µl of soil solution was added to LB medium (10 g Trypton, 5 g Yeast extract, 10g Sodium chloride) were dissolved in 1 liter of deionized water and adjusted to pH 7.0. The Petri dishes were incubated at 37°C for 24 hrs.

Preparation of DNA samples for PCR

a. Phenol extraction method.

Total DNA was extracted from bacterial cultures grown in LB medium for 24 hrs, 1 ml of culture was removed and centrifuged at 3000×*g* for 5 min Decanting the supernatants and resuspending the pellet in 467 µl TE buffer (10 mM Tris-HCl containing 1mM EDTA pH 8.0) with repeated pipetting was carried out followed by adding 30 µl of 10% SDS and 3µl 20 mg/ml proteinase K, incubated for 1 hr at 37°C then added 1/10 volume of sodium acetate (5 M pH 5.2). The DNA was extracted with (phenol-chloroform-isoamylalcohol) (25:24:1) precipitated with isopropanol followed by washing with 70% ethanol, and finally re-suspended in 200 µl of TE buffer (Sambrook *et al.,* 1989).

b. Boiling cell (BC)

In the BC method, bacteria were grown for 20 hrs then collected the cells by centrifuged (8000×rpm) for 2 min the pellet was resuspended in 100 µl distilled water, boiled for 15 min and centrifuged at 8000×rpm for 3 min.

Polymerase Chain Reaction (PCR)

a. The RAPD analysis

The RAPD-PCR technique was carried out using ten oligonucleotides primers (OPERON Technologies, Alameda. CA). These primers are listed in Table (2).

Table (2). The sequences of the RAPD-PCR primers

Primer	Sequence
OPB.04	5′-GGACTGGAGT-3′
OPB.05	5′-TGCGCCCTTC-3′
OPB.06	5′-TGCTCTGCCC-3′
OPB.07	5′-GGTGACGCAG-3′
OPB.08	5′-GTCCACACGG-3′
OPB.09	5′-TGGGGGACTC-3′
OPB.13	5′-TTCCCCCGCT-3′
OPB.14	5′-TCCGCTCTGG-3′
OPB.15	5′-GGAGGGTGTT-3′
OPB.18	5′-CCACAGCAGT-3′

The RAPD-PCR was carried out according to the procedure of Williams *et al.* (1990) with minor modifications. Amplification reaction was carried out in a volume of 25 µl. Each reaction mixture contained 3µl genomic DNA, 2µl decamer oligonucleotide primer, 0.5µl of Taq DNA polymerase, 3µl of 10X buffer] 500 mM KCl, 100 mM Tris-HCl (pH 9.0) and 1% triton X-100[3µl MgCl2, 3µl dNTPs (dATP, dCTP, dTTP, dGTP) and 11.5 ml distilled water. The PCR amplification was performed for 40 cycles after an initial denaturation step for 3 min at 94°C. Each cycle consisted of denaturation at 94°C for 1 min annealing at 35°C for 1 min extension at 72°C for 1 min An extension step was performed for 5 min at 72°C in the final cycle. The PCR products were analyzed in 1.5% agarose gels stained with ethidium bromide (Bio-Rad). The levels of similarity between DNA profiles were calculated using Dice coefficient. Dendrograms were constructed using the unweighted pair group method with average linkages (UPGMA).

b. Trehalose PCR primer

Specific primer was synthesized for amplification of the trehalose -6 phosphate syntheses, (TPS *otsA* as listed in Table (3). The PCR was performed in a 25µl reaction volume containing 2 µl DNA template, 3µl buffer 10x (10 mM Tris- HCl pH 8.3, 50 mM KCl), 3µl MgCl2 (25 mM), 3µl dNTPs, 2.5mM (dATP, dCTP, dGTP, dTTP), 0.5µl *Taq* DNA polymerase (5U/ µl), 2µl primer F (50 pmol), 2 µl primer R (50 pmol), and 9.5 distilled water.

The target DNA sequences were amplified in a programmed thermal cycler by using the stop-cycle program as follows: denaturation of the DNA at 94°C for 1min, annealing of 49.5°C for 1 min, extension at 72°Cfor 1 min and final extension at 72°C for 5 min.

3. Results and Discussion

Phenotypic characterization

The studied bacterial isolates were taken from six soil samples from Yemen state and purified. Developing colonies were pre-characterized based on their phenotypic and morphological characteristics. Cells were stained with Gram and endospore stains and then examined microscopically as seen in Fig (1).

Molecular characterization of bacterial isolates (RAPD-PCR)

In the present study, RAPD-PCR technique was used to characterize and differentiate the isolates exhibited the same morphological characteristics. The RAPD analysis was performed using ten random primers differed in their GC contents. In this context, six primers gave positive results and were used accordingly for RAPD analysis (Fig. 2). totaling 30 cycles. The PCR products were analyzed by agarose gel electrophoresis. A total of 10 µl of each PCR reaction mix was electrophorized on a 1% agarose gel and stained with ethidium bromide.

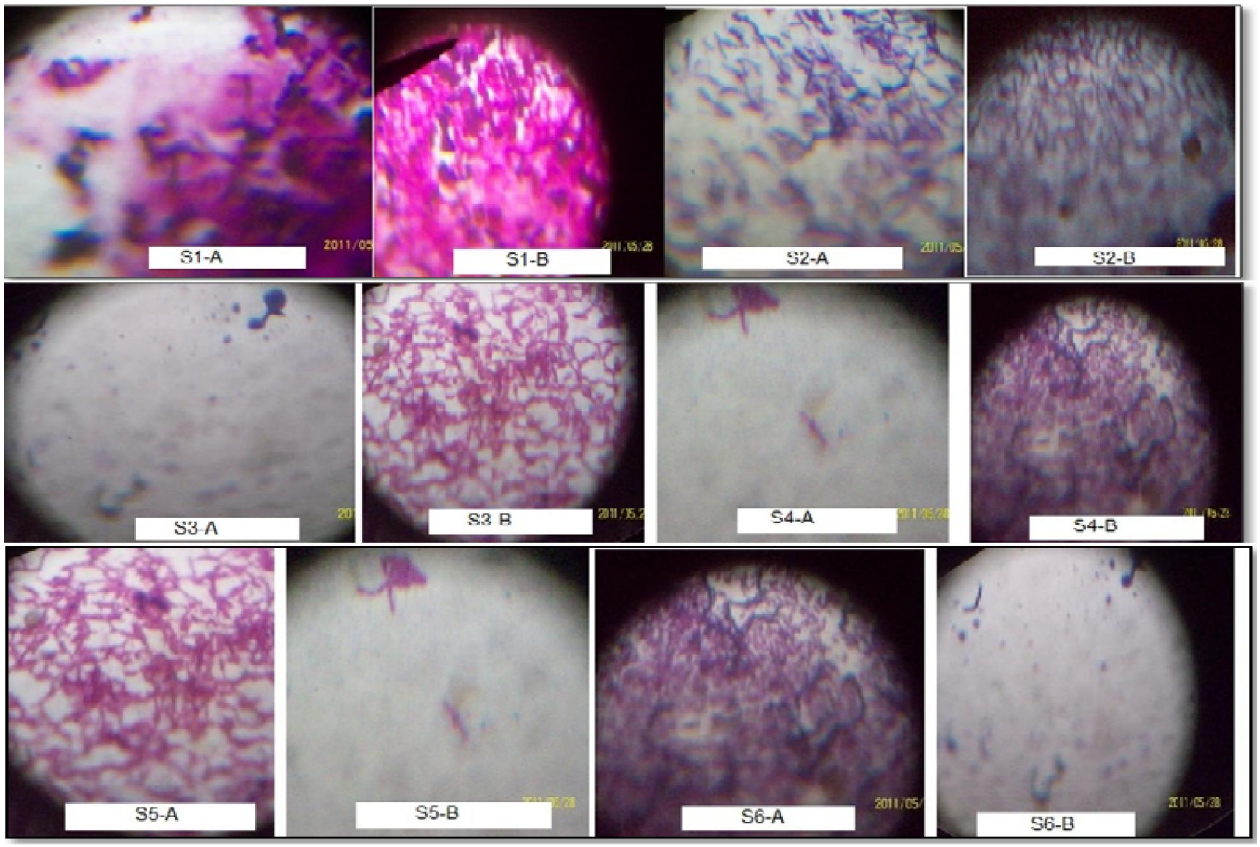

Fig (1). Morphology of the bacterial isolates stained with Gram stain. The all bacteria strain tested were Gram positive except strains S1-A and S1-B were Gram negative**.**

Table (3). The sequence of the PCR primers

Primer	sequence	Length
F	5′-TCTAGAATGAGTCGTTTAGTCGTAGT-3	26
R	5′-CCCGGGCTACR (G/A)CAAGCTTW (T/A)GGAAAGG-3′	28

The genetic relationships among the twelve isolates were determined by Dice coefficient (Table 4). The matrix of similarity index ranged from 48.1 % as recorded between S4-A and S5-B to 97.1 % as recorded between S3-A and S3-B isolates. However, the similarity recorded between S1-A and S1-B isolates was 93.8%. The values of similarity between the different pairs of genotypes are specific as recorded in Table (5).

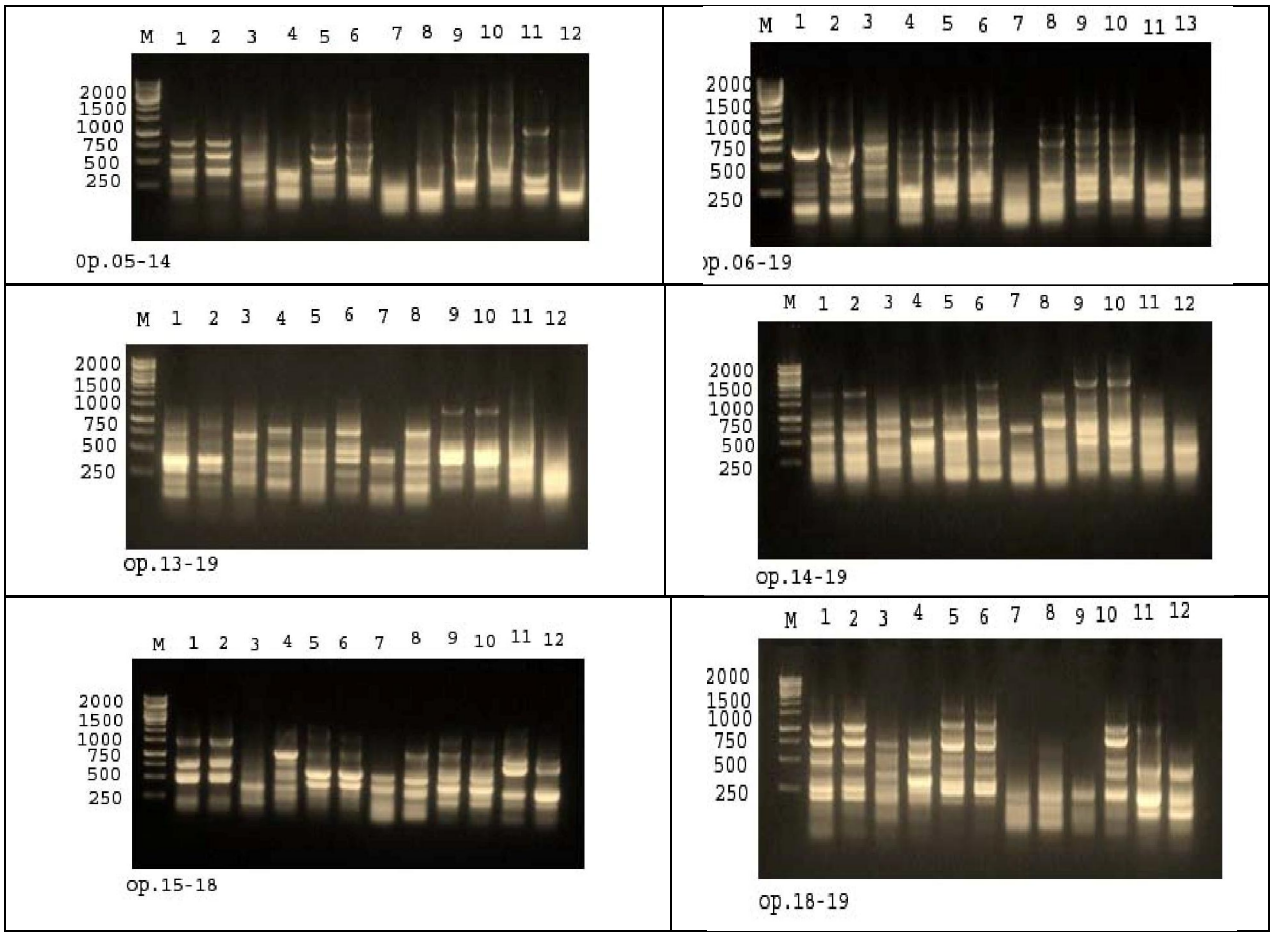

Fig (2). Product PCR-RAPD using six operon primers for the twelve strains, genetic similarity as revealed by RAPD markers

Table 4 Similarity coefficient among the twelve isolates as estimated by RAPD data

Species	1	2	3	4	5	6	7	8	9	10	11	12
1	100											
2	93.8	100										
3	70.0	73.3	100									
4	76.9	76.9	72.1	100								
5	76.9	80.0	68.9	81.8	100							
6	74.6	77.6	66.7	79.4	97.1	100						
7	56.0	52.0	65.2	66.7	54.9	52.8	100					
8	64.3	64.3	65.4	73.7	59.6	57.6	85.7	100				
9	65.6	71.9	56.7	73.8	70.8	68.7	52.0	64.3	100			
10	76.5	79.4	62.5	81.2	81.2	78.9	48.1	64.3	65.6	100		
11	68.8	68.8	60.0	83.1	73.8	71.6	64.0	71.4	65.6	73.5	100	
12	64.4	67.8	61.8	80.0	66.7	64.5	62.2	70.6	67.8	66.8	81.4	100

1=S1A, 2=S1B, 3=S2A, 4=S2B, 5=S3A, 6=S3B, 7=S4A, 8=S4B, 9=S5A, 10=S5B, 11=S6A, 12=S6B

The dendrogram illustrating the distance between the examined isolates is presented in Fig. (3). the dendrogram revealed two clusters. Cluster no.1 (S1-A, S1-B, S2-A, S2-B, S3-A, S3-B, S5-A, S5-B, S6-A, S6-B), Cluster two included (S4-A and S4-B) which divided into two subclusters one of them included S2-A, while the other divided into two groups. One group included S2-B, S6-A and S6-B. The two groups were divided into two subgroups. One subgroup included S5-A and S5-B. The second subgroup contained two groups, one contained S3-A and S3-B, while the other one included S1-A and S1-Bgenotypes.

Genotype identification by RAPD marker

A total of 54 bands were detected among the studied genotypes. In this context, 40 bands showed polymorphism. Out of these polymorphic bands, 6 unique bands were scored (Table 4). However, the primer OPB.15 showed 100% polymorphism. While the primer OPB.05, OPB.06, OPB.13, OPB.14, and OPB.18 showed 87.5, 81.81,75,80 and 88.88% polymorphism, respectively. The size of DNA ranged from 129.214 bp to 2274.062 bp (Fig 3).

The RAPD assay permitted the molecular identification of the twelve isolates by the presence of unique positive or the absence of negative markers, as well as the polymorphic markers (Table 4 and Fig. 4).

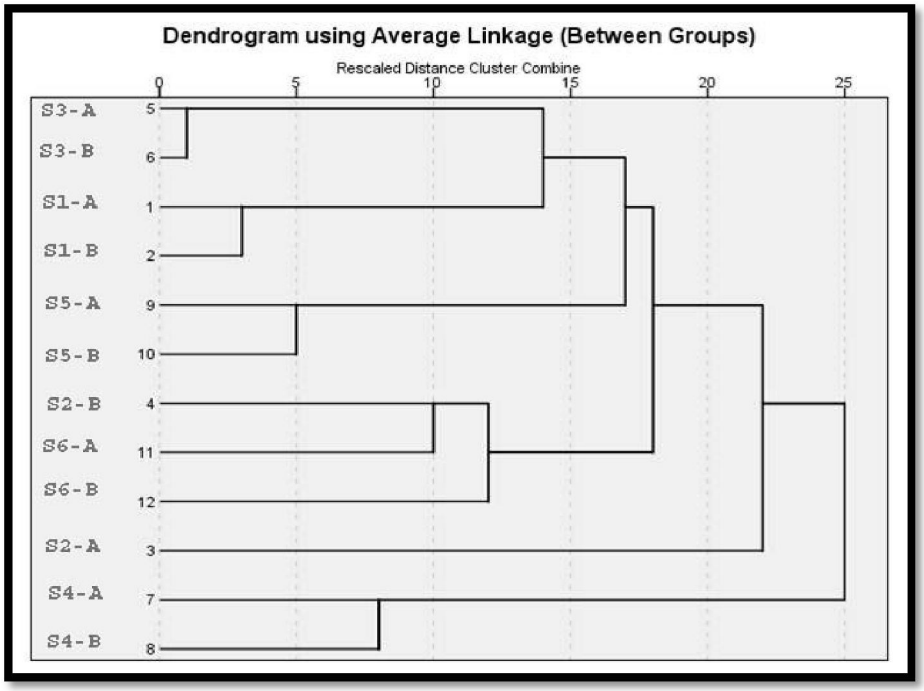

Fig (3) Dendrogram illustrating the distance between the twelve isolates constructed from RAPD data using the UPGMA and similarity matrix computed according to the Dice coefficient.

Table 5 Genotype specific RAPD markers of the twelve isolates

Species	Primer code	Positive unique markers	Total unique markers/species
1	OPB-O5	811.429 268.294	2
3	OPB-13	1041.874	1
4	OPB-14	1970.075 1603.149	2
6	OPB.18	386.873	1
Total	-		6

Table 6 Polymorphism data as detected by RAPD markers, total number of amplicons, monomorphic and polymorphic amplicons and the percentage of polymorphism among the twelve isolates

Primer code	Total Amplicons	Monomorphic	Polymorphic	Unique	Polymorphism %
OPB.05	8	1	5	2	87.50
OPB.06	11	2	9	0	81.81
OPB.13	8	2	5	1	75.00
OPB.14	10	2	6	2	80.00
OPB.15	8	0	8	0	100.00
OPB.18	9	1	7	1	88.88
Total	54	8	40	6	
Average	7.7	1.1	5.7	0.86	85.18

Characterization and isolation of the trehalose gene.

Trehalose gene was characterized and isolated by designing a specific primer for the trehalose gene in E. *coli* using the Genbank databases. The PCR products were screened on gel electrophoresis and the bacterial isolates S1-A and S1-B gave a unique band with molecular size about 1425 bp as shown in Fig. (5).

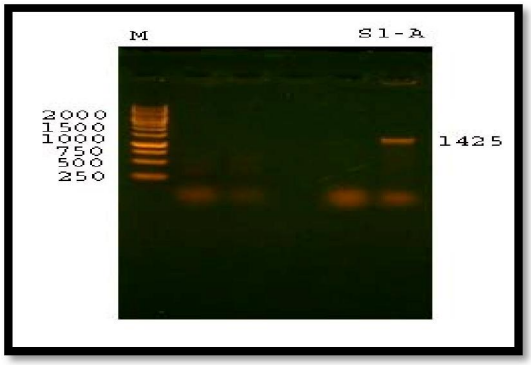

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Fig (5) The positive and negative control of the bacterial isolates for the trehalose gene. (P1 *E.coli DH 10β*, N1 *Pseudomunas* spp, P2 S1-A, N2 *Bacillus subtills*, P3 S1-B).

FT.ab1394
GAATTCATGAGTCGTTTAGTCGTAGTATCTAA
CCGGATTGCACCACCAGACGAGCACGCCGCC
AGTGCCGGTGGCCTTGCCGTTGGCATACTGGG
GGCACTGAAAGCCGCAGGCGGACTGTGGTTT
GGCTGGAGTGGTGAAACAGGGAATGAGGATC
AGCCGCTAAAAAAGGTGAAAAAAGGTAACAT
TACGTGGGCCTCTTTTAACCTCAGCGAACAGG
ACCTTGACGAATACTACAACCAATTCTCCAAT
GCCGTTCTCTGGCCCGCTTTTCATTATCGGCTC
GATCTGGTGCAATTTCAGCGTCCTGCCTGGGA
CGGCTATCTACGCGTAAATGCGTTGCTGGCAG
ATAAATTACTGCCGCTGTTGCAAGACGATGAC
ATTATCTGGATCC

Fig (6) The 394 bp from trehalose as a forword frame.

RT.ab1404
GATATCCTACGCAAGCTTTGGAAAGGTAGCA
ACTTTATCGCGCTGCTGGCTTTCCGCGCTTCG
CGGAACTATCTGCTTTAGGTCGCTAATGAAGC
ACTCCTGCCAGTGGTTAATATCGTTTTTCACG
ATAACGTCCAGCATTTCTGCATGACGGGAAAT
ACGTTCCGCCAGCGACATAGTCAATGCACGAT
CCAGCGCAGCTGCAACTTCGTCACGATCGTAG
GGGTTAACAATTAACGCCGACGTTAACTCGTT
TGCCGCTCCCGCAAATTGCGAAAGAACAAGA
ACGCCCGGATTGGCTGGGTCCTGAGCAGCAA
CATACTCTTTTGCTACCAGGTTCATCCCGTCA
CGCAGTGGCGTCACTAAGCCCACGTCAGAGT
AGCGGAATATTTTCATCAGTAATT

Fig (7) The 404 bp from trehalose gene as reverse frame

In order to confirm that the DNA band at 1425 bp is equivalent to DNA of the proposed trehalose gene, DNA sequencing was performed by Macrogen Company (Germany). The results gave the reading frame for 398 bp as forward and 404 bp as reverse frame Fig (6, 7, and 8).

The DNA sequencing alignment for the forward and reverse frames with database of the trehalose gene in Genbank was carried out as shown in (Fig. 8). Results showed 100% similarity for 26 strains of *E. coli*. However, 99 and 98% were scored for other 5 and 25 strains, respectively. In this respect, trehalose, synthesized by *otsA* gene in *E. coli,* is broadly used as a protecting of enzymes and membranes in many microorganisms under adverse environmental stresses such as drought, salt and cold so that it can enhance the resistant ability of plants (Crowe *et al.,* 1990; Drennan *et al.,* 1993; Strom and Kaasen, 1993). In recent years, many transgenic plants with *otsA* gene, for example tobacco (*Nicotiana tabacum)*, potato (*Solanum tuberosum*) and sugarcane (*Saccharum*) were developed through the application of new bio-techniques such as electroporation, microprojectile bombardment and *Agrobacterium-mediated* transformation (Goddijn *et al.,* 1997; Yeo *et al.,* 2000; Wang *et al.,* 2003). Among them, *Agrobacterium-mediated* transformation is often preferred over other plant transformation systems because of its simplicity, low cost, high transformation efficiency and low transgene copies integrated into the plant genome (Ishida *et al.,* 1996; Matzke *et al.*, 2001; Dong and Qu, 2005). The improvement of a biotic stress tolerance by transforming rice with a trehalose-6-phosphate synthesis phosphates (TPSP) fusion gene was reported by Seo, *et al.,* (2000). It includes the coding regions of *E.coli otsA* and *otsB* genes (encoding TPS and TPP, respectively). This approach has the dual advantages of necessitating only a single

transformation event and producing a higher net catalytic efficiency for trehalose formation (Seo, *et al.,* 2000)). Because Indian, rice varieties represent 80% of rice grown worldwide, we chose to transform the economically valuable indica rice, Pusa Basmati-1 (PB-1), even though transformation and regeneration are more difficult than in japonica rice varieties.

CLUSTAL 2.1 multiple sequence alignment trehalose N-terminal amino acids

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New isolate                    MSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNED  50
gi|417712824|ref|ZP_12361800.1 MSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNED  50
gi|420346177|ref|ZP_14847599.1 MSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNED  50
gi|331642512|ref|ZP_08343647.1 MSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNED  50
gi|260855834|ref|YP_003229725. MSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNED  50
gi|417231400|ref|ZP_12032798.1 MSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNED  50
gi|195939598|ref|ZP_03084980.1 MSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNED  50
gi|417639432|ref|ZP_12289582.1 MSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNED  50
gi|432602418|ref|ZP_19838662.1 MSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNED  50
gi|429061333|ref|ZP_19125399.1 MSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNED  50
gi|187734011|ref|YP_001879686. MSRLVVVSNRIAPPDEHAASAGGLAVGILGALKAAGGLWFGWSGETGNED  50
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New isolate                    QPLKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPAFHYRLDLVQFQR  100
gi|417712824|ref|ZP_12361800.1 QPLKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPAFHYRLDLVQFQR  100
gi|420346177|ref|ZP_14847599.1 QPLKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPAFHYRLDLVQFQR  100
gi|331642512|ref|ZP_08343647.1 QPLKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPAFHYRLDLVQFQR  100
gi|260855834|ref|YP_003229725. QPLKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPAFHYRLDLVQFQR  100
gi|417231400|ref|ZP_12032798.1 QPLKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPAFHYRLDLVQFQR  100
gi|195939598|ref|ZP_03084980.1 QPLKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPAFHYRLDLVQFQR  100
gi|417639432|ref|ZP_12289582.1 QPLKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPAFHYRLDLVQFQR  100
gi|432602418|ref|ZP_19838662.1 QPLKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPAFHYRLDLVQFQR  100
gi|429061333|ref|ZP_19125399.1 QPLKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPAFHYRLDLVQFQR  100
gi|187734011|ref|YP_001879686. QPLKKVKKGNITWASFNLSEQDLDEYYNQFSNAVLWPAFHYRLDLMQFQR  100
                               **************************************:***********

New isolate                    PAWDGYLRVNALLADKLLPLLQDDDIIWI  129
gi|417712824|ref|ZP_12361800.1 PAWDGYLRVNALLADKLLPLLQDDDIIWI  129
gi|420346177|ref|ZP_14847599.1 PAWDGYLRVNALLADKLLPLLQDDDIIWI  129
gi|331642512|ref|ZP_08343647.1 PAWDGYLRVNALLADKLLPLLQDDDIIWI  129
gi|260855834|ref|YP_003229725. PAWDGYLRVNALLADKLLPLLQDDDIIWI  129
gi|417231400|ref|ZP_12032798.1 PAWDGYLRVNALLADKLLPLLQDDDIIWI  129
gi|195939598|ref|ZP_03084980.1 PAWDGYLRVNALLADKLLPLLQDDDIIWI  129
gi|417639432|ref|ZP_12289582.1 PAWDGYLRVNALLADKLLPLLQDDDIIWI  129
gi|432602418|ref|ZP_19838662.1 PAWDGYLRVNALLADKLLPLLQDDDIIWI  129
gi|429061333|ref|ZP_19125399.1 PAWDGYLRVNALLADKLLPLLQDDDIIWI  129
gi|187734011|ref|YP_001879686. PAWDGYLRVNALLADKLLPLLQDDDIIWI  129
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Fig 8 Multiple amino acid sequence alignment of trehalose protein using ClustalW2 which is a general purpose multiple sequence alignment program for DNA or proteins

Trehalose is that functions as a compatible solute in the stabilization of biological structures under heat and desiccation stress in bacteria, fungi, and some "resurrection plants". In the plant kingdom, trehalose is biosynthesized by trehalose-6-phosphate synthase (TPS) and trehalose-6-phosphate phosphatase (TPP). Over-expression of exogenous and endogenous genes encoding TPS and TPP is reported to be effective for improving abiotic stress tolerance in tobacco, potato, tomato, rice, and *Arabidopsis* (Jiang *et al.* 2010).

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