ISSN 1682-296X (Print)
ISSN 1682-2978 (Online)

Bio Technology

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Biotechnology 7 (2): 258-265, 2008
ISSN 1682-296X
© 2008 Asian Network for Scientific Information

Transformation of Maize with Trehalose Synthase Gene Cloned from *Saccharomyces cerevisiae*

Dan Tao, Yu Mu, Feng-Ling Fu and Wan-Chen Li
Maize Research Institute/Education Ministry Key Laboratory of Crop Genetic Resources and Improvement, Sichuan Agricultural University, Ya'an, Sichuan 625014, China

Abstract: A new sequence of trehalose synthase gene *TPS*1 was cloned from strain AS.1416 of *Saccharomyces cerevisiae* by the method of homologous amplification. Sequence analysis showed that its similarity with formerly reported sequence of gene *TPS*1 (X68496) was as high as 99.3%. The putative protein of this sequence had the same conserved contigs with the protein sequences of trehalose synthases in many eukaryotic and prokaryotic organisms. This sequence was used as exotic gene to construct a stress-inducible expression vector and transform embryonic calli of maize mediated by agrobacterium. After screening and regeneration, one fertile plant was detected to be positive by specific PCR amplification and sequencing of the amplified product.

Key words: Transgenics, maize, *TPS*1, *Saccharomyces cerevisiae*, drought tolerance

INTRODUCTION

Drought is the most widespread abiotic constraint to maize production (Bartels and Nelson, 1994; Edmaedes *et al*., 1992; Rosen and Scott, 1992). The most effective way to stabilize and improve maize production under drought conditions is to improve varieties for drought tolerance (Bruce *et al*., 2002; Ceccarelli and Grando, 1996). However, this effort heavily depends on the development and utilization of drought-tolerant germplasm resources, which is far from plentiful and bottlenecks maize improvement. Therefore, germplasm enhancement and development is foundationally important for maize improvement (Smith *et al*., 2004; Wu, 1983; Zhang *et al*., 2000). Transgenic operation is a useful technology to overcome reproductive isolation among species and utilize beneficial exotic genes. *SOD*, *DREB* and some other stress-tolerant genes have been used to transform maize for drought-tolerance improvement. The tolerance of the transgenic offspring is not strong to meet the requirement of maize production, due to the mechanism of these exotic genes are not adaptive to physiological metabolism of maize. It is the key step to explore new exotic gene with strong tolerance and adaptive mechanism to maize (Ingram and Bartels, 1996; Song and Wang, 2005; Wang and Fang, 2002).

Microorganisms survive for a long time under stress conditions such as drought, heat and high salt, due to high concentration of trehalose in their cells. Trehalose is an innocuous, scentless and melliferous non-reducing disaccharide containing two glucose residues bound in an α,α-1,1 glycosidic linkage. It is the non-reducing of trehalose to determine its high stability to acid, alkali and heat. Trehalose can become glass state structure by combining two water molecules. Its hydroscopic property is more than 3 times of sucrose, maltose, glucose and fructose. In cells, the high tolerance of trehalose to dehydration provides protection to proteins and biomembranes from drying, freezing and heating (Colaco *et al*., 1995; de Virgilio *et al*., 1994; Eleutherio *et al*., 1993; Thevelein, 1996; van Leeuwenhoek, 1990). A recent research shows that trehalose provides protection in fruit fly and mammal cells under anaerobic condition (Chen and Gabriel, 2004).

In yeast, Trehalos-6-Phosphate Synthase (TPS), encoded by gene *TPS*1, catalyzes the combination of UDP-glucose and glucose-6-phosphate by α,α-1,1 glycosidic linkage to form trehalose-6-phosphate, which is further dephosphorylated to trehalose by Trehalose-6-Phosphate Phosphatase (TPP), encoded by gene *TPS*2. These reactions are catalyzed by trehalose synthesis complex containing TPS, TPP as well as regulatory subunits TSL1 and TPS3. However, TPS alone shows activity of trehalose synthesis (Bell *et al*., 1998). In plant, UDP-glucose and glucose-6-phosphate are intermediate products of normal carbohydrate metabolism. The introduction of exotic TPS encoding gene can bring about synthesis of trehalose-6-phosphate. The dephosphorylation of trehalose-6-phosphate can be catalyzed by non-specific phosphoesterase, which is

Corresponding Author: Wan-Chen Li, Maize Research Institute, Sichuan Agricultural University, Xinkang Road 36, Ya'an, Sichuan 625014, China Tel: +86-835-2882096 Fax: +86-835-2883124

familiar in plant cells. The introduction of TPP encoding gene is not indispensable to trehalose accumulation. Similar situation was found in the transformation of tobacco by mannitol-1-phosphate dehydrogenase gene (Tarczynski *et al*., 1992). Dai *et al*. (2001), Goddijn *et al*. (1997), Holmstrom *et al*. (1996), Pilon-Smits *et al*. (1998) and Romero *et al*. (1997) transformed tobacco and potato with TPS encoding gene from *E. coli* and yeast, or together with TPP encoding gene and obtained transgenic plants with improved drought tolerance. No report has been found about transformation of trehalose synthase gene in graminaceous crops.

In this study, trehalose synthase gene *TPS*1 was cloned from brewer's yeast (*Saccharomyces cerevisiae*). A stress-inducible expression vector was constructed and used to transform embryonic calli of maize. It was attempted to improve maize for drought tolerance by the synthesis and accumulation of trehalose under drought stress.

MATERIALS AND METHODS

This study was conducted at Molecular Biology Laboratory of Maize Research Institute, Sichuan Agricultural University from year 2005 to 2007.

Cloning of gene *TPS*1 from *Saccharomyces cerevisiae*: Genomic DNA of *Saccharomyces cerevisiae* was extracted from strain AS.1416 by the method introduced by Adams *et al.* (1998). According to the sequence of gene *TPS*1 (GenBank accession number: X68496), a pair of specific PCR primers (P1: 5'-CGACTAGTGCTAAGT AAGCAACAAAGCAGGC-3'/P2: 5'-AGGGGCCC GAAAACCGGACCAGGAATAGACG-3') was designed using primer design software Premier 5.0 (http://www.premierbiosoft.com). At the 5' ends of the forward and reverse primers, recognition sites of restriction endonucleases SpeI and ApaI (ACTAGT and GGGCCC) were introduced. With these primers, the sequence of gene *TPS*1 was amplified with the following temperature cycle: 94°C 2 min; 30 cycles of 94°C 30 sec, 60°C 30 sec and 72°C 2 min; 72°C 10 min. The specific amplified fragment was separated by agarose gel electrophoresis and inserted into cloning vector pGem-T Easy. The inserted vector was denominated as pT-TPS1 (Fig. 1) and sequenced at TaKaRa Biotechnology co., Ltd. (Dalian, China).

Sequence analysis of gene *TPS*1: Sequence similarity between the amplified fragment and gene *TPS*1 (X68496) reported by McDougall *et al.* (1993) was aligned using DNAMAN software (http://www.lynnon.com). The putative amino acid sequence encoded by the amplified fragment was deduced using DNA Star software (www.dnastar.com) and aligned with amino acid sequence of TPS proteins reported in different species in NCBI database (http://www.ncbi.nlm.nih.gov). The conserved contigs were analysed by DNAMAN software.

Construction of stress-inducible expression vector for monocotyledons: By the steps shown in Fig. 1, plasmid pT-TPS1 containing gene *TPS*1 and plasmid pWDREB containing stress-inducible promoter *mwcs*120 of monocotyledon (Du *et al*., 2005), were digested by restriction endonucleases SpeI and ApaI, respectively. Fragments of gene *TPS*1 and plasmid pWDREB without gene *DREB* were separated by low melting-point agarose gel electrophoresis and ligated by T4DNA ligase to form plasmid pWTPS1. This plasmid was digested by EcoRV and HindIII. The expression structure P-*mwcs*120-*TPS*1-T-nos' was recovered and inserted into plasmid pCAMBIA1300 digested by SmaI and HindIII to form stress-inducible expression vector pCWTPS1300 containing gene *TPS*1. The ligated product was used to transform strain EHA105 of agrobacterium (*Agrobacterium tumefaciens*) and screened on kalamycin plate. According to its multiple cloning sites, the expression structure of plasmid pCWTPS1300 was confirmed by restriction digestion of SpeI and ApaI.

Transformation and screening: In November, 2005, the transformed agrobacterium was incubated twice for activation on YEB solid medium with kalamycin and rifamycin in dark at 28°C and then incubated in YEB liquid medium antibiotic free in dark at 28°C with shaking at 180 rpm for 16-18 h (van Larebeke *et al*., 1977). The cultured was inoculated in YEB liquid medium containing kalamycin and rifamysin by 1:50 and incubated in dark at 28°C with shaking at 225 rpm for 3-4 h until OD_{600} = 0.5. The bacterium cells were collected by centrifugation at 5000 rpm for 10 min and suspended in inoculation solution (improved N6 medium + sucrose 68.4 g L^{-1} + glucose 36 g L^{-1} + acetosyringone 100 μmol L^{-1}, pH 5.2) of the same volume. Maize calli were induced from immature embryos of inbred lines 18-599R and 18-599W, which are parents of several commercial hybrids widely planted in southwest China (Fu *et al*., 2000). Embryonic calli were cut into 5 mm diameter, immersed in the suspended bacterium for 8-10 min, dipped with aseptic filter paper, co-cultured on improved N6 medium in dark at 20°C for three days, washed with aseptic water for 3-5 times, immersed in 500 mg L^{-1} cephamycin over night, dipped with aseptic filter paper, cultured for recovering on N6 medium for

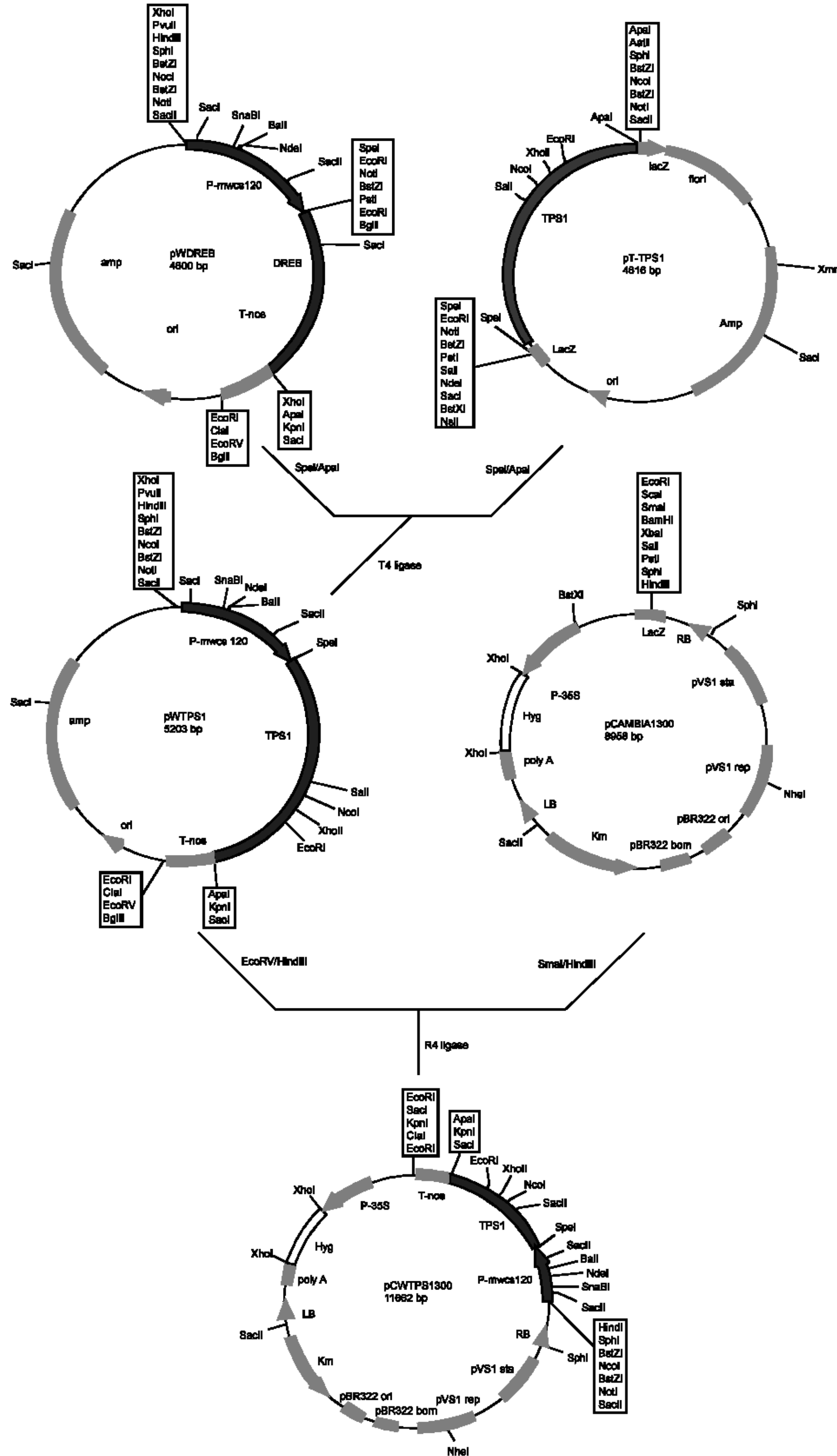

Fig. 1: Construction of stress-inducible expression vector pCWTPS1300 containing gene *TPS1* for monocotyledon

4-5 days and then screened on N6 medium containing hygromycin of gradient concentration (15 and 20 mg L^{-1}) for two times (20 days each) (Fu *et al*., 2000, 2005; Yu *et al*., 2005).

Regeneration and identification of T_0 plants: After screening, the calli were transferred to N6 differentiation medium and cultured at 27°C and 2000 lx illumination. Until the gemmules grew to 2-3 cm, the calli were transferred to N6 rooting medium and cultured under the same temperature and illumination. The regenerated plantlets were planted in vermiculite and perlite (3:1) and grown in greenhouse. Three weeks after new buds and roots germinated, the plantlets were transferred to field in March, 2006 (Fu *et al*., 2000; Yu *et al*., 2005).

Leaves were sampled from each regenerated T_0 plant and used to extract genomic DNA by the method introduced by Saghai-Maroof *et al*. (1984). A pair of specific PCR primers (P3: 5'-GACTACGGATAA CGCTAAGGC-3'/P4: 5'-CATCACGAGTGGACGAGACC-3') was designed based on the sequence of the amplified gene *TPS*1 and used to amplify the exotic gene. The expression vector pCWTPS1300 was used as positive control and the non-transformed receptor inbred line 18-599R was used as negative control. The amplified fragment was cloned and sequenced at TaKaRa Biotechnolgy co., Ltd. (Dalian, China).

RESULTS AND DISCUSSION

Sequence of gene *TPS*1 in *Saccharomyces cerevisiae*: The fragment amplified by primers P1/P2 from genomic DNA of strain AS.1416 of *Saccharomyces cerevisiae* was 1603 bp long (Fig. 2). Sequence analysis showed that this fragment had 99.3% similarity with the sequence of gene *TPS*1 (X68496) reported by McDougall *et al*. (1993). Although eleven single nucleotide mutations were found during this fragment and ten of them were included in the encoding region, nine of the ten were samesene mutations. They did not change the amino acid sequence of the encoding protein. Only the transition from G to A at site 1135 changed the glycine at site 356 of the encoding protein to asparamide (Fig. 3 and 4). Therefore, the sequence of this fragment was identified as gene *TPS*1 of *Saccharomyces cerevisiae* and registered at Genbank with accession number EF110520.

According to the result deduced by DNA Star software, the gene *TPS*1 we cloned from *Saccharomyces cerevisiae* encoded a protein of 495 amino acid residues (Fig. 4). This protein was identified as an acidic protein with molecular weight of 56205 Da and isoionic point of pH 5.8 and speculated as a hydrophilic protein since its

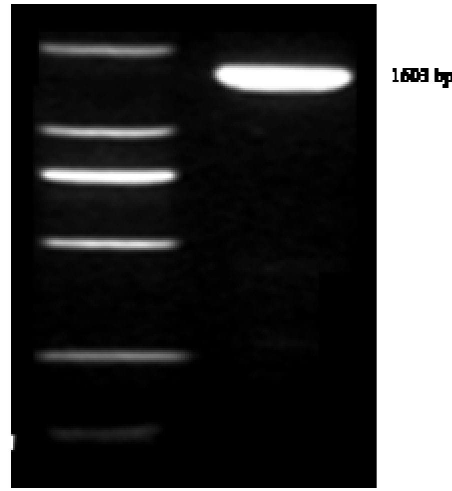

Fig. 2: Amplified fragment of gene *TPS1* in *Saccharomyces cerevisiae*

proportions of leucine, valine, serine and glutamic acid were as high as 9.3, 9.1, 7.9 and 7.1%, respectively. Alignment by DNAMAN software showed that the putative protein sequence had the same conserved contigs with the protein sequences of trehalose synthases in many eukaryotic and prokaryotic organisms such as rock lily (*Selaginella lepidophylla*), upland rice (*Medicago truncatula*), arabidopsis (*Arabidopsis thaliana*), mycetozoan (*Dictyostelium discoideum*), rice blast bug (*Magnaporthe grisea*) and rice aspergillus (*Aspergillus oryzae*) (underlined in Fig. 4).

Expression structure of plasmid pCWTPS1300: Two specific fragments of 1603 and 10059 bp were separated by agarose gel electrophoresis from plasmid pCWTPS1300 digested by restriction endonucleases SpeI and ApaI. They were the same long as gene *TPS*1 and the backbone of plasmid pCWTPS1300 (Fig. 5). This result showed that gene *TPS*1 had been inserted into vector pCAMBIA1300 and plasmid pCWTPS1300 had the expression structure as designed.

Rates of positive calli, regeneration and transformation: After two times of screening on gradient hygromycin medium, 340 and 607 pieces of positive calli were screened from inbred lines 18-599R and 18-599W, respectively. The rates of positive calli were 6.4 and 10.2%. No significant difference was found between these two rates. However, the regeneration ability of 18-599R was higher than 18-599W. The regeneration rate of 18-599R was more than three times of 18-599W. Some of the regenerated plantlets aborted during the planting

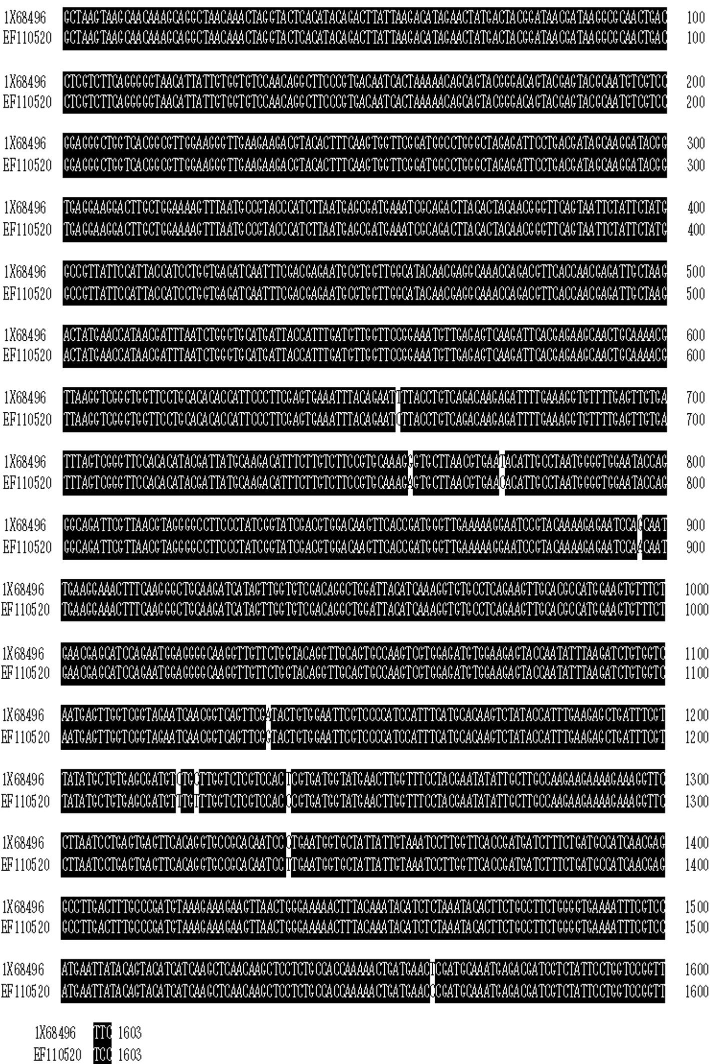

Fig. 3: Sequence alignment between the amplified fragment (EF110520) and gene *TPS*1 (X68496) reported by McDougall

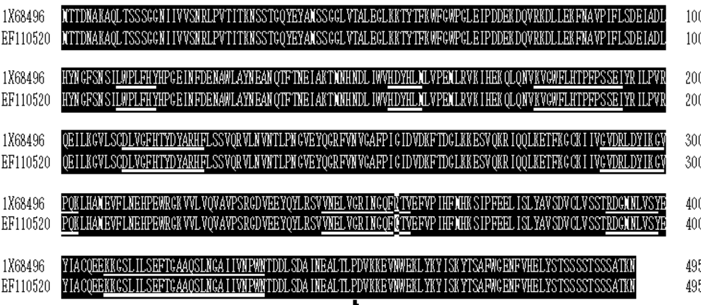

Fig. 4: Amino acid sequence of the putative protein based on the amplified fragment (EF110520) and gene *TPS*1 (X68496) reported by McDougall

Table 1 Rates of positive calli, regeneration and transformation

Inbred line	Piece of transformed calli	Piece of positive calli	Rate of positive calli (%)	No. of regenerated plants	Regeneration rate (%)	No. of PCR positive plants	Transformation rate (‰)
18-599R	5323	340	6.4	54	15.9	1	0.19
18-599W	5935	607	10.2	31	5.1	0	0.00
Total	11258	947	8.4	85	9.0	1	0.09

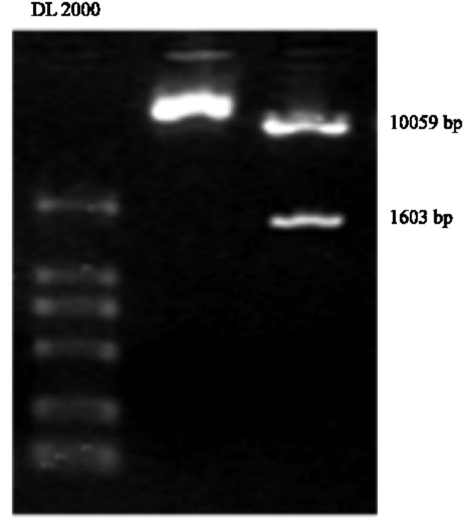

Fig. 5: Restriction endonuclease analysis of expression vector pCWTPS1300

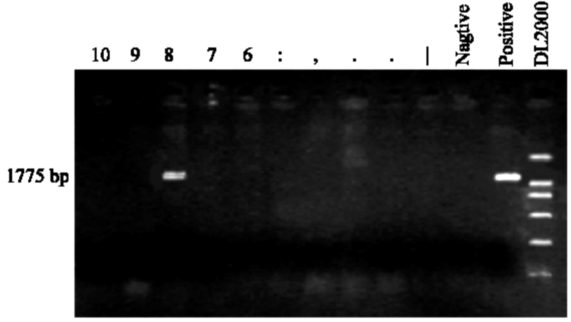

Fig. 6: Transformed regenerated plants identified by specific PCR amplification

(Table 1). A target fragment of 1175bp was amplified and separated from one fertile 18-599R plant of the 85 survived plants by specific PCR amplification (Fig. 6). Sequence analysis showed that this fragment had the same sequence with the exotic gene *TPS*1 inserted in the expression vector.

Possible mechanism of trehalose to improve drought tolerance: In the past, trehalose accumulation was not found in angiospermae, except a kind of extremely drought-tolerant myrothamnus plant (*Myrothamnus flabellifolius*) in south of Africa (Bianchi *et al*., 1993; Drennan *et al*., 1993; Gussin, 1972). In 1998, the genes encoding trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase were cloned from arabidopsis and their bioactivities were identified by yeast functional complementation (Blazquez *et al*., 1998; Vogel *et al*., 1998). Thevelein and Hohmann (1995) found that trehalose-6-phosphate inhibited hexokinase activity and prevented intermediate products of carbohydrate synthesis from entering glycolytic pathway in yeast. Goddijn and van Dun (1999) and Goddijn and Smeekens (1998) speculated that trehalose in angiospermae have the similar physiological functions. In their opinion, the improvement of the exotic gene *TPS*1 on drought tolerance of transgenic plant should be due to the change of trehalose metabolism pathway and its regulation to growth and development (Goddijn and Smeekens, 1998; Serrano *et al*., 1999). The increase of osmoregulatory ability was not the major reason. Goddijn *et al*. (1997) detected significant increase of trehalose accumulation in transgenic plant lines and non-transgenic control, after inhibiting trehalase activity with validamycin A. It was concluded that the activity of trehalase be higher than trehalose synthase encoded by exotic gene. The trehalose was hydrolyzed by trehalase before it could be accumulated in transgenic plant. Under ordinary conditions, clear physiological function has not been found in maize as well as other higher plants. Therefore, we used tress-inducible promoter *mwcs120* to promote the expression of gene *TPS*1 under drought stress.

CONCLUSIONS

In this study, a new sequence of trehalose synthase gene *TPS*1 we cloned from strain AS.1416 of *Saccharomyces cerevisiae*. Its similarity with formerly reported sequence of gene *TPS*1 (X68496) was as high as 99.3%. The putative protein of this sequence had the same conserved contigs with the protein sequences of trehalose synthases in many eukaryotic and prokaryotic organisms. This sequence was used as exotic gene to construct stress-inducible expression vector pCWTPS1300 and transform embryonic calli of maize mediated by agrobacterium. After screening and regeneration, one fertile plant was detected to be positive by specific PCR amplification and sequencing of the amplified product. More detail researches should be conducted to breed transgenic inbred line from this positive plant and investigated the effect of the expression of the exotic gene to improvement of drought tolerance.

ACKNOWLEDGMENTS

This study was supported by the National Natural Science Foundation of China (30571172 and 30671309), Rockefeller Foundation (2004 FS 047) and Program for Changjiang Scholar and Innovative Research Team in University (PCSIRT, IRT0453).

REFERENCES

Adams, A., D.E. Gottschling and C.A. Kaiser, 1998. Methods in Yeast Genetics. In: Cold Spring Harbor Laboratory Course Manual. Cold Spring Harbor Laboratory Press, New York.

Bartels, D. and D.E. Nelson, 1994. Approaches to improve stress tolerance using molecular genetics. Plant Cell Environ., 17 (5): 659-667.

Bell, W., W. Sun, S. Hohmann, S. Wera, A. Reinders, C. de Virgilio, A. Wiemken and J.M. Thevelein, 1998. Composition and functional analysis of the *Saccharomyces cerevisiae* trehalose synthase complex. J. Biol. Chem., 273 (50): 33311-33319.

Bianchi, G., A. Gamba, R. Limiroli, N. Pozzi, R. Elster, F. Salamini and D. Bartels, 1993. The unusual sugar composition in leaves of the resurrection plant *Myrothamnus flabellifolia*. Physiol. Plant, 87 (2): 223-226.

Blazquez, M.A., E. Santos, C.L. Flores, J.M. Martinez-Zapater, J. Salinas and C. Gancedo, 1998. Isolation and molecular characterization of the Arabidopsis TPS1 gene, encoding trehalose-phosphate synthase. Plant J., 13 (5): 685-689.

Bruce, W.B., G.O. Edmeades and T.C. Barker, 2002. Molecular and physiological approaches to maize improvement for drought tolerance. J. Exp. Bot., 53 (366): 13-25.

Ceccarelli, S. and S. Grando, 1996. Drought as a challenge for the plant breeder. Plant Grow. Regul., 20 (2): 149-155.

Chen, Q.F. and G.H. Gabriel, 2004. Role of trehalose phosphate synthase and trehalose during hypoxia: From flies to mammal. J. Exp. Biol., 207 (18): 3125-3129.

Colaco, C., J. Kampinga and B. Roser, 1995. Amorphous stability and trehalose. Sci., 268 (5212): 788.

Dai, X.Y., Y.Q. Wang, B. Yang and J. Zhou, 2001. Expression of *otsA* gene in tobacco and improvement stress tolerance. Acta Microbiol. Sin., 41 (4): 427-431.

de Virgilio, C., T. Hottiger, J. Dominguez, T. Boller and A. Wiemken, 1994. The role of trehalose synthesis for the acquisition of thermotolerance in yeast. I. Genetic evidence that trehalose is a thermoprotectant. Eur. J. Biochem., 219 (1-2): 179-186.

Drennan, P.M., M.T. Smith, D. Goldsworthy and J. van Staden, 1993. The occurrence of trehalose in the leaves of the desiccation-tolerant angiosperm *Myrothamnus flabellifolius* Welw. J. Plant Physiol., 142 (4): 493-496.

Du, J., Z. Zhu and W.C. Li, 2005. Cloning and expression properties of plant stress inducible promoter *mwcs120*. Acta Agron. Sin., 31 (10): 1328-1332.

Edmaedes, G.O., J. Bolanos and H.R. Lafitte, 1992. Progress of Breeding for Drought Tolerance in Maize. In: Proceedings of the 47th Annual Corn and Sorghum Industry and Research Conference. Wilkinson, D. (Ed.). ASTA, Washington, pp: 93-111.

Eleutherio, E.C., P.S. Araujo and A.D. Panek, 1993. Role of the trehalose carrier in dehydration resistance of *Saccharomyces cerevisiae*. Biochem. Biophysiol. Acta, 1156 (3): 263-266.

Fu, F.L., L.P. Zhang and Z. Zhu, 2000. The establishment, selection and regeneration of acceptors systems with excellent inbreds in maize. J. Sichuan Agric. Univ., 18 (2): 97-99.

Fu, F.L., W.C. Li and T.Z. Rong, 2005. Effect of Ca^{2+} and uniconazole appended in N6 medium on immature embryos culture in maize. Acta Agron. Sin., 31 (5): 634-639.

Goddijn, O.J.M., T.C. Verwoerd, E. Voogd, R.W. Krutwagen, P.T. de Graaf, K. van Dun, J. Poels, A.S. Ponstein, B. Damm and J. Pen, 1997. Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. Plant Physiol., 113 (1): 181-190.

Goddijn, O.J.M. and S. Smeekens, 1998. Sensing trehalose biosynthesis in plants. Plant J., 14 (2): 143-146.

Goddijn, O.J.M. and K. van Dun, 1999. Trehalose metabolism in plants. Trends Plant Sci., 4 (8): 315-319.

Gussin, A.E.S., 1972. Does trehalose occur in angiospermae? Phytochemistry, 11 (5): 1827-1828.

Holmstrom, K.O., E. Mantyla, B. Welin, A. Mandal, E.T. Palva, O. Tunnela and J. Londesborough, 1996. Drought tolerance in tobacco. Nature, 379 (4): 683-684.

Ingram, J. and D. Bartels, 1996. The molecular basis of dehydration tolerance in plant. Annu. Rev. Plant Physiol. Plant Mol. Biol., 47: 377-403.

McDougall, J., I. Kaasen and A.R. Strom, 1993. A yeast gene for trehalose-6-phosphate synthase and its complementation of an *Escherichia coli otsA* mutant. FEMS. Micobiol. Lett., 107 (1): 25-30.

Pilon-Smits, E.A.H., N. Terry, T. Sears, H. Kim, A. Zayed, S. Hwang, K. van Dun, E. Voogd, T.C. Verwoerd, R.W. Krutwagen and O.J.M. Goddijn, 1998. Trehalose-producing transgenic tobacco plants show improved growth performance under drought stress. J. Plant Physiol., 152 (4-5): 525-532.

Romero, C., J.M. Belles, J.L. Vaya, R. Serrano and F.A. CulianezMacia, 1997. Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: Pleiotropic phenotypes include drought tolerance. Planta, 201 (3): 293-297.

Rosen, S. and L. Scott, 1992. Famine grips sub-Saharan Africa. Agric. Outlook, 191 (1): 20-24.

Saghai-Maroof, M.A., K.M. Soliman, R.A. Jorgesen and R.W. Allard, 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA., 81 (24): 8014-8018.

Serrano, R., F.A. Culianz-Macia and V. Moreno, 1999. Genetic engineering of salt and drought tolerance with yeast regulatory genes. Sci. Hortic., 78 (1-4): 261-269.

Smith, J.S.C., D.N. Duvick, O.S. Smith, M. Cooper and L.Z. Feng, 2004. Changes in pedigree background of pioneer brand maize hybrids widely grown from 1930 to 1999. Crop Sci., 44 (6): 1935-1946.

Song, F.B. and X.B. Wang, 2005. Maize Physiology and Ecology Under Abiotic Stress. Science Press, Beijing, pp: 10-120.

Tarczynski, M.C., R.G. Jensen and H.J. Bohnert, 1992. Expression of a bacterial mtlD gene in transgenic tobacco leads to production and accumulation of mannitol. Proc. Natl. Acad. Sci. USA., 89 (7): 2600-2604.

Thevelein, J.M. and S. Hohmann, 1995. Trehalose synthase: Guard to the gate of glycolysis in yeast. Trends Biochem. Sci., 20 (1): 3-10.

Thevelein, J.M., 1996. Regulation of Trehalose Metabolism and its Relevance to Cell Growth and Function. In: The Mycota, Brambl, R. and G.A. Marzluf (Eds.). Springer-Verlag, Berlin, pp: 395-420.

van Larebeke, N., J.P. Hernalsteens, A. Depicker, I. Zaenen, E. Messens, M. van Montagu and J. Schell, 1977. Transfer of Ti-plasmids between Agrobacterium strains by mobilization with the conjugative plasmid RP4. Mol. General Genet., 152 (2): 119-124.

van Leeuwenhoek, A., 1990. Trehalose in yeast, stress protectant rather than reserve carbohydrate. J. Gen. Microbiol., 58 (3): 209-217.

Vogel, G., R.A. Aeschbacher, J. Muller, T. Boller and A. Wiemken, 1998. Trehalose-6-phosphate phosphatases from *Arabidopsis thaliana*: Identification by functional complementation of the yeast tps2 mutant. Plant J., 13 (5): 673-683.

Wang, G.L. and H.J. Fang, 2002. Plant Gene Engineering. 2nd Edn. Science Press, Beijing, pp: 61-73.

Wu, J.F., 1983. A review on the germplasm bases of the main corn hybrids in China. Sci. Agric. Sin., 16 (1): 1-8.

Yu, H.Q., B.Y. Qu, F.L. Fu and W.C. Li, 2005. Study on *CpTi* transgenic insect resistant maize with removable selective marker. J. Maize Sci., 13 (1): 44-46.

Zhang, S.H., Z.B. Peng and X.H. Li, 2000. Heterosis and germplasm enhancement, improvement and development of maize. Sci. Agric. Sin., 33 (sup.): 34-39.