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Optimizing a Screening Protocol for Trehalose Gene (*OsTPS1*) in Different Traditional and Improved Rice Varieties (*Oryza sativa* L. ssp indica) in Sri Lanka

K.G.T.T. PERERA[1], D.P.D.C. DE SILVA[2], N.R.M.K.N.D. RATHNAYAKE[2], S.N.T. DE SILVA[1] and S.G. SENARATNE[2]

[1]*Department of Biotechnology, Faculty of Agriculture and Plantation Management, Wayamba University of Sri Lanka, Makandura, Gonawila (NWP)*

[2]*Biotechnology Unit, Industrial Technology Institute, Colombo 07*

ABSTRACT

Trehalose is a glucose disaccharide which plays an important role in metabolism in variety of organisms including rice (*Oryza sativa*) during adverse abiotic conditions. To optimize a screening protocol for *OsTPS1* gene, three different local rice varieties were used to determine its expression levels. Total RNA was extracted from leaves of one improved and two traditional rice varieties *viz*: Bg 94-1, Dik wee and SuduHeenati respectively. Each variety was given salinity and drought conditions before the extraction, to induce Trehalose synthesis. Approximately equal amounts of RNA from each variety were reverse transcribed, amplified using *OsTPS1* gene specific primers and analyzed on Agarose gel electrophoresis. Results were normalized with a housekeeping gene *eEF-1α* (Eukaryotic elongation factor) semi-quantitatively. A trend of increment in *OsTPS1* gene expression was observed with the salinity and drought. Further experiments with more varieties, optimum sample size and quantitative PCR analysis are required to confirm the accurate expression levels of *OsTPS1* gene.

KEYWORDS: *Oryza sativa*, Trehalose, Trehalose-6-phosphote synthase., *OsTPS1*

INTRODUCTION

Majority of Sri Lankans consume Rice (*Oryza sativa*) as their staple food. Therefore, it is a very important and most widely grown food crop in Sri Lanka. Rice is cultivated approximately in 1218000 ha of the total land area and it shares 1.5% of the National GDP (Central Bank Annual Report, 2011).

The production yield of rice is affected by both abiotic and biotic stresses. Apart from the biotic stresses, heat, cold, drought, salinity are the major abiotic stresses that determine the present yield (Krasensky and Claudia, 2012). Therefore, breeding plants to withstand these stress conditions will lead to a better production. Within rice cultivars, several characteristics or mechanisms were observed, which can reduce the effect from abiotic stresses. Trehalose production is one of those mechanisms.

Trehalose is a non-reducing glucose disaccharide with unique abilities to protect bio molecules by accumulating upon abiotic stresses. Mainly, Trehalose can be seen in bacteria, fungi and some of the invertebrates (Elbein *et al.*, 2003). At sufficient levels, Trehalose can functions as an osmolyte and stabilize proteins and membranes (Paul *et al.*, 2008). When an adverse osmotic pressure builds, Trehalose preserves the cell membranes from drying out during drought periods (Crowe *et al.*, 1984). Trehalose is also tolerant to extreme heat and desiccation (Hounsa *et al.*, 1998).

Trehalose is produced in a two step path way. First, UDP-glucose is converted to Trehalose-6-phospate when Trehalose-6-phosphate synthase (TPS) is present. Then it is converted to Trehalose disaccharide in the presence of Trehalose-6-phosphate phosphatase (TPP) (Goddijn and van Dun, 1999). Synthetic pathway of Trehalose in plants is not very different from the pathway of bacteria or fungi (Iturriaga *et al.*, 2009)

Recently, 11 Trehalose-6-phosphate (OsTPS) genes have been identified in *O. sativa*. Therefore, it confirms that *O. sativa* genome also contains genes, which are responsible to produce Trehalose. However from the identified genes, only *OsTPS1* gene shows the TPS activity (Zang *et al.*, 2011).

The objective of this study was to screen *OsTPS1* gene in selected rice varieties. Moreover, this study focused on observing the expression level of *OsTPS1* gene with reference to *eEF-1α* (Eukaryotic elongation factor) housekeeping gene by a semi quantitative method.

MATERIALS AND METHODS

This research was carried out from January 2013 to April 2013, at Biotechnology Unit, Industrial Technology Institute, Colombo 07, Sri Lanka. Seeds of Bg 94-1, Dik wee and

Sudu Heenati were obtained from Rice Research and Development Institute, Bathalegoda, Ibbangamuwa, Sri Lanka.

Plant Materials

Equal amount of viable *O. sativa seeds* were exposed to running water for 30 min. Then they were treated with 50 mL of 50% Clorox for 30 min and 50 mL of 70% Ethanol for 5 min. Seeds were soaked in autoclaved distilled water overnight, wrapped around sterilized cloths, and kept under pressure for 6-7 days. Germinated, healthy seedlings were transferred to sterilize Petri dishes. Seeds were sprayed with 1% carbendazim fungicide for once in three days. Petri dishes were wetted twice a day with 3 mL of sterilized distilled water for one and a half weeks. Plastic pots were filled with about 2 kg of paddy field soil. Water was added about 5 cm above the soil, mixed well and kept for overnight. Healthy seedlings were transferred to pots. One variety was planted in four pots. Pots were equally maintained for 7 days. Plants were watered with 50 mL of water twice a day (9.00 AM and 4.00 PM).

Treatment for Salinity and Drought

From 8th day after planting, pots were divided into two sets. Each set consisted of two pots from each variety. One set was given salinity condition and the other set was given drought condition. Each set included one control pot and one treated pot per variety.

Initially, soil salinity level was measured. Then 6 dsm^{-1} saline condition was given on 8th day after potting. 12 dsm^{-1} salinity was given on 11th day. After that, until 28th day, only 50 mL of water was applied twice a day, per each pot.

For drought condition, on 8th day all pots were given 100 mL of water. Then, water was applied once in seven days. First drought cycle started from 9^{h} day to 14th day. Then water was applied on 14th day evening. Second drought cycle was started from 15th day to 20th day. Water was applied on 20th day evening. Third drought Cycle was started from 21st to 28th day. On 29th day plants were uprooted, washed thoroughly, wiped and stored at -20 ^{0}C.

Total RNA Extraction

RNA was extracted from *O. sativa* leaves using TRIcol extraction reagent (UC Biotech, University of Colombo, Sri Lanka)

100 mg of sample was crushed in liquid nitrogen (Ceylon Oxygen Limited) and homogenized in 10 volumes of TRIcol reagent. The homogenate was kept for 10 min at room temperature and vortexed for 10 min. Zero point two volumes of chloroform (MERCK) was added and vortexed for 10 min. The sample was centrifuged at 12000 rpm for 10 min at 4 ^{0}C using an HERMLE Labortechnik GnbH centrifuge (Z216MK). The supernatant was pipetted out to a fresh tube and added 10 volumes of chloroform and centrifuged at 12000 rpm for 10 min at 4 ^{0}C. The supernatant was transferred to a new tube and added 10 volumes of 100% ethanol (Fisher Scientific), mixed gently and incubated at -20 ^{0}C for One hr, vortexed for 2 min and centrifuged at 12000 rpm for 15 min at 4 ^{0}C. The supernatant was discarded, added 500 µL of 70% ethanol and centrifuged at 12000 rpm for 5 min at 4 ^{0}C. RNA pellet then dissolved in 30 µL of PCR water (Sigma).

One micro liter of RNA was spotted in a 0.8% Agarose gel to estimate the RNA quantity with reference to 113 ng/µL, 56.5 ng/µL and 28.25 ng/µL standard DNA solutions (Figure 1a). Quality was checked in 1% Agarose gel electrophoresis.

Analysis of OsTPS1Gene Expression with RT-PCR

The first strand cDNA was synthesized by using Random primers (IDT) and M-MLV reverse transcriptase (Promega) at 37 ^{0}C, in a reaction mix (25 µL) composed of 150 ng of total RNA, 5x reverse transcriptase buffer (Promega), 10 nM of dNTPs (Promega), 25 units of ribonuclease inhibitor (Promega), 200 units of reverse transcriptase and 5 µM Random primers (IDT).

cDNAs were used as templates for PCR amplification of *OsTPS1* gene using *OsTPS1* gene specific primers 5' TTGAAGTTCGGTCTGTCG 3' and 5' CTGCCTATCCAAGAACATG 3' (Zang *et al.*, 2011) and for amplification of *eEF-1α* housekeeping gene using gene specific primers 5' TTTCACTCTTGGTGTGAAGCAGAT 3' and 5' GACTTCCTTCACGATTTCATCGTAA 3' (Mukesh *et al.*, 2006). The expected amplicon product sizes were 689 bp band for *OsTPS1* gene and 103 bp band for eEF-1α housekeeping gene.

One micro liter of cDNA was spotted in a 0.8% Agarose (Vivantis) gel to estimate the cDNA quantity with reference to 113 ng/µL, 56.5 ng/µL and 28.25 ng/µL standard DNA solutions (Figure 1b).

Amplifications were performed in a 25 µL reaction volume, composed with 5 µL cDNA, 10 nM of dNTP, 1.5 mM $MgCl_2$ (UC Biotech), 1x PCR buffer (UC Biotech), 2 units of Taq polymerase (UC Biotech) and 5 µM each of forward and reverse primers (IDT).

The PCR amplification parameters for *OsTPS1* gene was 94 ^{0}C for 5 min followed by 35 cycles of 94 ^{0}C for 30 sec, 52 ^{0}C for 30 sec, 72 ^{0}C for 45 sec and final extension at 72 ^{0}C for 5 min. For eEF-1α gene 94 ^{0}C for 5 min followed by 35 cycles of 94 ^{0}C for 30 sec, 57 ^{0}C for 30 sec, 72 ^{0}C for 30 sec and final extension at 72 ^{0}C for 5 min (Bio-Rad Mycycler – 563BR1406).

PCR products were analyzed on 1% Agarose gel (Figure 2a and 2b) and obtained the relative expression values by ImageLab Gel Documentation software (Bio-rad). First, bands of eEF-1α gene and *OsTPS1* gene expressions were normalized with 500 bp band of 100 bp DNA marker (Promega) and relative values were taken. Subsequently, *OsTPS1* gene expression values were normalized with eEF-1α gene expression values. Results were plotted in a bar chart (Figure 3).

RESULTS AND DISCUSSION

Plant Materials

Life span of rice is generally from three months to four months (Anon, 2013). During the early period of life, plants tend to perform higher activity in growth and development. Susceptibility to stress conditions in young plants is comparatively high (Anon, 2002). Therefore, activity of protective mechanisms is eventually become higher. Young plants essentially need to have better protection against unfavorable abiotic conditions to gain a better plant growth and development before reproductive stage begins. Therefore, higher gene expression rate can be expected in young plants.

Therefore, more Trehalose synthesis can be expected, in early stages than latter stages of plant growth and development.

Analysis with RT-PCR

RNA quantity was estimated as 100 ng/μL in a spot and 150 ng from each sample was used to proceed cDNA synthesis (Figure 1a).

Figure 1b shows the amount of cDNA synthasized.

Garg *et al.*, (2008) confirms the actual Trehalose amounts in several *O. Sativa* indica, japonica and japonica tropical varieties using HPLC analysis. It is explained, unlike bacteria or fungi, higher plants like rice, contain less amount of Trehalose. The primary objective of the present study was to optimize a screening protocol and semi-quantitatively determine the *OsTPS1* gene expression. However, further studies are recommended to determine the actual Trehalose amount in local rice varieties prior to be used in breeding programmes.

Increased level of *OsTPS1* gene expression can be seen in both salinity and drought conditions compared to control for Bg 94-1 and Sudu Heenati. However, for Dik wee, an increment can be observed only under drought condition (Figure 3).

Pramanik and Imai (2005) suggested that Trehalose bio synthesis is differently regulated with multiple stress cues. This study was specifically focused only on the expression of *OsTPS1* gene, considering one stress factor per time. A trend can be seen to increase the level of expression of the gene. However, further studies are recommended to confirm the *OsTPS1* gene expression level with increased sample size.

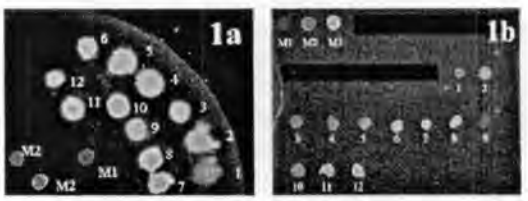

Figure 1(a) RNA spotting on 1% Agarose gel (b) cDNA spotting on 1% Agarose gel

Spot 1 – Bg 94-1 salinity control sample, Spot 2 – Bg 94-1 salinity treated sample, spot 3 – Dik wee salinity control sample, spot 4 – salinity treated sample, spot 5 – Sudu Heenati salinity control sample, spot 6 – Sudu Heenati salinity treated sample, Spot 7 – Bg 94-1 drought control sample, Spot 8 – Bg 94-1 drought treated sample, spot 9 – Dik wee drought control sample, spot 10 – drought treated sample, spot 11 – Sudu Heenati drought control sample, spot 12 – Sudu Heenati drought treated sample, spot M1 – Standard 28.125 ng/μL, spot M2 – standard 56.5 ng/μL, spot M3 -112 ng/μL

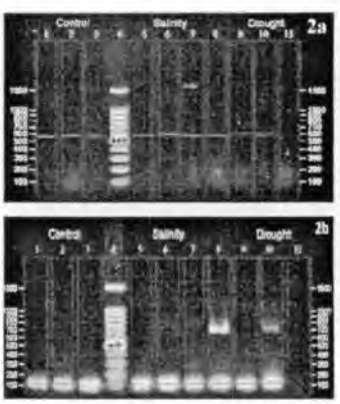

Figure 2. Agarose gel electrophoresis of PCR products; (a) Amplified with *OsTPS1* gene specific primers, (b) Amplified with eEF-1α gene specific primers

Lane 1, 5 and 8 – Bg 94-1, Lane 2,6 and 9 – Dik wee, Lane 3, 7 and 10 – Sudu Heenati, Lane 4 – 100 bp marker, Lane 11 – Negative

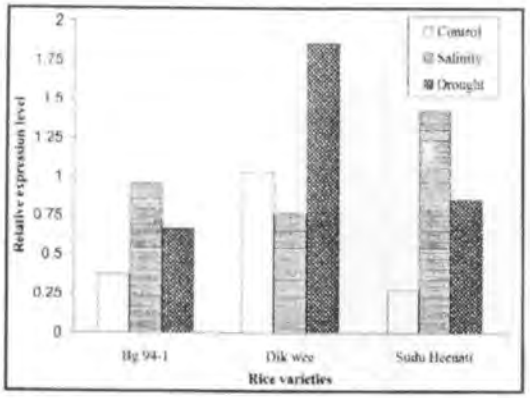

Figure 3. Relative expression levels of rice varieties

CONCLUSIONS

The expression of *OsTPS1* gene was high in salinity and drought conditions for two rice varieties Bg 94-1 and Sudu Heenati. However during the present analysis we could not observe increase level of *OsTPS1* gene expression in variety Dikwee under salinity condition.

This study successfully optimized the screening protocol for *OsTPS1*gene for local rice varieties. Due to limited time span this study was performed with only one sample of each variety hence the level of expression of *OsTPS1* gene could not be statistically analyzed or quantified. Therefore, further studies should be conducted with more varieties and more replications to confirm the results. The varieties with positive results can be used in crop improvement in relation to marker assisted breeding.

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