

The effect of drought and salinity on the expressional levels of sucrose transporters in rice (*Oryza sativa* Nipponbare) cultivar plants

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Abstract

Rice, like other plants, contains a number of sucrose transporter genes. However knowledge of their expression and regulation during environmental stress conditions is limited. Using expressional analysis, the role of sucrose transporter genes was examined during drought and salinity stress conditions in 3-week-old rice (*Oryza sativa* L. cv Nipponbare) plants, over a time-course of 1 to 10 days of stress treatment. Among the 5 rice sucrose transporter genes identified, only OsSUT2 was up-regulated during drought and salinity treatments. OsSUT1, OsSUT4 and OsSUT5 were only expressed at low levels, while OsSUT3 showed no significant expression. The transport of sucrose is essential for long distance sucrose transport necessary to meet the cellular energy demands and also for osmoprotectant activities during drought and salinity stresses. It therefore indicates that OsSUT2, which facilitates transport of sucrose from photosynthetic cells, is essential for rice plants to cope with drought and salinity stresses, and cultivars with higher expression should be able to tolerate these environmental stresses better than those with lower expression.

Keywords: Metabolism, Osmoprotectant, Plant Development, Quantitative Real-Time PCR, Source-Sink Relationship.

Introduction

The plant life cycle is composed of a variety of tissues and cells, whose autotrophy/heterotrophy for reduced carbon, nitrogen and sulphur vary spatially and temporarily. The autotrophic growth of plants depends on their capacity to manufacture carbohydrate photosynthetically. Glucose, sucrose and their derivatives are required by plants as a source of energy and as a carbon-skeleton for cellular and metabolic activities; hence access to sugars is considered as a central factor for plant growth. Consequently, plant physiology and productivity will be subject to the proper expression and regulation of transporters that facilitate direct exchanges between cells, tissues and organs. These transporters are encoded by multigenic families whose members differ slightly by their substrate specificity, cellular localization or their expressional regulation. Previous studies have reported the expression of sucrose transporter (SUT) gene transcripts during development. The SUT proteins are involved in the movement of sucrose into and out of the source and sink tissues, and through the phloem via apoplastic pathways (Hirose et al., 1997; Hirose et al., 1999; Aoki et al., 2003; Scofield et al., 2007a). Experimental physiological data have shown that long distance transport of sucrose is controlled by plant development and environmental factors such as light, temperature and osmotic potential (Delrot et al., 2000). The participation of sucrose in osmotic regulation of guard cells was examined in *Commelina* (Reddy and Das, 1986), *Vicia* (Talbot and Zeiger, 1996), *Pisum* (Ritte et al., 1999) and *Arabidopsis*

(Meyer et al., 2004), thus connecting a possible role for SUT's in these cells. In the current cropping system, irrigation is increasingly used exclusively for highly demanded food and economic crops, causing a number of other crops to be grown under rain fed or on dry land. This trend is increasing rapidly due to global development and climate change, thus crops which require little water supply are becoming more widely adopted and grown. This phenomenon may result in the use of recycled or brackish water, which leads to soil salinization and accumulation of toxic elements. The recent demand for cereals for biofuel production may further encourage such forms of irrigation. Sucrose has been identified as one of the major osmoprotectant molecules, which are collectively involved in regulating osmotic and ionic potential of plants, by helping plant cells to maintain their hydrated state, and consequently offering resistance against drought and high salinity stresses (Rontein et al., 2002). Thus the participatory role of sucrose transport mechanisms in plants is essential to general plant physiology, and most importantly during environmental stress conditions. The aim of this study is therefore to advance the understanding of sucrose assimilation and the role of sucrose transporters during drought and salinity stresses, with the further aim of more effectively exploiting this information to improve and stabilize crop yields under water-limited and/or salinity conditions. Previous experimental work has reported the participation of sucrose transporters in germination, the early phase of plant develop-

Table 1. Rice Sucrose Transporter Primer Pairs

Gene	Accession No	Primer	Start Position	End Position	Length	Sequence	T _m (GC+AT) value
OsSUT1	AF280050	SUT1-f	7323	7341	19	5'AGTTCGGTTCGGTCAGCAT3'	60
		SUT1-r	7565	7548	18	5'ACCGAGGTGGCAACAAAG3'	56
OsSUT2	AB091672	SUT2-f	1561	1581	21	5'AGGAGGAGAGGTCACCGATAA3'	64
		SUT2-r	1800	1778	23	5'CCAACATCCAATGTACAACAGCA3'	66
OsSUT3	AB071809	SUT3-f	1507	1524	18	5'GCCATGGCGTCCGTGTTC3'	60
		SUT3-r	1679	1699	21	5'GCCTGCTATAGTACCCGCTCT3'	66
OsSUT4	AB091673	SUT4-f	1819	1838	20	5'TTGGCTGAGCAGAACACCA3'	60
		SUT4-r	2067	2048	20	5'ATGCATTCCGGCAGAGCTT3'	60
OsSUT5	AB091674	SUT5-f	1616	1636	21	5'CTAGTGCGAACTCCATCAAA3'	60
		SUT5-r	1865	1843	23	5'AAAATATTGGGTTTCCTGAGAT3'	60

Where: f is the forward primer and r is the reverse primer.

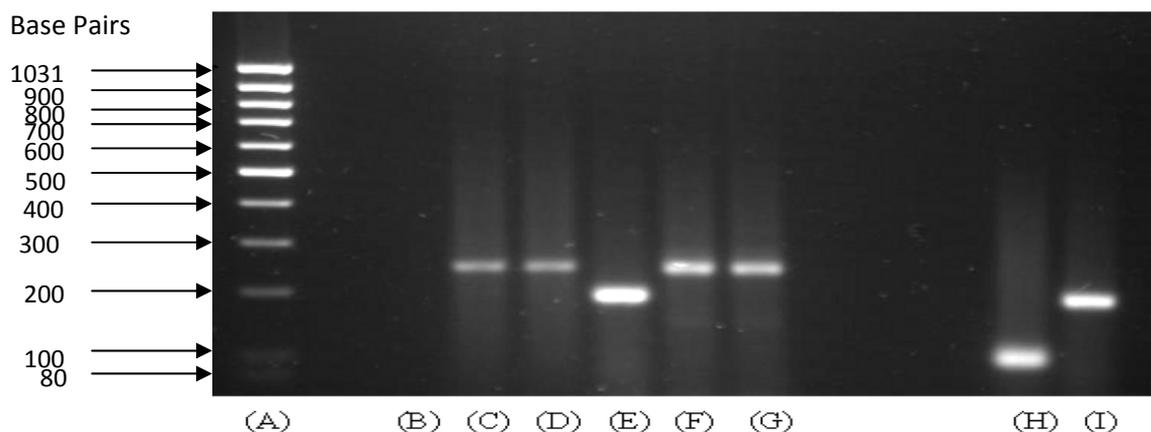


Fig 1. Confirmation of RT-PCR amplification by 1% agarose gel: (A) Fermentas Low Range Mass Ruler DNA Ladder (B) No template Control, (C) OsSUT 1, 245bp; (D) OsSUT 2, 243bp; (E) OsSUT 3, 194bp; (F) OsSUT 4, 249bp; (G) OsSUT5, 250bp; (H) RubQ1, 100bp; (I) Actin, 195bp.

ment and in grain filling (Hirose et al., 1997, Aoki et al., 2003, Scofield et al. 2007a, Scofield et al. 2007b). However data on their role during environmental stress conditions is limited.

Materials and methods

Cultivation of plant materials and treatment

Cultivation of rice (*Oryza sativa* L. cv *Nipponbare*) at optimum conditions

Seeds of rice (*Oryza sativa* L. cv *Nipponbare*) cultivar were obtained from CSIRO Plant Industry, Canberra, ACT2601, Australia. The rice seeds were germinated according to the method of Scofield et al. (2007a). Rice plants were grown to maturity (3 weeks old at the 4 leaf stage) in flooded paddy tanks in a greenhouse, in 75% compost and 25% perlite. Plant growth was under natural light with a day-time temperature of 28°C and a night-time temperature of 25°C.

Treatment of rice (*Oryza sativa* L. cv *Nipponbare*)

Rice plants cultivated as above were exposed to drought and salinity stress conditions as follows:

Drought Treatment

Twelve rice plants were grouped in triplicate to give four test experimental groups, with similar grouping for the control experiment. Drought was induced by withholding water and allowing the water in the soil to drain out. Treated seedlings' leaves were harvested as grouped at 24hrs (Day 1), 72hrs (Day 3), 120hrs (Day 5) and 240hrs (Day 10) post treatment. Leaves from control plants were harvested the same number of times at each time point, without subjecting the plants to drought treatment. Leaves harvested from the test and control experiments were quickly submerged in liquid nitrogen, stored at -80°C and used for total RNA extraction.

Salinity Treatment

Twelve rice plants were grouped in triplicate to give four test experimental groups, with similar grouping for the control experiment. The experimental rice plants were watered with 100mM NaCl, 200mM NaCl and 300mM respectively. Treated seedlings leaves were harvested as grouped at 24hrs (Day 1), 72hrs (Day 3), 120hrs (Day 5) and 240hrs (Day 10) post treatment. Leaves from control plants were harvested the same number of times at each time point without watering with NaCl. Leaves harvested from the test and control

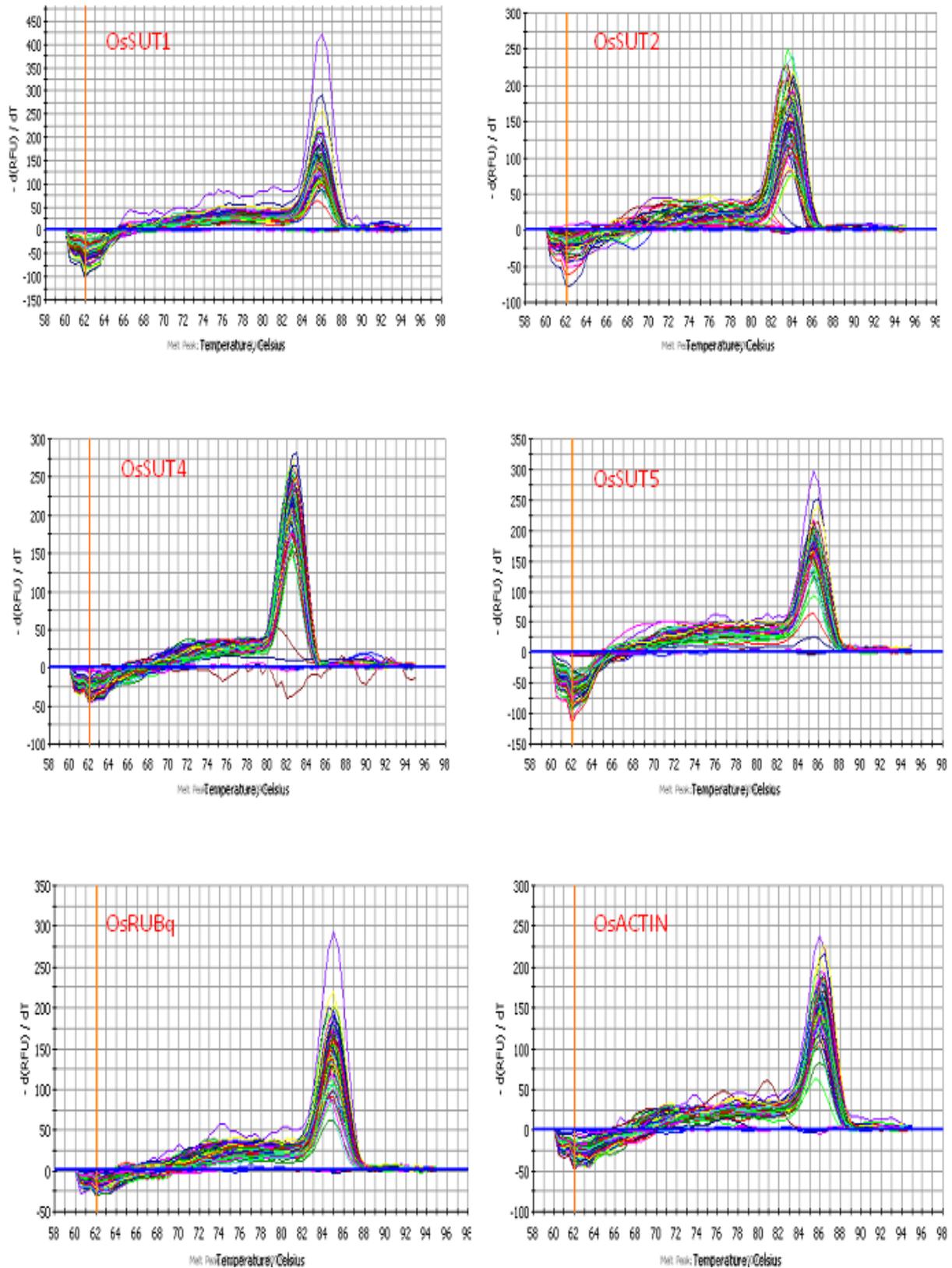


Fig 2. Melt curve analysis of the OsSUT genes, indicating single products for SUT1, 2, 4 and 5, and for the reference genes Polyubiquitin 1 and actin. SUT3 primers gave no clear peak indicating that no specific product was formed as a result of the overall poor expression of SUT3 during the drought and salinity treatments (data not shown).

experiments were quickly submerged in liquid nitrogen, stored at -80°C and used for total RNA extraction.

Analysis of differentially expressed sucrose transporters

Isolation of total RNA from controls and treated rice plants

All equipment used for the RNA isolation was made RNase free by autoclaving and rinsing thoroughly in RNase AWAY reagent solution (Invitrogen, USA). Leaves harvested from the groups of triplicate plants were pooled to minimize inter plant variability and were ground to powder in liquid nitrogen prior to RNA extraction. RNA from the ground tissues was extracted using the SV 96 Total RNA isolation system (Promega, USA), following the manufacturer's instructions. Extracted RNA was quantified using a PerkinElmer Lambda 35 UV/VIS Spectrophotometer at 260nm and electrophoresed on 1% agarose gel to verify its integrity.

Amplification of sucrose transporters (SUT) genes by reverse transcriptase polymerase chain reaction (RT-PCR) for primer pair confirmation:

The RT-PCR reactions were carried out using an Eppendorf AG 22331, Hamburg Master Cycler, and Qiagen® one step RT-PCR kit (Qiagen, USA), following the manufacturer's instructions. Total RNA template was used at concentrations varying from 50 – 1000ng, in a 50µl RT-PCR reaction volume. A specific region of each of the rice SUT genes was amplified using primers (Table 1) designed by Scofield et al. (2007a), who confirmed the specificity of the primer pairs by sequencing the amplified bands. The thermal cycling conditions used were as follows: reverse transcription: 50°C (30 min), initial PCR activation: 95°C (15 min), PCR cycling {denaturation: 95°C (30 sec), annealing: 55°C or 59°C (30 sec), extension: 72°C (1 min); 40 cycles}, final extension: 72°C (10 min). At the annealing step, 59°C was used for OsSUT3, while 55°C was used for SUT1, 2, 4 and 5.

Relative quantification of amplified SUT genes by Real-Time PCR:

qPCR was carried out using a two step method. Firstly, 1µg of total RNA from each sample was converted to cDNA using the iScript™ cDNA synthesis kit (BIO-RAD Laboratories, Inc. USA), following the manufacturer's instructions. This kit primes cDNA synthesis using a mixture of oligodT and random hexamers. Secondly, quantification of the amplified SUT genes was performed using the same gene-specific primers as used for RT-PCR (Table 1). Aliquots of the cDNA corresponding to 50ng of each sample input RNA were used as templates for the Real-Time PCR analysis. Reactions were carried out on a Bio-Rad iCycler Real-Time PCR Detection System with iQ™ SYBR® Green Super mix (BIO-RAD Laboratories, Inc. USA), according to the manufacturer's instructions. The thermal cycling conditions used were as follows: initial PCR activation: 95°C (15 min), PCR cycling {denaturation: 95°C (30 sec), annealing: 60°C (30 sec), extension: 72°C (30 sec); 40 cycles}, final extension: 72°C (10 min). PCR products were detected by monitoring the increase in fluorescence of the SYBR Green during the extension phase of each cycle when the SYBR Green bound to double stranded DNA. The results obtained for the different cDNAs were normalized against the expression levels of two stable reference genes: rice

polyubiquitin 1 (RubQ1; Accession number OsU37687) and rice actin (Accession number: NM_001072362.1) using the qBasePlus programme produced by BioGazelle (version 1.3; <http://www.biogazelle.com>). The Polyubiquitin 1 gene is reported to be a highly constitutively expressed gene in rice tissues (Wang et al., 2000), while actin is a generally stable gene in plants. The primer pairs used were: *RubQ1*-forward ^{5'}GGAGCTGCTGCTGTTCTTGG^{3'}, *RubQ1*-reverse ^{5'}CA - CAATGAAACGGGACACGA^{3'}, *Actin*-forward ^{5'}TGTTGGACTCTCTGGTGATGGTGT^{3'}, *Actin*-reverse ^{5'}CAAGCTTCTCCTTGATGTCCCT^{3'}. The relative mean fold expression level and standard error of each of the SUT genes was determined for the treatment and control groups at each time point by reference to the sample with lowest expression for each gene, using qBasePlus (Version 1.3).

Results

Effect of stress treatment on rice plants

A gradual and marked physical symptom of growth retardation was observed in rice plants subjected to drought and salinity stresses, as compared to the control. At Day 10 the plants' leaves all appeared withered in both treatments; a phenomenon also observed in the field (data not shown).

Total RNA extraction and confirmation of OsSUTs' primer pairs

All the total RNA extracts yielded two major bands on 1% agarose gel representing 28S and 18S rRNA. The A_{260nm}/A_{280nm} ratio for each extract was above 2, indicating the high purity of the extracts. All primer pairs amplified a single product of the expected size, which was confirmed on 1% agarose gel (Fig. 1) and analysis of the melt-curves after qPCR (Fig. 2), which gave a single band or single peak respectively; with the exception of OsSUT3 which showed no clear peak in the melt-curve analysis as a result of its overall poor expression during the drought and salinity treatments (data not shown). Despite this, the one-step RT-PCR reaction was able to amplify the portion of the OsSUT3 mRNA leading to the observed clear band of the expected size.

Quantitative real-time PCR

qPCR analysis of rice sucrose transporter genes revealed differential expression of the genes (Fig. 3 and Fig. 4). In all genes, changes of transcripts were observed in the drought and salinity treated plants when compared with the controls.

Drought Treatment:

A gradual increase in SUT2 expression level relative to control was observed in the drought treated plants, with a final relative expression of more than 4 fold increase observed at day 10, compared to control (Fig. 3). A relative increase in expression levels of SUT1 was observed at days 5 and 10 when compared to control, however only a 2 fold increase was observed at day 10 (Fig. 3a). A decrease in SUT5 expression levels relative to control was observed for days 1, 3, 5 and 10, with a final decrease of approximately 3 fold observed at day 10 (Fig. 3d). Interestingly, a 6 fold decrease in expression level of SUT5 was observed between day 1 and 5. No observable difference in expression levels relative to the control was observed for SUT4 at day 1, 3 and

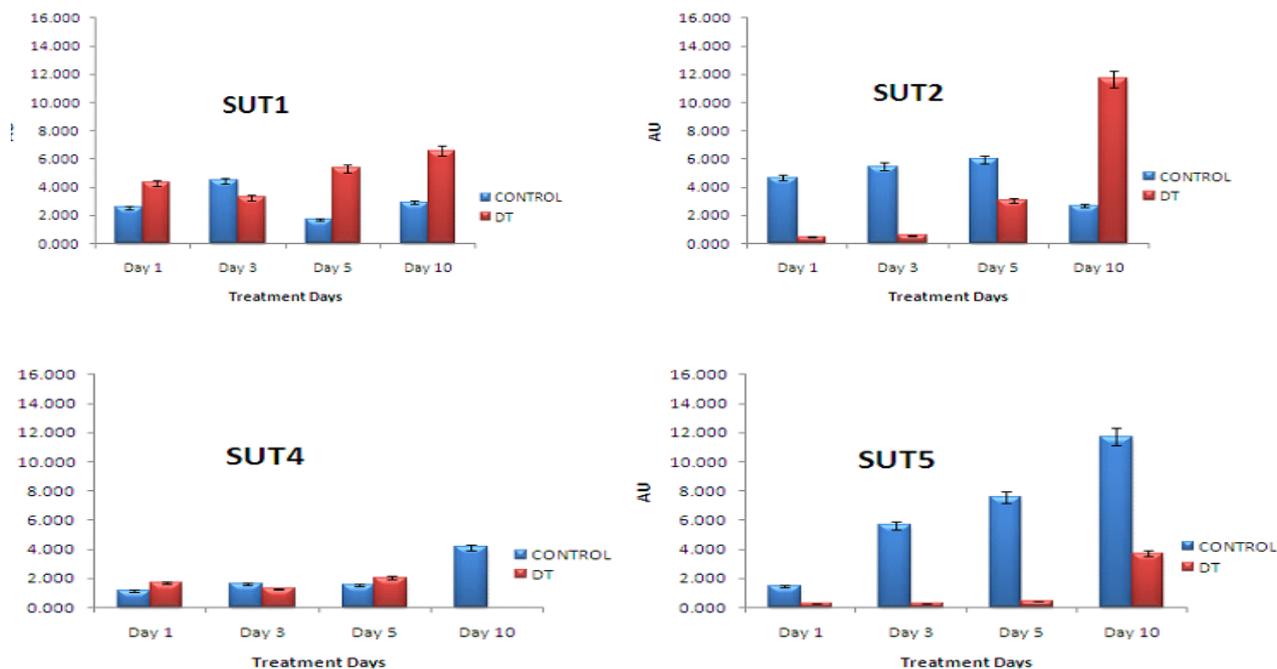


Fig 3. Quantitative PCR analysis of OsSUT1, OsSUT2, OsSUT4 and OsSUT5 genes during drought treatment of rice (*Orzya sativa* L. cv Nipponbare) cultivar plants. All values were normalized to the relative expressional levels of rice Polyubiquitin 1 and rice actin genes. The average of pooled tissue from triplicate sets of plant material tested in duplicate is shown. Error bars representing the standard error were obtained from the qBase Plus (Version 1.3) analysis. DT represents drought treatment. AU represents the fold change in expression relative to the minimum value obtained for each gene. OsSUT3 gave no significant expression during this treatment (data not shown).

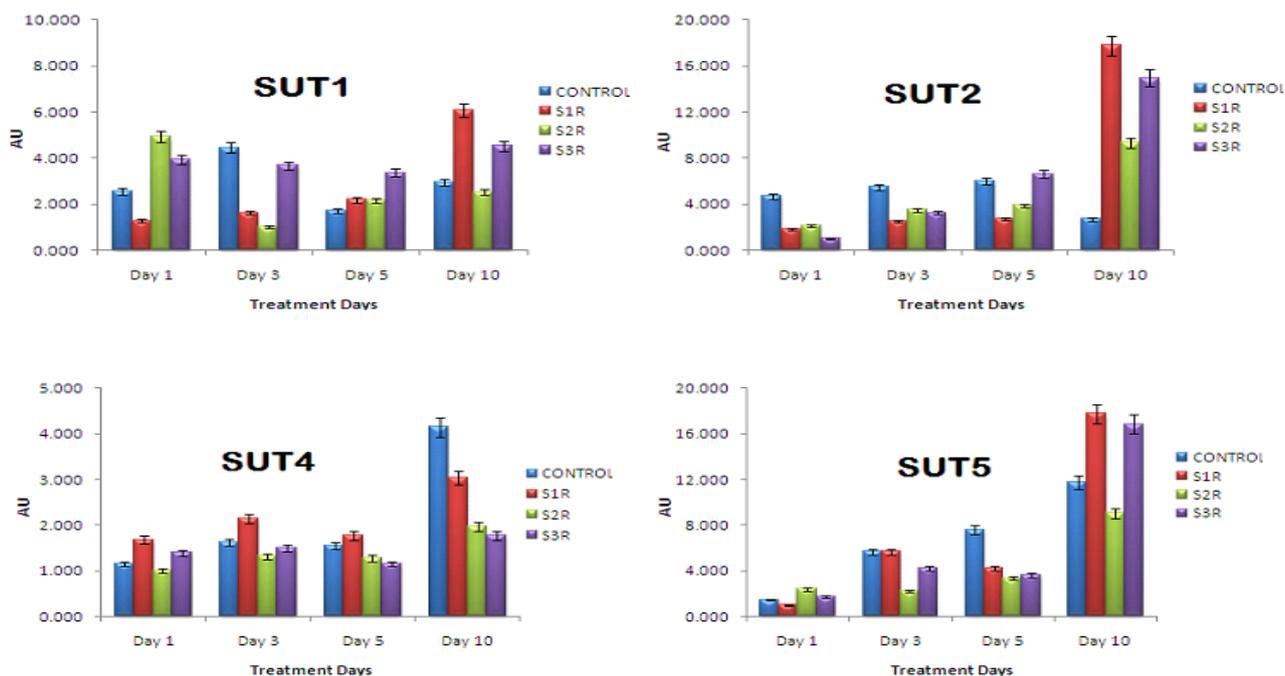


Fig 4. Quantitative PCR analysis of OsSUT1, OsSUT2, OsSUT4 and OsSUT5 genes during salinity treatment of rice (*Orzya sativa* L. cv Nipponbare) cultivar plants. All values were normalized to the relative expressional levels of rice Polyubiquitin 1 and rice actin genes. The average of pooled tissue from triplicate sets of plant material tested in duplicate is shown. Error bars representing the standard error were obtained from the qBase Plus (Version 1.3) analysis. S1R, S2R and S3R represent salinity treatment with respect to 100mM, 200mM and 300mM; NaCl respectively. AU represents the fold change in expression relative to the minimum value obtained for each gene. OsSUT3 gave no significant expression during the treatment (data not shown).

5. At day 10 no expression of SUT4 in the drought stressed plants was observed. No clear SUT3 expression was detected in either the control or drought treated plants (data not shown).

Salinity Treatment

A 2-8 fold increase in SUT2 expression level relative to control was observed in response to 100mM, 200mM and 300mM NaCl treatment of the rice plants. With all three treatments, there was a progressive increase in gene expression over time, with a more prominent increase with 200mM and 300mM NaCl treatment at day 10 (See Fig. 4). SUT5 showed a considerable difference in expression in treated rice plants as compared to the control in response to 100mM and 300mM NaCl treatment at Day10; evidence of probable up-regulation. SUT1 and SUT4 were expressed at low levels in control and salinity treated rice plants. SUT4 showed no change in expression in the control compared to the treated plants during the first five days of treatment. However after 10 days, the control plants showed up regulation of SUT4, whilst SUT4 expression in the plants treated with higher concentrations of NaCl was unchanged. There was no clear pattern of change in SUT1 transcript levels in response to salinity treatment. There was no detectable transcript expression of SUT3 in both the control and salinity treated rice plants (data not shown).

Discussion

Drought and salinity stress are environmental conditions in which plants have to cope with both declining water availability and ion toxicity as a result of a decrease in cellular osmotic potential. An excessive amount of salt entering the plant will result in an increase in toxic levels in older transpiring leaves, causing early senescence. This will further reduce the quantity of assimilates produced by the plant, and assimilates transported to the growing tissue, consequently limiting plant growth. This may well explain the poor growth of the plants observed in this study after 10 days of the drought and salinity stress treatments. Yeo et al. (1991) reported that the growth reduction in rice (*Orzya sativa* L.) after both long term and short term periods of salinity was as a result of a decrease in water supply to the plants, caused by increased salinization. Salt accumulation in soil due to excessive evapotranspiration induced by drought is now a generalized phenomenon as a result of global climate change. This greatly affects plant growth and development. Increased salt concentration has been shown to reduce seed germination in many plant species; it antagonistically affects shoots and roots and also affects the development of plant reproductive organs (Van Hoorn, 1991). Among the 5 sucrose transporter genes analyzed in the present study, OsSUT2 was found to be progressively up-regulated during the 10 day course of drought and salinity treatment of 3 week old rice (*Orzya sativa* L. cv Nipponbare) plants. Additional work needs to be done in order to identify the changes in sucrose metabolism and more precisely to describe the transporters involved at protein level, as well as confirm which respective promoters and transcription factors are involved in their expression. The putative *cis-acting* regulatory elements in the promoter regions of these transporters have been identified *in silico* by Ibraheem et al. (2010). Out of the 5 OsSUTs identified in rice, OsSUT2 had the highest drought and salinity related *cis-acting* elements (Ibraheem et al. 2010). This suggests the up-regulation of transcription of the SUT2 during drought and salinity stresses

reported in this study. Alternatively, the changes observed may be due to down regulation of sucrose for grain filling, since the major task of the plant will be channeled to overcome dehydration induced during drought and salinity stress. As a result, OsSUTs involved in transport of sucrose to sink organs like flower, anther, and grain may be down regulated. Rice sucrose transporter genes have been reported to be expressed in sink and source organs, with lower expression in the source organs than sink (Hirose et al. 1997; Aoki et al. 2003; Scofield et al. 2007a). Noiraud et al. (2000) found that AgSUT1 expression was decreased in all organs and distinctly in roots of celery plant when subjected to salt stress (30 days watering with 300mM NaCl). Although AgSUT1 was found to be expressed in matured leaves and in petioles of celery, it is also expressed in sink organs such as roots. Thus the low expression and regulation of OsSUT1, OsSUT3, OsSUT4 and OsSUT5 observed in the present study show that they may not be fully involved in sucrose transport during plant dehydration induced by drought or increase in salinity. However, more comprehensive reporter gene expression studies need to be carried out to fully understand the control of gene expression in different tissues during drought and salinity stresses. Although increases in mannitol and other polyols were considered to be a response to salt stress (Pardossi et al., 1998; Stoop and Pharr, 1994), our results indicate that sucrose transport is certainly not repressed under these conditions. Mannitol synthesis has been reported to increase at the expense of sucrose (Everard, et al., 1994) during salinity stress in celery plants; however the flux of sugar during these stress conditions still needs to be further studied in a less salt tolerant and economic crop such as rice. Celery plants were reported to grow new leaves when cultivated on a 300mM NaCl solution; a concentration equivalent to half that of sea water (Everard, et al., 1994). Furthermore, Everard et al. (1994) reported a 4-fold increase in mannitol/sucrose ratio in salt stressed celery plants as compared to controls. Mannitol dehydrogenase was found to be repressed in order to maintain a high level of mannitol, which acts as an osmoprotectant. Sucrose metabolism on the other hand is channeled to meet the energy need of the stressed tissues (Everard et al., 1994). This could therefore indicate that sucrose transport has to be maintained, which conforms to the observed up-regulation of OsSUT2 in source-exporting leaves in order to supply more sucrose for the increased energy for cellular activities and for osmoprotection. Using clustalW multiple sequence alignment, OsSUT2 (Protein Id: BAC67163) protein was found to have 61% and 83% similarity with AtSUT4 (Protein Id: AAG09191) and HvSUT2 (Protein Id: CAB75881) proteins respectively (data not shown). AtSUT4 is a low-affinity/high capacity saturable sucrose transporter (Weise et al., 2000), while HvSUT2 is a vacuolar sucrose transporter (Endler et al., 2006). AtSUT4 and HvSUT2 were reported to be expressed mainly in mesophyll cells and minor veins of source leaves, where high-capacity sucrose transport is needed for long distance sucrose phloem loading and for vacuolar storage of photosynthetically derived sucrose respectively. Therefore the transport of sucrose facilitated by OsSUT2 may be essential for long distance sucrose transport to meet the osmoprotectant activity of sucrose for the plant and for the cellular energy demands during drought and salinity stresses. Transport activities of osmolytes may be of great significance in plants, because they are only manufactured in the leaves. Hence, studies should be focused more on the elucidation of the molecular mechanism that links the effect of diverse stress stimuli to source-sink regulation. Coordination of development with the availability

of nutrients such as sucrose may aid in ensuring a sufficient supply of nutrient and energy essential in carrying out specific developmental activities and also to withstand environmental stresses. Attempts to develop environmental stress tolerant plants through genetic transformation have lead to several significant successes; but the complex stress response mechanism of plants to enable abiotic stress tolerance hampers progress. Thus, a comprehensive understanding of physiological and molecular processes underlying drought and salt tolerance in the major economic crop plants is needed and more research should be directed towards osmoprotectant metabolism and transport in crop plants and in transgenic plants engineered for salt tolerance. The differential regulation of sucrose transporter genes during drought and salinity stresses reported here may provide a link to the previous reports of Flowers and Yeo (1981) and Lutts et al. (1995). Sensitivity of rice plants to salinity stress was shown to vary at different growth stages. Plants were more sensitive to salinity stress at the young seedling stage than at the reproductive stage. Therefore improvement of drought and salt tolerance in rice should be targeted at the specific growth stage that is most sensitive to drought and salinity stresses, and which affects plant productivity and yield. This study therefore reveals the need for and the involvement of OsSUT2 in the adaptation of rice plants to cope with drought and salinity stresses, thus cultivars with a higher expression of this gene should show increased capacity to survive these environmental stresses.

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