Trehalose producing transgenic rice plants with improved salinity and cold tolerance: Progress and future prospects

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Rice is the most important food crop in the world and a model system for plant biology. Transgenic approaches offer new opportunities to improve salinity and cold tolerance in rice plants by incorporating genes that are involved in stress tolerance. Trehalose is a dimer of glucose that functions as both a compatible solute and in the stabilization of biological structures under abiotic stress in bacteria, fungi and invertebrates. With the notable exception of the desiccation-tolerant "resurrection plants", trehalose does not accumulate to significant levels in the vast majority of plants under normal conditions, in spite of the proliferation of plant trehalose pathway genes. However, recent studies show that trehalose metabolism is of immense importance in plant biotechnology and its manipulation has great potential in crop improvement. Here, we report our results on stress-inducible overproduction of trehalose in rice and other monocot plants for the purposes of improving abiotic stress tolerance and other agronomic traits.

Rice (*Oryza sativa* L.) feeds more than three billion people worldwide and is the number one staple food in Asia, where it provides 40-70% of the total food calories consumed. Of the 155 million hectares of land where rice is grown, about 20 percent contain levels of salt too high to allow optimal rice yield. Another 10 percent of locations where rice is grown occasionally experience temperatures that are too low for healthy plant development. Despite focused efforts to improve tolerance of rice against salinity and cold stresses by traditional breeding, success has been limited. This lack of desirable progress is attributable to the fact that abiotic stress tolerance is a complex trait that is influenced by coordinated and differential expression of a network of genes. Fortunately, recent developments in transgenic research offer new opportunities for elucidating the function of many useful candidate genes from numerous organisms that will lead to the improvement of stress tolerance of rice plants. Moreover, developing salt-tolerant transgenic rice plants will allow for the reclamation of millions of hectares of land with

salt content that is currently unsuitable for cultivating rice. Thus, improved transgenic rice varieties could ultimately help to combat world hunger and poverty.

In general, plants respond to environmental stresses (such as excessive salinity and low temperature) through a wide variety of biochemical and physiological adaptive changes, such as the accumulation of compatible solutes (glycine betaine, proline and polyamines) and the synthesis of proteins for overproducing regulatory compounds. One such compound is trehalose (α-D-glucopyranosyl-1, 1-α-D-glucopyranoside), a non-reducing disaccharide of glucose, which plays an important role in stress protection in a large variety of organisms, including bacteria, fungi and invertebrate animals (Crowe et al. 1992). The mechanisms by which trehalose protects biological molecules include water replacement, chemical stability of macromolecules and the regulation of carbohydrate metabolism. Multiple trehalose biosynthetic pathways have been identified in bacteria, but only one has been found in eukaryotes, including plants (Figure 1). The prevalent pathway for trehalose synthesis includes two enzymatic reactions. Trehalose-6-phosphate (Tre6P) is generated from UDP-glucose and glucose-6-phosphate (Glc6P) in a reaction catalyzed by trehalose-6-phosphate synthase (TPS). Tre6P is then dephosphorylated to form trehalose via trehalose-6-phosphate phosphatase (TPP).

Figure 1. The trehalose biosynthetic pathway in bacteria and plants.

Despite the wide distribution of trehalose in microorganisms and invertebrates, trehalose had until recently been found only in a few plant species, such as highly desiccation-tolerant, resurrection plants (*Selaginella lepidophylla* and *Myrothamnus flabellifolius*) (Wingler 2002). The question then arises as to whether the trehalose pathway is omnipresent and omnipotent in plants. The answer is that although the presence of trehalose biosynthesis genes in higher plants has been demonstrated, details of both the physiological functions and the regulation of this pathway remain largely unknown. In general, only one or two copies of TPS and TPP genes, with highly conserved substrate binding and catalytically relevant amino acid residues, exist in most bacteria, fungi and insects. On the other hand, similar genes in higher plants constitute a large gene family,

but lack several of the catalytically relevant residues. For example, genome sequencing of *Arabidopsis* and rice has revealed complex genomic organization of plant trehalose biosynthesis genes (Leyman et al. 2001, Paul et al. 2001). Eleven putative TPS genes were identified within the *Arabidopsis* and rice genome, whereas ten and eleven putative TPP genes were found within the *Arabidopsis* and rice genome, respectively. However, only one putative trehalase (TRE) gene is known to exist in both model species (Table 1). Interestingly, trehalose does not accumulate to any appreciable level in either species. Notably, there are more putative genes for the synthesis of trehalose than for sucrose. It now appears that even though the chemistries of trehalose and sucrose are similar, the biological functions performed by these sugars are quite different in crop plants.

Table 1. The genes encoding putative trehalose metabolism enzymes in rice and *Arabidopsis* plants.

Class*	Gene name	Locus name	Chr. #	Exons	Gene name	Locus name	Chr. #	Exons
TPS	*OsTPS1*	OsJ_009600	3	5	*AtTPS1*	At1g78580	1	17
	OsTPS2	OsJ_028127	9	8	*AtTPS2*	At1g16980	1	17
	OsTPS3	OsJ_026217	8	2	*AtTPS3*	At1g17000	1	16
	OsTPS4	OsJ_028025	9	3	*AtTPS4*	At4g27550	4	17
TPS/ TPP	*OsTPS5*	OsJ_003252	1	3	*AtTPS5*	At4g17770	4	3
	OsTPS6	OsJ_003366	1	3	*AtTPS6*	At1g68020	1	3
	OsTPS7	OsJ_018429	5	3	*AtTPS7*	At1g06410	1	4
	OsTPS8	OsJ_008378	2	3	*AtTPS8*	At1g70290	1	4
	OsTPS9	OsJ_026390	8	3	*AtTPS9*	At1g23870	1	4
	OsTPS10	OsJ_028286	9	3	*AtTPS10*	At1g60140	1	4
	OsTPS11	OsJ_018440	5	17	*AtTPS11*	At2g18700	2	3
TPP	*OsTPPA*	OsJ_008128	2	11	*AtTPPA*	At5g51460	5	11
	OsTPPB	OsJ_019796	6	10	*AtTPPB*	At1g78090	1	11
	OsTPPC	OsJ_026183	8	10	*AtTPPC*	At1g22210	1	11
	OsTPPD	OsJ_027978	9	8	*AtTPPD*	At1g35910	1	8
	OsTPPE	OsJ_010639	3	5	*AtTPPE*	At2g22190	2	12
	OsTPPF	OsJ_024197	7	11	*AtTPPF*	At4g12430	4	12
	OsTPPG	OsJ_007567	2	9	*AtTPPG*	At4g22590	4	9
	OsTPPH	OsJ_015060	4	11	*AtTPPH*	At4g39770	4	12
	OsTPPI	OsJ_031133	10	11	*AtTPPI*	At5g10100	5	11
	OsTPPJ	OsJ_023309	7	9	*AtTPPJ*	At5g65140	5	11
	OsTPPK	OsJ_034109	12	8				
TRE	*OsTRE1*	OsJ_030919	10	11	*AtTRE1*	At4g24040	4	11

* TPS = Trehalose-6-phosphate synthase, TPP = Trehalose-6-phosphate phosphatase domains, Phosphatase boxes and TRE = trehalase signature sequences were identified using ClustalW sequence analysis from EMBL-EBI.

Recently, several research groups have been trying to genetically modify trehalose biosynthetic pathways in plants to enable the study of its effect on plant growth, development and abiotic stress tolerance (Table 2). However, in most cases, constitutive overexpression of TPS and/or TPP encoding genes from yeast or *Escherichia coli* in

model plants resulted in enhanced trehalose levels, but also caused stunted plant growth, lancelet leaves, altered roots and changes in carbohydrate metabolism under normal growth conditions (Goddijn et al. 1997, Romero et al. 1997, Pilon-Smits et al. 1998, Yeo et al. 2000, Dai et al. 2001).

As an alternative strategy for engineering enhanced trehalose accumulation in rice, we used an ABA stress-inducible ABRC promoter (Su et al. 1998) to drive the overexpression of *Escherichia coli* trehalose biosynthetic genes (*otsA* and *otsB).* The resulting fusion gene (TPSP) has the dual advantages of requiring only a single transformation event to introduce both genes simultaneously into the rice genome, while at the same time imitating naturally occurring putative bipartite TPS/TPP-like genes in plants (Table 1). We introduced the TPSP gene into an economically important rice variety (Pusa Basmati 1) via *Agrobacterium*-mediated gene transfer and created a large number of transgenic rice plants that grow well under normal growth conditions and are completely fertile (Garg et al. 2002).

To assess whether trehalose accumulation in transgenic rice affected mineral nutrition during salt stress, we performed elemental analysis on the shoots and roots of ten independent transgenic and two non-transgenic plants via simultaneous inductively coupled argon-plasma emission spectrometry (ICP trace analyzer, US Plant, Soil, and Nutrition Laboratory, USDA-ARS, Cornell University, Ithaca, NY 14853, USA). In non-transgenic salt stressed plants (NTS) as well as transgenic plants a large increase in Na^+ content in both shoots and roots was noted after exposure to 100 mM NaCl: the NTS plants showed a 60-fold higher shoot Na^+ content compared to the same plants without salt stress. In contrast, transgenic plant shoots showed only 24-fold higher levels of Na^+ during stress compared to the same transgenic plants without salt stress. The observed differences in shoot Na^+ content between transgenic and non-transgenic plants could be due to a faster growth rate accompanied by a pronounced exclusion of Na^+ via better cellular compartmentation. Comparison between non-transgenic plants with or without stress showed an order of magnitude less in root K^+ content under salt stress. Furthermore, extreme ratios for Na^+/K^+, Ca^{2+}/Mg^{2+}, Zn^{2+}/Mn^{2+} and Fe^{3+}/Cu^{3+} were found in shoots, which is reflective of disruptions in the regulation of ion uptake and transport.

On the other hand, maintenance of the K^+/ Na^+ ratio in both shoots and roots of transgenic plants appears to be critical for normal plant growth, and may be the result of a protective effect of trehalose on plasma membrane integrity and its associated transporter proteins, as well as free radical scavenging. The role of ionic balance during salinity stress, especially the K^+/ Na^+ ratio, in several crop plants is well documented (Rus et al. 2001, Munns et al. 2006). In general, the relationship between salt stress and plant mineral content is complex, and the links between elevated trehalose content and improved mineral status during salt stress are as yet unknown. Nevertheless, we found a balanced mineral status of essential nutrients such as ions of K, Ca, Mg, P, S, Zn, Mn, Cu, Fe and B, in transgenic rice plants after salt treatment. The evidence supporting the role of superoxide dismutases (SOD), such as Zn-Cu, Fe and Mn-SOD, in scavenging free hydroxyl radicals during abiotic stress is emerging from many transgenic studies (Alscher et al. 2002).

Furthermore, the homozygous fifth generation transgenic rice plants exhibited sustained plant growth, less photo-oxidative damage and more favorable mineral balance under both salt and low-temperature stress conditions, as compared to non-transgenic plants. Depending on growth conditions, transgenic rice lines accumulate trehalose (50–200 μg g^{-1} FW) in shoots under non-stress conditions, and after salt stress the range of trehalose accumulation was significantly higher (150–550 μg g^{-1} FW). It is clear that many factors, in addition to endogenous trehalose levels, function to regulate plant stress responses. In non-transgenic salt-stressed plants, a considerable decline in soluble carbohydrates was observed in shoots vis-à-vis non-transgenic, non-stressed plants. In contrast, transgenic plants showed a significant increase in the accumulation of trehalose, glucose, sucrose and soluble starch in shoot tissue in response to abiotic stresses. The level of stress-induced trehalose accumulation in transgenic rice plants was far below that observed in resurrection plants. These data indicate that the enhanced stress tolerance we observed in transgenic plants was not a direct effect of trehalose acting as an osmoprotectant. Rather, the correlation between enhanced stress tolerance and total soluble carbohydrate levels suggests that trehalose may be acting as a general regulator of carbon metabolism, as previously reported for some microorganisms (Thevelein and Hohmann, 1995).

Table 2. Genetic manipulation of trehalose biosynthetic pathway in plants.

Plant species	Promoter/ gene	Impact of genetic modification on traits	Reference
Tobacco	RBCS/ *TPS1*	Increased trehalose levels; transgenic plants showed less water loss upon leaf detachment	Holmström et al. (1996)
Tobacco Potato	35S/ *OtsA* 35S/ *OtsB*	Low levels of trehalose in leaves of tobacco; no detectable level of trehalose in potato; inhibition of trehalase activity improves trehalose accumulation	Goddijn et al. (1997)
Tobacco	35S/ *TPS1*	Higher levels of trehalose; phenotypic alterations (stunted growth; lancet-shaped leaves); improved drought tolerance	Romero et al. (1997)
Tobacco	35S/ *OtsA*	Phenotypic alterations (larger leaves and shorter stems); higher growth under drought stress	Pilon-Smits et al. (1998)
Potato	35S/ *TPS1*	Phenotypic alterations (dwarfism); drought tolerance	Yeo et al. (2000)
Tobacco	35S/ *OtsA*	Altered phenotype (stunted growth); transgenic plants showed less water loss upon leaf detaching	Dai et al. (2001)
Rice	ABRC/ *TPSP* RBCS/ *TPSP*	Higher trehalose levels; sustained plant growth under drought, salt and cold stress; less photo-oxidative damage; favorable mineral balance under abiotic stress; increased photosynthetic capacity under both stress and non-stress conditions	Garg et al. (2002)
Rice	Ubi/ *TPSP*	Increased trehalose levels; absence of phenotypic alterations; salinity and drought stress tolerance	Jang et al. (2003)
Tobacco	35S/ *OtsA- OtsB*	Increased photosynthetic capacity per leaf unit area; increased growth rate and whole-plant biomass	Pellny et al. (2004)
Arabidopsis	AtTPS1/ *OtsA* AtUbi/ *AtTPS1*	Normal vegetative growth and transition to flowering	Van Dijken et al. (2004)
Tomato	35S/ *TPS1*	Higher trehalose content; altered phenotypes (dwarfism and lancet shaped leaves); tolerance to drought, salt and oxidative stress	Cortina and Culiáñez-Macià (2005)
Tobacco	35S/ *TP*	Higher trehalose content; no morphological alteration; tolerance to water deficit	Han et al. (2005)
Tobacco	35S/ *AtTPS1*	Tolerance to osmotic stress; plants smaller than wild type; absence of lancet-shaped leaves	Almeida et al. (2005)
Arabidopsis	35S/ *OtsA- OtsB*	Increased starch content in leaves	Kolbe et al. (2005)
Tobacco	35S/ *AtTPS1*	Plantlets able to grow in media containing glucose (glucose-insensitive phenotypes)	Leyman et al. (2006)
Sugarcane	35S/ *Gf TSase*	Very high levels of trehalose accumulation in transgenic plants (9-13 mg/g FW); improved drought tolerance	Zhang et al. (2006)
Tobacco *Arabidopsis*	RBCS/*ScTPS1*+ RBCS/*ScTPS2* RAB18/*ScTPS1*+ RBCS/*ScTPS2*	Higher trehalose content; alteration of root development in *Arabidopsis*; improved drought tolerance	Karim et al. (2007)
Maize Wheat Rice	ABRC/ *TPSP*	Higher levels of trehalose and other soluble carbohydrates; increased capacity for photosynthesis; differences in plant growth, development and grain yield per plant; multiple stress tolerance	Garg et al. (2007) (unpublished)

35S = Cauliflower mosaic virus 35S promoter; Ubi = Ubiquitin promoter; TP = Trehalose phosphorylase; RBCS = Rubisco small unit promoter; ABRC = Absicisic acid inducible promoter; RAB18 = drought-inducible promoter; AtTPS1 = *Arabidopsis* TPS1 promoter; AtUBQ10 = *Arabidopsis* Ubiquitin promoter; *TPS1* = Trehalose-6-phosphate synthase; *OtsA* = Trehalose-6-phosphate synthase (*E. coli*); *OtsB* = Trehalose-6-phosphate phosphatase (*E. coli*); *Gf TSase* = Trehalose-6-phosphate synthase (*Grifola frondosa*); *ScTPS1 + ScTPS2* = Trehalose-6-phosphate synthase and Trehalose-6-phosphate phosphatase (Yeast), respectively.

The discovery of a plethora of trehalose metabolism enzymes in higher plants, and its role in modulating photosynthesis, carbon metabolism and stress protection, has led to a new series of scientific surprises and offers new challenges for researchers in this field. Furthermore, it has been demonstrated that trehalose has a fundamental role in embryo development (Eastmond et al. 2002, Gómez et al. 2005), and in abscisic acid and sugar signaling (Avonce et al. 2004) in *Arabidopsis*. Therefore, analysis of the tissue-specific expression of trehalose biosynthesis and degradation should shed light on the role of trehalose in abiotic stress tolerance, plant metabolism, growth and development, plant-pathogen interactions and seed development. In view of the latest findings, plant trehalose research should be seen as an opportunity to use multidisciplinary approaches for the dissection of plant metabolic networks, including the interface between sugar sensing-signaling and carbohydrate metabolism.

In conclusion, engineering of trehalose overproduction in rice can be achieved by stress-inducible expression of a bifunctional TPSP fusion gene without any detrimental effect on plant growth or grain yield. During abiotic stress, transgenic plants accumulated increased amounts of trehalose and showed high levels of tolerance to salt, drought and low-temperature stresses, as compared to non-transgenic plants. These results demonstrate the potential for utilizing our transgenic approach to develop new rice cultivars with increased abiotic stress tolerance and enhanced rice productivity. In principle, this same technique can be used to confer stress tolerance on other economically important cereal crops such as maize, sorghum and wheat.

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