## 13. HPLC analysis of trehalose and other soluble carbohydrates from the leaf tissue of *indica and japonica* rice varieties

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Trehalose [α-D-glucopyranosyl-(1→1)-α-D-glucopyranoside] is a non-reducing disaccharide of glucose that serves as a protectant against a variety of stresses in different organisms. It is proposed that this sugar is involved in osmoregulation, removal of free radicals and stabilization of the hydrated structure of proteins to maintain membrane integrity and protein stability under various stress conditions (Crowe et al. 1992). The common trehalose biosynthetic pathway in bacteria and plants includes two enzymatic reactions. Trehalose-6-phosphate (T6P) is generated from UDP-glucose (UDP-Glu) and glucose-6-phosphate (G6P) in a reaction catalyzed by trehalose-6-phosphate synthase (TPS, EC 2.4.1.15). T6P is then dephosphorylated to form trehalose via trehalose-6-phosphate phosphatase (TPP, EC 3.1.3.12). Finally, trehalose is degraded by another enzyme, trehalase (Tre, EC 3.2.1.28) into two glucose molecules. However, trehalose has, so far, not been conclusively identified as an endogenous compound in vast majority of vascular plants, except for the two well-documented cases of "resurrection plant" (Adams et al. 1990, Drennan et al. 1993). Nonetheless, the level of endogenous trehalose in different rice varieties has not been previously investigated. Here, we present our HPLC data in several rice varieties to unambiguously show the presence of endogenous trehalose accumulation in an effort to obtain a better insight into its role in sugar metabolism and stress protection in rice.

In this paper we report our results on eleven different rice varieties representing from *indica*, *japonica* and/or tropical *japonica* group that were aseptically grown *in vitro* without or with Validamycin A (a potent inhibitor of trehalase) for determination of endogenous trehalose and other soluble carbohydrates by high performance liquid chromatography (HPLC) coupled with pulsed amperometric detection (HPAE-PAD). Sterilized rice seeds were germinated and grown in Magenta boxes on ½ MS medium with a 14 h/10 h of light-dark cycle at 150 μmoles photons $m^{-2}$ $s^{-1}$ at 27°C/22°C. To prevent the breakdown of trehalose by endogenous trehalase activity, 100 μM of the trehalase inhibitor Validamycin A (Duchefa Biochemie BV, Haarlem, The Netherlands) was added to the medium where indicated. The nutrient medium was replenished every week with a fresh sterilized liquid medium without or with Validamycin A to grow seedlings aseptically. Three weeks after germination, the leaves were immediately frozen in liquid nitrogen, and homogenized leaf tissue sample was extracted in boiling water for 10 min. and subsequently incubated at 70°C for 20 min. After centrifugation (10 min, 4,000 rpm) the supernatants were removed and the pellet was re-extracted twice at 70°C for 30 min. The supernatants were combined and eluted through poly-prep columns containing anion and cation

exchange resins (Amberlite IRA-67 and Dowex-50W, 1:1v/v) to remove charged compounds. The supernatant was lyophilized and dissolved in 0.5-5 ml HPLC grade water. The trehalose, glucose, fructose and sucrose content was measured from 0.5 ml sample using Dionex DX-500 (HPAE-PAD) liquid chromatograph equipped with a 4 x 250 mm Dionex 35391 CarboPac PA1 analytical column and a 4 x 50 mm Dionex 43096 CarboPac PA1 guard column. The column was eluted with 100 mM NaOH at a flow rate of 1 mL/min. Sugars were detected with an electrochemical detector (Dionex). Commercially available carbohydrates (Sigma) were used as standards. To confirm the identity of trehalose in the leaf extracts, samples were incubated with porcine kidney-derived trehalase (Sigma). Reactions were initiated by adding 20 µl (0.19 unit/mL) of this preparation to 500 µl of extract at 37°C. Samples were taken at different times, boiled for 5 min, and subsequently analyzed as described.

The HPLC method was tested for linearity of the detector response by analyzing the working standard solutions of mono- and disaccharides at different concentrations. Based on the replicated analysis of standards over a range of 1-100 µg/ml, the column and detector performance showed good retention and selectivity of sugars (trehalose, glucose, fructose and sucrose). We were able to detect unambiguously low amount of trehalose in the rice leaf extracts, and significantly higher levels of trehalose in the corresponding samples that were treated with Validamycin A (Table 1). To confirm the identity of the trehalose in the extracts, samples were digested with trehalase (Sigma) and found disappearance of the putative trehalose peak after digestion. The range of trehalose concentration detected in the rice seedlings was 10-20 µg/g FW, and with Validamycin A treatment increased from about 20-43 µg/g FW. Taken together, these results suggest that 1.6-2.6 –fold increase in trehalose accumulation in Validamycin A treated samples may be due to differences in trehalase activity of *indica* and *japonica* rice varieties. In contrast, regulated overexpression of *E. coli* trehalose biosynthetic genes (*ostA* and *ostB*) as a fusion gene in *indica* rice exhibited elevated trehalose accumulation from 50-200 µg/g FW in different transgenic lines and conferred high tolerance levels to different abiotic stresses (Garg et al. 2002).

Thus, our results suggest that rice has the capacity to synthesize and accumulate low amount of trehalose. Furthermore, the synthesis of trehalose may be wide spread in plant kingdom. It is generally accepted that trehalose or T6P function in sugar sensing and can modulate carbohydrate metabolism in plants. We found a ratio of about 1.3-1.5 between glucose and fructose content (data not shown), whereas the ratio between sucrose and trehalose with and without Validamycin A treatment is not always the same in different varieties (Table1). However, higher sucrose content was found in the Validamycin A treated seedlings, indicating that even a low amount of trehalose accumulation can alter the soluble carbohydrate pool of the plants. Similarly, exogenous trehalose treatment specifically increased the activities of sucrose synthase and alkaline invertase in soyabean roots (Muller et al. 1998) and two stress response

enzymes, phenylalanine ammonia-lyase and peroxidase, in wheat leaves (Reignault et al. 2001). These findings supported the suggestion that trehalose may function as an elicitor of genes involved in abiotic and biotic stresses.

We thank Kyle Shibuya and Sabrina Mustopo for technical assistance.

Table 1. HPLC analysis of trehalose and sucrose from the leaf tissue of different rice varieties.

| Rice Variety | Trehalose content (µg/g FW)* | | B/A | Sucrose content (mg/g FW)* | | D/C |
|---|---|---|---|---|---|---|
| | No Validamycin A (A) | 100 µM Validamycin A (B) | | No Validamycin A (C) | 100 µM Validamycin A (D) | |
| ***Indica*** | | | | | | |
| IR 36 | 14.0 ± 1.6 | 26.8 ± 1.0 | 1.91 | 14.1 ± 1.0 | 22.1 ± 0.4 | 1.57 |
| IR 64 | 15.6 ± 1.3 | 30.1 ± 1.6 | 1.93 | 13.4 ± 0.8 | 27.0 ± 1.0 | 2.01 |
| IR 72 | 10.1 ± 0.9 | 19.4 ± 0.3 | 1.92 | 12.2 ± 0.6 | 27.5 ± 2.4 | 2.25 |
| PB-1 | 11.4 ± 0.3 | 19.6 ± 1.0 | 1.73 | 12.0 ± 0.6 | 17.5 ± 1.4 | 1.46 |
| ***Japonica*** | | | | | | |
| Nipponbare | 14.7 ± 0.9 | 23.6 ± 0.2 | 1.61 | 12.4 ± 0.7 | 17.2 ± 1.3 | 1.39 |
| Kaybonnet | 16.7 ± 0.6 | 33.0 ± 1.3 | 1.97 | 18.2 ± 0.5 | 24.0 ± 1.7 | 1.32 |
| TNG 67 | 16.4 ± 1.5 | 42.6 ± 1.2 | 2.59 | 14.2 ± 0.7 | 18.3 ± 0.2 | 1.29 |
| ***Tropical Japonica*** | | | | | | |
| Madison | 20.9 ± 1.2 | 40.8 ± 0.9 | 1.96 | 15.3 ± 1.3 | 13.5 ± 0.6 | 0.88 |
| Dixiebelle | 16.7 ± 0.9 | 40.1 ± 0.9 | 2.40 | 13.5 ± 1.0 | 31.5 ± 0.9 | 2.33 |
| Jefferson | 19.4 ± 1.6 | 36.1 ± 2.1 | 1.87 | 11.5 ± 1.0 | 12.4 ± 1.3 | 1.08 |
| Cypress | 17.9 ± 0.8 | 37.0 ± 0.5 | 2.06 | 18.0 ± 1.1 | 21.0 ± 0.9 | 1.17 |

* Data represents mean ± SE of two independent analyses.

**References**

Adams R.P., E. Kendall and K.K. Kartha, 1990. Comparison of free sugars in growing and dessiccated plants of *Selaginella lepidophylla*. Biochem. Syst. Ecol. 18: 107-110.

Crowe J.H., F. A. Hoekstra and L.M. Crowe, 1992. Anhydrobiosis. Annu Rev Physiol. 54: 579-599.

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

Garg A.K., J.K. Kim, T.G. Owens, A.P. Ranwala, Y.D. Choi, L.V. Kochian and R.J. Wu, 2002. Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. Proc. Natl. Acad. Sci. USA 99: 15898-15903.

Muller J., T. Boller and A. Wiemken, 1998. Trehalose affects sucrose synthase and invertase activities in soybean (*Glycine max* [M.] Merr.) roots. J. Plant Physiol. 150: 255-257.

Reignault Ph., A. Cojan, J, Muchembled, A.L.-H. Sahouri, R. Durand, and M. Sancholle, 2001. Trehalose induces resistance to powdery mildew in wheat. New Phytol. 149: 519-529.