

The selection of a sugar for transport and storage of carbon in plants

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Sugars perform two vital functions in plants: as compatible solutes protecting the cell against osmotic stress and as mobile source of immediate and long-term energy requirement for growth and development. The two sugars that occur commonly in nature are sucrose and trehalose. Sucrose comprises one glucose and one fructose molecule; trehalose comprises two glucose molecules. Trehalose occurs in significant amounts in insects and fungi which greatly outnumber the plants. Surprisingly, in plants trehalose has been found in barely detectable amounts, if at all, raising the question ‘why did nature select sucrose instead of trehalose as the mobile energy source and as storage sugar for the plants?’ Modelling revealed that when attached to the ribbon-shaped β -1,4 glucan a trehalose molecule is shaped like a hook. This suggests that the β -1,4 glucan chains with attached trehalose will fail to align to form inter-chain hydrogen bonds and coalesce into a cellulose microfibril, as a result of which in trehalose-accumulating plant cells, the cell wall will tend to become leaky. Thus in plants an evolutionary selection was made in favour of sucrose as the mobile energy source. Genetic engineering of plant cells for combating abiotic stresses through microbial trehalose-producing genes is fraught with risk of damage to plant cell walls.

Keywords: Abiotic stress, cell wall, cellulose, *Cuscuta*, genetic engineering, trehalose.

A FASCINATING problem in biology is the analysis as to why nature selected a particular molecule over another molecule. Here we attempt to analyse why the disaccharide sucrose, having the chemical formula $C_{12}H_{22}O_{11}$, was chosen for plants in preference to the more widespread disaccharide trehalose having the same chemical formula.

Trehalose versus sucrose

Indeed, the problem is vexing considering that trehalose is far more common than sucrose; yet for some reason nature selected sucrose for the plants. Trehalose is the blood sugar in the insects which outnumber all other life-forms.

Trehalose is also found in fungi which rank next only to the insects, and also in species of protozoa, bacteria, actinomycetes, nematodes and crustaceans^{1,2}. Chemically, trehalose is α -D-glucopyranosyl- α -D-glucopyranoside, comprising two glucose molecules (Figure 1). A related sugar is sucrose (α -D-glucopyranosyl- β -D-fructofuranoside) comprising one each of glucose and fructose molecules. Both trehalose and sucrose are non-reducing sugars. However, they do not occur together: sucrose occurs in organisms which have cell walls; trehalose occurs in significant amounts only in organisms which either lack cell wall altogether or have cell wall that lacks the (homo) polysaccharide cellulose.

The two exceptions in the plant kingdom where trehalose occurs are *Selaginella lepidophylla* – a pteridophyte related to ferns³, and *Myrothamnus flabellifolius*⁴, a higher plant native to Africa. These plants (see Google images) are commonly known as ‘resurrection plants’ because they appear as dead when dry but revive when moistened. Whether trehalose has a role in the resurrection requires research. Study has shown that trehalose can protect membranes from melting by hydrogen bonding with the polar head groups of phospholipid molecules⁵, and the proteins against heat-denaturation by promoting their refolding^{6,7}. The idea that trehalose acts as a stress protectant⁸ has generated considerable interest in plant genetic engineering for building tolerance to temperature and drying using the microbial trehalose-synthesizing genes (Figure 2). However, except for trehalose-producing and

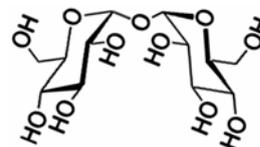


Figure 1. Schematic structure of trehalose. From Elbein *et al.*¹.



Figure 2. Trehalose biosynthesis genes. UDPG, uridine diphosphate glucose; G 6-P, glucose 6-phosphate; TPS, trehalose 6-phosphate synthase; T 6-P, trehalose 6-phosphate phosphatase; TPP, trehalose 6-phosphate photophatase and P_i , inorganic phosphate.

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RESEARCH ACCOUNT

stress-resistant transgenic rice⁹, no other trehalose-synthesizing crop is yet reported and its performance under field conditions is awaited. The available data have presented a picture of undesirable side effects of trehalose in plants. We recall a fortuitous observation made over 30 years ago which directly bears on the question why trehalose^{10–12}, though occurring in the kingdoms of the Monera, the Protista, the Fungi and the Animalia, trehalose is surprisingly absent in the Kingdom Plantae.

The initial goal of our experiments was to identify the sugar which best supports the *in vitro* growth of excised shoot tips of *Cuscuta reflexa* (dodder), an angiosperm parasite (Figure 3). In a medium containing 2% (w/v) trehalose, nearly all shoot-tip explants blackened within six days of culture with microdrops oozing from the blackened region of the vine, suggesting physical damage of the cell wall. The toxic syndrome was delayed if the culture medium was supplemented with glucose, fructose, sucrose, maltose, lactose or cellobiose. Our published findings have largely been ignored, possibly because the stabilizing properties of trehalose in the animal systems were so appealing to the trehalose lobbyists. We had used different batches of trehalose over a period of more than four years and the results were always the same. Trehalose clearly had a damaging effect on the plants.

Plant versus animal systems

Based on the results with animal systems where exogenously applied trehalose protects membranes and proteins against freezing and/or dehydration damage, attempts are being made to engineer microbial trehalose-synthesizing genes into plants for combating stresses. Although some trehalose-synthesizing plants produced have shown improved resistance to drought stress^{9,13,14}, growth defects such as stunted root and stem growth, altered leaf morphology or delayed flowering have been observed^{13,15–19}.



Figure 3. Vines of leafless and rootless *Cuscuta reflexa* growing as a parasite on *Tecoma stans* in the campus of the Indian Institute of Science, Bangalore. Photo: Prof. K. Sankara Rao.

It is noteworthy that except in some lower plants, known as resurrection plants, which can withstand drying to approximately 10% water content^{3,4,20}, trehalose has not been found in plants. Gussin²¹ had dismissed the occurrence of trehalose in higher plants due to microbial contamination. Wingler *et al.*²² reported that *Arabidopsis* seedlings did not develop primary leaves; their cotyledons become dark green with a red rim, and root growth ceases when supplied with trehalose. Schlupepmann *et al.*¹⁹ reported that on treatment with 100 mM trehalose, *Arabidopsis* seedlings ‘stop growing’. All these reports, taken with our own, indicate that trehalose inhibits new growth (Table 1).

A crucial observation

We used aseptic culture techniques throughout. When a 15–30 cm long dodder vine was fed trehalose through the cut end, the terminal 2.5 cm shoot tip blackened (Figure 4). This region corresponded to the zone of elongation¹⁰. Addition of gibberellic acid (GA₃) – a plant growth regulator that promotes marked elongation of excised *Cuscuta* shoot tips²³, hastened blackening of nearly all shoot-tip explants. Killing of the apical zone released a dormant bud below from apical dominance²⁴, but this bud too was killed as soon as it began elongation growth, showing that



Figure 4. Killing (blackening) of apical region of vines of *C. reflexa* grown in medium containing trehalose. TRE, Medium containing 2% trehalose; BM, Basal medium (without carbon source).

Table 1. Some toxic, inhibitory or damaging effects of trehalose in plants

Plant	Method	Effect	Reference
<i>Cuscuta reflexa</i>	Feeding trehalose to <i>in vitro</i> cultured shoot-tip explants	Blackening (killing) of terminal region. Toxicity reduced by addition of a metabolizable sugar.	10
<i>Phaseolus radiatus</i>	Feeding trehalose to cultured hypocotyl explants	Wilting of leaves.	10
<i>Lemna paucicostata</i>	Trehaloseamine, an inhibitor of trehalase added to growth medium	Irreversible loss of growth potential.	10
<i>Nicotiana tabacum</i>	Transgenic plants expressing yeast or <i>Escherichia coli</i> trehalose-synthesizing enzymes	Decreased growth rate.	15
<i>Arabidopsis thaliana</i>	Transgenic plants expressing <i>E. coli</i> trehalose 6-phosphate gene	Bleaching and delayed leaf expansion. Delayed flowering and poor seed set. Growth inhibition reversed if plants are engineered to overexpress <i>E. coli</i> trehalase.	19
Unnamed	Unspecified	Unspecified detrimental effects.	13, 22
<i>Arabidopsis thaliana</i>	<i>Sweetie</i> mutant isolated by T-DNA insertion contains increased level of trehalose	Severe dwarfism, lancet-shaped leaves, early senescence and flower sterility.	31

trehalose interferes with a process that is linked to cell elongation. Based on results with several plant tissues cultured *in vitro* and the measurement of cytosolic trehalase activity, we inferred that the toxic effect of trehalose is related to the low activity of endogenous trehalase. Since at the time of doing our experiments specific inhibitors of trehalase were not available commercially, we obtained a small quantity of semi-synthetic trehaloseamine as a gift. Using the tiny aquatic angiosperm (duckweed) *Lemna paucicostata*, which could be cultured in small volumes of growth medium, we demonstrated that the growth potential of *Lemna* is irreversibly lost if trehaloseamine – an inhibitor of trehalase – is added to the culture medium containing trehalose, implying that the presence of trehalase enzyme serves to detoxify trehalose that may be encountered in nature – derived from death of insects or of fungi.

Clue from localized killing

The site of trehalose action was revealed by feeding radio-labelled glucose to cut *Cuscuta* shoot tips and chasing the label by the addition of cold (non-radioactive) trehalose. An analysis of distribution of radioactivity¹² suggested that trehalose affects the cell-wall synthesis in elongating cells, wherein cellulose is expected to be the major and indispensable component of plant cell walls. Molecular modelling of the interaction between cellulose and trehalose reveals that because of the bent configuration about the glycoside bond, a trehalose molecule joined to the reducing end of a linear β -1,4 glucan results in a stereochemical bend at the site of its attachment (Figure 5), due to an inherent bend in a trehalose molecule. An *in vivo* consequence of this would be that the β -1,4 glucan chains will not self-associate via inter-chain

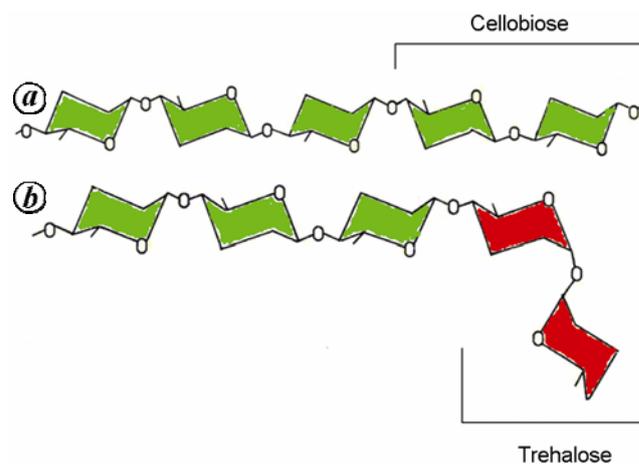


Figure 5. *a.* Schematic representation of a β -1,4 glucan chain composed of repeating units of glucose or cellobiose. *b.* Bending of chain with a trehalose molecule linked at its reducing end, assuming that polymerization of glucan occurs from the reducing end. The α , α -1,1 configuration is crucial for trehalose to exert its damaging effect as shown in Figure 4.

H-bonds to form crystalline cellulose microfibrils. This inference is in accord with oozing from the growing apical region. It explains that although trehalose accumulates in a trehalose-fed vine, it is only the terminal growing region where new cellulose synthesis occurs that is killed. Killing of only the growing apical region was a strong clue that trehalose affects cellulose synthesis in the plant cell wall.

Does trehalose occur in plants?

The repercussion of trehalose interfering with cellulose chain polymerization will be staggering. Indeed, Gussin²¹ stated that trehalose does not occur in the angiosperms

(higher plants), dismissing the earlier reports on its occurrence as due to microbial contamination. However, since homologues of microbial trehalose-synthesizing genes, *TPS* and *TPP*, have been found in the model plant *Arabidopsis thaliana*²⁵, a new hypothesis is that trehalose is synthesized in all plants, but the presence of cytosolic hydrolysing enzyme trehalase does not allow trehalose to accumulate^{16,22}. The presence of trehalose-synthesizing genes in plants has been rationalized by postulating that trehalose 6-phosphate derived from trehalose regulates the flux of carbon into glycolysis¹⁹.

Trehalose hydrolytic enzyme

Despite the apparent or real absence of trehalose in plants, the hydrolyzing enzyme trehalase is ubiquitous in plants. We found constitutive trehalase activity in aseptically grown plants or callus tissue cultures¹⁰. The near ubiquitous presence of trehalase in plants most likely serves to detoxify trehalose absorbed by the roots from the soil, where the sugar could be derived following the death of a myriad of arthropods and fungi, including the near ubiquitous mycorrhizal fungal mycelium. While no one has apparently looked for the presence of trehalase in the soil, the presence of trehalase in plants would be a safeguard mechanism for detoxification of trehalose that may be absorbed by the plant roots from the soil. Perhaps this was the selective pressure for the conservation of trehalase genes in plants.

Nature's choice of a translocatory sugar

The hypothesis that trehalose inhibits cellulose synthesis explains why nature selected sucrose as the translocatory and storage sugar in higher plants. According to Arnold²⁶, a translocatory sugar is ideally a 'protected' derivative of glucose, allowing the sugar to reach the cellular site of its utilization. Although the caloric yield of sucrose and trehalose is similar, Arnold reported activation energy (kcal/mol) of 25.8 and 40.2 for hydrolysis of sucrose and trehalose respectively. This suggests that in terms of its utilization after translocation, the glycoside bond of sucrose is less refractory than that of trehalose, and nature selected sucrose to serve as the translocatory sugar in plants. In plants the UDP-glucose required as an activated donor of glucose for cellulose biosynthesis is derived from sucrose²⁷. With a decrease in sucrose content in the trehalose-fed *Cuscuta* vine¹⁰, the trehalose-induced injury may be exacerbated since repair of injury to cell wall in the growing region will be jeopardized due to the lack of sucrose-derived UDP-glucose. Fujii *et al.*²⁷ have identified sucrose synthase as a component of the catalytic unit of cellulose biosynthesis proteins, implying that biosynthesis of sucrose is linked with that of cellulose, or, sucrose will be absent in organisms which lack cellulose,

such as fungi. Indeed, we are not aware of any fungus – either in the plant-parasitic types or in the saprophytes or in the mycorrhizal fungi – that contains sucrose. We can resolve the dilemma why despite the absence of trehalose, plants benefit from preserving the trehalase gene²⁸. Nature protected plants from trehalose injury to the indispensable cell wall composed mainly of cellulose by conserving trehalase genes. Based on the relative position of organisms in the evolutionary scale, biosynthesis of trehalose is older than that of sucrose, but ultimately nature selected sucrose as the storage and translocatory sugar in plants²⁹.

Why do plants make trehalase if there is no trehalose?

The ubiquitous presence of trehalase activity in plants despite no well-documented proof of the occurrence of trehalose can now be easily explained. The trehalase enzyme serves an ancestral and important function of countering the potentially toxic trehalose that the roots absorb from the soil. The widespread occurrence of trehalase enzyme in plants is an important chemical detoxification mechanism in all plants rooted and anchored in the soil, which is a habitat of a diversity of creatures containing trehalose.

Is trehalose genetic engineering in plants futile?

Prompted by the occurrence of trehalose in the drought-tolerant resurrection plants, there is an increasing interest in genetic engineering of crop plants for overexpression of stress-inducible *TPS-TPP* genes^{9,13}. Although some of the transgenic plants were reported to be free of growth defects, the expression levels did not correlate with the trehalose content. Genetic knockout of the trehalase genes in *Arabidopsis*³⁰, which will allow trehalose accumulation, will be useful to evaluate the effects of trehalose in plants. It is not clear from the published reports whether *AtTRE* knockout has been evaluated for its phenotypic effect in *Arabidopsis*. Achieving knock-out of multiple *AtTRE* genes in *Arabidopsis* is difficult. In this context, dodder, *Cuscuta reflexa*, emerges as a natural 'knockout' model with inherent lack of trehalase activity. Perhaps, because dodder is rootless, existing as a parasite on other plants, it never directly encounters trehalose in nature and may not possess genes either for the synthesis or hydrolysis of trehalose.

Risk exists of even small amounts of trehalose perturbing development of inflorescence or delayed flowering in transgenic plants^{13,31}. Recently, a trehalose-expressing *sweetie* mutant of *Arabidopsis* that accumulates trehalose was reported to show precocious senescence³². Taking into account that the cell wall in plants controls several processes encompassing growth and development, and

that trehalose can interact harmfully with some crucial component of the plant cell wall, putatively cellulose – much as we want it to, the available data suggest caution in genetic engineering of crop plants for trehalose accumulation for combating abiotic stresses. The mechanisms involved in the transfer and attachment of trehalose to β -1,4 glucan present new questions for research.

Conclusion

Our observations are directly relevant to the present debate whether trehalose is a friend or a foe of plants³². More work is required to assess genetic engineering of the plants founded on effects of trehalose in the animal cells.

- Elbein, A. D., Pan, Y. T., Pastuszak, I. and Carroll, D., New insights into trehalose: a multifunctional molecule. *Glycobiology*, 2003, **13**, 17R–27R.
- Crowe, J. H., Trehalose as a chemical chaperone: fact and fantasy. *Adv. Exp. Med. Biol.*, 2007, **595**, 143–158.
- Zentella, R. *et al.*, A *Selaginella lepidophylla* trehalose-6-phosphate synthase complements growth and stress-tolerance defects in a yeast *tps1* mutant. *Plant Physiol.*, 1999, **119**, 1473–1482.
- Bianchi, G., Gamba, A., Limiroli, R., Pozi, N., Elster, R., Salamini, F. and Bartels, D., The unusual sugar composition in leaves of the resurrection plant *Myrothamnus flabellifolia*. *Physiol. Plant.*, 1993, **87**, 223–226.
- Pereira, C. S., Lins, R. D., Chandrasekhar, I., Freitas, L. C. G. and Hünenberger, P. H., Interaction of the disaccharide trehalose with a phospholipid bilayer: A molecular dynamics study. *Biophys. J.*, 2004, **86**, 2273–2285.
- Colaco, C., Sen, S., Thangavelu, M., Pinder, S. and Roser, B., Extraordinary stability of enzymes dried in trehalose. Simplified molecular biology. *Bio/Technology*, 1992, **10**, 1007–1010.
- Kaushik, J. K. and Bhat, R., Why is trehalose an exceptional protein stabilizer? An analysis of thermal stability of protein in the presence of the compatible osmolyte trehalose. *J. Biol. Chem.*, 2003, **278**, 26458–25465.
- Wiemken, A., Trehalose in yeast, stress protectant rather than reserve carbohydrate. *Antonie van Leeuwenhoek*, 1990, **58**, 209–217.
- Garg, A. K., Kim, J.-K., Owens, T. G., Ranwala, A. P., Choi, Y. D., Kochian, L. V. and Wu, R. J., Trehalose accumulation in rice plants confers high tolerance levels to different abiotic stresses. *Proc. Natl. Acad. Sci. USA*, 2002, **99**, 15898–15903.
- Veluthambi, K., Mahadevan, S. and Maheshwari, R., Trehalose toxicity in *Cuscuta reflexa*: correlation with low trehalase activity. *Plant Physiol.*, 1981, **68**, 1369–1374.
- Veluthambi, K., Mahadevan, S. and Maheshwari, R., Trehalose toxicity in *Cuscuta reflexa*: sucrose content decreases in shoot tips upon trehalose feeding. *Plant Physiol.*, 1983, **69**, 1247–1251.
- Veluthambi, K., Mahadevan, S. and Maheshwari, R., Trehalose toxicity in *Cuscuta reflexa*. Cell wall synthesis is inhibited upon trehalose feeding. *Plant Physiol.*, 1983, **70**, 686–688.
- Karim, S. *et al.*, Improved drought tolerance without undesired side effects in transgenic plants producing trehalose. *Plant Mol. Biol.*, 2007, **64**, 371–386.
- Miranda, J. A., Avonce, M. N., Suarez, R., Thevelein, J. M., Van Dijk, P. and Iturriaga, G., A bifunctional TPS–TPP enzyme from yeast confers tolerance to multiple and extreme abiotic stress conditions in transgenic *Arabidopsis*. *Planta*, 2007, **226**, 1411–1421.
- Holmström, K., Mäntylä, E., Welln, B., Mandal, A. and Palva, E. T., Drought tolerance in tobacco. *Nature*, 1996, **379**, 683–684.
- Goddijn, O. J. M. *et al.*, Inhibition of trehalase activity enhances trehalose accumulation in transgenic plants. *Plant Physiol.*, 1997, **113**, 181–190.
- Romero, C., Belles, J. M., Vaya, J. Â. L., Serrano, R. and CulliaÂñez-Macia, F. A., Expression of the yeast trehalose-6-phosphate synthase gene in transgenic tobacco plants: pleiotropic phenotypes include drought tolerance. *Planta*, 1997, **201**, 293–297.
- Yeo, E. T., Kwon, H. B., Han, S. E., Lee, J. T. and Ryu, J. C., Genetic engineering of drought resistant potato plants by introduction of the trehalose-6-phosphate synthase (*TPS1*) gene from *Saccharomyces cerevisiae*. *Mol. Cell*, 2000, **10**, 263–268.
- Schluepmann, H., Pellny, T., van Dijken, A., Smeeckens, S. and Paul, M., Trehalose 6-phosphate is indispensable for carbohydrate utilization and growth of *Arabidopsis thaliana*. *Proc. Natl. Acad. Sci. USA*, 2004, **100**, 6849–6854.
- Bianchi, G., Gamba, A., Limiroli, R., Pozzi, N., Elster, R., Salamini, F. and Bartels, D., The unusual sugar composition in leaves of the resurrection plant *Myrothamnus flabellifolia*. *Physiol. Plant.*, 1993, **87**, 223–226.
- Gussin, A. E. S., Does trehalose occur in angiospermae? *Phytochemistry*, 1972, **11**, 1827–1828.
- Wingler, A., The function of trehalose biosynthesis in plants. *Phytochemistry*, 2002, **60**, 437–440.
- Maheshwari, R., Shailini, C., Veluthambi, K. and Mahadevan, S., The interaction of gibberellic acid and indole-3-acetic acid in the growth of excised *Cuscuta* shoot tips *in vitro*. *Plant Physiol.*, 1980, **65**, 186–192.
- Maheshwari, R. and Sreekrishna, S., The apical control of lateral bud development in excised shoot tips of *Cuscuta reflexa* cultured *in vitro*. *Physiol. Plant.*, 1981, **56**, 474–481.
- Grennan, A. K., The role of trehalose biosynthesis in plants. *Plant Physiol.*, 2007, **144**, 3–5.
- Arnold, W. N., The selection of sucrose as the translocate of higher plants. *J. Theor. Biol.*, 1968, **21**, 13–20.
- Fujii, S., Hayashim, T. and Mizuno, K., Sucrose synthase is an integral component of the cellulose synthesis machinery. *Plant Cell Physiol.*, 2010, **51**, 294–301.
- Müller, J., Trehalose and trehalase in plants: recent developments. *Plant Sci.*, 1995, **112**, 1–9.
- Salerno, G. L. and Curatti, L., Origin of sucrose metabolism in higher plants: when, how and why? *Trends Plant Sci.*, 2003, **8**, 63–69.
- Bouche, N. and Bouchez, D., *Arabidopsis* gene knockout: phenotype wanted. *Curr. Opin. Plant Biol.*, 2001, **4**, 111–117.
- Veyres, N. *et al.*, The *Arabidopsis sweetie* mutant is affected in carbohydrate metabolism and defective in the control of growth, development and senescence. *Plant J.*, 2008, **55**, 665–686.
- Fernandez, O., Béthencourt, L., Quero, A., Sangwan, R. S. and Clément, C., Trehalose and plant stress responses: friend or foe? *Trends Plant Sci.*, 2010, **15**, 409–417.

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