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**"ENGINEERING THE PRODUCTION OF SUGAR ALCOHOLS IN TRANSGENIC PLANTS:
EXTENDING THE LIMITS OF PHOTOSYNTHESIS?"**

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Summary of Results. In the different tobacco lines expressing different polyols, we have investigated how the presence of polyols affects ion uptake during short periods of stress. In addition, we began investigations on recovery from short periods of stress, e.g. eight days of drought and/ or five days in 400 mM NaCl. The transgenic plants take up sodium more slowly. The next set of experiments, modeled after the experiments done with *Mesembryanthemum* (Adams et al. 1994) will investigate ion transport and partitioning in control and transgenic tobacco.

Through a collaboration with Dr. G. Edwards (WSU, Pullman, WA) photosynthetic activities of drought-stressed mannitol/ ononitol tobacco were investigated. Measurements of fluorescence, carbon fixation rates and electron transport indicated that the polyol-containing plants lose photosynthetic competence more slowly than controls. For example, if it took controls six days to reach a certain low rate of carbon fixation, the mannitol-containing plants reached this value after eight to nine days. Re-watering at this point led to a recovery (to 100% of the unstressed control) of the transgenic tobacco within 1 day, while the controls took two to three days to regain 80 - 90% of the original rate.

Transfer of the *mtlD* gene (mannitol production) into *Arabidopsis* has been accomplished. The transgenic plants are phenotypically normal. They survive 300 mM NaCl when the stress is started when the plants are mature - in contrast to wild type which is killed at 150 mM. Seeds from mannitol-containing plants germinate (100 %) in 100 mM NaCl while germination rate of wild type is about 20%. In 200 mM NaCl no wild type germinates, while in some transgenic lines still 50 % of the seeds germinated. At 250 mM NaCl during germination, the transgenic seeds are severely impaired, only 10 to 20 % begin germination (Thomas JC, M Sepahi, HJ Bohnert, Ref).

Use of a sorbitol dehydrogenase gene led to a number of tobacco transformants that contained sorbitol at exorbitantly high levels (up to 80 times more than sucrose), while most transformants contained sorbitol and sucrose at approximately the same concentration based on total cell water. These plants seem to be osmotically stressed, they root poorly and, up until now, have not set seeds (but they can be maintained as cuttings). Grafting is attempted at present to obtain seeds. We have managed to obtain one backcross into wild type SR1 tobacco (STL⁺ as the pollen donor). The phenotype is intriguing - it appears that sorbitol cannot be transported out of the cells in which it is made. This material is probably the most interesting resulting from our work from a general mechanism standpoint. We have established suspension cultures and hope to investigate these cells in more detail.

Conclusions. If we put our knowledge about polyol functions in context and include the comparison in the salt-/drought-stress responses of transgenic glycophytes and a halophyte model, it seems obvious that the tolerance syndrome is based on a complex interaction of several factors, each of which contributes synergistically to tolerance, while each has also some protective value

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on its own. In general terms, the different factors involve (i) control over ion influx, (ii) control over ion partitioning, (iii) osmotic adjustment and (iv) protection of transcription, mRNA stability, translation and protein half-life. Together these factors allow the plants to maintain meristem-activity and to complete their life cycle under stress (see also: Gorham et al. 1985; Cheeseman 1988).

Our hypothesis is that the presence of polyols (and likely glycine-betaine and proline) has several effects on the tissue in which the metabolite(s) is (are) found. In high concentrations these metabolites generate an osmotic potential, while at lower concentrations they act as osmoprotectants. As a third function, we think, their presence may be sensed and this sensing leads to changes in gene expression. It has, for example, been shown that, in contrast to sugars and acetate, polyols do not affect the expression of genes for carbon metabolism enzymes - transcription was measured (Sheen 1990; Sheen 1994). We think, however, that either directly (*via* specific receptors) or indirectly (*via* stretch-sensitive channels, for example) polyols will lead to changes in gene expression and that these changes are related to the stress-protecting functions of these osmoprotectants.

Significance. During the previous funding period we began to understand the pathways that lead to polyol biosynthesis in naturally polyol-producing and in transgenic plants into which genes for polyol production had been introduced. During this time, we began to understand more about the complexity of the problem, which is: in what function may polyols be involved so that they protect plants against high salinity and drought stress conditions?

We have shown that polyols do protect plants. The demonstration was achieved both by correlation (Vernon & Bohnert 1992; Vernon et al. 1993; Adams et al. 1993; Adams et al. 1994) and by the analysis of transgenic plants which had been made to contain polyols (Tarczynski et al. 1992; Tarczynski et al. 1993). Other laboratories have provided data suggesting a protective value of polyols (Bielecki 1982; Ford 1984; Smirnoff & Cumbe 1989; Richter & Popp 1990; Ball & Munns 1992). This was based mainly on observations of the presence of polyols in stressed plants or the induction of polyol biosynthesis coinciding with stress. But we still do not understand in detail why they should be protective! Now we do have the plant lines to address this question.

Protection of plants by whatever means must entail the protection of photosynthetic functions, as we argued in the previous proposal. As a possible function of polyols their accumulation might lead to prolonged carbon fixation under stress (with polyols accumulating as relatively inert metabolites). This may be true, but in our most protected plant lines, barely 1 % of the carbon is found in the polyol fraction.

In contrast, sorbitol accumulation in some tobacco lines can be up to around 10% of the carbon fixed which leads to a stress syndrome - these plants are stunted, root miserably, and do not flower. We think the reason for this phenotype is that sorbitol is located exclusively in the cytoplasm and not in chloroplasts and that the transgenic tobacco cells cannot export sorbitol. Apart from simple synthesis, it seems that the time of synthesis, transport, and final location are also important.

We do not expect our work to result in plants that will be able to grow with sea water or in the arid desert around Tucson. The principles and mechanisms which are revealed by our analyses, however, will lead to an understanding of which functions must be present to protect plants during short-term stress episodes, such as a one-week drought in the field. Of all factors that reduce potential yield, losses due to drought and salinity have been estimated to amount to

70% of the total yield reduction - far higher than losses which are for example due to insects predation (USDA statistical yearbook 1982). Some preliminary data have been obtained indicating that transgenic, mannitol and ononitol-producing, tobaccos lose photosynthetic competence more slowly than control plants during drought. Carbon assimilation, electron transport and stomatal control are maintained longer when water is withheld (Edwards G, personal communication). Recovery after, for example, eight days of drought is faster in the transgenic plants - although these plants contain less than 100 mM of mannitol or ononitol.

The effect produced by such relatively low polyol amounts may be indicative of a function of these metabolites (and a function also for proline and glycinebetaine!) that does not simply involve osmotic balance. An important function of polyols may be the control of ion homeostasis. Our results are preliminary on this topic, but they are consistent between *Mesembryanthemum* (a halophyte which accumulates pinitol) (Adams et al. 1994; Marquez S, Jones W, Bohnert HJ, Jensen RG, unpublished) and in the transgenic tobacco which produce polyols (Marquez S, Jensen RG, unpublished). There is a correlation between the amount of NaCl and the amount of pinitol produced in *Mesembryanthemum*. There is also a correlation between the amount of potassium and the amount of pinitol: if the plants are grown on very little (< -0.1 mM) potassium, pinitol synthesis is increased. We think that pinitol under those circumstances is used to replace the missing potassium in the cytosol. We wish to focus more on this aspect during the next funding period.

Photosynthesis protection during stress is essential: protein import, continued synthesis of photosynthesis-related transcripts, protection of these transcripts, and protein expression have to be maintained. *Mesembryanthemum* does this efficiently (Köster & Anderson 1989; Bohnert et al. 1994; Breiteneder et al. 1994). Our transgenic tobacco plants are not equally efficient. Is this due to the fact that tobacco does not contain ononitol and mannitol in the chloroplast while *Mesembryanthemum* chloroplasts contain up to 500 mM pinitol (Demmig & Winter, 1986; Adams et al. 1994)? Is high concentration of an osmolyte/ osmoprotectant a signal for continued gene expression, an osmoticum that protects, or do polyols - as suggested by others (Smirnov & Cumbes 1989; Sommer et al. 1990) - act as hydroxyl radical scavengers? The answer to this will be a significant contribution to understanding the functions of compatible solutes. Understanding will enable utilization of the principle in plant protection.

Yet another, completely unexpected, avenue has come up during the current funding period. *Mesembryanthemum* accumulates oxalate, mainly in the form of Ca-oxalate raphids in specialized cells. In addition, young plants may contain more than 120 mM of free oxalate (Adams et al. 1994). However, towards the end of the plant's life under stress, the free oxalate disappears with the calcium apparently deposited in cell walls. Oxalate may be cleaved, resulting in two moles of CO_2 and one mole of H_2O_2 . We find an increase of a putative oxalate oxidase in old tissue (transcript level) and a dramatic increase in catalase and SOD activity (Michalowski CB, unpublished). The putative oxalate oxidase, known as germin, was originally found as a transcript in roots of *Mesembryanthemum* (Michalowski & Bohnert, 1993). Germin has now been recognized as oxalate oxidase (Lane et al. 1993). This could mean that oxalate may be a carbon dioxide storage form, to be mobilized under certain conditions, possibly under abiotic stress. The question may be asked whether oxalate deposition and mobilization is a carbon-concentrating pathway similar to the well-known C4 and CAM pathways.

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M. C. Tarczynski, R. G. Jensen and H. J. Bohnert, "Stress Protection of Transgenic Tobacco by Production of the Osmolyte Mannitol" *Science* 259: 508-510 (1993).

D. M. Vernon, M. C. Tarczynski, R. G. Jensen and H. J. Bohnert, "Cyclitol Production in Transgenic Tobacco" *Plant Journal* 4: 199-205 (1993).

H. J. Bohnert, D. M. Vernon, M. C. Tarczynski and R. G. Jensen, "The Engineering of Salt-Tolerant Transgenic Glycophytes" In T. J. Mabry, H. T. Nguyen, R. A. Dixon, M. S. Bonness, Editors, Biotechnology for Aridland Plants, Univ. of Texas, Austin, TX, p. 117-133, (1993).

P. Adams, A. Zegeer, H. J. Bohnert and R. G. Jensen, "Anion Exchange Separation and Pulsed Amperometric Detection of Inositols from Flower Petals", *Analyt. Biochem.* 214:321-324 (1993).

H. J. Bohnert, J. C. Thomas, E. J. DeRocher, C. B. Michalowski, H. Breiteneder, D. M. Vernon, W. Deng, S. Yamada and R. G. Jensen, "Responses to Salt Stress in the Halophyte *Mesembryanthemum crystallinum*", In J. Cherry, Editor, Biochemical and Cellular Mechanisms of Stress Tolerance in Plants, NATO ASI Series, Vol. H 86, Springer Verlag, Berlin, pp 415-428, (1994).

H.J. Bohnert, D. E. Nelson and R. G. Jensen, "Adaptions to Environmental Stresses", *The Plant Cell* 7:1099-1111 (1995).

G. Rammesmayer, H. Pichorner, P. Adams, R. G. Jensen and H. J. Bohnert. "Characterization of IMT1, *myo*-Inositol O-methyltransferase, from Mesembryanthemum crystallinum" *Arch. Biochem. Biophys.* 322: 183-188 (1995).