Plant, Cell and Environment (1997) **20,** 609–616

Salinity and drought tolerance of mannitol-accumulating transgenic tobacco

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ABSTRACT

Tobacco plants (*Nicotiana tabacum* L.) were transformed with a mannitol-1-phosphate dehydrogenase gene resulting in mannitol accumulation. Experiments were conducted to determine whether mannitol provides salt and/or drought stress protection through osmotic adjustment. Non-stressed transgenic plants were 20–25% smaller than non-stressed, non-transformed (wild-type) plants in both salinity and drought experiments. However, salt stress reduced dry weight in wild-type plants by 44%, but did not reduce the dry weight of transgenic plants. Transgenic plants adjusted osmotically by 0·57 MPa, whereas wild-type plants did not adjust osmotically in response to salt stress. Calculations of solute contribution to osmotic adjustment showed that mannitol contributed only 0·003–0·004 MPa to the 0·2 MPa difference in full turgor osmotic potential (π_o) between salt-stressed transgenic and wild-type plants. Assuming a cytoplasmic location for mannitol and that the cytoplasm constituted 5% of the total water volume, mannitol accounted for only 30–40% of the change in π_o of the cytoplasm. Inositol, a naturally occurring polyol in tobacco, accumulated in response to salt stress in both transgenic and wild-type plants, and was 3-fold more abundant than mannitol in transgenic plants.

Drought stress reduced the leaf relative water content, leaf expansion, and dry weight of transgenic and wild-type plants. However, $\pi_o$was not significantly reduced by drought stress in transgenic or wild-type plants, despite an increase in non-structural carbohydrates and mannitol in droughted plants. We conclude that (1) mannitol was a relatively minor osmolyte in transgenic tobacco, but may have indirectly enhanced osmotic adjustment and salt tolerance; (2) inositol cannot substitute for mannitol in this role; (3) slower growth of the transgenic plants, and not the presence of mannitol *per se,* may have been the cause of greater salt tolerance, and (4) mannitol accumulation was enhanced by drought stress but did not affect π_o or drought tolerance.

Key-words: *Nicotiana tabacum* L. cv. Wisconsin 31; tobacco; drought tolerance, genetic engineering; mannitol; osmotic adjustment; plant sciences; polyols; salt tolerance.

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INTRODUCTION

The environmental stresses of salinity and drought reduce growth and agricultural productivity more than any other factors. Plants commonly react to these stresses by accumulation of solutes in cells, or osmotic adjustment, which has resulted in improved environmental stress tolerance (Greenway & Munns 1980; O'Neill 1983; Mathews *et al.* 1984). Under mild stress conditions, osmotic adjustment allows turgor maintenance at water potentials which would eliminate turgor in non-acclimated cells, and therefore impair stomatal function, expansive growth (Mathews *et al.* 1984) and other turgor-dependent processes. During severe stress, osmotic adjustment reduces the amount of cellular dehydration as water potential declines. It has been shown that cell death occurs at a given level of dehydration rather than at some specific water potential (Flower & Ludlow 1986). Thus, the difference between sensitive and tolerant plants may be the level of the stress that must be applied to produce the same amount of cellular dehydration.

Solutes involved in osmotic adjustment are typically sugars, amino acids, inorganic ions and organic acids (Morgan 1984). However, in some plants, polyols (sugar alcohols) such as mannitol and sorbitol accumulate during stress, functioning as osmolytes or compatible cytoplasmic solutes (Loescher 1987). Tarczynski *et al.* (1992) showed that transformation of tobacco with a gene encoding mannitol-1-phosphate dehydrogenase (mtlD) resulted in mannitol accumulation. Transformed tobacco plants survived, whereas non-transformed plants were severely stressed or killed when exposed to 250 mol m^{-3} NaCl, suggesting a role for mannitol in salt tolerance (Tarczynski *et al.* 1993). A positive correlation between carbon partitioning into mannitol and salt stress was also found in celery, which produces mannitol naturally (Kann *et al.* 1993). Similarly, sorbitol accumulation is associated with adaptation to salt stress in *Plantago* (Briens & Lahrer 1983). Myo-inositol has been reported to increase in maritime pine (Nguyen & Lamant 1988) and strawberry (Zhang & Archibold 1993) during drought stress. However, high levels of sorbitol are found in Rosaceous trees which are relatively intolerant of salt and drought stresses, indicating that the function of polyols may be species- or compound-specific.

Recent studies on salt stress (Tarczynski *et al.* 1993; Willamson *et al.* 1995) have assumed that mannitol acts as

an osmolyte, although the contribution of mannitol to osmotic adjustment has not been evaluated. Our objective was to determine whether mannitol acts as an osmolyte, and whether transgenic tobacco plants could tolerate drought as well as salinity through the common mechanism of mannitol accumulation.

MATERIALS AND METHODS

Plant material and growth conditions

Transgenic tobacco (cultivar 'Wisconsin 31') was produced during the summer of 1994 using the modified protocol of Mira *et al.* (1993). *Agrobacterium tumefaciens* (strain LBA4404) with the pCaMVMTLDS cassette subcloned into the binary vector pBin 19 (Tarczynski *et al.* 1992) was obtained from the University of Arizona. Tobacco leaf discs were co-cultivated for 2 d with the *Agrobacterium*, and selected on media containing 100 mg dm^{-3} kanamycin for 3 months. Surviving shoots were transferred to media containing 300 mg dm^{-3} kanamycin, and successfully rooted plants were transferred to 1:1 (volume:volume) perlite:vermiculite mix and moved to the greenhouse in November 1994. Leaf tissue from about 60 putatively transformed lines was screened for mannitol using gas chromatography, and lines showing mannitol were selfed to produce seed. Seeds were sterilized and placed on media containing 100 mg dm^{-3} kanamycin to select against plants losing the gene through segregation, and plants germinated at the expected ratio of 3:1. Two independent lines of first generation transgenic (+mtlD) plants were used in subsequent experiments, selected for > 10% of leaf soluble carbohydrates as mannitol. Subsequent analysis showed no significant differences between these two lines in any of the parameters measured in this study. Non-transformed wild-type plants (–mtlD) were regenerated from leaf discs without cocultivation or antibiotic selection.

Transgenic plants germinated and grew more slowly in tissue culture than –mt1D plants, and to compensate for this, the +mt1D plants were sown about 2 weeks prior to –mt1D plants. Thus, when plants were transferred from tissue culture to soil, the +mt1D and –mt1D plants were of comparable size, having 6–8 leaves and heights of 3–6 cm.

Salinity treatments and experimental design

Forty uniform individuals of both –mt1D and +mt1D plants were transferred from tissue culture to 1:1 perlite:vermiculite media, and placed in a greenhouse in September 1995. The greenhouse yielded ≈70% integrated daily light transmission and temperatures ranged from 22 °C (minimum) to 35 °C (maximum). The photoperiod was extended to maintain vegetative growth using 100 W lights above the greenhouse bench.

On 5 October 1995, 32 +mtlD (two lines, 16 plants of each line) and 32 –mtlD plants were transferred to 8 dm^3 hydroponic containers filled with full-strength nutrient solution (Jones 1985). The experiment was a factorial combination of two plant types (–mtlD, +mtlD) and two salt levels (0 and 150 mol m^{-3} NaCl). The experimental design was a randomized complete block of four treatments with eight replications, each replicate consisting of two plants in a single container. Plants were allowed to acclimate to hydroponic conditions for 2 weeks, and salt stress was applied by adding 25 mol m^{-3} NaCl the first week, increasing by 25 mol m^{-3} per week up to 150 mol m^{-3} NaCl. The hydroponic growth period lasted a total of 11 weeks. Nutrient solutions were made fresh every week.

Drought treatments and experimental design

On 5 March 1996, 20 +mtlD and 20 –mtlD plants were transferred to 20 dm^3 pots filled with composted pine bark. This experiment was also a factorial combination of two plant types (+mtlD and –mtlD) and two stress levels (well-watered and drought-stressed), yielding a randomized complete block design of four treatments and 10 single-plant replications. All plants were initially watered and fertilized for 2 weeks to overcome transplant shock, and water was withheld from 19 March 1996. Drought-stressed plants were rewatered periodically when severe wilting was observed by adding 1 dm^3 of water to a basin in which the pot was placed; this prevented plant death while maintaining relatively dry soil conditions. Non-stressed plants were watered from the top of the pot every other day. The water added to each drought-stressed plant was recorded over the 4 week drought stress period; the –mtlD and +mtlD plants received 4·2 ± 1·0 and 3·2 ± 1·1 dm^3 $week^{-1}$, respectively.

Measurements

Growth

In the salt and drought experiments, plants were destructively harvested after 9 and 4 weeks of treatment exposure, respectively. Plants were divided into leaves, stems and roots, and oven-dried at 70 °C until a constant weight was reached.

Osmotic adjustment

Osmotic potentials at full turgor (π_o) of expanding and fully expanded leaves were measured using a thermocouple psychrometer (Decagon SCA-10, Decagon Devices, Pullman, WA) at the end of each experiment. Leaf discs *c*. 20 mm in diameter were removed from each plant and wrapped in parafilm and foil for transport to the laboratory, where they were floated on deionized water for 2 h. Preliminary tests showed that leaf discs reached a relative water content (RWC) of 100% in 1·5–2 h. Discs were then frozen at –20 °C and stored until measured. NaCl standards bracketing the range of expected π_o were included with each run of samples, and a regression developed from these

standards was used to calculate the water potentials of the samples. Since the freezing process destroys membranes and eliminates turgor, the water potential equaled π_o. It was assumed that the dilution of cell sap by apoplastic water was similar for all treatments.

Mannitol and non-structural carbohydrates

Non-structural carbohydrates were quantified at the end of each study in leaf tissue using gas chromatography (Rieger & Marra 1994). Methanol (80%) containing 0·22 mg cm^{-3} phenyl-ß-D-glucopyranose as internal standard was used for extraction. One hundred mg of freeze-dried, ground leaves was homogenized for 1 min followed by centrifugation for 5 min at 7000 r.p.m. One to two cm^3 of supernatant, which constituted the soluble carbohydrate and polyol fraction, was pipetted into vials and stored at 4 °C. The remainder of supernatant was discarded, and the pellet was washed and centrifuged again with 80% methanol. This supernatant was also discarded, 10 cm^3 of methanol was added, and tubes were placed in a water bath at 100 °C for 1 h to gelatinize the starch. The tubes were refilled with 10 cm^3 distilled, deionized water, 1 cm^3 acetate buffer and 100 mm^3 of amyloglucosidase enzyme solution and placed in a water bath at 55 °C for 48 h. Afterwards, 5 cm^3 of internal standard was added (xylitol, 0·2 mg cm^{-3} H_2O), and a 1–2 cm^3 aliquot of the supernatant was stored for starch analysis. Samples (100 mm^3) were dried in GC vials and derivatized for injection on a Hewlett Packard 5890 A gas chromatograph (Avondale, Pa).

Sodium, chloride and nutrient element concentrations of leaf tissue (salinity experiment only)

Oven-dried leaves were finely ground and used for analysis of Na^+ and Cl^- concentrations. Sodium and all other nutrient elements except chloride were determined by using ICP emission spectroscopy (Jones 1977). Chloride was determined using a Cl^- selective electrode, after the method of Islam *et al.* (1983). Briefly, 0·5 g of ground tissue was dissolved in 50 cm^3 of 0·5 HNO_3 and allowed to stand for at least 5 min. Stable values were obtained 1–2 min after inserting the electrode into the sample mixture, which was gently stirred. The electrode was calibrated using NaCl standards yielding 10, 100 and 1000 mg dm^{-3} Cl^-.

Leaf expansion (drought experiment only)

This parameter was estimated by measuring the length and width of a newly emerging leaf. The relationship between leaf area and length*width was obtained by destructive sampling of similar plants not included in the experiment: leaf area = 0·76(L*W) + 2·0, r^2 = 0·98. A newly emerging leaf from each plant was tagged and measured every other day until it stopped enlarging. The relative leaf expansion rate (RLER) was calculated as RLER = $[(LA_2-LA_1)/LA_1]/2$, yielding units of d^{-1}.

Relative water content (drought experiment only)

Relative water content was measured at approximately weekly intervals after withholding water, except during the third week when inclement weather prevented measurement. Measurements were made before re-watering stressed plants, and so reflected the minimum RWC that the stressed plants experienced. Leaf discs 1 cm in diameter were taken from a recently expanded leaf, weighed, and then floated on distilled water for 2 h so that they became fully turgid. Discs were weighed at full turgidity and again after drying at 70 °C. The RWC was calculated as [(fresh weight – dry weight)/(turgid weight – dry weight)] * 100.

Statistical analyses were performed using PROC GLM of SAS (SAS institute, Cary, NC), using Tukey's studentized comparison or LSD to separate means.

RESULTS

Salt stress experiment

Growth

Salinity reduced the dry weight of –mtlD plants by 44%, whereas the dry weight of +mtlD plants was not significantly reduced by salinity (Table 1). In absolute terms, the dry weights of the –mtlD and +mtlD plants given NaCl were similar. Thus, the difference in salt response between –mtlD and +mtlD plants could be accounted for by differences in growth without added NaCl, i.e. the growth

Table 1. Dry weight of wild-type (-mtlD) and transgenic (+mt1D) tobacco plants 9 weeks after NaCl treatments were applied

NaCl	Plant Type	Root (g)	Shoot (g)	Root/shoot ratio	Total (g)
Yes	–mtlD	9·2 b[z]	33·4 b	0·29 a	40·4 b
	+mtlD	9·6 b	33·1 b	0·31 a	41·5 b
No	–mtlD	12·5 a	65·3 a	0·19 b	73·6 a
	+mtlD	10·2 b	46·4 b	0·23 b	55·0 b
NaCl effect[y]		0·0260	0·0001	0·0001	0·0001

[z] Means followed by the same lower case letter within a column are not significantly different at $P < 0·05$, by Tukey's Studentized Comparison Test.
[y] Probability > F value for the main effect of salt treatment for a given variable.

of +mtlD plants was significantly reduced in comparison with –mt1D plants under non-saline conditions.

Osmotic potential

Osmotic potentials of expanding leaves did not differ with either plant type or salt treatment (Fig. 1). However, π_o of fully expanded leaves of +mtlD plants was reduced by 0·57 MPa in response to salinity, whereas the π_o of fully expanded leaves of –mtlD plants did not differ between stressed and non-stressed plants. Thus, osmotic adjustment occurred only in fully expanded leaves of transgenic plants.

Non-structural carbohydrates

Total non-structural carbohydrates were increased by salt stress in both –mtlD and +mtlD plant types (Table 2). This was partially due to increases in inositol and fructose in salt-stressed plants. In +mtlD plants, 6–10% (depending on treatment) of the total non-structural carbohydrates was mannitol. Starch was not influenced by salt stress, although sucrose was reduced by salt stress in –mtlD plants only.

Mannitol was the only compound affected by plant type, being present in +mt1D and absent in –mt1D plants (Table 2). Mannitol synthesis did not seem to occur at the expense of the other carbohydrates, although the small amount of mannitol present probably would not significantly reduce concentrations of other carbohydrates.

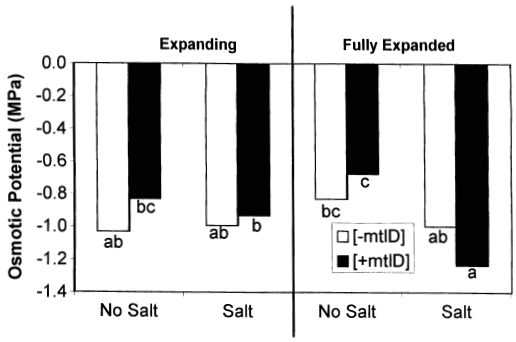

Figure 1. Osmotic potentials at full turgor of expanding and fully expanded leaves of wild-type (–mtlD) and transgenic (+mtlD) tobacco plants 9 weeks after NaCl was applied in the salinity experiment.

Na, K and Cl concentrations of leaves

Leaf Na^+ and Cl^- concentrations were increased by salinity for both plant types as expected (Table 3). However, neither Na^+ nor Cl^- was influenced by plant type. This suggests that transgenic tobacco plants did not merely avoid salt stress by excluding ions from leaves, but tolerated the same amount of ions in leaves (Levitt 1980). Leaf K^+ concentration was higher for +mtlD plants in both salt treatments, although the concentrations of other nutrient elements were unaltered by the mtlD gene (data not shown).

Drought stress experiment

Growth and leaf expansion

Drought stress reduced the dry weight of –mtlD plants by 61% and that of +mtlD plants by 56% (Table 4). The dry weights of drought-stressed –mtlD and +mtlD plants were not significantly different. Under well-watered conditions, however, –mtlD plants had 20% greater dry weight than +mtlD plants, similar to the results of the salinity experiment.

The leaf expansion rates of both –mtlD and +mtlD plants were reduced by drought stress initially, but expansion rates were similar between stressed and non-stressed plants later when growth rates had become very slow (Fig. 2). This resulted in smaller leaves in drought-stressed than non-stressed plants. Leaf expansion was reduced by drought stress to the same extent in +mt1D and –mt1D plants. The leaf production rate (number of leaves produced per week) was also reduced by drought stress by 20%, but did not differ between –mtlD and +mtlD plants (data not shown).

Relative water content (RWC)

Drought stress reduced RWC of both plant types on all dates measured (Fig. 3). RWC of stressed plants reached 50–60% by the end of the experiment, indicating moderate

Table 2. Nonstructural carbohydrate and mannitol concentrations (dry weight basis) in leaves of wild type (-mtlD) and transgenic (+mtlD) tobacco plants 9 weeks after applying NaCl treatments

NaCl	Plant Type	Mannitol (mg g^{-1})	Fructose (mg g^{-1})	Inositol (mg g^{-1})	Sucrose (mg g^{-1})	Glucose (mg g^{-1})	Starch (mg g^{-1})	Total (mg g^{-1})
Yes	–mtlD	0·0 b[z]	7·4 a	13·7 a	3·6 b	7·5 a	19·6a	53·0 a
	+mtlD	3·5 a	8·0 a	12·0 a	5·2 b	8·8 a	18·8 a	56·2 a
No	–mtlD	0·0 b	4·7 b	6·1 b	8·2 a	4·8 b	16·2 a	40·3 b
	+mtlD	3·8 a	5·4 b	5·3 b	7·1 ab	5·4 b	13·2 a	40·3 b
NaCl effect [y]		0·3800	0·0001	0·0001	0·0001	0·0001	0·1300	0·0012

[z] Means followed by the same lower case letter within a column are not significantly different at $P < 0·05$, by Tukey's Studentized Comparison Test.
[y] Probability > F value for the main effect of salt treatment for a given variable.

Table 3. Leaf sodium, chloride and potassium concentration (dry weight basis) of wild-type (+mtlD) and transgenic (–mtlD) tobacco plants 9 weeks after NaCl treatments were applied

NaCl	Plant type	Na^+ (%)	Cl^- (%)	K^+ (%)
Yes	–mtlD	3·65 a[z]	9·35 a	3·73 d
	+mtlD	3·57 a	10·42 a	4·40 c
No	–mtlD	0·01 b	0·29 b	4·79 b
	+mtlD	0·01 b	0·24 b	5·24 a
NaCl effect[y]		0·0001	0·0001	0·0250

[z] Means followed by the same lower case letter within a column are not significantly different at $P < 0·05$, by Tukey's Studentized Comparison Test.
[y] Probability > F value for the main effect of salt treatment for a given variable.

to severe desiccation. RWC was 6–8% lower in –mt1D than +mt1D plants on the third and fourth measurement dates, indicating greater stress for the –mt1D plants. This occurred despite the fact that –mtlD plants were given 24% more water than +mtlD plants during the drought period. As the differences in RWC did not translate into differences in leaf expansion or initiation between –mtlD and +mtlD plants, and these processes are extremely sensitive to drought stress (Bradford & Hsiao 1982), the biological impact of differences in RWC between –mtlD and +mtlD plants appeared to be minimal.

Osmotic potential

Osmotic potentials did not differ between –mtlD and +mtlD plants except for expanding leaves of the drought-stressed plants, where –mt1D plants had π_o 0·35 MPa lower than +mt1D plants (Fig. 4). However, within a plant type, the change in π_o between stressed and non-stressed plants was not significant, indicating a lack of osmotic adjustment in response to drought stress for both –mtlD and +mtlD plants. Statistically, the main effect of drought stress was significant, indicating that drought stress caused a general reduction of π_o when averaged over all leaf and plant types.

Non-structural carbohydrates

Drought stress increased total soluble carbohydrates of both –mtlD and +mtlD plant types (Table 5). Fructose, glucose and starch were increased whereas inositol and sucrose were not affected by drought stress. Mannitol concentration was doubled by drought stress, unlike the salinity experiment where mannitol concentration was unaffected by salt stress.

DISCUSSION

Transformation with the mt1D gene reduced the growth of non-stressed plants by 20–25% in both experiments. We also observed slower growth of +mtlD plants in the tissue culture phase in these and other experiments (Karakas 1996). To adjust for the slower growth rate, we germinated the +mtlD plants about 2 weeks before the –mtlD plants, but commonly found that –mtlD plants were of comparable size or larger when transferred to the greenhouse. Thus, regardless of cultural conditions, transgenic line of +mt1D, or time of year, the slow growth trait was associated with the mt1D gene. This conflicts with observations of Tarcyznski *et al.* (1993) who reported no difference in growth between +mtlD and –mtlD tobacco plants without stress.

The difference in potential growth was eliminated by stress in both experiments, creating ambiguity in interpretation. In a relative sense, +mtlD plants could be considered more salt tolerant, because their growth was not significantly reduced by salt stress, unlike the 44% reduction in growth observed for the –mtlD plants. However, in an absolute sense, the difference in salt tolerance between +mtlD and –mtlD plants could be considered equivocal, because dry weights were similar for salt-stressed plants. The drought experiment was less confounded in this way because, by both relative and absolute measures, growth of +mt1D and –mt1D plants responded similarly to drought stress. Regardless of the effect of the mtlD gene on growth without stress, it is clear that no additional reduction in growth occurred once NaCl was added, and therefore we conclude that transgenic plants had improved tolerance to salt, but not drought stress.

The slower growth of +mtlD plants without stress could be due to the mtlD gene diverting carbohydrate

Table 4. Dry weight of wild type (–mtlD) and transgenic (+mtlD) tobacco plants 4 weeks after drought stress imposition

Drought stress	Plant type	Root (g)	Shoot (g)	Root/shoot ratio	Total (g)
Yes	–mtlD	15·9 c	22·0 b	0·73 a	37·9 c
	+mtlD	13·4 c	20·5 b	0·65 ab	33·8 c
No	–mtlD	38·9 a	57·0 a	0·67 ab	95·9 a
	+mtlD	27·2 b	49·8 a	0·55 b	76·9 b
Drought effect[y]		0·0001	0·0001	0·043	0·0001

[z] Means followed by the same lower case letter within a column are not significantly different at $P < 0·05$, by Tukey's Studentized Comparison Test.
[y] Probability > F value for the main effect of drought treatment for a given variable.

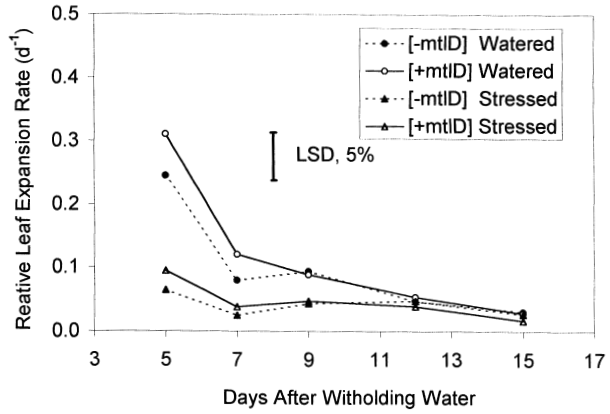

Figure 2. Relative leaf expansion rates (RLER) of wild-type (–mtlD) and transgenic (+mtlD) tobacco plants during the first 2 weeks after withholding water in the drought experiment.

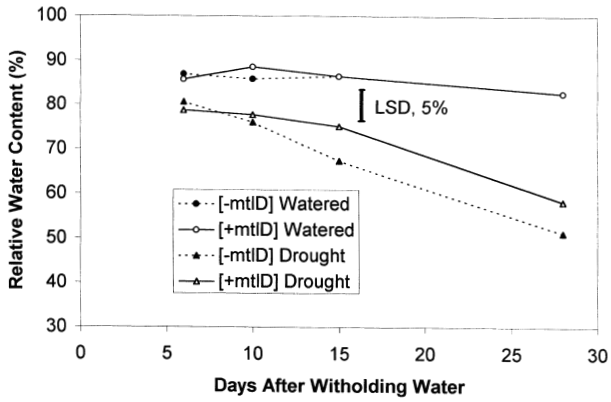

Figure 3. Relative water contents (RWC) of fully expanded leaves of wild-type (–mtlD) and transgenic (+mtlD) tobacco plants after water was withheld during the drought experiment.

metabolism (Tarzcynski *et al.* 1992), sequestering carbon in an unusable form with respect to growth. However, as the mannitol concentration was very low, and the total carbohydrate pools were similar between –mtlD and +mtlD types in both experiments (Tables 2 & 5), growth does not appear to have been carbohydrate-limited. It is possible that the selective marker gene (NPTII, kanamycin resistance) caused the difference in growth, although this is unlikely based on reports of unaltered plant growth and/or yield following transformation with the NPTII marker gene (Arnoldo *et al.* 1992; Dale & McPartlan 1992; Caligari *et al.* 1993; Tarcyznski *et al.* 1993).

The inherently slower growth of the +mt1D plants, not the presence of mannitol, may have been the cause of the improved salt tolerance. Slower growth may allow plants to sequester harmful ions in vacuoles at a rate consistent with ion uptake, and thereby prevent salt build-up in the cytoplasm (Greenway & Munns 1983). This slow growth hypothesis was recently illustrated by El-Khashab *et al.* (1997 and references therein), who found that plants were more tolerant of salinity when sprayed with the growth retardant paclobutrazol. In their study, the percentage reduction in growth due to NaCl stress was halved by applying paclobutrazol, largely due to slower growth and hence slower rates of ion accumulation in leaf tissue. If slower growth is indeed the cause of improved salt tolerance, then incorporation of the mt1D gene may simply be one of many possible strategies that could serve this purpose.

Transgenic plants adjusted osmotically to a greater extent than –mt1D plants in the salinity experiment, but not in the drought experiment (Figs 1 & 4). However, the difference in osmotic adjustment in the salt experiment could not be attributed to mannitol accumulation alone. Using the Van't Hoff equation, $\Pi = cRT$ [where c is the concentration of solute (mol dm^{-3}), $R = 0{\cdot}00821$ dm^3 MPa (mol °K)$^{-1}$, and T is temperature (°K)], and a saturated leaf water concentration of 92–95%, one can estimate the π_o generated by a given amount of solute. Calculations show that 3·5 mg g^{-1} mannitol would contribute 0·003–0·004 MPa (assuming 95 and 92% water content, respectively) to the overall π_o difference of 0·2 MPa between salt-stressed +mtlD and –mtlD plants. Even if we assumed that all of the mannitol was cytoplasmic, and that cytoplasmic water constituted 5% of the total water volume (as did Nguyen & Lamant 1988 in a similar analysis), then mannitol could contribute 0·06–0·08 MPa to the overall change of 0·2 MPa in the salt experiment, or roughly 30–40%. This assumes that there are solutes present in the vacuole which balance the cytoplasmic mannitol, presumably Na^+ and Cl^- ions.

Accumulation of other solutes would affect π_o to a greater extent than accumulation of mannitol. The drought-induced increase in mannitol of 4·3 mg g^{-1} (Table 5) would have contributed only 0·005 MPa to the (non-significant) drop in π_o of about 0·3 MPa, less than half the π_o contribution caused by the associated increase in fructose. The difference in K^+ concentration between +mtlD and –mtlD plants in the salt experiment (Table 2) would generate 0·036 MPa of π_o difference, about 9-fold greater than the mannitol contribution. Thus, mannitol itself does not appear to act as a significant osmolyte, yet its presence was

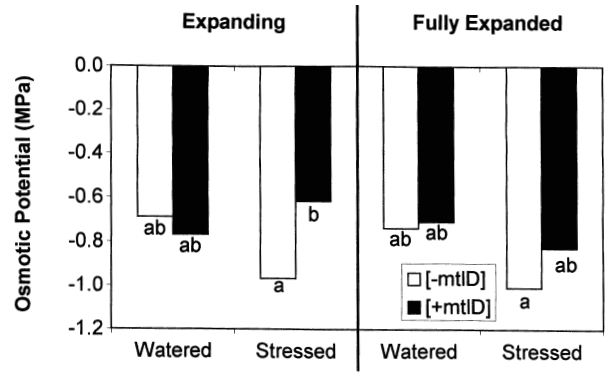

Figure 4. Osmotic potentials at full turgor of expanding and fully expanded leaves of wild-type (–mtlD) and transgenic (+mtlD) tobacco plants 4 weeks after drought stress imposition.

Table 5. Non-structural carbohydrate and mannitol concentrations (dry weight basis) in leaves of wild-type (–mtlD) and transgenic (+mtlD) tobacco plants 4 weeks after drought stress imposition

Drought stress	Plant type	Mannitol (mg g^{-1})	Fructose (mg g^{-1})	Inositol (mg g^{-1})	Sucrose (mg g^{-1})	Glucose (mg g^{-1})	Starch (mg g^{-1})	Total (mg g^{-1})
Yes	–mtlD	0·0 c[z]	30·1 a	10·6 b	20·4 a	32·7 a	190·5 b	284·4 b
	+mtlD	9·1 a	25·5 a	10·7 b	23·4 a	23·4 b	245·2 b	334·4 b
No	–mtlD	0·0 c	15·0 b	12·5 a	22·8 a	22·0 b	312·4 a	384·7 a
	+mtlD	4·8 b	15·3 b	10·3 b	23·0 a	23·8 b	334·8 a	412·0 a
Drought effect[y]		0·0001	0·0001	0·1500	0·7000	0·0890	0·0003	0·0011

[z] Means followed by the same lower case letter within a column are not significantly different at $P < 0{\cdot}05$, by Tukey's Studentized Comparison Test.
[y] Probability > F value for the main effect of drought treatment for a given variable.

associated with greater osmotic adjustment and greater salt tolerance.

The inositol concentration in leaves of +mtlD plants was almost 3-fold higher than that of mannitol in the salt experiment (Table 2), and about the same as the mannitol concentration in the drought experiment (Table 5). Since inositol and mannitol are both hexitols, the contribution of inositol to π_o would be equal to or greater than that of mannitol, but also of questionable biological significance. The concentration of inositol increased with salt stress, but did not differ between plant types, and therefore was unrelated to the difference in salt tolerance between +mtlD and –mtlD plants. The role of mannitol in stress tolerance remains unknown, but may be more specific than previously suggested (Loescher 1987), because inositol cannot substitute for mannitol despite having a nearly identical structure and being present in equal or higher amounts.

Others have measured osmolyte accumulation in plant stress studies and reached similar conclusions. Nguyen & Lamant (1988) found that pinitol accumulated in drought-tolerant lines of maritime pine more than in drought-sensitive lines, but pinitol could only account for 0·01 MPa of osmotic adjustment. Similarly, micromolar quantities of mannitol and arabitol accumulate in the growing points of endophyte-infected tall fescue plants, which are known to be more drought tolerant than non-infected counterparts (West 1994); however, the calculated contribution of these molecules to π_o was less than 1 kPa. Pilon-Smits *et al.* (1995) and Holstrom *et al.* (1996) observed greater drought tolerance in fructan-accumulating and trehalose-accumulating transgenic tobacco, respectively, but both suggested that solute concentrations were too low to have a significant osmotic effect. In a closely related study, Kishor *et al.* (1995) reported greater drought tolerance in tobacco plants transformed with a gene promoting proline accumulation. However, their plants did not undergo osmotic adjustment following stress (Blum *et al.* 1996), suggesting that proline accumulation, like mannitol accumulation, does not necessarily result in osmotic adjustment.

Our data cast doubt on the hypothesis that mannitol can accumulate to levels allowing biologically significant osmotic adjustment in transgenic tobacco. However, we recognize that polyols may act as osmolytes in other biological systems during stress (Gorham *et al.* 1981; Briens & Larher 1983; Ranney *et al.* 1991; Voetberg & Sharp 1991) and that mannitol may play a role in stress tolerance other than as an osmolyte (Loescher 1987). Our results and those of related studies suggest that genetic alteration of plants for solute accumulation does not directly cause osmotic adjustment or result in broad-spectrum environmental stress tolerance.

ACKNOWLEDGMENTS

This research was supported by the Program in Biological Resources & Biotechnology of the University of Georgia, and by the Georgia Seed Development Commission. We thank Hans Bohnert for providing the gene construct, and Paula Litvin and Hazel Wetzstein for technical assistance.

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Received 24 September 1996; received in revised form 2 January 1997; accepted for publication 9 January 1997