Stress Protection of Transgenic Tobacco by Production of the Osmolyte Mannitol

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The accumulation of sugar alcohols and other low molecular weight metabolites such as proline and glycine-betaine is a widespread response that may protect against environmental stress that occurs in a diverse range of organisms. Transgenic tobacco plants that synthesize and accumulate the sugar alcohol mannitol were engineered by introduction of a bacterial gene that encodes mannitol 1-phosphate dehydrogenase. Growth of plants from control and mannitol-containing lines in the absence and presence of added sodium chloride was analyzed. Plants containing mannitol had an increased ability to tolerate high salinity.

Drought, low temperature, and high salinity are environmental factors that may dramatically limit plant growth and crop productivity (*1*). In response to these abiotic stresses, which all disturb the intracellular water balance, many plants and bacteria synthesize and accumulate osmotically active, low molecular weight compounds such as sugar alcohols, proline, and glycine-betaine (*2, 3*). Collectively, these compounds have been referred to as osmolytes, osmoprotectants, or compatible solutes. Although their exact function in plants is unknown, numerous studies [(*2*) and references therein] suggest these osmolytes may protect the plant from abiotic stress. For example, osmolytes accumulate in plant cells in response to water or salinity stress and are subsequently degraded or lost after stress relief (*2*). Other studies indicate a macromolecular protective effect: in vitro incubation of osmolytes with protein extracts from plants often alleviates the adverse effects of electrolytes and temperature stress on enzymatic activity (*4–7*). However, several "nonprotective" roles for many of these compounds have also been suggested (*8–11*), such as storage products during stress. Additionally, accumulation of these compounds may be the result of a stress-induced metabolic impairment (*12*).

An example of such an osmolyte is the sugar alcohol mannitol, which occurs widely in plants and animals (*2, 3*). We previously introduced a metabolic branch point into transgenic tobacco by transformation with a 35*S* mtlD gene construction (*13*). Mannitol accumulation in leaves and roots of these transformed plants was estimated at a maximum concentration of 100 mM (*13*), if a cytosolic location is assumed. Here we determine whether the tobacco plant, which does not normally produce and accumulate mannitol, is protected from salinity stress by this introduced osmolyte.

The growth of 6-week-old plants in the absence and presence of added NaCl was assessed. We evaluated plant growth by measuring the percent of change in height and in fresh weight (*14*). Initial heights and weights for all plants of each experiment were measured just before NaCl addition (*15*). For nonstressed plants, final measurements were taken at the onset of flowering (after 9 to 10 days of culture). Final heights and fresh weights for stressed plants were measured after 14 and 30 days of culture because these plants did not flower. No differences were observed between control and mannitol-containing plants after 10 days of NaCl treatment. Plants were evaluated from five different lines: the two controls, SR1, wild-type tobacco cv. SR1 and SR1^{km}, kanamycin-resistant tobacco, which was transformed with a gene construction identical to that used for mannitol expression (*13*) except for the absence of the mtlD gene, and three lines of manitol-containing transformants, 1-mtl, 2-mtl, and 3-mtl (*16*). Plants were hydroponically cultured under controlled conditions (*17*). All plants from mtl lines contained mannitol, whereas mannitol was not detected in plants from control lines (*13*).

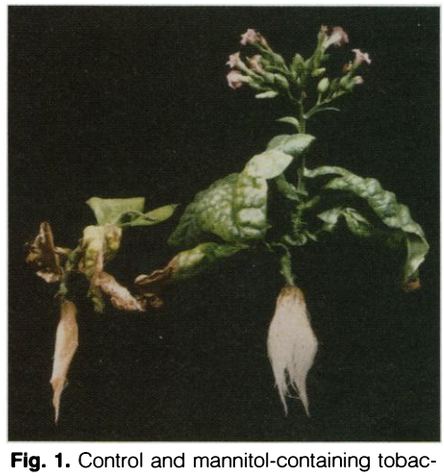

Fig. 1. Control and mannitol-containing tobacco plants after 30 days of culture in the presence of added NaCl (250 mM). Plants were cultured as described (*17*). Left plant, wild type (SR1); right plant, 35*S* mtlD transformant (1-mtl).

When cultured without added NaCl, both the control and mtl plants increased about six- to sevenfold in height and more than twofold in fresh weight over the 9- to 10-day interval before flowers appeared (Table 1). Statistical analyses of percent change in height and fresh weight [single degree of freedom contrasts from a combined analysis of variance (ANOVA) of two experiments] indicated no significant differences in these variables between the two control lines or between the control lines and the three mtl lines ($P = 0.17$ to 0.63). Thus, neither the transformation protocol nor mannitol accumulation influenced plant growth in the absence of excess NaCl.

To determine the NaCl concentrations at which mannitol may affect growth, we conducted pilot studies with 6-week-old plants exposed to various concentrations of NaCl (100 to 300 mM) for 30 days. Primarily on the basis of visual analysis, growth of plants containing mannitol could be distin-

Table 1. Growth of tobacco plants in the absence of added NaCl. All data from two separate experiments are shown. Seeds were germinated and plants were cultured in an equal mixture of vermiculite, potting soil, and sand under greenhouse conditions for 3 weeks. Intact plants were then transferred to a hydroponics system and cultured in a growth room for ~3 weeks (±1 day) (*17*). Height of the aerial portion (in centimeters) and total fresh weight of individual plants (in grams) were measured (initial measurement, *I*). These two traits were measured again for the same plants just before flowering, 9 to 10 days later (final measurement, *F*). Roots were blotted dry before weight measurements were taken. Percent change was calculated as [(*F*/*I*) − 1] 100. Lines evaluated (*16*): SR1, wild type; SR1^{km}, kanamycin-resistant; and 1-mtl, 2-mtl, and 3-mtl (three individual transformants), kanamycin-resistant and expressing 35*S* mtlD (*13*). Evaluations were performed blindly. Data are mean values ± SEM; *n*, total number of plants.

Plant line	Height (% change)	Fresh weight (% change)	*n*
	Experiment 1		
SR1	748.8 ± 110.9	301.5 ± 29.4	8
SR1^{km}	718.1 ± 95.6	248.0 ± 20.1	6
1-mtl	791.4 ± 78.6	330.8 ± 26.3	8
2-mtl	871.9 ± 91.4	276.1 ± 31.6	6
3-mtl	615.7 ± 36.2	270.6 ± 17.6	8
	Experiment 2		
SR1	577.3 ± 26.1	199.7 ± 5.7	8
SR1^{km}	546.8 ± 33.2	202.7 ± 6.5	8
1-mtl	560.8 ± 48.1	200.5 ± 13.1	8
2-mtl	643.9 ± 29.5	250.3 ± 9.8	8
3-mtl	582.3 ± 40.3	203.0 ± 12.4	8

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guished from that of control plants at high salinity (for example, 250 mM). Thus, we pursued this initial observation by assessing the growth of plants cultured under the extreme, or shock, condition of 250 mM NaCl.

After 14 days of treatment with 250 mM NaCl, plant growth from both control and mtl lines was severely inhibited (Table 2). Stem elongation of salt-treated plants was generally less than 10% of that for the nonstressed plants after 9 to 10 days of culture. Additionally, plants typically did not flower after 14 days of stress. The fresh weight of plants from most lines decreased because of necrosis and dehydration of lower leaves. A comparison of the different lines for each experiment shows that after 14 days of stress there were no clear differences between the growth of plants from control and mtl lines.

Fig. 2. Roots from control and mannitol-containing tobacco plants after 30 days of culture in the absence and presence of added NaCl (250 mM). Plants were cultured as described (*17*). From left to right: wild-type plant (SR1) cultured without added NaCl, 35*S* mtlD transformant (1-mtl) cultured without added NaCl, SR1 plant cultured in 250 mM NaCl, and 1-mtl plant cultured in 250 mM NaCl.

After 30 days of exposure to high salinity, however, this difference could be distinguished, with plants that contained mannitol showing increased tolerance (Table 2). In both experiments, plants from mtl lines had decreased weight loss relative to that for plants of control lines. Additionally, mtl transformants increased in height a mean of 80% (range of 53 to 121%) compared with their height at 14 days. Conversely, control plants increased in height only a mean of 22% (range of 3 to 32%) over the same interval. Statistical analyses (single degree of freedom contrasts from a combined ANOVA for both experiments) documented significant differences between control and mtl lines in mean height ($P = 0.0006$) and fresh weight ($P = 0.0001$). No such differences were observed between the two control lines. Thus, on the basis of height and fresh weight, plants that contained mannitol showed increased tolerance to high salinity relative to control plants. Variability in results (for example, with 3-mtl plants) may reflect subtle differences in plant development among the lines just before NaCl treatment or in the experimental conditions (*18*).

Control and transgenic plants also appeared different after 30 days of stress with 250 mM NaCl (Figs. 1 and 2 and Table 3). With all plants, roots exposed to 250 mM NaCl were apparently unable to survive treatment after about 10 days and appeared dark brown in color (salt-stressed control roots, Fig. 2). However, plants that contained mannitol often produced new roots and then new leaves (Figs. 1 and 2 and Table 3). New root and leaf growth occurred in a mean of 75% (range of 60 to 88%) of plants from mtl lines, whereas such growth occurred in only a mean of 33% (range of 13 to 50%) of plants from control lines (Table 3). These new roots typically appeared after about 14 days of NaCl treatment. Flowering (Fig. 1) was observed in 73% of plants that generated new roots and leaves. Although it is not clear how intracellular mannitol accumulation may lead to new root growth, the osmolyte may affect processes at the cellular level that are involved in formation of new roots.

The increased production of new roots and the subsequent emergence of new leaves and then flowers indicate that the relative increases in height and fresh weight of plants containing mannitol are due to new growth rather than to a reallocation of resources. Additional experiments with dry weight analyses (which were not carried out in our time course studies because of their destructive nature) should confirm this observation.

Sugar alcohols may contribute to tolerance at the cellular level by adjustment of the cytosolic osmotic potential when the concentration of electrolytes is lower in the cytosol than in the vacuole (*2*). These compounds may also protect membranes and proteins in the presence of high concentrations of electrolytes (*4*, *19*). An assumption in both mechanisms is that the osmolytes are localized in the cytosol. Our data do not distinguish between these mechanisms. Depending on the cell type, its normal responses to stress, and environ-

Table 2. Growth of tobacco plants in the presence of 250 mM NaCl. All data from two separate experiments are shown. Plant height and fresh weight were measured from ~6-week-old plants (±1 day) just before salt addition (added all at once) and then at 14 and 30 days after addition. Evaluations were performed blindly. Procedures and abbreviations as in Table 1. Data are mean values ± SEM.

Plant line	Height (% change)		Fresh weight (% change)		*n*
	14 days	30 days	14 days	30 days	
			Experiment 1		
SR1	45.9 ± 3.9	60.3 ± 9.5	−14.0 ± 5.8	−48.4 ± 11.1	8
SR1^{km}	66.9 ± 8.5	68.7 ± 10.8	−9.5 ± 9.7	−44.7 ± 16.8	8
1-mtl	59.4 ± 4.2	113.4 ± 29.8	−1.7 ± 12.2	−7.7 ± 24.9	6
2-mtl	54.4 ± 9.6	83.3 ± 14.9	−1.6 ± 4.9	−14.8 ± 10.4	8
3-mtl	74.5 ± 10.2	129.3 ± 12.0	+16.3 ± 5.2	+27.6 ± 12.0	8
			Experiment 2		
SR1	23.1 ± 2.8	28.0 ± 3.0	−9.1 ± 5.7	−34.0 ± 8.2	10
SR1^{km}	36.5 ± 3.5	48.2 ± 5.4	−7.3 ± 5.6	−36.2 ± 7.5	10
1-mtl	29.4 ± 3.1	55.1 ± 7.1	−6.5 ± 3.6	−18.4 ± 10.4	10
2-mtl	19.2 ± 2.8	42.5 ± 7.3	−6.9 ± 5.6	−14.7 ± 9.3	10
3-mtl	29.0 ± 2.3	44.8 ± 3.4	−4.3 ± 3.4	−14.8 ± 9.8	10

Table 3. Visual assessment of new root and leaf growth in tobacco plants after 30 days of exposure to 250 mM NaCl. All data from two separate experiments are shown. Evaluations were performed blindly. Procedures and abbreviations as in Table 1.

Plant line	Plants with new roots and leaves	*n*
	Experiment 1	
SR1	1	8
SR1^{km}	2	8
1-mtl	4	6
2-mtl	6	8
3-mtl	7	8
	Experiment 2	
SR1	5	10
SR1^{km}	4	10
1-mtl	8	10
2-mtl	8	10
3-mtl	6	10

mental conditions, a contribution in tobacco cells of 100 mM mannitol may be consistent with both mechanisms. However, uniform distribution of mannitol at the subcellular level would likely preclude cytosolic osmoregulation. Determination of both the subcellular location of mannitol and the concentrations of the sugar alcohol in tissues will help to distinguish between these two mechanisms.

Alternatively, mannitol may metabolically predispose tobacco cells to stress tolerance. Thus, the cellular accumulation of mannitol, which is normally foreign in tobacco cells, may increase the response of metabolic pathways normally involved in stress tolerance, which thereby allow cells to withstand stress.

On the basis of pilot experiments, we studied the growth response of plants of a defined age that were exposed to the extreme, or shock, condition of 250 mM NaCl. Additional experiments that vary plant age (development) and NaCl concentration (added all at once or incrementally), as well as experiments that investigate the effects of other environmental stresses such as drought and cold, will help to explain the function of mannitol in higher plants. We have demonstrated that for tobacco, the presence of mannitol in vivo protects against high salinity. Sugar alcohol accumulation may also enhance stress tolerance in other plants.

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14. Data for percent changes in height and fresh weight collected over time for the same individual plants were used to determine the inherent variability in initial measurements and were used in all statistical analyses. Data for percent changes in both height and fresh weight were shown to be normally distributed ($P \leq 0.01$) by calculation of the Shapiro-Wilk statistic that permitted our evaluation with ANOVA.
15. Initial heights/weights for experiment 1; and those from experiment 2, respectively, in percent change, for each line. Plants not receiving NaCl: SR1, 6.1 ± 0.4/21.6 ± 1.1, 7.3 ± 0.5/26.9 ± 0.9; SR1^{km}, 6.7 ± 0.5/27.2 ± 1.2, 7.6 ± 0.3/27.6 ± 0.9; 1-mtl, 6.4 ± 0.6/23.7 ± 1.5, 7.3 ± 0.6/26.0 ± 1.1; 2-mtl, 5.9 ± 0.3/27.3 ± 1.1, 6.4 ± 0.4/24.9 ± 0.8; 3-mtl, 6.3 ± 0.3/21.9 ± 1.9, 7.1 ± 0.5/27.1 ± 1.5. Plants subsequently receiving NaCl treatment: SR1, 7.1 ± 0.4/27.9 ± 2.0, 7.4 ± 0.2/26.1 ± 1.3; SR1^{km}, 9.4 ± 0.4/30.8 ± 1.1, 7.1 ± 0.3/25.5 ± 1.0; 1-mtl, 7.4 ± 0.4/26.4 ± 1.4, 7.2 + 0.3/28.1 ± 0.7; 2-mtl, 6.8 ± 0.3/27.5 ± 1.4, 6.6 ± 0.3/27.4 ± 0.7; 3-mtl, 7.3 ± 0.4/26.3 ± 1.6, 7.1 ± 0.2/27.9 ± 0.8. Values are mean ± SEM; for number of plants evaluated; see Tables 1 and 2.
16. For lines that express 35*S* mtlD, 30 primary transformants were chosen randomly and self-pollinated, and the progeny were selected for single-locus inserts on the basis of expression of kanamycin resistance. Of the lines that contain single locus inserts, three were randomly chosen (1-mtl, 2-mtl, 3-mtl), self-pollinated again, and determined to be homozygous on the basis of expression of kanamycin resistance. This procedure was also used for control SR1^{km}, except that five primary transformants were chosen. Lines analyzed were SR1, wild type; SR1^{km}, kanamycin-resistant; and 1-mtl, 2-mtl, and 3-mtl, three different kanamycin-resistant lines that express 35*S* mtlD.
17. The hydroponics system consisted of two genetically identical plants cultured in containers with half-strength Hoagland's nutrient solution (5 liters). (For Figs. 1 and 2, plants from SR1 and 1-mtl were cultured together in 250 mM NaCl in hydroponic containers.) The nutrient solution was changed every 2 weeks. Plants were inserted into the nutrient solution through two holes in the container lid. The growth room housed eight to ten plots of five randomly placed containers (one for each line). For each experiment, half of the plots were cultured in the absence of added NaCl and the other half were cultured in the presence of 250 mM NaCl. To minimize variability in growth and development of plants before stress, all plants (for the data reported in Tables 1 to 3) were approximately the same age (6 weeks) and were selected from larger groups for inclusion in each experiment for their similar heights and weights. For the two experiments, plants were visually identical before NaCl treatment. To eliminate potential experimenter bias, evaluations were performed blindly. Growth-room conditions were 18° and 25°C, night and day temperatures, respectively; photoperiod, 12 hours light (500 μE m^{-2} s^{-1}).
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Altered Specificity of DNA-Binding Proteins with Transition Metal Dimerization Domains

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The bZIP motif is characterized by a leucine zipper domain that mediates dimerization and a basic domain that contacts DNA. A series of transition metal dimerization domains were used to alter systematically the relative orientation of basic domain peptides. Both the affinity and the specificity of the peptide-DNA interaction depend on domain orientation. These results indicate that the precise configuration linking the domains is important; dimerization is not always sufficient for DNA binding. This approach to studying the effect of orientation on protein function complements mutagenesis and could be used in many systems.

Active sites of proteins are typically composed of recognition elements guided into proximity and appropriate orientation by the native protein fold. Individual recognition elements may be remote in primary structure or may be located on different polypeptide chains in multisubunit proteins. With site-directed mutagenesis, amino acids that constitute individual recognition elements can be changed without affecting the overall orientation of the recognition domain. Yet present technology does not allow predictable and routine changes in the orientation of the domains themselves.

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The transcriptional activator protein GCN4 is one of a large family of DNA-binding proteins identified by a bZIP structural motif (*1*); this motif contains a DNA contact domain characterized by conserved basic and hydrophobic residues (b domain), and a dimerization domain identified by a heptad repeat of leucine residues (ZIP domain) (*2*, *3*). The two domains are separated by a six–amino acid linker whose length, but not sequence is conserved across bZIP families (*1*). Previous work has demonstrated that the active DNA-binding entity is generated when the ZIP domains of two protein monomers assemble (*4*) into a parallel coiled coil (*5*, *6*). The scissors grip (*1*) and induced helical fork (*7*) models propose that the coiled coil, the natural dimeriza-