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Increased Salt and Drought Tolerance by D-Ononitol Production in Transgenic *Nicotiana tabacum* L.[1]

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A cDNA encoding *myo*-inositol *O*-methyltransferase (IMT1) has been transferred into *Nicotiana tabacum* cultivar SR1. During drought and salt stress, transformants (I5A) accumulated the methylated inositol D-ononitol in amounts exceeding 35 μmol g^{-1} fresh weight In I5A, photosynthetic CO_2 fixation was inhibited less during salt stress and drought, and the plants recovered faster than wild type. One day after rewatering drought-stressed plants, I5A photosynthesis had recovered 75% versus 57% recovery with cultivar SR1 plants. After 2.5 weeks of 250 mM NaCl in hydroponic solution, I5A fixed 4.9 ± 1.4 μmol CO_2 m^{-2} s^{-1},whereas SR1 fixed 2.5 ± 0.6 μmol CO_2 m^{-2} s^{-1}. *myo*-Inositol, the substrate for IMT1, increases in tobacco under stress. Preconditioning of I5A plants in 50 mM NaCl increased D-ononitol amounts and resulted in increased protection when the plants were stressed subsequently with 150 mM NaCl. Pro, Suc, Fru, and Glc showed substantial diurnal fluctuations in amounts, but D-ononitol did not. Plant transformation resulting in stress-inducible, stable solute accumulation appears to provide better protection under drought and salt-stress conditions than strategies using osmotic adjustment by metabolites that are constitutively present.

The accumulation of high amounts of specific metabolites in transgenic plants increases tolerance to salt and water stress (Bohnert and Jensen, 1996). This effect has been observed in tobacco after the transfer of genes or cDNAs encoding enzymes that lead to the biosynthesis of mannitol, Gly betaine, Pro, trehalose, or fructan (Tarczynski et al., 1993; Kishor et al., 1995; Nomura et al., 1995; Pilon-Smits et al., 1995; Holmström et al., 1996). Because many naturally salt- or drought-tolerant plants accumulate such compounds when stressed, these metabolites are considered to be compatible solutes that act by providing osmotic adjustment and by lowering the osmotic potential, i.e. increasing the capacity to retain water (LeRudelier and Boullard, 1983). Other mechanisms by which these metabolites might enhance resistance to stress are, however, still not well understood. Some of these metabolites could act, for example, as scavengers of hydroxyl radicals—extremely toxic, short-lived active oxygen species that have no known enzymatic systems for breakdown (Smirnoff and Cumbes, 1989; Smirnoff, 1993; Asada, 1994; Shen et al., 1997)—or their protective function might be in maintaining the hydration sphere of proteins under water-stress conditions (Galinski, 1993; Papageorgiou and Murata, 1995).

We have previously shown that tobacco, transformed to accumulate mannitol, survives high salinity better when stressed at the beginning of the exponential growth phase (Tarczynski et al., 1993). However, the amount of mannitol accumulated was usually not greater than approximately 6 to 8 μmol g^{-1} fresh weight, possibly because of the fact that the *Escherichia coli mtlD* gene product catalyzed an equilibrium reaction that could also result in a conversion of mannitol-6-P to Fru-6-P, owing to the equal affinity of MtlD for either mannitol-6-P or Fru-6-P (Teschner et al., 1990). The direction of the reaction is determined by a pH-dependent change in affinity for NAD/NADH. We decided to generate tobacco plants with a capacity for higher polyol production by exploiting a pathway that is induced in the halophyte ice plant (*Mesembryanthemum crystallinum*) following salt stress (Vernon and Bohnert, 1992). This pathway shunts carbon from Glu-6-P to *myo*-inositol and then to methylated inositols. Methylated inositols are formed by a specific *O*-methyltransferase, IMT1, which seems to be absent in tobacco. IMT1 methylates *myo*-inositol to form D(+)-ononitol (1D-1-*O*-methyl-*myo*-inositol). Our expectation was that D-ononitol, which is dependent on the concentration of *myo*-inositol, might accumulate and provide stress tolerance, but it also seemed possible that the activity of the methyltransferase would generate *myo*-inositol deficiency.

Tobacco (*Nicotiana tabacum* L., cv SR1) plants, transformed with the IMT1 cDNA from *M. crystallinum* producing the enzyme D-*myo*-inositol methyltransferase (Vernon et al., 1993), were phenotypically normal. We compare here the physiology of the transformed line, I5A, with untransformed tobacco cv SR1. When the I5A plants were salt stressed or water stressed, D-ononitol accumulated to amounts that could exceed 35 μmol g^{-1} fresh weight. The plants showed enhanced tolerance to drought and salt stress compared with control tobacco. Following stress, leaves of I5A plants were slower to lose turgor and their photosynthetic rates were less affected than in wild type. After several days of stress, leaves of I5A plants regained turgor to a much greater extent than those of SR1 plants, in

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Abbreviation: IMT1, *myo*-inositol *O*-methyltransferase; F_v/F_m, variable fluorescence to maximum fluorescence ratio.

which either whole leaves or parts of leaves remained without turgor and eventually became necrotic. When the stress was terminated, photosynthetic rates of I5A plants returned faster to rates typical of the unstressed condition than those of the controls. High accumulation of D-ononitol was most likely due to the fact that, in tobacco, *myo*-inositol increases during stress, thus providing additional substrate for IMT1. Fortuitously, this led to the stress-dependent increase of D-ononitol and demonstrated that the inducibility of osmoprotectant synthesis may provide better protection than constitutive presence of osmoprotectants.

MATERIALS AND METHODS

Plant Transformation and Growth

Tobacco (*Nicotiana tabacum* L., cv SR1) was transformed with the IMT1 cDNA expressed under control of a cauliflower mosaic virus 35S promoter/enhancer to produce the enzyme D-*myo*-inositol methyltransferase (Vernon et al., 1993). Seeds of the T4 generation after repeated selfing were used. Plants of wild-type tobacco and the transformed line I5A were germinated and grown in vermiculite for approximately 3 weeks before they were transferred either to soil or to hydroponic culture. In drought-stress experiments the plants were grown in soil in 1-gallon pots in a growth room (light intensity, 400 μmol m^{-2} s^{-1}; photoperiod, 12 h; day/night temperature, −27/23°C; RH, approximately 20%). Plants were watered with 0.5× Hoagland nutrient solution daily. When the plants were 6 weeks old, water stress was initiated by withholding water.

Two hydroponic systems, one with salt added and one without, were set up for plant cultivation. The plants were irrigated in 0.25× Hoagland solution in a greenhouse, with midday light intensity of 1600 μmol quanta m^{-2} s^{-1}, 60 to 80% RH, and 28 ± 3°C. The inorganic ion content of the hydroponic solutions was determined each week by ion-exchange chromatography and depleted compounds were added. Six-week-old plants were subjected to salt stress in six tubs with a total volume, including the reservoir, of 460 L with five plants per tub. The second system was stocked similarly but without addition of NaCl.

Leaves were designated by leaf number counting the true leaves from the base of the plant. The area of the top leaf was about 5 to 10% of the fifth leaf down, which was the youngest fully mature leaf on each plant.

Analytical Methods

Tissue was extracted in ethanol:chloroform:water (12:5:3, v/v) and analyzed by HPLC separation with pulsed amperometric detection of carbohydrates and Pro (Adams et al., 1992, 1993). Anions were analyzed by suppressed anion-exchange chromatography using a 10-μL injection loop. A Dionex (Sunnyvale, CA) Ion Pac ATC-1 column (for the removal of carbonate) preceded the injector, which was followed by a guard column and an analytical column (Dionex Omni Pac-100). Ions were eluted in 30 mM NaOH/5% methanol at a flow rate of 1.0 mL min^{-1}. A suppressor anion micromembrane was used and regenerated with 25 mM H_2SO_4 at a flow rate of 7 to 10 mL min^{-1}. Background conductivity was 4 to 6 μS. Cations were analyzed similarly with a 50-μL injection loop. The cation profile was separated on a Universal Cation Column (Alltech Associates, Deerfield, MI) without a guard column with 3 mM methanesulfonic acid at a flow rate of 1.0 mL min^{-1}.

For determination of IMT1 protein, 1 g of leaf tissue from transformed and untransformed plants was ground in 5 mL of 15 mM Hepes, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 10 mM PMSF, centrifuged, and the proteins in the supernatant were precipitated with acetone. The proteins were solubilized in the same buffer and separated by SDS/PAGE on 12% polyacrylamide gels. The bands were blotted onto nitrocellulose membranes using a semi-dry blotter. Immunological detection of IMT1 protein was performed with antibodies raised in rabbits against the purified protein (Rammesmayer et al., 1995; D.E. Nelson, G. Rammesmayer, and H.J. Bohnert, unpublished data). Net CO_2 assimilation rates were measured for attached leaves using an IR gas analyzer (LI-6400; Li-Cor, Lincoln, NE) with CO_2 at 360 ppm in a growth room at 27°C or in the greenhouse at 28°C.

RESULTS

A full-length cDNA for *Imt1*, encoding IMT1 from the halophyte *M. crystallinum*, was transferred into *N. tabacum* cv SR1. The construct contained a caulifower mosaic virus 35S promoter including an enhancer segment, the entire IMT1 coding sequence, and a cauliflower mosaic virus polyadenylation signal. The construct was introduced by *Agrobacterium* spp.-mediated transformation (Vernon et al., 1993). Under nonstressed conditions the plants were very similar to SR1 with respect to growth, numbers of internodes, biomass, flowering time, and seed set. All experiments reported here were conducted three times using 6 to 10 plants for each set. Each observation reported here is from a single experiment that typified the overall response.

Water-Deficit Stress

Both water-deficit and salt stress caused the D-ononitol pool in I5A plants to increase. Water was withheld from 6-week-old SR1 and I5A plants growing in soil in a growth room (light intensity, 400 μmol m^{-2} s^{-1}) and the amounts of *myo*-inositol and D-ononitol were measured (Fig. 1). In all leaves of both lines the concentration of *myo*-inositol increased for the first 4 d of drought stress and then decreased, whereas the amounts of sugars showed less variability. After 8 d without water, stressed SR1 plants had accumulated 16.4 ± 2.8 mM *myo*-inositol in contrast to 3.65 ± 0.12 mM *myo*-inositol and 35.9 ± 4.6 mM D-ononitol in I5A plants. At any time, the total concentration of polyols in line I5A was approximately twice that of the control line (Fig. 1).

Rates of photosynthesis, measured as CO_2 exchange at 400 μmol m^{-2} s^{-1} in a growth room, decreased similarly in both lines after the last watering, so that by 48 h the rates had declined to 5% of rates under well-watered conditions.

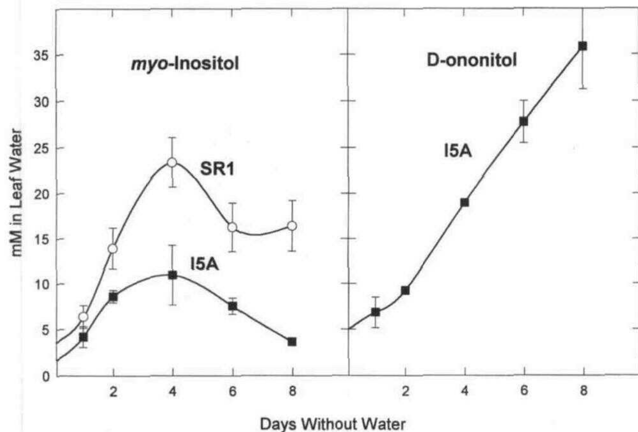

Figure 1. Accumulation of *myo*-inositol and D-ononitol in SR1 and I5A tobacco during water-deficit stress. Water was withheld for 8 d. Partially developed leaves, leaf 10, in identical developmental stages were used for both lines. This leaf began at 10% of the size of a mature leaf and expanded during the stress period to 30 to 50% of the size of a mature leaf. The ambient humidity was 10 to 15%. The plants were grown in a walk-in growth room with a light intensity of 400 μmol m^{-2} s^{-1}.

After 4 d of withholding water, neither line exhibited net CO_2 fixation. Twenty-four hours after rewatering, the photosynthetic rates of the youngest mature leaves of the I5A plants had recovered to 75% (7.3 ± 0.9 μmol CO_2 fixed m^{-2} s^{-1}), whereas these same leaves from SR1 had recovered to 57% (5.5 ± 1.0 μmol CO_2 fixed m^{-2} s^{-1}) of the unstressed rates (data not shown).

Expression of *Imt1* seemed unaltered by conditions of water stress or salt stress, which was likely due to the expression of the cDNA under control of the constitutive 35S cauliflower mosaic virus promoter. Likewise, the amount of IMT1 protein per total protein remained constant, as judged by immunological blot analysis of the protein (Fig. 2).

Salt Stress

Salt stress, at the concentrations of NaCl chosen, was always less severe than withholding water. Plants were grown hydroponically to control NaCl at the roots. Stressing the plants by increasing the salt concentration to 250 mM NaCl in 0.25× Hoagland solution had similar effects in both lines, causing the leaves to become necrotic. Photosynthesis (at 1600 μmol m^{-2} s^{-1} in a greenhouse), stomatal conductance, and the contents of sugars, Pro, and cyclitols were measured from six replicates of each plant type (Table I). During the first 2 weeks after adding 250 mM NaCl, the youngest mature leaves (leaves 10) from both plants displayed no net CO_2 fixation. By 2.5 weeks new leaves appeared and photosynthesis resumed. Rates measured with the developing leaf 12 of I5A plants were about twice that of the same leaf from SR1 plants. This difference in CO_2 fixation rates between the two lines was maintained over at least 2 weeks (Fig. 3). After 4 weeks in 250 mM NaCl, photosynthesis of the same leaves (12 and 13) was similar in both plant types and *myo*-inositol had increased 3- to 4-fold in both lines, whereas D-ononitol amount had more than doubled in I5A compared with the amount present in the corresponding leaf before the start of the experiment.

The changes in photosynthesis correlated with changes in stomatal conductance. The photochemical efficiency of PSII of the two plant types was measured as the F_v to F_m ratio (Krause and Weis, 1991; Endo et al., 1995). Even after 10 d of drought or up to 8 d of salt stress, F_v/F_m had not changed in either line (data not shown), indicating that the efficiency of the light-trapping reactions was not significantly affected over the period of the experiments. The main factor regulating photosynthesis during salt stress in our experiments appeared to be the aperture of the stomata and not the photochemistry.

The photosynthetic response at 150 mM NaCl showed distinct differences between the two plant types (Fig. 3). The CO_2 assimilation rates of leaves 10, which were similar for the two plant types under no-salt conditions, declined immediately after applying NaCl to the hydroponic solution. Although the rate of net CO_2 assimilation of both plant types had dropped considerably within 20 to 25 min, the rate maintained by the I5A plants was twice as high as in the SR1 plants. In this experiment, leaf 10 of SR1 contained 3.3 ± 0.2 mM *myo*-inositol, whereas the same leaf of I5A contained 1.0 ± 0.2 mM *myo*-inositol and 10.5 ± 1.4 mM D-ononitol.

Preconditioning to Salt Stress

Preconditioning of plants by a two-step addition of NaCl to hydroponic solutions in the greenhouse showed most reproducibly the protective effects of D-ononitol against salt stress (Fig. 4). From a collection of young plants, individual SR1 and I5A were selected for identical biomass and grown for 4 weeks in hydroponic culture with 0.25× Hoagland solution. Plants were placed randomly to minimize differences in growth conditions between the lines. Photosynthesis and leaf samples from the top three to four leaves were taken for the "no salt" control (leaves 7–10; Fig. 4A). All plants were adapted for 4 d to 50 mM NaCl in 0.25× Hoagland solution. Addition of 50 mM NaCl in the evening when light was low allowed the plants to adapt during the night, but addition of 50 mM NaCl in high light during the day was less well tolerated and photosynthesis declined.

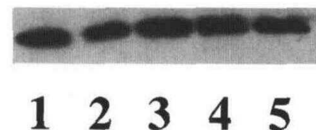

Figure 2. IMT1 in I5A tobacco following water and salt stress. Equal total protein extracted from leaves with 50 to 70% of the size of mature leaves was separated by gel electrophoresis. IMT1 was detected using antibodies to IMT1 (D.E. Nelson, G. Rammesmayer, and H.J. Bohnert, unpublished data) and chemiluminescence. IMT1 migrates with the apparent molecular weight of 42,000 on SDS gels. Lane 1, Six-week-old plants grown in soil without stress in a growth room; lane 2, 7-week-old plants grown in soil without stress; lane 3, 6-week-old plants after 4 d of withholding water; lane 4, 6-week-old plants after 7 d of withholding water; lane 5, 6-week-old plants irrigated with 400 mM NaCl for 7 d.

Table I. *Photosynthesis and carbohydrate status of SR-1 and I5A tobacco before and during salt stress with 250 mM NaCl*

Plants were grown in a greenhouse in hydroponic culture in one-fourth Hoagland solution. At 6 weeks 250 mM NaCl was added to start the salt stress. Expanding leaves, about 50 to 70% of the size of mature leaves, were used for measurement of photosynthetic CO_2 exchange rates and then detached for analysis of carbohydrates. Leaves from six visually identical plants were frozen in N_2 and ground in a mortar and pestle, then extracted in ethanol/chloroform/water. The mean of the six photosynthetic rates and their standard deviations are given.

	Before Salt Stress	2½ Weeks Stress	4 Weeks Stress
Leaf analyzed	10	12	12 or 13
		μmol CO_2 fixed m^{-2} s^{-1}	
SR-1 Photosynthetic CO_2 exchange	14.6 ± 1.3	2.5 ± 0.58	6.76 ± 1.25
		μmol m^{-2}	
Stomatal conductance	0.378 ± 0.031	0.017 ± 0.004	0.066 ± 0.012
Suc, Glc, Fru		*μmol g^{-1} fresh wt*	
Proline	15.5	—[a]	13.34
myo-Inositol	2.3	—	5.6
Ononitol	3.9	—	11.2
	0.0	—	0.0
		μmol CO_2 fixed m^{-2} s^{-1}	
I5A Photosynthetic CO_2 exchange	13.8 ± 0.8	4.93 ± 1.35	7.7 ± 1.01
		μmol m^{-2}	
Stomatal conductance	0.350	0.040 ± 0.011	0.093 ± 0.019
		μmol g^{-1} fresh wt	
Suc, Glc, Fru	12.0	—	11.23
Proline	1.2	—	3.9
myo-Inositol	1.0	—	4.5
Ononitol	9.5	—	21.8

[a] —, Not tested.

Once adapted, photosynthesis changed little between the SR1 and I5A plants (Fig. 4, A and B). This behavior could be expected because tobacco plants can tolerate 50 mM NaCl.

When NaCl was increased to 150 mM in 0.25× Hoagland solution, leaves 8 to 11 from both plant types lost turgor. Within 24 h leaves from all 10 of the I5A plants had regained turgor, whereas the upper portions of leaves 9 through 11 of SR1 (8 out of 10 plants) nearest to the petiole became turgid, leaving the rest of the leaf without turgor. Those wilted leaf areas that did not regain turgor within 2 d eventually underwent necrosis. After 2 d in 150 mM NaCl, photosynthesis in turgid portions of leaves 8 through 11 of SR1 plants was greatly reduced, whereas photosynthesis in I5A had declined less (Fig. 4C). After 5 d in 150 mM NaCl, photosynthetic rates of SR1 began to recover, whereas rates of the lower leaves (leaves 10 and lower) of the I5A plants slowly decreased (Fig. 4D). Photosynthesis of the youngest leaf in both plant types was little affected by salt. Measurements took into account that the plants continued to grow and, thus, the top leaf of the plants represented in Figure 4A was leaf 10, in Figure 4B was leaf 12, and in Figure 4, C and D, was leaf 13. With the I5A plants between d 2 and 5 in 150 mM NaCl (Fig. 4, C and D), the median rates of photosynthesis of leaves 11 and 12 increased, whereas leaves 8 to 10 decreased and approached the rates shown by the SR1 leaves.

The rates of photosynthesis of individual leaves at the same position on different plants were widely different, especially when stressed. Because the mean or average of the rates would be highly susceptible to extremely large or small values, we chose to represent the rates in Figure 4 by the median value of the rates of 10 plants and the dispersion by the interquartile range. Leaf samples were taken from the plants recorded in Figure 4, which were pooled, and the content of organic osmolytes (sugars, Pro, *myo*-inositol, and D-ononitol) was determined (Table II). The youngest leaves usually had the highest concentration of osmolytes. Even though leaves from the I5A plants had less *myo*-inositol than those from SR1, the sum of all cyclitols (*myo*-inositol and D-ononitol) was about twice as high. Inorganic ions were measured for the samples also used for osmolyte determinations. Lowest amounts of Na^+ were present in the youngest leaves. We were unable to detect significant differences in ion uptake between the two lines; the concentrations of Na^+, K^+, NH_4^+, Cl^-, SO_4^{2-}, NO_3^-, and PO_4^{3-} were approximately the same for SR1 and I5A (data not shown). In both lines, the amounts of Na^+ increased approximately equally.

Light/Dark Changes in Osmolytes

Changes in the pool sizes of Pro, sugars, polyols, and inorganic ions were determined at the end of the light period and after 15 h of darkness. Large changes were observed for Pro, Glc, Fru, and Suc. The amounts of these metabolites decreased during the dark treatment by 60% in

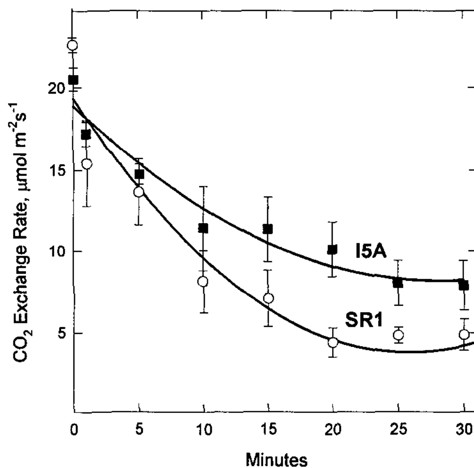

Figure 3. Net photosynthetic CO_2 assimilation rates of leaf 10 from I5A and SR1 plants. The leaves collected from 10 individual plants were 50 to 70% of the size of mature leaf 8 when applying 150 mM NaCl in water culture. ○, SR1 leaves; ■, I5A leaves. During this time all leaves lost turgor but regained turgor by the next morning.

leaf 11 and more than 70% in the lower, older leaf 7 (Table III). Fluctuations of *myo*-inositol, D-ononitol, K^+, Na^+, and Cl^- were much smaller, showing a decline during the night phase by approximately 20% in leaf 11 and approximately 30% in leaf 7.

DISCUSSION

We present data from the analyses of transgenic tobacco expressing the ice plant IMT1 and accumulating the methylated sugar alcohol, D-ononitol, which in long-term water-stressed plants collected up to 36 μmol g^{-1} fresh weight. Under the assumption that the cauliflower mosaic virus 35S promoter leads to uniform expression of IMT1 in all leaf cells, this is equivalent to a concentration of more that 170 mM when the D-ononitol is confined to the cytosol and chloroplast stroma compartments, or 640 mM if the polyol is confined to the cytosol only, based on measurements of compartment sizes according to the method of Winter et al. (1994). The gene encoding IMT1 is absent from wild-type tobacco, as is D-ononitol. Following salt stress, I5A plants showed a distinctly different behavior from wild type in that they maintained higher rates of photosynthesis, and all plants in several experiments recovered when the stress was removed. In contrast, 8 out of 10 SR1 plants contained leaf areas without turgor, which eventually became necrotic. This result is similar to previous reports that have indicated a protective effect by even a modest accumulation of metabolites, including Pro, mannitol, trehalose, and Gly betaine (Delauney and Verma, 1993; Tarczynski et al., 1993; Pilon-Smits et al., 1995; Holmstrom et al., 1996). Although the protection reported in these experiments is small, the observed effect agrees with physiological studies that established a correlation between metabolite accumulation and stress tolerance in many species (Flowers et al., 1977; Jefferies, 1981; Ford, 1982; Yancey et al., 1982).

D-ononitol and its epimer, D-pinitol, accumulate in a number of systematically unrelated species, such as *M. crystallinum* (Paul and Cockburn, 1989), nodules of *Pisum sativum* and *Glycine max* (Streeter, 1985), *Vigna* spp. (Ford, 1982), and *Pinus lambertiana* Dougl. (Wiley, 1891). In *M. crystallinum*, the transcript encoding the IMT1 enzyme is induced by salt stress, and the amounts of D-ononitol and pinitol are correlated with the amount of NaCl found in the leaves and epidermal bladder cells after stress (Vernon and Bohnert, 1992; Adams et al., 1992). When *Imt1* is expressed in tobacco, IMT1 protein can be detected and its amount seems to be unchanged under stress and nonstress conditions, as judged by immunological analysis of protein blots. Because of the choice of the promoter, more transcript was found in young tissues, which also contain the highest amount of protein (data not shown), and the amount of D-ononitol is highest in immature leaves. In stressed I5A plants, the substrate for IMT1, *myo*-inositol, can be 2- to 4-fold lower than in SR1, but still higher than in the absence of stress in SR1 or I5A (Table II; Fig. 1). The increase in D-ononitol after stress (Fig. 1) was unexpected because in plants engineered to produce mannitol (Tarczynski et al., 1992; E. Sheveleva, unpublished data), stress usually caused no change in the concentration of this polyol. Withholding water generally induced larger amounts of D-ononitol than salt stress (compare Fig. 1 and Tables I–III),

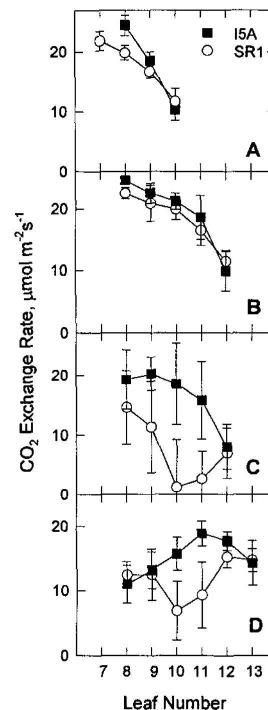

Figure 4. Mean and interquartile range of CO_2 gas-exchange rates of leaves of SR1 and I5A. A, Unstressed plants; B, after 4 d of 50 mM NaCl; C, after 4 d 50 mM NaCl plus 2 d 150 mM NaCl; D, after 4 d 50 mM NaCl plus 5 d of 150 mM NaCl. Plants were grown in a greenhouse in hydroponic culture and at 6 weeks were stressed with NaCl. The top leaves were 5 to 10% of the size of a mature leaf (leaf 8). The measurements were done on the corresponding leaves from 10 plants of each line.

Table II. *Leaf contents of 6-week-old SR-1 and I5A lines before and during salt stress*

The plants shown in Figure 4 were used for analysis. Leaves are numbered from the base excluding the cotyledons. The first fully developed leaf was leaf 8 before addition of NaCl to the hydroponic solution and leaf 9 after 4 d of 50 mM NaCl and after 5 d of 150 mM NaCl.

	Leaf No.	Sugars	Proline	*myo*-Inositol	D-ononitol
			µmol g⁻¹ fresh wt		
Before NaCl					
SR-1					
	8	6.2	1.3	0.8	
	9	7.1	3.0	1.0	
	10[a]	8.5	3.9	1.6	
I5A					
	8	4.0	1.2	0.5	1.1
	9	6.3	2.5	0.5	1.7
	10[a]	16.5	4.0	1.6	4.0
After 4 d of 50 mM NaCl					
SR-1					
	9	10.2	1.5	2.4	
	10	20.3	7.1	5.2	
	11	37.5	10.9	5.7	
	12[a]	32.8	15.3	5.8	
I5A					
	9	12.9	2.4	1.8	6.0
	10	16.0	5.6	1.6	8.1
	11	18.1	11.4	2.1	9.2
	12[a]	24.0	12.9	2.0	8.3
After 4 d of 50 mM NaCl plus 5 d of 150 mM NaCl					
SR-1					
	9	8.3	4.3	5.8	
	10	13.8	6.4	7.2	
	11	18.6	15.1	8.3	
	12	19.3	17.5	8.5	
	13[a]	27.8	30.8	10.3	
I5A					
	9	8.1	4.0	3.4	6.9
	10	11.2	5.0	3.3	9.7
	11	10.5	6.9	3.3	12.6
	12	17.5	12.6	3.1	17.1
	13[a]	25.1	31.4	2.9	16.1

[a] Indicates the top leaf.

which is possibly the reflection of greater loss of leaf water under drought conditions.

From preliminary $^{14}CO_2$ pulse:$^{12}CO_2$ chase experiments with intact transgenic plants, we have estimated the turnover of the carbohydrate pools. Between 4 and 24 h after the start of the $^{12}CO_2$ chase, the half-life of Suc was 6 to 10 h, whereas *myo*-inositol half-life measured 10 to 23 h. According to Figure 1, D-ononitol accumulated at 4.5 μmol g^{-1} fresh weight d^{-1} with no detectable turnover. D-ononitol amounts depend on *myo*-inositol, which is derived from Glc-6-P from enzymes located in the cytosol (Ishitani et al., 1996). During long-term stress *myo*-inositol increases in SR1 and I5A (Table I) for as-yet-unknown reasons. Conceivably, this increase led to higher amounts of D-ononitol in I5A.

Regulation of *myo*-Inositol Synthesis

The pathway of *myo*-inositol synthesis in higher plants is likely to be similar to that in yeast (Loewus and Loewus, 1983). Control is on the first enzyme in the pathway, *myo*-inositol 1-phosphate synthase (INPS; termed INO1 in yeast), which converts Glu-6-P into *myo*-inositol 1-P. Expression of INPS is repressed by *myo*-inositol and choline (Carmen and Henry, 1989) and the activities of several enzymes for phospholipid synthesis, including INPS, are coordinately regulated in *Saccharomyces cerevisiae*. The structural genes encoding a number of these enzymes have been cloned. Regulation in response to *myo*-inositol and choline has been shown to occur at the transcriptional level (Paltauf et al., 1992).

In contrast, not much is known about the regulation of the *myo*-inositol pathway in plants. Transcripts with homology to yeast INO1 have been found in *A. thaliana, Citrus paradisi, Spirodela polyrrhiza,* and *M. crystallinum* (Smart and Fleming, 1993; Abu-Abied and Holland, 1994; Johnson, 1994; Ishitani et al., 1996). The increase in the amount of *myo*-inositol in wild-type tobacco following salt stress may be a consequence of a retardation in growth under stress. Labeling data and pool-size measurements to investigate

Table III. *Oscillation of osmolytes between day and night in I5A tobacco*

I5A tobacco plants were grown for 6 weeks in soil in a greenhouse. Water was withheld from the plants during the 4th and 5th weeks and they were watered again during the 6th week when they were sampled. From leaves 7 and 11 (top, leaf 12) two disks were punched each of two different plants, pooled, frozen in liquid N_2, and extracted in ethanol:chloroform:water. The day values were obtained at 6 PM, the plants were covered, and sampled at 9 AM the next day, still in dark. Night values are given as a percent of the day values.

Osmolyte	Leaf 11		Leaf 7	
	Day	Night	Day	Night
	μmol g⁻¹ fresh wt	*% of d*	*μmol g⁻¹ fresh wt*	*% of d*
Proline	4.2	39%	1.6	17%
Glc	7.0	7%	5.7	25%
Fru	7.6	5%	15.7	27%
Suc	8.4	19%	6.8	12%
K^+	352	79%	236	72%
Na^+	24	113%	11.1	96%
Cl^{-1}	200	91%	129	88%
myo-Inositol	6.4	137%	3.8	88%
Ononitol	34.6	89%	25.8	77%

this hypothesis in tobacco are not yet available, but we consider that the stress-dependent increases in *myo*-inositol and D-ononitol are linked. The K_m for *myo*-inositol of 1.3 mM for ice plant IMT1 (Rammesmayer et al., 1995) could result in the increases in D-ononitol observed. As well, the increase would have to be supported by increased availability of methyl groups, which we hypothesized previously could be derived from increased photorespiration (Bohnert and Jensen, 1996). Increased photorespiration accompanying a stress strong enough to affect stomatal conductance would be a logical candidate for increased provision of methyl groups. Hypothetically, increased *myo*-inositol biosynthesis and increased methyl groups from photorespiration or another unknown methyl donor, and the osmotic adjustment that is provided by the accumulation of D-ononitol and pinitol in the ice plant fulfill requirements for salinity tolerance (Adams et al., 1992; Bohnert et al., 1995; Ishitani et al., 1996). We think that the capacity for D-ononitol synthesis and accumulation in the I5A line protects against salinity stress by operating as an osmolyte, balancing the osmotic stress that accompanies Na^+ uptake. Similarly, water loss in droughted plants might be retarded, owing to the presence of D-ononitol. Most likely, D-ononitol accumulates in the cytosol where it would contribute approximately 600 mM, which would be sufficient to offset the osmotic potential of the vacuole upon uptake of Na^+.

The differences in photosynthetic rates between I5A and SR1 under stress depended on plant developmental age at the beginning of stress, and on the growth conditions. Protection was most clearly observed when the plants were preconditioned to stress, for example, by an adaptation to 50 mM NaCl for 4 d, followed by the transfer to 150 mM NaCl, rather than direct addition of 150 mM NaCl. While photosynthesis was not affected in either SR1 or I5A in the presence of 50 mM NaCl, D-ononitol increased in I5A from 4.0 to 9.2 μmol g^{-1} fresh weight (Table II). The subsequent shift to 150 mM NaCl affected SR1 plants more severely, whereas I5A plants increased D-ononitol to 17.1 μmol g^{-1} fresh weight with greater stomatal apertures and less inhibition of photosynthesis than in SR1 (Tables I and II; Fig. 3). Leaves remained turgid longer and there was less damage due to permanent wilting of leaves or sectors of leaves.

Net CO_2 uptake during the light period and loss at night contrasts with the uptake of Na^+, which is connected to water movement and, hence, transpiration. Whereas intermediates in carbohydrate metabolism vary in a diurnal fashion, the amount of Na^+ in the plant fluctuates much less, requiring constant amounts of compensating osmolytes during both light and dark. In such a function, D-ononitol could provide an advantage in contrast to Pro, Glc, Fru, and Suc that drastically fluctuated between day and night (Table III). These metabolites might be less significant for osmotic adjustment with the changing amounts reflecting their connection to carbohydrate metabolism in the cell (Geiger and Servaites, 1994). The accumulation of D-ononitol did not affect ion uptake, however. Na^+ in particular accumulated to similar amounts in SR1 and I5A (data not shown). Our observations suggest a role for D-ononitol in osmotic adjustment. The high concentrations estimated in the cytosol and stroma from 36 μmol D-ononitol g^{-1} fresh weight and quoted earlier support this hypothesis.

The function for osmolytes in osmotic adjustment is an accepted dogma, but this is probably not their only purpose. Several polyols act as scavengers of reactive oxygen species, and methylated inositols were particularly effective and more active than, for example, Gly betaine (Smirnoff and Cumbes, 1989; Orthen et al., 1994). The presence of D-ononitol and *myo*-inositol could protect sensitive enzymes and membranes from damage by reactive oxygen species, in particular the highly toxic hydroxyl radicals. The rate constants for reactions with hydroxyl radicals in aqueous solution for mannitol and *myo*-inositol are similar, suggesting that both are effective scavengers, whereas Pro, for example, is four times slower (Buxton et al., 1988). Mannitol has recently been shown to act in the chloroplast as a scavenger of hydroxyl radicals in vivo (Shen et al., 1997). It has also been shown in vitro that *myo*-inositol was most effective as a heat protectant in

preventing loss of Gln synthase activity, compared with Gly betaine and Pro, the last being ineffective (Laurie and Stewart, 1990).

In IMT1-expressing tobacco, the fortuitous increases in *myo*-inositol shown by tobacco under water-stress conditions lead to stress-dependent accumulation of a compatible osmolyte with a low turnover, which is removed from the major flux of carbohydrates and exhibits no diurnal fluctuations. High accumulation with stress induction may be superior to the constitutive accumulation at low concentrations of, for example, mannitol, which we have reported earlier (Tarczynski et al., 1993). Since D-ononitol probably does not enter the vacuole, its accumulation provides an osmolyte for osmotically balancing Na^+, which is partitioned to the vacuole. A viable strategy for achieving tolerance against salt stress and drought may be engineering the presence of osmolytes that are not part of the normal metabolism in the plant that is to be transformed and that are not part of the normal stress response repertoire of this species.

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