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Cyclitol production in transgenic tobacco

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Summary

High levels of cyclic sugar alcohols (cyclitols) correlate with tolerance to osmotic stress in a number of plant species. A gene encoding a cyclitol biosynthesis enzyme from a halophyte, *Mesembryanthemum crystallinum* has been introduced into tobacco. The gene, *Imt1*, encodes a *myo*-inositol *O*-methyl transferase that, in *M. crystallinum*, catalyzes the first step in the stress-induced accumulation of the cyclitol pinitol. Tobacco transformed with the *Imt1* cDNA under the control of the CaMV 35S promoter appeared phenotypically normal and exhibited IMT1 enzyme activity. Transformants accumulated a carbohydrate product not detectable in non-transformed control plants. This product was identified by HPLC and NMR as ononitol (1-D-4-*O*-methyl *myo*-inositol). Ononitol was a major carbohydrate constituent in leaf tissue of plants expressing the *Imt1* gene, accumulating to up to 25% the level of sucrose in transformant seedlings. The identification of ononitol as the IMT1 product and the specific accumulation of this compound in transformed tobacco support a role for ononitol as a stable intermediate in pinitol biosynthesis and indicate that an epimerization activity lacking in tobacco is responsible for the conversion of ononitol to pinitol in *M. crystallinum*. The production of ononitol in tobacco indicates that plant carbohydrate metabolism is flexible and can accommodate the synthesis and accumulation of non-endogenous metabolites. The transgenic system described here will serve as a useful model to test the ability of cyclitols such as ononitol to confer tolerance to environmental stress in a normally glycophytic plant.

Introduction

Accumulation of low molecular weight metabolites is a virtually universal response to environmental stress, common to both prokaryotes and eukaryotes (Bieleski, 1982; Csonka and Hanson, 1991; Hellebust, 1976; Yancey *et al.*, 1982). Examples of such metabolites include amino acids, such as proline, quaternary ammonia compounds such as glycine-betaine, and a variety of polyhydroxylated sugar alcohols (polyols) (Bieleski, 1982; Hanson *et al.*, 1991; Wyn Jones and Gorham, 1983; Yancey *et al.*, 1982). Polyols are among the most widespread of the low molecular weight metabolites that accumulate in osmotically stressed organisms. Their presence correlates with osmotolerance in salt-tolerant bacteria, marine algae, fungi from environments of fluctuating osmolarity, and osmotolerant higher plants (Bieleski, 1982; Csonka and Hanson, 1991; Hellebust, 1976; Warr *et al.*, 1988; Yancey *et al.*, 1982). Two prevailing hypotheses exist explaining the importance of these molecules in plant stress tolerance. One view is that they serve primarily as osmolytes, helping cells to osmotically adjust when faced with low exterior water potentials (Hellebust, 1976; McCue and Hanson, 1990; Yancey *et al.*, 1982). An alternate view is that polyols may act as protectants which serve to stabilize membranes and shield macromolecules from the adverse effects of high intracellular ion concentrations or excessive water loss (Schobert, 1977).

One subclass of sugar alcohols, the cyclic sugar alcohols (cyclitols), has, until recently, been neglected as being important in osmotic stress tolerance in higher plants. Cyclitols are highly soluble, non-reactive, and relatively metabolically inert (Loewus and Loewus, 1980). These qualities allow them to accumulate to high levels without interfering with cellular structures or metabolism. Most cyclitols are synthesized from the ubiquitous plant carbohydrate *myo*-inositol (Loewus and Dickinson, 1982); the group includes various methyl *myo*-inositol isomers and their epimerized derivatives. One of the most common *myo*-inositol-derived cyclic sugar alcohols is pinitol (1-D-3-*O*-methyl *chiro*-inositol). Pinitol is thought to be synthesized in a two-step pathway involving the methylation of *myo*-inositol followed by epimerization of the methylated intermediate (Dittrich and Korak, 1984; Loewus and Dickinson, 1982). High levels of pinitol and a closely related epimer, ononitol, are found in a number of osmotolerant plant species, including maritime pine, halophytic mangroves, and the desert shrub jojoba (Dittrich and Korak, 1984; Ford, 1982, 1984; Nguyen and Lamant, 1988; Popp, 1984). Pinitol also undergoes a stress-inducible accumulation in a facultative halophyte, *Mesembryanthemum crystallinum* (Paul and Cockburn, 1989) during this plant's adaptation to high salinity.

Recently, we reported the cloning and identification of a cDNA encoding a methyl transferase which carries out a

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key step in stress-induced pinitol accumulation in *M. crystallinum* (Vernon and Bohnert, 1992). This enzyme, IMT1, catalyzes a position-specific hydroxymethylation of *myo*-inositol. The activity of IMT1, and the expression of the *Imt1* mRNA, increase dramatically during adaptation to salt stress and in response to low temperature in *M. crystallinum* (Vernon and Bohnert, 1992; Vernon *et al.*, 1993). Here, we report the stable introduction of the *Imt1* gene into tobacco, a glycophyte. We demonstrate that *Imt1* transformants contain *myo*-inositol *O*-methyl transferase activity and accumulate substantial levels of a methyl *myo*-inositol, ononitol, which is not detected in control plants. The production and accumulation of ononitol in transgenic tobacco confirm the view (Tarczynski *et al.*, 1992) that plant carbohydrate pathways can be genetically manipulated to produce novel metabolic branch points, without apparent detrimental effects on plant growth or development. The accumulation of ononitol observed in plants expressing IMT1 suggests a role for this compound as an intermediate in pinitol synthesis, and indicates that an epimerization activity not present in tobacco is necessary for the final step in pinitol biosynthesis in *M. crystallinum.*

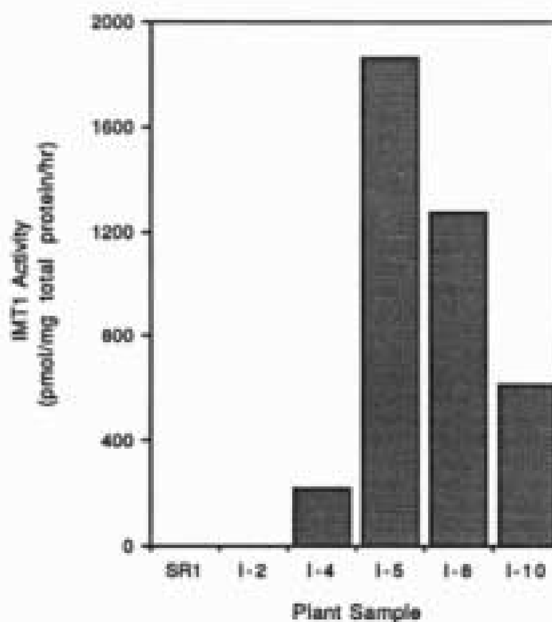

Figure 2. Inositol methyl transferase activity in transformants.
IMT1 enzyme activity was measured in crude soluble protein extracts from SR1 controls and five transformant lines (I-2, I-4, I-5, I-8, I-10). Leaf and shoot material pooled from four Kan^R T_1 progeny from each of five distinct primary transformants was pooled for each protein sample. Detection of activity was dependent on addition of *myo*-inositol to extracts (data not shown).

Results

Transgenic plants express the IMT1 enzyme

The *Imt1* gene, encoding the *myo*-inositol *O*-methyl transferase from the facultative halophyte *M. crystallinum* (Vernon and Bohnert, 1992), was modified for expression in tobacco (Figure 1). The expression construct contained a CaMV 35S promoter element, the full IMT1 amino acid coding sequence, and a CaMV polyadenylation signal. The construct was introduced into tobacco by *Agrobacterium*-mediated transformation, and transformants were identified by selection on kanamycin. Visual examination of more than 40 primary transformants indicated no obvious phenotypic differences as compared with control

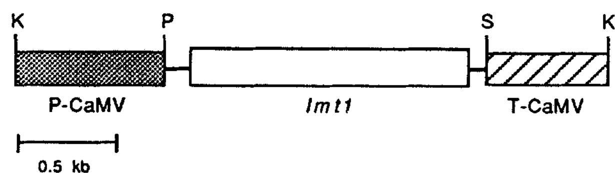

Figure 1. Gene construction.
The construct used for plant transformation was made by inserting the full-length *M. crystallinum Imt1* cDNA behind the double CaMV 35S promoter in the plant expression cassette pJit117 (Guerineau *et al.*, 1988) to create pJitImt1, as described in Experimental procedures. A *Kpn*I fragment containing the entire gene construction was transferred to pBin19 for transfer into tobacco. K, *Kpn*I; P, *Pst*I; S, *Sma*I; P-CaMV, double 35S promoter; T-CaMV, polyadenylation sequence. Black lines: original 5′ and partial 3′ non-coding regions of *M. crystallinum Imt1* cDNA.

(SR1) plants, during both regeneration in sterile culture and after transfer to soil and growth under greenhouse conditions. Southern blots of genomic DNA isolated from transformants and controls were probed with a *M. crystallinum Imt1* cDNA, verifying the presence of the *Imt1* gene in kanamycin-resistant (Kan^R) plants (data not shown). Five independent Kan^R primary transformants (T_0) were arbitrarily selected and self-pollinated. Progeny from T_0 transformants (I-2, I-4, I-5, I-8, I-10) were germinated on kanamycin. Ratios of Kan^R to kanamycin-sensitive seedlings indicated that the number of insertions in different T_0 transformants ranged from one to three independent insertion events. Kanamycin-resistant progeny (T_1) were used for IMT1 activity assays and carbohydrate analyses described below.

To determine whether transformed plants expressed active IMT1 enzyme, protein extracts from T1 progeny from each of the five original transformants were assayed for IMT1 activity (Figure 2). Kan^R seedlings exhibited SAM-dependent *myo*-inositol methyl transferase activity. This activity was not detected in soluble protein extracts from wild-type control plants. Kanamycin-resistant progeny of one regenerant, I-2, did not express measurable levels of the IMT1 activity, possibly due to insertional position effects or alterations to the transferred gene during transformation.

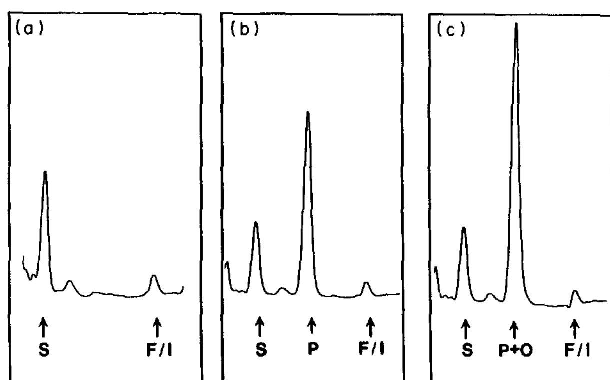

Figure 3. Accumulation of a novel carbohydrate in transformants.
Soluble carbohydrates were extracted from leaf tissue of control plants (a) and tobacco transformed with the *Imt1* construct (b) and resolved by calcium-form partition-exchange HPLC. Extracts from transformed plants were also spiked with 1.5 nmol of a purified methyl-*myo*-inositol (ononitol) standard (c) prior to HPLC analysis. S, sucrose; F/I, combined fructose and endogenous *myo*-inositol peak; P, product; O, ononitol. Retention times were 8.5 min, 11.1 min, and 13.5 min, for sucrose, ononitol, and fructose/*myo*-inositol, respectively. Representative traces from a mature transformant and control are shown.

A novel carbohydrate accumulates in transformed plants

The carbohydrate content of transformants and wild-type controls was analyzed by HPLC (Figure 3). A product with a retention time of 11.1 min was present in carbohydrate extracts from IMT1 transformants, but not in extracts from controls. This retention time is identical to that of the ^{14}C-labeled product formed in IMT1 enzyme assays carried out with IMT1 protein expressed in *E. coli* (Vernon and Bohnert, 1992). A purified methyl *myo*-inositol standard, ononitol, was used to spike extracts (Figure 3c). This standard exhibited a retention time identical to that of the IMT1 product, suggesting that transformed tobacco is producing and accumulating ononitol.

Confirmation of ononitol as the methyl myo-*inositol IMT1 product*

Myo-inositol can be methylated at different positions to form ononitol, sequoyitol, or bornesitol (Loewus and Loewus, 1980; 1983). Both ononitol and sequoyitol have been proposed as intermediates in the pinitol biosynthetic pathway, the pathway in which IMT1 is involved in its native species, *M. crystallinum* (Figure 4) (Dittrich and Brandl, 1987; Dittrich and Korak, 1984; Loewus and Dickinson, 1982; Ruis and Hoffmann-Ostenhof, 1969). To confirm the identity of the methyl *myo*-inositol produced in the transgenic tobacco, further HPLC and NMR analyses were carried out. The HPLC retention time of ononitol was compared with those of sequoyitol and pinitol standards (Figure 5). Ononitol was easily distinguished from both sequoyitol and pinitol, being detected as a distinct peak

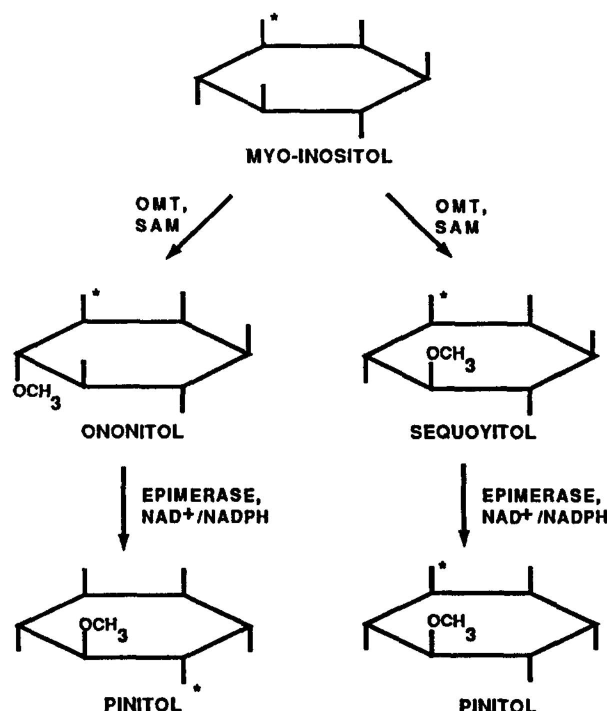

Figure 4. Proposed pathways of pinitol biosynthesis.
The common cyclitol pinitol is synthesized from *myo*-inositol in a two-step process consisting of a methylation step carried out by a position-specific S-adenosyl methionine (SAM)-dependent *O*-methyl transferase, followed by an epimerization reaction which may require NAD^+ and/or NADPH (Loewus and Dickinson, 1982). Two different methyl-*myo*-inositol isomers, ononitol and sequoyitol, have been proposed as intermediates. Vertical bars shown on ring structures represent hydroxyl groups. One hydroxyl position is marked with an asterisk for reference.

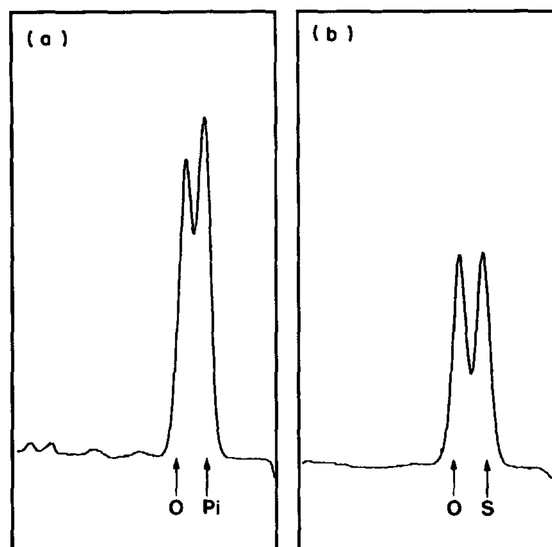

Figure 5. HPLC retention times of methyl-*myo*-inositol isomers.
The retention times of sequoyitol and pinitol were compared with that of the putative IMT1 product, ononitol. Purified cyclitol standards (1.5 nmol) were mixed and resolved under the conditions described in Experimental procedures. (a) Ononitol (O) plus pinitol (Pi); (b) ononitol and sequoyitol (S). Retention times: Ononitol, 11.1 min; pinitol and sequoyitol, 11.5 min.

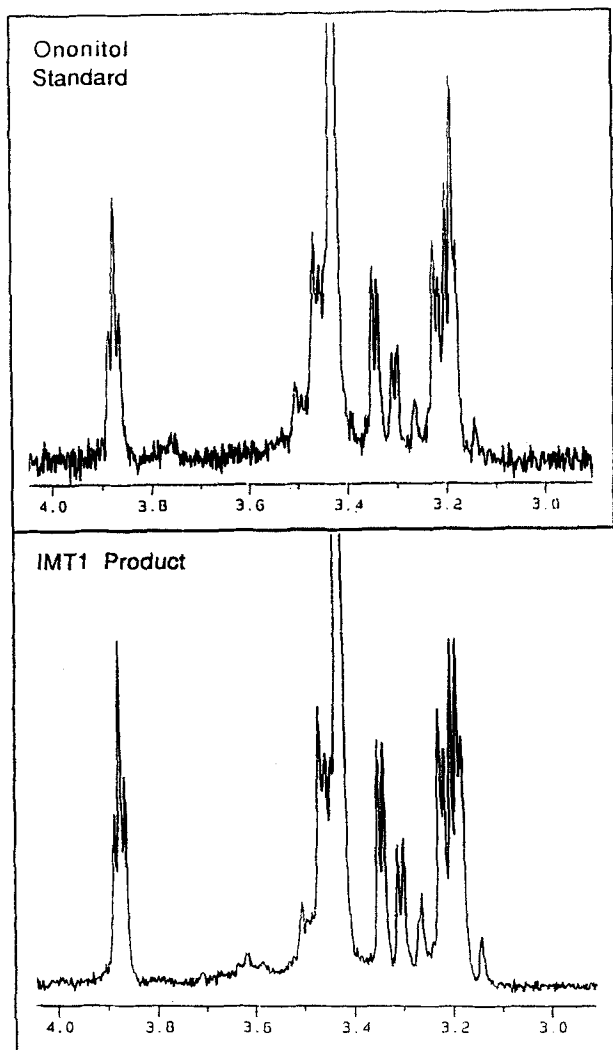

Figure 6. ^{1}H-NMR spectra of purified IMT1 product and ononitol standard.
The proton NMR spectrum of an ononitol standard was compared with that of the IMT1 product purified from leaf extracts of transgenic plants by HPLC. Chemical shift scales (in p.p.m., relative to water) are shown under each panel.

on HPLC traces. Its retention time differed from those of both sequoyitol and pinitol by approximately 0.5 min under our HPLC conditions. A bornesitol standard exhibited a distinct retention time of 12.2 min (data not shown). Only ononitol, therefore, co-migrated with the IMT1 product (see Figure 3c). Proton NMR performed on an ononitol standard and on the IMT1 product purified by HPLC from tobacco transformant leaf extracts verified the identity of this cyclitol as ononitol (Figure 6).

Levels of ononitol in seedlings from the five transgenic lines

Levels of the IMT1 product in Kan^R T_1 progeny of the five selected primary transformants were compared (Table 1).

Table 1. Cyclitol accumulation in tobacco transformed with *Imt1*

Transgenic line	Ononitol (μmol g^{-1} FW)	Sucrose (μmol g^{-1} FW)
SR1 (control)	ND	3.1
I-2	$<$0.0002	4.2
I-4	0.3	4.0
I-5	1.0	6.0
I-8	0.7	2.8
I-10	0.2	1.8

Carbohydrates were extracted from leaf and shoot tissue and resolved by HPLC as described in Experimental procedures. Leaf and shoot material from four Kan^R T_1 progeny seedlings was pooled for each sample. ND, not detected; FW, fresh weight. Quantifiable detection limit is approximately 25 pmol per injection, which for these samples was approximately 0.0002 μmol g^{-1} FW. In the I-2 sample, a smaller peak which could not be integrated was visible on traces.

As observed with IMT1 enzyme activity measurements, amounts of ononitol varied between transgenic lines. There was a general correlation between IMT1 activity detected in crude extracts (Figure 2) and accumulation of product *in vivo*. For example, I-2 seedlings, which did not contain detectable IMT1 activity, did not accumulate ononitol to significant levels, while I-5 seedlings accumulated the most ononitol on a per gram of fresh weight basis. Levels of ononitol were lower (in relation to sucrose levels) in these T_1 seedlings than in leaves of mature plants (see Figure 3b). Regardless, ononitol was a major carbohydrate constituent of the I-4, I-5, I-8, and I-10 seedlings, accumulating to levels ranging from 7.5% (I-4) to 25% (I-8) the levels of sucrose in these plants (Table 1). Sucrose levels per gram of fresh weight varied greatly between T_1 lines; however, there was no apparent relationship between sucrose levels and variation in ononitol levels.

Discussion

Cyclic sugar alcohols accumulate to high levels in a number of drought- and salinity-tolerant plant species. The chemical properties and metabolically inert nature of these compounds make them ideally suited to serve as cytoplasmically compatible solutes in environmentally stressed tissues. We have introduced a gene encoding a cyclitol biosynthesis enzyme from a facultative halophyte, *M. crystallinum*, into a glycophyte, tobacco. This gene, *Imt1*, encodes a position-specific *myo*-inositol *O*-methyl transferase that, in *M. crystallinum*, is dramatically induced by both salt stress and low temperature (Vernon and Bohnert, 1992; Vernon *et al.*, 1993).

The introduction of the *Imt1* gene into tobacco created a novel branchpoint in carbohydrate metabolism in this plant. The production in tobacco of another non-native carbohydrate, mannitol, was recently reported (Tarczynski

et al., 1992). As with the ononitol-producing plants described here, tobacco that accumulated mannitol did not display any obvious phenotypic differences from wild-type controls under standard growth conditions. These results, taken together, indicate that new pathways that produce novel carbohydrates can be accommodated by plants, and suggest that there is a certain degree of flexibility, and therefore room for genetic manipulation, in plant carbohydrate metabolism.

With the identification of IMT1 from *M. crystallinum*, the first cyclitol biosynthesis enzyme has become available for detailed studies. The introduction of the *Imt1* gene into tobacco has clarified certain aspects of the biosynthetic pathway of pinitol, the most common methyl *myo*-inositol. For example, the identification of ononitol as the IMT1 product (Figures 3, 5, and 6), along with the observation that neither sequoyitol nor bornesitol are detected in salt-stressed *M. crystallinum* (Adams and Vernon, unpublished data), supports a role for ononitol as an intermediate in pinitol biosynthesis (see Figure 4). This is in agreement with the pathway proposed by Dittrich and Brandl (1987), who suggested that ononitol, and not sequoyitol (Loewus and Dickinson, 1982; Ruis and Hoffmann-Ostenhof, 1969), was the precursor to pinitol, at least in most angiosperms.

The expression of IMT1 in tobacco has also shed light on a second aspect of pinitol biosynthesis, the epimerization of ononitol to pinitol. Although some studies of plant cyclitol epimerases have been carried out (Hoffmann-Ostenhol *et al.*, 1978), specific enzymes have not been identified, nor have co-enzyme requirements been established (Loewus and Dickinson, 1982). Our own observations of pinitol biosynthesis in *M. crystallinum* have indicated that the conversion of ononitol to pinitol is slow (possibly taking days), suggesting that the epimerization of ononitol to pinitol is inefficient, perhaps being carried out by a non-specific enzyme. The accumulation of ononitol and the absence of any detectable pinitol in transgenic tobacco expressing active IMT1 suggests that the conversion of ononitol to pinitol normally seen in *M. crystallinum* is carried out by an epimerase not present in tobacco leaf tissue.

It has been suggested (Binzel *et al.*, 1988) that the difference in stress tolerance between halophytes and glycophytes may be due chiefly to quantitative differences in mechanisms common to both types of plants, such as vacuolar ion transport capabilities. The absence in tobacco of detectable inositol methyl transferase and epimerase activities suggests that this glycophyte is lacking both components of a cyclitol biosynthesis pathway that is present in leaves of the halophyte *M. crystallinum*. It appears, then, that pathways specific to halophytes may also contribute to osmotolerance in halophytic species.

The transgenic system described here will serve as a useful model to investigate the role of cyclitols in providing protection from environmental stress. The production of ononitol in tobacco will allow direct assessment of the ability of cyclitols to enhance tolerance to osmotic stress in a normally glycophytic species. Although a role in osmotic adjustment would require that cyclitols accumulate to high enough concentration to contribute significantly as cytoplasmic osmolytes, these compounds would likely be able to function as macromolecular protectants when present at much lower levels. Assuming a strictly cytoplasmic localization, the accumulation of ononitol observed in T_1 seedlings (Table 1) can be estimated (Tarczynski *et al.*, 1992) to reach intracellular concentrations of 50–100 mM in the I-5 and I-8 transgenic lines. Accumulation of cyclitols to this level may be sufficient to provide significant protection against exposure to high salinity (Tarczynski *et al.*, 1993). Preliminary experiments indicate that ononitol levels are considerably higher in leaf tissue of mature plants than in seedlings (see Figure 3), probably due to the inability of tobacco to catabolize the compound. Mature transformants may therefore represent the most attractive system for evaluating the ability of ononitol to provide protection from osmotic stress.

Experimental procedures

Plasmid construction

Standard techniques were used for gene construction (Maniatis *et al.*, 1982). A 1.3 kbp *Pst*I–*Eco*RV fragment of the *M. crystallinum Imt1* cDNA containing the entire IMT1 coding region (Vernon and Bohnert, 1992) was subcloned into the *Pst*I-*Sma*I site of a modified pJIT117 vector (Guerineau *et al.*, 1988), from which the transit peptide coding sequence had been deleted by digestion with *Hin*dIII and *Sph*I, followed by blunt-ending with T4 DNA polymerase and religation. The vector containing *Imt1*, pJitImt1, thus contained a 35S CaMV promoter, the *Imt1* structural gene, and the CaMV polyadenylation signal. A *Kpn*I fragment from pJitImt1 containing the gene construction was then subcloned into the disarmed binary vector Bin19 (Bevan, 1984) for transformation into tobacco.

Plant material

Agrobacterium-mediated plant transformation and subsequent regeneration of tobacco (*Nicotiana tabacum*, cv. SR1) were performed essentially as described (Tarczynski *et al.*, 1992). Primary transformants (T_0) were allowed to flower and were selfed. Seeds from five distinct selfed primary transformants were germinated on rooting medium containing kanamycin. Ratios of Kan^R to kanamycin-sensitive progeny indicated that the various primary T_0 transformants contained from one to three unlinked inserts. Surviving Kan^R seedlings (T_1) were transferred to soil and grown under conditions previously described (Tarczynski *et al.*, 1992) for use in carbohydrate analyses and IMT1 activity assays. Wild-type SR1 was used for controls.

IMT1 activity

Leaf soluble proteins were extracted and IMT1 activity was measured as described previously (Vernon and Bohnert, 1992). Briefly: proteins were extracted from leaf tissue of F_1 plants according to Ostrem *et al.* (1987) in buffer containing 150 mM Tris-HCl, pH 8, 100 mM NaCl, 20 mM EDTA, 5 mM leupeptin and 10 mM β-mercaptoethanol. Assays were carried out in a 200 μl volume consisting of 50 mM Tris-HCl pH 8, 10 mM $MgCl_2$, 10 mM *myo*-inositol (except in controls where indicated in the Results section), and S-adenosyl-L-methionine (SAM) at a concentration of 0.5 mM. SAM stock solution contained unlabeled SAM (Sigma Chemicals, St. Louis, MO) and ^{14}C-methyl SAM (45 mCi $mmol^{-1}$; ICN Biochemicals, Irvine, CA) at a 25:1 ratio. Assays were carried out at 30°C for 2 h and terminated by transfer to ice and phenol extraction. Remaining ^{14}C-SAM was removed by desalting column (AG50WX4; BioRad, Richmond, CA), and samples were subjected to HPLC analysis (see below). Fractions were collected at 0.5-min intervals and scintillation counting was used to determine ^{14}C content and product retention time.

Carbohydrate analysis

Soluble carbohydrates were extracted and HPLC analyses were carried out using a 300 × 7.8 mm HPX87C calcium-form partition-exchange column (BioRad, Richmond, CA) as described previously (Vernon and Bohnert, 1992), with the exception that columns were run at 30°C rather than 85°C. The lower temperature allowed the resolution of ononitol from pinitol (see Results), which co-migrates with ononitol at 85°C. Standards were resolved under identical conditions. Traces were obtained using a pulsed amperometric detector (Dionex, Sunnyvale, CA) at 35°C. A Spectrophysics SP4290 integrator (Spectrophysics Analytical, San Jose, CA) was used for determination of retention time and peak areas. Ononitol levels were quantified by comparison of peak areas to those of standards of known concentration (Tarczynski *et al.*, 1992).

For NMR analysis, ononitol was purified from leaf tissue of transformants by HPLC, and washed extensively in ^2H_2O. The NMR spectrum was obtained using a WM-250 spectrometer (Bruker Instruments, Billerica, MA).

Acknowledgments

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References

Bevan, M. (1984) Binary *Agrobacterium* vectors for plant transformation. *Nucl. Acids Res.* **12**, 8711–8721.

Bieleski, R.L. (1982) Sugar alcohols. In *Encyclopedia of Plant Physiology: Plant Carbohydrates I – Intracellular Carbohydrates*, Volume 13A (Loewus, F.A. and Tanner, W., eds). Berlin: Springer-Verlag, pp. 158–192.

Binzel, M.L., Hess, F.D., Bressan, R.A. and Hasegawa, P.M. (1988) Intracellular compartmentation of ions in salt adapted tobacco cells. *Plant Physiol.* **86**, 607–614.

Csonka, L.N. and Hanson, A.D. (1991) Prokaryotic osmoregulation: genetics and physiology. *Ann. Rev. Microbiol.* **45**, 569–606.

Dittrich, P. and Brandl, A. (1987) Revision of the pathway of D-pinitol formation in leguminasae. *Phytochemistry*, **26**, 1925–1926.

Dittrich, P. and Korak, A. (1984) Novel biosynthesis of D-pinitol in *Simmondsia chinensis*. *Phytochemistry*, **23**, 65–66.

Ford, C.W. (1982) Accumulation of *O*-methyl inositols in water-stressed *Vigna* species. *Phytochemistry*, **21**, 1149–1151.

Ford, C.W. (1984) Accumulation of low molecular weight solutes in water-stressed tropical legumes. *Phytochemistry*, **23**, 1007–1015.

Guerineau, F., Woolston, S., Brooks, L. and Mullineaux, P. (1988) An expression cassette for targeting foreign proteins into chloroplasts. *Nucl. Acids Res.* **16**, 11380.

Hanson, A.D., Rathinasabapathi, B., Chamberlin, B. and Gage, D.A. (1991) Comparative physiological evidence that β-alanine betaine and choline-*O*-sulfate act as compatible osmolytes in halophytic *Limonium* species. *Plant Physiol.* **97**, 1199–1205.

Hellebust, J.A. (1976) Osmoregulation. *Ann. Rev. Plant Physiol.* **27**, 485–505.

Hoffmann-Ostenhoff, O., Pittner, F. and Koller, F. (1978) Some enzymes of inositol metabolism, their purification and their mechanism of action. In *Cyclitols and Phosphoinositides*, (Wells, W.W. and Eisenberg, F., eds). New York: Academic Press, pp. 233–247.

Loewus, F. and Dickinson, D.B. (1982) Cyclitols. In *Encyclopedia of Plant Physiology: Plant Carbohydrates I: Intracellular Carbohydrates*, Volume 13A (Loewus, F.A. and Tanner, W., eds). Berlin: Springer-Verlag, pp. 193–206.

Loewus, F. and Loewus, M. (1980) Biochemistry of *myo*-inositol. In *Biochemistry of Plants*, Volume 3 (Stumpf, P. and Conn E., eds). New York: Academic Press, pp. 43–76.

Loewus, F. and Loewus, M. (1983) *Myo*-inositol: Its biosynthesis and metabolism. *Ann. Rev. Plant Physiol.* **34**, 137–161.

Maniatis, T., Fritsch, E. and Sambrook, J. (1982) *Molecular Cloning, A Laboratory Manual.* Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

McCue, K.F. and Hanson, A.D. (1990) Drought and salt tolerance: towards understanding and application. *Trends Biotech.* **8**, 358–362.

Nguyen, A. and Lamant, A. (1988) Pinitol and *myo*-inositol accumulation in water-stressed seedlings of maritime pine. *Phytochemistry*, **27**, 3423–3427.

Ostrem, J.A., Olsen, S.W., Schmitt, J.M. and Bohnert, H.J. (1987) salt stress increases the level of translatable mRNA for phosphoenolpyruvate carboxylase in *Mesembryanthemum crystallinum*. *Plant Physiol.* **84**, 1270–1275.

Paul, M.J. and Cockburn, W. (1989) Pinitol, a compatible solute in *Mesembryanthemum crystallinum* L? *J. Exp. Bot.* **40**, 1093–1098.

Popp, M. (1984) Chemical composition of Australian mangroves II. Low molecular weight carbohydrates. *Z. Pflanzenphysiol.* **113**, 411–421.

Ruis, H. and Hoffmann-Ostenhoff, O. (1969) Enzymatic epimerization of sequoyitol to D-pinitol in *Trifolium incarnatum*. *European J. Biochem.* **7**, 442–448.

Schobert, B. (1977) Is there an osmotic regulatory mechanism in algae and higher plants?, *J. Theor. Biol.* **68**, 17–26.

Tarczynski, M.C., Jensen, R.G. and Bohnert, H.J. (1992) Expression of a bacterial mt1D gene in transgenic tobacco leads to production and accumulation of mannitol. *Proc. Natl Acad. Sci. USA*, **89**, 2600–2604.

Tarczynski, M.C., Jensen, R.G. and Bohnert, H.J. (1993) Stress protection in transgenic tobacco producing a putative osmoprotectant, mannitol. *Science*, **259**, 508–510.

Vernon, D.M. and Bohnert, H.J. (1992) A novel methyl transferase induced by osmotic stress in the facultative halophyte *M. crystallinum. EMBO J.* **11**, 2077–2085.

Vernon, D.M., Ostrem, J.A. and Bohnert, H.J. (1993) Stress perception and response in a facultative halophyte: the regulation of salinity-induced genes in *Mesembryanthemum crystallinum. Plant Cell Environ.* in press.

Warr, S.R.C., Reed, R.H. and Stewart, W.D.P. (1988) The compatibility of osmotica in cyanobacteria. *Plant Cell Environ.* **11**, 137–142.

Wyn Jones, R.G. and Gorham, J. (1983) Osmoregulation. In *Encyclopedia of Plant Physiology: Responses to the Environment*, Volume 12C, (Lange, O.L., Nobel, P.S., Osmond, C.B., and Ziegler, H., eds). Berlin: Springer-Verlag, pp. 35–58.

Yancey, P., Lark, M., Hand, S., Bowlus, R. and Somero, G. (1982) Living with water stress: evolution of osmolyte systems. *Science*, **217**, 1214–1222.