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Expression of a bacterial *mtlD* gene in transgenic tobacco leads to production and accumulation of mannitol

(metabolic engineering/polyol/compatible solute/sugar alcohol)

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ABSTRACT **A bacterial gene encoding mannitol-1-phosphate dehydrogenase, *mtlD*, was engineered for expression in higher plants. Gene constructions were stably incorporated into tobacco plants. The *mtlD* gene was expressed and translated into a functional enzyme in tobacco, resulting in the synthesis and accumulation of mannitol, which was identified by NMR and mass spectroscopy. Mannitol concentrations exceeded 6 μmol/g (fresh weight) in the leaves and in the roots of some transformants, whereas this sugar alcohol was not detected in these organs of wild-type tobacco plants or of untransformed tobacco plants that underwent the same regeneration scheme. These experiments demonstrate that branch-points in plant carbohydrate metabolism can be generated by which novel gene products can utilize endogenous substrates to divert metabolic energy into novel compounds. Additionally, the system described here allows for physiological studies in which the responses of wild-type and transgenic tobacco to various environmental stimuli can be compared directly. Such studies will facilitate our understanding of the roles of sugar alcohols (e.g., in stress tolerance) in higher plants.**

Metabolic engineering (1) was examined as an approach to gain insight into the roles of sugar alcohols or polyols in higher plants. These simple carbohydrates are widely distributed among organisms including bacteria, algae, fungi, higher plants, insects, and mammals (2–5). In plants, the importance of sugar alcohols is reflected by the estimation that metabolism of these compounds, rather than that of sugars, contributes to about 30% of the annual global primary carbon production (4). Some sugar alcohols, notably mannitol and sorbitol, are major photosynthetic products of, and can accumulate to high levels in, various higher plant species (3, 4, 6). These sugar alcohols are relatively widespread in higher plants, with mannitol, for example, having been detected in over 50 families (3). Mannitol and sorbitol are also translocated by some higher plant species (e.g., ref. 7), indicating a role as a storage compound. Other sugar alcohols, such as dulcitol and ribitol, are considerably less prevalent in higher plants and are apparently characteristic of species representing only a few families (3, 4).

The functions of sugar alcohols in higher plants (and organisms in general) are not clear. A commonly held belief is that these compounds may confer beneficial traits on those species where they are found, rather than being simply intermediates of carbohydrate metabolism. Suggested physiological roles for sugar alcohols include osmoregulation (3, 8) and service as compatible solutes (5, 9), storage of reduced carbon and energy (3), regulation of coenzymes (3, 10), and neutralization of hydroxyl radicals (11). Evidence supporting these roles for sugar alcohols has been obtained primarily with fungi and animals; only limited studies exist regarding the functions of these compounds in higher plants (12–17).

It seemed possible, by means of gene engineering, to attempt genetic alterations in carbohydrate metabolic pathways that would result in the partial diversion of carbon and energy flow from metabolites recognized by the host organism to products foreign to the organism. Gene engineering of higher-plant carbohydrate metabolism has been virtually unexplored, with the exception of the overproduction of a yeast invertase (18–20).

In this report, we demonstrate the stable introduction of the *Escherichia coli mtlD* gene into tobacco. This gene encodes mannitol-1-phosphate dehydrogenase (EC 1.1.1.17) and its expression in *E. coli* leads to mannitol catabolism. We show that mannitol-1-phosphate dehydrogenase functions anabolically in transgenic tobacco, resulting in the production and the accumulation of mannitol in these plants. This sugar alcohol was not detected in control plants. These data indicate that basic changes in native carbohydrate metabolic pathways of higher plants can lead to the production of novel compounds. Additionally, the system described here will provide insight into sugar-alcohol function in higher plants by allowing the direct comparison of various physiological responses from transgenic plants with those of control plants.

MATERIALS AND METHODS

Plasmid Construction. Standard techniques were used for gene construction (21). The 1.5-kilobase-pair (kbp) *Nsi* I–*Pst* I fragment containing the coding region for the *E. coli mtlD* gene and a 150-bp untranslated leader from plasmid pCD7.5 (22) was subcloned into the *Pst* I site of the expression vector pUC8CaMVCAT [unpublished, but similar to pCaMVNEO (23)]. The resulting plasmid, pCaMVMTLDL, thus contained a 35S cauliflower mosaic virus promoter (P_{CaMV}), the *mtlD* structural gene with a 5′ leader, and a nopaline synthase termination signal (T_{NOS}). In a second construction, the 150-bp 5′ untranslated leader of *mtlD* was decreased to 15 bp by blunt-ended ligation of the *Ava* I–*Pst* I fragment of pCaMVMTLDL to the *Pst* I site of pUC8CaMVCAT, creating pCaMVMTLDS. A similar set of expression vectors (containing the *mtlD* gene) was constructed as above, except that expression was under the control of the nopaline synthase promoter (P_{NOS}) of the expression vector pNOSCAT-1 [unpublished, but similar to pNOSCAT (24)], creating pNOSMTLDL and pNOSMTLDS. The *Hin*dIII fragments of pCaMVMTLDL(S) and the *Hin*dIII–*Eco*RI fragments of pNOSMTLDL(S), respectively, were subcloned into the disarmed binary vector Bin19 (25).

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Abbreviations: P_{CaMV}, 35S promoter of cauliflower mosaic virus; P_{NOS}, nopaline synthase promoter; fwt, fresh weight; HPAE–PAD, high-performance anion-exchange chromatography coupled to pulsed amperometric detection.

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Plant Transformation and Regeneration. The Bin19 expression vectors (see above) were separately introduced in *Agrobacterium tumefaciens* (strain LBA-4404) via triparental mating (25). Tobacco (*Nicotiana tabacum* cv. SR1) transformation and regeneration were performed as described (26, 27). Following rooting and selection by kanamycin in axenic culture, some plantlets were transferred to soil and greenhouse-cultured (21/24°C night/day temperature), whereas others were assayed directly for carbohydrates (see below). Plants in soil were watered daily and supplemented weekly with Hoagland's nutrient solution. Primary transformants (F_0) containing P_{CaMV} gene constructs were allowed to flower, and the transformants were then selfed. Seeds (n = 100) from the selfed transformants were selected by resistance to kanamycin in root-inducing medium, and surviving plantlets (F_1) were transferred to soil and cultured as before. This cycle was repeated with F_1 plants to obtain F_2 transformants. In all experiments, SR1 plants (original stock) and regenerated, untransformed plants derived from SR1 were used as controls.

Tissue Harvesting. Leaf tissue was obtained primarily from F_0 plants following rooting in axenic culture and from F_0 plants cultivated for 6 weeks in a greenhouse. Plants in culture were 5–6 cm high, and excised leaves from these plants were 2–3 cm long. Samples of a given greenhouse-grown F_0 plant were taken from five different leaves, representing different developmental stages along the central axis of the plant. In all cases, following sampling, leaf tissue was immediately frozen and stored in liquid nitrogen until carbohydrate analysis. Leaf samples from F_1 and F_2 plants were treated and harvested similarly, except that only two leaf samples per greenhouse-grown plant were harvested—i.e., expanding (6–7 cm long) and fully expanded (19–21 cm long). These experiments were performed to compare with data obtained from F_0 plants.

In experiments with root tissue, F_2 plants transformed with pCaMVMTLDL were selected by resistance to kanamycin in sterile culture. Immediately following rooting, plantlets were transferred to a hydroponics system. Plants were then cultured for six weeks in a growth room [21/24°C night/day temperature; light, 500 $\mu E \cdot m^{-2} \cdot s^{-1}$; 1 E (einstein) = 1 mol of photons]. The hydroponics system consisted of Hoagland's nutrient solution (5 liters) in a light-tight container with a small hole in the lid for plantlet insertion. The nutrient solution was changed weekly under darkened conditions. Root material (30 cm long) was harvested and immediately frozen as either whole, intact roots or as 10-cm segments taken along the length of the root.

Carbohydrate Extraction. Carbohydrates were extracted from leaf tissue and from root tissue by grinding 100–300 mg [fresh weight (fwt)] of the frozen tissue in a Ten-Broeck homogenizer containing 1–3 ml of methanol/chloroform/water, 12:5:3 (vol/vol), at 4°C. Tissue samples greater than 300 mg were ground in liquid nitrogen prior to extraction. An equal volume of water (4°C) was then added to the extract. The extract was centrifuged at ≈1000 × g for 5 min, and 100 μl of supernatant was collected and vacuum dried. The resulting pellet was resuspended in 100 μl of water and passed through a C_{18} solid-phase extraction cartridge (Alltech Associates) to remove hydrophobic compounds. The cartridge was washed once with purified water and the two fractions were combined.

Soluble Carbohydrate Analysis. Carbohydrates were separated by high-performance anion-exchange chromatography coupled to pulsed amperometric detection (HPAE–PAD; ref. 28). Samples were injected into a HPAE–PAD system (SP8700 pump; Spectra-Physics) by a model 7125 injector with a 100-μl sample loop (Rheodyne, Cotati, CA). The system was operated isocratically at 1.0 ml/min with degassed 0.15 M NaOH as the mobile phase. Separation of the sugars was achieved by a CarboPac PA-1 analytical column (250 mm × 4 mm i.d.; Dionex) maintained at 25°C. Detection was by a Dionex pulsed amperometric detector. A Spectra-Physics SP4290 integrator was used for quantification of peak area and determination of retention time. Authentic sugars were used as standards.

NMR Analysis. Mannitol was collected from leaf tissue of four F_0 plants (axenically grown) transformed with pCaMVMTLDL, following sugar extraction and HPAE separation. The NMR spectrum for mannitol (200 μg) was obtained in ^2H_2O with a WM-250 spectrometer (Bruker Instruments, Billerica, MA).

RESULTS

Vector Construction and Transformation of Tobacco. Four genes were constructed for plant transformation (Fig. 1). The gene elements that distinguished the different constructions were the promoter element (P_{CaMV} or P_{NOS}), and the length of the bacterial 5′ untranslated leader sequence. Promoter selection was based on transcriptional efficiency. All constructions contained the unaltered bacterial *mtlD* coding sequence and an identical terminator element, T_{NOS}. Tobacco was transformed with plasmids containing these constructions, and transformants were selected by resistance to kanamycin. Visual examination of plants resistant to kanamycin (≈200 total) indicated no apparent phenotypic differences as compared with control plants (≈40 total) during regeneration in axenic culture. In some experiments, RNA was extracted from leaf material of untransformed and transformed plants. Only transformed plants showed a hybridization signal to a single band of the expected length (≈1350 bp) after hybridization of the radioactively labeled bacterial gene probe to filters containing leaf RNA (data not shown).

Mannitol Detection in Transgenic Tobacco. Leaf material (2–3 cm long) from young, axenically grown plants, which were transformed with constructions containing P_{CaMV} and selected by kanamycin resistance, was extracted. The sugars were then separated by HPAE–PAD. Fig. 2 compares the soluble carbohydrate profile of an extract from an untransformed regenerated plant (control, profile A) with that of a transformant (profile C). Authentic mannitol (M) added to the control leaf extract identified the migration position for this sugar alcohol (profile B). The carbohydrate profile of the leaf extract from transformed tobacco showed a peak with a retention time identical to that for mannitol (cf. M in profiles

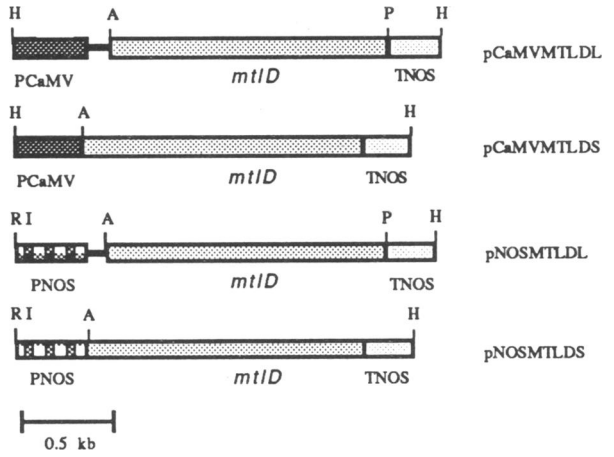

FIG. 1. Gene constructions. For use in plant transformation, four plasmids (pCaMVMTLDL, pCaMVMTLDS, pNOSMTLDL, and pNOSMTLDS) were constructed in which the promoter element and the length of the 5′ leader were varied. Black bar, authentic leader region of the *E. coli mtlD* gene; A, *Ava* I; H, *Hin*dIII; P, *Pst* I; RI, *Eco*RI; kb, kilobase; TNOS, nopaline synthase terminator.

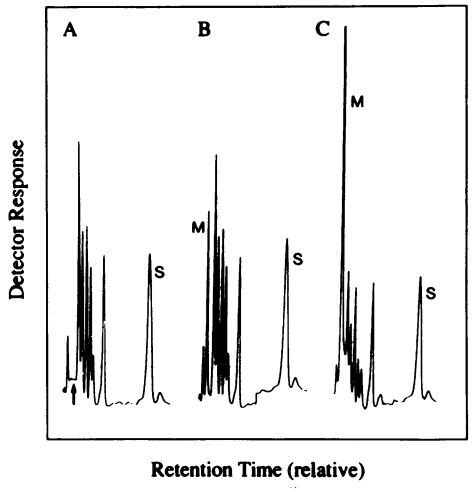

FIG. 2. HPAE–PAD separation of soluble carbohydrates extracted from leaf material of transgenic tobacco. Extracts were of leaf material (100 mg, fwt) from young, axenically grown plants. Profiles: A, leaf extract from an untransformed, regenerated plant (arrow indicates migration time of mannitol, ≈2.30 min); B, same as A, except that authentic mannitol (0.25 nmol) was added to the extract; C, leaf extract from a plant transformed with pCaMVMTLDL. M, mannitol; S, sucrose.

B and C). No peak corresponding to the retention time for mannitol was detected in extracts of control leaves (profile A, arrow). Because mannitol is an indirect product of the reaction catalyzed by mannitol-1-phosphate dehydrogenase, we confirmed by NMR (Fig. 3) and mass spectroscopy (data not shown) that the peak corresponding to the retention time for mannitol in extracts of transformants was mannitol. Mannitol was detected in leaf extracts of virtually all primary transformants examined (40 total), whereas the sugar alcohol was not detected in leaf extracts of control plants (30 plants examined).

For the transformant represented in Fig. 2, the mannitol content in the leaf sample was 3.8 μmol/g (fwt) and was >25% of the total peak area; the sucrose content of the leaf was lower, at 2.3 μmol/g (fwt). The mannitol contents of leaf material in young plants transformed with P_{CaMV} gene constructs were variable and ranged from <1 μmol/g (fwt) to >6 μmol/g (fwt) (n = 20). A comparison of profiles A and C of Fig. 2 indicated that these two extracts were qualitatively similar, except for the presence of mannitol. We observed no appreciable differences in the levels of mannitol with regard to the length of the bacterial 5′ leader (data not shown).

HPAE–PAD analyses of leaf material from 10 transformants containing P_{NOS} showed that with this promoter, mannitol levels were generally either not detected or 2- to 3-fold lower than in the transformants containing P_{CaMV} (data not shown). Because the higher levels of mannitol observed with P_{CaMV} gene constructs did not appear to affect tobacco adversely, we report on transgenic plants containing this promoter.

Mannitol Accumulation in Transgenic Tobacco. Primary transformants with P_{CaMV} gene constructs were transferred to soil and cultured in a greenhouse for 6 weeks. Fig. 4 shows typical levels found for mannitol and for sucrose in tobacco leaf tissues harvested at various developmental stages (as a function of leaf size). Mannitol contents were severalfold lower than those of sucrose at all leaf developmental stages. However, the trends in the levels of mannitol and sucrose among the different leaf developmental stages were similar; i.e., mannitol and sucrose levels were higher in younger, sink leaves and lower in the more mature, source leaves (fwt basis). In no case was mannitol detected in leaves of control plants.

Leaf and root samples from three F_2 plants transformed with pCaMVMTLDL and cultured for 6 weeks in a hydroponics system were harvested and extracted. Extracts were then analyzed by HPAE–PAD and quantified. A comparison of the mannitol contents from expanding leaves (6–7 cm long) and from fully expanded leaves (19–21 long) (Table 1) indicated that the trend observed for mannitol accumulation in leaves of F_0 plants (Fig. 4) also occurred in subsequent generations (data not shown for F_1 plants). Mannitol was not

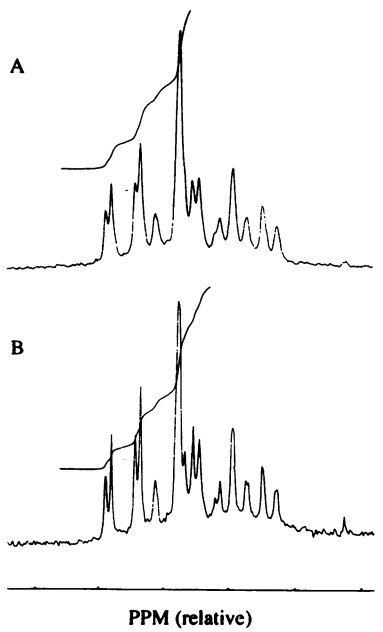

FIG. 3. ^1H NMR spectrum of mannitol isolated from leaf tissue of transgenic tobacco plants. About 200 μg of material corresponding to the retention time for mannitol was analyzed in ^2H_2O. Spectrum A, material isolated from leaf extract; spectrum B, authentic mannitol standard (250 μg).

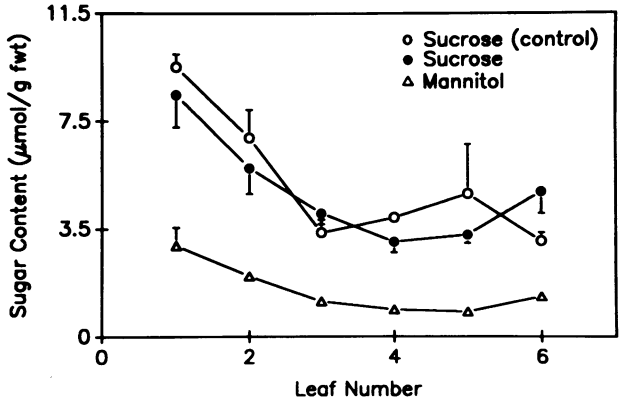

FIG. 4. Mannitol and sucrose contents of leaf material harvested from transgenic tobacco during different developmental stages. Extracts of soluble sugars were prepared from leaves of untransformed, regenerated plants or from leaves of plants transformed with P_{CaMV} gene constructs. Leaf material (100 mg, fwt) was harvested from 6-week-old soil-grown plants, following regeneration by tissue culture. Separation of soluble carbohydrates was by HPAE–PAD. Quantitation of the sugars was by peak integration and comparison with sugar standards. Young leaves (≈3 cm long) were harvested from the plant apex, while more mature leaves were excised from lower down the plant. Leaf number corresponds to the leaf position along the central axis (1 = apex, 6 = bottom). Approximate leaf lengths: leaf 1, 3 cm; leaf 2, 7 cm; leaf 3, 10 cm; leaf 4, 20 cm; leaf 5, 30 cm; leaf 6, 20 cm. Values are the means ± SEs (n = 2, control; n = 4, experimental).

Table 1. Mannitol in leaf and root tissues of F_2 plants transformed with pCaMVMTLDL

Tissue	Concentration, μmol/g (fwt)	
	Mannitol	Sucrose
Leaf (≈7 cm long)	3.76 ± 0.24	4.08 ± 0.14
Leaf (≈20 cm long)	1.05 ± 0.02	2.13 ± 0.10
Root A*	1.02 ± 0.17	1.23 ± 0.23
Root B	1.22 ± 0.11	1.48 ± 0.28
Root C	1.20 ± 0.16	1.46 ± 0.19

Values are means ± SEs (n = 3).
*Root tissue was divided into three 10-cm segments along the length of the root; A, increment closest to root/shoot interface.

detected in extracts of root material from control plants (data not shown); however, this sugar alcohol was similar in concentration to sucrose in root extracts of transgenic plants (Table 1). Mannitol represented up to 25% (area basis) of the total soluble carbohydrate profile obtained for root material from transgenic plants. No gradient in mannitol or sucrose accumulation was observed along the length of the roots (fwt basis).

DISCUSSION

Metabolic engineering is an approach by which cellular activities can be altered through the use of recombinant DNA technology (1). It seemed possible that this approach could be used to evaluate an apparent environmental adaptation in a nonadapted organism. To examine this possibility, we applied metabolic engineering toward evaluating the roles of sugar alcohols in higher plants by introducing the *E. coli* gene *mtlD* into tobacco. As a result of this basic genetic manipulation in tobacco, reduced carbon and cellular energy (i.e., NADH) were partially diverted to produce mannitol. To our knowledge, this sugar alcohol has not previously been detected in tobacco. This study demonstrates that branchpoints in higher-plant carbohydrate metabolism can be generated by which novel gene products can utilize endogenous substrates to divert metabolic energy into novel compounds.

In *E. coli, mtlD* expression leads to mannitol catabolism. The pathway involves phosphorylation of mannitol to mannitol 1-phosphate, which is oxidized to fructose 6-phosphate by the *mtlD* product (Fig. 5). Fructose 6-phosphate then enters glycolysis. In transgenic tobacco, however, *mtlD* expression leads to mannitol biosynthesis. Cytoplasmic fructose 6-phosphate and NADH of tobacco are utilized by mannitol 1-phosphate dehydrogenase to form mannitol 1-phosphate and NAD^+. The accumulation of significant levels of mannitol in transgenic tobacco indicates that a phosphatase, probably a nonspecific enzyme, is present and results in mannitol 1-phosphate dephosphorylation. Although mannitol metabolism in higher plants is generally not well understood, the pathway for mannitol biosynthesis in celery has been characterized (29). In this plant, mannitol is produced by the reactions of mannose-6-phosphate isomerase, mannose-6-phosphate reductase, and mannitol-1-phosphate phosphatase. Thus, mannitol biosynthesis in celery proceeds via a different pathway, which requires an additional enzyme-catalyzed step, than that in transgenic tobacco.

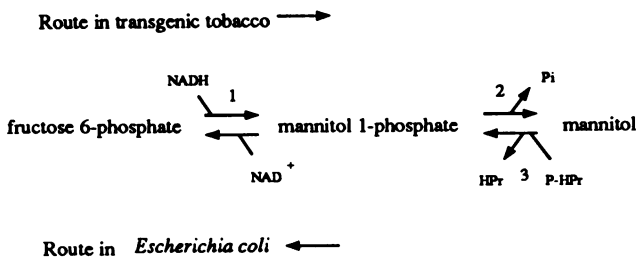

FIG. 5. Mannitol metabolism in *E. coli* and transformed tobacco plants. Arrows: 1, mannitol-1-phosphate dehydrogenase; 2, nonspecific phosphatase(s); 3, mannitol-specific enzyme II. P-HPr, phosphorylated heat-stable protein.

The fate of mannitol in transgenic tobacco is unknown; however, several possibilities exist: (*i*) mannitol is oxidized to fructose 6-phosphate or to fructose, (*ii*) mannitol is translocated via the phloem, or (*iii*) mannitol is a "dead-end" product. Although mannitol is presumably synthesized in the cytoplasm of transgenic tobacco, the subcellular location of the sugar alcohol has not been proven.

All gene constructions resulted in mannitol production in transgenic tobacco. Mannitol concentrations were typically greater in leaves of young plants transformed with P_{CaMV} constructions than in corresponding leaves of plants transformed with P_{NOS} constructions. This finding may be a result of the increased transcriptional efficiency associated with P_{CaMV} in young leaf tissue (30). Mannitol accumulated in all leaves examined of 6-week-old plants transformed with P_{CaMV} (Fig. 4, Table 1). On the basis of fwt, the levels of mannitol (and sucrose) decreased in the more mature leaves. However, in some experiments where leaf soluble protein was measured, expression of mannitol on this basis indicated that the sugar alcohol and sucrose levels were relatively constant in leaves of different ages, which supports the presumption that this sugar alcohol is localized in the cytosol. The accumulation of mannitol observed in roots of plants transformed with P_{CaMV} constructs suggested that the introduced mannitol-1-phosphate dehydrogenase is also functional in this organ.

The introduction of mannitol biosynthesis in tobacco may lead to predictable physiological responses associated with this sugar alcohol (e.g., mitigation of water stress). Although few studies exist regarding the function of mannitol (or sugar alcohols in general) in higher plants, there are indications that mannitol may contribute to osmotic adjustment (31) or may serve as a compatible solute (13, 14). Studies of sugar alcohols in general have shown that these compounds accumulate in many higher plant species in response to water or salt stress (see ref. 8 for review). Furthermore, *in vitro* experiments with enzyme extracts of higher plants have indicated that sugar alcohols may alleviate the effects of temperature on protein lability (14, 15). However, such physiological experiments, by their design, rely on pathways that are endogenous to the plants studied. The results can give only correlative, circumstantial evidence for the role of sugar alcohols in, for example, environmental stress protection. The tobacco model system described here makes possible the evaluation of the significance of sugar-alcohol production in plants, because tobacco has stress responses typical of glycophytes.

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