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Inhibition of Trehalase Activity Enhances Trehalose Accumulation in Transgenic Plants

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As a first step toward the exploitation of the disaccharide trehalose as a stress-protective and preservative agent in plants, we engineered trehalose biosynthesis in tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) by introducing the *otsA* and *otsB* genes from *Escherichia coli*, which encode trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, respectively. In leaves of transgenic tobacco plants, very low levels of trehalose accumulation were obtained (0.11 mg g^{-1} fresh weight), whereas in transgenic potato tubers, no trehalose accumulated at all. Plant trehalase activity was shown to affect the accumulation of trehalose in these plants. An increase in trehalose accumulation, up to 0.41 and 4.04 mg g^{-1} fresh weight in tobacco leaves and potato microtubers, respectively, was noted when the potent trehalase inhibitor validamycin A was added to in vitro plants and to hydroponically grown greenhouse plants. Stunted growth and the formation of lancet-shaped leaves by trehalose-accumulating tobacco plants suggest a negative effect of trehalose biosynthesis on *N. tabacum* development. It is surprising that experiments with wild-type plants cultured in the presence of validamycin A indicate that, despite current belief, the capacity to synthesize trehalose may not be restricted to primitive phyla of vascular plants and certain "resurrection plants," but may exist throughout the angiosperms.

The nonreducing disaccharide trehalose (α-D-glucopyranosyl-[1–1]-α-D-glucopyranoside) is commonly found in a wide variety of fungi, bacteria, yeasts, and algae, as well as in some invertebrates and vascular plants (Elbein, 1974). Its chemical structure resembles that of Suc but its physical properties are very different, trehalose being only slightly sweet and not hydrolyzing to reducing sugars at high temperatures (Lee, 1980).

In nature, trehalose serves as a protectant against a variety of stresses in different organisms (Eleutherio et al., 1993; Ström and Kaasen, 1993). It is proposed that this sugar enables so-called resurrection plants to tolerate complete dehydration and to spring back to life upon rehydration (Young, 1985; Drennan et al., 1993). Although many sugars can protect proteins under dehydration-induced stress conditions (Colaço et al., 1992), the mechanism by which trehalose is able to stabilize biological molecules during desiccation is still under debate (Crowe et al., 1987). Because of its stability, nontoxicity, and chemical inertness, trehalose may be an ideal preservative for individual biological molecules such as proteins, enzymes, flavors, fragrances, or complex mixtures thereof, such as food items.

It was shown by the Quadrant Research Foundation (Cambridge, UK) that the characteristic color, aroma, and flavor of many foods, especially fresh fruits and herbs, were much better conserved when trehalose was added during dehydration (Roser and Colaço, 1993). Commercial application of trehalose in food conservation, however, has been limited due to its price on the world market (approximately $200/kg) (Kidd and Devorak, 1994). The price, combined with the amount required for food-drying processes, restricts the use of trehalose to high-value products such as pharmaceuticals and reagents. Currently, microorganisms such as yeasts are used for the production of trehalose. One of the drawbacks of this system is the very high activity in yeast cells of trehalase, an enzyme that hydrolyzes trehalose into two Glc molecules during extraction. To prevent a rapid decrease of trehalose (up to 50% in 2–3 min), trehalase from *Saccharomyces cerevisiae* must be heat-inactivated immediately after harvesting the yeast cells (Yoshikawa et al., 1994), thereby increasing the cost of trehalose production.

Although exogenously supplied trehalose has been found to be toxic for plant tissues (Veluthambi et al., 1981), we evaluated whether plants would be a suitable organism for the accumulation of trehalose, both to exploit plants as production systems (Goddijn and Pen, 1995) and to evaluate the capacity of trehalose to protect plant tissues against various stresses (Holmström et al., 1996). To this end, *Escherichia coli* genes involved in trehalose biosynthesis were introduced in plants. In *E. coli*, biosynthesis of trehalose is a two-step process consisting of the conversion of UDP-Glc and Glc-6-P into trehalose-6-phosphate by TPS and subsequent dephosphorylation by TPP (Giaever et al., 1988). An operon (*ots*BA) was identified that harbors the *ots*A and *ots*B genes encoding TPS and TPP, respectively (Kaasen et al., 1992). Since the substrates for TPS are readily available

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Abbreviations: de35SCaMV, double-enhanced 35S cauliflower mosaic virus; HPAEC-PED, high-performance anion-exchange chromatography-pulsed electrochemical detector; PM, postculture medium; PotPiII, potato proteinase inhibitor II; TPP, trehalose-phosphate phosphatase; TPS, trehalose-phosphate synthase.

in the cytosol of plant cells, introduction of TPS activity in this cell compartment is expected to lead to the formation of trehalose-6-phosphate. Because plant cells contain a wide array of nonspecific phosphatases, this intermediate may be converted to trehalose without the simultaneous introduction of TPP activity; this is similar to the synthesis of mannitol in tobacco (*Nicotiana tabacum*) plants expressing a bacterial mannitol-1-phosphate dehydrogenase only (Tarczynski et al., 1992).

Both single *ots*A constructs and double *ots*A *ots*B constructs were introduced in tobacco and potato (*Solanum tuberosum*) under the control of a constitutive or a tissue-specific promoter. Despite the expression of both genes, very limited amounts of trehalose were detected in leaves of transgenic tobacco plants, and no trehalose was detected in potato tubers. We investigated whether enzymes hydrolyzing trehalose could be responsible for this lack of trehalose accumulation. A number of reports describe the presence of trehalase activity in a variety of plants, plant cells, and tissue cultures, possibly as a detoxifying mechanism (Kendall et al., 1990; Müller et al., 1995). Particularly high activities were found in nitrogen-fixing root nodules of soybean and other legumes (Müller et al., 1994). To study the effect of plant trehalases on the accumulation of trehalose in our transgenic plants, validamycin A, a secondary metabolite from *Streptomyces hygroscopicus*, was evaluated. This compound has been found to be a highly specific inhibitor of trehalases from various sources with IC_{50} values ranging from 10^{-6} to 10^{-10} M (Asano et al., 1987; Kameda et al., 1987). Except for trehalase, it does not significantly inhibit any α- or β-glycohydrolase activity.

MATERIALS AND METHODS

Strains

Escherichia coli K-12 strain DH5α was used for cloning procedures. The *Agrobacterium tumefaciens* strain EHA105 (Hood et al., 1993) was used in tobacco (*Nicotiana tabacum*) and potato (*Solanum tuberosum*) transformation experiments.

DNA Manipulations

All DNA procedures (DNA isolation from *E. coli*, restriction, ligation, transformation, etc.) were performed according to standard protocols (Sambrook et al., 1989).

Plasmid Constructs

pMOG799

DNA was prepared from a lysate of λ clone 7F11 (Kohara et al., 1987). The 5′ end of the *ots*A gene was tailored by PCR using the synthetic oligonucleotides TPS1 (5′-GAGAAAATACCCGGGGTGATGAC-3′) and TPS2 (5′-GATAATCGTGGATCCAGATAATGTC-3′), thereby introducing a *Sma*I site 5′ of the coding sequence. The synthetic oligonucleotide duplex TPS6/7 (TPS6: 5′-AGCTGGCGTGAGGAGCGGTTAATAAGCTTGAGCT-3′; TPS7: 3′-CCGCACTCCTCGCCAATTATTCGAAC-5′) tailored the 3′ coding region introducing a *Hin*dIII site. After assembly, the full-length *ots*A coding sequence was cloned in the expression vector pMOG747, which is derived from pMOG180. The plant expression cassette of pMOG180, flanked by an *Eco*RI (5′) and *Hin*dIII (3′) site, comprises the de35SCaMV (Guilley et al., 1982), the alfalfa mosaic virus RNA4 leader sequence (Brederode et al., 1980), a unique *Bam*HI cloning site, and the nopaline synthase transcription terminator sequence from *A. tumefaciens*. pMOG180 was tailored to harbor the *ots*A gene by replacing the *Hin*dIII site by *Sst*I and inserting a polylinker comprising *Sma*I, *Sph*I, and *Hin*dIII in the *Bam*HI cloning site, resulting in pMOG747. The entire expression cassette harboring the *ots*A coding region in the correct orientation was cloned as a 2.5-kb *Eco*RI/*Sst*I fragment into the binary vector pMOG402, resulting in pMOG799. pMOG402 is derived from pMOG23 (Sijmons et al., 1990) and harbors a restored neomycin phosphotransferase gene (Yenofsky et al., 1990).

pMOG851

A set of oligonucleotides, TPP1 (5′-CTCAGATCTGGCCACAAA-3′) and TPP2 (5′-GTGCTCGTCTGCAGGTGC-3′), complementary to the sequence of the *E. coli ots*B gene, was used to PCR-amplify a DNA fragment of 375 bp harboring the 3′ part of the coding region of *ots*B by introducing a *Pst*I site 10 bp downstream of the stop codon using the 2.9-kb *Hin*dIII fragment of λ clone 7F11 as a template. The resulting fragment was digested with *Bgl*II-*Pst*I and hooked up to PotPiII (An et al., 1989). The PotPiII terminator was isolated by PCR amplification using chromosomal DNA isolated from the potato cultivar Desiree as a template and the following set of oligonucleotides: 5′-GTACCCTGCAGTGTGACCCTAGAC-3′ and 5′-TCGATTCATAGAAGCTTAGAT-3′.

A TPP expression cassette was generated after a three-way ligation with a *Afl*III-*Bgl*II fragment of 514 bp harboring the 5′ part of the coding region of *ots*B and the de35SCaMV promoter (*Eco*RI-*Nco*I fragment). From the resulting vector, a 2.8-kb fragment was isolated and cloned in a three-way ligation with a synthetic linker formed by TPP3 (5′-AGCT GTCGAC GC TCTAGA C-3′) and TPP4 (3′-CAGCTG CG AGATCT GTTAA-5′) into binary vector pMOG799 linearized with *Eco*RI, resulting in pMOG851.

Isolation of a Patatin Promoter

A 1123-bp patatin promoter fragment was PCR-amplified from chromosomal DNA of *S. tuberosum* cv Bintje using a set of oligonucleotides, 5′-AAG CTT ATG TTG CCA TAT AGA GTA G-3′ and 5′-GTA GTT GCC ATG GTG CAA ATG TTC-3′, complementary to the sequence of the upstream region of the λpat21 patatin gene (Bevan et al., 1986). The fragment amplified showed a high degree of similarity to the λpat21 patatin sequence and was cloned using *Eco*RI linkers into a pUC18 vector, resulting in plasmid pMOG546.

pMOG845

Plasmid pMOG546 was digested with *Nco*I-*Kpn*I and incubated with *E. coli* DNA polymerase I in the presence of dATP and dCTP, thereby destroying the *Nco*I and *Kpn*I site, and was subsequently religated. From the resulting vector a 1.1-kb *Eco*RI-*Sma*I fragment containing the patatin promoter was isolated and cloned into pMOG798 (PCT 6037–2.IND) linearized with *Sma*I-*Eco*RI, consequently exchanging the de35SCaMV promoter for the patatin promoter. The resulting vector was linearized with *Hin*dIII and ligated with the following oligonucleotide duplex: (*Hin*dIII) *Pst*I *Kpn*I *Hin*dIII

The resulting vector is linearized with *Hin*dIII and ligated with the following oligonucleotide duplex:

(*Hin*dIII)	*Pst*I		*Kpn*I	*Hin*dIII
5′ AGCT	CTGCAG	TGA	GGTACC	A 3′
3′	GACGTC	ACT	CCATGG	TTCGA 5′

After checking the orientation of the introduced oligonucleotide duplex, a 950-bp *Pst*I-*Hin*dIII fragment harboring PotPiII was inserted. The TPS expression cassette was subsequently cloned as a *Eco*RI-*Hin*dIII fragment into the binary vector pMOG402, resulting in pMOG845.

pMOG852

Similar to the construction of pMOG851, this vector was generated after a three-way ligation with an *Afl*III-*Bgl*II fragment of 514 bp harboring the 5′ part of the coding region of *ots*B and a *Eco*RI-*Nco*I fragment comprising the patatin promoter. From the resulting vector a 2.9-kb fragment was isolated and cloned in a three-way ligation with a synthetic linker formed by TPP3 and TPP4 into binary vector pMOG845 linearized with *Eco*RI, resulting in pMOG852.

Triparental Matings

Binary vectors were mobilized in triparental matings with *E. coli* strain HB101 containing plasmid pRK2013 (Ditta et al., 1980) into *A. tumefaciens* strain EHA105, and used for transformation.

Plant Transformation

N. tabacum cv Petit Havana SR1 and cv Samsun NN were transformed by co-cultivating tobacco leaf discs with *A. tumefaciens* containing the binary vector of interest (Horsch et al., 1985). Transgenic plants were regenerated from shoots that grew on selection medium containing kanamycin. Once they had rooted, they were transferred to soil. The transformation frequency was calculated as the percentage of explants giving at least one transgenic plant.

S. tuberosum cv Kardal was transformed with *A. tumefaciens* as follows. Tubers were peeled and surface-sterilized by burning them in 96% ethanol for 5 seconds. Discs from approximately 2-mm-thick slices were cut from the vascular tissue with a bore and incubated in MS30R3 medium containing 1×10^8 to 5×10^8 *A. tumefaciens* bacteria/mL. MS30R3 medium consists of Murashige and Skoog salts (Murashige and Skoog, 1962), R3 vitamins (Ooms et al., 1987), 30 g/L Suc, and 0.5 g/L Mes, with a final pH of 5.8 (adjusted with KOH) and solidified, when necessary, with 8 g/L Daishin agar. After 20 min of incubation, tuber discs were washed with MS30R3 medium and transferred to solidified PM (consisting of M30R3 medium supplemented with 3.5 mg/L zeatin riboside and 0.03 mg/L IAA). Two days later, discs were transferred to fresh PM with 200 mg/L cefotaxime and 100 mg/L vancomycin. After 3 d, the tuber discs were transferred to shoot-induction medium (consisting of PM medium with 250 mg/L carbenicillin and 100 mg/L kanamycin). Shoots emerging from the discs were excised after 4 to 8 weeks and placed on rooting medium (MS30R3 medium with 100 mg/L cefotaxim, 50 mg/L vancomycin, and 50 mg/L kanamycin). Validamycin A was obtained from Duchefa (Haarlem, The Netherlands). Transgenic shoots were propagated axenically by meristem cuttings.

Induction of Microtubers

Stem segments of in vitro potato plants harboring an auxiliary meristem were transferred to microtuber-inducing medium, which contained 1× Murashige and Skoog salts supplemented with R3 vitamins, 0.5 g/L Mes (final pH = 5.8, adjusted with KOH), 60 g/L Suc, and 2.5 mg/L kinetin solidified with 8 g/L Daishin agar. After 3 to 5 weeks of growth in the dark at 24°C, microtubers were formed.

Detection of Trehalose

Trehalose was determined quantitatively by HPAEC-PED. Extracts were prepared by adding 1 mL of water to 1 g of frozen material, which was subsequently heated for 15 min at 100°C. When known amounts of trehalose were added to plant extracts, 100 ± 5% of the total amount was recovered after extraction. Samples (25 μL) were analyzed on a liquid chromatograph (model DX 300, Dionex, Breda, The Netherlands) equipped with a 4 × 250 mm Dionex 35391 Carbopac PA-1 column and a 4 × 50 mm Dionex 43096 Carbopac PA-1 precolumn. Elution was with 100 mM NaOH at 1 mL/min. Sugars were detected with a pulsed electrochemical detector (Dionex). Commercially available trehalose (Sigma) was used as a standard. To confirm the identity of trehalose in plant extracts, samples were incubated with porcine kidney-derived trehalase (Sigma), which was dialyzed against 10 mM sodium phosphate, pH 6.5. Reactions were initiated by adding 20 μL (0.19 unit/mL) of this preparation to 300 μL of extract at 37°C. Samples were taken at different times, boiled for 5 min, and subsequently analyzed as described.

Enzyme Assays

TPP Assay

TPP was assayed at 37°C by measuring the production of [^{14}C]trehalose from [^{14}C]trehalose-6-phosphate (Londesborough and Vuorio, 1991). Crude extracts were prepared in 25 mM Tris-HCl, pH 7.4, containing 5.5 mM $MgCl_2$. Samples were diluted to a protein concentration of 1

mg/mL in extraction buffer containing 1 mg/mL BSA. Standard assay mixtures (50 μL final volume) contained 27.5 mM Tris-HCl, pH 7.4, 5.5 mM $MgCl_2$, 1 mg/mL BSA, and 0.55 mM trehalose-6-phosphate (specific activity 854 cpm/nmol). Reactions were initiated by the addition of 5 μL of enzyme and terminated after 1 h by heating for 5 min in boiling water. AG1-X8 (formate) anion-exchange resin (Bio-Rad) was added and the reaction mixtures were centrifuged after 20 min of equilibration at room temperature. The radioactivity in the supernatant of the samples (400 μL) was measured by liquid scintillation counting.

Trehalase Activity Assay

Trehalase activity was measured using a disaccharidase assay (Dahlqvist, 1964). As substrate solution, 50 mM trehalose in 0.1 M NaPi (pH 6.5) was used. After color development samples were analyzed in microtiter plates on an ELISA reader at 405 nm.

Extraction of RNA and Northern Blot Analysis

Total RNA was extracted from frozen plant material (Verwoerd et al., 1989). RNA was denatured in 40% (v/v) formamide and 12% (v/v) formaldehyde, subjected to agarose gel electrophoresis (1.5% [w/v] agarose, 15% [v/v] formaldehyde), and blotted onto nylon membranes (Hybond N, Amersham). Membranes were hybridized at 65°C in a buffer containing 6× SSC (1× SSC = 0.15 M NaCl, 0.015 M sodium citrate), 5× Denhardt's solution (100× Denhardt's solution = 2% [w/v] BSA, 2% [w/v] Ficoll, 2% [w/v] PVP), 0.5% SDS, and 100 μg/mL denatured salmon sperm DNA. Radioactive labeling of DNA probes was performed using a random-primer DNA labeling kit (Prime-It II, Stratagene). Filters were washed three times for 20 min at 65°C with 0.1× SSC/0.1% SDS before exposure to x-ray films with an intensifying screen at −80°C.

Sequence Determination

DNA sequencing was performed on double-stranded templates using a DNA polymerase sequencing kit (Sequenase T7, United States Biochemical). Data were analyzed using the University of Wisconsin Genetics Computer Group programs (Devereux et al., 1984).

RESULTS

Cloning of the *E. coli otsA* and *otsB* Gene

In *E. coli*, TPS is encoded by the *otsA* gene located on the operon *otsBA*. The location and the direction of transcription of this operon on the *E. coli* chromosome are known (Kaasen et al., 1992). The *otsA* gene is located at 42′, and, according to Kaasen et al., is confined on an 18.8-kb fragment present in the EMBL4 genomic clone designated 7F11 (Kohara et al., 1987). A 2.9-kb *Hin*dIII fragment was isolated from a lysate of λ clone 7F11 harboring part of the *ara*H gene of the Ara transport operon (Scripture et al., 1987), the *otsB* gene encoding TPP, and part of the *otsA* gene encoding TPS. To complete the coding sequence of *otsA*, an overlapping *Bam*HI/*Eco*RI fragment was isolated and partially sequenced. Sequence data of the *otsB* gene did not unequivocally reveal its start codon in *E. coli*. A construct was generated wherein the ATG at position 618, as enumerated in a published sequence of the *otsBA* operon (Kaasen et al., 1994), was used as translational start codon. Single (*otsA* only) and double (*otsA* and *otsB*) constructs were generated that harbored the respective genes under control of the constitutive de35SCaMV promoter (pMOG799 and pMOG851) or the tuber-specific patatin promoter (pMOG845 and pMOG852). Details of the constructions are given in "Materials and Methods."

Transformation of Tobacco and Potato Plants with the *otsA* Gene

Transgenic tobacco and potato plants were generated harboring the *otsA* gene driven by the de35SCaMV promoter (pMOG799) and a tuber-specific patatin promoter (pMOG845), respectively.

Transformation experiments of *N. tabacum* with pMOG799 resulted in low transformation frequencies compared with the empty-vector control (data not shown). After 6 weeks of growth in vitro, rooted shoots were transferred to the greenhouse to set seed. A number of plantlets (approximately 10%) maintained in vitro showed a remarkable phenotype after several weeks of growth. The development of a normal root system was completely disturbed (Fig. 1). Short, thick roots were formed, and light microscopy of cross-sections revealed swollen root hair cells and highly vacuolated cells at the outer layers of the cortex, separated by large intercellular cavities. After transfer to

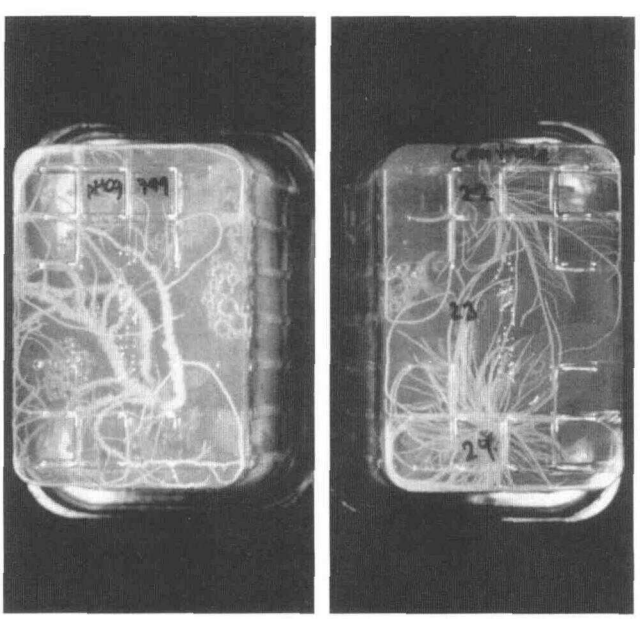

Figure 1. Aberrant root development of *otsA* transgenic tobacco plants cultured in vitro. A, Roots of a pMOG799 transgenic plant; B, roots of nontransgenic control plants.

the greenhouse, a root system developed that was indistinguishable from that of nontransgenic control plants. Leaves of some soil-grown plants did not fully expand in the lateral direction, leading to a lancet-shaped morphology. Furthermore, apical dominance was reduced, resulting in stunted growth and formation of several axillary shoots. Seven out of 32 plants showed severe growth reduction, reaching plant heights of 4 to 30 cm at the time of flowering (Fig. 2; Table I). Control plants reached heights of 60 to 70 cm at the time of flowering. Less seed was produced by transgenic lines with the stunted-growth phenotype. These phenomena were also observed in the S_1 generation, indicating that their transgenic nature, and not tissue-culture artifacts, form the basis for these effects.

Transgenic potato lines harboring the patatin-driven TPS construct were obtained with transformation frequencies comparable to those of empty-vector controls. All plants obtained were phenotypically indistinguishable from the wild type.

Table I. *Trehalose accumulation and plant heights of otsA transgenic tobacco plants*

Leaf samples of *ots*A transgenic tobacco plants were analyzed by HPAEC-PED for trehalose accumulation. Plant length was measured at the time of flowering. Trehalose accumulation data represent the average of two independent measurements. ND, Not determined.

Plant Line	Trehalose	Height
	mg/g^{-1} fresh wt	*cm*
Control	0.00	60–70
799-1	0.04	ND
799-3	0.02	10
799-5	0.08	4
799-15	0.055	30
799-24	0.02	12
799-26	0.05	25
799-32	0.055	30
799-40	0.11	25

Analysis of *ots*A Transgenic Plants

Northern blot analysis showed a correlation between the presence of *ots*A mRNA and the accumulation of trehalose and stunted growth in tobacco (Fig. 3A), although no strict correlation was found between the steady-state *ots*A mRNA level and the amount of trehalose detected (Table I). There was also no direct correlation found between aberrant root morphology in vitro and either reduced growth or trehalose synthesis of transgenic plants in the greenhouse. A very small amount of trehalose could be detected (0.013 mg g^{-1} fresh weight) in the aberrant roots of only one transformant grown in vitro. Carbohydrate analyses of leaf material from 32 transgenic, greenhouse-grown tobacco plants revealed the presence of 0.02 to 0.11 mg g^{-1} fresh weight trehalose in plants reduced in length (Table I). Further proof of the accumulation of trehalose in tobacco was obtained by treating crude extracts with porcine trehalase. Prolonged incubation of a tobacco leaf extract with trehalase resulted in complete degradation of trehalose (data not shown). The concomitant increase in Glc could not be measured accurately, since large amounts of this monosaccharide were already present in the extract.

Trehalose was not found in control plants, transgenic tobacco plants without an aberrant phenotype, or any of the tubers formed by pMOG845 transgenic potato plants. The precursor of trehalose, trehalose-6-phosphate, was not detected in any of the extracts of leaves, roots, or tubers of transgenic lines.

Analysis of Plants Harboring the *ots*A *ots*B Double Construct

To determine whether simultaneous expression of *ots*A and *ots*B enhances trehalose accumulation, pMOG851 (de35SCaMV-driven expression) was introduced in tobacco, and pMOG852 (patatin-driven expression) was introduced in potato. Five transgenic tobacco lines of the pMOG851 double construct were analyzed in more detail. Roots of three transformants displayed an aberrant mor-

Figure 2. Stunted phenotype of greenhouse-grown *ots*A transgenic tobacco plants. Plant lines transgenic for pMOG799 accumulating trehalose (foreground, lines 15, 32, and 40) are shown, in combination with two nonaccumulating control lines (background).

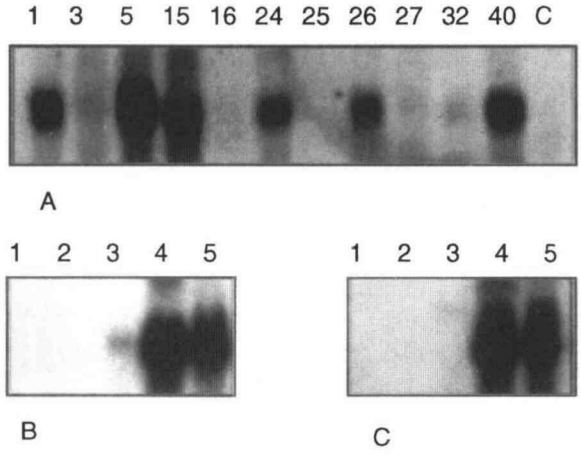

Figure 3. Northern blot analysis of *ots*A and *ots*A *ots*B transgenic tobacco plants. A, Expression of *ots*A mRNA in leaves of individual pMOG799 transgenic tobacco plants. The control lane (C) contains total RNA from a nontransformed *N. tabacum* plant. B and C, *ots*A and *ots*B mRNA levels of pMOG851 transgenic plants, respectively. The corresponding control lanes did not reveal any signal after prolonged exposure (not shown).

phology after continuous growth in vitro, as noted with pMOG799 transgenic plants. After transfer to the greenhouse, normal root systems developed. Strikingly, interveinal tissue of mature leaves of pMOG851 transgenic tobacco plants in the greenhouse bleached out, whereas leaves of control plants remained green. This phenomenon was not observed with tobacco plants transgenic for *ots*A only. Additionally, the stunted-growth phenotype was less pronounced compared with the pMOG799 transgenic plants. Leaves of greenhouse-grown pMOG851 plants accumulated 0.005 to 0.07 mg g^{-1} fresh weight trehalose (Table II). Northern blot analysis revealed the presence of high levels of both *ots*A and *ots*B mRNA in lines 4 and 5 (Fig. 3, B and C). Leaf samples of plants that accumulated trehalose showed enhanced (5–10 times) TPP activities compared with control plants (Table II). Four out of 12 potato lines transgenic for pMOG852 accumulated very small amounts of trehalose, 0.003 to 0.02 mg g^{-1} fresh weight, in (greenhouse-grown) tuber tissue. Seven of these lines failed to produce any tubers. No trehalose was detected in the tuber material of wild-type potato plants.

Table II. *Trehalose accumulation and trehalose-6-phosphate phosphatase activities in leaf samples of otsA otsB transgenic tobacco plants*

Trehalose accumulation data represent the average of two independent measurements.

Plant Line	Trehalose	TPP Activity
	mg g^{-1} fresh wt	*nmol mg^{-1} protein h^{-1}*
Control	0	16
851-1	0	37
851-2	<0.01	17
851-3	0	6
851-4	0.065	224
851-5	0.03	127

Plant Trehalase Activity and Its Inhibition by Validamycin A

To find an explanation for the absence or low level of trehalose accumulation in our transgenic lines, an assay was performed to analyze the trehalose-hydrolyzing activity in tobacco leaf and potato tuber tissue. Trehalose added to crude extracts of potato microtuber material was rapidly degraded into Glc when the extracts were incubated at 37°C. This degrading activity increased approximately 12-fold when samples were desalted prior to analysis. The trehalose-hydrolyzing activity observed could be destroyed by boiling the plant extracts. For a number of different plant tissues, the trehalose-hydrolyzing activity was subsequently determined (Table III).

To establish whether the observed hydrolysis of trehalose was due to trehalase activity, a specific inhibitor of trehalase, validamycin A, was added to the assay mixture. Validamycin A at 10^{-8} M inhibited 50% of the trehalose-hydrolyzing activity. At 10^{-4} M, over 99% of the trehalose-degrading activity was inhibited, suggesting that trehalases are mainly responsible for the hydrolysis of trehalose in plant extracts.

Enhanced Accumulation of Trehalose in Leaves of Hydroponically Grown Tobacco Plants

Since validamycin A is able to inhibit plant trehalase activity in vitro, we investigated if this compound is taken up by the plant root system and transported to other tissues, thereby inhibiting trehalase activity in planta. Greenhouse-grown, nontransgenic tobacco plants (cv Petit Havana SR1) were transferred with their root systems intact into tap water supplemented with 10^{-3} M validamycin A. After 24 h, leaf samples were analyzed using HPLC for the presence of validamycin A. In top, middle, and lower leaf samples, approximately 100 μM validamycin A was detected. No validamycin A was detected in control plants transferred to tap water.

Subsequently, transgenic tobacco plants were grown hydroponically in the greenhouse on a solution of pokon (Pokon and Chrysal, Naarden, Holland) in tap water supplemented with 10^{-3} M validamycin A. Tobacco S_1 seedlings transgenic for pMOG799 and pMOG851 were germinated on kanamycin-containing medium and transferred to hydroponic beads (Asef, Didam, Holland) after the formation of a small root system. After 4 weeks, samples of mature leaves were taken and analyzed for trehalose (Fig. 4). A significant increase in the level of trehalose accumu-

Table III. *Trehalose hydrolyzing activity in plant tissues*

Species	Tissue	Activity
		μmol h^{-1} mg^{-1} protein
N. tabacum (cv Petit Havanna SR1)	Leaf	0.679 ± 0.002
N. tabacum (cv Samsun NN)	Leaf	0.667 ± 0.008
N. tabacum (cv White Burley)	Leaf	0.442 ± 0.012
S. tuberosum (cv Kardal)	Tuber	0.125 ± 0.003
	Microtuber	0.071 ± 0.006
	Leaf	0.048 ± 0.006

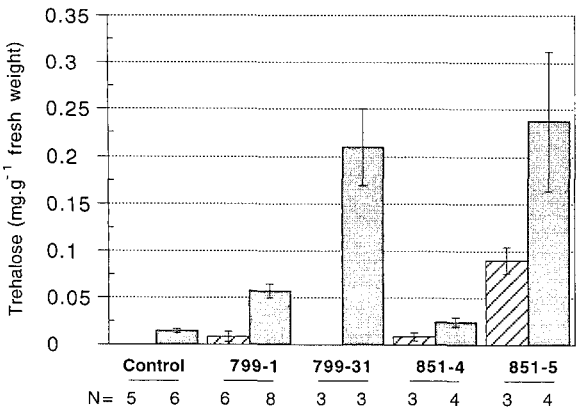

Figure 4. Trehalose accumulation in leaf samples of hydroponically grown tobacco plants. Plants were grown with (solid bars) and without (striped bars) validamycin A. N, Number of plants sampled. Error bars indicate SE.

lation was noted in leaves of both pMOG799 and pMOG851 transgenic plants grown on 10^{-3} M validamycin A compared with the controls. Surprisingly, in both experiments wild-type plants also seemed to accumulate very small amounts of trehalose, up to 0.021 mg g^{-1} fresh weight, when cultured on validamycin A. We did not detect any trehalose in control plants cultured without inhibitor.

Trehalose Accumulation in Potato Microtubers Grown on Validamycin A

Microtubers induced in vitro on stem explants of wild-type controls, pMOG845, and pMOG852 transgenic potato plants do not accumulate any trehalose. To evaluate the effect of plant trehalase activity on trehalose accumulation in these tubers, the experiment was repeated supplementing the microtuber medium with 10^{-3} M validamycin A (Table IV, Fig. 5). Eight out of 11 pMOG845 lines accumulated trehalose in microtubers up to 1.12 mg g^{-1} fresh weight. Thirteen out of 16 explants transgenic for pMOG852 formed microtubers accumulating trehalose up to 4.04 mg g^{-1} fresh weight (Table IV). Only very small microtubers were formed on explants transgenic for pMOG852, irrespective of the presence of validamycin A in the culture medium.

Considerable variation in trehalose accumulation was noted between microtubers induced on explants of identical lines in three independent experiments. Part of this variation can probably be explained by the nonsynchronous induction of the microtubers and by the fact that the inhibition of trehalase depends on the uptake of the inhibitor validamycin A. Nontransgenic control microtubers cultured in the presence of validamycin A accumulated on average 0.01 mg trehalose g^{-1} fresh weight (10 out of 17 lines). The more than 100-fold higher levels measured in transgenic lines, therefore, clearly can be attributed to the introduction of *E. coli* trehalose biosynthesis genes.

DISCUSSION

In *E. coli*, trehalose biosynthesis is the result of the activities of TPS and TPP encoded by two genes, *ots*A and *ots*B, respectively. To synthesize trehalose in plants, *N. tabacum* and *S. tuberosum* were transformed with constructs harboring either *ots*A (pMOG799/845) or both *ots*A and *ots*B (pMOG851/852) under the control of de35SCaMV- or tuber-specific patatin promoter sequences. After construction of these vectors, N-terminal protein sequence data were published, which indicated that the coding region of *ots*B starts with a GTG start codon that can be recognized by *N*-formyl-Met $tRNA^{fMet}$ (Kaasen et al., 1994). In our studies we have used an *ots*B gene construct in plants in which an ATG codon, located 57 nucleotides upstream, is used as translational start, resulting in a protein with 19 additional N-terminal amino acids. Expression of both the *ots*A as the modified *ots*B gene under control of

Table IV. *Trehalose accumulation in microtubers of pMOG845 and pMOG852 transgenic potato lines cultured in vitro on validamycin-A-containing medium*

All lines were cultured in vitro in the presence of validamycin A. No trehalose accumulation was detected in transgenic and wild-type control lines cultured without validamycin A. I, II, and III represent three independent microtuber induction experiments. nd, Not determined.

pMOG845	Trehalose			pMOG852	Trehalose		
	I	II	III		I	II	III
	mg g^{-1} fresh wt				*mg g^{-1} fresh wt*		
2	0.16	0.15	0	1	nd	0.19	0.39
8	0.51	0.14	nd	3	nd	2.60	0.35
11	0.05	0.96	0.15	4	0.06	<0.01	0
13	0.05	0.11	0	5	nd	1.12	0
15	0.07	nd	nd	6	nd	0.48	0
22	1.12	nd	nd	7	0.05	0.05	nd
25	0.02	nd	nd	8	nd	nd	2.25
28	0	1.09	0	9	nd	4.04	0
				10	nd	nd	0.78
				11	nd	2.48	1.51
				14	nd	nd	0.06
				15	nd	0.03	0
				16	nd	0.04	0

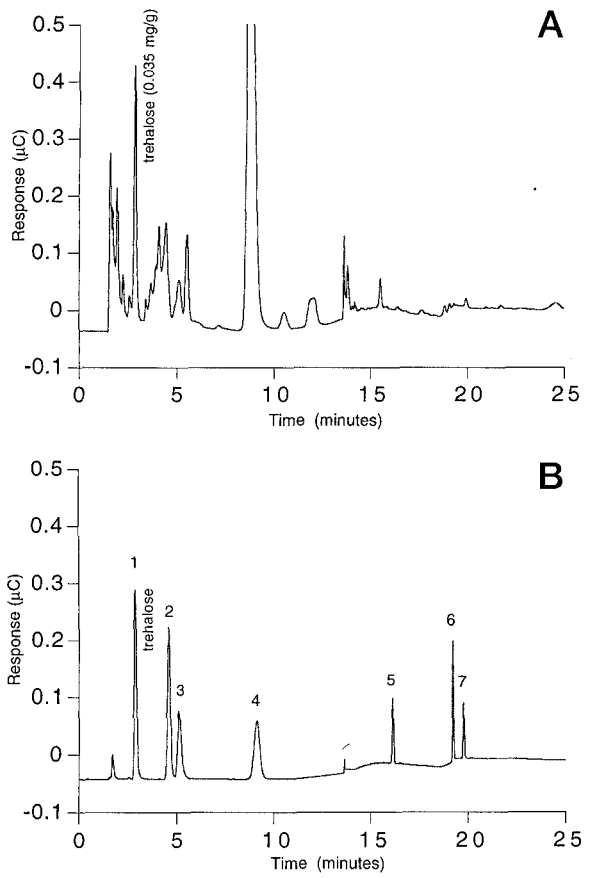

Figure 5. HPAEC-PED analysis of trehalose accumulation in a potato microtuber. The chromatograms show the PED-response profile of an extract of a microtuber transgenic for pMOG845 and cultured in the presence of validamycin A containing 0.035 mg g^{-1} fresh weight trehalose (A) and a standard containing known concentrations of several saccharides (B). The standard solution contains trehalose (1), Glc (2), Fru (3), Suc (4), trehalose-6-phosphate (5), Glc-6-P (6), and Fru-6-P (7).

de35SCaMV and the patatin promoter sequences in plants leads to detectable mRNA levels and active TPP enzymes capable of dephosphorylating trehalose-6-phosphate. Nevertheless, constructs using the TPP gene with the GTG as the start codon might improve the activity of the TPP enzyme formed.

Several pMOG799 and pMOG851 tobacco plantlets cultured in vitro developed thick, short roots. This growth phenotype was also observed in the S_1 generation, excluding tissue culture artifacts. Cross-sections of the aberrant roots show swollen epidermal and cortex cells separated by large intercellular cavities. The phenomena observed are not likely to be caused by the cultivar used in our experiments. Both *N. tabacum* cv Petit Havana SR1 and cv Samsun NN gave this phenotype. Probably, trehalose biosynthesis affects plant development when expressed constitutively. Surprisingly, no correlation was found between aberrant root development in vitro and reduced growth and trehalose accumulation in the greenhouse. Lack of an aberrant root system in soil may indicate that the in vitro growth conditions in agar inflict a problem on *ots*A-expressing plants. Production of osmotically active saccharides in the cytosol of root cells might influence the uptake of Suc from the synthetic medium. This situation is different in the greenhouse, where plants are dependent on Suc generated by photosynthesis. The lack of a root phenotype in vitro or stunted growth in the greenhouse with transgenic potato shoots transgenic for pMOG845 or pMOG852 may be explained by the use of the tuber-specific patatin promoter. Using these constructs, no trehalose or trehalose-6-phosphate accumulation, which could cause aberrancies in root morphology, would be expected.

Our data show that trehalose can be synthesized in plants through the cytosolic expression of a single TPS from *E. coli*. Most likely, the trehalose-6-phosphate formed by TPS is dephosphorylated by nonspecific phosphatases present in the cytosol. The simultaneous expression of TPP increases the level of trehalose accumulation only slightly. The data presented here from tobacco do not reveal a significant difference, whereas in potato microtubers grown on validamycin A, trehalose accumulation increased approximately 4-fold when *ots*B was included in the construct. These data suggest that the endogenous phosphatase activity present in the cytosol does not limit the formation of trehalose. The absence of trehalose-6-phosphate in trehalose-accumulating transgenic pMOG799 plants supports this view; nevertheless, trace amounts of trehalose-6-phosphate may be responsible for the observed effects on plant development. Trehalose-6-phosphate has been shown to play an important role in regulating glycolysis, mainly through the inhibition of hexokinase II in yeast cells (Blázquez et al., 1993). Similar regulatory processes may influence the growth and development of plants.

Potato lines transgenic for pMOG852 reveal a clear negative correlation between the accumulation of trehalose and the fresh weight of the microtubers formed on validamycin A. Since the microtuber fresh weight yield is not reduced for the pMOG845 lines, this phenomenon is most likely the result of TPP expression. The reduction in tuber yield is also observed with greenhouse-grown pMOG852 plants. Currently, we do not know if the TPP enzyme is able to dephosphorylate substrates other than trehalose-6-phosphate. Such nonspecific activities may interfere with tuber initiation, formation, or sink strength, thus reducing tuber yield.

In cryptobiotic plants, trehalose can constitute as much as 10% of the dry mass of an almost completely dehydrated plant (Müller et al., 1995). Only a fraction of that level is obtained in transgenic plants expressing trehalose-synthesizing genes. One of the reasons for this difference may be that the trehalose formed is rapidly degraded by hydrolyzing enzymes. Our experiments with a specific inhibitor of plant trehalase activity, validamycin A, indicate the presence of trehalases in leaf and tuber tissue of tobacco and potato. This activity clearly interferes with the accumulation of trehalose, since higher levels are obtained when plant material is cultured in the presence of validamycin A. Tobacco plants transgenic for pMOG799 or 851 accumulate 2- to 4-fold more trehalose when cultured on

validamycin A (Fig. 5). In transgenic potato microtubers, trehalases affect trehalose accumulation even more, since no trehalose is detected when microtubers are grown in the absence of validamycin A. Another reason for the limited accumulation of trehalose may be that the substrates necessary for trehalose biosynthesis are in short supply. The kinetics of the enzyme Suc-P synthase competing for the substrate UDP-Glc may favor Suc biosynthesis. As shown for the formation of fructan polymers (Ebskamp et al., 1994), targeting of trehalose biosynthesis to other cellular compartments of plant cells may improve the level of trehalose accumulation.

Trehalose has been found to be toxic for certain plants and plant cell cultures (Veluthambi et al., 1981) when supplied exogenously. In contrast, young soybean plantlets have been shown to take up trehalose and accumulate it strongly in roots and leaves in the presence of validamycin A, without apparent effects on growth or survival (Müller and Boller, unpublished data). Our data suggest that production of trehalose in planta has a negative effect on growth and development when the *ots*A gene is driven by the constitutive de35SCaMV promoter. As demonstrated by the tuber-specific expression of trehalose biosynthesis in potato, negative effects on plant development can be circumvented by using promoter sequences that drive expression in specific cell types or plant tissues.

The activity of trehalases may protect plants from the toxic effects of trehalose accumulation. Although trehalose occurs rarely in angiosperms, trehalase activity has been found in a number of higher plants (Kendall et al., 1990). The presence of trehalase is thought to protect the plant from the toxic effects of trehalose, which it may encounter in soil or during association with fungi or insects. It was surprising that we could demonstrate significant amounts of trehalose in wild-type tobacco plants and wild-type potato microtubers if they were cultured in the presence of validamycin A. Since this phenomenon was observed using in vitro material, a possible bacterial or fungal origin can be excluded. This would suggest that, in contrast to current belief, angiosperms have the capacity to synthesize trehalose. It is possible that this has never been demonstrated due to the trehalose-hydrolyzing activities present in plants. Further evidence for this hypothesis is the fact that in Arabidopsis, expressed sequence tag clones have been identified recently that are homologous to *ots*A and *ots*B (O. J. M. Goddijn, unpublished data). If angiosperms are indeed able to synthesize trehalose, what role does it play in plant development and how is its synthesis regulated? One approach to answering these questions may be to inhibit the action of trehalases via an antisense gene expression strategy.

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