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Altered Physiology in Trehalose-Producing Transgenic Tobacco Plants: Enhanced Tolerance to Drought and Salinity Stresses

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Transgenic tobacco (*Nicotiana tabacum* L. var. SR1) plants that over-express the *Escherichia coli* trehalose-6-phosphate synthase (TPS) gene (*otsA*) synthesized small amounts of trehalose (<400 μg g^{-1} leaf) while non-transformants produced no detectable trehalose. Some transgenic plants expressing a high level of *otsA* exhibited stunted growth and morphologically altered leaves. We tested F_2 homozygous plants devoid of phenotypic changes to determine their physiological responses to dehydration and salinity stresses. All transgenic plants maintained better leaf turgidity under a limited water supply or after treatment with polyethylene glycol (PEG). Furthermore, fresh weight was maintained at higher levels after either treatment. The initial leaf water potential was higher in transgenic plants than non-transformants, but, in both plant types, was decreased to a comparable degree following dehydration. When grown with 250 mM NaCl, transgenic plants exhibited a significant delay in leaf withering and chlorosis, as well as more efficient seed germination. Our results suggest that either trehalose or trehalose-6-phosphate can act as an osmoprotective molecule without maintaining water potential, in contrast to other osmolytes. Furthermore, both appear to protect young embryos under unfavorable water status to ensure subsequent germination.

Keywords: dehydration, salinity, stress tolerance, transgenic tobacco, trehalose

Trehalose is widely distributed in nature, from bacteria to higher organisms, both plants and mammals. It is a non-reducing disaccharide composed of two glucose units linked by an α(1 → 1) bond, and is synthesized by the sequential action of trehalose-6-phosphate synthase (TPS; EC 2.4.1.15) and trehalose-6-phosphate phosphatase (TPP; EC 3.1.3.12) (Cabib and Leloir, 1958; Elbein, 1974). Because of several physical properties, e.g., high hydrophilicity, chemical inertness, and non-hygroscopic glass formation, trehalose works as a protective molecule under various abiotic stresses, especially desiccation and high temperatures, in organisms such as bacteria, yeast, nematodes, fungi, and primitive plants (Thevelein, 1984; Larsen et al., 1987; Crowe et al., 1992, 1998; Bianchi et al., 1993; Strom and Kaasen, 1993; de Virgilio et al., 1994; Argüelles, 2000). In higher plants, however, its occurrence and role are dubious. Nevertheless, in the presence of validamycin A, an inhibitor of trehalase, small quantities of trehalose have been detected in potato and tobacco (Goddijn et al., 1997). Furthermore, endogenous production of trehalose, even in higher plants, has been implicated by the identification of functional homologues for genes involved in trehalose synthesis in *Selaginella* and *Arabidopsis*, suggesting its functional role in higher plants as well (Blázquez et al., 1998; Goddijn and Smeekens, 1998; Vogel et al., 1998; Müller et al., 1999; Zentella et al., 1999).

Engineered tobacco plants that express yeast *TPS* or *Escherichia coli otsA* show enhanced dehydration tolerance by retaining higher fresh weights and recovering after being air-dried (Holmström et al., 1996; Romero et al., 1997; Pilon-Smits et al., 1998). These plants also exhibit some prominent physiological changes, including stunted growth and morphological alterations, in parallel with this enhanced tolerance (Goddijn et al., 1997; Romero et al., 1997; Pilon-Smits et al., 1998). Similar results were also observed in trehalose-producing transgenic rice plants without accompanying stunted growth, extending its protective role to monocots (Garg et al., 2002; Jang et al., 2003).

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Nonetheless, most studies on improved water-stress tolerance have merely tested changes in external shape or fresh weight without examining the role of trehalose in leaf water potential and status. In addition, most research has been confined to the dehydration response only, even though various environmental stresses that also induce dehydration, such as drought, salt, and chilling, have been assessed in rice plants (Garg et al., 2002; Jang et al., 2003).

Therefore, the object of our current study was, first, to re-evaluate the earlier data regarding drought stress under physiologically more relevant conditions by comparing the effects of dehydration when administered through air-drying, limits on water supply, and treatment with PEG. Changes in leaf potential, as well as the photosynthetic capacity of trehalose-producing tobacco plants, were examined, in parallel, to diagnose the osmotic effect of trehalose. In addition, we compared the growth and germination efficiency of transgenic tobacco under salinity stress.

MATERIALS AND METHODS

Plant Material and Growing Conditions

Tobacco (*Nicotiana tabacum* L. var. SR1) plants were grown on soil for three to four weeks in a growth chamber maintained at 25±1°C, with a 16-h photoperiod, and under a light intensity of 150 μmol m^{-2} s^{-1}.

Plasmid Construct and Plant Transformation

The *TPS* overexpressor was constructed as follows. First, the coding region of the *otsA* gene (Kassen et al., 1992) was obtained by PCR using Vent polymerase (New England Biolabs, USA), with primers corresponding to its 5′- and 3′-ends with the *Xba*I and *Eco*RI sites (5′-GCTCTAGACTATGAGTCGTTTAGTCG-3′ and 5′-CGGAATTCCTACGCAAGCTTTGGAAAGG-3′), respectively. Cloning was done into the pWP90 vector that contains a double 35S *CaMV* promoter and a *CaMV* poly(A) terminator. The entire fragment was again cloned into the pBIN90 vector as a plant expression cassette, using the *Kpn*I and *Sal*I sites. Transgenic tobacco plants were generated by leaf-disc transformation (Horsch et al., 1985), and were selected on an MS medium containing kanamycin. Homozygous plants from the F_2 generation were then used for subsequent physiological experiments.

Water- and Salt-Stress Treatments

Dehydration was induced by 1) withholding irrigation, 2) air-drying, or 3) immersing the roots of whole plants in a Hoagland solution containing 10% (w/v) PEG-6000. Polyethylene glycol was chosen for its chemical inertness (Michel, 1970). Salt stress was applied by supplementing 250 mM NaCl twice a week during the irrigation of 4-week-old plants.

RNA Gel Blot Analysis

Total RNA was isolated according to the procedure of Chomczynski and Sacchi (1987). RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde, and transferred to a Hybond N membrane (Amersham, USA) by the capillary method, using 10x SSC as the transfer buffer. The membrane was hybridized with a ^{32}P-labeled fragment in 6× SSC, 0.2% (w/v) SDS, and 5× Denhardt's solution at 62°C. The entire coding region of *otsA* was used as a probe for RNA gel blot analysis.

Determination of Trehalose Content

Trehalose was determined by HPLC, using Aminex HPX-87C (Bio-Rad, USA). Two large leaves were removed from 4-week-old tobacco plants in the middle of their photoperiod. After they were de-ribbed, their soluble sugars were extracted by boiling 1 g of fresh tissue in 0.5 ml DW for 15 min. The 20-μL samples were then injected for HPLC analysis.

Measurement of Leaf Water Potential

Leaf water potential was measured psychrometrically with an HR-33T Dewpoint Microvoltmeter (Wescor, USA) in a C-52 sample chamber at room temperature (Boyer and Knipling, 1965). Discs (0.7-cm diam.) were placed in the chamber and allowed to equilibrate for 15 min, then cooled for 45 s before the voltage was recorded. Water potential was calculated from this voltage value and corrected for temperature.

Measurement of Photosynthesis

O_2 evolution and Chl fluorescence were measured simultaneously from 3.5-cm-diameter leaf discs in a Hansatech LD2 chamber (Kings Lynn, UK), using a Clark type electrode and a Walz PAM Chl fluorometer (Effeltrich, Germany). Each disc was presumed to

have an approximately equal Chl content when measured by a Chl meter (SPAD-502; Minolta, Japan), but was then normalized to the control.

Germination Test

Tobacco seeds were surface-sterilized and imbibed for 2 d in the refrigerator for stratification. To measure salinity tolerance, 100 seeds each were placed on plates containing three layers of filter paper soaked with 10 mL of various concentrations of NaCl solutions. Germination was defined as the emergence of the radicle from the seed coat.

RESULTS

Generation of Trehalose-Producing Transgenic Tobacco Plants

Through leaf-disc transformation, we generated tobacco plants over-expressing the *E. coli TPS* gene, *otsA*. Transgenic plants were first identified by PCR, then confirmed by northern hybridization. Levels of *otsA* expression varied depending on the line. The amount of trehalose detected in transgenic leaves, however, was not necessarily correlated with the amount of *otsA* transcript, but ranged from 130 to 370 μg g^{-1} fresh weight (Fig. 1). In contrast, no trehalose was detected in the leaves of non-transformants. Interestingly, phenotypic alterations were more related to the *otsA* expression level than was trehalose content. For example, plants in Line 8, which exhibited the greatest *otsA* expression but the lowest accumulation of trehalose, showed the most distinct morphological changes at maturation. Schluepmann et al. (2003) have also noted that the phenotypes of transgenic *Arabidopsis* expressing *otsA* depend upon T6P content rather than trehalose content. Therefore, we selected 4 out of 21 transgenic lines for subsequent experiments, based on our results from the northern hybridization instead of the trehalose quantification. These included Lines 4 and 5 for weak expression and Lines 6 and 8 for strong expression (Fig. 1). Plants from Lines 4 and 5 showed no distinct phenotypic changes throughout their life span, while the changes in those from Lines 6 and 8 included stunted growth and altered leaf morphology at the maturation stage. After 5 to 6 weeks of growth, the leaves became longer and narrower, manifesting a lancet shape in the mature plants. Occasionally, some leaves were severely irregular-shaped, with curls and wrinkles. In addition, some transgenic plants had an increased number of leaves with multiple vestigial branches along the main stem. The generation time for these transgenic plants nearly doubled, and was accompanied by slower growth and retarded senescence (data not shown). However, because no such phenotypic alterations were observed in 4-week-old plants, we reserved these somewhat smaller specimens for physiological experiments.

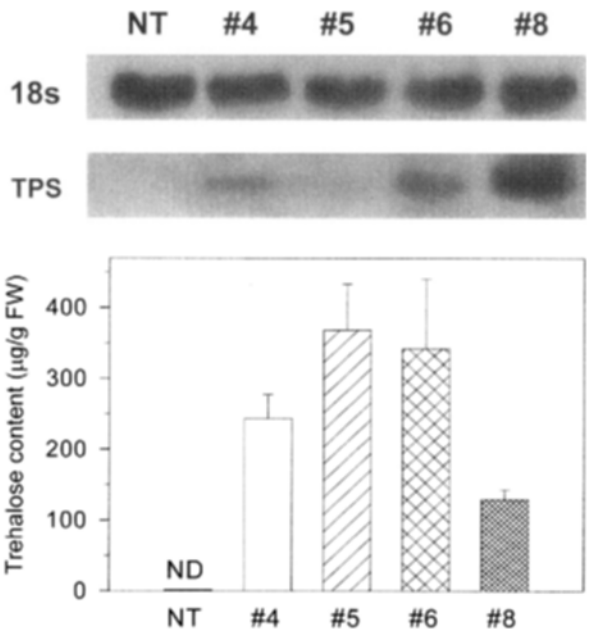

Figure 1. Northern hybridization of *otsA* expression, and trehalose amounts in leaves of transgenic tobacco plants. Numbers indicate transgenic lines. Data are mean values ± SE for 4 measurements. NT, non-transformants; ND, not detected.

Response to Water Stress

To re-evaluate the effectiveness of trehalose production in improving tolerance against water stress, we placed our non-transformed and transgenic tobacco plants under various water-deficit conditions. Non-transformant leaves showed apparent signs of wilting beginning 3 d after irrigation was first withheld, while leaves of plants from Lines 6 and 8 remained turgid during that period. Leaves from Lines 4 and 5 appeared less turgid but without clear wilting. This difference became more prominent after 5 d without watering, with leaves from transgenic Lines 6 and 8 maintaining their turgidity while those of the non-transformants, and even those of Lines 4 and 5, showing evident wilting (Fig. 2A).

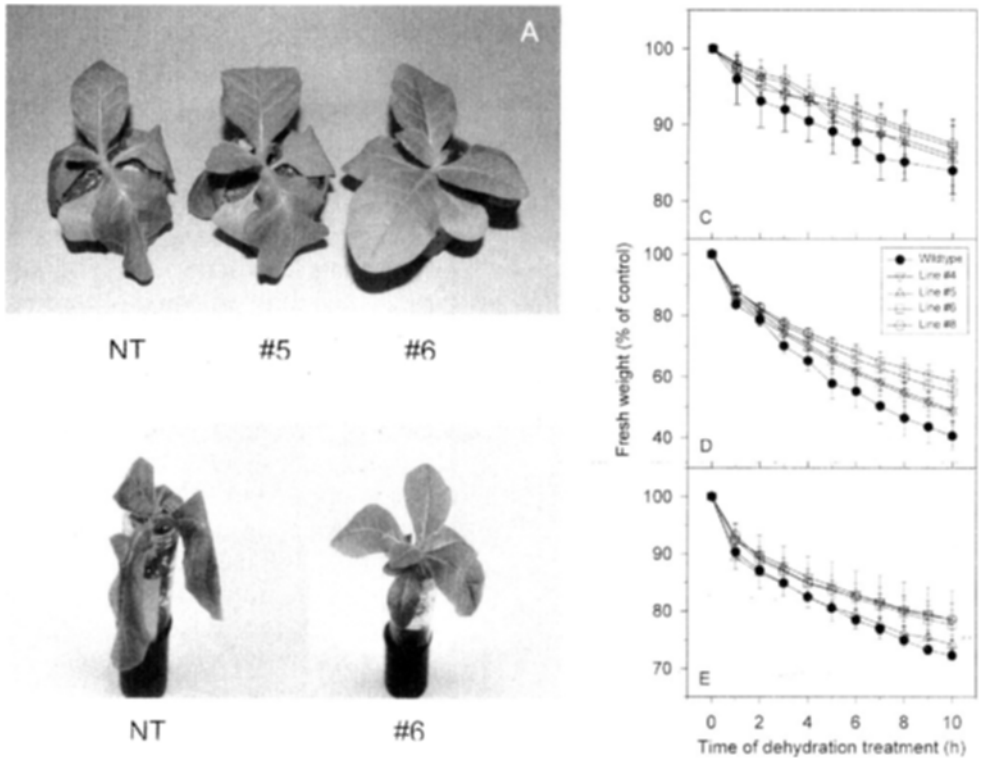

Figure 2. Increased tolerance to dehydration in trehalose-producing plants. Photographs of 4-week-old tobacco plants after 5 d without water supply **(A)** and after PEG treatment for 3 h **(B)**. Note the maintenance of leaf turgidity in transgenic plant from Line 6 compared with withered leaves in non-transformants (NT). Decrease in fresh weights of detached leaves **(C)** and whole plants **(D, E)** after dehydration treatment. Water deficit was induced by air-drying **(C, D)** or by immersing roots of whole plants in 10% (w/v) PEG solution **(E)**. Data are mean values ± SE (n = 5).

The response was similar with our PEG treatment. When dehydration was induced by 10% (w/v) PEG, the trehalose-producing tobacco plants maintained leaf turgidity better than the non-transformants. After 3 h of treatment, leaves from Lines 6 and 8 remained firm while those of the non-transformants were severely withered (Fig. 2B). Leaves from Lines 4 and 5 showed an intermediate response despite containing at least as much trehalose as that measured from Lines 6 and 8. Finally, when detached leaves were air-dried, those of the non-transformants wilted faster than the transgenic tissues. A similar trend was observed with the whole plants (data not shown).

Interestingly, the degree of tolerance against dehydration was more related to the level of *otsA* expression than to trehalose content. This suggests that T6P, not trehalose, acts as the protective molecule. Furthermore, when whole plants were re-hydrated after 12 h of air-drying or 24 h of PEG treatment, all the non-transformants failed to recover and eventually died whereas most of the transgenic plants revived and fully regained their turgidity (data not shown).

Changes in Fresh Weights and Leaf Water Potential after Dehydration

To compare water-retaining capabilities under dehydration, we monitored changes in fresh weight following the stress treatments. When detached leaves were air-dried, the decrease in fresh weight after 10 h was lower for transgenic plants than for the non-transformants, i.e., 11-13% reduction versus >15% reduction (Fig. 2C). For the whole plants, non-transformants lost nearly 60% of their fresh weight compared with a loss of only 40 to 50% for the transgenics (Fig. 2D). Treatment with 10% PEG yielded similar results, with non-transformants losing about 28% of their fresh weight after 10 h. In contrast, all transgenic fresh weights, except for plants from Line 5, were only about 20% lower (Fig. 2E). For those from Line 5, fresh weight losses were nearly as much as those recorded for the air-dried non-transformants, even though these transgenics had the lowest level of *TPS* expression among all transformed plants (Fig. 2E).

We measured changes in leaf water potential to

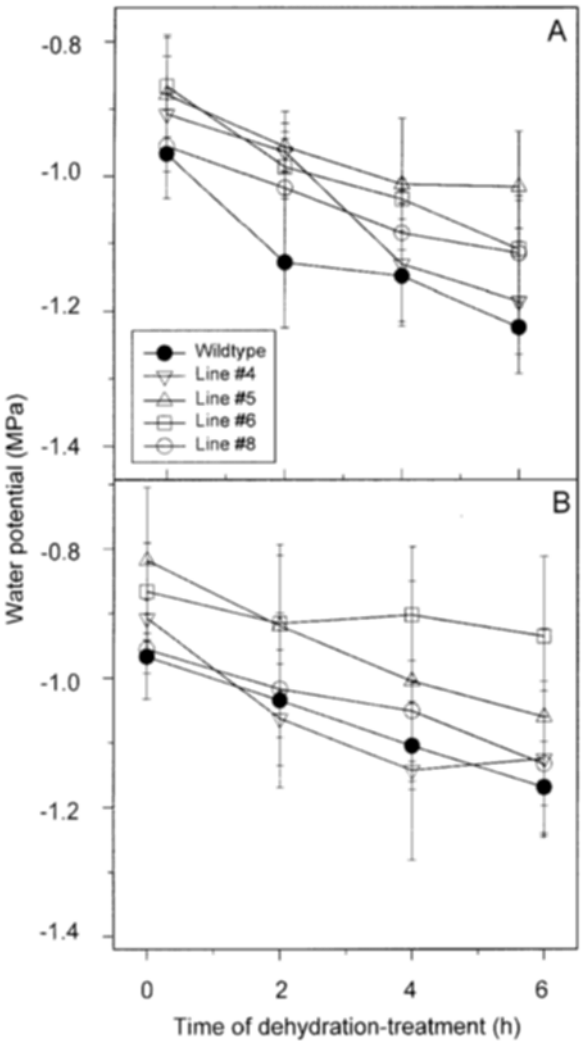

Figure 3. Changes in leaf water potential after air-drying **(A)** or PEG treatment **(B)** of whole plants. Note that initial leaf water potential in transgenic plants remains higher than in non-transformants. Data are mean values ± SE (n = 4).

explore the possibility that trehalose works as an osmolyte. The initial values measured in all the transgenic lines were at least equal to those determined for the non-transformant leaves. However, decreases that were induced by dehydration (via air-drying or PEG) were linear and time-dependent (Fig. 3A and B). Furthermore, leaf water potential declined to a comparable degree in both the non-transformants and the transgenics. Although these values varied somewhat because of the difficult nature in measuring the water status, similar trends were noted in three separate experiments. Our results indicated that trehalose may not have acted as an osmolyte to maintain constant leaf water potentials under dehydration. Furthermore, we demonstrated that the amount of trehalose accumulated in the leaves (<400 mg) was too small to be effective as an osmolyte (Fig. 1).

HPLC analysis revealed no significant increases in soluble sugar content from the leaves of transgenic plants (data not shown). Nevertheless, trehalose production appeared to induce an increase in leaf water potential by improving the retention of cellular water. Alternatively, T6P may have acted indirectly to maintain this cellular water content.

Because our leaf water potential data were somewhat variable, we used calculations of photosynthetic capacities in leaves following PEG treatment as an additional means for monitoring changes in potential. When accompanied by lowered leaf water potential, dehydration reduces the photosynthetic activity of chloroplasts (Boyer and Bowen, 1970; Mohanty and Boyer, 1976; Lee et al., 1998). Here, the maximal photosynthetic rate of O_2 evolution (Pmax) in both non-transformants and transgenics was almost linearly decreased, by about 60%, after 2 h of PEG treatment (Table 1). Even when the plants were dehydrated by curtailing their irrigation, the changes in Pmax were almost identical to those seen with PEG (data not shown). However, Chl fluorescence parameters (Fo and Fv/Fm) did not change for any of the plants (Table 1). Therefore, the photosynthetic data support our conclusion that leaf water potential after dehydration decreases in all transgenic plants to a degree comparable to that measured in the nontransformants. Furthermore, the production of neither trehalose nor T6P can rescue the inhibition of photosynthesis because of lowered leaf water poten-

Table 1. Changes in Pmax (mmol m^{-2} s^{-1}) of O_2 evolution and maximal photochemical efficiency of PSII (Fv/Fm) after dehydration, which was induced by PEG treatment for 2 h.

Treatment	Pmax			Fv/Fm		
	NT	#4	#6	NT	#4	#6
Control	8.41 ± 1.51	7.55 ± 0.68	10.07 ± 1.01	0.81	0.80	0.81
Dehydration	3.25 ± 0.29	2.55 ± 0.34	4.12 ± 0.57	0.81	0.81	0.82

*Data presented are mean values±SD for 3 to 5 measurements. Standard deviations for Fv/Fm are not shown, but are less than 2%.

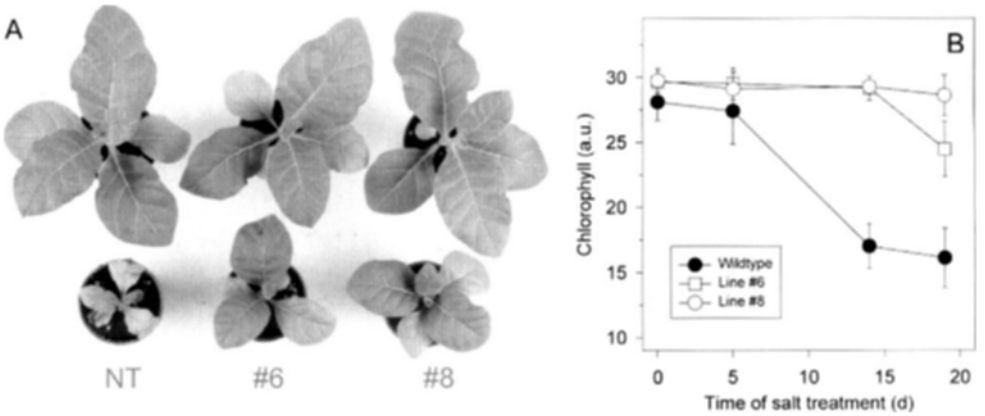

Figure 4. Increased tolerance to salinity in transgenic plants. **A)** Photographs are of non-transformant (NT) and transgenic tobacco plants grown with or without the supplementation of 250 mM NaCl. Top row shows tobacco plants grown without salinity stress; bottom row, those under salinity stress for 20 d. Note the smaller size of plants after sustained growth under salinity, although extensive chlorosis and necrosis occurred only in leaves of non-transformants. **B)** Changes in Chl content in leaves after sustained growth under 250mM NaCl. Chl content was measured with Minolta Chl meter. Data are mean values ± SE (n = 3).

tial, thereby proving that neither of them functions as an osmolyte.

Growth under Salt Stress

Dehydration is often imposed by high salinity. Therefore, to test their tolerance against salt stress, 4-week-old plants initially reared in the absence of salt were allowed to grow continuously under irrigation supplemented with 250 mM NaCl. Growth was apparently inhibited in all plant lines (compare upper and lower panels of Fig. 4A). The negative effects of salinity, including wilting, bleaching, and necrosis of leaves, was initiated significantly earlier in non-transformants, i.e., by Day 10, while the growth of the transgenic plants appeared normal. After 3 weeks of NaCl treatment, the non-transformant leaves manifested extensive chlorosis and necrosis while the leaves from transgenic Lines 6 and 8 were just beginning to show slight bleaching (Fig. 4A). Transgenics that expressed low levels of *TPS* (Lines 4 and 5) were less tolerant of the salt treatment than those of Lines 6 and 8, but were still better than the non-transformants (data not shown). Therefore, over-expression of *otsA* confers increased tolerance against salt stress, and its protective effect seems to be correlated with the *TPS* expression level rather than trehalose content.

Changes in Chl Content and PSII Function after Salt Stress

Because leaf-bleaching was associated with the NaCl treatment, we monitored changes in Chl contents in leaves with no apparent damage or necrosis. Chlorophyll content in the leaves of non-transformants was maintained for 5 d after the salt treatment, but sharply declined by about 40% on Day 15 despite there being no apparent damage or chlorosis (Fig. 4B). In contrast, no significant reduction in Chl content was observed in the transgenic plants of Lines 6 and 8 (Fig. 4B). However, those that expressed low *TPS* levels (Lines 4 and 5) were less efficient in maintaining their leaf Chl content (data not shown). Thus, trehalose or T6P appears to protect the thylakoid membranes by delaying the chlorosis normally induced by high salinity.

As additional parameters for thylakoidal integrity, we monitored initial Chl fluorescence (Fo) and the maximal photochemical efficiency of PSII (Fv/Fm), both of which are often used to assess PSII function (Renger and Schreiber, 1986). After salt treatment for up to 20 d, the Fv/Fm and Fo values did not change significantly for either non-transformants or transgenic plants, indicating that NaCl causes no substantial decline in PSII function (data not shown).

Changes in Germination Efficiency after Salt Stress

Germination is either blocked or delayed under unfavorable conditions because environmental stresses, such as high salinity or temperature, often lead to embryo mortality. Therefore, to test whether the protective effect of trehalose extends to the embryos, we monitored germination efficiency under various salt

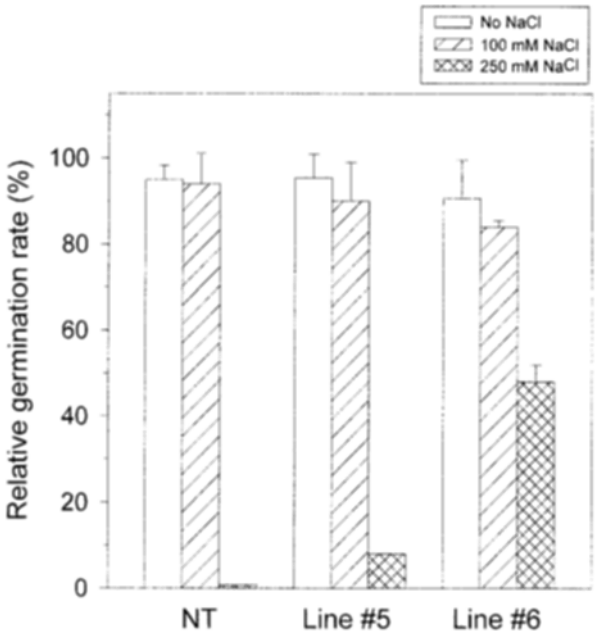

Figure 5. Germination efficiency in presence of NaCl. After imbibition, seeds were allowed to germinate on filter paper soaked with NaCl solution. After 5 d of incubation under same growing conditions, seeds were considered germinated when radicles were exposed. Each treatment had 3 plates with 100 seeds each. Experiments were repeated three times. Data are mean values ± SE (n = 3).

concentrations. Treatment with up to 100 mM NaCl did not negatively affect the germination for any of our tested lines, but a concentration of 250 mM NaCl was sufficient to completely block the germination of non-transformant seeds. That same concentration resulted in germination rates of about 10% for seeds from Lines 4 and 5, and 50% for those from Lines 6 and 8 (Fig. 5). At that level, survival was greater than 10% for seedlings produced from germinants of Lines 6 and 8 (data not shown). Finally, in the presence of 500 mM NaCl, all seed lines failed to germinate. Therefore, germination efficiency under saline conditions also seems to be correlated with the *TPS* expression level, a result that implies that trehalose or T6P may help to maintain the ionic balance in developing young embryos to alleviate the toxic effect of NaCl in addition to the osmoprotective effect.

DISCUSSION

Morphological Features of Trehalose-Producing Tobacco Plants

The identification of two functional homologs of trehalose (*TPS* and *TPP*) in *Arabidopsis* strongly supports the idea of its existence and synthesis in higher plants, at least in that species (Blázquez et al., 1998; Vogel et al., 1998). In a separate study, we also identified another functional *Arabidopsis TPS* homologue (*AtTPS4*), strengthening the functional importance of trehalose (unpublished data). Furthermore, albeit very small quantities of trehalose have been detected in tobacco leaves, potato microtubers, and *Arabidopsis* plants in the presence of validamycin A, a specific inhibitor for trehalase (Goddijn et al., 1997; Vogel et al., 2001). Therefore, it is probable that trehalose is a natural ingredient in higher plants, even if its role there is still unclear.

Trehalose functions as a stress protectant against dehydration, salinity, and heat in bacteria, yeast, nematodes, and resurrection plants (Giaever et al., 1988; Serrano, 1996; Argüelles, 2000). Trehalose-producing transgenic human cells, tobacco, and rice also show enhanced tolerance against water stress and salt (Holmström et al., 1996; Romero et al., 1997; Pilon-Smits et al., 1998; Guo et al., 2000; Jun et al., 2001; Garg et al., 2002; Jang et al., 2003). Trehalose is usually synthesized in two steps, requiring the action of both TPS and TPP. However, the amount of trehalose synthesized in the presence of TPS alone is comparable to that synthesized with both TPS and TPP in transgenic tobacco (Goddijn et al., 1997). Notably, trehalose contents in our transgenic tobacco leaves were not proportional to the expression level of the inserted *E. coli otsA*. For example, plants from Line 8, with the most *otsA* expression, accumulated the least trehalose while those in Line 5 accumulated the greatest amount despite having the lowest level of *otsA* transcript.

The degree of phenotypic alterations by our transgenic plants depended on the level of *otsA* expression, but not on trehalose content. Lines with low expression (4 and 5) maintained a normal phenotype throughout their life span, whereas those with high expression (Lines 6 and 8) exhibited various morphological features not observed in the non-transformants, including an altered leaf shape and abnormal branching patterns at the maturation stage. These plants typically showed retarded growth but longer survival, with delayed senescence and full fertility. Size differences were evident from the early stage on, while changes in leaf shape became apparent when plants were about 8 weeks old. Leaves gradually became thicker, but also narrower and longer, taking a lancet form. In addition, some transgenic plants had more leaves and vestigial branches. These phenotypic

alterations were fairly similar to those described in earlier reports (Goddijn et al., 1997; Romero et al., 1997). It appears that trehalose may act as a regulatory molecule that affects not only leaf development but also the senescing process, which coincides with a previously suggested role as a signaling molecule (Goddijn and Smeekens, 1998; Müller et al., 1999). However, in our own study, the severity of those phenotypic changes was closely related to *otsA* expression level rather than trehalose content. Therefore, the altered phenotype was more dependent on *TPS* than trehalose.

T6P, rather than trehalose, has previously been credited with various biological activities in *Arabidopsis* (Schluepmann et al., 2003, 2004). Although we failed to quantify T6P in our study, probably due to its presence in only minute quantities and the low sensitivity of our assay procedure, it is likely that its content is more directly correlated with *TPS* expression than the trehalose content, thus supporting earlier statements that T6P is more responsible for phenotypic alterations. However, the fact that T6P has not been detected in other *otsA* tobacco plants (Goddijn et al., 1997) and that the growth of transformed potato plants is stunted even in the presence of a bifunctional fusion enzyme (Jang et al., 2003), which presumably depleted T6P, do not preclude the possibility that trehalose, rather than T6P, is the effective molecule.

Enhanced Tolerance to Dehydration

Trehalose is dramatically effective in protecting bacteria, yeasts, nematodes, human cells, and resurrection plants against dehydration (Giaever et al., 1988; Drennan et al., 1993; Argüelles, 2000; Guo et al., 2000). However, transgenic tobacco and rice plants engineered to produce trehalose show variable degrees of tolerance to water stress (Holmström et al., 1996; Romero et al., 1997; Pilon-Smits et al., 1998; Jun et al., 2001; Garg et al., 2002; Jang et al., 2003). In our experiments, all transformants apparently exhibited enhanced tolerance, maintaining their leaf turgidity regardless of the type of treatment applied. The particular degree of tolerance was correlated with the expression level of *otsA*, with higher tolerance exhibited in lines with greater expression. Our results imply that T6P is the effective molecule, contrary to those from other studies where increased tolerance depends on trehalose content (Garg et al., 2002; Jang et al., 2003). However, the amount of trehalose accumulated in any transgenic leaves (<400 μg) was not sufficient to act as an osmolyte, and, according to our HPLC data, no major changes in other soluble sugars could account for this osmoprotective response. Earlier research has also shown that increases in the soluble sugars from trehalose-producing plants are not adequate for them to function as osmolytes (Romero et al., 1997; Pilon-Smits et al., 1998). Therefore, regardless of which one is indeed the effective molecule, neither T6P nor trehalose seems to directly exert protection as an osmolyte.

To further explore the osmoprotective effect of trehalose, we monitored changes in leaf water potential. Before dehydration was induced, the water potential for transgenic plants, especially Lines 5 and 6, was higher than that of the non-transformants, and was evidenced by their thicker leaves. This differs from observations by Pilon-Smits et al. (1998), who found significantly lower initial osmotic potentials in *otsA* plants due to a higher level of soluble sugars. However, that increased sugar amount was too little to explain the difference in osmotic potentials (>0.5 MPa). How trehalose or T6P can elevate leaf water potential is still unclear, but their presence seems to aid in maintaining higher cellular water content, thereby increasing leaf water potential rather than decreasing it. It is possible that trehalose retards the export of water from the cells by preserving the properties of the hydrated membrane (Singer and Lindquist, 1998). In our study, the onset of dehydration resulted in similar decreases in water potential for both non-transformants and transgenic plants. This suggests that enhanced tolerance to water stress in transgenic plants via trehalose production is not by its action as an osmolyte, but because of something else. Although trehalose may initially elevate leaf water potential by increasing the cellular water content, it fails to maintain a high potential during the water-stress period.

To confirm the validity of our water potential data, we also measured photosynthetic activity after the dehydration treatments. Photosynthesis is directly and negatively affected by water stress because of the decline in leaf water potential (Boyer and Bowen, 1970; Mohanty and Boyer, 1976; Lee et al., 1998). Our trehalose-producing transgenic plants should have maintained higher activities if trehalose were able to sustain greater potentials. However, because those values decreased similarly in both non-transformed and transgenic plants after PEG treatment, the Pmax of O_2 evolution declined to a comparable degree in both plant types, as expected. Likewise, the Chl fluorescence parameters (Fo and Fv/Fm), did not

change significantly after PEG treatment in any of our plants due to the negligible effect of water stress on PSII function. Nevertheless, our results further support the conclusion that the production of trehalose or T6P does not prevent the decrease in leaf water potential normally imposed by dehydration.

Enhanced Tolerance to Salt Stress

Salt stress usually evokes dehydration. Consequently, transgenic plants that over-produce osmolytes such as glycinebetaine, mannitol, proline, or fructan show enhanced tolerance (Tarczynski et al., 1993; Kishor et al., 1995; Pilon-Smits et al., 1995; Hayashi et al., 1997). The same is true for trehalose-producing transgenic rice (Garg et al., 2002; Jang et al., 2003). Here, we tested salt tolerance by continuously irrigating transgenic plants with 250 mM NaCl. As with the dehydration treatments, our transgenics were less negatively affected by wilting and necrosis. In addition, leaf Chl content was only slightly reduced in the transgenics, compared with the sharp decline measured in the non-transformants. The degree of tolerance against salinity also depended upon the *otsA* expression level, indicating that T6P was the protective molecule. These results demonstrate the membrane-stabilizing effect of trehalose or T6P because high salt normally destabilizes the thylakoid membrane structure by affecting the water-splitting complex, leading to Chl-bleaching. To confirm this, we monitored changes in Chl fluorescence, but found that PSII function was not significantly affected by salinity, with few changes measured in Fo or Fv/Fm.

As an additional criterion for salt tolerance, we tested germination efficiency under salinity. As expected, the germination of non-transformant seeds was completely blocked in the presence of 250 mM NaCl while, under the same conditions, that of the transgenic seeds was partially rescued. Furthermore, most germinated seedlings of the non-transformants did not survive while some from the transgenic lines did. Therefore, trehalose or T6P appears to protect embryos from excessive salt. The germination efficiency under saline stress was dependent upon the expression level of *otsA*, as was also observed with our dehydration treatments. In trehalose-producing rice leaves, the ion balance is maintained by effluxing Na^+ when 100 mM NaCl is supplemented (Garg et al., 2002). Therefore, in addition to membrane stabilization, the maintenance of this balance may be essential to the survival of embryos under salt stress, and a high amount of trehalose or T6P may be a prerequisite for this.

Trehalose and T6P: Signaling Molecules or Multiple Stress Protectants?

We have demonstrated that *otsA* over-expressing tobacco plants exhibit increased tolerance to drought, salt, and heat stresses, but not to chilling stress (unpublished data). Moreover, we have found that *AtTPS1*, a functional homologue of *TPS* in *Arabidopsis*, is up-regulated by various abiotic stresses, including drought, salt, and heat (unpublished data). Although it is not conclusive whether trehalose or T6P is the acting molecule for this protection, we favor trehalose for the following reasons. First, senescence was significantly delayed in our *otsA* over-expressing plants due to the membrane-stabilizing effect of trehalose (unpublished data). Second, PSII was better protected in trehalose-producing plants after heat treatment at 45°C in the dark (unpublished data). All of these results indicate that the thylakoid membranes in the chloroplasts are apparently protected by the cytoplasmic production of trehalose. Finally, trehalose production in the chloroplasts is equally effective as in the cytosol in conferring tolerance against dehydration and salt stresses (Garg et al., 2002). Trehalose is likely to move across the chloroplast membrane more easily than T6P.

If we conclude that trehalose is indeed the effective molecule, it is very likely that this disaccharide acts as a multiple stress protectant in higher plants as it does in microorganisms. Trehalose stabilizes the membrane structure to enable tolerance against heat or salinity, in addition to its osmoprotective properties (Crowe et al., 1984; Iwahashi et al., 1995; Singer and Lindquist, 1998). However, the different natures of the stresses applied here make it difficult for us to regard it as a simple stress protectant, thereby suggesting it as a regulatory molecule in the signaling network of various abiotic stresses. T6P levels are positively correlated with stress responsive genes in *Arabidopsis*, as demonstrated by microarray studies (Schluepmann et al., 2003, 2004). Nevertheless, the correlation between the degree of tolerance and *TPS* expression level or trehalose content implies that it may be working directly as a multiple stress protectant. In addition, the modulation of *AtTPS1* under abiotic stresses, as seen in our own study, suggests that trehalose production is closely related to stress responses. Therefore, although we cannot exclude the possibility of trehalose acting as a signaling molecule, as has been postulated by Goddijn and Smeekens (1998) and Müller et al.

(1999), we believe that trehalose may function simply as a multiple stress protectant. Because the nature of that protection may depend upon the given stress, its elucidation requires further investigation. In contrast, T6P, appears to be involved primarily in the regulation of various developmental processes.

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