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Transgenic Tobacco Plants Expressing the Bacterial Levansucrase Gene Show Enhanced Tolerance to Osmotic Stress

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Abstract Fructans are polyfructose molecules that function as nonstructural storage carbohydrates in several plants. In addition, it has been suggested that, due to their solubility, they can play an important role in helping plants survive periods of osmotic stress. In order to study the effect of levan synthesis on plant growth, the coding region of the levansucrase gene, which was isolated from *Zymomonas mobilis*, was introduced into tobacco plants using *Agrobacterium tumefaciens*-mediated transformation. The presence of the levansucrase gene in transgenic plants was verified by genomic DNA gel blot analysis. RNA gel blot and immunoblot analyses showed an accumulation of the corresponding transcript and protein product of the bacterial levansucrase gene in transgenic plants. Furthermore, a thin layer chromatography analysis revealed that fructans were synthesized and deposited in transgenic tobacco plants. When T_1 seeds were germinated and grown under polyethylene glycol-mediated drought stress or cold stress, the transgenic seedlings displayed a substantially higher level of growth than that of untransformed plants. These results suggest that fructans may play a significant role in the tolerance of plants under osmotic stress.

Key words: Levansucrase, osmotic stress, tobacco, transgenic plant, *Zymomonas mobilis*

Plant growth and productivity are dramatically reduced by drought, low temperature and high salinity [3]. Many studies have been performed in order to develop genetically engineered plants with enhanced tolerance to environmental stresses [2, 20, 24]. Many species of plants and bacteria synthesize and accumulate osmotically active, low molecular weight compounds such as sugar alcohols, proline, and glycinebetaine that are known collectively as osmolytes or compatible solutes. Although their exact function in plants is unknown, transgenic plants which synthesize the compatible solutes are enhanced in stress tolerance [6, 11, 20, 24].

In addition to low molecular weight organic compounds, it has been suggested that high molecular weight fructans also serve as an osmoprotectant because of their solubility [16, 18]. Fructans are produced as a prevalent storage carbohydrate in some plants and bacteria. The degree of polymerization of plant fructans varies from 10 to 250 fructose units [17], while bacterial fructans can range over 100,000 fructose units [5]. Differences in fructan polymerization degree arise not only due to taxonomic variation, but also due to the consequence of environmental influence [18]. Despite these findings, the functional significance of fructans in plants remains to be clarified. The biosynthetic pathways for fructans have been characterized in plants and in microorganisms. In plants, fructans are synthesized from sucrose in the vacuole by at least two fructosyl transferases [4, 18]. However, bacterial fructan biosynthesis involves only one enzyme [5].

To elucidate the function of fructan, Pilon-Smits *et al.* [16] produced transgenic plants containing the *sacB* gene from *Bacillus subtilis* [23], which encodes a levansucrase, and showed that the transgenic plants exhibited an increased tolerance level to drought stress. In a similar approach, we introduced another bacterial levansucrase gene isolated from *Z. mobilis* [22] into tobacco plants, and made an investigation to determine the tolerance level of transgenic plants to drought and cold stress.

MATERIALS AND METHODS

Chemicals

Restriction enzymes and DNA modifying enzymes were purchased from Boehringer Mannheim (Mannheim,

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Germany) and Promega Co. (Madison, U.S.A.). α-^{32}P-dCTP was purchased from Amersham (Little Chalfont, U.K.) and other chemicals were from Sigma Chemical Co. (St. Louis, U.S.A.).

Vector Construction and Plant Transformation

The levansucrase gene (*levU*) was obtained from plasmid pZL8 [22]. To isolate the entire coding region of the *levU* gene, PCR was performed by using two primers: *levU* 5' (5'-CGCCGGATCCACATGTTGAATAAAGCAGGC-3) and *levU* 3' (5'-CGCGGATCCACATGTGCATAATCA-GAAACGTC-3'). The amplified PCR product (1.3 kb) was ligated with the pMBP1 plant expression vector containing a CaMV 35S promoter [10]. This construct was introduced into *Nicotiana tabacum cv.* WI38 by the *Agrobacterium*-mediated plant transformation method [8] using an *A. tumefaciens* strain LBA 4404.

Plant Growth Conditions

Tobacco plants were grown either on Murashige and Skoog (MS) [15] plates at 25°C in a growth chamber or in a green house under conditions of 70% room humidity and a 16 h/8 h light/dark cycle. For drought stress, 8% polyethylene glycol (PEG) 8,000 was added to MS plates. For the cold treatment, four-week-old plants that had been grown on MS agar plates were subjected to stress caused by the low temperature. To this end, plants were transferred and maintained in a growth chamber preset at 2°C under a 16 h/8 h light/dark cycle for a period of 7 days. Then they were allowed to grow under normal growth conditions.

DNA and RNA Analyses

Genomic DNA was isolated from four-week-old tobacco leaves by the method of Ausubel *et al.* [1]. To prepare for Southern blot, 10 µg of genomic DNA was digested with 50 units of restriction enzymes overnight. DNA fragments were separated by electrophoresis overnight in a 0.8% agarose gel in 1×TBE buffer [21] and transferred to Nytran Plus membrane (Schleicher and Schuell, Dassel, Germany) in 10×SSC (1×SSC=0.15 M NaCl, 0.015 M sodium citrate, pH 7.2). Total RNA was prepared from four-week-old leaves as described by Ausubel *et al.* [1]. Twenty micrograms of total RNA were electrophoresed in a 1.5% agarose gel containing 6% formaldehyde in MOPS buffer (pH 7.0) and transferred to Nytran Plus membrane. The PCR-amplified product (1.3 kb), which represents the complete coding sequence of the levansucrase gene from plasmid pZL8, was radiolabeled with α-^{32}P-dCTP and used as a probe.

Immunoblot Analysis

Samples of leaf tissues were ground in a mortar pre-chilled with liquid nitrogen and homogenized in 50 mM Tris-HCl, 2 mM EDTA, pH 7.0. The extracts were centrifuged at 13,000 rpm for 10 min at 4°C, and the supernatant was collected. The total protein extracts (30 µg) were subjected to SDS-PAGE according to Laemmli [13], and transferred onto a polyvinylidene difluoride (PVDF) membrane (Schleicher & Schuell, Dassel, Germany) by the semi-dry blotting method [7]. Immunostaining was carried out with the use of the polyclonal anti-LevU serum, which was produced from rabbits injected with LevU recombinant protein that was expressed in bacteria [12]. The antiserum was diluted 1:1000 before being applied as a primary antibody in immunostaining. As a secondary antibody, the horseradish peroxidase-conjugated anti-rabbit IgG antibody (Amersham, Little Chalfont, U.K.) was used. Detection of the immunoreactive protein in protein gel blot was carried out with the ECL detection system (Amersham, Little Chalfont, U.K.) according to the procedures described by Kim *et al.* [9].

Fructan Extraction and Analysis by Thin-Layer Chromatography

Leaf tissues (0.2 g) were harvested from four-week-old transgenic and untransformed control plants, ground in liquid nitrogen, and homogenized in 500 µl of water. The homogenate was centrifuged at 16,000 rpm for 10 min and the supernatant containing soluble carbohydrates was precipitated overnight in two volumes of 90% ethanol. Fructan was cleaved in 200 mM H_2SO_4 at 60°C for 1 h and neutralized with NaOH. Six microliter aliquots from 100 µl were spotted on a thin layer chromatography plate (TLC silica gel, Schleicher and Schuell, Dassel, Germany). The TLC was performed in a solution of 15:12:13 chloroform: methanol:acetic acid and the chromatogram was visualized by spraying with naphthoresorcinol-sulfuric acid.

RESULTS AND DISCUSSION

Introduction of Levansucrase Gene into Tobacco Plants

Tobacco plants do not normally contain fructan as a storage carbohydrate [14]. To investigate whether fructan-accumulating plants are more tolerant than non-accumulating plants to water stress, the bacterial gene encoding levansucrase protein isolated from *Z. mobilis* was introduced into tobacco plants. *Z. mobilis* is a bacterium that is used in fermenting sugars to alcohol [19], and has been employed as an alternative source for isolating the levansucrase gene [22]. The construction of plant transformation vector containing p35S-*levU* is presented in Fig. 1. To ensure the uniform expression of the bacterial enzyme throughout different plant parts and cellular compartments, the construct was fused to the cauliflower mosaic virus 35S promoter. A previous study had shown that another bacterial levansucrase gene placed under the control of the same 35S promoter was targeted to vacuoles [25].

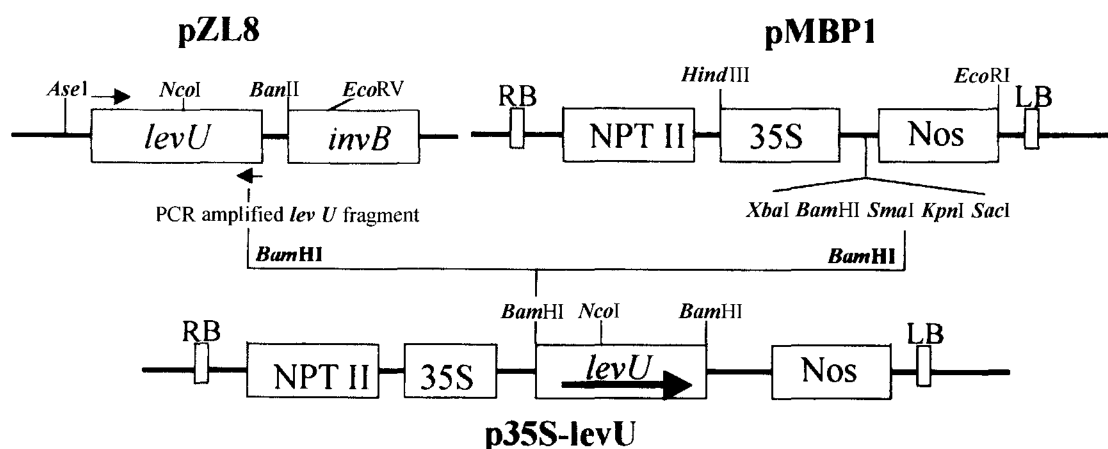

Fig. 1. Construction of plant expression vector containing levansucrase gene.
The *levU* gene was placed under the control of a CaMV 35S promoter and terminated by the nopaline synthase (*nos*) terminator.

After *Agrobacterium*-mediated transformation of tobacco with the levansucrase gene construct, transgenic plants were selected for kanamycin resistance and five independent primary transformants (T_0) were obtained. These T_0 plants were self-fertilized to yield T_1 progenitor lines, two of which were selected as showing elevated levels of drought tolerance through a preliminary screen. For the purpose of estimating the number of DNA copies integrated into the individual plant genome, genomic DNA gel blot analysis was performed (Fig. 2). Genomic DNAs isolated from plants of the T_1 transgenic lines and control plants were digested with *Bam*HI and *Eco*RI. The hybridization result showed that a single copy of the *levU* gene was incorporated into the tobacco genome.

Expression of the Levansucrase Gene in Transgenic Plants

To monitor the expression level of the *levU* gene in transgenic plants, RNA gel blot analysis was performed (Fig. 3A). In contrast to the untransformed control plants in which transcripts homologous to the *levU* gene were

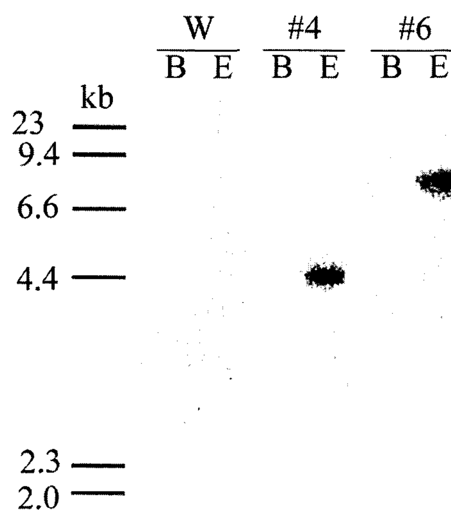

Fig. 2. Genomic DNA gel blot analysis of transgenic tobacco plants.
Genomic DNA (10 μg) was restricted with *Bam*HI (B) and *Eco*RI (E). The 1.3-kb *levU* coding region was amplified by PCR and used as a probe. W denotes untransformed control plant, and the numbers indicate independent lines of transgenic plants.

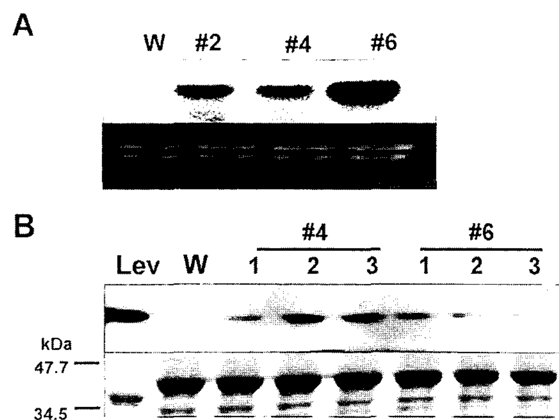

Fig. 3. RNA gel blot and immunoblot analyses of transgenic tobacco plants.
A. Total RNA was isolated from the transgenic plants and analyzed by RNA gel blot hybridization using the entire coding region *levU* as a probe (top). This permitted identification of the *levU* mRNA of approximately 1.6 kb in size. Equal loading was verified by visualizing rRNA in the gel stained with ethidium bromide (bottom). B. Immunoblot analysis of protein extracts from leaves of transgenic plant (T_1) (top). Equal amounts of protein (30 μg) were loaded in each lane, as shown by visualizing Coomassie blue-stained SDS gel. In both A and B, W denotes total RNA or protein extract samples from untransformed control plant. The numbers #2, #4, and #6 indicate independent lines of transgenic plant, and the lanes 1, 2, and 3 represent their progeny (T_1). Lev: purified levansucrase protein (10 ng).

undetectable, leaves of the T_1 transgenic plants were detected to contain substantial amounts of the steady-state *levU* transcripts of approximately 1.6 kb in size. In order to examine whether these transcripts indeed led to the synthesis of the protein product, an immunoblot analysis was carried out with an antiserum raised against levansucrase. According to the result, it was revealed that a 43 kDa protein accumulated in the leaves of transgenic plants (Fig. 3B). The size of the detected protein was identical to that of the bacterial levansucrase protein. These results demonstrate that the *levU* gene is expressed and maintained in a stable condition in T_1 generation of the *LevU* transgenic plants, thus providing a first direct piece of evidence for the expression of the introduced bacterial levansucrase gene in transgenic plants at both RNA and protein levels.

Accumulation of Fructans in Transgenic Plants

In order to investigate the accumulation of fructans in transgenic plants, fructans were extracted from leaves and analyzed by TLC (Fig. 4). For this purpose, fructans were isolated from the transgenic and untransformed control plants by ethanol precipitation of water extracts. The average degree of polymerization of the fructan precipitable under this condition is at least 6 [14]. To completely hydrolyze fructan, the precipitated fructan was dissolved and incubated in sulfuric acid at 60°C for 1 h. The depolymerized fructan samples were then spotted on the TLC plate. As shown in Fig. 4, TLC analysis revealed that not only did the transgenic plants produce fructans, but those fructans consisted of polymerized fructoses exclusively. These results are supported by our foregoing finding that the introduced levansucrase gene was transcribed and translated correctly in the tobacco cells (see above). It is therefore interpreted that the observed accumulation of fructans in transgenic tobacco plants is the consequence of the novel activity of the levansucrase protein that polymerizes sucrose units into oligomers. These results are also in agreement with previous reports that introduction of the *B. subtilis* levansucrase gene into potato [25] and tobacco [16] significantly increased the amount of fructans that were synthesized in many different plant tissues including leaves and tubers.

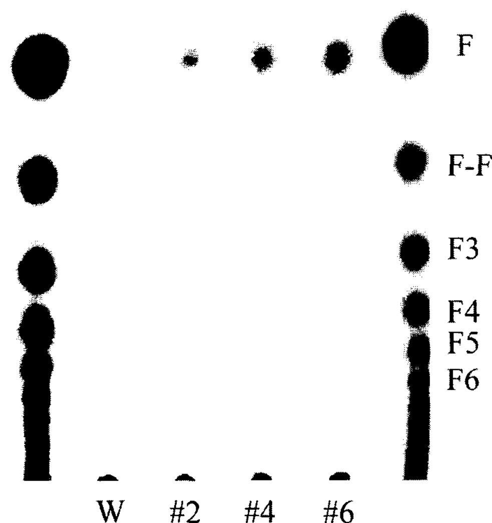

Fig. 4. TLC analysis of fructan accumulation in transgenic tobacco plants.

Fructan was isolated from untransformed (W) and three independent transgenic plants (#2, #4, and #6), and were subjected to depolymerization by chemical cleavage. Samples were spotted on TLC plates and the chromatogram was stained with naphthoresorcinol-sulphuric acid. The positions of fructose monomer (F) and oligomers (F-F to F6) are indicated.

Tolerance of Transgenic Plants to Drought and Cold Stress

To analyze the effect of transformation of the levansucrase gene on tobacco, seeds of the transgenic and untransformed plants were germinated in the growth medium and seedlings were analyzed for growth. The early growth rate of the transgenic plants was slightly faster than that of untransformed control plants (Fig. 5A). However, there was no significant difference in the phenotype and the timing of the flowering between the two in the later stages of development (data not shown). To examine the effect of stress conditions, these plants were exposed to drought and cold stress, respectively. Figure 5B shows the effect of the PEG-mediated drought stress on the growth of the untransformed and two independent lines of transgenic plants. Seeds were germinated and grown for the length of 3 weeks on MS agar plates containing 8% PEG 8,000. Under this condition, most kanamycin resistant transformants grew as well as unstressed plants. But on the other hand, untransformed control plants could not grow in the same condition. To investigate the protective effects of the fructan at a low temperature, four-week-old untransformed and transgenic plants were also exposed to 2°C for one week and then they were allowed to grow under normal growth conditions (Fig. 5C). Unfortunately, this treatment caused all untransformed plants to not survive. By contrast, the transgenic plants still contained chlorophyll in their young leaves, although their old leaves had some noticeable damage, such as bleaching of chlorophyll and necrosis. These results indicate that fructan synthesized in transgenic tobacco conferred tolerance against drought and cold stress.

Fructan metabolism has been suggested to play a role in the toleration of drought or cold stress [17, 18]. The drought-related increase in fructan concentration was also reported under a low temperature [16]. In addition, a higher dry matter percentage of the transgenic plants relative to untransformed control plants has been associated with fructan-induced increase in the levels of nonstructural carbohydrates such as cell-wall components [25]. However, the mechanism underlying the improved performance of

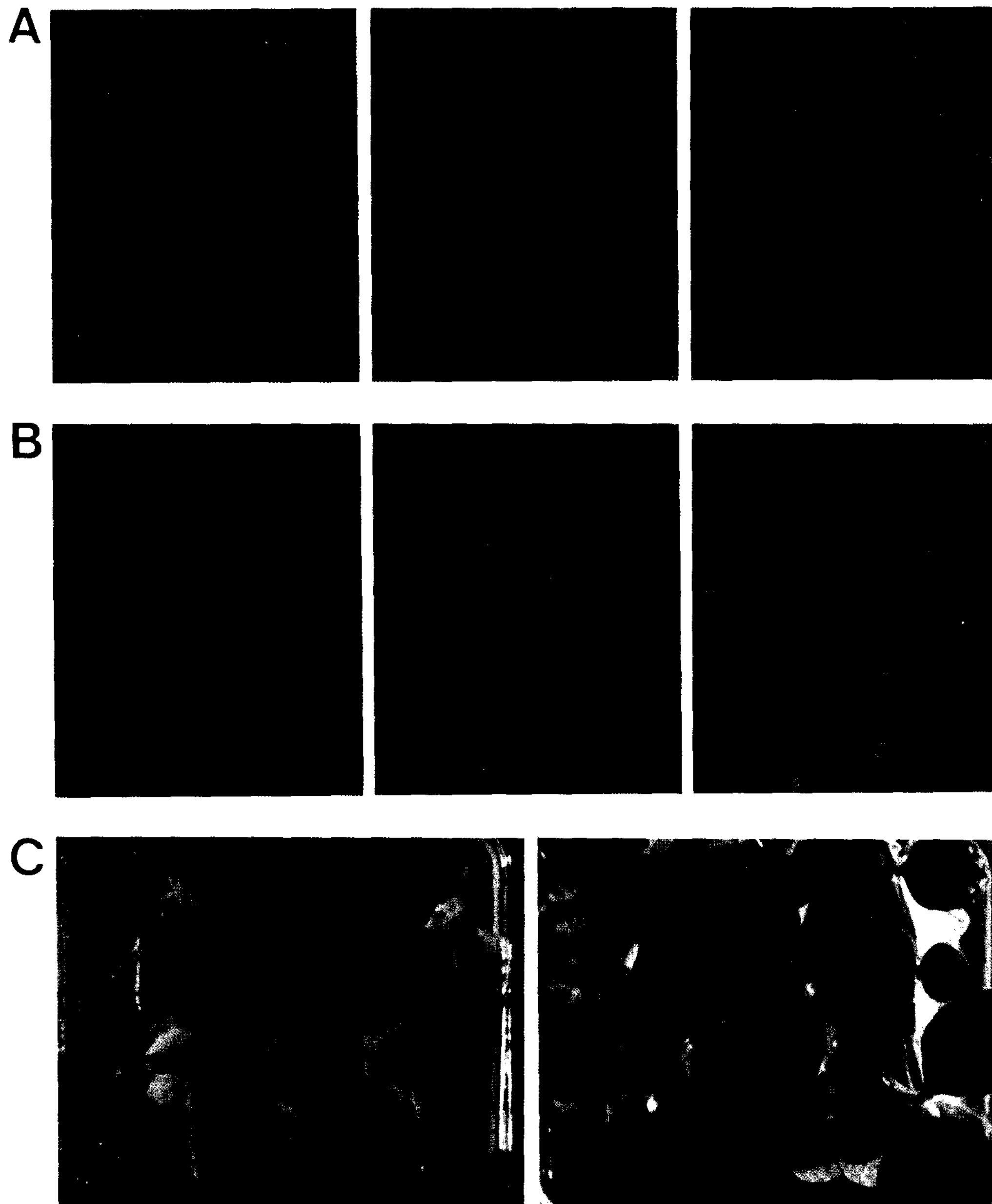

Fig. 5. Phenotypes of transgenic tobacco plants under drought and cold stress.
A. Comparison of untreated tobacco plants three weeks after germination on MS agar plates. a: untransformed control plants; b and c: transgenic plants containing levansucrase gene. B. Growth performance of untransformed and transgenic tobacco plants under PEG-induced drought stress. Untransformed (a) and transgenic tobacco plants (b and c) were germinated and grown on MS agar plates containing 8% PEG 8,000 in a growth chamber at 25°C. C. Response of untransformed and transgenic tobacco plants to cold treatment. Four-week-old untransformed (a) and transgenic tobacco plants (b) were transferred to a growth chamber at 2°C and maintained for 7 days.

the fructan-accumulating plants under drought and cold stress remains to be clarified.

Acknowledgments

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