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Improved Performance of Transgenic Fructan-Accumulating Tobacco under Drought Stress[1]

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Fructans are polyfructose molecules produced by approximately 15% of the flowering plant species. It is possible that, in addition to being a storage carbohydrate, fructans have other physiological roles. Owing to their solubility they may help plants survive periods of osmotic stress induced by drought or cold. To investigate the possible functional significance of fructans, use was made of transgenic tobacco (*Nicotiana tabacum*) plants that accumulate bacterial fructans and hence possess an extra sink for carbohydrate. Biomass production was analyzed during drought stress with the use of lines differing only in the presence of fructans. Fructan-producing tobacco plants performed significantly better under polyethyleneglycol-mediated drought stress than wild-type tobacco. The growth rate of the transgenic plants was significantly higher (+55%), as were fresh weight (+33%) and dry weight (+59%) yields. The difference in weight was observed in all organs and was particularly pronounced in roots. Under unstressed control conditions the presence of fructans had no significant effect on growth rate and yield. Under all conditions the total nonstructural carbohydrate content was higher in the transgenic plants. We conclude that the introduction of fructans in this non-fructan-producing species mediates enhanced resistance to drought stress.

Fructans are polyfructose molecules that are produced by many plants and bacteria. Approximately 45,000 plant species use fructans as their main storage carbohydrate (Nelson and Smith, 1986; Hendry, 1987, 1993). Some prominent families of the fructan flora are Poaceae (e.g. wheat, barley), Liliaceae (e.g. onion, tulip), and Asteraceae (e.g. Jerusalem artichoke, chicory). In plants the synthesis of fructans from Suc involves at least two enzymes. The first enzyme catalyzes the production of a Glc-Fru-Fru trisaccharide that can be extended with Fru residues in various ways by one or more other enzymes (Pollock and Cairns, 1991). There is substantial variation in both linkage type and length of plant fructans. The functionality, if any, of the different fructan structures is as yet not clear. The DP of plant fructans varies from 10 to 200 Fru units. Differences in fructan length not only result from taxonomic variation but are also subject to environmental influences: plants have been reported to respond to changing conditions by shifting the average length of their fructan pool (Pontis and Del Campillo, 1985).

In addition to plants, several bacteria synthesize fructans. Bacterial fructan biosynthesis from Suc involves only one enzyme. Most bacterial fructans have a very high DP (up to 100,000) and a 2–6-linkage type with occasional 2–1 branching (Dedonder, 1966).

One of the goals of our studies is to elucidate why some plant taxa use fructans as the predominant storage carbohydrate instead of starch, which is ubiquitous in the plant kingdom. In other words, what is the functional significance of fructans and under which selection pressure has fructan metabolism evolved? The most obvious differences between starch and fructan are the location and solubility. Fructans are located in the vacuole and are soluble, in contrast to the insoluble plastidic starch. A possible advantage of vacuoles as storage organelles could be that the storage capacity of vacuoles may be larger than that of the plastids, since the vacuole constitutes 95% of the protoplast volume. Fructan storage capacity in plants may be further increased by the formation of specialized organs like bulbs (onion, tulip), tubers (Jerusalem artichoke), or succulent stems (*Agave*). Fructan is indeed often accumulated to higher quantities than starch (Brocklebank and Hendry, 1989).

Since fructans are soluble, they may play a role in the osmotic adjustment of natural fructan accumulators to changing environmental conditions via variation in the DP of the fructan pool. An example of osmotic adaptation via the use of fructans is the rapid conversion of fructan into low-DP products as a mechanism to sustain petal expansion in the daylily (Bieleski, 1993). Also, fructan metabolism may play a role in the toleration of drought or cold stress (Pontis and Del Campillo, 1985; Pollock, 1986). A role in drought resistance was suggested by Hendry (1993), who argued that (a) the historical increase of fructan-producing taxa (30–15 million years ago) corresponds with a climatological shift toward seasonal drought and (b) the

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Abbreviations: DP, degree of polymerization; RGR, relative growth rate; WT, wild type.

distribution of present-day fructan flora corresponds with regions of seasonal drought. Functions of fructans in cold stress probably involve enhanced growth at low temperature rather than freezing tolerance (Pollock et al., 1988). Fructan-producing species often show growth during late winter or early spring (Brocklebank and Hendry, 1989). As a means of osmotic adjustment in *Helianthus tuberosus* the average DP of the fructan pool declines with decreasing temperature (Pontis and Del Campillo, 1985).

Our approach to investigating the functional significance of fructans has been to use transgenic plants differing from the WT only in the capacity to produce fructans. This was achieved by introducing a new gene into a non-fructan-accumulating plant (gain of function). Since no plant genes involved in fructan metabolism have yet been isolated, the *SacB* gene from *Bacillus subtilis* was used, encoding levansucrase (Steinmetz et al., 1985). Transgenic plants have been produced that accumulate bacterial fructans using this construct (Ebskamp et al., 1994; Van der Meer et al., 1994). The isogenic tobacco (*Nicotiana tabacum*) lines differing in the presence of fructans only provide ideal material to investigate the effect of fructans on growth performance and stress resistance. We have investigated growth characteristics of these lines under conditions of reduced water supply to obtain better insight into the physiological function of fructans.

MATERIALS AND METHODS

Plant Material

Nicotiana tabacum var petit Havana plants were transformed with the pKP construct as described by Ebskamp et al. (1994). This construct contains the *SacB* gene from *Bacillus subtilis* fused to the carboxypeptidase Y vacuolar sorting signal from yeast, placed under the constitutive 35S cauliflower mosaic virus promoter. Whether the levansucrase protein and fructan produced are indeed present in the vacuole is not yet certain. Transformant KP12, which had a high fructan level, was bred to homozygosity, and plants from the homozygous fructan-accumulating line KP12–9 were used for the experiments described here.

Growth Conditions and Stress Induction

Tobacco WT and transgenic (KP) plants were grown either in a growth chamber under continuous light (PAR 42 μmol m^{-2} s^{-1}) at 25°C or in a greenhouse under natural conditions of light and temperature (June/July, Gent, Belgium). The data presented are from growth chamber experiments unless stated otherwise. Similar experimental procedures were followed in the experiments carried out in the growth chamber and the greenhouse. The plants were grown hydroponically on vermiculite that was covered with plastic to prevent evaporation, under a constant supply of nutrient solution containing 2.93 mM KNO_3, 0.73 mM KH_2PO_4, 1.6 mM $MgSO_4 \cdot 7H_2O$, 0.75 mM $(NH_4)2SO_4$, 2.86 mM $Ca(NO_3) \cdot 4H_2O$, 82 μM NaFeEDTA, 1.05 μM H_3BO_3, 0.78 μM KCl, 0.27 μM $MnSO_4 \cdot H_2O$, 20 nM $ZnSO_4$, 4.7 nM $(NH_4)6Mo7O_{24} \cdot 4H_2O$, and 11.25 nM $CuSO_4 \cdot 5H_2O$.

To impose drought stress PEG 10,000 was added to the nutrient solution in both drought stress experiments. PEG 10,000 is too large to be taken up by intact plant roots (Lawlor, 1970) and enables the imposition of uniform and controllable drought stress. Ten plants of both WT and KP tobacco were stressed, and equal numbers were kept non-stressed as controls. The experiments were started with 3-week-old plants. After 9 d, drought stress was imposed on the treated groups by adding PEG 10,000 to a final concentration of 5% (w/v, −0.4 MPa) stepwise throughout a period of 3 d to allow the plants to adjust gradually. Eight days later the drought stress imposed on the treated groups was further increased to 10% PEG (−0.8 MPa). Subjection to 10% PEG caused reversible wilting of older leaves. Plant growth was followed both by measuring stem height and dry weight (RGR). The plants were harvested just before flowering. The fresh weight of the different organs was determined, as was the dry weight after 2 d at 80°C.

Analysis of Carbohydrates

Samples for carbohydrate analysis were taken at the end of each PEG treatment (0, 5, and 10% PEG). From the 10 plants per treatment three pooled samples were taken from the youngest mature leaves. The samples were ground in liquid nitrogen. Soluble carbohydrates were extracted from approximately 300-mg samples, in succession, with 1 mL g^{-1} fresh weight of water, 80% ethanol, and water, respectively, and extracts were pooled. The water extractions were performed at 90°C for 5 min, and the ethanol extractions were performed at room temperature. Starch was solubilized by a 1-h incubation in 20 mM NaOH at 75°C and, after neutralization with 4.5 μL 1 M H_2SO_4, further degraded into Glc by overnight incubation at 37°C with 5 units of amyloglucosidase at pH 4.6. The levels of the extracted Suc, Glc, Fru, and starch-derived Glc were analyzed by HPLC on an Aminex HPX-87C column (Bio-Rad) at 85°C, using water as eluent and added Man as a standard.

For fructan analysis the extracted soluble carbohydrates were separated by TLC (TLC Silica-gel, Schleicher & Schuell, Dassel, Germany). After two runs in 90% (v/v) acetone, Fru-containing sugars were visualized specifically with urea spray at 80°C (Wise et al., 1955). The fructans (at the application spot) were quantified by laser scanning (LKB Gelscan XL) using levan (Sigma) as a standard.

Statistical data were obtained by one-way analysis of variation using the program Number Cruncher Statistical System (Dr. J.L. Hintze, 865 East 400 North, Kaysville, UT 84037).

RESULTS

Growth Performance

We investigated the growth performance of WT and transgenic (KP) tobacco plants under PEG 10,000-induced drought stress. Growth under 10% PEG stress was significantly faster in KP plants than in WT plants, both when expressed in terms of change in stem height (Fig. 1; Table

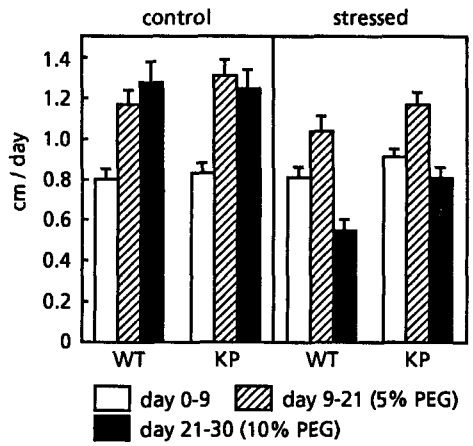

Figure 1. Growth rates (cm/d) of transgenic fructan accumulating (KP) and WT tobacco plants, grown in a growth chamber, either nonstressed (control) or drought stressed by PEG 10,000. Day 0 to 9, 0% PEG; d 9 to 21, 5% PEG; d 21 to 30, 10% PEG. The growth rate represents the average ± SE of 10 plants.

I) and as RGR (Table II). Under unstressed control conditions no significant difference in growth was observed between KP and WT plants.

There was no difference in the onset of flowering between WT and KP plants; nor was any difference in phenotype observed, other than size. At the time of harvest the drought-stressed KP plants yielded significantly more fresh and dry weight than stressed WT plants (Fig. 2; Table I). The differences in dry weight were especially pronounced because the KP plants contained a higher dry weight content than WT plants, in particular under drought stress (Table II). In fact, the dry weight of stressed KP plants was not significantly different from that of unstressed KP plants (Fig. 2). The observed difference in weight between drought-stressed KP and WT plants was especially pronounced for the roots: KP plant roots increased 73% in weight under drought stress compared to unstressed plants, whereas WT roots showed a decrease (Fig. 2; Table I). This is also reflected by the shoot/root ratios (Table II). Under control conditions fresh and dry weight yields of KP and WT plants did not differ significantly.

Similar results were obtained under greenhouse conditions, in which plants were grown at natural (summer) photoperiod and temperature. Drought-stressed KP plants yielded 19% higher fresh weight and 32% higher dry weight than WT plants (results not shown). Under unstressed conditions no significant differences were observed. These results were obtained with pooled plant material and, therefore, the statistical significance of the differences could not be assessed. Under greenhouse conditions, the KP plants accumulated half as much fructan as in the growth chamber, perhaps because of the higher growth rates.

Carbohydrate Composition

Leaf samples were taken for carbohydrate analysis at the end of each PEG level period (Fig. 3). In WT and KP plants similar Suc, Glc, and Fru patterns were observed: under nonstressed conditions amounts increased with age by a factor of 2 to 3, whereas drought stress caused an extra 2- to 3.5-fold increase (Fig. 3, A–C). The starch pattern was different from the soluble sugars and also differed between WT and KP ($P \leq 0.04$ at d 28). In WT plants the starch level did not increase with age, whereas it increased 2.6-fold in KP (Fig. 3D). Drought stress did not have any influence on starch level in KP or WT plants. The fructan levels in control KP plants increased about 2-fold with age. Drought stress induced an additional 7-fold increase in fructan concentration (Fig. 3E). The total concentration of nonstructural carbohydrates (sum of A–E) was higher in KP plants than in WT plants under all conditions (Fig. 3F). The increase with age was larger in KP than in WT plants, whereas the additional increase with drought was relatively smaller.

Since the difference in growth performance between WT and KP plants was most pronounced for the roots, we tested whether there was any difference in carbohydrate composition between WT and KP plants in roots, as compared to stem and leaf material. The carbohydrate composition of root, stem, and leaf material was very similar in nonstressed WT and KP plants (results not shown). The roots contained predominantly Suc, whereas the aboveground parts contained comparable amounts of Suc, Glc, and Fru. In KP plants fructans were found in all organs in about equal amounts.

DISCUSSION

Previously transgenic tobacco plants have been produced that express the bacterial *SacB* gene, leading to the

Table I. *Growth performance of KP tobacco as percentage of wild type, either nonstressed or grown under PEG-induced drought stress in a growth chamber*

RGR was measured during the total growing period. Stem growth shown is during the last 10 d of the experiment (10% PEG). Fresh weight and dry weight were measured after the entire growth period.

Sample	Fresh Wt			Dry Wt			Stem Growth (d 21–30)	RGR (d 0–30)
	Shoot	Root	Total	Shoot	Root	Total		
Control	106	89	105	109	96	109	98	104
P	NS[a]	NS	NS	NS	NS	NS	NS	NS
Drought stressed	131	161	133	155	190	159	155	118
P	0.03	0.04	0.02	0.003	0.005	0.002	0.003	0.02

[a] NS, Not shown.

Table II. *Growth characteristics of WT and transgenic (KP) tobacco plants grown in a growth chamber, either nonstressed or drought stressed*

% DW = dry weight/fresh weight × 100%. Values are the averages of 10 plants.

Sample	RGR	Percent DW	Shoot/Root	
			Fresh wt	Dry wt
	$kg\ kg^{-1}d^{-1}$			
WT nonstressed	0.090	5.3	24.2	8.6
KP nonstressed	0.094	5.4	28.8	9.8
WT drought stressed	0.077	6.9	17.8	5.9
KP drought stressed	0.091	8.2	14.3	4.8

accumulation of fructans in a species with no native fructan (Ebskamp et al., 1992). These plants have enabled us to investigate the effect of fructans on plant growth and stress resistance, with the aim to obtain more insight into the function of fructans. We conclude that the introduction of fructan synthesis in tobacco leads to enhanced resistance to PEG-induced drought stress. These data are, therefore, consistent with the hypothesis that water availability may be the selection pressure that has driven the evolution of fructan metabolism in plants.

The novelty of these results is that they provide the first experimental data suggesting that accumulation of fructans in plants is effective as a device against water stress. These findings are of significance considering that the function of fructans has been subject to discussion but also in view of the fact that water availability is the most stringent factor limiting plant distribution and productivity (Boyer, 1982). Our data are not the first report of a conference of stress protection by a foreign compound. Tarczynski et al. (1993) demonstrated enhanced salt-stress tolerance following the introduction of a bacterial gene encoding mannitol 1-phosphate dehydrogenase, which resulted in mannitol accumulation in tobacco.

WT tobacco cannot produce or degrade fructan (Ebskamp et al., 1994). Thus, the fructan present in these transgenic plants is synthesized by a fructosyltransferase of bacterial origin as encoded by the *SacB trans*-gene. Indeed the fructan in these plants is of the bacterial levan type, with a DP > 25,000 and a 1H NMR pattern similar to levan from *B. subtilis* (Ebskamp et al., 1994). Also these fructans are recognized by a monoclonal antibody directed against the β-2–6-linkage type (our unpublished results), which is not found in dicotyledons that accumulate fructans. The presence of the bacterial gene in these plants was established by Southern blotting, but no mRNA could be detected in transgenic tobacco or potato, in spite of the 35S promoter used and in spite of the high fructan levels obtained in some of these plants (Ebskamp et al., 1994; Van der Meer et al., 1994). Fructan biosynthetic enzyme activity can be detected in these transgenic species but only by using a sensitive assay (Van der Meer et al., 1994, and our unpublished results).

Drought resistance is a complex trait and the question arises whether factors other than the fructan introduced are responsible for the resistance displayed in the transgenic plants, e.g. the position of integration of the *trans*-gene in the tobacco genome. The plants seem perfectly normal in every respect except drought resistance, and the drought resistance observed correlates well with the amount of fructan accumulated. Therefore, we favor the explanation that fructan biosynthesis is somehow involved in the enhanced performance under drought stress.

The mechanism of the improved performance of the fructan-accumulating plants under drought stress is not yet clear. While trying to explain the results obtained we have to keep in mind that the fructan concentration is quite low compared to other sugar levels. Also the cellular distribution of the fructan, which of course has important implications for fructan concentration and mode of action, is not yet certain. The amount of fructan accumulated in the leaf

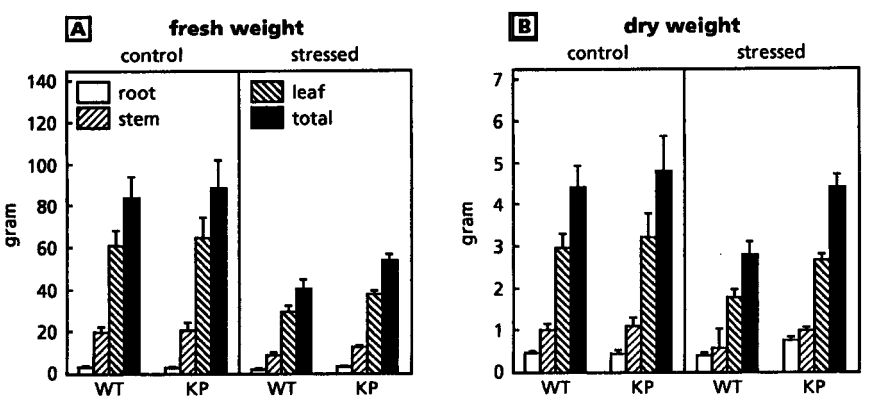

Figure 2. Fresh weight (A) and dry weight (B) of WT and KP tobacco plants grown in a growth chamber under low continuous light at 25°C. Left, Controls; right, drought stressed. Values are the averages ± SE of 10 plants.

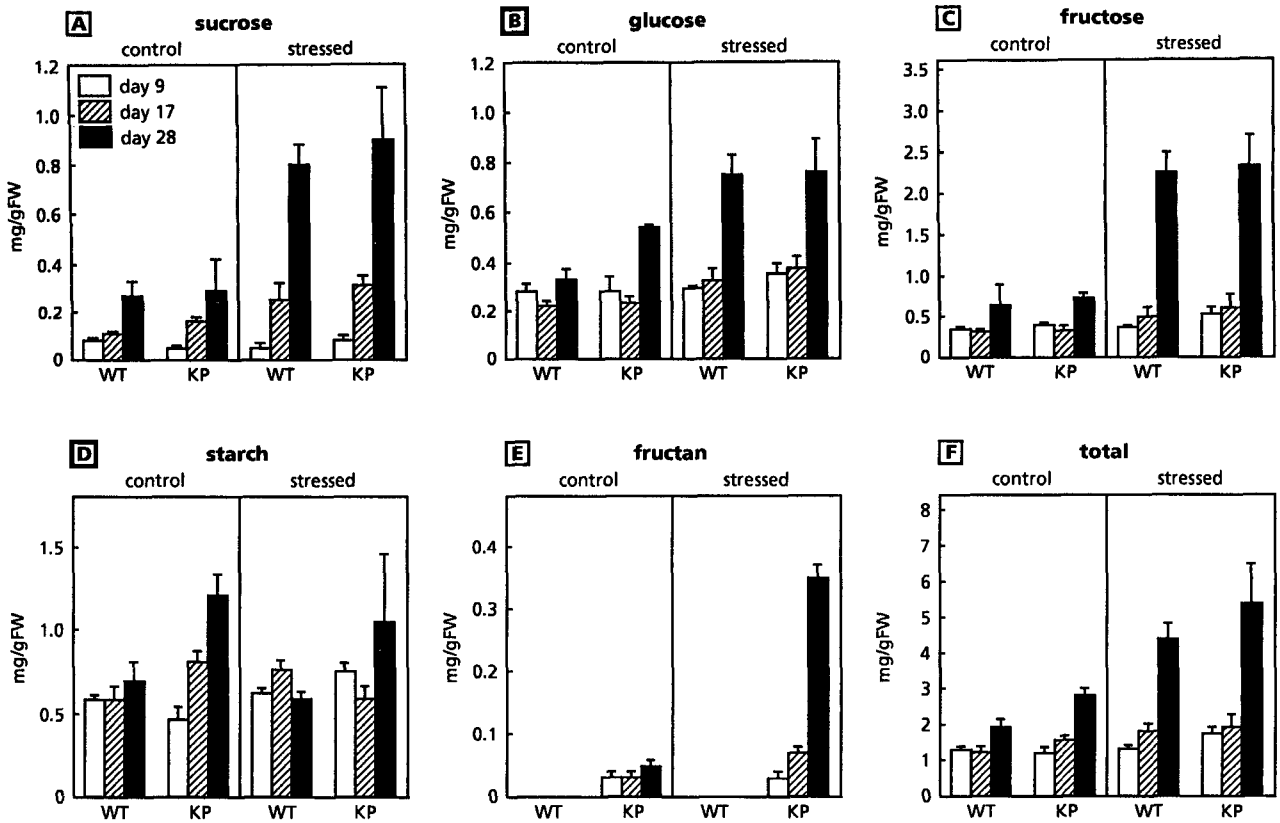

Figure 3. Nonstructural carbohydrate composition of WT and KP tobacco plants grown in a growth chamber, either drought stressed or under control conditions. A, Suc; B, Glc; C, Fru; D, starch; E, fructan; F, total. Leaf samples were taken at d 9 (0% PEG), d 17 (5% PEG), and d 28 (10% PEG). Values are the means ± SE of three samples taken from 10 plants.

seems too low to have an osmotic effect. Perhaps fructan accumulation is high enough to have an effect in specific organs (e.g. roots) or in specific cellular compartments. Perhaps fructans can protect membranes or other cellular components against the adverse effects of drought. Alternatively, the biosynthesis of fructans may influence the process of cell-wall hardening. This is one of the first reactions to water stress (Chazen and Neumann, 1994) and functions to limit cell expansion and thus to reduce water demand. Since fructan accumulation in these tobacco plants induces an increase in the levels of other nonstructural carbohydrates, it may also enhance the synthesis of structural carbohydrates, like cell-wall components. The higher dry matter percentage of KP plants relative to WT plants may be an indication of such an increase in nonstructural carbohydrate content.

Since KP plants produce larger roots when drought stressed than under nonstressed conditions, enhanced root development seems to be an adaptation to drought and a possible basis for the better growth performance of the total plant. Similarly, in mannitol-accumulating tobacco (Tarczynski et al., 1993) root growth was favored more in the transgenic plants than in the WT plants under salt stress, leading to the speculation that mannitol might affect cellular processes involved in root formation. Fructans may have a similar effect. For instance, fructans may promote the process of root branching, thus increasing root surface and water uptake. Less likely, the higher levels of soluble compounds in KP plants may provide a higher capacity for osmotic adjustment. The carbohydrate gained may lead to deeper rooting and greater water uptake, as has been reported for wheat (Morgan and Condon, 1986; Sharp and Davies, 1989). As yet no data are available to substantiate any hypothesis on the mechanism promoting enhanced root growth in the presence of fructans.

The drought-related increase in fructan concentration has also been observed under other types of stress, namely, low temperature (12°C) and salt stress (200 mM). Because of the lower growth rate under these unfavorable conditions, fructans can accumulate to higher levels. Since photosynthesis is less inhibited than growth, Suc concentrations will increase (substrate for fructan synthesis), which may further enhance fructan biosynthesis. No significant difference in growth performance was observed between WT and KP plants grown at low temperature or under salt stress (our unpublished results).

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