Fructan as a New Carbohydrate Sink in Transgenic Potato Plants

Ingrid M. van der Meer, Michel J. M. Ebskamp, Richard G. F. Visser, Peter J. Weisbeek, and Sjef C. M. Smeeckens

Department of Molecular Cell Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands
Department of Plant Breeding, Agricultural University, P.O. Box 386, 6700 AJ Wageningen, The Netherlands

Fructans are polyfructose molecules that function as nonstructural storage carbohydrates in several plant species that are important crops. We have been studying plants for their ability to synthesize and degrade fructans to determine if this ability is advantageous. We have also been analyzing the ability to synthesize fructan in relation to other nonstructural carbohydrate storage forms like starch. To study this, we induced fructan accumulation in normally non-fructan-storing plants and analyzed the metabolic and physiological properties of such plants. The normally non-fructan-storing potato plant was modified by introducing the microbial fructosyltransferase genes so that it could accumulate fructans. Constructs were created so that the fructosyltransferase genes of either Bacillus subtilis (sacB) or Streptococcus mutans (ftf) were fused to the vacuolar targeting sequence of the yeast carboxypeptidase Y (cpy) gene. These constructs were placed under the control of the constitutive cauliflower mosaic virus 35S promoter and introduced into potato tissue. The regenerated potato plants accumulated high molecular mass (>5 x 10^6 D) fructan molecules in which the degree of polymerization of fructose units exceeded 25,000. Fructan accumulation was detected in every plant tissue tested. The fructan content in the transgenic potato plants tested varied between 1 and 30% of dry weight in leaves and 1 and 7% of dry weight in microtubers. Total nonstructural neutral carbohydrate content in leaves of soil-grown plants increased dramatically from 7% in the wild type to 35% in transgenic plants. Our results demonstrated that potato plants can be manipulated to store a foreign carbohydrate by introducing bacterial fructosyltransferase genes. This modification affected photosynthesize partitioning in microtubers and leaves and increased nonstructural carbohydrate content in leaves.

INTRODUCTION

Plant growth and development are dependent upon the fixation of carbon dioxide and its reduction into carbohydrates; these processes require energy generated by light in the chloroplast. The major products of photosynthesis are plastidic starch and sucrose, which is synthesized in the cytosol. Leaf starch serves mainly as a temporary deposit for the products of carbon fixation, but in other organs (e.g., seeds and tubers) it can be synthesized and stored for longer periods in the amyloplast. Sucrose represents not only a carbohydrate storage form but also plays a central role in the distribution of photoassimilates in many plant species.

Besides sucrose and starch, which are ubiquitous, a third storage carbohydrate can be found in plants; this carbohydrate is found less frequently. Fructans (polyfructosylsucroses) are the dominant carbohydrate reserve in several orders of the plant kingdom. Among the angiosperms that store fructans, the Asterales (dicotyledons) are most noted, as well as the Cyperales and Liliales (monocotyledons) (Nelson and Smith, 1986; Hendry, 1987; Pollock and Cairns, 1991). Among the fructan-storing plants are important crops, such as wheat and barley (in which fructans are stored in leaves and stems), bulb-forming plants, such as tulip and onion (fructan accumulation in bulbs), and also chicory and Jerusalem artichoke (storage in roots and tubers).

In plants, fructans are thought to be synthesized from sucrose in the vacuole by the concerted action of at least two fructosyltransferases (Pontis and del Campillo, 1985; Pollock and Cairns, 1991). Fructans can be stored in sink as well as in source tissue, depending on the environmental conditions and developmental stage. Next to their role as a plant carbohydrate reserve, fructans may have other functions, including involvement in dry and cold tolerance (protection against cold-induced dessication; Pontis and del Campillo, 1985). Unlike starch, which is insoluble, vacuolar fructans are soluble and can contribute to the osmotic potential of this compartment.

Our goal is to understand the significance of fructan metabolism for plants by studying the advantages of fructan-metabolizing plants over plants unable to synthesize and degrade...
fructan. We are also interested in determining how this reserve carbohydrate affects physiological processes like photosynthetic partitioning.

In this study, we describe the induction of fructan accumulation in potato plants. Because the plant genes encoding the fructosyltransferase enzymes involved in fructan synthesis are not available, we used bacterial genes. Besides plants, many microorganisms also have the ability to synthesize fructans from sucrose. These fructans can have a very high molecular mass and may consist of more than 100,000 fructose units (Dedonder, 1966). In contrast, the length of plant fructan chains seldom exceeds a degree of polymerization (DP) of fructose molecules of 100, and DP values are usually smaller. Fructans produced by bacteria are branched polymers in which fructose units are connected by β(2-1)- and β(2-6)-glycosidic linkages. Different bacterial strains can produce different fructan structures in which the ratio between the two types of linkages can vary.

Two chimeric genes were constructed and introduced into potato. These genes contained either the sacB (levansucrase) gene from *Bacillus subtilis* (Steinmetz et al., 1985) or the *ftf* (fructosyltransferase) gene from *Streptococcus mutans* (Shiroza and Kuramitsu, 1988). The enzymes encoded by these genes use sucrose as a substrate for the transfructosylation reactions. The linkage type found in the fructan produced by the *B. subtilis* levansucrase is mainly of the β(2-6) type with β(2-1) branch points (Han, 1991). The *S. mutans* enzyme produces fructan with a very high DP value in which sucrose units are mainly β(2-1) linked with β(2-6) branching (Shiroza and Kuramitsu, 1988). A vacuolar targeting signal was added to the constructs because the vacuole is the compartment where fructans normally are stored in fructan-synthesizing plants (Frehner et al., 1984; Darwen and John, 1989). The vacuolar targeting sequence used was derived from yeast preprocarboxypeptidase Y (Valls et al., 1987). This vacuolar targeting signal has been investigated extensively in yeast and may also be able to direct proteins to the vacuole in plants.

To test whether the fusion between the cpy vacuolar targeting signal and the fructosyltransferases yields active proteins, both chimeric genes were expressed in *Escherichia coli* under the control of the lacZ promoter, and the activity of the proteins produced was analyzed in an in vitro assay (see Methods).

Both chimeric fructosyltransferase enzymes proved to be enzymatically active (data not shown). The chimeric genes were inserted between the CaMV 35S promoter with the enhancer duplication and the alfalfa mosaic virus (AIMV) RNA 4 translational enhancer leader sequence, terminated by the nopaline synthetase (nos) terminator. These constructs were ligated into p8bin19-derived plant transformation vector pMOG23 (Sijmons et al., 1990), resulting in the plasmids pKP (containing the sacB gene) and pTP (containing the *ftf* gene).

**RESULTS**

**Construction of Chimeric Fructosyltransferase Genes**

Two bacterial genes, one originating from *B. subtilis* (*sacB*) and the other from *S. mutans* (*ftf*), were selected for introduction into potato to analyze whether fructan accumulation can be induced. The construction scheme of the chimeric genes used for potato transformation is presented in Figure 1. Because in fructan-storing plants fructans normally are present in the vacuole, both fructosyltransferase genes were fused to a sequence encoding the vacuolar targeting signal from yeast carboxypeptidase Y (*cpy*) (Valls et al., 1987). This vacuolar targeting signal has been investigated extensively in yeast and may also be able to direct proteins to the vacuole in plants.

Figure 1. Representation of the Chimeric Fructosyltransferase Genes. A detailed description of the construction of the fusion genes is presented in the Methods section. The genes were placed under the control of a CaMV 35S promoter with enhancer duplication and the alfalfa mosaic virus (AIMV) RNA 4 translational enhancer leader sequence, terminated by the nopaline synthetase (nos) terminator. Both constructs were ligated in the p8bin19-derived plant transformation vector pMOG23 (Sijmons et al., 1990), resulting in the plasmids pKP (containing the *sacB* gene) and pTP (containing the *ftf* gene). **A**, AccI; **B**, BamHI; **H**, HindIII; **N**, Ncol; **P**, PstI; **Xb**, XbaI; **Xh**, Xhol.

To test whether the fusion between the cpy vacuolar targeting signal and the fructosyltransferases yields active proteins, both chimeric genes were expressed in *Escherichia coli* under the control of the (noninduced) lacZ promoter, and the activity of the proteins produced was analyzed in an in vitro assay (see Methods).

Figure 1. Representation of the Chimeric Fructosyltransferase Genes.
mediation (Visser, 1991). Transgenic plants were selected for kanamycin resistance, and ~35 independent transformants per construct were raised. The number of T-DNA inserts varied from one to five copies per genome as determined by DNA gel blot analysis (data not shown). Plants containing the sacB coding region were designated KP; those containing the ttf coding region were designated TP.

**Transgenic Potato Plants Produce Fructans**

A convenient method to screen for fructan accumulation in the transformants is by analysis of leaf homogenates by thin-layer chromatography (TLC). In this system, neutral sugars are separated according to size and subsequently visualized by treating the TLC foil with a urea phosphoric acid spray that preferentially reacts with ketose residues (see Methods). Fructans with a DP of more than 15 did not migrate on the TLC foil and stayed at the point of origin.

Figure 2 shows the TLC analysis of leaf samples taken from in vitro–grown transgenic potato plants containing the sacB chimeric construct. More than 90% of the transformants containing the sacB chimeric construct showed a spot at the application site, indicating that they accumulated fructans. We also analyzed fructan accumulation in ttf-containing transformants by TLC; however, fructan levels were much lower. In only 20% of the ttf-containing transformants could fructan accumulation be detected (data not shown). Fructose polymers were absent from wild-type plants and in plants containing an "empty" plant vector. The TLC analysis of fructan accumulation revealed greatly varying levels of fructose and sucrose among the transformants. However, no conclusions about a possible correlation between fructan accumulation and fructose or sucrose content can be drawn from these TLC results because, for this experiment, leaf tissue was taken from in vitro–grown plant material grown under conditions that were not standardized. Fructan-accumulating transgenic potato plants were put in soil. The phenotypes of these plants were indistinguishable from nontransformed control plants. However, we failed to raise a mature plant from transformant KP11, which after TLC analysis showed an above average fructan level (Figure 2). The variation of fructan expression levels between mature transgenic plants containing the same construct was not correlated with the number of inserted transgenes but was probably caused by plant sequences that surrounded the insertion site (Weising et al., 1988).

**A High Molecular Mass Fructan Accumulates In Transgenic Potato Plants**

We investigated whether the nonmigrating spot observed by TLC analysis of the transformants consisted of polyfructose molecules only. For this purpose, partial and complete chemical degradation products were analyzed. Fructan produced by the transformants was isolated and subjected to acid hydrolysis by incubation with 200 mM sulfuric acid at 60°C. At several time points, a sample was taken and analyzed by TLC, as shown in Figure 3. The time course shows a shift from large molecules at the origin at 0 min to almost complete hydrolysis to fructose at 30 min. Several fructan molecules with differing DP values were observed at the intermediate time points. A similar degradation pattern was observed with fructan isolated from ttf-containing transformants and with commercially available bacterial fructan (data not shown). A complete hydrolysate was analyzed on an Aminex HPX-87C column equipped with a refractive index detector (see Methods), and the hydrolysate product was found to elute with a similar retention time as the fructose standard (data not shown).

Bacterial fructan produced by *B. subtilis* and *S. mutans* can have a very high degree of polymerization of more than 100,000 fructose units. We were interested in determining whether fructan synthesized by bacterial fructosyltransferase enzymes in transgenic plants has a similarly high degree of polymerization. The molecular mass distribution of the fructan produced by the transgenic plants was determined by fast protein liquid chromatography analysis using a Superose 6 gel filtration column. All fractions from the column were spotted on a TLC foil and analyzed for the presence of fructan, as shown in Figure 4. The fructan isolated from sacB-containing potato leaves eluted in the same fractions as dextran blue, which is an indicator of the void volume of the column. Fructan isolated from an ttf-containing plant also eluted in the void volume. Because the size exclusion limit for this type of column is $5 \times 10^6$ D
Figure 3. Time Course of the Acid Hydrolysis of Transgenic Potato Plant Fructan.

Degradation products of fructan isolated from a sacB-containing transformant (KP29) were analyzed by TLC. At different time points (given in minutes) during acid hydrolysis, samples were taken and spotted on a TLC foil. H.t., fructans isolated from Helianthus tuberosus tubers that were used as a marker; t, time; F, fructose; S, sucrose; I, 1-kestose; DP4 to DP15, degrees of polymerization of 4 to 15.

for globular proteins, the degree of polymerization of the potato fructan probably exceeds 25,000 fructose units.

These results showed that the high molecular mass carbohydrates produced by the transgenic plants are polyfructose molecules and that the size of this fructan is similar to that of bacterial fructans.

Fructosyltransferase Activity Can Be Detected in Transgenic Leaf Protein Extract

We tested whether microbial fructosyltransferase activity can be detected in the transgenic plants. Leaf extracts were incubated with sucrose, and the products formed were analyzed by TLC. By using this method, however, we could not detect enzyme activity in the plant extracts.

A more sensitive method for detecting fructosyltransferase activity was applied; it involved the use of radioactive sucrose as a substrate combined with concentrated protein extracts of the transgenic plants (see Methods). Reaction products were separated by TLC, and the TLC foil was exposed to an x-ray film. As shown in Figure 5A, the autoradiogram of the TLC shows production of fructan with a high degree of polymerization for sacB and ftf plant extracts. This in vitro fructosyltransferase activity was especially prominent in enzyme extracts from the sacB-containing plant, but low activity could also be found in the ftf-containing plant. This activity was not present in control plant extracts even though some radioactive "debris" can be seen at the origin (Figure 5, wild type). Figure 5B shows the other half of the TLC foil on which markers are visualized with an acid urea spray.

With this more sensitive technique, it was possible to detect fructosyltransferase activity in transgenic potato plants containing either the sacB or ftf gene after prolonged incubation, although the ftf-containing plants showed considerably lower activity levels. The need to use this sensitive technique for detection of fructosyltransferase activities produced by the transgenic plants suggests either a high turn over or a low catalytic activity for these enzymes in the in vitro assay used.

All Plant Organs Accumulate Fructans

In our experiments, we used the constitutive CaMV 35S promoter to drive expression of the chimeric fructosyltransferase genes. Therefore, we expected to find fructans in every cell type of the transgenic potato plants. Different types of tissue (leaf, stem, root, and induced microtuber) from several transformants were analyzed by TLC for fructan accumulation.

An in vitro-grown representative sacB transformant showed the following fructan content in various plant organs: leaf, 160; stem, 42; root, 75; microtuber, 8.1 (mg per g fresh weight). These data show that in the in vitro-grown transformant, all of the tissues analyzed contained fructan. The fructan content was significantly higher in leaf tissue compared to other organs. This could have been due to differences in fructosyltransferase protein stability or to differences in sucrose metabolism in these organs.

Reduced Starch Content in Fructan-Accumulating Microtubers

The tuberization process in potato plants can be induced in vitro by incubating potato explants on high sucrose-containing...
Carbohydrate Partitioning into Fructan

Figure 5. Fructosyltransferase Enzyme Activity in Plant Extracts from ftf and sacB Transformants.

Partially purified and concentrated protein extracts from an ftf-containing transformant (TP), a sacB-containing transformant (KP), and a wild-type plant (WT) were incubated with 14C-labeled sucrose, and their products were analyzed by TLC.

(A) Autoradiogram exposed to the TLC foil.

(B) The other half of the TLC foil on which markers were spotted. H.t., fructans isolated from Helianthus tuberosus tubers that were used as a marker; F, fructose; S, sucrose; I, 1-kestose; DP4, DP5, and DP15, degrees of polymerization of 4, 5, and 15.

medium in the dark (Paiva et al., 1982; Peterson et al., 1985; Hovenkamp-Hermelink et al., 1988). The resulting microtubers represent a physiological system for studying tuber development. For example, carbohydrate and protein content in microtubers are similar to soil-grown tubers (Paiva et al., 1982; Ewing, 1985; Peterson et al., 1985; Hovenkamp-Hermelink et al., 1988).

Because microtubers are a good model system for tuber formation, we analyzed the effect of fructan accumulation on carbohydrate metabolism in this tissue. Microtubers are starch-storing organs, and it is of interest to determine whether starch levels are affected by the additional fructan sink. Microtubers were induced on transformants and wild-type plants; these were subsequently analyzed for their carbohydrate content with respect to starch, sucrose, glucose, fructose, and fructan. In these experiments, microtubers from ftf-containing transformants (TP; "low" expressers) and sacB-containing transformants (KP; "high" expressers) were analyzed.

First, the starch content in microtubers of control plants and transformants was compared and related to the fructan content of the transformants. As shown in Figure 6, the starch content of microtubers that accumulated high levels of fructan (KP transformants) was severely reduced, with amounts being only about one-third of the level seen in control plants (an average of 64.9 mg of starch per g fresh weight of tissue in wild-type microtubers compared to 18.9 mg/g in sacB-containing microtubers). The ftf-containing plants had a lower fructan content and starch levels were less reduced.

No significant effect on the low molecular mass neutral sugar content (sucrose, glucose, and fructose) was observed (data not shown). In fructan-accumulating microtubers, the total non-structural carbohydrate content (sucrose, glucose, fructose, starch, and fructan) was significantly reduced. Because monosaccharides and disaccharides constitute a small fraction of total sugars, this reduction was most likely due to decreased starch content. In conclusion, we determined that the effect of fructan accumulation on carbohydrate partitioning in potato microtubers is mainly due to a decreased flow of sugars into starch.

The tuberization process in potato is controlled by developmental and environmental processes (Ewing, 1985). It was of interest for us to investigate whether fructan accumulation in combination with the resulting shift in carbohydrate metabolism affects microtuber formation and properties. Microtubers with a high fructan content had an average of almost twofold lower fresh weight than untransformed microtubers (23 mg for fructan-containing tubers versus 43 mg for control tubers). Also, the dry weight as a percentage of fresh weight was reduced from 24% in microtubers derived from a wild-type plant to 16% in microtubers derived from a sacB-containing plant. Furthermore, the average number of microtubers induced per six explants was also lower compared to the wild type (three for fructan-containing explants versus seven for control explants).

These results showed that introduction of fructan synthesis in potato strongly influences carbohydrate partitioning in a

Figure 6. Comparison of Starch and Fructan Levels in Microtubers Induced on Wild-Type and Transgenic Explants.

Starch and fructan levels were determined in microtubers from wild-type potato plants (WT1 to WT4), potato plants containing ftf (TP11, TP15, TP25, and TP26), and plants carrying sacB (KP1, KP15, KP18, KP24, KP25, and KP29) as described in Methods. The data are presented in milligram per gram fresh weight of tissue.
strong sink such as tuber tissue and that fructan accumulation and/or the resulting decrease in starch storage leads to changes in microtuber physiology.

**Fructan Accumulation Dramatically Raises the Total Nonstructural Carbohydrate Content in Leaves**

The highest fructan accumulation level was observed in leaf tissue of transgenic potato plants grown in vitro (described previously). We were interested in whether the effect of fructan accumulation on total nonstructural carbohydrate synthesis and partitioning, as seen in transgenic microtubers, could also be observed in leaves of mature potato plants. Therefore, the carbohydrate content of young, middle-aged, and old (still fully green, but in which senescence is about to be initiated) leaves was determined in a representative sacB-containing plant (KP29) and compared to wild-type potato.

Levels of starch, soluble sugars (sucrose, glucose, and fructose), and fructan were quantitated in leaves at the developmental stages, as indicated in Figure 7A. In wild-type potato, the middle-aged leaf exhibited the highest starch and soluble sugar content. This leaf was photosynthetically active and would be an exporter of carbohydrates. In old leaves, starch levels were reduced, probably due to initiation of senescence.

Analysis of the fructan content in leaves of the sacB-expressing KP29 transformant revealed that fructans accumulate during leaf development. Old leaves have the highest fructan content, but young and middle-aged leaves also have significant fructan levels. The fructan accumulation in an old leaf can reach up to 30% of the dry weight. The effect of fructan production on the starch content in leaves was comparable to the effect in microtubers. Also, in leaves a threefold reduction was found. The sucrose content increased twofold, whereas the total soluble sugars content (sucrose, glucose, and fructose combined) did not change much compared to wild-type leaves.

Fructan accumulation dramatically affected total nonstructural carbohydrates in potato leaves (Figure 7B). Comparison of total carbohydrate contents (sucrose, glucose, fructose, starch, and fructan) in transgenic potato leaves to control leaves showed an increase from 53 mg/g dry weight of tissue in old control leaves to 353.8 mg/g dry weight of tissue in old fructan-accumulating leaves. The dry weight-to-fresh weight ratio in these leaves increased from 20% in WT1 to 35% in KP29. In conclusion, these data showed that in leaves carbohydrate metabolism is also affected. As was also found in microtubers, leaf starch levels were reduced. But levels of total nonstructural sugars increased significantly in fructan-accumulating leaves, in contrast to what was found in microtubers.

Figure 7A shows that fructan accumulated during leaf development and was maximal in old leaves. Leaf senescence was accompanied by the mobilization of useful metabolites to other parts of the plant, and in this process biopolymers were degraded. The observation that fructans were still present in old leaves and were also detectable in dried out leaves suggested that fructan-degrading enzymes are either absent or have little activity. This is in agreement with our previous results (M.J.M. Ebskamp, unpublished results) in which protein extracts from several non-fructan-storing plants were incubated with microbial fructan. In these experiments, no released fructose could be measured even after prolonged periods of time. Apparently in non-fructan-storing plants, specific or nonspecific fructan hydrolases are absent or are inactive.

**DISCUSSION**

**Fructan Accumulation in Transgenic Potato Plants**

In this study, we describe the introduction of bacterial fructosyltransferase genes in potato plants. This modification
established an extra carbohydrate sink that affected photosynthetic partitioning in microtubers and leaves and increased nonstructural carbohydrate content in leaves.

Microbial fructosyltransferases utilize sucrose to synthesize high molecular mass fructans with a DP that can exceed 100,000. The expression of the bacterial fructosyltransferase genes (sacB and ftf) in transgenic potato plants resulted in the accumulation of considerable amounts of soluble fructan with a high degree of polymerization. Based on chemical degradation analysis (Figure 3) and sizing on a Superose column (Figure 4), we concluded that these fructans are similar if not identical to fructans produced by bacteria.

In protein extracts of the transgenic potato plants, we could detect fructosyltransferase activity only by using a sensitive radioactive assay (Figure 5). The levansucrase activity was higher than the FTF activity, which is in accordance with the higher fructan accumulation that was observed in sacB-transformed plants. Using immunological methods (protein gel blot analysis), we have been unable to detect the fructosyltransferase activities in transgenic plants (I.M. van der Meer, M.J.M. Ebkskamp, R.G.F. Visser, P.J. Weisbeek, and S.C.M. Smeekens, unpublished results) and hypothesize that low activity is probably due to a low level of enzyme.

In most plant cells, the vacuole is by far the largest compartment, and fructans accumulated in this compartment would be metabolically isolated from cytosolic intermediary carbohydrate metabolism. Because fructans are also found in the vacuole of fructan-storing plants, we attempted to direct fructosyltransferase activity to this compartment by using the vacuolar targeting sequence of the cpy gene of yeast (Valls et al., 1987). This vacuolar targeting signal has been investigated extensively in yeast and may also be able to direct proteins to the vacuole in plants. We are currently localizing the enzymatic activity within the cell. Evidence suggesting that the bacterial enzymes are targeted to the vacuole instead of following the default pathway to the apoplast was provided by differences in phenotype by transformed sacB-containing tobacco plants; in these plants the fructosyltransferase enzyme was targeted to either the vacuole or the apoplast. Tobacco plants containing a construct with the vacuolar targeting signal sequence exhibited severe necrosis (M.J.M. Ebkskamp, E.A.H. Pilon-Smits, I.M. van der Meer, P.J. Weisbeek, and S.C.M. Smeekens, manuscript in preparation).

To ensure expression of fructosyltransferase genes in every cell type of source and sink tissue, the constitutive CaMV 35S promoter was used. Analysis of different tissue extracts from in vitro–grown potato plants showed that all tissues accumulated fructans. The highest fructan level was found in leaf tissue. Differences in sucrose metabolism and compartmentalization or differences in fructosyltransferase activities in the tissues investigated could explain this observation. The distribution of fructan accumulation in tissue-cultured plants is comparable to the situation in soil-grown plants. Comparison of fructan levels in leaf and stem tissue of soil-grown plants showed that leaves produce severfold more fructan when compared to stems (data not shown).

We did not investigate the turnover of accumulated fructans in potato plants in detail. However, the absence of fructan-degrading activities in normally non-fructan-storing plants and the increase with age of fructan levels in the potato leaves up to senescence strongly suggest that levels of hydrolytic activities are low or not present. Consequently, fructan turnover in potato is anticipated to be low.

**Fructan Accumulation Alters Carbohydrate Partitioning in Microtubers and Affects the Tuberization Process**

The tuberization process in potato can be induced in vitro by incubating potato explants on high sucrose–containing medium in the dark (Paiva et al., 1982; Peterson et al., 1985; Hovenkamp-Hermelink et al., 1988). The resulting microtubers represent a physiological system for studying tuber development. For example, carbohydrate and protein content in microtubers is similar to soil-grown tubers (Paiva et al., 1982; Ewing, 1985; Peterson et al., 1985; Hovenkamp-Hermelink et al., 1988). Once tuber formation is induced, these organs constitute a strong sink in which imported carbohydrates are converted into starch (Mares et al., 1989). We observed that bacterial fructosyltransferase activity can compete with this process and divert normal carbohydrate flow. Increasing fructan levels in microtubers was clearly correlated with decreasing starch levels. Starch and fructan levels were about equal in microtubers with the highest fructan content (Figure 6).

When compared to leaves, fructan content in microtubers is low. The microtuber might not be a good system for obtaining high fructan accumulation because the direct substrate for fructan biosynthesis, sucrose, must enter the growing tuber through diffusion and can be subject to degradation during this process. Alternatively, the accumulating fructan itself or the in vitro tuberization process with its hormonal requirements may inhibit higher levels of fructan accumulation. In contrast, leaves of soil-grown plants are photosynthetically active, and the sucrose generated by photosynthesis is apparently shuttled efficiently into the new pathway.

Carbohydrates have been discussed as potential inducers of the tuberization process (Hawker et al., 1979). Therefore, we were interested in determining whether fructan accumulation and the resulting decrease in starch content influenced tuberization, as has been found in sugar-storing tubers in which starch formation was inhibited by using an antisense ADP-glucose pyrophosphorylase gene (Müller-Röber et al., 1992). Analysis of microtuber induction showed a twofold reduction of the number of microtubers per explant in fructan-accumulating KP microtubers. Also, the fresh weight of fructan-accumulating KP microtubers was reduced twofold.

Starch in tubers serves as the major reserve carbohydrate supporting the growth of the new plant. It will be of interest to analyze how starch reduction and the accumulation of seemingly nondegradable fructan influence physiological
and developmental processes in mature tubers (e.g., the sweetening of cold-stored potato tubers and formation and development of new sprouts).

Fructan Accumulation Dramatically Increases Total Carbohydrate Content in Leaves

The effect of fructan accumulation on carbohydrate partitioning was also analyzed in leaves of potato plants where the fructan content was much higher than in microtubers both in tissue culture and in soil-grown plants. The effect on the starch content of potato leaves was comparable to the effect in microtubers (reduction to one-third of the wild-type plant starch content), but seemed less dramatic because leaves did not store much starch (Figure 7A). However, fructan accumulation in old leaves of the sacB-expressing KP29 transformant had a dramatic effect on the total nonstructural carbohydrate content. Fructan production raised the total carbohydrate content from 7% of the dry weight in untransformed plants to 35% of the dry weight in KP29 (Figure 7B).

A potentially limiting factor in carbon assimilation in leaves is the accumulation of sucrose. It is thought that in many plant species the accumulation of sucrose in the source cell results in inhibition of photosynthesis. A possible cause of sucrose inhibition of photosynthesis is limited utilization of sucrose in the sinks ("sink regulation; Herold, 1980). This theory of sink regulation of photosynthesis has been tested by decreasing inhibition of photosynthesis. A possible cause of sucrose accumulation is the accumulation of fructan. It is thought that in many plant species the accumulation of fructan-accumulating plants to normally non-fructan-storing plants. The degree of polymerization of the fructan produced in these plants by the microbial enzymes is more than 25,000 and therefore exceeds the normal degree of polymerization range observed in fructan-storing plants (Pollock and Cairns, 1991) by more than two orders of magnitude. These transgenic plants showed surprisingly high fructan levels, thereby making them interesting tools for investigating both the regulation of carbohydrate utilization in plants and the possible functions fructan may have besides its function as a reserve carbohydrate.

METHODS

DNA Methodology

Standard procedures were used for DNA isolation, subcloning, restriction analysis, and sequence analysis (Maniatis et al., 1982). Isolation of DNA from individual potato transformants and DNA gel blot analysis were performed as described previously by DellaPorta et al. (1983).

Construction of Chimeric Fructosyltransferase Genes and Transformation of Potato

Two chimeric fructosyltransferase genes were constructed using the levansucrase (sacB) gene from Bacillus subtilis (Steinmetz et al., 1985) and the fructosyltransferase (fft) gene from Streptococcus mutans (Shiroza and Kuramitsu, 1988). Both genes were fused to a sequence encoding the vacuolar targeting signal from yeast carboxypeptidase Y (cpy) (Valls et al., 1987). To construct the cpy-sacB and the cpy-fft fusions, Ncol sites were introduced by site-directed mutagenesis with the appropriate primers (Kramer et al., 1984). An Ncol site was created near the mature processing site downstream of the vacuolar targeting sequences of the cpy gene at nucleotide position 330 (Valls et al., 1987), which is near the mature processing site of the sacB gene at nucleotide position 550 (Steinmetz et al., 1985) and the mature processing site of the fft gene at position 738 (Shiroza and Kuramitsu, 1988). The cpy portion containing the vacuolar targeting signal sequence was isolated by restriction with Ncol/HindIII, and the sacB or fft portion containing the mature fructosyltransferase sequences was isolated by restriction with Ncol and PstI. The parts were fused in the EMBL8 vector, which was digested with HindIII and PstI. The correct reading frames of the fusion genes were confirmed by sequence analysis. The resulting chimeric genes were digested with AccI and PstI and inserted in vector pMOG18 (Sijmons et al., 1990) from which the Escherichia coli uidA coding sequence was deleted. This vector contains the enhanced cauliflower mosaic virus (CaMV) 35S promoter, the alpha factor mosaic virus RNA 4 leader sequence, and the nopaline synthase polyadenylation sequence (Sijmons et al., 1990). The chimeric constructs were digested with XbaI (partial) and Xhol and ligated in the plant binary vector pMOG23 (Sijmons et al., 1990). The resulting plasmids were called pKP (chimeric sacB gene) and pTP (chimeric fft gene).

The constructs were transferred to Agrobacterium tumefaciens LBA4404 by triparental mating (Ditta et al., 1980). Exconjugants of the Agrobacterium cells carrying the chimeric fructosyltransferase constructs were used to transform diploid potato (Solanum tuberosum, genotype A16, 2n = 2x = 2n) stem explants as described previously (Visser, 1991). Plants grown in vitro were transferred to soil and grown in plant growth cabinets under fluorescent light (3000 lux) at a 23°C and a 16:8 hr light/dark cycle. Leaves from mature plants (0.75 to 1.00 m height, >10 leaves), in which photosynthesis was the only source of carbon, were used for analysis. Microtubers were induced as described by van der Steege et al. (1992).

Fructan Extraction and Analysis by Thin-Layer Chromatography

Transgenic plant material (leaf, stem, root, and tuber tissue) was harvested at the end of the light period and homogenized in 250 to 1000 µL of water, incubated for 5 min at 90°C, and centrifuged at 12,000g for 5 min. This extraction procedure was performed three times and the supernatants were combined. The fructan concentration of the plant extracts was determined by thin-layer chromatography (TLC) of 1 µL of extract in the presence of a range of concentration standards.

TLC was performed on silica gel TLC foils (Schleicher and Schuell) as described by Wagner and Wiemken (1987). The TLC foil was
developed three times in acetone:water, 9:1 and then stained with a fructose-specific urea phosphoric acid spray according to Wise et al. (1955). The TLC system used separates neutral sugars according to molecular mass. Fructans with a degree of polymerization (DP) of more than 15 do not migrate and stay on the application site. Fructan concentration was determined by scanning developed and stained TLC foil (Gelscan; Pharmacia-LKB) on which standard concentrations of commercially available fructans (levan; Sigma) were spotted.

**Analysis of Fructan Composition and Molecular Mass**

The composition of the synthesized fructans was analyzed by acid hydrolysis. Isolated plant fructan was incubated with 200 mM sulfuric acid at 60°C, and at several time points a sample was taken which was analyzed by TLC. After neutralization, a completely hydrolyzed sample was also analyzed on an Aminex HPX-67C column (Bio-Rad, see below).

The molecular mass of the fructans produced by the transgenic plants was determined by sizing on a fast protein liquid chromatography Superose 6 HR10/30 gel filtration column (Pharmacia) with 50 mM sodium phosphate, pH 6.5, 50 mM NaCl as elution buffer at a 0.5 mL/min flow rate. The Superose elution pattern was standardized with dextran blue, ovalbumin (M, of 45 kD), myoglobin (M, of 17.8 kD), and N-dinitrophenyl-L-alanine (M, of 255 D).

**In Vitro Fructosyltransferase Enzyme Activity Assay**

Five grams of mature transgenic leaf material was ground in a mortar with liquid nitrogen and dissolved in 5 mL of 50 mM citrate phosphate buffer, pH 5.7. The homogenate was centrifuged at 10,000 g, and the supernatant was saturated to 80% with ammonium sulfate and incubated for 2 hr at 4°C. Precipitated proteins were collected by centrifugation at 10,000 g in 0.5 mL of 20 mM sodium phosphate buffer, pH 7.0; 0.4 mM phenylmethylsulfonyl fluoride was added to the buffers. The protein solution was dialyzed against 20 mM sodium phosphate buffer, pH 7.0, for 20 hr at 4°C. The precipitate was removed by centrifugation, and 10 μL of enzyme preparation was incubated with 30 μL of 20 mM sodium phosphate buffer, pH 7.0, containing 30 mM sucrose and 5 μCi of [14C]-labeled sucrose as substrate. The reaction mixture was incubated 20 hr at 30°C. Products from the in vitro enzyme assay were precipitated with 90% ethanol and analyzed by TLC.

**Determination of Starch and Soluble Sugars Content**

Plant material (100 to 300 mg of leaf, stem, root, or microtubers) was extracted three times with 300 μL of water at 90°C. The three supernatants were combined and used for determination of sucrose, glucose, and fructose content after filtration through a 0.22-μm membrane filter. Sugars were quantified via HPLC using an Aminex HPX-67C column (Bio-Rad) and water as the mobile phase (flow rate 0.6 mL/min at 85°C) in conjunction with a refractive index detector (model 2142; Pharmacia-LKB). Mannose was used as an external standard for quantification of sugars in the samples. Starch was extracted by incubating the pellet with 20 mM NaOH for 1 hr at 75°C. After neutralization with 1 M H2SO4, the extracts were incubated in 20 mM sodium acetate, pH 4.6, with five units of amyloglucosidase (Boehringer Mannheim) at 37°C overnight. The amount of glucose released was determined by HPLC analysis as described above.

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**REFERENCES**


