Accumulation of Short-chain Fructo-oligosaccharides in Excised Stem and Root Tissues of Asparagus Cultured on a Carbohydrate-rich Medium

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Summary

To understand the production of fructo-oligosaccharides in asparagus storage roots, the accumulation of exogenous carbohydrates and changes in the amount of short-chain fructo-oligosaccharides (degree of polymerization (DP) = 3 and 4) were examined in excised stem and root tissues of young asparagus plantlets that had been cultured on a carbohydrate-rich medium. Analyses of the carbohydrates and water content of cultured segments showed that exogenous carbohydrates from the medium gradually penetrated each tissue, regardless of the type of carbohydrate. Short-chain fructo-oligosaccharides accumulated in segments that had been cultured for more than 12 hr on a medium that contained glucose, fructose or sucrose but not mannitol or sorbitol. Furthermore, not only root tissue but also stem tissue synthesized short-chain fructo-oligosaccharides in vitro. No increase in starch occurred in any segments with or without a carbohydrate in the medium. Short-chain fructo-oligosaccharides in cultured tissues started to increase at a lower concentration of exogenous carbohydrate in root segments than in stem segments, accumulated to a greater level in the former than they did in the latter after 96 hr of culture. The threshold level for fructo-oligosaccharide synthesis triggered by the accumulated fructose, glucose and sucrose may be lower in root tissue than in stem tissue, that accounts for the difference in fructo-oligosaccharide content between stems and storage roots of intact plantlets.

Key Words: Asparagus officinalis L., fructan, storage root, sugar accumulation, tissue culture.

Introduction

Asparagus (Asparagus officinalis L.) is a perennial plant in which much of the carbohydrate produced in aerial regions by photosynthesis is translocated and stored in storage roots as fructo-oligosaccharides (DP 3 - 15), rather than as starch or inulin (Shiom, 1989). The fructo-oligosaccharide content in storage roots changes throughout the year with the growth of plantlets and/or the harvesting of spears (Kim and Sakiyama, 1989; Pressman et al., 1993). Little fructo-oligosaccharides can, however, be detected in parts of the aerial stem, namely, the spear, stalk, lateral branch and cladophyll, in which the levels of fructose, glucose and sucrose are relatively high (King et al., 1988; Pressman et al., 1994; Suzuki et al., 2002). The reason for the difference in fructo-oligosaccharide content between the storage root and the aerial stem is unclear. In asparagus (Shiom, 1989) and chicory (Van den Ende and Van Laere, 1996) fructo-oligosaccharides are usually synthesized from sucrose by various fructosyl transferases. Pollock (1984) first noted the relationship between accumulation of sucrose and biosynthesis of fructo-oligosaccharides in a study of Lolium temulentum. Wagner et al. (1986) subsequently revealed that the treatment of barley leaves with a high concentration of sucrose enhanced the activities of fructosyl transferases with the resultant accumulation of fructo-oligosaccharides. Thus, it seems possible that the accumulation of some carbohydrates in specific plant tissues might induce the biosynthesis of fructo-oligosaccharides. If so, two possibilities may explain the difference in fructo-oligosaccharide content between the storage root and the aerial stem: a) the concentration of carbohydrates might be higher in the storage root than in the aerial stem; or b) the response of tissues to the accumulation of carbohydrates in the biosynthesis of fructo-oligosaccharides may be more sensitive in storage roots than in the aerial stem.

To understand the production of fructo-oligosaccharides in the asparagus storage roots we examined the effects of a high concentration of various exogenous carbohydrates on the fructo-oligosaccharide content of stem and root tissues of young asparagus plantlets cultured in vitro. Tissues of young plantlets were cultured in vitro on a carbohydrate-rich medium because those from field-grown adult plants, in particular the roots, are not amenable to such culture. We also ex-
amined differences in fructo-oligosaccharide content and carbohydrate concentration between aerial stems and storage roots of actively growing young plantlets on soil. Then we attempted to relate the changes in carbohydrate content that occurred in cultured tissues in vitro with those in tissues of the intact plant.

**Materials and Methods**

**Cultivation of seedlings**

Seeds of asparagus (*A. officinalis* L. cv. Mary Washington 500W) were rehydrated overnight in tap water and incubated on wet filter paper at 25°C in darkness for several days. After germination, seeds were sown in soil in plastic pots [55 cm × 37 cm × 16 cm (height)]. The aqueous soil extract had a pH of 6.5 and very little nutrients. Ammonium sulfate (82.6 mg), calcium phosphate (20.0 mg) and potassium sulfate (28.1 mg) were added per 100 g of dry soil and thoroughly mixed. The soil mixture was autoclaved for 1 hr at 120°C (1.1 kg/cm²) before use. All seedlings were grown from June to September in a greenhouse in which the air temperature ranged from 20.7°C (daily minimum) to 34.3°C (daily maximum). The length of the longest shoot of 16-week-old seedlings was more than 50 cm.

**Tissue culture on carbohydrate-rich media**

Cultures were prepared in vitro as follows. Above-mentioned seeds of asparagus were surface-sterilized for 20 min with a solution of sodium hypochlorite (containing 1% (w/v) active chlorine) that had been supplemented with 0.05% (v/v) polyoxyethylene (20) sorbitan monolauroate (Tween-20). Then they were rinsed three times with sterilized double-distilled water and cultured at 25°C under a 16-h photoperiod (illuminated from fluorescent tubes at 60 μmol·m⁻²·s⁻¹) on a medium that contained half of the nitrogen source and all other ingredients of the MS medium (Murashige and Skoog, 1962) plus 0.7% (w/v) agar (pH adjusted to 5.7). The medium was dispensed into grass bottles (8 cm in diam. and 12 cm in height) and then autoclaved for 10 min at 120°C (1.1 kg·cm⁻²) before use. After 12 weeks of culture segments of stems (0.5–0.7 mm in diam. without the cladophyll) and of storage roots (1.0–2.0 mm in diam. without feeder root, referred to hereafter as root) of approximately 10 mm in length were prepared from the plantlets (the length of the shoot was more than 20 cm) and used as plant materials for subsequent experiments.

The segments were cultured on carbohydrate-rich media by the following procedure. The freshly prepared medium, as described above, supplemented with glucose, fructose, sucrose, mannitol or sorbitol at 0.5 M was autoclaved for 10 min at 120°C (1.1 kg·cm⁻²) and then dispensed into sterilized petri dishes (12 cm in diam.). Upon cooling, the segments were placed on a piece of sterilized filter paper (Qualitative No. 1, Advantec Toyo, Tokyo) that was placed on top of the medium, and cultured for 3, 6, 12, 24, 48 or 96 h under the above conditions (with lights on for the first 16 hr of the experiment). This precaution was taken to prevent segments from coming directly into contact with agar-solidified medium that contained a high concentration of carbohydrate, as well as to prevent errors in the subsequent quantitation of carbohydrates, starch and water. An advantage of the method is that changes of the amount of carbohydrates in excised plant tissues are independent of the translocation of carbohydrates from source to sink.

**Quantitation of carbohydrates**

Samples were prepared for the analysis of carbohydrates as described previously (Suzuki et al., 1997). Samples (approximately 200 mg FW) of stem (0–10 cm from the base) and root (5–6 cm from the base) were obtained 4–16 weeks after germination from soil grown plantlets and analyzed for carbohydrates. Likewise, stem and root segments (approximately 200 mg FW) cultured on medium with or without 0.5 M of various carbohydrates were blotted with filter paper and analyzed. Samples were homogenized in a mortar at 0°C with 2 ml of 0.2 N perchloric acid, 200 mg of sea sand and 1 ml of 20 mM lactose (as an internal standard). After the homogenates were centrifuged at 4°C for 5 min at 14,000 × g, the supernatant was decanted and the solution was adjusted to pH 4.0 with solid potassium hydrogen carbonate and lyophilized. The residue was dissolved in 0.5 ml of distilled water and the solution was centrifuged for 5 min at 14,000 × g. A 10-μl aliquot of the supernatant was injected into a high-performance liquid chromatograph (HPLC) apparatus. The conditions for HPLC were as follows: mobile phase, 75% acetonitrile (Kanto Chemical, Tokyo) and 25% water (v/v); pump (L-6200; Hitachi, Tokyo); column (NH2P-50 4E; Shodex, Tokyo); detector, RI-monitor (L-3300; Hitachi); temperature, 30°C (L-5020 column oven; Hitachi); and flow rate, 0.7 ml·min⁻¹. The samples of authentic neokestose (inulin neo-series, F(β→6)G(1→2)β), 1-kestose (trisaccharide of inulin type fructo-oligosaccharides) and nystose (tetrasaccharide of inulin types) had been purified as described by Shiomi et al. (1991). We previously confirmed that authentic 1-kestose and nystose dissolved and incubated for 30 min in 0.2 N perchloric acid at 4°C had 98.5% recovery, and that the carbohydrate recovery of a sample extracted by the method was the same as that extracted with 80% ethanol at 70°C. The former perchloric extraction was preferable for carbohydrate extraction because of its simplicity.

**Quantitation of starch**

Samples for the analysis of starch were prepared as described by McCready et al. (1950). The pellet obtained after two centrifugations of the homogenate was
finally rinsed in 4 ml of distilled water to remove residual soluble carbohydrates and then re-
suspended in 3 ml of 52% (w/v) aqueous perchloric acid and incubated at room temperature for 30 min. The suspension containing the solubilized starch was diluted with 17 ml of distilled water and centrifuged at 4°C for 15 min at 5,000 \( \times \) g. A 5-ml aliquot of the supernatant was stored at \(-20°C\) until analyzed for starch as described by Dubois et al. (1956). The starch content of each sample was estimated from the absorbance of the solution by reference to the standard curve generated with glucose. Three replicates were used for each determination.

**Water determination**

Water content was determined by drying samples similar to those used for the analyses of carbohydrates and starch (approximately 200 mg FW) at 70°C for three days. The data are expressed as a percentage of FW.

**Statistical analysis**

The levels of carbohydrates and water in plantlets were obtained from two separate experiments with three replicates for each determination. Levels of carbohydrates, starch and water in segments cultured in vitro were obtained from two determinations. Results are given as averages \( \pm \) SE. Analysis of variance was performed to examine the statistical significance of differences in respective levels of short-chain fructo-oligosaccharides, starch and water in segments that had been cultured on carbohydrate-rich media.

**Results**

**Carbohydrate and water content of plantlets**

Seven carbohydrates were detected in stem and root tissues of young plantlets grown on soil (Fig. 1). Nystose and an analogue composed of 4 monosaccharides were quantified as nystose because the peaks could not be separated. The amounts of more highly polymerized oligomers (DP = 5 and 6) were detectable in our HPLC system (Fig. 2), but they existed in trace quantities in these experiments; furthermore, no authentic reagents of such fructo-oligosaccharides were available to us so that accurate measurements of such carbohydrates were not possible. Therefore, we ignored the amounts of fructo-oligosaccharides with DP \( \geq 5 \) and defined the total amount of short-chain fructo-oligosaccharides as the sum of the amounts of neokestose, 1-kestose and nystose. The total amount of short-chain fructo-oligosaccharides was always larger in root tissue than in stem tissue, regardless of the duration of growth of the plantlets (Fig. 3). The total amount of carbohydrates in stem and in root tissue increased gradually after germination and then decreased in tissues of 16-week-old plantlets. The relative levels of each carbohydrate in each tissue showed little changes after germination. Respective levels of fructose and fructo-oligosaccharides were higher, while those of glucose, inositol and sucrose were lower in root than in stem tissue. The water content decreased gradually from 90 to 70% in stem and from 90 to 80% in root with the growth of plantlets. The molar concentration of carbohydrates in both tissues, calculated from the ratio of the carbohydrate to water content, increased from 0.10 M to 0.16 M during the 12-week growth period; no differences were apparent in the concentration of carbohydrates between the stem and the root, except in samples from 16-week-old plantlets.

**Carbohydrate, starch and water content of tissues cultured on carbohydrate-rich media**

The time course of changes in carbohydrate content of cultured stem and root segments (Fig. 4) show that fresh segments of stem (83.2 \( \mu \) mol \cdot g\(^{-1}\) FW) and of root (63.9 \( \mu \) mol \cdot g\(^{-1}\) FW) remained unchanged or tended to decrease gradually when segments were cultured on the control medium. Contrarily, large amounts of carbohydrates gradually accumulated in segments that were cultured on carbohydrate-rich media, even after as little as 3 hr. The relative levels of carbohydrates in segments changed with the type of carbohydrate in the medium; the total amount of carbohydrates was larger in stem segments than in root segments. Fructose, glucose, inositol and sucrose were always detected in all segments with or without culture. In segments cultured on carbohydrate media, a large amount of the same carbohydrate as that supplemented to the medium was detected. Furthermore, although the levels of fructose, glucose and sucrose increased in segments that were
Fig. 2. HPLC profiles of authentic carbohydrates (A) and carbohydrates in a sample extracted from the storage roots collected in winter from an adult asparagus plantlet in which the levels of the fructo-oligosaccharides of DP 5–6 are relatively high (B). Lactose was the internal standard. Authentic neokestose, 1-kestose and nystose were added to standard solution at 1/3 strength in molar concentration of other authentic carbohydrates. No inositol was detected.

cultured on media supplemented with fructose, glucose or sucrose (except for fructose in roots cultured on the glucose medium), they did not increase in segments cultured on media supplemented with sorbitol or mannitol.

Water content of fresh root segments (Fig. 5) was 89% compared with 80% in the fresh stem. In both types of segment it ranged from 83 to 90% for 3 to 6 hr, regardless of the type of medium. The water content decreased to below 82% after 96 hr of culture in segments on media that contained glucose, fructose or sucrose.

The calculated concentrations of carbohydrates in stem and root tissues cultured on carbohydrate rich-media increased from ≤ 0.1 M to 0.3–0.4 M (in stems) and to 0.2–0.3 M (in roots) during 96 hr of culture (Fig. 3).

Fig. 3. Changes in the water content (A), the total concentration of soluble carbohydrates (B) and the amount of short-chain fructo-oligosaccharides (C) in stem (△) and root (○) tissues of young intact asparagus plantlets. Values of the carbohydrate concentration were calculated from the carbohydrate content (represented in Fig. 1) and water content (total carbohydrate (μ mol·g⁻¹ FW)/water (%)) x 10. The amount of short-chain fructo-oligosaccharides represents the total amount of neokestose, 1-kestose and nystose. Values are averages of two experiments with three replicates each. Where no error bar is presented in water content and carbohydrate concentration, the SE was smaller than the symbol.
6). By contrast, the concentration of carbohydrates decreased gradually in control segments.

Short-chain fructo-oligosaccharides were not detected in fresh stem segments, but its concentration was 6.7 mg·g⁻¹ FW in root segments (Fig. 7). Their level significantly increased in both tissues after a culture of 12-96 hr in stems or 24-96 hr in roots on media supplemented with glucose, fructose or sucrose; by contrast, no increases in the levels were detected in tissues on the control medium or on media supplemented with mannitol or sorbitol.

The starch contents of segments on carbohydrate-supplemented media ranged from 3.9 to 6.5 mg·g⁻¹FW in stem and from 4.5 to 9.8 mg·g⁻¹FW in root. The starch levels of original stem and root segments (4.1 and 6.6 mg·g⁻¹FW, respectively) were unaffected by the types of carbohydrate in medium and the duration of culture.

Discussion

Analyses of levels of carbohydrates and water in segments cultured in vitro revealed that exogenous carbohydrates were gradually absorbed from the medium during culture, regardless of the type of carbohydrate. Suzuki et al. (1997) reported that the concentration of carbohydrates in asparagus shoot apices that had been cultured for 48 hr on carbohydrate-rich medium corresponded to that of the 0.5 M carbohydrate in the media, except for sucrose. In the present study, the final concentrations of total carbohydrates in each tissue, 0.3-0.4 M in stems and 0.2-0.3 M in roots after 96 hr on carbohydrate-rich media, were lower than the 0.5 M carbohydrates of the medium. This difference in carbohydrate concentration between shoot apices and segments might be attributed to the difference in the volume of plant material. The diameter was greater in the root segments than in the stem segments so that the ratio of surface area to volume was larger in stem segments than in root segments. Consequently, penetration of exogenous carbohydrates into stem segments might occur more rapidly than into root segments.

With respect to the metabolism of carbohydrates, Suzuki et al. (1997) confirmed that some fructo-oligosaccharides accumulated in asparagus shoot apices that
Fig. 5. Changes in the water content in stem and root segments of asparagus cultured on a carbohydrate-rich medium. Symbols represent the type of carbohydrate in the medium: △, glucose; ○, fructose; □, sucrose; △, mannitol; ◊, sorbitol. • represents "no carbohydrate". Values are averages ± SE of results from two determinations. Where no error bar is visible, the SE is smaller than the symbol. An asterisk indicates that promotion of the reduction of water content by a carbohydrate (△, ○, □) vs. absence of promotion by a carbohydrate (△, ○, •) was significant at *P < 0.05. ns: Not significant.

had been cultured for 48 hr on media supplemented with sucrose, fructose or glucose, although no fructo-oligosaccharide was detected in fresh shoot apices. In the present study, some of the fructose, glucose and sucrose taken up by both types of tissue were converted to other carbohydrates, while mannitol and sorbitol were not metabolized to other carbohydrates. Asparagus appears to lack the enzymes required for the metabolism of mannitol and sorbitol. We also found that the level of starch in segments did not increase under our test conditions even though the accumulation of fructo-oligosaccharides occurred in segments cultured with glucose, fructose or sucrose. It has been shown that sucrose might be a substrate for fructo-oligosaccharides in barley (Wagner et al., 1986) and chicory (Van den Ende and Van Laere, 1996). However, our results indicated that not only sucrose but also glucose and fructose can be converted to fructo-oligosaccharides, albeit indirectly, to the same extent in asparagus stems and roots; moreover, the accumulation of these carbohydrates into asparagus tissues induces the biosynthesis of short-chain fructo-oligosaccharides. Actually, altered gene expression of asparagine synthetase was observed in asparagus cultures supplemented with a high concentration of carbohydrates (Irving et al., 2001). The accumulation of carbohydrates occurred even in segments that were cultured for a mere 3 hr, while levels of short-chain fructo-oligosaccharides increased in segments that were cultured for more than 12 hr. Three possibilities can explain the delay between the start of the accumulation of carbohydrates and the start of fructo-oligosaccharide biosynthesis: a) no exogenous carbohydrate was present at the early stage of culture in the cytoplasm where carbohydrates are probably metabolized. Any carbohydrates that had entered tissues might still be in the apoplast and/or in vesicles of cells formed by fluid-phase endocytosis (Oparka et al., 1990; Jitsuyama et al., 2001). The uptake of exogenous carbohydrates into the cytosol is a gradual process that involves the sugar transporter in the plasmalemma (Beruter and Studer Feusi, 1995; Marquart et al., 1997). b) The activation and/or biosynthesis of fructosyltransferases take longer than the penetration of carbohydrates into the tissue (Winters et al., 1994). c) The delay reflects the time required for the carbohydrate concentration to reach the threshold level at which fructo-oligosac-
and, thus, the threshold level to accumulated carbohydrates might be lower in root tissue than in stem tissue (Fig. 6, 7). Indeed, the amount of fructo-oligosaccharides was greater in storage roots than in aerial stems of intact plantlets, in which the concentrations of carbohydrates were below 0.2 M (Fig. 3). The fact that the fructo-oligosaccharide content was greater in root segments than in stem segments after 96 hr of culture (Fig. 7) supports the idea that the fructo-oligosaccharide-biosynthetic response to the carbohydrate accumulation is greater in storage roots than in aerial stems. The fact that the amount of glucose plus fructose as a percentage of total carbohydrates was smaller in root segments than in stem segments that had been cultured on sucrose-containing medium, regardless of the duration of culture (Fig. 4), is attributed to a difference in the ability to utilize sucrose as the substrate for formation of fructo-oligosaccharides.

Haas et al. (1991) reported high levels of fructo-oligosaccharides in callus tissue of Symphytum officinale that had been cultured on medium supplemented with zeatin, regardless of the presence of other plant growth regulators. Vieira et al. (1995) reported the effects of different relative levels of auxin and cytokinin on the fructo-oligosaccharide content of callus tissue of Gomphrena macrocephala. Matsubara (1980) reported a gradient in levels of abscisic acid in tissues of asparagus. Thus, it is possible that a gradient of concentrations and/or the combination of endogenous phytohormones may have been responsible for the difference in fructo-oligosaccharide content among asparagus tissues, even though no phytohormone was used in our studies.

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Literature Cited


炭水化物高濃度添加培地で培養したアスパラガス茎および根組織片における短鎖フルクトオリゴ糖の集積

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摘要

アスパラガス貯蔵根におけるフルクトオリゴ糖の生成を理解するため、幼植物体から切り出し、数種の可溶性炭水化物高濃度添加培地で培養した茎および根組織における、外与の炭水化物の集積並びに短鎖フルクトオリゴ糖（重合度3および4）含量の経時的変化を調べた。含水率および炭水化物分析の結果は、外与の炭水化物がいずれも組織中に徐々に浸透したことを示した。短鎖フルクトオリゴ糖の集積は、ブドウ糖、果糖またはシロ糖添加培地で12時間以内培養した組織に認められたが、ソルビトールまたはマンニトール添加培地で培養した組織では確認されなかった。根組織だけでなく、茎組織も、in vitroで短鎖フルクトオリゴ糖を生成した。組織中のデンプン含量は、培養の有無にかかわらず変化しなかった。根組織では、外与された炭水化物の組織中濃度が茎組織のそれより低いレベルで短鎖フルクトオリゴ糖含量が増加し始め、培養96時間後における根組織のフルクトオリゴ糖含量は、茎組織のそれより多かった。集積した果糖、ブドウ糖およびシロ糖に対する根組織のフルクトオリゴ糖生成反応の関値が茎組織のそれよりも低いことが、植物体における茎および貯蔵根組織のフルクトオリゴ糖含量の違いを生む原因と考えられる。