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(Title Page)

Confirmation of fructans biosynthesized *in vitro* from [1-¹³C]glucose in asparagus tissues using MALDI-TOF MS and ESI-MS

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Summary

Accumulation of fructans was confirmed in asparagus tissues that had been cultured for 2 days on media supplemented with glucose. It is very common that fructans are biosynthesized from sucrose. We hypothesized however that fructans could also be biosynthesized from glucose. Stem tissues of *in vitro*-cultured asparagus were subcultured for 72 h on a medium containing 0.5 M of [^{13}C]glucose. A medium containing 0.5 M of normal (^{12}C) glucose was used as control. Carbohydrates were extracted from the tissues and analyzed using HPLC, MALDI-TOF MS and ESI-MS. HPLC results indicated that the accumulation of short-chain fructans was similar in both ^{13}C -labeled and control samples. Short-chain fructans of DP=3-7 were detected using MALDI-TOF MS. The molecular mass of each oligomer in the ^{13}C -labeled sample was higher than the mass of the natural sample by 1 m/z unit per sugar moiety. The results of ESI-MS on the HPLC fractions of neokestose and 1-kestose showed that these oligomers (DP=3) were biosynthesized from exogenous glucose added to the medium. We conclude that not only exogenous sucrose but glucose can induce fructan biosynthesis; fructans of both inulin type and inulin neoserries are also biosynthesized from glucose accumulated in asparagus tissues; the glucose molecules (or its metabolic products) were incorporated into fructans as structural monomers.

Key words: *Asparagus officinalis*, Carbon isotopes, Fructan biosynthesis, Mass spectrometry, Oligosaccharides.

Abbreviations: ESI-MS, electrospray ionization mass spectrometry; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

1 **Introduction**

2 Fructans (fructooligo- and fructopolysaccharides) are carbohydrates found in families of
3 plants such as Asteraceae, Poaceae and Liliaceae (Pollock and Cairns, 1991). Fructans have
4 been well known as carbohydrate reserves and are now attracting the attention of researchers
5 in the field of abiotic stress tolerance in plants (Livingston et al., 2009). They are composed
6 of multiple fructose molecules with or without a glucose molecule, usually biosynthesized
7 from sucrose by several fructosyl transferases (Wagner and Wiemken, 1987; Shiomi, 1989;
8 Obenland et al., 1991; Cairns, 1993; Van den Ende and Van Laere, 1993; St. John et al.,
9 1997). Pollock (1984) first noted the relationship between the accumulation of sucrose and
10 biosynthesis of fructans in a study of *Lolium temulentum*. Wagner et al. (1986) subsequently
11 revealed that the treatment of barley leaves with a high concentration of sucrose enhanced the
12 activities of fructosyl transferases with the resultant accumulation of fructans. Cairns and
13 Pollock (1988) showed activity of fructosyl transferases in *Lolium temulentum* could be
14 reduced by inhibitors for gene expression. These facts indicate that exogenous sucrose
15 accumulated in those plant tissues induced gene expression of fructosyl transferases (Van der
16 Meer, et al., 1998; Ueno et al., 2005; Lasseur et al., 2006; Van den Ende et al., 2006) followed
17 by increased fructan content. On sugar-mediated fructan biosynthesis, Müller et al. (2000)
18 showed that gene expression of 6-SFT (EC 2.4.1.10) in barley leaves was induced by
19 disaccharides. Small GTPases (Ritsema et al., 2009) and TaMYB13 (Xue et al., 2011) are
20 candidates for the signal controlling sugar-mediated fructan biosynthesis. However,
21 mechanisms that lead to biosynthesis of fructans were not clarified.

22 In a study for successful cryopreservation of asparagus shoot apices, Suzuki et al. (1997)
23 found accumulated fructans in the tissue that had been cultured for 2 days on media
24 supplemented with fructose, glucose or sucrose. Suzuki et al. (2004) further revealed that this

1 accumulation could occur in stem segments as well as in root segments that had been cultured
2 for more than 48 h on media containing 0.5 M of fructose, glucose or sucrose, although
3 fructans could be detected mainly from storage roots of an intact asparagus plant. Since no
4 accumulation of fructans was confirmed in segments treated with sorbitol or mannitol, but
5 accumulation was found in those treated with fructose, glucose or sucrose, the fructans
6 appeared to be biosynthesized from exogenous glucose as well as fructose and sucrose. To
7 understand the mechanisms of fructan biosynthesis, it is important to understand whether
8 fructans are biosynthesized from glucose. Glucose must be converted to fructose and/or
9 sucrose before being used for biosynthesis of various asparagus fructans (Shiomi, 1993);
10 inulin type or inulin neoseries (Fig. 1). In addition, accumulation of glucose may also be a
11 trigger for fructan biosynthesis as well as for sucrose. It is widely accepted that external
12 glucose can be imported to build up internal sucrose by the action of hexokinase (EC 2.7.1.1),
13 phosphoglucose isomerase (EC 5.3.1.9), SPS (EC 2.4.1.14) and SPP (EC 3.1.3.24):
14 conversion from glucose to glucose-6-phosphate (G6P) is catalyzed by hexokinase;
15 conversion from G6P to fructose-6-phosphate (F6P) is catalyzed by phosphoglucose
16 isomerase; sucrose is synthesized from UDP-glucose and F6P in a sequence of two reactions
17 catalyzed by SPS and SPP. Alternatively, sucrose may be synthesized from UDP-glucose and
18 fructose in a reversible reaction catalyzed by SuSy (EC 2.4.1.13). However, since natural
19 carbohydrates were also in the original asparagus tissues (Suzuki et al., 1997; 1998), the
20 origin of accumulated fructans is unclear.

21 Recent analysis of fructans was performed using reversed-phase HPLC (St. John et al.,
22 1997) and anion exchange chromatography combined with amperometric detection (Shiomi et
23 al., 1991; Wang et al., 1999; Hinch et al., 2007). However, this technique is insufficient to
24 separate long-chain fructan molecules. Mass spectrometry techniques are useful for analysing

fructans (Stahl et al., 1997; Losso and Nakai, 1997; Wang et al., 1999; Ravenscroft et al., 2009; Harrison et al., 2011). Mass spectrometers are also powerful tools for examining translation of carbohydrates into structures of biomolecules and biosynthetic pathways when combined with isotopes. Amiard et al. (2003) used exogenous ^{13}C -labeled fructose to examine fructan distribution in *Lolium perenne* plants. Harrison et al. (2012) used linear ion trap MSⁿ for estimating the molecular structure of enzymatically synthesized ^{13}C -labeled fructans. Asparagus fructans have been previously analyzed using MALDI-TOF MS (Suzuki et al., 2011). This study was designed to identify fructans biosynthesized from exogenous glucose in asparagus tissues using mass spectrometry (MALDI-TOF MS and ESI-MS) combined with [$1\text{-}^{13}\text{C}$]glucose.

Materials and methods

Plant material

Seeds of asparagus (*Asparagus officinalis* L. cv. Mary Washington 500W) were surface-sterilized and incubated at 25 °C with 16 h of illumination daily ($60\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$, from fluorescent tubes) on a medium in glass bottles (8 cm in diameter and 12 cm in height), that contained half of the nitrogen source and all other ingredients of MS medium (Murashige and Skoog, 1962) plus 0.7% (w/v) agar (pH adjusted to 5.7), but no sucrose (seeds have starch as carbohydrate reserve). Segments of stems (0.5-0.7 mm in diameter without the cladophyll) of approximately 10 mm in length were prepared from the resultant 12-week-old seedlings and used as plant material for experiments.

Culture on glucose-rich medium

Culturing procedures were the same as those described previously (Suzuki et al., 2004). The segments were inoculated on a freshly prepared above-mentioned medium supplemented with [$1\text{-}^{13}\text{C}$]glucose (99% in purity, Cambridge Isotope Lab. Inc., Andover, MA, USA) at 0.5 M and cultured for 72 h at 25 °C with 16 h of illumination daily by light from fluorescent tubes at $60\text{ }\mu\text{mol m}^{-2}\text{ s}^{-1}$ (with lights on for the first 16 h of the experiment). A medium containing 0.5 M of normal (^{12}C) glucose was used as control. Media were autoclaved for 15 min at 120 °C (1.1 kg cm^{-2}) and then dispensed into sterilized petri dishes (12 cm in diameter) in a laminar air-flow cabinet. To prevent segments from coming directly into contact with agar-solidified medium that contained a high concentration of glucose, as well as to prevent errors in the subsequent quantification of carbohydrates, a piece of sterilized filter paper (Qualitative No. 1, Advantec Toyo, Tokyo, Japan) was placed on top of the medium before inoculation.

Quantification of carbohydrates using HPLC

Samples were prepared for the analysis of carbohydrates as described previously (Suzuki et al., 1997). Stem segments cultured for 72 h on glucose-rich medium, blotted with dried filter paper and weighed (200 mg FW) were homogenized in a mortar at 0 °C in the presence of 2 mL of 0.2 N perchloric acid, 200 mg of sea sand and 1 mL of 20 mM lactose as an internal standard. Fresh stem segments, before culturing on glucose-rich medium, were examined as a blank. After centrifugation of each homogenate at 4 °C for 5 min at $14,000 \times g$,

the supernatant was adjusted to pH 4 (using the whole range test paper, Advantec Toyo) with solid potassium hydrogen carbonate (this step was finished within 30 min after homogenization to avoid hydrolysis of fructans) and lyophilized. The residue was dissolved in 0.5 mL of distilled water and the solution was centrifuged for 5 min at $14,000 \times g$ prior to analysis of carbohydrates. To confirm the effect of perchloric acid extraction on hydrolysis of fructans authentic 1-kestose (DP = 3) was extracted by the same procedure. Levels of carbohydrates were determined by HPLC. The conditions for HPLC were as follows: mobile phase, 75% (v/v) acetonitrile; pump (L-6200; Hitachi, Tokyo, Japan); column (NH2P-50 4E; Shodex, Tokyo, Japan); detector, refract intensity (RI)-monitor (L-3300; Hitachi); temperature, 30 °C (L-5020 column oven; Hitachi); flow rate, 0.7 mL min⁻¹; and sample volume, 10 µL.

Detection of fructans using MALDI-TOF MS and ESI-MS

The extracts used for MALDI-TOF MS were prepared as those for HPLC without adding lactose to the sample as an internal standard. Three µL of each extract was placed on a piece of Parafilm® sheet and mixed quickly with 3.0 µL of matrix solution (10 g L⁻¹ DHB in distilled and deionized water) using a micropipette according to the previous report (Suzuki et al., 2011). Then, 1.2 µL of the mixture was mounted on a sample slide for MALDI-TOF MS and air-dried in a desiccator. Analyses were performed using a MALDI-TOF MS instrument (Voyager-DE® STR; Applied Biosystems, Foster City, CA, USA) equipped with a nitrogen laser (337 nm) producing 3 ns pulses at repetition rate to 20 Hz, with an accelerating voltage of 15 kV and the linear positive-ion mode with delayed extraction at 100 µs. All mass spectra were generated by collecting 50 laser shots, that representing average masses.

HPLC fractions of sucrose, neokestose, 1-kestose and nystose were collected

independently from both 1-¹³C-labeled and control (¹²C) samples with 10 time-repetition, and concentrated fractions were analyzed in negative ion mode using an ESI-MS instrument (JMS-SX102A, JEOL Ltd., Tokyo, Japan) with a needle voltage of 2 kV, a ring lens voltage of 60V, an ion guide voltage of 3V, a secondary electron multiplier (SEM) voltage of 1 kV, a resolution of R = 2,000 and mobile phase of methanol. An advantage of ESI-MS is that mono-isotopic masses could be separated due to the high resolution.

Results

Availability of perchloric acid extraction for fructan analysis

The effect of perchloric acid extraction on hydrolysis of authentic 1-kestose (the shortest fructan) was examined first (Fig. 2). The HPLC profile of perchloric acid solution neutralized to pH 4 with potassium hydrogen carbonate had two peaks at around 6-8 min of retention time except for a large peak of water at 3.9 min (Fig. 2A). Since the solution contained no carbohydrate, the peaks based on refraction of light were likely inorganic salts formed by neutralization. In addition, since all peaks formed by salts appeared prior to the 8 min mark, they would not disturb the peaks of fructose, glucose and sucrose that had appeared later than 8 min. The HPLC profile of dissolved 1-kestose, incubated at 4 °C for 1 h in 0.2 N perchloric acid had only peaks of 1-kestose, salts and water, except for the peak of lactose added after neutralization as internal standard (Fig. 2C). Furthermore, recovery of 1-kestose was same as that of the sample without perchloric acid extraction (Fig. 2B). Thus, it was confirmed that no hydrolysis of 1-kestose had occurred during 1 h of the incubation at 4 °C in 0.2 N perchloric

acid.

Amounts of accumulated carbohydrates in asparagus tissue cultured on glucose-rich medium

HPLC results indicated that small amounts of fructose, glucose and sucrose, but no short-chain fructan, were contained in the original stem tissue (Fig. 3). Accumulation of glucose, fructose, sucrose and short-chain fructans (DP = 3 and 4) were confirmed in the segments cultured for 72 h on glucose-supplemented medium, and HPLC profiles were similar in both ^{13}C -labeled and control (^{12}C) samples. Except for glucose, the supplemented carbohydrate, the molar content of sucrose was greater than those of fructose and short-chain fructans (neokestose, 1-kestose and nystose) in cultured tissues (Table 1).

Detection of fructans biosynthesized from $[1\text{-}^{13}\text{C}]$ glucose using MALDI-TOF MS and ESI-MS

In MALDI-TOF MS, in the positive ion mode, only singly-charged molecules can be detected. The fructan molecules that originate from the asparagus tissues usually form singly-charged ions by binding with a potassium ion, since sample extracts neutralized with potassium hydrogen carbonate are rich in potassium ions. Thus, as was shown in previous research (Suzuki et al., 2011), the expected average m/z value of fructans with a DP value of n in MALDI-TOF MS could be calculated as follows:

$$[\text{C}_{6n} \text{H}_{10n+2} \text{O}_{5n+1} + \text{K}]^+, \text{ where } n \geq 3$$

Since average atomic weights of carbon, hydrogen, oxygen and potassium are 12.0107, 1.00794, 15.9994 and 39.0983, respectively, the above equation could be shown as:

$$m/z = 12.0107 \times 6n + 1.00794 \times (10n + 2) + 15.9994 \times (5n + 1) + 39.0983, \text{ where } n \geq 3$$

According to this equation theoretical m/z values of DP = 3 to DP = 7 fructans are 543.5, 705.6, 867.8, 1,029.9 and 1,192.0, respectively. In MALDI-TOF MS, sucrose and short-chain fructans of DP=3-7 were detected from the glucose-treated samples (Fig. 4). Several weaker peaks (such peaks as m/z values of 365.6, 525.5 and 687.5 in control sample) appeared just left of the peaks of $[C_{6n} H_{10n+2} O_{5n+1} + K]^+$ representing sucrose and fructan molecules binding with a sodium ion (atomic weight: 22.9898). Strong ion-intensities (the strongest value was at around 50,000 in both samples) of fructan molecules achieved without adding ethanol to the sample in MALDI-TOF MS were likely due to potassium ions added. The average m/z value of each fructan in the control (^{12}C) sample showed a number close to the theoretical value calculated. In contrast, the average m/z value of each fructan in the ^{13}C -labeled sample was higher than that of the oligomer with the same DP value in the control (^{12}C) sample. In this case, the differences in average molecular mass between the ^{13}C -labeled and control (^{12}C) sample were 0.3 (sucrose), 2.4 (DP=3), 3.4 (DP=4), 5.4 (DP=5), 6.9 (DP=6) and 8.1 (DP=7), respectively.

In ESI-MS, in the negative ion mode, the fructan molecules usually form singly-charged ions by losing a proton. Thus, the expected average m/z value of fructans with a DP value of n in ESI-MS could be calculated as follows:

$$[C_{6n} H_{10n+2} O_{5n+1} - H]^{-}, \text{ where } n \geq 3$$

Thus, the theoretical m/z values of neokestose and 1-kestose (DP=3 fructans) and nystose (DP=4 fructans) were 503.4 and 665.6 in ESI-MS, respectively. The theoretical m/z value of sucrose is 341.3 in ESI-MS. The m/z value of sucrose in the HPLC fractions from the control sample had a peak at 340.8 m/z that was close to the theoretical value (Fig. 5). Furthermore, a peak originating from natural isotope was also confirmed at around 342. In contrast, the m/z values of sucrose in the ^{13}C -labeled sample was higher than that of the control. The strongest

1 peak was at 342.9, but two additional weaker mono-isotopic peaks were confirmed at around
2 342 and 344. The difference in molecular mass of the strongest peak between the ^{13}C -labeled
3 and control sample was 2.1 in sucrose.

4 On fructans the m/z value of neokestose and 1-kestose in the HPLC fractions from the
5 control sample had a peak at 503.1 m/z that was close to the theoretical value (Fig. 6).
6 Furthermore, a peak originating from natural isotope was also confirmed at around 504. In
7 contrast, the m/z values of each fructan in the ^{13}C -labeled sample was stronger than those of
8 the control. The strongest peak was at 506.1, but additional two weaker mono-isotopic peaks
9 were confirmed at around 505 and 507. The difference in molecular mass of the strongest
10 peak between the ^{13}C -labeled and control sample was 3.0 in both neokestose and 1-kestose.
11 Similarly, the m/z value of nystose in the HPLC fractions from the control sample had a peak
12 at 665.2 m/z that was close to the theoretical value (Fig. 7). Furthermore, a peak originating
13 from a natural isotope was also confirmed at around 666. In contrast, the m/z value of each
14 fructan in the ^{13}C -labeled sample was higher than that of the control. The strongest peak was
15 at 669.2, but additional weaker mono-isotopic peaks were confirmed at around 667, 668, 670
16 and 671. The difference in molecular mass of the strongest peak between the ^{13}C -labeled and
17 control sample was 4.0 in nystose.

20 Discussion

21
22 Plant tissue culture systems are useful for examining metabolism of biomolecules (Irving
23 et al., 2001). In the present study, stem tissues of asparagus cultures (not root) were used for
24 the plant material, since no fructan was contained in the original stem tissues; this makes it

possible to confirm accumulation of biosynthesized fructans (Fig. 3). Although neutral sugars were usually extracted with 80%(v/v) ethanol at 70 °C to avoid hydrolysis, fructans were extracted with 0.2 N perchloric acid, because the ionization of fructan molecules extracted with 80%(v/v) ethanol was not adequate in MALDI-TOF MS (Suzuki et al., 2011). In a preliminary test, ionization was better when extracted with perchloric acid followed by the addition of potassium hydrogen carbonate for neutralization. In this case, since the occurrence of hydrolysis makes the analyses doubtful, we first confirmed the effect of perchloric acid extraction on hydrolysis of naturally occurring 1-kestose, the shortest-chain fructan (DP = 3). As a result, no hydrolysis of 1-kestose was found after incubation for 1 h at 4 °C in 0.2 N perchloric acid (Fig. 2). Neutralization of extracts (adjusted to pH 4) was always finished within 30 min after homogenization of the asparagus tissues for the following experiments.

HPLC results indicated the accumulation of fructose, glucose, sucrose and short-chain fructans (neokestose, 1-kestose and nystose) occurred similarly in both ¹³C-labeled and control samples (Fig. 3 and Table 1). Short-chain fructans of DP=3-7 were detected using MALDI-TOF MS (Fig. 4). According to the pathway of fructan biosynthesis in asparagus shown in Fig. 1, there are many isomers with the same DP value of fructan; the number of fructan isomers with a DP value of n ($n \geq 3$) is $n - 1$. If the largest DP value of fructans biosynthesized in the asparagus cultures is 7 based on the results here (Fig. 6), there could be a maximum of 20 different fructan molecules. The average molecular mass of each oligomer in the ¹³C-labeled sample was higher than the mass of the natural sample by 1 m/z unit per sugar moiety (Fig. 4), indicating incorporation of [1-¹³C]glucose (or its metabolic products) into the fructan oligomers. Recently Harrison et al. (2012) showed that sizes of the fragment ions from ¹³C-labelled inulin-type fructans were different from those of levan-types by using

LC-MSⁿ, indicating differences in biosynthetic mechanisms of each type of fructans. In the present study, however, conformational isomers of fructans could not be differentiated.

The results of ESI-MS on the HPLC fractions of sucrose showed that most of the sucrose was biosynthesized from exogenous [1^{13}C]glucose, because the m/z of the strongest peak in the ESI-mass spectrum from ^{13}C -labeled samples corresponded to 2.1 m/z units (1 per sugar moiety) higher than the natural molecular mass (Fig. 5). The result indicates that most of the sucrose molecule was composed of a glucose molecule and a fructose molecule that each had a ^{13}C -atom. Similarly, for the results of ESI-MS on the HPLC fractions of 1-kestose and neokestose, the primary fructans (DP=3) of inulin type and inulin neoserries in asparagus, showed that these oligomers were biosynthesized from exogenous glucose added to the medium, because m/z of the strongest peak in each ESI-mass spectrum of both fructans from ^{13}C -labeled samples corresponded to 3.0 m/z units (1 per sugar moiety) higher than their natural molecular masses of 503.4 (Fig. 6). A similar result was also confirmed in the case of nystose, one of the DP=4 fructans (Fig. 7). These facts indicate that most of the DP=3 and 4 fructan molecules were biosynthesized in the asparagus tissue from exogenous glucose, and that they may have been composed of monomers that had a ^{13}C -atom in each molecule. In other words, each fructan molecule was composed of a glucose molecule (containing a ^{13}C -atom) and some fructose molecules (each containing a ^{13}C -atom). Most of the fructose molecules seem to have been converted from supplemented glucose molecules in the tissue. Since the concentration of sucrose was higher than that of fructose in the tissue cultured for 72 h on glucose-supplemented medium (Table 1), it is likely that external glucose molecules were used immediately for biosyntheses of sucrose via G6P and F6P prior to biosynthesis of fructans. In other words external glucose may be imported to build up internal sucrose by actions of hexokinase, phosphoglucose isomerase, SPS and SPP, not by SuSy. The reason is

that in the case of SuSy-involved sucrose biosynthesis high fructose/sucrose ratios should be maintained to drive the reaction in that direction (Lunn, 2002), but rather high sucrose/fructose ratios were observed in the asparagus tissue. Furthermore, this scenario is supported by the fact that the increase in G6P concentration activates SPS by inhibiting the kinase SnRK1, which lead to sucrose accumulation. Obenland et al. (1993) showed that three constitutive acid invertases in barley leaves were found to act also as sucrose:sucrose fructosyltransferases when supplied with high concentrations of sucrose, forming 1-kestose as the principal product. However, biosynthesis of fructans in the asparagus tissues may not be catalyzed by invertases but by fructosyl transferases, since invertase-related biosynthesis would not occur at high glucose concentrations. Furthermore, the fact that 1-kestose, neokestose and nystose were biosynthesized from [1^{13}C]glucose indicates that all asparagus fructosyl transferases (1-SST, 1-FFT and 6G-FFT) were activated by supplying glucose. Recently, Xue et al. (2011) showed that TaMYB13 activated transcription of fructosyltransferase genes in wheat. Similar *MYB*-like genes may also control biosynthetic pathways of asparagus fructans. The metabolism of exogenous glucose might be related to ultrastructural changes in plastids, since numerous plastids with starch granules had developed immediately from pro-plastids in asparagus apical meristematic cells during 48 h of the culture growth on medium supplemented with 0.5 M glucose (Suzuki et al., 1997).

A small amount of fructose that contained two or more ^{13}C -atoms in a molecule had also been formed when converted from [1^{13}C]glucose, since the 344 m/z peak (3 m/z heavier than that of the control) was confirmed in the ESI-mass spectrum of sucrose from the ^{13}C -labeled samples (Fig. 5). Similarly the 507 m/z peak (4 m/z heavier than that of the control) of both neokestose and 1-kestose, and the 670 m/z peak (5 m/z heavier than that of the control) of nystose were confirmed in the ESI-mass spectrum of these short-chain fructans from the

¹³C-labeled samples (Figs. 6 and 7). This means that one of the structural monomers (except for glucose) in the 344 m/z sucrose, the 507 m/z fructan molecule (DP=3) and the 670 m/z fructan molecule (DP=4) had at least two ¹³C-atoms (Fig. 8). The difference of the m/z value of ¹³C-labeled fructans from the theoretical value (in case that each structural monomer has a ¹³C-atom) on MALDI-TOF MS increased gradually depending on DP: -0.6 (DP=3), -0.6 (DP=4), +0.4 (DP=5), +0.9 (DP=6) and +1.1 (DP=7). This could mean there were more monomers possessing two or more ¹³C-atoms in longer-chain fructans (Fig. 8). In contrast, the reason why the average m/z value (381.9) of sucrose from ¹³C-labeled sample on MALDI-TOF MS was less than the theoretical value (383.4) is that a part of sucrose was made of glucose and fructose without ¹³C-atom which was originally contained in the tissue. This was supported by the fact that the 341.8 m/z peak was detected from ¹³C-labeled sucrose on ESI-MS (Fig. 5). The 505.1 m/z peak and the 668.2 m/z peak were detected from ¹³C-labeled DP=3 fructans (neokestose and 1-kestose) and DP=4 fructans (nystose) on ESI-MS respectively, which may have resulted from a structural monomer without ¹³C in the molecule (Figs. 6 and 7).

In the present study it is concluded that fructan biosynthesis in the asparagus tissue can be induced by accumulation of glucose, as well as that of sucrose as reported for *Lolium temulentum* (Pollock, 1984) and barley (Wagner et al., 1986). Furthermore, fructans (DP=3-7) of both inulin type and inulin neoseries are also biosynthesized within 72 h from accumulated glucose. On the glucose accumulation-related fructan biosynthesis, it was shown by Müller et al. (2000) that fructosyl transferase gene expression was induced in barley leaves by external trehalose, an analogue of sucrose, but could not be catalyzed by invertases, and thus fructan biosynthesis might have been induced by sucrose. If so, external glucose must be converted to sucrose promptly, since accumulation of short-chain fructans (DP=3 and 4) was observed in

the asparagus stem tissue cultured for more than 48 h on both glucose- and
sucrose-supplemented media without a time lag (Suzuki et al., 2004).

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Figure Legends

Fig. 1. A simple scheme of the biosynthetic pathway for fructan in asparagus adapted from Shiomi, 1993. Suc, sucrose; Glc, glucose; and 1-Kes, 1-kestose. 1-SST, sucrose:sucrose 1-fructosyltransferase; 6G-FFT, fructan:fructan 6G-fructosyltransferase; and 1-FFT, fructan:fructan 1-fructosyl transferase.

Fig. 2. Stability of naturally occurring 1-kestose (DP=3 fructan) incubated at 4 °C for 30 min with 0.2 N perchloric acid: A, 200 µL of DDW + 200 µL of 0.2 N HClO₄; B, 100 µL of 0.15 M 1-kestose + 300 µL of DDW; C, 100 µL of 0.15 M 1-kestose + 100 µL of DDW + 200 µL of 0.2 N HClO₄; D, naturally occurring fructose (Fru, 0.03 M), glucose (Glc, 0.03 M), sucrose (Suc, 0.03 M), lactose (Lac, 0.03 M), neokestose (Neo, 0.01 M), 1-kestose (1-Kes, 0.01 M) and nystose (Nys, 0.01 M). Blended solutions of charts A and C were incubated at 4 °C for 30 min, adjusted to pH 4 with solid KHCO₃, and then solutions in charts B and C were supplemented with 100 µL of 0.15 M lactose as an internal standard. Arrows in chart C represent the absence of fructose, glucose and sucrose.

Fig. 3. High performance liquid chromatograms of carbohydrates in the asparagus tissue before and after culturing for 72 h on medium supplemented with 0.5 M glucose labeled with or without (control) ¹³C. Naturally occurring fructose, glucose, sucrose, lactose, neokestose, 1-kestose and nystose are the same as those used in Fig. 2.

Fig. 4. MALDI-TOF mass spectra of the fructans from ¹³C-labeled and control samples.

Digits in the parentheses represent theoretical average molecular mass of fructans at each

DP. Digits underlined show the difference of m/z value of fructans at each DP from those of control.

Fig. 5. ESI-mass spectrums of the sucrose from ^{13}C -labeled and control samples. Digits in the parenthesis represent theoretical average molecular mass of sucrose. Digits underlined show the difference of m/z value of sucrose from that of control.

Fig. 6. ESI-mass spectrums of the DP=3 fructans (1-kestose and neokestose) from ^{13}C -labeled and control samples. Digits in the parentheses represent theoretical average molecular mass of fructans at each DP. Digits underlined show the difference of m/z value of fructans at each DP from those of control.

Fig. 7. ESI-mass spectrums of the DP=4 fructans (nystose) from ^{13}C -labeled and control samples. Digits in the parenthesis and underlined are the same as those in Fig. 6.

Fig. 8. Possible combinations of glucose and fructose molecule(s) in terms of the number of ^{13}C in sucrose and short-chain fructans from ^{13}C -labeled samples which had 1 or 2 m/z heavier values (the 344.3 m/z sucrose, the 507.4 m/z DP=3 fructans, the 670.6 m/z DP=4 fructans and the 671.6 m/z DP=4 fructans) than expected values on ESI-MS shown in Figs. 5-7. The hypothesis assumes that fructose molecules possessing one or two ^{13}C -atoms had been formed during conversion from glucose. Digits on the figures of glucose and fructose molecules represent the number of ^{13}C -atoms possessed. Arrows represent the most probable combinations in each group. Squares with broken lines show combinations of fructose molecules with two ^{13}C -atoms.

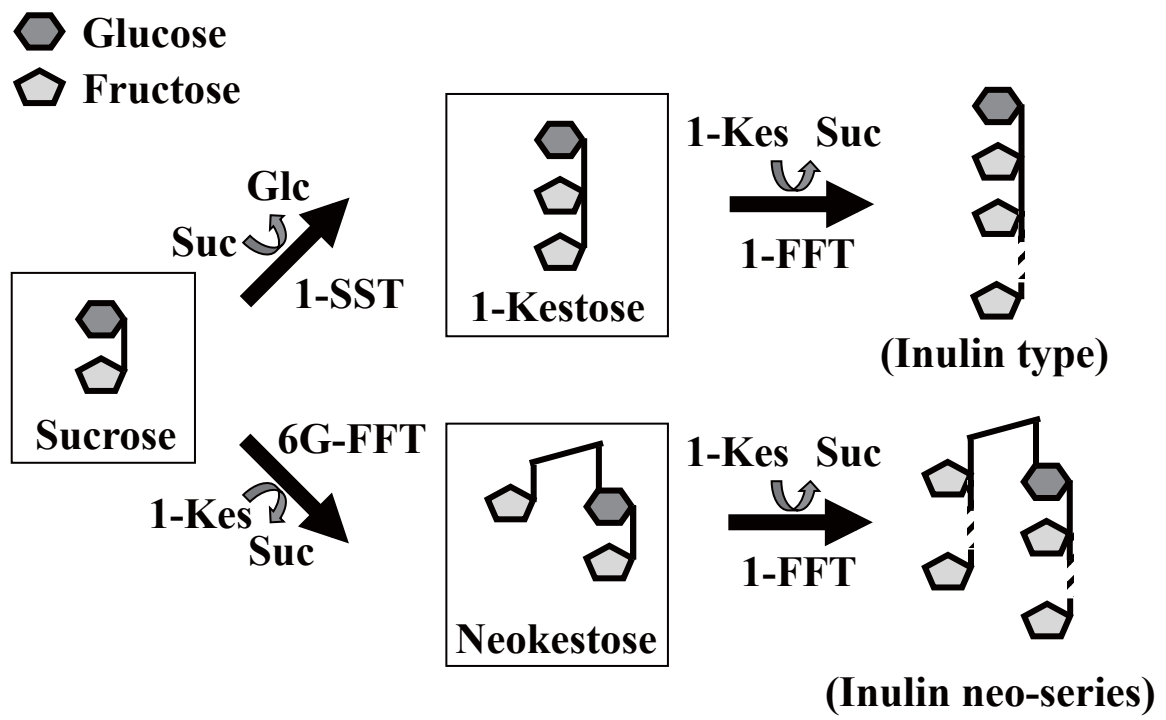


Fig. 1

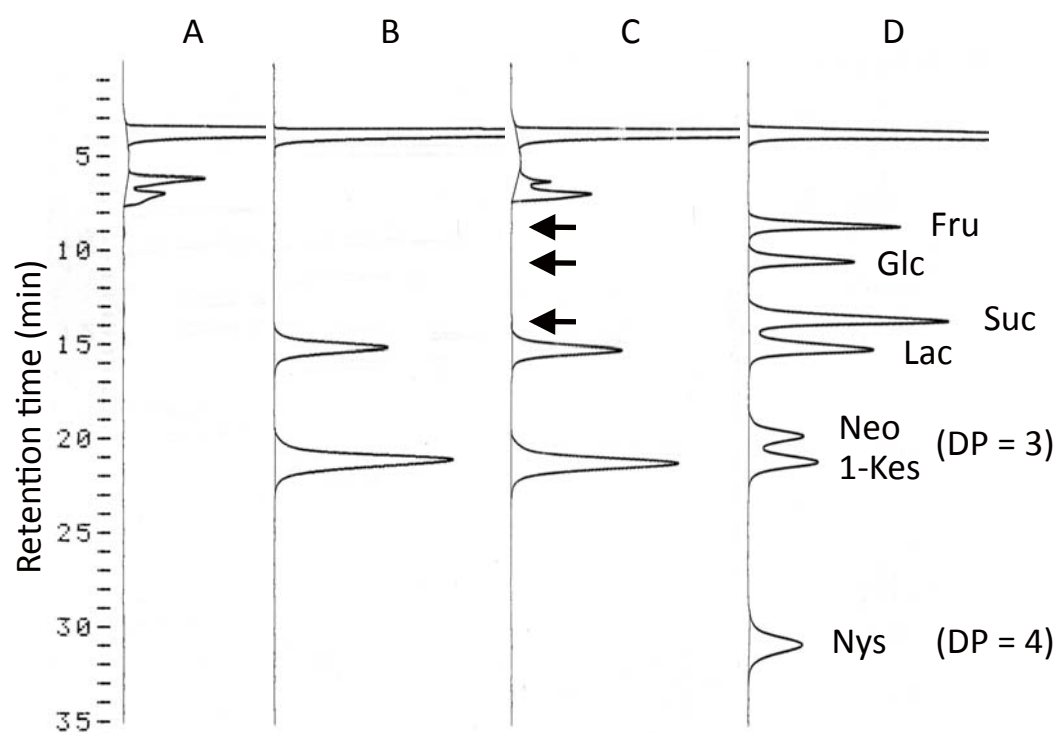


Fig. 2

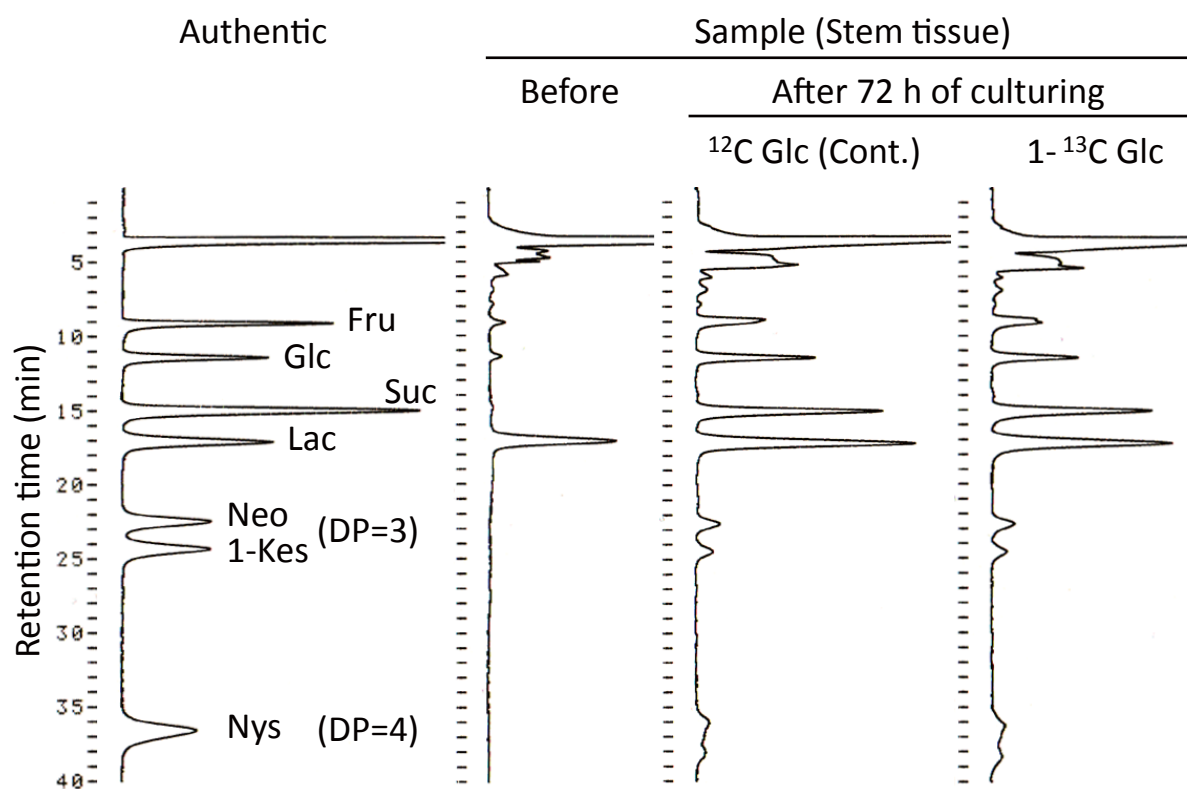


Fig. 3

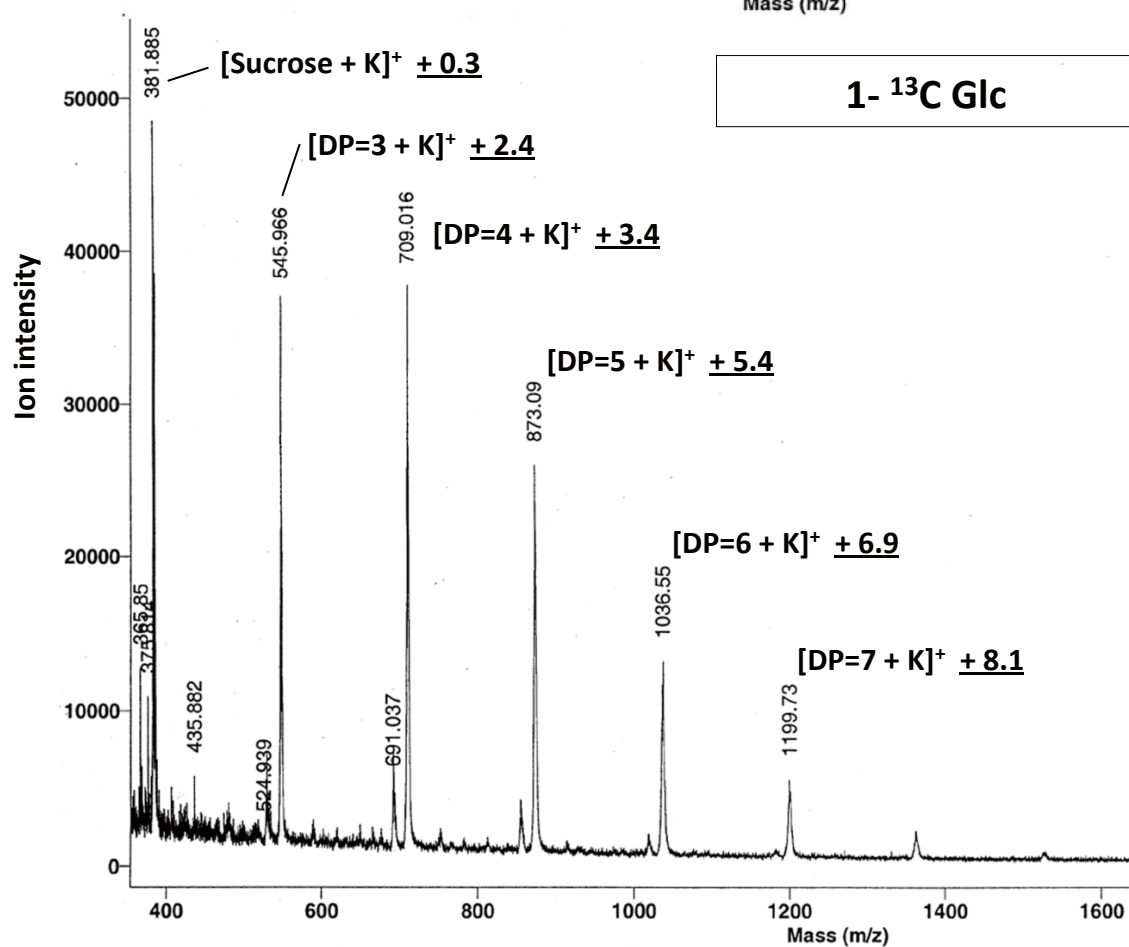
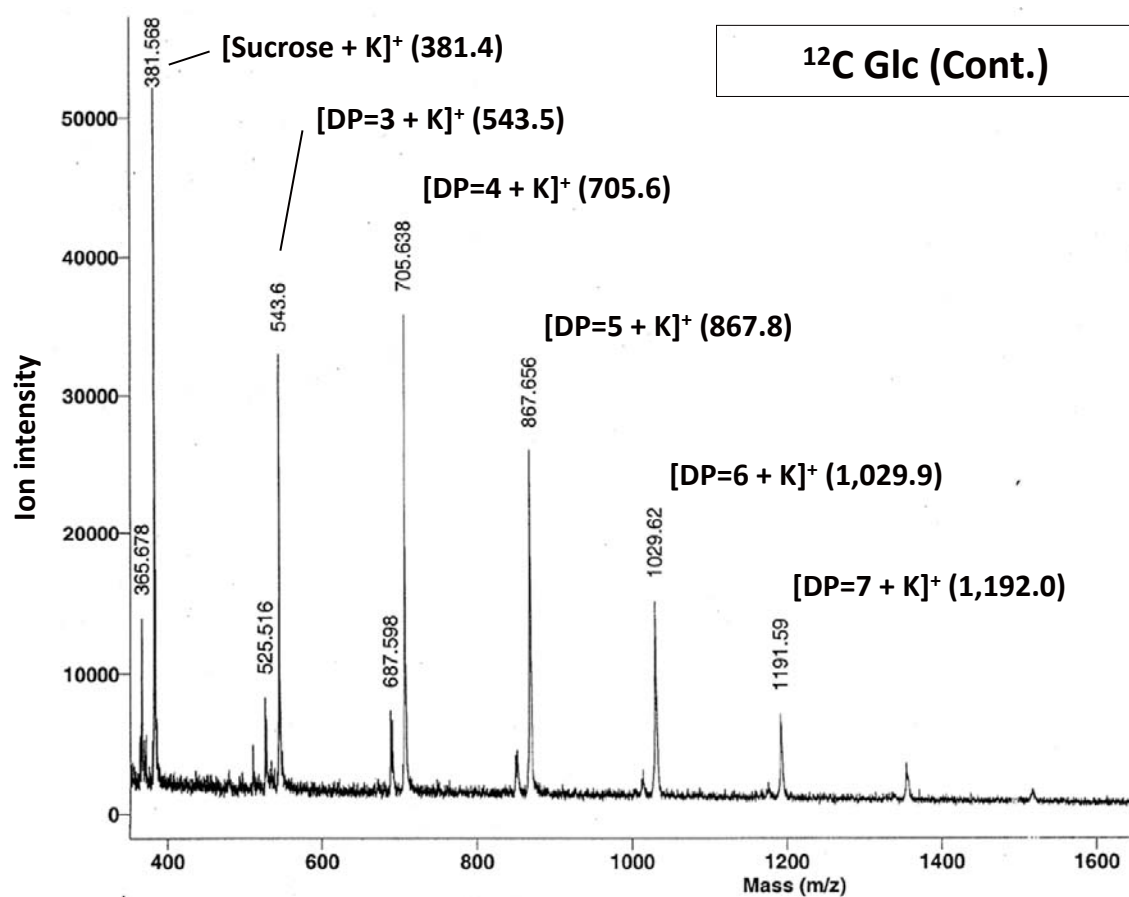


Fig. 4

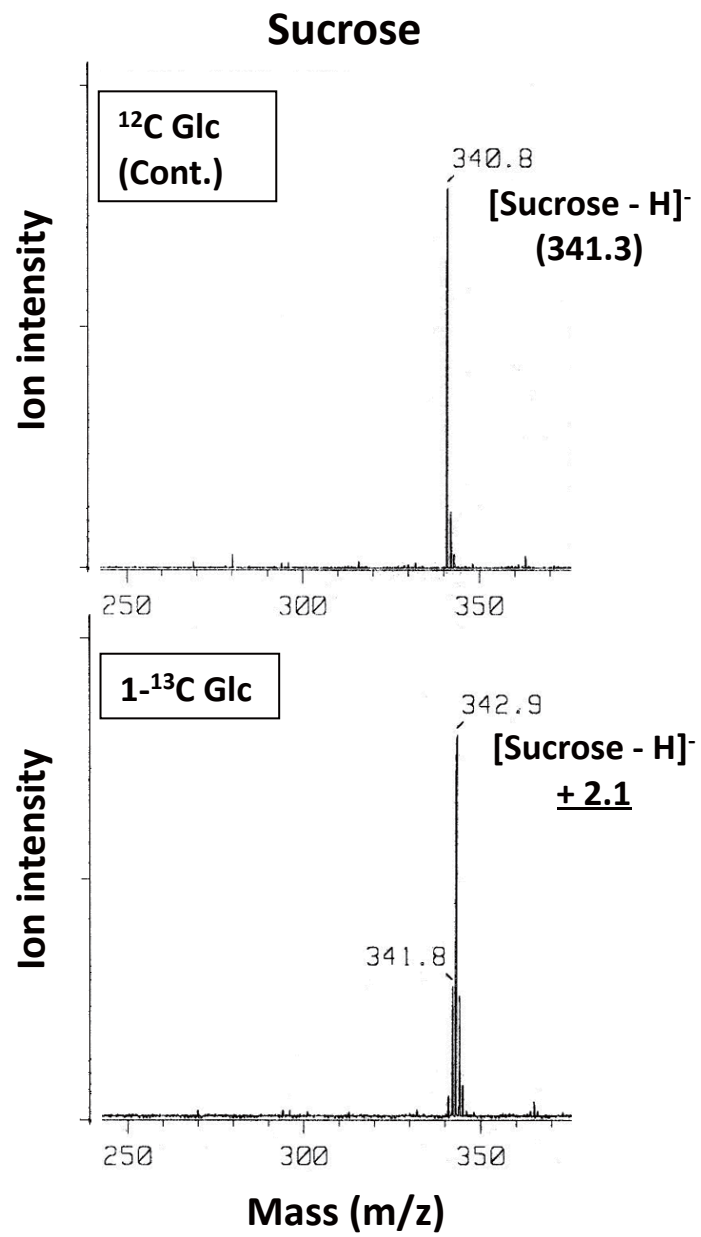


Fig. 5

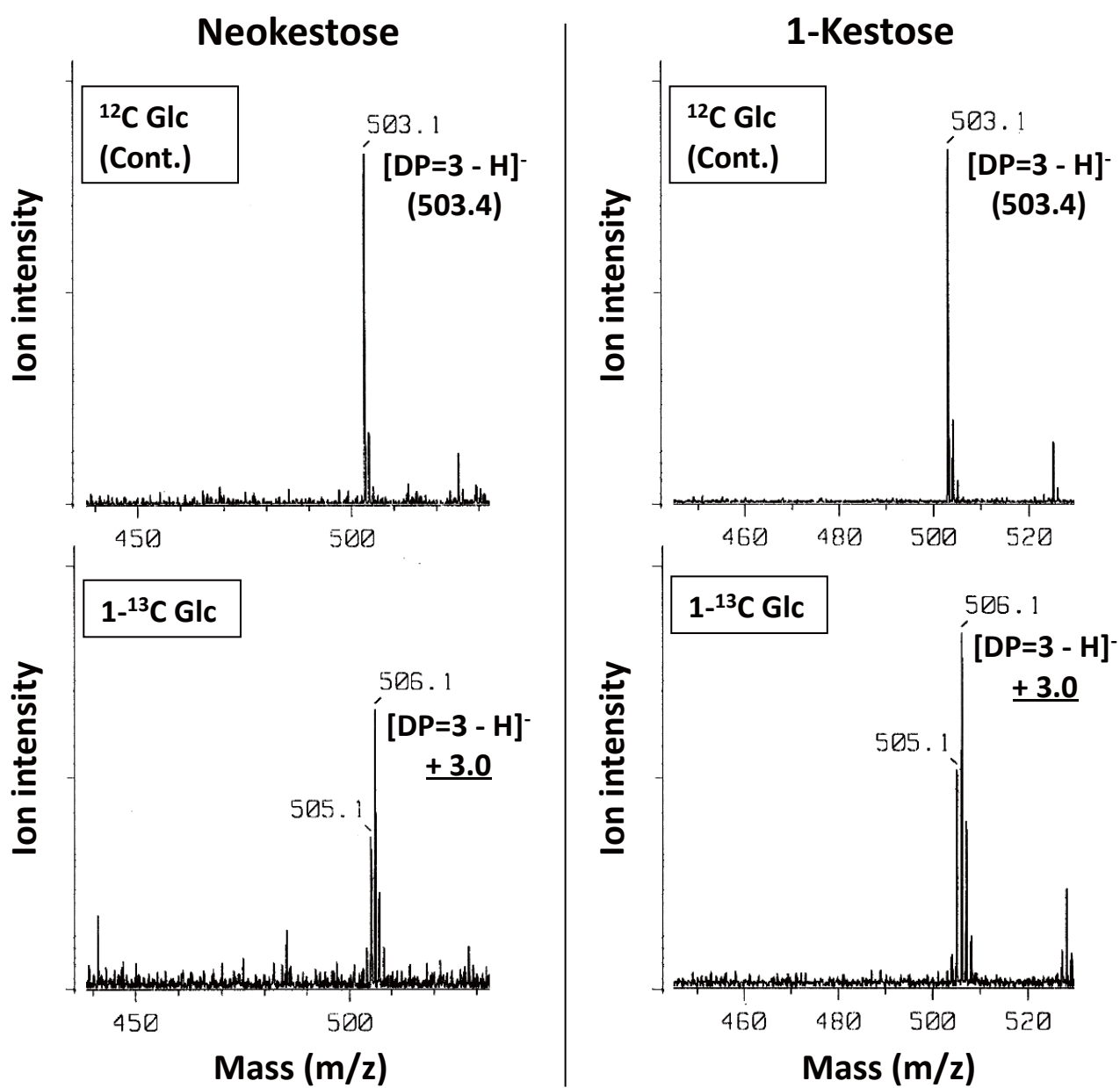


Fig. 6

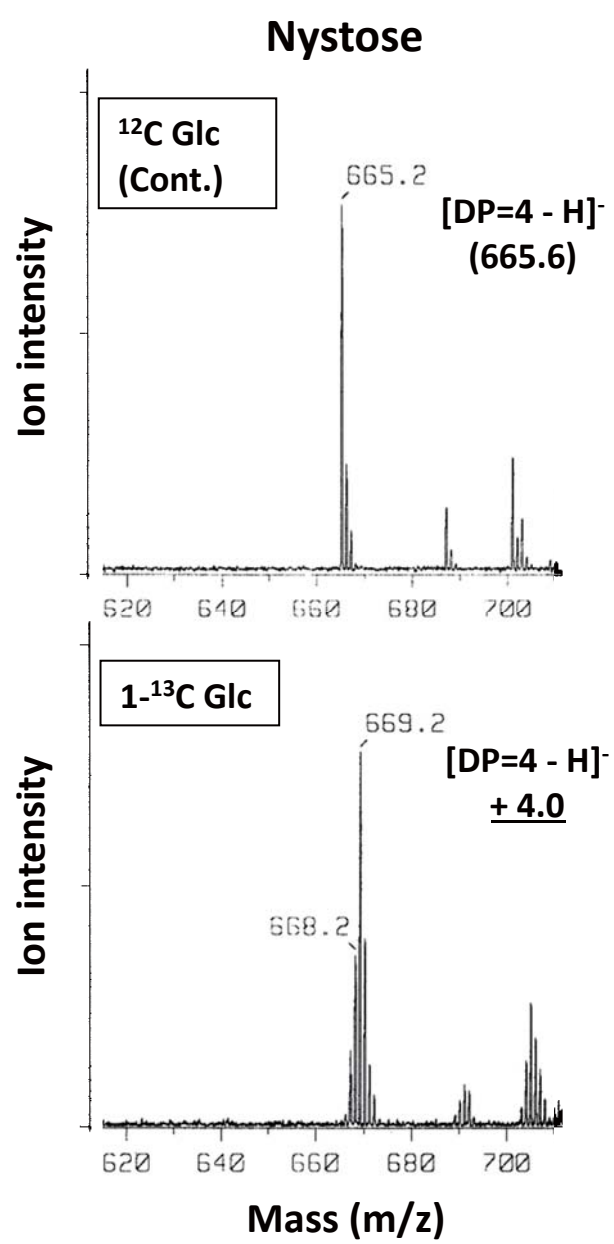


Fig. 7

 Glucose
  Fructose

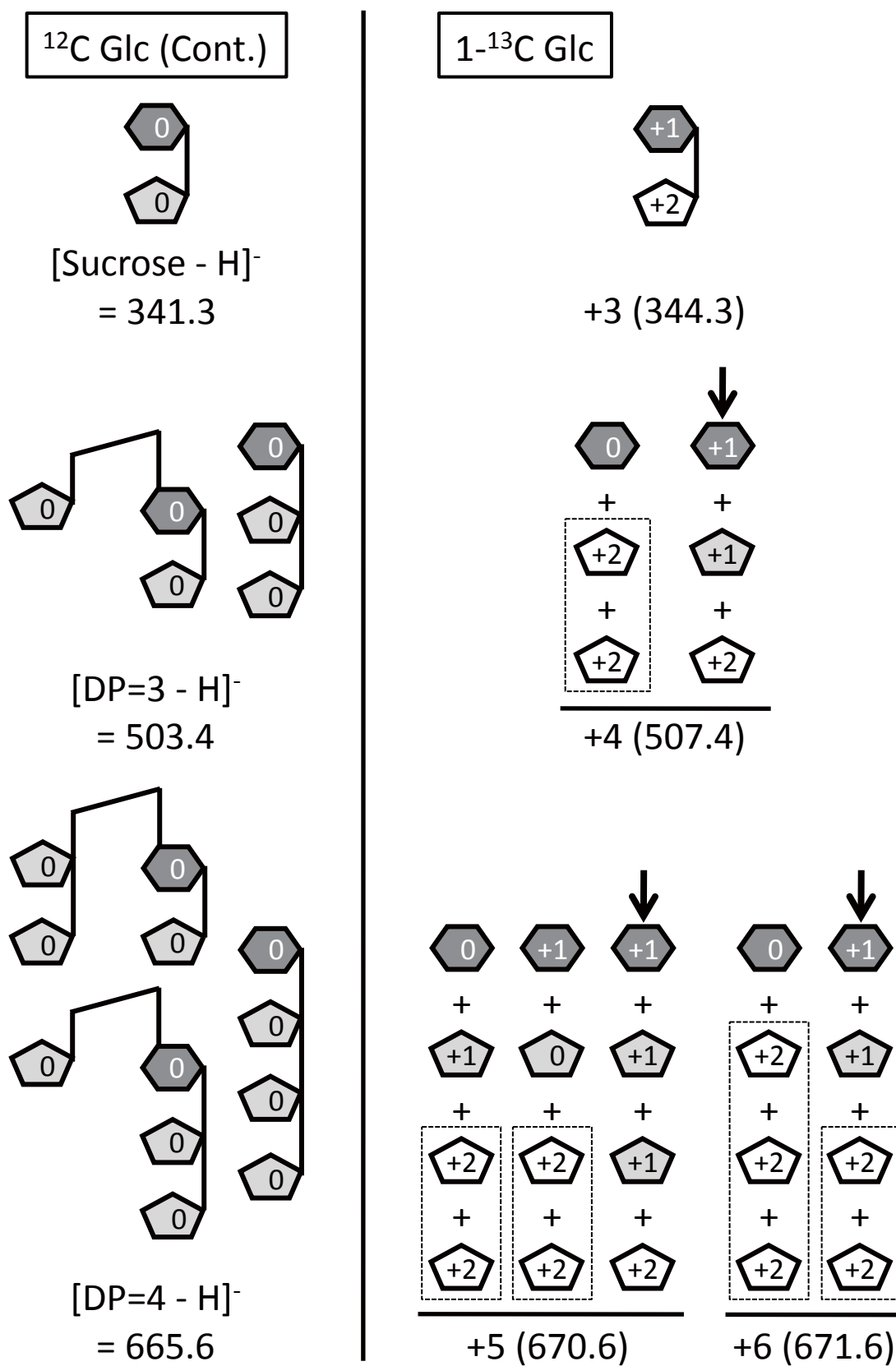


Fig. 8

Table 1. Carbohydrate content in asparagus tissues calculated on HPLC profiles shown in Fig. 3

Carbohydrates	Content (μmol/g FW)		
	Before	After 72 h of culturing	
		¹² C Glucose (Cont.)	[1- ¹³ C]Glucose
Fructose	10.1	22.8	20.2
Glucose	11.5	56.0	49.4
Sucrose	1.5	43.4	44.4
Neokestose	- ^z	6.3	7.2
1-Kestose	-	4.4	4.9
Nystose	-	4.6	5.5

^zNot detected.