Dynamic Biochemistry, Process Biotechnology and Molecular Biology ©2009 Global Science Books

Analysis and Characterization of Fructan Oligosaccharides and Enzymatic Activities in the Leaves of *Agave tequilana* (Weber) var. 'Azul'

Iván Saldaña Oyarzábal[1,2*] • Tita Ritsema[3,4] • Stephen R. Pearce[2]

[1] R&D Laboratory, Casa Pedro Domecq/Pernod Ricard Mexico, Carr. Fed. Mex-Pue Km 17.5, 56400, Los Reyes La Paz, Edo. Mex, Mexico
[2] Department of Biology and Environmental Science School of Life Sciences, John Maynard-Smith Building, University of Sussex, Falmer, Brighton, BN1 9QG, UK
[3] Botanical Institute, Hebelstrasse 1, CH-4056 Basel, Switzerland
[4] Plant-Microbe Interactions, Institute of Environmental Biology, Utrecht University Kruijtgebouw, W308 Padualaan 8 3584 CH Utrecht, The Netherlands

Corresponding author: * **Ivan.Saldana@casa-pedro-domecq.com**

ABSTRACT

The ability of *Agave* species to synthesize fructans has been poorly investigated in the past. *Agave tequilana*, a CAM plant of Mexican origin, accumulates fructans in their false stem or *piña* that are harvested and used as a source of sugars for the production of tequila. Synthesis of fructans occurs in the agave leaf and in the past it has been suggested that they are transported through the phloem to the storage organ. In this work the structural characteristics of oligosaccharides present in plant leaves are examined by different methods such as TLC, MALDI-TOF and HPAEC-PAD. Also, leaf protein extracts were tested for enzyme activities by incubation with different sugars to identify the machinery responsible for agave fructan structures. Results showed that sucrose represents the largest contributor (67%) followed by fructans (20%) up to DP 12 and monosaccharides (13%), respectively. Leaf oligofructan structures are comprised of a mixture of inulin, neoseries and branched fructans. All DP3 and DP4 fructans were linear molecules of the inulin and neoseries type, with the notable absence of 6-kestose or bifurcose, which is considered to be the primer oligosaccharide in grasses. Enzymatic assays confirmed the presence of the synthetic activities 1-SST, 1-FFT and 6G-FFT, but could not detect any 6-SFT activity, generally considered responsible of the branching fructans and synthesizer of 6-kestose or bifurcose that could not be found. From this study we conclude that the branching activity in *Agave* spp. is of different nature than 6-SFT already characterized in grasses. We proposed that an unknown 6-FFT activity may be responsible for the branched structure in *Agave* fructan.

Keywords: carbohydrate metabolism, Crasulacean Acid Metabolism, fructosyltransferase, MALDI-TOF
Abbreviations: DP, degree of polymerization; **KH**, kestose hydrolase; **1-FFT**, fructan:fructan 1-fructosyltransferase; **1-SST**, sucrose: sucrose 1-fructosyltransferase; **6-FFT**, fructan:fructan 6-fructosyltransferase; **6G-FFT**, fructan:fructan 6G-fructosyltransferase; **6-SFT**, sucrose:fructan 6-fructosyltransferase; **6-SST**, sucrose:sucrose 6-fructosyltransferase

INTRODUCTION

Plants containing fructans can be found in both monocots and dicots, which suggests that the enzymatic machinery involved in their synthesis and degradation has appeared independently many times in evolution (Hendry 1993). However, fructans are not the same in all plants, as they can exhibit strong differences in concentrations, sizes and structures between species (Cairns and Ashton 1993). Also, the physiological role that fructans play is different depending on the specie and the organ where they are shytetized and/or stored. Even plants from the same species but at different developmental stages (such as age, flowering or growth season) grow in different environmental conditions (such as temperature, light conditions and nutrients in the soil) and can present large variations in their fructan concentrations (Pollock and Jones 1979; Demeulemeester *et al.* 1998; see Ritsema and Smeekens 2003 for review).

Agave spp. are strong fructan accumulators during their life cycle, building an important reserve at the base of the plant, a false stem or *piña.* Fructans are remobilized from there and used to fuel the reproductive activities in this plant. In asexual reproduction, fructans are utilized for the establishment of clonal offspring or *hijuelos* derived from the rhizomes of the mother plant (Tissue and Nobel 1988). In sexual reproduction, fructans are used to build the inflorescence, a tall central floral structure that serves for breeding and the production of seeds, which requires such a large amount of resources that generally its formation implies the sacrifice of the whole plant (Arizaga and Ezcurra 1995; Mclaughlin *et al.* 2000).

Agave tequilana is considered one of the most representative plants of the Mexican flora, because it is the source carbohydrate for the production of tequila, Mexico's national spirit. It is a member of the Agavaceae family, an arid adapted group of plants distributed along North and Central America (Gentry 1982). Historically, agaves from different species have been domesticated and grown by traditional Native American cultures and tribes due to their medicinal, nutritional and utilitarian properties (Colunga and Maypat 1993). The carbon assimilation in these plants is based on CAM (Crassulacean Acid Metabolism) which allows them to obtain CO_2 at night, thus reducing water loss due to transpiration by closing the stomata during the day(see Nobel 1988 for review). The mature agave *piña* possesses a large concentration of fructans, which makes this crop a potential source for industrial purposes. *A. tequilana*'s cropping system has been well developed in size and technology in the past decades and has advantages compared with other plants used for fructan production such as chicory or Jerusalem artichoke. For instance, *A. tequilana* is available all year long and can be grown in warmer environments with less of a requirement for water. Initial investigations on the prebiotic functionality of agave fructans provide evidence

Received: 1 May, 2009. Accepted: 27 October, 2009.

Original Research Paper

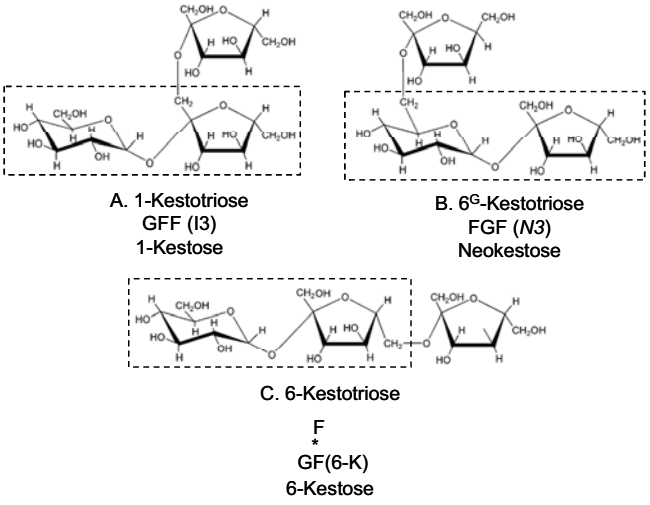


Fig. 1 Molecular structure of the three trisaccharide precursors to plant fructans. All structures results from the addition of a fructose residue to a sucrose molecule (in the box). (**A**) Inulin basic trisaccharide; (**B**) Neoseries basic trisaccharide, (**C**) Levan basic trisaccharide. Structure's technical names using the Waterhouse and Chatterton (1993) nomenclature; peak identification tags (in parenthesis) following Ernst *et al.* (1998). Common names are written below each structure. Black numbers indicates linkage types.

to believe that it behaves similarly to other inulins in the market (Urias-Silvas *et al.* 2008; for a review in prebiotic effect of fructans see Roberfroid 2002).

Although fructans only have three different types of linkages: fru-β(2-1), fru-β(2-6) and glu-β(2-6), a large diversity in types and structures can be found in plants (Pollock and Cairns 1991). For instance, fructan molecules containing only fructosyl β(2-1) between fructose residues are known as inulin. Its simplest example and the precursor for all other inulin fructans is the trisaccharide 1-kestose (**Fig. 1A**) followed by the tetrasaccharide nystose (**Fig. 2A**). Fructans containing only fructosyl β(2-6) are know as levan, and its simple case is the trisaccharide 6-kestose (**Fig. 1C**). Fructans containing a fructose unit attached to the glucose residue via a glucosyl β(2-6) linkage of the original sucrose acceptor are named as neoseries, independently if they have inulin and/or levan types of fructosyl linkages. The trisaccharide neokestose is the simplest of all (**Fig. 1B**). Some plants only produce a single type of fructans such as chicory (*Cichorium intybus*) or Jerusalem artichoke (*Helianthum tubersum*) which have only the inulin type, or the grass *Poa ampla* which has levan only (Chatterton and Harrison 1997; van Laere and van Den Ende 2002). Some other plants, such as onion, garlic or asparagus (*Allium cepa*, *Allium sativa* and *Asparagus officianalis*, respectively) also have fructans with the neoseries internal glucosyl linkage, where fructans have fru-β(2-1) and glu-β(2-6) linkages these are commonly classified as the inulin neoseries type (Shiomi 1989; Shiomi 1993; Ernst *et al.* 1998). The simplest examples of inulin neoseries are the oligosaccharides 1&6^{G}-Kestotetraose and 1,6^{G}-Kestotetraose (**Fig. 2B** and **2C**, respectively). Interestingly, neoseries fructans can be elongated by the addition of new fructosyl residues in both directions of the original sucrose acceptor, while pure inulin or levan presents a single elongation point (see **Fig. 2**).

Plant fructan pools can be composed of a mixture of different independent types of fructan molecules, but generally one is more abundant than the others. In *Avena sativa*, inulin and levan neoseries are present but the latter is more abundant (Livingston *et al.* 1993). Fructan molecules can also contain a mixture of fructosyl β(2-1) and β(2-6) linkages in the same structure, with or without the neoseries internal glucose. It may be branched if the both fructosyl linkages types are present in the same single fructose residue, allowing new elongation points for new fructose chains in the molecules (see examples in **Fig. 2D** and **2E**). Well characterized examples of plants containing branched fruc-

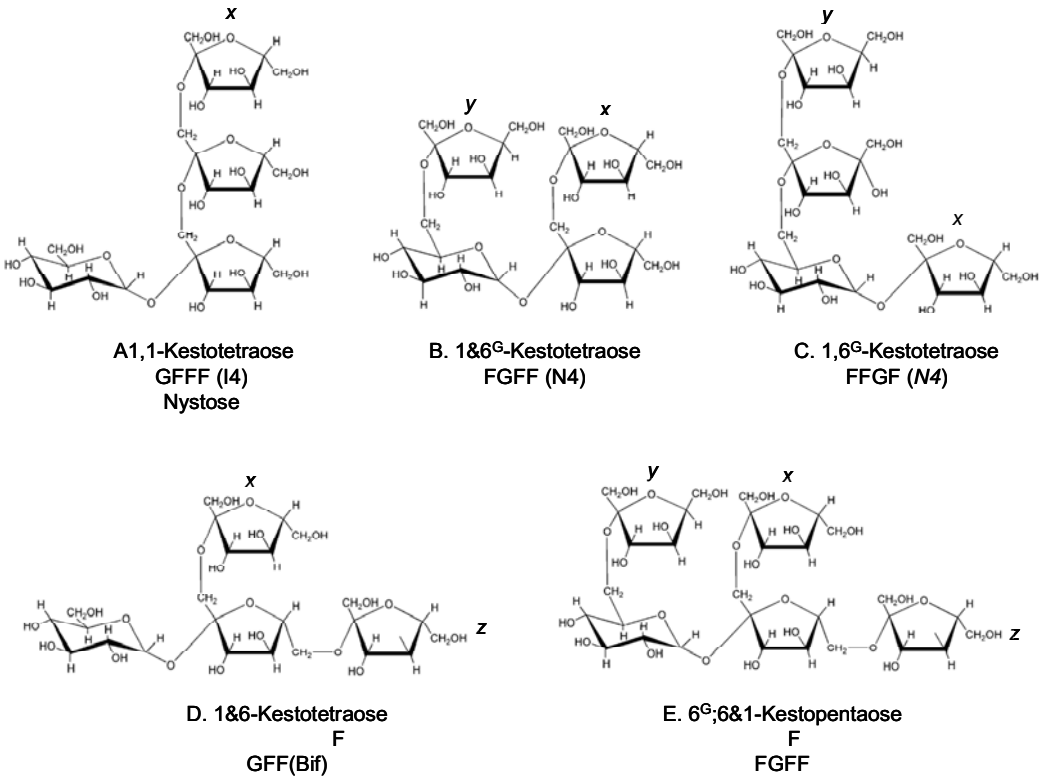


Fig. 2 Structure of oligosacharides present in inulin, neoseries, branched inulin-levan and branched neoseries-levan fructan types. All structures result from the addition of fructose residues to a precursor trisaccharide (see **Fig. 1**). (**A**) Inulin, (**B, C**) neoseries, (**D**) branched inulin-levan, (**E**) branched neoseries-levan. Elongation points are described by *x*, *y* and *z*. Inulin presents only *x*; neoseries presents *x* and *y*; branched neoseries-levan presents *x*, *y* & *z*. Structure's technical names using the Waterhouse and Chatterton (1993) nomenclature, peak identification tags (in parenthesis) adapting Ernst *et al.* (1998). Common names (if any) are written below each structure.

tan with an inulin-levan mixture are barley (*Hordeum vulgare*), wheat (*Triticum aestivum*) and *Bromus tectorum* (Chatterton *et al.* 1993a; Roth *et al.* 1997). The simplest example of a branched fructan is the tetrasacharide bifurcose (**Fig. 2D**). Other species, mainly in the gramineae and Asteraceae have branched fructans of the inulin-levan neoseries type. Examples are *Lolium temulentum*, *Lolium perene*, *Phleum pratense*, *Fetusca arundinacea*, *Fetusca novae-zelandie*, *Phormium tenax*, *Phormium cookianum*, *Cordyline australis*, *Dactylis glomerata* and *Urginea maritima* (Brasch *et al.* 1988; Chatterton *et al.* 1990; Sims *et al.* 1992; Spies *et al.* 1992; Chatterton *et al.* 1993b; Sims *et al.* 2001; Pavis *et al.* 2001b; Sims 2003; Clark *et al.* 2004). *Agave* spp. exhibit this last type of fructans in their *piña*, where all three types of linkages can be found (López *et al.* 2003; Mancilla-Margalli and López 2006).

The synthesis and degradation of plant fructans occurs by the participation of fructosyltransferases (FT) and fructan exohydrolases (FEH), respectively. FTs transfer fructosyl units from a donor to an acceptor molecule forming a β linkages bond to join them. FEHs release fructosyl units from fructan molecules, sequentially, by destroying the molecular β linkage and releasing free fructose (for a review see Ritsema and Smeekens 2003). Different fructan types are produced by the combined action of different FT activities events. In a few plants such as chicory (inulin type fructans), onion (inulin neoseries fructans), and barley (branched inulin-levan) enough FTs recombinant gene products and/or purified protein enzymes had been characterized to explain their complete synthetic enzymology (Simmen *et al.* 1993; Duchateau *et al.* 1995; Sprenger *et al.* 1995; van den Ende and van Laere 1996; van den Ende *et al.* 1996; Sprenger *et al.* 1997; Ritsema *et al.* 2003; Fujishima *et al.* 2005; Shiomi *et al.* 2005; Ueno *et al.* 2005). However, in most others species and particularly in those presenting more complex fructan patterns such as branched inulin-leavan neoseries containing species (mainly within the Poaceae and Asteraceae orders) the enzymology behind their structure is not very well established yet.

Only four enzymatic activities have been fully characterized in purified and/or recombinant proteins: 1-SST (EC 2.4.1.99), 1-FFT (EC 2.4.1.100), 6G-FFT (EC 2.4.1.243) and 6-SFT (EC 2.4.1.10; see abbreviations). 1-SST is responsible for the transfer of a fructosyl residue from a donor sucrose to another acceptor sucrose forming 1-kestose (see **Fig. 1A**), the simplest of all fructans. 1-FFT is responsible for the elongation of fructans by the transfer of additional fructosyl residues donated from a fructan molecule to another fructan molecule that serves as an acceptor forming a β(2-1) linkage. The simplest example is the formation of nystose (**Fig. 2A**), obtained by the transfer of a fructosyl unit to a kestose acceptor molecule. 6G-FFT is responsible for transferring a fructosyl unit from a fructan donor to a sucrose or fructan acceptor molecule at the carbon 6 of the terminal glucosyl unit forming a β(2-6) linkage. The simplest example is neokestose (**Fig. 1B**), were a sucrose molecule served as an acceptor, forming a trisaccharide, but other examples having larger fructans as acceptors also occurs (**Fig. 2B**). Finally, 6-SFT is responsible for the transfer of a fructosyl unit from a sucrose molecule to fructan or sucrose acceptor by the creation of a fructosyl β(2-6) linkage and may produce branches. This enzyme has shown to synthesize both bifurcose and 6-kestose (**Fig. 1C** and **1D**, respectively). Although all these activities have been produced by specific single proteins, they generally exhibit more than a single activity, with invertase being the most common secondary activity (for a review see Vijn and Smeekens 1999).

Other FTs activities that have been postulated in some plant species are 6-SST, 6-FFT, however, their existence is still a matter of debate because no complete characterization or purification of proteins presenting these activities has been done so far. These enzymes are believed to have the ability of transferring fructosyl residues to an acceptor molecule forming a β(2-6) linkage from and to sucrose or from and to fructan molecules (6-SST and 6-FFT, respectively). The highly branching patterns in species such as some *Lolium* spp. and members of the order Asparagales (and of the synthesis linear levan in species such as *Poa ampla* where no 6-SFT activities seems to be present) could be explained by the action of these enzymes (Chatterton and Harrison 1997; Pavis *et al.* 2001a, 2001b; Sims 2003).

So far it is not clear how synthesis of fructans occurs in agave leaves in terms of its enzymology and oligosaccharides involved. The presence of fructan has been reported in the leaves of *Agave deserti*, *Agave americana*, and *Agave veracruz* (Dorland *et al.* 1977; Nandra and Bhatia 1980; Wang and Nobel 1998). Although in most plants leaf fructans serve as a transient non-transported carbon pool (Amiard *et al.* 2004), mainly when sucrose concentrations are abundant (Cairns *et al.* 1997), in agave leaves substantial amounts of fructans have been detected in phloem sap, suggesting that they are transported through the phloem (Wang and Nobel 1998) to other tissues, such as the *piña*. This unique feature implies that fructans are not fully synthesized from sucrose in the storage organ as in most other fructan-accumulating plants, but that its synthetic pathway is distributed between the two organs. The complexity of *Agave tequilana piña* fructan is due to its highly branched structure which requires the action of different enzyme activities. Structural analysis of carbohydrates extracted from *A. veracruz* leaf showed the presence of oligosaccharides with a degree of polymerization (DP) higher than 5 that can have branching points, suggesting that branching activity can be present in the leaves, too (Dorland *et al.* 1977).

This study investigates concentrations, sizes and structure types of basic oligofructans, as well as the enzymatic machinery responsible for their synthesis in the leaf. In this report carbohydrate composition of *A. tequilana* leaves growing in Sussex University's greenhouse is presented. The naturally occurring leaf oligofructans are characterized by different chromatographic methods such as TLC, MALDI-TOF-MS and HPAEC-PAD and comparisons with other plant fructans with different structures are made. Also, this report analyzes the FTs and FEH activities present in desalted protein extracts from the leaves and compares *in vitro* synthesized oligosaccharides with those detected *in vivo*. Finally, we discuss the results considering previous investigations on Agave fructans and propose a model for the synthesis of agave fructans.

MATERIALS AND METHODS

Plant material and growth conditions

Several *Agave tequilana* (Weber) var. 'Azul' plants were obtained from a commercial plantation located within the municipality of Atotonilco el Alto, in the state of Jalisco, Mexico (20° 28.500 N, 102° 35.711 W). The greenhouse in the University of Sussex was prepared to receive 20 of these plants, which at the time of their arrival weighed between 15 and 20 kg. They were air freighted to the UK and placed to grow in the greenhouse within a total time period of 7 days. The plants were allowed to acclimatize to the new conditions for at least 6 months before any of these studies were performed. The agaves were placed in pots filled with a 3: 2: 1 mix of John Innes soil: sand: expanded vocanic rock (Fargro Ltd., Littlehampton, West Sussex, UK) and watered with 0.2X Hoagland nutrient solution No. 1 once a week, allowing the soil to dry between each watering events. Average temperatures were maintained at 25°C/15°C at day/night with relative humidity varying between 45 to 70%. The photosynthetic photon flux (PPF) was variable throughout the year but artificial light was supplied to maintain acceptable PPF for the development of these plants in a controlled environment (13-25 mol m^2 s^{-1}). Only well illuminated exposed leaves presenting an angle of 40 to 60° were used in this study.

Extraction of *Agave tequilana* leaf carbohydrates for quantification and for chromatographic characterization

To investigate the concentrations of the different carbon pools contributing to the water-soluble carbohydrates in the agave leaf, nine leaf samples were taken at different times of the day from the upper half of leaves of plants growing in the greenhouse. Samples were frozen in liquid nitrogen, weighed and lyophilized in a freeze dryer chamber at -20°C. After this, dry weight was recorded and water content calculated. Samples were then pulverized using a mortar and pestle. A subsample of 100 mg DW was used for sugar extraction. The plant material was placed in a 15 ml centrifuge tube containing 250 µl methanol: chloroform: water (12: 5: 3) and tissue was strongly mixed. After homogenizing, 6 ml of boiling H_2O was added and the tube was immediately incubated in a water bath at 100°C for 4 min. Heating destroys any enzyme activity in the sample ensuring no further sugar break down will occur. The samples were frozen in dry ice for 10 min and allowed to melt at room temperature to break down the tissue even more to maximize the extraction recoveries. The tubes were vigorously mixed and centrifuged at 7,000 × *g* for 15 min in a bench centrifuge and the supernatant collected. The samples were then passed through a C_{18} cartridge (Alltech Associates, Deerfield, IL) to remove lipids and no polar components and filtered through a 0.2 µm nylon filter (Sigma-Aldrich, Poole, Dorset) to retain proteins. The extracts were stored at -20°C and until carbohydrates were quantified.

A single and abundant leaf sugar extract was produced using leaf samples obtained from 3 randomly selected plants from the greenhouse at the end of the day (18 hrs), when maximal accumulation of sugars is expected. All the harvested tissue was taken from the middle region of the leaves sampled. To make an average representative sample the leaf samples of both sets were chopped into small squares and extracted together (40 g approximately). The material was placed together into a kitchen blender with 100 ml of distilled water and homogenized. Then, the homogenate was heated at 95°C for 15 min with constant mixing to extract the sugars. The extracted material was filtered through a 1mm sieve and the aqueous part kept apart. The blended tissue was re-extracted with 100 ml of ethanol by heating it to 70°C for 10 min with constant mixing, and the extract filtered as above. The water and ethanol extract were independently centrifuged at 10,000 × *g* during 15 min to remove any insoluble traces, and supernatant mixed together. Finally the total extract was concentrated almost to dryness in a vacuum-driven rotary evaporator system using a water bath at 70°C. The thick slurry obtained was resuspended in distilled water until a 30ml volume was reached and passed through a 0.2 µm filter membrane (Millipore Limited, Consett, UK) using a vacuum driven device. For Thin Layer Chromatography (TLC) analysis, the extract was used with no further processing. For matrix-assisted laser deabsorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) and for high-performance anion exchange chromatography coupled with pulsed amperometric detector (HPAEC-PAD) analysis, an aliquot of 4 ml was desalted by passing the extract through anion and cation exchange cartridges (Alltech Associates, Deerfield, IL) using a syringe and following vendor specifications. Unsalted and filtered extracts were stored in a -20°C freezer until further analysis was performed.

Quantification of total soluble carbohydrates, glucose, fructose, sucrose, oligosaccharides and total fructan content

To measure monosaccharide glucose and sucrose, disaccharide sucrose and the oligosaccharides, kestose and neokestose in all leaf extracts sampled for sugar quantification, high-performance anion exchange chromatography with pulsed amperometric detection (HPEAC-PAD, Dionex Corp., USA) was used as previously described (Lüscher *et al.* 2000a). Samples integrated peak areas from chromatograms obtained were compared with commercially available standards from Fluka, Buchs, Switzerland and previously purified onion neokestose obtained in the lab as described by Duchateau *et al.* (1995). 10 µl of each sample was injected and passed through a Dionex CarboPac PA100 column (Dionex Corp., Olten, Switzerland) using a gradient program with two elution solvents: Solvent A, 1M NaOH and Solvent B, 1M Na-acetate, a flow rate of 1 ml min^{-1}. Start (0 min), 10% A, 0% B; 0-5 min, linear increase to 30% A, 0% B; 5-15 min, linear increase to 30% A, 20% B; 15-25 min, 10% A, 0% B. The detector settings were: T_1 = 0.0 sec, 0.05 V; 0.2 sec, 0.05 V; 0.4 sec, 0.05 V; T_2=0.41 sec, 0.75 V; 0.6 sec, 0.75 V; T3=0.61 sec, -0.15 V; 1.0 sec, -0.15 V; integration: 0.2-0.4 sec.

To measure total soluble carbohydrates, complete acid hydrolysis with heat treatment was performed. A volume of 30 µl of all nine samples extracted for carbohydrate quantification was placed in Eppendorf tubes with screw caps and 735 µl of a 20 mM HCl solution added to all of them. The tubes were place in a heating block for 1 h at 98°C to produce the hydrolysis. After this, the tubes were allowed to cool down and 735 µl of a 20 mM NaOH solution was added to neutralize the pH. Controls were used in all hydrolysis procedures to ensure complete breakdown of polysaccharides. Hydrolytically released monosaccharides were measured in a total volume of 250 µl of reaction media containing a final concentration of 125 mM HEPES pH 7.6, 2 mM NAD, 5 mM ATP and 4 mM $MgCl_2$, 1 U hexokinase (EC 2.7.1.1). For glucose quantification, baseline was measured, and then 1 unit of glucose-6-phosphate dehydrogenase (EC 1.1.1.49) was added, the mix was incubated at 28°C for 15 min and the absorbance measured at 340 nm. For fructose, 1 unit of phosphoglucose isomerase (EC 5.3.1.9) was added to the same reaction media after glucose quantification, incubated again at 28°C for another 15 min and final absorbance measured at 340 nm. Fructan content in the agave leaf extract was quantified by subtracting all other sugars (glucose, fructose and sucrose) from the total soluble carbohydrate value obtained.

Qualitative analysis of leaf fructans by Thin Layer Chromatography (TLC)

The analysis of fructan by TLC was performed following the indications described by Cairns (1988). An aliquot of the agave leaf extract was diluted 1 to 5, mixing 100 µl extract in 400 µl of distilled water. Firstly, linear regions for the separation of the fructans in 20 × 20 silica gel TLC plates (Merck Co., Darmstadt, Germany) were established with a ruler and a pencil. Because the relative proportion of fructans in the sample was unknown, the same sample was applied three times in different amounts (20, 40 or 60 µl of the diluted extract) and left to dry. As a standard for DP determination, *Heliantum tuberosus* inulin was analyzed next to the sample of interest. Secondly, the silica plate was placed in a glass chamber containing a separation solvent (butan-1-ol: propan-1-ol: water [3: 12: 4 v/v]) taking care not to allow a direct contact between the solvent and the area where the samples were placed. After the solvent reached the superior edge, the silica plate was taken out of the chamber and allowed to air-dry. The introduction in the chamber and the dry steps were repeated once more to improve the separation of fructans. Finally the plate was developed by spraying phosphoric acid-urea solution containing 1 M phosphoric acid in butanol saturated water (≈80% butanol, w/w), 4.5 g of urea and 7.5 ml of ethyl alcohol, in a uniform manner using a compress-air driven appliance. The plate was let to dry and treated with heated air using a hair drier. The separated fructans were then visualized and the chromatogram produced was immediately scanned into a digital format.

Qualitative analysis of leaf fructans by MALDI-TOF

The presence of DP of fructans by Matrix-Assisted Laser Desorption/ionization coupled with a Time of flight Mass Spectrometry (MALDI-TOF-MS) on the leaf sugar extract was analyzed. An OmniFLEX MALDI-TOF equipment was used (Bruker Daltonik GmbH, Bremmer, Germany) in the positive ion mode. DHB matrix (2,5-dihydroxybenzoic acid, Fluka, Buchs, Switzerland) dissolved 10 g L^{-1} in distilled water, was used as recommended by Stahl *et al.* (1997). The matrix and the leaf sugar extract (diluted 1: 5) was serially applied to the metallic sample carrier by applying volumes of 1 or 2 µl and immediately drying with a warm air current. The equipment was previously calibrated with a variety of standards such as maltodextrins. The laser used operated at 74% of its maximum capacity (8 J) with a pulse width of 2.5 ns. The

spectrum obtained was the result of the accumulation of 25 single spectra independently obtained.

Qualitative analysis by HPAEC-PAD and comparison of agave leaf fructan with others plant fructans of the inulin, neoseries and branched type

Agave leaf fructans were compared with fructans from a variety of different plant sources. Chromatograms of fructans extracted from agave *piña*, chicory roots, onion and barley basal stem were obtained using by HPAEC-PAD as previously described above. The agave *piña* fructans sample was a generous donation from the company Agaviótica S.A. de C.V (Guadalajara, Mexico). Chicory inulin was bought from Sigma Aldrich (UK). Onion fructans were obtained as described by Vijn *et al.* (1998). Barley fructans were extracted by grinding 100 mg of three week-old plants tissue in the presence of 1 ml of distilled water in an Eppendorf grinder and centrifuged at 15,000 × *g* for 5 min, supernatant was kept and analysed. Peak identification was made by running standards (Glu, Fru, Suc, Kest, Nystose, Bifurcose and Neokestose) in the chromatographic equipment and/or by analysing mixtures of fructan samples analysed. Published patterns were also used to corroborate identification. The peak identification tags were adapted as proposed by Shiomi (1991, 1993, 1997) and Ernst *et al.* (1998) and the nomenclature as proposed by Waterhouse and Chatterton (1993).

Enzyme activity extractions and assays

To elucidate the enzymatic machinery behind agave leaf fructans, enzyme activities in desalted protein extracts were investigated. Samples taken from the middle part of the leaf were obtained at the end of the day, when fructan concentrations are expected to be higher. Invertase, 1-SST, 1-FFT, 6G-FFT, KH and FEH activities were quantified as explained below. Approximately 20 mg of freeze-dried material was extracted with 250 µl of ice-cold 50 mM citrate (Na+) buffer (pH 5.8) by mechanical homogenization in a microfuge tube. The tubes were centrifuged for 10 min at 13,000 × *g* and an aliquot of 70 µl of supernatant was desalted through a micro-column (MoBiTec, Göttingen, Germany) filled with 1 cm^3 of Bio-Gel P-6 (Bio-Rad Laboratories, CA, USA) using centrifugal force of 5,000 × *g* for 1 min at 4°C. The column size and the conditions used were optimized to remove all sugars in the same purification step (Nagaraj *et al.* 2004). The volume of the final purified protein was diluted with 20 µl of extra citrate buffer and an aliquot was stored for protein quantification by the Bradford assay (Sigma protein assays reagent).

The Invertase and 1-SST assay mixture (final volume 20 µl) consisted of 18 µl of the enzyme extract and 2 µl of 1 M Suc (final concentration 100 mM) and was incubated at 27°C. The reaction was stopped after 4 h by heating for 1 min at 97°C and analyzed by HPAEC-PAD as described above. The accumulated production of 1-kestose was used as a measure of 1-SST activity. The accumulation of free fructose was used as a measurement for Invertase activity. The original extraction buffer (50 mM citrate, pH 5.8) serves to keep reaction conditions right.

For KEH and 1-FFT activities assays, 18 µl of the enzyme extract and 2 µl of 1 M kestose (final concentration 100 mM) were incubated together for 4 h at 27°C, and then heated for 1 min at 97°C. 1-FFT activity was calculated on the basis of nystose accumulation and KH activity was estimated by quantifying the final concentration of fructose. The original extraction buffer (50 mM citrate, pH 5.8) serves to keep reaction conditions right.

6G-FFT and 6-SFT were estimated in 16 µl of the extract which was mixed with 2 µl 1 M sucrose (final concentration 100 mM) and 2 µl of 1 M 1-kestose (final concentration 100 mM), incubated and stopped as previously described. 6G-FFT activity was measured as the formation of glucosyl β(2-6) linkage in the assay, as the sum of the formation of neokestose and DP4 neoseries FGFF. To quantify FGFF its chromatographic area per µmol of sugars was estimated by subtracting all other sugars from a sample where the total carbohydrate was known, except for FGFF. 6-SFT activity is measured by quantifying the amount of bifurcose formed during the incubation, however no bifurcose was formed.

For FEH activity assay, 18 µl of the enzyme extract and 2 µl of a 150 mg/ml high molecular weight agave fructans solution (>15 DP; final concentration 15 mg/ml) were incubated together for 4 h at 27°C and then heated for 1 min at 95°C. To estimate FEH the hydrolyzed fructose accumulation was quantified. The high molecular weight agave fructans substrate was prepared from commercially available food grade *A. tequilana* fructans generously donated by Mercantil Orgánica S.A. de C.V. (Guadalajara, Mexico) by ethanol precipitations (Ku *et al.* 2003). To ensure no small oligosaccharides were contaminating the substrate, the high DP fructans were precipitated 3 times and checked using HPEAC-PAD.

Statistical analysis

The concentrations of the different carbon pools contributing to the water-soluble carbohydrates in the agave leaf are expressed as the mean of nine analysis performed (see carbohydrate extraction for quantification) as mg of carbohydrate per g of tissue (fresh weight and dry weight). Enzymatic activities where calculated as the mean of 8 independent leaf samples analyzed. All leaf samples for enzyme activities were obtained at dusk (18 hrs). The variability between set of samples investigated is expressed as the Standard Error of the Mean (SEM).

RESULTS

Water-soluble carbohydrates and fructan content in the *Agave tequilana* leaf

In the leaf, variations in sugar pools can be significant, due to changes in the general physiological plant framework or produced by environmental conditions. To have a better estimate of the leaf's mean concentrations and variability of different WSC pools several leaves (n=9) at different times of the day (see materials and methods), were sampled. The data obtained, was averaged and variability analyzed (SEM is used a measure of variability). **Table 1** shows the carbohydrate concentrations of glucose, fructose, sucrose, total fructans and DP3 fructans such as kestose and neokestose. Values are presented on the basis of dry weight and fresh weight because of the high water content of the leaves.

Sucrose was the most abundant carbon pool representing 67.54% ± 9.6 of WSC. The total fructan pool corresponded to 20.19 ± 7.6% WSC, whilst the monosaccharides, glucose and fructose accounted for 11.78 ± 1.48% with glucose being 1.81 times higher than fructose. While monosaccharide concentrations were not very variable between samples, the sucrose and fructan pools were. Although fructan levels are variable in general, when sucrose concentration was high, the concentrations of fructans were also high. In a similar way, higher fructan concentrations presented higher DP. Of the total fructan pool, approximately 21.4 ± 6.17% correspond to trisaccharides (kestose and neokestose) while the rest correspond to higher DP fructans up to

Table 1 Water-soluble carbohydrate (WSC) content in *Agave tequilana* leaves. Values are calculations of measurements taken in samples obtained at random times during the day from 3.5 years-old plants growing at greenhouse conditions. Mean ± SEM are shown. n=9.

Carbohydrates	Concentration[a] (mg g FW^{-1})	Concentration[b] (mg g DW^{-1})
Simple carbohydrates		
Glucose	4.10 ± 0.68	15.23 ± 2.54
Fructose	2.26 ± 0.44	8.38 ± 1.63
Sucrose	36.07 ± 5.16	133.93 ± 19.15
Fructans		
Kestose	1.07 ± 0.27	3.98 ± 0.99
Neokestose	1.18 ± 0.41	4.38 ± 1.63
Higher DP4~DP12	8.40 ± 4.12	31.19 ± 15.28
Total fructans	10.65 ± 3.75	39.54 ± 13.91
Total WSC	80.67 ± 15.33	299.56 ± 56.92

[a] Expressed as a function of fresh weight tissue
[b] Expressed as a function of dry weight tissue

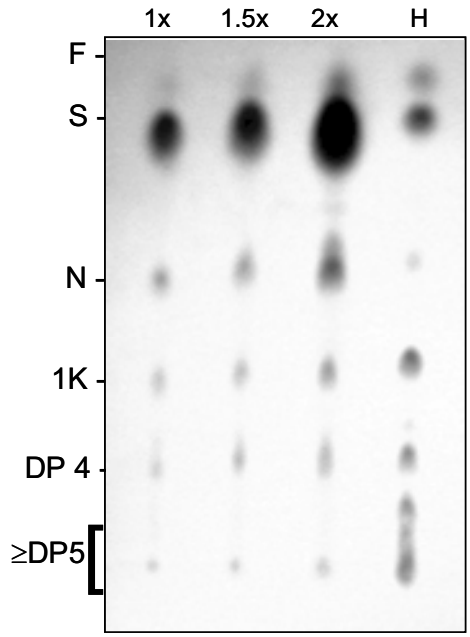


Fig. 3 Analysis of water-soluble carbohydrates (WSC) extracted from *Agave tequilana* leaves by thin-layer chromatography. The same sample was analyzed at 1, 1.5 and 2 times the original extract concentration and was compared with inulin; (H) used as standard to determine degree of polymerization. Markers represent the mobilities of fructose (F), sucrose (S), neokestose (N), 1-kestose (1K), inulin tertasacharide (DP4), pentasaccharide (DP5) and >DP5.

12. Kestose and neokestose were found in almost the same concentrations, but neokestose was more variable between samples. Average total WSC was 80.67 ± 15.33 mg per g FW^{-1} or 299.56 ± 56.92 mg per g DW^{-1} in the leaves sampled. Water content was very high accounting for 78.55 ± 2.43%.

Chromatographic analysis of *Agave tequilana* leaf fructans by TLC and MALDI-TOF

Water-soluble carbohydrates (WSC) from leaves collected at dusk were extracted for analysis by TLC and MALDI-TOF. The sampling time was selected at dusk because at this time maximal fructan concentrations are expected to occur in the leaf. **Fig. 3** presents the TLC spot pattern of the agave leaf extract confirming the presence of fructans. The extract was applied at three different concentrations to allow a better detection of spots in the upper and lower range of detection and separation. Linear inulin from Jerusalem artichoke was used as a standard. While the lines with 1 and 1.5X concentration shows well-differentiated spots of sucrose and fructose in the sample, oligosaccharides spots are difficult to visualize. In the line with 2X, neokestose and kestose (structures showed in **Fig. 3.1A** and **B**, respectively) were successfully separated; the presence of DP4 and DP5 oligosaccharides was confirmed. Small amounts of ≥DP5 were also present in the sample and no 6-kestose (see **Fig. 1C**) was detected. In **Fig. 4** a second chromatographic analysis performed by MALDI-TOF-MS also showed a similar picture. The integrated detection signal is very strong for sucrose and decreases as oligofructans increase. Both chromatographic methods confirm the presence of fructans higher than >DP5 in leaves of *A. tequilana* plants.

Fructan patterns from *A. tequilana* leaf compared with fructan from Agave *piña* origin, onion, barley and chicory

The agave leaf extract was analyzed by a third method: HPEAC-PAD. The oligosaccharide present in the sample was identified and compared with those present in the *piña* and in other plants. Chromatograms from WSC extracted from agave leaf, agave *piña*, onion, barley and chicory are presented in **Fig. 3A, B, C, D** and **E**, respectively. Four structural types of fructans are compared: inulin (chicory), inulin neoseries (onion), branched inulin-levan (barley) and branched inulin-levan neoseries (agave *piña*). Only the levan (i.e. *Poa ampla*)-type of fructans was not analyzed in this report.

The agave leaf fructans presented a chromatogram with a DP of approximately DP3 to 12 (**Fig. 5A**). Smaller oligosaccharides seem to be better separated than larger ones. However DP3 (1-kestose and neokestose) and DP4 (nystose, 1&6^G-kestotetraose and 1,6G-kestotetraose) oligosaccharides are fully and clearly resolved in independent peaks that show a correct differentiation between isomers. DP5 fructans and other larger ones cannot be resolved completely; nevertheless certain co-elutions were established based on the other species chromatograms. No 6-kestose or bifurcose was detected in the agave leaf extracts.

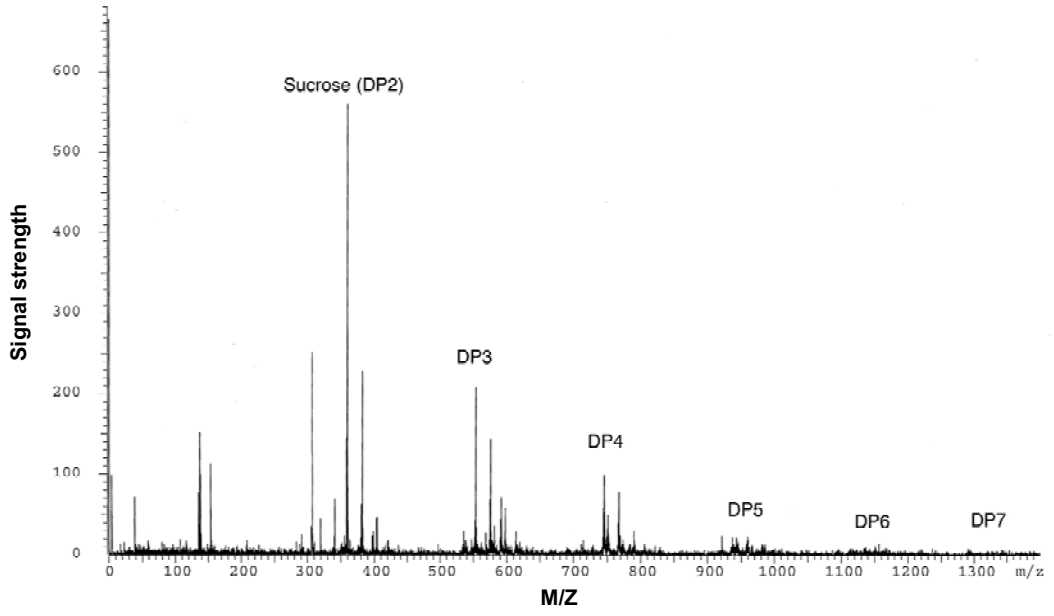


Fig. 4 Analysis of water-soluble carbohydrates (WSC) extracted from Agave tequilana leaves by matrix-assisted laser desorption time-of-flight mass spectrometry (MALDI-TOF-MS). The different degree of polymerization are indicated, the M/Z values correspond to the molecular weight of the signal detected.

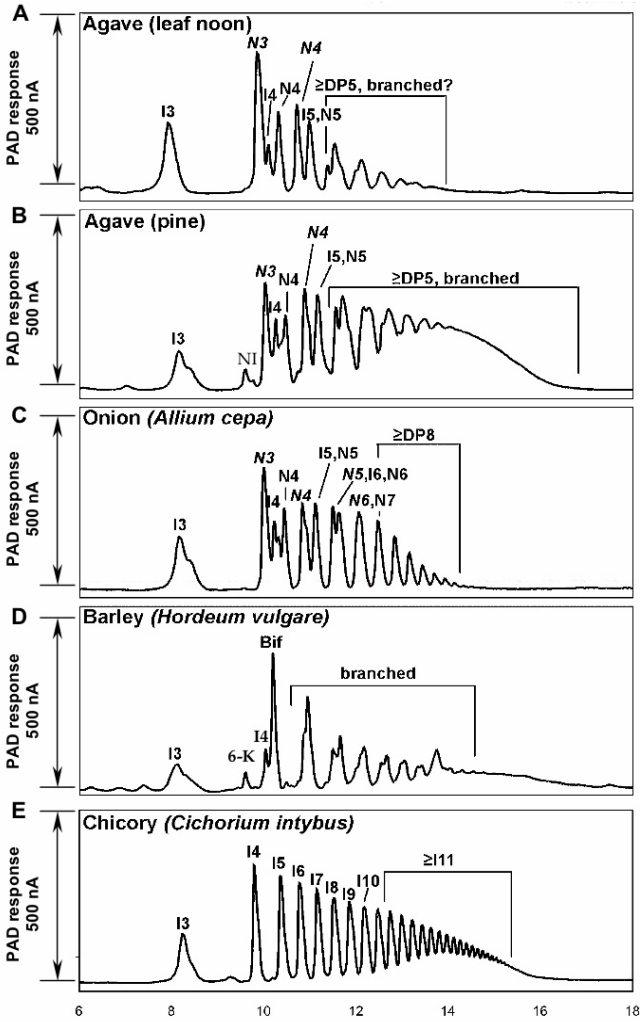


Fig. 5 High-performance anion exchange chromatography with pulsed amperometric detection of fructan extracted from different plants. (**A**) *A. tequilana* leaf (at noon), (**B**) *A. tequilana* pine, (**C**) onion bulb, (**D**) barley base leaf, and (**E**) chicory root. Peak annotation adapted from Ernst *et al*. (1998): I3, 1, kestotriose (1-kestose); *N3*, 6G-kestotriose (Neokestose); I4, 1,1-kestotetraose (Nystose); *N4*, 1,6G-kestotetraose; N4, 1&6^{G}-kestotetraose; I5, 1,1,1-kestopentaose; *N5*, 1,1,6G-kestopentaose; N5, mixture of DP5 fructans elongated on both sides of the sucrose; I6, 1,1,1,1-kestohexaose; N6, mixture of DP6 fructans with chain elongation on both sites of the sucrose; *N6*, 1,1,1,6G-kestohexaose; N7, mixture of neo-series inulin DP7; 6-K, 6-kestotriose; 6-K, 6-Kestose; Bif, 1&6-kestotetraose (Bifurcose). In agave pine fructan chromatogram we detected a NI, not identified peak. Although the elution point of NI is very similar to 6-K they are different compound, as we found by coelunting mixed samples of fructan (data not shown).

Agave *piña* fructans have been reported to be highly branched in structure like the inulin-levan neoseries type fructan (López *et al.* 2003). All oligosaccharides from DP3 and DP4 were coincident with those found in the leaf with the exception of a peak detected just before neokestose (*N3*, in **Fig. 1B**) named NI (not identified) in **Fig. 5B**. This NI peak was carefully analyzed to establish if it was 6-kestose or not, as the elution time was very similar to this component (see Bif in **Fig. 5D**). By analyzing chromatograms of mixtures of agave *piña* fructan + 6-K standard and agave *piña* + barley fructan it was finally established that this peak does not correspond to 6-kestose as they elute as two clearly differentiated peaks (data not shown). The DP5 oligosaccharides 1,1,1-kestopentaose (I5) of the inulin type and those of the neoseries type (N5) seem also to be present in both, leaf and *piña*, however for other DP5 fructans and larger ones this is impossible to establish because separation becomes unclear and different components elute together. Fructans DPs go up to a maximal ~DP35, based on the DP and elution times detected for the chicory inulin (see **Fig. 5E**). Larger fructans are present in the agave *piña* in considerable amounts.

Onion inulin and neoseries oligosaccharides reach an approximate maximal DP of 13 (see **Fig. 5C**). Interestingly all the onion oligosaccharides that go up to DP5 (I3, *N3*, I4, N4, *N4*, I5, N5) are also present in agave *piña* and agave leaf fructans. Onion fructans can contain internal glucose residues (all neoseries) having two elongation points (see **Fig. 2B**). In the chromatogram elution peaks corresponding to onion fructans from DP6 to DP13 present significant differences from those found in the agave leaf.

In barley, only branched inulin-levan type fructans are present (Roth *et al.* 1997; see **Fig. 5D** for chromatogram). It contains 6-ketose (6-K) and bifurcose (Bif) oligosaccharides (see **Fig. 1C** and **Fig. 2D**, respectively for structures). Nystose (I4) is also present in small amounts. All other fructans with DP5 to ~DP11 present complex thick peaks that do not differentiate between oligasaccharides. The shapes of these peaks have similar properties to the ones detected in agave leaf, such as their irregularity and thickness, what may be a signal of co-elution.

Finally, chicory fructans were analyzed. Peaks represent a single linear inulin-type DP. All 1-kestose (I3), nystose (I4) and 1,1,1-kestopentaose (I5) were identified in the agave leaf. The chromatogram presents a clear DP pattern up to an approximate DP.

Characterization of enzymatic synthetic and degradative activities in protein extracts from *A. tequilana* leaf

To understand the enzymatic machinery involved in the agave fructan metabolism of the leaf, desalted protein extracts were assayed to identify the major fructans synthetic and degrading enzymes activity present. The *in vitro* assays need to be considered with caution as *in vivo* conditions such as enzyme-substrate concentrations relationships, pH, and compartmentalization are destroyed by extraction. In **Table 2** all different activities found are listed with the average activities detected at dusk, when sugar concentrations are expected to be the highest. Different FTs such as 1-SST, 1-FFT and 6G-FFT were detected, all of them being necessary enzymes for the synthesis of inulin- and neoseries-type fructans. Nevertheless, the enzyme activity traditionally responsible for pattern via β(2-6) linkage type (levan structure), the 6-SFT was not detected in any of the leaves sampled. With the exception of a DP5 fructan that could not be identified, all products generated during the enzyme assays were confirmed to be only of the inulin and neoseries types (**Fig. 6**). Fructan exohydrolase activity was investigated in two substrates: 1-kestose and high molecular weight agave fructans and both KH and FEH activities were detected. Finally, acid invertase was also detected.

All sucrose-utilizing enzymes were investigated together by analyzing their products in chromatograms obtained

Table 2 Enzyme activities detected in Agave tequilana leaves. Enzyme activities were obtained from desalted protein extracts obtained from leaves sampled at dusk (18:00 hrs). Mean ± SEM are presented in the table. n=8.

	Enzyme activity[a] (nmol g FW^{-1} min^{-1})	Enzyme activity[b] (nmol g DW^{-1} min^{-1})
Degrading enzymes		
Acid Invertase	20.71 ± 1.80	76.9 ± 6.68
KH	5.33 ± 0.35	19.8 ± 1.31
FEH	1.57 ± 0.16	5.8 ± 0.59
Synthesis enzymes		
1-SST	6.29 ± 0.90	23.3 ± 3.34
1-FFT	5.37 ± 0.41	19.9 ± 1.51
6G-FFT	1.31 ± 0.31	4.9 ± 1.14
6-SFT	undetected	undetected

[a] Expressed as a function of fresh weight. Protein content: 9.79 ± 0.86 μg g FW^{-1}
[b] Expressed as a function of dry weight. Protein content: 36.3 ± 3.20 μg g DW^{-1}

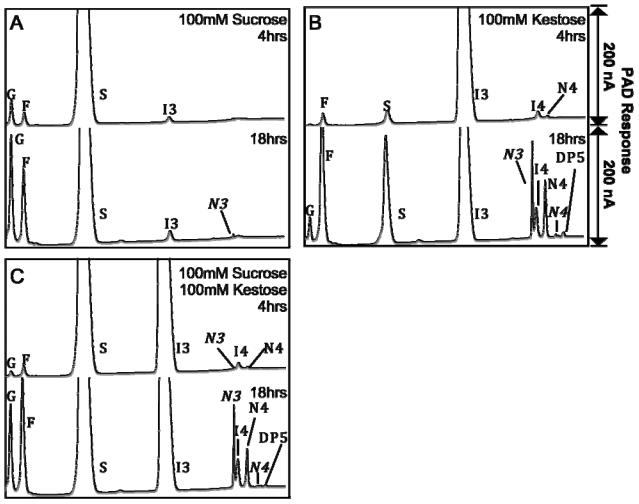


Fig. 6 High-performance anion exchange chromatography with pulsed amperometric detection of *in vitro* synthesized fructans by desalted protein extracts of *Agave tequilana* leaves. Incubation during 4 and 18 hrs with (**A**) sucrose, (**B**) 1-kestose, and (**C**) sucrose + kestose are presented. Peak annotation adapted from Ernst *et al.* 1998: G, glucose; F, fructose; S, sucrose; I3, 1, kestotriose (1-kestose); *N3*, 6G-kestotriose (neokestose); I4, 1,1-kestotetraose (nystose); *N4*, 1,6G-kestotetraose; N4, 1&6^{G}-kestotetraose and a DP5 of unknown identity.

at 4 and 18 hrs of reaction (see **Fig. 6A**). In the reaction, sucrose can be hydrolyzed via acid invertase or used for the synthesis of 1-kestose by 1-SST. Invertase activity showed to be 3.29 times stronger than 1-SST activity. As the reaction time increases, the reaction products in the media also increase (in this case 1-kestose) becoming available to other enzymes activities present in the crude extract assayed. As we can see at 18 hrs of reaction time, when very high concentrations of sucrose are still present and a relatively small amount of 1-kestose can be found, a new product of a secondary enzyme can be seen: neokestose (*N3*).

To evaluate 1-FFT and FH the desalted protein extract was incubated in 1-kestose (see **Fig. 6B**). Fructan synthesis activity via 1-FFT was measured as the rate of synthesis of nystose (I4) in the reaction. Degradation of 1-kestose via KH was established by determining the fructose release in the reaction. Both activities showed to have similar strength *in vitro* (see **Table 2**). Also, 6G-FFT activity using 1-kestose as both donor and acceptor was detected at 4 hrs incubation time as 1&6^{G}-kestotetraose (N4) was synthesized. Later on, after 18 hrs of incubation, important concentrations of neokestose (*N3*) were detected, most probably synthesized by the utilization of new available sucrose as acceptor and the donation of fructosyl units from 1-kestose. Similarly, small amounts of 1,6^{G}-kestotetraose (*N4*) were detected, which implies that 1-FFT activity was elongating the glucose chain of 1&6^{G}-kestotetraose (N4) previously synthesized. Neoseries fructans were mostly represented after 18 hrs, with some nystose (I4) and a DP5 fructan that could not be identified.

To establish the existence of 6G-FFT and 6-SFT activities in the leaf, incubations with 1-kestose and sucrose were performed (see **Fig. 6C**). No 6-SFT activity was detected as no bifurcose (Bif, 1&6-Kestotetraose) or 6-kestose (6-K) were found at any time of the incubation. Nevertheless, the formation of neoseries fructans such as neokestose was clearly demonstrated. A very similar oligosaccharide pattern to the one observed in incubation with kestose alone was observed, however it seems that overall concentrations of fructans were higher in kestose incubation than in kestose + sucrose incubation after 18 hrs.

Finally, FEH activity was analyzed in purified high DP agave fructan isolated by ethanol precipitation (see materials and methods; chromatograph is not shown). FEH activity in the crude extract released 3.39 times less fructose molecules per unit of time than the KH previously described (see **Table 2**). Interestingly the hydrolase activity on both substrates kept this proportionality among all individual samples analyzed.

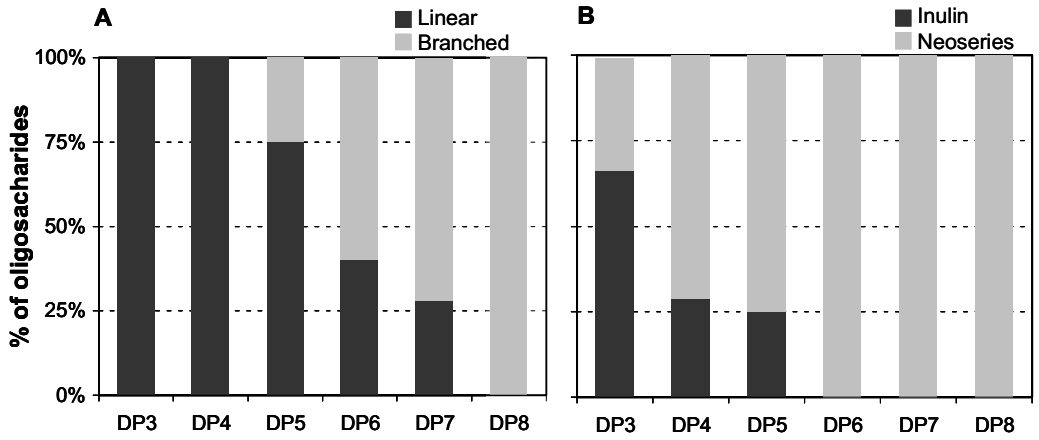
DISCUSSION

Branched fructans of the inulin-neoseries type are common in the Asparangales order (Sims 2003) where *A. tequilana* is grouped. Analysis of sugars showed that sucrose is the most abundant sugar in the leaves of *A. tequilana* at all times accounting for 65.7%, followed by fructans with 21.5% and monosaccharides with 12.9% (see **Table 1**). Fructan content was variable between samples analyzed. In *A. americana* plant growing in similar conditions, sucrose accounted for the highest WSC measured, representing 75.9% of the carbohydrates in the extract, followed by glucose with 18.8% and fructose with 5.25% (calculated from Raveh *et al.* 1998). Unfortunately no fructan measurements were performed in this study. In *A. deserti* tissue specific analysis showed that on the chlorenchyma, the most abundant in the leaf sections sampled, sucrose accounted for 66% of WSC, followed by glucose and fructose representing 32% of WSC. A small amount of oligofructans were detected in this tissue, only accounting for 2%. In the vascular tissue however, sucrose had lower relative concentrations and fructan concentrations were as high as 44% (Wang and Nobel 1998). Sucrose has been shown to serve as the main source for the regeneration of phosphoenolpyruvate in Crassulacean Acid Metabolism (CAM) in agaves (Christopher and Holtum 1996; Raveh *et al.* 1998; Wang and Nobel 1998). This molecule serves as an acceptor during nocturnal fixation in this plants (see Dodd *et al.* 2002 for a review).

Chromatographic techniques provided different information on WSC extracted from *A. tequilana* leaf. Fructan content in this plant's leaf was confirmed by MALDI and TLC. While TLC has been traditionally used for characterization of fructans in many species with different types of fructans (Cairns and Ashton 1993; Bonnett *et al.* 1997; Sims 2003), MALDI-TOF technology has not been extensively used to study fructan polysaccharides until recently. MALDI fructan characterization of onion, garlic and chicory has been previously reported (Losso and Nakai 1997; Stahl *et al.* 1997) as well as for *A. tequilana piña* fructans (López *et al.* 2003), showing that it can be a suitable technique to elucidate qualitatively the different DPs present in a mixed fructan pool. Nevertheless, this technology has limitations. MALDI cannot distinguish between fructans of the same DP and normally it does not provide quantitative information. Also, MALDI signal strength can become weaker in the presence of certain ions and salts (Stahl *et al.* 1997) which can reduce the accuracy and strength of the DP pattern detected. TLC has advantages such as resolution, which is mostly limited to DP3 oligosaccharides. Using HPAEC-PAD analysis it is possible to resolve agave oligofructans in a clearer way. This analysis showed that leaf and *piña* fructan patterns were similar but differences in DP were clear.

While agave *piña*'s fructans presented a maximal DP of around 30 units, based on inulin elution, the agave leaf's fructans presented a maximal length of only up to DP12. HPAEC-PAD was more sensitive to higher DP/lower concentration oligofructans than MALDI, as DP6, DP7 and larger fructans were almost unresolved by this technique. Fructan oligosaccharide patterns obtained from chicory, onion and barley fructans by HPAEC-PAD where used as comparative examples of inulin, inulin neoseries and branched inulin-levan fructans, respectively. The patterns achieved corroborated those previously reported in the literature (Roth *et al.* 1997; Sprenger *et al.* 1997; Ernst *et al.* 1998; Ritsema *et al.* 2003). None of the fructan types analyzed showed complete similarity with leaf or *piña* agave.

The comparison of chromatographic peaks up to DP4 obtained from the agave leaf extract were equivalent to onion, which demonstrates that agave DP3 and DP4



C

Size	Linear: Inulin	Linear: Neoseries	Branched (all types)	Method of detection
DP3	I3	N3	no detected (6-K)	TLC, HPAEC
DP4	I4	*N4,* N4	no detected (Bif)	HPAEC-PAD
DP5	I5	N5, *N5*	Detected *	HPAEC-PAD
≥ DP6	Uresolved peaks		Detected *	HPAEC-PAD

* Coelution in large chromatographic peaks not corresponding to inulin/neoseries

Fig. 7 Oigosaccharides presenting different structures in specific DP pools of fructans isolated from *Agave tequilana* and *Agave veracruz*. Information presented here was integrated from Dorland *et al.* (1977a, 1977b) and from this study. The figure shows the abundance of (**A**) linear or branched structure; and (**B**) inulin or neoseries composition of the oligosaccharides separated by specific DP pools in *A. veracruz*. The table (**C**) present results on oligosaccharides detected and/or quantified in this study. **A** and **B** were calculated as relative percentage values based on linkage quantification made by Dorland *et al.* (1977a, 1977b).

oligosaccharides are of the neoseries and inulin type. Oligosaccharides of DP≥5 showed more complex and less defined peaks in HPAEC-PAD, not following the onion pattern. These peak irregularities are typical for branched structures such as the ones found in barley indicating an incremental branched pattern in larger oligofructans (**Fig. 6A** and **6D**).

Agave fructans have been reported as highly branched structures (Dorland *et al.* 1977; López *et al.* 2003; Mancilla-Margalli and Lopez 2006). In this study no 6-kestose or bifurcose was identified (structures in **Fig. 1C** and **2C**), typical products of the 6-SFT enzyme considered responsible of the branching activity in some monocot plants such as barley (Sprenger *et al.* 1997).

Dorland *et al.* (1977) analyzed the linkage present in specific DP fractions pools of naturally occurring fructans from *A. veracruz piña*. Using paper and thin-layer chromatography they were able to successfully separate and purify DP3 to DP15 fructans. Methylation analysis of linkages occurring in each DP fraction was reported. This data was quantitatively analyzed to establish the amount of oligofructan molecules at each DP fraction belonging to different fructan types. **Fig. 7** integrates these calculations with those found in this study. This shows how structures and fructan types become dominant as DP increases. While DP3 and DP4 are formed by linear molecules only, branching structures become more and more abundant from DP5 (25%) to DP7 (73%) to DP8 when all oligofructans present have at least 1 branching point in their structure (**Fig. 7A**). Similarly, inulin oligosaccharides become less common as neoseries incremented (**Fig. 7B**). From DP6 upward all fructan molecules found presented an internal glucose residue. DP3 to DP5 inulin molecules seems to be efficiently utilized as fructosyl acceptors by 6G-FFT. These findings maintain coherence with data presented here on *A. tequilana* fructans (**Fig. 7C**). Firstly, no 6-kestose or bifurcose was detected in *A. vercruz* as in the leaf and *piña* extracts analyzed in this report. Secondly, the branched molecules found in *A. veracruz* from DP5 upwards correspond well with the irregular peak detection we obtained by HPAEC-PAD. It is highly possible that ≥DP5 *A. tequilana* and other members of this genus exhibit similar structures.

There are other examples in the order Asparangales that resemble agave fructans, formed by β(2-1) fructosyl linkage but with an strong branching β(2-6) linkages (Sims *et al.* 2001; Sims 2003). *Phormium tenax* and *Phormium cookienum*, as well as *Cordyline australis* and *Urginea maritima* have shown to have inulin and neoseries oligofructans up to DP4 and then to present branching linkages in other larger fructans (Brasch *et al.* 1988; Spies *et al.* 1992; Sims *et al.* 2001; Sims 2003). Unfortunately no enzymatic studies investigating their synthetic pathways have been reported in these species.

All FTs activities responsible for the synthesis of inulin and neoseries fructans were detected in protein leaf extracts and the *in vitro* synthesized oligofructan patterns showed all DP3 and DP4 molecules in similar proportions as found *in vivo* including a neokestose derived DP5. 1-SST, 1-FFT and 6G-FFT activities were detected but no 6-SFT activity was detected. This evidence, together with the absence of 6-kestose and bifurcose in naturally occurring fructan, strengthen the idea that this enzyme may not be present in these plants or that its function may be modified. Invertase activity was found to be strong in agave leaf extracts, which could have a physiological explanation as at dusk, when leaf tissue was sampled, CAM metabolism demands a high amount of carbon structures derived from the sucrose pool (Wang and Nobel 1998). Enzyme FTs assays performed in *A. veracruz piña* tissue do not exhibit hydrolase activity (Satyanarayana 1976; Dorland *et al.* 1977), even when high concentrations of sucrose were used in the assay (more than 1 M). Nevertheless, the protein extract assayed was capable of synthesizing *de novo* fructans with maximal DP6, all of them of the inulin or neoseries types (Dorland *et al.* 1977) with no branched pattern detected. *In vitro* patterns in *A. tequilana* seems to be the same than *A. veracruz* products with a maximal DP5 and not being able to synthesize branched fructans. KH and FEH hydrolytic activities present important differences in leaf protein extracts, this may be explain by the affinity of the exohydrolases to substrates (1-kestose

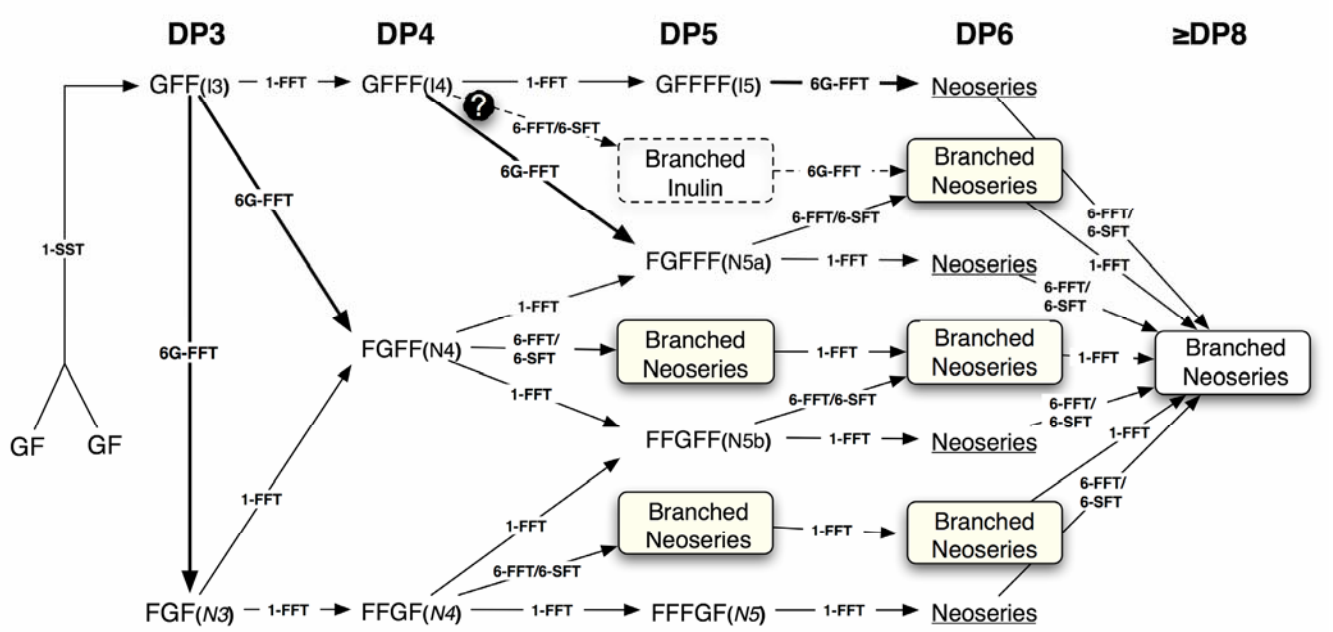


Fig. 8 Schematic proposed model of fructan syntetic pathway for *Agave* species. Arrows represent a fructosyltransferse event and are named by the fructosytranferse involved (1-SST, 1-FFT, 6G-FFT, 6-SFT and/or 6-FFT). Linear DP3, DP4 and DP5 are represented as proposed by Ernst *et al.* (1998). GF, sucrose; GFF (I3), 1-kestose; FGF (*N3*), 6G-kestotriose; GFFF (I4), 1,1-kestotetraose; FFGF (*N4*), 1,6G-kestotertaose; FGFF (N4), 1&6G-kestotetraose; GFFFF (I5), 1,1,1-kestopentaose; FFFGF (*N5*), 1,1,6G-kestopentaose; FGFFF (N5a), 1,1 and 6G-kestopentaose; FFGFF (N5b), 1 and 1,6G-kestopentaose. Branched fructans and linear DP6 neoseries specific names are not presented. Note that all DP6 fructans are either neoseries or branched neoseries, but not inulin is present. All ≥DP8 fructans have at least one branching point. The utilization of GFFF(I4) for the generation of branched inulin is unclear (see ? symbol and discontinues lines) as all other reactions are based on neoseries fructans.

and High DP *Agave* fructans).

Agave species are not the only plant species where branched fructans without the presence of measurable 6-SFT activity and/or the absence of their adjudicated initial products (6-kestose and/or bifurcose) has been reported. In *Lolium* species, where bifurcose does not naturally occur (although 6-kestose does) it is unclear how β(2-6) linkages are synthesized (Pavis *et al.* 2001b) as this activity has not been detected in protein extracts (Pavis *et al.* 2001b), as it has in *A. tequilana* and *A. veracruz*. The authors suggest that the branching activity may not be a product of 6-SFT, but of the hypothetically proposed 6-FFT. They also speculate on the existence of a different form of 6-SFT which does not have the ability of using sucrose or DP3 fructans as fructosyl acceptors, probably due to low affinity to these substrates, but have the capacity of utilizing larger ones. Fructan products with a maximal DP6 have been obtained from *Lolium rigidum* protein extracts incubations with a variety of substrates such as 1-kestose, neokestose and 1,1,1- kestopentaose (St. John *et al.* 1997). All products did not have a branched structure after linkage analysis; in contrast to naturally occurring oligosaccharides where branches are detected in ≥DP5. Despite the unsuccessful utilization of any of these substrates the possibility that an existing 6-SFT can perform a branching reaction β(2-6) on larger fructans but not on smaller ones is still open Considering the high proportion of DP4 and DP5 fructan of the neoseries type over inulin. found in *A. veracruz,* it may be possible that a 6-SFT activity may prefer this type of acceptors than those of inulin type.

FT *in vitro* assays must always be interpreted with caution because activities are not necessarily related to a single protein (more than one 1-SST in barley, Lüscher *et al.* 2000a) and a single protein is not necessarily responsible for one single activity (*i.e.* Onion 6G-FFT enzyme shows 1-FFT activity; Ritsema *et al.* 2003; Fujishima *et al.* 2005) as has been extensively documented in purified and recombinant proteins (Sprenger *et al.* 1995; van den Ende and van Laere 1996; van den Ende *et al.* 1996; Hochstrasser *et al.* 1998; Vijn *et al.* 1998; Lüscher *et al.* 2000b; Chalmers *et al.* 2003). For this reason activities in this study are conceptualized as the plant capability to perform certain FTs reactions, and not as specific proteins acting alone. *In vivo* physiological context is destroyed during the extraction process, meaning that the results can only represent tendencies based in the measurable potential of protein extracts to achieve certain reactions. The physiological significance of *in-vitro* FT assays has also been questioned as ineludible evidence showing invertases ability to synthesize fructans in high concentrations of sucrose has been well documented (Cairns and Ashton 1991).

Based on what has been discussed here, a general hypothetical model is proposed for the synthesis of fructans in *Agave* spp. (see **Fig. 8**). This model may also describe what could be occurring in other members of Asparagales presenting similar structural characteristics and enzymatic β(2-6) branching dilemmas. We propose 1-SST to be responsible of the synthesis of 1-kestose, from which neoseries and inulin fructans are initially elongated. Inulin oligosaccharides tend to disappear as DPs becomes larger (see also **Fig. 7A**), due to the action of 6G-FFT (bold arrows), while 1-FFT activity increases the size of fructosyl chains at the elongation points available on both sides of the internal glucose residue and on introduced branches.

Although β(2-6) linked fructosyl units of the levan type with no branching have also been detected in agave (Dorland *et al.* 1977; López *et al.* 2003), their relative amount is small (6% of all fructosyl units), which is half of the branching fructosyl units detected in *A. veracruz* fructans (Sims *et al.* 2003) based on Dorland *et al.* (1977) what suggests that continous chains of levan are very improbable. The activity of 1-SST, 1-FFT and 6G-FFT has been corroborated, however it is not clear if the β(2-6) activity corresponds to a 6-SFT insensitive to DP3 and sucrose molecules or to another hypothetical 6-FFT, as suggested in *Lolium* spp. (Pavis *et al.* 2001a, 2001b). The enzymatic mechanism that produced β(2-6) needs further investigation.

All DP3 and DP4 oligofructans presented in this model have been identified in naturally occurring and *in vitro* synthesized fructans. Linear DP5 oligosaccharides have been identified *in vivo* and DP5 products presumably of neoseries unbranched type were also synthesized. Additional information based on previous reports show identical results in *A. veracruz* than the ones obtained in *A. tequilana* and

provide data that establishes the structure presents in oligosaccharides *in vivo*. The model proposes that the ability to produce branched fructans using inulin DP4 inulin fructans is a possibility but it is not confirmed (see interrogation symbol in **Fig. 8**). Nevertheless, the data available clearly demonstrates that the branching activity must have the ability to use ≥DP4 neoseries fructans as acceptors branching fructosyl units, as from DP6 to DP8 implies the activity in fructan pool that corresponds to neoseries only. Furthermore, the relative concentrations of branched fructans found in DP5 and DP6 fructan pools in *A. veracruz* (25 and 60%, respectively) could be produced from neoseries acceptors only, as the DP4 and DP5 concentrations of this fructan type is sufficiently abundant (70 and 75% of neokestose, respectively). Despite its limitations, this model may serve as a theoretical framework to understand agave fructan synthesis.

ACKNOWLEDGEMENTS

This work was supported by a PhD CONACyT scholarship (num. 161764) granted to ISO. We recognize the generous financial support provided by Pernod Ricard Mexico/Casa Pedro Domecq for covering various traveling expenses. We are also thankful to Prof. Andres Wiemken for allowing us to perform several experimental activities within his lab at the University of Basel.

REFERENCES

Amiard W, Morvan-Bertrand A, Cliquet JB, **Billard JP, Huault C, Sandstrom JP, Prud'homme MP** (2004) Carbohydrate and amino acid composition in phloem sap of *Lolium perenne* L before and after defoliation. *Canadian Journal of Botany* **82**, 1594-1601

Arizaga S, Ezcurra E (1995) Insurance against reproductive failure in a semelparous plant – bulbil formation in *Agave macroacantha* flowering stalks. *Oecologia* **101**, 329-334

Bonnett GD, Sims IM, Simpson RJ, Cairns AJ (1997) Structural diversity of fructan in relation to the taxonomy of the *Poaceae*. *New Phytologist* **136**, 11-17

Brasch DJ, Fankhauser BL, McDonald AG (1988) A study of the glucofructofuranan from the New Zealand cabbage tree *Cordyline australis*. *Carbohydrate Research* **180**, 315

Cairns AJ, Ashton JE (1991) The interpretation of *in vitro* measurements of fructosyl transferase activity – an analysis of patterns of fructosyl transfer by fungal invertase. *New Phytologist* **118**, 23-34

Cairns AJ, Ashton JE (1993) Species-dependent patterns of fructan synthesis by enzymes from excised leaves of oat, wheat, barley and timothy. *New Phytologist* **124**, 381-388

Cairns AJ, Bonnett GD, Gallagher JA, Simpson RJ, Pollock CJ (1997) Fructan biosynthesis in excised leaves of *Lolium temulentum*. *New Phytologist* **136**, 61-72

Chalmers J, Johnson X, Lidgett A, Spangenberg G (2003) Isolation and characterisation of a sucrose, sucrose 1-fructosyltransferase gene from perennial ryegrass (*Lolium perenne*). *Journal of Plant Physiology* **160**, 1385-1391

Chatterton NJ, Harrison PA, Thornley WR, Bennett JH (1993a) Structure of fructan oligomers in cheatgrass (*Bromus tectorum* L). *New Phytologist* **124**, 389-396

Chatterton NJ, Harrison PA, Thornley WR, Bennett JH (1993b) Structures of fructan oligomers in orchardgrass (*Dactylis glomerata* L). *Journal of Plant Physiology* **142**, 552-556

Chatterton NJ, Harrison PA, Thornley WR, Draper EA (1990) Oligosaccharides in foliage of *Agropyron*, *Bromus*, *Dactylis*, *Festuca*, *Lolium* and *Phleum*. *New Phytologist* **114**, 167-171

Chatterton NJ, Harrison PA (1997) Fructan oligomers in *Poa ampla*. *New Phytologist* **136**, 3-10

Christopher JT, Holtum JAM (1996) Patterns of carbon partitioning in leaves of Crassulacean acid metabolism species during deacidification. *Plant Physiology* **112**, 393-399

Clark GT, Zuther E, Outred HA, McManus MT, Heyer AG (2004) Tissue-specific changes in remobilisation of fructan in the xerophytic tussock species *Festuca novae-zelandiae* in response to a water deficit. *Functional Plant Biology* **31**, 377-389

Colúnga GM, Maypat F (1993) Agave studies in Yucatan, México. 1 Past and present germplasm diversity and uses. *Economic Botany* **47**, 312-327

Demeulemeester MAC, Verdoodt V, De Proft MP (1998) Interaction between physiological age and cold treatment on the composition and concentration of carbohydrates in chicory roots (*Cichorium intybus* L.). *Journal of Plant Physiology* **153**, 437-445

Dodd AN, Borland AM, Haslam RP, Griffiths H, Maxwell K (2002) Crassulacean acid metabolism, plastic, fantastic. *Journal of Experimental Botany* **53**, 569-580

Dorland L, Kamerling JP, Vliegenthart JFG, Satyanarayana MN (1977) Oligosaccharides isolated from *Agave vera cruz*. *Carbohydrate Research* **54**, 275-284

Duchateau N, Bortlik K, Simmen U, Wiemken A, Bancal P (1995) Sucrose-fructan 6-fructosyltransferase, a key enzyme for diverting carbon from sucrose to fructan in barley leaves. *Plant Physiology* **107**, 1249-1255

Ernst M, Chatterton NJ, Harrison PA, Matitschka G (1998) Characterization of fructan oligomers from species of the genus *Allium* L. *Journal of Plant Physiology* **153**, 53-60

Fujishima M, Sakai H, Ueno K, Takahashi N, Onodera S, Benkeblia N, Shiomi N (2005) Purification and characterization of a fructosyltransferase from onion bulbs and its key role in the synthesis of fructo-oligosaccharides *in vivo*. *New Phytologist* **165**, 513-524

Gentry HS (1982) *Agaves from Continental North America*, The University of Arizona Press, Tucson, 670 pp

Hendry GAF (1993) Evolutionary origins and natural functions of fructans. A climatological, biogeographic and mechanistic appraisal. *New Phytologist* **123**, 3-14

Hochstrasser U, Luscher M, De Virgilio C, Boller T, Wiemken A (1998) Expression of a functional barley sucrose-fructan 6- fructosyltransferase in the methylotrophic yeast *Pichia pastoris*. *FEBS Letters* **440**, 356-360

Livingston DP, Chatterton NJ, Harrison PA (1993) Structure and quantity of fructan oligomers in oat (*Avena* spp.). *New Phytologist* **123**, 725-734

López MG, Mancilla-Margalli NA, Mendoza-Díaz G (2003) Molecular structures of fructans from *Agave tequilana* Weber var *azul*. *Journal of Agricultural and Food Chemistry* **51**, 7835-7840

Losso DN, Nakai S (1997) Molecular size of garlic fructooligosaccharides and fructopolysaccharides by matrix-assisted laser desorption ionization mass spectrometry. *Journal of Agricultural and Food Chemistry* **45**, 4342-4346

Lüscher M, Hochstrasser U, Boller T, Wiemken A (2000a) Isolation of sucrose, sucrose 1-fructosyltransferase (1-SST) from barley (*Hordeum vulgare*). *New Phytologist* **145**, 225-232

Lüscher M, Hochstrasser U, Vogel G, Aeschbacher R, Galati V, Nelson CJ, Boller T, Wiemken A (2000b) Cloning and functional analysis of sucrose, sucrose 1-fructosyltransferase from tall fescue. *Plant Physiology* **124**, 1217-1227

Mancilla-Margalli NA, Lopez MG (2006) Water-soluble carbohydrates and fructan structure patterns from *Agave* and *Dasylirion* species. *Journal of Agricultural and Food Chemistry* **54**, 7832-7839

McLaughlin SP, Williams RR, Anouti AR, Ravetta DA, Nelson JM (2000) Allocation of resources to flowering and fruit production in *Hesperaloe funifera* (Agavaceae). *Journal of Arid Environments* **45**, 99-110

Nagaraj VJ, Altenbach D, Galati V, Lüscher M, Meyer AD, Boller T, Wiemken A (2004) Distinct regulation of sucrose, sucrose-1-fructosyltransferase (1-SST) and sucrose, fructan-6-fructosyltransferase (6-SFT), the key enzymes of fructan synthesis in barley leaves, 1-SST as the pacemaker. *New Phytologist* **161**, 735-748

Nandra KS, Bhatia IS (1980) *In vivo* biosynthesis of glucofructosans in *Agave americana*. *Phytochemistry* **19**, 965-966

Nobel PS (1988) *Environmental Biology of Agaves and Cacti*, Cambridge University Press, Cambridge, 270 pp

Pavis N, Boucaud J, Prud'homme MP (2001a) Fructans and fructan-metabolizing enzymes in leaves of *Lolium perenne*. *New Phytologist* **150**, 97-109

Pavis N, Chatterton NJ, Harrison PA, Baumgartner S, Praznik W, Boucaud J, Prud'homme MP (2001b) Structure of fructans in roots and leaf tissues of *Lolium perenne*. *New Phytologist* **150**, 83-95

Pollock CJ Cairns AJ (1991) Fructan metabolism in grasses and cereals. *Annual Review of Plant Physiology and Plant Molecular Biology* **42**, 77-101

Pollock CJ, Jones T (1979) Seasonal patterns of fructan metabolism in forage grasses. *New Phytologist* **83**, 9-15

Raveh E, Wang N, Nobel PS (1998) Gas exchange and metabolite fluctuations in green and yellow bands of variegated leaves of the monocotyledonous CAM species *Agave americana*. *Physiologia Plantarum* **103**, 99-106

Ritsema T, Joling J, Smeekens S (2003) Patterns of fructan synthesized by onion fructan, fructan 6G-fructosyltransferase expressed in tobacco BY2 cells – is fructan, fructan 1-fructosyltransferase needed in onion? *New Phytologist* **160**, 61-67

Ritsema T, Smeekens S (2003) Fructans, beneficial for plants and humans. *Current Opinion in Plant Biology* **6**, 223-230

Roberfroid MB (2002) Functional foods, concepts and application to inulin and oligofructose. *British Journal of Nutrition* **87**, S139-S143

Roth A, Luscher M, Sprenger N, Boller T, Wiemken A (1997) Fructan and fructan-metabolizing enzymes in the growth zone of barley leaves. *New Phytologist* **136**, 73-79

Satyanarayana MN (1976) Biosynthesis of oligosaccharides and fructans in *Agave vera cruz* 2. Biosynthesis of oligosaccharides. *Indian Journal of Biochemistry and Biophysics* **13**, 398-407

Shiomi N (1989) Properties of fructosyltransferases involved in the synthesis of fructan in Liliaceous plants. *Journal of Plant Physiology* **124**, 151-155

Shiomi N, Onodera S, Chatterton J, Harrison PA (1991) Separation of fructooligosaccharides isomers by anion-exchange chromatography. *Agricultural and Biological Chemistry* **55**, 1427-1428

Shiomi N (1993) Structure of fructopolysaccharide (asparagosin) from roots of asparagus (*Asparagus officinalis* L.). *New Phytologist* **123**, 263-270

Shiomi N, Benkeblia N, Onodera S (2005) The metabolism of the fructooligosaccharides in onion bulbs, a comprehensive review. *Journal of Applied Glycoscience* **52**, 121-127

Shiomi N, Onodera S, Sakai H (1997) Fructo-oligosaccharide content and fructosyltransferase activity during growth of onion bulbs. *New Phytologist* **136**, 105-113

Simmen U, Obenland D, Boller T, Wiemken A (1993) Fructan synthesis in excised barley leaves – Identification of 2 sucrose:sucrose fructosyltransferases induced by light and their separation from constitutive invertases. *Plant Physiology* **101**, 459-468

Sims IM (2003) Structural diversity of fructans from members of the order Asparagales in New Zealand. *Phytochemistry* **63**, 351-359

Sims IM, Cairns AJ, Furneaux RH (2001) Structure of fructans from excised leaves of New Zealand flax. *Phytochemistry* **57**, 661-668

Sims IM, Pollock CJ, Horgan R (1992) Structural analysis of oligomeric fructans from excised leaves of *Lolium temulentum*. *Phytochemistry* **31**, 2989-2992

Spies T, Praznik W, Hofinger A, Altmann F, Nitsch E, Wutka R (1992) The structure of the fructan sinistrin from *Urginea maritima*. *Carbohydrate Research* **235**, 221-230

Sprenger N, Bortlik K, Brandt A, Boller T, Wiemken A (1995) Purification, cloning, and functional expression of sucrose:fructan 6-fructosyltransferase, a key enzyme of fructan synthesis in barley. *Proceedings of the National Academy of Sciences USA* **92**, 11652-11656

Sprenger N, Schellenbaum L, van Dun K, Boller T, Wiemken A (1997) Fructan synthesis in transgenic tobacco and chicory plants expressing barley sucrose:fructan 6-fructosyltransferase. *FEBS Letters* **400**, 355-358

St John JA, Sims IM, Bonnett GD, Simpson RJ (1997) Identification of products formed by a fructan:fructan fructosyltransferase activity from *Lolium rigidum*. *New Phytologist* **135**, 249-257

Stahl B, Linos A, Karas M, Hillenkamp F, Steup M (1997) Analysis of fructans from higher plants by matrix-assisted laser desorption/ionization mass spectrometry. *Analytical Biochemistry* **240**, 195-204

Tissue DT, Nobel PS (1988) Parent-ramet connections in *Agave deserti* – influences of carbohydrates on growth. *Oecologia* **75**, 266-271

Ueno K, Onodera S, Kawakami A, Yoshida M, Shiomi N (2005) Molecular characterization and expression of a cDNA encoding fructan:fructan 6^G-fructosyltransferase from asparagus (*Asparagus officinalis*). *New Phytologist* **165**, 813-824

Urias-Sílvas JE, Cani PD, Delmee E, Neyrinck A, López MG, Delzenne NM (2008) Physiological effects of dietary fructans extracted from *Agave tequilana* Gto and *Dasylirion* spp. *British Journal of Nutrition* **99**, 254-261

van den Ende W, van Laere A (1996) *De novo* synthesis of fructans from sucrose in vitro by a combination of two purified enzymes (sucrose:sucrose 1-fructosyl transferase and fructan:fructan 1-fructosyl transferase) from chicory roots (*Cichorium intybus* L). *Planta* **200**, 335-342

van den Ende W, van Wonterghem D, Dewil E, Verhaert P, DeLoof A, van Laere A (1996) Purification and characterization of 1-SST, the key enzyme initiating fructan biosynthesis in young chicory roots (*Cichorium intybus*). *Physiologia Plantarum* **98**, 455-466

van Laere A, van den Ende W (2002) Inulin metabolism in dicots, chicory as a model system. *Plant Cell and Environment* **25**, 803-813

Vijn I, Smeekens S (1999) Fructan, More than a reserve carbohydrate? *Plant Physiology* **120**, 351-359

Vijn I, van Dijken A, Luscher M, Bos A, Smeets E, Weisbeek P, Wiemken A, Smeekens S (1998) Cloning of sucrose, Sucrose 1-fructosyltransferase from onion and synthesis of structurally defined fructan molecules from sucrose. *Plant Physiology* **117**, 1507-1513

Wang N, Nobel PS (1998) Phloem transport of fructans in the crassulacean acid metabolism species *Agave deserti*. *Plant Physiology* **116**, 709-714

Waterhouse AL, Chatterton NJ 1993 Glossary of fructan terms. In: Suzuki M, Chatterton NJ (Eds) *Science and Technology of Fructans*, CRC Press, Boca Raton, FL, pp 1-8