Fructans: from natural sources to transgenic plants

REVIEW

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ABSTRACT

In nature, fructans are synthesized from sucrose by various microbial and plant species. Depending on the source, fructans can contain from two to more than a hundred thousand fructose units linked by β-(2→1) (inulin-type) or β-(2→6) (levan-type) glycosidic bonds. Bacteria produce levan or inulin polymers via a one-enzyme process aimed to confer competitiveness in the interaction with the host plant or animal, protection against abiotic and biotic stress, and a circumstantial energy source. Plant fructans are shorter and have diverse structures. They are synthesized in the cell vacuoles as reserve carbohydrates by the concerted action of at least two enzymes with distinct substrate specificities. Plant fructosyltransferases evolved from vacuolar invertases, a process likely connected with the independent adaptation of unrelated families to cold and arid environments. Fructans of short and medium sizes are prebiotics with increasing demand in the functional food market. Large polyfructans also have potential applications in the non-food industry. Current production systems are restricted either to the recovery of linear inulin stored in the roots of the low-yielding chicory plant or to the more costly industrial conversion of sucrose into fructooligosaccharides using immobilized fungal enzymes. The introduction of the appropriate fructosyltransferase genes in agro-industrial productive crops that naturally store high concentrations of sucrose, such as sugar beet or sugar cane, offers a plausible alternative to produce different types of fructans at lower production costs.

Keywords: Fructan, levan, inulin, fructooligosaccharides, FOS, prebiotic, fructosyltransferase

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RESUMEN

Fructanos: producción natural y plantas transgénicas. En la naturaleza, la síntesis de fructanos ocurre a partir del sustrato sacarosa en varias especies de microorganismos y plantas. Los fructanos pueden tener desde dos hasta cientos de miles de residuos de fructosa unidos por enlaces β-(2→1) (inulina) o β-(2→6) (levana). Los fructanos vegetales son de menor tamaño y en ocasiones de mayor complejidad estructural que las levanas e inulinas bacterianas. En bacterias, una sola enzima (levanasacarasa o inulosacarasa) cataliza las reacciones de transfructosilación de la sacarosa y polimerización del fructano. En plantas, este proceso requiere de la acción de al menos dos fructosiltransferasas con diferente especificidad de sustrato que evolucionaron a partir de las invertasas. Los fructanos vegetales se sintetizan y acumulan en las vacuolas como carbohidratos de reserva, y se les relaciona con la adaptación a ambientes fríos y áridos. Desde el punto de vista comercial, los fructanos son prebióticos y presenta una demanda creciente en el mercado de alimentos funcionales. Los actuales sistemas de producción están restringidos a la obtención de inulina a partir de raíces de achicoria y la conversión industrial de sacarosa en fructooligosacáridos con el empleo de enzimas de hongos. La introducción individual o combinada de genes de fructosiltransferasas en cultivos de alta productividad agro-industrial y que de forma natural acumulen altas concentraciones del sustrato sacarosa, como la remolacha azucarera o la caña de azúcar, ofrece una alternativa menos costosa para la producción de diferentes tipos de fructanos con interés comercial.

Palabras claves: Fructano, levana, inulina, fructooligosacáridos, FOS, prebiótico, fructosiltransferasa

*I*ntroduction

Fructans are sucrose-derived sugars consisting of several fructose units and a common glucose residue. Fructan synthesis occurs in a wide spectrum of bacteria of differing physiologies, a limited number of fungi and about 15% of flowering plant species belonging to selected families of both monocots and dicots. Fructans of distinct origin can differ by the degree of polymerization (DP), the presence of branches, the type of linkage between adjacent fructose units, and the position of the glucose residue. Microbial fructans have protective and temporally energetic functions. In plants, fructans are used as reserve carbohydrates and are likely involved in the adaptation of unrelated families to cold and arid environments.

Bacterial levansucrases (EC 2.4.1.10) and inulosucrases (EC 2.4.1.9) convert sucrose into high DP fructans with a predominance of either β-(2→6) linkages (levan) or β-(2→1) linkages (inulin). Fungi synthesize linear β-(2→1) linked fructans, with a predominant occurrence of fructooligosaccharides (FOS) of DP 3-10. Unlike bacteria and fungi, plants synthesize fructans by the concerted action of two or more enzymes exhibiting distinct specificities for the fructosyl-donor and fructosyl-acceptor substrates. The enzyme sucrose:sucrose 1-fructosyltransferase (1-SST) initiates fructan synthesis by producing the intermediary 1-kestose from two sucrose molecules, with the consequent release of glucose. Depending on the plant species, the fructan stored is synthesized by the sole or combined action of the enzymes fructan:fructan 1-fructosyltransferase (1-FFT), fructan:fructan 6G-fructosyltransferase (6G-FFT) and sucrose:fructan 6-fructosyltransferase (6-SFT) (for review see [1]).

Fructans have several potential applications in the food and non food industries, but they are especially

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attractive because of their health-promoting effect as prebiotics. A prebiotic can be defined as a nondigestible food ingredient that beneficially affects the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon, thus improving host health [2]. The commercially available fructans (FOS and inulin) are the typical examples of prebiotics for bifidobacteria, the predominant group of the colonic microflora. The selective increase in the number of bifidobacteria and lactobacilli in the human and animal large intestine due to the ingestion of fructans confer several benefits to their host, such as the competitive exclusion of intestinal pathogens, the reduction of serum cholesterol, an increase in calcium and magnesium absorption, the prevention of colon cancer, and the production of B-vitamins (for review, see [3]).

At present, FOS are produced at an industrial-scale from sucrose using immobilized fungal cells. Inulin is recovered from chicory roots with relatively poor yields and frequently deteriorated quality due to endogenous degradation during and after harvest. In both commercial processes production costs are high, hindering the large-scale use of fructans. Novel attempts to achieve more cost-effective productions of fructans are focused on the use of transgenic plants.

Structure and origin

The term fructan comprises both oligosaccharides and polysaccharides that mainly have fructose residues. Fructans synthesized in nature are water-soluble and non-reducing sugars in which from one up to more than a hundred thousand fructose units are attached to the precursor sucrose molecule. The addition of one fructosyl residue to sucrose produces 1-kestose (G1↔2F1←2F), 6-kestose (G1↔2F6←2F) or neokestose (F2→6G1↔2F) (Figure 1). The successive enlargement of the trisaccharide by one or more types of linkages results in the formation of linear or branched polyfructans, respectively. Fructans are classified depending on the predominant linkage type and chain size. Inulin-type fructans contain mostly or exclusively β-(2→1) fructosyl-fructose linkages, whereas levan-type fructans are β-(2→6) linked molecules with the occasional presence, or not, of β-(2→1) bonds. Fructans with degrees of polymerization (DP) from 2 to 10 are commonly known as FOS (fructooligosaccharides) [4].

Fructan synthesis occurs in a broad range of microorganisms of differing physiologies and a limited number of plant species mainly in temperate and arid climates. Many Gram-positive and Gram-negative bacteria produce levan, while inulin synthesis has been reported so far only in the Gram-positive species *Streptococcus mutans*, *Lactobacillus reuteri* and *Leuconostoc citreum*. Bacterial levan and inulin are the largest fructans in nature, with a DP ranging from 10^4 to 10^6. Fungal species that produce fructans are basically included in the genera *Aspergillus*, *Aureobasidium*, *Penicillium*, *Fusarium*, *Pestalotiopsis*, *Myrothecium*, *Trichoderma*, and *Phytophthora*. Fungal fructans consist of a linear β-(2→1) linked chain, with the predominant occurrence of FOS. Chain size can vary between species in the same genus. For instance, *Aspergillus sydawi* converts sucrose into inulin with a DP above 30, whereas *A. niger*, *A. phoenicis*, *A. foetidus*, and *A. oryzae* produce a mixture of FOS containing 3 to 8 monosaccharide units [5-8].

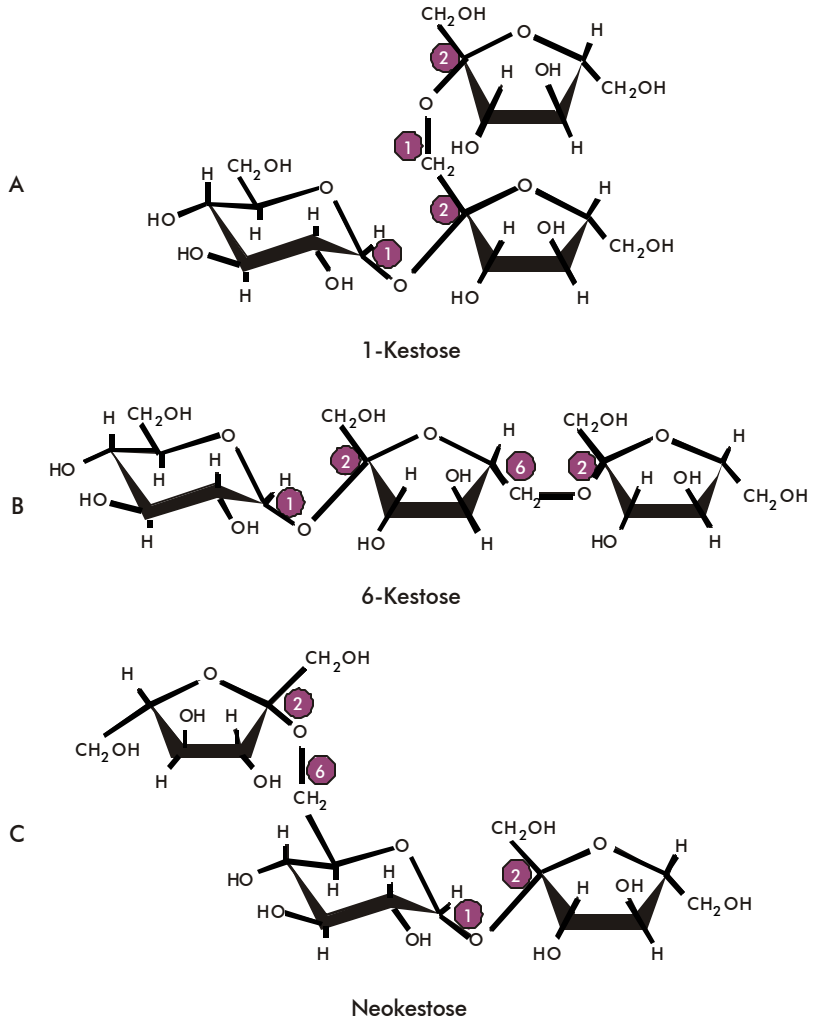

Figure 1. Structural representation of three kestrioses containing all the disaccharide linkages found in natural polyfructans. A) 1-Kestose [O-β-D-fructofuranosyl-(2→1)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside], B) 6-Kestose [O-β-D-fructofuranosyl-(2→6)-β-D-fructofuranosyl-(2→1)-α-D-glucopyranoside], and C) Neokestose [O-β-D-fructofuranosyl-(2→6)-α-D-glucopyranosyl-(1↔2)-β-D-fructofuranoside].

In plants, fructans are synthesized and stored in the vacuole of about 15% of flowering species, including dicotyledons and monocotyledons. Dicot plants (family Asteraceae) synthesize linear inulin consisting of one terminal glucose residue and a variable number of fructose residues exclusively linked by β-(2→1) bonds. The chain length of the inulin deposited in storage organs varies between species. The inulin stored in chicory (*Cichorium intybus*) taproots and Jerusalem artichoke (*Helianthus tuberosus*) tubers has a rather low mean DP of about 10 to 30. The highest DP inulin in Asteraceae has been found in globe artichoke (*Cynara scolymus*) roots reaching up to 200 fructose residues [9, 10]. Monocot plants (families Poaceae, Alliaceae, Asparagaceae, Agavaceae, Amaryllidaceae, Haemodoraceae, and Iridaceae) produce more complex fructans. Temperate grasses (Poaceae) form structures that vary from li-

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near levan, referred to as phlein, for instance in big bluegrass (*Poa secunda*) [11, 12], to highly branched levan having both β-(2→6) and β-(2→1) fructosyl-fructose linkages, referred to as graminan, for instance in wheat (*Triticum aestivum*) and barley (*Hordeum vulgare*) [13]. Graminans may also contain an oligoinulin chain attached to the C6 of the glucosyl residue of sucrose, for instance in oat (*Avena sativa*), tall fescue (*Festuca arundinacea*) and Lolium species [14-16]. The members of Alliaceae and Asparagaceae, for instance onion (*Allium cepa*) and asparagus (*Asparagus officinalis*), produce inulin neoseries, also referred to as neoinulins, consisting of two linear β-(2→1) linked fructosyl chains, one attached to the fructose residue of the sucrose starter, the other attached to the glucose residue at the C6 position [17, 18]. In Agavaceae diverse fructan structures have been reported. Inulin was identified as the principal reserve carbohydrate in *A*gave *americana*. The stems of *Agave vera cruz* and *Agave tequilana* store a complex mixture of fructooligosaccharides, inulins, neoinulins, and branched fructans containing both β-(2→1) and β-(2→6) fructosyl-fructose linkages [19, 20].

Function in nature

Bacterial fructans are present as part of extracellular polysaccharides, having specific habitat-related functions and occasionally serving as energy reserves. For instance, inulin and levan produced by oral streptococci are involved in the adherence of bacteria between themselves and to the tooth surface, contributing to the formation of dental plaques and cavities [21, 22]. Levan is a barrier to plant recognition and prevents a defense response during the early phase of infection of pathogens *Erwinia amylovora* and *Pseudomonas syringae* [23, 24]. Levan produced by *Paenibacillus polymyxa* is involved in the aggregation of root-adhering soil on wheat [25]. Alternatively, fructans may protect microbial cells against abiotic and biotic stress, such as: desiccation, freezing, antibiotics or toxic compounds, and attacks of parasites and predators.

In plants, fructans are used as reserve carbohydrates and, in that respect, they work much like starch and sucrose. In contrast to starch, which is stored in the plastids, fructans are synthesized, stored and hydrolyzed in cell vacuoles. Fructans accumulate during growth if carbon production exceeds demands and are mobilized when energy is needed. For instance, fructans are hydrolyzed during the regrowth of leaves and for sprouting after defoliation and winter dormancy [26, 27]. Fructan degradation has also been associated with petal expansion in the flowers of *Hemerocallis* [28] and *Campanula rapunculoides* [29]. There is strong evidence that fructans protect the plant against drought and freezing [30, 31], most likely by stabilizing cell membranes [32, 33].

Applications

Fructans have applications in the food, nutraceuticals and non-food industries. Different size fructans potentially have different uses. However, only FOS and inulin are currently produced at a commercial scale. These fructans are marketed under the category of functional foods with an increasing demand in developed countries. By concept, functional foods have an added health value above their nutritional properties [34].

Fructans are considered the typical representative of prebiotics, with a proven bifidogenic effect in animals and humans. These sugars have a low caloric value and dietary fiber-like properties, due to the fact that the β-fructosyl linkages can not be hydrolyzed by the digestive enzymes in the upper part of the human gastrointestinal track. Once in the colon, fructans are selectively metabolized by resident bacteria that produce β-fructofuranosidases, including bifidobacteria and lactobacilli, main representatives of the beneficial colonic microflora. The proliferation of healthy bacteria in the gut results in the competitive exclusion of pathogens, such as *Escherichia coli*, *Clostridium* sp. and *Salmonella* sp. The release of short-chain fatty acids and lactic acids by bifidobacteria and lactobacilli provokes other important associated benefits to human and animal health, such as the reduction of serum cholesterol, an increase of calcium and magnesium absorption, the prevention of colon cancer and the production of B-vitamins.

Fructans are considered to be prebiotics regardless of their size and type of linkages. Short-chain fructans are more convenient substrates for rapid growth of bifidobacteria [35, 36], whereas branched fructans are claimed to provide for a long-lasting source of energy [37]. Although fructans with a low and medium DP are important primarily because of their functional properties, they have additional applications in the food industry. The trisaccharide 1-kestose has a natural sweet taste, and in a blend with other low calorie sweeteners it can replace sucrose in certain specific uses. Inulin recovered from chicory is extensively used as a food ingredient due to its neutral taste and excellent characteristics as a fat replacer and dietary fiber.

Levan and inulin produced by bacteria have other potential food and non-food applications. In the food industry, these high DP fructans are ideal substrates for the production of High Fructose Syrups (HFS), because of the very low glucose content. Levan is more soluble than inulin and produces viscous solutions in water. In the industry, this property makes levan especially attractive as an emulsifier or encapsulating agent in a wide range of products, including biodegradable plastics, cosmetics, glues, textile coatings and detergents. Levan may also have medical applications. The polymer could substitute dextran as a blood plasma volume extender, and it was found to have anti-tumor and immunomodulatory activities in mice [38, 39].

Enzymes for fructan synthesis

Fructans are synthesized from sucrose by a double-displacement mechanism that involves the formation and subsequent hydrolysis of a covalent fructosyl-enzyme intermediate. The reaction occurs with an overall retention of the anomeric configuration of the fructosyl residue. Within the sequence-derived classification of glycoside hydrolases and trans-glycosidases, bacterial fructosyltransferases are classified in family GH68, whereas fungal and plant fructosyltransferases are grouped together with

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invertase, fructanases and sucrose-6-phosphate hydrolases in family GH32 (http://afmb.cnrs-mrs.fr/CAZY). Enzymes from the families GH32 and GH68 comprise clan GH-J and have a five-bladed β-propeller structure with a fully conserved catalytic triad of acidic residues placed at the bottom of a deep central cavity. These residues are involved in the cleavage of the glycosidic bond of the fructosyl-donor substrate (ex. sucrose) functioning as nucleophile (Asp), general acid/base catalyst (Glu) and transition state stabilizer (Asp) [40-43]. The structural determinants responsible for the polymerization reaction of microbial and plant fructosyltransferases remain unknown.

Bacterial fructosyltransferases

In bacteria, fructan synthesis occurs by the action of a multifunctional enzyme capable of directly converting sucrose into FOS and high DP fructans. The enzyme is named inulosucrase (EC 2.4.1.9) when it synthesizes inulin-type fructans, and levansucrase (EC 2.4.1.10) when the polymerization product is levan. Many levansucrases have the dual ability to form β-(2→6) and β-(2→1) fructosyl-fructose linkages. The majority of fructan-synthesizing bacteria produce levansucrase. Only some lactic acid species have been identified to produce inulosucrase, sometimes in combination with a levansucrase. Fructosyltransferases from related genera are rather similar in amino acid sequence, but the average percentage of sequence identity between levansucrases from Gram-positive and Gram-negative bacteria is only 23%.

All bacterial fructosyltransferases catalyze the transfer of the fructosyl residue from the donor substrate sucrose to a variety of acceptor substrates, such as water (sucrose hydrolysis), sucrose (kestose synthesis), fructan (fructan polymerization), glucose (sucrose synthesis), and fructose (bifructose synthesis). Depending on the enzyme origin, there are remarkable differences in the affinity for sucrose and the other fructosyl acceptors emerging during the reaction. In general, levansucrases from Gram-positive bacteria (for instance, *Bacillus subtilis* and *Streptococcus salivarius*) catalyze the formation of high DP levan without transient accumulation of FOS, whereas the enzymes from Gram-negative species (for instance, *Gluconacetobacter diazotrophicus* and *Zymomonas mobilis*) render high levels of β-(2→1) linked tri- and tetra-saccharide (1-kestose and nystose) with a lower yield of levan [44-47]. The fact that the trisaccharide 6-kestose, the initial intermediary fructan in levan formation, and larger β-(2→6) linked FOS are not accumulated during sucrose transformation by levansucrases, indicates that the growing fructan chain remains bound to the enzyme and is directly elongated in successive transfructosylation steps. In contrast to levansucrases, inulosucrase from *L. reuteri* catalyzes a non-processive reaction converting sucrose into a range of inulin-type oligofructans with progressively increased sizes. These products are released from the enzyme after every fructosyl transfer [48].

Levasucrases display Michaelis-Menten type of kinetics for both the hydrolase and polymerase activities [45, 46, 48]. In contrast, inulosucrases appear not to follow the Michaelis-Menten reaction kinetics. The overall activity of *L. reuteri* inulosucrase rose proportionally with increasing concentrations of sucrose, showing the absence of substrate-saturation kinetics [48]. The ratio of hydrolysis versus transfructosylation activities of both levansucrases and inulosucrases is highly dependent on the reaction conditions. Sucrose hydrolysis occurs optimally at about 50-60 ºC, but fructan formation is favored at lower temperatures and with the increase of sucrose concentration. In the absence of sucrose, levansucrases and inulosucrases can degrade the synthesized fructans by releasing the terminal fructose unit in a consecutive manner. Fructosyltransferases from several Gram-positive species require the presence of Ca^{2+} for optimum activity (for review, [49]). This ion connects catalytic residues placed distantly in the amino acid sequence of *B. subtilis* levansucrase [40]. A similar fold-stabilizing role is accomplished by a disulfide bridge in *G. diazotrophicus* levansucrase, lacking the Ca^{2+} binding site like other fructosyltransferases from Gram-negative bacteria [43].

Fructosyltransferase genes often form part of a chromosomal operon. Their expression is constitutive in bacteria that reside in sucrose-containing habitats [50-52]. In the soil bacterium *B. subtilis*, the levansucrase gene is strongly controlled by a sucrose-inducible antitermination mechanism [53].

All known bacterial fructosyltransferases are extracellular or cell-bound proteins, although they follow different secretion routes. Levansucrase secretion in Gram-positive bacteria involves the cleavage of signal-peptide containing precursors [54-56], whilst the vast majority of Gram-negative bacteria secrete the enzyme by a signal-peptide-independent pathway [23, 57-59]. Exceptionally, *G. diazotrophicus* levansucrase requires a type-II secretory machinery for translocation across the outer membrane [60, 61].

Plant fructosyltransferases

Plant fructans are synthesized by the concerted action of at least two fructosyltransferases exhibiting distinct fructosyl-donor and fructosyl-acceptor specificities (for review, [3]). Sucrose:sucrose 1-fructosyltransferase (1-SST) initiates fructan synthesis by catalyzing the transfer of the fructosyl residue from one sucrose to another sucrose molecule, resulting in the formation of the intermediary trisaccharide 1-kestose and glucose. To a much lesser extent, 1-SST also uses 1-kestose as the acceptor substrate to form β-(2→1) linked tetra- and penta-saccharides, named nystose and fructosylnystose, respectively. The 1-SST activity is present in all fructan-producing plants and the encoding gene has been isolated from different species [62-65]. Other fructosyltransferase enzymes are required for fructan elongation and ramification.

In dicots (family Asteraceae) two enzymes are responsible for fructan synthesis. The enzyme fructan:fructan 1-fructosyltransferase (1-FFT) elongates 1-kestose and nystose into linear inulins with 10 to 200 fructose residues depending on the plant species [66]. Monocots require additional fructosyltransferase enzymes to form fructans with different type of linkages. In the closely-related families Alliaceae and Asparagaceae, the enzyme fructan:fructan 6G-fructosyltransferase (6G-FFT) produces neokestose by transferring the terminal fructose residue of 1-kestose to the glucose residue of sucrose via a β-(2→6) linkage, although it also has a side

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β-(2→1) activity [18, 67]. The combined action of 1-SST and 6G-FFT is sufficient for the production of onion neoinulins, whereas a three-enzyme system (1-SST/1-FFT/6G-FFT) is present in asparagus [68, 69]. In the Poaceae family, the distinctive enzyme is sucrose:fructan 6-fructosyltransferase (6-SFT) that produces the tetrasaccharide bifurcose by coupling a fructose residue to the internal glucose moiety of 1-kestose via the β-(2→6) linkage [70]. 6-SFT is also able to synthesize 6-kestose from sucrose when the substrate 1-kestose is not available, functioning as a key enzyme for diverting carbon to fructans in barley leaves [71]. Other fructan-modifying/polymerizing activities detected in members of Poaceae are 6G-FFT and 1-FFT. Recently, Lasseur et al. [72] cloned a 6G-FFT/1-FFT that accounts for the prevailing neokestose-based fructan synthesis in perennial ryegrass. The combination of 1-SST, 1-FFT, 6-SFT and 6G-FFT activities should give rise to the complex mixture of fructans found in Agavaceae.

A remarkable property of plant fructosyltransferases is their lack of substrate-saturation kinetics. Differing from invertases that have a K_m for sucrose in the millimolar range, 1-SST enzymes do not show Michaelis-Menten kinetics and their activity *in vitro* progressively increases at least up to 1 M, the maximum concentration of sucrose usually tested [64, 73, 74].

Each plant fructosyltransferase is encoded by a single gene whose expression is controlled at the transcriptional level. The first enzyme in fructan synthesis (1-SST) is induced under conditions leading to a high sucrose concentration, such as high light and cold or drought stress [75, 76]. A similar expression profile has been observed in 6-STF, another fructan-synthesizing enzyme that can use sucrose as a fructosyl-donor substrate [77]. The presence of 1-kestose and other short-chain FOS is likely to induce the expression of the fructan-elongating enzymes.

Enzymes for fructan degradation

A wide range of bacteria and fungi metabolize fructans as an alternative carbon source when more energetic sugars (for instance, glucose and sucrose) are limiting or exhausted. Microbial levan and inulin are hydrolyzed by the action of extracellular endo and exofructanases. Most levanases characterized so far function as fructose or levanbiose-producing exohydrolases. Fructose-releasing levanases have been identified in the species *Bacillus subtilis*, *Actinomyces viscosus*, *Bacteroides fragilis*, *Paenibacillus polymyxa*, *Bacillus stearothermophilus*, and *Gluconacetobacter diazotrophicus* [78-82]. In addition to levan, these enzymes commonly hydrolyze inulin, raffinose and sucrose, although with different substrate preferences. In all cases the enzyme attacks the substrate molecule from the fructose end and releases fructose as the sole reaction product. By contrast, levanbiose-producing levanases split levan mostly into levanbiose and hardly hydrolyze the β-(2→1) linkages of inulin, raffinose, or sucrose [83-85]. Endolevanases and endoinulinases have absolute substrate specificity for levan and inulin, respectively. These enzymes split at random the internal β-linkages of the polymer yielding a mixture of oligofructans of different sizes [86]. Transcriptional studies have revealed that the expression of bacterial fructanase genes has basically two levels of control:

1. Specific induction by low concentrations of the degradation product of levan or inulin.

2. Repression by glucose as a global regulation of cell catabolism [53, 87, 88].

In plants, the breakdown of fructans is accomplished by a complex of fructan exohydrolases (FEHs). Two isoforms of 1-FEH have been identified to degrade inulin in chicory roots [27]. More recently, three cDNAs from wheat encoding two 1-FEHs and one 6&1-FEH were associated with the breakdown of branched graminan-type fructans containing both β-(2→1) or β-(2→6) fructosyl linkages [89]. The 6&1-FEH type of enzyme in wheat crowns was suggested to fulfill a crucial role in the modulation of fructan content and DP under cold stress.

The expression of FEH genes is mainly induced after defoliation or by freezing temperature [90-92]. Hormones like gibberellin and ABA have been found to be important for FEH regulation [93]. Plant FEHs, in contrast to microbial exofructanases, are unable to hydrolyze sucrose. This sugar is, otherwise, a strong competitive inhibitor of FEH enzymes and may regulate their activity *in vivo* avoiding fructan hydrolysis when more energetic substrates are available.

Surprisingly, functional 6-FEH genes have been isolated from the non-fructan plants *Arabidopsis thaliana* and sugar beet [94, 95]. Since no endogenous substrates are present in these plants, it was proposed that these enzymes may have a defense-related function against pathogenic or endophytic bacteria, which produce levan as a virulent factor [96].

Evolution

Fructosyltransferases and fructanases are evolutionarily, structurally, and mechanistically related to invertases. In bacteria, the sequence similarities between fructan-synthesizing enzymes (levansucrase and inulosucrase) and invertases are mainly restricted to few specific domains located at the active site. By contrast, fructan-degrading enzymes (levanase and inulinase) and invertases share extensive similarities at their amino acid sequences and thus have a closer evolutionary relationship. Considering that many fructan-producing bacteria do not have an invertase, it is reasonable to think that fructosyltransferase genes have been primarily transferred between different species sharing a common habitat. The genes encoding the enzymes for fructan synthesis and degradation form an operon in some species (for instance, *G. diazotrophicus*) [82], suggesting the occurrence of the dual acquisition by a single horizontal transfer event.

In higher plants, invertase is ubiquitous. Phylogenetic studies based on the comparison of protein sequences have shown that invertases and fructan-metabolizing enzymes from one species or family are often more closely related than enzymes that catalyze the same reaction but originate from different species or families. It is suggested that fructosyltransferases arose by few mutations of acid vacuolar invertases, whereas fructan exohydrolases (FEHs) evolved from cell-wall invertases that later gained a vacuolar targeting signal and a low isoelectric point [96, 97]. Recently, Ritsema *et al.* [98] succeeded increasing the 1-kestose synthesis capability of a

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vacuolar invertase by exchanging a strictly conserved Trp in the sucrose-binding box with another aromatic residue. Such a replacement corresponds with a natural variation and might represent an initial mutational step in the evolution of vacuolar invertases to fructosyltransferases.

Current production systems

Meiji Seika Kaisha Ltd. started the commercial production of fructans about twenty years ago. This Japanese company produces β-(2→1) linked FOS (DP 3 to 5) from sucrose in a continuous process by using a biocatalyzer composed of *Aspergillus niger* cells entrapped in calcium alginate gels. With the same purpose, other Asian companies currently use different fungi, for instance *Aureobasidium pullulans*. All fungal fructosyltransferases catalyze both transfructosylation and hydrolysis reactions although at different rates. Regardless the enzyme origin, the reaction product obtained is a blend of FOS, sucrose, glucose, and fructose. The reaction is started with sucrose at 60-70% (w/v), which is initially transformed to trisaccharide 1-kestose and monosaccharides glucose and fructose. At the end of the reaction, the maximum FOS content can reach 55-60% (w/v) with a shift from DP 3 to DP 4-5. The next steps of chromatographic purification, concentration and sterilization are performed to obtain an enriched FOS product that is sold as a syrup, a white powder or crystals. At present, Meiji Seika Kaisha sends to the market about 4000 tons of FOS per year. The range of commercial FOS products covers from concentrated non-purified syrups to crystalline 1-kestose.

The worldwide production of inulin is basically accomplished by three companies located in Western Europe. Linear inulin with DP of 10 to 30 is commercially recovered from chicory roots where the fructan content can reach about 20% of the fresh weight (about 80% of the dry weight). The first processing step is an extraction with hot water followed by a filtration to eliminate colloidal materials. The resulting liquid (dark and bitter) is further refined using ion exchange resins. Since inulin cannot be crystallised the final step is always spray drying to obtain a product in the form of a powder. The extracted inulin can be subjected to complete or partial hydrolysis to produce fructose or oligofructans, which are sold as syrups. Chicory is a low-yielding crop, in agronomic terms, and expresses exohydrolases that can severely degrade the inulin stored after harvesting the roots.

No commercially attractive technology is currently available for the industrial production of branched fructans with a high DP or various types of linkages. The enzymatic conversion of sucrose to levan using bacterial levansucrases in industrial reactors is highly expensive. The elevated viscosity of the reaction mixtures and the fact that the synthesized polymer remains attached to the enzyme hinders the implementation of continuous production systems. On the other hand, natural crops like onion, asparagus, agaves and temperate grasses appear not to fit the agronomic characteristics required for the cost-effective production of neofructans, phleins and graminans.

Transgenic plants

From the mid nineties, several groups motivated by academic and biotechnological reasons have transferred bacterial and plant fructosyltransferase genes into different plant species, most of which do not naturally produce fructans. The synthesis of the transgenic fructan as an additional sucrose sink in leaves and reserve organs offered the opportunity for accomplishing original carbon partitioning studies. From an applied viewpoint, transgenesis has been focused on to improve crop resistance to environmental stress, as well as for creating novel sources for the cost-effective production and commercialization of different fructans.

Plants transformed with bacterial fructosyltransferase genes

Levansucrase genes from different bacteria have been placed under the control of constitutive or organ-specific promoters and engineered for protein targeting to different subcellular plant locations: cytoplasm, apoplasm, vacuole, and plastid (Table 1). From the first transgenic experiments, the vacuole was focused as the most compatible compartment to achieve high levan yields. In this compartment the substrate sucrose

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Table 1. Fructan accumulation in plants transformed with bacterial levansucrase

Host	Organ / putative compartment	Promoter / targeting signal / gene source	Endpoint fructan* (mg g-1)	Reference
Tobacco	Leaf / vacuole	35S / CPY / *Bs*	2.8	[100]
Potato	Leaf / vacuole Tuber / vacuole	35S / CPY / *Bs* 35S / CPY / *Bs*	160 10	[101]
Tobacco	Leaf / vacuole	35S / CPY / *Bs*	0.35	[30]
Potato	Leaf / vacuole Tuber / vacuole	35S / CPY / *Bs* 35S / CPY / *Bs*	5 11	[102]
Potato	Tuber / vacuole Tuber / apoplasm	B33 / NTRP / *Ea* B33 / own signal / *Ea*	28 17	[103]
Maize	Seed / cytoplasm Seed / vacuole Seed / vacuole	Zein / no signal / *Ba* Zein / lectin / *Ba* Zein / sporamin / *Ba*	18 9 9	[104]
Tobacco Potato	Leaf / cytoplasm Tuber / cytoplasm	M 2-2 / no signal / *Ba* B33 / no signal / *Ba*	4 10	[105]
Tobacco	Leaf / vacuole	35S / sporamin / *Bs*	6	[106]
Sugar beet	Leaf / vacuole	35S / CPY / *Bs*	1	[107]
Tobacco Potato	Leaf / plastid Tuber / plastid	35S / ferredoxin / *Bs* 35S / ferredoxin / *Bs*	20 66	[108]
Ryegrass	Leaf / vacuole Leaf / vacuole	Ubi / sporamin / *Bs* Ubi / CPY / *Bs*	< 0.1	[109]

Promoter: 35S, Cauliflower mosaic virus 35S; B33, Potato tuber specific; Zein, Maize seed specific; M 2-2, Maize inducible; Ubi, Maize ubiquitin 1.

Vacuolar targeting signal: CPY, yeast carboxipeptidase Y; NTRP, N-terminal region of patatin (92 amino acids); lectin, Barley lectin; sporamin, Sweet potato sporamin.

Plastid targeting signal: ferredoxin, Silene pratensis ferredoxin.

Gene source: *Bs, Bacillus subtilis; Ba, Bacillus amyloliquefaciens; Ea, Erwinia amylovora*.

*Adapted from Cairns [110]. Where several values were reported, the maximum is presented. Fructan concentrations are expressed on a fresh mass basis.

would be constantly available and the synthesized polymer would remain sequestered, minimizing its potential disruptive effects. On the other hand, recombinant experiments in yeast suggest that the potential occurrence of glycosylation during the transit to the vacuole is not likely to alter the catalytic performance of levansucrase [99]. However, in many cases the attempted vacuolar targeting of the bacterial enzyme has been unsuccessful. Pilon-Smits *et al.* [102] showed that levan was present at the cell perimeter in leaves of potatoes transformed with the *Erwinia amylovora* levansucrase fused to a yeast CPY vacuolar-targeting sequence. In tobacco transformants, the vacuolar-targeting signal of the sweet potato sporamin failed to translocate the *Bacillus subtilis* levansucrase beyond the endomembrane system [106]. Rober *et al.* [103] succeeded in producing levan in the vacuoles of the potato tubers by using the patatin signal sequence; although Cairns [110] argued that the polymer was also associated to the cell wall. Using a seed-specific promoter and the vacuolar-targeting sequence of either the sweet potato sporamin or a barley lectin, Caimi *et al.* [104] observed that the maize seeds developed normally despite the accumulation of levan. The synthesis of levan in the cytoplasm was lethal in tobacco, potato and maize [104, 105].

In terms of endpoint concentrations of levan, reports varied between 0.04 and 160 mg g^{-1}, (0.02-80% of dry biomass) with the majority lying between 5-20 mg g^{-1}, (2.5-10% of dry biomass) [110]. The maximum value was obtained for leaves of plants grown *in vitro*, presumably with sucrose as the exogenous carbon source [101]. For greenhouse plants the highest levan production (66 mg g^{-1}) was surprisingly achieved in tubers of potatoes transformed with the *B. subtilis* levansucrase fused to a ferredoxin chloroplast targeting sequence [108]. This value is lower than the natural inulin accumulation in tubers of *H. tuberosus* (150 mg g^{-1}) [111].

The primary explanation for the relatively low rates of levan accumulation in transgenic crops is the low expression level of the levansucrase gene, based on the fact that the transgene product, regardless of its origin, has not been detected in western blot experiments. On the other hand, it is speculated that higher rates of levan accumulation, even in the vacuoles, might cause cell toxicity and prevent the recovery of high-expressing transformants.

Levan accumulation in transgenic tobacco and sugar beet increased drought tolerance [30,107], suggesting that the novel trait could be useful for crop improvement. However, in a general view, plant expressing bacterial fructosyltransferase genes exhibited aberrant phenotypes such as stunting and leaf bleaching. In transgenic potato, there was a reduction in the number and weight of tubers, as well as in their starch content [101, 103, 108].

Plants transformed with plant fructosyltransferase genes

Plant fructosyltransferase genes were cloned and used for transformation later than their bacterial counterparts. The transgenic production of plant-derived fructans has two main reasons:

1. The study of the catalyzing specificities of distinctive fructan biosynthesis pathways.

2. The creation of novel fructan-producing crops with biotechnological interest.

In this sense, the cloned fructan enzymes of a defined species were constitutively expressed in the vacuoles of a non-fructan host (for instance: petunia, tobacco, potato and sugar beet) or used to modify the inulin pattern of chicory (Table 2). The *1-SST* and *1-FFT* genes from *Helianthus tuberosus* were introduced alone or combined into petunia [62]. The *1-SST* transformants accumulated mainly 1-kestose and nystose, whilst the plants expressing the two genes yielded linear inulin chains of 9-40 fructose units in yellow senescent leaves. Similarly, the independent (*1-SST*) and combined (*1-SST/1-FFT*) expression of the *Cinara scolymus* genes in potato tubers resulted in the accumulation of short-chain FOS and inulin (DP up to 40), respectively [112]. These findings confirm the two-enzyme model proposed by Edelman and Jefford [115] for the synthesis of the linear β-(2→1) linked fructans observed in members of Asterales. However, the ability of 1-SST to produce oligomers with a DP higher than 3 contradicts the clear distinction of the fructosyltransferase activities established in this model.

The *6-SFT* gene from barley was expressed in tobacco and chicory plants [113]. As predicted, with sucrose as the sole substrate, the transgenic tobacco plants accumulated low amounts of 6-kestose and a series of unbranched fructans of the phlein type. In addition to the endogenous inulin, the chicory transformants produced traces of the tetrasaccharide bifurcose and graminan-type fructans in illuminated leaves, but not in roots. In a similar approach, the *6G-FFT* gene from onion was expressed in tobacco and chicory [18]. Due to the enzyme requirement

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Table 2. Fructan accumulation in plants transformed with plant fructosyltransferases

Host	Organ	Promoter / Gene(s) and source	Endpoint fructan* (mg g^{-1})	Reference
Potato	Tuber	B33 / *1SST Cs*	9.7	[112]
Chicory	Leaf	35S / *6SFT Hv*	Trace	[113]
Tobacco	Leaf	35S / *6SFT Hv*	0.06	
	Root	35S / *6SFT Hv*	0.6	
Chicory	Leaf	35S / *6G-FFT Ac*	n.d	[18]
Sugar beet	Leaf	35S / *1SST Ht*	0.6	[114]
	Root	35S / *1SST Ht*	62.3	
Petunia	Leaf	35S / *1SST Ht*	0.47	[62]
	Leaf	35S / *1SST+1FFT Ht*	0.08	
Potato	Tuber Leaf Tuber	35S / *1SST Cs*	4.9	[10]
		35S / *1SST Cs*	0.1	
		35S / *1SST+1FFT Cs*	6.1	
Sugar beet	Root	Ubi / *1SST+6G-FFT Ac*	0.1	[37]

Promoter: 35S, Cauliflower mosaic virus 35S; B33, Potato tuber specific; Ubi, Arabidopsis thaliana ubiquitin 3

Gene source: *Cs, Cynara scolymus; Hv, Hordeum vulgare; Ac, Allium cepa; Ht, Helianthus tuberosus.*

n.d: not determined

*Adapted from Cairns [110]. Where several values were reported, the maximum is presented. Fructan concentrations are expressed on a fresh mass basis.

for 1-kestose as a donor substrate, the tobacco extracts were incubated *in vitro* with the trisaccharide resulting in the formation of neokestose and low DP fructans of the inulin neoseries. The illuminated leaves of the transgenic chicory produced the endogenous inulin and 6G-linked fructans (neoinulins) with DP from 3 to 14.

In an attempt to create a novel source for the cost-effective production of commercial FOS, Sévenier *et al.* [114] constitutively expressed the *H. tuberosus 1-SST* gene in sugar beet. In the taproot of a greenhouse-grown transformant, the concentration of GF_2, GF_3, and GF_4 reached up to 73.8, 33.7 and 5.7 μmol g^{-1} (fresh weight), respectively. The sum of these values represents more than 40% of the taproot dry weight. Over 90% of the stored sucrose was channeled into fructan synthesis, but the process was accompanied by a slight decrease (8%, expressed in hexose equivalents) in the total content of soluble carbohydrates. More recently, Weyens *et al.* [37] were able to produce inulin neoseries (DP ranging 3-5) in sugar beet by the constitutive expression of the onion *1-SST* and *6G-FFT* genes. The content of the fructans accumulated in the transgenic taproots reached about 90 mg g^{-1} (fresh weight), without the loss of overall storage carbohydrates.

With the exception of the fructans accumulated in taproots of the transformed sugar beet plants [37, 114], the endpoint concentrations of transgenic fructans were generally below 10 mg g^{-1}, a value similar to that of the levan transgenics but lower than the maxima found for endogenous reserve accumulation (60-150 mg g^{-1}), as reviewed by Cairns [110]. A plausible cause for the limited fructan accumulation observed in crops other than sugar beet is the low sucrose affinity (apparent K_m >250 mM) of plant fructosyltransferases, contrasting with the substrate competitor invertases (K_m <50 mM). The sucrose concentration in the sugar beet vacuoles may reach 500 mM, but in other transformed plants it is below 40 mM [110]. Thus, the fructan enzymes were severely substrate-limited in the latter trans-formants. Another potential factor that could affect fructan accumulation is the degradation of 1-kestose and other short-chain FOS by the endogenous invertases.

Unlike levan-producing transgenics, the plants transformed with plant-derived fructosyltransferase genes showed neither phenotypic aberrations nor a reduction of agronomic yield. The conversion of highly productive crops that naturally accumulate sucrose, such as sugar beet and sugar cane, into fructan crops emerges as an attractive alternative for a more cost-effective production and wider commercialization of these increasingly demanded prebiotics.

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