Title: COMPOSITIONS AND METHODS FOR PRODUCING FERMENTABLE CARBOHYDRATES IN PLANTS

Abstract: Provided herein are methods for producing fermentable sugar obtained from a plant tissue. The methods include providing transgenic plant material comprising one or more locked carbohydrates and contacting plant material with an enzyme capable of converting the locked carbohydrate into a fermentable sugar. The methods are useful for providing sugar or sugar precursors for several industrial purposes including ethanol production. The invention also encompasses plants and plant parts that produce a lock enzyme to yield a locked carbohydrate, with the consequence of accumulating the locked carbohydrate in the plant. The invention also encompasses providing a key enzyme able to convert locked carbohydrates to fermentable sugars. Key enzymes can be provided by transgenic plants or plant parts, transgenic microbes, transgenic yeast, microbes or yeast.
COMPOSITIONS AND METHODS FOR PRODUCING FERMENTABLE CARBOHYDRATES IN PLANTS

REFERENCE TO SEQUENCE LISTING SUBMITTED ELECTRONICALLY

The official copy of the sequence listing is submitted concurrently with the specification as a text file via EFS-Web, in compliance with the American Standard Code for Information Interchange (ASCII), with a file name of "71825USPSP2 sequence Hsting.txt, created June 10, 2009, and a size of 313KB. The sequence listing filed via EFS-Web is part of the specification and is hereby incorporated in its entirety by reference herein.

FIELD OF THE INVENTION

This invention relates to plant molecular biology, particularly to methods and compositions for improving plants for obtaining commercially desirable harvested plant material, particularly for ethanol production.

BACKGROUND OF THE INVENTION

Plant biomass is comprised of sugars and represents the greatest source of renewable hydrocarbon on earth. Unlike other renewable energy sources, biomass can be converted directly into liquid fuels. The two most common types of biofuels are ethanol (ethyl alcohol) and biodiesel. Ethanol is an alcohol, which can be produced by fermenting any biomass high in carbohydrates (starches, sugars, or celluloses) once fermentable sugars have been obtained from the biomass material. Sugars generated from degradation of plant biomass could provide plentiful, economically competitive feedstocks for fermentation to produce chemicals, plastics, and fuels or any other product of interest.

Fuel ethanol could be made from crops which contain starch such as feed grains, food grains, and tubers, such as potatoes and sweet potatoes. Crops containing sugar,
such as sugar beets, sugarcane, and sweet sorghum also could be used for the production of ethanol. Sugar, in the form of raw or refined sugar, or as sugar in molasses requires no pre-hydrolysis (unlike corn starch) prior to fermentation. Consequently, the process of producing ethanol from sugar is simpler than converting corn starch into ethanol.

The yield and concentration of desired carbohydrates in plants are key determinants of the technical and economic feasibility of downstream industrial processes. However, the metabolic networks of plants for biosynthesis of sugars show substantial internal buffering and redundancy, with the consequence that alteration to a key gene in metabolism of a sugar commonly results in no useful change to the harvestable yield of the sugar (Moore, Australian Journal of Plant Physiology 22: 661-679 (1995); Nguyen-Quoc and Foyer, J of Experimental Botany 52: 881-889 (2001); Fernie et al., Trends in Plant Science 7: 35-41 (2002)).

SUMMARY OF THE INVENTION

Provided herein are methods for producing locked carbohydrates in a plant tissue by providing one or more carbohydrate-metabolizing enzymes that catalyze the conversion of an endogenous carbohydrate to a non-native carbohydrate. The invention encompasses plants and plant parts that produce one or more carbohydrate-metabolizing enzymes to yield a locked carbohydrate, with the consequence of increasing the total locked carbohydrate content in the plant. Further provided are hydrolytic enzymes (key enzymes) for converting the locked carbohydrate into a fermentable sugar. Fermentable sugars are used for a variety of industrial purposes including the production of ethanol.

DETAILED DESCRIPTION OF THE INVENTION

Overview

Plants accumulating large amounts of sugar are valuable as fermentation feedstocks for the downstream production of commercially-useful products. However, plants have various mechanisms to regulate the flow of sugars, therefore, sugar accumulation is limited in many plants. Plants contain both internal receptors and membrane-bound external receptors for monitoring sugar biosynthesis, transport, and
uptake (reviewed in Lalonde et al. (1999) Plant Cell 11:707-726). Intracellular receptors modulate metabolic processes such as photosynthesis. Extracellular receptors sense external sugar concentrations in order to control sugar influx from the surrounding environment. Thus, the plant cells are capable of maintaining sufficient levels of sucrose by regulating metabolic processes and sugar uptake.

Provided herein is a method for producing locked storage carbohydrates in plants so that they cannot be metabolized by the plant. The methods comprise introducing into the plant or plant part one or more enzymes capable of converting an endogenous sugar into a locked carbohydrate. By "endogenous sugar" or "native sugar" is intended a sugar that is normally produced by a particular variety of plant. In contrast, a "locked carbohydrate" or a "locked sugar" is one that is not produced under normal conditions of growth or development of that variety of plant or in a particular plant part or plant organelle. Expression of an enzyme capable of converting the endogenous sugar into a locked carbohydrate (which is herein referred to as a "lock enzyme") in a plant will allow accumulation of the locked carbohydrates in the plant. Because these locked carbohydrates are not metabolized in plants, they are unlikely to be subject to "futile cycles" of degradation and synthesis in the mature storage tissues, which have the potential to decrease storage efficiency and harvestable yield. Many of these oligosaccharides, polysaccharides, or monosaccharides will also evade the plant's carbohydrate detecting mechanisms, such as sucrose sensing, such that native and non-native carbohydrate synthesis may occur to compensate for decreases in endogenous carbohydrates which have been diverted into the locked carbohydrate storage pathway.

Recently, Wu and Birch, *infra*, have demonstrated that converting sucrose to the non-metabolized sucrose isomer isomaltulose allows accumulation of isomaltulose and sucrose providing combined sugar production in sugarcane. Isomaltulose is currently used to manufacture sugar alcohols consumed as low-calorie sweeteners (Schiweck et al. (1991) In F. W. Lichtenthaler (ed.), Carbohydrates as organic raw materials. Wiley-VCH, Weinheim, Germany), and it is an attractive renewable starting material for the manufacture of biosurfactants and biocompatible polymers (Lichtenthaler (2002) Accounts Chem. Res. 35:728-737).
The invention also comprises expressing hydrolytic enzymes capable of hydrolyzing the locked carbohydrates into fermentable sugars. These enzymes are herein referred to as "key enzymes." These enzymes may be of plant, bacterial, fungal, archean, or other origin; may be provided exogenously in an enzyme preparation, may be expressed in a separate line of plants or the same line of plants, or in yeast or other microbes, or may be provided in microbes that are used in a fermentative process converting fermentable sugars, carbohydrates or di, tri, oligo or polymeric saccharides to useful fermentation products. Fermentable sugars are carbohydrates which can be metabolized by conventional organisms such as yeast. Fermentation is the process of energy production in a cell and is not limited to the production of alcohols. Fermentation refers to the breakdown and re-assembly of biochemicals for industry in either aerobic or anaerobic growth conditions. It generally is the process of energy production in a cell and is not limited to the production of alcohols. Commonly known fermentable sugars include but are not limited to sucrose, glucose and fructose.

Commercial applications of the invention include the production of sugarcane, sugar beet, or other plants capable of producing locked carbohydrates. In some embodiments, accumulation of the normal storage carbohydrates (e.g., sucrose) is not affected in these plants. These plants or their extracts are then treated with enzyme preparations or with microbes or plant materials expressing key enzymes capable of hydrolyzing locked carbohydrates into fermentable sugar. These sugars could then be used in fermentation for many purposes including ethanol production or any other product of interest.

Thus, the methods of the invention find particular use in the integration of current practices for the cultivation of crop plants for the purpose of obtaining a commercially desired plant material with increased accumulation of carbohydrates (locked or native) in a plant, and the use of the crop plant or plant part as a source of biomass for the production of fermentable sugars, or for agricultural and/or human consumption.

By a "crop plant" is intended any plant that is cultivated for the purpose of producing plant material that is sought after by man for either oral consumption, or for utilization in an industrial, pharmaceutical, or commercial process. The invention may be applied to any of a variety of plants, including, but not limited to maize, wheat, rice,
barley, soybean, cotton, sorghum, oats, tobacco, strawberry, Miscanthus grass, Switch grass, trees, beans in general, rape/canola, alfalfa, flax, sunflower, safflower, millet, rye, sugarcane, sugar beet, cocoa, tea, Brassica, cotton, coffee, sweet potato, flax, peanut, clover; vegetables such as lettuce, tomato, cucurbits, cassava, potato, carrot, radish, pea, lentils, cabbage, cauliflower, broccoli, Brussels sprouts, peppers, and pineapple; tree fruits such as citrus, apples, pears, peaches, apricots, walnuts, avocado, banana, and coconut; and flowers such as orchids, carnations and roses.

As used herein, the term "plant part" or "plant tissue" includes plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, and plant cells that are intact in plants or parts of plants such as embryos, pollen, ovules, seeds, leaves, flowers, branches, fruit, kernels, ears, cobs, husks, stalks, roots, root tips, anthers, and the like.

The article "a" and "an" are used herein to refer to one or more than one (i.e., to at least one) of the grammatical object of the article. By way of example, "an element" means one or more element. Throughout the specification the word "comprising," or variations such as "comprises" or "comprising," will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

"Isolated" means altered "by the hand of man" from its natural state; i.e., that, if it occurs in nature, it has been changed or removed from its original environment, or both. For example, a naturally occurring polynucleotide or a polypeptide naturally present in a living animal in its natural state is not "isolated", but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. For example, with respect to polynucleotides, the term isolated means that it is separated from the chromosome and cell in which it naturally occurs. A sequence is also isolated if separated from the chromosome and cell in which it naturally occurs in but inserted into a genetic context, chromosome, or cell in which it does not naturally occur.
Locked carbohydrates

Sucrose is the major intermediary in carbon flux between source (photosynthetic) tissues and sink (growth and storage) tissues within plants, and it is the primary storage product in certain plants such as sugarcane and sugar beet. Plants have highly adapted sensors and transporters for sucrose, but it is generally considered that these sucrose sensors and transporters are not able to respond in the same way to locked carbohydrates (Loreti et al., Plant Physiol 123: 939-948 (2000); Sinha et al., Plant Physiol 128: 1480-1489 (2002)). In stark contrast with sucrose, plants are unable to metabolize these locked carbohydrates as a source of carbon and energy (Sinha et al, 2002).

While not bound by any particular theory or mechanism, specific alterations to metabolism, involving the conversion of a carbohydrate normally sensed by the plant into a locked carbohydrate that is not perceived in an equivalent manner, can shift metabolism and result in the accumulation of higher concentrations of locked carbohydrates or, in some cases, accumulation of higher concentrations of total carbohydrates.

Thus, provided herein are methods for the expression in a plant of an enzyme capable of converting an endogenous sugar into a locked sugar. The endogenous sugars produced by different plants may differ and as such an endogenous sugar of one plant may be non-native to another. Where the sugar is non-native to a particular plant, that plant is a candidate for production of a locked carbohydrate using the methods of the invention. Also, a non-native carbohydrate may also refer to a carbohydrate that is not normally produced in a particular subcellular compartment, or in a particular plant part of the native plant. In this embodiment, the subcellular compartment or the plant part would normally not be capable of metabolizing or transporting out of the compartment or plant part any non-native carbohydrate produced therein. Thus, it is essential to determine which carbohydrates are endogenously produced by a chosen plant or plant part to thereby deduce which carbohydrates are non-native to the plant and the type of carbohydrate-metabolizing enzyme(s) that could be useful for producing a locked carbohydrate in the plant.

For example, amylose (i.e., a type of starch) is a polysaccharide consisting of glucosyl residues linked by alpha-(1-4) bonds and is the primary carbohydrate storage compound found in most plants. Producing starch in plants that use sucrose as their
primary carbohydrate storage compound, such as sugarcane, may permit the
accumulation of starch which would behave as a "locked" sugar (i.e., sugar that cannot be
metabolized by the plant).

The types of carbohydrates endogenously produced by plants can be determined
using methods well known to persons of skill in the art. These methods include
separation of sugars or sugar derivatives by electrophoresis or chromatography (including
paper chromatography, thin layer chromatography, gas chromatography, gas-liquid
chromatography and high-performance liquid chromatography) techniques. The separated
components are typically identified by comparison of separation profiles with standards
of known identity, or by analytical techniques such as mass spectrometry and nuclear
magnetic resonance spectroscopy. See, for example, reference may be made to Robinson
1980, The Organic Constituents of Higher Plants, Cordus Press, North Amherst, USA;
et al. 2001, Carbohydrate Res. 331:149-161; each of which is incorporated by reference
herein for their teachings regarding analysis of sugar content.

The endogenous or the non-native carbohydrates may include monosaccharides,
oligosaccharides, sugar alcohols, sugar acids, amino sugars or other variants such as
deoxy sugars, methyl sugars and the like. Examples of monosaccharides include
compounds with formula (CH₂O)ₙ where n=3 or more but suitably less than 10;
including compounds comprising tetroses (e.g., erythrose, threose, erythrulose), pentoses
(e.g., ribose, arabinose, xylose, lyxose, ribulose, xylulose), hexoses (e.g., allose, altrose,
glucose, mannose, gulose, idose, galactose, talose, psicose, fructose, sorbose, tagatose),
and longer molecules such as sedoheptulose or mannoheptulose. Oligosaccharides, which
are formed by linking together two or more monosaccharide units through glycosidic
bonds, may be selected from disaccharides (e.g., maltose, lactose, gentibiose, melibiose,
trehalose, sophorose, primoverose, rutinose, sucrose, isomaltulose, trehalulose, turanose,
maltulose, leucrose, 2-keto-sucrose) and longer oligomers such as raffinose, melezitose,
isobemisiose or stachyose. Examples of sugar alcohols include, but are not limited to,
erythritol, ribitol, mannitol, sorbitol. Non-limiting examples of sugar acids include
gluconic acid, glucaric acid, glucuronic acid. Non-limiting examples of amino sugars
include glucosamine, galactosamine. Endogenous or non-native sugars may also be
selected from other variants such as deoxy sugars and methyl sugars. Further
encompassed are isobemisiose, tagatose, isomaltotriose, dextrin, cyclodextrins, lactose,
verbascoe, amylose, and rhamnose.

*Isomaltulose and trehalulose*

In certain embodiments, the locked carbohydrate is an isomer of the endogenous
carbohydrate. In one example of this embodiment, the endogenous sugar is sucrose and
the sugar-metabolizing enzyme is a sucrose isomerase, which converts the sucrose by
isomerization to a locked sugar selected from isomaltulose and trehalulose. Isomaltulose
\( \alpha \)-D-glucopyranosyl-1,6-D-fructofuranose (also called palatinose) is a nutritive
disaccharide, with sweetness and bulk similar to sucrose. Several characteristics make
isomaltulose advantageous over sucrose for some applications in the food industry: 1) noncariogenic (not causing dental decay); 2) low glycemic index (useful for diabetics); 3) selective promotion of growth of beneficial bifidobacteria among human intestinal microflora; 4) greater stability of isomaltulose-containing foods and beverages; 5) less hygroscopic; 6) simple conversion into sugar alcohols with other useful properties as foods.

Sucrose isomerases (E.C. 5.4.99.1) are enzymes produced by organisms
including various microbes, with the capability to convert the disaccharide sucrose into
isomers such as isomaltulose (palatinose) or trehalulose. Sucrose isomerases vary in their
properties including the disaccharide reaction products, the proportion of
monosaccharides such as glucose and fructose in the reaction products, the kinetic
properties of the enzymes, the optimal reaction conditions, and the sensitivity of the
enzyme to variations from the optimal conditions (Veronese and Perlot, Enzyme. Microb.
Technol 24: 263-269 (1999)). An isolate of *Pantoea dispersa* designated UQ68J is
Microbiol. 97:93-103). Another exemplary sucrose isomerase has been isolated from
*Erwinia carotovora* (GENBANK Accession No. YP049947).

*Dexiram andfructam*
This invention also comprises transforming plants with one or more genes involved in the synthesis of fructans or dextrans. These genes may come from plant, bacterial, or fungal sources and should catalyze the formation of fructose and glucose polysaccharides or polysaccharides comprised of mixed sugars that are found in cane or sugar beet, sweet sorghum, mangel-wurzel or other sugar crops. The oligo- or polysaccharides produced may also comprise mixed sugar monomers, for example glucose, fructose, mannose and galactose.

By producing these fructan, dextran and mixed fructan and dextran carbohydrates in plants whose primary storage carbohydrate is sucrose, such as sugarcane and sugarbeet, a method for sequestering carbohydrates is provided in a form that is non-metabolizable for the plant. Such compounds may evade the sucrose sensing mechanisms of the plant so that they can be accumulated for later enzymatic hydrolysis to fermentable sugars.

Dextran is a collective name for high-molecular-weight polymers composed of D-glucose units connected with alpha-1,6 linkages and various amounts of side branches linked with alpha-1,2, alpha-1,3, or alpha-1,4 to the main chains. The enzymes that synthesize these glucans from sucrose are known by the generic term dextransucrase (1^-alpha-D-glucan-6-alpha-glucosyltransferase, EC2.4.1.5.). The biosynthesis of dextran has been demonstrated in numerous bacteria, especially in Streptococcus mutans, Leuconostoc mesenteroides ssp. mesenteroides and Leuconostoc mesenteroides ssp. dextranicum. Leuconostoc produce the enzyme dextran sucrase and secrete it into the culture medium in the presence of sucrose. This enzyme, dextran sucrase, then synthesizes dextran from the sucrose substrate. Dextran has applications in several fields. It is used especially in biochemistry as a support for filtration chromatography on a gel of the Sephadex type. Additionally, in the field of therapeutics, it is used as a substitute for blood plasma (Biochimie generale (General Biochemistry)-- J. H. WEIL-Masson, 6th edition--1990-p. 171).

Exemplary dextransucrase enzymes include (but are not limited to): the dextransucrase from Streptococcus downei, gFB gene (Gilmore et al. (1990) Infect. Immun. 58 (8), 2452-2458; GENBANK Accession No. P29336); the dextranase from Streptococcus mutans, gF gene, produces a 1.3 glucose soluble dextrans (Shiroza

There is no common class of enzymes identified as "Leucrose synthases." Instead leucrose \( O\text{-alpha-D-glucopyranosyl-(1} \rightarrow 5\text{-)} \)\( D\)-fructopyranoside] is generally a byproduct of dextranucrase enzyme (EC 2.4.1.5) activity. These enzymes act as glucosyltransferases, and normally transfer a glucose unit hydrolyzed from a sucrose molecule to a growing dextran chain, or in the case of leucrose to a pyranosyl-fructose molecule yielding leucrose. Glucose can also serve as an acceptor for the transglucosylase reaction resulting in isomalto (O-\( \alpha\text{-D-glucopyranosyl-} \alpha[1-6]\text{-} \alpha\text{-D-glucopyranoside}) production. Since the 1950's leucrose has been made enzymatically typically using the *Leuconostoc mesenteroides* dextranucrase (The Preparation, Properties and Structure of the Disaccharide Leucrose Journal of the American Chemical Society, Stodola et. al; (1956) 78: 2415) followed by chemical purification.

Dextranucrases can be mutated to produce more leucrose and or turanose. This has been shown for the dextranucrase of *Streptococcus oralis* (Engineering the Glucansucrase GTFR Enzyme Reaction and Glycosidic Bond Specificity: Toward Tailor-Made Polymer and Oligosaccharide Products, *Biochemistry* 2008, 47, 6678-6684, Hendrik Hellmuth et. al). Since dextranucrases can be mutated to produce leucrose it is reasonable to assume that other related enzymes (e.g. amylosucrases EC 2.4.1.4) or unrelated enzymes that also produce sucrose isomers could be mutated to produce leucrose. Leucrose synthase activity is attributed to any enzyme that produces leucrose by any mechanism, i.e. isomerization, transglycosylation, hydrolysis, dehydrogenation, reduction, etc.

The production of leucrose can be assayed using HPAE chromatography with pulsed amperometric detection (PAD). This technique is widely accepted as a preferred method for separating carbohydrates and is effective in separating sucrose isomers. Comparison of peak elution times with known standards is one method for determining the presence of leucrose. Full verification of the bond arrangements in the carbohydrate molecules can be determined either by methylation and acetylation of leucrose followed
by GC-MS, or directly by NMR spectroscopy if the samples are of sufficient quantity and purity.

Sucrose:sucrose fructosyltransferase (SST) (EC 2.4.1.99), 1,2-β-fructan 1-fructosyltransferase (FFT) (EC 2.4.1.100), 2-β-fructan 1-fructosyltransferase (FFT) (EC 2.4.1.100), glucan sucrase, and levan sucrase (EC 2.4.1.10) are enzymes within the larger class of fructosyl transferases. The fructosyl transferase enzymes catalyze the formation of fructans composed of fructose linked by β(2→1) and/or β(2→6) glucoside bonds. Fructosyl transferases may be identified and isolated from plant, bacterial, or fungal sources. These enzymes may be expressed in plants to accumulate fructans as storage carbohydrates. Accumulation of this polysaccharide (fructan) in sugarcane or other plants may allow the accumulation of excess carbohydrates.

Inulin is a fructan type carbohydrate polymer which occurs as a polydisperse composition in many plants and can also be produced by certain bacteria and fungi. Inulin from plant origin consists of a polydisperse composition of mainly linear chains composed of fructose units, mostly terminating in one glucose unit, which are linked to each other through β(2-1) fructosyl-fructose linkages.

Inulin molecules are synthesised by the concerted action of two enzymes: sucrose:sucrose 1-fructosyltransferase (in short 1-SST enzyme or 1-SST, used interchangeably) and fructan:fructan 1-fructosyltransferase (in short 1-FFT enzyme or 1-FFT, used interchangeably) (Koops and Jonker, J of Experimental Botany 45: 1623-1631 (1994); and Koopos and Jonker, Plant Physiol 110: 1167-1 175 (1996)). Both 1-SST and 1-FFT are active during the period of inulin synthesis and accumulation: 1-SST catalyses the initial reaction of inulin biosynthesis, the conversion of sucrose into the smallest inulin molecule, the trisaccharide kestose (GFF). 1-FFT catalyzes the redistribution of terminal fructosyl units (-F) between inulin molecules, which results in a stepwise increase in chain length.

**Amylose**

This invention further comprises transforming plants with one or more genes involved in the synthesis of novel carbohydrates such as amylosucrase (E.C. 2.4.1.4) to produce amylose in order to accumulate carbohydrates for later fermentation into ethanol.
Examples of enzymes that may catalyze the desired conversions include isomerases, epimerases, mutases, kinases, aldolases, transferases, transketolases, phosphatases, synthases, carboxylases, dehydrogenases and hydrolases. An exemplary amylosucrase includes the enzyme produced by *Neisseriapolysacharea* (GENBANK Accession number Q9ZEU2), which catalyzes the conversion of sucrose to a linear alpha-1,4-linked glucan.

*Alieman*

*Alternan* is a polysaccharide consisting of glucosyl residues linked by alternate alpha-(1-3)/alpha-(1-6) bonds. This polymer is highly soluble and has very low viscosity. Accumulation of this polysaccharide in sugarcane or other plants may allow the accumulation of excess carbohydrates.

*Alteransucrase* is an enzyme which catalyzes the conversion of sucrose to alternan. *Alteransucrase* is encoded by the *Asr* gene of *Leuconostoc mesenteroides* described in Jeannes et al. (1954) Am Chem Soc 76:5041-5052.

**Key enzymes**

The invention also comprises expressing hydrolytic enzymes capable of hydrolyzing the locked carbohydrates into fermentable sugars. These enzymes are herein referred to as "key enzymes." These enzymes may be of plant, bacterial, fungal, archael, or other origin; may be provided exogenously in an enzyme preparation, may be expressed in a separate line of plants or the same line of plants, or in yeast or other microbes, or may be provided in microbes that are used in a fermentative process to convert the locked carbohydrates into fermentable sugars. Yeast or microbes used in the fermentative process may also be identified or engineered to convert locked carbohydrates to energy. Furthermore, the locked carbohydrates may be converted to a fermentable sugar by chemical methods, e.g., by one or more chemicals capable of converting a locked carbohydrate into a fermentable sugar. The chemical(s) can be added prior to fermentation, or during the fermentation process.

Key enzymes can be isolated from, produced by, provided by a wide range of sources. Recombinant organisms such as plants, microbes or yeast, can be engineered to
express a key enzyme. The recombinant organism can be used directly in a method of converting locked carbohydrates to fermentable sugars without further purification of the enzyme. Alternatively, key enzymes may be isolated from recombinant organisms for further use in the processing of locked carbohydrates. Native sources for key enzymes may also be used either directly (such as yeast or microbes which express a key enzyme normally) or by further isolation of the key enzyme. A key enzyme may be provided by a source selected from the group consisting of transgenic plant expressing one or more key enzymes, recombinant microbe expressing one or more key enzymes, transgenic yeast expressing one or more key enzymes, microbe expressing one or more key enzymes, and yeast expressing one or more key enzymes.

Isomaltulose and trehalulose can be hydrolyzed by alpha-1,6-glucosidase enzymes. Exemplary glucosidase enzymes are set forth in SEQ ID NO: 1-6 herein. Additional sequences are described in United States Patent 5,786,140, and in Børnke et al (2001) Journal of Bacteriology 183(8):2425-2430, each of which is herein incorporated by reference in its entirety.

Dextran-degrading enzymes form a diverse group of different carbohydrates and transferases. These enzymes have often been classified as endo- and exodextranases based on the mode of action and commonly called dextranases and include enzymes such as dextranases (EC3.2.1.1), glucan-1,6-alpha-D-glucosidases (EC3.2.1.70), glucan-1,6-alpha-isomaltosidases (EC3.2.1.94), dextran 1,6-alpha-isomaltotriosidases (EC3.2.1.95), and branched-dextran exo-1,2-alpha-glucosidases (EC3.2.1.15).

Exodextranases, such as glucodextranase (EC3.2.1.70; glucan 1,6-alpha-glucosidase), catalyze stepwise hydrolysis of the reducing terminus of dextran and derived oligosaccharides to yield solely alpha-D-glucose; i.e., hydrolysis is accompanied by inversion at carbon-1 in such a way that new reducing ends are released only in the alpha-configuration. Some bacteria and yeasts are known to produce glucodextranases. Dextran-inducible extracellular glucodextranase occurs in Arthrobacter globiformis strains 142 and T-3044 (Oguma and Kobayashi (1996) J. Appl. Glycosci. 43:73-78; Oguma et al. (1999) Biosci. Biotechnol. Biochem. 63:2174-2182).

Intracellular dextran glucosidases (EC3.2.1.) producing alpha-D-glucose from dextran exist in several strains of Streptococcus mitis (Linder and Sund (1981) Caries
The soil bacterium *A. globiformis* T6 isomaltodextranase (EC 3.2.1.94; 1,6-alpha-D-glucan isomaltohydrolase) is an extracellular exoenzyme capable of hydrolyzing dextran by removing successive isomaltose units from the nonreducing ends of the dextran chains (Sawai and Yano (1974) J. Biochem. 75:105-112; Sawai and Nawa (1976) Agric. Biol. Chem. 40:1246-1250).

Branched dextran exo-1,2-alpha-glucosidase (EC 3.2.1.15) was found in the culture supernatant of the soil bacterium *F. javohacterium* sp. strain M-73 by Mitsuishi et al. (1979) Agric. Biol. Chem. 43:2283-2290. The enzyme had a strict specificity for 1,2-alpha-D-glucosidic linkage at the branch points of dextrans (containing 12 to 34% of 1,2-alpha linkages) and related polysaccharides producing free D-glucose as the only reducing sugar.

A list of additional exemplary microbial dextran-hydrolyzing enzymes and their substrate specificities and hydrolysis products is provided in Khalikova et al. (2005) Microbiology and Molecular Biology Reviews 2005:306-325, which is herein incorporated by reference as it describes and lists various dextran-hydrolyzing enzymes.

Fructanases are fructosydases which catalyze the hydrolysis of fructosidic linkages in fructans to break the fructan down into simpler sugar molecules. Fractans can be hydrolyzed to fermentable sugars through the catalytic activity of fructanases. For example, the fructanase 2,1-β-D-fructan fructanohydrolase [EC 3.2.1.7] can hydrolyze fructan polymers into fructose monosaccharides which can be fermented to form ethanol.

Inulin can be converted to a fermentable carbohydrate using one or more inulase enzymes. Microbial inulinases (2,1-3-D-fructan fructanohydrolase [EC 3.2.1.7]) are usually inducible and exo-acting enzymes, which catalyze the hydrolysis of inulin by splitting off terminal fructosyl units (D-fructose).

Alternans can be hydrolyzed to form fermentable sugars by the activity of a alpha-1,6-glucosidase or alpha-1,3-glucosidase.

**Methods**
Provided herein are methods for improving the yield of carbohydrate in plants by expressing an enzyme capable of converting endogenous carbohydrate into locked carbohydrate. The locked carbohydrates accumulated in the plants described herein can be converted to fermentable carbohydrates using one or more of the key enzymes disclosed herein, which can then be used as fermentation feedstocks for ethanol, propanol, butanol or other fuel alcohol, ethanol-containing beverages (such as malted beverages and distilled spirits), and other fermentation products such as foods, nutraceuticals, enzymes and industrial materials. The methods for fermentation using plant-derived carbohydrate feedstocks are well known to those skilled in the art, with established processes for various fermentation products (see for example Vogel et al. 1996, Fermentation and Biochemical Engineering Handbook: Principles, Process Design, and Equipment, Noyes Publications, Park Ridge, NJ., USA and references cited therein). Key enzyme proteins could also be incorporated into the ethanol production process downstream of the feedstock step. It is envisioned that locked carbohydrates could be harvested and, in the process of making ethanol, the key enzyme is added during the production process. Key enzyme proteins could also be incorporated into the fermentable sugar production process downstream of the feedstock step. It is envisioned that locked carbohydrates could be harvested and, in the process of making fermentable sugar, the key enzyme is added during the production process.

In one embodiment, the use of the methods disclosed herein results in a substrate that leads to higher ethanol yields compared to the ethanol yield from plant material not accumulating locked carbohydrates. The increase in ethanol yield can be at least about 1%, at least about 2%, at least about 3%, at least about 4%, at least about 5%, at least about 6%, at least about 7%, at least about 8%, at least about 9%, at least about 10%, at least about 20%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, or greater. Even small increases in ethanol yield will translate to large volumes of ethanol produced over time in a commercial-scale fermentation process. Such improvements in ethanol production could result in a significant increase in profit to the ethanol producer.
In one embodiment, the use of the methods disclosed herein results in a substrate that leads to higher carbohydrate yields compared to the carbohydrate yield from plant material not accumulating locked carbohydrates. The increase in carbohydrate yield can be at least about 1%, at least about 2%, at least about 3%, at least about 4%, at least about 5%, at least about 6%, at least about 7%, at least about 8%, at least about 9%, at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least about 100%, at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, or greater. Even small increases in carbohydrate yield will translate to large volumes of carbohydrate produced over time in a commercial-scale fermentation process. The carbohydrate may be sucrose or a combination of sucrose and a locked sugar.

In another embodiment, the plants accumulating locked carbohydrates can be used in various other downstream products other than ethanol production. Locked carbohydrates can be converted into fermentable sugars which are used in many commercial fermentation processes including growing recombinant yeast which produce important chemicals such as insulin, antibodies, or enzymes. Isomaltulose is currently used to manufacture sugar alcohols consumed as low-calorie, non-cariogenic sweeteners. Fructose also has value as a sweetener in high fructose syrups such as high fructose corn syrup. Plants engineered to produce fructans as a locked sugar may be used as a source of fructans which, after hydrolysis by a fructanase enzyme, produce a solution with a high fructose concentration. In such plants the yield of fructan may be increased by expressing an additional enzyme (e.g., glucose isomerase) to catalyze the conversion of glucose to fructose. The glucose isomerase (invertase) could be expressed in maize endosperm, or expressed in microbes. The purified enzyme could be used to produce fructans, glucans and alternans.

Sweeter plant products can be generated by expressing in plants a combination of enzymes that first allow for the accumulation of fructans in the plant and then convert the fructans directly or indirectly to fructose. Expressing invertase (glucose isomerase) in plants accumulating fructans will lead to a higher sweetness index in the plant.
In another embodiment, plants accumulating locked carbohydrates as described herein are useful for providing protection of the plant against disease. While not being bound by any particular theory or mechanism, plants accumulating locked sugars may be more tolerant or resistant to microbial infection due to the presence of carbohydrates other than sucrose, since infection by some microbes depends upon the content of sucrose in the plant.

**Enzyme extracts for key enzyme**

In various embodiments of the present invention, the enzyme capable of converting the locked carbohydrate to a fermentable carbohydrate (referred to herein as the "key" enzyme) is provided as a purified or partially-purified preparation of the enzyme. The exogenously-added key enzyme may be *de novo* synthesized, or may be isolated from an organism expressing the enzyme prior to addition of the enzyme to the locked carbohydrate-containing plant material.

A purified or semi-purified preparation of enzyme will contain at least one class of key enzyme, but may also contain one or more additional enzymes of the same or different class. The preparation may further comprise one or more additional enzymes useful in the starch conversion method, such as amylase or glucoamylase. A "semi-purified" enzyme preparation will contain one or more key enzymes, one or more additional enzymes useful in the starch conversion process, or may contain other buffers or stabilizing agents (e.g., glycerol). Furthermore, the semi-purified enzyme preparation may also be culture supernatant or crude extract collected from a cell population expressing and/or secreting the enzyme. The preparation may also be a lyophilized formulation of enzyme that is reconstituted upon addition to the locked carbohydrate-containing plant material.

The various key enzymes discussed herein can be expressed in and isolated from any number of eukaryotic and prokaryotic organisms. Appropriate expression cassettes, vectors, transformation, and transfection techniques for a particular organism of interest will be evident to one of skill in the art.

In one embodiment, bacterial cells, such as *E. coli*, *Streptomyces*, *Bacillus subtilis*; and various species within the genera *Escherichia*, *Pseudomonas*, *Serratia*,
Streptomyces, Corynebacterium, Brevibacterium, Bacillus, Microbacterium, and Staphylococcus can be used as a host to express one or more classes of key enzymes encompassed herein. Methods for transformation of bacterial hosts are described in, for example, U.S. Patent Publication No. 2003/0135885.

In another embodiment, fungal hosts, such as fungal host cells belonging to the genera Aspergillus, Rhizopus, Triehoderma, Newospora, Mucor, Penicillium, etc., such as yeast belonging to the genera Kluyveromyces, Saccharomyces, Schizosaccharomyces, Trichosporon, Schwanniomyces, etc. may be used. Transformation of fungus may be accomplished according to Gonni et al. Agric. Biol. Chem., 51:2549 (1987).

Another suitable host includes any number of eukaryotic cells, for example, insect cells such as Drosophila S2 and Spodoptera Sf9; animal cells such as CHO, COS or Bowes melanoma, C127, 3T3, CHO, HeLa and BHK cell lines. Any host can be used insofar as it can express the gene of interest. The American Type Culture Collection (http://www.atcc.org/) maintains cell lines from a wide variety of sources and many of these cultures can be used to generate a transgenic cell line capable of expressing a heterologous enzyme. Transformation vectors appropriate for eukaryotic cells are available commercially such as pXT1, pSG5 (Stratagene) pSVK3, pBPV, pMSG, and pSVLSV40 (Pharmacia). Techniques for transformation and selection of transgenic eukaryotic cells are well known in the art. Exemplary methods are also described elsewhere herein.

In another embodiment, the key enzymes can be isolated from an organism that endogenously expresses the enzyme, or the organism expressing the enzyme can be used in one or more fermentation steps without the need for purification or isolation of the enzyme from the organism.

Transgenic plants

In one embodiment of the present invention, the locked carbohydrate-containing plant material comprises plant parts derived from at least one variety of a transgenic plant expressing at least one polynucleotide encoding a lock enzyme. In another embodiment, the transgenic plant material expresses more than one lock enzyme, resulting in the accumulation of more than one type of locked carbohydrate. In yet another embodiment, both the lock and the key enzymes are expressed in plant material. Where both the lock and the key enzymes are provided as transgenic plant material, each class of enzyme may be expressed in the same plant variety, or may be expressed in different plant varieties.

As used herein the term "transgenic" refers to plants that include an exogenous polynucleotide (e.g., gene) that is stably maintained in the transformed plant and is stably inherited by progeny in successive generations. The term "transgenic plant" can refer either to the initially transformed plant or to the progeny of the initially transformed plant. Techniques for transforming plants, plant cells or plant tissues can include, but are not limited to, transformation with DNA employing A. tumefaciens or A. rhizogenes as the transforming agent, electroporation, DNA injection, microprojectile bombardment, and particle acceleration. See, for example, EP 295959 and EP 138341. As used herein, the terms "plant material" or "plant part" includes plant cells, plant protoplasts, plant cell tissue cultures from which plants can be regenerated, plant calli, plant clumps, and plant cells that are intact in plants or parts of plants such as embryos, pollen, ovules, seeds, leaves, flowers, branches, fruit, kernels, ears, cobs, husks, stalks, roots, root tips, anthers, tubers, rhizomes and the like.

Where both the lock and the key enzymes are provided by transgenic plant material, it is not necessary for the plant material expressing the key enzyme to be 100% transgenic for the key enzyme. Rather, it is only necessary for the plant material to contain an amount of key enzyme that is sufficient for the downstream use (e.g., for conversion of locked carbohydrates to fermentable sugars). For example, for fermentation purposes, a sufficient amount of the key enzyme may be provided in the fermentation process by less than 100% key enzyme-expressing plant material. For example, a sufficient amount of key enzyme may be provided to the fermentation process when only about 0.1% of the locked carbohydrate-containing plant material expresses the
key enzyme, or only about 1%, about 2%, about 3%, about 4%, about 5%, about 6%, about 7%, about 8%, about 9%, about 10%, about 11%, about 12%, about 13%, about 14%, about 15%, about 16%, about 17%, about 18%, about 19%, or about 20%, of the plant material. However, it is contemplated that the percentage of plant material expressing the key enzyme could be as much as 100%, including, for example, about 25%, about 30%, about 35%, about 40%, about 50%, about 60%, about 65%, about 70%, about 80%, about 90%, about 95%, or about 99% of the plant material.

The methods of the invention are particularly useful in plants producing high amounts of sugar, such as (for example), sugarcane, sugar beet, and sorghum. However, the plant material can be derived from any plant, including but not limited to plants producing edible flowers such as cauliflower (Brassica oleracea), artichoke (Cynara scoivmus), and safflower (Carthamus, e.g. tinctorius); fruits such as apple (Malus, e.g. domesticus), banana (Musa, e.g. acuminata), berries (such as the currant, Ribes, e.g. rubrum), cherries (such as the sweet cherry, Prunus, e.g. avium), cucumber (Cucumis, e.g. sativus), grape (Vitis, e.g. vinifera), lemon (Citrus limon), melon (Cucumis melo), nuts (such as the walnut, Juglans, e.g. regia; peanut, Arachis hypoaeae), orange (Citrus, e.g. maxima), peach (Prunus, e.g. persica), pear (Pyra, e.g. communis), pepper (Solanum, e.g. capsicum), plum (Prunus, e.g. domestica), strawberry (Fragaria, e.g. moschata), tomato (Lycopersicon, e.g. esculentum); leafs, such as alfalfa (Medicago, e.g. sativa), sugar cane (Saccharum), cabbages (such as Brassica oleracea), endive (Cichoreum, e.g. endivia), leek (Allium, e.g. porrum), lettuce (Lactuca, e.g. sativa), spinach (Spinacia e.g. oleracea), tobacco (Nicotiana, e.g. tabacum); roots, such as arrowroot (Maranta, e.g. arundinacea), beet (Beta, e.g. vulgaris), carrot (Daucus, e.g. carota), cassava (Manihot, e.g. esculenta), turnip (Brassica, e.g. rapa), radish (Raphanus, e.g. sativus) yam (Dioscorea, e.g. esculenta), sweet potato (Ipomoea batatas); seeds, such as bean (Phaseolus, e.g. vulgaris), pea (Pisum, e.g. sativum), soybean (Glycine, e.g. max), wheat (Triticum, e.g. aestivum), barley (Hordeum, e.g. vulgare), corn (Zea, e.g. mays), rice (Oryza, e.g. sativa); grasses, such as Miscanthus grass (Miscanthus, e.g., giganteus) and switchgrass (Panicum, e.g. virgatum); trees such as poplar (Populus, e.g. tremula), pine (Pinus); shrubs, such as cotton (e.g., Gossypium hirsium); and tubers, such as kohlrabi (Brassica, e.g. oleracea), potato (Solanum, e.g. tuberosum), and the like.
The locked carbohydrate-containing plant material may also comprise one or more varieties of plants having naturally-occurring genetic variability resulting in altered starch metabolism. Many such plants carry mutations in genes encoding isoforms of starch synthesis or starch degradation enzymes. For example, plants have been identified which are heterozygous or homozygous for one or more of the waxy (wx), amylose extender (ae), dull (du), horny (h), shrunken (sh), brittle (bt), floury (fl), opaque (o), or sugary (su) mutant alleles. See, for example, US Patent Nos. 4,428,972; 4,767,849; 4,774,328; 4,789,738; 4,789,557; 4,790,997; 4,792,458; 4,798,735; and 4,801,470, herein incorporated by reference.

**Dual expression of lock enzymes**

The invention also comprises the simultaneous expression of two lock enzymes such as two sucrose isomerases, one that produces predominantly isomaltoolose, and one that produces predominantly trehalulose, so that both isomers of sucrose may be accumulated in the same plant. Sugarcane possesses an excess capacity for carbohydrate synthesis, however, there is a continuous "futile cycle" of sucrose synthesis and breakdown in sugarcane. By diverting carbohydrates into a form that is not metabolized by the plant, these carbohydrates may be removed from that futile cycle, and the plant may make up for the loss by producing more sucrose. The fact that Wu and Birch have seen isomaltoolose accumulate to the same level as sucrose, without decreasing the amount of sucrose, suggests that this excess capacity of sugarcane for sugar synthesis has not been exhausted. By genetically modifying sugarcane with two or more lock enzymes that produce more than one isomers of sucrose (isomaltoolose, trehalulose, leucrose, etc.) at equivalent levels it may be possible to significantly increase the total sugar content in sugarcane, or to increase the level of locked sugar in the sugarcane.

In one embodiment, the total carbohydrate content, or the total locked carbohydrate content, or both, is increased at least about 10%, at least about 20%, at least about 50%, at least about 100%, at least about 125%, at least about 150%, at least about 2-fold, at least about 3-fold, at least about 4-fold or greater when compared to the same variety of plant that does not accumulate locked carbohydrate according to the methods of the invention.
Sucrose isoraerase enzymes producing predominantly isoraaltulose include, for example, the \textit{P. dispersa} UQ68J enzyme described in U.S. Patent No. 7,250,282, which is herein incorporated by reference in its entirety. Other enzymes producing predominantly trehalulose include, for example, the whitefly enzyme characterized by Salvucci (2003) Comp. Biochem. Physiol. B 135:385-395. While not to be limited by theory, the whitefly enzyme may be a representative of the lock enzyme trehalulose synthase.

\textit{Subcellular targeting}

For the purpose of producing starch in a transgenic plant, it may be advantageous to target the lock enzyme in the plant to subcellular compartments that have high concentrations of sucrose, such as the vacuole of sugarcane. Another target may be the vacuole of the maize endosperm. Targeting an enzyme capable of synthesizing starch from sucrose to the vacuole of maize endosperm cells may permit the accumulation of more starch in the maize endosperm as naturally occurring enzymes do not produce starch in the vacuoles of maize endosperm cells. Alternatively targeting to the apoplast is another way to achieve conversion of sucrose into locked sugars such as starch or isomaltulose. In plants such as maize, sucrose accumulates in the leaf and is transported to the ear during grain filling which provides a carbon sink.

In one embodiment, the lock enzyme is targeted to the amyloplast, where locked carbohydrate can accumulate, and the key enzyme (when expressed in the same plant) is targeted to the apoplast. The key enzyme can be targeted to the apoplast using, for example, the maize Gamma zein N-terminal signal sequence, which confers apoplast-specific targeting of proteins. The lock enzyme may be targeted to the amyloplast by, for example, fusion to the waxy amyloplast targeting peptide (Klosgen et al., 1986) or to a starch granule. For example, the polynucleotide encoding the lock enzyme may be operably linked to a chloroplast (amyloplast) transit peptide (CTP) and a starch binding domain, e.g., from the waxy gene.

Directing the key enzyme to the apoplast will allow the enzyme to be localized in a manner that it will not come into contact with the locked carbohydrate substrate. In this manner the enzymatic action of the enzyme will not occur until the enzyme contacts its
substrate. The enzyme can be contacted with its substrate by the process of milling (physical disruption of the cell integrity), or heating the cells or plant tissues to disrupt the physical integrity of the plant cells or organs that contain the enzyme. For example the key enzyme can be targeted to the apoplast or to the endoplasmic reticulum so as not to come into contact with the locked carbohydrate in the amyloplast. Milling of the grain will disrupt the integrity of the grain and the key enzyme will then contact the starch granules. In this manner the potential negative effects of co-localization of an enzyme and the locked carbohydrate can be circumvented.

*Locked carbohydrates as selectable markers*

Plant transformation requires the use of positive selectable marker genes for identification and propagation of transformed tissue and the elimination of non-transformed tissue. One advantage of this system would be the ability to select and/or screen for expression and/or accumulation of the key enzyme involved in the breakdown of the locked carbohydrates, from the very earliest stages of the plant transformation process. A transformation system using the desired enzyme end product as a means of initial selection would permit early screening for position effects or genomic insertion sites that lead to high level or constitutive expression of the transgene. Also, the use of the desired end product as the selectable marker can reduce the number of genes that must be transferred into the plant. This will reduce the size of the T-DNA needed for transformation and be useful in the production of "molecular stacks" in which multiple transgenes are desired in a single transgenic plant, i.e., eliminate the need for an extraneous selectable marker gene such as PMI, or antibiotic resistance genes that are necessary for production of transgenic plants, but are no longer useful to the plant after transformation/selection. However, it is contemplated that multiple selectable markers can be used in the methods of the invention, including those used solely for selection.

In one embodiment, an alpha-1,6-glucosidase enzyme may be used to cleave the alpha-1,6-glucoside linkage between glucose and fructose in the disaccharide isomaltulose. This enzyme is desirable for converting isomaltulose produced by transgenic sugarcane plants into fermentable sugar or ethanol and may be useful as a novel selectable marker for sugarcane transformation.
Expression Cassettes

A plant or plant part expressing a lock and/or key enzyme can be obtained by introducing into the plant or plant part a heterologous nucleic acid sequence encoding the enzyme. The heterologous nucleic acid sequences may be present in DNA constructs or expression cassettes. "Expression cassette" as used herein means a nucleic acid molecule capable of directing expression of a particular nucleotide sequence in an appropriate host cell, comprising a promoter operatively linked to the heterologous nucleotide sequence of interest (i.e., lock and/or key enzyme) which is operatively linked to termination signals. It also typically comprises sequences required for proper translation of the nucleotide sequence. The expression cassette comprising the lock and/or key enzyme may be chimeric, meaning that at least one of its components is heterologous with respect to at least one of its other components. The expression cassette may also be one that is naturally occurring but has been obtained in a recombinant form useful for heterologous expression. Typically, however, the expression cassette is heterologous with respect to the host. The expression of the nucleotide sequence in the expression cassette may be under the control of a constitutive promoter or of an inducible promoter that initiates transcription only when the host cell is exposed to some particular external stimulus. Additionally, the promoter can also be specific to a particular tissue or organ or stage of development.

The expression cassette may optionally comprise a transcriptional and translational termination region (i.e. termination region) functional in plants. In some embodiments, the expression cassette comprises a selectable marker gene to allow for selection for stable transformants. Expression constructs of the invention may also comprise a leader sequence and/or a sequence allowing for inducible expression of the lock and/or key enzyme. See, Guo et al. (2003) Plant J. 34:383-92 and Chen et al. (2003) Plant J. 36:731-40 for examples of sequences allowing for inducible expression.

The regulatory sequences of the expression construct are operably linked to the nucleic acid sequence encoding the lock and/or key enzyme. By "operably linked" is intended a functional linkage between a first sequence and a second sequence for instance, the first sequence may be a promoter sequence which is operably linked to a
second sequence wherein the promoter sequence initiates and mediates transcription of the DNA sequence corresponding to the second sequence. Generally, operably linked means that the nucleotide sequences being linked are contiguous; however, the sequences may have linking sequences that join them together, thus the operably linked sequences may not be directly linked.

**Promoter**

Any promoter capable of driving expression in the plant of interest may be used in the practice of the invention. The promoter may be native or analogous or foreign or heterologous to the plant host. The terms "heterologous" and "exogenous" when used herein to refer to a nucleic acid sequence (e.g. a DNA or RNA sequence) or a gene, refer to a sequence that originates from a source foreign to the particular host cell or, if from the same source, is modified from its original form. Thus, a heterologous gene in a host cell includes a gene that is endogenous to the particular host cell but has been modified through, for example, the use of DNA shuffling. The terms also include non-naturally occurring multiple copies of a naturally occurring DNA sequence. Thus, the terms refer to a DNA segment that is foreign or heterologous to the cell, or homologous to the cell but in a position within the host cell nucleic acid in which the element is not ordinarily found. Exogenous DNA segments are expressed to yield exogenous polypeptides.

The choice of promoters to be included depends upon several factors, including, but not limited to, efficiency, selectability, inducibility, desired expression level, and cell- or tissue-preferential expression. For example, where expression in specific tissues or organs is desired, tissue-specific promoters may be used. In contrast, where gene expression in response to a stimulus is desired, inducible promoters are the regulatory elements of choice. Where continuous expression is desired throughout the cells of a plant, constitutive promoters are utilized. It is a routine matter for one of skill in the art to modulate the expression of a sequence by appropriately selecting and positioning promoters and other regulatory regions relative to that sequence.

A number of plant promoters have been described with various expression characteristics. Examples of some constitutive promoters which have been described include the rice actin 1 (Wang et al., Mol. Cell. Biol, 12:3399 (1992); U.S. Pat. No.
5,641,876), CaMV 35S (Odell et al., Nature, 313:810 (1985)), CaMV 19S (Lawton et al., 1987), nos (Ebert et al., 1987), Adh (Walker et al., 1987), sucrose synthase (Yang & Russell, 1990), and the ubiquitin promoters.

Vectors for use in tissue-specific targeting of genes in transgenic plants will typically include tissue-specific promoters and may also include other tissue-specific control elements such as enhancer sequences. Promoters which direct specific or enhanced expression in certain plant tissues will be known to those of skill in the art in light of the present disclosure. These include, for example, the rbcS promoter, specific for green tissue; the ocs, nos and smas promoters which have higher activity in roots or wounded leaf tissue; a truncated (-90 to +8) 35S promoter which directs enhanced expression in roots, an α-tubulin gene that directs expression in roots and promoters derived from zein storage protein genes which direct expression in endosperm.

Tissue specific expression may be functionally accomplished by introducing a constitutively expressed gene (all tissues) in combination with an antisense gene that is expressed only in those tissues where the gene product is not desired.

Moreover, several tissue-specific regulated genes and/or promoters have been reported in plants. Some reported tissue-specific genes include the genes encoding the seed storage proteins (such as napin, cruciferin, beta-conglycinin, and phaseolin) zein or oil body proteins (such as oleosin), or genes involved in fatty acid biosynthesis (including acyl carrier protein, stearoyl-ACP desaturase, and fatty acid desaturases (fad 2-1)), and other genes expressed during embryo development (such as Bce4, see, for example, EP 255378 and Kridl et al., Seed Science Research, 1:209 (1991)). Examples of tissue-specific promoters, which have been described include the lectin (Vodkin, Prog. Clin. Biol. Res., 138:87 (1983); Lindstrom et al., Der. Genet., 11:160 (1990)), corn alcohol dehydrogenase 1 (Vogel et al., EMBO J., 11:157 (1989)); Dennis et al., Nucleic Acids Res., 12:3983 (1984)), corn light harvesting complex (Simpson, 1986; Bansal et al., Proc. Natl. Acad. Sci. USA, 89:3654 (1992)), corn heat shock protein (Odell et al., Nature, 313: 810 (1985)); pea small subunit RuBP carboxylase ((Poulsen et al., Mol. Gen. Genet. 205:193 (1986)); Ti plasmid mannopine synthase ((Langridge et al., Cell 34:1015 (1989)), Ti plasmid nopaline synthase ((Langridge et al., Cell 34: 1015 (1989)), petunia chalcone isomerase (vanTunen et al., EMBO J., 7:1257 (1988)), bean glycine rich protein

In various embodiments, the lock and/or key enzyme is active in the fruit of the plant. A class of fruit-specific promoters expressed at or during anthesis through fruit development, at least until the beginning of ripening, is discussed in U.S. Pat. No. 4,943,674, the disclosure of which is hereby incorporated by reference. cDNA clones that are preferentially expressed in cotton fiber have been isolated (John et al., Proc. Natl. Acad. Sci. USA, 89:5769 (1992). cDNA clones from tomato displaying differential expression during fruit development have been isolated and characterized (Mansson et al., Gen. Genet, 200:356 (1985), Slater et al., Plant MoI. Biol., 5:137 (1985)). The promoter for polygalacturonase gene is active in fruit ripening. The polygalacturonase gene is described in U.S. Pat. No. 4,535,060, U.S. Pat. No. 4,769,061, U.S. Pat. No. 4,801,590, and U.S. Pat. No. 5,107,065, which disclosures are incorporated herein by reference. The fruit specific E8 promoter is described in Deikman et al. (1988, EMBO J. 2: 3315-3320) and DellaPenna et al. (1989, Plant Cell 1: 53-63). In another embodiment, promoters that selectively express coding sequences in sucrose storage tissues (such as the mature stems of sugarcane and the tubers of sugar beet) may be used. For example, promoters specific for the mature stems of sugarcane are described in International Publication WO 01/1821 1.
In another embodiment, the expression of the lock enzyme is under the control of a sink tissue-specific promoter. By "sink tissue-specific promoter" is meant a promoter that preferentially directs expression of an operably linked transcribable sequence in the sink tissue of a plant as compared to expression in other tissues of the plant, including source tissues (e.g., leaf). "Sink cell" and "sink tissue" as used herein, refer to cells, tissues or organs which at the time of harvest comprise organic carbon that has entered the cells by net inflow in a form other than carbon dioxide. In plants, sink tissues include all non-photosynthetic tissues, as well as photosynthetic tissues with a net inflow of organic carbon fixed by other photosynthetic cells or otherwise obtained from the surrounding medium or environment by means other than direct fixation of carbon dioxide.

Other examples of tissue-specific promoters include those that direct expression in leaf cells following damage to the leaf (for example, from chewing insects), in tubers (for example, patatin gene promoter), and in fiber cells (an example of a developmentally-regulated fiber cell protein is E6 (John et al., Proc. Natl. Acad. Sci. USA, 89:5769 (1992). The E6 gene is most active in fiber, although low levels of transcripts are found in leaf, ovule and flower. Other tissue-specific promoters can be isolated by one skilled in the art (see U.S. Pat. No. 5,589,379).

Several inducible promoters have been reported. Many are described in a review by Gatz, in Current Opinion in Biotechnology, 7:168 (1996) and Gatz, C , Annu. Rev. Plant Physiol. Plant Mol Biol., 48:89 (1997). Examples include tetracycline repressor system, Lac repressor system, copper-inducible systems, salicylate-inducible systems (such as the PRIa system), glucocorticoid-inducible (Aoyama T. et al., N-H Plant Journal, 11:605 (1997)) and ecdysone-inducible systems. Other inducible promoters include ABA- and turgor-inducible promoters, the promoter of the auxin-binding protein gene (Schwob et al., Plant J., 4:423 (1993)), the UDP glucose flavonoid glycosyltransferase gene promoter (Ralston et al., Genetics, 119:185 (1988)), the MPI proteinase inhibitor promoter (Cordero et al, Plant J., 6:141 (1994)), and the glyceraldehyde-3-phosphate dehydrogenase gene promoter (Kohler et al., Plant Mol. Biol., 29;1293 (1995); Quigley etal, J. Mol. Evol., 29:412 (1989); Martinez et al., J. Mol. Biol, 208:551 (1989)). Also included are the benzene sulphonamide-inducible (U.S. Pat. No. 5,364,780)
and alcohol-inducible (WO 97/06269 and WO 97/06268) systems and glutathione S-transferase promoters.

Other studies have focused on genes inducibly regulated in response to environmental stress or stimuli such as increased salinity, drought, pathogen and wounding. (Graham et al., J. Biol. Chem., 260:6555 (1985); Graham et al., J. Biol. Chem., 260:6561 (1985), Smith et al., Planta, 168:94 (1986)). Accumulation of metallocarboxypeptidase-inhibitor protein has been reported in leaves of wounded potato plants (Graham et al., Biochem. Biophys. Res. Comm., 101:1164 (1981)). Other plant genes have been reported to be induced by methyl jasmonate, elicitors, heat-shock, anaerobic stress, or herbicide safeners.

Preferably, in the case of a multicellular organism, the promoter can also be specific to a particular tissue, organ or stage of development. Examples of such promoters include, but are not limited to, the Zea mays ADP-gpp and the Zea mays Gamma zein promoter and the Zea mays globulin promoter.

Expression of a gene in a transgenic plant may be desired only in a certain time period during the development of the plant. Developmental timing is frequently correlated with tissue specific gene expression. Timing the expression of carbohydrate-metabolizing enzymes advantageously takes into consideration the change in carbohydrate concentration that occurs during plant development. The importance of a carbohydrate within tissue may also change with time and, in this regard, sink tissue may undergo changes in sucrose concentrations during development. For example, sucrose concentration in certain fruits such as sweet melons changes as the fruit matures. Hexose sugars accumulate early in development, followed by high levels of sucrose at later stages (Schaffer et al., 1987, Phytochemistry 26: 1883-1887). In developing corn endosperm, sucrose concentration increases from 8 to 12 days after pollination and then drops more than ten fold 28 days after pollination (Tsai et al., 1970, Plant Phys. 46: 299-306). Additionally, sucrose concentration in soybean seed changes significantly during development as raffinose saccharides content increases dramatically, 53 days after anthesis (Amuti, 1977, Phytochemistry 16: 529-532). In pea seed, sucrose content falls dramatically with continued development (Holl and Vose, Can. 1980, J. Plant Sci. 60: 1109-1 114). These examples illustrate the desirability of promoter selection for specific
expression of an enzyme gene timed to take advantage of fluctuating sucrose pools. Thus, in various embodiments, the promoter is an inducible promoter which is capable of driving expression of the enzyme-encoding polynucleotide at an appropriate developmental stage of the plant. In this embodiment, the transcriptional control element is suitably a developmentally regulated promoter to control the timing of expression.

Localization signals

The polynucleotide sequences encoding the lock and/or key enzyme of the present invention may be operably linked to polynucleotide sequences encoding localization signals or signal sequence (at the N- or C-terminus of a polypeptide), e.g., to target the enzyme to a particular compartment within a plant. Examples of such targets include, but are not limited to, the vacuole, endoplasmic reticulum, chloroplast, amyloplast, starch granule, or cell wall, or to a particular tissue, e.g., seed. The expression of a polynucleotide encoding a lock and/or key enzyme having a signal sequence in a plant, in particular, in conjunction with the use of a tissue-specific or inducible promoter, can yield high levels of localized enzyme in the plant. Targeting or signal sequences can be used to localize a lock or key enzyme such that the enzyme does not come into contact with a specific substrate during the growth and development of the plant. For instance, key enzymes expressed in plants that accumulate locked sugars may be targeted away from the plant organelle or compartment which contains the locked sugar. At the time of harvest, the plant tissue may be physically disrupted in order to combine the key enzyme with the locked sugar during the processing of the plant tissue.

Thus, vectors may be constructed and employed in the intracellular targeting of a specific gene product within the cells of a transgenic plant or in directing a protein to the extracellular environment. This will generally be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of a particular gene. The resultant transit, or signal, peptide will transport the protein to a particular intracellular or extracellular destination, respectively, and will then be post-translationally removed. Transit or signal peptides act by facilitating the transport of proteins through intracellular membranes, e.g., vacuole, vesicle, plastid and
mitochondrial membranes, whereas signal peptides direct proteins through the extracellular membrane.

Numerous signal sequences are known to influence the expression or targeting of a polynucleotide to a particular compartment or outside a particular compartment. Suitable signal sequences and targeting promoters are known in the art and include, but are not limited to, those provided herein.

In one embodiment, the lock enzyme carbohydrate can accumulate, and the key enzyme is targeted to the apoplast. The key enzyme can be targeted to the apoplast using, for example, the maize Gamma zein N-terminal signal sequence, which confers apoplast-specific targeting of proteins. The lock enzyme may be targeted to the amyloplast by, for example, fusion to the waxy amyloplast targeting peptide (Klosgen et al., Mol Gen Genet 203: 237-244 1986) or to a starch granule. For example, the polynucleotide encoding the lock enzyme may be operably linked to a chioroplast (amyloplast) transit peptide (CTP) and a starch binding domain, e.g., from the waxy gene. Alternatively, the maize Brittle 1 transit peptide sequence (Btlts, Sullivan and Kaneko, Planta 196: 477-484 (1995)) can be used for amyloplast targeting. In other embodiments, the total carbohydrate content or sweetness or the endogenous carbohydrate content of the sink tissue is increased by targeting the carbohydrate-metabolizing enzyme to a sub-cellular compartment used for carbohydrate storage in the plant cells (e.g., vacuole or apoplastic space).

A signal sequence such as the maize Gamma zein N-terminal signal sequence for targeting to the endoplasmic reticulum and secretion into the apoplast may be operably linked to a polynucleotide encoding the key enzyme in accordance with the present invention (Torrent et al., Plant Mol. Biol. 34:139 (1997)). Another signal sequence is the amino acid sequence SEKDEL (SEQ ID NO:7) for retaining polypeptides in the endoplasmic reticulum (Munro et al. Cell 48:899 (1987)).

Enhancers

Numerous sequences have been found to enhance gene expression from within the transcriptional unit and these sequences can be used in conjunction with the genes of this invention to increase their expression in transgenic plants.
Various intron sequences have been shown to enhance expression. For example, the introns of the maize Adhl gene have been found to significantly enhance the expression of the wild-type gene under its cognate promoter when introduced into maize cells. Intron 1 was found to be particularly effective and enhanced expression in fusion constructs with the chloramphenicol acetyltransferase gene (Callis et al., Genes Develop. 1: 1183-1200 (1987)). In the same experimental system, the intron from the maize bronze 1 gene had a similar effect in enhancing expression. Intron sequences have been routinely incorporated into plant transformation vectors, typically within the non-translated leader.

A number of non-translated leader sequences derived from viruses are also known to enhance expression. Specifically, leader sequences from Tobacco Mosaic Virus (TMV, the "W-sequence"), Maize Chlorotic Mottle Virus (MCMV), and Alfalfa Mosaic Virus (AMV) have been shown to be effective in enhancing expression (e.g. Gallie et al. Nucl. Acids Res. 15: 8693-8711 (1987); Skuzeski et al. Plant Molec. Biol. 15: 65-79 (1990)). Other leader sequences known in the art include but are not limited to: picomavirus leaders, for example, EMCV leader (Encephalomyocarditis 5' noncoding region) (Elroy-Stein, O., Fuerst, T. R., and Moss, B. PNAS USA 86:6126-6130 (1989)); potyviruses leaders, for example, TEV leader (Tobacco Etch Viru) (Allison et al., Virology 154: 9-20 (1986)); MDMV leader (Maize Dwarf Mosaic Virus); Virology 154:9-20); human immunoglobulin heavy-chain binding protein (BiP) leader, (Macejak, D. G., and Samow, P., Nature 353: 90-94 (1991); untranslated leader from the coat protein mRNA of alfalfa mosaic virus (AMV RNA 4), (Jobling, S. A., and Gehrke, L., Nature 325:622-625 (1987); tobacco mosaic virus leader (TMV), (Gallie, D. R. et al., Molecular Biology of RNA, pages 237-256 (1989); and Maize Chlorotic Mottle Virus leader (MCMV) (Lommel, S. A. et al., Virology 81:382-385 (1991). See also, Della-Cioppa et al., Plant Physiology 84:965-968 (1987).

**Regulatory sequences**

The polynucleotides of the present invention, in addition to processing signals, may further include other regulatory sequences, as is known in the art. "Regulatory sequences" and "suitable regulatory sequences" each refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding...
sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences include enhancers, promoters, translation leader sequences, introns, and polyadenylation signal sequences. They include natural and synthetic sequences as well as sequences that are a combination of synthetic and natural sequences.

A variety of transcriptional terminators are available for use in expression cassettes. These are responsible for the termination of transcription beyond the transgene and correct mRNA polyadenylation. The termination region may be native with the transcriptional initiation region, may be native with the operably linked DNA sequence of interest, may be native with the plant host, or may be derived from another source (i.e., foreign or heterologous to the promoter, the DNA sequence of interest, the plant host, or any combination thereof). Appropriate transcriptional terminators are those that are known to function in plants and include the CAMV 35S terminator, the tml terminator, the nopaline synthase terminator and the pea rbcS E9 terminator. These can be used in both monocotyledons and dicotyledons. In addition, a gene's native transcription terminator may be used.

**Selectable markers**

Generally, the expression cassette will comprise a selectable marker gene for the selection of transformed cells. Selectable marker genes are utilized for the selection of transformed cells or tissues. Selectable markers may also be used in the present invention to allow for the selection of transformed plants and plant tissue, as is well-known in the art. One may desire to employ a selectable or screenable marker gene as, or in addition to, the expressible gene of interest. "Marker genes" are genes that impart a distinct phenotype to cells expressing the marker gene and thus allow such transformed cells to be distinguished from cells that do not have the marker. Such genes may encode either a selectable or screenable marker, depending on whether the marker confers a trait which one can select for by chemical means, i.e., through the use of a selective agent (e.g., a herbicide, antibiotic, or the like), or whether it is simply a trait that one can identify through observation or testing, i.e., by screening (e.g., the R-locus trait). Of
course, many examples of suitable marker genes are known in the art and can be employed in the practice of the invention.

In one embodiment, both the lock and the key enzymes are expressed in the same plant, and the expression of the key enzyme is used as a selectable marker. In one example, the selection system is based on the expression of alpha-1,6-glucosidase in a plant accumulating isomaltulose. In such a system a means of breaking down isomaltulose into a substrate for fermentation is necessary, and may be provided in the form of sugarcane, sugarbeet, etc. plants engineered to express an alpha-1,6-glucosidase (isomaltulase, palatinase, etc.). Such a selectable marker system would be useful in screening for high level expression of alpha-1,6-glucosidase from the very earliest steps of plant transformation, this would be helpful in identifying integration events that are stable, highly expressed, and resistant to gene silencing. Also, this system could be used to select alpha-1,6-glucosidases with improved activity and in selecting for variants that increase protein or siRNA stability, localization to specific subcellular locations etc.

Also included within the terms selectable or screenable marker genes are also genes which encode a "secretable marker" whose secretion can be detected as a means of identifying or selecting for transformed cells. Examples include markers which encode a secretable antigen that can be identified by antibody interaction, or even secretable enzymes which can be detected by their catalytic activity. Secretable proteins fall into a number of classes, including small, diffusible proteins detectable, e.g., by ELISA; small active enzymes detectable in extracellular solution (e.g., β-lactamase, phosphinothricin acetyltransfersase); and proteins that are inserted or trapped in the cell wall (e.g., proteins that include a leader sequence such as that found in the expression unit of extensin or tobacco PR-S).

With regard to selectable secretable markers, the use of a gene that encodes a protein that becomes sequestered in the cell wall, and which protein includes a unique epitope is also encompassed herein. Such a secreted antigen marker would ideally employ an epitope sequence that would provide low background in plant tissue, a promoter-leader sequence that would impart efficient expression and targeting across the plasma membrane, and would produce protein that is bound in the cell wall and yet
accessible to antibodies. A normally secreted wall protein modified to include a unique epitope would satisfy all such requirements.

One example of a protein suitable for modification in this manner is extensin, or hydroxyproline rich glycoprotein (HPRG). For example, the maize HPRG (Steifel et al., The Plant Cell, 2:785 (1990)) molecule is well characterized in terms of molecular biology, expression and protein structure. However, any one of a variety of extensins and/or glycine-rich wall proteins (Keller et al., EMBO Journal, 8:1309 (1989)) could be modified by the addition of an antigenic site to create a screenable marker.

Possible selectable markers for use in connection with the present invention include, but are not limited to, a neo or nptI gene (Potrykus et al., MoI. Gen. Genet., 199:183 (1985)) which codes for kanamycin resistance and can be selected for using kanamycin, G418, and the like; a bar gene which confers resistance to the herbicide phosphinothricin; a gene which encodes an altered EPSP synthase protein (Hinchee et al., Biotech., 6:915 (1988)) thus conferring glyphosate resistance; a nitrilase gene such as bxn from Klebsiella ozaenae which confers resistance to bromoxynil (Stalker et al., Science, 242:419 (1988)); a mutant acetolactate synthase gene (ALS) which confers resistance to imidazolinone, sulfonylurea or other ALS-inhibiting chemicals (European Patent Application 154,204, 1985); a methotrexate-resistant DHFR gene (Thillet et al., J. Biol. Chem., 263:12500 (1988)); a dalapon dehalogenase gene that confers resistance to the herbicide dalapon; a phosphomannose isomerase (PMI) gene; a mutated anthranilate synthase gene that confers resistance to 5-methyl tryptophan; the hph gene which confers resistance to the antibiotic hygromycin; or the mannose-6-phosphate isomerase gene (also referred to herein as the phosphomannose isomerase gene), which provides the ability to metabolize mannose (U.S. Pat. Nos. 5,767,378 and 5,994,629). One skilled in the art is capable of selecting a suitable selectable marker gene for use in the present invention.

An illustrative embodiment of a selectable marker gene capable of being used in systems to select transformants are the genes that encode the enzyme phosphinothricin acetyltransferase, such as the bar gene from Streptomyces hygroscopicus or the pat gene from Streptomyces viridochromogenes. The enzyme phosphinothricin acetyl transferase (PAT) inactivates the active ingredient in the herbicide bialaphos, phosphinothricin (PPT). PPT inhibits glutamine synthetase, (Murakami et al., MoI. Gen. Genet, 205:42
(1986); Twell et al., Plant Physiol., 91:1270 (1989)) causing rapid accumulation of ammonia and cell death. The success in using this selective system in conjunction with monocots was particularly surprising because of the major difficulties which have been reported in transformation of cereals (Potrykus, Trends Biotech., 7:269 (1989)).

Where one desires to employ a bialaphos resistance gene in the practice of the invention, a particularly useful gene for this purpose is the bar or pat genes obtainable from species of Streptomyces (e.g., ATCC No. 21,705). The cloning of the bar gene has been described (Murakami et al., Mol. Gen. Genet., 205:42 (1986); Thompson et al., EMBO Journal, 6:2519 (1987)) as has the use of the bar gene in the context of plants other than monocots (De Block et al., EMBO Journal, 6:2513 (1987); De Block et al., Plant Physiol., 91:694 (1989)).

Screenable markers that may be employed include, but are not limited to, a β-glucuronidase or uidA gene (GUS) which encodes an enzyme for which various chromogenic substrates are known; an R-loeus gene, which encodes a product that regulates the production of anthocyanin pigments (red color) in plant tissues (Dellaporta et al., in Chromosome Structure and Function, pp. 263-282 (1988)); a β-lactamase gene (Sutcliffe, PNAS USA, 75:3737 (1978)), which encodes an enzyme for which various chromogenic substrates are known (e.g., PADAC, a chromogenic cephalosporin); a xyle gene (Zukowsky et al., PNAS USA, 80:1101 (1983)) which encodes a catechol dioxygenase that can convert chromogenic catechols; a tyrosinase gene (Katz et al., J. Gen. Microbiol., 129:2703 (1983)) which encodes an enzyme capable of oxidizing tyrosine to DOPA and dopaquinone which in turn condenses to form the easily detectable compound melanin; a β-galactosidase gene, which encodes an enzyme for which there are chromogenic substrates; a luciferase (lux) gene (Ow et al., Science, 234:856 (1986)), which allows for bioluminescence detection; or an aequorin gene (Prasher et al., Biochem. Biophys. Res. Comm., 126:1259 (1985)), which may be employed in calcium-sensitive bioluminescence detection, or a green fluorescent protein gene (Niedz et al., Plant Cell Reports, 14: 403 (1995)).

Genes from the maize R gene complex are contemplated to be particularly useful as screenable markers. The R gene complex in maize encodes a protein that acts to regulate the production of anthocyanin pigments in most seed and plant tissue. A gene
from the R gene complex is suitable for maize transformation, because the expression of this gene in transformed cells does not harm the cells. Thus, an R gene introduced into such cells will cause the expression of a red pigment and, if stably incorporated, can be visually scored as a red sector. If a maize line carries dominant alleles for genes encoding the enzymatic intermediates in the anthocyanin biosynthetic pathway (C2, Al, A2, BzI and Bz2), but carries a recessive allele at the R locus, transformation of any cell from that line with R will result in red pigment formation. Exemplary lines include Wisconsin 22 which contains the rg-Stadler allele and TR1 12, a K55 derivative which is r-g, b, P1. Alternatively any genotype of maize can be utilized if the C1 and R alleles are introduced together. A further screenable marker contemplated for use in the present invention is firefly luciferase, encoded by the lux gene. The presence of the lux gene in transformed cells may be detected using, for example, X-ray film, scintillation counting, fluorescent spectrophotometry, low-light video cameras, photon counting cameras or multiwell luminometry. It is also envisioned that this system may be developed for popililational screening for bioluminescence, such as on tissue culture plates, or even for whole plant screening.

Additional agronomic traits

The plants disclosed herein may further exhibit one or more agronomic traits that primarily are of benefit to a seed company, a grower, or a grain processor, for example, herbicide resistance, virus resistance, bacterial pathogen resistance, insect resistance, nematode resistance, and fungal resistance. See, e.g., U.S. Pat. Nos. 5,569,823; 5,304,730; 5,495,071; 6,329,504; and 6,337,431. Such trait may also be one that increases plant vigor or yield (including traits that allow a plant to grow at different temperatures, soil conditions and levels of sunlight and precipitation), or one that allows identification of a plant exhibiting a trait of interest (e.g., selectable marker gene, seed coat color, etc.). Various traits of interest, as well as methods for introducing these traits into a plant, are described, for example, in U.S. Pat. Nos. 5,569,823; 5,304,730; 5,495,071; 6,329,504; 6,337,431; 5,767,366; 5,928,937; 4,761,373; 5,013,659; 4,975,374; 5,162,602; 4,940,835; 4,769,061; 5,554,798; 5,879,903; 5,276,268; 5,561,236; 4,810,648; and 6,084,155; in European application No. 0 242 246; in U.S. Patent Application No.
20010016956; and on the worldwide web at
www.lifesci.sussex.ac.uk/home/Neil_Crickmore/Bt/.

**Plant Transformation**

Once a nucleic acid sequence encoding the lock and/or key enzyme has been cloned into an expression system, it is transformed into a plant cell. The word "plant" refers to any plant, particularly to seed plant, and "plant cell" is a structural and physiological unit of the plant, which comprises a cell wall but may also refer to a protoplast. The plant cell may be in form of an isolated single cell or a cultured cell, or as a part of higher organized unit such as, for example, a plant tissue, or a plant organ. The term "transformation" refers to the transfer of a nucleic acid fragment into the genome of a host cell, resulting in genetically stable inheritance. Host cells containing the transformed nucleic acid fragments are referred to as "transgenic" cells, and organisms comprising transgenic cells are referred to as "transgenic organisms."

Examples of methods of transformation of plants and plant cells include Agrobacterium-mediated transformation (De Blaere et al., 1987) and particle bombardment technology (Klein et al. 1987; U.S. Pat. No. 4,945,050). Whole plants may be regenerated from transgenic cells by methods well known to the skilled artisan (see, for example, Fromm et al., 1990).

The expression cassettes of the present invention can be introduced into the plant cell in a number of art-recognized ways. The term "introducing" in the context of a polynucleotide, for example, a nucleotide encoding an enzyme disclosed herein, is intended to mean presenting to the plant the polynucleotide in such a manner that the polynucleotide gains access to the interior of a cell of the plant. Where more than one polynucleotide is to be introduced, these polynucleotides can be assembled as part of a single nucleotide construct, or as separate nucleotide constructs, and can be located on the same or different transformation vectors.

Accordingly, these polynucleotides can be introduced into the host cell of interest in a single transformation event, in separate transformation events, or, for example, in plants, as part of a breeding protocol. The methods of the invention do not depend on a particular method for introducing one or more polynucleotides into a plant, only that the
polynucleotide(s) gains access to the interior of at least one cell of the plant. Methods for introducing polynucleotides into plants are known in the art including, but not limited to, transient transformation methods, stable transformation methods, and virus-mediated methods.

"Transient transformation" in the context of a polynucleotide is intended to mean that a polynucleotide is introduced into the plant and does not integrate into the genome of the plant.

By "stably introducing" or "stably introduced" in the context of a polynucleotide introduced into a plant is intended the introduced polynucleotide is stably incorporated into the plant genome, and thus the plant is stably transformed with the polynucleotide.

"Stable transformation" or "stably transformed" is intended to mean that a polynucleotide, for example, a nucleotide coi

plant integrates into the genome of the plant; progeny thereof, more particularly, by the progeny of multiple successive generations.

Numerous transformation vectors available for plant transformation are known to those of ordinary skill in the plant transformation arts, and the genes pertinent to this invention can be used in conjunction with any such vectors. The selection of vector will depend upon the preferred transformation technique and the target species for transformation. For certain target species, different antibiotic or herbicide selection markers may be preferred as discussed elsewhere herein.

Methods for regeneration of transformed plants are well known in the art. For example, Ti plasmid vectors have been utilized for the delivery of foreign DNA, as well as direct DNA uptake, liposomes, electroporation, microinjection, and microprojectiles. In addition, bacteria from the genus Agrobacterium can be utilized to transform plant cells. Below are descriptions of representative techniques for transforming both dicotyledonous and monocotyledonous plants, as well as a representative plastid transformation technique.

Many vectors are available for transformation using Agrobacterium tumefaciens. These typically carry at least one T-DNA border sequence and include vectors such as pBIN19 (Bevan, Nucl. Acids Res. (1984)). For the construction of vectors useful in
Agrobacterium transformation, see, for example, US Patent Application Publication No. 2006/026001, herein incorporated by reference.

Transformation without the use of Agrobacterium tumefaciens circumvents the requirement for T-DNA sequences in the chosen transformation vector and consequently vectors lacking these sequences can also be utilized. Transformation techniques that do not rely on Agrobacterium include transformation via particle bombardment, protoplast uptake (e.g. PEG and electroporation) and microinjection. The choice of vector depends largely on the preferred selection for the species being transformed. For the construction of such vectors, see, for example, US Application No. 2006026001, herein incorporated by reference.

Transformation techniques for dicotyledons are well known in the art and include Agrobacterium-based techniques and techniques that do not require Agrobacterium. Non-Agrobacterium techniques involve the uptake of exogenous genetic material directly by protoplasts or cells. This method can be accomplished by PEG or electroporation mediated uptake, particle bombardment-mediated delivery, or microinjection. Examples of these techniques are described by Paszkowski et al., EMBO J. 3: 2717-2722 (1984), Potrykus et al., Mol. Gen. Genet. 199: 169-177 (1985), Reich et al., Biotechnology 4: 1001-1004 (1986), and Klein et al., Nature 327: 70-73 (1987). In each case the transformed cells are regenerated to whole plants using standard techniques known in the art.

Agrobacterium-mediated transformation is a preferred technique for transformation of dicotyledons because of its high efficiency of transformation and its broad utility with many different species. Agrobacterium transformation typically involves the transfer of the binary vector carrying the foreign DNA of interest to an appropriate Agrobacterium strain which may depend on the complement of vir genes carried by the host Agrobacterium strain either on a co-resident Ti plasmid or chromosomally (Uknes et al. Plant Cell 5: 159-169 (1993)). The transfer of the recombinant binary vector to Agrobacterium is accomplished by a trip parental mating procedure using E. coli carrying the recombinant binary vector, a helper E. coli strain which carries a plasmid that is able to mobilize the recombinant binary vector to the target Agrobacterium strain. Alternatively, the recombinant binary vector can be
transferred to Agrobacterium by DNA transformation (Hofgen & Willmitzer, Nucl. Acids Res. 16: 9877 (1988)).

Transformation of the target plant species by recombinant Agrobacterium usually involves co-cultivation of the Agrobacterium with explants from the plant and follows protocols well known in the art. Transformed tissue is regenerated on selectable medium carrying the antibiotic or herbicide resistance marker present between the binary plasmid T-DNA borders.

Another approach to transforming plant cells with a gene involves propelling inert or biologically active particles at plant tissues and cells. This technique is disclosed in U.S. Pat. Nos. 4,945,050, 5,036,006, and 5,100,792. Generally, this procedure involves propelling inert or biologically active particles at the cells under conditions effective to penetrate the outer surface of the cell and afford incorporation within the interior thereof. When inert particles are utilized, the vector can be introduced into the cell by coating the particles with the vector containing the desired gene. Alternatively, the target cell can be surrounded by the vector so that the vector is carried into the cell by the wake of the particle. Biologically active particles (e.g., dried yeast cells, dried bacterium or a bacteriophage, each containing DNA sought to be introduced) can also be propelled into plant cell tissue.

Transformation of most monocotyledon species has now also become routine. Preferred techniques include direct gene transfer into protoplasts using PEG or electroporation techniques, and particle bombardment into callus tissue. Transformations can be undertaken with a single DNA species or multiple DNA species (i.e. co-transformation) and both of these techniques are suitable for use with this invention. Co-transformation may have the advantage of avoiding complete vector construction and of generating transgenic plants with unlinked loci for the gene of interest and the selectable marker, enabling the removal of the selectable marker in subsequent generations, should this be regarded desirable.

and Fromm et al. (Biotechnology 8: 833-839 (1990)) have published techniques for transformation of A188-derived maize line using particle bombardment. Furthermore, WO 93/07278 and Koziel et al. (Biotechnology 11: 194-200 (1993)) describe techniques for the transformation of elite inbred lines of maize by particle bombardment. This technique utilizes immature maize embryos of 1.5-2.5 mm length excised from a maize ear 14-15 days after pollination and a PDS-IOOOHe Biolistics device for bombardment.

The plants obtained via transformation with a nucleic acid sequence of the present invention can be any of a wide variety of plant species, including those of monocots and dicots; however, the plants used in the method of the invention are preferably selected from the list of agronomically important target crops set forth supra. The expression of a gene of the present invention in combination with other characteristics important for production and quality can be incorporated into plant lines through breeding. Breeding approaches and techniques are known in the art. See, for example, Welsh J. R., Fundamentals of Plant Genetics and Breeding, John Wiley & Sons, NY (1981); Crop Breeding, Wood D. R. (Ed.) American Society of Agronomy Madison, Wis. (1983); Mayo O., The Theory of Plant Breeding, Second Edition, Clarendon Press, Oxford (1987); Singh, D. P., Breeding for Resistance to Diseases and Insect Pests, Springer-Verlag, NY (1986); and Wricke and Weber, Quantitative Genetics and Selection Plant Breeding, Walter de Gruyter and Co., Berlin (1986).

The genetic properties engineered into the transgenic seeds and plants described above are passed on by sexual reproduction or vegetative growth and can thus be maintained and propagated in progeny plants. Generally, maintenance and propagation make use of known agricultural methods developed to fit specific purposes such as tilling, sowing or harvesting.

The lock and/or key enzymes disclosed herein may also be incorporated into or maintained in plant lines through breeding or through common genetic engineering technologies. Breeding approaches and techniques are known in the art. See, for example, Welsh J. R., Fundamentals of Plant Genetics and Breeding, John Wiley & Sons, NY (1981); Crop Breeding, Wood D. R. (Ed.) American Society of Agronomy Madison, Wis. (1983); Mayo O., The Theory of Plant Breeding, Second Edition, Clarendon Press, Oxford (1987); Singh, D. P., Breeding for Resistance to Diseases and Insect Pests,

The relevant techniques are well known in the art and include but are not limited to hybridization, inbreeding, backcross breeding, multi-line breeding, dihaploid inbreeding, variety blend, interspecific hybridization, aneuploid techniques, etc. Hybridization techniques also include the sterilization of plants to yield male or female sterile plants by mechanical, genetic (including transgenic), chemical, or biochemical means.

The following examples are offered by way of illustration and not by way of limitation.
EXPERIMENTAL


Example 1: Enzymes that can produce locked sugars

1A: Bacterial expression system of His-tagged enzymes

Selected genes coding for specific enzymes were cloned into an *Escherichia coli* expression vector, pET24b (Novagen), using restriction sites that place the coding sequence in-frame downstream of an inducible T71ac promoter. Polynucleotide sequences coding for specific enzymes were generated by back translating the polypeptide sequence of the enzyme using the codon preference for *E. coli*. The expression plasmids were introduced into an *E. coli* expression strain, BL21 Star (DE3) (Invitrogen). Recombinant *E. coli* isolates containing the modified pET24b expression vector were selected on standard LB agar containing 50 μg/mL kanamycin.

Recombinant *E. cod* isolates were grown with shaking at 37 degrees C for 8 hours to overnight in 20 mL of LB media containing 50 μg/mL kanamycin. The 20 mL of *E. coli* culture was transferred to 1 L of autoinduction media (9.57 g trypton, 4.8 g yeast extract, 2 ml of 1 M MgSO4, 1 mL of 100X trace metals, 20 ml of 50X 5052, 20 mL of 50X M) (100X trace metals: 36 mL sterile water, 50 mL of 0.1M FeCB in 0.12M HCl, 2 mL of 1M CaCl2, 1 mL of 1M MnCl2 4 H2O, 1 mL of 1M ZnSO4 7 H2O, 1 mL of 0.2M CoCl2 6 H2O, 2 mL of 0.1M CuCl2 2 H2O, 1 mL of 0.2M MC12 6 H2O, 2 mL of 0.1M Na2MoO4 2 H2O, 2 mL of 0.1M H3BO3) (50X 5052: 25 g glycerol, 73 mL H2O, 2.5 g glucose 10 g alpha-lactose monohydrate) (50X M: 80 mL H2O, 17.75 g Na2HPO4, 17.0 g KH2PO4, 13.4 g NH4Cl, 3.55 g Na2SO4) with 25 μg/mL kanamycin and grown.
with shaking at 28 degrees C overnight. The E. coli cells were harvested out of the autoinduction media by centrifugation at 10,000Xg for 15 minutes and the collected cells were frozen at -80 degrees C.

IB: Sucrose isomerase (E.C. 5.4.99.1)

The amino acid sequence for a sucrose isomerase expressed by Erwinia carotovora has been listed in GeneBank under the accession number YP049947 (SEQ ID NO: 14). The amino acid sequence of this sucrose isomerase was back translated into a polynucleotide coding sequence using the codon preference of E. coli. The polynucleotide sequence was generated by gene synthesis (GeneArt) and cloned into the expression vector pET24b (Novagen) using restriction sites that place the coding sequence in-frame downstream of an inducible T7lac promoter. This expression plasmid was introduced into an E. coli expression strain, BL21, harboring a λDE3 lysogen. After growing for 3 hours in LB media containing 50microgram/microliter kanamycin, the cells were induced to produce the E. carotovora sucrose isomerase enzyme with IPTG at a final concentration of 1mM. The E. coli cells were harvested 3 hours after induction by centrifugation at 10,000Xg for 10 min and the supernatant was removed. Cells were lysed by resuspending the cell pellet in BugBuster reagent (Novagen) containing lysozyme (1KU/mL BugBuster) and benzonase (25 units/1 mL BugBuster) followed by incubation for 10 min on a shaking platform. Insoluble debris was removed by centrifugation at 16,000Xg for 20 min at 4 degrees C. Supernatant containing total soluble protein and the recombinant enzyme was transferred to a fresh 1.5 mL Eppendorf tube and aliquots were stored at 4 degrees C and -20 degrees C for further characterization.

Sucrose isomerase enzyme activity was assayed by combining the enzyme with the substrate, sucrose, and measuring the production of isomaltulose and trehalulose. The total soluble protein extract from the recombinant E. coli was assayed for sucrose isomerase activity by incubating 10 microliters of supernatant E. coli lysate, as described above, with 90 microliters of 292 mM sucrose 50 mM sodium phosphate buffer (pH 6.0) at 30 degrees C for 20 hours. The reaction product was screened for the presence of
isomaltulose and trehalulose by thin layer chromatography (TLC) and high pressure liquid chromatography (HPLC).

TLC was performed by spotting 3 microliters of the supernatants of the growth media onto AL SIL G silica gel plates (Whatman) and developed twice in a solvent consisting of 3 parts ethylacetate : 3 parts acetic acid : 1 part distilled water. After drying, the plates were sprayed with a dye mixture consisting of 4 milliliters aniline, 4 g diphenylamine, 200 milliliters acetone, and 30 milliliters 80% phosphoric acid. Isomaltulose and trehalulose were distinguished from other sugars, such as sucrose, by their relative mobility and by the distinct colors produced when they reacted with aniline dye. Greenish yellow indicates the presence of isomaltulose, red indicates the presence of trehalulose, and brown/black indicates the presence of sucrose. The monosaccharides, glucose and fructose, produced by hydrolysis of sucrose were blue or red-orange respectively.

Identification of the sugars present in each lane of the developed TLC plate was possible by comparing both the relative mobility of the sugars present in the samples and the staining color with aniline dye to the relative mobility and staining color of sugar standards. The reaction product of sucrose isomerase incubated with sucrose as described above was three colored bands. The highest mobility band had a purple color and migrated with the same mobility as both glucose and fructose standards blue and red colored respectively and is therefore interpreted to be a mixture of co migrating glucose and fructose released by hydrolysis of one of the disaccharides: sucrose, isomaltulose, or trehalulose. The middle band corresponded with the isomaltulose standard in both coloration and relative mobility and is therefore identified as isomaltulose. The slowest migrating band had a red coloration and migrated slower than either the isomaltulose, or sucrose standards. The relative mobility of this sugar band corresponds well with published reports on the migration of trehalulose in similar TLC assays (Cho et al. Biotechnology Letters (2007) 29:453-458; an isomaltulose-producing microorganism isolated from traditional Korean food.) Therefore this sugar band was concluded to be trehalulose. No trehalulose standard was available at the time of the TLC assay, however, subsequent HPLC (Dionex) analysis of sucrose isomerase reaction products and standards obtained later indicate that this band was definitely trehalulose. Also, it is
important to note that the reaction product did not contain any sucrose which has a higher relative mobility than isomaltulose and trehalulose and slower mobility than the monosaccharides glucose and fructose. The absence of sucrose was expected due to the complete conversion of sucrose into isomaltulose and trehalulose due to the activity of the sucrose isomerase enzyme.

Alternatively, supernatants were separated by running a 50 mM sodium phosphate, 500 mM NaCl, 10 mM Imidazole, pH 8 containing protease inhibitors (Roche Complete EDTA-free protease inhibitor tablets). Cells were lysed by 2 passages through a FRENCH Press (Thermo IEC). Cell lysate was centrifuged for 30 minutes at 10,000Xg at 4 degrees C. Supernatant was filtered using 0.45 micron vacuum filter devices (Millipore) to generate a clarified lysate. A HisTrap FF 5 mL column (GE Healthcare) was equilibrated with extraction buffer. The clarified lysate was loaded onto the equilibrated column at 5 mL/min. Bound his-tagged sucrose isomerase was eluted in a linear imidazole gradient from 50 mM sodium phosphate, 500 mM NaCl, 10 mM Imidazole, pH 8 to 50 mM sodium phosphate, 500 mM NaCl, 200 mM Imidazole over 100 mL. Fractions containing the enzyme were collected and diluted in 50 mM Tris-HCl, pH 8. Diluted sample was loaded onto a 5 mL HiTrap Q HP anion exchange column (GE Healthcare). Bound proteins were eluted from the column by running a linear NaCl gradient from 50 mM Tris-HCl, pH 8 to 50 mM Tris-HCl, 500 mM NaCl, pH 8 over 100 mL. Active sucrose isomerase was detected in the flow through and fractions that eluted at approximately 100 mM NaCl. These fractions were pooled and concentrated to a final protein concentration of 0.8 mg/mL. Samples were aliquoted and stored at -80 degrees C.

His-tagged sucrose isomerase (SEQ ID NO: 14)

Recombinant BL21(DE3) cell pellets expressing his-tagged sucrose isomerase (SEQ ID NO: 14) were generated essentially as described in Example IA. The recombinant BL21 cell pellets were brought up to a volume of 40 mL in extraction buffer (50 mM sodium phosphate, 500 mM NaCl, 10 mM Imidazole, pH 8 containing protease inhibitors (Roche Complete EDTA-free protease inhibitor tablets)). Cells were lysed by 2 passages through a FRENCH Press (Thermo IEC). Cell lysate was centrifuged for 30 minutes at 10,000Xg at 4 degrees C. Supernatant was filtered using 0.45 micron vacuum filter devices (Millipore) to generate a clarified lysate. A HisTrap FF 5 mL column (GE Healthcare) was equilibrated with extraction buffer. The clarified lysate was loaded onto the equilibrated column at 5 mL/min. Bound his-tagged sucrose isomerase was eluted in a linear imidazole gradient from 50 mM sodium phosphate, 500 mM NaCl, 10 mM Imidazole, pH 8 to 50 mM sodium phosphate, 500 mM NaCl, 200 mM Imidazole over 100 mL. Fractions containing the enzyme were collected and diluted in 50 mM Tris-HCl, pH 8. Diluted sample was loaded onto a 5 mL HiTrap Q HP anion exchange column (GE Healthcare). Bound proteins were eluted from the column by running a linear NaCl gradient from 50 mM Tris-HCl, pH 8 to 50 mM Tris-HCl, 500 mM NaCl, pH 8 over 100 mL. Active sucrose isomerase was detected in the flow through and fractions that eluted at approximately 100 mM NaCl. These fractions were pooled and concentrated to a final protein concentration of 0.8 mg/mL. Samples were aliquoted and stored at -80 degrees C.
Sucrose isomerase enzyme activity was measured in the samples by combining 6 ug/mL his-tagged sucrose isomerase, 70 mM 0.1 M Citrate-phosphate buffer, pH 6 and 584 mM sucrose at 30 degrees C for 2 hours. Sample was analyzed by Dionex essentially as described in Example IG. Table 1 outlines the sucrose isomerase activity detected in recombinant E. coli cells expressing sucrose isomerase (SEQ ID NO: 14). Activity is demonstrated by the accumulation of the locked sugars trehalulose and isomaltulose.

<table>
<thead>
<tr>
<th>Time</th>
<th>Glucose (mM)</th>
<th>Fructose (mM)</th>
<th>Sucrose (mM)</th>
<th>Trehalulose (mM)</th>
<th>Isomaltulose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose isomerase</td>
<td>5.98</td>
<td>4.97</td>
<td>0.61</td>
<td>227.96</td>
<td>248.45</td>
</tr>
<tr>
<td>Negative control</td>
<td>0</td>
<td>0</td>
<td>512</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

1C: Dextransucrase enzyme (E.C. 2.4.1.5)

Dextransucrases (E.C. 2.4.1.5) are glucosyl transferase enzymes capable of transferring glucose from a sucrose molecule to form glucose homopolymers known as dextrans. This type of enzymatic reaction is an example of transglycosylation. The dextran is composed of mainly 1,6 alpha D glucose linkages of varying length. The dextran can also contain a variety of 1,4 alpha D glucose linkages which form branch points in the dextran molecule. These branching points have a direct impact on the physiochemical properties (such as solubility) of the dextran molecules. The polynucleotide sequence coding for a dextransucrase enzyme will be generated that uses the codon preference for E.coli. This polynucleotide sequence will be synthesized, cloned into an expression vector and expressed in E. coli as described in Example IA.

Dextransucrase enzyme activity will be monitored using a colorimetric assay to detect the rate of fructose release from sucrose (Kobayashi, M et al. (1980) Biochimica et Biophysica Acta vol 614, pp 46-62). Dextran accumulation will be monitored using methods similar to those described in Zhang, S., et al. (2007) Transgenic Res. 16:467-478 in combination with HPLC techniques such as size exclusion chromatography. Dextransucrase enzyme activity assays will be validated by comparing dextransucrase
activity recovered from recombinant *E coli* with commercially available dextranucrase enzyme.

Dextranucrase activity will be measured using sugarcane juice as the source of sucrose. Selected *E* coli expressed dextranucrases will be incubated in a similar fashion as described above, however sucrose will be replaced with sugarcane juice as the substrate. These experiments will be designed to test the ability of the expressed enzymes to produce dextrans from sucrose in the presence of other proteins and unknown compounds found in sugarcane juice.

A mutant dextranucrase has been characterized by Hellmuth et al. Biochemistry 47: 6678-6684 (2008) which alters the activity of the enzyme such that it can catalyze the conversion of sucrose to isomaltulose or leucrose. This dextranucrase variant has leucrose synthase activity due to the ability of the variant enzyme to catalyze the conversion of sucrose to leucrose.

Analysis of His-tagged dextranucrase with leucrose synthase activity (SEQ ID NO: 29).

Recombinant BL21[DE3] cell expressing a His-tagged dextranucrase with leucrose synthase activity (SEQ ID NO: 29) was generated essentially as described in Example IA. Frozen cell pellets were brought up to a volume of 30-40 mL in extraction buffer (50 mM sodium phosphate, 500 mM NaCl, 10 mM Imidazole, pH 7.2 containing protease inhibitors (Roche Complete EDTA-free protease inhibitor tablets)). Cells were lysed by 2 passages through a FRENCH Press (Thermo IEC). Cell lysates were centrifuged for 30 minutes at 10,000Xg at 4 degrees C. Supernatants were filtered using 0.45 micron vacuum filter devices (Millipore). A HisTrap FF 5 ml column (GE Healthcare) was equilibrated with extraction buffer and the clarified lysates were loaded at 5 mL/min. Bound his-tagged enzymes were eluted in 50 mM sodium phosphate, 500 mM NaCl, containing 300 mM Imidazole, pH 7.2. All samples were buffer exchanged into 50 mM HEPES, 50 mM NaCl, pH 7 using a HiPrep 26/10 desalting column (GE Healthcare). 50% Glycerol was added to such that the final buffer was 40 mM HEPES, 40 mM NaCl, 10% glycerol, pH 7. Protein concentrations were estimated by Bradford assay. Samples were stored at -80 degrees C.
As a negative control, BL21 [DE3] cell pellets expressing the empty pET24b vector were processed as above except for elution from HisTrap in 50 mM sodium phosphate, 500 mM NaCl, containing 500 mM Imidazole, pH 7.2.

His-tagged dextranucrase with leucrose synthase activity was diluted to 0.1 mg/mL in 40 mM HEPES, 40 mM NaCl, 10% glycerol, pH 7.2. - 100 uL reactions were set up for the leucrose synthase and the negative control with the following conditions:

<table>
<thead>
<tr>
<th>Sample (0.1 mg/ml)</th>
<th>#1</th>
<th>#2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer (200 mM Sorensen’s Buffer + 500 mM CaCl2, pH 7)</td>
<td>60.8</td>
<td>60.8</td>
</tr>
<tr>
<td>2 M Sucrose</td>
<td>14.6</td>
<td>14.6</td>
</tr>
<tr>
<td>2M Fructose</td>
<td>0</td>
<td>14.6</td>
</tr>
<tr>
<td>Water</td>
<td>14.6</td>
<td>0</td>
</tr>
<tr>
<td>Total Reaction Volume</td>
<td>100</td>
<td>100</td>
</tr>
</tbody>
</table>

Volumes in column #1 and #2 are in microliters

Table 2 outlines data demonstrating that his-tagged dextranucrase (SEQ ID NO: 29) with leucrose synthase activity is enzymatically active and converts sucrose to leucrose and isomaltose. Dextranucrase enzymes catalyze the conversion of sucrose to locked sugars through a transglycosylation reaction. Table 2, comparing sample 1 and sample 2, demonstrates that dextranucrase with leucrose synthase activity has altered specificity toward producing leucrose versus isomaltose dependent on the addition of fructose as a secondary substrate.

Table 2. Dionex analysis of carbohydrate products from microbially expressed His-tagged dextranucrase with leucrose synthase activity. Enzyme activity indicated by the change in percent sugar determined by comparing samples collected at time 0 and time 24 hours.

<table>
<thead>
<tr>
<th>Sample set up</th>
<th>Glucose (% total sugar)</th>
<th>Fructose (% total sugar)</th>
<th>Sucrose (% total sugar)</th>
<th>Isomaltose (% total sugar)</th>
<th>Isomaltulose (% total sugar)</th>
<th>Leucrose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.99</td>
<td>20.55</td>
<td>-37.46</td>
<td>3.16</td>
<td>0.66</td>
<td>4.09</td>
</tr>
<tr>
<td>2</td>
<td>1.40</td>
<td>-0.29</td>
<td>-6.57</td>
<td>0.12</td>
<td>0.57</td>
<td>4.77</td>
</tr>
<tr>
<td>1 (Negative control)</td>
<td>0.08</td>
<td>0.14</td>
<td>-0.22</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>-0.01</td>
<td>0.63</td>
<td>-0.62</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. Negative control contains bacterial fractions collected as described in Example IA from cells containing an empty pET24 vector.

ID: Levan sucrase, fructosyl transferase (E.C. 2.4.1.10, E.C. 2.4.1.99, E.C. 2.4.1.100)

Sucrose:sucrose fructosyltransferase (SST) (EC 2.4.1.99), 1,2-β-fructan 1-fructosyltransferase (FFT) (EC 2.4.1.100), and levan sucrase (EC 2.4.1.10) are enzymes within the larger class of fructosyl transferases. The fructosyl transferase enzymes catalyze the formation of fructans composed of fructose linked by β(2→1) and/or β(2→6) glucoside bonds. Fructosyl transferases may be identified and isolated from plant, bacterial, or fungal sources. These enzymes may be expressed in plants to accumulate fructans as storage carbohydrates. Accumulation of this polysaccharide (fructan) in sugarcane or other plants may allow the accumulation of excess carbohydrates.

The polynucleotide sequence coding for a fructosyltransferase enzyme will be generated that uses the codon preference for E.coli. This polynucleotide sequence will be synthesized, cloned into an expression vector and expressed in E. coli essentially as described in Example IA.

Fructosyl transferase activity will be estimated by TLC and HPLC similar to the procedures described above for sucrose isomerase and the Dionex analysis described in Example IB. Modifications to the protocol in order to increase the sensitivity for fructans may include development in a solution of propanol:butanol:water (12:3:4) and the use of a urea-phosphoric acid dye mixture (Wise et al., 1955, Anal Chem 27:33-36). Long polymers of fructose have low mobility in the TLC assay and will remain in the location where they are spotted on the silica gel plate. Hydrolysis of fructans to fructose by HCl solution will allow specific identification of fructose using the aniline dye described above. Alternatively a fructanase enzyme may be used to hydrolyze fructans to fructose. This technique will be useful in determining that large polymers are indeed fructans as only fructans would be hydrolyzed by a fructanase enzyme.
Fructose, as the sweetest naturally occurring sugar, also has value as a sweetener in high fructose syrups such as high fructose corn syrup. Plants engineered to produce fructans as a locked sugar may be used as a source of fructans which, after hydrolysis by a fructanase enzyme, produce a solution with a high fructose concentration. In such plants the yield of fructan may be increased by expressing an additional enzyme glucose isomerase to catalyze the conversion of glucose to fructose. The glucose isomerase (invertase) could be expressed in maize endosperm, or expressed in microbes. The purified enzyme could be used to produce fructans, glucans and alternans.

Sweeter plant products can be generated by expressing in plants a combination of enzymes that first allow for the accumulation of fructans in the plant and then convert the fructans directly or indirectly to fructose. Expressing invertase (glucose isomerase) in plants accumulating fructans will lead to a higher sweetness index in the plant.

Endogenous sucrose synthase activity in the endosperm will create additional sucrose which may be used as a substrate for further fructan synthesis.

IE: Alternansucrase

Alternan is a polysaccharide consisting of glucosyl residues linked by alternate alpha-(1-3)/alpha-(1-6) bonds. This polymer is highly soluble and has very low viscosity. Accumulation of this polysaccharide in sugarcane or other plants may allow the accumulation of excess carbohydrates. Alternansucrase is an enzyme which catalyzes the conversion of sucrose to alternan.

Alternansucrase is encoded by the Asr gene of Leuconosloc mesenleroides NRRL B-1355, 1498, and 1501 (Jeannes et al. Am Chem Soc 76:5041-5052, 1954). The Asr gene may be synthesized, cloned into an expression vector and expressed in E. coli essentially as described in Example IA.

Alternansucrase activity may be detected by enzyme-linked immunosorbent assay (ELISA) as described by Kok-Jacon et al. J. Plant Physiol 160: 765-777 (2005). Alternans can be hydrolyzed to form fermentable sugars by the activity of a alpha-1,6-glucosidase or alpha-1,3-glucosidase or a combination of the two enzymes.

IF: Amylosucrase (E.C. 2.4.1.4)
Amylose or starch, is a polysaccharide consisting of glucosyl residues linked by alpha-(1-4) bonds and is the primary carbohydrate storage compound found in most plants. Producing starch in plants that use sucrose as their primary carbohydrate storage compound, such as sugarcane, may permit the accumulation of starch which would behave as a locked sugar.

*Neisseria polysacharea* produces an amylosucrase enzyme (GenBank Accession number Q9ZEU2) which catalyzes the conversion of sucrose to a linear alpha-1,4-linked glucart. For the purpose of producing starch in a transgenic plant, it may be advantageous to target the amylosucrose enzyme in the plant to subcellular compartments that have high concentrations of sucrose, such as the vacuole of sugarcane. Another target may be the vacuole of the maize endosperm. Targeting an enzyme capable of synthesizing starch from sucrose to the vacuole of maize endosperm cells may permit the accumulation of more starch in the maize endosperm as naturally occurring enzymes do not produce starch in the vacuoles of maize endosperms cells. Targeting such an enzyme to endosperm vacuoles may be expected to create up to 10% more starch because of starch accumulation in a subcellular compartment that normally does not accumulate starch. Alternatively targeting to the apoplast is another way to achieve conversion of sucrose into locked sugars such as starch or isomaltulose. In plants such as maize, sucrose accumulates in the leaf and is transported to the ear during grain filling which provides a carbon sink. Table 3 outlines the sugar content of maize tissue with and without removal of the ear. Note that when the ear is removed, excess sugar accumulates in the leaf tissue.

Table 3: Sugar content of maize with and without ears.

<table>
<thead>
<tr>
<th>Sugar, mg/mL</th>
<th>Earless maize</th>
<th>Maize with Ear</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sucrose</td>
<td>7.42</td>
<td>2.6</td>
</tr>
<tr>
<td>Glucose</td>
<td>1.34</td>
<td>1.05</td>
</tr>
<tr>
<td>Fructose</td>
<td>1.32</td>
<td>0.95</td>
</tr>
<tr>
<td>Total, mg/mL</td>
<td>10.08</td>
<td>4.6</td>
</tr>
</tbody>
</table>
A codon optimized polynucleotide sequence coding for the *N. pofysacharea* amylosucrase enzyme may be synthesized, cloned into an expression vector and expressed in *E. coli* essentially as described in Example IA.

His-tagged amylosucrase

Recombinant BL21 cells expressing an amylosucrase will be generated essentially as described in Example IA. Frozen BL21[DE3] cell pellets expressing amylosucrase will be recovered from a 30 mL overnight culture in autoinduction media and will be resuspended in 3 mL BugBuster HT (Novagen) containing Complete EDTA-free protease inhibitors (Roche). Samples will be incubated at room temperature for 10 minutes with occasional mixing to lyse cells. Cell lysate will be centrifuged at 10,000Xg for 10 minutes at 4 degrees C. 10 uL of supernatant will be incubated in a 500 uL reaction containing IX PBS and 100 mM sucrose overnight at 30 degrees C. The presence of a visible white precipitate indicates amylosucrase activity. Determination that this precipitate is starch can be done by washing the precipitate in 80% ethanol several times, followed by solubilization in DMSO and gel permeation chromatography. Susceptibility to digestion by amylase enzyme would further demonstrate the precipitate is composed of starch.

IG: Dionex HPAEC analysis of carbohydrates

Carbohydrate separation and detection was analyzed utilizing a Dionex IC3000 system with a Dionex AS autosampler, a Dionex DC detection compartment (pulsed amperometric detection (PAD) using a disposable Dionex carbohydrate certified gold surface electrode), and a Dionex SP pump system. For high resolution separation, one Carbopac PA1 4x50mM Guard Column followed by two Carbopac PA1 4x250mM analytical columns were used for all analysis. The electrode potentials were set to the carbohydrates standard quad with AgCl reference electrode as specified by Dionex Corporation. The eluent system utilized an isocratic mobile phase consisting of 100mM NaOH and 2mM NaOAc with a 38 min run time. Peak identification was based on standard retention times of glucose, fructose, sucrose (Sigma), leucrose (Carbosynth),
isomaltulose (Fischer) and trehalulose. Peak analysis utilized Chromeleon version 6.80 software (Dionex Corp., Sunnyvale, CA).

Example 2: Enzymes that unlock locked sugars

2A: Fructanase (EC 3.2.1.80, E.C. 3.2.1.7)

Fructanases are fructosydases which catalyze the hydrolysis of fructosidic linkages in fractans to break the fructan down into simpler sugar molecules. Fructans can be hydrolyzed to fermentable sugars through the catalytic activity of fructanases. For Example, the fructanase 2,1-β-D-fructan fructanohydrolase [EC 3.2.1.7] can hydrolyze fructan polymers into fructose monosaccharides which can be fermented to form ethanol.

A codon optimized polynucleotide sequence coding for a fructanase enzyme may be synthesized, cloned into an expression vector and expressed in *E. coli* essentially as described in Example IA.

Fructanase activity may be estimated by incubating a fructanase enzyme with a solution of fructan. Hydrolysis of fructan by the fructanase will release the monosaccharide fructose which may be detected by TLC or HPLC as described above for sucrose isomerase (Example IB).

2B: Glucosidase

Gene sequences for alpha-1,6-glucosi search the NCBI database for genes homolog polypeptide sequences (SEQ ID NOs: 1 - 6) were back translated (using Vector NTI program) into polynucleotide sequences using the codon preference of *E. coli*. The *E. coli* codon optimized polynucleotide sequences were synthesized by GeneArt and expressed in *E. coli* essentially as described in Example IB.

Alpha-1,6-glucosidase activity was assayed by measuring the production of glucose from hydrolysis of the alpha-1,6-glucoside bond of isomaltulose. 13 microliters of crude *E. coli* extract was added to 37 microliters of isomaltulose reaction buffer (100mM isomaltulose and 30 mM HEPES (pH 7.5)) at 30 degrees, 40 degrees, 50 degrees, 60 degrees, 70 degrees, or 80 degrees C depending on the enzyme; for 10
minutes, 20 minutes, 30 minutes, or 40 minutes. 20 microliters of the reaction product was added to a 96 well microplate, then 250 microliters of glucose oxidase reagent (Pointe Scientific) was added and the mixture was incubated at 37 degrees C for 10 minutes. After this incubation, the Absorbance at 500 nm was read using a SpectraMax plus 384. Sample absorbance was compared with the absorbance at 500 nm of controls which were 13 microliters each of a set of glucose standards that were also allowed to react with the glucose oxidase reagent. A standard curve was created from the controls and the production of glucose from the hydrolysis of isomaltulose by the samples was estimated by comparing the absorbance at 500 nm for the samples to the standard curve.

Using this method, the alpha-1,6-glucosidase enzymes described by SEQ ID NOs: 1 - 6 were screened and found to have activities at temperatures ranging from 30 degrees C to 80 degrees C. Table 4 describes the alpha-1,6-glucosidase activity measured in total cell lysate of an E. coH strain expressing the Bacillus thermoamyloHquefaciem enzyme (SEQ ID NO:5).
Table 4: Alpha-1,6-glucosidase (SEQ ID NO: 5) activity measured using sucrose as the substrate.

His tagged enzyme recovery from recombinant E. coli

Recombinant BL21 E. coli cells expressing an alpha-1,6-glucosidase (SEQ ID NOs: 1, 3, 5 and 6) were generated essentially as described in Example IA. The frozen cell pellets expressing the his-tagged alpha-1,6-glucosidase key enzymes were brought up to a volume of 40 mL in extraction buffer (50 mM sodium phosphate, 500 mM NaCl, 10 mM Imidazole, pH 7.2-8 containing protease inhibitors (Roche Complete EDTA-free protease inhibitor tablets)). Cells were lysed by 2 passages through a FRENCH Press (Thermo IEC). Cell lysates were centrifuged for 30 minutes at 10,000Xg at 4 degrees C. Supernatants were collected and filtered using 0.45 micron vacuum filter device (Millipore).

A His Trap FF column was used to recover the his-tagged enzymes from the supernatant. A HisTrap FF 5 mL column (GE Healthcare) was equilibrated with
extraction buffer. The clarified lysates were loaded at 5 mL/tnin. Bound his-tagged enzymes were eluted in 50 mM sodium phosphate, 500 tnM NaCl, containing 150-500 mM Imidazole, pH 7.2-8.

The negative control was BL21[DE3] cell pellets transformed with empty pET24b vector essentially as described in Example IA. Negative control cell pellets were extracted essentially as described above for the his-tagged alpha-1,6-glucosidase enzymes; however, the extraction buffer and elution buffers were at pH 7.2.

All samples collected from the HisTrap FF column were buffer exchanged into 50 mM HEPES, 50 mM NaCl, pH 7 using either Bio-Rad Econo-Pac 10-DG desalting column or HiPrep 26/10 desalting column (GE Healthcare). 50% Glycerol was added such that the final buffer was 40 mM HEPES, 40 mM NaCl, 10% glycerol, pH 7. Protein concentrations were estimated by Bradford assay. Samples were stored at -80 degrees C.

7. *ethanolicus* alpha-1,6-glucosidase (SEQ ID NO: 6):

His-tagged *T. ethanolicus* alpha-1,6-glucosidase (SEQ ID NO: 6) was recovered from recombinant BL21 *E. coli* cells essentially as described above (Example 2B "His tagged enzyme recovery from recombinant *E. coli*”). Frozen samples derived from the HisTrapFF column were combined with 3 M ammonium sulfate, 50 mM ammonium phosphate, pH 7 to a final ammonium sulfate concentration of 1 M. This sample was applied to a 5 mL HiTrap Phenyl HP column (GE Healthcare). Bound proteins were eluted from the column by washing the column with a linear ammonium sulfate gradient over 100 ml from 50 mM Sodium phosphate, 1.5 M ammonium sulfate, pH 7 to 50 mM sodium phosphate buffer pH 7 containing no ammonium sulfate. Fractions containing the enzyme were pooled and concentrated using Centri-prep YM-30 concentrator device (Amicon).

*B. thuringiensis* alpha-1,6-glucosidase (SEQ ID NO: 3):

His-tagged *B. thuringiensis* alpha-1,6-glucosidase (SEQ ID NO: 3) was recovered from recombinant BL21 *E. coli* cells essentially as described above (Example 2B "His tagged enzyme recovery from recombinant *E. coli*”). Fractions containing his-tagged enzyme were pooled and diluted in 50 mM HEPES, pH 6. Sample was applied to a 5 mL
HiTrap Q HP column (GE Healthcare). Bound proteins were eluted by washing the column with a linear NaCl gradient over 100 mL from 50 mM HEPES, pH 6 to 50 mM HEPES, 1 M NaCl, pH 6. The fractions containing the enzyme were pooled.

\textit{G. thermogluco\textit{c}osidasi\textit{s}} alpha-1,6-glucosidase (SEQ ID NO: 1):

His-tagged \textit{G. thermogluco\textit{c}osidasi\textit{s}} alpha-1,6-glucosidase (SEQ ID NO: 1) was recovered from recombinant BL21 \textit{E. coli} cells essentially as described above (Example 2B "His tagged enzyme recovery from recombinant \textit{E. coli}"). Fractions containing his-tagged enzyme were pooled and diluted in 50 mM Tris-HCl, pH 7. Sample was applied to a 5 mL HiTrap Q HP column (GE Healthcare). Bound proteins were eluted by washing the column with a linear NaCl gradient over 100 mL from 50 mM HEPES, 10 mM NaCl, pH7 to 50 mM HEPES, 1 M NaCl, pH 7. The fractions containing the enzyme were pooled and concentrated to 1 mL Centri-prep YM-30 concentrator device (Amicon). Sample was applied to a HiPrep 26/60 S-100 HR size exclusion column and eluted with 20 mM Tris-HCl, 250 mM NaCl, pH 7. Fractions containing the enzyme were pooled and diluted in 1.5 M Ammonium Sulfate, 50 mM Sodium phosphate, pH 7. Sample was applied to a 5 mL HiTrap Phenyl HP column (GE Healthcare). Bound proteins were eluted by washing the column with a linear ammonium sulfate gradient over 100 mL from 50 mM Sodium phosphate, 1.5 M ammonium sulfate, pH 7 to 50 mM sodium phosphate buffer pH 7 containing no ammonium sulfate. Fractions containing the enzyme were pooled.

\textit{B. thermoamyloliquefaci\textit{e}} alpha-1,6-glucosidase (SEQ ID NO: 5):

His-tagged \textit{i?. thermoamyloliquefaci\textit{e}} alpha-1,6-glucosidase (SEQ ID NO: 5) was recovered from recombinant BL21 \textit{E. coli} cells essentially as described above (Example 2B "His tagged enzyme recovery from recombinant \textit{E. coli}"). Fractions containing his-tagged enzyme were pooled and diluted in 20 mM Tris-HCl, pH 7. Sample was applied to a 5 mL HiTrap Q HP column (GE Healthcare). Bound proteins were eluted by washing the column with a linear NaCl gradient over 100 mL from 20 mM Tris-HCl, 50 mM NaCl, pH 7 to 50 mM HEPES, 1 M NaCl, pH 7. Fractions containing the enzyme were pooled and concentrated to 1 mL Centri-prep YM-30
concentrator device (Araicon). Sample was applied to a HiPrep 26/60 S-100 HR size exclusion column and eluted with 50 mM HEPES, 50 mM NaCl, pH 7.4. Fractions containing the enzyme were pooled in 1.5 M Ammonium Sulfate, 50 mM Sodium phosphate, pH7. Sample was applied to a 5 mL HiTrap Phenyl HP column (GE Healthcare). Bound proteins were eluted by washing the column with a linear ammonium sulfate gradient over 100 mL from 50 mM Sodium phosphate, 1.5 M ammonium sulfate, pH 7 to 50 mM sodium phosphate buffer pH 7 containing no ammonium sulfate. Fractions containing the enzyme were pooled.

Activity of His-tagged alpha-1,6-glucosidase key enzymes

The enzyme activity of the alpha-1,6-glucosidase enzymes (SEQ ID NOs: 1, 3, 5 and 6) recovered from recombinant BL2 1 E. coli cells was measured. Samples collected from the purification schemes described above (Example 2B) were diluted to 0.2 mg/mL in 50 mM HEPES, 50 mM NaCl, pH 7. Reactions were initiated by mixing samples with an equal volume of 100 mM HEPES, 4 mM EDTA, 0.04% Tween-20, 200 mM Isomaltulose, pH 7. For buffer controls, 100 mM HEPES, 4 mM EDTA, 0.04% Tween-20, pH 7 was combined with an equal volume of 200 mM Isomaltulose. Reactions were incubated at optimal temperature for the enzyme (37, 45, or 60 degrees C) for 40 minutes in a Biorad Tetrad 2 thermocycler for the appropriate time. Reactions were terminated by heating samples at 95 degrees C for 5 minutes. Glucose concentrations in reactions were estimated using the GOPOD assay. Enzyme activity is detected as the conversion of isomaltulose to glucose.

The GOPOD assay was performed by combining 20 uL aliquots of reaction samples, or glucose standards of known concentrations, with 250 uL GlucoseOx Reagent (Pointe Scientific) in a 96-well assay plate (Costar 3370) and incubated for 10 minutes at 37 degrees C. Absorbance at wavelength of 500 nm was measured using SpectraMax 384 Plus plate reader. Absorbance values of sample reactions were converted to glucose concentrations using the equation from a glucose standard curve generated by plotting the absorbance value versus the known glucose standard concentration. The activity of the various alpha-1,6-glucosidase enzymes is described in Table 5.
Table 5: Activity data for alpha-1,6-glucosidase enzymes

<table>
<thead>
<tr>
<th>Sample (SEQ ID NO)</th>
<th>Glucose (mM)</th>
<th>Reaction temperature in degrees C</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. ethanolicus (6)</td>
<td>19.72</td>
<td>60</td>
</tr>
<tr>
<td>G. thermoglucosidasius (1)</td>
<td>29.16</td>
<td>60</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.07</td>
<td>60</td>
</tr>
<tr>
<td>Buffer only negative control</td>
<td>0.03</td>
<td>60</td>
</tr>
<tr>
<td>B. thurgiensis (3)</td>
<td>23.35</td>
<td>37</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.07</td>
<td>37</td>
</tr>
<tr>
<td>Buffer only negative control</td>
<td>0.01</td>
<td>37</td>
</tr>
<tr>
<td>B. thermoamyloliquefaciens (5)</td>
<td>1.17</td>
<td>45</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.09</td>
<td>45</td>
</tr>
<tr>
<td>Buffer only negative control</td>
<td>0.01</td>
<td>45</td>
</tr>
</tbody>
</table>
Activity Analysis of His-tagged α-1,5-glucosidase and α-1,1-glucosidase key enzymes

Extracts of his-tagged enzymes were generated essentially as described above and were diluted to 0.08 mg/mL in 40 mM HEPES, 40 mM NaCl, 10% glycerol, pH 7. Enzyme activity assays were initiated by mixing samples with an equal volume of 100 mM HEPES, 4 mM EDTA, 0.04% Tween-20, 200 mM leucrose (for α-1,5-glucosidase key enzymes (SEQ ID NOs: 30-33)) or 135 mM trehalulose/67 mM isomaltulose mixture (for α-1,1-glucosidase key enzyme (SEQ ID NO: 34)), pH 7. Reactions were incubated at optimal temperature (70 degrees C for α-1,5-glucosidase enzymes and 80 degrees C for α-1,1-glucosidase key enzyme) for 40 minutes in a rate time. Reactions were terminated by the conversion of a locked substrate (leucrose or trehalulose and/or isomaltulose) to glucose. Glucose concentrations in reactions were estimated using GOPOD assay essentially as described above. Table 6 outlines data which demonstrates that his-tagged α-1,5-glucosidase enzymes and α-1,1-glucosidase enzyme are active and convert locked sugar substrates to fermentable sugar.

Table 6: Conversion of locked sugars to glucose by his-tagged key enzymes.

<table>
<thead>
<tr>
<th>Sample name (SEQ ID NO:)</th>
<th>GK24 N-de (30)</th>
<th>GK24 (31)</th>
<th>HB27 (32)</th>
<th>HB8 (33)</th>
<th>Negative Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose Conc. (mM)</td>
<td>0.94</td>
<td>1.01</td>
<td>0.42</td>
<td>1.56</td>
<td>0.05</td>
</tr>
<tr>
<td>Sample name (SEQ ID NO:</td>
<td>SAM1606 (34)</td>
<td>Negative control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Glucose concentration (mM)</td>
<td>8.67</td>
<td>0.46</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2C: Dextranase (E.C. 3.2.1.11)
Dextranases are glycosidases which catalyze the exo or endohydrolysis of 1, 6 alpha D glucosidic linkages in dextrans thus converting the dextran to smaller sugar molecules. A codon optimized polynucleotide sequence coding for a dextranase enzyme may be synthesized, cloned into an expression vector and expressed in E. coli essentially as described in Example IA.

Dextranase enzyme activity assays will monitor the rate of isomaltose released from a dextran molecule during a hydrolysis reaction. HPLC size exclusion chromatography will also be employed to determine the level of dextran hydrolysis achieved by measuring the release of individual sugars.

Assays will be validated using a commercially available dextranase from PeniaUiwn sp I.U.B.: 3.2.1. 11(Worthington Biochemical Corporation, NJ 08701). The dextran hydrolysis can be measured by incubating 0.1 mL of 5-20 micrograms/mL of dextranase with 1.9 mL of commercially available dextran solution (substrate). Thermostability of dextranases will be tested in experiments performed at 60 to 70 degrees C which are temperatures relevant to sugar mill sugarcane juice processing. Validated assays will be further optimized for detection of functional dextranases cloned and expressed in E. coli.

Example 3: Transgenic plants

3A: Transgenic sugarcane

Embryogenic callus was produced from the immature leaf tissue of sugarcane. In greenhouse, cane was harvested by cutting off immature shoots at or above ground level and outer leaves and leaf sheaths were stripped. Basal nodes and emergent leaves were trimmed. In the laboratory (laminar flow cabinet), excess leaf sheaths were unfurled, nodes were trimmed and cane was sterilized (sprayed with 70% ethanol or immersed in 20% bleach for 20 minutes). Additional outer leaf sheaths were removed to expose inner 4-6 leaf rolls and leaf roll was cut to manageable size (12-15 mm in length). Remaining basal nodes and internodes were removed to expose the leaf roll region just above the apical meristem.
Transverse sections of the leaf roll were cut to form discs 0.5 - 1.0 mm in thickness, using not more than a 3.0 cm length of the leaf roll material. Leaf roll discs were plated onto MS media containing 2 -3 mg/L of 2, 4-D and cultured in the dark for 3-4 weeks. Leaf roll discs were cut or split apart at the time of initiation or 2 weeks following initiation and the resulting pieces spread across media to promote a more consistent and prolific embryogenic/proto-embryogenic culture response. After 3-4 weeks of culture, embryogenic callus was selectively excised from leaf disc rolls and sub-cultured on same (MS +2, 4-D) media. Further selective subcultures were performed every 2-3 weeks, dependent upon growth and development to produce additional cultures, until cultures reach 8 -10 weeks of age.

Gene Delivery using the Biolistics PDS 2000 Particle Delivery Device for Sugarcane Transformation

Target embryogenic cultures were prepared for gene delivery by selecting high quality target tissue pieces and preculturing them for 3-6 days on fresh media before gene delivery.

At 2-5 hours prior to gene delivery, target tissues were arranged in a target pattern on high osmotic potential media containing MS basal salts and B5 Vitamins supplemented with sucrose 30 g/L and 0.2 M sorbitol and 0.2 M mannitol plus 2 mg/l 2,4-D.

To prepare DNA for bombardment, gold particles (0.6 micrometer size, Bio-Rad) were re-suspended in 50% sterile glycerol by vortexing. An aliquot of the glycerol – gold particle suspension was combined by gentle mixing with 2 x 10^14mol DNA of the gene encoding the selectable marker (PMI) and genes of interest outlined in Table 29 of Example 12. The mixture was combined with 2.5M CaC12 and cold 1M spermidine to precipitate the DNA onto the gold particles. The gold particles with precipitated DNA were washed with ethanol. The gold particles were repeatedly re-suspended in ethanol and aliquots of DNA/particle suspension were placed evenly onto the center of individual macrocarrier membrane disks and allowed to dry. The macrocarrier was loaded into the gene gun above the stopping screen. Bombardment of embryos was performed with a PDS - 1000 Helium gene gun. A rupture disc of 1300 psi was used and the distance from
the rupture disc and the macrocarrier was set at 8 mm with a stopping screen at 10 mm. The distance between the stopping screen and the embryos was about 7 cm. The pressure on the helium tank was set at about 1400 psi. Target tissues (embryogenic cultures) were bombarded with 2 shots before being transferred to the dark at 28 degrees C for about 12 hours.

After recovery, the bombarded cultures were transferred to maintenance medium and cultured at 28 degrees C in the dark. After 7 days, the bombarded cultures were transferred to fresh selection medium containing mannose (7-9 grams/L), 5g/L sucrose plus 2 mg/L 2,4-D and incubated for 4-5 weeks in dark. Growing callus pieces were then subcultured to fresh selection media every 2 weeks until they were large enough for analysis. Typically, 2 to 3 rounds of subculture were required.

Regeneration of plants from transgenic callus lines

After 4-5 weeks on mannose selection media, surviving embryogenic callus colonies are selectively isolated from original cultures and transferred onto regeneration media (MS salts and B5 vitamins, 30g/L sucrose, supplemented with 3-6 g/L mannose and 2 mg/L BAP) at 28 degrees C in dark in Flambeau boxes.

One week later, the cultures are transferred to a light room for shoot development under 16 hours light at 28 degrees C. After 3-4 weeks in the regeneration media, the visible green buds or shoots are sub-cultured on elongation media (MS basal salts and B5 vitamins, sucrose 30 g/L with hormone-free)

Regenerated shoots are rooted in the i

rooting cultures are kept at 28 degrees C under light for another 2 weeks before transfer to the greenhouse and soil. Any of the genes described in Example 1, Example 2 or Example 12 can be transformed into sugarcane to generate transgenic plants using the above described protocol. Agrobacterium mediated genetic transformation is also possible and methods are described in the literature such as Arencibia, Ariel D. and Carmona, Elva R. Sugarcane (Saccharum spp.) Methods in Molecular Biology (Totowa, NJ, United States) (2006), 344(Agrobacterium Protocols (2nd Edition), Volume 2), 227-235
3B: Transgenic sugarcane expressing dextransucrase activity

Selected dextransucrases are sequence optimized based upon the codon preference for sugarcane. The sugarcane codon optimized sequence is cloned into transformation vectors for sugarcane transformation. One of skill in the art is able to select the appropriate promoter and terminator for the dextransucrase gene as well as select an appropriate selectable marker for sugarcane transformation. Targeting sequences are incorporated into the expression construct for dextransucrases to target the enzyme to the vacuolar compartment of parenchyma cells where sucrose is stored.

Transgenic sugarcane plants are generated as described in Example 3A. Transformed plants are analyzed using routine methods for DNA analysis of transgenic plants in order to determine if the expression construct has been incorporated into the nuclear DNA of the sugarcane plant.

Transgenic sugarcane plants are evaluated for dextransucrase enzyme activity. Mature plant tissue is crushed and the juice will be collected and chilled prior to assaying for dextran accumulation using the detection methods described in Example 1C. Enzyme assay methods described in Example 1C are used to determine the functionality of the expressed enzyme in transgenic plants.

3C: Generation of transgenic plants expressing dextranase activity.

Selected dextranases are codon optimized for expression in sugarcane using the codon preference for sugarcane. The sugarcane optimized gene sequence is cloned into a transformation vector designed for sugarcane transformation. One of skill in the art is able to select the appropriate promoter and terminator for the dextranase as well as select an appropriate selectable marker for sugarcane transformation. The dextranase enzyme is targeted to the ER subcellular compartment of parenchyma cells using the appropriate targeting sequences. The dextranase enzyme is targeted away from the sucrose and dextran storage compartment of the sugarcane plant.

Transgenic plants are generated as described in Example 3A. Enzyme activity is evaluated in mature plant tissue by crushing and extracting juice from the transgenic plant and performing the assays for dextranase activity as described in Example 2C. Enzyme assay methods described in Example 2C are used to determine the functionality
of the expressed enzyme in sugarcane juice:

Expression cassettes described in Example 12 were cloned into either a binary vector or a binary vector also containing an origin of replication from BCTV, beet curly top virus, (SEQ ID NO: 8). The binary vectors without the origin of replication from BCTV were transferred into Agrobacterium tumefaciens strain LBA4404 using the freeze-thaw method (An et al., Binary vector. In: Gelvin SB, Schilproot RA (eds), Plant molecular biology manual. Kluwer Academic Publishers, Dordrecht, pp A3 (1988)). The binary vectors containing the origin of replication from BCTV (BCTV binary vectors) were transferred into Agrobacterium tumefaciens strain LBA4404 containing a helper plasmid containing a replicase sequence from BCTV (SEQ ID NO: 9) using the freeze-thaw method (An et al., Binary vector. In: Gelvin SB, Schilproot RA (eds), Plant molecular biology manual. Kluwer Academic Publishers, Dordrecht, pp A3 1-19 (1988)).

Leaves from sugar beet or tobacco were used for the transient expression of enzymes in plant tissue. Tobacco leaves from transgenic TEV-B tobacco plants (made in the tobacco cultivar Xanthi) containing a mutated PI/HC-Pro gene from TEV that suppresses post-transcriptional gene silencing (Mallory et al., Nat Biotechnol 20:622 (2002)) were used for transient expression of selected enzymes. Preparation of Agrobacterium cultures and infiltration of tobacco or sugar beet leaves was carried out as described by Azhakanandam et al., Plant Mol. Biol. 63: 393-404 (2007). In brief, the genetically modified agrobacteria were grown overnight in 50 mL of LB medium containing 100 µM acetosyringone and 10 µM MES (pH 5.6), and subsequently were pelleted by centrifugation at 4000Xg for 10 min. The pellets were resuspended in the infection medium [Murashige and Skoog salts with vitamins, 2% sucrose, 500 µM MES (pH 5.6), 10 µM MgSO₄, and 100 µM acetosyringone] to OD₆₀₀ = 1.0 and subsequently held at 28 degrees C for 3 hours. Infiltration of individual leaves was carried out on sugar beet (about 3 weeks old) and TEV-B tobacco plants (about 4 weeks old) using a 5 mL syringe by pressing the tip of the syringe (without a needle) against the abaxial surface of the leaf. Infiltrated plants were maintained at 22-25 degrees C with a photoperiod of 16 hours light and 8 hours dark. Plant tissue was harvested after 5 days post infiltration for subsequent analysis.
To ensure that enzyme activity measured was due to plant expression of the enzymes, the expression constructs also incorporated an intron in the polynucleotide sequence coding for the enzyme. The presence of the intron ensures that expression of the enzyme is due to plant expression (able to process out the intron and therefore express a fully processed enzyme) versus agrobacterium expression (unable to process the intron and thus not able to express a functional enzyme).

3D: Transient expression in tobacco and sugar beet leaves

Expression cassettes described in Example 12 were cloned into either a binary vector or a binary vector also containing an origin of replication from BCTV, beet curly top virus (SEQ ID NO: 8). The binary vectors without the BCTV origin of replication were transferred into Agrobacterium tumefaciens strain LBA4404 using the freeze-thaw method (An et al., Binary vector. In: Gelvin SB, Schilproot RA (eds), Plant molecular biology manual. Kluwar Academic Publishers, Dordrecht, pp A3 1-19 (1988)). The BCTV containing binary vectors were transferred into Agrobacterium tumefaciens strain LBA4404 containing a helper plasmid containing a BCTV replicase sequence (SEQ ID NO: 9) using the freeze-thaw method (An et al., Binary vector. In: Gelvin SB, Schilproot RA (eds), Plant molecular biology manual. Kluwar Academic Publishers, Dordrecht, pp A3 1-19 (1988)).

Leaves from sugar beet or tobacco were used for transient expression of enzymes. Transgenic TEV-B tobacco plants (made in the tobacco cultivar Xanthi) containing a mutated Pl/HC-Pro gene from TEV that suppresses post-transcriptional gene silencing (Mallory et al., Nat Biotechnol 20:622 (2002)) were used for transient expression of selected enzymes in tobacco leaves. Preparation of Agrobacterium cultures and infiltration of tobacco or sugar beet plants was carried out as described by Azhakanandam et al., Plant Mol. Biol. 63: 393-404 (2007). In brief, the senetically modified agrobacteria were grown overnight

acetylsyringone and 10 µM MES (pH 5.6), anu auuacqucuuy weic µuctcu uy

centrifugation at 4000Xg for 10 min. The pellets were resuspended in the infection medium [Murashige and Skoog salts with vitamins, 2% sucrose, 500 µM MES (pH 5.6), 10 µM MgSO₄, and 100 µM acetylsyringone] to OD₆₀₀ = 1.0 and subsequently held at 28
degrees C for 3 hours. Infiltration of individual leaves was carried out on sugar beet (about 3 weeks old) and TEV-B tobacco plants (about 4 weeks old) using a 5 mL syringe by pressing the tip of the syringe (without a needle) against the abaxial surface of the leaf. Infiltrated plants were maintained at 22-25 degrees C with a photoperiod of 16 hours light and 8 hours dark. Plant tissue was harvested after 5 days post infiltration for subsequent analysis.

3E. Maize transient expression system

Expression cassettes described in Example 12 were cloned into a binary vector. The constructs were transferred into Agrobacterium tumefaciens strain LBA4404 containing helper plasmid (pSBI) using a freeze-thaw method (An et al., Binary vector. In: Gelvin SB, Schilproot RA (eds), Plant molecular biology manual. Kluwar Academic Publishers, Dordrecht, pp A3 1-19 (1988)).

The maize transient expression system was established using young maize seedlings (5-12 d old). Preparation of Agrobacterium cultures and infiltration of maize leaves was carried out as described by Azhakanandam et al., Plant Mol. Biol. 63: 393-404 (2007). In brief, the genetically modified agrobacteria were grown overnight in 50 mL of LB medium containing 100 µM acetosyringone and 10 µM MES (pH 5.6), and subsequently were resuspended in the infection medium (Murashige and Skoog salts with vitamins, 2% sucrose, 500 µM MES (pH 5.6), 10 µM MgSO₄, and 100 µM acetosyringone) to OD₅₉₀ = 1.0 and subsequently held at 28 degrees C for 3 hours. Infiltration of individual leaves was carried out on maize seedlings using a 5 mL syringe, without a needle, by pressing the tip of the syringe against the abaxial surface of the leaf. Infiltrated plants were maintained at 22-25 degrees C with a photoperiod of 16 hours light and 8 hours dark. Plant tissue was harvested after 5-7 days post infiltration for subsequent analysis.

To ensure that enzyme activity measured was due to plant expression of the enzymes, the expression constructs also incorporated an intron in the polynucleotide sequence coding for the enzyme. The presence of the intron ensures that expression of the enzyme is due to plant expression (able to process out the intron and therefore express
a fully processed enzyme) versus agrobacteriuni expression (unable to process the intron and thus not able to express a functional enzyme).

3F. Transgenic maize callus and plants

Transformation of maize callus was performed using a biolistic transformation method. Maize embryos were collected from maize kernels about 8 to 11 days after pollination. The ears were collected and sterilized in 20% Germicidal Clorox for 20 minutes on an orbital shaker set at 120 rpm followed by extensive rinsing of the ear in sterile water. Embryos were collected from the kernels and kept on culture media in the dark for 3 to 7 days.

To prepare DNA for bombardment, gold particles (0.6 to 1 micrometer size, Bio-Rad) were resuspended in 50% sterile glycerol by vortexing. An aliquot of the glycerol – gold particle suspension was combined by gentle mixing with 2 x 10^-6 mol DNA of the gene encoding the selectable marker (PMI) and gene of interests outlined in Table 29 of Example 12. The mixture was combined with 2.5M CaCl2 and cold 1M spermidine to precipitate the DNA onto the gold particles. The gold particles with precipitated DNA were washed in ethanol. The washed gold particles were re-suspended in ethanol and aliquots of DNA suspension were placed evenly onto the center of individual macrocarrier membrane disks and allowed to dry. The macrocarrier was loaded into the gene gun above the stopping screen. Bombardment of embryos was performed with a PDS Helium – 1000 gene gun. A rupture disc in the range of 650 - 1800 psi was used and the distance from the rupture disc and the macrocarrier was set at 8 mm with a stopping screen at 10 mm. The distance between the stopping screen and the embryos was about 7 cm. The pressure on the helium tank was set at about 1200 psi. Target tissues (embryos) were bombarded 3 times before being transferred to the dark at 28 degrees C to recover for 3 days.

After recovery, the bombarded embryos were transferred to maintenance medium and cultured at 28 degrees C in the dark. After 3 days, the bombarded embryo tissue was transferred to fresh callus induction medium and incubated for 1 week to induce callus formation. The calli were then transferred to selection medium containing mannose for three weeks at 28 degrees C in the dark.
Selection of transgenic calli was performed by transferring living callus tissue to selection medium and cultured at 28 degrees C in the dark for 3 weeks. Surviving calli were transferred to fresh selection medium and cultured an additional 2 weeks at 28 degrees C in the dark. Surviving calli were then transferred to regeneration medium and cultured at 28 degrees C in the dark for 2 weeks.

Callus tissues will be incubated under 16 hours of light at 24 degrees C to encourage shoot development. Once shoot development starts, callus with shoots will be transferred to rooting medium and cultured at 24 degrees C with light for another week prior to transplanting to soil for the remainder of the maize growing cycle.

3G: Analysis of key enzymes in plant tissue

Whole leaves from tobacco or sugar beet transiently expressing an enzyme were frozen at -80 degrees C in 24-well blocks containing 3/16” chrome ball bearings. The frozen material was shaken at setting 9 for 2 min in a Kleco Titer plate/Microtube Grinding Mill creating a powder. Buffer (50mM HEPES, 2mM EDTA, 0.02% Tween-20, 100mM locked sugar (isomaltulose, leucrose, or trehalulose depending upon the enzyme), pH 7) was added to the powdered samples to give a thick slurry. Samples were incubated in a Glas-Col rotator at 80% speed for 30 min. Samples were transferred by wide-bore P200 pipet to PCR tubes at 100 uL per tube and incubated at the appropriate temperature for the enzyme (50, 60, 70, 80 degrees C depending on enzyme) in a Biorad Tetrad 2 thermocycler. The sample was transferred to either a Millipore Biomax 5KD MW membrane spin filter and centrifuged at 12,000Xg for 20 min or a Millipore Multiscreen-HV filter plate and filtered at 20 were diluted in MiIH-Q water as necessary ar vials with split caps for carbohydrate analysis by Dionex HPAEC.

3H: Analysis of locking enzymes in plant tissue

Whole leaves from tobacco, sugar beet, or maize were rolled and placed into filtration baskets (DNA IQ Spin Basket) and the filled filtration baskets placed into 1.5mL eppendorf tubes. The filled filtration baskets and eppendorf tubes were frozen on dry ice for 5-8 min (or until frozen) followed by thawing on ice for 5-8 min (or until
thawed). The thawed filled filtration baskets and eppendorf tubes were then centrifuged at 10,000Xg for 15 min at 4 degrees C and the filtrate collected.

The filtrate was boiled at 100 degrees C for 5 min followed by centrifugation at 16,000Xg for 20 min. The boiled filtrate was further filtered by transferring the boiled filtrate to either a Millipore Biomax 5KD MW membrane spin filter and centrifuged at 12,000Xg for 20 min or a Millipore Multiscreen-HV filter plate and filtered at 20 InHg. The filtrate was collected and diluted in Milli-Q water as necessary and placed into either 0.3 or 1.5mL sample vials with split caps for analysis.

Example 4: Plant expressed sucrose isomerase enzyme

4A: Transient expression of sucrose isomerase in sugar beet and tobacco leaves

The transformation vector 17588, as described in Example 12, was used to transiently expressing enzymes in tobacco or sugar beet leaves essentially as described in Example 3D. Tobacco or sugar beet leaves transiently expressing a sucrose isomerase were generated using the vector 17588 which contains a dicot optimized polynucleotide sequence encoding a sucrose isomerase (SEQ ID NO: 16). Leaves transiently expressing sucrose isomerase were harvested and extracted essentially as described in Example 3H and analyzed by Dionex for carbohydrates essentially as described in Example 1G.

Dionex HPAE chromatography utilized pure sugar standards as a reference for retention time and standard curve production for determining sugar concentrations. Sugar concentrations were based on the total sugar consisting of glucose, fructose, sucrose, trehalulose and isomaltulose when present. These five sugars represent >98% of the total peak area of the chromatograms with the remainder coming from minor unknown peaks from the biological extraction milieu of the leaf.

Sucrose isomerase activity in transiently infiltrated leaves was directly detected by the formation of the two major products of the enzymatic conversion of sucrose to the locked sugars, trehalulose and isomaltulose. Neither of the locked sugars were present in control leaves. Tables 7 - 10 summarize the analysis of tobacco and sugar beet transiently expressing a sucrose isomerase (vector 17588) and demonstrate that tobacco and sugar beet plants are able to express an active sucrose isomerase which catalyzes the
conversion of sucrose to the locked sugars isomaltulose and trehalulose and accumulate the locked sugars in the leaves.

Table 7: Carbohydrate analysis (HPAEC) of tobacco leaves expressing a sucrose isomerase (SEQ ID NO: 16).

<table>
<thead>
<tr>
<th>sample</th>
<th>Sucrose (mM)</th>
<th>Trehalulose (mM)</th>
<th>Isomaltulose (mM)</th>
<th>Total Disaccharide (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17588</td>
<td>3.6</td>
<td>17.7</td>
<td>6.4</td>
<td>27.7</td>
</tr>
<tr>
<td>17588</td>
<td>6.8</td>
<td>34.3</td>
<td>14.1</td>
<td>55.2</td>
</tr>
<tr>
<td>17588</td>
<td>4.2</td>
<td>23.9</td>
<td>8.1</td>
<td>36.2</td>
</tr>
<tr>
<td>17588</td>
<td>14.7</td>
<td>33.1</td>
<td>13.8</td>
<td>61.6</td>
</tr>
<tr>
<td>Negative control</td>
<td>11.9</td>
<td>0.0</td>
<td>0.0</td>
<td>11.9</td>
</tr>
<tr>
<td>Negative control</td>
<td>11.8</td>
<td>0.0</td>
<td>0.0</td>
<td>11.8</td>
</tr>
<tr>
<td>Negative control</td>
<td>6.3</td>
<td>0.0</td>
<td>0.0</td>
<td>6.3</td>
</tr>
<tr>
<td>Negative control</td>
<td>4.2</td>
<td>0.0</td>
<td>0.0</td>
<td>4.2</td>
</tr>
</tbody>
</table>

Table 8: Carbohydrate analysis (HPAEC) of tobacco leaves transiently expressing sucrose isomerase.

<table>
<thead>
<tr>
<th>sample</th>
<th>Glucose + Fructose (% total sugar)</th>
<th>Sucrose (% total sugar)</th>
<th>Trehalulose (% total sugar)</th>
<th>Isomaltulose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17588</td>
<td>39.2</td>
<td>7.9</td>
<td>38.8</td>
<td>14.1</td>
</tr>
<tr>
<td>17588</td>
<td>51.4</td>
<td>6.0</td>
<td>30.2</td>
<td>12.4</td>
</tr>
<tr>
<td>17588</td>
<td>47.9</td>
<td>6.0</td>
<td>34.4</td>
<td>11.7</td>
</tr>
<tr>
<td>17588</td>
<td>51.7</td>
<td>11.5</td>
<td>26.0</td>
<td>10.8</td>
</tr>
<tr>
<td>Negative control</td>
<td>40.6</td>
<td>59.4</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Negative control</td>
<td>58.5</td>
<td>41.5</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Negative control</td>
<td>45.7</td>
<td>54.3</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Negative control</td>
<td>53.3</td>
<td>46.7</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Table 9: Carbohydrate analysis (HPAEC) of sugar beet leaves transiently expressing sucrose isomerase (SEQ ID NO: 16).
Table 10: Carbohydrate analysis (HPAEC) of sugar beet leaves transiently expressing sucrose isomerase (SEQ ID NO: 16).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Sucrose (mM)</th>
<th>Trehalulose (mM)</th>
<th>Isomaltulose (mM)</th>
<th>Total disaccharide (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17588</td>
<td>8.5</td>
<td>9.9</td>
<td>3.1</td>
<td>21.5</td>
</tr>
<tr>
<td>17588</td>
<td>16.6</td>
<td>0.7</td>
<td>0.1</td>
<td>17.3</td>
</tr>
<tr>
<td>17588</td>
<td>15.1</td>
<td>2.5</td>
<td>1.3</td>
<td>18.9</td>
</tr>
<tr>
<td>17588</td>
<td>31.8</td>
<td>0.5</td>
<td>0.3</td>
<td>32.6</td>
</tr>
<tr>
<td>Negative control</td>
<td>10.0</td>
<td>0.0</td>
<td>0.0</td>
<td>10.0</td>
</tr>
<tr>
<td>Negative control</td>
<td>15.3</td>
<td>0.0</td>
<td>0.0</td>
<td>15.3</td>
</tr>
<tr>
<td>Negative control</td>
<td>17.6</td>
<td>0.0</td>
<td>0.0</td>
<td>17.6</td>
</tr>
<tr>
<td>Negative control</td>
<td>7.8</td>
<td>0.0</td>
<td>0.0</td>
<td>7.8</td>
</tr>
</tbody>
</table>

Table 10: Carbohydrate analysis (HPAEC) of sugar beet leaves transiently expressing sucrose isomerase (SEQ ID NO: 16).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose + fructose (% of total sugar)</th>
<th>Sucrose (% of total sugar)</th>
<th>Trehalulose (% of total sugar)</th>
<th>Isomaltulose (% of total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17588</td>
<td>28.2</td>
<td>28.5</td>
<td>33.1</td>
<td>10.2</td>
</tr>
<tr>
<td>17588</td>
<td>43.2</td>
<td>54.2</td>
<td>2.3</td>
<td>0.3</td>
</tr>
<tr>
<td>17588</td>
<td>56.5</td>
<td>34.7</td>
<td>5.8</td>
<td>3.0</td>
</tr>
<tr>
<td>17588</td>
<td>42.4</td>
<td>56.1</td>
<td>0.9</td>
<td>0.6</td>
</tr>
<tr>
<td>Negative control</td>
<td>50.4</td>
<td>49.6</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Negative control</td>
<td>42.9</td>
<td>57.1</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Negative control</td>
<td>39.8</td>
<td>60.2</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Negative control</td>
<td>74.4</td>
<td>25.6</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

4B: Transient expression of enzymes in maize leaves

Transient expression of enzymes in maize leaves was performed essentially as described in Example 3E using the binary vector pEB47 (described in Example 12) comprising a monocot optimized polynucleotide sequence encoding a sucrose isomerase (SEQ ID NO: 24). Maize leaves were harvested and analyzed for the presence of isomaltulose and trehalulose (products of sucrose isomerase activity within the maize leaf) essentially as described above for tobacco and sugar beet leaves transiently.
expressing sucrose isomerase. Table 1 outlines data that demonstrates sucrose isomerase is actively expressed in maize leaves transiently expressing sucrose isomerase and leads to the accumulation of the locked sugars, isomaltulose and trehalulose within the maize leaf.

Table 1: Carbohydrate analysis (HPAEC) of maize leaves transiently expressing sucrose isomerase (SEQ ID NO: 24).

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose + fructose (% of total sugar)</th>
<th>Sucrose (% of total sugar)</th>
<th>Trehalulose (% of total sugar)</th>
<th>Isomaltulose (% of total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>47-6 (pEB47)</td>
<td>78.9</td>
<td>17.2</td>
<td>2.4</td>
<td>1.5</td>
</tr>
<tr>
<td>47-7 (pEB47)</td>
<td>63.7</td>
<td>33.3</td>
<td>2.1</td>
<td>0.9</td>
</tr>
<tr>
<td>47-8 (pEB47)</td>
<td>73.1</td>
<td>16.0</td>
<td>7.3</td>
<td>3.6</td>
</tr>
<tr>
<td>Negative control (GUS containing construct)</td>
<td>69.4</td>
<td>30.6</td>
<td>0.0</td>
<td>0.0</td>
</tr>
<tr>
<td>Negative control leaf tissue</td>
<td>58.2</td>
<td>41.8</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

4C: Transgenic maize callus expressing sucrose isomerase

Transgenic maize callus expressing sucrose isomerase was generated by bombarding maize embryos with linear polynucleotide sequence. The method of embryo transformation and generation of callus was essentially as described in Example 3F; however, two polynucleotide sequences were bombarded at the same time. One of the polynucleotide sequences contained the selectable marker, PMI, which allows for selection of transgenic maize cells by growth on mannose. The second polynucleotide sequence, pEB38, contained a maize optimized polynucleotide sequence encoding a sucrose isomerase (SEQ ID NO: 20). The sucrose isomerase was targeted to the vacuole. Table 12 outlines data which demonstrates that transgenic maize callus which expresses sucrose isomerase accumulated the locked sugars trehalulose and isomaltulose.
Table 12: Carbohydrate analysis (HPAEC) of transgenic maize callus tissue expressing sugarcane callus expressing sucrose isomerase.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose + Fructose % total sugar</th>
<th>Sucrose % total sugar</th>
<th>Trehalulose % total sugar</th>
<th>Isomaltulose % total sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 pEB38</td>
<td>14.8</td>
<td>0.95</td>
<td>38.2</td>
<td>46.0</td>
</tr>
<tr>
<td>2 pEB38</td>
<td>25.0</td>
<td>0.69</td>
<td>35.3</td>
<td>39.0</td>
</tr>
<tr>
<td>3 pEB38</td>
<td>32.0</td>
<td>5.13</td>
<td>34.8</td>
<td>28.1</td>
</tr>
<tr>
<td>Negative control</td>
<td>70.0</td>
<td>30.0</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. The negative control is transgenic maize callus generated by bombardment with the polynucleotide sequence encoding PMI only.

4D: Transgenic sugarcane callus expressing sucrose isomerase

Transgenic sugarcane callus expressing sucrose isomerase was generated essentially as described in Example 3A; however, two polynucleotide sequences were bombarded at the same time. One of the polynucleotide sequences contained the selectable marker, PMI, which allows for selection of transgenic sugarcane cells by growth on mannose. The second polynucleotide sequence, pEB38, contained a monocot optimized polynucleotide sequence encoding a sucrose isomerase (SEQ ID NO: 20). The sucrose isomerase was targeted to the vacuole. Table 13 outlines data which demonstrates that transgenic sugarcane callus which expresses sucrose isomerase accumulated the locked sugars trehalulose and isomaltulose.

Table 13: Carbohydrate analysis (HPAEC) of transgenic sugarcane callus tissue expressing sucrose isomerase.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose + Fructose % total sugar</th>
<th>Sucrose % total sugar</th>
<th>Trehalulose % total sugar</th>
<th>Isomaltulose % total sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 pEB38</td>
<td>44.13</td>
<td>37.70</td>
<td>8.87</td>
<td>9.30</td>
</tr>
<tr>
<td>Negative control</td>
<td>34.61</td>
<td>65.39</td>
<td>0.0</td>
<td>0.0</td>
</tr>
</tbody>
</table>

Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. Negative control is transgenic sugarcane callus generated by bombardment with a polynucleotide sequence encoding the selectable marker PMI.

4E: Transgenic sugar beet expressing sucrose isomerase (SEQ ID NO: 16)
Transgenic sugar beet plants containing the expression cassette 17588 (described in Example 12) were generated essentially as described in patent application WO02/14523 which is a multiple shoot method of transformation. The transgenic sugar beet callus was selected using mannose selection (the selectable marker gene was PMI) which was performed essentially as described in patent application WO94/20627.

The transgenic sugar beet plants were analyzed by PCR to determine if the selectable marker (PMI) and the sucrose isomerase gene (SEQ ID NO: 16) were present in the plant. In addition, the transgenic sugar beet plants were analyzed for the accumulation of locked sugars.

To analyze the sugar content of the transgenic sugar beet plants, leaves from the transgenic sugar beet plants were sampled into a Costar 96-well box. The box was placed on ice during the sampling procedure. After filling the box with glass balls the leaf samples were placed into the wells and 100µL sterile ddH₂O was added. The wells were closed using strip caps or a lock and the box shaken in a Tissue lyser (25-30 s, 30 Hz.) to pulverize the tissue in the water. The locks covering the wells were pierced and the samples were boiled on a water bath for 10 min. After boiling, an additional 100µL sterile ddH₂O was added followed by centrifugation (10 min, 3000 rpm). The supernatants were transferred to Millipore spin filter and centrifuged at 12000 rpm, 5 min. The filtered supernatants were stored at -20 degrees C or in 4 degrees C if the analysis was performed directly.

The samples were diluted 100 times with distilled water prior to analysis using the Dionex HPAE-system. The Dionex HPAE-system, ICS-3000 was used to separate the carbohydrates. The instrument was equipped with a temperature regulated auto sampler, CarboPac PA20 3x30 mm guard column, CarboPac PA20 3x15 mm analytical column and pulsed amperometric detector (PAD). The mobile phase used was 200 mM NaOH solution and water in following gradient program: 8min/16%NaOHsolution//2min 16-100%NaOHsolution//3min 100%NaOHsolution//2min 100-16%//7min 16% NaOHsolution. The column temperature was set at 30 degrees C and the flow 0.43 mL/min. The approximate retention times were glucose 7.7 min, fructose 9.3 min, sucrose 11.0 min, trehalulose 13.1 min and isomaltulose 14.5 min. The peaks were identified
using the standard solutions. Table 14 outlines data which demonstrates transgenic sugar beet plants expressing a sucrose isomerase enzyme and the subsequent accumulation of the locked sugars, isomaltulose and trehalulose. Locked sugars are detected in transgenic sugar beet plants expressing sucrose isomerase indicating that the enzyme is both expressed and is able to perform the enzymatic activity which converts sucrose to isomaltulose and trehalulose.

Table 14: Transgenic sugar beet plants expressing sucrose isomerase.

<table>
<thead>
<tr>
<th>Event</th>
<th>PCR PMI</th>
<th>PCR GOI</th>
<th>Dionex - isomaltulose</th>
<th>Dionex - trehalulose</th>
</tr>
</thead>
<tbody>
<tr>
<td>0851 B:1 A</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>++</td>
</tr>
<tr>
<td>biennial</td>
<td></td>
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<td></td>
</tr>
<tr>
<td>0851 B:2 A</td>
<td>+</td>
<td></td>
<td>+</td>
<td>++</td>
</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>+</td>
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<td>08511:1 B</td>
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<td>-</td>
<td>+</td>
<td>++</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0851 K:2 A</td>
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<td>+</td>
<td>++</td>
<td>+++</td>
</tr>
<tr>
<td>biennial</td>
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<td></td>
</tr>
<tr>
<td>0851 K:2 B</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>biennial</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>0851 K:2 C</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
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<td>biennial</td>
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<td></td>
</tr>
<tr>
<td>0851 K:4 A</td>
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<td>-</td>
<td>+</td>
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<tr>
<td>0851 N:1 A</td>
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<td>+</td>
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<td>+</td>
<td>++</td>
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</tr>
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<td>+</td>
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<tr>
<td>0903R1 C</td>
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<td>++</td>
</tr>
<tr>
<td>annual</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Example 5: Transgenic plants expressing dextransucrase with leucrose synthase activity

5A: Transient expression of dextransucrase (SEQ ID NO: 35) in tobacco leaves

The transformation vector 902195, as described in Example 12, was used to generate tobacco leaves transiently expressing dextransucrase essentially as described in Example 3D. Transient expression of dextransucrase in tobacco leaves was performed using the vector 902195 which contains a dicot optimized polynucleotide sequence encoding a dextransucrase with leucrose synthase activity (SEQ ID NO: 35). Transiently expressing leaves were harvested and extracted essentially as described in Example 3H and analyzed by Dionex for carbohydrates essentially as described in Example 3G.

Dionex HPAE chromatography utilized pure sugar standards as a reference for retention time and standard curve production for determining sugar concentrations. Sugar concentrations were based on the total sugar consisting of glucose, fructose, sucrose, and locked sugars when present. These sugars represent >98% of the total peak area of the chromatograms with the remainder coming from minor unknown peaks from the biological extraction milieu of the leaf.

Dextranucrase with leucrose synthase activity transiently expressed in leaves was directly detected by the formation of the locked sugar leucrose. Leucrose was not present in control leaves. Table 15 summarizes the analysis of tobacco leaves transiently expressing a dextransucrase with leucrose synthase activity (vector 902195) and demonstrates that tobacco leaves are able to express an active dextranucrase which catalyzes the conversion of sucrose to the locked sugar leucrose which accumulates in the leaf.

5B: Transient expression of dextranucrase (SEQ ID NO: 24) in maize leaves.

Maize leaves transiently expressing dextranucrase with leucrose synthase activity were generated essentially as described in Example 3E using the vector pEB47 (described in Example 12) comprising a monocot optimized polynucleotide sequence encoding a dextranucrase (SEQ ID NO: 47). Maize leaves were harvested and extracted
essentially as described in Example 3H. The extract was analyzed for carbohydrate content essentially as described in Example IG. Table 15 outlines data that demonstrates dextranase is actively expressed in maize leaves and leads to the accumulation of the locked sugar leucrose within the maize leaf.

5C: Transgenic sugarcane callus expressing dextranucrase (SEQ ID NO: 37)

Transgenic sugarcane callus expressing dextranucrase with leucrose synthase activity (SEQ ID NO: 37) was generated essentially as described in Example 3A; however, two polynucleotide sequences were bombarded at the same time. One of the polynucleotide sequences contained the selectable marker, PMI, which allows for selection of transgenic sugarcane cells by growth on mannose. The second polynucleotide sequence, pEB28, contained a monocot optimized polynucleotide sequence encoding a dextranucrase (SEQ ID NO: 37). The dextranucrase was targeted to the vacuole. Table 15 outlines data which demonstrates that transgenic sugarcane callus which expresses sucrose isomerase accumulated the locked sugar leucrose.

Table 15: Plant tissue expressing dextranucrase accumulates leucrose and/or isomaltose.

<table>
<thead>
<tr>
<th>dextranucrase</th>
<th>tobacco</th>
<th>maize</th>
<th>sugar cane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leucrose</td>
<td>Leucrose</td>
<td>Leucrose and isomaltose</td>
</tr>
<tr>
<td>Negative control</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Leucrose synthase activity is determined by the accumulation of leucrose above 10x signal:noise on a Dionex IC.

Example 6: Transgenic plants expressing amylosucrase

6A: Total starch analysis of amylosucrase-expressing maize and sugarcane callus

The effectiveness of the amylosucrase gene, when expressed in either maize or sugar cane callus, can be evaluated by comparing the total starch content of the amylosucrase expressing calli to control calli that have not been transformed with the gene. The total starch content of any plant tissue of interest can be measured using a protocol similar to that of the Megazyme Total Starch Assay kit. In this assay, the starch contained in a plant sample is broken down into glucose monomers through digestion by
both an alpha-amylase and an amylglucosidase. The resulting solution of glucose can be enumerated by a glucose oxidase-peroxidase (GOPOD) reaction essentially as is described in Example 2B. In this reaction, the glucose oxidase enzymes break down glucose to hydrogen peroxide which the peroxidase then digests, releasing oxygen which reacts with the 4-aminoantipyrine in solution to evolve a pink color. The pink color can be measured with a spectrophotometer and, when compared with the absorbance of a glucose standard, can give a measure of the amount of glucose and therefore, the amount of starch in a given sample.

To accurately measure the production of carbohydrate polymers by the amylosucrase gene in callus, several controls and conditions will need to be established. For every calli that is transformed with the amylosucrase gene, a duplicate calli should be transformed with an empty vector that can act as a control sample. Both transformed and control calli should initially be grown on sucrose media to provide amylosucrase with its natural substrate and raise the overall starch content in the calli. After sufficient growth, some calli (both AMS and control) should be transferred to sorbitol media where the natural metabolism of the tissue will lower the background of transient starch and, theoretically, leave the amylosucrase produced carbohydrate polymer. In tissue culture, sorbitol is assimilated and metabolized by plants to a much lesser degree than sucrose. With sorbitol as a carbon source, plant cells are expected to deplete transient and storage starch reserves leaving an amylosucrase derived starch to accumulate.

Once the calli are harvested from the media, similar events can be pooled into wells of a 24-well block to bulk up the amount of tissue and lyophilized so that calculations can be made on a dry weight basis. Lyophilized tissue can be easily ground in the 24-well blocks using a Kleco. As mentioned previously, the Megazyme total starch protocol can be used to effectively measure the total starch content of tissue samples. The following is an example of a slightly modified protocol that could be employed to analyze lyophilized callus material. Approximately 30-70mg of the ground tissue should be washed with 5mL of 80-90% ethanol for 30-60 minutes and centrifuged for 5 minutes at 3000rpm to wash away any soluble sugars or other soluble compounds. Additional ethanol washes may be added as necessary, as long as all samples are treated identically. The pelleted material should then be washed in 5mL of cold water and centrifuged again
for 5 minutes at 3000rpm to remove any remaining ethanol. At this stage, the pellet should be completely resuspended in 3mL of a 1:30 dilution of alpha-amylase (Megazyme) in 50mM MOPS buffer pH=7 and incubated for 6 minutes in a 100 degree C water bath. Samples should then be transferred to a 50 degree C water bath where 4mL of NaOAc buffer pH=4.5 and 0.1mL of amyloglucosidase (Megazyme) will be added and then incubated for 30 minutes at 50 degree C. After incubation, all samples should be brought to 10 mL with water, vortexed, and centrifuged for 10 minutes at 3000rpm. This supernatant contains the solubilized glucose monomers that remain from the digestion of the carbohydrate polymers that were extracted from the lyophilized tissue samples. To enumerate the glucose in this mixture, 2 mL should be added in duplicate to glass test tubes, mixed with 3 mL of GOPOD reagent, and incubated for 20 minutes at 50 degree C. Once cooled to room temperature, the optical density of the samples can be read at 510nm. Based on the OD reading of the samples and its comparison to a known standard, the amount of glucose, and therefore starch, in the original dry weight sample can be calculated.

Upon completion of total starch content analysis, it is expected that calli expressing the amylosucrase gene will show an increased level of total starch over the negative control calli due to the additional production of carbohydrate polymers by the enzyme. Additionally, targeted expression of the amylosucrase enzyme to the vacuole or apoplast of transgenic plant cells would serve to isolate the de novo starch from the endogenous starch metabolizing enzymes allowing for accumulation of a locked carbohydrate. Therefore, when the calli are depleted of transient starch after growth on sorbitol media, the total starch content would be expected to fall slightly, but remain at an increased level over the negative controls.

6B: Starch Structure: Amylose/Amylopectin Differentiation by Iodine Binding

The structure of the carbohydrate polymers produced by the amylosucrase enzyme can potentially be identified by developing a method to enumerate the proportions of amylose and amylopectin in plant material. The comparison of control samples with samples expressing the amylosucrase gene could identify structural composition changes that may be present in the polymers produced by amylosucrase
expressing events, suggesting that a carbohydrate polymer lock is being produced. One possible method for accomplishing this is through an iodine binding assay. In this assay, the plant produced carbohydrate polymers are solubilized from the tissue and then stained with iodine. The resulting iodine-starch complexes will absorb at different wavelengths depending on the proportions of amylose and amyllopectin present in the extract. Through comparison with known standards and mixtures of amylose and amyllopectin, both the total amount of starch present and the proportions of amylose and amyllopectin present in the starch produced in the tissue can be calculated.

The following is an example of a starch extraction and iodine staining procedure that could be used to analyze lyophilized, ground tissue samples. Approximately 100-200mg of ground, lyophilized tissue should be washed with 5mL of 90% ethanol, incubated for 15 minutes in a 100 degree C water bath, and centrifuged for 5 minutes at 3000rpm to remove the supernatant. This wash step should be repeated at least two more times to ensure sufficient removal of soluble sugars and other potential iodine binding compounds from the samples. To the sample material, 5mL of 100% ethanol should be added and incubated again for 15 minutes at 100 degree C. Prior to centrifuging the sample, 5mL of acetone should be added to the mixture. The pellet should then be suspended once more in 5mL of acetone to ensure the complete removal of any residual ethanol, centrifuged for 5 minutes at 3000rpm, and the pellet allowed to dry overnight.

To solubilize the starch from the dried pellet, 5mL of 0.5M KOH should be added and incubated for 2-3 hours at 100 degree C. Debris may be pelleted by centrifugation for 10tnin at 3000rpm. For the staining of the solubilized carbohydrate polymers, 1mL of the KOH extract should first be neutralized with 5mL of 0.1M HCl, then 0.5mL of Lugol's Iodine solution should be added and diluted to between 25 and 50mL with water to bring the absorbance into an appropriate range. The color should be allowed to develop for about 15 minutes and then samples can be added to a microtiter plate for measuring the optical density along with pure amylose and pure amyllopectin stained standards. The spectra of the samples and standards should be measured first to determine at which wavelength the maximum absorbance occurs for each sample, since this is indicative of the proportions of amylose and amyllopectin in the samples. To analyze the sample spectra, a system of equations will be set up using Beer's law based on the absorbance
values at 6 different wavelengths. Measurements of the absorbance will be recorded at 504nm, the wavelength of greatest difference between the amylose and amylopectin peaks where amylopectin's absorbance is greater than amylose's absorbance; 548nm, the wavelength of the pure amylopectin peak; 630nm, the wavelength of the pure amylose peak; 700nm, the wavelength of greatest difference between the amylose and amylopectin peaks where amylose's absorbance is greater than amylopectin's absorbance; 800nm, the wavelength of greatest absorbance due to amylose where amylopectin's absorbance approaches zero; and the wavelength determined to be the location of the sample spectra's maximum (Jarvis and Walker J. Sci. Food Agric. 63: 53-57 (1993)). The results of this system of equations will give a concentration value of the amount of amylose and the amount of amylopectin present in the sample extract, from which a ratio of the two starch forms can be determined.

Upon successful completion of the iodine binding assay, it is expected that the assay data will support the total starch assay data in showing an overall starch increase in the samples expressing the amylosucrase gene. In addition, it is expected that the amylosucrase expressing events will produce a carbohydrate polymer that is more closely related to amylose than amylopectin, therefore a larger proportion of amylose when compared to control samples should be observed. This shift in composition of the starch produced in amylosucrase expressing events will also support the successful production of a locked substrate in plant tissue.

6C: Digestion of Plant Produced Carbohydrate Polymers with Plant-Expressed Enzymes

The ability of a plant produced key enzyme to digest a plant produced locked substrate can be exemplified using the principle underlying the glucose oxidase-peroxidase (GOPOD) reaction. If the plant purified key enzyme acts on the plant produced locked sugar, glucose monomers should be liberated from the locked sugar which can be enumerated by the GOPOD reaction. In order to complete this digestion, however, an appropriate plant expressed ky enzyme must be purified and a carbohydrate polymer produced by the amylosucrase enzyme must be solubilized in an appropriate buffer. Alpha-amylase can be collected from transgenic maize plants expressing alpha-amylase in the seed through laboratory established FPLC methods yielding a purified plant-expressed key enzyme (alpha-amylase). Locked sugars produced in tobacco or
another plant system by the amylosucrase gene can be extracted in boiling water from lyophilized plant material after washing with 80-90% ethanol to remove any soluble sugars or compounds (Spoehr and Milner J. Biol. Chem. 111 (3): 679-687. (1935)). The alpha-amylase will not yield strictly glucose in its digest, the amount of glucose produced should be sufficient to be detected by the GOPOD reaction assay when compared to a control sample of the undigested locked sugar. It is expected that a difference in glucose levels would be detected in this type of digestion assay, verifying that plant expressed key enzymes are, indeed, capable of digesting plant produced locks.

Additionally, in the process of performing HPSEC on debranched amylosucrose polymer mixture, sample fractions could be collected, and a plant expressed alpha amylase or glucoamylase key enzyme could be used to hydrolyze the starch in the collected fractions to glucose. A GOPOD reaction assay could be used to detect the glucose liberated from the amylosucrose locked-carbohydrate fraction.

6D: Detection of Amylosucrase activity in stably transformed plants or plants transiently expressing amylosucrase.

Amylosucrase may be expressed either transiently or through stable transformation of maize, cane, beets, tobacco or other plants with a promoter that drives expression in the appropriate target tissue (leaf, endosperm, embryo, etc.) and with targeting sequences that direct the amylosucrase to the desired subcellular location (vacuole, chloroplast, cytoplasm, apoplast, etc.). A variety of techniques may be used to detect the activity of the amylosucrase gene in plants.

For instance, plant tissue samples expressing the amylosucrase polypeptide may be incubated in the dark for 24 to 48 hours in order for transient starch produced in the chloroplast to be broken down by the plant. Leaf or other tissue may be excised from the plant and dipped into boiling water for one minute to heat kill the tissue. After heat killing plant tissue samples may be incubated in hot ethanol to remove the chlorophyll, repeated washing with hot ethanol may be necessary to remove all the chlorophyll. Once the chlorophyll has been removed, the tissue can be rinsed with cold water and placed on a petri dish. Lugol's solution (5g iodine (I₂) and 10 g potassium iodide (KI) mixed with
85 ml distilled water), then be poured over the sample and allowed to incubate at room temperature. Control samples that have been in the dark for 24 hours should contain no starch and should not stain black in Lugols solution. Samples expressing the amylosucrase gene should stain black due to starch that is produced in the vacuole or other organelles targeted for expression of the Amylosucrase enzyme.

Leaves contain a variety of unique cell types such as the pavement cells that are highly specialized cells making up the majority of the leaf surface. These are easily identified by their puzzle piece shapes (in dicots) and are only found at the leaf surface. They contain no chloroplasts or amyloplasts, so if pavement cells are found to have what appeared to be dark staining "amyloplasts" and these are not observed in pavement cells from "vector only" controls, this would be good evidence that the construct is working and that starch is being produced.

6E: Analysis of Locked Amylosucrose Carbohydrates by HPSEC

Another means of analyzing structural composition changes that may be present in the polymers produced by amylosucrase expressing events is by the use of High-performance size exclusion chromatography, HPSEC. Using HPSEC, a locked amylosucrase carbohydrate polymer could be identified and characterized based on its molecular weight or chain length distribution.

The extraction of starch from plant material for analysis by HPSEC could be carried out essentially as described by Santacruz et al. J. Agric. Food Chem. 2004, 52 (7): 1985-1989. Starch could be extracted from plant material such as leaf or callus by lyophilizing and grinding plant material. Powdered lyophilized plant tissue could be mixed with 90% ethanol (v/v) and placed in a boiling water bath for 15 minutes. After centrifugation at 1000g for 10 minutes, the pellet could be washed three more times with hot 90% ethanol. The pellet can be washed again with 100% ethanol, boiled for 15 minutes. After centrifugation, the supernatant can be discarded and the pellet washed further with acetone, centrifuged and supernatant discarded. The pellet can be dried overnight at room temperature. The dried plant material can be further extracted by addition of 0.2% EDTA to the dried residual pellet and mixed overnight with shaking at room temperature. After centrifugation, the resulting starch pellet can be further
extracted by addition of 90% ethanol and boiled for 30 minutes. After centrifugation, the supernatant can be saved and the pellet extracted again with 90% ethanol. The supernatants can be combined and mixed with 100% ethanol in a ratio of 1 part DMSO to 9 parts ethanol. The solution can be incubated at room temperature for 15 minutes, centrifuged to obtain a starch pellet. The starch pellet can then be solubilized in 90% DMSO with boiling for 15 minutes. The starch could be done debranched for GPC analysis essentially as described by Yao et al Carbo. Research. 2005, 340:701-710.

Debranching of starch can be carried out in a 50mM Sodium Acetate, pH 4.0 buffer which has been warmed to 42-50C. A reaction which combines 880ul of warm NaAc buffer, 120 ul of the DMSO solubilized starch pellet can be prepared. To keep the starch solubilized, the reaction can be heated to 100C for 10 minutes and then cooled to 22-42C before addition of IU/ml of isoamylase (Megazyme Inc., Ireland.) The digestion reaction can be incubated at 37-42C with constant agitation for 16-24 hours. After digestion, the debranching reaction can be heated in a boiling water bath for 10 minutes. The starch dispersion can then be concentrated in a Speed-Vac vacuum evaporator.

Gel permeation chromatography or HPSEC could be carried out on this concentrated starch sample to characterize the starch structure of the locked amylosucrose carbohydrate. Starch samples can be diluted up to 30 fold in DMSO in preparation for analysis by the HPSEC system.

Using an HPSEC system such as a Waters Breeze 717 system. 50ul of debranched starch polymer could be injected into a Ultrahydrogel-6x40mm Guard column (WAT 011565) and Ultrahydrogel 250 A-7.8 x300mm column (WATOI 1525) with Waters 1515 isocratic HPLC pump and a differential refractometer such as Waters Model 410 for detection. A flow rate of 0.5 mL/min at a column, column temperature of 35 C and detector temperature of 40 C may be used. The molecular weight standards for column calibration could be maltotriose (Sigma), maltohepatose (Sigma), and pullulan standards (P-5, MW 5800; P-IO,MW 12,200; P-20, MW 23,700; P-50, MW 48,000, from Shodex, Japan). On the chromatogram the differential refractive index (DRI) value on the y-axis will be the mass response to the carbohydrate at a particular retention time (RT).
Within the separation range of the HPSEC media, the RT on the x-axis will be approximately proportional to the logarithm of the molecular weight (or chain length), and using standards the precise relationship may be determined to generate a standard curve. In this way, the chain length of an amylosucrose polymer may be determined and characterized.

Example 7: Transgenic plants expressing key enzymes

7A: Transient transgenic tobacco and sugar beet expressing alpha-1,6-glucosidase

Tobacco and sugar beet leaves transiently expressing an alpha-1,6-glucosidase enzyme were generated essentially as described in Example 3D. Leaves transiently expressing alpha-1,6-glucosidase were generated using the binary vector 902525 or the BCTV binary vector 902526. Both of the binary vectors contain expression cassettes encoding an alpha-1,6-glucosidase (SEQ ID NO: 11) which has been targeted through the ER and is expected to accumulate in the apoplast. Infiltrated tobacco and sugar beet leaves were harvested, extracted and enzyme activity assayed essentially as described in Example 3G. The key enzyme, alpha-1,6-glucosidase, catalyzes the conversion of isomaltulose to the fermentable sugars fructose and glucose and was assayed at 60 degrees C. Carbohydrate analysis of the final filtrate was performed using the Dionex system essentially as described in Example 1G. Tables 16 - 17 outline data demonstrating transient expression of an alpha-1,6-glucosidase in tobacco and sugar beet leaves.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose (% total sugar)</th>
<th>Fructose (% total sugar)</th>
<th>Isomaltulose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>902525</td>
<td>11.97</td>
<td>12.46</td>
<td>-24.43</td>
</tr>
<tr>
<td>binary</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>902526</td>
<td>22.66</td>
<td>26.95</td>
<td>-49.61</td>
</tr>
<tr>
<td>BCTV</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>-1.67</td>
<td>3.75</td>
<td>-2.08</td>
</tr>
</tbody>
</table>

Table 16. Carbohydrate analysis of tobacco leaves transiently expressing an alpha-1,6-glucosidase enzyme (SEQ ID NO: 11). Enzyme activity is indicated by the change in abundance of each sugar as a percentage of the total sugars over a 24 hour period.
Table 17. HPAEC analysis of carbohydrate products from sugar beet leaves transiently expressing an alpha-1,6-glucosidase enzyme (SEQ ID NO: 11). Enzyme activity is indicated by the change in abundance of each sugar as a percentage of the total sugars over a 24 hour period.

<table>
<thead>
<tr>
<th>sample</th>
<th>Glucose (% total sugar)</th>
<th>Fructose (% total sugar)</th>
<th>Isomaltulose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>902525 binary</td>
<td>19.73</td>
<td>19.10</td>
<td>-38.83</td>
</tr>
<tr>
<td>902526 BCTV</td>
<td>14.05</td>
<td>11.91</td>
<td>-25.96</td>
</tr>
<tr>
<td>Negative control</td>
<td>6.14</td>
<td>6.61</td>
<td>-12.74</td>
</tr>
</tbody>
</table>

Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. The negative control is sugar beet leaves transiently expressing a binary vector containing an origin of replication from beet curly top.

7B: Transgenic maize callus expressing alpha-1,6-glucosidase

Transgenic maize callus expressing an alpha-1,6-glucosidase enzyme was generated by bombarding maize embryos with linear polynucleotide sequence. The method of embryo transformation and generation of callus was essentially as described in Example 3F; however, two polynucleotide sequences were bombarded at the same time. One of the polynucleotide sequences contained the selectable marker, PMI, which allows for selection of transgenic maize cells by growth on mannose. The second polynucleotide sequence, 902435 or 902425, contained a maize optimized polynucleotide sequence encoding an alpha-1,6-glucosidase (SEQ ID NO: 54 or SEQ ID NO: 56). The alpha-1,6-glucosidase was targeted to the endoplasmic reticulum (902435) or to the chloroplast (902425).

Analysis of alpha-1,6-glucosidase enzyme activity in transgenic maize calli was performed by extracting the enzyme from the transgenic calli and incubating the extract with isomaltulose. If alpha-1,6-glucosidase enzyme activity is present, the isomaltulose is converted to glucose and fructose. Essentially, maize calli expressing the alpha-1,6-
glucosidase were collected 8 calli per well in Slicprep 96 device. Samples were frozen at -80 degrees C and thawed at room temperature. Thawed samples were centrifuged at 1770Xg and flow-through extract collected. Extracts were heated at 60 degrees C for 10 minutes. Extracts were centrifuged at 1770Xg 30 minutes at 4 degrees C to pellet denatured proteins in samples. Equal volumes of clarified extract and reaction buffer (200 mM Isomaltulose, 100 mM HEPES, 0.04% Tween-20, 4 mM EDTA, 40 nM NaOH, 2X protease inhibitor (Roche Complete EDTA-free)) were combined and incubated at 60 degrees C in BioRad Tetrad 2 thermocycler. Samples were collected at times 0 and 24 hours. Collected samples were incubated at 95 degrees C for 5 minutes before freezing at -20 degrees C. Samples were analyzed by Dionex. Table 18 outlines data which demonstrates that transgenic maize callus expresses an active alpha-1,6-glucosidase enzyme.

Table 18. HPAEC analysis of carbohydrate products from transformed maize callus tissue expressing alpha-1,6-glucosidase enzymes. Enzyme activity is indicated by the change in abundance of each sugar as a percentage of the total sugars over a 24 hour period.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose (% total sugar)</th>
<th>Fructose (% total sugar)</th>
<th>Isomaltulose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>902435 ER</td>
<td>14.28</td>
<td>18.03</td>
<td>-32.31</td>
</tr>
<tr>
<td>902425 (plastid)</td>
<td>7.24</td>
<td>9.26</td>
<td>-16.50</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.49</td>
<td>-0.18</td>
<td>-0.31</td>
</tr>
</tbody>
</table>

Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. Negative control is maize callus transformed with a vector that contains the PMI selectable marker only.

7C: Transgenic sugarcane callus expressing alpha-1,6-glucosidase

Transgenic sugarcane callus expressing an alpha-1,6-glucosidase enzyme was generated essentially as described in Example 3A; however, two polynucleotide sequences were bombarded at the same time. One of the polynucleotide sequences contained the selectable marker, PMI, which allows for selection of transgenic sugarcane cells by growth on mannose. The second polynucleotide sequence, 902425, contained a
polynucleotide sequence encoding an alpha-1,6-glucosidase (SEQ ID NO: 56). The alpha-1,6-glucosidase was targeted to the chloroplast.

Sugarcane calli expressing the alpha-1,6-glucosidase were collected 1 callus per well in 96-well 2 mL plates (Whatman) containing one 3/16" chrome ball bearing per well. The plate was shaken at setting 9 for 2 min in a Kleco Titer plate/Microtube Grinding Mill creating a powder. Buffer (100 mM HEPES, 4 mM EDTA, 0.04% Tween-20, pH 7) was added to the powdered samples to give a thick slurry. Samples were incubated in a Glas-Col rotator at 80% speed for 30 min. Samples were transferred by wide-bore P200 pipet to a 96 well PCR at 100 uL per well and incubated at 60 degrees C for 20 minutes. Extracts were centrifuged at 1770Xg for 30 mins to pellet denaturated proteins in samples. Equal volumes of clarified extract and 271 mM trehalulose/134 mM isomaltulose were combined and incubated at 60 degrees C in BioRad Tetrad 2 thermocycler. Samples were collected at times 0 and 24 hours. Collected samples were incubated at 95 degrees C for 5 minutes before freezing at -20 degrees C. Samples were analyzed by HPAE chromatography essentially as described in Example IG. Table 19 demonstrates that sugarcane callus expresses an active alpha-1,6-glucosidase that also shows alpha-1,1-glucosidase activity.

Table 19. Carbohydrate analysis (HPAE chromatography) of products from transformed sugarcane callus tissue expressing an alpha-1,6-glucosidase enzyme. Enzyme activity is indicated by the change in abundance of each sugar as a percentage of the total sugars over a 24 hour period.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose (% total sugar)</th>
<th>Fructose (% total sugar)</th>
<th>Isomaltulose (% total sugar)</th>
<th>Trehalulose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>902425 (plastid)</td>
<td>8.98</td>
<td>9.59</td>
<td>-6.86</td>
<td>-9.60</td>
</tr>
<tr>
<td>Negative control</td>
<td>2.53</td>
<td>3.70</td>
<td>-2.82</td>
<td>-2.15</td>
</tr>
</tbody>
</table>

Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. Negative control is wildtype sugarcane callus.

7D: Transient expression of alpha-1,1-glucosidase (SEQ ID NO: 27) enzyme in sugar beet or tobacco leaves
Tobacco and sugar beet leaves transiently expressing an alpha-1,1-glucosidase (SEQ ID NO: 27) enzyme were generated essentially as described in Example 3D. The vector for transient expression was 901612 or 902522 which are described in Example 12. The binary vector 901612 contains an expression cassette encoding an alpha-1,1-glucosidase (SEQ ID NO: 27) targeted to the chloroplast. The binary vector 902522 contains an expression cassette encoding an alpha-1,1-glucosidase (SEQ ID NO: 27) targeted to pass through the endoplasmic reticulum and accumulate in the apoplast. Infiltrated tobacco and sugar beet leaves were harvested, extracted and enzyme activity assayed essentially as described in Example 3G. The key enzyme, alpha-1,1-glucosidase, catalyzes the conversion of isomaltulose or trehalulose to the fermentable sugars fructose and glucose and was assayed at 70 degrees C. Carbohydrate analysis of the final filtrate was performed using the Dionex system essentially as described in Example 1G. Tables 20 - 21 outline data demonstrating transient expression of an alpha-1,1-glucosidase in tobacco and sugar beet leaves.

Table 20. HPAEC analysis of carbohydrate products from tobacco leaves transiently expressing an alpha-1,1-glucosidase enzyme. Enzyme activity is indicated by the change in abundance of each sugar as a percentage of the total sugars over a 24 hour period.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose (% total sugar)</th>
<th>Fructose (% total sugar)</th>
<th>Trehalulose (% total sugar)</th>
<th>Isomaltulose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>901612</td>
<td>21.61</td>
<td>23.38</td>
<td>-22.57</td>
<td>-22.41</td>
</tr>
<tr>
<td>Negative control</td>
<td>1.47</td>
<td>1.55</td>
<td>1.93</td>
<td>-4.95</td>
</tr>
</tbody>
</table>

Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. The negative control is tobacco leaves transiently expressing empty binary vector.

Table 21. HPAEC analysis of carbohydrate products from sugar beet leaves transiently expressing alpha-1,1-glucosidase enzymes. Enzyme activity is indicated by the change in abundance of each sugar as a percentage of the total sugars over a 24 hour period.

<table>
<thead>
<tr>
<th>sample</th>
<th>Glucose (% total sugar)</th>
<th>Fructose (% total sugar)</th>
<th>Trehalulose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>901612 chloroplast</td>
<td>12.48</td>
<td>13.70</td>
<td>-13.59</td>
</tr>
<tr>
<td>902522 apoplast</td>
<td>18.73</td>
<td>19.51</td>
<td>-22.46</td>
</tr>
<tr>
<td>Negative</td>
<td>6.94</td>
<td>7.45</td>
<td>-5.49</td>
</tr>
</tbody>
</table>
Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. The negative control is sugar beet leaves transiently expressing empty binary vector.

7E: Transgenic maize callus expressing alpha-1,1-glucosidase

Transgenic maize callus expressing alpha-1,1-glucosidase enzyme was generated by bombarding maize embryos with two binary vectors. The method of embryo transformation and generation of callus was essentially as described in Example 3F; however, two polynucleotide sequences were bombarded at the same time. One of the polynucleotide sequences contained the selectable marker, PMI, which allows for selection of transgenic maize cells by growth on mannose. The second polynucleotide sequence, 902429, contained a maize optimized polynucleotide sequence encoding an alpha-1,1-glucosidase (SEQ ID NO: 49). The alpha-1,1-glucosidase was targeted to be retained by the endoplasmic reticulum.

Maize calli expressing the alpha-1,1-glucosidase was collected 1 callus per well in 96-well 2 mL plates (Whatman) containing one 3/16" chrome ball bearing per well. The plate was shaken at setting 9 for 2 min in a Kleco Titer plate/Microtube Grinding Mill. Sets of 4 pulverized callus tissue samples were combined and transferred to microfuge tubes. The samples were centrifuged at 20,000Xg 30 minutes at 4 degrees C. The supernatants containing protein extract were transferred to new tubes and extracts with volumes < 20 uL were pooled such that all samples were > 30 uL in volume. Equal volume of extract and reaction buffer (~185 nM trehalulose, 93 mM isomaltulose, 100 mM HEPES, 0.04% Tween-20, 4 mM EDTA, 40 mM NaOH, Roche protease inhibitors) were combined and incubated at 70 degrees C in BioRad Tetrad 2 thermocycler. Samples were collected at times 0 and 24 hours. Collected samples were incubated at 95 degrees C for 5 minutes before freezing at -20 degrees C. Samples were analyzed by Dionex essentially as described in Example IG. Table 22 demonstrates that maize callus expresses an active alpha-1,1-glucosidase.

Table 22: HPAEC analysis of carbohydrate products from transformed maize callus tissue expressing an alpha-1,1-glucosidase enzyme. Enzyme activity is indicated by the
change in abundance of each sugar as a percentage of the total sugars over a 24 hour period.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Glucose (% total sugar)</th>
<th>Fructose (% total sugar)</th>
<th>Trehalulose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>902429</td>
<td>10.02</td>
<td>11.32</td>
<td>-6.47</td>
</tr>
<tr>
<td>Negative control</td>
<td>3.51</td>
<td>3.46</td>
<td>1.50</td>
</tr>
</tbody>
</table>

Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. The negative control was transgenic maize callus generated by transformation with the binary vector expressing the selectable marker (PMI) only.

7F: Transient expression of alpha-1,5-glucosidase by tobacco leaves

Tobacco leaves transiently expressing an alpha-1,5-glucosidase (SEQ ID NO: 46) enzyme were generated essentially as described in Example 3D. The vector for transient expression was BCTV binary vector 902550 which is described in Example 12. BCTV binary vector 902550 contains an expression cassette encoding an alpha-1,5-glucosidase (SEQ ID NO: 46) which is targeted to the chloroplast. Infiltrated tobacco and sugar beet leaves were harvested, extracted and enzyme activity assayed essentially as described in Example 3G. The key enzyme, alpha-1,5-glucosidase, catalyzes the conversion of leucrose to the fermentable sugars glucose and fructose and was assayed at 80 degrees C.

Table 23 outlines data demonstrating tobacco leaves transiently expressed the alpha-1,5-glucosidase enzyme.

Table 23. HPAEC analysis of carbohydrate products from tobacco leaves transiently expressing an alpha-1,5-glucosidase enzyme. Enzyme activity is indicated by the change in abundance of each sugar as a percentage of the total sugars over a 24 hour period.

<table>
<thead>
<tr>
<th>sample</th>
<th>Glucose (% total sugar)</th>
<th>Fructose (% total sugar)</th>
<th>Leucrose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>902550</td>
<td>18.07</td>
<td>20.36</td>
<td>-38.43</td>
</tr>
<tr>
<td>Negative control</td>
<td>3.30</td>
<td>1.50</td>
<td>-4.80</td>
</tr>
</tbody>
</table>

Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. The negative control is tobacco leaves transiently expressing empty BCTV vector.

7G: Transgenic maize callus expressing alpha-1,5-glucosidase (SEQ ID NO: 43)
Transgenic maize callus expressing alpha-1,5-glucosidase enzyme was generated by bombarding maize embryos with two binary vectors. The method of embryo transformation and generation of callus was essentially as described in Example 3F; however, two polynucleotide sequences were bombarded at the same time. One of the polynucleotide sequences contained the selectable marker, PMI, which allows for selection of transgenic maize cells by growth on mannose. The second polynucleotide sequence, 902423, contained a maize optimized polynucleotide sequence encoding an alpha-1,5-glucosidase (SEQ ID NO: 43). The alpha-1,5-glucosidase was targeted to the chloroplast.

Maize calli expressing an alpha-1,5-glucosidase (SEQ ID NO: 43) was collected 1 callus per well in 96-well 2 mL plates (Whatman) containing one 3/16” chrome ball bearing per well. Samples were frozen at -80 degrees C. The frozen material was shaken at setting 9 for 4 min in a Kleco Titer plate/Microtube Grinding Mill. 200 uL of extraction buffer (100 mM HEPES, 4 mM EDTA, 0.04% Tween-20, pH 7) was added to each sample. Extracts were incubated in a Glas-Col rotator at 80% speed for 10 min. Extract was centrifuged at 12700Xg for 10 minutes at 4 degrees C in Eppendorf 5810R swing bucket centrifuge. Extract was frozen at -80 degrees C. Extract was later thawed and transferred to a 96-well PCR plate (Thermo Sci). Samples were heated at 80 degrees C for 15 minutes in BioRad Tetrad 2 thermocycler. Plates were again centrifuged at 12700Xg for 10 minutes at 4 degrees C in Eppendorf 5810R swing bucket centrifuge. Supernatants were filtered using a Millipore Multiscreen-HV filter plate. Filtered extracts of 8 callus samples were combined. Combined samples were concentrated from 0.6 mL to 100-500 uL using Microcon concentrators with MWCO 3k membrane filters (Amicon). An equal volume of 200 mM leucrose and extract was added to 96-well PCR plate and incubated at 80 degrees C in the thermocycler. Samples were collected at times 0 and 24 hours. Collected samples were incubated at 95 degrees C 5 minutes before freezing at -20 degrees C. Samples were analyzed by Dionex essentially as described in Example IG. Alpha-1,5-glucosidase activity was confirmed by measuring the conversion of the locked sugar, leucrose, to the fermentable sugars glucose and fructose. Table 24 demonstrates that maize callus expressed an active alpha-1,5-glucosidase enzyme.
Table 24. HPAEC analysis of carbohydrate products from transformed maize callus tissue expressing an alpha-1,5-glucosidase enzyme. Enzyme activity is indicated by the change in abundance of each sugar as a percentage of the total sugars over a 24 hour period.

<table>
<thead>
<tr>
<th>sample</th>
<th>Glucose (% total sugar)</th>
<th>Fructose (% total sugar)</th>
<th>Leucrose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>902423</td>
<td>6.86</td>
<td>12.71</td>
<td>-19.57</td>
</tr>
<tr>
<td>Negative control</td>
<td>0.48</td>
<td>0.73</td>
<td>-1.21</td>
</tr>
</tbody>
</table>

Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. Negative control consisted of maize callus transformed with the binary vector containing the selectable marker (PMI) only.

Example 8: Combining plant expressed locking and key enzymes

Tobacco leaves transiently expressing enzymes were generated essentially as described in Example 3D. Leaves were generated by transiently expressing two binary vectors simultaneously. One of the binary vectors was 17588 (described in Example 12) which contains a polynucleotide sequence encoding a sucrose isomerase (SEQ ID NO: 16). The second binary vector was 902526 (described in Example 12) which contains a polynucleotide sequence encoding an alpha-1,6-glucosidase (SEQ ID NO: 11). Both binary vectors were infiltrated into the same tobacco leaf.

Essentially as described in Example 3D, whole leaves from tobacco were co-infiltrated with both binary vectors 17588 and 092526. Co-infiltration was performed essentially as described in Example 3D except that two strains of Agrobacterium, each containing one of the two vectors, were infiltrated into the tobacco leaf. Infiltrated leaves were collected and frozen at -80 degrees C in 24-well blocks containing 3/16" chrome ball bearings. The frozen material was shaken at setting 9 for 2 min in a Kleco Titer Plate/Microtube Grinding Mill creating a powder. Powder samples were transferred to 30 mL centrifuge tubes and centrifuged at 20,000Xg for 20 minutes at 4 degrees C. The supernatants were transferred to new tubes and adjusted to 50 mM HEPES, 0.02% Tween-20, 2 mM EDTA and 20 mM NaOH resulting in a mixture with pH between 7 and 8. Samples were then transferred to PCR tubes and incubated at 60 degrees C in a Biorad Tetrad 2 thermocycler. Samples were collected from the thermocycler at times 0,
Table 25 demonstrates that plants transiently expressing both sucrose isomerase and alpha-1,6-glucosidase expressed an active sucrose isomerase. Sucrose isomerase activity was demonstrated by the accumulation of trehalulose and isomaltulose in both the negative control (17588) and the sample (17588 and 902526). It is noted that the sample (17588 and 902526) accumulated less trehalulose and isomaltulose than the negative control (17588). While not to be limited by theory, this observation suggests that the alpha-1,6-glucosidase enzyme is active in the sample (17588 and 902526) and thus leads to the conversion of the trehalulose and isomaltulose to fermentable sugars.

Table 25 - 26 demonstrate that plants transiently expressing both sucrose isomerase and alpha-1,6-glucosidase expressed active enzymes. Alpha-1,6-glucosidase activity was demonstrated by comparing time 0 samples with samples collected at 48 hours which demonstrated the conversion of the locked sugars, trehalulose and isomaltulose, to the fermentable sugars, glucose and fructose.

Data outlined in Table 25 - 26 demonstrates the co-expression of a locking enzyme (sucrose isomerase) and an key enzyme (alpha-1,6-glucosidase) in a plant.

Table 25: HPAEC analysis of carbohydrate products from tobacco leaves transiently expressing both sucrose isomerase and an alpha-1,6-glucosidase enzyme. Accumulation of sucrose isomers in a plant co-expressing both lock and key enzymes before incubating for key activity. (T. ethanolicus)

<table>
<thead>
<tr>
<th>sample</th>
<th>Glucose + Fructose % total sugar</th>
<th>Sucrose % total sugar</th>
<th>Trehalulose % total sugar</th>
<th>Isomaltulose % total sugar</th>
</tr>
</thead>
<tbody>
<tr>
<td>17588 and 902526</td>
<td>75.88</td>
<td>0</td>
<td>15.91</td>
<td>8.21</td>
</tr>
<tr>
<td>Negative control</td>
<td>80.99</td>
<td>19.01</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. Negative control consisted of non-infiltrated tobacco leaves.

Table 26. HPAEC analysis of carbohydrate products from tobacco leaves transiently expressing both sucrose isomerase and an alpha-1,6-glucosidase enzyme. Table 254 converts hydrolysis of the lock sugars by key activity after incubation. Enzyme activity is
indicated by the change in abundance of each sugar as a percentage of the total sugars over a 24 hour period.

<table>
<thead>
<tr>
<th>sample</th>
<th>Glucose (% total sugar)</th>
<th>Fructose (% total sugar)</th>
<th>Isomaltulose (% total sugar)</th>
<th>Trehalulose (% total sugar)</th>
</tr>
</thead>
<tbody>
<tr>
<td>17588 and 902526</td>
<td>0.15</td>
<td>10.34</td>
<td>-4.20</td>
<td>-6.30</td>
</tr>
<tr>
<td>Negative control</td>
<td>-8.18</td>
<td>3.58</td>
<td>1.19</td>
<td>3.41</td>
</tr>
</tbody>
</table>

Total sugar = total amount of identifiable sugars in sample based on retention times of pure sugar standards. Extraneous peaks in samples are indeterminate and not included in sample analysis. Negative control consisted tobacco leaves transiently expressing sucrose isomerase and an empty control vector.

Example 9: Production of fermentable sugars and/or ethanol

9A: Glucose production using both dextranucrase and dextranase

Dextranucrase and dextranase form a pair of enzymes that are a lock and key combination. The dextranucrase catalyzes the formation of dextrans which are a locked form of sugar or carbohydrate. The dextranase is a key enzyme which can be used to convert the dextran back to a fermentable form of sugar.

The dextranucrase is expressed in transgenic sugarcane plants such that dextrans accumulate in the sugarcane plant. Dextrans produced from dextranucrase reactions in sugarcane juice (Example 1C) or dextrans produced by transgenic plants expressing dextranucreases (Example 3B) are harvested. These dextrans are used as substrate for dextranase activity assays to demonstrate the ability of the selected dextranases to convert the dextrans back into glucose, maltose and other small reducing sugars. The dextranase is provided as either transgenic plant produced enzyme (Example 3C) or as microbially produced enzyme (Example 2C).

9B: Isomaltulose fermented to produce ethanol

Yeast, Saccharomyces cerevisiae, strains were screened for the ability to ferment isomaltulose into ethanol. Strains were grown in a media containing 10 g yeast extract, and 20 g peptone per liter of media. This media was supplemented with glucose or isomaltulose to the appropriate final concentration.
Single yeast colonies were inoculated into 5mL 2% glucose media and incubated for 24 hours at 30 degrees C cells were centrifuged at 3000Xg for 5 minutes, supernatant was discarded, cells were washed by resuspending the cells in 5 mLs of distilled water, washed cells were centrifuged at 3000Xg for 5 minutes, supernatant was discarded, cells were resuspend in 5 mLs of yeast media containing 1% isomaltulose media and incubated for 12 hours at 30 degrees C. After 12 hours cells were centrifuged at 3000Xg for 5 minutes, supernatant was discarded, cells were washed by resuspending in 5 mLs of distilled water, washed cells were centrifuged at 3000Xg for 5 minutes, supernatant was discarded, cells were resuspend in 5 mLs of 4% isomaltulose media or 4% glucose media for fermentation. Samples for ethanol and sugar analysis were removed every hour for six hours and stored at -20 degrees C. After all samples were collected they were thawed and filtered in 0.45 Micron nylon SpinX columns by centrifugation at 7000 rpm for 5 minutes. Filtered solution was then subjected to HPLC to determine the concentration of ethanol and the sugar composition of the solution which is shown in table 27. The graph below outlines the ethanol produced by various yeast strains grown in the presence of glucose or isomaltulose over time.

Table 27: Ethanol yield from yeast strains grown with isomaltulose or glucose

<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Sugar</th>
<th>Percentage Ethanol Yield</th>
<th>Percentage of Theoretical Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>Glucose</td>
<td>2.1</td>
<td>80.1</td>
</tr>
<tr>
<td>B</td>
<td>Isomaltulose</td>
<td>1.49</td>
<td>57.4</td>
</tr>
<tr>
<td>C</td>
<td>Glucose</td>
<td>2.14</td>
<td>82.0</td>
</tr>
<tr>
<td>C</td>
<td>Isomaltulose</td>
<td>0.35</td>
<td>13.6</td>
</tr>
<tr>
<td>A</td>
<td>Glucose</td>
<td>1.9</td>
<td>72.4</td>
</tr>
<tr>
<td>A</td>
<td>Isomaltulose</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>
Example 10: Transfer of ethanol producing genes between yeast strains

Not all yeast strains, including commercial yeast strains used in the ethanol industry, possess the capacity for isomaltulose fermentation. Genes needed for isomaltulose fermentation can be introduced into commercial strains by mating, mutagenesis or transformation. These genes may include an alpha glucosidase enzyme in addition to a receptor which senses the presence of isomaltulose and induces the expression of an alpha-glucoside transporter which transports isomaltulose and other alpha glucosides into the cell. Genes involved with these functions occur at the melezitose locus in *S. cerevisiae* and may be introduced into other strains of yeast by mating techniques known to skilled practitioners in the art (Hwang & Lindegren Nature vol 203 no 4946, pp79 1-792 (1964)). Alternatively, the coding sequence of a highly efficient alpha-1,6-glucosidase enzyme may be introduced into yeast in place of the alpha glucosidase gene at the melezitose locus by homologous recombination or they may be inserted elsewhere in the genome. By replacing the endogenous alpha-glucosidase gene with a gene that more efficiently hydrolyzes isomaltulose or other locked sugars it may
be possible to improve the rate of fermentation of these sugars. Similarly, genes for alpha-glucoside transporters and receptors may be overexpressed or altered by site directed mutagenesis in order to increase the rate of isomaltulose uptake by yeast strains to improve the efficiency of isomaltulose fermentation. Another approach may be to identify strains which constitutively express the genes necessary for isomaltulose fermentation or to mutagenize or engineer yeast strains so that they constitutively express the genes necessary for isomaltulose fermentation. The techniques necessary for these approaches are widely known to skilled practitioners of the art.

1OA: Transgenic yeast expressing key enzymes

A yeast codon optimized gene for Bacillus SAM 1606 (ScJSAM 1606) glucosidase (GeneBank Accession CAA54266) was cloned into the Xhol/Xbal sites of pGEM30 (ATCC 53345), which contains an N-terminus DEX4 secretion signal. This created a DEX4-Sc_SAM1606 glucosidase fusion protein.

The URA3 marker was replaced with the kanMX locus, which confers resistance to the antibiotic Geneticin (G418) (Wach et al. Yeast 10: 1793-1808 (1994)). The URA3 cassette was excised with Smal and CiaI and the backbone was gel-purified. The kanMX cassette was amplified from a yeast insertional library (ATCC number GSA-7) using Phusion High Fidelity DNA polymerase (Finnzymes) with primers bearing 30 bp of homology to the ends of the Smal/ClaI backbone fragment.

The Smal/ClaI backbone fragment and the kanMX cassette were recombined using SLIC recombination (Li and Elledge, Nature Methods 4: 251-256 (2007)). Briefly, both fragments were treated with T4 DNA polymerase at room temperature to create single stranded DNA, the reaction was stopped after 15 minutes with dCTP, and the fragments were co-transformed into E. coli TOP10 competent cells (Invitrogen). Plasmids isolated from recombinant E. coli cells were sequenced and analyzed by restriction enzymes. The resulting vector was named pEB68.

A second yeast vector containing the Bacillus thuringiensis alpha-1,6-glucosidase gene was generated by cloning a yeast codon optimized polynucleotide sequence encoding the alpha-1,6-glucosidase into the pEB68 backbone by SLIC recombination to create pEB77.
An 'empty-vector' control consisting of the pEB68 backbone but lacking any
gene behind the TP1 promoter was made by cutting pEB68 with Xhol/Xbal, purification
of the backbone, blunting the ends, and self-ligation. This vector was named pEB70.

Saccharomyces cerevisiae strain X1049-9C (ATCC number 204802) was
transformed with the vectors pEB68, pEB77, and pEB70. Yeast competent cells were
made and transformed using the S. c. EasyComp™ Transformation kit (Invitrogen).
Transformed yeast cells were recovered by holding them at 30 degreesC for 4-5 hours
after transformation and then plated on YPD medium containing 200 ug/mL of G418.

Glucosidase enzyme activity associated with vector pEB69 was measured in
transformed yeast cells by selected three yeast clones expressing DEX4-Sc_SAM1606
fusion protein and three untransformed yeast clones which were inoculated on 5 mL of
YPD with G418 (untransformed yeast was inoculated in YPD without selection). After
24 hours of growth, cells were pelleted and the media was separated and used for enzyme
analyses.

Sc-SAM1606 activity was measured at 70 degreesC for 16 hours by combining 10
uL of yeast media, 25 uL of buffer (100 raM HEPES, 4 mM EDTA, 0.04 % Tween-20, pH
7.0), and 15 uL of a sugar solution containing 280 mM trehalulose, 100 mM
isomaltulose, 70 mM citrate. Enzyme activity was estimated by measuring the amount of
glucose released from the conversion of locked sugar (trehalulose and isomaltulose) to
glucose using a GO-POD assay essentially as described in Example 2B. Table 27
outlines data demonstrating the transformed yeast expressed an active glucosidase
enzyme.

Glucosidase enzyme activity associated with vector pEB77 was demonstrated by
isolating two clones of each transformation (pEB77 and pEB70) and inoculated into
medium containing 10 g yeast extract, 20 g peptone, 4 g isomaltulose, and 0.5% glucose
per liter of medium. Cultures were grown until glucose was exhausted (24 hours). After
24 hours, the cells were spun and 1 mL of medium was saved for enzyme activity.
To evaluate glucosidase activity on isomaltulose the following reaction was set up:
25 ul of 2X Buffer (100 mM HEPES pH: 7.0, 4 mM EDTA, 0.04 % Tween-20, protease
inhibitors), 10 ul isomaltulose (500 mM), and 15 ul medium obtained as described above.
The 50 uL reaction was incubated overnight at 37 degrees C. 20 uL of the above reaction were added to 250 uL of Glucose oxidase reagent (GOPOD assay essentially as described in Example 2B) and incubated at 37 degrees C for 10 minutes. The reactions consisted of three technical replicates. The glucose concentration measured was termed GlucoseA. To account for any glucose left in the medium after 24 hours of yeast growth, the same GOPOD assay was conducted by diluting 15 uL of medium with 35 uL of water (no isomaltulose) and using 20 uL of this dilution to the Glucose oxidase reagent. All the glucose measured this way is considered background noise and must come from the medium. This was termed GlucoseB.

The amount of glucose produced by hydrolysis of isomaltulose was calculated as GlucoseA minus GlucoseB and correspond to the values shown in Table 29.

Table 28: Glucose Cone of samples (mM): Transformed raw data from yeast expressing glucosidase using equation from glucose standard curve.

<table>
<thead>
<tr>
<th>Sample Replicate</th>
<th>pEB68</th>
<th>pEB68</th>
<th>pEB68</th>
<th>pEB68</th>
<th>Negative control</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>4.74</td>
<td>7.19</td>
<td>4.21</td>
<td>4.73</td>
<td>1.49</td>
</tr>
<tr>
<td>B</td>
<td>4.81</td>
<td>3.86</td>
<td>4.26</td>
<td>4.59</td>
<td>1.65</td>
</tr>
<tr>
<td>C</td>
<td>4.83</td>
<td>4.50</td>
<td>4.47</td>
<td>4.90</td>
<td>1.63</td>
</tr>
</tbody>
</table>

Table 29: Transgenic yeast containing plasmid pEB77 demonstrate glucosidase activity.
Example 11: Improvement of molecules to increase activity, thermostability, and catalytic efficiency and product specificity

Improvement of sucrose isomerase enzymes can be achieved through rational design of the enzyme. For example, the product of the paf1 gene (GenBank accession number AY040843) contains a product specificity domain 325RLDRD329 which influences the proportion of trehalulose or isomaltulose produced by the enzyme. By mutating these four charged amino acid residues (Arg325, Arg328, Asp327 and Asp329) trehalulose formation can be increased by 17-61% and formation of isomaltulose can be decreased by 26-67% (Zhang et al. FEBS Letters 534 (2003) 151-155). An aromatic clamp formed by Phe 256 and Phe280 has also been identified as important in substrate recognition and product specificity. (Ravaud et al. The Journal of Biological Chemistry VOL. 282, NO. 38, pp. 28126-28136, September 21, 2007).

Example 12: Constructs for transient expression

Table 1 outlines expression constructs used for generation of stable, transgenic plants as well as for the expression of enzymes transiently in plant tissues. The DNA sequences encoding proteins were codon optimized for the appropriate host; for example, expression constructs designed for tobacco and sugarbeet transient and stable transgenic plant expression were codon optimized for dicots while expression constructs designed for sugarcane or maize transient and stable transgenic plant expression were codon optimized for monocots. Codon optimization tables are available through commercial software applications such as Vector NTI 9.0.

Standard cloning techniques such as restriction enzyme digestion, gel electrophoresis and subsequence fragment purification, DNA ligation, bacterial cell transformation and selection, and the like were used to generate the vectors described in Table 29. Some of the components of the expression vectors described in Table 1 were synthesized by GeneArt (Germany), additionally, some of the vectors were cloned by GeneArt (Germany).
The binary vector 17588 contains an expression cassette with the following components operatively linked together in this order: the Arabidopsis ubiquitin promoter (SEQ ID NO: 7); GY1 ER targeting sequence (SEQ ID NO: 13), which targets the polypeptide encoded by the sucrose isomerase coding region through the endoplasmic reticulum; the sporamin vacuolar targeting sequence (SEQ ID NO 15) which directs the sucrose isomerase polypeptide from the endoplasmic reticulum to the vacuole; a dicot optimized polynucleotide sequence encoding a sucrose isomerase (SEQ ID NO: 16); and a NOS termination sequence.

The binary vector pEB47 contains an expression cassette with the following components operatively linked together in this order: an FMV enhancer (SEQ ID NO: 22); a 35S enhancer (SEQ ID NO: 23); a maize ubiquitin promoter (SEQ ID NO: 18); a maize gamm-zein ER targeting sequence (SEQ ID NO: 19) which directs the sucrose isomerase polypeptide to the ER; a sporamin vacuolar targeting sequence (SEQ ID NO: 15) which directs the sucrose isomerase polypeptide from the ER to the vacuole; a maize optimized polynucleotide sequence encoding a sucrose isomerase (SEQ ID NO: 24); a NOS terminator.

The vector pEB38 contains an expression cassette with the following components operatively linked together in this order: maize ubiquitin promoter (SEQ ID NO: 18); maize gamma zein signal sequence (SEQ ID NO: 19) which targets the polypeptide encoded by the sucrose isomerase polynucleotide sequence to the endoplasmic reticulum; sporamin vacuolar targeting sequence (SEQ ID NO: 15) which directs the polypeptide encoded by the sucrose isomerase polynucleotide sequence from the endoplasmic reticulum to the vacuole; monocot optimized polynucleotide sequence encoding sucrose isomerase (SEQ ID NO: 20); and the NOS terminator.

The binary vector 902525 contains an expression cassette with the following components operatively linked together in this order: Arabidopsis ubiquitin promoter (SEQ ID NO: 7); GY1 ER targeting sequence (SEQ ID NO: 13), which targets the polypeptide encoded by the sucrose isomerase coding region through the endoplasmic reticulum; dicot optimized polynucleotide sequence encoding sucrose isomerase polypeptide (SEQ ID NO: 11); NOS terminator. The sucrose isomerase enzyme
expressed by this expression cassette is expected to accumulate in the apoplast of the
transgenic plant cell comprising the expression cassette.

The BCTV binary vector 902526 contains an expression cassette with the
following components operatively linked together in this order: Agrobacterium NOS
promoter (SEQ ID NO: 10); GY1 ER targeting sequence (SEQ ID NO: 13), which targets
the polypeptide encoded by the sucrose isomerase coding region through the endoplasmic
reticulum; dicot optimized polynucleotide sequence encoding sucrose isomerase
polypeptide (SEQ ID NO: 11); NOS terminator. The sucrose isomerase enzyme
expressed by this expression cassette is expected to accumulate in the apoplast of the
transgenic plant cell comprising the expression cassette.

The binary vector 901612 contains an expression cassette with the following
components operatively linked together in this order: Arabidopsis ubiquitin promoter
(SEQ ID NO: 7); FNR plastid targeting sequence (SEQ ID NO: 26) which directs the
alpha-1,1-glucosidase polypeptide to the chloroplast; dicot optimized polynucleotide
sequence encoding alpha-1,1-glucosidase (SEQ ID NO: 27); NOS terminator. The alpha-
1,1-glucosidase enzyme expressed by this expression cassette is expected to accumulate
in the chloroplast of the transgenic plant cell comprising the expression cassette.

The binary vector 902195 contains an expression cassette with the following
components operatively linked together in this order: Agrobacterium NOS promoter
(SEQ ID NO: 10); GY1 ER targeting sequence (SEQ ID NO: 13) which targets the
dextranucrase polypeptide to the endoplasmic reticulum; sporamin vacuolar targeting
sequence (SEQ ID NO: 15) which directs the polypeptide encoded by the dextranucrase
polynucleotide sequence from the endoplasmic reticulum to the vacuole; dicot optimized
polynucleotide sequence encoding a dextranucrase with leucrose synthase activity (SEQ
ID NO: 35); NOS terminator.

The vector pEB28 contains an expression cassette with the following components
operatively linked together in this order: maize ubiquitin promoter (SEQ ID NO: 18);
maize gamma zein signal sequence (SEQ ID NO: 19) which targets the polypeptide
encoded by the dextranucrase polynucleotide sequence to the endoplasmic reticulum;
sporamin vacuolar targeting sequence (SEQ ID NO: 15) which directs the polypeptide
encoded by the dextranucrase polynucleotide sequence from the endoplasmic reticulum
to the vacuole; monocot optimized polynucleotide sequence encoding a dextranucrase with leucrose synthase activity (SEQ ID NO: 37); NOS terminator.

The binary vector 902550 contains an expression cassette with the following components operatively linked together in this order: Arabidopsis ubiquitin promoter (SEQ ID NO: 7); chloroplast targeting sequence (SEQ ID NO: 42); dicot optimized polynucleotide sequence encoding an alpha-1,5-glucosidase (SEQ ID NO: 46); NOS terminator.

The vector 902423 contains an expression cassette with the following components operatively linked together in this order: maize ubiquitin promoter (SEQ ID NO: 39); TMV enhancer (SEQ ID NO: 40); chloroplast targeting sequence (SEQ ID NO: 41) which directs the alpha-1,5-glucosidase polypeptide encoded by the polynucleotide sequence (SEQ ID NO: 43) to the chloroplast; maize optimized polynucleotide sequence encoding alpha-1,5-glucosidase (SEQ ID NO: 43); terminator from maize ubiquitin (SEQ ID NO: 45).

The binary vector 90522 contains an expression cassette with the following components operatively linked together in this order: Arabidopsis ubiquitin promoter (SEQ ID NO: 7); GY1 ER targeting sequence (SEQ ID NO: 13) which targets the alpha-1,1-glucosidase polypeptide to the endoplasmic reticulum; dicot optimized polynucleotide sequence encoding an alpha-1,1-glucosidase (SEQ ID NO: 52); NOS terminator. The expectation is that the alpha-1,1-glucosidase polypeptide will be processed through the endoplasmic reticulum and accumulate in the apoplast.

The vector 902435 contains an expression cassette with the following components operatively linked together in this order: maize ubiquitin promoter (SEQ ID NO: 29); TMV enhancer sequence (SEQ ID NO: 40); maize optimized polynucleotide sequence encoding an alpha-1,6-glucosidase (SEQ ID NO: 54); ER retention sequence (SEQ ID NO: 51); maize ubiquitin termination sequence (SEQ ID NO: 45).

The vector 902425 contains an expression cassette with the following components operatively linked together in this order: maize ubiquitin promoter (SEQ ID NO: 29); TMV enhancer sequence (SEQ ID NO: 40); chloroplast targeting sequence (SEQ ID NO: 26); monocot optimized polynucleotide sequence encoding an alpha-1,6-glucosidase (SEQ ID NO: 56); maize ubiquitin termination sequence (SEQ ID NO: 45).
<table>
<thead>
<tr>
<th>Vector number</th>
<th>Promoter</th>
<th>Regulatory elements</th>
<th>Enzyme</th>
<th>crop</th>
</tr>
</thead>
<tbody>
<tr>
<td>17588 (binary vector)</td>
<td>Arabidopsis ubiquitin promoter (SEQ ID NO: 7)</td>
<td>GY1 ER targeting sequence (SEQ ID NO: 13); sporamin vacuolar targeting sequence (SEQ ID NO: 15)</td>
<td>Sucrose isomerase (SEQ ID NO: 16)</td>
<td>Sugar beet and tobacco</td>
</tr>
<tr>
<td>pEB47 (binary vector)</td>
<td>maize ubiquitin promoter (SEQ ID NO: 18)</td>
<td>FMV enhancer (SEQ ID NO: 22); 35S enhancer (SEQ ID NO: 23); Maize ? gamma zein ER targeting sequence (SEQ ID NO: 19); sporamin vacuolar targeting sequence (SEQ ID NO: 15)</td>
<td>Sucrose isomerase (SEQ ID NO: 24)</td>
<td>Maize and sugarcane</td>
</tr>
<tr>
<td>pEB38</td>
<td>maize ubiquitin promoter (SEQ ID NO: 18)</td>
<td>Maize gamma zein ER targeting sequence (SEQ ID NO: 19); sporamin vacuolar targeting sequence (SEQ ID NO: 15)</td>
<td>Sucrose isomerase (SEQ ID NO: 20)</td>
<td>Maize and sugarcane</td>
</tr>
<tr>
<td>902525 binary</td>
<td>Arabidopsis ubiquitin promoter (SEQ ID NO: 7)</td>
<td>GY1 ER targeting sequence (SEQ ID NO: 13)</td>
<td>T. ethanolicus alpha-1,6-glucosidase (SEQ ID NO: 11)</td>
<td>Sugar beet and tobacco</td>
</tr>
<tr>
<td>902526 (BCTV binary)</td>
<td>NOS promoter (SEQ ID NO: 10)</td>
<td>GY1 ER targeting sequence (SEQ ID NO: 13)</td>
<td>T. ethanolicus alpha-1,6-glucosidase (SEQ ID NO: 11)</td>
<td>Sugar beet and tobacco</td>
</tr>
<tr>
<td>902195</td>
<td>NOS promoter (SEQ ID NO: 10)</td>
<td>GY1 ER targeting sequence (SEQ ID NO: 13); sporamin vacuolar targeting sequence (SEQ ID NO: 15)</td>
<td>Dextranucrase (SEQ ID NO: 35)</td>
<td>Tobacco and sugarbeet</td>
</tr>
<tr>
<td>pEB28</td>
<td>maize ubiquitin promoter (SEQ ID NO: 18)</td>
<td>Maize gamma zein ER targeting sequence (SEQ ID NO: 19); sporamin vacuolar targeting sequence (SEQ ID NO: 15)</td>
<td>Dextranucrase (SEQ ID NO: 37)</td>
<td>Maize and sugarcane</td>
</tr>
<tr>
<td>902435</td>
<td>maize ubiquitin promoter (SEQ ID NO: 18)</td>
<td>ER retention sequence (51); maize ubiquitin terminator (SEQ ID NO: 45); TMV enhancer (SEQ ID NO: 40)</td>
<td>Alpha-1,6-glucosidase (SEQ ID NO: 54)</td>
<td>Maize and sugarcane</td>
</tr>
<tr>
<td>NO: 39)</td>
<td>maize ubiquitin promoter (SEQ ID NO: 39)</td>
<td>TMV enhancer (SEQ ID NO: 40); FNR chloroplast targeting sequence (SEQ ID NO: 41); maize ubiquitin terminator (SEQ ID NO: 45)</td>
<td>Alpha-1,6-glucosidase (SEQ ID NO: 56)</td>
<td>Maize and sugarcane</td>
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<td>901612</td>
<td>Arabidopsis ubiquitin promoter (SEQ ID NO: 7)</td>
<td>Plastid targeting sequence FNR (SEQ ID NO: 26)</td>
<td>Bacillus alpha-1,1-glucosidase (SEQ ID NO: 27)</td>
<td>Sugar beet and tobacco</td>
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<td>Arabidopsis ubiquitin promoter (SEQ ID NO: 7)</td>
<td>GY1 ER targeting sequence (SEQ ID NO: 13)</td>
<td>Alpha-1,1-glucosidase (SEQ ID NO: 52)</td>
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<td>902429</td>
<td>maize ubiquitin promoter (SEQ ID NO: 39)</td>
<td>TMV enhancer (SEQ ID NO: 40); ER targeting sequence (SEQ ID NO: 48); ER retention sequence (51); maize ubiquitin terminator (SEQ ID NO: 45)</td>
<td>Alpha-1,1-glucosidase (SEQ ID NO: 49)</td>
<td>Maize and sugarcane</td>
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<td>902550</td>
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<td>Plastid targeting sequence FNR (SEQ ID NO: 26)</td>
<td>Alpha-1,5-glucosidase (SEQ ID NO: 46)</td>
<td>Sugar beet and tobacco</td>
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<td>902423</td>
<td>maize ubiquitin promoter (SEQ ID NO: 39)</td>
<td>TMV enhancer (SEQ ID NO: 40); FNR chloroplast targeting sequence (SEQ ID NO: 41); maize ubiquitin terminator (SEQ ID NO: 45)</td>
<td>Alpha-1,5-glucosidase (SEQ ID NO: 43)</td>
<td>Maize and sugarcane</td>
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</table>

The following embodiments are encompassed by the present invention:
1. A method for producing fermentable sugar comprising:
   a) providing transgenic plant material comprising one or more locked carbohydrates; and
   b) contacting said transgenic plant material with one or more key enzymes wherein said contacting is under conditions sufficient for conversion of said locked carbohydrate to fermentable sugar.

2. The method of claim 1, wherein the one or more locked carbohydrate is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextrans, fructans, maltulose, turanose and isomaltose.

3. The method of claim 1, wherein the one or more key enzyme is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

4. The method of claim 1, wherein the one or more key enzyme is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

5. The method of claim 1, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

6. A method for producing fermentable sugar comprising:
   a) providing transgenic plant material comprising one or more locked enzymes and one or more locked carbohydrates; and
   b) contacting said transgenic plant material with one or more key enzymes wherein said contacting is under conditions sufficient for conversion of said locked carbohydrate to fermentable sugar.
7. The method of claim 6, wherein the one or more locked carbohydrate is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltulose, turanose and isomaltose.

8. The method of claim 6, wherein the one or more lock enzymes is selected from the group consisting of dextranucrase, levan sucrose, alternansucrase, sucrose isomerase and amylosucrase.

9. The method of claim 6, wherein the one or more key enzymes is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

10. The method of claim 6, wherein the one or more key enzymes is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

11. The method of claim 6, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

12. A method for producing alcohol comprising:
   a) providing transgenic plant material comprising one or more locked carbohydrates;
   b) contacting said transgenic plant material with one or more key enzymes wherein said contacting is under conditions sufficient for conversion of said one or more locked carbohydrates to fermentable sugar; and
   c) fermenting said fermentable sugar to form alcohol.

13. The method of claim 12, wherein the locked carbohydrate is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltulose, turanose and isomaltose.
14. The method of claim 12, wherein the one or more key enzyme is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

15. The method of claim 12, wherein the one or more key enzyme is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

16. The method of claim 12, wherein the alcohol is selected from the group consisting of ethanol and butanol.

17. The method of claim 12, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

18. A method for producing alcohol comprising:
   a) providing transgenic plant material comprising one or more lock enzymes and one or more locked carbohydrates;
   b) contacting said transgenic plant material with one or more key enzymes wherein said contacting is under conditions sufficient for conversion of said one or more locked carbohydrates to fermentable sugar; and
   c) fermenting said fermentable sugar to form alcohol.

19. The method of claim 18, wherein the one or more locked carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltulose, turanose and isomaltose.

20. The method of claim 18, wherein the one or more lock enzymes is selected from the group consisting of dextransucrase, levan sucrose, alternansucrase, sucrose isomerase and amylosucrase.
21. The method of claim 18, wherein the one or more key enzymes is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

22. The method of claim 18, wherein the one or more key enzymes is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

23. The method of claim 18, wherein the alcohol is selected from the group consisting of ethanol and butanol.

24. The method of claim 18, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

25. A method for producing fermentable sugar comprising:
   a) providing transgenic plant material comprising one or more locked carbohydrates and one or more key enzymes; and
   b) processing said transgenic plant material under conditions sufficient for one or more key enzymes to convert one or more locked carbohydrates to fermentable sugar.

26. The method of claim 25, wherein the one or more key enzymes is targeted away from the one or more locked carbohydrates.

27. The method of claim 25, wherein the one or more key enzymes is targeted to an organelle selected from the group consisting of chloroplast, vacuole, cytoplasm, apoplast and endoplasmic reticulum.
28. The method of claim 25, wherein the one or more locked carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltulose, turanose and isomaltose.

29. The method of claim 25, wherein the one or more key enzymes is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

30. The method of claim 25, wherein the one or more key enzymes is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

31. The method of claim 25, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

32. A method for producing fermentable sugar comprising:
   a) providing transgenic plant material comprising one or more lock enzymes, one or more locked carbohydrates and one or more key enzymes; and
   b) processing said transgenic plant material under conditions sufficient for said one or more key enzymes to convert said one or more locked carbohydrates to fermentable sugar.

33. The method of claim 32, wherein the one or more lock enzymes is selected from the group consisting of dextranulose, levan sucrose, alternansucrase, sucrose isomerase and amylosucrase.

34. The method of claim 32, wherein the one or more key enzymes is targeted away from the one or more locked carbohydrates.
35. The method of claim 32, wherein the one or more key enzymes is targeted to an organelle selected from the group consisting of chloroplast, vacuole, cytoplasm, apoplast and endoplasmic reticulum.

36. The method of claim 32, wherein the one or more locked carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltulose, turanose and isomaltose.

37. The method of claim 32, wherein the one or more key enzymes is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

38. The method of claim 32, wherein the one or more key enzymes is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

39. The method of claim 32, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

40. A transgenic plant comprising one or more heterologous lock enzymes and one or more heterologous key enzymes.

41. The transgenic plant of claim 40, wherein the one or more lock enzymes is selected from the group consisting of dextransucrase, levan sucrose, alternansucrase, sucrose isomerase and amylosucrase.

42. The transgenic plant of claim 40, wherein the one or more key enzymes is targeted away from the locked carbohydrate.
43. The transgenic plant of claim 40, wherein the one or more key enzymes is targeted to an organelle selected from the group consisting of chloroplast, vacuole, cytoplasm, apoplast and endoplasmic reticulum.

44. The transgenic plant of claim 40, wherein the locked carbohydrate is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltose, turanose and isomaltose.

45. The transgenic plant of claim 40, wherein the one or more key enzyme is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

46. The transgenic plant of claim 40, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

47. A transgenic plant comprising one or more locked carbohydrates and one or more key enzymes.

48. The transgenic plant of claim 47, wherein the one or more key enzymes is targeted away from the one or more locked carbohydrates.

49. The transgenic plant of claim 47, wherein the key enzyme is targeted to an organelle selected from the group consisting of chloroplast, vacuole, cytoplasm, apoplast and endoplasmic reticulum.

50. The transgenic plant of claim 47, wherein the one or more locked carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltose, turanose and isomaltose.
51. The transgenic plant of claim 47, wherein the one or more key enzyme is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

52. The transgenic plant of claim 47, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

53. A method for producing fermentable sugar comprising:
   a) providing transgenic plant material wherein said transgenic plant material is selected from the group consisting of sugar beet, sorghum, maize, and sugarcane, and wherein said transgenic plant material comprises:
      i) one or more lock enzymes wherein said one or more lock enzymes is selected from the group consisting of dextranucrase, levan sucrose, alternansucrase, sucrose isomerase and amylosucrase,
      ii) one or more locked carbohydrates wherein said one or more locked carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextrans, fructans, maltose, turanose and isomaltose,
      iii) one or more key enzymes wherein said one or more key enzymes is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase; and wherein said one or more key enzymes is targeted away from said one or more locked carbohydrates; and
   b) processing said transgenic plant material under conditions sufficient for said one or more key enzymes to convert said one or more locked carbohydrates to fermentable sugar.

54. A transgenic plant comprising:
   a) one or more lock enzymes wherein said one or more lock enzymes is selected from the group consisting of dextranucrase, levan sucrose, alternansucrase, sucrose isomerase and amylosucrase,
b) one or more locked carbohydrates wherein said one or more locked carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextrans, fructans, maltose, turanose and isomaltose,

c) one or more key enzymes wherein said one or more key enzymes is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase; and wherein said one or more key enzymes is targeted away from the one or more locked carbohydrates, and

d) wherein said transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

55. A method for producing fermentable sugar derived from a plant comprising:
   a) providing plant material comprising locked carbohydrate; and,
   b) contacting said plant material with one or more enzymes capable of converting the locked carbohydrate into fermentable sugar (key enzyme), wherein said contacting is under conditions sufficient for said conversion.

56. The method of embodiment 55, wherein said plant material comprising locked carbohydrate is derived from a transgenic plant expressing one or more enzymes capable of converting an endogenous carbohydrate of said transgenic plant into said locked carbohydrate (lock enzyme).

57. The method of embodiment 55 or 56, wherein the key enzyme is provided as a purified or semi-purified enzyme preparation.

58. The method of embodiment 55 or 56, wherein at least one of the key enzymes is provided as plant material derived from a plant expressing said key enzyme.

59. The method of embodiment 58, wherein at least one of the key enzymes is expressed in the same plant as the plant comprising the locked carbohydrate.
60. The method of embodiment 55, wherein the locked carbohydrate is selected from the group consisting of isomaltulose, trehalulose, dextran, fractan, amylose, leucrose and alternan.

61. The method of embodiment 56, wherein the transgenic plant expresses at least two sucrose isomerase enzymes, wherein at least the first sucrose isomerase enzyme catalyzes the conversion of sucrose primarily into isomaltulose, and wherein at least the second sucrose isomerase enzyme catalyzes the conversion of sucrose primarily into trehalulose.

62. The method of embodiment 55, wherein said plant material comprising the locked carbohydrate is derived from a plant selected from the group consisting of maize, wheat, rice, barley, soybean, cotton, sorghum, oats, tobacco, Miscanthus grass, Switch grass, trees, beans, rape/canola, alfalfa, flax, sunflower, safflower, millet, rye, sugarcane, sugar beet, cocoa, tea, Brassica, cotton, coffee, sweet potato, flax, peanut, clover; vegetables such as lettuce, tomato, cucurbits, cassava, potato, carrot, radish, pea, lentils, cabbage, cauliflower, broccoli, Brussels sprouts, peppers, and pineapple; tree fruits such as citrus, apples, pears, peaches, apricots, walnuts, avocado, banana, and coconut; and flowers such as orchids, carnations and roses.

63. The method of embodiment 62, wherein said plant material comprising the locked carbohydrate is derived from sugarcane, sugar beet, or sweet sorghum.

64. The method of embodiment 55, wherein the key enzyme is derived from a microorganism.

65. The method of embodiment 64, wherein the key enzyme is endogenous to said microorganism.

66. The method of embodiment 64, wherein the key enzyme is a recombinant enzyme expressed in the microorganism.
67. The method of embodiment 65, wherein the microorganism is a *Saccharomyces* strain capable of fermenting isomaltulose.

68. A method of selecting a transformed plant comprising:
   a) introducing into said plant or part thereof:
      i) an expression cassette comprising a nucleotide sequence encoding an enzyme capable of converting an endogenous sugar in said plant to a locked carbohydrate; and,
      ii) an expression cassette comprising a nucleotide sequence encoding an enzyme capable of converting the locked carbohydrate into a fermentable sugar;
   b) maintaining said plant or part thereof under conditions sufficient for the expression of the lock enzyme and the key enzyme; and,
   c) evaluating the sugar profile of said plant;
wherein the presence of one or more of the fermentable sugars produced by said key enzyme is indicative of a transformed plant.

69. A transgenic plant useful for the production of ethanol, wherein said plant comprises:
   a) a nucleotide sequence encoding an enzyme capable of converting an endogenous sugar in said plant to said locked carbohydrate; and,
   b) a nucleotide sequence encoding an enzyme capable of converting the locked carbohydrate into a fermentable sugar.

70. The plant of embodiment 69, wherein the locked carbohydrate is selected from the group consisting of isomaltulose, trehalulose, dextran, fructan, amylose, leucrose and alternan.

71. The plant of embodiment 70, wherein the transgenic plant expresses at least two sucrose isomerase enzymes, wherein at least the first sucrose isomerase enzyme catalyzes
the conversion of sucrose primarily into isomaltulose, and wherein at least the second sucrose isomerase enzyme catalyzes the conversion of sucrose primarily into trehalulose.

72. The transgenic plant of embodiment 69 selected from the group consisting of maize, wheat, rice, barley, soybean, cotton, sorghum, oats, tobacco, Miscanthus grass, Switch grass, trees, beans, rape/canola, alfalfa, flax, sunflower, safflower, millet, rye, sugarcane, sugar beet, cocoa, tea, Brassica, cotton, coffee, sweet potato, flax, peanut, clover; vegetables such as lettuce, tomato, cucurbits, cassava, potato, carrot, radish, pea, lentils, cabbage, cauliflower, broccoli, Brussels sprouts, peppers, and pineapple; tree fruits such as citrus, apples, pears, peaches, apricots, walnuts, avocado, banana, and coconut; and flowers such as orchids, carnations and roses.

73. The plant of embodiment 62, wherein said plant is sugarcane, sugar beet, or sorghum.

All publications and patent applications mentioned in the specification are indicative of the level of skill of those skilled in the art to which this invention pertains.

All publications and patent applications are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although the foregoing invention has been described in some detail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and modifications may be practiced within the scope of the appended claims.
THAT WHICH IS CLAIMED:

1. A method for producing fermentable sugar comprising:
   a) providing transgenic plant material comprising one or more locked carbohydrates; and
   b) contacting said transgenic plant material with one or more key enzymes wherein said contacting is under conditions sufficient for conversion of said locked carbohydrate to fermentable sugar.

2. The method of claim 1, wherein the one or more locked carbohydrate is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextrans, fructans, maltulose, turanose and isomaltose.

3. The method of claim 1, wherein the one or more key enzyme is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

4. The method of claim 1, wherein the one or more key enzyme is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

5. The method of claim 1, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

6. A method for producing fermentable sugar comprising:
   a) providing transgenic plant material comprising one or more locked enzymes and one or more locked carbohydrates; and
   b) contacting said transgenic plant material with one or more key enzymes wherein said contacting is under conditions sufficient for conversion of said locked carbohydrate to fermentable sugar.
7. The method of claim 6, wherein the one or more locked carbohydrate is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltulose, turanose and isomaltose.

8. The method of claim 6, wherein the one or more lock enzymes is selected from the group consisting of dextransucrase, levan sucrose, alternansucrase, sucrose isomerase and amylosucrase.

9. The method of claim 6, wherein the one or more key enzymes is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

10. The method of claim 6, wherein the one or more key enzymes is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

11. The method of claim 6, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

12. A method for producing alcohol comprising:

   a) providing transgenic plant material comprising one or more locked carbohydrates;
   b) contacting said transgenic plant material with one or more key enzymes wherein said contacting is under conditions sufficient for conversion of said one or more locked carbohydrates to fermentable sugar; and
   c) fermenting said fermentable sugar to form alcohol.
13. The method of claim 12, wherein the locked carbohydrate is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltulose, turanose and isomaltose.

14. The method of claim 12, wherein the one or more key enzyme is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

15. The method of claim 12, wherein the one or more key enzyme is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

16. The method of claim 12, wherein the alcohol is selected from the group consisting of ethanol and butanol.

17. The method of claim 12, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

18. A method for producing alcohol comprising:
   a) providing transgenic plant material comprising one or more lock enzymes and one or more locked carbohydrates;
   b) contacting said transgenic plant material with one or more key enzymes wherein said contacting is under conditions sufficient for conversion of said one or more locked carbohydrates to fermentable sugar; and
   c) fermenting said fermentable sugar to form alcohol.

19. The method of claim 18, wherein the one or more locked carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltulose, turanose and isomaltose.
20. The method of claim 18, wherein the one or more lock enzymes is selected from the group consisting of dextranucrase, levan sucrose, alternansucrase, sucrose isomerase and amylosucrase.

21. The method of claim 18, wherein the one or more key enzymes is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

22. The method of claim 18, wherein the one or more key enzymes is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

23. The method of claim 18, wherein the alcohol is selected from the group consisting of ethanol and butanol.

24. The method of claim 18, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

25. A method for producing fermentable sugar comprising:
   a) providing transgenic plant material comprising one or more locked carbohydrates and one or more key enzymes; and
   b) processing said transgenic plant material under conditions sufficient for one or more key enzymes to convert one or more locked carbohydrates to fermentable sugar.

26. The method of claim 25, wherein the one or more key enzymes is targeted away from the one or more locked carbohydrates.
27. The method of claim 25, wherein the one or more key enzymes is targeted to an organelle selected from the group consisting of chloroplast, vacuole, cytoplasm, apoplast and endoplasmic reticulum.

28. The method of claim 25, wherein the one or more locked carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltulose, turanose and isomaltose.

29. The method of claim 25, wherein the one or more key enzymes is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

30. The method of claim 25, wherein the one or more key enzymes is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

31. The method of claim 25, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

32. A method for producing fermentable sugar comprising:
   a) providing transgenic plant material comprising one or more lock enzymes, one or more locked carbohydrates and one or more key enzymes; and
   b) processing said transgenic plant material under conditions sufficient for said one or more key enzymes to convert said one or more locked carbohydrates to fermentable sugar.

33. The method of claim 32, wherein the one or more lock enzymes is selected from the group consisting of dextranucrase, levan sucrose, alternansucrase, sucrose isomerase and amylosucrase.
34. The method of claim 32, wherein the one or more key enzymes is targeted away from the one or more locked carbohydrates,

35. The method of claim 32, wherein the one or more key enzymes is targeted to an organelle selected from the group consisting of chloroplast, vacuole, cytoplasm, apoplast and endoplasmic reticulum.

36. The method of claim 32, wherein the one or more locked carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltulose, turanose and isomaltose.

37. The method of claim 32, wherein the one or more key enzymes is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

38. The method of claim 32, wherein the one or more key enzymes is provided by a source selected from the group consisting of transgenic plant material expressing a key enzyme, recombinant microbe expressing a key enzyme, transgenic yeast expressing a key enzyme, microbe expressing a key enzyme and yeast expressing a key enzyme.

39. The method of claim 32, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

40. A transgenic plant comprising one or more heterologous lock enzymes and one or more heterologous key enzymes.

41. The transgenic plant of claim 40, wherein the one or more lock enzymes is selected from the group consisting of dextranucrase, levan sucrose, alternansucrase, sucrose isomerase and amylosucrase.
42. The transgenic plant of claim 40, wherein the one or more key enzymes is targeted away from the locked carbohydrate.

43. The transgenic plant of claim 40, wherein the one or more key enzymes is targeted to an organelle selected from the group consisting of chloroplast, vacuole, cytoplasm, apoplast and endoplasmic reticulum.

44. The transgenic plant of claim 40, wherein the locked carbohydrate is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltose, turanose and isomaltose.

45. The transgenic plant of claim 40, wherein the one or more key enzyme is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

46. The transgenic plant of claim 40, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

47. A transgenic plant comprising one or more locked carbohydrates and one or more key enzymes.

48. The transgenic plant of claim 47, wherein the one or more key enzymes is targeted away from the one or more locked carbohydrates.

49. The transgenic plant of claim 47, wherein the key enzyme is targeted to an organelle selected from the group consisting of chloroplast, vacuole, cytoplasm, apoplast and endoplasmic reticulum.

50. The transgenic plant of claim 47, wherein the one or more locked carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextran, fructan, maltose, turanose and isomaltose.
51. The transgenic plant of claim 47, wherein the one or more key enzyme is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase.

52. The transgenic plant of claim 47, wherein the transgenic plant is selected from the group consisting of maize, sugar beet, sorghum and sugarcane.

53. A method for producing fermentable sugar comprising:
   a) providing transgenic plant material wherein said transgenic plant material is selected from the group consisting of sugar beet, sorghum, maize, and sugarcane, and wherein said transgenic plant material comprises:
      i) one or more lock enzymes wherein said one or more lock enzymes is selected from the group consisting of dextranucrase, levan, sucrose, alternansucrase, sucrose isomerase and amylosucrase,
      ii) one or more locked carbohydrates wherein said one or more locked carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose, starch, dextrans, fructans, maltose, turanose and isomaltose,
      iii) one or more key enzymes wherein said one or more key enzymes is selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase; and wherein said one or more key enzymes is targeted away from said one or more locked carbohydrates; and
   b) processing said transgenic plant material under conditions sufficient for said one or more key enzymes to convert said one or more locked carbohydrates to fermentable sugar.

54. A transgenic plant comprising:
a) one or more lock enzymes wherein said one or more lock enzymes is
selected from the group consisting of dextranucrase, levan sucrose, alternansucrase,
sucrose isomerase and amylosucrase,

b) one or more locked carbohydrates wherein said one or more locked
carbohydrates is selected from the group consisting of isomaltulose, trehalulose, leucrose,
starch, dextrans, fructans, maltose, turanose and isomaltose,

c) one or more key enzymes wherein said one or more key enzymes is
selected from the group consisting of dextranase, alpha-amylase, glucoamylase, alpha-
1,5-glucosidase, alpha-1,1-glucosidase and alpha-1,6-glucosidase; and wherein said one
or more key enzymes is targeted away from the one or more locked carbohydrates, and

d) wherein said transgenic plant is selected from the group consisting of
maize, sugar beet, sorghum and sugarcane.
# INTERNATIONAL SEARCH REPORT

**A. CLASSIFICATION OF SUBJECT MATTER**

**INV.** C12N 5/82

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database consulted during the international search (name of database and, where practical, search terms used)

EPO-Internal, BIOSIS, WPI Data

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

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<th>Relevant to claim No.</th>
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<td>WO 03/018766 A (SYNGENTA PARTICIPATIONS AG [CH]; LANAHAN MICHAEL B [US]; BASU SHIB SAN) 6 March 2003 (2003-03-06) page 6 - page 11; claims 82,85,150,178,180,181,185; figures 1,3,5; examples 14-17,32-41</td>
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<td>WO 2004/099403 A (UNIV QUEENSLAND [AU]; BIRCH ROBERT GEORGE [AU]; WU LUGUANG [AU]) 18 November 2004 (2004-11-18) paragraphs [0001], [0005] - [0009], [0179]; claims 54,66,72</td>
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[X] Further documents are listed in the continuation of Box C. [X] See patent family annex

Special categories of cited documents:

- **A** document defining the general state of the art which is not considered to be of particular relevance
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T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

S document member of the same patent family

Date of the actual completion of the international search: 2 September 2009

Date of mailing of the international search report: 17/09/2009

Name and mailing address of the ISA:

European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016

Authorized officer: Krüger, Julia

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## DOCUMENTS CONSIDERED TO BE RELEVANT

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