

METHODS OF PRODUCING NATURAL SWEETENERS

RELATED APPLICATION/S

This application claims the benefit of priority of U.S. Provisional Patent Application No.
5 63/525,322 filed on July 6, 2023, the contents of which are incorporated herein by reference in
their entirety.

SEQUENCE LISTING STATEMENT

The XML file, entitled 100786 Sequence Listing.XML, created on July 4, 2024, comprising
10 256,208 bytes, submitted concurrently with the filing of this application is incorporated herein by
reference.

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods of producing
15 modified cucurbitane-type triterpenoids that can be utilized in the production of sweet glycosylated
triterpenoids known as mogrosides and compositions comprising same and uses thereof.

Mogrosides are triterpene-derived specialized secondary metabolites found in the fruit of
the *Cucurbitaceae* family plant *Siraitia grosvenorii* (Luo Han Guo). Their biosynthesis in fruit
involves the synthesis of mogrol, a tetra-hydroxy cucurbitane triterpenoid, followed by a number
20 of consecutive glucosylations of the aglycone mogrol to the final sweet products mogroside IV and
mogroside V (Figure 6).

The parent aglycone compound mogrol is derived by successive hydroxylations of
cucurbitadienol, the initial product of the stereospecific triterpene synthase, cucurbitadienol
synthase. Cucurbitadienol subsequently undergoes hydroxylations, by a combination of
25 epoxidation and action of epoxide hydrolase, at the C24 and C25 positions, and an additional
hydroxylation at C11 by a cytochrome P450 enzyme, leading to mogrol, as described in Itkin et al,
2016 (Figure 1). The mogrol is subsequently glucosylated at the C3 and C24 positions to varying
degrees, from 1 to 6 glucosyl groups, in a temporally successive pattern during fruit development
and the glucosylated mogrol compounds are termed mogrosides. The sweetness strength of the
30 mogrosides increases with the additional glucose moieties such that M6 (with 6 glucosyl groups)
is sweeter than M5, followed by M4, respectively (Kasai R., et al., Sweet cucurbitane glycosides
from fruits of *Siraitia siamensis* (chi-zi luo-han-guo), a Chinese folk medicine. Agric Biol Chem
1989, 53(12):3347-3349). The purified mogroside V, has been approved as a high-intensity
sweetening agent in Japan (Jakinovich, W., Jr., Moon, C., Choi, Y. H., & Kinghorn, A. D. 1990.

Evaluation of plant extracts for sweetness using the Mongolian gerbil. Journal of Natural Products, 53, 190–195) and the extract has gained generally recognized as safe (GRAS) status in the USA as a non-nutritive sweetener and flavor enhancer.

Mogroside V has been known in the food industry as a natural non-sugar food sweetener, with a sweetening capacity of ~250 times that of sucrose (Kasai R., et al., Sweet cucurbitane glycosides from fruits of *Siraitia siamensis* (chi-zi luo-han-guo), a Chinese folk medicine. Agric Biol Chem 1989, 53(12):3347-3349.). Moreover, additional health benefits of mogrosides have been revealed in recent studies (Li et al., Chemistry and pharmacology of *Siraitia grosvenorii*: a review. Chin J Nat Med. 2014 12(2):89-102.).

Extraction of mogrosides from the *Siraitia* fruit can yield a product of varying degrees of purity, often accompanied by undesirable aftertaste. In addition, yields of mogroside from cultivated *Siraitia* fruit are limited due to low plant yields and particular cultivation requirements of the plant. It is therefore advantageous to be able to produce sweet mogroside compounds in an alternative plant species, amenable to high yield cultivation.

Fruit of other species in the Cucurbitaceae family accumulate members of the cucurbitane-type triterpenoid family, such as cucurbitacins. The cucurbitacins, however, are extremely bitter. Non-glycosylated mogrol and non-glycosylated cucurbitacin differ from each other in the number and positions of oxygenations (either as hydroxyl groups, or as carbonyl groups), by dehydrogenations and by acetylations of hydroxyl groups.

Mogrol synthesis from squalene precursors proceeds from squalene to diepoxysqualene via successive epoxidations by squalene epoxidase. The diepoxysqualene (two epoxy groups, one each at each of the penultimate terminal positions of the 30-carbon squalene molecule) is further transformed to mogrol as described above.

Cucurbitacins are a family of triterpenoid compounds comprising over 20 members, differing in the number of hydroxyl, carbonyl, and acetyl groups on the cucurbitadienol skeleton. Squalene epoxide undergoes cyclization to the cucurbitadienol skeleton via cucurbitadienol synthase. The cucurbitadienol skeleton may undergo further chemical modifications, including numerous hydroxylations, acetylations, dehydrogenations and reductions, the combination of which determines the final cucurbitacin compound. For example, Cucurbitacin E, which is the major cucurbitacin in wild bitter watermelon is shown in Fig. 2 and contains various oxygenations as well as acetylation at C25, dehydrogenation at C1-C2, reduction of hydroxyl groups to carbonyl groups, as well as possible glucosylation at C2, producing cucurbitacin E-glucoside. Cucurbitacin C, which is the major cucurbitacin in wild bitter cucumbers differs in possessing a hydroxyl group at C19 rather than C2, a hydroxyl at C3, in place of a carbonyl, and hydrogenation at C1-C2, in

pace of a double bond. The major cucurbitacin of wild bitter melon (*Cucumis melo*) is Cucurbitacin B, differing from cucurbitacin E only in the hydrogenation at C1-C2. The chemical modifications of the cucurbitacin family members are shown in Figure 1, (derived from Ielciu, I.I., et al, 2016.. *Farmacia*, 64(3)).

While mogrosides have received copious attention, primarily for their value as low-calorie sweeteners, interest in cucurbitacins has been mostly focused on their ability to activate or inhibit pro- or anti-apoptotic proteins, via JAK/STAT inhibition, modulation of the MAPK pathway, PARP cleavage, caspase-3 expression, and effects on other downstream STAT3 targets (Alghasham, Int J Health Sci 2013, 7:77-89, Dai et al, Pharm Res 2023, 187, US20230000924), for use in anti-inflammatory and anti-cancer medicine (see, for example, US20220000872, US20200390786, US20200282051, US20230001193 and US20170106003), treatment of infections (US 20210268103), in traditional Chinese medicine for treatment of hepatic disease (Yang et al, Basic and Clin Pharm 2020, 127:371-379), and as both a feed attractant and natural pesticide in agriculture (US20230044077, US Patent No. 11553703 and US20160309720).

Additional relevant publications include Shang, Y, et al, 2014 Science 346(6213); 1084-1088 and Zhou, Y et al., 2016, Nature Plants 2(12), 1-8, Che, G. et al. 2019, Curr Opin Plant Biol 47: 38-46 Kim, Y, et al, 2020 Commun Biol 3(1) 444, Chen JC, et al, Cucurbitacins and cucurbitane glycosides: structures and biological activities. Natural Product Reports. 2005 Jun;22(3):386-399. DOI: 10.1039/b418841c. PMID: 16010347; Miro, M., 1995. Cucurbitacins and their pharmacological effects. *Phytotherapy research*, 9(3), pp.159-168; and Dong, L. et al, 2021. An independent evolutionary origin for insect deterrent cucurbitacins in *Iberis amara*. *Molecular Biology and evolution*, 38(11), pp.4659-4673; PCT Publications WO2024/064695, WO2024/064694 and WO2021/202513.

SUMMARY OF THE INVENTION

According to an aspect of some embodiments of the present invention, there is provided a method of producing a plant or plant cell with a modified cucurbitacin content, the method comprising down-regulating expression of at least one cucurbitacin biosynthetic pathway gene in the plant or plant cell, thereby modifying cucurbitacin expression in the plant or plant cell.

According to an aspect of some embodiments of the present invention, there is provided a method of producing a plant or plant cell with a modified cucurbitacin content, the method comprising growing the plant or plant cell of having modified expression of the at least one cucurbitacin biosynthetic pathway gene.

According to an aspect of some embodiments of the present invention the down-regulation is by genome editing.

According to an aspect of some embodiments of the present invention, there is provided a plant or plant cell modified to have reduced expression of at least one gene of the cucurbitacin biosynthetic pathway, wherein the plant or plant cell is obtainable according to the methods of the invention.

According to an aspect of some embodiments of the present invention the plant or plant cell of the invention is an elite plant or plant cell.

According to an aspect of some embodiments of the present invention the plant or plant cell is a hybrid plant or plant cell.

According to an aspect of some embodiments of the present invention the plant or plant cell is an inbred plant or plant cell.

According to an aspect of some embodiments of the present invention there is provided an inbred plant or plant cell having a nucleic acid sequence alteration of at least one gene of the cucurbitacin biosynthetic pathway.

According to an aspect of some embodiments of the present invention there is provided an elite plant or plant cell having a nucleic acid sequence alteration of at least one gene of the cucurbitacin biosynthetic pathway.

According to an aspect of some embodiments of the present invention there is provided a hybrid plant or plant cell having a nucleic acid sequence alteration of at least one gene of the cucurbitacin biosynthetic pathway.

According to an aspect of some embodiments of the present invention the plant or plant cell comprises at least one tetracyclic triterpene capable of glucosylation by a UDP-glucuronosyltransferase (UGT).

According to an aspect of some embodiments of the present invention the UGT is a plant UGT.

According to an aspect of some embodiments of the present invention the UGT is selected from the group consisting of *S. grosvenorii* UGTs selected from the group consisting of UGT74-345-2, UGT73-348-2, UGT94-289-1, UGT73-327-2, UGT73-251-5, UGT73-251-6, UGT75-281-2, UGT85-269-4, UGT85-269-1, UGT94-289-2 and UGT94-289-3.

According to an aspect of some embodiments of the present invention the UGT is a UGT having an amino acid sequence selected from the group consisting of SEQ ID NOs. 139, 140, 141, 142, 143, 144, 145, 146, 147, 148 and 149.

According to an aspect of some embodiments of the present invention the UGT is a UGT encoded by a polynucleotide having a nucleotide sequence selected from the group consisting of SEQ ID NOs. 128, 129, 130, 131, 132, 133, 135 and 137.

5 According to an aspect of some embodiments of the present invention there is provided an extract of the plant or plant cell of the invention, comprising at least one tetracyclic triterpene capable of glucosylation by a UGT.

According to an aspect of some embodiments of the present invention the plant or plant cell is of a bitter cucurbit species.

10 According to an aspect of some embodiments of the present invention the plant or plant cell is of a species naturally expressing the at least one cucurbitacin biosynthetic pathway gene.

According to an aspect of some embodiments of the present invention the plant or plant cell is of a species genetically modified to express said at least one cucurbitacin biosynthetic pathway gene.

15 According to an aspect of some embodiments of the present invention the plant or plant cell is an *Iberis amara* plant or plant cell.

According to an aspect of some embodiments of the present invention the plant is selected from the group consisting of cultivated bitter cucurbits and non-cultivated cucurbits.

According to an aspect of some embodiments of the present invention the cultivated bitter cucurbit is *Citrillus vulgaris* (Hawkesbury watermelon) or *Cucurbita pepo*.

20 According to an aspect of some embodiments of the present invention the non-cultivated bitter cucurbit is selected from the group consisting of non-cultivated bitter melon, non-cultivated bitter cucumber and non-cultivated bitter watermelon.

25 According to an aspect of some embodiments of the present invention the non-cultivated bitter cucurbit is selected from the group consisting of *Cucumis* species, *Citrullus* species, *Momordica* species and *Cucurbita* species.

According to an aspect of some embodiments of the present invention the at least one cucurbitacin biosynthetic pathway gene is a gene selected from the genes of Table 2.

According to an aspect of some embodiments of the present invention the at least one cucurbitacin biosynthetic pathway gene is a (2OG) and Fe(II)-dependent oxygenase gene.

30 According to an aspect of some embodiments of the present invention the (2OG) and Fe(II)-dependent oxygenase gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 64, 67 and 70.

According to an aspect of some embodiments of the present invention the down-regulation of the (2OG) and Fe(II)-dependent oxygenase gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 66, 69 and 72.

According to an aspect of some embodiments of the present invention the at least one
5 cucurbitacin biosynthetic pathway gene is a cytochrome P450 gene.

According to an aspect of some embodiments of the present invention the cytochrome p450 gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 43, 46, 49, 52, 55, 58 and 61.

According to an aspect of some embodiments of the present invention the cytochrome p450
10 gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 7, 28, 31, 34, 37 and 49.

According to an aspect of some embodiments of the present invention the down-regulation of the cytochrome p450 gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54,
15 57, 60 and 63.

According to an aspect of some embodiments of the present invention the down-regulation of the cytochrome p450 gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 9, 30, 33, 36, 39 and 51.

According to an aspect of some embodiments of the present invention the at least one
20 cucurbitacin biosynthetic pathway gene is an FAD-binding Berberine gene.

According to an aspect of some embodiments of the present invention the FAD-binding Berberine gene has the nucleic acid sequence as set forth in SEQ ID NO: 85.

According to an aspect of some embodiments of the present invention the down-regulation of the FAD-binding Berberine gene is effected by targeting the nucleic acid sequence selected as
25 set forth in SEQ ID NO: 87.

According to an aspect of some embodiments of the present invention the at least one cucurbitacin biosynthetic pathway gene is an NAD(P)-binding Rossmann-fold gene.

According to an aspect of some embodiments of the present invention the NAD(P)-binding Rossmann-fold gene has the nucleic acid sequence as set forth in SEQ ID NO: 88 or 91.

According to an aspect of some embodiments of the present invention the NAD(P)-binding
30 Rossmann-fold gene has the nucleic acid sequence as set forth in SEQ ID NO: 88.

According to an aspect of some embodiments of the present invention the down-regulation of the NAD(P)-binding Rossmann-fold gene is effected by targeting the nucleic acid sequence selected as set forth in SEQ ID NO: 91 or 94.

According to an aspect of some embodiments of the present invention the down-regulation of the NAD(P)-binding Rossmann-fold gene is effected by targeting the nucleic acid sequence selected as set forth in SEQ ID NO: 91.

According to an aspect of some embodiments of the present invention the at least one
5 cucurbitacin biosynthetic pathway gene is an HXXXX-type acyl-transferase-like protein gene.

According to an aspect of some embodiments of the present invention the HXXXX-type acyl-transferase-like protein gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 118, 121 and 124.

According to an aspect of some embodiments of the present invention the HXXXX-type
10 acyl-transferase-like protein gene has the nucleic acid sequence as set forth in SEQ ID NOs: 124.

According to an aspect of some embodiments of the present invention the down-regulation of the HXXXX-type acyl-transferase-like protein gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 120, 123 and 126.

According to an aspect of some embodiments of the present invention the down-regulation
15 of the HXXXX-type acyl-transferase-like protein gene is effected by targeting the nucleic acid sequence of SEQ ID NOs: 126.

According to an aspect of some embodiments of the present invention the at least one cucurbitacin biosynthetic pathway gene is a polyketide cyclase/hydrase gene.

According to an aspect of some embodiments of the present invention the polyketide
20 cyclase/hydrase gene has the nucleic acid sequence as set forth in SEQ ID NO: 109.

According to an aspect of some embodiments of the present invention the down-regulation of the polyketide cyclase/hydrase gene is effected by targeting the nucleic acid sequence selected as set forth in SEQ ID NO: 111.

According to an aspect of some embodiments of the present invention the at least one
25 cucurbitacin biosynthetic pathway gene is a proline dehydrogenase gene.

According to an aspect of some embodiments of the present invention the proline dehydrogenase gene has the nucleic acid sequence as set forth in SEQ ID NO: 112.

According to an aspect of some embodiments of the present invention the down-regulation of proline dehydrogenase gene is effected by targeting the nucleic acid sequence selected as set
30 forth in SEQ ID NO: 114.

According to an aspect of some embodiments of the present invention the at least one cucurbitacin biosynthetic pathway gene is a short-chain dehydrogenase/reductase gene.

According to an aspect of some embodiments of the present invention the short-chain dehydrogenase/reductase gene has the nucleic acid sequence as set forth in SEQ ID NO: 115.

According to an aspect of some embodiments of the present invention the down-regulation of the short-chain dehydrogenase/reductase gene is effected by targeting the nucleic acid sequence selected as set forth in SEQ ID NO: 117.

According to an aspect of some embodiments of the present invention the at least one
5 cucurbitacin biosynthetic pathway gene is an alcohol dehydrogenase gene.

According to an aspect of some embodiments of the present invention the alcohol dehydrogenase gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 73, 76, 79 and 82.

According to an aspect of some embodiments of the present invention the alcohol
10 dehydrogenase gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 73 and 79.

According to an aspect of some embodiments of the present invention the down-regulation of the alcohol dehydrogenase gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 75, 78, 81 and 84.

According to an aspect of some embodiments of the present invention the down-regulation
15 of the alcohol dehydrogenase gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 75 and 81.

According to an aspect of some embodiments of the present invention the at least one cucurbitacin biosynthetic pathway gene is a peroxidase gene.

According to an aspect of some embodiments of the present invention the peroxidase gene
20 has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 94, 97, 100, 103 and 106.

According to an aspect of some embodiments of the present invention the down-regulation of the peroxidase gene is effected by targeting a nucleic acid sequence selected from the group
25 consisting of SEQ ID NOs: 96, 99, 102, 105 and 108.

According to an aspect of some embodiments of the present invention there is provided a method of producing a glucosylated tetracyclic triterpene, comprising glucosylating the at least one tetracyclic triterpene capable of glucosylation of the invention by contacting the at least one tetracyclic triterpene with a UGT, thereby producing a glucosylated tetracyclic triterpene.

According to an aspect of some embodiments of the present invention, the glucosylated
30 tetracyclic triterpene is a non-bitter glucosylated tetracyclic triterpene.

According to an aspect of some embodiments of the present invention the glucosylated tetracyclic triterpene is a mogroside.

According to an aspect of some embodiments of the present invention the glucosylating is effected in a cell.

According to an aspect of some embodiments of the present invention the cell is a plant cell.

5 According to an aspect of some embodiments of the present invention the cell is not a plant cell.

According to an aspect of some embodiments of the present invention the glucosylating is effected in a cell-free system.

10 According to an aspect of some embodiments of the present invention the UGT is a plant UGT.

According to an aspect of some embodiments of the present invention the UGT is selected from the group consisting of *S. grosvenorii* UGTs selected from the group consisting of UGT74-345-2, UGT73-348-2, UGT94-289-1, UGT73-327-2, UGT73-251-5, UGT73-251-6, UGT75-281-2, UGT85-269-4, UGT85-269-1, UGT94-289-2 and UGT94-289-3.

15 According to an aspect of some embodiments of the present invention the UGT is a UGT having an amino acid sequence selected from the group consisting of SEQ ID NOs. 139, 140, 141, 142, 143, 144, 145, 146, 147, 148 and 149.

According to an aspect of some embodiments of the present invention the UGT is a UGT encoded by a polynucleotide having a nucleotide sequence selected from the group consisting of
20 SEQ ID NOs. 128, 129, 130, 131, 132, 133, 135 and 137.

According to an aspect of some embodiments of the present invention the UGT is a recombinant UGT.

According to an aspect of some embodiments of the present invention there is provided a composition comprising a plant or plant cell having modified cucurbitacin biosynthesis pathway
25 gene expression, the plant or plant cell comprising tetracyclic triterpene capable of glucosylation by a UGT.

According to an aspect of some embodiments, the composition is enriched in tetracyclic triterpene glucosides.

Unless otherwise defined, all technical and/or scientific terms used herein have the same
30 meaning as commonly understood by one of ordinary skill in the art to which the invention pertains. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of embodiments of the invention, exemplary methods and/or materials are described below. In case of conflict, the patent specification, including definitions, will control. In

addition, the materials, methods, and examples are illustrative only and are not intended to be necessarily limiting.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Some embodiments of the invention are herein described, by way of example only, with reference to the accompanying drawings. With specific reference now to the drawings in detail, it is stressed that the particulars shown are by way of example and for purposes of illustrative discussion of embodiments of the invention. In this regard, the description taken with the drawings makes apparent to those skilled in the art how embodiments of the invention may be practiced.

In the drawings:

FIG. 1 shows the base structure of cucurbitacins, and the specific modifications of the individual cucurbitacin variants;

FIG. 2 shows a structural diagram of an exemplary cucurbitacin (cucurbitacin E), showing targets (circled) for modification by down-regulation of genes of the cucurbitacin biosynthesis pathway;

FIG. 3 shows a structural diagram of mogrol, the aglycone of sweet mogrosides;

FIG. 4 shows a structural diagram of cucurbitacin E glycoside (1) and cucurbitacin I glycoside (2), glycosylated at C2;

FIG. 5 shows the divergent biosynthetic pathways of cucurbitacin and mogrol synthesis from cucurbitadienol, the common precursor of mogrol and cucurbitacins;

FIG. 6 shows the detailed biosynthetic pathway of mogrosides, from the squalene precursors to aglycone mogrol, and subsequent glycosylations of mogrol to sweet mogrosides IV and V (from Itkin et al, 2016);

FIGs. 7A-7D are chromatograms showing the results of silencing of expression of the C1G06g001610 gene in transgenic hairy roots, with the appearance of a new peak (arrows) of non-acetylated cucurbitacin I (7A). Figures 7B and 7C are mass spectrograms showing the new peak of putative non-acetylated C30H42O7. Figure 7D shows the native, C-25 acetylated cucurbitacin I, and the novel, deacetylated form of the cucurbitacin;

FIG. 8 is a schematic of the plasmid vectors for silencing of candidate genes, including C1G06g001600 coding for cucurbitadienol synthase, the first committed step in cucurbitane-type triterpenoid synthesis and C1G06g001610 (acetyltransferase) gene in the hairy roots;

FIGs. 9A-9B show the hairy root – tissue culture model of bitter mutant watermelon (9A), stained with Red Fluorescent Protein (RFP) (9B), a marker for transgenic cells/tissue;

FIG. 10 is a graph of cucurbitacin levels in transformed mutant bitter watermelon hairy root culture, without (WT) and with cucurbitadienol synthase gene silencing (CLCG06g001600). CLCG06g001600 encodes the first committed step in triterpenoid cucurbitane synthesis.);

FIG. 11 shows the fruit of bitter (upper) and non-bitter (lower) watermelon, cucurbitacin levels (microgram/gram fresh weight) and bHLH transcription factor CICG01G003370 expression levels (as FPKM) in anthesis stage fruit;

FIGs. 12A-12E are chromatograms showing the results of silencing of expression of the acyltransferase C1G06g001610 gene in transgenic hairy roots, with the appearance of a new peak which coelutes with non-acetylated cucurbitacin I (C30H42O7)(12A, arrow). Figures 12B-12D are mass spectrograms of cucurbitacin E (Fig. 12B), cucurbitacin I (Fig. 12C) and the modified cucurbitacin peak appearing with acetyltransferase silencing, coeluting with cucurbitacin I (Fig. 12D). Figure 12E shows the native, C-25 acetylated cucurbitacin E, and the deacetylated form of cucurbitacin I. The bottom chromatogram (Fig. 12A) shows the peaks of a mixture of purified cucurbitacin B, D, E and I;

FIGs. 13A-13H are chromatograms showing the results of silencing of expression of three dehydrogenase genes CICG01G018250 (13B), CICG03G002490 (13C) and CICG09G009760 (13D), in transgenic hairy roots, with the appearance of an intensified peak of a modified, non-dehydrogenated cucurbitacin (C32H46O8) coeluting with cucurbitacin B (13B-13D, arrow). Figures 13E-13H are mass spectrograms of cucurbitacin E (13E) and the spectrograms of the modified cucurbitacin peak appearing with dehydrogenase silencing in the hairy roots, showing the intensified peak of the non-dehydrogenated cucurbitacin coeluting with cucurbitacin B (C32H46O8);

FIGs. 14A-14L are chromatograms showing the results of silencing of expression of five cytochrome P450 genes CICG06G001590 (14B), CICG10G012530 (14C), CICG06G001580 (14D), CICG06G001620 (14E) and CICG01G014540 (14F), in transgenic hairy roots, with the appearance of modified cucurbitacins (C30H46O5, C30H46O4, C30H44O3 and C30H46O2). Figures 14G-14L are mass spectrograms of peaks 1, 2, 3, 4, 5 and 6 from the chromatographic separation, identified as modified cucurbitacin C30H46O4 (peak 1, 14G and peak 3, 14H), cucurbitacin C30H46O5 (peak 2, 14I), cucurbitacin C30H44O3 (peak 4, 14J and peak 5, 14K) and cucurbitacin C30H46O2 (peak 6, 14L);

FIGs. 15A-15F are chromatograms showing the results of overexpression of cytochrome P450 CICG06G001570 in yeast expressing the precursor cucurbitadienol (Cuc, C30H50O). Chromatograms 15A and 15B show that expression of CICG06G001570 (15A) results in production of hydroxylated cucurbitadienol products (peaks 1, and overlapping peaks 2 and 3).

Figs. 15C- 15E show the mass spectrograms of cucurbitadienol (15C) and the hydroxylated modified products C₃₀H₄₈O₃ (peak 1, 15D), C₃₀H₅₀O₂ (peak 2, 15E) and C₃₀H₄₈O₂ (peak 3, 15F).

5 DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to methods for modifying the cucurbitacin biosynthetic pathway in plant species, by down-regulating expression of cucurbitacin biosynthetic genes, to modified plant species having tetracyclic triterpenes capable of glucosylation by UDP-glucuronosyltransferase (UGT) to produce sweet rather than bitter cucurbitane secondary
10 metabolites, and to methods of producing glucosylated tetracyclic triterpenes by glucosylating the tetracyclic triterpenes from the modified plant species or extracts thereof.

Before explaining at least one embodiment of the invention in detail, it is to be understood that the invention is not necessarily limited in its application to the details set forth in the following description or exemplified by the Examples. The invention is capable of other embodiments or of
15 being practiced or carried out in various ways.

The bitter tetracyclic triterpene cucurbitacin and the sweet tetracyclic triterpene mogrosides have a common precursor, cucurbitadienol, the stereospecific product of the cyclization of the squalene epoxide molecule. Through multiple hydroxylations, acetylations and dehydrogenations, cucurbitadienol is transformed into the bitter cucurbitacins of bitter melons, cucumbers, squash and
20 other cucurbits, as well as cucurbitacin-producing non-cucurbits (such as *Iberis amara*) which presumably contribute to their defense against plant-eating wildlife. A different set of enzymatic reactions transform cucurbitadienol into mogrol, which, although also bitter, can be glucosylated by UGTs to the sweet mogrosides IV-V, which are currently popular sugar substitutes. However, extraction of mogrosides of consistent quality from the monkfruit (*Siraitia grosvenorii*) is
25 inefficient and costly.

Though the biosynthetic pathway for synthesis of mogrol and mogroside from cucurbitadienol is well defined, the enzymatic reactions responsible for the production of the cucurbitacins are poorly understood.

Whilst conceiving and reducing to practice embodiments of the invention, the present
30 inventors have identified target genes for modification of the cucurbitacin biosynthetic pathway in plants. By inhibiting expression of candidate enzymes in bitter plants (such as the bitter watermelon), analyzing the appearance of novel tetracyclic triterpenes, and the corresponding diminution of end-product cucurbitacins, the present inventors have begun to unravel the

component enzymatic reactions of cucurbitacin biosynthesis from cucurbitadienol (Examples 1 and 2) and redirect the pathway towards novel substrates for eventual glycosylation (Examples 3-6).

The inventors have identified candidate cucurbitacin biosynthetic pathway genes amenable to modification, and have shown that targeted silencing of some of these candidate genes results in actual modification of the profile of cucurbitacin and cucurbitacin derivatives in bitter species. Silencing of acetyltransferases in bitter species resulted a non-acetylated derivative of cucurbitacin E (see Example 4), silencing of dehydrogenases in bitter species resulted in a cucurbitacin derivative with additional protons (see Example 5) and silencing of cytochrome P450 genes in bitter species resulted in modified cucurbitacin derivatives with reduced oxidation (see Example 6).

It will be appreciated that the present teachings contemplate the production of cucurbitacin-producing plants having modified cucurbitacin and cucurbitacin derivatives, including cucurbitacin and cucurbitacin derivatives having reduced bitterness due to the modifications in the cucurbitacin biosynthetic pathways.

Concurrent reduction in bitterness of the modified bitter cucurbit and other cucurbitacin-producing plants, and identification of such tetracyclic triterpenoids capable of glucosylation to sweet rather than bitter molecules, can provide novel sources of useful non-bitter plants and plant products, as well as previously unattainable methods for synthesis of tetracyclic triterpene-based molecules having useful industrial and pharmacological properties, including sweet glucosylated tetracyclic triterpenes.

Thus, according to one aspect of the present invention there is provided a method for producing a plant or plant cell with a modified cucurbitacin content, the method comprising down-regulating expression of at least one cucurbitacin biosynthetic pathway gene in the plant or plant cell, thereby modifying cucurbitacin expression in the plant or plant cell.

Definitions

So that the invention may be more readily understood, certain terms are first defined.

As used herein, the term "plant" refers to an entire plant, its organs (i.e., leaves, stems, roots, flowers etc.), seeds, plant cells, and progeny of the same. The term "plant cell" includes without limitation cells within seeds, suspension cultures, embryos, meristematic regions, callus tissue, leaves, shoots, gametophytes, sporophytes, pollen, and microspores. According to a specific embodiment, the plant is a plant line.

According to a specific embodiment the plant or plant cell is an elite plant or plant cell.

According to a specific embodiment the plant or plant cell is a hybrid plant or plant cell.

According to a specific embodiment the plant or plant cell is an inbred plant or plant cell. As used herein, the term “inbred” refers to a relatively true-breeding strain resulting from at least five successive generations of controlled self-fertilization or of backcrossing to a recurrent parent with selection, or its equivalent, for specific characteristics.

5 The phrase “plant part” refers to a part of a plant, including single cells and cell tissues such as plant cells that are intact in plants, cell clumps, and tissue cultures from which plants can be regenerated. Examples of plant parts include, but are not limited to, single cells and tissues from pollen, ovules, leaves, embryos, roots, root tips, anthers, flowers, fruits, stems, shoots, and seeds; as well as scions, rootstocks, protoplasts, calli, and the like.

10 The present invention also envisions modification of hairy root culture of the cucurbit or other cucurbitacin-producing plant(s), both for screening and production of components of the cucurbitacin biosynthetic pathway.

Any technique able to induce the formation of hairy roots in a plant can be used in the invention.

15 Hairy roots is a type of proliferating root that emerges at the wounding site of a plant, following an infection caused by *Agrobacterium rhizogenes*, a gram negative soil bacterium. The hairy root phenotype is characterized by fast hormone-independent growth, lateral branching, genetic stability and lack of geotropism.

It should be noted that *Agrobacterium rhizogenes* has been renamed *Rhizobium rhizogenes* following taxonomic changes to the genus *Agrobacterium* and the family of the *Rhizobiaceae*. *Rhizobium rhizogenes* can also be identified by the name *Agrobacterium rhizogenes*.

20 Hairy roots was first identified as a disease in select plants caused by *Rhizobium rhizogenes*, which can be isolated from the soil. The gram-negative bacterium transfers DNA from its root-inducing (Ri) plasmid into the genome of the infected plant cell which results in the formation of roots. In particular, the rol genes containing genes rolA, rolB and rolC genes (F. F. White et al., 1983) are present in the T-DNA of *Rhizobium rhizogenes* Ri plasmid and expression of these genes induce the formation of hairy roots.

25 In a particular embodiment, formation of hairy roots is induced by transforming the plant with a bacterial strain comprising the rol genes, wherein the bacterial strain is able to infect the plant.

30 The expression “rol genes” as used herein has its general meaning in the art. It refers to the group of bacterial genes which are capable of inducing the formation of hairy roots (Schmiilling et al, 1988; Bulgakov et al, 2008). Typically, the rol genes are harbored by a plasmid such as a pRi plasmid.

In a particular embodiment, the bacterial strain naturally comprises rol genes in its genome, or is modified by introduction of heterologous rol genes.

In a particular embodiment, the bacterial strain belongs to the *Rhizobium* genus.

In a preferred embodiment, a strain of *Rhizobium rhizogenes* is used.

5 Several strains of *Rhizobium rhizogenes* can be used for carrying out the invention. Suitable strains include, but are not limited to, the *Rhizobium rhizogenes* strain TR7, also known as ATCC 25818 and the strains LBA 9402, A4T, A4, LBA1334, ATCC 11325, ATCC 15834, LMG 155, HRI, TR105, ATCC 39207, R1000, LBA 9422, strain 1072, BL311, R1600, R1601, C58C1, A4RS, MSU440, ARqual, 8194, TR101, 2659, LBA8490, NIAES1724, C8 (MAFF03-10268) and DC-
10 AR2.

In a particular embodiment, said strain of *Rhizobium rhizogenes* is the strain K599 or GV3101.

In specific embodiments, the strain of *Rhizobium rhizogenes* is the strain K599.

In another embodiment, a strain of *Agrobacterium tumefaciens* is used. In a particular
15 embodiment, the strain of *Agrobacterium tumefaciens* that is used has been modified in order to introduce in its genome the rol genes. In a particular embodiment, *Agrobacterium tumefaciens* has been modified by transformation with a pRi plasmid comprising the rol genes.

In another embodiment, the strain of *Agrobacterium tumefaciens* that is used does not comprise the rol genes. In this embodiment, transforming the plant with *Agrobacterium*
20 *tumefaciens* induces the formation of callus tissues. Said callus tissues are then differentiated in hairy roots following the addition of one or more hormonal substance(s). In a particular embodiment said hormonal substance is a hormone of the auxin family such as 1-Naphthaleneacetic acid (NAA), Indole-3-acetic acid (IAA) or Indole-3-butyric acid (IBA).

Several strains of *Agrobacterium tumefaciens* can be used for carrying out the invention.
25 Suitable strains include, but are not limited to, *A. tumefaciens* C58, C58C1, LBA4404, GV2260, GV3100, A136, GV3101, GV3850, EHA101, EHA105, AGL-1.

Transformation by the bacterial strain such as *Rhizobium rhizogenes* and/or *Agrobacterium tumefaciens* is a known technique in the art. The skilled person is familiar with the different techniques commonly employed for carrying out said transformation step. According to the plant
30 species to be transformed, different parts of the plant can be used for the infection. Such plant parts can include, for example and without limitation, seed, plant stem, leaves, petiole, cotyledonary node, hypocotyl, or other plant parts or cells.

Typically, infection by *Rhizobium rhizogenes* and/or *Agrobacterium tumefaciens* is carried out by applying a *Rhizobium rhizogenes* and/or *Agrobacterium tumefaciens* inoculum to plant tissues which has been previously wounded.

In specific embodiments, the hairy root culture is watermelon hairy root culture, established using decoated watermelon (*Citrullus lanatus*) seeds. Seeds are sterilized (e.g. by hypochlorate), rinsed, dried and incubated on plates with ½ Murashige and Skoog media (MS) agar supplemented with 1.5% sucrose for 10 days.

Rhizobium rhizogenes strain K599 transformed with the relevant construct by electroporation is grown and transformants selected, grown in liquid medium, and then used to transform watermelon cotyledons by bruising with a sterile syringe needle dipped in the *R. rhizogenes* suspension. Inoculated cotyledons are grown on agar, and transformed hairy roots emerging after three weeks of tissue culture are excised from the cotyledons and subcultured every two weeks.

According to a specific embodiment, the plant, plant part or plant cell has modified cucurbitacin content. As used herein, the term “modified cucurbitacin content” refers to alteration(s) in the identity and amounts of cucurbitacins in the plant, plant part or plant cell, relative to the cucurbitacin profile of the same plant, plant part or plant cell without down-regulation of the cucurbitacin biosynthetic pathway gene(s) of the invention. It will be understood that “modified cucurbitacin content” can also include the term “modified cucurbitacin expression”, inasmuch as cucurbitacin is the product of catalytic activity of the cucurbitacin biosynthesis pathway enzymes.

According to a further embodiment, the plant, plant part or plant cell has a nucleic acid alteration in at least one gene of the cucurbitacin biosynthetic pathway.

As used herein, the phrase “nucleic acid alteration” refers to any mutation in the DNA sequence of a plant (e.g., of a plant cell) which can result in downregulation of the expression level and/or activity of isolated polypeptide of some embodiments of the invention (e.g., the polypeptide encoded by the isolated polynucleotide of the invention). Non-limiting examples of such nucleic acid alteration include a missense mutation, *i.e.*, a mutation which changes an amino acid residue in the protein with another amino acid residue and thereby abolishes the enzymatic activity of the protein; a nonsense mutation, *i.e.*, a mutation which introduces a stop codon in a protein, e.g., an early stop codon which results in a shorter protein devoid of the enzymatic activity; a frameshift mutation, *i.e.*, a mutation, usually, deletion or insertion of nucleic acid(s) which changes the reading frame of the protein, and may result in an early termination by introducing a stop codon into a reading frame (e.g., a truncated protein, devoid of the enzymatic activity), or in a longer amino acid

sequence (e.g., a readthrough protein) which affects the secondary or tertiary structure of the protein and results in a non-functional protein, devoid of the enzymatic activity of the “wild-type” or non-mutated polypeptide; a readthrough mutation due to a frameshift mutation or a modified stop codon mutation (*i.e.*, when the stop codon is mutated into an amino acid codon), with an abolished enzymatic activity; a promoter mutation, *i.e.*, a mutation in a promoter sequence, usually 5' to the transcription start site of a gene, which results in down-regulation of a specific gene product; a regulatory mutation, *i.e.*, a mutation in a region upstream or downstream, or within a gene, which affects the expression of the gene product; a deletion mutation, *i.e.*, a mutation which deletes coding nucleic acids in a gene sequence and which may result in a frameshift mutation or an in-frame mutation (within the coding sequence, deletion of one or more amino acid codons); an insertion mutation, *i.e.*, a mutation which inserts coding or non-coding nucleic acids into a gene sequence, and which may result in a frameshift mutation or an in-frame insertion of one or more amino acid codons; an inversion, *i.e.*, a mutation which results in an inverted coding or non-coding sequence; a splice mutation (*i.e.*, a mutation which results in abnormal splicing or poor splicing); and a duplication mutation, *i.e.*, a mutation which results in a duplicated coding or non-coding sequence, which can be in-frame or can cause a frameshift.

According to a specific embodiment, the plant part is a seed. According to a specific embodiment, the plant part is a hybrid seed.

As used herein, the phrases “progeny plant” refers to any plant resulting as progeny from a vegetative or sexual reproduction from one or more parent plants or descendants thereof. For instance, a progeny plant can be obtained by cloning or selfing of a parent plant or by crossing two parental plants and include selfings as well as the F₁ or F₂ or still further generations. An F₁ is a first-generation progeny produced from parents at least one of which is used for the first time as donor of a trait, while progeny of second generation (F₂) or subsequent generations (F₃, F₄, and the like) are specimens produced from selfings, intercrosses, backcrosses, or other crosses of F₁s, F₂s, and the like. An F₁ can thus be (and in some embodiments is) a hybrid resulting from a cross between two true breeding parents (*i.e.*, parents that are true-breeding are each homozygous for a trait of interest or an allele thereof, e.g., in this case male sterile having long stigma as described herein and a restorer line), while an F₂ can be (and in some embodiments is) a progeny resulting from self-pollination of the F₁ hybrids.

As used herein “cultivated” refers to a plant species (e.g. a cucurbitacin-producing species, a cucurbit species, a non-cucurbit species producing cucurbitacin) that has undergone a process of domestication and is therefore endowed with agriculturally desirable characteristics, e.g., higher yield, resistance to biotic/abiotic stress, reproducibility, etc. As used herein, the term “cultivated”

refers to plants whose origin or selection is primarily due to intentional human activity, whereas “non-cultivated” are plants growing without the intervention of intentional human activity. Some non-limiting examples of human activity contributing to cultivation of plants includes tilling and preparing the soil, planting and nurturing the seeds or plants, selecting and breeding plants, pollenating and grafting the plants. In specific embodiments, producing the plant or plant cell with a modified cucurbitacin content comprises growing a plant or plant cell with the modified cucurbit content. As used to herein, the term “growing” refers to the cultivation of the plant or plant cells, including but not limited to planting, nurturing, selecting and breeding the plant or plant cells.

As used herein the term “cucurbit” refers to any member of the Cucurbitaceae family, which cultivated genera and species include but are not limited to gourds (e.g. snake, wax, yellow-flowered gourds), cucumbers (e.g. bur, musk, cucumbers, gherkins), bryony, colocynth, watermelon, melon, zucchini, squash, calabazilla and loofa.

According to a specific embodiment, the plant is of the Cucurbitaceae family. Exemplary species are provided below.

15 **Subfamily Zanonioideae** (small striate pollen grains)

- Tribe Zanonieae
 - Subtribe Fevilleinae: *Fevillea*
 - Subtribe Zanoninae: *Alsomitra Zanonina Siolmatra Gerrardanthus Zygosicyos Xerosicyos Nealsomitra*
 - 20○ Subtribe Gomphogyninae: *Hemsleya Gomphogyne Gynostemma*
 - Subtribe Actinostemmatinae: *Bolbostemma Actinostemma*
 - Subtribe Sicydiinae: *Sicydium Chalema Pteropepon Pseudosicydium Cyclantheropsis*

Subfamily Cucurbitoideae (styles united into a single column)

- Tribe Melothriaceae
 - 25○ Subtribe Dendrosicyinae: *Kedrostis Dendrosicyos Corallocarpus Ibervillea Tumamoca Halosicyos Ceratosanthes Doyerea Trochomeriopsis Seyrigia Dieterlea Cucurbitella Apodanthera Guraniopsis Melothrianthus Wilbrandia*
 - Subtribe Guraniinae: *Helmontia Psiguria Gurania*
 - Subtribe Cucumerinae: *Melancium Cucumeropsis Posadaea Melothria Muellarargia Zehneria*
 - 30 *Cucumis* (including: *Mukia, Dicaelospermum, Cucumella, Oreosyce, and Myrmecosicyos*).
 - Subtribe Trochomeriinae: *Solena Trochomeria Dactyliandra Ctenolepsis*
 - Tribe Schizopeponae: *Schizopepon*
 - Tribe Joliffieae
 - Subtribe Thladianthinae: *Indofevillea Siraitia Thladiantha Momordica*

- Subtribe Telfairiinae: *Telfaria*
- Tribe Trichosantheae
- Subtribe Hodgsoniinae: *Hodgsonia*
- Subtribe Ampelosicyinae: *Ampelosicyos Peponium*
- 50 ○ Subtribe Trichosanthisae: *Gymnopetalum Trichosanthes Tricyclandra*
- Subtribe Herpetosperminae: *Cephalopentandra Biswarea Herpetospermum Edgaria*
- Tribe Benincaseae
- Subtribe Benincasinae: *Cogniauxia Ruthalicia Lagenaria Benincasa Praecitrullus Citrullus Acanthosicyos Eureiandra Bambekea Nothoalsomitra Coccinia Diplocyclos Raphidiocystis*
- 10 *Lemurosicyos Zombitsia Ecballium Bryonia*
- Subtribe Luffinae: *Luffa*
- Tribe Cucurbiteae (pantoporate, spiny pollen): *Cucurbita Sicana Tecunumania Calycophysum Peponopsis Anacaona Polyclathra Schizocarpum Penelopeia Cionosicyos Cayaponia Selysia Abobra*
- 15 • Tribe Sicyeae (trichomatous nectary, 4- to 10-colporate pollen grains)
- Subtribe Cyclantherinae: *Hanburia Echinopepon Marah Echinocystis Vaseyanthus Brandegea Apatzingania Cremastopus Elateriopsis Pseudocyclanthera Cyclanthera Rytidostylis*
- Subtribe Sicyinae: *Sicyos Sicyosperma Parasicyos Microsechium Sechium Sechiopsis Pterosicyos*
- incertae sedis: *Odosicyos*
- 20 **Alphabetical list of genera:** *Abobra Acanthosicyos Actinostemma Alsomitra Ampelosycios Anacaona Apatzingania Apodanthera Bambekea Benincasa Biswarea Bolbostemma Brandegea Bryonia Calycophysum Cayaponia Cephalopentandra Ceratosanthes Chalema Cionosicyos Citrullus Coccinia Cogniauxia Corallocarpus Cremastopus Ctenolepis Cucumella Cucumeropsis Cucumis Cucurbita Cucurbitella Cyclanthera Dactyliandra Dendrosicyos Dicaelospermum*
- 25 *Dieterlea Diplocyclos Doyerea Ecballium Echinocystis Echinopepon Edgaria Elateriopsis Eureiandra Fevillea Gerrardanthus Gomphogyne Gurania Guraniopsis Gymnopetalum Gynostemma Halosicyos Hanburia Helmontia Hemsleya Herpetospermum Hodgsonia Ibervillea Indofevillea Kedrostis Lagenaria Lemurosicyos Luffa Marah Melancium Melothria Melothrianthus Microsechium Momordica Muellerargia Mukia Myrmecosicyos Neoalsomitra*
- 30 *Nothoalsomitra Odosicyos Oreosyce Parasicyos Penelopeia Peponium Peponopsis Polyclathra Posadaea Praecitrullus Pseudocyclanthera Pseudosicydium Psiguria Pteropepon Pterosicyos Raphidiocystis Ruthalicia Rytidostylis Schizocarpum Schizopepon Sechiopsis Sechium Selysia Seyrigia Sicana Sicydium Sicyos Sicyosperma Siolmatra Siraitia Solena Tecunumania Telfairia*

Thladiantha Trichosanthes Tricyclandra Trochomeria Trochomeriopsis Tumacoca Vaseyanthus Wilbrandia Xerosicyos Zanonia Zehneria Zombitsia Zygosicyos.

Cucurbita genus refers to genus in the gourd family Cucurbitaceae native to and originally cultivated in the Andes and Mesoamerica. The Cucurbita species may be domesticated or non-domesticated.

Exemplary species include, but are not limited to:

- *C. argyrosperma* (synonym *C. mixta*) – pipian, cushaw pumpkin; origin-Panama, Mexico
- *C. kellyana*, origin-Pacific coast of western Mexico
- *C. palmeri*, origin-Pacific coast of northwestern Mexico
- 10○ *C. sororia*, origin-Pacific coast Mexico to Nicaragua, northeastern Mexico
- *C. digitata* – fingerleaf gourd; origin-southwestern USA, northwestern Mexico
- *C. californica*
- *C. cordata*
- *C. cylindrata*
- 15○ *C. palmata*
- *C. ecuadorensis*, origin-Ecuador's Pacific coast
- *C. ficifolia* – figleaf gourd, chilacayote; origin-Mexico, Panama, northern Chile and Argentina
- *C. foetidissima* – stinking gourd, buffalo gourd; origin-Mexico
- *C. scabridifolia*, likely a natural hybrid of *C. foetidissima* and *C. pedatifolia*
- 20• *C. galeottii* is little known; origin-Oaxaca, Mexico
- *C. lundelliana*, origin-Mexico, Guatemala, Belize
- *C. maxima* – winter squash, pumpkin; origin-Argentina, Bolivia, Ecuador
- *C. andreana*, origin-Argentina
- *C. moschata* – butternut squash, 'Dickinson' pumpkin, golden cushaw; origin-Bolivia, Colombia,
- 25 Ecuador, Mexico, Panama, Puerto Rico, Venezuela
- *C. okeechobeensis*, origin-Florida
- *C. martinezii*, origin-Mexican Gulf Coast and foothills
- *C. pedatifolia*, origin-Querétaro, Mexico
- *C. moorei*
- 30• *C. pepo* – field pumpkin, summer squash, zucchini, vegetable marrow, courgette, acorn squash; origin-Mexico, USA
- *C. fraterna*, origin-Tamaulipas and Nuevo León, Mexico
- *C. texana*, origin-Texas, USA
- *C. radicans* – calabacilla, calabaza de coyote; origin-Central Mexico

o *C. gracilior*

The *Cucumis* genus refers to twining, tendril-bearing plants which include the cucumber (*Cucumis sativus*), true melons (*Cucumis melo*), the horned melon (*Cucumis metuliferus*) and the West Indian gherkin (*Cucumis anguria*). The *Cucumis* species may be wild or domesticated.

5 Exemplary *Cucumis* species include, but are not limited to:

- *Cucumis aculeatus* Cogn.
- *Cucumis aetheocarpus*
- *Cucumis africanus* L.f.
- *Cucumis althaeoides* (Ser.)
- 10 • *Cucumis anguria* L.
- *Cucumis argenteus* (Domin)
- *Cucumis asper* Cogn.
- *Cucumis baladensis* Thulin
- *Cucumis bryoniifolius* (Merxm.)
- 15 • *Cucumis canoxyi* Thulin
- *Cucumis carolinus* J.H.Kirkbr.
- *Cucumis cinereus* (Cogn.)
- *Cucumis clavipetiolatus*
- *Cucumis costatus*
- 20 • *Cucumis debilis*
- *Cucumis dipsaceus*
- *Cucumis engleri*
- *Cucumis ficifolius*
- *Cucumis globosus*
- 25 • *Cucumis gracilis*
- *Cucumis hastatus*
- *Cucumis heptadactylus*
- *Cucumis hirsutus* Sond.
- *Cucumis humifructus* Stent (as *Cucumis humofructus*)
- 30 • *Cucumis hystrix*
- *Cucumis indicus*
- *Cucumis insignis*
- *Cucumis javanicus*
- *Cucumis jeffreyanus*

- *Cucumis kalahariensis*
- *Cucumis kelleri* (Cogn.)
- *Cucumis kirkbridei*
- *Cucumis leiospermus*
- 5 • *Cucumis maderaspatanus* L.
- *Cucumis meeusei*
- *Cucumis melo* L.
- *Cucumis messorius* (C.Jeffrey)
- *Cucumis metuliferus* E.Mey. ex Naudin
- 10 • *Cucumis myriocarpus* Naudin
- *Cucumis omissus* Thulin
- *Cucumis picrocarpus* F.Muell.
- *Cucumis prophetarum* L.
- *Cucumis pubituberculatus* Thulin
- 15 • *Cucumis pustulatus* Naudin ex Hook.f.
- *Cucumis queenslandicus* I.Telford
- *Cucumis quintanilhae* R.Fern. & A.Fern.
- *Cucumis reticulatus* (A.Fern. & R.Fern.)
- *Cucumis rigidus* E.Mey. ex Sond.
- 20 • *Cucumis ritchiei* (C.B.Clarke)
- *Cucumis rostratus* J.H.Kirkbr.
- *Cucumis rumphianus* (Scheff.)
- *Cucumis saclexii* Paill. & Bois
- *Cucumis sagittatus* Wawra & Peyr.
- 25 • *Cucumis sativus* L., cucumber
- *Cucumis setosus* Cogn.
- *Cucumis silentvalleyi*
- *Cucumis thulinianus*
- *Cucumis umbellatus*
- 30 • *Cucumis variabilis*
- *Cucumis zambianus*
- *Cucumis zeyheri* So

The *Citrullus* genus refers to several species of desert vines which include the watermelon (*Citrullus lanatus*). The *Citrullus* species may be wild or domesticated.

Exemplary *Citrullus* species and sub-species include, but are not limited to:

- *Citrullus amarus* Schrad. – citron melon
- 5 • *Citrullus colocynthis* (L.) Schrad. – colocynth
- *Citrullus ecirrhosus* Cogn. – tendrill-less melon
- *Citrullus lanatus* – desert watermelon
- *Citrullus lanatus* subsp. *vulgaris* var. *cordophanus* (Ter-Avan.) Fursa
- *Citrullus lanatus* var. *lanatus*
- 10 • *Citrullus mucospermus* (Fursa) Fursa – egusi melon
- *Citrullus naudinianus* (Sond.)
- *Citrullus rehmanii*

According to a specific embodiment the cucurbit is a cultivated cucurbit.

- 15 According to a further embodiment the cucurbit is a bitter cucurbit, and, in specific embodiments, a bitter cultivated cucurbit. As used herein, a bitter cucurbit is a cucurbit species in which the cucurbitacin biosynthetic pathway is operative, producing cucurbitacin. The amount of cucurbitacins, and the degree of bitterness can vary from species to species.

- 20 Cucurbits accumulating cucurbitacins may be bitter, and commonly non-bitter cucurbits may accumulate cucurbitacins as a result of hybrid formation, stress, adverse growing conditions or insect or other pest infestations.

It will be appreciated that the bitter cucurbit may be a product of an interspecific cross within genera.

- 25 In particular embodiments, the “cultivated cucurbit” refers to bitter cultivars of watermelon, including the bitter Hawkesbury watermelon available via USDA PI673137 or PI274035 and cultivated as a source of bitter cucurbitacins (Chambliss, O.L. and Jones, C.M., 1966. Cucurbitacins: specific insect attractants in Cucurbitaceae. *Science*, 153(3742), pp.1392-1393.

- 30 In particular embodiments, the “cultivated cucurbit” refers to the bitter melon (*Momordica charantia* L.). As used herein, the term bitter melon is interchangeable with bitter gourd, bitter squash, Goya melon, karela and balsam pear.

In other embodiments, the cultivated cucurbit plant is *Citrullus vulgaris* (a bitter form of Hawkesbury watermelon) or *Cucurbita pepo* (pumpkin) or *Cucumis sativus* (cucumber) or

Cucumis melo (melon). In some embodiments, the bitter cucurbit may be the product of an interspecific cross within genera.

In some embodiments, the non-cultivated cucurbit is selected from the group consisting of non-cultivated bitter melon, non-cultivated bitter cucumber, and non-cultivated bitter watermelon.

- 5 In other embodiments, the non-cultivated bitter cucurbit can be at least one of a Cucumis species, Citrullus species, Momordica species and Cucurbita species.

In some embodiments, the cultivated cucurbit is not a naturally mogroside-accumulating cucurbit, for example, not *Siraitia grosvenorii* (Luo Han Guo, monkfruit).

- 10 Although cucurbitacins were originally isolated from Cucurbitaceae, they were also later detected in plants of the families Brassicaceae, Scrophulariaceae, Begoniaceae, Elaeocarpaceae, Datisceae, Desfontainiaceae, Polemoniaceae, Primulaceae, Rubiaceae, Sterculiaceae, Rosaceae and Thymelaeaceae, in several genera of mushroom, including Russula and Hebeloma, and even in shell-less marine mollusks (doris nudibranchs) (Chen et al, Natural Product Reports 2005 22:386-399). For an extensive listing of all the cucurbitacin-producing species, and their respective
15 cucurbitacins, see Miro et al, Phytotherapy Research, 1995. Thus, in other embodiments, the plant is a non-cucurbit plant naturally producing cucurbitacin. In specific embodiments, the non-cucurbit plants producing cucurbitacin is selected from the group consisting of plants of the families Brassicaceae, Scrophulariaceae, Begoniaceae, Elaeocarpaceae, Datisceae, Desfontainiaceae, Polemoniaceae, Primulaceae, Rubiaceae, Sterculiaceae, Rosaceae and Thymelaeaceae.

- 20 It will be appreciated that the present invention also envisions plants modified to produce cucurbitacins, or other cucurbitacin-like (non-sweet) tetracyclic triterpenes, which tetracyclic triterpenes may be modified to be capable of glucosylation by the methods of the invention.

Within each subspecies and type, there are many cultivars, each favored for particular purposes or regions.

- 25 The term “crossed” or “cross” in the context of this invention means the fusion of gametes via pollination to produce progeny (i.e., cells, seeds or plants). The term encompasses both sexual crosses (the pollination of one plant by another) and selfing (self-pollination, i.e., when the pollen and ovule are from the same plant or from genetically identical plants).

- “Backcrossing” is a process in which a breeder repeatedly crosses hybrid progeny back to
30 one of the parents, for example, crossing a first-generation hybrid F₁ with one of the parental genotypes of the F₁ hybrid. The parent to which the hybrid is backcrossed is the “recurrent parent.”

Marker assisted selection may be used to augment or replace the phenotypic selection.

As used herein, “outcross” and “outcrossing” refers to cross-pollinations with a plant of differing genetic constitution, as opposed to self-pollination i.e., selfing. Preferably, the two plants

are of a same species, sub-species, e.g., bitter melon, e.g., cultivated bitter melon of the same subspecies. However, intercrossing between different plant species is also contemplated.

As used herein the term “heterosis” refers to hybrid vigor, or outbreeding enhancement, that is the improved or increased function of any biological quality in a hybrid offspring. An offspring exhibits heterosis if its traits are enhanced as a result of mixing the genetic contributions of its parents.

“Yield” describes the amount of fruit produced by a plant or a group, or crop, of plants. Yield can be measured in several ways, e.g. t ha^{-1} , and average fruit yield per plant in grams.

As used herein, “introgression” means the movement of one or more genes, or a group of genes, from one plant variety into the gene complex of another as a result of breeding methods (e.g. outcrossing). Introgression also refers to movement of a trait encoded by one or more genes, or a group of genes, from one plant variety into the another.

“Converted” refers to a plant that has been introgressed with a trait of another plant.

A plant having “essentially all the physiological and morphological characteristics” of a specified plant refers to a plant having the same general physiological and morphological characteristics, except for those characteristics derived from a particular converted gene or group of genes (e.g., long stigma).

The term “associated with” or “associated” in the context of this invention refers to, for example, a QTL and a phenotypic trait (e.g., bitterness), that are in linkage disequilibrium, i.e., the QTL and the trait are found together in progeny plants more often than if the nucleic acid and phenotype segregated independently.

The term “marker” or “molecular marker” or “genetic marker” refers to a genetic locus (a “marker locus”) used as a point of reference when identifying genetically linked loci such as a QTL.

A “probe” is an isolated nucleic acid to which is attached a conventional detectable label or reporter molecule, e.g., a radioactive isotope, ligand, chemiluminescent agent, or enzyme. Such a probe is complementary to a strand of a target nucleic acid, in the case of the present invention, to a strand of genomic DNA of a cucurbitacin biosynthetic pathway gene, whether from a plant of interest (e.g. cucurbit or other cucurbitacin-producing plant) or from a sample that includes DNA from the plant. Probes according to the present invention include not only deoxyribonucleic or ribonucleic acids but also polyamides and other probe materials that bind specifically to a target DNA sequence and can be used to detect the presence of that target DNA sequence.

“Primers” are isolated nucleic acids that are annealed to a complementary target DNA strand by nucleic acid hybridization to form a hybrid between the primer and the target DNA strand,

then extended along the target DNA strand by a polymerase, e.g., a DNA polymerase. Primer pairs of the present invention refer to their use for amplification of a target nucleic acid sequence, e.g., by the polymerase chain reaction (PCR) or other conventional nucleic-acid amplification methods.

Probes and primers are generally 11 nucleotides or more in length, preferably 18
5 nucleotides or more, more preferably 24 nucleotides or more, and most preferably 30 nucleotides or more. Such probes and primers hybridize specifically to a target sequence under high stringency hybridization conditions. According to some embodiment, probes and primers according to the present invention have complete sequence similarity with the target sequence, although probes differing from the target sequence and that retain the ability to hybridize to target sequences may
10 be designed by conventional methods.

Methods for preparing and using probes and primers are described, for example, in Molecular Cloning: A Laboratory Manual, 2nd ed., vol. 1-3, ed. Sambrook et al., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1989 (hereinafter, "Sambrook et al., 1989"); Current Protocols in Molecular Biology, ed. Ausubel et al., Greene Publishing and Wiley-
15 Interscience, New York, 1992 (with periodic updates) (hereinafter, "Ausubel et al., 1992"); and Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press: San Diego, 1990. PCR-primer pairs can be derived from a known sequence, for example, by using computer programs intended for that purpose such as Primer (Version 0.5, .COPYRGT. 1991, Whitehead Institute for Biomedical Research, Cambridge, Mass.).

20 The term "specific for (a target sequence)" indicates that a probe or primer hybridizes under stringent hybridization conditions only to the target sequence in a sample comprising the target sequence.

As used herein, "amplified DNA" or "amplicon" refers to the product of nucleic-acid amplification of a target nucleic acid sequence that is part of a nucleic acid template.

25 As used herein the term "polynucleotide" refers to a single or double stranded nucleic acid sequence which is isolated and provided in the form of an RNA sequence, a complementary polynucleotide sequence (cDNA), a genomic polynucleotide sequence and/or a composite polynucleotide sequence (e.g., a combination of the above).

The term "isolated" refers to at least partially separated from the natural environment e.g.,
30 from a plant part or plant cell.

As used herein "homologous" or "orthologous" sequences refer to naturally occurring or synthetic nucleic acid sequences (or polypeptides encoded thereby) which comprise at least the functional portion of the polynucleotides/polypeptides of the invention, and are capable of imparting a plant with modified cucurbitacin content.

Such homologues or orthologues can be, for example, at least 80 %, at least 81 %, at least 82 %, at least 83 %, at least 84 %, at least 85 %, at least 86 %, at least 87 %, at least 88 %, at least 89 %, at least 90 %, at least 91 %, at least 92 %, at least 93 %, at least 94 %, at least 95 %, at least 96 %, at least 97 %, at least 98 %, at least 99 % or 100 % identical to the nucleotide sequences of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 33, 35, 37, 39, 41, 43, 45, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, 77, 79, 81 or 83, or the amino acid sequences SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 34, 36, 38, 40, 42, 44, 46, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, 78, 80, 82 or 84 encoded thereby , as determined using the BestFit software of the Wisconsin sequence analysis package, utilizing the Smith and Waterman algorithm and default parameters.

As used herein, the term cucurbitacin refers to any one of a class of triterpene compounds produced by some plants – inter alia, members of the pumpkin and gourd family, Cucurbitaceae – and which function as a defense against herbivores. Cucurbitacins are chemically classified as triterpenes, formally derived from cucurbitane, a triterpene hydrocarbon – specifically, from the unsaturated variant cucurbit-5-ene, or 19(10→9β)-abeo-10α-lanost-5-ene. Most of the cucurbitacins are tetracyclic triterpenes, but some (e.g. cucurbitacin S and T) have an extra ring from cyclization between C-16 and C-24. In some embodiments, the cucurbitacins are cucurbitacin glycosides. Cucurbitacins differ in the number and location of their keto-, hydroxyl- and acetyl-groups.

A non-limiting list of cucurbitacins includes cucurbitacin A-L and O-S, and their glycoside derivatives. Table 1 shows a non-limiting list of cucurbitacins and their chemical formulae, and the Table of Figure 1 provides the details of the differences in keto-, hydroxyl- and acetyl-groups between the different cucurbitacins A-L and O-S.

Table 1:

Cucurbitacin	Formula
A	C ₃₂ H ₄₆ O ₉
B	C ₃₂ H ₄₆ O ₈
C	C ₃₂ H ₄₈ O ₈
D	C ₃₀ H ₄₄ O ₇
E	C ₃₂ H ₄₄ O ₈
F	C ₃₀ H ₄₆ O ₇
G	C ₃₀ H ₅₂ O ₉
H	C ₃₀ H ₄₆ O ₈
I	C ₃₀ H ₄₂ O ₇

J	$C_{30}H_{44}O_8$
K	$C_{30}H_{44}O_8$
L	$C_{30}H_{44}O_7$
O	$C_{30}H_{46}O_7$
P	$C_{30}H_{48}O_7$
Q	$C_{32}H_{48}O_8$
S	$C_{30}H_{42}O_6$

According to some embodiments of the present invention, the plant or plant cell has reduced expression of one or more cucurbitacin selected from the group consisting of cucurbitacin A-L and O-S. In specific embodiments the plant or plant cell has reduced expression of one or more cucurbitacin selected from the group consisting of cucurbitacin B, cucurbitacin I, cucurbitacin E.

Modifications in the cucurbitacin resulting from down-regulation of cucurbitacin biosynthesis pathway gene expression according to the present invention can include, but are not limited to reduction in any one or more of hydroxylation of carbons C2, C16, C20 and C23 of the tetracyclic triterpene molecule, reduction in oxidation and/or dehydrogenation of carbons C3, C11, C21, C1-C2 and C22-C23 of the tetracyclic triterpene molecule and reduction in glycosylation of carbon C2 (in cucurbitacin E-glucoside).

As used herein, the phrase “cucurbitacin biosynthetic pathway” or “cucurbitacin biosynthesis pathway” refers to at least one, or more enzymatic reactions catalyzing the synthesis of cucurbitacin from precursor molecules (e.g. cucurbitadienol). The phrase “cucurbitacin biosynthetic pathway gene” refers to at least one or more genes encoding enzyme proteins catalyzing the production of cucurbitacin from cucurbitacin precursor molecules.

The present inventors have compiled a group of candidate watermelon genes encoding enzyme proteins likely catalyzing one or more of the steps in the cucurbitacin biosynthetic pathway. Table 2 below is a non-limiting list of watermelon genes, grouped by the annotated catalytic activity of their respective encoded enzyme proteins, and which can serve as a target for down-regulation of the cucurbitacin biosynthetic pathway, according to the methods of the invention.

Table 2:

Watermelon gene (Charleston Grey genome V2.5)	Watermelon gene (Var. 97103 genome V2.5)	Annotation	Watermelon Gene SEQ ID NO.	Watermelon Protein SEQ ID NO.
CICG01G003400	Cla97C01G003480	Cytochrome P450	1	2
CICG01G003700	Cla97C01G003790	Cytochrome P450	4	5
CICG01G014540	Cla97C01G013560	Cytochrome P450	7	8
CICG01G014560	Cla97C01G013570	Cytochrome P450	10	11
CICG01G017130	Cla97C01G016270	Cytochrome P450	13	14
CICG01G024550	Cla97C01G024490	Cytochrome P450	16	17
CICG02G019010	Cla97C02G044950	Cytochrome P450	19	20
CICG05G019420	Cla97C05G101020	Cytochrome P450	22	23
CICG05G019890	Cla97C05G101520	Cytochrome P450	25	26
CICG06G001570	Cla97C06G110850	Cytochrome P450	28	29
CICG06G001580	Cla97C06G110860	Cytochrome P450	31	32
CICG06G001590	Cla97C06G110870	Cytochrome P450	34	35
CICG06G001620	Cla97C06G110900	Cytochrome P450	37	38

CICG09G012200	Cla97C09G17507 0	Cytochrome P450	40	41
CICG09G012210	Cla97C09G17508 0	Cytochrome P450	43	44
CICG10G005830	Cla97C10G19000 0	Cytochrome P450	46	47
CICG10G012530	Cla97C10G19534 0	Cytochrome P450	49	50
CICG03G015220	Cla97C03G06544 0	Cytochrome P450	52	53
CICG10G015150	Cla97C10G19801 0	Cytochrome P450	55	56
CICG11G002820	Cla97C11G20928 0	Cytochrome P450	58	59
CICG11G017020	Cla97C11G22307 0	Cytochrome P450	61	62
CICG01G014610	Cla97C01G01362 0	oxygenase	64	65
CICG01G024160 a	Cla97C01G02410 0	oxygenase	67	68
CICG01G024160 b	Cla97C01G02411 0	oxygenase	70	71
		DEHYDROGENASE AND OXIDOREDUCTASE FAMILY (<i>italics</i>)		
CICG09G009760	Cla97C09G17257 0	<i>Alcohol dehydrogenase</i>	73	74
CICG01G014590	Cla97C01G01360 0	<i>Alcohol dehydrogenase</i>	76	77
CICG01G018250	Cla97C01G01752 0	<i>Alcohol dehydrogenase,</i>	79	80
CICG09G009780	Cla97C09G17258 0	<i>Alcohol dehydrogenase,</i>	82	83

CICG03G002490	Cla97C03G05354 0	<i>FAD-binding Berberine family</i>	85	86
CICG01G014570	Cla97C01G01358 0	<i>NAD(P)-binding Rossmann-fold</i>	88	89
CICG09G004750	Cla97C09G16713 0	<i>NAD(P)-binding Rossmann-fold</i>	91	92
CICG01G017400	Cla97C01G01657 0	<i>Peroxidase</i>	94	95
CICG02G023760	Cla97C02G04992 0	<i>Peroxidase</i>	97	98
CICG02G023770	Cla97C02G04993 0	<i>Peroxidase</i>	100	101
CICG02G023780	Cla97C02G04994 0	<i>Peroxidase</i>	103	104
CICG03G004740	Cla97C03G05589 0	<i>Peroxidase</i>	106	107
CICG07G007250	Cla97C07G13468 5	<i>Polyketide dehydrase</i>	109	110
CICG11G005180	Cla97C11G21209 0	<i>Proline dehydrogenase</i>	112	113
CICG03G016930	Cla97C03G06730 0	<i>Short-chain dehydrogenase/reductase</i>	115	116
CICG01G014530	Cla97C01G01355 0	<i>HXXXD-type acyl-transferase-like</i>	118	119
CICG05G020040	Cla97C05G10169 0	<i>HXXXD-type acyl-transferase-like</i>	121	122
CICG06G001610	Cla97C06G11089 0	<i>HXXXD-type acyl-transferase-like</i>	124	125

Thus, in some embodiments, the at least one cucurbitacin biosynthetic pathway gene is a gene having a coding sequence selected from SEQ ID NOS: 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31,

34, 37, 40, 43, 46, 49, 52, 55, 58, 61, 64, 67, 70, 73, 76, 79, 82, 85, 88, 91, 94, 97, 100, 103, 106, 112, 115, 118, 121 and 124 or a functional ortholog thereof from a different cucurbit species. In specific embodiments, the at least one cucurbitacin biosynthetic pathway gene is a (2OG) and Fe(II)-dependent oxygenase gene. In some embodiments, the (2OG) and Fe(II)-dependent oxygenase gene is selected from the group consisting of C1CG01G014610, C1CG01G014610a and C1CG01G014610b, having a coding sequence selected from the group consisting of SEQ ID NO: 64, 67 and 70, encoding an enzyme protein having an amino acid sequence selected from the group consisting of SEQ ID NO: 65, 68 and 71.

In other embodiments, the at least one cucurbitacin biosynthetic pathway gene is a cytochrome p450 gene. In some embodiments, the cytochrome p450 gene is selected from the group consisting of C1CG01G003400, C1CG01G003790, C1CG01G014540, C1CG01G014560, C1CG01G017130, C1CG01G024550, C1CG02G019010, C1CG05G019420, C1CG05G019890, C1CG06G001570, C1CG06G001580, C1CG06G001590, C1CG06G001620, C1CG09G012200, C1CG09G012210, C1CG10G005830, C1CG10G012530, C1CG03G015220, C1CG10G015150, C1CG11G002820 and C1CG11G017020, having a coding sequence selected from the group consisting of SEQ ID NO: 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 43, 46, 49, 52, 55, 58 and 61 encoding an enzyme protein having an amino acid sequence selected from the group consisting SEQ ID NO: 2, 5, 8, 11, 14, 17, 20, 23, 26, 29, 32, 35, 38, 41, 44, 47, 50, 53, 56, 59 and 62. In specific embodiments the cytochrome p450 gene is selected from the group consisting of C1CG01G014540, C1CG06G001570, C1CG06G001580, C1CG06G001590, C1CG06G001620, C1CG10G012530, having a coding sequence selected from the group consisting of SEQ ID NO: 7, 28, 31, 34, 37 and 49 encoding an enzyme protein having an amino acid sequence selected from the group consisting SEQ ID NO: 8, 29, 32, 35, 38 and 50, respectively.

In other embodiments, the at least one cucurbitacin biosynthetic pathway gene is a FAD-binding Berberine gene (member of the dehydrogenase-oxidoreductase family). In some embodiments, the FAD-binding Berberine gene is C1CG03G002490, having the coding sequence as set forth in SEQ ID NO: 85 encoding an enzyme protein having an amino acid sequence as set forth in SEQ ID NO: 86.

In other embodiments, the at least one cucurbitacin biosynthetic pathway gene is an NAD(P)-binding Rossmann-fold gene (member of the dehydrogenase-oxidoreductase family). In some embodiments, the NAD(P)-binding Rossmann-fold gene is selected from the group consisting C1CG01G014570, and C1CG01G01004750 having the coding sequence as set forth in SEQ ID NO: 88 and 91 encoding an enzyme protein having an amino acid sequence selected from the group consisting SEQ ID NO: 89 and 92, respectively. In specific embodiments, the NAD(P)-binding

Rossmann-fold gene is C1CG01G014570 having the coding sequence as set forth in SEQ ID NO: 88, encoding an enzyme protein having the amino acid sequence of SEQ ID NO: 89.

In other embodiments, the at least one cucurbitacin biosynthetic pathway gene is an HXXXX-type-acyl-transferase-like protein gene. In some embodiments, the HXXXX-type-acyl-transferase-like protein gene is selected from the group consisting of C1CG01G014530, C1CG05G020040, C1CG06G001610, and having a coding sequence selected from the group set forth in SEQ ID NO: 118, 121 and 124, encoding an enzyme protein having an amino acid sequence selected from the group consisting SEQ ID NO: 119, 122 and 125, respectively. In specific embodiments, the HXXXX-type-acyl-transferase-like protein gene is C1CG06G001610 having the coding sequence of SEQ ID NO: 124, encoding an enzyme protein having an amino acid sequence of SEQ ID NO: 125.

In other embodiments, the at least one cucurbitacin biosynthetic pathway gene is a polyketide cyclase/dehydrase gene (member of the dehydrogenase-oxidoreductase family). In some embodiments, the polyketide cyclase/dehydrase gene is C1CG07G007250, and having a coding sequence as set forth in SEQ ID NO: 109 encoding an enzyme protein having an amino acid sequence as set forth in SEQ ID NO: 110.

In other embodiments, the at least one cucurbitacin biosynthetic pathway gene is a short-chain dehydrogenase/reductase gene (member of the dehydrogenase-oxidoreductase family). In some embodiments, the short-chain dehydrogenase/reductase gene is C1CG03G016930, and having a coding sequence as set forth in SEQ ID NO: 115 encoding an enzyme protein having an amino acid sequence selected as set forth in SEQ ID NO: 116.

In other embodiments, the at least one cucurbitacin biosynthetic pathway gene is a proline dehydrogenase gene (member of the dehydrogenase-oxidoreductase family). In some embodiments, the proline dehydrogenase gene is C1CG11G005180, and having a coding sequence as set forth in SEQ ID NO: 112 encoding an enzyme protein having an amino acid sequence as set forth in SEQ ID NO: 113.

In other embodiments, the at least one cucurbitacin biosynthetic pathway gene is an alcohol dehydrogenase gene (member of the dehydrogenase-oxidoreductase family). In some embodiments, the alcohol dehydrogenase gene is selected from the group consisting of C1CG09G009760, C1CG01G014590, C1CG01G018250 and C1CG01G009780 and having a coding sequence selected from the group consisting of SEQ ID NO: 73, 76, 79 and 82 encoding an enzyme protein having an amino acid sequence selected from the group consisting SEQ ID NO: 74, 77, 80 and 83. In specific embodiments, the alcohol dehydrogenase gene is selected from the group consisting of C1CG09G009760 and C1CG01G018250 and having a coding sequence

selected from the group consisting of SEQ ID NO: 73 and 79 encoding an enzyme protein having an amino acid sequence selected from the group consisting SEQ ID NO: 74 and 80, respectively.

In other embodiments, the at least one cucurbitacin biosynthetic pathway gene is a peroxidase gene (member of the dehydrogenase-oxidoreductase family). In some embodiments, the peroxidase gene is selected from the group consisting of C1CG01G017400, C1CG02G023760, C1CG02G023770, C1CG02G023780, C1CG03G004740 and having a coding sequence selected from the group consisting of SEQ ID NO: 94, 97, 100, 103 and 106 encoding an enzyme protein having an amino acid sequence selected from the group consisting SEQ ID NO: 95, 98, 101, 104 and 107.

Thus, in some embodiments of the present invention, the plant or plant cell with a modified cucurbitacin content is a plant cell of a bitter cucurbit having reduced expression of one or more of any one of a (2OG) and Fe(II)-dependent oxygenase gene, a cytochrome p450 gene, a FAD-binding Berberine gene, an NAD(P)-binding Rossmann-fold gene, an HXXXD-type-acyl-transferase-like protein gene, a polyketide cyclase/hydrase gene, a short-chain dehydrogenase/reductase gene, a proline dehydrogenase gene, an alcohol dehydrogenase gene and a peroxidase gene.

In particular embodiments, the at least one cucurbitacin biosynthetic pathway gene is a bitter cucurbit functional ortholog of the at least one cucurbitacin biosynthetic pathway gene identified in watermelon. In specific embodiments, the functional ortholog is a bitter melon functional ortholog of a watermelon cucurbitacin biosynthetic pathway gene.

Cucurbitacins include, but are not limited to the common cucurbitacins that have been isolated from plant families (including Brassicaceae, Cucurbitaceae, Scrophulariaceae, Begoniaceae, Elaeocarpaceae, Datisceae, Desfontainiaceae, Polemoniaceae, Primulaceae, Rubiaceae, Sterculiaceae, Rosaceae, and Thymelaeaceae), fungi (including *Russula* and *Hebeloma*) and some marine mollusks. In specific embodiments, the cucurbitacins are cucurbitacins from the cucurbit family Cucurbitaceae.

It will be appreciated that homologs and orthologs of the cucurbitacin biosynthetic pathway genes of interest can be identified in any species using known bioinformatics tools such as BLAST (NIH) and UniProt BLAST (Expasy.org).

Table 2a is a compilation of non-limiting examples of cucurbitacin biosynthetic pathway gene orthologs of the *Citrullis lanatus* (Charleston Grey watermelon) genes from *Cucumis melo* (melon), *Cucumis sativus* (cucumber) and *Cucurbita pepo* (pumpkin, squash).

Table 2a

Citrullis lanatus gene (Charleston Grey genome V2.5)	Cucumis melo gene (DHL92 genome v.4)	Cucumis sativus (Gy14 genome v2)	Cucurbita pepo (MU-CU-16 genome v4)
<i>OXIDOREDUCTASES (E.C. 1.14)</i>			
CICG01G003400	MELO3C005602	CsGy5G003450	Cp4.1LG15g01120
CICG01G003700	MELO3C005576	CsGy5G003700	Cp4.1LG04g01050
CICG01G014540	MELO3C002192	CsGy1G007640	Cp4.1LG09g10760
CICG01G014560	MELO3C002192	CsGy1G007640	Cp4.1LG09g10760
CICG01G017130	MELO3C007590	CsGy6G027940	Cp4.1LG10g02250
CICG01G024550	MELO3C007140	CsGy6G035600	Cp4.1LG10g05990
CICG02G019010	MELO3C019585	CsGy6G010830	Cp4.1LG05g10140
CICG05G019420	MELO3C018259	CsGy3G039690	Cp4.1LG06g01430
CICG05G019890	MELO3C016752	CsGy7G007210	Cp4.1LG02g06980
CICG06G001570	MELO3C022377	CsGy6G007150	Cp4.1LG17g09460
CICG06G001580	MELO3C022376	CsGy6G007160	Cp4.1LG03g08700
CICG06G001590	MELO3C022375	CsGy6G007170	Cp4.1LG17g09460
CICG06G001620	MELO3C022372	CsGy6G007210	Cp4.1LG17g09580
CICG09G012200	MELO3C003387	CsGy3G044340	Cp4.1LG05g02130
CICG09G012210	MELO3C003385	CsGy3G044370	Cp4.1LG15g03520
CICG10G005830	MELO3C025434	CsGy5G011470	Cp4.1LG00g00200
CICG10G012530	MELO3C023960	CsGy3G027660	Cp4.1LG02g15440
CICG03G015220	MELO3C015511	CsGy1G027690	Cp4.1LG07g11530
CICG10G015150	MELO3C009864	CsGy3G036500	Cp4.1LG07g11530
CICG11G002820	MELO3C004527	CsGy2G001530	Cp4.1LG11g10900
CICG11G017020	MELO3C016687	CsGy4G020810	Cp4.1LG07g11530
CICG01G014610	MELO3C000410	CsGy1G007600	Cp4.1LG20g01950
CICG01G024160a	MELO3C007174	CsGy6G035280	Cp4.1LG19g06550
CICG01G024160b	MELO3C007175	CsGy6G035280	Cp4.1LG19g06550
<i>DEHYDROGENASE AND OXIDOREDUCTASE FAMILY</i>			
CICG09G009760	MELO3C023685	CsGy7G012090	Cp4.1LG02g08870
CICG01G014590	MELO3C002189	CsGy1G007610	Cp4.1LG20g01940

CICG01G018250	MELO3C007702	CsGy6G029120	Cp4.1LG10g01530
CICG09G009780	MELO3C023685	CsGy7G012090	Cp4.1LG06g07690
CICG03G002490	MELO3C004984	CsGy1G013140	Cp4.1LG01g04280
CICG01G014570	MELO3C002191	CsGy1G007630	Cp4.1LG00g01860
CICG09G004750	MELO3C023618	CsGy7G016930	Cp4.1LG18g00670
CICG01G017400	MELO3C007618	CsGy3G042080	Cp4.1LG19g10870
CICG02G023760	MELO3C014655	CsGy4G012840	Cp4.1LG05g05170
CICG02G023770	MELO3C014657	CsGy4G012820	Cp4.1LG05g05170
CICG02G023780	MELO3C014658	CsGy4G012810	Cp4.1LG05g05170
CICG03G004740	MELO3C002457	CsGy1G010600	Cp4.1LG20g03530
CICG07G007250	MELO3C015004	CsGy5G011240	Cp4.1LG01g07720
CICG11G005180	MELO3C022076	CsGy5G029220	Cp4.1LG01g22230
CICG03G016930	MELO3C015326	CsGy3G016130	Cp4.1LG18g00670
<i>ACYL TRANSFERASES</i>			
CICG01G014530	MELO3C002193	CsGy1G007650	Cp4.1LG09g10640
CICG05G020040	MELO3C016768	CsGy4G022520	Cp4.1LG06g01710
CICG06G001610	MELO3C022373	CsGy6G007200	Cp4.1LG17g09410

The present invention envisions production of a plant or plant cell having modified cucurbitacin content, the modified expression effected by down-regulating expression of at least one cucurbitacin biosynthesis pathway gene. Downregulation of expression of at least one cucurbitacin biosynthesis pathway gene can, in turn, modify the hydroxylation, oxidation-reduction, dehydrogenation, acetylation and/or glucosylation of cucurbitacin or cucurbitacin precursors. As used herein the phrase “downregulating expression” refers to downregulating the expression of a protein (e.g. cucurbitacin biosynthetic pathway enzyme) at the genomic (e.g. homologous recombination and site specific endonucleases) and/or the transcript level using a variety of molecules which interfere with transcription and/or translation (e.g., RNA silencing agents).

For the same cultivation conditions, the expression is generally expressed in comparison to the expression in a plant or plant cell of the same species but not contacted with the agent or contacted with a vehicle control, also referred to as control.

Down regulation of expression may be either transient or permanent.

According to specific embodiments, down regulating expression refers to the absence of mRNA and/or protein, as detected by RT-PCR or Western blot, respectively.

According to other specific embodiments down regulating expression refers to a decrease in the level of mRNA and/or protein, as detected by RT-PCR or Western blot, respectively. The reduction may be by at least a 10 %, at least 20 %, at least 30 %, at least 40 %, at least 50 %, at least 60 %, at least 70 %, at least 80 %, at least 90 %, at least 95 % or at least 99 % reduction.

Non-limiting examples of agents capable of down regulating cucurbitacin biosynthetic pathway gene expression are described in detail hereinbelow.

Down-regulation at the nucleic acid level

Down-regulation at the nucleic acid level is typically effected using a nucleic acid agent, having a nucleic acid backbone, DNA, RNA, mimetics thereof or a combination of same. The nucleic acid agent may be encoded from a DNA molecule or provided to the cell *per se*.

According to specific embodiments, the downregulating agent is a polynucleotide.

According to specific embodiments, the downregulating agent is a polynucleotide capable of hybridizing to a gene or mRNA encoding a cucurbitacin biosynthetic pathway enzyme.

According to specific embodiments, the downregulating agent directly interacts with the cucurbitacin biosynthetic pathway enzyme gene.

According to specific embodiments, the agent directly binds the cucurbitacin biosynthetic pathway enzyme gene.

According to specific embodiments, the agent indirectly binds the cucurbitacin biosynthetic pathway enzyme gene (e.g. binds an effector of the cucurbitacin biosynthetic pathway enzyme gene).

According to specific embodiments the downregulating agent is an RNA silencing agent or a genome editing agent.

Thus, downregulation of expression of cucurbitacin biosynthetic pathway genes can be achieved by RNA silencing. As used herein, the phrase "RNA silencing" refers to a group of regulatory mechanisms [e.g. RNA interference (RNAi), transcriptional gene silencing (TGS), post-transcriptional gene silencing (PTGS), quelling, co-suppression, and translational repression] mediated by RNA molecules which result in the inhibition or "silencing" of the expression of a corresponding protein-coding gene. RNA silencing has been observed in many types of organisms, including plants, animals, and fungi.

As used herein, the term "RNA silencing agent" refers to an RNA which is capable of specifically inhibiting or "silencing" the expression of a target gene. In certain embodiments, the RNA silencing agent is capable of preventing complete processing (e.g. the full translation and/or

expression) of an mRNA molecule through a post-transcriptional silencing mechanism. RNA silencing agents include non-coding RNA molecules, for example RNA duplexes comprising paired strands, as well as precursor RNAs from which such small non-coding RNAs can be generated. Exemplary RNA silencing agents include dsRNAs such as siRNAs, miRNAs and shRNAs.

In one embodiment, the RNA silencing agent is capable of inducing RNA interference.

In another embodiment, the RNA silencing agent is capable of mediating translational repression.

According to an embodiment of the invention, the RNA silencing agent is specific to the target RNA (e.g., cucurbitacin biosynthetic pathway enzyme gene transcript) and does not cross-inhibit or silence other targets or a splice variant which exhibits 99% or less global homology to the target gene, e.g., less than 98%, 97%, 96%, 95%, 94%, 93%, 92%, 91%, 90%, 89%, 88%, 87%, 86%, 85%, 84%, 83%, 82%, 81% global homology to the target gene; as determined by PCR, Western blot, Immunohistochemistry and/or flow cytometry.

RNA interference refers to the process of sequence-specific post-transcriptional gene silencing in animals and plants mediated by short interfering RNAs (siRNAs).

Following is a detailed description on RNA silencing agents that can be used according to specific embodiments of the present invention.

DsRNA, siRNA and shRNA - The presence of long dsRNAs in cells stimulates the activity of a ribonuclease III enzyme referred to as dicer. Dicer is involved in the processing of the dsRNA into short pieces of dsRNA known as short interfering RNAs (siRNAs). Short interfering RNAs derived from dicer activity are typically about 21 to about 23 nucleotides in length and comprise about 19 base pair duplexes. The RNAi response also features an endonuclease complex, commonly referred to as an RNA-induced silencing complex (RISC), which mediates cleavage of single-stranded RNA having sequence complementary to the antisense strand of the siRNA duplex. Cleavage of the target RNA takes place in the middle of the region complementary to the antisense strand of the siRNA duplex.

Accordingly, some embodiments of the invention contemplate use of dsRNA to downregulate protein expression from mRNA.

According to one embodiment dsRNA longer than 30 bp are used. Various studies demonstrate that long dsRNAs can be used to silence gene expression without inducing the stress response or causing significant off-target effects - see for example [Strat et al., Nucleic Acids Research, 2006, Vol. 34, No. 13 3803–3810; Bhargava A et al. Brain Res. Protoc. 2004;13:115–

125; Diallo M., et al., *Oligonucleotides*. 2003;13:381–392; Paddison P.J., et al., *Proc. Natl Acad. Sci. USA*. 2002;99:1443–1448; Tran N., et al., *FEBS Lett*. 2004;573:127–134].

The term "siRNA" refers to small inhibitory RNA duplexes (generally between 18-30 base pairs) that induce the RNA interference (RNAi) pathway. Typically, siRNAs are chemically synthesized as 21mers with a central 19 bp duplex region and symmetric 2-base 3'-overhangs on the termini, although it has been recently described that chemically synthesized RNA duplexes of 25-30 base length can have as much as a 100-fold increase in potency compared with 21mers at the same location. The observed increased potency obtained using longer RNAs in triggering RNAi is suggested to result from providing Dicer with a substrate (27mer) instead of a product (21mer) and that this improves the rate or efficiency of entry of the siRNA duplex into RISC.

It has been found that position of the 3'-overhang influences potency of an siRNA and asymmetric duplexes having a 3'-overhang on the antisense strand are generally more potent than those with the 3'-overhang on the sense strand (Rose et al., 2005). This can be attributed to asymmetrical strand loading into RISC, as the opposite efficacy patterns are observed when targeting the antisense transcript.

The strands of a double-stranded interfering RNA (e.g., an siRNA) may be connected to form a hairpin or stem-loop structure (e.g., an shRNA). Thus, as mentioned, the RNA silencing agent of some embodiments of the invention may also be a short hairpin RNA (shRNA).

The term "shRNA", as used herein, refers to an RNA agent having a stem-loop structure, comprising a first and second region of complementary sequence, the degree of complementarity and orientation of the regions being sufficient such that base pairing occurs between the regions, the first and second regions being joined by a loop region, the loop resulting from a lack of base pairing between nucleotides (or nucleotide analogs) within the loop region. The number of nucleotides in the loop is a number between and including 3 to 23, or 5 to 15, or 7 to 13, or 4 to 9, or 9 to 11. Some of the nucleotides in the loop can be involved in base-pair interactions with other nucleotides in the loop. Examples of oligonucleotide sequences that can be used to form the loop include 5'-CAAGAGA-3' and 5'-UUACAA-3' (International Patent Application Nos. WO2013126963 and WO2014107763). It will be recognized by one of skill in the art that the resulting single chain oligonucleotide forms a stem-loop or hairpin structure comprising a double-stranded region capable of interacting with the RNAi machinery.

Synthesis of RNA silencing agents suitable for use with some embodiments of the invention can be effected as follows. First, the cucurbitacin biosynthetic pathway gene or gene transcript (e.g. mRNA) sequence is scanned downstream of the AUG start codon for AA dinucleotide sequences. Occurrence of each AA and the 3' adjacent 19 nucleotides is recorded as potential

siRNA target sites. Preferably, siRNA target sites are selected from the open reading frame, as untranslated regions (UTRs) are richer in regulatory protein binding sites. UTR-binding proteins and/or translation initiation complexes may interfere with binding of the siRNA endonuclease complex [Tuschl ChemBiochem. 2:239-245]. It will be appreciated though, that siRNAs directed at untranslated regions may also be effective, as demonstrated for GAPDH wherein siRNA directed at the 5' UTR mediated about 90 % decrease in cellular GAPDH mRNA and completely abolished protein level ([www\(dot\)ambion\(dot\)com/techlib/tn/91/912\(dot\)html](http://www.ambion.com/techlib/tn/91/912.html)).

Second, potential target sites are compared to an appropriate genomic database (e.g., cucurbit) using any sequence alignment software, such as the BLAST software available from the NCBI server ([www\(dot\)ncbi\(dot\)nml\(dot\)nih\(dot\)gov/BLAST/](http://www.ncbi.nlm.nih.gov/BLAST/)). Putative target sites which exhibit significant homology to other coding sequences are filtered out.

Qualifying target sequences are selected as template for siRNA synthesis. Preferred sequences are those including low G/C content as these have proven to be more effective in mediating gene silencing as compared to those with G/C content higher than 55 %. Several target sites are preferably selected along the length of the target gene for evaluation. For better evaluation of the selected siRNAs, a negative control is preferably used in conjunction. Negative control siRNA preferably include the same nucleotide composition as the siRNAs but lack significant homology to the genome. Thus, a scrambled nucleotide sequence of the siRNA is preferably used, provided it does not display any significant homology to any other gene.

For example, suitable siRNAs directed against cucurbitacin biosynthetic pathway enzyme gene expression can be as described in Fusaro AF et al. EMBO Rep. 2006 Nov;7(11):1168-75. doi: 10.1038/sj.embor.7400837. Epub 2006 Oct 13. PMID: 17039251; PMCID: PMC1679793. Briefly, expression constructs comprising a target sequence (i.e. cucurbitacin biosynthetic pathway enzyme gene coding sequence or portion thereof) and an inverted version of the target sequence (reverse orientation), connected via a 308 bp long Arabidopsis thaliana polyubiquitin intron (SEQ ID NO: 127), with a 5' promoter sequence can be employed, which, when expressed in host cells create hairpin transcripts (hpRNA), which are subsequently digested by DICERs into short, double stranded siRNA fragments (see illustration in Figure 8, Tripathi, P.K. et al *Plant Growth Regul* (2023). [www\(dot\)doi\(dot\)org/10.1007/s10725-023-01013-0](https://doi.org/10.1007/s10725-023-01013-0)). Suitable expression constructs can be synthesized using target sequences from any cucurbitacin biosynthetic pathway enzyme gene or functional homologue thereof. In specific embodiments, the target sequence is selected from any of the gene sequences, or portions thereof of the genes in Table 2. In further embodiments, the target sequences are selected from any of SEQ ID NOs: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36,

39, 42, 45, 48, 51, 54, 57, 60, 63, 66, 69, 72, 75, 78, 81, 84, 87, 90, 93, 96, 99, 102, 105, 108, 111, 114, 117, 120, 123 and 126.

In specific embodiments, the target gene is a cytochrome p450 gene is selected from the group consisting of C1CG01G014540, C1CG06G001570, C1CG06G001580, C1CG06G001590, 5 C1CG06G001620, C1CG10G012530 and the target sequences are selected from the group consisting of SEQ ID NO: 9, 30, 33, 36, 39 and 51.

In other embodiments, the target gene is a FAD-binding Berberine gene (member of the dehydrogenase-oxidoreductase family) and the target sequences is as set forth in SEQ ID NO: 87.

In specific embodiments, the target gene is an NAD(P)-binding Rossman-fold gene and the 10 target sequence is as set forth in SEQ ID NO: 90.

In specific embodiments, the target gene is a HXXXD-type-acyl-transferase-like protein gene and the target sequence is as set forth in SEQ ID NO: 126.

In further specific embodiments, the target gene is an alcohol dehydrogenase gene selected from the group consisting of C1CG09G009760 and C1CG01G018250 and the target sequence is 15 selected from the group consisting of SEQ ID NO: 75 and 81, respectively.

In specific embodiments, target sequences for silencing cucurbitacin biosynthetic pathway enzyme genes include, but are not limited to the sequences listed in Table 3 herein, corresponding to target sequences of cucurbitacin biosynthetic pathway enzyme genes of Table 2.

Table 3:

Wermelon gene	Melon gene	Annotation	Watermelo n Gene SEQ ID NO.	Watermelo n Gene Silencing Target SEQ ID NO.
C1CG01G003400	Cla97C01G003480	Cytochrome P450	1	3
C1CG01G003700	Cla97C01G003790	Cytochrome P450	4	6
C1CG01G014540	Cla97C01G013560	Cytochrome P450	7	9

CICG01G014560	Cla97C01G01357 0	Cytochrome P450	10	12
CICG01G017130	Cla97C01G01627 0	Cytochrome P450	13	15
CICG01G024550	Cla97C01G02449 0	Cytochrome P450	16	18
CICG02G019010	Cla97C02G04495 0	Cytochrome P450	19	21
CICG05G019420	Cla97C05G10102 0	Cytochrome P450	22	24
CICG05G019890	Cla97C05G10152 0	Cytochrome P450	25	27
CICG06G001570	Cla97C06G11085 0	Cytochrome P450	28	30
CICG06G001580	Cla97C06G11086 0	Cytochrome P450	31	33
CICG06G001590	Cla97C06G11087 0	Cytochrome P450	34	36
CICG06G001620	Cla97C06G11090 0	Cytochrome P450	37	39
CICG09G012200	Cla97C09G17507 0	Cytochrome P450	40	42
CICG09G012210	Cla97C09G17508 0	Cytochrome P450	43	45
CICG10G005830	Cla97C10G19000 0	Cytochrome P450	46	48
CICG10G012530	Cla97C10G19534 0	Cytochrome P450	49	51
CICG03G015220	Cla97C03G06544 0	Cytochrome P450	52	54
CICG10G015150	Cla97C10G19801 0	Cytochrome P450	55	57

CICG11G002820	Cla97C11G20928 0	Cytochrome P450	58	60
CICG11G017020	Cla97C11G22307 0	Cytochrome P450	61	63
CICG01G014610	Cla97C01G01362 0	oxygenase	64	66
CICG01G024160 a	Cla97C01G02410 0	oxygenase	67	69
CICG01G024160 b	Cla97C01G02411 0	oxygenase	70	72
		DEHYDROGENASE AND OXIDOREDUCTASE FAMILY (<i>italics</i>)		
CICG09G009760	Cla97C09G17257 0	<i>Alcohol dehydrogenase</i>	73	75
CICG01G014590	Cla97C01G01360 0	<i>Alcohol dehydrogenase</i>	76	78
CICG01G018250	Cla97C01G01752 0	<i>Alcohol dehydrogenase,</i>	79	81
CICG09G009780	Cla97C09G17258 0	<i>Alcohol dehydrogenase,</i>	82	84
CICG03G002490	Cla97C03G05354 0	<i>FAD-binding Berberine family</i>	85	87
CICG01G014570	Cla97C01G01358 0	<i>NAD(P)-binding Rossmann-fold</i>	88	90
CICG09G004750	Cla97C09G16713 0	<i>NAD(P)-binding Rossmann-fold</i>	91	93
CICG01G017400	Cla97C01G01657 0	<i>Peroxidase</i>	94	96
CICG02G023760	Cla97C02G04992 0	<i>Peroxidase</i>	97	99
CICG02G023770	Cla97C02G04993 0	<i>Peroxidase</i>	100	102

CICG02G023780	Cla97C02G04994 0	<i>Peroxidase</i>	103	105
CICG03G004740	Cla97C03G05589 0	<i>Peroxidase</i>	106	108
CICG07G007250	Cla97C07G13468 5	<i>Polyketide dehydrase</i>	109	111
CICG11G005180	Cla97C11G21209 0	<i>Proline dehydrogenase</i>	112	114
CICG03G016930	Cla97C03G06730 0	<i>Short-chain dehydrogenase/reductase</i>	115	117
CICG01G014530	Cla97C01G01355 0	HXXXD-type acyl- transferase-like	118	120
CICG05G020040	Cla97C05G10169 0	HXXXD-type acyl- transferase-like	121	123
CICG06G001610	Cla97C06G11089 0	HXXXD-type acyl- transferase-like	124	126

It will be appreciated that, and as mentioned hereinabove, the RNA silencing agent of some embodiments of the invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

5 *miRNA and miRNA mimics* - According to another embodiment the RNA silencing agent may be a miRNA.

The term "microRNA", "miRNA", and "miR" are synonymous and refer to a collection of non-coding single-stranded RNA molecules of about 19-28 nucleotides in length, which regulate gene expression. miRNAs are found in a wide range of organisms (viruses, fow, humans) and
10 have been shown to play a role in development, homeostasis, and disease etiology.

Below is a brief description of the mechanism of miRNA activity.

Genes coding for miRNAs are transcribed leading to production of an miRNA precursor known as the pri-miRNA. The pri-miRNA is typically part of a polycistronic RNA comprising multiple pri-miRNAs. The pri-miRNA may form a hairpin with a stem and loop. The stem may
15 comprise mismatched bases.

The hairpin structure of the pri-miRNA is recognized by Drosha, which is an RNase III endonuclease. Drosha typically recognizes terminal loops in the pri-miRNA and cleaves approximately two helical turns into the stem to produce a 60–70 nucleotide precursor known as the pre-miRNA. Drosha cleaves the pri-miRNA with a staggered cut typical of RNase III endonucleases yielding a pre-miRNA stem loop with a 5' phosphate and ~2 nucleotide 3' overhang. It is estimated that approximately one helical turn of stem (~10 nucleotides) extending beyond the Drosha cleavage site is essential for efficient processing. The pre-miRNA is then actively transported from the nucleus to the cytoplasm by Ran-GTP and the export receptor Exportin-5.

The double-stranded stem of the pre-miRNA is then recognized by Dicer (or Dicer-like proteins (DCL)), which is also an RNase III endonuclease. Dicer may also recognize the 5' phosphate and 3' overhang at the base of the stem loop. Dicer then cleaves off the terminal loop two helical turns away from the base of the stem loop leaving an additional 5' phosphate and ~2 nucleotide 3' overhang. The resulting siRNA-like duplex, which may comprise mismatches, comprises the mature miRNA and a similar-sized fragment known as the miRNA*. The miRNA and miRNA* may be derived from opposing arms of the pri-miRNA and pre-miRNA. miRNA* sequences may be found in libraries of cloned miRNAs but typically at lower frequency than the miRNAs.

Although initially present as a double-stranded species with miRNA*, the miRNA eventually becomes incorporated as a single-stranded RNA into a ribonucleoprotein complex known as the RNA-induced silencing complex (RISC). Various proteins can form the RISC, which can lead to variability in specificity for miRNA/miRNA* duplexes, binding site of the target gene, activity of miRNA (repress or activate), and which strand of the miRNA/miRNA* duplex is loaded in to the RISC.

When the miRNA strand of the miRNA:miRNA* duplex is loaded into the RISC, the miRNA* is removed and degraded. The strand of the miRNA:miRNA* duplex that is loaded into the RISC is the strand whose 5' end is less tightly paired. In cases where both ends of the miRNA:miRNA* have roughly equivalent 5' pairing, both miRNA and miRNA* may have gene silencing activity.

The RISC identifies target nucleic acids based on high levels of complementarity between the miRNA and the mRNA, especially by nucleotides 2-7 of the miRNA.

A number of studies have looked at the base-pairing requirement between miRNA and its mRNA target for achieving efficient inhibition of translation (reviewed by Bartel 2004, Cell 116-281). In mammalian cells, the first 8 nucleotides of the miRNA may be important (Doench & Sharp 2004 GenesDev 2004-504). However, other parts of the microRNA may also participate in

mRNA binding. Moreover, sufficient base pairing at the 3' can compensate for insufficient pairing at the 5' (Brennecke et al, 2005 PLoS 3-e85). Computation studies, analyzing miRNA binding on whole genomes have suggested a specific role for bases 2-7 at the 5' of the miRNA in target binding but the role of the first nucleotide, found usually to be "A" was also recognized (Lewis et al 2005 Cell 120-15). Similarly, nucleotides 1-7 or 2-8 were used to identify and validate targets by Krek et al. (2005, Nat Genet 37-495).

The target sites in the mRNA may be in the 5' UTR, the 3' UTR or in the coding region. Interestingly, multiple miRNAs may regulate the same mRNA target by recognizing the same or multiple sites. The presence of multiple miRNA binding sites in most genetically identified targets may indicate that the cooperative action of multiple RISCs provides the most efficient translational inhibition.

miRNAs may direct the RISC to downregulate gene expression by either of two mechanisms: mRNA cleavage or translational repression. The miRNA may specify cleavage of the mRNA if the mRNA has a certain degree of complementarity to the miRNA. When a miRNA guides cleavage, the cut is typically between the nucleotides pairing to residues 10 and 11 of the miRNA. Alternatively, the miRNA may repress translation if the miRNA does not have the requisite degree of complementarity to the miRNA. Translational repression may be more prevalent in animals since animals may have a lower degree of complementarity between the miRNA and binding site.

It should be noted that there may be variability in the 5' and 3' ends of any pair of miRNA and miRNA*. This variability may be due to variability in the enzymatic processing of Drosha and Dicer with respect to the site of cleavage. Variability at the 5' and 3' ends of miRNA and miRNA* may also be due to mismatches in the stem structures of the pri-miRNA and pre-miRNA. The mismatches of the stem strands may lead to a population of different hairpin structures. Variability in the stem structures may also lead to variability in the products of cleavage by Drosha and Dicer.

The term "microRNA mimic" or "miRNA mimic" refers to synthetic non-coding RNAs that are capable of entering the RNAi pathway and regulating gene expression. miRNA mimics imitate the function of endogenous miRNAs and can be designed as mature, double stranded molecules or mimic precursors (e.g., or pre-miRNAs). miRNA mimics can be comprised of modified or unmodified RNA, DNA, RNA-DNA hybrids, or alternative nucleic acid chemistries (e.g., LNAs or 2'-O,4'-C-ethylene-bridged nucleic acids (ENA)). For mature, double stranded miRNA mimics, the length of the duplex region can vary between 13-33, 18-24 or 21-23 nucleotides. The miRNA may also comprise a total of at least 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15,

16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39 or 40 nucleotides. The sequence of the miRNA may be the first 13-33 nucleotides of the pre-miRNA. The sequence of the miRNA may also be the last 13-33 nucleotides of the pre-miRNA.

Preparation of miRNAs mimics can be effected by any method known in the art such as
5 chemical synthesis or recombinant methods.

It will be appreciated from the description provided herein above that contacting cells with a miRNA may be effected by transfecting the cells with e.g. the mature double stranded miRNA, the pre-miRNA or the pri-miRNA.

The pre-miRNA sequence may comprise from 45-90, 60-80 or 60-70 nucleotides.

10 The pri-miRNA sequence may comprise from 45-30,000, 50-25,000, 100-20,000, 1,000-1,500 or 80-100 nucleotides.

Antisense – Antisense is a single stranded RNA designed to prevent or inhibit expression of a gene by specifically hybridizing to its mRNA. Downregulation of a cucurbitacin biosynthetic pathway enzyme can be effected using an antisense polynucleotide capable of specifically
15 hybridizing with an mRNA transcript encoding the cucurbitacin biosynthetic pathway enzyme.

Design of antisense molecules which can be used to efficiently downregulate a cucurbitacin biosynthetic pathway enzyme expression must be effected while considering two aspects important to the antisense approach. The first aspect is delivery of the oligonucleotide into the cytoplasm of the appropriate cells, while the second aspect is design of an oligonucleotide which specifically
20 binds the designated mRNA within cells in a way which inhibits translation thereof.

The prior art teaches of a number of delivery strategies which can be used to efficiently deliver oligonucleotides into a wide variety of cell types [see, for example, Jääskeläinen et al. Cell Mol Biol Lett. (2002) 7(2):236-7; Gait, Cell Mol Life Sci. (2003) 60(5):844-53; Martino et al. J Biomed Biotechnol. (2009) 2009:410260; Grijalvo et al. Expert Opin Ther Pat. (2014) 24(7):801-
25 19; Falzarano et al, Nucleic Acid Ther. (2014) 24(1):87-100; Shilakari et al. Biomed Res Int. (2014) 2014: 526391; Prakash et al. Nucleic Acids Res. (2014) 42(13):8796-807 and Asseline et al. J Gene Med. (2014) 16(7-8):157-65]

In addition, algorithms for identifying those sequences with the highest predicted binding affinity for their target mRNA based on a thermodynamic cycle that accounts for the energetics of
30 structural alterations in both the target mRNA and the oligonucleotide are also available [see, for example, Walton et al. Biotechnol Bioeng 65: 1-9 (1999)]. Such algorithms have been successfully used to implement an antisense approach in cells.

In addition, several approaches for designing and predicting efficiency of specific oligonucleotides using an in vitro system were also published (Matveeva et al., Nature Biotechnology 16: 1374 - 1375 (1998)).

Thus, the generation of highly accurate antisense design algorithms and a wide variety of oligonucleotide delivery systems, enable an ordinarily skilled artisan to design and implement antisense approaches suitable for downregulating expression of known sequences without having to resort to undue trial and error experimentation.

Suitable antisense oligonucleotides can be constructed to target the gene transcripts (e.g. mRNA) of any one of the cucurbitacin biosynthesis pathway genes detailed in Table 2 hereinabove, and/or fragments thereof.

Nucleic acid agents can also operate at the DNA level as summarized infra.

Downregulation of any of the cucurbitacin biosynthetic pathway genes can also be achieved by inactivating the gene (e.g., including, but not limited to the target genes listed in Table 2) via introducing targeted mutations involving loss-of function alterations (e.g. point mutations, deletions and insertions) in the gene structure.

As used herein, the phrase "loss-of-function alterations" refers to any mutation in the DNA sequence of a gene (e.g., cucurbitacin biosynthetic pathway gene) which results in downregulation of the expression level and/or activity of the expressed product, i.e., the mRNA transcript and/or the translated protein. Non-limiting examples of such loss-of-function alterations include a missense mutation, *i.e.*, a mutation which changes an amino acid residue in the protein with another amino acid residue and thereby abolishes the enzymatic activity of the protein; a nonsense mutation, *i.e.*, a mutation which introduces a stop codon in a protein, e.g., an early stop codon which results in a shorter protein devoid of the enzymatic activity; a frame-shift mutation, *i.e.*, a mutation, usually, deletion or insertion of nucleic acid(s) which changes the reading frame of the protein, and may result in an early termination by introducing a stop codon into a reading frame (e.g., a truncated protein, devoid of the enzymatic activity), or in a longer amino acid sequence (e.g., a readthrough protein) which affects the secondary or tertiary structure of the protein and results in a non-functional protein, devoid of the enzymatic activity of the non-mutated polypeptide; a readthrough mutation due to a frame-shift mutation or a modified stop codon mutation (*i.e.*, when the stop codon is mutated into an amino acid codon), with an abolished enzymatic activity; a promoter mutation, *i.e.*, a mutation in a promoter sequence, usually 5' to the transcription start site of a gene, which results in down-regulation of a specific gene product; a regulatory mutation, *i.e.*, a mutation in a region upstream or downstream, or within a gene, which affects the expression of the gene product; a deletion mutation, *i.e.*, a mutation which deletes coding

nucleic acids in a gene sequence and which may result in a frame-shift mutation or an in-frame mutation (within the coding sequence, deletion of one or more amino acid codons); an insertion mutation, *i.e.*, a mutation which inserts coding or non-coding nucleic acids into a gene sequence, and which may result in a frame-shift mutation or an in-frame insertion of one or more amino acid codons; an inversion, *i.e.*, a mutation which results in an inverted coding or non-coding sequence; a splice mutation *i.e.*, a mutation which results in abnormal splicing or poor splicing; and a duplication mutation, *i.e.*, a mutation which results in a duplicated coding or non-coding sequence, which can be in-frame or can cause a frame-shift.

According to specific embodiments loss-of-function alteration of a gene may comprise at least one allele of the gene.

The term "allele" as used herein, refers to any of one or more alternative forms of a gene locus, all of which alleles relate to a trait or characteristic. In a diploid cell or organism, the two alleles of a given gene occupy corresponding loci on a pair of homologous chromosomes.

According to other specific embodiments loss-of-function alteration of a gene comprises both alleles of the gene. In such instances the e.g. modified cucurbitacin biosynthetic pathway gene may be in a homozygous form or in a heterozygous form. According to this embodiment, homozygosity is a condition where both alleles at the e.g. modified cucurbitacin biosynthetic pathway gene locus are characterized by the same nucleotide sequence. Heterozygosity refers to different conditions of the gene at the e.g. modified cucurbitacin biosynthetic pathway gene locus.

Methods of introducing nucleic acid alterations to a gene of interest are well known in the art [see for example Menke D. *Genesis* (2013) 51: - 618; Capecchi, *Science* (1989) 244:1288-1292; Santiago et al. *Proc Natl Acad Sci USA* (2008) 105:5809-5814; International Patent Application Nos. WO 2014085593, WO 2009071334 and WO 2011146121; US Patent Nos. 8771945, 8586526, 6774279 and UP Patent Application Publication Nos. 20030232410, 20050026157, US20060014264; the contents of which are incorporated by reference in their entireties] and include targeted homologous recombination, site specific recombinases, PB transposases and genome editing by engineered nucleases. Agents for introducing nucleic acid alterations to a gene of interest can be designed publically available sources or obtained commercially from Transposagen, Addgene and Sangamo Biosciences.

Following is a description of various exemplary methods used to introduce nucleic acid alterations to a gene of interest and agents for implementing same that can be used according to specific embodiments of the present invention.

Genome Editing using engineered endonucleases - this approach refers to a reverse genetics method using artificially engineered nucleases to cut and create specific double-stranded breaks at

a desired location(s) in the genome, which are then repaired by cellular endogenous processes such as, homology directed repair (HDR) and non-homologous end-joining (NHEJ). NHEJ directly joins the DNA ends in a double-stranded break, while HDR utilizes a homologous sequence as a template for regenerating the missing DNA sequence at the break point. In order to introduce specific nucleotide modifications to the genomic DNA, a DNA repair template containing the
5 desired sequence must be present during HDR. Genome editing cannot be performed using traditional restriction endonucleases since most restriction enzymes recognize a few base pairs on the DNA as their target and the probability is very high that the recognized base pair combination will be found in many locations across the genome resulting in multiple cuts not limited to a desired
10 location. To overcome this challenge and create site-specific single- or double-stranded breaks, several distinct classes of nucleases have been discovered and bioengineered to date. These include the meganucleases, Zinc finger nucleases (ZFNs), transcription-activator like effector nucleases (TALENs) and CRISPR/Cas system.

Meganucleases – Meganucleases are commonly grouped into four families: the
15 LAGLIDADG family, the GIY-YIG family, the His-Cys box family and the HNH family. These families are characterized by structural motifs, which affect catalytic activity and recognition sequence. For instance, members of the LAGLIDADG family are characterized by having either one or two copies of the conserved LAGLIDADG motif. The four families of meganucleases are widely separated from one another with respect to conserved structural elements and, consequently,
20 DNA recognition sequence specificity and catalytic activity. Meganucleases are found commonly in microbial species and have the unique property of having very long recognition sequences (>14bp) thus making them naturally very specific for cutting at a desired location. This can be exploited to make site-specific double-stranded breaks in genome editing. One of skill in the art can use these naturally occurring meganucleases, however the number of such naturally occurring
25 meganucleases is limited. To overcome this challenge, mutagenesis and high throughput screening methods have been used to create meganuclease variants that recognize unique sequences. For example, various meganucleases have been fused to create hybrid enzymes that recognize a new sequence. Alternatively, DNA interacting amino acids of the meganuclease can be altered to design sequence specific meganucleases (see e.g., US Patent 8,021,867). Meganucleases can be designed
30 using the methods described in e.g., Certo, MT et al. Nature Methods (2012) 9:973-975; U.S. Patent Nos. 8,304,222; 8,021,867; 8, 119,381; 8, 124,369; 8, 129,134; 8,133,697; 8,143,015; 8,143,016; 8, 148,098; or 8, 163,514, the contents of each are incorporated herein by reference in their entirety. Alternatively, meganucleases with site specific cutting characteristics can be obtained using

commercially available technologies e.g., Precision Biosciences' Directed Nuclease Editor™ genome editing technology.

ZFNs and TALENs – Two distinct classes of engineered nucleases, zinc-finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs), have both proven to be effective at producing targeted double-stranded breaks (Christian *et al.*, 2010; Kim *et al.*, 1996; Li *et al.*, 2011; Mahfouz *et al.*, 2011; Miller *et al.*, 2010).

Basically, ZFNs and TALENs restriction endonuclease technology utilizes a non-specific DNA cutting enzyme which is linked to a specific DNA binding domain (either a series of zinc finger domains or TALE repeats, respectively). Typically a restriction enzyme whose DNA recognition site and cleaving site are separate from each other is selected. The cleaving portion is separated and then linked to a DNA binding domain, thereby yielding an endonuclease with very high specificity for a desired sequence. An exemplary restriction enzyme with such properties is FokI. Additionally FokI has the advantage of requiring dimerization to have nuclease activity and this means the specificity increases dramatically as each nuclease partner recognizes a unique DNA sequence. To enhance this effect, FokI nucleases have been engineered that can only function as heterodimers and have increased catalytic activity. The heterodimer functioning nucleases avoid the possibility of unwanted homodimer activity and thus increase specificity of the double-stranded break.

Thus, for example to target a specific site, ZFNs and TALENs are constructed as nuclease pairs, with each member of the pair designed to bind adjacent sequences at the targeted site. Upon transient expression in cells, the nucleases bind to their target sites (in some embodiments, target sites within the sequences of any of the cucurbitacin biosynthesis pathway genes, e.g. the genes detailed in Table 2) and the FokI domains heterodimerize to create a double-stranded break. Repair of these double-stranded breaks through the nonhomologous end-joining (NHEJ) pathway most often results in small deletions or small sequence insertions. Since each repair made by NHEJ is unique, the use of a single nuclease pair can produce an allelic series with a range of different deletions at the target site. The deletions typically range anywhere from a few base pairs to a few hundred base pairs in length, but larger deletions have successfully been generated in cell culture by using two pairs of nucleases simultaneously (Carlson *et al.*, 2012; Lee *et al.*, 2010). In addition, when a fragment of DNA with homology to the targeted region is introduced in conjunction with the nuclease pair, the double-stranded break can be repaired via homology directed repair to generate specific modifications (Li *et al.*, 2011; Miller *et al.*, 2010; Urnov *et al.*, 2005).

Although the nuclease portions of both ZFNs and TALENs have similar properties, the difference between these engineered nucleases is in their DNA recognition peptide. ZFNs rely on

Cys2- His2 zinc fingers and TALENs on TALEs. Both of these DNA recognizing peptide domains have the characteristic that they are naturally found in combinations in their proteins. Cys2-His2 Zinc fingers typically found in repeats that are 3 bp apart and are found in diverse combinations in a variety of nucleic acid interacting proteins. TALEs on the other hand are found in repeats with a one-to-one recognition ratio between the amino acids and the recognized nucleotide pairs. Because both zinc fingers and TALEs happen in repeated patterns, different combinations can be tried to create a wide variety of sequence specificities. Approaches for making site-specific zinc finger endonucleases include, e.g., modular assembly (where Zinc fingers correlated with a triplet sequence are attached in a row to cover the required sequence), OPEN (low-stringency selection of peptide domains vs. triplet nucleotides followed by high-stringency selections of peptide combination vs. the final target in bacterial systems), and bacterial one-hybrid screening of zinc finger libraries, among others. ZFNs can also be designed and obtained commercially from e.g., Sangamo Biosciences™ (Richmond, CA).

Method for designing and obtaining TALENs are described in e.g. Reyon et al. Nature Biotechnology 2012 May;30(5):460-5; Miller et al. Nat Biotechnol. (2011) 29: 143-148; Cermak et al. Nucleic Acids Research (2011) 39 (12): e82 and Zhang et al. Nature Biotechnology (2011) 29 (2): 149–53. A recently developed web-based program named Mojo Hand was introduced by Mayo Clinic for designing TAL and TALEN constructs for genome editing applications (can be accessed through [www\(dot\)talendesign\(dot\)org](http://www(dot)talendesign(dot)org)). TALEN can also be designed and obtained commercially from e.g., Sangamo Biosciences™ (Richmond, CA).

CRISPR-Cas system - Many bacteria and archaea contain endogenous RNA-based adaptive immune systems that can degrade nucleic acids of invading phages and plasmids. These systems consist of clustered regularly interspaced short palindromic repeat (CRISPR) genes that produce RNA components and CRISPR associated (Cas) genes that encode protein components. The CRISPR RNAs (crRNAs) contain short stretches of homology to specific viruses and plasmids and act as guides to direct Cas nucleases to degrade the complementary nucleic acids of the corresponding pathogen. Studies of the type II CRISPR/Cas system of *Streptococcus pyogenes* have shown that three components form an RNA/protein complex and together are sufficient for sequence-specific nuclease activity: the Cas9 nuclease, a crRNA containing 20 base pairs of homology to the target sequence, and a trans-activating crRNA (tracrRNA) (Jinek et al. *Science* (2012) **337**: 816–821.). It was further demonstrated that a synthetic chimeric guide RNA (gRNA) composed of a fusion between crRNA and tracrRNA could direct Cas9 to cleave DNA targets that are complementary to the crRNA in vitro. It was also demonstrated that transient expression of Cas9 in conjunction with synthetic gRNAs can be used to produce targeted double-stranded breaks

in a variety of different species (Cho *et al.*, 2013; Cong *et al.*, 2013; DiCarlo *et al.*, 2013; Hwang *et al.*, 2013a,b; Jinek *et al.*, 2013; Mali *et al.*, 2013).

The CRISPR/Cas system for genome editing contains two distinct components: a gRNA and an endonuclease e.g. Cas9.

5 The gRNA is typically a 20 nucleotide sequence encoding a combination of the target homologous sequence (crRNA) and the endogenous bacterial RNA that links the crRNA to the Cas9 nuclease (tracrRNA) in a single chimeric transcript. The gRNA/Cas9 complex is recruited to the target sequence by the base-pairing between the gRNA sequence and the complement genomic DNA. For successful binding of Cas9, the genomic target sequence must also contain the
10 correct Protospacer Adjacent Motif (PAM) sequence immediately following the target sequence. The binding of the gRNA/Cas9 complex localizes the Cas9 to the genomic target sequence so that the Cas9 can cut both strands of the DNA causing a double-strand break. Just as with ZFNs and TALENs, the double-stranded breaks produced by CRISPR/Cas can undergo homologous recombination or NHEJ.

15 The Cas9 nuclease has two functional domains: RuvC and HNH, each cutting a different DNA strand. When both of these domains are active, the Cas9 causes double strand breaks in the genomic DNA.

A significant advantage of CRISPR/Cas is that the high efficiency of this system coupled with the ability to easily create synthetic gRNAs enables multiple genes to be targeted
20 simultaneously. In addition, the majority of cells carrying the mutation present biallelic mutations in the targeted genes.

However, apparent flexibility in the base-pairing interactions between the gRNA sequence and the genomic DNA target sequence allows imperfect matches to the target sequence to be cut by Cas9.

25 Modified versions of the Cas9 enzyme containing a single inactive catalytic domain, either RuvC- or HNH-, are called 'nickases'. With only one active nuclease domain, the Cas9 nickase cuts only one strand of the target DNA, creating a single-strand break or 'nick'. A single-strand break, or nick, is normally quickly repaired through the HDR pathway, using the intact complementary DNA strand as the template. However, two proximal, opposite strand nicks
30 introduced by a Cas9 nickase are treated as a double-strand break, in what is often referred to as a 'double nick' CRISPR system. A double-nick can be repaired by either NHEJ or HDR depending on the desired effect on the gene target. Thus, if specificity and reduced off-target effects are crucial, using the Cas9 nickase to create a double-nick by designing two gRNAs with target

sequences in close proximity and on opposite strands of the genomic DNA would decrease off-target effect as either gRNA alone will result in nicks that will not change the genomic DNA.

Modified versions of the Cas9 enzyme containing two inactive catalytic domains (dead Cas9, or dCas9) have no nuclease activity while still able to bind to DNA based on gRNA specificity. The dCas9 can be utilized as a platform for DNA transcriptional regulators to activate or repress gene expression by fusing the inactive enzyme to known regulatory domains. For example, the binding of dCas9 alone to a target sequence in genomic DNA can interfere with gene transcription.

There are a number of publically available tools available to help choose and/or design target sequences as well as lists of bioinformatically determined unique gRNAs for different genes in different species such as the Feng Zhang lab's Target Finder, the Michael Boutros lab's Target Finder (E-CRISP), the RGEN Tools: Cas-OFFinder, the CasFinder: Flexible algorithm for identifying specific Cas9 targets in genomes and the CRISPR Optimal Target Finder.

Thus, in some embodiments, downregulation of the cucurbitacin biosynthetic pathway gene is by genome editing, and, in particular, by CRISPR.

In order to use the CRISPR system, both gRNA and Cas9 should be expressed in a target cell. The insertion vector can contain both cassettes on a single plasmid or the cassettes are expressed from two separate plasmids. CRISPR plasmids are commercially available such as the px330 plasmid from Addgene.

“Hit and run” or “in-out” - involves a two-step recombination procedure. In the first step, an insertion-type vector containing a dual positive/negative selectable marker cassette is used to introduce the desired sequence alteration. The insertion vector contains a single continuous region of homology to the targeted locus and is modified to carry the mutation of interest. This targeting construct is linearized with a restriction enzyme at a one site within the region of homology, electroporated into the cells, and positive selection is performed to isolate homologous recombinants. These homologous recombinants contain a local duplication that is separated by intervening vector sequence, including the selection cassette. In the second step, targeted clones are subjected to negative selection to identify cells that have lost the selection cassette via intrachromosomal recombination between the duplicated sequences. The local recombination event removes the duplication and, depending on the site of recombination, the allele either retains the introduced mutation or reverts to wild type. The end result is the introduction of the desired modification without the retention of any exogenous sequences.

The “double-replacement” or “tag and exchange” strategy - involves a two-step selection procedure similar to the hit and run approach, but requires the use of two different targeting

constructs. In the first step, a standard targeting vector with 3' and 5' homology arms is used to insert a dual positive/negative selectable cassette near the location where the mutation is to be introduced. After electroporation and positive selection, homologously targeted clones are identified. Next, a second targeting vector that contains a region of homology with the desired mutation is electroporated into targeted clones, and negative selection is applied to remove the selection cassette and introduce the mutation. The final allele contains the desired mutation while eliminating unwanted exogenous sequences.

Site-Specific Recombinases - The Cre recombinase derived from the P1 bacteriophage and Flp recombinase derived from the yeast *Saccharomyces cerevisiae* are site-specific DNA recombinases each recognizing a unique 34 base pair DNA sequence (termed "Lox" and "FRT", respectively) and sequences that are flanked with either Lox sites or FRT sites can be readily removed via site-specific recombination upon expression of Cre or Flp recombinase, respectively. For example, the Lox sequence is composed of an asymmetric eight base pair spacer region flanked by 13 base pair inverted repeats. Cre recombines the 34 base pair lox DNA sequence by binding to the 13 base pair inverted repeats and catalyzing strand cleavage and religation within the spacer region. The staggered DNA cuts made by Cre in the spacer region are separated by 6 base pairs to give an overlap region that acts as a homology sensor to ensure that only recombination sites having the same overlap region recombine.

Basically, the site-specific recombinase system offers means for the removal of selection cassettes after homologous recombination. This system also allows for the generation of conditional altered alleles that can be inactivated or activated in a temporal or tissue-specific manner. Of note, the Cre and Flp recombinases leave behind a Lox or FRT "scar" of 34 base pairs. The Lox or FRT sites that remain are typically left behind in an intron or 3' UTR of the modified locus, and current evidence suggests that these sites usually do not interfere significantly with gene function.

Thus, Cre/Lox and Flp/FRT recombination involves introduction of a targeting vector with 3' and 5' homology arms containing the mutation of interest, two Lox or FRT sequences and typically a selectable cassette placed between the two Lox or FRT sequences. Positive selection is applied and homologous recombinants that contain targeted mutation are identified. Transient expression of Cre or Flp in conjunction with negative selection results in the excision of the selection cassette and selects for cells where the cassette has been lost. The final targeted allele contains the Lox or FRT scar of exogenous sequences.

Transposases – As used herein, the term "transposase" refers to an enzyme that binds to the ends of a transposon and catalyzes the movement of the transposon to another part of the genome.

As used herein the term “transposon” refers to a mobile genetic element comprising a nucleotide sequence which can move around to different positions within the genome of a single cell. In the process the transposon can cause mutations and/or change the amount of a DNA in the genome of the cell.

5 A number of transposon systems that are able to also transpose in cells e.g. vertebrates have been isolated or designed, such as Sleeping Beauty [Izsvák and Ivics *Molecular Therapy* (2004) 9, 147–156], piggyBac [Wilson et al. *Molecular Therapy* (2007) 15, 139–145], Tol2 [Kawakami et al. *PNAS* (2000) 97 (21): 11403–11408] or Frog Prince [Miskey et al. *Nucleic Acids Res.* Dec 1, (2003) 31(23): 6873–6881]. Generally, DNA transposons translocate from one DNA site to
10 another in a simple, cut-and-paste manner. Each of these elements has their own advantages, for example, Sleeping Beauty is particularly useful in region-specific mutagenesis, whereas Tol2 has the highest tendency to integrate into expressed genes. Hyperactive systems are available for Sleeping Beauty and piggyBac. Most importantly, these transposons have distinct target site preferences, and can therefore introduce sequence alterations in overlapping, but distinct sets of
15 genes. Therefore, to achieve the best possible coverage of genes, the use of more than one element is particularly preferred. The basic mechanism is shared between the different transposases, therefore we will describe piggyBac (PB) as an example.

PB is a 2.5 kb insect transposon originally isolated from the cabbage looper moth, *Trichoplusia ni*. The PB transposon consists of asymmetric terminal repeat sequences that flank a
20 transposase, PBase. PBase recognizes the terminal repeats and induces transposition via a “cut-and-paste” based mechanism, and preferentially transposes into the host genome at the tetranucleotide sequence TTAA. Upon insertion, the TTAA target site is duplicated such that the PB transposon is flanked by this tetranucleotide sequence. When mobilized, PB typically excises itself precisely to reestablish a single TTAA site, thereby restoring the host sequence to its
25 pretransposon state. After excision, PB can transpose into a new location or be permanently lost from the genome.

Typically, the transposase system offers an alternative means for the removal of selection cassettes after homologous recombination quit similar to the use Cre/Lox or Flp/FRT. Thus, for example, the PB transposase system involves introduction of a targeting vector with 3' and 5'
30 homology arms containing the mutation of interest, two PB terminal repeat sequences at the site of an endogenous TTAA sequence and a selection cassette placed between PB terminal repeat sequences. Positive selection is applied and homologous recombinants that contain targeted mutation are identified. Transient expression of PBase removes in conjunction with negative

selection results in the excision of the selection cassette and selects for cells where the cassette has been lost. The final targeted allele contains the introduced mutation with no exogenous sequences.

For PB to be useful for the introduction of sequence alterations, there must be a native TTAA site in relatively close proximity to the location where a particular mutation is to be inserted.

5 Genome editing using recombinant adeno-associated virus (rAAV) platform - this genome-editing platform is based on rAAV vectors which enable insertion, deletion or substitution of DNA sequences in the genomes of live mammalian cells. The rAAV genome is a single-stranded deoxyribonucleic acid (ssDNA) molecule, either positive- or negative-sensed, which is about 4.7 kb long. These single-stranded DNA viral vectors have high transduction rates and have a unique
10 property of stimulating endogenous homologous recombination in the absence of double-strand DNA breaks in the genome. One of skill in the art can design a rAAV vector to target a desired genomic locus and perform both gross and/or subtle endogenous gene alterations in a cell. rAAV genome editing has the advantage in that it targets a single allele and does not result in any off-target genomic alterations. rAAV genome editing technology is commercially available, for
15 example, the rAAV GENESIS™ system from Horizon™ (Cambridge, UK).

It will be appreciated that the agent can be a mutagen that causes random mutations and the cells exhibiting downregulation of the expression level and/or activity of cucurbit biosynthesis pathway genes may be selected.

The mutagens may be, but are not limited to, genetic, chemical or radiation agents. For
20 example, the mutagen may be ionizing radiation, such as, but not limited to, ultraviolet light, gamma rays or alpha particles. Other mutagens may include, but not be limited to, base analogs, which can cause copying errors; deaminating agents, such as nitrous acid; intercalating agents, such as ethidium bromide; alkylating agents, such as bromouracil; transposons; natural and synthetic alkaloids; bromine and derivatives thereof; sodium azide; psoralen (for example, combined with
25 ultraviolet radiation). The mutagen may be a chemical mutagen such as, but not limited to, ICR191, 1,2,7,8-diepoxy-octane (DEO), 5-azaC, N-methyl-N-nitrosoguanidine (MNNG) or ethyl methane sulfonate (EMS).

Methods for qualifying efficacy and detecting sequence alteration are well known in the art and include, but not limited to, DNA sequencing, electrophoresis, an enzyme-based mismatch
30 detection assay and a hybridization assay such as PCR, RT-PCR, RNase protection, in-situ hybridization, primer extension, Southern blot, Northern Blot and dot blot analysis.

Sequence alterations in a specific gene can also be determined at the protein level using e.g. chromatography, electrophoretic methods, immunodetection assays such as ELISA and western blot analysis and immunohistochemistry.

In addition, one ordinarily skilled in the art can readily design a knock-in/knock-out construct including positive and/or negative selection markers for efficiently selecting transformed cells that underwent a homologous recombination event with the construct. Positive selection provides a means to enrich the population of clones that have taken up foreign DNA. Non-limiting
5 examples of such positive markers include glutamine synthetase, dihydrofolate reductase (DHFR), markers that confer antibiotic resistance, such as neomycin, hygromycin, puromycin, and blasticidin S resistance cassettes. Negative selection markers are necessary to select against random integrations and/or elimination of a marker sequence (e.g. positive marker). Non-limiting
10 examples of such negative markers include the herpes simplex-thymidine kinase (HSV-TK) which converts ganciclovir (GCV) into a cytotoxic nucleoside analog, hypoxanthine phosphoribosyltransferase (HPRT) and adenine phosphoribosyltransferase (ARPT).

It will be appreciated that the RNA silencing agent of some embodiments of the invention need not be limited to those molecules containing only RNA, but further encompasses chemically-modified nucleotides and non-nucleotides.

15 In some embodiments, the RNA silencing agent provided herein can be functionally associated with a cell-penetrating peptide." As used herein, a "cell-penetrating peptide" is a peptide that comprises a short (about 12-30 residues) amino acid sequence or functional motif that confers the energy-independent (i.e., non-endocytotic) translocation properties associated with transport of the membrane-permeable complex across the plasma and/or nuclear membranes of a cell. The cell-
20 penetrating peptide used in the membrane-permeable complex of some embodiments of the invention preferably comprises at least one non-functional cysteine residue, which is either free or derivatized to form a disulfide link with a double-stranded ribonucleic acid that has been modified for such linkage. Representative amino acid motifs conferring such properties are listed in U.S. Pat. No. 6,348,185, the contents of which are expressly incorporated herein by reference. The cell-
25 penetrating peptides of some embodiments of the invention preferably include, but are not limited to, penetratin, transportan, pIsl, TAT(48-60), pVEC, MTS, and MAP.

Reducing the expression of a cucurbitacin biosynthetic pathway gene or genes in a bitter cucurbit, as taught herein, can result in the accumulation of tetracyclic triterpenes lacking chemical motifs (hydroxyl groups, acyl groups, carbonyl groups, etc) which interfere with glucosylation by
30 UDP- glucuronosyl transferase (UGT), thus allowing their glucosylation by the UGTs. Thus, in some embodiments of the present invention, there is provided a plant or plant cell of (e.g. a bitter cucurbit plant or plant cell, or a plant or plant cell of a non-cucurbit expressing cucurbitacin biosynthetic pathway gene(s)) having reduced expression of at least one gene of the cucurbitacin biosynthetic pathway, the plant or plant cell comprising at least one tetracyclic triterpene capable

of glucosylation by a UDP- glucuronosyl transferase (UGT). In some embodiments the UGT is a plant UGT. In particular embodiments, the plant UGT is a cucurbit UGT. In specific embodiments, the cucurbit UGT is a *Siraitia grosvenorii* UGT. A non-limiting disclosure of UGTs suitable for glucosylation of the tetracyclic triterpene capable of glucosylation is available in PCT 5 IL2015/050933.

A non-limiting list of the amino acid sequences of *S. grosvenorii* UGT enzyme proteins, and the nucleic acid sequences encoding them is provided in Table 3a.

Table 3a:

UGT	SEQ ID	SEQ ID
	Nucleic	Enzyme
	Acid	Protein
>UGT74-345-2	128	139
>UGT73-348-2	129	140
>UGT94-289-1	130	141
>UGT73-327-2	131	142
>UGT73-251_5	132	143
>UGT73-251-6	133	144
>UGT75-281-2	134	145
>UGT85-269-4	135	146
>UGT85-269-1	136	147
>UGT94-289-2	137	148
>UGT94-289_3	138	149

Thus, in some embodiments, the cucurcubit plant or plant cell of the invention comprises at least one tetracyclic triterpene capable of glycosylation by a UGT, the UGT selected from the group of UGTs having the amino acid sequence as set forth in any one of SEQ ID NOs. 139, 140, 141, 142, 143, 144, 145, 146, 147, 148 and 149, or functional equivalents thereof. In other
5 embodiments, the UGT is selected from the group of UGT enzymes encoded by a polynucleotide having any one of the nucleic acid sequences of SEQ ID NOs. 128, 129, 130, 131, 132, 133, 134, 135, 136, 137 and 138. In specific embodiments, the UGT is a UGT having the amino acid sequence selected from the group consisting of SEQ ID NOs. 139, 140, 141, 142, 143, 144, 147 and 149.

10 It will be appreciated that plants having endogenous UGT enzymatic activity may be capable of catalyzing glucosylation of the tetracyclic triterpene capable of glucosylation newly produced by the method of the invention. In the event of such a glucosylation, the previously bitter plant (e.g. bitter cucurbit) can be converted into a non-bitter plant (e.g. non- bitter cucurbit), by virtue of accumulation of non-bitter, tetracyclic triterpene glucosides.

15 Likewise, it will be appreciated that cells (plant or non-plant cells) transformed to express, or over-express UGT enzymatic activity may also be capable of catalyzing glucosylation of the tetracyclic triterpene capable of glucosylation newly produced by the method of the invention. Thus, the plant UGT can be endogenous to the plant or plant cell, or may be a heterologous UGT expressed in a transformed plant or plant cell.

20 Thus, in some embodiments, there is provided a method of producing a glucosylated tetracyclic triterpene, comprising glucosylating the at least one tetracyclic triterpene capable of glucosylation of the plant or plant cell newly produced by the methods of the invention by contacting the at least one tetracyclic triterpene with a UGT. When glucosylated by the UGT, the tetracyclic thus becomes a glucosylated tetracyclic triterpene.

25 Some glucosylated tetracyclic triterpenes retain the bitterness associated with cucurbitacin-like molecules- for example, the mogroside precursor mogrol, and mogrosides I and II, although glucosylated, are bitter-tasting. Additional glucosylation of the mogroside precursors, (e.g. to mogroside IV, V or VI), results in a sweet-tasting rather than bitter glucosylated tetracyclic triterpene. Thus, in some embodiments, the glucosylated tetracyclic triterpene of the invention is a
30 non-bitter glucosylated tetracyclic triterpene. In particular embodiments, the glucosylated tetracyclic triterpene of the invention is a mogroside, e.g. any one of mogrosides I, II, III, IV, V, VI, including but not limited to a single or combination of mogrosides including but not limited to mogroside I-A1, mogroside I-E1, mogroside IIA, mogroside IIB, mogroside HE, 7-oxomogroside HE, 11- oxomogroside A1, mogroside III, mogroside III A1, mogroside III A2, mogroside IIIx, 11-

deoxymogroside III, mogroside IV, mogroside IV-A, 11-oxomogroside IV-A, siamenoside I, mogroside V, 7-oxomogroside V, 11-oxomogroside V, mogroside VI, and mogroside VII.

In some embodiments of the method, the one or more mogrosides is selected from mogrol, mogroside I-A1, mogroside I-E1, mogroside IIA, mogroside IIB, mogroside HE, 7-oxomogroside HE, 11-oxomogroside A1, mogroside III, mogroside III A1, mogroside III A2, mogroside IIIx, 11-
5 deoxymogroside III, mogroside IV, mogroside IV-A, 11-oxomogroside IV-A, siamenoside I, mogroside V, 7-oxomogroside V, 11-oxomogroside V, mogroside VI, and mogroside VII.

In certain embodiments of the method, the one or more mogrosides is mogrol.

In certain embodiments of the method, the one or more mogrosides is mogroside I-A1.

10 In certain embodiments of the method, the one or more mogrosides is mogroside I-E1.

In certain embodiments of the method, the one or more mogrosides is mogroside IIA.

In certain embodiments of the method, the one or more mogrosides is mogroside IIB.

In certain embodiments of the method, the one or more mogrosides is mogroside HE.

In certain embodiments of the method, the one or more mogrosides is 7-oxomogroside HE.

15 In certain embodiments of the method, the one or more mogrosides is 11-oxomogroside A1.

In certain embodiments of the method, the one or more mogrosides is mogroside III.

In certain embodiments of the method, the one or more mogrosides is mogroside III A1.

In certain embodiments of the method, the one or more mogrosides is mogroside III A2. In certain embodiments of the method, the one or more mogrosides is mogroside IIIx.

20 In certain embodiments of the method, the one or more mogrosides is 11-deoxymogroside III.

In certain embodiments of the method, the one or more mogrosides is mogroside IV.

In certain embodiments of the method, the one or more mogrosides is mogroside IV-A.

25 In certain embodiments of the method, the one or more mogrosides is 11-oxomogroside IV-A.

In certain embodiments of the method, the one or more mogrosides is siamenoside I.

In certain embodiments of the method, the one or more mogrosides is mogroside V.

In certain embodiments of the method, the one or more mogrosides is 7-oxomogroside V.

In certain embodiments of the method, the one or more mogrosides is 11-oxomogroside V.

30 In certain embodiments of the method, the one or more mogrosides is mogroside VI.

In certain embodiments of the method, the one or more mogrosides is mogroside VII.

Plants or plant cells of the present invention can be propagated. Following identification of down-regulation of expression of the cucurbitacin biosynthetic pathway in a plant or plant cell of the invention, plant propagation is exercised. The most common method of plant propagation

is by seed. Regeneration by seed propagation, however, has the deficiency that due to heterozygosity there is a lack of uniformity in the crop, since seeds are produced by plants according to the genetic variances governed by Mendelian rules. Basically, each seed is genetically different and each will grow with its own specific traits. Therefore, it is preferred that the plant be
5 produced such that the regenerated plant has the identical traits and characteristics of the modified parent plant. Therefore, it is preferred that the modified plant be regenerated by micropropagation which provides a rapid, consistent reproduction of the transformed plants.

Micropropagation is a process of growing new generation plants from a single piece of tissue that has been excised from a selected parent plant or cultivar. This process permits the mass
10 reproduction of plants having the preferred tissue expressing the fusion protein. The new generation plants which are produced are genetically identical to, and have all of the characteristics of, the original plant. Micropropagation allows mass production of quality plant material in a short period of time and offers a rapid multiplication of selected cultivars in the preservation of the characteristics of the original modified plant. The advantages of cloning plants are the speed of
15 plant multiplication and the quality and uniformity of plants produced.

Micropropagation is a multi-stage procedure that requires alteration of culture medium or growth conditions between stages. Thus, the micropropagation process involves four basic stages: Stage one, initial tissue culturing; stage two, tissue culture multiplication; stage three, differentiation and plant formation; and stage four, greenhouse culturing and hardening. During
20 stage one, initial tissue culturing, the tissue culture is established and certified contaminant-free. During stage two, the initial tissue culture is multiplied until a sufficient number of tissue samples are produced to meet production goals. During stage three, the tissue samples grown in stage two are divided and grown into individual plantlets. At stage four, the transformed plantlets are transferred to a greenhouse for hardening where the plants' tolerance to light is gradually increased
25 so that it can be grown in the natural environment.

Although stable modification is presently preferred, transient modification of leaf cells, meristematic cells or the whole plant is also envisaged by some embodiments of the invention.

In some embodiments, there is provided a cell lysate of the modified plant or plant cell. Such a cell lysate can comprise both the modified cucurbitacin biosynthetic pathway enzymes of
30 the present invention, the tetracyclic triterpenes of the present invention, and/or the products of further enzymatic reactions of the tetracyclic triterpenes (e.g. glucosylation to tetracyclic triterpene glucosides by UGTs). Thus, the cell lysate can be used either for recovery of the products of the modified cucurbitacin biosynthetic pathway or recovery of modified cucurbitacin biosynthetic

pathway enzyme polypeptides. Methods for extraction of active enzyme polypeptides and tetracyclic triterpenes are well known in the art.

Glucosylation of the tetracyclic triterpenes of the invention capable of glucosylation can be carried out in plant or other cells, either naturally expressing one or more UGTs catalyzing the glucosylation of the tetracyclic triterpene, or in plant or other cells modified to express one or more UGTs catalyzing the glucosylation. Modification of cells to express UGTs suitable for glucosylating tetracyclic triterpene substrates in the mogroside biosynthetic pathway, for example, is described in detail in, inter alia, PCT IL2015/050933.

Further, glucosylation of the tetracyclic triterpenes of the invention capable of glucosylation by UGTs can be carried out in cell-free synthesis systems. In some embodiments, the glucosylation is carried out in multiple steps (e.g. enzymatic reactions), and can be effected wholly in plant or other cells, wholly in a cell free system, or in steps combining enzymatic reactions occurring in plants or plant cells as well as cell-free reactions.

Thus, in some embodiments, the tetracyclic triterpene of the invention is glucosylated in a cell, for example, in a plant cell, or in a non-plant (e.g. yeast, bacteria, insect or animal cell). In other embodiments, the tetracyclic triterpene of the invention is glucosylated in a cell-free system.

Cell lysates of the invention can also be used for cell-free synthesis of the modified tetracyclic triterpenes of the invention, alone or in combination with other suitable substrates or enzymes.

Further, tetracyclic triterpenes resulting from the down-regulation of cucurbitacin biosynthesis pathway gene expression can be identified, and assayed for desirable biological or chemical properties other than as substrates for glucosylation by UGTs.

Thus, there is provided a composition enriched in the tetracyclic triterpenes resulting from the down-regulation of cucurbitacin biosynthesis pathway gene expression of the invention. In some embodiments, the composition is enriched in tetracyclic triterpene glucosides resulting from the down-regulation of cucurbitacin biosynthesis pathway gene expression of the invention. In other embodiments, the composition is enriched in tetracyclic triterpene glucosides produced by contacting with UGT enzymes according to the methods of the invention.

In some embodiments, the compositions can be used as sweeteners and flavor modifiers.

Sweeteners and flavor modifiers disclosed herein can have a range of concentration, e.g. 0.1% and up to 99%. In some embodiments the sweeteners and/or flavor modifiers comprise the compositions disclosed herein in concentrations of about 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55,

56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98 and 99% and intervening concentrations.

The compounds and compositions (e.g. sweeteners and flavor enhancers) can be obtained
5 from any part of the plant, such as the fruit, leaf, root, which can be hand or mechanically harvested and sorted, preserved (chilled, pH change, dried) or immediately processed. The compositions and compounds may also be obtained from cell or tissue culture, and then processed as detailed herein.

The following are typical processing methods, utilized as single processes or in various combinations, targeting the production of a specific ingredient (e.g. sweetener and/or flavor
10 enhancer) fit for the required applications:

The plant or parts thereof is crushed, juiced, squeezed, dried, powdered, extracted (water, water-ethanol blends, sonication, Soxhlet, super critical fluid, etc.) followed by optimized combination of known to the trade of at least one of gross separation/filtration, fine separation (Micro/Ultra/Nano filtration, centrifugation, decantation, etc.), concentration (membrane, porous
15 columns, evaporation etc.), isolation and purification (affinity chromatography, pH modifications, etc.) concentration, stabilization, formulation including drying (heat, vacuum, freeze drying, powdering etc.) or wet formulations.

In some embodiments, the composition of the invention is a consumable composition.

Consumables include all food products, including but not limited to, cereal products, rice products, tapioca products, sago products, baker's products, biscuit products, pastry products, bread
20 products, confectionery products, desert products, gums, chewing gums, chocolates, ices, honey products, treacle products, yeast products, baking-powder, salt and spice products, savory products, mustard products, vinegar products, sauces (condiments), tobacco products, cigars, cigarettes, processed foods, cooked fruits and vegetable products, meat and meat products, jellies, jams, fruit sauces, egg products, milk and dairy products, yoghurts, cheese products, butter and butter
25 substitute products, milk substitute products, soy products, edible oils and fat products, medicaments, beverages, carbonated beverages, alcoholic drinks, beers, soft drinks, mineral and aerated waters and other non-alcoholic drinks, fruit drinks, fruit juices, coffee, artificial coffee, tea, cocoa, including forms requiring reconstitution, food extracts, plant extracts, meat extracts, condiments, sweeteners, nutraceuticals, gelatins, pharmaceutical and non-pharmaceutical gums,
30 tablets, lozenges, drops, emulsions, elixirs, syrups and other preparations for making beverages, and combinations thereof.

Compositions of the invention can be used in various consumables including but not limited to water-based consumables, solid dry consumables and dairy products, dairy-derived products and dairy-alternative products. In some embodiments the composition is a foodstuff.

Water-based consumables include but are not limited to beverage, water, aqueous drink, enhanced/slightly sweetened water drink, mineral water, carbonated beverage, non-carbonated beverage, carbonated water, still water, soft drink, non-alcoholic drink, alcoholic drink, beer, wine, liquor, fruit drink, juice, fruit juice, vegetable juice, broth drink, coffee, tea, black tea, green tea, oolong tea, herbal tea, cacao (water-based), tea-based drink, coffee-based drink, cacao-based drink, syrup, frozen fruit, frozen fruit juice, water-based ice, fruit ice, sorbet, dressing, salad dressing, sauce, soup, and beverage botanical materials (whole or ground), or instant powder for reconstitution (coffee beans, ground coffee, instant coffee, cacao beans, cacao powder, instant cacao, tea leaves, instant tea powder). In some embodiments, the composition can be a beverage such as Coca-Cola® and the like.

Solid dry consumables include but are not limited to cereals, baked food products, biscuits, bread, breakfast cereal, cereal bar, energy bars/nutritional bars, granola, cakes, cookies, crackers, donuts, muffins, pastries, confectioneries, chewing gum, chocolate, fondant, hard candy, marshmallow, pressed tablets, snack foods, and botanical materials (whole or ground), and instant powders for reconstitution as mentioned above.

In certain products a higher sweetener concentration is usually necessary to reach similar sweetness intensity, for example in dairy products, dairy-derived products and dairy-alternative products. Dairy-derived food products contain milk or milk protein. Dairy-alternative products contain (instead of dairy protein derived from the milk of mammals) protein from botanical sources (soy, rice, and other protein-rich plant materials). Dairy products, dairy-derived products and dairy-alternative products include but are not limited to milk, fluid milk, cultured milk product, cultured and noncultured dairy-based drinks, cultured milk product cultured with lactobacillus, yoghurt, yoghurt-based beverage, smoothy, lassi, milk shake, acidified milk, acidified milk beverage, butter milk, kefir, milk-based beverage, milk/juice blend, fermented milk beverage, icecream, dessert, sour cream, dip, salad dressings, cottage cheese, frozen yoghurt, soy milk, rice milk, soy drink, rice milk drink.

Milk includes, but is not limited to, whole milk, skim milk, condensed milk, evaporated milk, reduced fat milk, low fat milk, nonfat milk, and milk solids (which may be fat or nonfat).

The composition of the invention can also include one or more additional flavor ingredients, such as additional sweeteners. A non-limiting list of suitable flavor ingredients useful with the composition of the invention includes sucrose, fructose, glucose, high fructose corn syrup, xylose,

arabinose, rhamnose, erythritol, xylitol, mannitol, sorbitol, inositol, AceK, aspartame, neotame, sucralose, saccharine, naringin dihydrochalcone (NarDHC), neohesperidin dihydrochalcone (NDHC), rubusoside, rebaudioside A, stevioside, stevia and trilobtain.

As used herein the term "about" refers to $\pm 10\%$.

5 The terms "comprises", "comprising", "includes", "including", "having" and their conjugates mean "including but not limited to".

The term "consisting of" means "including and limited to".

10 The term "consisting essentially of" means that the composition, method or structure may include additional ingredients, steps and/or parts, but only if the additional ingredients, steps and/or parts do not materially alter the basic and novel characteristics of the claimed composition, method or structure.

As used herein, the singular form "a", "an" and "the" include plural references unless the context clearly dictates otherwise. For example, the term "a compound" or "at least one compound" may include a plurality of compounds, including mixtures thereof.

15 Throughout this application, various embodiments of this invention may be presented in a range format. It should be understood that the description in range format is merely for convenience and brevity and should not be construed as an inflexible limitation on the scope of the invention. Accordingly, the description of a range should be considered to have specifically disclosed all the possible subranges as well as individual numerical values within that range. For example,
20 description of a range such as from 1 to 6 should be considered to have specifically disclosed subranges such as from 1 to 3, from 1 to 4, from 1 to 5, from 2 to 4, from 2 to 6, from 3 to 6 etc., as well as individual numbers within that range, for example, 1, 2, 3, 4, 5, and 6. This applies regardless of the breadth of the range.

25 Whenever a numerical range is indicated herein, it is meant to include any cited numeral (fractional or integral) within the indicated range. The phrases "ranging/ranges between" a first indicate number and a second indicate number and "ranging/ranges from" a first indicate number "to" a second indicate number are used herein interchangeably and are meant to include the first and second indicated numbers and all the fractional and integral numerals therebetween.

30 As used herein the term "method" refers to manners, means, techniques and procedures for accomplishing a given task including, but not limited to, those manners, means, techniques and procedures either known to, or readily developed from known manners, means, techniques and procedures by practitioners of the chemical, pharmacological, biological, biochemical and medical arts.

When reference is made to particular sequence listings, such reference is to be understood to also encompass sequences that substantially correspond to its complementary sequence as including minor sequence variations, resulting from, e.g., sequencing errors, cloning errors, or other alterations resulting in base substitution, base deletion or base addition, provided that the frequency of such variations is less than 1 in 50 nucleotides, alternatively, less than 1 in 100 nucleotides, alternatively, less than 1 in 200 nucleotides, alternatively, less than 1 in 500 nucleotides, alternatively, less than 1 in 1000 nucleotides, alternatively, less than 1 in 5,000 nucleotides, alternatively, less than 1 in 10,000 nucleotides.

It is appreciated that certain features of the invention, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the invention, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable subcombination or as suitable in any other described embodiment of the invention. Certain features described in the context of various embodiments are not to be considered essential features of those embodiments, unless the embodiment is inoperative without those elements.

Various embodiments and aspects of the present invention as delineated hereinabove and as claimed in the claims section below find experimental support in the following examples.

EXAMPLES

Reference is now made to the following examples, which together with the above descriptions illustrate some embodiments of the invention in a non-limiting fashion.

Example I: ELUCIDATION OF CUCURBITACIN BIOSYNTHETIC PATHWAY GENES

Experimental methods: A novel line of bitter watermelon was used for the testing of candidate genes. A line of watermelon (PI673173, USDA) with yield and other horticultural characteristics of cultivated watermelon varieties, was identified in which the fruit was extremely bitter. This is due to the expression of the bHLH transcription factor causal to activation of the cucurbitacin metabolic pathway, in contrast to cultivated sweet watermelons. The silencing of the gene encoding the bHLH transcription factor was critical for the evolution of the edible cultivated watermelon *C. lanatus* and is considered to be a major domestication event in the evolution of edible watermelons (Zhou, Y. et al, 2016). Figure 11 shows both the bitter Hawkesbury fruit (upper) and the non-bitter cultivated sweet Hawksbury watermelons. Shown also is the expression level (FPKM, Fragments Per Kilobase of transcript per Million mapped reads. FPKM is a simple

expression level normalization method, which normalizes read count based on gene length and the total number of mapped reads), indicating that the controlling transcription factor determining cucurbitacin synthesis (encoded by ClCG01G003370) is expressed in the bitter Hawkesbury watermelon and not in the sweet variety.

5 In order to allow for the determination of genes responsible for the enzymatic steps leading to cucurbitacin E, the predominant cucurbitacin in bitter watermelon, gene expression was analyzed using the technique of RNAseq. Gene expression was studied in the fruit of the two watermelon lines (sweet Hawkesbury PI636601 and a bitter Hawkesbury mutant PI673137). In addition, the two lines were crossed, hybrid F1 plants were self-pollinated, F2 populations from the cross were
10 grown and fruit were sampled at ~30 days following anthesis. Fruit samples were analyzed for cucurbitacin levels. In brief, cucurbitacins are extracted from the plant tissue in methanol, which is passed through a 45 micron filter and analyzed by HPLC, as described in Itkin, M. et al 2016. *Proceedings of the National Academy of Sciences*, 113(47), pp.E7619-E7628, or in Matsuo, K. et al, 1999. *Journal of agricultural and food chemistry*, 47(7), pp.2755-2759.

15 Fruit from F2 plants with cucurbitacin E levels of >50 micrograms/gram fresh weight ("High Cucurbitacin") and fruit from F2 plants with no detectable cucurbitacin E levels ("Low Cucurbitacin") were chosen for extraction of RNA, as well as fruit of the two parental lines. Fruit from at least 6 segregants each were sampled for RNA. Samples of fruit used for the gene expression analysis comprised the following:

- 20 1. Fruit pulp tissue of the Hawkesbury watermelon (PI635601) at the developmental stages of anthesis, as well as about 30 days after anthesis;
2. Fruit pulp tissue of the bitter Hawkesbury watermelon (PI673173) at the developmental stages of anthesis, as well as about 30 days after anthesis;
3. Fruit pulp tissue of non-bitter F2 segregants, about 30 days after anthesis;
- 25 4. Fruit pulp tissue of bitter F2 segregants, about 30 days after anthesis, and
5. In addition, anthesis stage fruit of a wild species of bitter watermelon *Citrullus amarus* (PI 596653) was also sampled.

RNA was extracted using the Spectrum™ Plant RNA extraction kit (Merck, product #STRN50) and RNAseq library preparation, and Illumina sequencing was performed by MacroGen
30 Inc. (Seoul, South Korea). Analysis of the sequenced reads was performed using standard bioinformatics tools and reads were aligned to two watermelon reference genomes, Watermelon Charleston Gray genome v2.5 reference, listed in Table 2 as ClCG#, and to the Watermelon 97103 genome v2.5 reference, listed in table 2 as Cla# (both can be retrieved at [www\(dot\)cucurbitgenomics\(dot\)org/v2/](http://www(dot)cucurbitgenomics(dot)org/v2/)). In certain cases the gene sequences listed in the two

reference genomes are not identical and the actual listed sequences are the product of manual annotation, conducted in consideration of the RNA sequences derived from the gene expression analysis.

The extensive transcriptome analysis of cucurbitacin-accumulating bitter watermelon fruit, compared to non-bitter cultivated watermelon fruit allowed for the selection of a very limited number of candidate genes responsible for the additional hydroxylations, oxygenations, dehydrogenations and acetylations that characterize the molecular decorations that distinguish the cucurbitacins from the less decorated mogrol, precursor of sweet mogrosides. These candidates were selected from large gene families present in the watermelon genome and are presented in Table 4, together with the short annotation of the predicted gene function, along with the gene expression levels (FPKM) in the different non-bitter and bitter fruit, listed above.

Based on the differential expression between the non-bitter and bitter watermelon fruit (Table 4), only 21 genes coding for the cytochrome P450 enzymes were selected from a family size of over 200 annotated cyp450 genes in the watermelon genome. Similarly, 3 oxygenase genes were selected from a family size of over 30, and the oxido-reductase gene families including the dehydrogenases and related enzyme families comprise over 600 members, on the basis of differential expression (see Table 4, bolded candidate genes). In addition some candidate genes were selected on the basis of their general expression, and not differential expression between the non-bitter and bitter watermelon fruit. Chromosomal location, either within metabolic clusters or tandemly localized to other candidate genes, also served as a basis for selection of a few candidate genes. Basis for selection of each gene is denoted in Table 4 "Comments": Genes selected based on differential expression are bolded and categorized as "expressed, higher in bitter tissue". Genes selected based on general expression are italicized and categorized as "expressed, possible housekeeping enzyme contributing to cucurbitacins". Genes selected based on chromosomal location are either underlined and categorized as "part of a possible metabolic cluster of genes", or capitalized and categorized as "not expressed but tandem to candidate gene".

Using this strategy, a limited and focused list of candidates for functional characterization and selection for silencing, representing approximately 5% of the likely gene families involved in the pathway, and ~0.1% of the genes in the watermelon genome (Table 4) was constructed.

Table 4:

			RNA expression (FPKM) low cucurbitacin			RNA expression (FPKM) high cucurbitacin			
			Haw kesb ury mids ize	Non - bitte r F2 sesr egan ts mid size	Haw kesb ury anth esis	Bitte r Haw kesb ury mids ize	Bitt er F2 segr egan ts mid size	Bitte r Haw kesb ury anth esis	C. ama rus anth esis
Water melon gene	annotation	Comments	1	avg 7,8	36 haw k	4	avg 7,8	56 al9	29 ama rus
CICG01G 003400	Cytochrom e P450	expressed, higher in bitter tissue	0	0	2	1	0	11	7
CICG01G 003700	Cytochrom e P450	expressed, higher in bitter tissue	0	0	1	6	6	0	3
CICG01G 014540	Cytochrom e P450	expressed, higher in bitter tissue	0	2	0	406	254	55	74
CLCG01 G014560	Cytochrom e P450	NOT EXPRESSED BUT TANDEM TO CANDIDATE GENE	0	0	0	1	1	1	0
<i>CICG01G 017130</i>	Cytochrom e P450	<i>expressed, possible housekeeping enzyme contributing to cucurbitacins</i>	130	95	70	101	97	13	22
<i>CICG01G 024550</i>	Cytochrom e P450	<i>expressed, possible housekeeping enzyme contributing to cucurbitacins</i>	3	3	22	13	8	45	70
CICG02G 019010	Cytochrom e P450	expressed, higher in bitter tissue	4	19	182	9	108	508	310

<i>CICG05G</i> 019420	Cytochrom e P450	<i>expressed, possible housekeeping enzyme contributing to cucurbitacins</i>	78	49	38	27	30	20	18
CICG05G 019890	Cytochrom e P450	expressed, higher in bitter tissue	1	7	2	8	25	1	8
CICG06G 001570	Cytochrom e P450	expressed, higher in bitter tissue	0	1	5	193	68	59	387
CICG06G 001580	Cytochrom e P450	expressed, higher in bitter tissue	0	1	52	358	97	318	691
CICG06G 001590	Cytochrom e P450	expressed, higher in bitter tissue	0	0	6	65	31	38	89
CICG06G 001620	Cytochrom e P450	expressed, higher in bitter tissue	0	2	0	517	388	25	336
CICG09G 012200	Cytochrom e P450	expressed, higher in bitter tissue	0	6	0	0	19	0	0
<i>CICG09G</i> 012210	Cytochrom e P450	NOT EXPRESSED BUT TANDEM TO CANDIDATE GENE	0	0	0	0	0	0	0
CICG10G 005830	Cytochrom e P450	expressed, higher in bitter tissue	0	0	0	0	50	0	0
CICG10G 012530	Cytochrom e P450	expressed, higher in bitter tissue	0	0	1	17	4	7	21
<i>CICG03G</i> 015220	Cytochrom e P450	NOT EXPRESSED BUT TANDEM TO CANDIDATE GENE	0	0	0	0	0	0	0
<i>CICG10G</i> 015150	Cytochrom e P450	<i>expressed, possible housekeeping enzyme contributing to cucurbitacins</i>	24	23	23	11	22	13	3
<i>CICG11G</i> 002820	Cytochrom e P450	<i>expressed, possible housekeeping enzyme contributing to cucurbitacins</i>	1	1	6	0	3	9	6

<i>CICG11G</i> <i>017020</i>	Cytochrome P450	<i>expressed, possible housekeeping enzyme contributing to cucurbitacins</i>	25	23	20	18	18	17	28
<i>CICG01G</i> <i>014610</i>	oxygenase	<u>part of possible metabolic cluster of genes</u>	0	0	0	0	0	0	0
<i>CICG01G</i> <i>024160a</i>	oxygenase	<i>expressed, possible housekeeping enzyme contributing to cucurbitacins</i>	7	6	30	7	7	14	14
<i>CICG01G</i> <i>024160b</i>	oxygenase	<i>expressed, possible housekeeping enzyme contributing to cucurbitacins</i>	7	6	30	7	7	14	14
DEHYDROGENASE-OXIDOREDUCTASE FAMILY (<i>italics</i>)									
<i>CICG09G</i> <i>009760</i>	<i>Alcohol dehydrogenase</i>	expressed, higher in bitter tissue	47	71	270	159	243	22	58
<i>CICG01G</i> <i>014590</i>	<i>Alcohol dehydrogenase</i>	<i>expressed, possible housekeeping enzyme contributing to cucurbitacins</i>	13	10	12	5	13	14	19
<i>CICG01G</i> <i>018250</i>	<i>Alcohol dehydrogenase,</i>	expressed, higher in bitter tissue	6	12	43	3	122	66	35
<i>CICG09G</i> <i>009780</i>	<i>Alcohol dehydrogenase,</i>	<i>expressed, possible housekeeping enzyme contributing to cucurbitacins</i>	185	204	369	354	164	30	63
<i>CICG03G</i> <i>002490</i>	<i>FAD- binding Berberine family</i>	expressed, higher in bitter tissue	0	0	15	45	27	122	192
<i>CICG01G</i> <i>014570</i>	<i>NAD(P)- binding</i>	expressed, higher in bitter tissue	7	11	2	101	55	58	215

	Rossmann-fold								
CICG09G 004750	NAD(P)-binding Rossmann-fold	expressed, higher in bitter tissue	0	1	1	9	1	1	4
CICG01G 017400	Peroxidase	expressed, higher in bitter tissue	19	26	192	159	181	104	137
CICG02G 023760	Peroxidase	expressed, higher in bitter tissue	0	1	25	16	6	235	84
CICG02G 023770	Peroxidase	expressed, higher in bitter tissue	0	2	24	24	17	8	10
CICG02G 023780	Peroxidase	expressed, higher in bitter tissue	4	1	235	77	13	58	63
CICG03G 004740	Peroxidase	expressed, higher in bitter tissue	7	42	410	59	113	343	1226
CICG07G 007250	Polyketide dehydrase	expressed, higher in bitter tissue	0	5	0	431	134	4	0
CICG11G 005180	Proline dehydrogenase	expressed, higher in bitter tissue	0	3	3	2	9	6	5
CICG03G 016930	Short-chain dehydrogenase/reductase	expressed, possible housekeeping enzyme contributing to cucurbitacins	2	127	8	10	162	9	3
CICG01G 014530	HXXXXD-type acyl-transferase-like	expressed, higher in bitter tissue	0	1	0	60	35	16	36
CICG05G 020040	HXXXXD-type acyl-transferase-like	expressed, possible housekeeping enzyme contributing to cucurbitacins	35	9	6	18	10	6	3
CICG06G 001610	HXXXXD-type acyl-	expressed, higher in bitter tissue	5	1	2	112	50	30	167

	transferase-like								
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Notes:

“Watermelon gene” refers to candidate cucurbitacin biosynthetic pathway genes according to the Watermelon Charleston Gray genome, v2.5, available at the “cucurbitgenomics(dot)org” website.

“RNA expression (FPKM)” refers to the relative abundance of transcripts from each of the watermelon genes in the fruit pulp from anthesis stage (“anthesis”) or anthesis + 30 day (“mid-size”) of the low cucurbitacin (non-bitter) and high cucurbitacin (bitter) Hawkesbury melon fruit, as well as FPKM from anthesis + 30 days from bitter and non-bitter F2 (cross between the bitter- and non-bitter Hawkesbury watermelons) segregants, and from the anthesis stage of the wild, bitter-fruited watermelon *C. amarus*.

For example, the expression pattern of C1CG01G014540, encoding a cytochrome P450 candidate clearly shows that it is not expressed at detectable levels in the non-bitter parent or in the non-bitter F2 segregants, but is highly expressed in the bitter parent, the bitter F2 segregants and in the bitter wild watermelon *C. amarus*. Thus, using this approach, genes likely to be good candidates for downregulation of cucurbitacin biosynthesis enzymes can be identified.

Example 2: MODIFICATION OF CUCURBITACIN BIOSYNTHETIC PATHWAY GENES

Cucurbitacin biosynthetic pathway gene modification was investigated using different methodologies for determination of phenotype, specifically, gene function and the effect on metabolite accumulation:

a. Agrobacterium rhizogenes transformation of a cucurbit variety having high cucurbitacin accumulation in the roots leading to the formation of transgenically modified hairy roots. This may be for example the bitter watermelon PI673137 but may also include other watermelon varieties with non-bitter fruit (since cucurbitacins may accumulate in their root tissue). The control, unmodified hairy roots accumulate cucurbitacin E, the hexa-oxygenated, 25-acetylated cucurbitane compound (see Table in Figure 1).

b. Agrobacterium tumefaciens transformation of the high cucurbitacin accumulating variety (for example the bitter watermelon PI673137), leading to the formation of transgenically modified watermelon plants. Analysis of the cucurbitacins components show modification in

transgenic fruit, roots and other plant tissues in which the control non-transgenic plants and their respective tissues accumulate natural cucurbitacins.

- c. Candidate gene function can also be determined by overexpression of the candidate genes in a novel yeast line engineered to synthesize the precursor cucurbitadienol (see Figure 5). This yeast line and the identical strategy was successful in identifying the C11 Cyp450 hydroxylase of *Siratia grosvenorii* in Itkin et al., 2016, and is described in Davidovich-Rikenati et al., Yeast, 2015). Briefly, yeast cells are transformed with plasmids that express cucurbitadienol and a gene of interest. Single transformed yeast colonies are grown in SC -URA medium. After 2% w/v galactose induction for 2 days, cells are collected and the pellet is disrupted with hot 20% w/v KOH and 50% v/v EtOH and extracted twice with a similar volume of n-hexane. The hexane extract is evaporated and resuspended in 1 ml MeOH for analysis of the cucurbitadienol product, using a LC-TOF-APCIMS.

Hairy Root Culture:

- Decoated Watermelon (*Citrullus lanatus*) seeds were sterilized in a 50-ml falcon tube by 2% Sodium hypochlorate and shaken for 20 min. Seeds were then rinsed 3-4 time in ddH₂O. The wet seeds were placed on a sterile petri dish in a sterile bench to surface dry. Air dried seeds were placed on plates with ½ Murashige and Skoog media (MS) agar supplemented with 1.5% sucrose, sealed with parafilm (Merck KGaA, Darmstadt, Germany) and placed in a controlled climate chamber: light at 79 uE, 25°C, 80% humidity, 16-hour photoperiod for 10 days.

- Rhizobium rhizogenes* strain K599 are transformed with the plasmid containing the relevant silencing construct through electroporation and recovered on LB medium for 4 hours at 28°C before being plated on LB agar supplemented with spectinomycin (50 µg/ml). In the example described below the silencing construct for cucurbitadienol synthase was included in the following plasmid pOmega1:ClCG06g001600-RFP. A single colony was selected for each transformation and grown overnight in liquid LB, supplemented with spectinomycin at 28°C under constant agitation (240 rpm). Subsequently, 50 mL of the *R. rhizogenes* suspension was placed on solid YEB medium supplemented with spectinomycin, and grown for two days at 28°C and colonies resuspended into MS buffer supplemented with acetosyringone to a concentration of 100 mM. Finally the OD600 of the suspension was adjusted to 0.6.

Watermelon cotyledons were transformed with *R. rhizogenes* ten days after germination. Cotyledons were inoculated by bruising with a sterile syringe needle dipped in the *R. rhizogenes* suspension. Four incisions were made on the abaxial side of the cotyledon perpendicular to the central vein and were placed with the abaxial side down onto ½ MS (3% sucrose) agar plates

without antibiotics and incubated in the dark for two days. Afterwards, the inoculated cotyledons were transferred to ½ MS (3% sucrose) agar plates supplemented with cefotaxim (400 µg/ml) and kanamycin (100 µg/ml) and incubated under light for a week. Cotyledons were subsequently transferred to new plates where cefotaxim concentrations were halved.

5 Transformed hairy roots expressing mRFP would start to emerge after three weeks of tissue culture. These roots were excised from the cotyledons and subcultured every two weeks with cefotaxim and kanamycin. The concentrations of cefotaxim were reduced 50% in each subculturing step until no antibiotic was used, nevertheless kanamycin concentration remained constant.

Results: Each of the 42 genes are silenced using siRNA strategy followed by measurement
10 of accumulated metabolites. siRNA constructs were developed using the hairpin silencing strategy and constructed with standard GoldenBraid technology, (as described in Fusaro et al, EMBO Rep. 2006). Briefly, this method uses Type IIS restriction enzymes and T4 DNA ligase to assemble multiple DNA sequences in a single reaction. Type II restriction enzymes such as BsaI, BsmBI cut outside of their recognition site, creating unique non-palindromic overhangs. DNA fragments can
15 be assembled in specific order where fragments with complementary overhangs will ligate. The siRNA sequence was constructed by ligating a promoter sequence to an approximately 300 bp sequence corresponding to a section of the gene intended for silencing, followed by an intron sequence followed by a sequence which is a reverse complement of the gene fragment used before the intron. Gene specific target sequences were synthesized, based on the coding sequence of the
20 genes as listed in SEQ ID NOs. 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 43, 46, 49, 52, 55, 58, 61, 64, 67, 70, 73, 76, 79, 82, 85, 88, 91, 94, 97, 100, 103, 106, 112, 115, 118, 121 and 124, corresponding to the sequences listed in Table 3 herein.

Example 3: Use of hairy root culture to modify cucurbitacin production: silencing of 25 cucurbitadienol synthase

In order to develop the protocol for candidate gene silencing in bitter watermelon hairy roots the effect of silencing the first committed step of cucurbitadienol synthesis was tested, carried out by the enzyme cucurbitadienol synthase, and coded by C1CG06g001600 (SEQ ID NO: 150). The silencing construct comprised the unique gene target sequence (SEQ ID NO: 152), and the
30 *Agrobacterium rhizogenes* was constructed as described above. Hairy roots were cultivated essentially as described hereinabove and following ~6 weeks of growth roots were extracted in methanol and cucurbitacin levels were analyzed by LCMS, as described in Itkin et al., (2016)

Employing the hairy root culture assay, modification of the cucurbitacin biosynthesis pathway was observed. By silencing the gene for the cucurbitadienol synthase (C1CG06g001600),

significantly reduced cucurbitacin levels accumulated in the transgenic hairy roots (Fig. 10), confirming the efficacy of the hairy root culture for investigating cucurbitacin biosynthetic pathway gene silencing.

5 **Example 4: Use of hairy root culture to modify cucurbitacin production: silencing of candidate acetyltransferase gene**

10 The silencing of a candidate acetyltransferase gene (CICG06g001610, SEQ ID NO: 124), using the unique target gene sequence SEQ ID NO: 126, resulted in the appearance of previously undetected cucurbitacin I, the non-acetylated form of cucurbitacin E (Figures 7A-7D and 12A-12E).

Example 5: Use of hairy root culture to modify cucurbitacin production: silencing of candidate dehydrogenase genes

15 The silencing of the candidate alcohol dehydrogenase genes (CICG01g018250, SEQ ID NO: 79; CICG03G002490, SEQ ID NO: 85 and C1CG09G009760, SEQ ID NO: 73), using the unique target gene sequences SEQ ID NO: 81, SEQ ID NO: 87 and SEQ ID NO: 75, respectively, resulted in the enhancement of a previously poorly represented product (C32H46O8), corresponding to a cucurbitacin with 2 additional hydrogen atoms and coeluting with cucurbitacin B (Figures 13B-13H), which is a hydrogenated form of cucurbitacin E.

20

Example 6: Use of hairy root culture to modify cucurbitacin production: silencing of candidate cytochrome P450 genes

25 The silencing of the candidate cytochrome P450 genes (CICG01G014540, SEQ ID NO: 7; CICG06G001580, SEQ ID NO: 31; CICG06G001590, SEQ ID NO: 34; CICG06G001620, SEQ ID NO: 37 and CICG10G012530, SEQ ID NO: 49), using the unique target gene sequences SEQ ID NO: 9, SEQ ID NO: 33, SEQ ID NO: 36, SEQ ID NO: 39 and SEQ ID NO: 51, respectively, resulted in the appearance of previously poorly represented cucurbitacin compounds with modified mass, indicating a loss of oxygen atoms (putative molecular formulas are C30H46O5, C30H46O3, C30H44O3 and C30H46O2) (Figures 14B-14L).

30

Example 7: Use of yeast to test function of a cytochrome P450 gene

 The overexpression of a candidate cytochrome P450 gene (CICG06G001570, SEQ ID NO: 28) in the cucurbitadienol-producing yeast strain led to the production of oxygenated

cucurbitadienol products whose mass spectrum indicated putative molecular formulas C₃₀H₅₀O₂ and C₃₀H₄₈O₃ (Figs. 15A-15F).

Example 8: Modification of cucurbitacin production in bitter watermelon

5 Using the silencing methodologies proven effective for cucurbitacin biosynthetic pathway genes in the hairy root model, watermelon varieties accumulating cucurbitacin are transformed with silencing constructs targeting cucurbitacin biosynthetic pathway genes such as, inter alia, the cytochrome P450 genes, the dehydrogenase-oxidoreductase family genes (alcohol dehydrogenases, FAD-binding Berberine enzyme family genes, NAD(P)-binding Rossmann-fold enzyme genes, peroxidases, polyketide dehydrase enzyme genes, proline dehydrogenase genes and
10 short-chain dehydrogenase/reductase genes) and HXXXD-type acyltransferase-like enzyme genes.

The gene silencing can be achieved by production of transgenic watermelon fruit, via *Agrobacterium tumefaciens* or other transformation technologies of wounded plant tissue with constructs comprising siRNA sequences based on coding sequence of genes as listed in SEQ ID
15 NOs. 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 43, 46, 49, 52, 55, 58, 61, 64, 67, 70, 73, 76, 79, 82, 85, 88, 91, 94, 97, 100, 103, 106, 112, 115, 118, 121 and 124, corresponding to the sequences listed in Table 3 herein, as described hereinabove. Furthermore other silencing technologies including genome editing strategies may be used in order to silence expression of the target genes.

20 Gene silencing through CRISPR technology is achieved by designing guide RNAs against the genes of interest. Guide RNAs are designed to target regions within the promotor or coding sequence of the genes. Plasmids containing the appropriate guide RNA and Cas9 gene are transformed into watermelon plants in varying combinations. RNA-seq of the transformed tissue is performed in order to validate the silencing of the genes of interest.

25 Initial efforts are directed to silencing of the cucurbitacin biosynthetic pathway genes that have been successfully silenced in watermelon tissue using the hairy root model system. Thus, initially, silencing constructs are designed targeting watermelon cytochrome P450 genes C1CG01G014540 (SEQ ID NO: 7), C1CG06G001570 (SEQ ID NO: 28), C1CG06G001580 (SEQ ID NO: 31), C1CG06G001590 (SEQ ID NO: 34), CLCG06G001620 (SEQ ID NO: 37) and
30 C1CG10G012530 (SEQ ID NO: 49), watermelon alcohol dehydrogenase genes C1CG09G009760 (SEQ ID NO: 73) and C1CG03G002490 (SEQ ID NO: 79), watermelon FAD-binding Berberene family gene C1CG03G002490 (SEQ ID NO: 85), NAD(P)-binding Rossmann-fold gene C1CG01G014570 (SEQ ID NO: 88) and the HXXXD-type acetyl-transferase-like gene C1CG06G001610 (SEQ ID NO: 124).

Following transformation, the watermelon tissue is cultivated, and analyzed to identify modifications in the function of cucurbitacin biosynthetic pathway genes (e.g. chromatography and spectroscopy of tissue extracts) and characterization of the cucurbitacin and cucurbitacin-derived compound profile.

5 Successfully transformed watermelon tissue is then cultured to provide candidate plants and
fruits maintaining the gene silencing and displaying a modified cucurbitacin and cucurbitacin-
derived compound profile.

Plant tissue with desired modified cucurbitacin biosynthesis pathway gene function is then further modified, with one or more additional silencing constructs, and the effects of multiple cucurbitacin biosynthesis pathway gene silencings are evaluated to identify modified cucurbitacin and cucurbitacin-derived compound profiles providing potential substrates for glucosylation by UGT enzymes.

15 **Example 9: Modification of cucurbitacin production in other cucurbits and non-cucurbit plants**

Silencing constructs are designed against the orthologs of the genes of interest listed in Table 2 in species other than *Citrullus lanatus*. Examples of orthologs from *Cucumis melo*, *Cucumis sativus* and *Cucurbita pepo* listed in Table 2a are used for the design of silencing constructs for these species. Silencing in other species can be achieved using methodologies as in example 8, i.e. transformation via *A. tumefaciens* or other transformation technologies, and/or gene editing.

Thus, the results provided herein show that key cucurbitacin biosynthetic pathway genes can be identified, and downregulated by the methods of the invention, leading to modification of the tetracyclic triterpene profile of the cucurbit plant or plant cell with reduced expression of the cucurbitacin biosynthetic pathway gene(s). Silencing of key functional genes of the cucurbitacin biosynthetic pathway can provide tools for the modification of the tetracyclic triterpenoid molecules, producing novel substrates available for multiple glucosylations.

It is the intent of the applicant(s) that all publications, patents and patent applications referred to in this specification are to be incorporated in their entirety by reference into the specification, as if each individual publication, patent or patent application was specifically and individually noted when referenced that it is to be incorporated herein by reference. In addition, citation or identification of any reference in this application shall not be construed as an admission that such reference is available as prior art to the present invention. To the extent that section headings are

used, they should not be construed as necessarily limiting. In addition, any priority document(s) of this application is/are hereby incorporated herein by reference in its/their entirety.

WHAT IS CLAIMED IS:

1. A method of producing a plant or plant cell with a modified cucurbitacin content, the method comprising down-regulating expression of at least one cucurbitacin biosynthetic pathway gene in the plant or plant cell, thereby modifying cucurbitacin expression in said plant or plant cell.
2. A method of producing a plant or plant cell with a modified cucurbitacin content, the method comprising growing the plant or plant cell of claim 1 with modified expression of the at least one cucurbitacin biosynthetic pathway gene.
3. The method of claim 1 or 2, wherein said down-regulation is by genome editing.
4. A plant or plant cell modified to have reduced expression of at least one gene of the cucurbitacin biosynthetic pathway, wherein said cucurbit plant or plant cell is obtainable according to the method of claim 1.
5. The plant or plant cell of claim 4, wherein said plant or plant cell is an elite plant or plant cell.
6. The plant or plant cell of claim 4, wherein said plant or plant cell is a hybrid plant or plant cell.
7. The plant or plant cell of claim 4, wherein said plant or plant cell is an inbred plant or plant cell.
8. An inbred plant or plant cell having a nucleic acid sequence alteration of at least one gene of the cucurbitacin biosynthetic pathway.
9. An elite plant or plant cell having a nucleic acid sequence alteration of at least one gene of the cucurbitacin biosynthetic pathway.
10. A hybrid plant or plant cell having a nucleic acid sequence alteration of at least one gene of the cucurbitacin biosynthetic pathway.

11. The plant or plant cell of any one of claims 4-10, wherein said plant or plant cell comprises at least one tetracyclic triterpene capable of glucosylation by a UDP-glucuronosyltransferase (UGT).

12. The plant or plant cell of claim 11, wherein said UGT is a plant UGT.

13. The plant or plant cell of claim 11, wherein said UGT is selected from the group consisting of *S. grosvenorii* UGTs selected from the group consisting of UGT74-345-2, UGT73-348-2, UGT94-289-1, UGT73-327-2, UGT73-251-5, UGT73-251-6, UGT75-281-2, UGT85-269-4, UGT85-269-1, UGT94-289-2 and UGT94-289-3.

14. The plant or plant cell of claim 11, wherein said UGT is a UGT having an amino acid sequence selected from the group consisting of SEQ ID NOs. 139, 140, 141, 142, 143, 144, 145, 146, 147, 148 and 149.

15. The plant or plant cell of claim 11, wherein said UGT is a UGT encoded by a polynucleotide having a nucleotide sequence selected from the group consisting of SEQ ID NOs. 128, 129, 130, 131, 132, 133, 135 and 137.

16. An extract of the plant or plant cell of any one of claims 4-11, comprising at least one tetracyclic triterpene capable of glucosylation by a UGT.

17. The method of any one of claims 1-3, or plant or plant cell of any one of any one of claims 4-15, or the extract of claim 16, wherein said plant or plant cell is of a bitter cucurbit species.

18. The method of any one of claims 1-3, or plant or plant cell of any one of any one of claims 4-15, or the extract of claim 16, wherein said plant or plant cell is of a species naturally expressing said at least one cucurbitacin biosynthetic pathway gene.

19. The method of any one of claims 1-3, or plant or plant cell of any one of any one of claims 4-15, or the extract of claim 16, wherein said plant or plant cell is of a species genetically modified to express said at least one cucurbitacin biosynthetic pathway gene.

20. The method of any one of claims 1-3, or plant or plant cell of any one of any one of claims 4-15, or the extract of claim 16, wherein said plant or plant cell is an *Iberis amara* plant or plant cell.

21. The method of any one of claims 1-3, or plant or plant cell of any one of any one of claims 4-15, or the extract of claim 16, wherein said plant is selected from the group consisting of cultivated bitter cucurbits and non-cultivated cucurbits.

22. The method or plant or plant cell of claim 21, wherein said cultivated bitter cucurbit is *Citrullus vulgaris* (bitter Hawkesbury), *Cucumis sativus* or *Cucurbita pepo*.

23. The method or plant or plant cell of claim 21, wherein said non-cultivated bitter cucurbit is selected from the group consisting of non-cultivated bitter melon, non-cultivated bitter cucumber and non-cultivated bitter watermelon.

24. The method or plant or plant cell of claim 17, wherein said non-cultivated bitter cucurbit is selected from the group consisting of *Cucumis* species, *Citrullis* species, *Momordica* species and *Cucurbita* species.

25. The method of any one of claims 1-3, plant or plant cell of any one of any one of claims 4-15 and 21-24, or extract of claim 16, wherein said at least one cucurbitacin biosynthetic pathway gene is a gene selected from the genes of Table 2.

26. The method of any one of claims 1-3, plant or plant cell of any one of any one of claims 4-15 and 21-25, or extract of claim 16, wherein said at least one cucurbitacin biosynthetic pathway gene is a (2OG) and Fe(II)-dependent oxygenase gene.

27. The method or plant or plant cell of claim 26, wherein said (2OG) and Fe(II)-dependent oxygenase gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 64, 67 and 70.

28. The method or plant or plant cell of claim 26, wherein said down-regulation of said (2OG) and Fe(II)-dependent oxygenase gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 66, 69 and 72.

29. The method of any one of claims 1-3, plant or plant cell of any one of any one of claims 4-15 and 21-24, or extract of claim 16, wherein said at least one cucurbitacin biosynthetic pathway gene is a cytochrome P450 gene.

30. The method or plant or plant cell of claim 29, wherein said cytochrome p450 gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 1, 4, 7, 10, 13, 16, 19, 22, 25, 28, 31, 34, 37, 40, 43, 46, 49, 52, 55, 58 and 61.

31. The method or plant or plant cell of claim 29, wherein said cytochrome p450 gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 7, 28, 31, 34, 37 and 49.

32. The method or plant or plant cell of claim 29, wherein said down-regulation of said cytochrome p450 gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, 33, 36, 39, 42, 45, 48, 51, 54, 57, 60 and 63.

33. The method or plant or plant cell of claim 29, wherein said down-regulation of said cytochrome p450 gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 9, 30, 33, 36, 39 and 51.

34. The method of any one of claims 1-3, plant or plant cell of any one of any one of claims 4-15 and 21-24, or extract of claim 16, wherein said at least one cucurbitacin biosynthetic pathway gene is an FAD-binding Berberine gene.

35. The method or plant or plant cell of claim 34, wherein said FAD-binding Berberine gene has the nucleic acid sequence as set forth in SEQ ID NO: 85.

36. The method or plant or plant cell of claim 34, wherein said down-regulation of said FAD-binding Berberine gene is effected by targeting the nucleic acid sequence selected as set forth in SEQ ID NO: 87.

37. The method of any one of claims 1-3, plant or plant cell of any one of any one of claims 4-15 and 21-24, or extract of claim 16, wherein said at least one cucurbitacin biosynthetic pathway gene is an NAD(P)-binding Rossmann-fold gene.

38. The method or plant or plant cell of claim 37, wherein said NAD(P)-binding Rossmann-fold gene has the nucleic acid sequence as set forth in SEQ ID NO: 88 or 91.

39. The method or plant or plant cell of claim 37, wherein said NAD(P)-binding Rossmann-fold gene has the nucleic acid sequence as set forth in SEQ ID NO: 88.

40. The method or plant or plant cell of claim 37, wherein said down-regulation of said NAD(P)-binding Rossmann-fold gene is effected by targeting the nucleic acid sequence selected as set forth in SEQ ID NO: 91 or 94.

41. The method or plant or plant cell of claim 37, wherein said down-regulation of said NAD(P)-binding Rossmann-fold gene is effected by targeting the nucleic acid sequence selected as set forth in SEQ ID NO: 91.

42. The method of any one of claims 1-3, plant or plant cell of any one of any one of claims 4-15 and 21-24, or extract of claim 16, wherein said at least one cucurbitacin biosynthetic pathway gene is an HXXXD-type acyl-transferase-like protein gene.

43. The method or plant or plant cell of claim 42, wherein said HXXXD-type acyl-transferase-like protein gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 118, 121 and 124.

44. The method or plant or plant cell of claim 42, wherein said HXXXD-type acyl-transferase-like protein gene has the nucleic acid sequence as set forth in SEQ ID NO: 124.

45. The method or plant or plant cell of claim 42, wherein said down-regulation of said HXXXD-type acyl-transferase-like protein gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 120, 123 and 126.

46. The method or plant or plant cell of claim 42, wherein said down-regulation of said HXXXD-type acyl-transferase-like protein gene is effected by targeting a nucleic acid sequence as set forth in SEQ ID NO: 126.

47. The method of any one of claims 1-3, plant or plant cell of any one of any one of claims 4-15 and 21-24, or extract of claim 16, wherein said at least one cucurbitacin biosynthetic pathway gene is a polyketide cyclase/hydrase gene.

48. The method or plant or plant cell of claim 47, wherein said polyketide cyclase/hydrase gene has the nucleic acid sequence as set forth in SEQ ID NO: 109.

49. The method or plant or plant cell of claim 47, wherein said down-regulation of said polyketide cyclase/hydrase gene is effected by targeting the nucleic acid sequence selected as set forth in SEQ ID NO: 111.

50. The method of any one of claims 1-3, plant or plant cell of any one of any one of claims 4-15 and 21-24, or extract of claim 16, wherein said at least one cucurbitacin biosynthetic pathway gene is a proline dehydrogenase gene.

51. The method or plant or plant cell of claim 50, wherein said proline dehydrogenase gene has the nucleic acid sequence as set forth in SEQ ID NO: 112.

52. The method or plant or plant cell of claim 50, wherein said down-regulation of said proline dehydrogenase gene is effected by targeting the nucleic acid sequence selected as set forth in SEQ ID NO: 114.

53. The method of any one of claims 1-3, plant or plant cell of any one of any one of claims 4-15 and 21-24, or extract of claim 16, wherein said at least one cucurbitacin biosynthetic pathway gene is a short-chain dehydrogenase/reductase gene.

54. The method or plant or plant cell of claim 53, wherein said short-chain dehydrogenase/reductase gene has the nucleic acid sequence as set forth in SEQ ID NO: 115.

55. The method or plant or plant cell of claim 53, wherein said down-regulation of said short-chain dehydrogenase/reductase gene is effected by targeting the nucleic acid sequence selected as set forth in SEQ ID NO: 117.

56. The method of any one of claims 1-3, plant or plant cell of any one of any one of claims 4-15 and 21-24, or extract of claim 16, wherein said at least one cucurbitacin biosynthetic pathway gene is an alcohol dehydrogenase gene.

57. The method or plant or plant cell of claim 56, wherein said alcohol dehydrogenase gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 73, 76, 79 and 82.

58. The method or plant or plant cell of claim 56, wherein said alcohol dehydrogenase gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 73 and 79.

59. The method or plant or plant cell of claim 56, wherein said down-regulation of said alcohol dehydrogenase gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 75, 78, 81 and 84.

60. The method or plant or plant cell of claim 56, wherein said down-regulation of said alcohol dehydrogenase gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 75 and 81.

61. The method of any one of claims 1-3, plant or plant cell of any one of any one of claims 4-15 and 21-24, or extract of claim 16, wherein said at least one cucurbitacin biosynthetic pathway gene is a peroxidase gene.

62. The method or plant or plant cell of claim 61, wherein said peroxidase gene has the nucleic acid sequence selected from the group consisting of SEQ ID NOs: 94, 97, 100, 103 and 106.

63. The method or plant or plant cell of claim 61, wherein said down-regulation of said peroxidase gene is effected by targeting a nucleic acid sequence selected from the group consisting of SEQ ID NOs: 96, 99, 102, 105 and 108.

64. A method of producing a glucosylated tetracyclic triterpene, comprising glucosylating the at least one tetracyclic triterpene capable of glucosylation of the plant or plant cell of claim 11 by contacting said at least one tetracyclic triterpene with a UGT, thereby producing a glucosylated tetracyclic triterpene.

65. The method of claim 64, wherein said glucosylated tetracyclic triterpene is a non-bitter glucosylated tetracyclic triterpene.

66. The method of claim 64, wherein said glucosylated tetracyclic triterpene is a mogroside.

67. The method of any one of claims 64-66, wherein said glucosylating is effected in a cell.

68. The method of claim 67, wherein said cell is a plant cell.

69. The method of claim 67, wherein said cell is not a plant cell.

70. The method of any one of claims 64-66, wherein said glucosylating is effected in a cell-free system.

71. The method of any one of claims 64-70, wherein said UGT is a plant UGT.

72. The method of claim 71, wherein said UGT is selected from the group consisting of *S. grosvenorii* UGTs selected from the group consisting of UGT74-345-2, UGT73-348-2, UGT94-289-1, UGT73-327-2, UGT73-251-5, UGT73-251-6, UGT75-281-2, UGT85-269-4, UGT85-269-1, UGT94-289-2 and UGT94-289-3.

73. The method of claim 72, wherein said UGT is a UGT having an amino acid sequence selected from the group consisting of SEQ ID NOs. 139, 140, 141, 142, 143, 144, 145, 146, 147, 148 and 149.

74. The method of claim 71, wherein said UGT is a UGT encoded by a polynucleotide having a nucleotide sequence selected from the group consisting of SEQ ID NOs. 128, 129, 130, 131, 132, 133, 135 and 137.

75. The method of any one of claims 71-74, wherein said UGT is a recombinant UGT.

76. A composition comprising a plant or plant cell having modified cucurbitacin biosynthesis pathway gene expression, the plant or plant cell comprising tetracyclic triterpene capable of glucosylation by a UGT.

77. The composition of claim 76, being enriched in tetracyclic triterpene glucosides.