Title: METHODS OF PRODUCING ARTESININ IN NON-HOST PLANTS AND VECTORS FOR USE IN SAME

Abstract: Provided are methods for generating and/or increasing content of artemisinin in a cell, comprising exogenously expressing within the cell: (i) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,1,1-diene from farnesyl diphasate (FDP); and (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-1(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and (iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,1,1-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,1,1-diene and/or artemisinic alcohol. Also provided are nucleic acid constructs, systems, kits, and cells for generating and/or increasing content of artemisinin in a cell, and plants with increased artemisinin content.
METHODS OF PRODUCING ARTEMISININ IN NON-HOST PLANTS
AND VECTORS FOR USE IN SAME

FIELD AND BACKGROUND OF THE INVENTION

The present invention, in some embodiments thereof, relates to nucleic acid constructs, cells including plant cells, plants and methods of producing same for generating and/or increasing content of artemisinin in a cell including plant cells or a plant and, more particularly, but not exclusively, to artemisinin production in non-host cells including plant cells or plants for the preparation of therapeutic compositions.

The world's most severe infectious disease, malaria, causes more than 500 million infections and approximately 1 million deaths annually, the vast majority of which are of children under the age of 5 in developing countries.

Malaria is caused by protozoan parasites of the genus Plasmodium. Multi-drug-resistant strains in the genus P. falciparum constitute a major threat to controlling the disease. Artemisinins, produced from the traditional Chinese medicinal herb Artemisia annua (sweet wormwood), are the only class of drugs to which there is no known resistance. Unfortunately however, the drug is too expensive for most malaria sufferers in developing countries and is in short supply worldwide.

In the early 1970s, artemisinin was identified as the principle compound in A. annua extract with anti-malarial activity. Its structure was determined to be sesquiterpene lactone with an endoperoxide bridge. In addition to their anti-malarial properties, artemisinins have been recently shown to be cytotoxic for cancer cells [Efferth, T. (2006) Current Drug Targets 7: 407-421]. Artemisinin levels in A. annua are usually in the range of 0.01 to 1 % of total dry weight, which together with the fact that complete chemical synthesis of artemisinin is complex and inefficient at least partially accounts for the drug's high price.

Being an endoperoxide sesquiterpene lactone, artemisinin is a product of the isoprene pathway, one of the main biosynthetic pathways in plants. As illustrated in Figure 1A, the direct precursor of amorpha-4,1 l-diene, the first specific substrate in the biosynthesis of artemisinin, is farnesyl diphosphate (FDP), which is produced by the condensation of two molecules of isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP). In higher plants, these two compounds can be
derived from either the plastid-localized deoxyxylulose-5-phosphate (DXP) pathway or the cytosolic mevalonate pathway (MVA). The former includes seven reactions (catalyzed by seven enzymes) starting with pyruvate and glyceraldehyde 3-phosphate leading to the production of geranyl diphosphate (GDP). The MVA pathway starts with the condensation of acetyl-CoA and includes five steps for the production of IPP, following which DMAPP chain elongation leads to the formation of FDP, the universal precursor for the production of sesquiterpenes. These are generated by a cyclization reaction catalyzed by sesquiterpene synthases, such as amorphadiene synthase (ADS) forming amorpha-4,11-diene from FDP.

Previous studies carried out in A. annua, yielded biochemical information on the downstream reactions leading to artemisinin production from FDP (see Figure 1A). The identity of amorpha-4,11-diene as a biosynthetic intermediate was established with the cloning of the ADS gene. Biochemical evidence suggesting the involvement of amorpha-4,11-diene hydroxylation by a cytochrome P450 monoxygenase led to the molecular cloning and characterization of CYP71AV1, a multifunctional sesquiterpene oxidase that sequentially converts amorphadiene to artemisinic alcohol, artemisinic aldehyde and artemisinic acid. Reduction of artemisinic aldehyde by DBR2 in-vitro and when co-expressed with ADS and CYP71AV1 in yeast was shown to lead to production of dihydroartemisinic acid - the probable immediate precursor of artemisinin in plants [Brown, G. D. (2010) Molecules 15: 7603-7698; Zhang Y. et al. (2008) J. Biol. Chem. 283: 21501-21508]. Also, it was recently suggested that an aldehyde dehydrogenase (ALDH1) is involved in the oxidation of artemisinic and dihydroartemisinic aldehydes to the corresponding acids. Nafis et al. [Nafis et al. (2011) Plant Biotechnology Reports 5(1): 53-60] illustrated the importance of HMG-CoA reductase in artemisinin production. Specifically, they showed that overexpression of the 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR) gene (hmgr) from Catharanthus roseus L. in Artemisia annua L. resulted in higher levels of artemisinin as compared to non-transgenic plants. Several lines of evidence indicate that in Artemisia transformation of dihydroartemisinic acid to artemisinin is non-enzymatic and requires only the presence of light and molecular oxygen.

In view of the low concentrations of artemisinin produced naturally by plants, many attempts have been made to increase its yield. Modifying agricultural conditions
has had little success in raising artemisinin production to any significant extent. Some attempts have been made to breed high drug-yielding lines, but so far, they also have met with limited success. For example, Chen et al. [Chen et al. (2011) Planta Med. 77(15): 1759-65] showed increased artemisinin biosynthesis in transgenic *Artemisia annua* plants transformed with an antisense fragment for downregulation of β-caryophyllene synthase (a sesquiterpene synthase competing as a precursor of artemisinin). Although a genetic map of *A. annua* was recently published some of the main problems are still the long time needed for identifying and mapping genetic loci influencing relevant traits, breeding via crosses and the need for complicated high-throughput chemical screening of plants. One of the main factors hampering improvement in the production of artemisinin is the poor understanding of the regulation of the artemisinin pathway by endogenous *Artemisia* factors. For example, almost nothing is known about the mechanisms controlling spatial and temporal regulation of artemisinin production, which peaks just before and during blooming. A promising alternative is the generation of heterologous metabolically engineered plant hosts for artemisinin production that will allow to broaden the cultivation areas and to improve production yields through the use of better suited plant system, e.g. higher tissue mass per propagation area, shorter production cycles, season independent production etc.

Several attempts were made to produce artemisinin precursors in microbial heterologous hosts by expressing artemisinin pathway genes [Ro D.-K et al. (2006) Nature 440: 940-943; Zhang Y. et al. (2008) J. Biol. Chem. 283: 21501-21508]. These works demonstrated that part of artemisinin pathway (up to dihydroartemisinic acid) can be functionally reconstructed in an alternative host. There are also two reports attempting to produce artemisinin precursors in plants. In one instance the glycoside conjugate of artemisininic acid, a metabolic dead-end product was produced. Also in the second instance no artemisinin was produced and instead artemisinic and dihydroartemisinic alcohols accumulated probably due to strong endogenous reducing activities. As of to date, all these alternative approaches, whether utilizing microbial or plant systems for artemisinin production, allowed only biosynthesis of the precursors [Ro D.-K et al. (2006) supra; Zhang Y. et al. (2008) supra; Covello P. S. (2008)
US 2008261280 provides methods for engineering genetically transformed plants and microalgae for the mevalonate and isoprenoid biosynthetic pathways.

US 2009136925 provides methods for identifying terpenoid regulatory region-regulatory protein associations and for modulating expression of sequences of interest (e.g. a sequence encoding an enzyme involved in artemisinin biosynthesis, e.g., an enzyme such as amorpha-4,11-diene synthase or CYP71AV1).

US 2011300546 provides methods of utilizing the PTS gene and RNA interference of the ADS gene to increase patchouli alcohol content in *Artemisia annua* L. plants.

EP 2204449 provides nucleotide sequences from *Artemisia annua* encoding enzymes in biosynthesis of dihydroartemisinic acid. Specifically, EP 2204449 discloses artemisinic/dihydroartemisinic aldehyde dehydrogenase, DBR1, used in processes to produce dihydroartemisinic aldehyde, dihydroartemisinic acid or artemisinic acid in a host cell.

**SUMMARY OF THE INVENTION**

According to an aspect of some embodiments of the present invention there is provided a method of generating and/or increasing content of artemisinin in a cell, comprising exogenously expressing within the cell: (i) a polynucleotide comprising a nucleic acid sequence encoding amorphaadiene synthase (ADS) which catalyzes formation of amorpha-4,11-diene from farnesyl diphosphate (FDP); and (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and (iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,11-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,11-diene and/or artemisinic alcohol; thereby generating and/or increasing the content of artemisinin in the cell.

According to an aspect of some embodiments of the present invention there is provided a nucleic acid expression construct, comprising: (i) a polynucleotide comprising a nucleic acid sequence encoding amorphaadiene synthase (ADS) which...
catalyzes the formation of amorpha-4,1 l-diene from farnesyl diphosphate (FDP); and (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and (iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,1 l-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,1 l-diene and/or artemisinic alcohol.

According to an aspect of some embodiments of the present invention there is provided a cell comprising a heterologous polynucleotide, wherein the heterologous polynucleotide comprises: (i) a polynucleotide comprising a nucleic acid sequence encoding amorphaadiene synthase (ADS) which catalyzes the formation of amorpha-4,1 l-diene from farnesyl diphosphate (FDP); and (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and (iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,1 l-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,1 l-diene and/or artemisinic alcohol.

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

According to an aspect of some embodiments of the present invention there is provided a method of producing artemisinin, comprising: (a) generating and/or increasing content of the artemisinin in a cell according to the method of some embodiments of the invention, and (b) isolating the artemisinin from the cell, thereby producing the artemisinin.

According to an aspect of some embodiments of the present invention there is provided a method of producing artemisinin, comprising: (a) providing the plant cell of some embodiments of the invention, and (b) isolating the artemisinin from the plant cell, thereby producing the artemisinin.

According to an aspect of some embodiments of the present invention there is provided a method of generating and/or increasing content of artemisinin in a cell (e.g., a plant cell), comprising exogenously expressing within the cell a nucleic acid construct which comprises: (i) a polynucleotide comprising a nucleic acid sequence encoding amorphaadiene synthase (ADS) which is capable of forming amorpha-4,1 l-diene from
farnesyl diphtophosphate (FDP); (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; (iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,1 1-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,1 1-diene and/or amorpha-4,1 1-diene alcohol; (iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450; and (v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGR), thereby generating and/or increasing content of artemisinin in the cell (e.g., in the plant cell).

According to an aspect of some embodiments of the present invention there is provided an isolated artemisinin produced by the method of some embodiments of the invention.

According to an aspect of some embodiments of the present invention there is provided a polynucleotide system comprising: (i) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes formation of amorpha-4,1 1-diene from farnesyl diphtophosphate (FDP); and (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and (iii) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes formation of amorpha-4,1 1-diene from farnesyl diphtophosphate (FDP); and (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and (iii) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes formation of amorpha-4,1 1-diene from farnesyl diphtophosphate (FDP); and (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and (iii) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthas...
monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,11-diene and/or artemisinic alcohol;

According to an aspect of some embodiments of the present invention there is provided a kit comprising the system of any of some embodiments of the invention and instructions for use in transformation of a cell.

According to some embodiments of the invention, the cell is a plant cell.

According to some embodiments of the invention, the method further comprising subjecting the cell to light and/or to oxygen prior to the isolating of the artemisinin.

According to some embodiments of the invention, the method further comprising exogenously expressing within the cell: (iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

According to some embodiments of the invention, the method further comprising exogenously expressing within the cell: (v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

According to some embodiments of the invention, the nucleic acid construct further comprises: (iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

According to some embodiments of the invention, the nucleic acid construct further comprises: (v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

According to some embodiments of the invention, the heterologous polynucleotide further comprises: (iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

According to some embodiments of the invention, the heterologous polynucleotide further comprises: (v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).
According to some embodiments of the invention, at least two of the polynucleotides of (i)-(v) are comprised in a single nucleic acid construct.

According to some embodiments of the invention, polynucleotides (i) and (ii) are comprised in a single nucleic acid construct.

According to some embodiments of the invention, polynucleotides (i) and (iii) are comprised in a single nucleic acid construct.

According to some embodiments of the invention, polynucleotides (ii) and (iii) are comprised in a single nucleic acid construct.

According to some embodiments of the invention, polynucleotides (i)-(iii) are comprised in a single nucleic acid construct.

According to some embodiments of the invention, the polynucleotides wherein (i)-(iv) are comprised in a single nucleic acid construct.

According to some embodiments of the invention, polynucleotides wherein (i)-(v) are comprised in a single nucleic acid construct.

According to some embodiments of the invention, the nucleic acid sequence further comprises a nucleic acid sequence encoding a mitochondrial signal peptide to thereby direct localization of the polypeptide into the mitochondria of the cell.

According to some embodiments of the invention, the nucleic acid sequence comprises the nucleic acid sequence encoding an amorphadiene synthase (ADS).

According to some embodiments of the invention, the nucleic acid construct further comprises: (vi) a polynucleotide comprising a nucleic acid sequence encoding a polypeptide which enables selection of a cell expressing the nucleic acid construct.

According to some embodiments of the invention, the polypeptide which enables selection of a cell expressing the nucleic acid construct comprises a polypeptide which confers antibiotic resistance to a cell expressing the nucleic acid construct.

According to some embodiments of the invention, the polypeptide which confers the antibiotic resistance is jieomycin phosphotransferase II (nptII).

According to some embodiments of the invention, each of the polynucleotides further comprises a promoter sequence for directing expression of the nucleic acid sequence in the cell.
According to some embodiments of the invention, at least two of the promoters are not identical.

According to some embodiments of the invention, each of the polynucleotides further comprises a terminator sequence for controlling expression of the nucleic acid sequence in the cell.

According to some embodiments of the invention, the plant is a tobacco plant.

According to some embodiments of the invention, the plant is selected from the group consisting of tobacco, aspen, tomato, marguerite and lettuce.

According to some embodiments of the invention, the artemisinin is characterized by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) operated in multiple reaction monitoring (MRM) mode and monitoring MRM traces m/z 283.2 → 219 and 283.2 → 265.

According to some embodiments of the invention, the artemisinin is characterized by liquid chromatography-high resolution mass spectrometry (LC-HR-MS) m/z value of 283.1530 Da.

According to some embodiments of the invention, the polynucleotide system of some embodiments of the invention, further comprises: (iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

According to some embodiments of the invention, the polynucleotide system of some embodiments of the invention, further comprises: (v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

According to some embodiments of the invention, the polynucleotide system of some embodiments of the invention, further comprises: (vi) a polynucleotide comprising a nucleic acid sequence encoding a polypeptide which enables selection of a cell expressing the nucleic acid construct.

According to some embodiments of the invention, the nucleic acid construct system of some embodiments of the invention, further comprises: (iv) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.
According to some embodiments of the invention, the nucleic acid construct system of some embodiments of the invention, further comprises: (v) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (\textit{tHMG}).

According to some embodiments of the invention, the nucleic acid construct system of some embodiments of the invention, further comprises: (vi) a polynucleotide comprising a nucleic acid sequence encoding a polypeptide which enables selection of a cell expressing the nucleic acid construct.

According to some embodiments of the invention, the polypeptide which enables selection of a cell expressing the nucleic acid construct comprises a polypeptide which confers antibiotic resistance to a cell expressing the nucleic acid construct.

According to some embodiments of the invention, the polypeptide which confers the antibiotic resistance is tetracycline phosphotransferase II (\textit{nptII}).

Unless otherwise defined, all technical and/or scientific terms used herein have the same 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 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. 1A is a schematic illustration of the mevalonate and proposed artemisinin pathways. Engineered genes are marked in red.
FIG. IB is a schematic illustration of a mega vector comprising the gene constructs assembled to engineer artemisinin production. Arrows indicate genes, boxes indicate promoters and terminators, and the difference between the two constructs CYTART or MITART used to generate transgenic tobacco plants is in the use of ADS or mtADS.

FIG. 2A-J are photographs illustrating subcellular localization of ADS and mtADS. The photographs illustrate transient expression of EGFP fused to ADS (Figures 2A-E) or mtADS (Figures 2F-J) in A. thaliana protoplasts. Transient expression of RFP (for cytosol labeling) and MitoTracker-stained mitochondria are illustrated in red, EGFP fluorescence is illustrated in green, plastid autofluorescence is illustrated in blue. Micrographs of merged figures of RFP (Figure 2E) or MitoTracker (Figure 2J) with EGFP, and Figures 2D and 2I illustrate bright-field.

FIGs. 3A-B are photographs illustrating the expression of tHMG and artemisinin pathway genes in transgenic Nicotiana tabacum plants. Total RNA extracted from plants transformed with pRCS16F[kan][tHMG][CPR][ADS][CYP][DBR2] (Figure 3A, transgenic tobacco plant line 6) and pRCS16F[kan][tHMG][CPR][mtADS][CYP][DBR2] (Figure 3B, transgenic tobacco plant line 7) was analyzed for tHMG, ADS, mtADS, CYP71AV1, CPR and DBR2 by RT-PCR.

FIG. 4A-C illustrate identification of artemisinin in extracts from metabolically engineered tobacco by LC-HR-MS. LC/Orbitrap MS chromatograms of extracts from transgenic CYTART (transgenic tobacco plant line 6) and MITART (transgenic tobacco plant line 7) and control GFP and ADS-non-expressing transgenic tobacco plants. All samples were supplemented with labeled artemisinin (artemisinin-3D). Ion current m/z values of 283.1530 and 286.1733 Da, recorded for artemisinin and artemisinin-3D respectively, are characteristic for these molecules. Ion current m/z value of 283.1530 is shown for control GFP and ADS-non-expressing transgenic tobacco plants (Figure 3C).

FIGs. 5A-D illustrate identification of artemisinin in engineered tobacco extracts by LC-MS/MS. LC/triple quadrupole analysis in the multiple reactions monitoring (MRM) mode of extracts from metabolically engineered CYTART (transgenic tobacco plant line 6), MITART (transgenic tobacco plant line 7) and ADS-non-expressing
tobacco plants. Figure 4D shows pure artemisinin standard. MRM traces m/z 283.2 → 219 are shown in the upper chromatograms and 283.2 → 265 in lower chromatograms.

FIGs. 6A-C illustrate identification of amorpha-4,1 diene in engineered tobacco cell suspension cultures by GC-MS. Single ion 189 m/z chromatograms of dodecane extract from metabolically engineered MITART (Figure 6A) or GFP (Figure 6B) cell suspension cultures and amorpha,4-1,diene standard (Figure 6C). (1) amorpha,4-1,diene; (2-5) oxidized derivatives of amorpha,4,1 diene.

FIG. 7 is a pSAT2A.nosP.tHMG expression plasmid.
FIG. 8 is a pSAT5.1.hspP.CYP expression plasmid.
FIG. 9 is a pSAT4.1A.rbcP.CPR expression plasmid.
FIG. 10 is a pSAT6A.supP.DBR2 expression plasmid.
FIG. 11 is a pSAT5A.35SP.ADS expression plasmid.
FIG. 12 is a pSAT5A.35SP.mtADS expression plasmid.
FIG. 13 is a pSATI.A.ocsAocsP.nptll.ocsT expression plasmid.

DESCRIPTION OF SPECIFIC EMBODIMENTS OF THE INVENTION

The present invention, in some embodiments thereof, relates to polynucleotide systems, nucleic acid constructs, nucleic acid constructs systems, cells such as plant cells, plants and methods of producing same for generating and/or increasing content of artemisinin in a cell including a plant cell or plant and, more particularly, but not exclusively, to artemisinin production in non-host cells including plant cells or plants for the preparation of therapeutic compositions.

The principles and operation of the present invention may be better understood with reference to the drawings and accompanying descriptions.

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 being practiced or carried out in various ways. Also, it is to be understood that the phraseology and terminology employed herein is for the purpose of description and should not be regarded as limiting.
The present inventors have uncovered a method of producing artemisinin, a highly effective compound drug (e.g. anti-malaria compound drug) in plant cells or plants other than *Artemisia annua*, the host plant which naturally produces artemisinin.

Thus, the present inventors have generated novel vectors for expression of the artemisinin pathway genes from *A. annua* in various types of plants and have transformed plant cells and plants using these novel vectors. The present inventors have further shown artemisinin production from transgenic plants, including tobacco plants. The aforementioned validates beyond any doubt the value of the present methods in producing artemisinin from plants.

As is illustrated in the Examples section which follows, the present inventors have constructed vectors for expression of the artemisinin pathway genes from *A. annua* i.e. *ADS, CYP71AV1* and *DBR2*, as well as *CYP71AV1* reducing partner *CPR* (see Figure IB). *ADS* was cloned with *(mtADS)* or without *(ADS)* mitochondrial signal peptide to allow accumulation of the enzyme in the organelle or cytosol. Additionally an N' terminal truncated hydroxymethylglutaryl-CoA (HMG-CoA) reductase (*tHMG*) from yeast was placed into these vectors (Figure IB). The vectors of some embodiments of the invention were used to develop transgenic plants (e.g. *Nicotiana tabacum*) for production of artemisinin. Transgenic tobacco plants generated using the vectors of some embodiments of the invention expressed all artemisinin pathway genes (Figures 3A-B) and authentic artemisinin accumulated in the engineered plants. The identity of artemisinin was confirmed by two different liquid chromatography-mass spectrometry (LC-MS) detection techniques, namely LC-high-resolution-MS (LC-HR-MS, Figures 4A-C) and LC-MS/MS (Figures 5A-D) with multiple reactions monitoring (MRM) mode. These results demonstrate that the biosynthesis of artemisinin can be performed in a plant cell or a plant which does not have the endogenous machinery for biosynthesizing artemisinin. Taken together, the present teachings provide powerful tools in the field of agriculture transgenic technologies for generation of artemisinin producing plants.
Thus, according to an aspect of some embodiments of the invention, there is provided a method of generating and/or increasing content of artemisinin in a cell, comprising exogenously expressing within the cell:

(i) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes formation of amorpha-4,11-diene from farnesyl diphosphate (FDP); and

(ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and

(iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,11-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,11-diene and/or artemisinic alcohol;

thereby generating and/or increasing the content of artemisinin in the cell.

As used herein the term "artemisinin" refers to the compound having a chemical formula of C15H22O5 and a chemical structure as shown in Formula I below, which is highly effective as an anti-malaria drug. The biosynthesis of artemisinin is depicted in Figure 1.

Formula I:

As used herein the phrase "increasing content of artemisinin" refers to at least about 0.1 %, at least about 0.5 %, at least about 1 %, at least about 2 %, at least about 3 %, at least about 4 %, at least about 5 %, at least about 10 %, at least about 15 %, at least about 20 %, at least about 30 %, at least about 40 %, at least about 50 %, at least about 60 %, at least about 70 %, at least about 80 %, increase in the content of artemisinin in the cell (e.g. a plant cell) as compared to a native cell (i.e., a cell not modified with the polynucleotides of the invention, e.g., a non-transformed cell of the same species) which is grown (or cultured) under the same (e.g., identical) growth conditions.
According to some embodiments of the invention, the increase in the content of artemisinin in the cell is compared to (e.g., relative to) the content in native cell grown under the same (e.g., identical) growth conditions.

As used herein the phrase "generating artemisinin" refers to at least upregulating the biosynthesis of artemisinin within a cell.

According to some embodiments of the invention, generating artemisinin refers to producing artemisinin within a cell which does not produce artemisinin when non-transformed to express the exogenous polynucleotide of some embodiments of the invention.

As used herein the phrase "amorphadiene synthase (ADS)" refers to a polypeptide which catalyzes formation of amorpha-4,11-diene from farnesyl diphosphate (FDP), essentially as shown in Figure 1 and described in Example 1 of the Examples section which follows.

Non-limiting examples of coding sequences of amorphadiene synthase catalytic activity are provided in GenBank Accession NOs. Q9AR04.2 (SEQ ID NO: 11 for polypeptide) and GenBank Accession NO. HQ315833.1 (SEQ ID NO: 50 for polynucleotide) from Artemisia annua; GenBank Accession NOs. AEQ63683.1 (SEQ ID NO: 12 for polypeptide) and JF951730.1 (SEQ ID NO: 13 for polynucleotide) from a synthetic construct; and GenBank Accession NOs. AFA34434.1 (SEQ ID NO: 51 for polypeptide) and JQ319661.1 (SEQ ID NO: 52 for polynucleotide).

According to some embodiments of the invention, the polynucleotide comprises a nucleic acid sequence encoding a polypeptide having at least 80 %, at least 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, 99 %, e.g., 100 % sequence homology or identity to the polypeptide set forth in SEQ ID NO: 11 (GenBank Access No. Q9AR04.2), wherein the polypeptide catalyzes the formation of amorpha-4,11-diene from farnesyl diphosphate (FDP).

Homology (e.g., percent homology, identity + similarity) can be determined using any homology comparison software, including for example, the BlastP or TBLASTN software of the National Center of Biotechnology Information (NCBI) such as by using default parameters, when starting from a polypeptide sequence; or the tBLASTX algorithm (available via the NCBI) such as by using default parameters,
which compares the six-frame conceptual translation products of a nucleotide query sequence (both strands) against a protein sequence database.

For example, default parameters for tBLASTX include: Max target sequences: 100; Expected threshold: 10; Word size: 3; Max matches in a query range: 0; Scoring parameters: Matrix - BLOSUM62; filters and masking: Filter - low complexity regions;

According to some embodiments of the invention, the polynucleotide comprises a nucleic acid sequence having at least 80 %, at least 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, 99 %, e.g., 100 % sequence identity to the polynucleotide set forth in SEQ ID NO: 50 (GenBank Accession No. HQ315833.1), wherein the polynucleotide encodes a polypeptide which catalyzes the formation of amorpha-4,11-diene from farnesyl diphosphate (FDP).

According to some embodiments of the invention the nucleic acid sequence further comprises a nucleic acid sequence encoding a mitochondrial signal peptide to thereby direct localization of the polypeptide into the mitochondria of the cell.

Non-limiting examples of mitochondrial signal peptides which can be conjugated to the nucleic acid sequence of some embodiments of the invention (e.g., by recombinant techniques) include Nicotiana plubaginifolia atp2-l gene for mitochondrial ATP synthase: GenBank Access Nos. CAA26620.1/X02868.1 (SEQ ID NOs: 54 and 53), Mitochondrial import receptor subunit TOM20: GenBank Access Nos. NP_198909.1/NM_123458.4 (SEQ ID NOs: 56 and 55), Arabidopsis thaliana 2-oxoglutarate dehydrogenase subunit El: GenBank Access Nos. BAE99494.1/AK227494.1 (SEQ ID NOs: 58 and 57) and Saccharomyces cerevisiae COX4 mitochondrial targeting sequence (SEQ ID NO: 60 for the polypeptide; and SEQ ID NO: 59 for the polynucleotide).

According to some embodiments of the invention the mitochondria signal peptide which is conjugated to nucleic acid sequence of some embodiments of the invention (e.g., to the nucleic acid sequence encoding amorphadiene synthase) is the Saccharomyces cerevisiae COX4 mitochondrial targeting sequence (SEQ ID NO: 60 for the polypeptide; and SEQ ID NO: 59 for the polynucleotide).

According to some embodiments of the invention the nucleic acid sequence encoding the amorphadiene synthase (ADS) further comprises a nucleic acid sequence
encoding a mitochondrial signal peptide to thereby direct localization of the amorphadiene synthase into the mitochondria of the cell.

Thus, as shown in Figures 4A-C and 5A-D and described in Examples 3 and 4 of the Examples section which follows, using a construct which includes the mitochondria signal peptide conjugated to amorphadiene synthase resulted in significantly higher levels of artemisinin in the engineered plant.

As used herewith the phrase "artemisinic aldehyde delta-11(13) reductase (DBR2)" refers to a polypeptide which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde, essentially as shown in Figure 1 and is described in Example 1 of the Examples section which follows.

The coding sequence of artemisinic aldehyde delta-11(13) catalytic activity is provided in GenBank Accession NOs. ACH61780.1 (SEQ ID NO: 14 for polypeptide) and EU704257 (SEQ ID NO: 15 for polynucleotide) from Artemisia annua;

According to some embodiments of the invention, the polynucleotide comprises a nucleic acid sequence encoding a polypeptide having at least 80 %, at least 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, 99 %, e.g., 100 % sequence homology or identity to the polypeptide set forth in SEQ ID NO: 14 (GenBank Accession NO. ACH61780.1), wherein the polypeptide catalyzes the reduction of artemisinic aldehyde to dihydroartemisinic aldehyde.

According to some embodiments of the invention, the polynucleotide comprises a nucleic acid sequence having at least 80 %, at least 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, 99 %, e.g., 100 % sequence identity to the polynucleotide set forth in SEQ ID NO: 15 (GenBank Accession NO. EU704257), wherein the polypeptide catalyzes the reduction of artemisinic aldehyde to dihydroartemisinic aldehyde.

According to some embodiments of the invention the nucleic acid sequence encoding the artemisinic aldehyde delta-11(13) reductase (DBR2) further comprises a nucleic acid sequence encoding a mitochondrial signal peptide to thereby direct localization of the artemisinic aldehyde delta-11(13) reductase (DBR2) into the mitochondria of the cell.
As used herewith the phrase "amorpha-4,1 l-diene monooxygenase (CYP71AV1)" refers to a polypeptide which catalyzes oxidation of amorpha-4,1 l-diene and/or artemisinic alcohol, essentially as shown in Figure 1 and is described in Example 1 of the Examples section which follows.

Non-limiting examples of coding sequences of amorpha-4,1 l-diene monooxygenase (CYP71AV1) catalytic activity are provided in GenBank Accession NOs. ABC41927.1 (SEQ ID NO: 16 for polypeptide) and DQ315671.1 (SEQ ID NO: 17 for polynucleotide) from Artemisia annua; GenBank Accession NO. Q1PS23.1 (SEQ ID NO: 18 for polypeptide) from Artemisia annua; GenBank Accession NOs. ADU25498.1 (SEQ ID NO: 19 for polypeptide) and HQ315834.1 (SEQ ID NO: 20 for polynucleotide) from Artemisia annua; GenBank Accession NOs. ABI31728 (SEQ ID NO: 21 for polypeptide) and DQ872632.1 (SEQ ID NO: 22 for polynucleotide) from Artemisia annua; GenBank Accession NOs. ACF74516.1 (SEQ ID NO: 23 for polypeptide) and EU684540.1 (SEQ ID NO: 24 for polynucleotide) from Artemisia annua; GenBank Accession NOs. ABB82944.1 (SEQ ID NO: 25 for polypeptide) and DQ268763.1 (SEQ ID NO: 26 for polynucleotide) from Artemisia annua; GenBank Accession NOs. AEQ63684 (SEQ ID NO: 27 for polypeptide) and JF951731.1 (SEQ ID NO: 28 for polynucleotide) (synthetic construct).

According to some embodiments of the invention, the polynucleotide comprises a nucleic acid sequence encoding a polypeptide having at least 80 %, at least 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, 99 %, e.g., 100 % sequence homology or identity to the polypeptide set forth in SEQ ID NO: 16 (GenBank Accession NO. ABC41927.1), wherein the polypeptide catalyzes oxidation of amorpha-4,1 l-diene and/or artemisinic alcohol.

According to some embodiments of the invention, the polynucleotide comprises a nucleic acid sequence having at least 80 %, at least 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, 99 %, e.g., 100 % sequence homology or identity to the polynucleotide set forth in SEQ ID NO: 17 (GenBank Accession NO. DQ315671), wherein the polynucleotide encodes a polypeptide which catalyzes the catalyzes oxidation of amorpha-4,1 l-diene and/or artemisinic alcohol.
According to some embodiments of the invention the nucleic acid sequence encoding the amorpha-4,11-diene monooxygenase (CYP71AV1) further comprises a nucleic acid sequence encoding a mitochondrial signal peptide to thereby direct localization of the amorpha-4,11-diene monooxygenase (CYP71AV1) into the mitochondria of the cell.

According to some embodiments of the invention, the method further comprising exogenously expressing within the cell:

(iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

As used herewith the phrase "cytochrome P450 reductase (CPR)" refers to a polypeptide which catalyzes the reduction of cytochrome p450, essentially as shown in Figure 1 and is described in Example 1 of the Examples section which follows.

Cytochrome P450 reductase has been isolated from various organisms such as human, mouse, rat, dog, yeast, Drosophila melanogaster, flies, rabbit, Bos taurus, boar, guinea pig (Cavia porcellus), sea urchin, Pan troglodytes, chimpanzee (Pan troglodytes), Trypanosoma brucei, fungus (Aspergillus nidulans), green alga (Chlamydomonas reinhardtii), Toxoplasma gondii ME49, and Artemisia annua, and the coding sequences of Cytochrome P450 reductase catalytic activity can be found in various databases including the National Center for Biotechnology Information (NCBI). For example, Cytochrome P450 reductase from Homo sapiens is provided in GenBank Accession Nos. NM_000941.2 (SEQ ID NO: 29 for polynucleotide) and NP_000932.3 (SEQ ID NO: 30 for polypeptide); and the sequence of Cytochrome P450 reductase from Artemisia annua is provided in GenBank Accession NOs. ABI98819.1 (SEQ ID NO: 31 for polypeptide) and DQ984181 (SEQ ID NO: 32 for polynucleotide).

According to some embodiments of the invention, the polynucleotide comprises a nucleic acid sequence encoding a polypeptide having at least 80 %, at least 81 %, 82 %, 83 %, 84 %, 85 %, 86 %, 87 %, 88 %, 89 %, 90 %, 91 %, 92 %, 93 %, 94 %, 95 %, 96 %, 97 %, 98 %, 99 %, e.g., 100 % sequence homology or identity to the polypeptide set forth in SEQ ID NO: 31 (GenBank Accession NO. ABI98819.1), wherein the polypeptide catalyzes the reduction of cytochrome p450.

According to some embodiments of the invention, the polynucleotide comprises a nucleic acid sequence having at least 80 %, at least 81 %, 82 %, 83 %, 84 %, 85 %,
According to some embodiments of the invention the nucleic acid sequence encoding the cytochrome P450 reductase (CPR) further comprises a nucleic acid sequence encoding a mitochondrial signal peptide to thereby direct localization of the cytochrome P450 reductase (CPR) into the mitochondria of the cell.

According to some embodiments of the invention, the method further comprising exogenously expressing within the cell: (v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

Typically, the wild type (normal, non-mutated polypeptide) 3-hydroxy-3-methylglutaryl-coenzyme-A (HMG-CoA) reductase catalyzes the conversion of HMG-CoA to mevalonate and is one of the early steps in the mevalonic acid pathway leading to production of isoprenoids. It is also considered as the rate-limiting enzyme in this pathway in eukaryotic cells. HMGR is an integral membrane protein localized in the endoplasmic reticulum; its N-terminal region consists of a membrane-spanning domain and its catalytically active domain is located in the C-terminal region.

The sequences of the wild type (non-mutated) form of 3-hydroxy-3-methylglutaryl-coenzyme A reductase are known from various organisms including plants (e.g., *Artemisia annua*), rat, mouse, human, zebrafish, *Arabidopsis thaliana*, *Xenopus laevis*, *Nasonia vitripennis*, *Sus scrofa*, *Andida dubliniensis* CD36, *Drosophila melanogaster*, *Macaca mulatta*, *Salmo salar*, *Gallus gallus*, *Bos taurus*, *Aedes aegypti*, *Uncinocarpus reesii* 1704, *Candida tropicalis* MYA-3404, *Pediculus humanus corporis*, *Culex quinquefasciatus*, *Danio rerio*, and more (See via NCBI web site).

For example, the coding sequence of wild type 3-hydroxy-3-methylglutaryl-coenzyme A reductase is provided in GenBank Accession NOs. Q43319 (SEQ ID NO: 33 for polypeptide) from *Artemisia annua*; GenBank Accession NOs. AAB67527 (SEQ ID NO: 34 for polypeptide) and U22382.1 (SEQ ID NO: 35 for polynucleotide) and GenBank Accession NOs. AA09766.1 (SEQ ID NO: 62 for the polypeptide) and BK006946.2 (SEQ ID NO: 61 for the polynucleotide).
An N-terminal truncation (e.g., a truncation of amino acids 1-552 of HMG-CoA) removes the membrane-binding region which includes a sterol-sensing domain that is required for feedback regulation and hence forms a soluble deregulated enzyme.

It should be noted that by using the mutated form (hyperactive form) of 3-hydroxy-3-methylglutaryl-coenzyme A reductase the amount of precursors in the MVA pathway (e.g., FDP) increases.

As used herewith the phrase "a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG)" refers to an hyperactive form of 3-hydroxy-3-methylglutaryl-coenzyme A reductase, which comprises the catalytic portion of the enzyme but which is devoid of the domain causing feedback inhibition of the cytosolic mevalonate pathway (MVA) pathway.

Typically, in order to generate the truncated form of HMG-CoA and to prevent feedback inhibition, the membrane spanning domain of the HMG-CoA protein is removed (ca. 500-550 amino acids are removed from the N-terminal portion of the polypeptide), alternatively the sterol-sensing domain contained within this region can be mutated to be non-functional. An exemplary sequence of the N-terminal truncated 3-hydroxy-3-methylglutaryl-coenzyme A reductase is set forth in SEQ ID NOs: 63 and 64 for the polynucleotide and polypeptide sequences, respectively.

According to some embodiments of the invention any of the polynucleotides of (i)-(v) is comprised in a nucleic acid construct along with a promoter for directing transcription of the nucleic acid sequence in a host cell (e.g., in a plant cell).

According to some embodiments of the invention at least two of the promoters are not identical.

According to some embodiments of the invention each of the polynucleotides further comprises a terminator sequence for controlling expression of the nucleic acid sequence in the cell (e.g., the plant cell).

According to some embodiments of the invention at least two of the polynucleotides of (i)-(v) are comprised in a single nucleic acid construct.

According to some embodiments of the invention polynucleotides (i) and (ii) are comprised in a single nucleic acid construct.

According to some embodiments of the invention polynucleotides (i) and (iii) are comprised in a single nucleic acid construct.
According to some embodiments of the invention polynucleotides (ii) and (iii) are comprised in a single nucleic acid construct.

According to some embodiments of the invention polynucleotides (i)-(iii) are comprised in a single nucleic acid construct.

According to some embodiments of the invention polynucleotides wherein (i)-(iv) are comprised in a single nucleic acid construct.

According to some embodiments of the invention polynucleotides wherein (i)-(v) are comprised in a single nucleic acid construct.

According to some embodiments of the invention, the nucleic acid construct further comprises: (vi) a polynucleotide comprising a nucleic acid sequence encoding a polypeptide which enables selection of a cell expressing the nucleic acid construct.

According to some embodiments of the invention, any selection polypeptide may be used to distinguish cells transformed with the nucleic acid constructs from cells not transformed with the nucleic acid construct. Exemplary selection polypeptides include, but are not limited to, nptll, hpt, acc3, aadA (antibiotic selection); bar and pat (herbicide selection) or dhfr ( antimetabolite selection).

According to one embodiment, the polypeptide which enables selection of a cell expressing the nucleic acid construct comprises a polypeptide which confers antibiotic resistance to a cell expressing the nucleic acid construct.

According to one embodiment, the polypeptide which confers the antibiotic resistance is neomycin phosphotransferase II (nptll). A non-limiting example of the nptll sequence is provided in SEQ ID NO: 38 and SEQ ID NO: 37 (GenBank accession V01547).

According to some embodiments of the invention, the nucleic acid construct may further comprise additional enzymes. Exemplary enzymes which may be incorporated into the nucleic acid constructs of the present invention include, but are not limited to, aldehyde dehydrogenase 1 and farnesyl diphosphate synthase.
According to some embodiments of the invention the method of generating and/or increasing content of artemisinin in a cell (e.g., a plant cell), comprising exogenously expressing within the cell a nucleic acid construct which comprises:

(i) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which is capable of forming amorpha-4,11-diene from farnesyl diphosphate (FDP);

(ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde;

(iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,11-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,11-diene and/or artemisinic alcohol;

(iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450; and

(v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

The phrase "exogenously expressing within the cell" as used herein refers to upregulating the expression level of an exogenous polynucleotide within the cell (e.g., a plant cell) by introducing the exogenous polynucleotide into the cell and expressing the polynucleotide by recombinant means.

As used herein "expressing" refers to the expression at the ribonucleic acid (RNA) level and/or at the polypeptide level. The expression of the polynucleotide in the cell can be in a stable or transient manner, so as to produce the desired RNA and polypeptide molecules within the cell.

The phrase "exogenous" as used herein refers to a heterologous polynucleotide or to a polynucleotide which overexpression thereof is desired in a cell.

According to some embodiments of the invention, the heterologous polynucleotide may not be naturally expressed within the cell, e.g., can be derived from another cell of the same species, from another organism or from another species.

According to some embodiments of the invention, the heterologous polynucleotide comprises a nucleic acid sequence which is identical or partially
homologous to an endogenous nucleic acid sequence of the cell, which is present and/or naturally expressed within the cell.

According to some embodiments of the invention, the cell is a prokaryotic cell.
According to some embodiments of the invention, the cell is a eukaryotic cell.
According to some embodiments of the invention, the cell is a yeast cell, a microbial cell, a fungi cell, a mammalian cell, an animal cell, a frog cell, a human cell and the like.
According to some embodiments of the invention, the cell is a plant cell.
According to some embodiments of the invention each of the polynucleotides of some embodiments of the invention further comprises a promoter sequence for directing expression of the nucleic acid sequence in the cell.

As used herein, the term "promoter" refers to a region of DNA which lies upstream of the transcriptional initiation site of a gene to which RNA polymerase binds to initiate transcription of RNA. The promoter controls where (e.g., which tissue, e.g., which portion of a plant) and/or when (e.g., at which stage or condition in the lifetime of an organism) the gene is expressed.

Any suitable promoter sequence can be used by the nucleic acid construct of the present invention, and exemplary promoters are described hereinunder.

The nucleic acid construct (also referred to herein as an "expression vector") of some embodiments of the invention includes additional sequences which render this vector suitable for replication and integration in prokaryotes, eukaryotes, or preferably both (e.g., shuttle vectors). In addition, a typical cloning vector may also contain a transcription and translation initiation sequence, transcription and translation terminator and a polyadenylation signal. By way of example, such constructs will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA synthesis, and a 3' LTR or a portion thereof.

The nucleic acid construct of some embodiments of the invention typically includes a signal sequence for secretion of the peptide from a host cell in which it is placed. Preferably the signal sequence for this purpose is a mammalian signal sequence or the signal sequence of the polypeptide variants of some embodiments of the invention.
Eukaryotic promoters typically contain two types of recognition sequences, the TATA box and upstream promoter elements. The TATA box, located 25-30 base pairs upstream of the transcription initiation site, is thought to be involved in directing RNA polymerase to begin RNA synthesis. The other upstream promoter elements determine the rate at which transcription is initiated.


Enhancer elements can stimulate transcription up to 1,000 fold from linked homologous or heterologous promoters. Enhancers are active when placed downstream or upstream from the transcription initiation site. Many enhancer elements derived from viruses have a broad host range and are active in a variety of tissues. For example, the SV40 early gene enhancer is suitable for many cell types. Other enhancer/promoter combinations that are suitable for some embodiments of the invention include those derived from polyoma virus, human or murine cytomegalovirus (CMV), the long term repeat from various retroviruses such as murine leukemia virus, murine or Rous sarcoma virus and HIV. See, Enhancers and Eukaryotic Expression, Cold Spring Harbor Press, Cold Spring Harbor, N.Y. 1983, which is incorporated herein by reference.

In the construction of the expression vector, the promoter is preferably positioned approximately the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

Polyadenylation sequences can also be added to the expression vector in order to increase the efficiency of mRNA translation of the exogenous polynucleotide. Two
distinct sequence elements are required for accurate and efficient polyadenylation: GU or U rich sequences located downstream from the polyadenylation site and a highly conserved sequence of six nucleotides, AAUAAA, located 11-30 nucleotides upstream. Termination and polyadenylation signals that are suitable for some embodiments of the invention include those derived from SV40.

In addition to the elements already described, the expression vector of some embodiments of the invention may typically contain other specialized elements intended to increase the level of expression of cloned nucleic acids or to facilitate the identification of cells that carry the recombinant DNA. For example, a number of animal viruses contain DNA sequences that promote the extra chromosomal replication of the viral genome in permissive cell types. Plasmids bearing these viral replicons are replicated episomally as long as the appropriate factors are provided by genes either carried on the plasmid or with the genome of the host cell.

The vector may or may not include a eukaryotic replicon. If a eukaryotic replicon is present, then the vector is amplifiable in eukaryotic cells using the appropriate selectable marker. If the vector does not comprise a eukaryotic replicon, no episomal amplification is possible. Instead, the recombinant DNA integrates into the genome of the engineered cell, where the promoter directs expression of the desired nucleic acid.

The expression vector of some embodiments of the invention can further include additional polynucleotide sequences that allow, for example, the translation of several proteins from a single mRNA such as an internal ribosome entry site (IRES) and sequences for genomic integration of the promoter-chimeric polypeptide.

Examples for mammalian expression vectors include, but are not limited to, pcDNA3, pcDNA3.1 (+/-), pGL3, pZeocSV2(+/-), pSecTag2, pDisplay, pEF/myc/cyto, pCMV/myc/cyto, pCR3.1, pSinRep5, DH26S, DHBB, pNMT1, pNMT41, pNMT81, which are available from Invitrogen, pCI which is available from Promega, pMbac, pPbac, pBK-RSV and pBK-CMV which are available from Stratagene, pTRES which is available from Clontech, and their derivatives.

Expression vectors containing regulatory elements from eukaryotic viruses such as retroviruses can be also used. SV40 vectors include pSVT7 and pMT2. Vectors derived from bovine papilloma virus include pBV-lMTHA, and vectors derived from
Epstein Bar virus include pHEBO, and p205. Other exemplary vectors include pMSG, pAV009/A+, pMTO10/A+, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the SV-40 early promoter, SV-40 later promoter, metallothionein promoter, murine mammary tumor virus promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

As described above, viruses are very specialized infectious agents that have evolved, in many cases, to elude host defense mechanisms. Typically, viruses infect and propagate in specific cell types. The targeting specificity of viral vectors utilizes its natural specificity to specifically target predetermined cell types and thereby introduce a recombinant gene into the infected cell. Thus, the type of vector used by some embodiments of the invention will depend on the cell type transformed. The ability to select suitable vectors according to the cell type transformed is well within the capabilities of the ordinary skilled artisan and as such no general description of selection consideration is provided herein. For example, bone marrow cells can be targeted using the human T cell leukemia virus type I (HTLV-I) and kidney cells may be targeted using the heterologous promoter present in the baculovirus Autographa californica nucleopolyhedrovirus (AcMNPV) as described in Liang CY et al, 2004 (Arch Virol. 149: 51-60).

Recombinant viral vectors are useful for in vivo expression of the exogenous polynucleotide since they offer advantages such as lateral infection and targeting specificity. Lateral infection is inherent in the life cycle of, for example, retrovirus and is the process by which a single infected cell produces many progeny virions that bud off and infect neighboring cells. The result is that a large area becomes rapidly infected, most of which was not initially infected by the original viral particles. This is in contrast to vertical-type of infection in which the infectious agent spreads only through daughter progeny. Viral vectors can also be produced that are unable to spread laterally. This characteristic can be useful if the desired purpose is to introduce a specified gene into only a localized number of targeted cells.

Various methods can be used to introduce the expression vector of some embodiments of the invention into cells. Such methods are generally described in Sambrook et al., Molecular Cloning: A Laboratory Manual, Cold Springs Harbor

Introduction of nucleic acids by viral infection offers several advantages over other methods such as lipofection and electroporation, since higher transfection efficiency can be obtained due to the infectious nature of viruses.

Currently preferred in vivo nucleic acid transfer techniques include transfection with viral or non-viral constructs, such as adenovirus, lentivirus, Herpes simplex I virus, or adeno-associated virus (AAV) and lipid-based systems. Useful lipids for lipid-mediated transfer of the gene are, for example, DOTMA, DOPE, and DC-Choi [Tonkinson et al., Cancer Investigation, 14(1): 54-65 (1996)]. The most preferred constructs for use in gene therapy are viruses, most preferably adenoviruses, AAV, lentiviruses, or retroviruses. A viral construct such as a retroviral construct includes at least one transcriptional promoter/enhancer or locus-defining element(s), or other elements that control gene expression by other means such as alternate splicing, nuclear RNA export, or post-translational modification of messenger. Such vector constructs also include a packaging signal, long terminal repeats (LTRs) or portions thereof, and positive and negative strand primer binding sites appropriate to the virus used, unless it is already present in the viral construct. In addition, such a construct typically includes a signal sequence for secretion of the peptide from a host cell in which it is placed. Preferably the signal sequence for this purpose is a mammalian signal sequence or the signal sequence of the polypeptide variants of some embodiments of the invention. Optionally, the construct may also include a signal that directs polyadenylation, as well as one or more restriction sites and a translation termination sequence. By way of example, such constructs will typically include a 5' LTR, a tRNA binding site, a packaging signal, an origin of second-strand DNA synthesis, and a 3' LTR or a portion
thereof. Other vectors can be used that are non-viral, such as cationic lipids, polylysine, and dendrimers.

Other than containing the necessary elements for the transcription and translation of the inserted coding sequence, the expression construct of some embodiments of the invention can also include sequences engineered to enhance stability, production, purification, yield or toxicity of the expressed peptide. For example, the expression of a fusion protein or a cleavable fusion protein comprising the protein encoded by the exogenous polynucleotide of some embodiments of the invention (the "exogenous polypeptide" hereinafter) and a heterologous protein can be engineered. Such a fusion protein can be designed so that the fusion protein can be readily isolated by affinity chromatography; e.g., by immobilization on a column specific for the heterologous protein. Where a cleavage site is engineered between the exogenous polypeptide and the heterologous protein, the exogenous polypeptide can be released from the chromatographic column by treatment with an appropriate enzyme or agent that disrupts the cleavage site [e.g., see Booth et al. (1988) Immunol. Lett. 19:65-70; and Gardella et al, (1990) J. Biol. Chem. 265:15854-15859].

As mentioned hereinafter, a variety of prokaryotic or eukaryotic cells can be used as host-expression systems to express the exogenous polypeptide of some embodiments of the invention. These include, but are not limited to, microorganisms, such as bacteria transformed with a recombinant bacteriophage DNA, plasmid DNA or cosmide DNA expression vector containing the coding sequence; yeast transformed with recombinant yeast expression vectors containing the coding sequence; plant cell systems infected with recombinant virus expression vectors (e.g., cauliflower mosaic virus, CaMV; tobacco mosaic virus, TMV) or transformed with recombinant plasmid expression vectors, such as Ti plasmid, containing the coding sequence. Mammalian expression systems can also be used to express the polypeptides of some embodiments of the invention.

Examples of bacterial constructs include the pET series of E. coli expression vectors [Studier et al. (1990) Methods in Enzymol. 185:60-89].

In yeast, a number of vectors containing constitutive or inducible promoters can be used, as disclosed in U.S. Pat. Application No: 5,932,447. Alternatively, vectors can
be used which promote integration of foreign DNA sequences into the yeast chromosome.

Other expression systems such as insects and mammalian host cell systems which are well known in the art and are further described hereinbelow can also be used by some embodiments of the invention.

Following is a non-limiting description of nucleic acid constructs and methods for expressing the exogenous polynucleotide in a plant cell.


According to some embodiments of the invention, the promoter which is used to express the polynucleotide of some embodiments of the invention within a plant cell is
the octopine synthase gene (ocs) promoter (SEQ ID NO: 39); the nopalin synthase (nos) promoter (SEQ ID NO: 40); the hsp8.1 heat shock inducible promoter (HS) (SEQ ID NO: 43); the super promoter (sup) (SEQ ID NO: 44); rubisco promoter (rbc) (SEQ ID NO: 41); and/or the 35S CaMV promoter (SEQ ID NO: 42).

According to some embodiments of the invention, the terminator sequence which is used for controlled expression of the polynucleotide of some embodiments of the invention within a cell is the octopine synthase gene (ocs) terminator (SEQ ID NO: 45); the nopalin synthase (nos) terminator (SEQ ID NO: 46); the rubisco (rbc) terminator (SEQ ID NO: 47); and/or the 35S CaMV promoter (SEQ ID NO: 48) and the ags terminator (SEQ ID NO: 49).

The nucleic acid construct of some embodiments of the invention can further include an appropriate selectable marker and/or an origin of replication. According to some embodiments of the invention, the nucleic acid construct utilized is a shuttle vector, which can propagate both in E. coli (wherein the construct comprises an appropriate selectable marker and origin of replication) and be compatible with propagation in cells. The construct according to the present invention can be, for example, a plasmid, a bacmid, a phagemid, a cosmid, a phage, a virus or an artificial chromosome.

The nucleic acid construct of some embodiments of the invention can be utilized to stably or transiently transform plant cells. In stable transformation, the exogenous polynucleotide is integrated into the plant cell genome and as such it represents a stable and inherited trait. In transient transformation, the exogenous polynucleotide is expressed by the cell transformed but it is not integrated into the genome and as such it represents a transient trait.


The principle methods of causing stable integration of exogenous DNA into plant genomic DNA include two main approaches:


The Agrobacterium system includes the use of plasmid vectors that contain defined DNA segments that integrate into the plant genomic DNA. Methods of inoculation of the plant tissue vary depending upon the plant species and the Agrobacterium delivery system. A widely used approach is the leaf disc procedure which can be performed with any tissue explant that provides a good source for initiation of whole plant differentiation. See, e.g., Horsch et al. in Plant Molecular Biology Manual A5, Kluwer Academic Publishers, Dordrecht (1988) p. 1-9. A supplementary approach employs the Agrobacterium delivery system in combination with vacuum infiltration. The Agrobacterium system is especially viable in the creation of transgenic dicotyledonous plants.

There are various methods of direct DNA transfer into plant cells. In electroporation, the protoplasts are briefly exposed to a strong electric field. In microinjection, the DNA is mechanically injected directly into the cells using very small micropipettes. In microparticle bombardment, the DNA is adsorbed on microprojectiles
such as magnesium sulfate crystals or tungsten particles, and the microprojectiles are physically accelerated into cells or plant tissues.

Following stable transformation 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 transformed plant be produced such that the regenerated plant has the identical traits and characteristics of the parent transgenic plant. Therefore, it is preferred that the transformed 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 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 transgenic or transformed plant. The advantages of cloning plants are the speed of 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 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 so that it can be grown in the natural environment.
According to some embodiments of the invention, the transgenic plants are generated by transient transformation of leaf cells, meristematic cells or the whole plant.

Transient transformation can be effected by any of the direct DNA transfer methods described above or by viral infection using modified plant viruses.

Viruses that have been shown to be useful for the transformation of plant hosts include CaMV, Tobacco mosaic virus (TMV), brome mosaic virus (BMV) and Bean Common Mosaic Virus (BV or BCMV). Transformation of plants using plant viruses is described in U.S. Pat. No. 4,855,237 (bean golden mosaic virus; BGV), EP-A 67,553 (TMV), Japanese Published Application No. 63-14693 (TMV), EPA 194,809 (BV), EPA 278,667 (BV); and Gluzman, Y. et al, Communications in Molecular Biology: Viral Vectors, Cold Spring Harbor Laboratory, New York, pp. 172-189 (1988). Pseudovirus particles for use in expressing foreign DNA in many hosts, including plants are described in WO 87/06261.

According to some embodiments of the invention, the virus used for transient transformations is avirulent and thus is incapable of causing severe symptoms such as reduced growth rate, mosaic, ring spots, leaf roll, yellowing, streaking, pox formation, tumor formation and pitting. A suitable avirulent virus may be a naturally occurring avirulent virus or an artificially attenuated virus. Virus attenuation may be effected by using methods well known in the art including, but not limited to, sub-lethal heating, chemical treatment or by directed mutagenesis techniques such as described, for example, by Kurihara and Watanabe (Molecular Plant Pathology 4:259-269, 2003), Gal-on et al. (1992), Atreya et al. (1992) and Huet et al. (1994).

Suitable virus strains can be obtained from available sources such as, for example, the American Type culture Collection (ATCC) or by isolation from infected plants. Isolation of viruses from infected plant tissues can be effected by techniques well known in the art such as described, for example by Foster and Tatlor, Eds. "Plant Virology Protocols From Virus Isolation to Transgenic Resistance (Methods in Molecular Biology (Humana Pr), Vol 81)", Humana Press, 1998. Briefly, tissues of an infected plant believed to contain a high concentration of a suitable virus, preferably young leaves and flower petals, are ground in a buffer solution (e.g., phosphate buffer solution) to produce a virus infected sap which can be used in subsequent inoculations.

When the virus is a DNA virus, suitable modifications can be made to the virus itself. Alternatively, the virus can first be cloned into a bacterial plasmid for ease of constructing the desired viral vector with the foreign DNA. The virus can then be excised from the plasmid. If the virus is a DNA virus, a bacterial origin of replication can be attached to the viral DNA, which is then replicated by the bacteria. Transcription and translation of this DNA will produce the coat protein which will encapsidate the viral DNA. If the virus is an RNA virus, the virus is generally cloned as a cDNA and inserted into a plasmid. The plasmid is then used to make all of the constructions. The RNA virus is then produced by transcribing the viral sequence of the plasmid and translation of the viral genes to produce the coat protein(s) which encapsidate the viral RNA.

In one embodiment, a plant viral polynucleotide is provided in which the native coat protein coding sequence has been deleted from a viral polynucleotide, a non-native plant viral coat protein coding sequence and a non-native promoter, preferably the subgenomic promoter of the non-native coat protein coding sequence, capable of expression in the plant host, packaging of the recombinant plant viral polynucleotide, and ensuring a systemic infection of the host by the recombinant plant viral polynucleotide, has been inserted. Alternatively, the coat protein gene may be inactivated by insertion of the non-native polynucleotide sequence within it, such that a protein is produced. The recombinant plant viral polynucleotide may contain one or more additional non-native subgenomic promoters. Each non-native subgenomic promoter is capable of transcribing or expressing adjacent genes or polynucleotide sequences in the plant host and incapable of recombination with each other and with native subgenomic promoters. Non-native (foreign) polynucleotide sequences may be inserted adjacent the native plant viral subgenomic promoter or the native and a non-native plant viral subgenomic promoters if more than one polynucleotide sequence is
included. The non-native polynucleotide sequences are transcribed or expressed in the host plant under control of the subgenomic promoter to produce the desired products.

In a second embodiment, a recombinant plant viral polynucleotide is provided as in the first embodiment except that the native coat protein coding sequence is placed adjacent one of the non-native coat protein subgenomic promoters instead of a non-native coat protein coding sequence.

In a third embodiment, a recombinant plant viral polynucleotide is provided in which the native coat protein gene is adjacent its subgenomic promoter and one or more non-native subgenomic promoters have been inserted into the viral polynucleotide. The inserted non-native subgenomic promoters are capable of transcribing or expressing adjacent genes in a plant host and are incapable of recombination with each other and with native subgenomic promoters. Non-native polynucleotide sequences may be inserted adjacent the non-native subgenomic plant viral promoters such that the sequences are transcribed or expressed in the host plant under control of the subgenomic promoters to produce the desired product.

In a fourth embodiment, a recombinant plant viral polynucleotide is provided as in the third embodiment except that the native coat protein coding sequence is replaced by a non-native coat protein coding sequence.

The viral vectors are encapsidated by the coat proteins encoded by the recombinant plant viral polynucleotide to produce a recombinant plant virus. The recombinant plant viral polynucleotide or recombinant plant virus is used to infect appropriate host plants. The recombinant plant viral polynucleotide is capable of replication in the host, systemic spread in the host, and transcription or expression of foreign gene(s) (exogenous polynucleotide) in the host to produce the desired protein.

In addition to the above, the polynucleotide of the present invention can also be introduced into a chloroplast genome thereby enabling chloroplast expression.

A technique for introducing exogenous polynucleotide sequences to the genome of the chloroplasts is known. This technique involves the following procedures. First, plant cells are chemically treated so as to reduce the number of chloroplasts per cell to about one. Then, the exogenous polynucleotide is introduced via particle bombardment into the cells with the aim of introducing at least one exogenous polynucleotide molecule into the chloroplasts. The exogenous polynucleotides selected such that it is integratable into the chloroplast's genome via homologous recombination which is readily effected by enzymes inherent to the chloroplast. To this end, the exogenous polynucleotide includes, in addition to a gene of interest, at least one polynucleotide stretch which is derived from the chloroplast's genome. In addition, the exogenous polynucleotide includes a selectable marker, which serves by sequential selection procedures to ascertain that all or substantially all of the copies of the chloroplast genomes following such selection will include the exogenous polynucleotide. Further details relating to this technique are found in U.S. Pat. Nos. 4,945,050; and 5,693,507 which are incorporated herein by reference. A polypeptide can thus be produced by the protein expression system of the chloroplast and become integrated into the chloroplast's inner membrane.

According to some embodiments of the invention, the exogenous polynucleotides of some embodiments of the invention are introduced into a single host plant by co-introducing multiple nucleic acid constructs, each including a different exogenous polynucleotide, into a single plant cell. The transformed cell can then be regenerated into a mature plant using the methods described hereinabove. The regenerated transformed plants can then be cross-bred and resultant progeny selected using conventional plant breeding techniques for increased content of artemisinin.

Alternatively, expressing a plurality of exogenous polynucleotides in a single host plant can be effected by co-introducing into a single plant-cell a single nucleic-acid construct including a plurality of different exogenous polynucleotides. Such a construct can be designed with a single promoter sequence which can transcribe a polycistronic messenger RNA including all the different exogenous polynucleotide sequences. To enable co-translation of the different polypeptides encoded by the polycistronic
messenger RNA, the polynucleotide sequences can be inter-linked via an internal ribosome entry site (IRES) sequence which facilitates translation of polynucleotide sequences positioned downstream of the IRES sequence. In this case, a transcribed polycistronic RNA molecule encoding the different polypeptides described above will be translated from both the capped 5' end and the two internal IRES sequences of the polycistronic RNA molecule to thereby produce in the cell all different polypeptides. Alternatively, the construct can include several promoter sequences each linked to a different exogenous polynucleotide sequence.

It should be noted that once the polynucleotide has been introduced into the plant cell, the plant cell can be further cultured under conditions which enable production of artemisinin in plant cell cultures. Thus, as shown in Example 5 of the Examples section which follows, artemisinin can be produced in tobacco suspension cultures. Tobacco suspension cultures can be initiated e.g. from young leaf explants of transgenic plants (e.g. transformed with the vectors of the present teachings). Explants can be placed on solid growth media (e.g. Murashige and Skoog (MS) growth media) supplemented with various supplements (e.g. 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/l kinetin) and grown until calli are formed (e.g. for ca. 4 weeks). Calli are then transferred to a solid MS media supplemented with various supplements [e.g. benzylaminopurine (BA) 0.5 mg/l and 0.5 mg/l 1-naphthaleneacetic acid (NAA)] and are subcultured every approximately 3 weeks. Suspension cultures are then initiated by placing callus fragments in flasks with liquid media (e.g. media based on basal MS containing 200 mg/l KH₂PO₄, 1 mg/l thiamine, 100 mg/l myo-inositol, 0.4 mg/l 2,4-D and 3% sucrose) and grown with continuous shaking for several weeks (e.g. for about 4 weeks).

Methods of determining the level in the cell or the plant of the RNA transcribed from the exogenous polynucleotide are well known in the art and include, for example, Northern blot analysis, reverse transcription polymerase chain reaction (RT-PCR) analysis (including quantitative, semi-quantitative or real-time RT-PCR) and RNA-in situ hybridization.

Methods of determining the level in the cell or the plant of the polypeptide encoded by the exogenous polynucleotide are well known in the art and include, for
example, Western blot analysis, activity assay, immunostaining, immunohistochemistry, immunofluorescence and the like.

According to some embodiments of the invention, the plant cell forms part of a plant.

According to some embodiments of the invention, the increase in the content of artemisinin in the plant is compared to the content in native plant grown under the same (e.g., identical) growth conditions.

According to an aspect of some embodiments of the invention, there is provided a plant comprising the plant cell of some embodiments of the invention.

The term "plant" as used herein encompasses whole plants, ancestors and progeny of the plants and plant parts, including seeds, shoots, stems, roots (including tubers), flowers, and plant cells, tissues and organs. The plant may be in any form including suspension cultures, embryos, meristematic regions, callus tissue, leaves, gametophytes, sporophytes, pollen, and microspores. Plants that are particularly useful in the methods of the invention include all plants which belong to the superfamily Viridiplantae, in particular monocotyledonous and dicotyledonous plants including a fodder or forage legume, ornamental plant, food crop, tree, or shrub selected from the list comprising Cichorium spp., Acacia spp., Acer spp., Actinidia spp., Aesculus spp., Agathis australis, Albizia amara, Alsophila tricolor, Andropogon spp., Arachis spp., Areca catechu, Astelia fragrans, Astragalus cicer, Baikiaea plurijuga, Betula spp., Brassica spp., Bruguiera gymnorrhiza, Burkea africana, Butea frondosa, Cadaba farinosa, Calliandra spp, Camellia sinensis, Canna indica, Capsicum spp., Cassia spp., Centroema pubescens, Chacoomeles spp., Cinnamomum cassia, Coffea arabica, Colophospermum mopane, Coronillia varia, Cotoneaster serotina, Crataegus spp., Cucumis spp., Cupressus spp., Cyathea dealbata, Cydonia oblonga, Cryptomeria japonica, Cymbopogon spp., Cynthea dealbata, Cydonia oblonga, Dalbergia monetaria, Davallia divaricata, Desmodium spp., Dicksonia squarosa, Dibeteropogon amplectens, Dioclea spp, Dolichos spp., Dorycnium rectum, Echinochloa pyramidalis, Ehraffia spp., Eleusine coracana, Eragrestis spp., Erythrina spp., Eucalyptus spp., Euclea schimperi, Eulalia vi/losa, Pagopyrum spp., Feijoa sellowiana, Fragaria spp., Flemingia spp., Freycinetia banksii, Geranium thunbergii, GinAgo biloba, Glycine javanica, Gliricidia spp, Gossypium hirsutum, Grevillea spp., Guibourtia coleosperma, Hedysarum spp,

According to some embodiments of the invention the plant is a tobacco plant.

According to some embodiments of the invention the plant is Artemisia annua plant.

According to some embodiments of the invention the plant is an aspen, a tomato, a marguerite or a lettuce.

According to an aspect of some embodiments of the invention there is provided a method of producing artemisinin, comprising: (a) generating and/or increasing content of the artemisinin in a cell or plant according to the method of some embodiments of the
invention, and (b) isolating the artemisinin from the cell or plant, thereby producing the artemisinin.

According to some embodiments of the invention, the method of producing artemisinin is effected by: (a) providing the cell of some embodiments of the invention, and (b) isolating the artemisinin from the cell, thereby producing the artemisinin.

According to some embodiments of the invention, the method further comprises providing and/or maintaining conditions suitable for artemisinin production within the cell.

According to one embodiment, the method of producing artemisinin further comprises subjecting the cell or the plant to light and/or to oxygen prior to the isolating of the artemisinin. For example, the cell or plant may be subjected to intense light or to daylight, for e.g. several minutes, several hours or several days, in order to increase artemisinin content in the cell or plant.

Methods of isolating artemisinin from a plant or plant parts (e.g. leaves, roots, flowers) are known in the art and described in Lapkin AA, Plucinski PK, Cutler M. Comparative assessment of technologies for extraction of artemisinin. Journal of Natural Products (2006) 69: 1653-1664; El-Sohly HN, Croom EM, Elferaly FS, Elsherei MM. A large-scale extraction technique of artemisinin from Artemisia annua. Journal of Natural Products (1990) 53: 1560-1564; Xu JD, Luo JG, Kong LY. Single-step preparative extraction of artemisinin from Artemisia annua by charcoal column chromatography. Chromatographia74: 471-475; Lapkin AA, Peters M, Greiner L, Chemat S, Leonhard K, Liauw MA, Leitner W. Screening of new solvents for artemisinin extraction process using ab initio methodology. Green Chemistry12: 241-251. Briefly, 100 mg dried and ground tissue (from a plant e.g. tobacco, A. annua, aspen, tomato or marguerite) may be extracted by sonication for 15 minutes with 2 ml hexane. After partitioning into 1 ml methanol, phases may be separated and the methanolic layer concentrated to approximately 100 µl under a nitrogen stream.

In addition, artemisinin can be also isolated from a culture medium of the cells (e.g. plant cells), which produce artemisinin (e.g., from the natural artemisinin producing Artemisia annua (sweet wormwood) plant cells or from any cell (e.g., a plant cell) engineered to produce artemisinin by recombinant techniques according to the methods described herein). Briefly, artemisinin or its precursor amorpha diene can than be
purified from the cell liquid culture by, for example, in-situ product removal approach as previously described [Farhi, M. et al. (2011) *Metab. Eng.* 13: 474-481]. For example, a two-phase partitioning culture may be employed by adding a volume of a biocompatible solvent, e.g. 10%-20 % (v/v) n-dodecane, methyl oleate or isopropyl myristate, as the organic phase or a solid adsorbent e.g. Amberlite resin, Diaion HP-20 or activated charcoal.

Once produced and isolated, the purity, content, amount or yield of artemisinin can be determined using known methods.

As used herein the term "isolated" with respect to artemisinin refers to at least partially separated from the cell producing same. In a specific embodiment, isolated refers to free of pathogenic contaminants.

It should be noted that the content of artemisinin can be determined within the plant, in a cell culture medium (e.g., a plant cell culture medium), or in a plant extract, essentially as described in the general materials and experimental procedures section of the Examples section which follows.

Methods of determining the purity of artemisinin are known and in the art, and are also described in Examples 3 and 4 of the Examples section which follows.

For example, for determining artemisinin content in a plant, plant tissues are dried and ground, mixed with deuterium labeled artemisinin (Toronto Research Chemicals) and extracted by sonication (e.g., for 15 minutes) with 2 ml hexane. After partitioning into methanol (e.g., 1 ml) phases are separated and the methanolic layer is concentrated under a nitrogen stream to about 100 μl.

Liquid chromatography-mass spectrometry (LC-MS) analysis can be performed using Agilent 1200 series rapid resolution liquid chromatography system coupled to Agilent 6410 triple quadrupole mass spectrometer. For chromatographic separation two Zorbax Eclipse XDB-C18 (100x2.1 mm, 1.8 μm, Agilent Technologies) columns are connected in sequence followed by Synergy Fusion-RP (100x2 mm, 2.5 μm, Phenomenex). Columns temperature is maintained at 40 °C, with an injection volume of about 10 μl. Chromatographic analysis can be performed using a binary gradient (See e.g., Table 2 in the Examples section which follows). The mass spectrometer can be equipped with electrospray ionization ion source which is operated in positive mode upon the following parameters: capillary voltage 4000V, nebulizer pressure 241 kPa,
drying gas 10 l/min, gas temperature 350 °C, 99.5 % nitrogen is used as nebulizer and
drying gas and 99.999 % nitrogen is used as collision gas.

According to some embodiments of the invention, artemisinin can be detected by
liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) operated in
multiple reaction monitoring (MRM) mode by monitoring transitions, e.g. 283 [M H+]
→247, 283→265, 283→219, 283.2→151.1 and/or 265→247 under the following
optimized parameters: fragmentor voltage 80V and CID energy 4eV. The LC-MS
system is controlled and data can be analyzed using MassHunter software (Agilent
Technologies Inc.).

For the liquid chromatography-high resolution mass spectrometry (LC-HRMS)
analysis an Accela LC system coupled with an LTQ Orbitrap Discovery hybrid FT mass
spectrometer (Thermo Fisher Scientific Inc.) can be used. Chromatographic analysis is
performed using two LC columns as described for LC-MS/MS and following a binary
gradient program (See e.g., Table 3 in the Examples section which follows). Columns
temperature is maintained at 40 °C, flow rate was 250 µl/min and injection volume was
10 µl. The mass spectrometer is equipped with an Atmospheric-pressure chemical
ionization (APCI) ion source operated in positive ionization mode. The ion source
parameters can be as follows: corona discharge needle current 5 µA, capillary
temperature 250 °C, sheath gas rate 50 (arb), auxiliary gas rate 10 (arb), vaporizer
temperature 400 °C. 99.5 % nitrogen is used as sheath and auxiliary gas. Ion transfer
optic parameters are optimized for protonated artemisinin using the automatic tune
option. Mass spectra are acquired in m/z 200-800 Da range, resolution was 30000. The
LC-MS system is controlled and data can be analyzed using Xcalibur software (Thermo
Fisher Scientific Inc.).

According to some embodiments of the invention, artemisinin can be detected by
liquid chromatography-high resolution mass spectrometry (LC-HR-MS) with accurate
mass ions in the range of m/z 283.1000-283.4000.

According to some embodiments of the invention, the artemisinin is detected by
liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) operated in
multiple reaction monitoring (MRM) mode and monitoring MRM traces m/z 283.2
→219 and 283.2→265 or by liquid chromatography-high resolution mass spectrometry
(LC-HR-MS) m/z value of 283.1530 Da.
According to some embodiments of the invention, the artemisinin produced by the method of some embodiments of the invention, from the cell (e.g., from the plant cell) or plant of some embodiments of the invention has a pharmaceutical grade purity of at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, e.g., 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, e.g., 100% purity.

According to an aspect of some embodiments of the invention there is provided an isolated artemisinin produced by the method of some embodiments of the invention.

Any of the polynucleotides described hereinabove can be included in a system (e.g., a cocktail of polynucleotides).

Thus, according to an aspect of some embodiments of the invention there is provided a system of isolated polynucleotides.

The phrase "isolated polynucleotide" as used herein refers to at least partially separated form the natural environment, e.g., from the plant cell naturally expressing the same.

The system of some embodiments of the invention comprising: (i) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes formation of amorpha-4,11-diene from farnesyl diphosphate (FDP); and (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and (iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,11-diene monoxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,11-diene and/or artemisinic alcohol;

According to some embodiments of the invention, the system further comprises: (iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

According to some embodiments of the invention, the system further comprises: (v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

It should be noted that any of the polynucleotides comprised in the system can be ligated in a nucleic acid construct suitable for expression in a host cell.
According to an aspect of some embodiments of the invention there is provided a system of isolated nucleic acid constructs, comprising: (i) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes formation of amorpha-4,11-diene from farnesyl diphosphate (FDP); and (ii) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and (iii) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,11-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,11-diene and/or artemisinic alcohol;

According to some embodiments of the invention, the system further comprises:

(iv) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

According to some embodiments of the invention, the system further comprises:

(v) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

According to some embodiments of the invention, the system further comprises:

(vi) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding a polypeptide which enables selection of a cell expressing the nucleic acid construct.

According to an aspect of some embodiments of the invention, there is provided a kit comprising the system of any of some embodiments of the invention and instructions for use in transformation of a cell.

According to an aspect of some embodiments of the invention, there is provided a nucleic acid construct comprising:

(i) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes the formation of amorpha-4,11-diene from farnesyl diphosphate (FDP); and
(ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and

(iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,1 diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,1 diene and/or artemisinic alcohol.

According to some embodiments of the invention, the nucleic acid construct further comprises:

(iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

According to some embodiments of the invention, the nucleic acid construct further comprises:

(v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

According to some embodiments of the invention the nucleic acid construct further comprises:

(vi) a polynucleotide comprising a nucleic acid sequence encoding a polypeptide which enables selection of a cell expressing the nucleic acid construct as described hereinabove, e.g., a polypeptide which confers antibiotic resistance to a cell expressing the nucleic acid construct, such as the neomycin phosphotransferase II.

According to an aspect of some embodiments of the invention, there is provided a cell (e.g., a plant cell) comprising a heterologous polynucleotide, wherein the heterologous polynucleotide comprises:

(i) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes the formation of amorpha-4,1 diene from farnesyl diphosphate (FDP); and

(ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and

(iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,1 diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,1 diene and/or artemisinic alcohol;
According to some embodiments of the invention, the heterologous polynucleotide further comprises:

(iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

According to some embodiments of the invention, the heterologous polynucleotide further comprises:

(v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

According to some embodiments of the invention, the heterologous polynucleotide further comprises:

(vi) a polynucleotide comprising a nucleic acid sequence encoding a polypeptide which enables selection of a cell expressing the nucleic acid construct as described hereinabove, e.g., a polypeptide which confers antibiotic resistance to a cell expressing the nucleic acid construct, such as the neomycin phosphotransferase II.

The artemisinin produced from the cell or plant according to the teachings of the invention is a highly effective therapeutic agent. For example, the artemisinin produced according to the teachings of the present invention may be used for treatment of malaria, cancer (e.g. hepatoma cancer), parasites (e.g. helminth parasites, neospora caninum), and towards any effective therapeutic utility.

The artemisinin produced in accordance with the present teachings may be used for treatment is any subject in need thereof, e.g. in mammals (e.g., humans and animals).

For such applications artemisinin is typically derivatized. Because artemisinin itself has physical properties such as poor bioavailability that limit its effectiveness, semisynthetic derivatives of artemisinin have been developed. These include: Artesunate (water-soluble: for oral, rectal, intramuscular, or intravenous use), Artemether (lipid-soluble: for oral, rectal or intramuscular use), Dihydroartemisinin, Artelinic acid, Artenimol and Artemotil. Methods of producing artemisinin derivatives are well known in the arts [see e.g., Oliaro P., et al., 2001, Trends in Parasitology, 17: 122-126; Meshnick SR et al., 1993, Antimicrobial agents and chemotherapy, 37: 1108-1114; Meshnick SR., et al, Microbiological Review, June 1996, 301-315; and Meshnick SR., et al., 2002, International Journal for Parasitology, 32: 1655-1660; each of which is fully incorporated herein by reference in its entirety].
The artemisinin produced from the plant according to the teachings of the invention and derivatives of same is a highly effective therapeutic drug, e.g., an anti-malaria, anti-cancer and anti-parasites infection drug, which can be used *per se*, and/or can form part of a pharmaceutical composition where it is mixed with suitable carriers or excipients.

As used herein a "pharmaceutical composition" refers to a preparation of one or more of the active ingredients described herein with other chemical components such as physiologically suitable carriers and excipients. The purpose of a pharmaceutical composition is to facilitate administration of a compound to an organism.

Herein the term "active ingredient" refers to artemisinin accountable for the biological effect.

Hereinafter, the phrases "physiologically acceptable carrier" and "pharmaceutically acceptable carrier" which may be interchangeably used refer to a carrier or a diluent that does not cause significant irritation to an organism and does not abrogate the biological activity and properties of the administered compound. An adjuvant is included under these phrases.

Herein the term "excipient" refers to an inert substance added to a pharmaceutical composition to further facilitate administration of an active ingredient. Examples, without limitation, of excipients include calcium carbonate, calcium phosphate, various sugars and types of starch, cellulose derivatives, gelatin, vegetable oils and polyethylene glycols.

Techniques for formulation and administration of drugs may be found in "Remington's Pharmaceutical Sciences," Mack Publishing Co., Easton, PA, latest edition, which is incorporated herein by reference.

Suitable routes of administration may, for example, include oral, rectal, transmucosal, especially transnasal, intestinal or parenteral delivery, including intramuscular, subcutaneous and intramedullary injections as well as intrathecal, direct intraventricular, intracardiac, e.g., into the right or left ventricular cavity, into the common coronary artery, intravenous, intraperitoneal, intranasal, or intraocular injections.

Conventional approaches for drug delivery to the central nervous system (CNS) include: neurosurgical strategies (e.g., intracerebral injection or intracerebroventricular
infusion); molecular manipulation of the agent in an attempt to exploit one of the endogenous transport pathways of the blood brain barrier (BBB); pharmacological strategies designed to increase the lipid solubility of an agent (e.g., conjugation of water-soluble agents to lipid or cholesterol carriers); and the transitory disruption of the integrity of the BBB by hyperosmotic disruption (resulting from the infusion of a mannitol solution into the carotid artery or the use of a biologically active agent such as an angiotensin peptide). However, each of these strategies has limitations, such as the inherent risks associated with an invasive surgical procedure, a size limitation imposed by a limitation inherent in the endogenous transport systems, potentially undesirable biological side effects associated with the systemic administration of a chimeric molecule comprised of a carrier motif that could be active outside of the CNS, and the possible risk of brain damage within regions of the brain where the BBB is disrupted, which renders it a suboptimal delivery method.

Alternately, one may administer the pharmaceutical composition in a local rather than systemic manner, for example, via injection of the pharmaceutical composition directly into a tissue region of a patient.

Pharmaceutical compositions of some embodiments of the invention may be manufactured by processes well known in the art, e.g., by means of conventional mixing, dissolving, granulating, dragee-making, levigating, emulsifying, encapsulating, entrapping or lyophilizing processes.

Pharmaceutical compositions for use in accordance with some embodiments of the invention thus may be formulated in conventional manner using one or more physiologically acceptable carriers comprising excipients and auxiliaries, which facilitate processing of the active ingredients into preparations which, can be used pharmaceutically. Proper formulation is dependent upon the route of administration chosen.

For injection, the active ingredients of the pharmaceutical composition may be formulated in aqueous solutions, preferably in physiologically compatible buffers such as Hank's solution, Ringer's solution, or physiological salt buffer. For transmucosal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art.
For oral administration, the pharmaceutical composition can be formulated readily by combining the active compounds with pharmaceutically acceptable carriers well known in the art. Such carriers enable the pharmaceutical composition to be formulated as tablets, pills, dragees, capsules, liquids, gels, syrups, slurries, suspensions, and the like, for oral ingestion by a patient. Pharmacological preparations for oral use can be made using a solid excipient, optionally grinding the resulting mixture, and processing the mixture of granules, after adding suitable auxiliaries if desired, to obtain tablets or dragee cores. Suitable excipients are, in particular, fillers such as sugars, including lactose, sucrose, mannitol, or sorbitol; cellulose preparations such as, for example, maize starch, wheat starch, rice starch, potato starch, gelatin, gum tragacanth, methyl cellulose, hydroxypropylmethyl-cellulose, sodium carboxymethylcellulose; and/or physiologically acceptable polymers such as polyvinylpyrrolidone (PVP). If desired, disintegrating agents may be added, such as cross-linked polyvinyl pyrrolidone, agar, or alginic acid or a salt thereof such as sodium alginate.

Dragee cores are provided with suitable coatings. For this purpose, concentrated sugar solutions may be used which may optionally contain gum arabic, talc, polyvinyl pyrrolidone, carbopol gel, polyethylene glycol, titanium dioxide, lacquer solutions and suitable organic solvents or solvent mixtures. Dyestuffs or pigments may be added to the tablets or dragee coatings for identification or to characterize different combinations of active compound doses.

Pharmaceutical compositions which can be used orally, include push-fit capsules made of gelatin as well as soft, sealed capsules made of gelatin and a plasticizer, such as glycerol or sorbitol. The push-fit capsules may contain the active ingredients in admixture with filler such as lactose, binders such as starches, lubricants such as talc or magnesium stearate and, optionally, stabilizers. In soft capsules, the active ingredients may be dissolved or suspended in suitable liquids, such as fatty oils, liquid paraffin, or liquid polyethylene glycols. In addition, stabilizers may be added. All formulations for oral administration should be in dosages suitable for the chosen route of administration.

For buccal administration, the compositions may take the form of tablets or lozenges formulated in conventional manner.

For administration by nasal inhalation, the active ingredients for use according to some embodiments of the invention are conveniently delivered in the form of an aerosol
spray presentation from a pressurized pack or a nebulizer with the use of a suitable propellant, e.g., dichlorodifluoromethane, trichlorofluoromethane, dichlorotetrafluoroethane or carbon dioxide. In the case of a pressurized aerosol, the dosage unit may be determined by providing a valve to deliver a metered amount. Capsules and cartridges of, e.g., gelatin for use in a dispenser may be formulated containing a powder mix of the compound and a suitable powder base such as lactose or starch.

The pharmaceutical composition described herein may be formulated for parenteral administration, e.g., by bolus injection or continuous infusion. Formulations for injection may be presented in unit dosage form, e.g., in ampoules or in multidose containers with optionally, an added preservative. The compositions may be suspensions, solutions or emulsions in oily or aqueous vehicles, and may contain formulatory agents such as suspending, stabilizing and/or dispersing agents.

Pharmaceutical compositions for parenteral administration include aqueous solutions of the active preparation in water-soluble form. Additionally, suspensions of the active ingredients may be prepared as appropriate oily or water based injection suspensions. Suitable lipophilic solvents or vehicles include fatty oils such as sesame oil, or synthetic fatty acids esters such as ethyl oleate, triglycerides or liposomes. Aqueous injection suspensions may contain substances, which increase the viscosity of the suspension, such as sodium carboxymethyl cellulose, sorbitol or dextran.

Optionally, the suspension may also contain suitable stabilizers or agents which increase the solubility of the active ingredients to allow for the preparation of highly concentrated solutions.

Alternatively, the active ingredient may be in powder form for constitution with a suitable vehicle, e.g., sterile, pyrogen-free water based solution, before use.

The pharmaceutical composition of some embodiments of the invention may also be formulated in rectal compositions such as suppositories or retention enemas, using, e.g., conventional suppository bases such as cocoa butter or other glycerides.

Pharmaceutical compositions suitable for use in context of some embodiments of the invention include compositions wherein the active ingredients are contained in an amount effective to achieve the intended purpose. More specifically, a therapeutically effective amount means an amount of active ingredients (*i.e.* artemisinin or its derivative(s), e.g., a synthetic derivative) effective to prevent, alleviate or ameliorate
symptoms of a disorder (e.g., malaria, cancer or parasites infection) or prolong the survival of the subject being treated.

Determination of a therapeutically effective amount is well within the capability of those skilled in the art, especially in light of the detailed disclosure provided herein.

For any preparation used in the methods of the invention, the therapeutically effective amount or dose can be estimated initially from in vitro and cell culture assays. For example, a dose can be formulated in animal models to achieve a desired concentration or titer. Such information can be used to more accurately determine useful doses in humans.

Toxicity and therapeutic efficacy of the active ingredients described herein can be determined by standard pharmaceutical procedures in vitro, in cell cultures or experimental animals. The data obtained from these in vitro and cell culture assays and animal studies can be used in formulating a range of dosage for use in human. The dosage may vary depending upon the dosage form employed and the route of administration utilized. The exact formulation, route of administration and dosage can be chosen by the individual physician in view of the patient's condition. (See e.g., Fingl, et al., 1975, in "The Pharmacological Basis of Therapeutics", Ch. 1 p.l).

Dosage amount and interval may be adjusted individually to provide tissue levels of the active ingredient are sufficient to induce or suppress the biological effect (minimal effective concentration, MEC). The MEC will vary for each preparation, but can be estimated from in vitro data. Dosages necessary to achieve the MEC will depend on individual characteristics and route of administration. Detection assays can be used to determine plasma concentrations.

Depending on the severity and responsiveness of the condition to be treated, dosing can be of a single or a plurality of administrations, with course of treatment lasting from several days to several weeks or until cure is effected or diminution of the disease state is achieved.

The amount of a composition to be administered will, of course, be dependent on the subject being treated, the severity of the affliction, the manner of administration, the judgment of the prescribing physician, etc.

Compositions of some embodiments of the invention may, if desired, be presented in a pack or dispenser device, such as an FDA approved kit, which may
contain one or more unit dosage forms containing the active ingredient. The pack may, for example, comprise metal or plastic foil, such as a blister pack. The pack or dispenser device may be accompanied by instructions for administration. The pack or dispenser may also be accommodated by a notice associated with the container in a form prescribed by a governmental agency regulating the manufacture, use or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the compositions or human or veterinary administration. Such notice, for example, may be of labeling approved by the U.S. Food and Drug Administration for prescription drugs or of an approved product insert. Compositions comprising a preparation of the invention formulated in a compatible pharmaceutical carrier may also be prepared, placed in an appropriate container, and labeled for treatment of an indicated condition, as is further detailed above.

As used herein the term "about" refers to ± 10%.

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".

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.

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, 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.

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.

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.

As used herein, the term "treating" includes abrogating, substantially inhibiting, slowing or reversing the progression of a condition, substantially ameliorating clinical or aesthetical symptoms of a condition or substantially preventing the appearance of clinical or aesthetical symptoms of a condition.

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.

Generally, the nomenclature used herein and the laboratory procedures utilized in
the present invention include molecular, biochemical, microbiological and recombinant
DNA techniques. Such techniques are thoroughly explained in the literature. See, for
example, "Molecular Cloning: A laboratory Manual" Sambrook et al., (1989); "Current
Protocols in Molecular Biology" Volumes I-III Ausubel, R. M., ed. (1994); Ausubel et
al., "Current Protocols in Molecular Biology", John Wiley and Sons, Baltimore,
Sons, New York (1988); Watson et al., "Recombinant DNA", Scientific American
Books, New York; Birren et al. (eds) "Genome Analysis: A Laboratory Manual Series",
Vols. 1-4, Cold Spring Harbor Laboratory Press, New York (1998); methodologies as
set forth in U.S. Pat. Nos. 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057;
"Cell Biology: A Laboratory Handbook", Volumes I-III Cellis, J. E., ed. (1994);
(eds), "Basic and Clinical Immunology" (8th Edition), Appleton & Lange, Norwalk, CT
(1994); Mishell and Shiigi (eds), "Selected Methods in Cellular Immunology", W. H.
Freeman and Co., New York (1980); available immunoassays are extensively described
in the patent and scientific literature, see, for example, U.S. Pat. Nos. 3,791,932;
3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654;
3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and
5,281,521; "Oligonucleotide Synthesis" Gait, M. J., ed. (1984); "Nucleic Acid
Hybridization" Hames, B. D., and Higgins S. J., eds. (1985); "Transcription and
Translation" Hames, B. D., and Higgins S. J., Eds. (1984); "Animal Cell Culture"
Freshney, R. I., ed. (1986); "Immobilized Cells and Enzymes" IRL Press, (1986); "A
Academic Press, San Diego, CA (1990); Marshak et al, "Strategies for Protein
Purification and Characterization - A Laboratory Course Manual" CSHL Press (1996);
all of which are incorporated by reference as if fully set forth herein. Other general
references are provided throughout this document. The procedures therein are believed
to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

GENERAL MATERIALS AND EXPERIMENTAL PROCEDURES

Plant and chemical materials

Tobacco (var. Samsun), aspen (Populus tremula), tomato (Solarium lycopersicum cv. Micro-Tom and M82), marguerite (Argyranthemum frutescens) and lettuce (Lactuca sativa) plants were grown in a green house under natural light conditions at night/day temperatures of 25/40 °C.

Molecular biology reagents, enzymes and kits were from Fermentas International (Burlington, Ontario), Promega (Madison, Wisconsin) and New England Biolabs (Ipswich, MA). Deuterium labeled artemisinin was purchased from Toronto Research Chemicals (North York, Ontario) all other chemicals were from Sigma-Aldrich (Rehovot, Israel).

Expression vectors

Artemisinin pathway genes ADS, [GenBank accession Nos: Q9AR04.2 (SEQ ID NO: 11) for polypeptide and HQ315833.1 (SEQ ID NO: 50) for polynucleotide]; CYP71AV1 [GenBank accession Nos: ABC41927.1 (SEQ ID NO: 16) for polypeptide and DQ315671.1 (SEQ ID NO: 17) for polynucleotide]; DBR2 [GenBank accession Nos: ACH61780.1 (SEQ ID NO: 14) for polypeptide and EU704257.1 (SEQ ID NO: 15) for polynucleotide], as well as CPR [GenBank accession Nos: ABI98819.1 (SEQ ID NO: 31) for polypeptide and DQ984181.1 (SEQ ID NO: 32) for polynucleotide] were PCR amplified from Artemisia annua cDNA and cloned into pGEMT vector (Promega). Generation of mitochondrial targeted amorphadiene synthase (mtADS), cloned by fusing the Saccharomyces cerevisiae COX4 mitochondrial targeting sequence to the ADS coding sequence (CDS), and a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG) were carried out as reported previously [Farhi, M. et al. (2011) Metab. Eng. 13: 474-481].

The tHMG, CYP71AV1, DBR2 and CPR open reading frames (ORF) were then recloned into pSAT vectors [as previously described, see Tzfira, T. et al. (2005) Plant Mol. Biol. 57: 503-516] as PCR fragments using proof reading Taq polymerase (Pfu) as followed: tHMG was cloned as EcoRI/BamHI fragment into pSAT2A.nosP, producing pSAT2.nosP.tHMG, in which the tHMG ORF was expressed under the control of the
nopaline synthase promoter (nos) (as shown in Figure 7); *CYP71AV1* was cloned as
*XhoI/Kpnl* fragment into pSAT5.1A.hspP, producing pSAT5.1.hspP.CYP, in which the
*CYP71AV1* ORF was expressed under the hps18.1 heat shock inducible promoter (HS)
(as shown in Figure 8); *DBR2* was cloned as *EcoRII/Kpnl* fragment into pSAT6A.supP,
producing pSAT6A.supP.DBR2, in which the *DBR2* ORF was expressed under the
control of the super promoter (sup) (as shown in Figure 10); *CPR* was cloned as
*XhoI/BamHI* fragment into pSAT4.1A.rbcP, producing pSAT4.1A.rbcP.CPR in which the
*CPR* ORF was expressed under the control of the rubisco promoter (rbc) (as shown
in Figure 9). The *ADS* or mt*ADS* ORFs were cloned as *EcoRII/BamHI* fragments into
pSAT5A.35SP, producing pSAT5A.35SP.ADS and pSAT5A.35SP.mtADS, respectively, in which the mitochondria-targeted or free *ADS* ORF were expressed under the control of tandem constitutive 35S CaMV promoter (as shown in Figures 11 and 12, respectively).

For construction of plant binary transformation vectors, the *tHMG*, *CYP71AV1* and *DBR2* expression cassettes from pSAT2A.nosP.tHMG, pSAT5.1.hspP.CYP, pSAT6A.supP.DBR2, were successively cloned as *Ascl/I-Ppol, I-ClI/P1-Pspl, PI-
Pspl/PI-Pspl*, respectively, into their corresponding sites into pRCS16F [as previously
pRCS16F[tHMG][AaCYP][AaDBR2]. Next, the constitutive kanamycin resistance expression cassette was cloned, as *Ascl/Ascl* fragment from
pRCS16F[tHMG][AaCYP][AaDBR2], producing
pRCS16F[kan][tHMG][CYP][DBR2]. Next, the *CPR* expression cassette from
pSAT4.1A.rbcP.CPR as *I-CeuI/I-SceI* fragment was cloned into
pRCS16F[kan][tHMG][CYP][DBR2]. producing
pRCS16F[kan][tHMG][CPR][CYP][DBR2]. Finally, the *ADS* and mt*ADS* expression cassettes from pSAT5A.35SP.ADS and pSAT5A.35SP.mtADS, were cloned as *I-
CeuI/I-CeuI* fragments into pRCS16F[kan][tHMG][CPR][ADS][CYP][DBR2] (termed CYTART) and
pRCS16F[kan][tHMG][CPR][mtADS][CYP][DBR2] (termed MITART), respectively.
Localization of ADS and mtADS

ADS and mtADS were cloned in frame upstream of EGFP in pSAT6-EGFP-Cl vector as previously described [Chung S.M. (2005), supra]. The resulting plasmids were used for transformation of protoplasts isolated from Arabidopsis thaliana leaf mesophyll using the TAPE-Arabidopsis Sandwich protoplast isolation method previously described [Wu FH et al. (2009) Plant Methods 24: 16] and cellular localization of EGFP signal was analyzed as reported previously [Spitzer-Rimon B. et al. (2010) Plant Cell 22: 1961-1976].

Plant transformation and callus generation

Plant transformation vectors were mobilized into Agrobacterium tumefaciens strain AGL0 by electroporation. Nicotiana tabacum 'Samsun' was transformed using standard leaf disc transformation method as previously described [Marton, I. et al. (2010) Plant Physiology 154: 1079-1087]. To generate control GFP-transgenic tobacco plants Agrobacterium tumefaciens strain AGL0 carrying pRCS2-EYFP-CHS/DsRed-P/ECFP was used as previously described [Tzfira, T. et al. (2005) supra]. Tobacco suspension culture was initiated from young leaves explants of TO MITART transgenic plants producing artemisinin or control GFP-transgenic tobacco plants. Explants were placed on solid Murashige and Skoog (MS) growth media supplemented with 2 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.2 mg/l kinetin and grown for ca. 4 weeks until calli were formed. Calli were then transferred to a solid MS media supplemented with benzylaminopurine (BA) 0.5 mg/l and 0.5 mg/l 1-naphthaleneacetic acid (NAA) and subcultured every 3 weeks. Suspension cultures were initiated by placing callus fragments in flasks with liquid media based on basal MS containing 200 mg/l KH$_2$PO$_4$, 1 mg/l thiamine, 100 mg/l myo-inositol, 0.4 mg/l 2,4-D and 3% sucrose and grown with continuous shaking for up to 4 weeks.

To generate transgenic aspen, stem explants were transformed as previously described [Tzfira, T. et al. (1997) Plant Mol. Biol. Rep. 15: 219-235] with A. tumefaciens strain AGL0 carrying MITART vector.

Transgenic M82 and Micro-Tom tomatoes were regenerated after transformation with A. tumefaciens strain AGL0 carrying the MITART construct as previously described in Vishnevetsky et al. [Vishnevetsky, M. et al. (1999) The Plant Journal 20: 423-431].
Marguerite and lettuce transgenic plants were generated by direct adventitious shoot regeneration obtained from *in-situ* grown leaves as previously described [Glassner, H., (2006) M.Sc, Thesis, The Hebrew University of Jerusalem] and using *A. tumefaciens* carrying the MITART construct.

**RNA extraction and RT-PCR analysis**

Total RNA was extracted from tobacco plants using RNeasy kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. For reverse transcription polymerase chain reaction (RT-PCR) analyses RNA was treated with RNase-free DNase (Promega, Madison, WI, USA), cDNA was generated using oligo(dT)i5 primer and M-MLV RT (both from Promega). Control samples were generated without the addition of RT to the reaction. The primers used for RT-PCR analysis are listed in Table 1, below. cDNA amplification was conducted with an initial denaturation step of 94 °C for 3 min, followed by 30 cycles of 94 °C for 10 s, 58 °C for 10 s, 72 °C for 40 s, and a final elongation step of 72 °C for 10 min. To confirm that the analyzed samples were not contaminated with DNA, PCR amplification was also conducted with samples generated without RT.

<table>
<thead>
<tr>
<th>Primer number</th>
<th>Primer name</th>
<th>Sequence (5' to 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>ADS-F1</td>
<td>ATGTCACTTACAGAAGAAAAACCTATTCG (SEQ ID NO: 1)</td>
</tr>
<tr>
<td>2</td>
<td>ADS-R253</td>
<td>TGATCAATCTCCCGTTCAAGTGA (SEQ ID NO: 2)</td>
</tr>
<tr>
<td>3</td>
<td>COX4-F</td>
<td>GCGGCCGCAAAAAAATGCTTTTCACCTACGTAATCTA (SEQ ID NO: 3)</td>
</tr>
<tr>
<td>4</td>
<td>HMG-F2604</td>
<td>TGATGTTTCCGATTTGTTGA (SEQ ID NO: 4)</td>
</tr>
<tr>
<td>5</td>
<td>HMG-R3057</td>
<td>ACTTTGAACAAATGGCCGG (SEQ ID NO: 5)</td>
</tr>
<tr>
<td>6</td>
<td>DBR2-F1</td>
<td>GCGGCCCCAGATTCTGAAAAACCAACCTTG (SEQ ID NO: 6)</td>
</tr>
<tr>
<td>7</td>
<td>DBR-R726</td>
<td>ATCGATTGAGGAGAGATTCTAAATACCAAC TCGGTCTGTACCAATGGCTGATACCGCTTCGAC (SEQ ID NO: 7)</td>
</tr>
<tr>
<td>8</td>
<td>CYP-F829</td>
<td>CTCAAAAGACAGTGGCTGAATTCCGAT (SEQ ID NO: 8)</td>
</tr>
<tr>
<td>9</td>
<td>CYP-R1489</td>
<td>CTAGAAGACTGGAACGAGTAACACCTCA (SEQ ID NO: 9)</td>
</tr>
<tr>
<td>10</td>
<td>CPR-F1943</td>
<td>ATCTGGAAATTTTACTCTCTGAGGAGCA (SEQ ID NO: 10)</td>
</tr>
</tbody>
</table>
Primer Primer Sequence (5’ to 3’)

<table>
<thead>
<tr>
<th>Primer number</th>
<th>Primer name</th>
<th>Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>CPR-ATCTCGAGTTACCATACATCACGGAGATATCTT</td>
<td>R2133 (SEQ ID NO: 36)</td>
</tr>
</tbody>
</table>

Table 1.

**Chemical analysis of artemisinin**

Dried and ground leaf samples (tobacco, *A. annua*, aspen, tomato or marguerite), 100 mg, were supplemented with 10 ng deuterium labeled artemisinin (Toronto Research Chemicals) and extracted by sonication for 15 min with 2 ml hexane. After partitioning into 1 ml methanol, phases were separated and the methanolic layer was concentrated to approximately 100 µl under a nitrogen stream. For extraction from plant cells suspension, the cultures (cells and medium) were freeze-dried in a lyophilizer for approximately 24 hours before grinding and extraction as with the leaves samples.

LC-MS/MS analysis was performed using Agilent 1200 series rapid resolution liquid chromatography system coupled to Agilent 6410 triple quadrupole mass spectrometer. For chromatographic separation, two Zorbax Eclipse XDB-C18 (100 x 2.1 mm, 1.8 µm, Agilent Technologies) columns were connected in sequence followed by Synergy Fusion-RP (100 x 2 mm, 2.5 µm, Phenomenex). Column temperature was maintained at 40 °C, injection volume was 10 µl. Chromatographic analysis was performed using a binary gradient as follows:

<table>
<thead>
<tr>
<th>Table 2: The binary gradient used for LC-MS/MS analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time (min)</td>
</tr>
<tr>
<td>------------</td>
</tr>
<tr>
<td>0</td>
</tr>
<tr>
<td>1.5</td>
</tr>
<tr>
<td>18</td>
</tr>
<tr>
<td>28</td>
</tr>
<tr>
<td>28.1</td>
</tr>
<tr>
<td>38</td>
</tr>
</tbody>
</table>

Table 2.

The mass spectrometer was equipped with electrospray ionization ion source which was operated in positive mode upon the following parameters: capillary voltage 4000 V, nebulizer pressure 241 kPa, drying gas 10 l/min, gas temperature 350 °C, 99.5 % nitrogen was used as nebulizer and drying gas and 99.999 % nitrogen was used as a collision gas. Artemisinin was detected in MRM mode by monitoring three transitions
(283 [MH+] → 247, 283 → 265 and 283 → 219) under following optimized parameters: fragmentor voltage 80 V and CID energy 4eV. The LC-MS system was controlled and data was analyzed using MassHunter software (Agilent Technologies Inc.).

For the LC-HR-MS analysis an Accela LC system coupled with an LTQ Orbitrap Discovery hybrid FT mass spectrometer (Thermo Fisher Scientific Inc.) was used. Chromatographic analysis was performed using two LC columns as described for LC-MS/MS and following a binary gradient program:

Table 3: The binary gradient used for LC-HR-MS analysis

<table>
<thead>
<tr>
<th>Time (min)</th>
<th>Solvent A, % Water (with 0.05 % AcOH)</th>
<th>Solvent B, % Acetone</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>2.5</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>18</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>28</td>
<td>0</td>
<td>100</td>
</tr>
<tr>
<td>28.1</td>
<td>75</td>
<td>25</td>
</tr>
<tr>
<td>38</td>
<td>75</td>
<td>25</td>
</tr>
</tbody>
</table>

Column temperature was maintained at 40 °C, flow rate was 250 µl/min and injection volume was 10 µl. The mass spectrometer was equipped with an APCI ion source operated in positive ionization mode. The ion source parameters were as follows: corona discharge needle current 5 µA, capillary temperature 250 °C, sheath gas rate 50 (arb), auxiliary gas rate 10 (arb), vaporizer temperature 400 °C. 99.5 % nitrogen was used as sheath and auxiliary gas. Ion transfer optic parameters were optimized for protonated artemisinin using the automatic tune option. Mass spectra were acquired in m/z 200-800 Da range, resolution was 30000. The LC-MS system was controlled and data were analyzed using Xcalibur software (Thermo Fisher Scientific Inc.).

**GC-MS analysis of amorpha-4,11-diene and its derivatives**

Leaf samples of tobacco, A. annua, aspen, tomato or marguerite (500 mg from each independent line) were ground in liquid nitrogen and extracted twice by sonication for 30 min with 2 ml hexane and 600 ng of the sesquiterpene valencene as internal standard. The extract was partially purified on a silica gel column (100 mesh) and washed with hexane. The eluate was concentrated under nitrogen stream before analyzing a 1 µl aliquot by gas chromatography-mass spectrometry (GC-MS). For
analysis of amorpha-4,1 1-diene production by tobacco suspension cultures a two-phase partitioning culture was employed by adding 10%-15% (v/v) n-dodecane as the organic phase. Cultures were grown for 29 days and the organic layer was then sampled for GC-MS as previously described [Farhi, M. et al. (2011) supra].

The GC-MS system was composed of a TRACE GC 2000 gas chromatograph and a TRACE DSQ quadrupole mass spectrometer (ThermoFinnigan). GC was run in a 30 m Rtx-5Sil MS column with 0.25-μm film thickness (Restek). The injection temperature was set at 250 °C, with an initial oven temperature of 100 °C for 1 min, followed by a 5 °C/min ramp to 270 °C. MS was operated in EI mode (70 eV) in both scanning mode (40-325 m/z) and selected ion monitoring of the molecular and fragment ions (204 and 119, 161 and 189 m/z). Amorpha-4,1 1-diene was identified and quantified using the selected ions' responses compared to that of the valencene internal standard as described previously [Farhi, M. et al. (2011) supra].

EXAMPLE 1

Construction of plant transformation vectors for expression of the artemisinin pathway

HMG-R is the main rate-limiting step in the MVA pathway, its activity is regulated by feedback inhibition and its overexpression in plants enhances the accumulation of terpenoids. To elevate precursors supply via the MVA pathway, a mutated HMG-R enzyme (tHMG) was generated to overcome the negative regulation and tHMG was cloned into the plant transformation vectors.

For production of artemisinin in plants the artemisinin pathway genes ADS, CYP71AV1 and DBR2 were cloned from A. annua.

To enable efficient activity of the cytochrome 450, its native reductase CPR was cloned.

Furthermore, the present inventors and others [see Farhi et al. (2011) supra; Kappers I. F. et al. (2005) Science 309: 2070-2072] found that targeting of terpene synthases, including ADS, to the mitochondria significantly improves terpenes production. Therefore, a version of the vector was constructed in which a mitochondrial targeting signal was fused to ADS (Figure IB). To confirm localization of ADS to the
cytosol and mtADS to the mitochondria, localization assays were also carried out (see Figures 2A-J).

To avoid repeated sequences in the recombinant T-DNA, which may lead to silencing of promoters or instability by recombination, each gene was placed between different promoters and terminators (Figure IB).

**EXAMPLE 2**

*Gene expression analysis of Nicotiana tabacum transformed with CYTART or MITART constructs*

The two plant binary transformation vectors CYTART and MITART were used to generate transgenic *Nicotiana tabacum* plants. 7 lines generated with the CYTART vector carrying *ADS* and 8 lines generated with the MITART vector carrying *mtADS*, all from independent transformation events, were rooted and grown in a greenhouse. No phenotypic differences were observed between artemisinin-producing ADS and mtADS plants and control lines. Expression of all five genes was confirmed by RT-PCR analysis in these transgenic lines (Figures 3A-B).

**EXAMPLE 3**

*LC-HR-MS analysis reveals artemisinin accumulation in metabolically engineered tobacco plants*

To test whether artemisinin was produced in metabolically engineered transgenic tobacco plants, tobacco extracts were subjected to high resolution, high accuracy liquid chromatography coupled with Orbitrap mass spectrometry (LC/Orbitrap MS). Complex matrix of plant extracts significantly increased chemical noise which resulted in decreased signal-to-noise ratio of artemisinin. This was circumvented by concocting two LC columns in sequence, which greatly improved the detection of artemisinin in plant extracts. Ion currents with mass to charge ratio (m/z) corresponding to that of artemisinin were detected in extracts from lines generated with either CYTART or MITART vectors. Identity of artemisinin was established and validated by comparison of retention time and exact m/z, in the positive mode, to those of an
authentic standard and to an internal deuterium labeled artemisinin added to the extracts (Figures 4A-C). No artemisinin was detected in negative control, GFP transgenic plants. Also CYTART or MITART lines expressing all the genes except ADS or mtADS respectively did not accumulate artemisinin, as expected. Lines biosynthesizing artemisinin via mitochondrial amorphadiene, i.e. MITART containing construct, achieved better than CYTART derived transgenes, and up to eight-fold higher levels of artemisinin were generated by mtADS-expressing line as compared to ADS-expressing lines (e.g. transgenic tobacco lines with cytosolic ADS produced about 0.75, 0.88, 0.94 and 0.48 μg artemisinin/g dry weight, whereas tobacco lines with mtADS generated about 5.0, 5.9, 6.3 and 6.8 μg artemisinin/g dry weight).

EXAMPLE 4

**LC-MS/MS analysis validates artemisinin production by metabolically engineered tobacco plants**

To confirm the structural identity of the artemisinin molecular ion identified by LC-HR-MS, extracts were also submitted to tandem MS analysis using triple quadrupole MS operated in the positive mode. The typical fragmentation of artemisinin, exact masses and isotopic profiles corresponding to the calculated one and to the authentic standard were observed in extracts from CYTART or MITART metabolically engineered plants (Figures 5A-D). As was observed in the LC-HR-MS, artemisinin was identified in both plants engineered with the either ADS or mtADS. Control CYTART or MITART lines expressing all the genes except ADS or mtADS respectively, did not biosynthesize artemisinin, as expected.

EXAMPLE 5

**GC-MS analysis reveals high-levels accumulation of amorpha,4-ll,diene in metabolically engineered tobacco suspension culture**

To produce artemisinin and its precursors in bioreactors of plant cell suspension cultures, an *in vitro* dedifferentiated culture was initiated from a high artemisinin producing tobacco line carting the MITART vector. Once a stable callus was formed it
was used to obtain a suspension culture. To trap amorphadiene and its subsequent oxidized molecules from the plant cells' liquid culture an *in-situ* product removal approach was used as previously described [Farhi, M. et al. (2011) supra]. Terpenoids trapped in the organic solvent were then subjected to GC-MS analysis which revealed production of amorpha-4,1 l-diene by the suspension culture (Figures 6A-C): 4.6-6.7 mg/l were measured, depending on growth time. The terpenoid was identified by comparison of the retention time and MS to those obtained from amorpha-4,1 l-diene extracted from *A. annua* and yeast producing amorpha-4,1 l-diene. Additionally, MS analysis indicated that the engineered tobacco cell suspension culture was also able to biosynthesis oxidized derivatives of amorpha-4,1 l-diene. No amorpha-4,1 l-diene, or its derivatives, was accumulate in GFP control lines. Extracts from suspension cultures, grown with or without a dodecane overlay, are monitored for artemisinin production by LC-HR-MS.

**EXAMPLE 6**

*Metabolic engineering of different plant species for production of artemisinin*

The MITART and CYTART vectors were used to genetically transform several, systematically unrelated, plant species. Several transgenic lines, arising from the different transformation events, were obtained and regenerated from tomato, lettuce, aspen and marguerite. Plantlets were transferred to the greenhouse for growth and are being analyzed using LC-HR-MS and GC-MS analysis for production of artemisinin and its precursors in different plant tissues and under different growth conditions and treatments.

Although the invention has been described in conjunction with specific embodiments thereof, it is evident that many alternatives, modifications and variations will be apparent to those skilled in the art. Accordingly, it is intended to embrace all such alternatives, modifications and variations that fall within the spirit and broad scope of the appended claims.

All publications, patents and patent applications mentioned in this specification are herein incorporated in their entirety by into the specification, to the same extent as if
each individual publication, patent or patent application was specifically and
individually indicated 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.
WHAT IS CLAIMED IS:

1. A method of generating and/or increasing content of artemisinin in a cell, comprising exogenously expressing within the cell:
   (i) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes formation of amorpha-4,1 1-diene from farnesyl diphosphate (FDP); and
   (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and
   (iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,1 1-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,1 1-diene and/or artemisinic alcohol;

   thereby generating and/or increasing the content of artemisinin in the cell.

2. A nucleic acid construct, comprising:
   (i) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes the formation of amorpha-4,1 1-diene from farnesyl diphosphate (FDP); and
   (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and
   (iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,1 1-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,1 1-diene and/or artemisinic alcohol.

3. A cell comprising a heterologous polynucleotide, wherein said heterologous polynucleotide comprises:
   (i) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes the formation of amorpha-4,1 1-diene from farnesyl diphosphate (FDP); and
(ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and

(iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,11-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,11-diene and/or artemisinic alcohol.

4. The method of claim 1, or the cell of claim 3, wherein said cell is a plant cell.

5. A plant comprising the plant cell of claim 4.

6. A method of producing artemisinin, comprising:
   (a) generating and/or increasing content of the artemisinin in a cell according to the method of claim 1 or 4, and
   (b) isolating the artemisinin from said cell, thereby producing the artemisinin.

7. A method of producing artemisinin, comprising:
   (a) providing said plant cell of claim 4, and
   (b) isolating the artemisinin from said plant cell, thereby producing the artemisinin.

8. The method of claim 6 or 7, further comprising subjecting said cell to light and/or to oxygen prior to said isolating of said artemisinin.

9. The method of claim 1, 4, 6, 7 or 8, further comprising exogenously expressing within the cell:
   (iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.
10. The method of claim 1, 4, 6, 7, 8 or 9, further comprising exogenously expressing within the cell:
   (v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

11. The nucleic acid construct of claim 2, further comprises:
   (iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

12. The nucleic acid construct of claim 2, further comprises:
   (v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

13. The cell of claim 3 or 4, or the plant of claim 5, wherein said heterologous polynucleotide further comprises:
   (iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

14. The cell of claim 3 or 4, or the plant of claim 5, wherein said heterologous polynucleotide further comprises:
   (v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

15. The method of claim 1, 4, 6, 7, 8, 9, or 10, the cell of claim 3, 4, 13 or 14, or the plant of claim 5, 13 or 14, wherein at least two of said polynucleotides of (i)-(v) are comprised in a single nucleic acid construct.

16. The method, the cell, or the plant of claim 15, wherein polynucleotides (i) and (ii) are comprised in a single nucleic acid construct.

17. The method, the cell, or the plant of claim 15, wherein polynucleotides (i) and (iii) are comprised in a single nucleic acid construct.
18. The method, the cell, or the plant of claim 15, wherein polynucleotides (ii) and (iii) are comprised in a single nucleic acid construct.

19. The method, the cell, or the plant of claim 15, wherein polynucleotides (i)-(iii) are comprised in a single nucleic acid construct.

20. The method, the cell, or the plant of claim 15, wherein polynucleotides wherein (i)-(iv) are comprised in a single nucleic acid construct.

21. The method, the cell, or the plant of claim 15, wherein polynucleotides wherein (i)-(v) are comprised in a single nucleic acid construct.

22. A method of generating and/or increasing content of artemisinin in a cell, comprising exogenously expressing within the cell a nucleic acid construct which comprises:
   (i) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which is capable of forming amorpha-4,11-diene from farnesyl diphosphate (FDP);
   (ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde;
   (iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,11-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,11-diene and/or artemisinic alcohol;
   (iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450; and
   (v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG), thereby generating and/or increasing content of artemisinin in said cell.

23. The method of any of claims 1, 4, 6, 7, 8, 9, 10, and 15-22, the nucleic acid construct of any of claims 2, 11, and 12, the cell of any of claims 3, 4, 13, and 14-
21, or the plant of any of claims 5, 13, and 14-21, wherein said nucleic acid sequence further comprises a nucleic acid sequence encoding a mitochondrial signal peptide to thereby direct localization of said polypeptide into said mitochondria of said cell.

24. The method, nucleic acid construct, plant cell or plant of claim 23, wherein said nucleic acid sequence comprises said nucleic acid sequence encoding an amorphadiene synthase (ADS).

25. The method of any one of claims 1, 4, 6, 7, 8, 9, 10 and 15-23, the nucleic acid construct of any one of claims 2, 11, 12, and 23, the cell of any one of claims 3, 4, 13, 14-21 and 23, or the plant of any one of claims 5, 13, 14-21 and 23, wherein said nucleic acid construct further comprises:

(vi) a polynucleotide comprising a nucleic acid sequence encoding a polypeptide which enables selection of a cell expressing said nucleic acid construct.

26. The method, nucleic acid construct, cell or the plant of claim 25, wherein said polypeptide which enables selection of a cell expressing said nucleic acid construct comprises a polypeptide which confers antibiotic resistance to a cell expressing said nucleic acid construct.

27. The method, the nucleic acid construct, the cell, or the plant of claim 26, wherein said polypeptide which confers said antibiotic resistance is neomycin phosphotransferase II (nptII).

28. The method of any of claims 1, 4, 6, 7, 8, 9, 10 and 15-27, the nucleic acid construct of any of claims 2, 11, 12, and 23-27, the cell of any of claims 3, 4, 13, 14, 15-21, and 23-27, or the plant of any of claims 5, 13, 14, 15-21 and 23-27, wherein each of said polynucleotides further comprises a promoter sequence for directing expression of said nucleic acid sequence in said cell.

29. The method, the nucleic acid construct, the cell or the plant of claim 28, wherein at least two of said promoters are not identical.
30. The method of any of claims 1, 4, 6, 7, 8, 9, 10 and 15-29, the nucleic acid construct of any of claims 2, 11, 12, and 23-29, the cell of any of claims 3, 4, 13, 14, 15-21 and 23-29, or the plant of any of claims 5, 13, 14, 15-21 and 23-29, wherein each of said polynucleotides further comprises a terminator sequence for controlling expression of said nucleic acid sequence in said cell.

31. The method of any of claims 4, 6-10 and 15-30, the cell of any of claims 4,13-14, 15-21 and 23-30, or the plant of claim 5, 13, 14, 15-21 and 23-30, wherein the plant is a tobacco plant.

32. The method of any of claims 4, 6-10 and 15-30, the cell of any of claims 4,13-14, 15-21 and 23-30, or the plant of claim 5, 13, 14, 15-21 and 23-30, wherein the plant is selected from the group consisting of tobacco, aspen, tomato, marguerite and lettuce.

33. An isolated artemisinin produced by the method of claim 6 or 7.

34. The isolated artemisinin of claim 33, wherein said artemisinin is characterized by liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) operated in multiple reaction monitoring (MRM) mode and monitoring MRM traces m/z 283.2 → 219 and 283.2 → 265.

35. The isolated artemisinin of claim 33, wherein said artemisinin is characterized by liquid chromatography-high resolution mass spectrometry (LC-HR-MS) m/z value of 283.1530 Da.

36. A polynucleotide system comprising:
   (i) a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes formation of amorpha-4,11-diene from farnesyl diphosphate (FDP); and
(ii) a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and

(iii) a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,1 1-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,1 1-diene and/or artemisinic alcohol.

37. The polynucleotide system of claim 36, further comprises:

(iv) a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

38. The polynucleotide system of claim 36 or 37, further comprise:

(v) a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

39. The polynucleotide system of any of claims 36-38, further comprises:

(vi) a polynucleotide comprising a nucleic acid sequence encoding a polypeptide which enables selection of a cell expressing said nucleic acid construct.

40. A nucleic acid construct system comprising:

(i) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding amorphadiene synthase (ADS) which catalyzes formation of amorpha-4,1 1-diene from farnesyl diphosphate (FDP); and

(ii) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding artemisinic aldehyde delta-11(13) reductase (DBR2) which catalyzes reduction of artemisinic aldehyde to dihydroartemisinic aldehyde; and

(iii) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding amorpha-4,1 1-diene monooxygenase (CYP71AV1) which catalyzes oxidation of amorpha-4,1 1-diene and/or artemisinic alcohol.
41. The nucleic acid construct system of claim 40, further comprises:
   (iv) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding cytochrome P450 reductase (CPR) which catalyzes reduction of cytochrome p450.

42. The nucleic acid construct system of claim 40 or 41, further comprises:
   (v) a nucleic acid construct comprising a polynucleotide comprising a nucleic acid sequence encoding a mutated form of yeast 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMG).

43. The nucleic acid construct system of any of claims 40-42, further comprises:
   (vi) a polynucleotide comprising a nucleic acid sequence encoding a polypeptide which enables selection of a cell expressing said nucleic acid construct.

44. The system of claim 39 or 43, wherein said polypeptide which enables selection of a cell expressing said nucleic acid construct comprises a polypeptide which confers antibiotic resistance to a cell expressing said nucleic acid construct.

45. The system of claim 44, wherein said polypeptide which confers said antibiotic resistance is neomycin phosphotransferase II (nptII).

46. A kit comprising the system of any of claims 36-45 and instructions for use in transformation of a cell.

47. The method of claim 22, wherein said cell is a plant cell.
FIG. 4A

CYTART [tHMG/ADS/CYP71AV1/CPR/DBR2]

Relative abundance

FIG. 4B

MITART [tHMG/mtADS/CYP71AV1/CPR/DBR2]

FIG. 4C

ADS-nonexpressing [tHMG/CYP71AV1/CPR/DBR2]

GFP
beta-lactamase (ampicillin resistance)

ten promoter

EcoRI (735)

pSAT2A.nosP.tHMG

4849 bp

tHMG

nos terminator

BamHI (2319)

FIG. 7
FIG. 10

pSAT6A.supP, DBR2

5741 bp

Super Promoter

EcoRI (1865)

KpnI (3039)

beta-lactamase (ampicillin resistance)

AGT/Stp PolyA
FIG. 11

- beta-lactamase (ampicillin resistance)
- CaMV 35S promoter
- CaMV dual 35S promoter + TEV enhancer
- CaMV 35S promoter
- translational enhancer; 5'-UTR from tobacco etch virus
- EcoRI (1342)
- CaMV 35S terminator
- BamHI (2989)
- pSAT5A.35SP.ADS 5485 bp

**CaMV 35S terminator**
Fig. 12

- beta-lactamase (ampicillin resistance)
- CaMV 35S promoter
- CaMV dual 35S promoter + TEV enhancer
- CaMV 35S promoter
- translational enhancer; 5'-UTR from tobacco etch virus
- EcoRI (13,42)
- mtADS
- CaMV 35S terminator
- BamHI (2989)
- pSAT5A.35SP.mtADS 5485 bp
FIG. 13

pSAT1A.ocsAocsP.nptII.ocsT
4603 bp

beta-lactamase (ampicillin resistance)
oct activator
ocs promoter
hptII
ocs terminator
**INTERNATIONAL SEARCH REPORT**

**International application No**

PCT/IL2012/050177

<table>
<thead>
<tr>
<th>A. CLASSIFICATION OF SUBJECT MATTER</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>INV. C12N15/82</td>
<td></td>
</tr>
<tr>
<td>C12N9/88</td>
<td></td>
</tr>
<tr>
<td>C12N9/00</td>
<td></td>
</tr>
<tr>
<td>C12N5/04</td>
<td></td>
</tr>
<tr>
<td>A01H5/00</td>
<td></td>
</tr>
</tbody>
</table>

**ADD.**

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

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

<table>
<thead>
<tr>
<th>C12N</th>
<th>A01H</th>
</tr>
</thead>
</table>

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

**Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)**

EPO-Internal, BIOSIS, EMBASE, WPI Data, Sequence Search

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y</td>
<td>the whole document</td>
<td>9-14, 20-24, 37,38, 41,42</td>
</tr>
<tr>
<td>X</td>
<td>ZHANG YANSHENG ET AL: &quot;The producti on of artemisin precursors in tobacco&quot;, PLANT BIOTECHNOLOGY JOURNAL, vol. 9, no. 4, 18 August 2010 (2010-08-18), pages 445-454 URL, XP002682751, the whole document</td>
<td>1-8, 15-19, 28, 30-32, 36,40, 46,47</td>
</tr>
</tbody>
</table>

**[X] Further documents are listed in the continuation of Box C.**

**[X] See patent family annex.**

*Special categories of cited documents:

- **"A"** document defining the general state of the art which is not considered to be of particular relevance.
- **"E"** earlier application or patent but published on or after the international filing date.
- **"L"** document which may throw doubts on priority claim(s) on which is cited to establish the publication date of another citation or other special reason (as specified).
- **"O"** document referring to an oral disclosure, use, exhibition or other means.
- **"P"** document published prior to the international filing date but later than the priority date claimed.

**[X]** Later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention.

**[X]** Document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone.

**[X]** Document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

**[A]** Document member of the same patent family.

Date of the actual completion of the international search

4 September 2012

Date of mailing of the international search report

02/10/2012

Name and mailing address of the ISA

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

Authorized officer

Oderwald, Harald
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>WALEERAT BANYAI ET AL: &quot;Overexpression of farnesyl pyrophosphate synthase (FPS) gene affected artemisin content and growth of Artemisia annua L.\textsuperscript{,}\textsuperscript{1}\textsuperscript{,}\textsuperscript{2}\textsuperscript{,}\textsuperscript{3}\textsuperscript{,}\textsuperscript{4}\textsuperscript{-5}\textsuperscript{,}\textsuperscript{6}\textsuperscript{,}\textsuperscript{7}, PLANT CELL, TISSUE AND ORGAN CULTURE, vol. 103, no. 2, 4 June 2010 (2010-06-04), pages 255-265, XP019831295, KLUWER ACADEMIC PUBLISHERS, DOI: 10.1007/s11110-010-0114-0</td>
<td>33-35</td>
</tr>
<tr>
<td>X</td>
<td>HONG GA-JI ET AL: &quot;Increased accumulation of Artemisia annua Expressing the Arabidopsis Blue Light Receptor CRY1&quot;, PLANT MOLECULAR BIOLOGY REPORTER, vol. 27, no. 3, September 2009 (2009-09), pages 334-341, XP002682752, ISSN: 0735-9640</td>
<td>33-35</td>
</tr>
<tr>
<td>A</td>
<td>figure 5</td>
<td>8</td>
</tr>
<tr>
<td>X</td>
<td>GA0-BIN PU ET AL: &quot;Salicylic acid activates artemisin biosynthesis in Artemisia annua L.\textsuperscript{,}\textsuperscript{1}\textsuperscript{,}\textsuperscript{2}, PLANT CELL REPORTS, vol. 28, no. 7, 12 June 2009 (2009-06-12), pages 1127-1135, XP019709182, SPRINGER, BERLIN, DE, ISSN: 1432-203X</td>
<td>33-35</td>
</tr>
<tr>
<td>A</td>
<td>figures 2-5</td>
<td>8</td>
</tr>
<tr>
<td>A</td>
<td>figure 6, table 1</td>
<td>8</td>
</tr>
</tbody>
</table>

\textsuperscript{1} Retrieved on 2009-04-01
<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>X</td>
<td>ABDUL MANNAN ET AL: &quot;DMSO triggers the generation of ROS leading to an increase in artemisinin and dihydroartemisinic acid in Artemisia annua shoot cultures&quot;, PLANT CELL REPORTS, vol. 29, no. 2, 20 December 2009 (2009-12-20), pages 143-152, XP019779603, SPRINGER, BERLIN, DE</td>
<td>33-35</td>
</tr>
<tr>
<td>A</td>
<td>the whole document</td>
<td>8</td>
</tr>
<tr>
<td>X</td>
<td>JING FUYUAN ET AL: &quot;Abscisic acid (ABA) treatment increases artemisinin content in Artemisia annua by enhancing the expression of genes in artemisin biosynthetic pathway&quot;, BIOLOGIA (BRATISLAVA), vol. 64, no. 2, April 1 (2009-04), pages 319-323, XP002682753, A figures 1, 2</td>
<td>33-35</td>
</tr>
<tr>
<td>Y</td>
<td>RO DAE-KYUN ET AL: &quot;Induction of multiple pleiotropic drug resistance genes in yeast enginered to produce an increased level of anti-malarial drug precursor, artemisinic acid&quot;, BMC BIOTECHNOLOGY, vol. 8, no. 1, 4 November 2008 (2008-11-04), page 83, XP021043280, BIOMED CENTRAL LTD, LONDON, GB</td>
<td>9, 11, 13, 20, 22, 37, 41</td>
</tr>
<tr>
<td>Y</td>
<td>VAN HERPEN TEUN W J M ET AL: &quot;Ni cotiana benthamiana as a Product for Platform for Artemisin Precursors&quot;, PLOS ONE, vol. 5, no. 12, December 2010 (2010-12), XP002682754, ISSN: 1932-6203, the whole document</td>
<td>10, 12, 14, 21, 22, 38, 42</td>
</tr>
<tr>
<td>Category</td>
<td>Citation of document, with indication, where appropriate, of the relevant passages</td>
<td>Relevant to claim No.</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------------</td>
<td>----------------------</td>
</tr>
<tr>
<td>X,P</td>
<td>FARHI MORAN ET AL:  &quot;Generation of the potent anti-malarial drug artemisinin in tobacco. &quot;</td>
<td>1-47</td>
</tr>
<tr>
<td></td>
<td>NATURE BIOTECHNOLOGY, vol. 29, no. 12, December 2011 (2011-12)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>pages 1072-1074, XP002682756, ISSN: 1546-1696, DOI: 10.1038/NBT.2054</td>
<td></td>
</tr>
<tr>
<td></td>
<td>the whole document</td>
<td></td>
</tr>
<tr>
<td>Patent document cited in search report</td>
<td>Publication date</td>
<td>Patent family member(s)</td>
</tr>
<tr>
<td>----------------------------------------</td>
<td>------------------</td>
<td>-------------------------</td>
</tr>
<tr>
<td>WO 2009067784 Al</td>
<td>04-06-2009</td>
<td>CA 2706424 Al</td>
</tr>
<tr>
<td></td>
<td></td>
<td>US 2010299778 Al</td>
</tr>
<tr>
<td></td>
<td></td>
<td>WO 2009067784 Al</td>
</tr>
</tbody>
</table>