9-13-2011

Transformed Plants Accumulating Terpenes

Joe Chappell  
*University of Kentucky, chappell@uky.edu*

Shuiqin Wu  
*University of Kentucky*

Michel Schalk

Anthony Clark

*Click here to let us know how access to this document benefits you.*

Follow this and additional works at: [https://uknowledge.uky.edu/pss_patents](https://uknowledge.uky.edu/pss_patents)

Part of the [Plant Sciences Commons](https://uknowledge.uky.edu/pss_patents)

**Recommended Citation**

Chappell, Joe; Wu, Shuiqin; Schalk, Michel; and Clark, Anthony, "Transformed Plants Accumulating Terpenes" (2011). *Plant and Soil Sciences Faculty Patents*. 3.  
[https://uknowledge.uky.edu/pss_patents/3](https://uknowledge.uky.edu/pss_patents/3)

This Patent is brought to you for free and open access by the Plant and Soil Sciences at UKnowledge. It has been accepted for inclusion in Plant and Soil Sciences Faculty Patents by an authorized administrator of UKnowledge. For more information, please contact UKnowledge@lsv.uky.edu.
TRANSFORMED PLANTS ACCUMULATING TERPENES

Inventors: Joe Chappell, Lexington, KY (US); Shuqin Wu, Lexington, KY (US); Michel Schalk, Collonges-Sous-Saleve (FR); Anthony Clark, West Windsor, NJ (US)

Assignee: University of Kentucky Research Foundation, Lexington, KY (US)

Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 681 days.

PCT Filed: Apr. 18, 2006
PCT No.: PCT/IB2006/051198
§ 371(e)(1), (2), (4) Date: Apr. 25, 2008
PCT Pub. No.: WO2006/111924
PCT Pub. Date: Oct. 26, 2006

Prior Publication Data

Foreign Application Priority Data
Jun. 16, 2005 (EP) 05105281

Int. Cl.
C12N 15/29 (2006.01)
C12N 15/31 (2006.01)
C12N 15/52 (2006.01)
C12N 15/82 (2006.01)

U.S. Cl. .... 800/288; 800/298; 435/193; 435/320.1

Field of Classification Search None
See application file for complete search history.

References Cited

U.S. PATENT DOCUMENTS
2004/0161819 A1 8/2004 Aharoni et al. .. 435/69.1

FOREIGN PATENT DOCUMENTS

OTHER PUBLICATIONS

* cited by examiner

Primary Examiner — Russell Kallis
Attorney, Agent, or Firm — Winston & Strawn LLP

ABSTRACT

The present invention relates to transformed plants with an altered terpene content, preferably over-accumulating a mono- or sesqui-terpene. By transformation of plants with genes encoding terpene synthases (TS), and prenyl transferases (PRT), plants accumulating at least 1000 ng per g of fresh leaf of a specific terpene were obtained. The present invention provides an advantageous system for production of terpenes in that any desired mono- or sesqui-terpene at the choice of the skilled person can be produced in plants. Preferably, the transformed plants contain at least one recombinant plastid targeted TS and PRT.

10 Claims, 20 Drawing Sheets
Figure 1 (A and B)
Figure 2

ADS transgenic line

Amorpha-4,11-diene

Cubenol

WT
Figure 3

![Graph showing Patchoulol content for different conditions.](image)
Figure 5

<table>
<thead>
<tr>
<th></th>
<th>2PTS</th>
<th>2tpPTS</th>
<th>2FPS-PTS</th>
<th>2tpFPS-PTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTS</td>
<td>5</td>
<td>6</td>
<td>12</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>2PTS+FPS</td>
<td>2tpPTS+FPS</td>
<td>FPS</td>
<td>2tpFPS+FPS</td>
</tr>
<tr>
<td>PTS</td>
<td>4</td>
<td>13</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>2PTS+FPS</td>
<td>2tpPTS+FPS</td>
<td>FPS</td>
<td>2tpFPS+FPS</td>
</tr>
<tr>
<td>FPS</td>
<td>4</td>
<td>13</td>
<td>14</td>
<td>18</td>
</tr>
<tr>
<td></td>
<td>2PTS+FPS</td>
<td>2tpPTS+FPS</td>
<td>FPS</td>
<td>2tpFPS+FPS</td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>PTS</th>
<th>2PTS</th>
<th>2tpPTS</th>
<th>2PTS+FPS</th>
<th>2tpPTS+FPS</th>
<th>tpFPS</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>6</td>
<td>14</td>
<td>14</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>2PTS+FPS</td>
<td>2tpPTS+FPS</td>
<td>FPS</td>
<td>2tpFPS+FPS</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FPS</td>
<td>4</td>
<td>13</td>
<td>14</td>
<td>18</td>
<td>WT1</td>
</tr>
<tr>
<td></td>
<td>2PTS+FPS</td>
<td>2tpPTS+FPS</td>
<td>FPS</td>
<td>2tpFPS+FPS</td>
<td></td>
</tr>
<tr>
<td>FPS</td>
<td>13</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>
Figure 6
Figure 8

SphI
P-NOS
HPT
CaUTR
cm ccdB
T-NOS
RB
attp2
attp1
LB

pBDON

Figure 9 A

Ndel
EcoRI
Xhol
Ncol
Gene 1
attB1
Pcv
TNOS
attB2

pTMON

Figure 9 B

SphI
SgfI
EcoRI
Xhol
XbaI
SpeI
KpnI
SacI
Gene 1
Gene 2
attB1
Pcv
Tnos
Pca
Tnos
attB2

pTDUAL
Figure 11

```
(15)  HPT  CaUTR  
      |     |     |
      No   Xba   

(18)  pTHPT

Sph

(19)  P-NOS  HPT  CaUTR  
      |     |     |
      Sph  No  Xba   

(20)  P-NOS  HPT  CaUTR  
      |     |     |
      Sph  Sph   

(16)  Sph  No  Xba

(13)  Sph  

(14)  ccm  ccdB  T-NOS  

(21)  P-NOS  HPT  CaUTR  
      |     |     |
      Sp   Sph   Sst   

pBI101 (invitrogen, Carlsbad, CA)
```

pTRBP

pTRBP HPT TT

pBattp

pBDON

attP

attP
Figure 13 part I

- KpnI to Sacl: Tnos attB2
- XbaI to SpeI: pGTNosK
- KpnI to Sacl: pTPca
- XbaI to SpeI: pTHPO

References:
(29) KpnI to Sacl: XbaI to SpeI: Pca
(30) KpnI to Sacl: XbaI to SpeI: Pca
(31) KpnI to Sacl: XbaI to SpeI: Pca
(32) KpnI to Sacl: XbaI to SpeI: Gene
(33) KpnI to Sacl: XbaI to SpeI: Gene
(34) KpnI to Sacl: Tnos attB2
(35) KpnI to Sacl: Tnos attB2
(36) KpnI to Sacl: Tnos attB2

- pTPHPO
- Gene
- Pca
Figure 13 part II

Gene 1

attB1  Pcv  Tnos

SphI  EcoRI  XhoI  XbaI  SacI

Gene

Pca  attB2

XbaI  SpeI  KpnI  SacI

nTPHPOT

Gene

Pca  attB2

XbaI  SpeI  KpnI  SacI

pTPCVST

attB1  Pcv  Tnos

SphI  SfeI  EcoRI  XhoI  XbaI  SpeI  KpnI  SacI

Gene

Pca  Tnos  attB2

pTDUAL
Figure 14

pBDON

Pnos HPT Tca attP2 attP1

pTMON

attB1 Pcv 6xHis PTS Tnos attB2

pTDUAL

attB1 Pcv 6xHis PTS Tnos Pca FPS Tnos attB2

(41) (42) (43)
With transient expression of helper Vector pKO107 or pKO111

pLGUS

Figure 16
TRANSFORMED PLANTS ACCUMULATING TERPENES

TECHNICAL FIELD

The present invention relates to transformed plants over-accumulating a specific mono- or sesquiterpene. The present invention further relates to a transformed plant with an altered content of a specific terpene, a vector comprising at least one nucleotide sequence encoding a prenyl transferase (PRT) and/or a mono- or sesquiterpene synthase (TS), methods for altering the content of specific terpenes in a plant, a method for increasing the content of specific terpenes in a plant, a method of producing a specific terpene and the use of at least one nucleotide sequence for producing plants having an altered terpene content.

BACKGROUND OF THE INVENTION AND PROBLEM TO BE SOLVED

Terpenes and terpenoids are found in most organisms. Their important commercial value, which is constantly increasing, is linked to the diverse range of bioactivities and functionalities encompassed by different terpenes. Accordingly, many vitamins, hormones, insect repellents, drugs, flavors and fragrances are found amongst this very large class of compounds, which all are made starting from units of 5 carbons called isoprene units.

Terpenes can be classified by the number of isoprene units present in their structure: monoterpenes (C_{10}), sesquiterpenes (C_{15}), diterpenes (C_{20}), triterpenes (C_{30}), tetraterpenes (C_{40}) and polyterpenes (C_{n}, n equal to or greater than 45 carbons). The plant kingdom contains a high diversity of mono- and sesquiterpenes representing thousands of different structures.

The chemical synthesis of higher terpenes such as sesqui- and diterpenes is very complex and environmentally acceptable processes for their preparation have not yet been realized. Therefore, it is a first objective of the present invention to provide methods for efficiently producing or accumulating specific terpenes while avoiding multiple-step chemical synthesis.

Studies on the biosynthetic pathway of terpenes revealed that the common C_{5}-precursor to all terpenes is isopentenyl diphosphate (IPP). Two distinct pathways for IPP biosynthesis coexist in the plants. The mevalonate pathway (MVA) is found in the cytosol in association with the endoplasmic reticulum and the non-mevalonate pathway, also called deoxyxylulose or methyl-D-erythritol phosphate pathway (DOXP/MEP) is found in the plastids of higher plants. The starting products, the enzymes involved and the catalysed reactions are different in both pathways, and, in the cells of higher plants they operate in parallel and complement each other. Accordingly, the MVA pathway in the cytoplasm is responsible for the biosynthesis of sterols, sesquiterpenes, and polyterpenes, whereas the plastid (MEP pathway) provides C_{5}-units for the synthesis of monoterpenes, diterpenes, for example kaurene (C_{20}), and polyterpenes, for example carotenoids (C_{40}) and plastoquinone-9 (C_{48}).

Following the synthesis of IPP, it is repetitively condensed by prenyl transferases (PRT) to form the acyclic prenyl diphosphate terpene precursors for each class of terpenes, that is, geranyl-diphosphate (GGPP) for the monoterpenes, farnesyl-diphosphate (FPP) for the sesquiterpenes, geranygeranyl-diphosphate (GGGPP) for the diterpenes. These precursors in turn serve as substrate for the terpene synthases or cyclases, which are specific for each class of terpene, e.g. monoterpene, sesquiterpene or diterpene synthases. Terpene synthases can catalyze complex multiple step cyclizations to form the large diversity of carbon skeleton of the terpene compounds.

Attempts have been made to isolate specific terpene synthases and WO 2004/031376 reports the isolation of the genes encoding cubebol, valencene and germacrene synthases. When E. coli cells were transformed with plasmids containing these genes, the corresponding fragrance compounds could be found in the cultivating medium. Generally, in view prior art concerned with heterologous expression of terpene synthases, it is an objective to provide means and methods for accumulating specific terpenes in still higher amounts.

In U.S. Pat. No. 5,589,619, U.S. Pat. No. 5,365,017, U.S. Pat. No. 5,349,126 and U.S. Pat. No. 5,349,126 processes for increasing squalene and sterol accumulation in transgenic plants are disclosed. These references, however, are silent as to how the accumulation of other classes of terpenes, such as mono-sesqui- and diterpenes could be increased.

The preparation of transgenic plants is also the subject of U.S. Pat. No. 6,841,717, which relates to genes associated with the MEP-pathway. This reference teaches a DNA molecule encoding an FPPS Synthase (GGCE protein), which was linked to a chloroplastic transit peptide and was thus used to produce a transgenic plant. While this reference deals with the accumulation of tocopherol substrates, it is silent how other terpene compounds can effectively be accumulated.

In U.S. Pat. No. 6,653,530 a method for increasing carotenoid production in seed is disclosed, in which a host plant is transformed with nucleic acid sequence of Erwinia uredora encoding a phytoene synthase.

WO 00/22150 discloses methods creating or enhancing resistance to insects in plants by expressing the monoterpene synthases limonene-, carveol and S-linalool synthases in plants transformed with nucleotide sequences encoding these enzymes.

WO 02/3360 A2 discloses nucleic acid sequences and methods for producing plants and seeds having altered tocopherol content and compositions.

In WO 91/13078 DNA sequences encoding various enzymes of Erwinia herbicola are disclosed. Transformed host organisms producing GGPP and various carotenoids are also mentioned.

EP 1 063 297 provides cDNA sequences coding for farnesyl diphosphate synthase and transgenic plants expressing heterologous farnesyl diphosphate synthase.

WO 02/064764 discloses isolated or recombinant nucleic acid sequences capable of synthesizing a monoterpene linalool and/or a sesquiterpene nerolidol when contacted with the respective precursor. In example 11, the difficulty of producing sesquiterpenes in transgenic plants is acknowledged, and no concrete results in this respect are presented.

Similarly, in a publication of Aharoni et al. “Terpenoid Metabolism in Wild-Type and Transgenic Arabidopsis Plants”, the Plant cell, Vol. 15, 2866-2884, only very low levels of nerolidol were synthesized by linalool/nerolidol synthase targeted to the plastids.

The present inventors address the problem of producing or accumulating a specific, selected terpene. Preferably, a method is provided, which is suitable to produce not only a predetermined, but any terpene of interest. The objective is thus to provide a system which allows, for example, the accumulation of any of the above-indicated sesquiterpenes, such as cubebol, valencene, germacrene, patchoulol, but which is also suitable to accumulate other terpenes. This problem has so far not been solved by the prior art, and the latter basically suggesting recombinant organisms having modified properties in the MVA or MEP pathway, and observing that certain terpene end products get accumulated.
A further objective of the present invention is to provide means for generating any selected terpene, preferably a sesquiterpene, in a stereochemically pure form and with a reliable and cost effective production platform.

An important problem addressed by the present inventors is the increased accumulation of a selected terpene, preferably a sesquiterpene, in plants. In the prior art, yields of a maximum of about 10 μg terpene per g fresh weight plant material are reported. In particular with respect to sesquiterpenes, accumulation remains particularly low, in general in the order of micro-grams or below. It is thus an objective of the present invention to provide a possibility of accumulating more significant amounts of any terpene at the choice of the skilled person in a plant, and in particular of a sesquiterpene. Preferably, the plant can easily be cultivated. It is a further objective to accumulate the terpene in plant organs that provide a high biomass with respect to the total weight of the adult plant.

Another objective of the present invention is to provide plants accumulating sufficient amounts of terpenes for inhibiting growth of plant pathogens and attack by herbivores.

SUMMARY OF THE INVENTION

Remarkably, the present inventors were able to transform organisms with genes encoding a farnesyl diphosphate synthase (FPS) and a specific mono- or sesquiterpene synthase targeted to the plastids of the plant and obtained high yield of the terpene that is synthesized by the terpene synthase (TS).

For the first time, enzymes of the isoprenoid biosynthetic pathway typically present in the cytosol could be directed to another cell compartment, the plastid, and, surprisingly, the amount of selected terpene compounds could be increased by exploring precursors of a different pathway. An important advantage of the present invention is that any desired terpene can be accumulated in any plant, if the nucleotide sequence encoding the synthase of the selected terpene is known or susceptible to be isolated. This is possible by targeting to the plastids of a plant at least two gene products, that is a TS and the enzyme capable of synthesising the direct precursor for the TS, a prenyl transferase (PRT).

Accordingly, the present invention provides, in a first aspect, a transformed plant over-accumulating a specific terpene, preferably a mono- or sesquiterpene.

In a further aspect, the present invention provides a transformed plant with an altered content of a specific terpene, said transformed plant comprising structural genes comprising at least one nucleotide sequence encoding a prenyltransferase (PRT) and a terpene synthase (TS) targeted to the plastids of the plant.

In another aspect, the present invention provides a transformed plant accumulating a sesquiterpene, which is transformed to comprise at least one nucleotide sequence encoding a farnesyl diphosphate synthase (FPS) and a sesquiterpene synthase targeted to the plastid of the plant.

In a further aspect, the present invention provides a vector comprising at least one nucleotide sequence encoding a PRT and/or a TS, or a fusion protein of a PRT and a TS, said nucleotide sequence further comprising a plastid targeting sequence linked in frame to the nucleotide sequence encoding the PRT, the TS, and/or the fusion protein.

In still further aspects, the present invention provides methods for altering the content of specific terpenes in a plant and for increasing the content of specific terpenes in a plant, the methods comprising the steps of transforming plant material with at least one DNA construct comprising at least one nucleotide sequence encoding a farnesyl diphosphate synthase, FPS or, if the terpene is a monoterpen, a geranyldiphosphate synthase, and a sesquiterpene synthase, or, a monoterpen synthase, respectively, and, regenerating the transformed plant from the transformed plant material.

In other aspects, the present invention provides a method of producing a specific terpene, the method comprising the step of isolating the terpene from the plant according to the invention, and, the use of at least one nucleotide sequence encoding a plastid targeted functional PRT and/or TS for producing plants having an altered terpene content.

The present invention also relates to a method of producing a sesquiterpene, the method comprising the step of cultivating the plant of the present invention.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows GC-MS analysis of a control tobacco line (WT) and one transformed with a construct comprising plastid targeted genes encoding a sesquiterpene (patchoulol) synthase and a farnesyl diphosphate synthase (tpIPS +tpFPS). Peak 12 in chromatogram A (not shown) could be identified as patchoulol by comparison to authentic patchoulol (D). Peak 3 was used as an internal standard (3-ct-cedrene).

FIG. 2 shows GC-MS analysis of a control tobacco line (WT) and one transformed with a construct comprising plastid targeted genes encoding another sesquiterpene (amorphalin) synthase and a farnesyl diphosphate synthase (tpADSA +tpFPS). The main peak shows accumulation of amorphalin in transformed plants.

FIG. 3 shows patchoulol content in leaves of plants transformed with different constructs (see detail in FIG. 2). The comparison reveals that high yields of patchoulol (>10,000 ng/g of fresh leaf FW) were accumulated in plants transformed with plastid targeted PTW and FPS, be it in the form of distinct gene products or in the form of a fusion protein. Cytosol targeted constructs remained below 2000 ng/g FW.

FIG. 4 quantitatively shows the mRNA expression levels in regenerant transgenic plant lines overexpressing a farnesyl diphosphate synthase (FPS), a sesquiterpene (patchoulol) synthase (PTS), or both.

FIG. 5 shows Expression levels and processing of the patchoulol synthase (PTS) and farnesyl diphosphate synthase (FPS) proteins in transgenic plants as measured immunodetection.

FIG. 6 shows predicted 13C-labeling patterns in patchoulol bio-synthesised through the MEP or MVA pathway in seedlings grown on [1,13C]glucose.

FIG. 7 compares MS parent ions for patchoulol synthesized by plants fed [13C]glucose (upper panel), versus synthesis from [1,13C]glucose by plants engineered for cytosolic (middle) or plastidic (bottom) biosynthesis. FIG. 8 shows the organization of a vector ("pBDON") suitable for A. tumefaciens-mediated transformation of plants, in which the region between the attp2 and the attp1 sequence (cm ccdB) may in vitro and site-specifically be recombined to harbour a TS, and/or PRT targeted to the plastid of the plant. The vector comprises border regions for transformation of plants, promoters, terminators, and the attp1/2 recombination sequences, as discussed in further detail in the examples.

FIGS. 9 A and 9 B show helper vectors ("p1MON" and "p1DUAL") comprising, between the attp2 and the attp1 sequence, one or two structural genes, respectively, to be inserted by site-specific recombination into the vector pBDON of FIG. 6. Part A shows the helper vector suitable to insert one gene at a time into the pBDON vector, while part B
shows the helper vector suitable to insert two genes at a time into pBDON. The vectors comprises promoter, terminator, attP1/2 recombination and place-holder sequences CVS, HPO, as discussed in further detail in the examples.

FIGS. 10 A and 10 B show the organisation of the T-DNA region of different plant transformation vectors. The HPT region provides hygromycin resistance, PTSE encodes a terpene (patchoulol)-synthase and FPS a farnesyl diphosphate synthase (a PRT). In some vectors, genes are flanked by a His-tag, or a plastid-targeting sequence (tp). The vector further comprises suitable promoter and terminator sequences.

FIG. 11 schematically shows the construction of pBDON vector (FIG. 8).

FIG. 12 schematically shows the construction of pTMON helper vector (FIG. 9 A).

FIG. 13 part I and II schematically show the construction of pTDUAL helper vector (FIG. 9 B).

FIG. 14 shows the insertion of relevant fragments from helper vectors pTMON and pTDUAL (FIGS. 10 A and B) into the pBDON vector to obtain a family of suitable plant transformation vectors.

FIGS. 5 parts I-III show the details of the preparation of a vector for transformation of plant plastidic DNA with genes encoding sesquiterpene and farnesyl diphosphate synthases.

FIG. 16 shows the organisation of vectors pLGUS and pLFP4P4TS for transforming the plastid genome of plants with genes encoding GUS and FPS4P4TS, respectively. NtLA63 is a Nicotiana tabacum strain of which the integration site for the transgenes, attP1, is indicated.

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to a transformed plant accumulating a specific mono-, or sesquiterpene. The invention also relates to transformed plants with an altered content of a specific terpene, the plant comprising structural genes targeted to the plastids of the plant.

A "transformed plant", for the purpose of the present invention, is a plant, which was subjected to genetic engineering. A "transformed plant", includes axenically (vegetative) and sexually derived material from an individual plant that has been transformed. For example, plants obtained by crossing of a plant having individually been transformed with an untransformed plant are encompassed by the present invention, if the progeny contains at least a nucleotide sequence encoding a PRT and a terpene synthase targeted to the plastid of the plant, according to the invention. The plant may be any plant, preferably, it is a plant which is suitable to be transformed according to the present invention. Preferably, the plant is a plant, which naturally produces high amounts of terpenes. For example, the plant is selected from the family of Solanaceae, Poaceae or Lamiaceae. For example, the plant is selected from the genus Nicotiana, Solanum, Sorghum, Arabidopsis, Medicago (alfalfa), Gossypium (cotton), Brassica (rape). Preferably, the plant belongs to the species of Nicotiana tabacum.

While the present invention is preferably related to plants, its concept can equally well be used with other organelle-containing organisms. For example, the present invention also applies to algae and fungi. Preferably, it applies to plastid-containing organisms such as algae and plants.

The terms "altered content of a specific terpene" or "accumulating a specific terpene" means the content of a specific terpene of a plant transformed according to the present invention is higher than the content of the same terpene in the same plant not so transformed. The invention thus covers two main situations: First, the invention relates to plants, the wild-type of which does not produce the terpene of specific interest. By transformation according to the invention, these plants will accumulate the terpene of interest and thus have an altered content of the specific terpene. According to another situation encompassed by the invention, a plant already produces the terpene of interest. By transformation, the plant will produce more of the specific terpene and thus have an altered terpene content.

Similarly, the term "accumulating" or "over-accumulating" refers to a higher content of a specific terpene in a transformed plant if compared to the same plant not so transformed.

Accordingly, the term "expression" or "over-expression" of a PRT and/or TS gene refers to plants having a higher content of PRT and/or TS RNA in any plant organs, for example leaves, than compared to a control, non-transformed plant.

A "terpene", is a hydrocarbon based on or composed of isoprene units (C5H8), which may be acyclic or cyclic.

As used herein, a "monoterpene" is a terpene based on a C10 structure and includes monoterpene derivatives. Examples are menthol, limonene, α-pinene, β-pinene, S-linalool, just to mention specific examples for illustration.

As used herein, a "sesquiterpene" is a terpene based on a C15 structure. Examples are cyclosativene, cyclocopacaphene, cyclocopacaphenol epimers, cyclocopacaphenal epimers, cyclocopacaphenic acid epimers, cis-α-bergamotene, trans-α-bergamotene, (+)-epi-β-santalene, β-bisabolene, and trans-γ-bisabolene.

The terms "terpene", "mono",- and "sesquiterpenes", for the purpose of the present invention, also includes terpene derivatives, such as terpenoids, which are terpene derivatives including molecules that have undergone one or more steps of functionalization such as hydroxylations, isomerizations, oxido-reductions, dimethylation, or acylation, for example.

Within the context of this specification the word "comprises" is taken to mean "includes, among other things", it is not intended to be construed as "consists only of".

In a preferred embodiment, the plant of the present invention is transformed to comprise a structural gene comprising a nucleotide sequence encoding a prenyltransferase (PRT) targeted to the plastid of the plant.

According to another preferred embodiment, the plant of the present invention is transformed to comprise a structural gene comprising a nucleotide sequence encoding a terpene synthase (TS) targeted to the plastid of the plant.

The term "targeted to the plastids of the plant" refers to the fact that the PRT and/or the TS will be present in the plastids. These proteins may be targeted to the plastids by nuclear transformation with genes comprising plastid-targeting sequences resulting in gene products being actively transported into the plastids by the plant cell. Another possibility of targeting proteins to the plastids is by transforming the plastid genome directly, in case of which plastid-targeting sequences may no longer be necessary, for example. The plants of the present invention are preferably transformed to comprise genes encoding a PRT and a specific TS targeted to the plastids of the plant cell.

Preferably, the plants of the present invention express PRT and/or TS. Preferably, the plants over-express PRT and/or TS. Expression of PRT or TS is preferably determined by RNA extraction and quantitative RT-PCR analysis following the protocol given in the examples.

Prenyl transferases (PRT), also called polyisoprenyl diphosphate synthases or poly-prenyl pyrophosphate synthases are enzymes that catalyse alkylation steps involving dimethylallyl diphosphate (DMAPP) and one or more IPP residues, for
example farnesyl diphosphate (FPP), geranyl diphosphate (GPP), geranygeranylglycerophosphate (GGPP), or others. The term PRT also includes one or several distinct enzymes capable of catalysing one or a sequence of reactions leading to the polyprenyl diphosphate precursors for the various terpenoid families. Therefore, at least one nucleotide sequence encoding a PRT, for the purpose of the present invention, encompasses sequences encoding for polypeptides having quaternary structures comprising homo- and hetero mixtures of mono-, di-, tri-, tetra-, and oligomeric proteins. In particular, the PRT may be a monomer, a hetero- and/or a homodimer. The geranyl diphosphate synthase from oil glands of peppermint (*Mentha piperita*) may serve as an example for the complex genetic organisation of certain PRTs encompassed by the present invention, as this enzyme has been purified and was revealed to be a heterodimer, with both subunits required to produce GPP from IPP and DMAPP precursors.

Preferably, PRTs encompass enzymes classified under EC number EC 2.5.1.1, for example. In a preferred embodiment, the at least one nucleotide sequence encoding a PRT encodes one or more proteins having the activity of a geranyl-diphosphate (GPP) synthase, and/or a farnesyl-diphosphate (FPD) synthase. GPP and FPP are the precursors for mono-, and sesquiterpenes, respectively.

Geranyl-diphosphate synthases (GDS), also called dimethylallyl-transferases, are examples for PRTs.

According to a preferred embodiment, the PRT is a farnesyl diphosphate synthase (FPS). FPSs are enzymes that catalyse, for example, the condensations of geranyl diphosphate (GPP) with IPP to give farnesyl diphosphate. Preferably, the FPS is capable of catalysing the sequential condensation of isopentenyl diphosphate (IPP) with the allylic diphosphates, dimethylallyl diphosphate (DMAPP), and then with the resultant geranyl diphosphate (GPP) to the ultimate product farnesyl diphosphate. Preferably, the PRT present in the transformed plant is isolated from a species different from the transformed plant. According to a preferred embodiment, the PRT is a non-plant PRT. Preferably, the PRT is an animal or fungal PRT, more preferably an animal PRT. Preferably, the PRT vertebrate, more preferably from a bird. Preferably, the PRT is a non-plant farnesyl diphosphate synthase.

For the purpose of the present invention, the transformed plant preferably comprises genes encoding a specific TS and/or the PRT capable of synthesising the direct precursor of the specific TS as indicated above. For example, a plastid targeted sesquiterpene synthase and a plastid targeted FPS. This is a particularly preferred embodiment of the present invention, because it utilises the MEP pathway for producing sesquiterpenes, which has previously not been demonstrated. As another example, the plants is transformed to comprises genes encoding a plastid targeted GDS and a plastid targeted monoterpene synthase.

A large number of nucleotide sequences encoding PRTs isolated from various organisms are readily available to the skilled person and can be downloaded from public databases. It is an important advantage of the present invention that the nucleotide sequence of any PRT's capable of synthesising the precursor of any corresponding TS may be interchangeably employed in the plants of the present invention. Publicly available databases suitable for obtaining nucleotide sequences encoding PRTs or TS are, for example, the database of the European Bioinformatics Institute, (http://www.ebi.ac.uk/swissprot/index.html), the EXPASY database (http://www.expasy.org/enzyme/), the NCBI database (http://www.ncbi.nlm.nih.gov) and many others. For the mere purpose of illustrating the many possibilities of FPSs available to the skilled person that could be used for the purpose of the present invention, one could cite a geranyl diphosphate synthase isolated from *ips pini* (NCBI Accession number: AW 955081), a farnesyl diphosphate synthase isolated from *Vibrio fischeri* (NCBI: GN: YP 200660), and an avian farnesyl diphosphate synthase reported by Tarshis et al. (1994). Of course, any other PRT could be selected from any database.

TS are enzymes that catalyse the formation of a mono- or sesquiterpene from a given precursor compound. An important advantage of the present invention is the exchangeability of the specific terpene synthase. Depending on the particular sesquiterpene that the skilled person is interested in, any corresponding TS can be selected and the nucleotide sequence encoding it can be used for transforming the plant of the invention.

The term “specific” or “selected” terpene, TS or PRT refers to a TS or PRT at the chosen of the skilled person. Depending on the “specific” terpene of interest, the nucleotide sequences encoding the PRT and the TS that are capable of synthesising the respective precursor and the terpene of interest are used to prepare the transformed plants. Preferably, the nucleotide sequence encoding the specific TS or PRT, which may be any TS or PRT, is readily available or can be isolated by the skilled person. For example, a “specific” terpene may be a compound having desirable properties as a fragrance, a flavor, a medicinal compound, a vitamin, an insect control agent, a plant control agent, just to mention a few, and may be selected by the skilled person due to any of these properties. The present invention then provides a versatile system that allows for the production or accumulation of the very terpene of interest in plants.

Preferably, the specific TS is a terpene synthase which utilizes any of GPP and/or FPP as an intermediate or precursor up-stream in the terpene biosynthetic pathway in which it naturally occurs. In this case, the carbon flux is more extensively directed towards the accumulation of the specific terpene synthesised by the TS. For example, the TS has farnesyl diphosphate as its direct substrate.

In an embodiment, the TS of the present invention is preferably selected from the group consisting of a monoterpene and a sesquiterpene synthase. Any terpene synthase may be used for the purpose of the present invention. Examples of monoterpene synthases with available sequences are the limonene synthase (LS), (Ohara et al., 2003), S-linalool synthase (LIS), (Lucker et al. 2001). Genes or nucleotide sequences encoding terpene synthases isolated from various organisms can readily be downloaded from publicly accessible databases (see above for PRTs) and are also disclosed in the literature.

According to an embodiment, the TS is a sesquiterpene synthase. Any sesquiterpene synthase is suitable for the purpose of the present invention, as these enzymes often catalyse a series of reactions leading to a specific sesquiterpene.

Amongst the large number of known sesquiterpenes, one can differentiate different classes according to the intermediate carbocation of the diphosphate precursor before obtaining the final sesquiterpene. For example, TS include enzymes forming the trans-humulyl cation to synthesise f-caryophyllene and -humulene, amongst others. Other TSS form the intermediate E,E-germacadienyl cation to obtain germacrene A, B, C, D, valencene, aristolochene, vetiapinadiene, for example. Of course many other TS are known and can be employed for the purpose of the present invention.

In a preferred embodiment, the TS is a sesquiterpene synthase selected from the group consisting of a patchoulol syn-
thase, valencene synthase, and a cubebol synthase. Alternatively, the TS is a γ-curcumene, (−)-germacrene D, (−)-germacrene D, bicyclo-germacrene, cubebol and/or 6-cadiene synthase. Genes encoding such synthases have been disclosed in the international applications PCT/IB/ 2004/003836 and WO 2004/031376.

The above examples represent only very few, arbitrarily selected examples of the large number of published sequences suitable for the purpose of providing a sequence encoding a PRT or a TS for the present invention. Accordingly, these few examples of different TSs and PRTs suitably illustrate the wide applicability of the terpene production platform of the present invention, according to which a suitable way for producing any terpene at the discretion of the skilled person is provided.

Preferably, the structural genes in the plant of the present invention encode a functional FPS and a functional TS.

The gene encoding the PRT and/or the TS may be isolated from the plant to be transformed itself. In this case, the transformation of the plant results in additional copies of genes encoding the PRT and/or TS in the transformed plant. Preferably, however, the FPS and/or the TS are heterologously expressed enzymes in the transformed plant.

Preferably, the genes comprising a nucleotide sequence encoding a TS and/or a PRT are transgenes.

In a preferred embodiment, the transformed plant of the present invention accumulates at least 1.5 as much of a specific terpene that can be synthesized by the TS if compared to a native, untransformed plant. Preferably, the transformed plant accumulates at least twice, three times, more preferably at least four times and most preferably at least six times as much of the specific terpene.

According to another embodiment of the invention, the transformed plant accumulates at least 1000 ng (nanograms)/per g of fresh leaf of a specific terpene that can be synthesized by the TS. Preferably, the transformed plant accumulates at least 1000 ng, more preferably at least 4000 ng, even more preferably at least 5000 ng, still more preferably at least 7000 ng of the specific terpene that can be synthesized by the recombinant TS present in the transformed plant. According to a preferred embodiment, the transformed plant accumulates at least 10000 ng/g of a terpene, preferably a sesquiterpene, that can be synthesized by the TS. Preferably, the transformed plant accumulates at least 13,000 ng, more preferably at least 20,000 ng, even more preferably at least 25,000 ng and most preferably more than 30,000 ng of the specific terpene per g of fresh leaf. The quantity of the specific terpene may also be expressed in weight of dry matter. In this case, dry matter plant materials, and in particular leaves, corresponds to about 10% of the values for fresh weight. Accordingly, at least 10, 40, 50, 70, 100, 130, 200, 250, 300 μg terpene per g of dry leaf are accumulated.

In case the native, untransformed plant already produces the specific terpene, the above values valid for the transformed plant are added to the amount of the specific terpene natively present in the untransformed plant.

For determination of the content of the specific terpene in the plant of the present invention, any plant organ producing or accumulating terpenes may be taken as a reference. Preferably, green leaves having the same age are taken from the transformed and non-transformed plant, respectively, for comparison. In general, adult leaves are taken. The analysis is preferably conducted according to the protocol "Terpene analysis" outlined in the examples. Preferably, fresh leaves are analysed directly after cutting from the plant. Leaves may be frozen after harvesting and be analysed in the frozen state.

According to a preferred embodiment, the transformed plant according to the invention is the transformed N. tabacum. Seeds of the plant of the present invention have been deposited at the ATCC, 10801 University Blvd, Manassas, Va. 20110-2209, USA, under the depositor's sample references "pIP2PS2+pIPFPS2-4" and "pIP3PS2+pIPFPS2-4" and the Patent Deposit Designations ATCC PTA-6659 and PTA-6660, respectively.

Preferably, the transformed plants may comprise knock-out, deletion or other forms of deleterious mutations in selected wild-type genes participating to the terpene biosynthetic pathways suitable to deviate the carbon flux away from the TS of the present invention. For example, the plants of the present invention may have one or more non-functional genes downstream the IPP, GPP and/or GGPP synthases encoding genes leading to the synthesis of sterols, polypropenoids, phytoestrogens and carotenoids. In this way, carbon flux may more efficiently be directed towards the synthesis of the specific terpene.

The present invention provides for methods of altering and/or increasing the content of specific terpenes in a plant, said methods including a step of transforming plant material with at least one DNA construct comprising structural genes targeted to the plastids of the plant.

Generally, any method for transforming plants with the structural genes of the present invention may be employed. For example, plant cells that have been stripped of their protective cell walls take up pure DNA when treated with certain membrane-active agents or with electroporation. DNA can also be microinjected into target plant cells using very thin glass needles. A recently developed method of plant transformation, biolistics, involves accelerating very small particles of tungsten or gold coated with DNA into cells using an electrostatic pulse, air pressure, or gunpowder percussion.

For example, the structural genes may be directly engineered into the plastid, preferably along with a suitable plasmid expression vector. Suitable methods for transforming the plastid genome of plants are disclosed in Lutz K.A., Comelle S., Azaguirri A.K., Svab Z., Maliga P. (2004) A novel approach to plastid transformation utilizes the phiC31 phage integrase. Plant journal 37: 906-913.

Accordingly, the present invention provides, in a preferred embodiment, plants in which the structural genes encoding a PRT and a TS are present within the plastid genome and are operatively linked to a plastid expression promoter.

Alternatively, the gene products may be targeted to the plastids by transforming plant material on the level of its nuclear DNA. Accordingly, the invention provides, in a preferred embodiment, plants in which the structural genes comprising nucleotide sequences encoding a PRT and a TS are nuclear genes and comprise, linked to the nucleotide sequence encoding a PRT and/or a TS, a plastid targeting sequence.

According to a preferred embodiment of the invention, the terpene, preferably the sesquiterpene, is, at least in part biosynthesised via the MEP pathway. Preferably, at least 30% of the terpene that can be synthesised by the terpene synthase is bio-synthesised via the MEP pathway. Preferably, at least 50%, more preferably at least 60% and most preferably at least 80% are obtained through the MEP pathway. The MEP pathway generally uses glyceraldehyde and pyruvate as substrates for synthesising isopentenyl-diphosphate. This pathway is characterized by the intermediate 2-C-methyl-D-erythritol-4-phosphate. It generally takes place in the plastids. A further pathway for IPP, the MVA pathway, generally takes place in the cytosol.
In IPP synthesised by the MEP pathway, carbons of the IPP are differently arranged than if IPP was synthesised through the MEV pathway, resulting in a different arrangement of carbon atoms in the resulting terpene. The different way of arranging carbons in the MEP and the MVA pathways can be utilised for identifying the biosynthetic origin of a terpene in labelling experiments. For example, incorporation of [1-13C]glucose by the MEP pathway results in IPP having 2 13C-labelled carbon atoms, whereas 3 such atoms should be present in IPP resulting from the MVA pathway.

The present inventors have obtained good results with the Agrobacterium tumefaciens-mediated transformation. Preferably, plasmid constructs (vectors) comprising at least one of the structural genes of the present invention, flanked by nucleotide sequences that allow integration of the structural genes into the plant nuclear chromosome DNA are used.

In an aspect, the present invention provides a vector comprising a nucleotide sequence encoding a PRT and/or a TS, or a fusion protein of a PRT and a TS, said structural gene further comprising a plastid targeting sequence up-stream or down-stream the sequence encoding a PRT, TS or fusion protein. In other words, the vector may comprise only one or both of a nucleotide sequence encoding a PRT and a TS, or a nucleotide sequence encoding a fusion protein of both.

Suitable plastid targeting sequences include sequences encoding a plastid or chloroplast transit peptide. Plastid targeting sequences are known to the skilled person and can be linked in frame to the nucleotide sequence encoding the PRT and/or the TS. One out of many possible examples of available plastid targeting sequences are the Arabidopsis thaliana RUBiCO small unit gene (GenBank accession no. NM123102).

Preferably, the plastid targeting sequence is situated at the N-terminal end of the structural gene encoding a PRT and/or TS.

Preferably, the vector further comprises promoter and terminator sequences, preferably up-stream and downstream the nucleotide sequence including the plastid targeting sequence and the structural gene for the PRT and/or TS, respectively. Preferably, the vector comprises strong constitutive promoters. Examples for promoters are the MOS and NOS promoter. Preferably, the promoters are selected to be independent from control and/or feed back regulation mechanisms of the untransformed host plant. Preferably, the recombinant PRT and TS have up-stream promoter sequences. More preferably, they have each different promoter sequences.

Optionally, the vector may further comprise tags suitable for antibody binding, such as His-tags and/or myc tags.

Preferably, the vector of the present invention further comprises a marker suitable for selecting transformed plants. For example, the vector may comprise genes conferring a hygromycin or a kanamycin resistance, or any other kind of marker suitable to select for successfully transformed plants.

Preferably, the vector of the present invention further comprises a left and a right border region, flanking the PRT and/or TS including optional promoter, terminator, tagging and/or plastid targeting sequences, as well as plant resistance markers, in order to facilitate integration of the structural genes into the plant nuclear genome.

Accordingly, the vector of the present invention preferably comprises structural genes encoding a PRT and/or a TS, linked to at least one plastid targeting sequence, and a marker for selection of transformed plants, flanked by left and right border regions facilitating insertion into the plant genome.

Preferably, the vector further comprises, outside the left and right border region, a marker for selecting positive bacterial transformants used to clone bacteria comprising the vector or used for transformation of plants (A. tumefaciens).

Transformation of plant material with a DNA construct comprising structural genes targeted to the plastids of the plant may be performed by standard methods, see Schmid et al, Gene (1987) 61; 1-11; Berger et al, Proc Natl Acad Sci USA (1989) 86: 8402-8406; and Horsch et al, Science (1985) 27: 1229-1231, the latter method describing the “leaf disk method”, which is particularly suitable for transforming Nicotiana tabacum.

The methods of the present invention further comprise the step of regenerating the transformed plant from the transformed plant material. Methods for regenerating entire plants from transformed plant material are routinely applied by the skilled person. For example, the method disclosed by Horsch et al (1985) may be employed. In general, segments of leaves comprising transformed cells may be grown in selective media until callus and regenerated plant shoots are evident. Shoots of 1-3 cm in size may be transferred to T-Media (Schardl, 1987) containing antibiotics to stimulate root development. Once root systems are established, the plantlets may be transferred to commercially available potting soil and propagated in a greenhouse.

The present invention further comprises a method for altering the content of specific terpenes produced in a plant, the method comprising the steps of transforming a first plant material with a nucleic acid encoding PRT, transforming a second plant material with a nucleic acid encoding a TS, regenerating a first and a second plant from the first and the second transformed plant material, respectively, crossing the first and the second plant, and, selecting from progeny obtained by the crossing for plants over-expressing both, the recombinant PRT and the recombinant TS.

The above method is a variant method for obtaining the transformed plant of the invention, in that different plant material is separately transformed with a structural gene encoding a PRT or a TS, and transformed plants are regenerated comprising only one of the two recombinant genes.

In a following step, sexually derived progeny is obtained from crossing plants containing recombinant PRT DNA with plants comprising recombinant TS DNA. Preferably, crossing refers to genetic, also called Mendelian crossing. This may typically be done by cross-pollinating. Among the progeny, individuals expressing both, the recombinant PRT as well as the TS are selected.

The present invention further provides a method for producing a specific terpene, the method comprising the step of isolating the terpene from the transformed plant of the invention. The transformed plant may be cultivated and harvested, preferably in sufficiently high amount to render the process economically favourable. The specific terpenes of the invention may be isolated by any method used in the art including but not limited to chromatography, for example gas chromatography (GC) extraction and distillation.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice of the present invention, exemplary methods and materials are described for illustrative purposes. All publications mentioned in this application are incorporated by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.
The following examples are intended to illustrate the invention without limiting the scope as a result. Methods and protocols of the examples are generally performed following standard protocols supplied by the manufacturer of specific materials or kits, or by following well-established protocols defined by Sambrook et al. (1989) and Ausubel et al. (1987).

The attached sequence listings include nucleic acid sequences encoding sequiturpene synthases and an avian farnesyl diposphate synthase, coupled to trans peptide sequences.

The nucleotide sequence encoding a patchoulol synthase (bp187-bp1845) linked via a linker sequence to a plastid targeting sequence (bp1-bp171) is named tpPTS (SEQ. ID. NO:1).

The nucleotide sequence encoding an avian farnesyl diposphate synthase synthase (bp211-bp1314) linked via a linker sequence to a plastid targeting sequence (bp1-bp171) is named tpFPS (SEQ. ID. NO:2).

The nucleotide sequence encoding a fusion protein in which a PTS encoding region (bp1321-bp2979) is fused to an avian IPS-encoding region (bp211-bp1311) avian farnesyl diposphate synthase and further linked via a linker sequence to a plastid targeting sequence (bp1-bp171) is named tpFPS-PTS (SEQ. ID. NO:3).

The nucleotide sequence encoding a amorph-4,11-diene synthase (bp181-bp1821) linked via a linker sequence to a plastid targeting sequence (bp1-bp171) is named tpADS (SEQ. ID. NO:4).

Primers used in the PCR reactions described in the examples are listed under SEQ. ID. NO: 5-70.

Peptide fragments used for preparing antibodies for western blotting are listed under SEQ. ID. NO: 71-78.

For some method or protocol steps, references are made to the literature, which is listed further below in more detail.

The plant material used to produce plants having their nuclear DNA transformed are Nicotiana tabacum L. cv. Xanthi. Plants having their plastidic DNA transformed are Nicotiana tabacum L.A63.

Examples 1-5
Construction of Recombination and Plant Transformation Vectors

The hygromycin selection marker (Hajdukiewicz et al., 1994) was chosen for creating a selection marker for transformed plants. New vectors were engineered with appropriate recombination cloning sites as described by Hartley et al. (2000).

Example 1
Development of the pBDON Vector (FIGS. 8 and 11)

The pBI101 vector (Invitrogen, Carlsbad, Calif.) was digested with the restriction enzymes SphI and SstI and the DNA fragment corresponding to the plasmid vector (not including the RB border and NPTII gene cassette) was isolated by agarose gel purification (Sambrook et al., 1989) (13). In parallel, an attp recombination cassette including the ccdB gene and chloramphenicol resistance gene was amplified from the pDON221 vector (Invitrogen, Carlsbad, Calif.) using standard PCR conditions with primers Attp1-SstFL-FW and Attp2-SphR-R. The PCR amplified DNA fragment was restricted with the SphI/SstI enzymes, gel purified and ligated into the corresponding sites of the similarly digested pBI101 vector described above to yield the intermediate pBattp vector (14).

A hygromycin gene cassette was prepared in a 2-step process. First, the hygromycin gene and CaUTR (termination sequence) was PCR amplified from the pCAMBIA1301 (Cambia, Can berra, AU) vector using the PCR primers HPT-NotI-FD and HPT-SphI-REV, and T/A cloned (Taq-Amplified PCR products directly from the PCR reaction mix) into the pT7Blue vector (Novagen, Madison, Wis.) to yield pTHPT (15). The right border (RB) and the NOS-promoter (P-NOS) regions were amplified from pBI101 using the PCR primers TB-SphI-FD and TB-NotI-REV (16), then T/A cloned into the pT7Blue vector giving rise to vector pTRBP (17). The hygromycin resistance gene cassette was released from vector pTHPT via digestion with NotI/SphI (18), and cloned into the similarly digested pTRBP vector, resulting in vector pTRB-PHPTT (19). The NOS promoter-hygromycin-CaUTR cassette was then amplified from this vector using the primers TB-SphI-REV and HPT-SphR (20). The amplified product was digested with SphI and ligated into the corresponding site of pBattp, yielding the pBDON vector (21).

The pBDON T-vector (FIG. 8) contains an NPTII selection marker outside the T-DNA region for selection in bacteria and the hygromycin gene (HPT) for plant transformation selection. The embedded attp cassette hence provides for the easy insertion of target gene constructs flanked with attp sites into the pBDON vector.

Two different attp help vectors with attp sites (FIGS. 9 A and B) were constructed following the protocol given in FIGS. 12 and 13. One for single gene insertion (pTMON, FIG. 9 A) and the other for two target gene insertions into the pBDON vector (pTDBUAL, FIG. 9 B).

Example 2

Generation of the pTMON Vector (FIGS. 9A and 12)

The pTMON vector was constructed by first amplifying the cassaya mosaic virus promoter from a modified pBI101 vector with a forward PCR primer containing a NdeI restriction site and an attpB sequence embedded into the primer CSMV-ATTB1-SGFI-FD, and the reverse primer CSMV-ECORI-REV containing an EcoRI site (22). The PCR fragment was T/A cloned into the pGem-Teasy vector (Promega, Madison, Wis.) (23), then re-isolated as a NdeI/EcoRI digestion product (24). The isolated digestion product was ligated into the corresponding restriction sites of the pCVS vector, a pET28a derivative harboring a terpene synthase gene ("Gene 1"), to generate pEPCSV (25).

In parallel, the NOS terminator (TNOS) sequence of pBI101 was amplified with the forward primer Tnos-Xhol-FW and the reverse primer Tnos-attB2-REV (26), which incorporated an attpB recombination site downstream of the TNOS sequence. The PCR fragment was T/A cloned into the pGEM-Teasy vector, yielding the pTNTOS vector (27). An NdeI/XhoI digestion fragment of pEPCSV was subsequently ligated into the corresponding sites of pTNTOS to generate pTMON (FIG. 9 A).

The pTMON vector was constructed for insertion of a single target gene behind a cassaya mosaic virus promoter (Pcv) (Frey et al. 2001) and followed by the Nos terminator sequence.

A plastid targeting sequence was engineered into the pTMON vector. Accordingly, the plastid targeting sequence of the Arabidopsis small subunit RUBP carboxylase/oxygenase gene (GenBank accession NM123202) was amplified
using the primers TP-ASCf and TP-ASCr, digested with AscI, and ligated into the corresponding restriction site of the mutated pTMON vector.

Example 3

Generation of the pTDUAL Vector (Figs. 9 B and 11)

The pTDUAL vector was constructed in a multi-step process (Fig. 13, part I and II). First, the cauliflower mosaic virus promoter (Benfey et al., 1990) was amplified from the pHJ121 vector (Invitrogen) using the primers PCaMV-XbaI-FW and PCaMV-Spel-RV (29), and T/A cloned into the p17Blue vector (30). The promoter element was subsequently released from this vector by digestion with XbaI and SpeI (31). In parallel, another terpene synthase gene ("Gene 2") was amplified with the primers 7120D-Spel-FW and 7120D-KpnI-RV, followed by digestion with SpeI/KpnI and ligation into the corresponding site of the p17Blue vector and yielding pHJOP (32). pHJOP was then digested with XbaI/SpeI and the CaMV promoter fragment similarly released from p17CA were ligated together to give pTPHPO (33).

In parallel to building pHJPO, the NOS terminator sequence was amplified from the pGDNOS vector with primers Tnos-KpnI-FW and Tnos-attB2-V3, T/A cloned into the pGem-Teasy vector (34) followed by subsequent re-isolation of the fragment by digestion of the pGDNOSK plasmid with KpnI and SacI (25). This fragment was then cloned into the corresponding restriction sites of pHJOP to yield pTPHPO (35) (part I of Fig. 11).

In the final steps of constructing the pTDUAL vector, a fragment of the pTMON vector spanning from the attB1 site to the nos-terminator sequence downstream of the inserted terpene synthase gene was amplified using standard PCR conditions (37). The amplification product was obtained with primers CsMV-attB1-SgfI-TD and Tnos-XbaI-RV, which also engineered terminal SphI and XbaI sites onto the fragment. The PCR fragment was digested and ligated into the corresponding SphI/XbaI sites of the p17Blue vector, generating TPCVST (38). Finally, an XbaI to SacI digestion fragment (39) from pTPHPO was ligated into the corresponding sites of TPCVST (40) to create the pTDUAL vector, which allows for the insertion of 2 gene sequences downstream of strong, constitutive expression promoters (Figs. 13 part II).

The pTDUAL vector (Fig. 9 B) was designed for the insertion of two genes into transgenic plants. Expression of the first gene is directed by the cassava mosaic virus promoter (Frey et al., 2001), while expression of the second gene is directed by a cauliflower mosaic virus promoter (Benfey et al., 1990).

A plastid targeting sequence was engineered into the pTDUAL vectors as for the pTMON vector. In these cases, the plastid targeting sequence of the Arabidopsis small subunit RuBP carboxylase/oxygenase gene (GenBank accession NM123232) was amplified with the primer pair TP-Spel-FW, TP-Spel-RV, or TP-ASCf, TP-ASCr, then digested with SpeI or AscI, respectively, before ligating the targeting sequence into the corresponding sites of the pTDUAL vector.

Example 4

Construction of the Patchouliol Synthase (PTS) and Patchouliol Synthase+Farnesyl Diphosphate Synthase (FP) Over-Expression Vectors

Generation of the PTS and PTS+FPS expression vectors were greatly facilitated by the appropriate recombination cloning sites associated with the pTMON, pTDUAL and pBDON vectors (Fig. 14). The PTS (WHO 2004/031376) or FPP genes (Tarshis et al., 1994) and plastid targeting sequences were amplified with primer pairs PTS-AseI and PTS-XhoI, or FPP-Spel-FW and FPP-KpnI-RV, respectively (41), digested with either AscI/XhoI or SpeI/KpnI, then ligated into the corresponding sites of the pTMON (42) or pTDUAL (43) vectors. The FPP-PTS gene fusion was created by amplifying the FPP gene with primers FPP-AseI and FPP-AscI, digesting the resulting PCR fragment with AseI and ligating this fragment into the corresponding AscI site found 5' to the PTS gene in the pTMON and pTDUAL vectors. The resulting plasmids were then used to mobilize the corresponding PTS, PTS+FPS and FPP-PTS gene cassettes into the pBDON vector by standard recombination cloning (Hartley et al., 2000) generating a family of Ti-plasmid vectors (Fig. 8).

Example 5

Construction of an Amorpha-4,11-diene Synthase (ADS) and Farnesyl Diphosphate Synthase (FPP) Over-Expression Vectors

ADS cDNA (Genbank accession number AF138959) was kindly provided by Dr. Peter Brodelius, Kalmar University, Kalmar Sweden, was PCR amplified using the primers ADS-AseI and ADS-XhoI, and substitution cloned for the PTS gene in the pTDUAL vector also containing the tfl-FPS gene. DNA sequence encoding for the Arabidopsis transit peptide found associated with the small unit of ribulose-1,5-bisphosphate carboxylase (Rubis CO, GenBank accession no. NM23202) was PCR amplified with Ascl restriction sites associated with the primers (primer sequences TP-ASCf and TP-ASCr), digested with the AseI restriction enzyme and introduced into the same restriction site 5' to theADS cDNA gene, creating pTDUAL vector tpADSFPS. The resulting plasmid was then used to mobilize the corresponding tpADSFPS gene cassette into the pBDON vector by standard recombination cloning (Hartley et al., 2000) generating the corresponding Ti-plasmid vector (Fig. 10B, last construct).

Example 6

Plant Transformation and Regeneration

Individual pBDON vector constructs (Figs. 10 A and 10 B) were transformed into Agrobacterium tumefaciens strain GV3850 by electroporation (Mersereau et al., 1990) and transformants selected for kanamycin resistance. Selected colonies were verified for the transgene constructs by limited DNA sequencing and subsequently grown in 50 ml of LB media containing 100 mg kanamycin/L. Overnight cultures having an OD600 equal to 0.6-0.8 were concentrated by centrifugation, resuspended in a 30 mL of fresh LB medium (without antibiotic) and used for inoculation of leaf explants as described previous (Chappell et al., 1995; Horsch et al., 1985). In brief, leaves from plants grown under sterile conditions were placed into the Agrobacterium cultures, cut into approximate 1 cm segments, and the leaf segments plated on non-selective media plates (Murashige and Skoog, 1962). After 3 days, the leaf explants were transferred to media plates containing 15 µg/mL hygromycin (Invitrogen, Carlsbad, Calif.) and 500 µg/mL cefotaxime (Bisworld, Dublin, Ohio) and subsequently to the same selection media weekly until callus and re-generated plant shoots were evident.
Shoots of 1-3 cm in size were then transferred to T-media (Murashige and Skoog, 1962) (containing the same antibiotics) to stimulate root development. Once root systems were established, the plantlets were transferred to commercially available potting soil and propagated in a greenhouse.

**Example 7**

**Terpene Analysis**

Sesquiterpenes extracted from leaf material of transformed plants obtained in Example 6 were identified and quantified by GC-MS analysis. Frozen leaf samples of 250-500 mg were ground in liquid nitrogen, then extracted with 3 mL of a hexane:ethyl acetate mixture (v/v 85:15) containing 200 ng of α-cedrene as an external internal standard. The extract was partially purified by running the sample over a silica column eluted with the same 85:15 mixture of ethyl acetate:hexane. The eluate was concentrated under a stream of nitrogen to 30 μL before analyzing 1 μL aliquots by GC-MS (Yahhali, S., 2005). Samples were injected onto a Trace GC-MS (ThermoFinnigan, Somerset, N.J.) equipped with a Restec Rtx-5 capillary column (30 m × 0.32 mm, 0.25 μm phase thickness) operated in the splitless mode with an injector temperature of 250°C and an initial oven temperature of 70°C. For 1 min, followed by a 4°C per min gradient to 230°C. Mass spectra were recorded at 70 eV, scanning from 35 to 300 atomic mass units, and compared to library standards (NIST library) and authentic standards for verification.

FIG. 1 shows the results of terpene analysis of a control tobacco line (WT) and one transformed with a tptPS+tppPS gene construct for sesquiterpene content. In FIG. 1, a total ion chromatogram for a transgenic line harboring the tptPS+tppPS gene construct (A) is compared to that for a control plant (non-transformant) (B). Peaks were identified by comparison of their mass spectra to available standards or by spectral matches available in the NIST library. For example, the MS for peak 12 (C) is compared to the MS for authentic patchoulol (D). Other peak identifications: 1-β-patchoulene; 2-β-elemene; 3-α-cedrene (internal standard); 4-caryophyllene; 5-α-guaiene; 6-unknown; 7-α-patchoulene; 8-8-salvialene; 9-unknown, 10-δ-guaiene; 11-globulol; and 12-patchoulol.

FIG. 2 shows the results of terpene analysis of a control tobacco line (WT) and one transformed with a tptPS+tppPS gene construct for sesquiterpene content. Similar to FIG. 1, a total ion chromatogram of the transgenic line is compared to that for a control plant. The highest peak corresponds to Amorph-4,11-diene, while the second highest peak is cubenol. The arrow indicates the presence of α-cedrene, which was used as an internal standard.

FIGS. 1 and 2 are the result of a GC-MS analysis and illustrates that plants specifically transformed with plastid targeted genes encoding a specific terpene (patchoulol or amorpha-4,11-diene) synthase of interest and a PRT (farnesyl diphosphate synthase) (A) had an altered terpene content if compared to the wild type (B) and over-accumulated patchoulol and amorpha-4,11-diene, respectively, besides other terpenes synthetised by the same terpene synthase.

**Example 8**

**RNA Extraction and Quantitative RT-PCR Analysis**

The total RNA of each line was isolated from 500 mg of young leaves with Trizol reagent according to the manufacturer (Invitrogen Life Technologies, Carlsbad, Calif.). First-strand cDNA was synthesized in 20 μL reactions with 5 μg of total RNA, 200 ng of oligo(dT)12-16 primer and Reverse Transcriptase (SuperScript II, Invitrogen Life Technologies) in reaction buffer and conditions as recommended by the manufacturer. One μL of RNase H was subsequently added to the cDNA preparations and incubated at 37°C for 1 hour to remove complementary RNA.

A quantitative RT-PCR method was used to determine mRNA levels in different transgenic lines. Optimal concentrations for linear PCR amplification were determined by varying the amount of added cDNA template and using number of PCR cycles (10, 15, 20, 25 and 30). The primers PPS-1F and PPS-10R were used to amplify PPS gene, while FPP-1F and FPP-10R were used for the FPP gene. Another primer pair, RBCS-FW and RBCS-RV, was used to amplify the RUBiCOS CO small subunit gene as an internal standard. Typical PCR conditions consisted of 1× Taq buffer (as supplied by the manufacturer), 1.5 mM MgCl2, 0.2 mM dNTP mixture, 2 μM of each primers, 1.25 μL cDNA, and 1 unit Taq DNA polymerase in a total of 25 μL mixture. PCR amplifications was carried out for 20 temperature cycles, each consisting of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec, and extension at 72°C for 60 sec. PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and photographed.

The results of the PPS and FPP mRNA analysis is shown in FIG. 4. The figure shows RT-PCR products derived from mRNA isolated from plants transformed with constructs containing a patchoulol synthase (PPS) gene, or a plastid targeted PPS (tptPS) gene, or a construct containing a patchoulol synthase and a FPP genes (PPT+tppPS), or a construct containing distinct genes for a plastid targeted PPS and FPP (tptPS+tppPS), or a corresponding plastid targeted fusion protein (tptPS+tppPS). RBCS is the RT-PCR product.
derived from the Rubisco small subunit mRNA, present in the wild-type (WT) and transgenic plants, and serves as an internal standard. It can be seen that wild-type (WT) plants did not express PTS or FPs. Many of the lines harboring the his PTS, PTS4FP, PTS-FPS, and 2pHTS constructs expressed readily detectable levels of the PTS mRNA, but patchoulool accumulation was relatively modest and poorly correlated with the PTS mRNA expression levels. In contrast, expression of the plastid targeted pHTPS gene in combination with pHTPS, either as separate genes or as a fusion gene, resulted in similar expression levels of the PTS mRNA and yielded accumulation of significantly higher levels of patchoulool.

Example 9

Protein Expression Analysis

A mixture of 4 to 5 synthesized peptides was used as antigens to prepare antibodies of PTS and FPs. The antigenic peptides were predicted by a free software (http://bioinfo.hms.harvard.edu/Tools/anticog.html) and selected by direct 3D-structural analysis (PDB: 1FPS) for FPs or homology model comparison with an available structure EAS (PDB: 5EAS) for PTS. We designed 5 antigenic peptides (PTS46; PTS108; PTS553; PTS462; PTS475) for PTS, and 4 antigenic peptides (FPFP41; FPFP59; FPFP218; FPFP20) for FPs. All the peptides were synthesized in a 10 mg scale at immunological grade by Sigma-Aldrich (The Woodland, CA, US). Each of the peptide mixtures was then conjugated to KLH (Keyhole Limpet Hemocyanin) carrier protein and injected into rabbits by Strategic Biosolutions (Windham, ME.). Polyclonal antibodies for PTS and FPs were further purified by preparative antigenic affinity column prepared with CNBr-activated sepharose 4B resin coupled with purified PTS or FPs protein according to the manufacturer’s instruction (Amersham Biosciences, Buckinghamshire, England).

To measure PTS and FPs protein levels in transgenic plant material, 1 mg of young leave material was ground in liquid nitrogen, then extracted with 800 μL of 80 mM potassium phosphate (pH 7.0), 10% glycerol, 10 mM sodium metabisulfide, 15 mM MgCl2, 10 mM sodium ascorbate, 1% polyvinylpyrrolidone, and 14 mM o-mercaptoethanol. The crude protein extracts were centrifuged at 4°C for 20 min in full speed in a table top microfuge, and the resulting supernatant used as the protein source. Samples containing 50 μg of supernatant protein was electrophoresed in a 15% SDS-PAGE gel and blotted into a nitrocellulose membrane (BIO-RAD, Hercules, Calif.). Membranes were blocked with standard Tween 80% Tris-buffered saline (TTBS) blocking solution containing 5% dried milk before adding the purified PTS or FPs antibody. Membranes were then incubated for 3-7 hrs at room temperature with shaking, washed three times with TTBS, and then incubated with goat peroxidase labeled antirabbit IgG (Kirkegaard and Perry Laboratories, Gaithersburg, Md.) in TTBS blocking solution for another hour at room temperature. Chemiluminescent detection of the secondary antibody was performed with an ECL reagent kit according to the manufacturer’s instructions (Amersham Biosciences, Buckinghamshire, England).

Expression levels and processing of the patchoulool synthase (PTS) and farnesyl diphosphate synthase (FPS) proteins in transgenic plants are shown in Fig. 5. The numbers above lanes in Fig. 5 represent independent transgenic plant lines engineered to contain a patchoulool synthase (PTS) gene, or a plastid targeted PTS (pHTPS) gene, or a construct containing a patchoulool synthase and a FPS gene (PTS4FPS), or a construct containing distinct genes for a plastid targeted PTS and FPS (pHTPS+pFPS), or a construct encoding for a fusion protein of FPS and PTS (FPS-PTS), or a corresponding plastid targeted fusion protein (pFPS-PTS). The positive control used are PTS and FPs proteins or the corresponding protein containing an amino-terminal plastid targeting peptide (respectively lanes PTS, FPS, pHTPS, pFPS) purified from bacteria transformed to over-express these proteins. Lane WT1, extract from the leaves of a wild-type plant. Upper panels show membranes probed with the PTS antibodies and the two membranes in the lower part of the figure show membranes probed with the FPS antibodies. It can be seen in Fig. 5 that no protein was immunologically detected in the wild type plants. For most of the transgenic lines analysed, PTS and/or FPS proteins could be detected in the leaf extracts and the expression pattern was generally consistent with the patchoulool accumulation. Interestingly, the apparent molecular weight of the PTS and FPs proteins detected in plants transformed with constructs containing the corresponding genes linked to a plastid targeted sequence (for example the lines 2pHTPS and 2pHTPS+pFPS), are identical to the molecular weight of the same protein expressed in plants or bacteria without plastid targeting signal (for example PTS, 2PTS or 2PTS+FPS). In addition, expression of the PTS or FPS with plastid targeting peptide in bacteria (which can not cleave the targeting peptide) leads to proteins having clearly a higher molecular weight. These observations show that the chloroplast targeted proteins were effectively processed and thus, that these proteins are actually transported to the plastids.

Example 10

[1,14C]-Glucose Labelling for Showing Pathway Used for Terpene Synthesis in Plants of the Present Invention

Seeds of 8PTS10 (transgenic plant lines expressing PTS in the cytosol) and 2pHTPS+pFPS12 (plasmidic) were germinated on solid MS medium with the addition of hygromycin (15 mg/l). For GC-MS analysis, thirty 4-week-old seedlings were subsequently transferred to 25 ml flasks containing 5 ml of liquid MS medium without sucrose and incubated with gentle shaking (125 rpm) in the dark. One day later, 100 mg of [1,14C]-glucose (Sigma) was added into media and plant material collected at daily intervals. For GC analysis, 100 mg samples were extracted as described in Example 7.

For NMR analysis, 1000 seedlings of above two transgenic lines were used, with 100 seedling grown in a 250 ml flask containing 50 ml of liquid MS medium with the addition of 1 g [1,14C]-glucose. All labeled seedlings were collected after one week and extracted as above. Concentrated extracts (3 ml) were then purified by preparative TLC separations using silica TLC plates and hexane/ethylacetate (9:1) as the developing solvent. 250 μg of purified 14C-labeled patchoulool from line 2pHTPS+pFPS12 and 500 μg from 8PTS10 were subsequently analyzed by 13C-NMR (750 MHz, CDC1, by Bob Coates in the Chemistry Department, University of Illinois, Champaign, I1.). Carbon positions and enrichments were assigned relative to un-labeled patchoulool purified from control plants.

Fig. 6 shows the predicted 13C labelling patterns in patchoulool synthase from IPP emerging from the MVA and the MEP pathway, respectively, in transgenic seedlings fed on [1,14C]-glucose. Patchoulool synthase from IPP of the MVA pathway is predicted to have 9 13C-carbons at positions 1, 3, 5, 7, 9, 12, 13, 14 and 15, whereas its counterpart from the MEP pathway is expected to have only 6 13C-carbons, namely art positions 2, 6, 8, 12, 13 and 15.

Results of GC-MS analysis are shown in Fig. 7, in which Fig. 7 b shows MS parent ions for patchoulool synthesised by
plants fed $^{13}$C-glucose (control) and FIGS. 7 c and d plants fed on $^{13}$C-glucose producing pachoulol by the (cytosolic)
MVA and (plasticid) MEP pathway, respectively. It can be seen that pachoulol emerging from plants fed on $^{13}$C have
heavier ions, with the effect being less pronounced with the plasticid pachoulol: the mass of the major parent ion is shift
from 222 to 225, and an M+8 mass parent ion can be observed when the cytosolic pathway is engineered; and the mass of
the major parent ion is shifted to 224 and an M+6 mass parent ion can be observed when the chloroplast pathway is engineered.
These observations are consistent with the labelling prediction. Results of NMR-analysis are shown in Table 2 below.

### Table 2

<table>
<thead>
<tr>
<th>Carbon atom</th>
<th>De (ppm)</th>
<th>8PTS-10</th>
<th>2ppPTS + tpFPS-12</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>74.9</td>
<td>13.0</td>
<td>0.0</td>
</tr>
<tr>
<td>2</td>
<td>33.1</td>
<td>3.0</td>
<td>13.8</td>
</tr>
<tr>
<td>3</td>
<td>28.9</td>
<td>21.8</td>
<td>4.2</td>
</tr>
<tr>
<td>4</td>
<td>28.1</td>
<td>2.9</td>
<td>2.7</td>
</tr>
<tr>
<td>5</td>
<td>43.8</td>
<td>19.1</td>
<td>2.4</td>
</tr>
<tr>
<td>6</td>
<td>24.5</td>
<td>22.2</td>
<td>18.4</td>
</tr>
<tr>
<td>7</td>
<td>39.4</td>
<td>19.3</td>
<td>2.7</td>
</tr>
<tr>
<td>8</td>
<td>24.9</td>
<td>2.9</td>
<td>17.3</td>
</tr>
<tr>
<td>9</td>
<td>29.1</td>
<td>17.1</td>
<td>3.6</td>
</tr>
<tr>
<td>10</td>
<td>37.8</td>
<td>1.6</td>
<td>2.0</td>
</tr>
<tr>
<td>11</td>
<td>40.1</td>
<td>4.0</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>18.8</td>
<td>22.9</td>
<td>19.1</td>
</tr>
<tr>
<td>13</td>
<td>20.9</td>
<td>20.2</td>
<td>17.9</td>
</tr>
<tr>
<td>14</td>
<td>27.3</td>
<td>22.0</td>
<td>6.1</td>
</tr>
<tr>
<td>15</td>
<td>24.7</td>
<td>15.8</td>
<td>13.8</td>
</tr>
</tbody>
</table>

$^*$signal due to contaminant

In accordance with predicted labelling patterns shown in FIG. 6, Table 2 shows that in pachoulol from plants having
PTS and FPS targeted to the plastids, carbon atoms 2, 6, 8, 12, 13 and 15 are enriched with $^{13}$C. The labelling thus shows that the sesquiterpene is actually synthesised from IPP resulting
from the MEP-pathway and hence, in the plastids.

### Example 11

**Plant Plasticid Genomes Transformed with PTS and FPS Genes**

The system of recombination cloning into the plastid genome developed by the Maliga laboratory (Lutz et al 2004)
has been used and modified. We used *Nicotiana tabacum* L.a63 as the recipient tobacco host material. L.a63 has one
attB site integrated in chloroplast genome DNA allowing for foreign DNA insertion into this site. A suitable transformat
vector was re-constructed as shown in FIG. 15 I-III. We included an IPTG inducible Prn promoter based on previously
published work (Mullhauer and Koop 2005) for providing for regulated gene expression.

#### 11.1 Construction of the Plastid Transformation Vectors

A universal plastid transformation vector pL.vec (FIG. 15 I-III) was constructed, using the p17Blue (Novagen, Madison,
Wis.) vector as the backbone.

**FIG. 15 Part I**

The p17Blue vector DNA was digested with the HindIII restriction enzyme. Following treatment with T4 polymerase
and self-ligation, the HindIII restriction enzyme site was knocked out and the resulting plasmid designated as pHindIII
p17Blue.

A linker consisting of two primers (pLinf and pLinR) were introduced into the EcoRI/KpnI sites of p17blue to
create new multiple cloning sites SgII-BamHII-Spel-NotI-SmAl. The previous EcoRI site was simultaneously removed
by linker designing (the last C for the EcoRI recognition sequence was removed). The generated vector was designated as pLlink.

The attP recombination site was amplified by primer attP-XbaI and Attpr-SphRI and ligated into the XbaI-SphI sites of
plink vector and designated as pLattP.

An aada cassette (including Prn promoter and Trbc1 terminator) was amplified using Primer Prnr-XbaI and Trbc1-
KpnRI and ligated into the pLattP XbaI-KpnI site to make vector plattP-aadaA.

The Prm promoter was amplified a second time with primer Prnr-KpnI and Prm-SmaR. After digestion with the corre-
responding restriction enzymes, this fragment was ligated into the KpnI-SmaR sites of pLattP-aadaA to make pLattP-aadaA+
prn.

**FIG. 15 Part II**

The *Nicotiana tabacum* ATP synthase alpha subunit openon terminator (Tap) was amplified from tobacco with
primers Tapi-SmaF and Tapr-NotR. This fragment was ligated into SmaI-NotI site of vector plattP-aadaA+prnT api-
Tapi.

Lac repressor gene (LacI) gene was amplified from *E. coli* with primers LacF-SmaF and Lacr-SmaR. After digestion
with SmaI, this fragment was ligated into the SmaI site of plattP-aadaA+prnT api-Tap to make vector platP-aadaA+LacI.

A new terminator Trbc1 was amplified from tobacco with primer Trbc1-SpeI and Trbc1-NotR. The amplified fragment
was digested with SpeI and NotI, and ligated into the vector plattP-aadaA+LacI to make pLattP-aadaA+LacI-Trbc1.

Based on the publication of Mullhauer and Koop (2005), an IPTG inducible primer Prrlacl was generated by two-
successive PCR amplification with Prrlac-SDrIg as forward primer and Prrlacl-R1 inst following Prrlacl-BamHI RS as
reverse primer. After digestion, Prrlacl was ligated into the SgII-BamHII site of plattP-aadaA+LacI-Trbc1 to make the
universal vector pl vec (FIG. 15 parts II and III).

**FIG. 15 Part III**

GUS gene was amplified from Ti vector of pB121 with primer GUS-BamF and GUS-SpeR. After digestion with cor-
responding strict enzymes, the GUS gene was ligated into the pl vec vector to make construct plGUS.

A FPS-(Ascl)-PTS fragment was amplified from ptpFPS+ tpPTS construct for nuclear transformation with primers
FPS-BamF and FPS-SpeR. After digestion with corresponding strict enzymes, FPS-(Ascl)PTS fragment was ligated into pl vec to
make construct pLPJS-PTS.

A Trbc1-(SpeI)-Prrlacl construct was constructed in a T/A helper vector. Trbc1 was first PCR amplified using
Trbc1-AsclF and Trbc1-SpeR and T/A cloned into pGem-T Easy vector (Promega). To obtain sequence orien-
tation, a clone harboring the Trbc1 gene 5' to 3' from the T7
promoter to the SP6 promoter was identified and used for the
next ligation. This intermediate vector was designated as pTTrbc1. Another gene Prrlacl was amplified in a same way
as described above by using primers Prrlacl-SpeF and Prrlac-
Ascl-SpeR. After digestion with SpeI, Prrlacl was ligated into the SpeI site of pTTrbc1. A clone having the correct orientation was identified by strategic restriction
enzyme digestion and designated as pTTrbc1-(SpeI)-Prrlacl.

After digestion of pTTrbc1-(SpeI)-Prrlacl with Ascl, the
Trbc1-(SpeI)-Prrlacl fragment was ligated into the pLPJS-
PTS Ascl site to make the final construct LPFP+PTS.

**11.2 Methods for Plastid Transformation**

DNA Preparation and Gold Treatment

7.5 mg (sufficient for 9 shots) of 1.0 micron gold particles
(Bio-rad, Hercules, Calif.) were first sterilized with 1 ml
ethanol. After centrifuging, the ethanol was removed and
111 μl fresh ethanol was added. The gold particles were separated
into three equal sized aliquots, each with 55 μl ethanol/gold
mixture. 1.0 ml of sterile water was added to each of the ethanol/gold aliquots. After centrifugation 5 min at 200 rpm, the water/ethanol supernatant was removed and gold particles were ready for DNA coating. Five μl of plasmid DNA (pL-GUS tptFPS+PITS, ca. 1 μg/ml), 220 μl sterilized water, 250 μl CaCl₂ (2.5M), 100 μl spermidine (1M) were added in order and mixed thoroughly. The DNA/gold mixture was then incubated on ice for 2 min, followed by centrifugation (5 min, 1000 rpm) and removal of the supernatant. The DNA/gold pellet was then rinsed with 600 μl ethanol and resuspended in 36 μl of ethanol and placed on ice for 1 hour before using for shooting (bioballistic treatment).

During DNA/gold precipitation on ice, plant materials were prepared. One-month-old sterile tobacco leaves of line LA63 were cut and placed on water-wetted filter paper (5 cm I.D.) in a petri dish.

10 μl of ice-treated DNA/gold pellet in ethanol was transferred into microcarriers and shot into the leaf material using a particle delivery system (Gene Gun) (model PDS-1000, DuPont) following manufacturer’s instructions.

*Shot* leaf segments were transferred onto TOM medium and incubated in the dark for 24 hrs.

leaf segments were then cut into small pieces and transferred into TOM media containing spectinomycin (500 ng/l) for callus generation.

leaf segments were transferred to fresh selection plates every 15 days until plant shoots were visible.

Regenerated shoots were transferred into rooting medium with same spectinomycin selection.

Regenerated plantlets were assessed for GUS expression or terpene accumulation as described above. For GUS expression test, GUS staining methods were used. First, the regenerate leaf segments were transferred into TOM medium containing spectinomycin (500 ng/l) and also with 1 mM IPTG for induction for 3 days. The induced shoots were incubated in GUS staining solution (50 mM potassium phosphate buffer, 12 mM 2-mecaptoptothanol, 0.1% Triton X-100, 500 μg/ml 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (Research Products International Corp., Mt. Prospect, Ill.). 2.5 μM potassium ferriyanide, 2.5 μM potassium ferrocyanide) for another day of incubation, then destained in 70% ethanol.

Positive transformants of the plastidic genome showed blue staining. It was thus shown that plastidic genomic DNA of plants were transformed to harbour heterologous genes encoding a sesquiterpene synthase and a farnesyl diposphate synthase.

**Example 12**

**Insect Repellent Test**

Three healthy leaves of the approximate same developmental age (W 5 cm x L 8 cm) were collected for line 2pFPS+tptFPS-12 and wild type 14-2, then placed within an equal distance of one another (ca. 1 cm) within an enclosed box. Six commercially available hornworms (Manudaca sexta) (Carolina Biological Supply Company, Burlington, N.C.), at the second instar stage of development were placed onto each leaf evenly so that each hornworm had an equal opportunity to eat the leaf or move to another. The experiment was run for 6 hrs before counting the digested leaf areas and recording hornworm numbers on each leaf after migration.

**REFERENCES**


<160> NUMBER OF SEQ ID NOS: 79
<210> SEQ ID NO 1
<211> LENGTH: 1845
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURES:
<223> OTHER INFORMATION: Patchoulol synthase cDNA of Pogostemon cablin linked to a plastid transit peptide sequence
<400> SEQUENCE: 1
atggtctct catatgcttc ctcogcagct gtggtagacct ccccgccctca gcggccacag tcggcccttc 60
gcctgtcttt aatcctgcgg ccagggaccc gtaaacatgg cacagttaag cctggtttgc 120
agggcacca ctcctagacg aagaacaggg ggaagatctt tagcccagaa ggtaggtgcc gtggagctag 180
ggcgcatgg agttgatagc caaaggtggt tagtttctgc ttcgtcttcg cgcctcggtg 240
aatcttttc ctcattgctg cgagagacaa ttcattgctc caaaccccaa aatctgcgcag 300
gctggaga gcagagaggg tggagaagct aagaggtgctg tgggagacag gctgagagag 360
gcagcagaca aatcaactcgc gcaacttgca atgggtgact gatacacaag ttatggactt 420
gcatcatctt tgttgaagaa gctgtctgta acgtgttttc aatgtttgat 480
gccctctgca agataacactc tgcatacgac gcaactgcct cctgctggct ccttcctca 540
cacatctgac caagaggttt cctgctgatt gttgaacagtc ttaagagtac ccaggagatg 600
ttaaggccc caataaggg aagagcggtt gctgctccct aatcttctg aagcaacgc ac 660
tcagctcgc atggaagag aagccttgcg aatgttctct gtcacactcg 720
gcacagttt atgcaaccttc gcagatcca acggcaaacca gagaactcag caatgatgaa 780
gagtctctt ttggaagag attgcacgct gttgaagcc ggaagactat atcaacctc 840
gcagcaatacg atcatctaca caaagggctg cttcaaccctg ctagctggg ttttacttg 900
tgacagttt tcccacagaa caggtcgatt gatgctctgt tgcgtgtcgg 960
tgcctcagaa ggtcatctct ggttggatgt cagtttgtgg agtctctactt ctggtctgct 1020
ggagtcatc ccaagcaggct tttttctgt cggtagacta tttgcagcagt ttttacaaacc ggggttgtct 1080
gttatttcgt ttatgtgtag gccatgtgta ctgattggta aatctgctgta 1140
ttgcagatgt ccaagcagag atggtggtgt ctctgtttaga ataaacttc ggattttactg 1200
aaaatgtata cacaagcctg ttggagatgt ttgagagagag tggagatgaa ttaaactgaa 1260
taggtgcac ccatctgccg ctaacttgga aagagccagc tgggtaaagc cggagagatg 1320
tacatggag aagcccaagt gaggacaaa aagcaacaac ccaacaacag gatgacttag 1380
aagctgctca ccaagacatct gtagcatctc actttcatgactattact aatccttact 1440
gaagagcca ttagctgacca aagacgcct cagttgtgtt tctccgacaca ctctttcatc 1500
ggagtcatct ccatctgttc caggtgctgtt atagataata cagcaaaaag gttgagaaaa 1560
aatctgagac aatagtcgac ctagcttgaag cttgtagatt aaagagagaa aagggagagag 1620
cgcgaggttg gttgtaaagc atcaaccaag cggagagatg cggagagatg cttgagaaa 1680
gggtctcttc gacoagtgg aatcctgtgct cccctttctt tttattttact aaactgctc 1740
gaagagacag aagcacaaga caaagacagc gtcacagtag gatcacttca ccaactactg 1800
cgaactacttt ctagcatcgtt ttcacacttc ccggttcata tatta 1845
<210> SEQ ID NO 2
<211> LENGTH: 1314
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURES:
<223> OTHER INFORMATION: Farnesyl diprophosphate cDNA of Gallus gallus linked to a plastid transit peptide sequence

<400> SEQUENCE: 2
atggtcctct ctatgctctc ctccgcgcct gtgggttcat cccgcgctca ggccacactg 60
gtggctcatt cccgctccct gcaatcctc cggccacccg cagacgccag 120
aaggccacaa cctgccacgc aagccacggc ggaagatctta ggtgcctgaa gactgatag 180
cagcccccatc atcacatgaa agagggccgt atgcataaat ttactgttgct caagcgaag 240
ttcgcacggc ccggcttggag gaaacccacg cccgctgagg gttgagggga gaggaggag 300
ttcggggtgt ttcctccgca gactgccggtc gattgacccg aggacgcgcat ccggaccccg 360
gagcgtgggac acgtgggtgc gcggcgttgaag ggggcgggac aattcagccg tccccgggga 420
aagtccacac gtggctttgc gctgtggtctg cggccgctgg gctgcccccg 480
aaggattgag agaatcctgcg tgtgtgctggc gctgctggtgctg cttgtaccg 540
tcgctccctg tgtctgcttg ttagataag tgcacctgcg cagcgctcct ctctgcctcg 600
tcgtgcgact ctgcacggcg gaagggcgct tggcctgcgttg gcggcgcctgc 660
tggtggctct tcgctgcagc ccggctaccac agctgactcg gcgcagatcct gcggcctgca 720
acagtccgct ccggcctaccag gcggctctgc gccgacgccgt gccgacgccgt ggcagacac 780
gtgaacctca gactcctgc ctcctccacg tccgctgctg cattggttcg cccgctcctg ccgcagttc 840
acagtccgct ccggcctaccag gcggctctgc gccgacgccgt gccgacgccgt ggcagacac 900
gtggcgcagt acgtacacag aagcgcacag aagcgcacag cccgctcctg gcggagagag 960
gaatctccac gcggtgctcag gcgtactgtg gcggccacct gcggcctccg gacgctgcttg 1020
aaggttcgac gtcacgc gacgctcag gcgtccctgc gccggtgctg ccggcctcctg 1080
cggccgctccg gcgtactgcg gcgtactgcg gcgtactgcg gcgtactgcg gcgtactgcg 1140
aaggttcgac gtcacgc gacgctcag gcgtactgcg gcgtactgcg gcgtactgcg 1200
tcgcggtcag gcgtactgcg gcgtactgcg gcgtactgcg gcgtactgcg gcgtactgcg 1260
cggccgctccg ctccgccgct gcgggctctg gcgtcctgc ggagccagtcc acgcagcag 1320
<br>
<210> SEQ ID NO 3
<211> LENGTH: 2979
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURES:
<223> OTHER INFORMATION: Fused patchouliol synthase (Prospetemon cabilin) and farnesyl diprophosphate synthase (Gallus gallus) cDNA including a plastid transit peptide sequence

<400> SEQUENCE: 3
atggtcctct ctatgctctc ctccgcgcct gtgggttcat cccgcgctca ggccacactg 60
gtggctcatt cccgctccct gcaatcctc cggccacccg cagacgccag 120
aaggccacaa cctgccacgc aagccacggc ggaagatctta ggtgcctgaa gactgatag 180
cagcccccatc atcacatgaa agagggccgt atgcataaat ttactgttgct caagcgaag 240
ttcgcacggc ccggcttggag gaaacccacg cccgctgagg gttgagggga gaggaggag 300
ttcggggtgt ttcctccgca gactgccggtc gattgacccg aggacgcgcat ccggaccccg 360
gagcgtgggac acgtgggtgc gcggcgttgaag ggggcgggga aattcagccg tccccgggga 420
aagtccacac gtggctttgc gctgtggtctg cggccgctgg gctgcccccg 480
aaggattgag agaatcctgcg tgtgtgctggc gctgctggtgctg cttgtaccg 540
tcgctccctg tgtctgcttg ttagataag tgcacctgcg cagcgctcct ctctgcctcg 600
tcgtgcgact ctgcacggcg gaagggcgct tggcctgcgttg gcggcgcctgc 660
tggtggctct tcgctgcagc ccggctaccac agctgactcg gcgcagatcct gcggcctgca 720
acagtccgct ccggcctaccag gcggctctgc gccgacgccgt gccgacgccgt ggcagacac 780
gtgaacctca gactcctgc ctcctccacg tccgctgctg cattggttcg cccgctcctg ccgcagttc 840
acagtccgct ccggcctaccag gcggctctgc gccgacgccgt gccgacgccgt ggcagacac 900
gtggcgcagt acgtacacag aagcgcacag aagcgcacag cccgctcctg gcggagagag 960
gaatctccac gcggtgctcag gcgtactgtg gcggccacct gcggcctcctg 1020
aaggttcgac gtcacgc gacgctcag gcgtccctgc gccggtgctg ccggcctcctg 1080
cggccgctccg gcgtactgcg gcgtactgcg gcgtactgcg gcgtactgcg gcgtactgcg 1140
aaggttcgac gtcacgc gacgctcag gcgtactgcg gcgtactgcg gcgtactgcg 1200
tcgcggtcag gcgtactgcg gcgtactgcg gcgtactgcg gcgtactgcg gcgtactgcg 1260
cggccgctccg ctccgccgct gcgggctctg gcgtcctgc ggagccagtcc acgcagcag 1320
aaggtatgctg aagactgtgct gcggcggtgc ggctggtggt gtgtgcacgc gttgacccag 540
gcctctcttc tgcggcgctg gatattcatt gcacggccac tcacgcgcgc gggcgagctg 600
tgctgggtata agaagagggg ggtgctttgg ggcgactctc acaagactctt ctctgcgcag 660
tcctctggt tcacagagtc gagaagattc tcagggcgac ggcgactattca gtcgacatgt 720
tggcagctct tcggcagcag cgcctctcag acctgacgct gcgcagatgt gcgcctctgc 780
aagacgctcag tctccaaaag gatgttgagt cacttcagcg agaagaggta caaagcccact 840
gttgctaa cagactgtcct tctctcttcct ttcctacccag gggctggtgct gcgttatagt 900
gttgcagctcg gacagatctga agaagacacag aacctccaag ccacctgtgc ggagatgggg 960
gaactcttcg agatgagaga tgtgctgttg gggacccgcc gttcaggggg 1020
aaggtgggaac gacagatcca gcacaaaatt tgcagccgtgc tgctgtggcga tgcctcgacg 1080
cgcagcagcg cggcagcagcg gcgctccctcg gaggacactc aggccgtaaa gggccgccg 1140
aaggtggtgac aagatgtgag gatcgggagc tggaggtgcgc gttcagcagcg 1200
tacggagaga gacagctcgac ggcgctcgac gcacagatag agaagacacct gcacggtcct 1260
cggacgagaa tctctctcgt ccgggacagc aagacttaca aacggccgag aacggccgcg 1320
atgaggttgtg tgcgccaaga aggttggagt ggtgttctct gcgtcctctc tcgcagatgt 1380
ctctcgattg gcgctgagagc cacaatcttc gcaccacaac cacaactctg ccaggtcggc 1440
gagagagaga aagctgtgagc gccaagagcg tgtgcaaaaa gagagctggaag gagagacatca 1500
gaccaactaca tgcggcactgt gcagagttgct gtcgacatcc aagccattag ccgtctctat 1560
cttttctggtg aagattggtg tgaagcttgg agaatctgtg tggattagtt gtagctctcc 1620
tgcagata agatcaacct tgcggctcag cgcgctcctgc acacgcaacct tcacacgctg 1680
gctcccaagt ggtgctgggac ggtgctggct gcgctacatc tggacgcccgc gcctcctcag 1740
gttcagctg aaggtgggcc gcgtctgcctgc tggagggcgt gcctcctcag 1800
gtctctagtgagc aaggtgtcct gcagatctgc ttcacgctg ttcacgctg ttcacgctg 1860
gtctagtcaag tctctcgttgc tggaggggct cttctcagcgtgctgctgc ttcacgctg 1920
tttttctgaa gaggtagctg gcaaggtggg gcagggagtt cactacatatt ccggtctcttac 1980
tagcgatattc tccacaaaagc ttcgtactgac tgcgctcacaagc ttcaggttactgct 2040
gcttcgatcata gtttcgagcg tgcgtgcctgc tgcgctgctg tgcgctgctg 2100
aacaagcttat ctcgctcgtg gatcgcgtgct gcacgtgcctgc ttcgctctgtt gtcgttttcct 2160
ttctcttcagc gattataatc ggtatgaagg attagtgctgga aagagagttt ggtgatgatt 2220
ttctctcttg atgatgtgtct gatgtctgtgc tgcgcctctct gatgtctgtgc 2280
gatgcagatt caagagctgtgct gcgctgcgtc tgcgctgcgtc tgcgctgcgtc 2340
gtatacaagc ctttctgtgg tcgggtttgag ggggattgag caggttgtat ggcgatgcag 2400
gcaccacactgc gcggcgctgac tggaaaaaaga ggcagtatata tgcgctgctg gcacgctgctg 2460
ggcgagggcct acagagaggg gcacagccttac ccaagagatca tgcgctgctg 2520
gcaccacactgc gcgtggctgc tgcgctgctg gcacgctgctg gcacgctgctg 2580
gggtgttgag ccaagagcgg tctcggtgtg ggcgatgcctg gcgtgtctgtg gcgtgtctgtg 2640
acatactgctgc gcgtgagttg gtcgctctgtc tgcgctgctg gcgtgtctgtg gcgtgtctgtg 2700
gacgtaagttc gcagcgtgatg aatagatctg gatggagact gcacatgcagtc gggagagacaag 2760
tgcgctgctgc gcgtgagttg gtcgctctgtc tgcgctgctg gcgtgtctgtg gcgtgtctgtg 2820
cacgacagtc tcggcgctgctgc gcgtgagttg gtcgctctgtc tgcgctgctg gcgtgtctgtg 2880
cggaggtta tttacaaga gggagatcc gctataacag ttgggtcttg agactcaaacct ccatatgtt ccata ttt gggaggtta 2940
aggtcact acagtgactt tcaacctgct ccata ttt gggaggtta 2979

<210> SEQ ID NO 4
<211> LENGTH: 1821
<212> TYPE: DNA
<213> ORGANISM: Artificial
<222> FEATURE: OTHER INFORMATION: Amorpho-4,11-diene synthase encoding cDNA of Artemisia annua linked to a plastid transit peptide sequence

<400> SEQUENCE: 4

atggtctctct ctagtttctt ccacggcgtt tggggtgacct coccggtctt ccacggctac gggaggtta 60
gtgggtctct tcaaccggtctt gacggttacct gcggctactac cggctaccgc gcacgagacc 120
aaggagatca ctcctactgc acacatgga aggagatctct tttgcgttctt gctcagttct ttttcgctgccg 180
agttgctctt ctctgtgttt cagagacgcc gttggtgttgc ggtggttctt ctctgtgttt 240
gagagtgtgt tgtgtggtctt ggtggttctt ctctgtgttt cagagacgcc gttggttctt 300
gagtctctt ctctgtgttt cagagacgcc gttggttctt ctctgtgttt 360
gccacacttct tcacctctc gggagtgtgt tgtgtgtggtctt ggtggttctt ctctgtgttt 420
cggaggtta tttggaggtta tgtgtgtggtctt ggtggttctt ctctgtgttt 490
gagagtctct tcctgtgttt cagagacgcc gttggttctt ctctgtgttt 540
gtttctctt ctttggtctct cagagacgcc gttggttctt ctctgtgttt 600
gaagttgctct ttttgcgttctt ggtggttctt ctctgtgttt cagagacgcc gttggttctt 660
gagtctctt ctctgtgttt cagagacgcc gttggttctt ctctgtgttt 720
tggttacgacacacaccttctctgtgttt cagagacgcc gttggttctt ctctgtgttt 780
tggttacgacacacaccttctctgtgttt cagagacgcc gttggttctt ctctgtgttt 840
tggttacgacacacaccttctctgtgttt cagagacgcc gttggttctt ctctgtgttt 900
tggttacgacacacaccttctctgtgttt cagagacgcc gttggttctt ctctgtgttt 960
ttggagtgtgt tgtgtgtgtgt ttttgtgtgtgt tgtgtgtgtgt ttttgtgtgtgt 1020
ttt tgtgtgtgtgt ttttgtgtgtgt ttttgtgtgtgt 1080
acatgtctt gcgtgtgtgt ttttgtgtgtgt ttttgtgtgtgt ttttgtgtgtgt 1140
ttt tgtgtgtgtgt ttttgtgtgtgt ttttgtgtgtgt 1200
acggtgtgtgt ttttgtgtgtgt ttttgtgtgtgt ttttgtgtgtgt 1260
acggtgtgtgt ttttgtgtgtgt ttttgtgtgtgt 1320
gccacacctgtt cttcagttctt ctctgtgttt ggtggttctt ctctgtgttt 1380
gccacacctgtt cttcagttctt ctctgtgttt ggtggttctt ctctgtgttt 1440
ttttgtgtgt tgtgtgtgtgt ttttgtgtgtgt ttttgtgtgtgt 1500
ttttgtgtgt tgtgtgtgtgt ttttgtgtgtgt 1560
ttttgtgtgt tgtgtgtgtgt ttttgtgtgtgt 1620
acggtgtgtgt ttttgtgtgtgt ttttgtgtgtgt 1680
gccacacctgtt cttcagttctt ctctgtgttt ggtggttctt ctctgtgttt 1740
gccacacctgtt cttcagttctt ctctgtgttt ggtggttctt ctctgtgttt 1800
ttttgtgtgt tgtgtgtgtgt ttttgtgtgtgt 1821
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Attp1-SstI-FW (Primer)

<400> SEQUENCE: 5
agttcaagac tgttaaaacg aaggca

<210> SEQ ID NO 6
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Attp2-SphI-RV (Primer)

<400> SEQUENCE: 6
gctgcagcg aaacagcct gac

<210> SEQ ID NO 7
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: HPT-NotI-FD (Primer)

<400> SEQUENCE: 7
gggcgcgcat gaaaaagctt gaaatc

<210> SEQ ID NO 8
<211> LENGTH: 25
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: HPT-XbaI-RV (Primer)

<400> SEQUENCE: 8
tctagataat tcgggggatt tggat

<210> SEQ ID NO 9
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: TB-SphI- FD (Primer)

<400> SEQUENCE: 9
gtggttgcca tgcacataca aatgga

<210> SEQ ID NO 10
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: TB-NotI-RV (Primer)

<400> SEQUENCE: 10
ggcgcgcgc gaaacagctcc agatcc

<210> SEQ ID NO 11
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: HPT-SphI-RV (Primer)

<400> SEQUENCE: 11
ggggccagtct ctcgccggg gttcggat
<210> SEQ ID NO 12
<211> LENGTH: 56
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: CSVPN-ATB1-EGPI-FD (Primer)

<400> SEQUENCE: 56

gggaccaagt ttgtacaaaa aacgagcctg cagtcgcccc atgttcacaa atgaag

<210> SEQ ID NO 13
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: CSVPN-ECORI-FV (Primer)

<400> SEQUENCE: 31
tacgttctca caaatctctc tgaagtgta t

<210> SEQ ID NO 14
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Tnose-XhoI-FW (Primer)

<400> SEQUENCE: 31
cgygtgctcg agatcgcccc caacatctgg c

<210> SEQ ID NO 15
<211> LENGTH: 49
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Tnose-attB2-FV (Primer)

<400> SEQUENCE: 49

gggacccact ttgtacacaag aagctgggtg atctagtcac atagatgac

<210> SEQ ID NO 16
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: His-EcoP (Primer)

<400> SEQUENCE: 35

aattcgccgc gcgtcgcaca atcatacatca tcacg

<210> SEQ ID NO 17
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: His-EcoR (Primer)

<400> SEQUENCE: 36

aattcgatat agtgatgtatg atcagtcgcc gcggcgcgcg

<210> SEQ ID NO 18
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: mHisPTS-P (Primer)
<400> SEQUENCE: 18
cttcagagaa atttgtagaat tatacatcat ctctcactc acgg 44

<210> SEQ ID NO: 19
<211> LENGTH: 44
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: mHieFTR-R (Primer)

<400> SEQUENCE: 19
ccgatgatctatgctgctg tataatcctc aatatctctc gaag 44

<210> SEQ ID NO: 20
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: TP-ASCF (Primer)

<400> SEQUENCE: 20
ctgagcctc tataagcttcc tctatgtcct c 31

<210> SEQ ID NO: 21
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: TP-ASCR (Primer)

<400> SEQUENCE: 21
tgagcctcct ctcatgctc a 31

<210> SEQ ID NO: 22
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCamV-XbaI-PW (Primer)

<400> SEQUENCE: 22
tactttctta gacatatgact tataattc 31

<210> SEQ ID NO: 23
<211> LENGTH: 27
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PCamV-SpeI-RV (Primer)

<400> SEQUENCE: 23
accatgacta gttccctgtg ttctc 27

<210> SEQ ID NO: 24
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: 7120D-SpeI-PW (Primer)

<400> SEQUENCE: 24
actagtatc aatctccag cttggtt 28

<210> SEQ ID NO: 25
<211> LENGTH: 33

acccatgacta gttccctgtg ttttcttc

<210> SEQ ID NO: 26
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: 7120D-SpeI-PW (Primer)

<400> SEQUENCE: 26
actagtatc aatctccag cttggtt 28

<210> SEQ ID NO: 27
<211> LENGTH: 33

acccatgacta gttccctgtg ttttcttc

<210> SEQ ID NO: 28
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: 7120D-SpeI-PW (Primer)

<400> SEQUENCE: 28
actagtatc aatctccag cttggtt 28

<210> SEQ ID NO: 29
<211> LENGTH: 33

acccatgacta gttccctgtg ttttcttc

<210> SEQ ID NO: 30
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: 7120D-SpeI-PW (Primer)

<400> SEQUENCE: 30
actagtatc aatctccag cttggtt 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: T120D-KpnI-RV (Primer)

<400> SEQUENCE: 25
cgggtacct tacctcagag aaggtgcata aag

<210> SEQ ID NO 26
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Tnco-KpnI-FW (Primer)

<400> SEQUENCE: 26
cgggtacctg atcgttcaaa cattggcc

<210> SEQ ID NO 27
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Tnco-attB2-RV3 (Primer)

<400> SEQUENCE: 27
gagctctgggg accaccttctg a

<210> SEQ ID NO 28
<211> LENGTH: 56
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Crw-attB1-Sgf I-FD (Primer)

<400> SEQUENCE: 28
ggggcacagt tgtcataaaa aagcagcctg cgatgcccct atgttcaaaa atgaag

<210> SEQ ID NO 29
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: TnOS-XbaI-RV (Primer)

<400> SEQUENCE: 29
gtcttagaga tacagaataca tagagac

<210> SEQ ID NO 30
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: TP-SpeFW (Primer)

<400> SEQUENCE: 30
gggactagtt atgcttctct atagcttc

<210> SEQ ID NO 31
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: TP-SpeRV (Primer)

<400> SEQUENCE: 31
gggactagtt cttcatgcag ctagatcttc c
<210> SEQ ID NO 32
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220>FEATURE:
<223> OTHER INFORMATION: PTS-AscF (Primer)

<400> SEQUENCE: 32
gggagctcg gcggcggat ggagttgat gccc

<210> SEQ ID NO 33
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220>FEATURE:
<223> OTHER INFORMATION: PTS-XhoR (Primer)

<400> SEQUENCE: 33
gggctcgaag tttaatgga acagggggaa g

<210> SEQ ID NO 34
<211> LENGTH: 36
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220>FEATURE:
<223> OTHER INFORMATION: FPP-SpeFW (Primer)

<400> SEQUENCE: 34
gggactagt atgcagcccc atcatcatca taaag

<210> SEQ ID NO 35
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220>FEATURE:
<223> OTHER INFORMATION: FPP-KpnRV (Primer)

<400> SEQUENCE: 35
cgggtactct ctttcttggc gttttagat c

<210> SEQ ID NO 36
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220>FEATURE:
<223> OTHER INFORMATION: FPP-AscF (Primer)

<400> SEQUENCE: 36
tggcgcgcc ttagcagccc catcatc

<210> SEQ ID NO 37
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220>FEATURE:
<223> OTHER INFORMATION: FPP-AscR (Primer)

<400> SEQUENCE: 37
ttgccggcgc ttttcgggcg ttttagatc

<210> SEQ ID NO 38
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220>FEATURE:
<223> OTHER INFORMATION: PTS-8F (Primer)
<400> SEQUENCE: 38

gcgtgggtgc tgctctcgt cctc

<210> SEQ ID NO 39
<211> LENGTH: 24
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PTS-160R (Primer)

<400> SEQUENCE: 39

cctccatggac ctctgagatgc gttg

<210> SEQ ID NO 40
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: FPP-1P (Primer)

<400> SEQUENCE: 40

gcagcccccct ccattcata aagagg

<210> SEQ ID NO 41
<211> LENGTH: 23
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: RBCS-FW (Primer)

<400> SEQUENCE: 41

gagagctcga ggagaagga gtc

<210> SEQ ID NO 42
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: RBCS-RV (Primer)

<400> SEQUENCE: 42

gagaggtgt ggcaccaaat t

<210> SEQ ID NO 43
<211> LENGTH: 21
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: ADG-AscF (Primer)

<400> SEQUENCE: 43

ttagtagccct tcctgtgtgt a

<210> SEQ ID NO 44
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: ADG-AscP (Primer)

<400> SEQUENCE: 44

ttgcgcggcc gatgctacct acagaagaa aacc

<210> SEQ ID NO 45
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: ADS-XhoR (Primer)

<400> SEQUENCE: 45

ggggtogag tcaataactc ataggataaa ogag 34

<210> SEQ ID NO 46
<211> LENGTH: 43
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pLinkP (Primer)

<400> SEQUENCE: 46

aattgctagc gggacctagc tgaatccgcg gococggggg tac 43

<210> SEQ ID NO 47
<211> LENGTH: 35
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: pLinkR (Primer)

<400> SEQUENCE: 47

ccccgggggc gcgccagctag tgaatccgcg atcgc 35

<210> SEQ ID NO 48
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: attP-XbaF (Primer)

<400> SEQUENCE: 48

gctctagaga gcatactgcc ggggtg 26

<210> SEQ ID NO 49
<211> LENGTH: 26
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: attP-SphR (Primer)

<400> SEQUENCE: 49

gggggcatgc cccggctcaac acccttg 28

<210> SEQ ID NO 50
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Prrn-XbaF (Primer)

<400> SEQUENCE: 50

gctctagag agtgctacct tgaagtgg 28

<210> SEQ ID NO 51
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: TrbcL -XpnR (Primer)

<400> SEQUENCE: 51

ggggtacctg atccggctca atccttttag 30
<210> SEQ ID NO 52
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Prrn-KpnP (Primer)
<400> SEQUENCE: 52

ggggtaccag agtgtaacct tgaacgtgg

<210> SEQ ID NO 53
<211> LENGTH: 28
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Prrn-SmaR (Primer)
<400> SEQUENCE: 53
tcccccgaggs aatccctccc tcaactcg

<210> SEQ ID NO 54
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Tatp-SmaP (Primer)
<400> SEQUENCE: 54
tcccccgaggs gaactattga tcacttttg

<210> SEQ ID NO 55
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Tatp-NotR (Primer)
<400> SEQUENCE: 55
gggcgccgog cctttatattc catatatatt t

<210> SEQ ID NO 56
<211> LENGTH: 31
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LacI-SmaP (Primer)
<400> SEQUENCE: 56
tccccgaggs tgaaccagt aacgttatac g

<210> SEQ ID NO 57
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: LacI-SmaR (Primer)
<400> SEQUENCE: 57
tccccgagt caactgcgcc tttcagtcg

<210> SEQ ID NO 58
<211> LENGTH: 32
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Trbc1-SpeP (Primer)
<400> SEQUENCE: 58

```
ggactgtaa aacagtagac attagcagat aa
```

<210> SEQ ID NO: 59
<211> LENGTH: 34
<212> TYPE: DNA
<213> ORGANISM: Artificial
<222> FEATURES:
<223> OTHER INFORMATION: Trbc1-NotR (Primer)

<400> SEQUENCE: 59

```
ggggggcggc ggttattctgg cttcaatcctt ctag
```

<210> SEQ ID NO: 60
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<222> FEATURES:
<223> OTHER INFORMATION: PrnlacI-SgFP (Primer)

<400> SEQUENCE: 60

```
ggggggcatcg cagatgtcga ccttgacggtg gty
```

<210> SEQ ID NO: 61
<211> LENGTH: 60
<212> TYPE: DNA
<213> ORGANISM: Artificial
<222> FEATURES:
<223> OTHER INFORMATION: PrnlacI-Rinest (Primer)

<400> SEQUENCE: 61

```
attcggccgg agttgctgcc cagaaatata ttggtattcc gttcaatcctg ctaatccac
```

<210> SEQ ID NO: 62
<211> LENGTH: 59
<212> TYPE: DNA
<213> ORGANISM: Artificial
<222> FEATURES:
<223> OTHER INFORMATION: PrnlacI-BamHR (Primer)

<400> SEQUENCE: 62

```
ccggatccaa atccccctcc ccaactgtatat ccaaggccttc tgtttctggcc cggagttccg
```

<210> SEQ ID NO: 63
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial
<222> FEATURES:
<223> OTHER INFORMATION: GUS-BamP (Primer)

<400> SEQUENCE: 63

```
ccgggattccat gttcaagtcct gtagaaacc
```

<210> SEQ ID NO: 64
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<222> FEATURES:
<223> OTHER INFORMATION: GUS-SpeR (Primer)

<400> SEQUENCE: 64

```
ggactagtcct atgttggcct tccctgtgctgc
```

<210> SEQ ID NO: 65
<211> LENGTH: 33
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: FPS-BamF (Primer)

<400> SEQUENCE: 65
cggtattcat gcagccocat catcatcata aag 33

<210> SEQ ID NO 66
<211> LENGTH: 29
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PTS-SpeR (Primer)

<400> SEQUENCE: 66
ggactagttt aatggaac aggtgaag 29

<210> SEQ ID NO 67
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Trbcl-AscF (Primer)

<400> SEQUENCE: 67
ttggcgcc aaaaagtagg acatagcag 30

<210> SEQ ID NO 68
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Trbcl-SpeR (Primer)

<400> SEQUENCE: 68
ggactagtgt aatcgcatac atcccttttag 30

<210> SEQ ID NO 69
<211> LENGTH: 30
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PrrmlacI-SpeF (Primer)

<400> SEQUENCE: 69
ggactagtag aagtgtcaacct tgaaggtgtg 30

<210> SEQ ID NO 70
<211> LENGTH: 40
<212> TYPE: DNA
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: Prrmlac-AccI-SpeR (Primer)

<400> SEQUENCE: 70
ggactagtgg cggtccaaat cctccacct aacctgctcc 40

<210> SEQ ID NO 71
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial
<220> FEATURE:
<223> OTHER INFORMATION: PTS46 antigenic peptide

<400> SEQUENCE: 71
Glu Glu Leu Lys Val Glu Leu
His Ala Thr Ala Leu Ser Phe Arg Leu Leu Arg Gln His Gly Tyr Arg Val Ser Cys Glu
1 5 10 15
20

His Ala Thr Ala Leu Ser Phe Arg Leu Leu Arg Gln His Gly Tyr Arg Amp Glu Glu Leu Ile Lys Leu Gly Ala Pro Tyr Arg Ala
1 5 10

His Val Arg Thr Ala Val Glu Cys Tyr Met Glu Lys Val Gly Lys Gln Glu Val Val Ser Glu
1 5 10

Lys Val Gly Lys Gln Glu Val Val Ser Glu Glu Phe Val Gly Phe Phe Pro Gln Ile Val Arg
1 5 10

Glu Phe Val Gly Phe Phe Pro Gln Ile Val Arg Glu Glu Phe Val Gly Phe Phe Pro Gln Ile Val Arg
1 5 10
The invention claimed is:

1. A transformed plant accumulating patchoulool, which is transformed to comprise at least one nucleotide sequence encoding a farnesyl diphosphate synthase (FPS) and a patchoulool synthase, both targeted to plant plastids, wherein said nucleotide sequence comprises SEQ ID NO:1 and wherein said transformed plant accumulates at least 10,000 ng/g of fresh leaf of patchoulool.

2. The transformed plant according to claim 1, in which patchoulool is, at least in part, bio-synthesised via the MEP pathway.

3. The transformed plant according to claim 1, in which the FPS is a non-plant prenyl transferase.

4. The transformed plant according to claim 1, in which the at least one nucleotide sequence is integrated into plant plastidic DNA.

5. A vector comprising at least one nucleotide sequence comprising SEQ ID NO:1 and encoding a farnesyl diphosphate synthase (FPS) and a patchoulool synthase, or a fusion protein of a FPS and a patchoulool synthase, with the nucleotide sequence further comprising a plastid targeting sequence linked in frame to the nucleotide sequence encoding the FPS, the patchoulool synthase, or the fusion protein.

6. A method for altering the content of patchoulool in a plant so that said plant accumulates at least 10,000 ng/g of fresh leaf of patchoulool, which comprises: transforming plant material with at least one DNA construct comprising at least one nucleotide sequences encoding a FPS and a patchoulool synthase, targeted to plant plastids, said DNA construct comprising SEQ ID NO:1 and regenerating a transformed plant from the transformed plant material.

7. A method of producing patchoulool which comprises providing a transformed plant according to the method of claim 6 and isolating patchoulool from the transformed plant.

8. A method for producing a plant having an altered patchoulool content and accumulating at least 10,000 ng/g of fresh leaf, which comprises: transforming plant material with at least one DNA construct comprising at least one nucleotide sequences encoding a FPS and a patchoulool synthase, targeted to plant plastids, said DNA construct comprising SEQ ID NO:1 and regenerating a transformed plant from the transformed plant material.

9. A method of producing patchoulool which comprises providing a transformed plant according to the method of claim 8 and isolating patchoulool from the transformed plant.

10. A method of producing patchoulool, which comprises isolating patchoulool from the plant of claim 1.
UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 8,017,835 B2
APPLICATION NO. : 11/911660
DATED : September 13, 2011
INVENTOR(S) : Chappell et al.

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Title Page:
Item (75) Inventors: correct the spelling of the first name of inventor Clark from “Athony” to -- Anthony --. The inventor’s name will then correctly appear as “Anthony Clark”.

Add the following item before Item (30):

-- Related U.S. Application Data

(60) Provisional application no. 60/673,019, filed Apr. 19, 2005 --.

Signed and Sealed this
Twenty-ninth Day of May, 2012

David J. Kappos
Director of the United States Patent and Trademark Office