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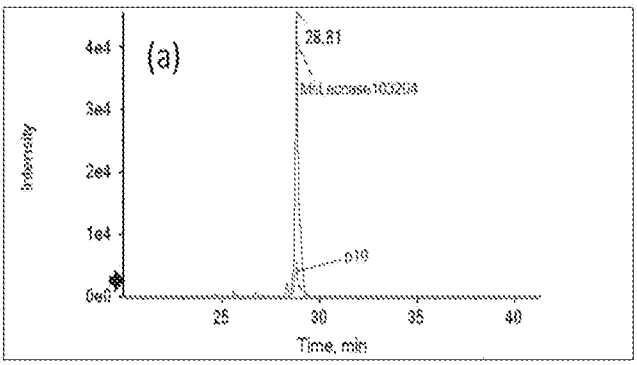
(54) **PRODUCTION OF NORNICOTINE ACYL-DERIVATIVES IN A PLANT HOST .**

(57) The present invention pertains to a process for preparing nornicotine acyl-derivatives in a plant host, through the transient expression of a plant laccase in said host. The present invention also pertains to nornicotine acyl-derivatives produced by a process according to the invention, and to compositions comprising said nornicotine acyl-derivatives.



PRODUCTION OF NORNICOTINE ACYL-DERIVATIVES IN A PLANT HOST

FIELD OF THE INVENTION

The present invention pertains to a process for preparing nornicotine acyl-derivatives in a plant host, through the transient expression of a laccase in said host. The present invention also pertains to nornicotine acyl-derivatives produced by a process according to the invention, and to compositions comprising said nornicotine acyl-derivatives.

The present invention therefore pertains to the field of recombinant production of alkaloid compounds of interest, compositions comprising said compounds as active agents and use of said alkaloid compounds.

TECHNICAL BACKGROUND

Nornicotine acyl-derivatives (*N*-acyl nornicotines or NacNN) are 1000-fold more toxic to the insect *Manduca sexta* than nicotine and are thus powerful insecticides (Laue G. *et al*. "*Fast track to the trichome: induction of N-acyl nornicotines precedes nicotine induction in Nicotiana repanda*" *Planta* 210, 510–514, (2000)).

Additionally, nornicotine acyl-derivatives were shown to exert inhibitory effects on the germination of tobacco seeds, suggesting their use in the development of new herbicides (Matsuzaki T. *et al.* "*Germination and Growth Inhibition of Acylnornicotines from Section Repandae of the Genus Nicotiana and Synthetic Acylnornicotines*". *Agricultural and Biological Chemistry* 52, 1899–1903, (1988); JP63126875).

From a pharmacological point of view, acyl-derivatives of nornicotine, such as *N*-octanoylnornicotine, are breast cancer aromatase inhibitors. These compounds have been isolated in very little amounts from cigarette smoke (Osawa, Y. *et al.* "Aromatase inhibitors in cigarette smoke, tobacco leaves and other plants." *J. Enzyme Inhib.* 4, 187–200 (1990); Kadohama *et al*. "*Tobacco alkaloid derivatives as inhibitors of breast cancer aromatase*". *Cancer Lett.* 75, 175–182 (1993)).

Aromatase inhibitors are also used in other applications, namely in the treatment of endometriosis (Wang, C. & Wilson, L. F. "*Methods and Compositions for the Treatment of Estrogen-Dependent Hyperproliferative Uterine Disorders*". (2014); US2014080794A1), in cosmetic products influencing hair growth or treating cellulite (US25685499A, US2020360734A1).

Due to their herbicide, insecticide and medical interest, there is a strong interest for developing a simple, fast and reliable process for producing nornicotine acyl-derivatives.

At present, the alkaloids nornicotine acyl-derivatives are found in low amount in some species only of wild tobacco, namely *Nicotiana* species *repanda*, *stocktonii* and *nesophila*. These compounds have also been found as minor components of cigarette smoke.

The biosynthesis of these compounds takes place in the trichomes, is induced upon elicitation with methyl jasmonate or wounding and this induction precedes that of their precursor nornicotine (Laue *et al.*, 2000).

Outchkourov *et al.* (Outchkourov *et al.* « *Control of anthocyanin and non-flavonoid compounds by anthocyanin-regulating MYB and bHLH transcription factors in Nicotiana benthamiana leaves*." *Front. Plant Sci.* 5, (2014)) discloses the production of *N*-hexanoylnornicotine and *N*-octanoylnornicotine upon agroinfiltration of *N. benthamiana* with two genes coding for the transcription factors ROS1 and DEL from snapdragon. ROS1 and DEL form a pair of transcription factor which specifically induce anthocyanin accumulation when expressed in tomato fruit.

The inventors have now developed a process for producing increased levels of nornicotine acyl-derivatives in a plant host cells by using the transient expression of a protein exhibiting a laccase enzymatic activity, in particular a laccase. In a particular embodiment, a process according to the invention for producing nornicotine acyl-derivatives involves the transient expression in a tobacco leaves of a plant laccase. In another particular embodiment, a process according to the invention involves the transient expression in tobacco leaves of a fungal laccase.

The present invention is based on the unexpected finding that tobacco leaves, transiently transformed with only one nucleotide sequence coding for a laccase, are able to produce at least one nornicotine acyl-derivative in higher quantity than when compared to the level of the same nornicotine acyl-derivative in control tobacco leaves.

The inventors defined a process comprising the production of at least one nornicotine acyl-derivative, said process comprising the steps of: i) transforming a plant host cell with a vector comprising a nucleotide sequence coding a protein exhibiting a laccase enzymatic activity, ii) cultivating said transformed host cell in appropriate culture conditions and iii) isolating at least one nornicotine acyl-derivative from said cultivated transformed host cells.

The nature and quantity of products obtained by a process according to the present invention are unexpected, as no prior data points to the role of laccases within the nornicotine acyl-derivative synthesis pathway.

A process according to the invention presents several advantages over processes disclosed in the prior art, as it involves the transient expression of only one gene and allows for the production of nornicotine acyl-derivatives of at least 1.5 fold, without requiring the separation of compounds from cigarette smoke nor eliciting, nor wounding, wild tobacco species. Indeed, in as short as a few days, for example 5-7 days, a high induction of *N*-acylnornicotine derivatives is obtained. Therefore, a process according to the invention is a fast and easy way for producing different and useful nornicotine acyl-derivatives in tobacco. A process according to the invention is furthermore safer and more ecological than processes of the prior art, and is less complicated, less labor-intensive, more user friendly than currently available processes.

SUMMARY OF THE INVENTION

In a first aspect, the present invention provides a process for the production of at least one nornicotine acyl-derivative, said process comprising the steps of:

- transforming a plant host cell with a vector comprising a nucleotide sequence coding a protein exhibiting a laccase enzymatic activity,
- cultivating said transformed host cell, and
- isolating at least one nornicotine acyl-derivative from said cultivated transformed host cell.

In a second aspect, the present invention provides a transformed plant host cell for the production of at least one nornicotine acyl-derivative. In a third aspect, the present invention provides the use of a nucleotide sequence coding for a protein exhibiting a laccase enzymatic activity, for the production of at least one nornicotine acyl-derivative in a plant host cell. In a fourth aspect, the present invention provides a composition comprising at least one nornicotine acyl-derivative obtained by a process according to the invention, said composition being preferably chosen among a pharmaceutical composition, a cosmetic composition, a herbicidal composition and an insecticidal composition.

Characteristics, advantages and uses of the subject-matter of the present invention are more detailed hereunder, in an illustrated and non-limiting way. When present, the disclosure of the ranges expressed as "from ... to ..." means that the limits are included in said ranges.

DESCRIPTION OF THE FIGURES AND TABLES

Figure 1(a) represents a chromatogram of LC-MS analysis of MsLaccase103204 after transient expression in *N. benthamiana;* leaves agro-infiltrated with the suppressor of silencing p19 were used as a negative control. The chromatogram is presented as selected ion plots of the *m/z* 275.2113 [M+H] ion.

Figure 1(b) represents the mass spectra of *N*-octanoylnornicotine.

DETAILED DESCRIPTION OF THE INVENTION

In a first aspect, the present invention relates to a process for the production of at least one nornicotine acyl-derivative, said process comprising the steps of:

i) transforming a plant host cell with a vector comprising a nucleotide sequence coding for a protein exhibiting a laccase enzymatic activity,

ii) cultivating said transformed host cell obtained at step i), and

iii) isolating at least one nornicotine acyl-derivative from said cultivated transformed host cell.

By "comprising" is meant that the element is present but that other elements may also be present. In the case of an amino acid sequence, the subject sequence may in particular further comprise additional amino acids, on the N-terminal or C-terminal side of said sequence, these additional amino acids making it possible in particular to facilitate the characterization and / or purification of the protein of interest. In the case of a nucleotide sequence, the subject sequence may in particular further comprise additional nucleotides, on the 3' or 5' side of said sequence.

By "consisting of" is meant that no element other than those mentioned is present. This limitation, however, includes possible post-translational modifications of the protein of interest.

By "cultivating said transformed host cell" it is intended providing to said transformed host cell an environment appropriate for its development, according to conditions and methods well known by a person skilled in the art. In a particular embodiment, said host cell is cultivated for 4, 5, 5, 6, 7 or 8 days. By "isolating" it is intended separating, or retrieving said at least one nornicotine acyl-derivative using any appropriate method well known by a person skilled in the art.

For the purpose of the invention, a "laccase" is a multicopper benzenediol oxygen oxidoreductase (EC 1.10.3.2) found in plants, fungi and bacteria. Laccases oxidize a variety of phenolic substrates through one-electron oxidation, coupled to a four-electron reduction of molecular oxygen to water. Laccases are classified for biochemical applications as low-, medium- and high-redox potential enzymes. This classification is based on the equilibrium

potentiometric titrations of the redox potential at the T1Cu site, where the substrate is oxidized. Said redox potential ranges from 0.43 to 0.79 V in reference to the normal hydrogen electrode (Mateljak *et al* "*Increasing redox potential, redox mediator activity and stability in a fungal laccase by computer-guided mutagenesis and directed evolution*", ACS Catalysis, 2019, 9, 4561-72). A high redox potential laccase is characterized by a redox potential comprised between 0.7 and 0.8 V, a medium redox potential laccase is characterized by a redox potential comprised between 0,5 and 0,6 V, a low redox potential laccase is characterized by a redox potential comprised between 0.4 and 0.5 V.

As a reference, by "laccase enzymatic activity" it is intended an enzymatic activity wherein a reference laccase substrate, for example 2,6 dimethoxy-phenol, is oxidized in defined experimental conditions known in the prior art (Mateljak *et al.*, "*Increasing redox potential, redox mediator activity, and stability in a fungal laccase by computer-guided mutagenesis and directed evolution*", ACS Catalysis, 9, 4561-4572).

In more specific embodiments, a process according to the invention comprises the step of transforming a plant host cell with a vector comprising a nucleotide sequence coding for a high redox potential laccase, a nucleotide sequence coding for a medium redox potential laccase or a nucleotide sequence coding for a low redox potential laccase.

Laccases from plant, fungi and bacteria are suitable for a process according to the invention. In more specific embodiments, a process according to the invention comprises the use of a vector comprising a nucleotide sequence coding for a plant laccase, a fungal laccase, or a bacteria laccase.

Among high-redox potential laccases, one can cite fungal laccases such as, for example laccases from *Pycnoporus sanguineus, Coriolopsis rigida, Ganoderma lucidum, Phanerochaete chrysosporium, Trametes versicolor, Polyporus* spp. and other *Basidiomycetes*.

More specifically, a process according to the invention uses a vector comprising a nucleotide sequence coding for a fungal laccase, said fungi being chosen among *Basidiomycetes*. Even more specifically, a process

according to the invention makes use of a vector comprising a nucleotide sequence coding for a *P. sanguineus* laccase.

Among low-redox potential laccases, one can cite plant laccases such as, for example MsLaccase103204 from *Medicago sativa* and laccase from the lacquer tree *Rhus vernicifera*.

The laccase Lac103204 extracted from *M. sativa*, also referred to as alfalfa, is coded by the nucleotide sequence SEQ ID N°1. M. *sativa* laccase Lac103204 has the amino acid sequence SEQ ID N°7

In a particular embodiment, a process of the present invention comprises the steps of:

- transforming a plant host cell with a vector comprising a nucleotide sequence coding for a protein exhibiting a laccase enzymatic activity, said nucleotide sequence being chosen among:
  - ✓ SEQ ID N°1,
  - ✓ a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1,
  - ✓ a fragment of at least 150 nucleotides of SEQ ID N°1 and
  - ✓ a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1,
- cultivating said transformed host cell, and
- isolating at least one nornicotine acyl-derivative from said cultivated transformed host cell.

By "exhibiting at least 80% identity" is meant that said sequence exhibits at least 80% identity after optimal overall alignment with another sequence, that is to say by global alignment between two sequences giving the highest percentage identity between them. The optimal global alignment of two sequences can in particular be carried out according to the Needleman-Wunsch algorithm, well known to those skilled in the art (Needleman & Wunsch, *"A general method applicable to the search for similarities in the amino acid sequences of two proteins"*, J. Mol. Biol., 48 (3): 443-53).

By "a sequence exhibiting at least 80% identity", it is intended a sequence exhibiting at least 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%,

89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or 100% identity with the cited sequence.

- ✓ By "a fragment of at least 150 nucleotides", it is intended a nucleic acid sequence comprising at least 150, 200, 250, 280, 290 or 300 nucleotides of said of SEQ ID N°1 or SEQ ID N°4 sequence.

In another particular embodiment, the present invention relates to a process for the production of at least one nornicotine acyl-derivative, said process comprising the steps of transforming a plant host cell with a vector comprising a nucleotide sequence coding for a protein exhibiting a laccase enzymatic activity, wherein said protein is chosen among:

- ✓ *M. sativa* laccase Lac103204 having the amino acid sequence SEQ ID N°7,
- ✓ a protein having an amino acid sequence exhibiting at least 80% identity with SEQ ID N°7,
- ✓ a protein having an amino acid sequence identical to a fragment of at least 50 amino acids of SEQ ID N°7 and
- ✓ a protein having an amino acid sequence of at least 50 amino acids, said sequence exhibiting at least 80% of identity with SEQ ID N°7.

The laccase extracted from *P. sanguineus BRFM66* is encoded by the nucleotide sequence SEQ ID N°4, its amino acid sequence is SEQ ID N°8, and is published as C9WKP8-1 in Uniprot Database. *Trametes sanguinea* and *P. sanguineus* are different names designating the same organism.

In another particular embodiment, a process according to the invention comprises the step of transforming a plant host cell with a vector comprising a nucleotide sequence chosen among:

- ✓ SEQ ID N°4,
- ✓ a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4,
- ✓ a fragment of at least 150 nucleotides of SEQ ID N°4 and
- ✓ a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4,

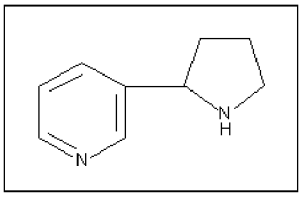
for the production of at least one nornicotine acyl-derivative in a plant host cell.

In another particular embodiment, the present invention relates to a process for the production of at least one nornicotine acyl-derivative, said process comprising the steps of transforming a plant host cell with a vector comprising a nucleotide sequence coding for a protein exhibiting a laccase enzymatic activity, wherein said protein is chosen among:

- ✓ *P. sanguineus* laccase having the amino acid sequence SEQ ID N°8,
- ✓ a protein having an amino acid sequence exhibiting at least 80% identity with SEQ ID N°8,
- ✓ a protein having an amino acid sequence identical to a fragment of at least 50 amino acids of SEQ ID N°8 and
- ✓ a protein having an amino acid sequence of at least 50 amino acids, said sequence exhibiting at least 80% of identity with SEQ ID N°8.

In a particular embodiment of this first aspect, the present invention relates to a process for producing at least one nornicotine acyl-derivative, said nornicotine acyl-derivative being preferably chosen among the following: *N*-hexanoylnornicotine, *N*-octanoylnornicotine, *N*-nonanoylnornicotine, and a combination thereof. In a particular embodiment, the present invention relates to a process for producing at least one nornicotine non hydroxylated acyl-derivative.

By "nornicotine" it is intended a de-methylated derivative of nicotine, represented as a compound of formula I.

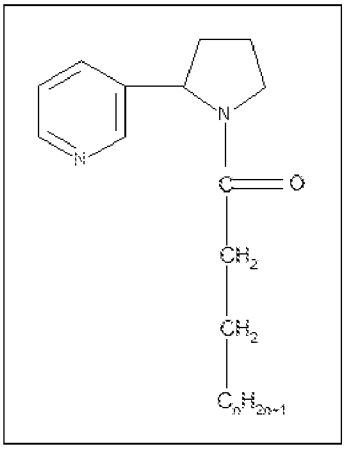


(Formula I)

By "nornicotine acyl-derivative" it is intended a nornicotine compound which is bound to an acyl group. Nornicotine acyl-derivatives comprise

hydroxylated and nonhydroxylated nornicotine acyl-derivatives, as shown for example in Figure 1 of Laue *et al*.

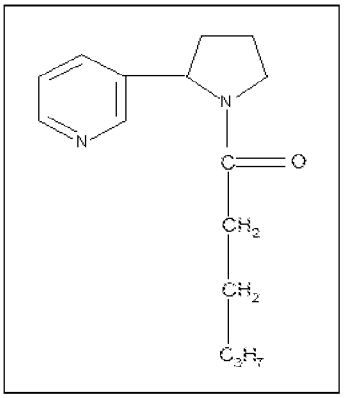
In the invention, non-hydroxylated nornicotine acyl-derivatives nornicotine acyl-derivatives are preferably represented by Formula II, wherein for example n=9 for *N*-dodecanoic acid nornicotine, were found to be induced after a transient expression of a plant or a fungal laccase.



(Formula II)

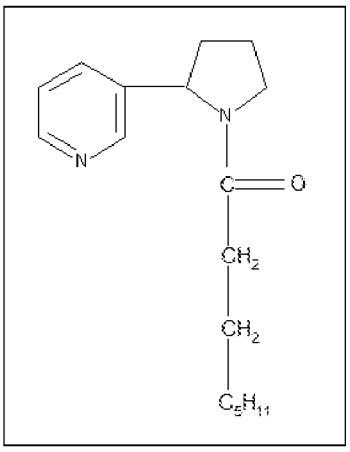
In a particular embodiment, a process according to the invention provides at least one non-hydroxylated nornicotine acyl derivative, said non-hydroxylated nornicotine acyl derivative being preferably chosen among: *N*-hexanoylnornicotine, *N*-octanoylnornicotine and *N*-nonanoylnornicotine.

By "*N*-hexanoylnornicotine" it is intended *N*-hexanoylnornicotine ($C_{15}H_{22}ON_2$) as represented by Formula III and mentioned in Outchkourov *et al.,* 2014.



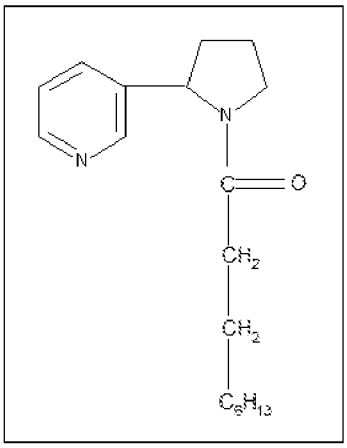
(Formula III)

By "*N*-octanoylnornicotine" it is intended *N'*-octanoylnornicotine ($C_{17}H_{26}ON_2$) as represented by formula IV and mentioned in Outchkourov *et al.,* 2014.



(Formula IV)

By "*N*-nonanoylnornicotine" it is intended *N*-nonanoylnornicotine ($C_{18}H_{28}ON_2$) as represented by formula V.



(Formula V)

In a more particular embodiment, a process according to the present invention comprises a step of transforming a plant host cell with a vector comprising a nucleotide sequence coding for a protein exhibiting a laccase enzymatic activity, wherein said vector is itself integrated into a plant pathogen. In a particular embodiment, said plant pathogen is *Agrobacterium tumefasciens,* or any plant pathogen known by a person skilled in the art to be able for this purpose. Depending on said plant pathogen used in a process of the invention, a person skilled in the art will select the appropriate vector, in particular a binary vector adapted to transform said plant pathogen.

Production of a suitable gene product may be achieved using recombinant techniques. For example, a suitable vector may be inserted into a host cell and expressed in that cell.

In a process according to the invention, the nucleotide sequence is functionally associated with the necessary means for the expression of the recombinant protein in said host cell. Said elements include a nucleotide sequence enabling the regulation of the protein expression, such as a promoter sequence, a transcription terminator, an origin of replication, and optionally a selection marker.

Such a nucleic acid may be in the form of a vector. As used herein, the term "vector" refers to a nucleic acid molecule capable of replicating into the host cell and of transporting another nucleic acid by means of insertion into e.g. a multiple cloning site. One type of vector is a "plasmid", which refers to a circular double stranded DNA loop into which additional DNA segments can be ligated. Another type of vector is a viral vector wherein additional DNA segments can be ligated into the viral genome. Certain vectors are capable of autonomous replication in a host cell into which they are introduced (e.g., bacterial vectors having a bacterial origin of replication and episomal mammalian vectors). Other vectors (e.g., non-episomal mammalian vectors) are integrated into the genome of a host cell upon introduction into the host cell, and thereby are replicated along with the host genome. Moreover, certain vectors, expression vectors, are capable of directing the expression of genes to which they are linked. In general, expression vectors of utility in recombinant DNA techniques are often in the form of plasmids (vectors). However, the invention is intended to include such other forms of expression vectors, such as binary vectors and viral vectors including, but not limited to, replication defective retroviruses, adenoviruses, lentiviruses and adeno-associated viruses (AAV), which serve equivalent functions.

Ex vivo introduction may be performed by any standard method well known by one skilled in the art, including, but not limited to, transfection, electroporation, microinjection, transduction, cell fusion, polyethylene glycol (PEG), diethylaminoethyl (DEAE) dextran, calcium phosphate precipitation, or use of a gene gun.

The polynucleotide coding for a laccase can also be introduced *ex vivo* or *in vivo* by lipofection. In certain embodiments, the use of liposomes and/or nanoparticles is contemplated for the introduction of the donor nucleic acid targeting system into host cells. Nanocapsules can generally entrap compounds in a stable and reproducible way. Liposomes are formed from phospholipids that are dispersed in an aqueous medium and spontaneously form multilamellar concentric bilayer vesicles, also termed multilamellar vesicles (MLVs). MLVs generally have diameters of from 25 nm. Sonication of MLVs results in the formation of small unilamellar vesicles (SUVs) with diameters in the range of 200 to 500 Å, containing an aqueous solution in the core.

In a more particular embodiment of this first aspect, the present invention relates to a process for the production of at least one nornicotine acyl-derivative, said process comprising the step of transforming a *Nicotiana benthamiana* host cell with a vector comprising a nucleotide sequence coding for a protein exhibiting a laccase enzymatic activity, wherein said vector is itself integrated in the plant pathogen vector *Agrobacterium tumefaciens,* and wherein said transforming step is a transient transformation.

More particularly, the present invention relates to a process for the production of at least one nornicotine acyl-derivative, wherein said transforming step is performed in the presence of a phenolic compound. In a particular embodiment, for the transformation step of a process according to the present invention, said phenolic compound is chosen among: acetosyringone (abbreviated as "acs"), coniferyl alcohol, cinnamic acid, coumarin and vanillin. In a process according to the invention, a preferred phenolic compound for transformation is acs.

In a second aspect, the present invention relates to a transformed plant host cell comprising a vector comprising a nucleotide sequence chosen among:

- ✓ SEQ ID N°1, a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1, a fragment of at least 150 nucleotides of SEQ ID N°1, a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1,

- ✓ SEQ ID N°4, a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4, a fragment of at least 150 nucleotides of SEQ ID N°4, a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4.

In a particular embodiment of this second aspect, the present invention relates to a transformed *N. benthamiana* host cell comprising *A. tumefaciens,* wherein said *A. tumefaciens* comprises a nucleotide sequence chosen among:

- ✓ SEQ ID N°1, a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1, a fragment of at least 150 nucleotides of SEQ ID N°1, a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1,
- ✓ SEQ ID N°4, a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4, a fragment of at least 150 nucleotides of SEQ ID N°4, a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4.

In a third aspect, the present invention relates to the use of a nucleotide sequence coding for a protein exhibiting a laccase enzymatic activity for the production of at least one nornicotine acyl-derivative in a plant host cell.

More particularly, the present invention relates to the use of a nucleotide sequence coding for a protein exhibiting a laccase enzymatic activity and chosen among:

- ✓ SEQ ID N°1, a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1, a fragment of at least 150 nucleotides of SEQ ID N°1, a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1,
- ✓ SEQ ID N°4, a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4, a fragment of at least 150 nucleotides of SEQ ID N°4, a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4,

for the production of at least one nornicotine acyl-derivative in a plant host cell.

In another aspect, the present invention relates to use of an amino acid sequence chosen among:

- ✓ SEQ ID N°7, an amino acid sequence exhibiting at least 80% identity with SEQ ID N°7, a fragment of at least 50 amino acids of SEQ ID N°7, a fragment of at least 50 amino acids of an amino acid sequence exhibiting at least 80% identity with SEQ ID N°7,
- ✓ SEQ ID N°8, an amino acid sequence exhibiting at least 80% identity with SEQ ID N°8, a fragment of at least 50 amino acids of SEQ ID N°8, a fragment of at least 50 amino acids of an amino acid sequence exhibiting at least 80% identity with SEQ ID N°8,

for the production of at least one nornicotine acyl-derivative in a plant host cell.

In a fourth aspect, the present invention pertains to a composition comprising at least one nornicotine acyl-derivative obtained by a process according to the invention. More particularly, said composition comprises at least one of the following nornicotine acyl-derivative: *N*-hexanoylnornicotine, *N*-octanoylnornicotine and *N*-nonanoylnornicotine.

In a particular embodiment of this fourth aspect, the present invention pertains to a composition comprising at least one nornicotine acyl-derivative obtained by a process according to the invention, wherein said composition is chosen among a pharmaceutical composition, a cosmetic composition, an herbicidal composition and an insecticidal composition.

In a more particular embodiment of this fourth aspect, the present invention pertains to a pharmaceutical composition comprising, as an active ingredient, at least one nornicotine acyl-derivative obtained by a process according to the invention, said at least one nornicotine acyl-derivative being chosen among: *N*-hexanoylnornicotine, *N*-octanoylnornicotine and *N*-nonanoylnornicotine, or a combination thereof.

The term "active agent" refers to a chemical compound or a composition that exhibits a desirable effect in the biological context, either when administered to a subject of evaluated *in vitro*. The term includes pharmaceutically acceptable derivatives of those active agents. A pharmaceutical composition according to the present invention comprises a

therapeutically effective amount of at least one nornicotine acyl-derivative. A "therapeutically effective amount" is intended for a minimal amount of active agent which is necessary to impart therapeutic benefit to a subject. For example, a "therapeutically effective amount" to a mammal is such an amount which induces, ameliorates or otherwise causes an improvement in the pathological symptoms, disease progression or physiological conditions associated with or resistance to succumbing to a disorder.

More particularly, the present invention relates to a pharmaceutical composition comprising, as an active ingredient, at least *N*-hexanoylnornicotine *N*-octanoylnornicotine and *N*-nonanoylnornicotine, or a combination thereof, obtained by a process according to the invention.

The pharmaceutical composition of the present invention may consist essentially of the active ingredient and at least one physiologically compatible carrier, excipient or diluent. Said carrier, excipient or diluent is a conventional pharmaceutically acceptable carrier, excipient or diluent, suitable for the desired mode of administration of said pharmaceutical composition.

Examples of dosage forms used for administration of the pharmaceutical composition of the present invention include tablets, granules, powders, capsules, syrups, suspensions, injections, and eye drops. Agents, ointments, patches and the like. These dosage forms can be manufactured using a technique widely used as a usual preparation method, for example, tablets, capsules, oral preparations such as granules, lactose, starch, crystalline cellulose as needed, vegetable oils and other bulking agents, magnesium stearate, lubricants such as talc, binders such as hydroxypropylcellulose and polyvinylpyrrolidone, disintegrators such as calcium carboxymethylcellulose, coating agents such as hydroxypropylcellulose, macrogol and silicone resin The active ingredient can be formulated using a gelatin coating agent.

For example, a pharmaceutical composition of the invention comprises 0.1 mg, 10 mg, 20 mg, 50 mg, or 100 mg or the like as a dosage unit amount of the active ingredient.

In a particular embodiment, the present invention relates to a pharmaceutical composition comprising at least *N*-hexanoylnornicotine, *N*-octanoylnornicotine, *N*-nonanoylnornicotine, or a combination thereof.

In a particular embodiment, the present invention relates to a pharmaceutical composition comprising, as an active ingredient, *N*-octanoylnornicotine obtained by a process according to the invention, for its use in the prevention or the treatment of breast cancer or of endometriosis.

In another particular embodiment, the present invention relates to a cosmetic composition comprising *N*-octanoylnornicotine for its use for influencing hair growth or treating cellulite.

The present invention also provides a method of prevention or treatment of a pathology chosen among breast cancer and endometriosis, said method comprising the administration, to a patient in need thereof, of pharmaceutical composition comprising *N*-octanoylnornicotine obtained by a process according to the invention.

For a method according to the present invention, said administration is for example chosen among oral, parenteral, intravenous, intramuscular, transdermal, eye drops, intra-auricular or subcutaneous. These administration methods and dosage forms are to be selected according to the patient's condition, age and treatment. The present invention is particularly for mammalian, preferably humans.

EXAMPLES

Example 1: Production of nornicotine acyl-derivatives in tobacco leaves using laccase from *M. sativa*

Materials and Methods

- *Cloning of MsaLac103204 from alfalfa and transient expression in tobacco leaves*

The construct for MsaLac103204 agroinfiltration in tobacco (*Nicotiana benthamiana*) was prepared by PCR amplification of Lac103204 cDNA sequence using Q5 DNA polymerase (NEB) with primers MsaLac103204 Fwd

(5′-CACCATGACACGATTTATGTTTTCTCTAGCATGG-3′) (SEQ ID N°2) and MsaLac103204 Rev (5′-ACACTTAGGAAGATTAGCTGGTGGAGGAGG-3′) (SEQ ID N°3) to generate an amplification product without stop codon. The PCR product was PCR purified after verification of the right size with gel electrophoresis and cloned into the Gateway pENTR/D-TOPO vector using the directional TOPO cloning kit (Invitrogen) following the manufacturer's instructions. The open reading frame of MsaLac103204 was thereafter recombined using the Gateway LR Clonase II Enzyme mix (Invitrogen) into the destination vector pEarleyGate103 to create a C-terminal fusion to GFP (Earley *et al*. "*Gateway compatible vectors for plant functional genomics and proteomics*", *Plant. J.* Feb(45):616-29, 2006). All constructs were checked by sequencing on an Applied Biosystems 3500 Genetic Analyser using the BigDye Terminator v3.1 Cycle Sequencing and the BigDye XTerminator Purification kits, according to the manufacturer's instructions.

The verified construct was transformed in *Agrobacterium tumefaciens* GV3101-pMP90 using heat shock. The two *A. tumefaciens* GV3101-pMP90 strains (transformed with p103::MsaLac103204 and the silencing suppressor pBIN61-p19, respectively) were grown in 50 ml of Luria-Bertani (LB) medium supplemented with gentamycin (30 mg l^{-1}), rifampicin (10 mg l^{-1}), kanamycin (50 mg l^{-1}) at 30°C. Bacteria were sedimented by centrifugation at 3000g for 15 min at room temperature and resuspended in infiltration solution (10 mM MES pH 5.6 and 10 mM $MgCl_2$) supplemented with 150 µg ml^{-1} acetosyringone. Bacterial suspensions were adjusted to a final OD_{600} of 1.0 for the silencing suppressor and 0.8 for p103::MsaLac10304. Cells were left in this medium in the dark for 2-3 h.

Tobacco plants *(Nicotiana benthamiana*) were grown in phytotrons under a 16h light at 25°C/8h dark at 20°C regime. Agroinfiltration (mixtures containing the same volume of strains transformed with the suppressor of silencing and MsaLac103204 were injected and, as a control, only the bacteria transformed with the suppressor were infiltrated) was carried out on plants showing 4 fully-expanded leaves (ca. 4 weeks-old plants) by injecting agrobacteria in the abaxial sides. After agroinfiltration, tobacco

plants were put back in the growth chambers for 5 days. After 5 days, the extraction of metabolites was carried out.

- *Extraction of metabolites from tobacco leaves*

Leaf tissue sample (100 mg) from 3 pooled leaves was homogenized in liquid nitrogen and extracted with 1 ml of methanol:water (4:1, v/v). This mixture was then homogenized using a vortex for 30 s, sonicated for 10 min and shaken for 4 h at room temperature. After centrifugation at 10000 g for 15 min, the supernatant was collected and evaporated to dryness using a centrifugal vacuum evaporator. Dried *N. benthamiana* extracts were resuspended in 600 µl of ethanol:water (1:1, v/v) and filtered through a syringe filter (0.2 µm, PTFE Millex-LG (Merck KGaA, Darmstadt, Germany).

- *UPLC-TripleTOF Analysis*

Extracts were analyzed with a Waters Acquity UPLC system (Milford, MA) hyphenated to a high resolution time of flight mass spectrometer (TripleTOF 6600+, AB Sciex, Concord, Ontario, Canada). The separation of 5 µl aliquot was performed on a reverse-phase Acquity UPLC BEH C18 column (2.1 × 100 mm, 1.7 µm particle size, Waters). In positive mode, the eluents were 0.1% formic acid in water (A) and 0.1% formic acid in acetonitrile (B). In negative mode, the solvents were (A) H_2O with 2.5 mM (v/w) ammonium acetate and (B) acetonitrile. The gradient was as follows: 0 min, 1% B; 4 min, 1% B; 16 min, 5% B; 35 min, 40% B; 45 min, 100% B; 50 min, 100% B; 53 min, 1% B; 60 min, 1% B. The flow rate was of 0.5 ml min^{-1} and the column temperature was 50°C. Analytes were positively ionized with electrospray ionization (ESI) source using the following parameter values for the positive and negative mode: source temperature, 650°C; ion spray voltage of 4.5 and – 4.5 kV, respectively, curtain gas (nitrogen) of 30, nebulizer gas (air) of 55, and turbine gas (air) of 50. Precursor charge state selection was set at 1. For information dependent acquisition (IDA in high sensitivity mode), survey scans were acquired in 175 ms and the 10 most abundant product ion scans were collected if exceeding a threshold of 100 counts per sec. The total cycle time was fixed at 2.25 s. Four time bins were summed for each scan at a pulser frequency value of 16.4 kHz. A sweeping collision energy setting of 15 eV in positive and – 15 eV in negative mode

and was applied to all precursor ions for collision-induced dissociation. The declustering potential was set at 60 eV and – 60 eV in positive and negative mode, respectively. Dynamic exclusion was set for 8 s after 2 occurrences, and then the precursor is refreshed off of the exclusion list. For MS1, full HR-MS spectra between 100 and 1300 mass-to-charge ratio (*m/z*) were recorded. MS2 scans were recorded between 25 and 1300 *m/z*.

- *Data Processing*

Data were first processed with MS Data Converter (Beta v1.3, AB SCIEX, Concord, Ontario, Canada). The software converts the raw data (*.wiff) into peak lists (*.mzML). The Proteowizard software (v3.0, Chambers et al., 2012) was then used to transform the files into *.mzXML. The *.mzXML files (containing MS1 data only) were processed using XCMS online (https://xcmsonline.scripps.edu/) for feature detection, alignment, and statistical analysis to highlight metabolites of interest. The software PeakView (v 1.2 0.3, AB SCIEX, Concord, Ontario, Canada) combined with the Metlin and PubChem database (https://pubchem.ncbi.nlm.nih.gov) as well as literature data were used for structure elucidation.

Results

A metabolic profiling was performed using UPLC-TripleTOF in both positive and negative mode. Figure 1 represents a chromatogram of LC-MS analysis of MsLaccase103204 after transient expression in *N. benthamiana*.

In positive mode, 12 metabolite features were identified that significantly changed after agroinfiltration with MsLaccase103204 (with fold change > 1.5). After removal of isotopes, fragment ions, and unclear mass spectra, 4 metabolites matched the PubChem and METLIN database (Table 1).

	Average area of the peak in XIC				Average area of the peak in XIC	
Putative identity, molecular formula and reference	103204 +acs	103204 -acs	P19 +acs	P19 -acs	FC 103204 +acs/p19+acs	FC 103204 -acs/p19-acs
Nornicotine C9H12N2 METLIN	2.36 10^6	1.80 10^6	1.39 10^6	8.95 10^5	1.7	2.0
N-hexanoyl nornicotine C15H22N2O Outchkourov, 2014	3.25 10^6	1.70 10^6	1.77 10^6	6.85 10^5	1.8	2.5
N-octanoyl nornicotine C17H26N2O Outchkourov, 2014	1.65 10^7	9.75 10^5	9.74 10^6	4.95 10^6	1.7	2.0
N-nonanoyl-nornicotine C18H28N2O	1.90 10^6	1.57 10^5	9.43 10^5	5.71 10^5	2.0	2.7

Table 1

Increases in the fold change (FC) of the average peak area in the extracted ion chromatographs (XIC) of nornicotine and acylated nornicotine derivatives were observed when comparing leaves expressing the alfalfa laccase *vs* the p19 silencing suppressor (Table 1). These conjugated molecules are strong defensive compounds against insect herbivores. The largest metabolite increase was observed for *N*-octanoyl-nornicotine and *N*-hexanoylnornicotine (Table 1).

It should be noted that the addition of the compound acetosyringone (acs), known to be a mediator of laccases and routinely used in agro-infiltration

assays to increase the virulence of the bacteria used for agro-infiltration, increased the average area of the peaks identified as compared to the samples obtained in the absence of acetosyringone.

Acylated nornicotine derivatives were not detected in the non-infiltrated (wild-type) leaves.

The inventors repeated this experiment and obtained an increase in the fold change of at least 1.5, in particular an increase with fold change of 4 or 5, when compared to appropriate controls, such as controls indicated in Table 1.

Example 2: Production of nornicotine acyl-derivatives in tobacco leaves using laccase from *Pycnoporus sanguineus*

Materials and Methods

- *Transient expression of Pycnoporus sanguineus laccase in tobacco leaves*

The construct for *Pycnoporus sanguineus laccase agroinfiltration in tobacco (N. benthamiana)* was prepared by PCR amplification of FJ858749 cDNA sequence using Q5 DNA polymerase (NEB) with primers *Psanguineus laccase* Fwd (5'-CACCATGTCGAGGTTCCAGTCTCTCCTCTC-3') (SEQ ID N°5) and *Psanguineus laccase* Rev (5'-GAGATCGCTAGGGTCAAGCGCGTCGTAGAT-3') (SEQ ID N°6) to generate an amplification product without stop codon.

The PCR product was then treated according to methods described in Example 1 and transformed in *Agrobacterium tumefaciens* GV3101-pMP90 using heat shock. The two *A. tumefaciens* GV3101-pMP90 strains (transformed with p103:: *Psanguineus laccase* and the silencing suppressor pBIN61-p19, respectively) were grown and infiltrated as described in example 1.

Extraction of metabolites from tobacco leaves, UPLC-TripleTOF analysis and data processing were performed as described in example 1.

Results

A metabolic profiling was performed using UPLC-TripleTOF in positive mode.

The compounds nornicotine, *N*-hexanoylnornicotine, *N*-octanoynornicotine and *N*-nonanoylnornicotine were specifically looked at (Table 2).

Putative identity, molecular formula and reference	Average of intensity of the ion at XIC peak apex (cps)		Average area of the peak in XIC		
	Psang lac+acs	p19+acs	Psang lac+acs	p19+acs	FC Psang lac+acs/p19+acs
Nornicotine C9H12N2 Metline	$2.04\ 10^5$	$2.27\ 10^4$	$2.45\ 10^6$	$2.81\ 10^5$	8.7
N-hexanoyl nornicotine C15H22N2O Outchkourov, 2014	$2.31\ 10^5$	$2.98\ 10^4$	$6.87\ 10^6$	$8.92\ 10^5$	7.7
N-octanoyl nornicotine C17H26N2O Outchkourov, 2014	$1.30\ 10^6$	$1.35\ 10^5$	$3.41\ 10^7$	$3.56\ 10^6$	9.6
N-nonanoyl-nornicotine C18H28N2O	$8.94\ 10^4$	$1.52\ 10^4$	$2.09\ 10^6$	$4.00\ 10^5$	5.2

Table 2

Increases in the fold change (FC) of the average peak area in the extracted ion chromatographs (XIC) of nornicotine and acylated nornicotine derivatives were observed when comparing leaves expressing the laccase vs the p19 silencing suppressor (Table 2). The highest FC was observed for *N*-octanoylnornicotine.

CLAIMS

1. Process for the production of at least one nornicotine acyl-derivative, said process comprising the steps of:
   i) transforming a plant host cell with a vector comprising a nucleotide sequence coding for a protein exhibiting a laccase enzymatic activity,
   ii) cultivating said transformed host cell obtained at step i), and
   iii) isolating at least one nornicotine acyl-derivative from said cultivated transformed host cell.

2. Process according to claim 1, wherein said protein exhibiting a laccase enzymatic activity is chosen in the group consisting of: high redox potential laccases, medium redox potential laccases and low redox potential laccases.

3. Process according to claim 1 or 2, wherein said nucleotide sequence is chosen among:
   - ✓ SEQ ID N°1,
   - ✓ a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1,
   - ✓ a fragment of at least 150 nucleotides of SEQ ID N°1 and
   - ✓ a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1.

4. Process according to claim 3, wherein said protein is *Medicago sativa* laccase Lac103204 having the amino acid sequence SEQ ID N°7.

5. Process according to claim 1 or 2, wherein said nucleotide sequence is chosen among:
   - ✓ SEQ ID N°4,
   - ✓ a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4,
   - ✓ a fragment of at least 150 nucleotides of SEQ ID N°4 and

- ✓ a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4.

6. Process according to claim 5, wherein said protein is *Pycnoporus sanguineus* BRFM66 laccase having the amino acid sequence SEQ ID N°8.

7. Process according to any one of claims 1 to 6, wherein said at least one nornicotine acyl-derivative is a nornicotine acyl-derivative chosen among: *N*-hexanoylnornicotine, *N*-octanoylnornicotine and *N*-nonanoylnornicotine.

8. Process according to any one of claims 1 to 7, wherein said vector is itself integrated into a plant pathogen.

9. Process according to claim 8, wherein:
   - ✓ said plant host cell is from *Nicotiana benthamiana,*
   - ✓ said plant pathogen is *Agrobacterium tumefaciens*, and
   - ✓ said transforming step is a transient transformation.

10. Process according to any one of claims 1 to 9, wherein said transforming step is performed in the presence of a phenolic compound, said phenolic compound being preferably chosen among: acetosyringone, coniferyl alcohol, cinnamic acid, coumarin and vanillin.

11. Transformed plant host cell comprising a vector comprising a nucleotide sequence chosen among:
   - ✓ SEQ ID N°1, a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1, a fragment of at least 150 nucleotides of SEQ ID N°1, a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1, and

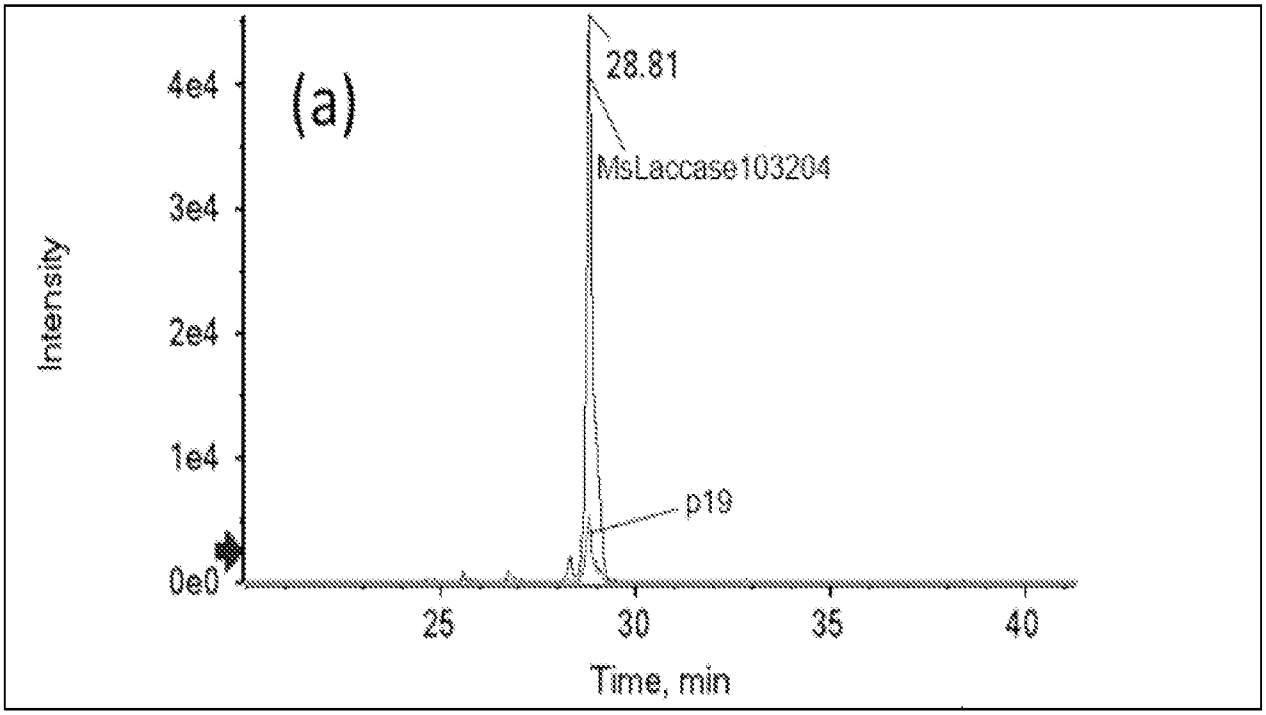
- ✓ SEQ ID N°4, a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4, a fragment of at least 150 nucleotides of SEQ ID N°4, a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4.

12. Transformed plant host cell according to claim 11, wherein said plant host cell is a *N. benthamiana* cell comprising *A. tumefaciens,* wherein said *A. tumefaciens* comprising said nucleotide sequence coding for a protein exhibiting a laccase enzymatic activity.

13. Use of a nucleotide sequence chosen among

- ✓ SEQ ID N°1, a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1, a fragment of at least 150 nucleotides of SEQ ID N°1, a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°1, and
- ✓ SEQ ID N°4, a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4, a fragment of at least 150 nucleotides of SEQ ID N°4, a fragment of at least 150 nucleotides of a nucleotide sequence exhibiting at least 80% identity with SEQ ID N°4,

for the production of at least one nornicotine acyl-derivative in a plant host cell.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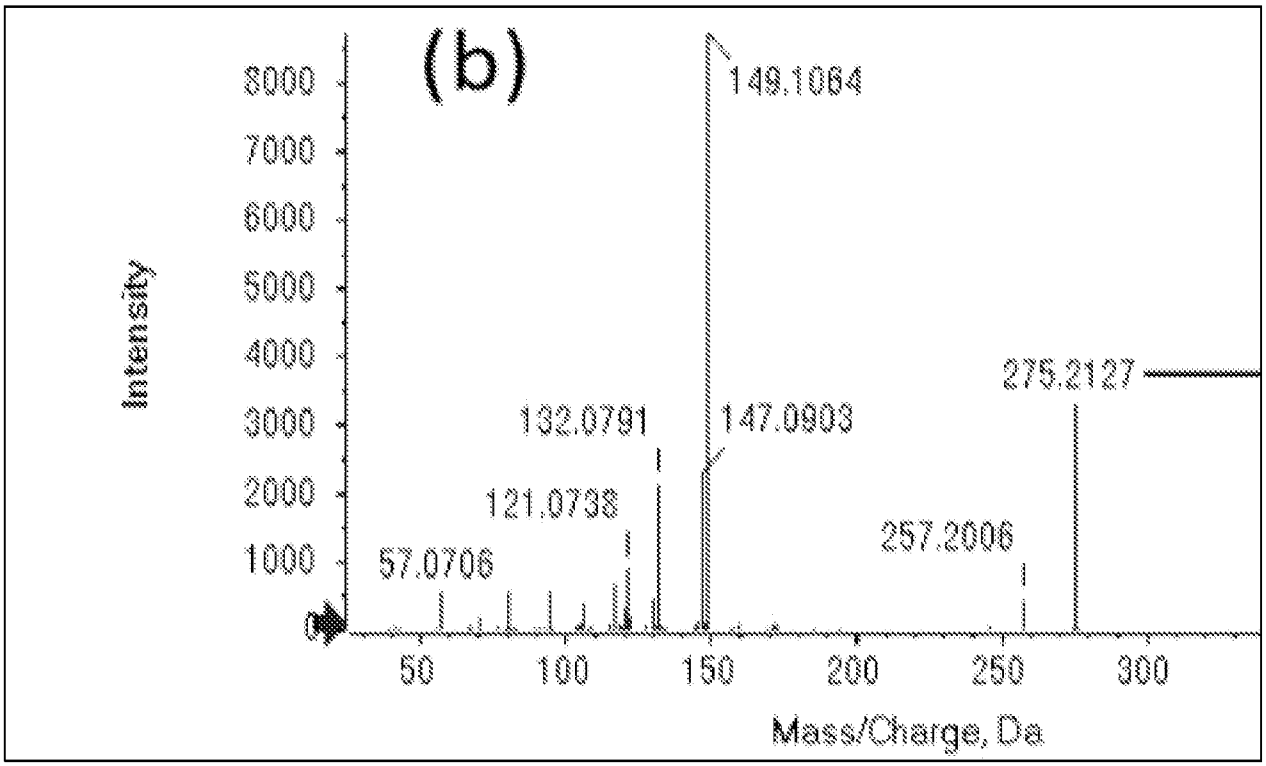
Time, min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Mass/Charge, Da

Figure 1