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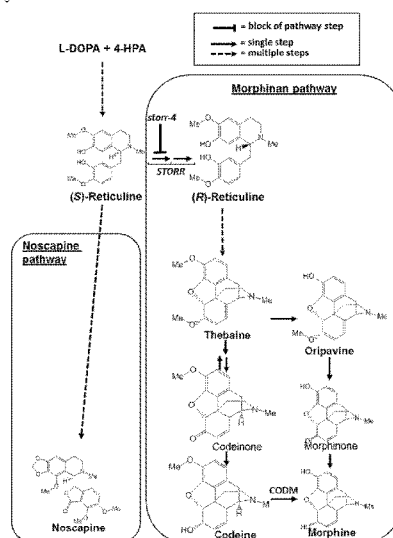
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(54) **Title:** PRODUCTION OF NOSCAPINE

Figure 3



(57) **Abstract:** This disclosure relates to high noscapine producing plants comprising functional or a non-functional or partially functional copy of a cytochrome P450-oxidoreductase catalysing the conversion of (S)-reticuline to (R)-reticuline in plants of the genus *Papaver*.

Production of Noscapine

Field of the Invention

5 This disclosure relates to high noscapine producing plants comprising functional, or a non-functional or partially functional copy of a cytochrome P450-oxidoreductase catalysing the conversion of (S)-reticuline to (R)-reticuline in plants of the genus *Papaver*.

10 Background to the Disclosure

Papaver somniferum, the opium poppy, is a source of clinically useful opiate alkaloids such as morphine, codeine, thebaine, noscapine and papaverine. Opiate alkaloids have broad clinical application ranging from analgesics to cough suppressants and anti-
15 spasmodics and are known for inducing physical dependencies.

Opiate alkaloids are structurally diverse with benzyloisoquinoline providing the structural backbone and include compounds such as papaverine, noscapine, codeine, apomorphine, benerine and sanguinarine. Benzyloisoquinoline alkaloids can be further
20 grouped based on their chemical structure into morphinans and phthalideisoquinoline alkaloids. Morphine, codeine, and thebaine are examples of morphinans, whereas papaverine and noscapine belong to the class of phthalideisoquinolines. Unlike morphinans noscapine has no analgesic properties but found its application as cough suppressant and acts as a potent antitumor agent binding to tubulin, inhibiting its
25 polymerisation, arresting cell division and inducing apoptosis. The anti-tumor activity of noscapine and its derivatives was shown in various forms of cancer. Interestingly, contrary to most other opiate alkaloids, noscapine does not induce physical dependencies.

30 Benzyloisoquinoline alkaloids are found in a number of species in the Papaveraceae family, but morphinan production has only been reported in opium poppy and the closely related *P. setigerum*. Total chemical synthesis, although possible, is not an economically viable means of production. Consequently, morphinan alkaloids are still exclusively sourced from the opium poppy plant. Whilst the biosynthesis of morphinans has been
35 extensively studied over the last 25 years and the majority of genes in the pathway have been identified, the genes involved in the first step of morphinan synthesis, the epimerization of (S)-to (R)-reticuline, required for the development of efficient and

economic viable morphinan production in microbial based systems have just recently been discovered in *P. somniferum* and are disclosed in patent application PCT/GB2015/051446 (WO2015173590). (S)-reticuline is transformed to (R)-reticuline via 1,2-Dehydroreticuline by a fusion protein encoding both a cytochrome P450 and
5 oxidoreductase module, the latter belonging to the aldo-keto reductase family. Metabolite analysis of mutant alleles and heterologous expression demonstrate that the P450 module is responsible for the conversion of (S)-reticuline to 1,2-dehydroreticuline while the oxidoreductase module converts 1,2-dehydroreticuline to (R)-reticuline rather than functioning as a P450 redox partner. A similar fusion protein catalyzing the conversion
10 from (S)- to (R)-reticuline has also been described in WO 2015/081437.

Likewise, noscapine synthesis has only been reported in a number of *Papaver* species. The level of noscapine in high noscapine varieties is typically less than 3 % of the capsule dry weight (which corresponds to a noscapine concentration of less than 60% of
15 the total benzyloquinoline alkaloid content) and relatively low and when compared to the morphinan levels which constitute over 41 % of the capsule dry weight. WO2009/143574 and WO2009/109012 discloses *P. somniferum* with noscapine concentrations based on the alkaloid concentration in poppy straw of between 0.1 to a maximum of 3.1 %. The production of noscapine was found to require the presence of a
20 complex cluster of co-expressed genes comprising mostly cytochrome P450 and methyltransferases converting scoulerine via several steps to noscapine and is disclosed in US2015/0004659, the content of which is incorporated by reference in its entirety.

This disclosure relates to the identification and characterization of high noscapine opium
25 poppy plants comprising a non-functional version of the P450-oxidoreductase fusion protein involved in the conversion of (S) to (R)-reticuline. Plants having the mutated gene have enhanced noscapine concentrations of over **3 % noscapine** of the capsule dry weight (over at least 50% noscapine of the total benzyloquinoline alkaloid content). Poppy plants comprising the modified fusion gene or engineered plants that are either
30 null or have reduced expression of the wild-type fusion gene provide an alternative, more efficient source of noscapine.

Statements of Invention

35 According to an aspect of the invention there is provided a plant of the genus *Papaver* wherein said plant is modified in a gene encoding a cytochrome P450-oxidoreductase catalysing the conversion of (S)-reticuline to (R)-reticuline wherein said modification is

stably inherited and reduces or abrogates the expression or activity of said cytochrome P450 domain and/or oxidoreductase domain of said modified polypeptide compared to a wild-type plant comprising a functional cytochrome P450-oxidoreductase.

- 5 In an embodiment of the invention said plant is modified in a gene comprising a nucleotide sequence selected from the group consisting of:
- i) a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 1, 2 or 3;
 - 10 ii) a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 4 or 33;
 - iii) a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 5;
 - iv) a nucleic acid molecule comprising a nucleic acid sequence as set forth in SEQ ID NO: 6, 7 or 8;
 - 15 v) a nucleic acid molecule the complementary strand of which hybridizes under stringent hybridization conditions to the nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33 and which encodes a polypeptide that has reduced or abrogated expression or activity of a cytochrome P450-oxidoreductase catalysing the conversion of (*S*)-reticuline to (*R*)-reticuline compared to a wild-type *Papaver* plant comprising a gene encoding a functional cytochrome P450-oxidoreductase
 - 20

Hybridization of a nucleic acid molecule occurs when two complementary nucleic acid molecules undergo an amount of hydrogen bonding to each other. Isolated nucleic acid molecules as referred herein include genomic DNA, cDNA molecules and RNA molecules. The stringency of hybridization can vary according to the environmental conditions surrounding the nucleic acids, the nature of the hybridization method, and the composition and length of the nucleic acid molecules used. Calculations regarding hybridization conditions required for attaining particular degrees of stringency are discussed in Sambrook et al., *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 2001); and Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology—Hybridization with Nucleic Acid Probes Part I*, Chapter 2 (Elsevier, New York, 1993). The T_m is the temperature at which 50% of a given strand of a nucleic acid molecule is hybridized to its complementary strand. The following is an exemplary set of hybridization conditions and is not limiting:

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Very High Stringency (allows sequences that share at least 90% identity to hybridize)

Hybridization: 5x SSC at 65°C for 16 hours
 Wash twice: 2x SSC at room temperature (RT) for 15 minutes each
 Wash twice: 0.5x SSC at 65°C for 20 minutes each

5

High Stringency (allows sequences that share at least 80% identity to hybridize)

Hybridization: 5x-6x SSC at 65°C-70°C for 16-20 hours
 Wash twice: 2x SSC at RT for 5-20 minutes each
 Wash twice: 1x SSC at 55°C-70°C for 30 minutes each

10

Low Stringency (allows sequences that share at least 50% identity to hybridize)

Hybridization: 6x SSC at RT to 55°C for 16-20 hours
 Wash at least twice: 2x-3x SSC at RT to 55°C for 20-30 minutes each.

15 In an embodiment of the invention said modified nucleic acid molecule is at least 50% identical to the nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33.

In an embodiment of the invention the modified nucleic acid molecule is at least 55%, 60%, 65%, 70%, 75%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33
 20 over the full nucleotide sequence.

In an embodiment of the invention said modified nucleic acid molecule is at least 99% identical to the nucleotide sequence set forth in SEQ ID NO 6, 7 or 8, over the full nucleotide sequence and wherein said modification is an addition, substitution or deletion of at least one nucleotide base between nucleotides 1280-1300 set forth in SEQ ID NO
 25 6, 7 or 8.

In an embodiment of the invention said modification is a substitution of at least one nucleotide base between nucleotides 1280-1300 set forth in SEQ ID NO 6, 7 or 8.

In an embodiment of the invention said modification is a substitution of at least one nucleotide base between nucleotides 1285-1295 set forth in SEQ ID NO 6, 7 or 8.

30 In an embodiment of the invention said modification is a C to T substitution of the nucleotide base at nucleotide 1291 of SEQ ID NO 6, 7 or 8.

In a preferred embodiment of the invention there is provided a modified polypeptide encoded by nucleic acid molecule comprising a nucleotide sequence as herein disclosed.

- 5 In an embodiment of the invention said plant expresses a modified P450-oxidoreductase polypeptide wherein said modified polypeptide has reduced or abrogated activity in the conversion of (*S*)-reticuline to (*R*)-reticuline.

10 In an embodiment of the invention said modified polypeptide is selected from the group consisting of:

- i) a polypeptide comprising a modified amino acid sequence of the amino acid sequence set forth in SEQ ID NO: 9, 10 or 11;
- ii) a polypeptide comprising a modified amino acid sequence of the amino acid sequence as set forth in SEQ ID NO: 12, 13 or 14;
- 15 iii) a polypeptide comprising a modified amino acid sequence that is at least 95% identical to the full length amino acid sequence as set forth in SEQ ID NO: 9, 10, 11, 12, 13 or 14, wherein said sequence is modified by addition, deletion or substitution of one or more amino acid sequences and wherein said polypeptide has reduced or abrogated enzyme activity in
20 the conversion of (*S*)-reticuline to 1,2-dehydroreticuline and/or to (*R*)-reticuline when compared to the unmodified polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 9, 10, 11, 12, 13 or 14.

25 In an embodiment of the invention said modified polypeptide is selected from the group consisting of:

- i) a modified polypeptide comprising a modified amino acid sequence of the amino acid sequence set forth in SEQ ID NO: 15;
- ii) a modified polypeptide comprising a modified amino acid sequence of the amino acid sequence set forth in SEQ ID NO: 16;
- 30 iii) a modified polypeptide comprising a modified amino acid sequence that is at least 95% identical to the full length amino acid sequence as set forth in SEQ ID NO: 15 or 16, wherein said sequence is modified by addition, deletion or substitution of one or more amino acid sequences and wherein said polypeptide has reduced or abrogated enzyme activity in the
35 conversion of 1,2-dehydroreticuline to (*R*)-reticuline when compared to the

unmodified polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 15 or 16.

5 In an embodiment of the invention said modified polypeptide comprises a deletion or substitution of amino acid residue 431 of SEQ ID NO: 9 wherein said modified polypeptide is at least 90% identical to the amino acid sequence set forth in SEQ ID NO: 9.

10 In an embodiment of the invention said modified polypeptide comprises a deletion or substitution of amino acid residue 432 of SEQ ID NO: 10 wherein said modified polypeptide is at least 90% identical to the amino acid sequence set forth in SEQ ID NO: 10.

In an embodiment of the invention said modified polypeptide comprises a deletion or substitution of amino acid residue 431 of SEQ ID NO: 11 wherein said modified polypeptide is at least 90% identical to the amino acid sequence set forth in SEQ ID NO: 11.

15 In an embodiment of the invention said modified polypeptide is 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence set forth in SEQ ID NO: 9 wherein said modified polypeptide comprises a deletion or substitution of amino acid residue 431 of SEQ ID NO: 9.

20 In an embodiment of the invention said modified polypeptide is 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence set forth in SEQ ID NO: 10 wherein said modified polypeptide comprises a deletion or substitution of amino acid residue 432 of SEQ ID NO: 10.

25 In an embodiment of the invention said modified polypeptide is 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence set forth in SEQ ID NO: 11 wherein said modified polypeptide comprises a deletion or substitution of amino acid residue 431 of SEQ ID NO: 11.

In an embodiment of the invention said modified polypeptide comprises a substitution or deletion at amino acid arginine 431 of the amino acid sequence set forth in SEQ ID NO: 9.

30 In an embodiment of the invention said modified polypeptide comprises a substitution or deletion at amino acid arginine 432 of the amino acid sequence set forth in SEQ ID NO: 10.

In an embodiment of the invention said modified polypeptide comprises a substitution or deletion at amino acid arginine 431 of the amino acid sequence set forth in SEQ ID NO: 11.

5 In an embodiment of the invention said modification is the substitution of arginine for tryptophan in at position 431 of the amino acid sequences set forth in SEQ ID NO: 9.

In an embodiment of the invention said modification is the substitution of arginine for tryptophan in at position 432 of the amino acid sequences set forth in SEQ ID NO: 10.

In an embodiment of the invention said modification is the substitution of arginine for tryptophan in at position 431 of the amino acid sequences set forth in SEQ ID NO: 11.

10 In an embodiment of the invention said modified polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 27.

In an embodiment of the invention said modified polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 28.

15 In an embodiment of the invention said modified polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 29.

In an embodiment of the invention said modified polypeptide is encoded by a nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 30.

In an embodiment of the invention said modified polypeptide is encoded by a nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 31.

20 In an embodiment of the invention said modified polypeptide is encoded by a nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 32.

In a further embodiment of the invention said plant is heterozygous for said modified gene.

25 In an alternative embodiment of the invention said plant is homozygous for said modified gene.

Mutagenesis as a means to induce phenotypic changes in organisms is well known in the art and includes but is not limited to the use of mutagenic agents such as chemical mutagens (e.g. base analogues, deaminating agents, DNA intercalating agents, alkylating agents, transposons, bromine, sodium azide] and physical mutagens [e.g. 30 ionizing radiation, psoralen exposure combined with UV irradiation). It is also common general knowledge that ablation of gene function can be obtained through genetic means

such as siRNA and antisense. The introduction of double stranded RNA, also referred to as small inhibitory/interfering RNA (siRNA) or short hairpin RNA [shRNA], into a cell which results in the destruction of mRNA complementary to the sequence included in the siRNA/shRNA molecule. This is typically via transfection of expression constructs, for example those disclosed in PCT/GB2015/051446, the content of which is incorporated in its entirety, which are stably integrated into the genome and inherited in a Mendelian manner.

In an embodiment of the invention said modified *Papaver* plant further comprises one or more genes comprising a nucleotide sequence selected from the group consisting of:

- i) a nucleotide sequence as represented by the sequence in SEQ ID NO: 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26;
- ii) a nucleotide sequence wherein said sequence is degenerate as a result of the genetic code to the nucleotide sequence defined in (i);
- iii) a nucleic acid molecule the complementary strand of which hybridizes under stringent hybridization conditions to the sequence in SEQ ID NO: 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26 wherein said nucleic acid molecule encodes polypeptides involved in the biosynthesis of *Papaver* noscapine or intermediates in the biosynthesis of noscapine.

In an embodiment of the invention said nucleic acid molecule is at least 55%, 60%, 65%, 70%, 75%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the nucleotide sequence set forth in SEQ ID NO: 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26 over the full nucleotide sequence.

In an embodiment of the invention said plant comprises enhanced levels of noscapine or intermediates in the synthesis of noscapine.

In an embodiment of the invention noscapine comprises at least 50% of the benzyloquinoline alkaloid content of said modified plant.

In a further embodiment of the invention said plant comprises a noscapine content that is at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the benzyloquinoline alkaloid content of said modified plant.

In an alternative embodiment of the invention said plant comprises a noscapine content that is at least 50% of the alkaloid content of a combination of alkaloids comprising morphine, codeine, oriparvine, thebaine and noscapine.

- 5 In a further embodiment of the invention said plant comprises a noscapine content that is at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the alkaloid content of a combination of alkaloids comprising morphine, codeine, oriparvine, thebaine and noscapine.
- 10 In an embodiment of the invention said plant comprises a benzyloquinoline content that comprises substantially noscapine.

In an embodiment of the invention said *Papaver plant* is *Papaver somniferum*.

- 15 According to an aspect of the invention there is provided a *Papaver* plant modified by transformation or transfection with an expression vector adapted to express an antisense, siRNA or shRNA designed with reference to a nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33 wherein noscapine comprises at least 50% of the benzyloquinoline alkaloid content of said modified plant.

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In an embodiment of the invention said modified plant comprises a noscapine content that is at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the of the benzyloquinoline alkaloid content of said modified plant.

- 25 In an embodiment of the invention said modified plant comprises a benzyloquinoline alkaloid content that comprises substantially noscapine.

- In an alternative embodiment of the invention said modified plant comprises a noscapine content that is at least 50% of the alkaloid content of a combination of alkaloids comprising morphine, codeine, oriparvine, thebaine and noscapine.
- 30

- In a further embodiment of the invention said modified plant comprises a noscapine content that is at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the alkaloid content of a combination of alkaloids comprising morphine, codeine, oriparvine, thebaine and noscapine.
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According to a further aspect of the invention there is provided a Papaver capsule obtained from the modified plant according to the invention.

According to a further aspect of the invention there is provided a Papaver straw obtained
5 from the modified plant according to the invention.

According to an aspect of the invention there is provided a Papaver seed obtained from the modified plant according to the invention.

10 According to a further aspect of the invention there is provided a modified polypeptide comprising an amino acid sequence wherein said amino acid sequence is at least 90% identical to the amino acid sequence set forth in SEQ ID NO: 27, 28 or 29.

According to a further aspect of the invention there is provided a modified polypeptide comprising an amino acid sequence wherein said amino acid sequence is at least 91%,
15 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the amino acid sequence set forth in SEQ ID NO: 27, 28 or 29.

According to an aspect of the invention there is provided a modified polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 27.

According to an aspect of the invention there is provided a modified polypeptide
20 comprising the amino acid sequence set forth in SEQ ID NO: 28.

According to an aspect of the invention there is provided a modified polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 29.

According to a further aspect of the invention there is provided a nucleic acid molecule comprising a nucleotide sequence wherein said nucleotide sequence is at least 90%
25 identical to the nucleotide sequence set forth in SEQ ID NO: 30, 31 or 32.

According to an aspect of the invention there is provided a nucleic acid molecule comprising a nucleotide sequence wherein said nucleotide sequence is at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the nucleotide sequence set forth in SEQ ID NO: 30, 31 or 32.

30 According to an aspect of the invention there is provided a nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 30.

According to an aspect of the invention there is provided a nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 31.

According to an aspect of the invention there is provided a nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 32.

According to a further aspect of the invention there is provided a method to obtain a *Papaver* plant comprising enhanced or elevated noscapine, or intermediates in the synthesis of noscapine, comprising the steps of:

- i) mutagenesis of wild-type seed from a *Papaver* plant that comprises a gene encoding a functional cytochrome P450-oxidoreductase catalysing the conversion of (*S*)-reticuline to (*R*)-reticuline;
- ii) cultivation of the seed in i) to produce first and subsequent generations of plants;
- iii) obtaining seed from the first generation plant and subsequent generations of plants;
- iv) determining if the seed from said first and subsequent generations of plants has altered nucleotide sequence of said gene encoding a cytochrome P450-oxidoreductase;
- v) obtaining a sample and analysing the nucleic acid sequence of a nucleic acid molecule selected from the group consisting of:
 - a) a nucleic acid molecule comprising a nucleotide sequence as represented in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33;
 - b) a nucleic acid molecule that hybridises to a complementary strand of said nucleic acid molecule in a) under stringent hybridisation conditions and that encodes a non-functional cytochrome P450-oxidoreductase; and optionally
- vi) comparing the nucleotide sequence of the nucleic acid molecule in said sample to a nucleotide sequence of a nucleic acid molecule of the original wild-type plant.

In an embodiment of the method of the invention said plants have an altered expression of said cytochrome P450-oxidoreductase.

In a further embodiment of the method of the invention said modified nucleic acid molecule is at least 55%, 60%, 65%, 70%, 75%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33 over the full nucleotide sequence.

In a yet further embodiment of the method of the invention said nucleic acid molecule is analysed by a method comprising the steps of:

- i) extracting nucleic acid from said mutated plants;
- 5 ii) amplification of a part of said nucleic acid molecule by a polymerase chain reaction;
- iii) forming a preparation comprising the amplified nucleic acid and nucleic acid extracted from wild-type seed to form heteroduplex nucleic acid;
- 10 iv) incubating said preparation with a single stranded nuclease that cuts at a region of heteroduplex nucleic acid to identify the mismatch in said heteroduplex; and
- v) determining the site of the mismatch in said nucleic acid heteroduplex.

15 In an embodiment of the method of the invention said modified plant is analysed for noscapine content and optionally compared to the original unmodified plant.

In an embodiment of the method of the invention said *Papaver* plant is *Papaver somniferum*.

20 According to a further aspect of the invention there is provided a plant obtained by the method according to the invention.

According to a further aspect of the invention there is provided the use of a gene encoding a cytochrome P450-oxidoreductase catalysing the conversion of (*S*)-reticuline
25 to (*R*)-reticuline in the identification of *Papaver* cultivars that have enhanced levels of noscapine or intermediates in noscapine synthesis.

In an embodiment of the invention said gene comprises a nucleotide sequence selected from the group consisting of:

- 30 i) a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 1, 2 or 3;
- ii) a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 4 or 33;
- 35 iii) a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 5;
- iv) a nucleic acid molecule comprising a nucleic acid sequence as set forth in SEQ ID NO: 6, 7 or 8;

- 5 v) a nucleic acid molecule the complementary strand of which hybridizes under stringent hybridization conditions to the nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8, 33 and which encodes a polypeptide that has reduced or abrogated expression or activity of a cytochrome P450-oxidoreductase catalysing the conversion of (*S*)-reticuline to (*R*)-reticuline compared to a wild-type *Papaver* plant comprising a gene encoding a functional cytochrome P450-oxidoreductase.
- 10 According to a further aspect of the invention there is provided a method to identify a *Papaver* plant comprising a modified cytochrome P450-oxidoreductase gene comprising the steps:
- i) obtaining a sample of plant material from a plant to be tested;
 - ii) extracting genomic nucleic acid from said plant material;
 - 15 iii) analysing the extracted genomic nucleic acid encoding a variant nucleotide sequence of the nucleotide sequences set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33; and optionally,
 - iv) comparing the sequence to a wild-type nucleotide sequence comprising SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33.

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Competitive allele specific PCR (KASP) allows the identification of single-nucleotide polymorphism in a DNA sequence. The method is based on primer extension of two allele-specific (forward) primers and amplification of the resulting product using a common reverse primer. The forward primers are designed specifically to bind and extend the primer to either the SNP or wild type allele which allows subsequent binding of the common reverse primer for amplification of the product. In a second step an oligonucleotide cassette complimentary to a specific 5' sequence of the first allele-specific forward primer and a second oligonucleotide cassette complimentary to a specific 5' sequence of the second allele-specific forward primer both comprising a different fluorescent label is added to the reaction allowing amplification of the SNP and wild type targets and product detection through fluorescent signalling. Alternatively, directly fluorescent labelled primers targeted to the common reverse primer can be used to amplify e.g. just the species with the SNP in a sample. The KASP technology is disclosed in US2016/0002712 which is incorporated by reference in its entirety.

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In a preferred method of the invention said analysis comprises the steps:

- i) preparing a reaction mixture comprising:
 - a) genomic DNA to be analysed,
 - b) a first allele specific forward oligonucleotide primer comprising a nucleotide sequence complementary to said cytochrome P450-oxidoreductase gene nucleotide sequence and a nucleotide sequence complementary to a nucleotide sequence of a first fluorescent reporter cassette,
 - c) a second allele specific forward oligonucleotide primer comprising a nucleotide sequence complementary to said cytochrome P450-oxidoreductase gene and a nucleotide sequence complementary to a nucleotide sequence of a second fluorescent reporter cassette wherein the nucleotide at the 3'-end of said second oligonucleotide primer sequence is complementary to a polymorphic nucleotide sequence variant of said cytochrome P450-oxidoreductase gene, and wherein the nucleotide sequence of said first and second oligonucleotide primers are complementary to the same upstream nucleotide sequence of said cytochrome P450-oxidoreductase gene;
 - d) a reverse oligonucleotide primer comprising a nucleotide sequence complementary to a nucleotide sequence downstream of said cytochrome P450-oxidoreductase gene nucleotide sequence; and
 - e) two reporter cassettes comprising double stranded nucleic acid comprising:
 - a first double stranded nucleic acid wherein one strand comprises a fluorescent label and is complementary to said first allele specific primer and a second strand comprising a quencher label that quenches said fluorescent label; and
 - a second double stranded nucleic acid wherein one strand comprises a fluorescent label different from the fluorescent label of i) and is complementary to said second allele specific primer and a second strand comprising a quencher label that quenches said second fluorescent label;
- ii) heating said reaction mixture to denature genomic nucleic acid and said double stranded reporters and providing conditions to anneal primers to their respective complementary nucleotide sequence, primer extending said annealed oligonucleotide primers and polymerase chain amplification of said mixture; and
- iii) detecting fluorescence emission.

In a preferred method of the invention said modified and wild type cytochrome P450-oxidoreductase gene nucleic acid sequence is as set forth SEQ ID NO: 6.

In a preferred method according of the invention said first forward primer comprises (SEQ ID NO: 35) CCAGGCAATCATCAAAGAATCAATGT (SEQ ID NO: 35), said second forward primer comprises CCAGGCAATCATCAAAGAATCAATGC (SEQ ID NO: 34) and said reverse primer comprises CACGGGGCTGGCTGGATACAA (SEQ ID NO 5 36).

In an embodiment of the invention said use or method includes analysis of the sequence or expression of a further gene selected from the group consisting of:

- 10 i) a nucleotide sequence as represented by the sequence in SEQ ID NO: 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26;
- ii) a nucleotide sequence wherein said sequence is degenerate as a result of the genetic code to the nucleotide sequence defined in (i);
- 15 iii) a nucleic acid molecule the complementary strand of which hybridizes under stringent hybridization conditions to the sequence in SEQ ID NO: 17, 18, 19, 20, 21 or 22, 23, 24, 25 or 26 wherein said nucleic acid molecule encodes polypeptides involved in the biosynthesis of *Papaver* noscapine or intermediates in the biosynthesis of noscapine.

In an embodiment of the invention said nucleic acid molecule is at least 55%, 60%, 65%, 20 70%, 75%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the nucleotide sequence set forth in SEQ ID NO: 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26 over the full nucleotide sequence.

It will be apparent to the skilled artisan that techniques that enable the analysis of 25 mutations in gene sequences and/or expression are available and include, but are not limited to genomic DNA sequencing, PCR based analysis of DNA via single polynucleotide polymorphisms (SNP analysis), single stranded conformational polymorphism (SSCP), denaturing gradient gel electrophoresis (DGGE), quantitative PCR and multiplex PCR to detect expression of multiple genes.

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According to a further aspect of the invention there is provided a method for the extraction of noscapine from a modified plant or capsule or straw according to the invention comprising the steps:

- 35 i) harvesting a modified plant, capsule or straw according to the invention and forming a reaction mixture of particulate plant material;
- ii) solvent extracting said reaction mixture to provide an alkaloid enriched fraction; and

- iii) concentrating said enriched alkaloid fraction to provide an enriched noscapine fraction.

Throughout the description and claims of this specification, the words “comprise” and “contain” and variations of the words, for example “comprising” and “comprises”, means “including but not limited to”, and is not intended to (and does not) exclude other moieties, additives, components, integers or steps. “Consisting essentially” means having the essential integers but including integers which do not materially affect the function of the essential integers.

Throughout the description and claims of this specification, the singular encompasses the plural unless the context otherwise requires. In particular, where the indefinite article is used, the specification is to be understood as contemplating plurality as well as singularity, unless the context requires otherwise.

Features, integers, characteristics, compounds, chemical moieties or groups described in conjunction with a particular aspect, embodiment or example of the invention are to be understood to be applicable to any other aspect, embodiment or example described herein unless incompatible therewith.

Definitions

“Benzylisoquinoline alkaloids” typically include papaverine, noscapine, codeine, morphine, apomorphine, berberine, protopine, tubocurarine and sanguinarine. The major alkaloids are morphine, codeine, oripavine, thebaine and noscapine.

“Substantially noscapine” means that other benzylisoquinoline alkaloids are trace alkaloids or undetectable benzylisoquinoline alkaloids as measured by techniques available to the skilled person.

“Stably inherited” refers to the transfer of relevant characteristics which remain unchanged after repeated propagation or, in the case of a particular cycle of propagation, at the end of each such cycle.

“Reduced or abrogated expression” refers to reduction of mRNA expression by 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% when compared to a wild-type plant expressing a

gene encoding an unmodified cytochrome p450-oxidoreductase. Quantitative mRNA expression methods are known to the skilled person such for example real time PCR.

“Functional cytochrome P450-oxidoreductase” refers to a non-modified enzyme which is capable of converting (S)- to (R)- reticuline via 1,2-dehydroreticuline. The measurement of cytochrome P450 and oxidoreductase activities are well known in the art, for example see Lenz & Zenk, 1995, FEBS 233:132-139. In addition heterologous expression of cytochrome P450 and oxidoreductase domains in addition to expression of cytochrome P450-oxidoreductase fusion protein in microbial systems such as yeast allows functional characterisation of functional and non-functional cytochrome P450-oxidoreductase enzymes.

The phrase “enhanced levels of noscapine and noscapine intermediates” means producing a higher level of noscapine or intermediates in the synthesis of noscapine compared to a wild-type plant comprising a functional cytochrome P450-oxidoreductase, for example, a 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99% increase in the level of noscapine or intermediates in noscapine biosynthesis.

“Wild-type plant” means a plant having a phenotype that is characteristic of the species in a natural breeding population.

“Reduced or abrogated activity in the conversion of (S)-reticuline to (R)-reticuline” refers to the reduction of the catalytic activity of the enzyme by 10, 20, 30, 40, 50, 60, 70, 80, 90 or 100% relative to an unmodified cytochrome P450-oxidoreductase.

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Variant as referred to under “SEQ ID Summary” refers to different polymorphic forms of the particular gene.

SEQ ID NO: 1 P450 domain nucleotide sequence; polymorphic variant a

30 SEQ ID NO: 2 P450 domain nucleotide sequence; polymorphic variant b

SEQ ID NO: 3 P450 domain nucleotide sequence; polymorphic variant c

SEQ ID NO: 4 oxidoreductase A nucleotide sequence, variant b

SEQ ID NO: 5 oxidoreductase B nucleotide sequence

35 SEQ ID NO: 6 cytochrome P450-oxidoreductase nucleotide sequence; polymorphic variant a

SEQ ID NO: 7 cytochrome P450-oxidoreductase nucleotide sequence; polymorphic variant b

- SEQ ID NO: 8 cytochrome P450-oxidoreductase nucleotide sequence; polymorphic variant c
- SEQ ID NO: 9 cytochrome P450-oxidoreductase amino acid, polymorphic variant a
- SEQ ID NO: 10 cytochrome P450-oxidoreductase amino acid; polymorphic variant b
- 5 SEQ ID NO: 11 cytochrome P450-oxidoreductase amino acid; polymorphic variant c
- SEQ ID NO: 12 P450 domain amino acid; polymorphic variant a
- SEQ ID NO: 13 P450 domain amino acid; polymorphic variant b
- SEQ ID NO: 14 P450 domain amino acid; polymorphic variant c
- SEQ ID NO: 15 oxidoreductase A amino acid
- 10 SEQ ID NO: 16 oxidoreductase B amino acid
- SEQ ID NO: 17 PSMT1
- SEQ ID NO: 18 PSMT2
- SEQ ID NO: 19 PSMT3
- SEQ ID NO: 20 CYP450 1 (CYP 82X1)
- 15 SEQ ID NO: 21 CYP450 2 (CYP 719A21)
- SEQ ID NO: 22 CYP450 3 (CYP 82X2)
- SEQ ID NO: 23 CYP450 4 (CYP 82Y1)
- SEQ ID NO: 24 PSAT1
- SEQ ID NO: 25 PSSDR1
- 20 SEQ ID NO: 26 PSCXE1
- SEQ ID NO: 27 cytochrome P450-oxidoreductase mutant amino acid, variant a
- SEQ ID NO: 28 cytochrome P450-oxidoreductase mutant amino acid, variant b
- SEQ ID NO: 29 cytochrome P450-oxidoreductase mutant amino acid, variant c
- SEQ ID NO: 30 cytochrome P450-oxidoreductase mutant nucleotide sequence variant a
- 25 SEQ ID NO: 31 cytochrome P450-oxidoreductase mutant nucleotide sequence variant b
- SEQ ID NO: 32 cytochrome P450-oxidoreductase mutant nucleotide sequence variant c
- SEQ ID NO: 33 Oxidoreductase A, nucleic acid sequence, variant a (and c)

An embodiment of the invention will now be described by example only and with
 30 reference to the figures, examples and tables:

Figure 1: Violin plot of the relative amount (% total) of noscapine and morphinan alkaloids in dried capsules of F2 populations segregating *storr-4*. HOM_STORR4, F2 material homozygous for *storr-4*; HET_STORR4, F2 material heterozygous for *storr-4*;
 35 WT_STORR, F2 material homozygous for the STORR wild type allele; HN4, control plants of NOSCAPINE CVS 4, one of the parental lines of the F2 material;

Figure 2: Violin plot of the absolute amount (% capsule dry weight) of noscapine and morphinan alkaloids in dried capsules of segregating F2 populations segregating *storr-4*. HOM_STORR4, F2 material homozygous for *storr-4*; HET_STORR4, F2 material heterozygous for *storr-4*; WT_STORR, F2 material homozygous for the STORR wild type allele; HN4, control plants of NOSCAPHINE CVS 4, one of the parental lines of the F2 material; DW, dry weight; and

Figure 3: Schematic overview of noscapine and morphinan biosynthetic pathways in opium poppy and the position of the pathway block conferred by *storr-4*. (S)-reticuline is a major branch point intermediate in alkaloid metabolism in opium poppy serving as a precursor for both noscapine and morphinan biosynthesis. The *STORR* locus controls the gateway reaction into morphinan biosynthesis, the two step epimerisation of (S)- to (R)-reticuline. Impairment of STORR, for example by means of knock out mutations such as *storr-4*, leads to a block of morphinan biosynthesis and increased production of noscapine in noscapine producing cultivars of opium poppy.

Materials & Methods

EMS mutagenesis

Prior to mutagenesis, M0 seeds from *P. somniferum* cultivar GSK NOSCAPHINE CVS2 were soaked in 0.1% (w/v) potassium chloride solution for approximately 24 hours. The seed was then imbibed for three hours with ethyl methanesulfonate (EMS) solution (200 mM EMS in 100 mM sodium hydrogen phosphate buffer, pH 5.0, supplemented with 700 mM dimethyl sulfoxide). The treated M1 seed was washed twice for 15 minutes each with 100 mM sodium thiosulphate solution and then twice for 15 minutes each with distilled water. A volume of 10 mL of the respective solutions was used per 1000 seeds and all steps were carried out on a rocking platform. The washed M1 seed was dried overnight on Whatman 3MM Blotting paper (Whatman/GE Healthcare Life Sciences, Little Chalfont, UK) and sown the following morning on top of a mixture of John Innes no 2 compost, vermiculite and perlite (4:2:1), then covered with a thin layer of compost. Seedlings were transplanted three to four weeks after germination into larger pots and grown in the glasshouse until maturity. Dried capsules were harvested by hand from the M1 population once capsules had dried to approximately 10% moisture on the plant. M2 seed was manually separated from the capsule, providing the M2 seed families that were used in the field-based trials described below.

Field-based trials of EMS-mutagenised lines

M2 seed was sown in in the field in Tasmania in the 2009/2010 growing season. M2 seed lines from the EMS mutagenised population were hand sown in 3m long rows. After
5 germination, plants were thinned to 75 mm apart. Typically 4 plants (designated A, B, C, D) per M2 line were self-pollinated (two immature buds per plant selected) by securing a linen bag over the bud. Open-pollinated rows were harvested at ~ 11% moisture, and assayed (as described below) for morphine (M), codeine (C), oripavine (O) and thebaine (T) content. Analysis of a selected self-pollinated capsules from an M2 plants indicated
10 significantly reduced morphinan assays, and significantly higher assays of noscapine. M3 and M4 seeds derived from a selected self-pollinated M2 capsule displaying the low morphinan alkaloids/high noscapine phenotype were sown in the following growing seasons in Tasmania to confirm this phenotype in both open-pollinated and self-pollinated capsules. Typically 4 plants (designated A, B, C, D) were self-pollinated per
15 M3 and M4 seed line as described above. GSK commercial cultivars were grown alongside the M2, M3 and M4 lines as a comparison. Depending on the season, they included GSK MORPHINE CULTIVARs 3 and 5, GSK THEBAINE CULTIVAR 3, GSK NOSCAPINE CULTIVARs 3 - 6.

20 Poppy straw analysis of field-based trials

Poppy capsules were harvested from the respective mutant lines by hand once capsules had dried to approximately 10% moisture on the plant. The seed was manually separated from the capsule, and capsule straw material (poppy straw) was then shipped
25 to the GSK extraction facility in Port Fairy, Australia.

The poppy straw samples were ground in a Retsch Model MM400 ball mill into a fine powder. Two gram samples of ground poppy straw (2 ± 0.003 g) were extracted in 50 mL of a 10% acetic acid solution. The extraction suspension was shaken on an orbital
30 shaker at 200rpm for a minimum of 10 minutes then filtered to provide a clear filtrate. The final filtrate was passed through a 0.22 μ m filter prior to analysis.

The solutions were analysed using a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA) fitted with a Waters Acquity BEH C18 column, 2.1mm x 100mm with
35 1.7 micron packing. The mobile phase used a gradient profile with eluent A consisting of 0.1% Trifluoroacetic acid in deionised water and eluent B consisting of 100% Acetonitrile. The mobile phase gradient conditions used are as listed in Table 1, the gradient curve

number as determined using a Waters Empower chromatography software package. The flow rate was 0.6 mL per minute and the column maintained at 45 degrees centigrade. The injection volume was 1 μ L injection volume and the alkaloids were detected using a UV detector at 285 nm.

5

The loss on drying (LOD) of the straw was determined by drying in an oven at 105 degrees centigrade for 16-20 hours.

Table 1 – Gradient Flow Program

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TIME (minutes)	% Eluent A	% Eluent B	Flow (mL/min)	Curve No
0.00	95.0	5.0	0.60	INITIAL
0.80	90.0	10.0	0.60	6
3.40	75.0	25.0	0.60	3
3.60	95.0	5.0	0.60	6
4.00	95.0	5.0	0.60	11

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Alkaloid concentrations for morphine, pseudomorphine, codeine, thebaine, oripavine and noscapine were quantified using external standard calibration and the results calculated on a dry weight basis. Reticuline was quantified using the thebaine calibration (assuming the thebaine response) and results calculated on a dry weight basis (% weight relative to thebaine; %WRT).

Typical retention times are as follows:

Compound	Retention Time (minutes)
Morphine	1.14
Pseudomorphine	1.26
Codeine	1.69
Oripavine	1.80
Reticuline	2.05
10-Hydroxythebaine	2.32
Thebaine	2.53
Noscapine	3.16

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Leaf latex analysis of glasshouse grown M4 generation (S-186668) derived from the self-pollinated MCS 746 C plant.

The latex alkaloid profile determined in M4 siblings of the plants used for sequencing the cytochrome P450-oxidoreductase (*STORR*) cDNA (see below). Latex was collected
5 when the first flower buds emerged (~7 week old plants) from cut petioles, with a single drop dispersed into 500 μ L of 10% acetic acid. This was diluted 10 $\times$ in 1% acetic acid to give an alkaloid solution in 2% acetic acid for further analysis. The samples were analysed by UPLC-MS and R-scripts as previously described ("the standard method"; (Winzer T. et al. (2012) Science 336, 1704-8)). Compounds were annotated by
10 generation of empirical formulae from exact masses (< 5 ppm mass accuracy) and comparison to authentic standards. Unknowns were annotated by a masstag in the format MxTy, where x is mass and y is retention time in seconds.

The relative content of alkaloids was measured as % peak area relative to total alkaloid peak area.
15

Isolation and sequencing the of the cytochrome P450-oxidoreductase cDNA in the M4 generation (S-186668) derived from the self-pollinated MCS 746 C plant.

20 RNA was isolated from M4 individuals derived from self-pollinated M3 individual MCS 746 C that displayed the low morphinan alkaloid/high noscapine phenotype. M4 plants were grown under glasshouse conditions to post-flowering stages (1-6 days after petal fall) and stem segments (2.5-3 cm long) immediately beneath the flowers were harvested and flash frozen in liquid nitrogen.

25 Total RNA was extracted from the stems using the pine tree RNA extraction method (Chang et al., 1993. Plant Mol. Biol. Rep. 11: 113-116) and further purified using the RNeasy Plus MicroKit (Qiagen, Crawley, UK).

30 Synthesis of cDNA was performed using 1 μ g of total RNA, 10 μ M oligo dT MW 4500 (Invitrogen/Life technologies, Paisley, UK) and 1 mM dNTP. Reactions were incubated for 5 min at 65°C to allow annealing of oligonucleotides. First strand synthesis reactions contained 1 X First Strand buffer (Invitrogen/Life Technologies), 20 mM DTT, 40 U RNase out (Invitrogen/Life technologies). Reactions were incubated at 42°C for 2 min
35 and then 200 U SuperScript II reverse transcriptase (Invitrogen/Life technologies, Paisley, UK) was added followed by a 50 min incubation at 42°C and heat inactivation at 70°C for 15 min. Samples were diluted 5 X in water and used for gene specific amplifications.

The full-length region encoding the cytochrome P450-oxidoreductase gene was amplified from the cDNA samples using the primers 5'- ATGGAGCTCCAATATATTTC-3', 5'- TCAAGCTTCATCATCCCACA-3' and the high fidelity Q5® Hot Start High-Fidelity DNA Polymerase (New England Biolabs, Hitchin, UK) according to the guidelines supplied by the manufacturer. Cycling conditions were as follows: 98°C for 30 s, 30 cycles of 98°C for 10 s, 60°C for 30 s, 72°C for 1.5 min and a final extension at 72°C for 2 min. The 3 kb-PCR product was then ligated into the pJET1.2 cloning vector using the CloneJET PCR Cloning Kit (Thermo Scientific) according to the manufacturer's protocol. Three individual clones were fully sequenced with the sequencing primer shown in the Table 1.

Table 1: Sequencing primers used to sequence the cytochrome P450-oxidoreductase cDNA M4 progeny of MCS 746 C.

SEQ ID NO	Oligonucleotides sequence (5' to 3')
pJET1.2 Forward Sequencing primer (SEQ ID NO 37)	CGACTCACTATAGGGAGAGCGGC
pJET1.2 Reverse Sequencing primer (SEQ ID NO 38)	AAGAACATCGATTTTCCATGGCAG
Primer 1 (SEQ ID NO 39)	GCAGTGCTTCATATTTCTCG
Primer 2 (SEQ ID NO 40)	CCTTATGGAAAATATTGGAGGGAGC
Primer 3 (SEQ ID NO 41)	GTCTCTCTCAGAGGCTGCT
Primer 4 (SEQ ID NO 42)	TTGTCTACATCCAGGCAATC

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RNA-seq of stems and capsules from GSK NOSCAPINE CVS 3.

Stem and capsule material was harvested from stems and capsules of GSK NOSCAPINE CVS 3 plants 1-2 days after petal fall. RNA was prepared individually from five plants. The harvested material was ground in liquid nitrogen using a mortar and pestle. RNA was isolated from the ground stem or capsule preparations as previously described (Chang et al., 1993, Plant Mol. Biol. Rep. 11: 113-116) with slight

20

modifications (three extractions with chloroform:isoamylalcohol, RNA precipitation with Lithium chloride at -20°C overnight). RNA was quantified spectrophotometrically before pooling equal amounts of RNA from five plants per cultivar, stage and organ. The pooled samples underwent a final purification step using an RNeasy Plus MicroKit (Qiagen, Crawley, UK) to remove any remaining genomic DNA from the preparations. RNA was eluted in 100 µL RNase-free water.

RNA sequencing was performed on one lane of the Illumina HiSeq 2000 platform (Illumina, Little Chesterford, UK) using the TruSeq RNA kit. TruSeq RNA library construction was carried out according to the manufacturer's description and included a poly-A selection step.

De novo sequence assembly of the raw sequence datasets was performed using the Trinity RNA-seq de novo assembly tool (Haas et al., 2013, Nature Protocols, 8: 1494–1512; <http://trinityrnaseq.github.io/>).

Mutation discovery in the *STORR* locus

The clonal *STORR* cDNA sequences obtained from M4 progeny of plant MCS 746 C displaying the low morphinan alkaloid/high noscapine phenotype were compared to the corresponding contiguous sequence assembled from RNA-seq data from GSK NOSCAPHINE CVS 3, the opium poppy cultivar used for EMA mutagenesis. The clonal and contiguous sequences were identical apart from a C to T transitions at position 1291 with respect to SEQ ID NO: 6. This C to T transition is consistent with the type of mutations introduced by EMS (Greene et al., 2003, Genetics, 164: 731-740). The mutation results in a arginine to tryptophan amino acid substitution at position 431 of the *STORR* protein (R431W).

Enzyme assays

General reaction set up was carried out as previously described (Lenz & Zenk, 1995, FEBS 233:132-139). Substrates (*S*)-reticuline, 1,2-dehydroreticuline and coclaurine were purchased from TRC Chemicals (Toronto, Canada) and codeinone was supplied by MacFarlan-Smith (Edinburgh, UK). Assay reactions contained 300 mM NADPH, 5 µg oxidoreductase A or B preparation, 100 mM buffer covering a range of pH values (potassium phosphate pH 6-8, glycine-NaOH pH 9) and 75 µM substrate. Reactions were incubated for 2 h at 37°C and immediately frozen at -80°C. Reactions were dried

down to powder in speedvac Gene Vac EZ-2 plus (Ipswich, UK) at 40°C and resuspended in 100 µL 1:1 Hexane:Ethanol (v/v), 0.1% Diethylamine (v/v).

Alternatively enzyme assays were set up in 250 µL volumes containing final
5 concentrations of 50 mM Bis-tris propane buffer, 1 mM NADPH and 100 µM of substrate
prepared from transformed yeast recombinantly expressing wild-type and mutant
cytochrome P450-oxidoreductases. The assays were started by the addition of 50 µL of
2 mg mL⁻¹ soluble or microsomal extract from transformed yeast, and incubated at 37 °C
for 3 hours. After this time, 50 µL of the extract was removed and quenched by the
10 additional of 50 µL of methanol + 1 % acetic acid containing 100 mM noscapine as an
internal standard. The reaction was then centrifuged at 20,000 × *g* for 2 min, and the
supernatant recovered for analysis of 1,2-dehydroreticuline and (*R/S*) -reticuline content
(i.e., non-chiral method). The remaining 200 µL of assay was used for chiral analysis.
The reaction was quenched by the addition of 0.5 M sodium carbonated buffer (pH 10.0).
15 Reticuline was then extracted from the reaction products by three sequential extractions
with 400 µL of dichloromethane. The solvent extracts were combined and evaporated to
dryness in a speedvac. The extract was then dissolved in 100 µL of 50:50:0.1
hexane/ethanol/diethylamine and analysed by the chiral HPLC method to determine the
relative amounts of (*R*)- and (*S*)-reticuline.

20 **EMS mutagenesis of a noscapine opium poppy cultivars leads to opium poppy
lines where noscapine constitutes more than 90% of the total alkaloid content.**

Among the plants derived from M2 seed line S-121435 one individual – designated plant
A – displayed a high noscapine and low morphinan alkaloid phenotype compared to
noscapine, thebaine and morphine cultivars (Table 3). The relative noscapine content
25 expressed as percentage of total major alkaloids (morphine, codeine, oripavine, thebaine
and noscapine) reached almost 85% in this plant. The relative content of noscapine
further increased and that of morphinan alkaloids further decreased in the M3 and M4
progeny both in self-and open-pollinated capsules indicating that the underlying mutation
is no longer segregating in these populations. Likewise, noscapine amounts to more than
30 90% of the relative alkaloid content in the latex of glasshouse-grown M4 siblings of
plants used for sequencing the cytochrome P450-oxidoreductase (STORR) cDNA (Table
2). This shows that the mutant phenotype is stable in the M4 generation and across
environmental and developmental conditions.

Generation of F2 populations population segregating *storr-4* in the background of high noscapine cvs 4 (HN4)

The impact of the *storr-4* allele on noscapine and morphinan alkaloid accumulation was assessed in segregating F2 populations. M4 mutant plants derived from seed batch S-186668 were crossed with high NOSCAPHINE CVS4 (HN4). The resulting F1 generation was self-pollinated to produce F2 seed lines segregating *storr-4* in a noscapine background. Three F2 lines (S-185973, S-185975, S-185976) were grown along HN4 control plants in the glasshouse to maturity. Dried capsule material was harvested and analysed as described below.

Genotyping of *storr-4*

KASP genotyping assays for *storr-4* were designed to genotype F2 populations. To design allele-specific primers, sequences of 50-100 nucleotides around the mutation site were submitted to LGC genomics (Hoddesdon, UK) to order KASP by Design (KBD) primer mix. The sequences of the allele-specific primers as well as the common primer are shown in Table 4.

Table 4. Primer sequences, in 5'-3' orientation.

Mutant allele	Primer	Primer sequence	Label
<i>storr-4</i>	Wild type allele (SEQ ID NO 34)	CCAGGCAATCATCAAAGAATCAATGC	FAM
	Mutant allele (SEQ ID NO 35)	CCAGGCAATCATCAAAGAATCAATGT	HEX
	Common primer (SEQ ID NO 36)	CACGGGGCTGGCTGGATACAA	

Leaf samples (30-50mg) for DNA extraction were harvested from 4-6 week old plants. Genomic DNA was extracted using the BioSprint 96 Plant kit on the BioSprint 96 Workstation (Qiagen, Crawley, UK) according to the manufacturer's protocol. Extracted DNA was quantified on the NanoDrop™ 8000 Spectrophotometer (Fisher Scientific, Wilmington, DE, USA) and normalized to 5 ng/μL.

KASP assay reactions were carried out in FrameStar® 384 well plates (4titude® Ltd, Wotton, UK) in a 5.07 μL volume per reaction, with the following reaction components: 2x KASP V4.0 mastermix (LGC genomics), 2.5 μL; KBD primer mix, 0.07 μL; HyClone molecular grade water (Thermo Scientific, Hemel Hempstead, UK), 0.5 μL; 5 ng/μL DNA, 2 μL. The plates were centrifuged for 2 minutes at 4500 x *g*, heat-sealed using the Kube™ heat-based plate sealer (LGC genomics), and thermal cycling was carried out in

a Hydrocycler™ (LGC genomics) water bath-based thermal cycler, with the following conditions: 94°C for 15 minutes; 10 cycles: 94°C for 20 seconds, 61-55°C for 60 seconds (dropping 0.6°C per cycle); 34 cycles: 94°C for 20 seconds, 55°C for 60 seconds. The plate reading was carried out on an Applied Biosystems ViiA7 instrument (Life Technologies), for 30 seconds at 25°C.

Capsule straw analysis of glasshouse grown F2 populations

Capsules from the F2 populations and HN4 controls were harvested once capsules had dried to approximately 10% moisture on the plants. Seed was manually separated from the capsule and single capsules were ground to a fine powder in a ball mill (Model MM04, Retsch, Haan, Germany). Samples of ground poppy straw were then weighed accurately to 10 ± 0.1 mg and extracted in 0.5 mL of a 10% (v/v) acetic acid solution with gentle shaking for 1h at room temperature. Samples were then clarified by centrifugation and a 50 µL subsample diluted 10× in 1% (v/v) acetic acid to give an alkaloid solution in 2% acetic acid for further analysis. All solutions were analysed as described for the poppy straw analysis from field grown plants. Likewise, all data analysis was carried out using the R programming language. Putative alkaloid peaks were quantified by their pseudomolecular ion areas using custom scripts. Alkaloids were identified by comparing exact mass and retention time values to those of standards. Where standards were not available, the Bioconductor rcdk package (Smith et al., 2006), Anal. Chem. 78 (3): 779-787) was used to generate pseudomolecular formulae from exact masses within elemental constraints C = 1 100, H = 1 200, O = 0 200, N = 0 3 and mass accuracy < 5ppm. The hit with the lowest ppm error within these constraints was used to assign a putative formula.

25

Examples

The *storr-4* allele co-segregates with the high noscapine zero morphinan phenotype.

F2 populations segregating *storr-4* in a high noscapine background were genotyped for *storr-4* and their relative and absolute alkaloid content determined in dried capsules. The *storr-4* mutant allele strictly co-segregated with extremely high noscapine and zero morphinan levels (Figure 1 and 2). The relative noscapine content in homozygous *storr-4* material reached 98 ± 0.7 % with the remainder being mainly reticuline (1.8 ± 0.7 %). Noscapine and morphinan levels of heterozygous F2 material were intermediate between those of homozygous *storr-4* F2 material and F2 plants homozygous for the *STORR* wild type allele (Figure 1 and 2). The alkaloid profile of F2 plants homozygous

for the *STORR* wild type allele was overall very similar to that of control plants from HN4, one of the parental varieties used to generate the F2 population.

The data clearly demonstrate that the shutdown of morphinan biosynthesis in *STORR* knock out mutations such as *storr-4* allows leads to increased metabolic flux into the noscapine branch pathway (Figure 3) thus resulting in extremely high levels of noscapine.

10 Table 2

Latex data from glasshouse-grown M4 progeny (S-186668) of plant MCS 746 C							
Alkaloid content % total alkaloids							
Morphine	Codeine	Codeinone	Oripavine	Thebaine	Noscapine	Laudanosin	Protopine
1.3 ± 2.32	0.34 ± 0.71	0.01 ± 0	0 ± 0	0.07 ± 0.1	92.34 ± 3.35	0.81 ± 0.38	0.2 ± 0.22
averages and standard deviation of 15 individual M4 plants are shown							

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Table 3: alkaloid content (% dry weight and % total alkaloid content) in different plant lines

Season	Line	Generation	Self-/open pollinated *	Alkaloid content % dry weight					Alkaloid content % total alkaloids**						
				Morphine	Codeine	Oripavine	Thebaine	Noscapine	Morphine	Codeine	Oripavine	Thebaine	Noscapine		
2012/13	GSK MORPHINE CVS 4		OP row	3.520	0.310	0.050	0.170	0.000	86.9	7.7	1.2	4.2	0.0		
	GSK NOSCAPINE CVS 3		OP row	2.630	0.180	0.015	0.030	2.550	48.7	3.3	0.3	0.6	47.2		
	S-121435	M2	OP row	1.780	0.140	0.010	0.060	2.850	36.8	2.9	0.2	1.2	58.9		
			SP cap A	0.590	0.060	0.020	0.080	4.220	11.9	1.2	0.4	1.6	84.9		
2013/14	GSK MORPHINE CVS 5		OP row	3.840	0.140	0.040	0.135	0.090	90.5	3.3	0.9	3.2	2.1		
	GSK THEBAINE CVS 3		OP row	0.970	0.210	1.150	2.970	0.060	18.1	3.9	21.5	55.4	1.1		
	GSK NOSCAPINE CVS 4		OP row	2.225	0.185	0.115	0.25	1.955	47.0	3.9	2.4	5.3	41.3		
	MC 746 (progeny of S-121435 self- pollinated capsule A)	M3	OP row	0.27	0.06	0.01	0.07	3.370	7.1	1.6	0.3	1.9	89.2		
	SP caps A-D		0.19 ± 0.295	0.033 ± 0.013	0.01 ± 0	0.07 ± 0.046	3.47 ± 1.39	4.05 ± 4.95	1.03 ± 0.74	0.31 ± 0.13	1.70 ± 0.56	92.91 ± 4.87			
			GSK NOSCAPINE CVS 3	OP row	2.125	0.145	0.025	0.065	1.885	50.1	3.4	0.6	1.5	44.4	
2014/15	GSK NOSCAPINE CVS 4		OP row	1.145	0.080	0.030	0.175	2.520	29.0	2.0	0.8	4.4	63.8		
	GSK NOSCAPINE CVS 5		OP row	2.325	0.095	0.01	0.035	1.995	52.1	2.1	0.2	0.8	44.7		
	GSK NOSCAPINE CVS 6		OP row	2.375	0.165	0.015	0.055	1.71	55.0	3.8	0.3	1.3	39.6		
	MD 2717 (progeny of MC 746 self- pollinated capsule A)	M4	OP row	0.020	0.020	0.010	0.080	3.610	0.530	0.530	0.270	2.140	96.520		
	SP caps A-D		0.028 ± 0.005	0.035 ± 0.013	0.02 ± 0	0.058 ± 0.019	4.108 ± 0.39	0.655 ± 0.152	0.833 ± 0.322	0.474 ± 0.046	1.377 ± 0.517	96.661 ± 0.717			
			* OP = open pollinated; SP-cap(s) = self-pollinated capsule(s),												
** based on main alkaloids: Morphine, Codeine, Oripavine, Thebaine and Noscapine															

Claims

1. A plant of the genus *Papaver* wherein said plant is modified in a gene encoding a cytochrome P450-oxidoreductase catalysing the conversion of (*S*)-reticuline to (*R*)-reticuline wherein said modification is stably inherited and reduces or abrogates the expression or activity of said cytochrome P450 domain and/or oxidoreductase domain of said modified polypeptide compared to a wild-type plant comprising a functional cytochrome P450-oxidoreductase.
2. The plant according to claim 1 wherein said plant is modified in a gene comprising a nucleotide sequence selected from the group consisting of:
- i) a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 1 or 2 or 3;
 - ii) a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 4 or 33;
 - iii) a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 5;
 - iv) a nucleic acid molecule comprising a nucleic acid sequence as set forth in SEQ ID NO: 6, 7 or 8;
 - v) a nucleic acid molecule the complementary strand of which hybridizes under stringent hybridization conditions to the nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33 and which encodes a polypeptide that has reduced or abrogated expression or activity of a cytochrome P450-oxidoreductase catalysing the conversion of (*S*)-reticuline to (*R*)-reticuline compared to a wild-type *Papaver* plant comprising a gene encoding a functional cytochrome P450-oxidoreductase
3. The plant according to claim 1 or 2 wherein said modified nucleic acid molecule is at least 50% identical to the nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33.
4. The plant according to claim 3 wherein said modified nucleic acid molecule is at least 55%, 60%, 65%, 70%, 75%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33 over the full nucleotide sequence.

5. The plant according to claim 4 wherein said modified nucleic acid molecule is at least 99% identical to the nucleotide sequence set forth in SEQ ID NO 6, 7 or 8, over the full nucleotide sequence and wherein said modification is an addition, substitution or deletion of at least one nucleotide base between nucleotides 1280-1300 set forth in SEQ ID NO 6, 7 or 8.
6. The plant according to claim 5 wherein said modification is a substitution of at least one nucleotide base between nucleotides 1280-1300 set forth in SEQ ID NO 6, 7 or 8.
7. The plant according to claim 6 wherein said modification is a substitution of at least one nucleotide base between nucleotides 1285-1295 set forth in SEQ ID NO 6, 7 or 8.
8. The plant according to claim 7 wherein said modification is a C to T substitution of the nucleotide base at nucleotide 1291 of SEQ ID NO 6, 7 or 8.
9. The plant according to any one of claims 1 to 8 wherein said plant expresses a modified P450-oxidoreductase polypeptide wherein said modified polypeptide has reduced or abrogated activity in the conversion of (*S*)-reticuline to (*R*)-reticuline.
10. The plant according to claim 9 wherein said modified polypeptide is selected from the group consisting of:
- i) a polypeptide comprising a modified amino acid sequence of the amino acid sequence set forth in SEQ ID NO: 9, 10 or 11;
 - ii) a polypeptide comprising a modified amino acid sequence of the amino acid sequence as set forth in SEQ ID NO: 12, 13 or 14;
 - iii) a polypeptide comprising a modified amino acid sequence that is at least 95% identical to the full length amino acid sequence as set forth in SEQ ID NO: 9, 10, 11, 12, 13 or 14, wherein said sequence is modified by addition, deletion or substitution of one or more amino acid sequences and wherein said polypeptide has reduced or abrogated enzyme activity in the conversion of (*S*)-reticuline to 1,2-dehydroreticuline and/or to (*R*)-reticuline when compared to the unmodified polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 9, 10, 11, 12, 13 or 14.

11. The plant according to claim 9 wherein said modified polypeptide is selected from the group consisting of:

- i) a modified polypeptide comprising a modified amino acid sequence of the amino acid sequence set forth in SEQ ID NO: 15;
- 5 ii) a modified polypeptide comprising a modified amino acid sequence of the amino acid sequence set forth in SEQ ID NO: 16;
- 10 iii) a modified polypeptide comprising a modified amino acid sequence that is at least 95% identical to the full length amino acid sequence as set forth in SEQ ID NO: 15 or 16, wherein said sequence is modified by addition, deletion or substitution of one or more amino acid sequences and wherein said polypeptide has reduced or abrogated enzyme activity in the conversion of 1,2-dehydroreticuline to (*R*)-reticuline when compared to the unmodified polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 15 or 16.

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12. The plant according to claim 9 or 10 wherein said modified polypeptide comprises at least an addition, deletion or substitution of an amino acid residue in the region of amino acids 386-486 as set forth in SEQ ID NO: 9, 10 or 11.

13. The plant according to claim 12 wherein said modified polypeptide comprises at least an addition, deletion or substitution of an amino acid residue in the region of amino acids 426-436 as set forth in SEQ ID NO: 9, 10 or 11 and wherein said modified polypeptide is at least 90% identical to the amino acid sequence set forth in SEQ ID NO: 9, 10 or 11.

14. The plant according to any one of claims 9 or 10 wherein said modified polypeptide comprises a deletion or substitution of amino acid residue 431 of SEQ ID NO: 9 wherein said modified polypeptide is at least 90% identical to the amino acid sequence set forth in SEQ ID NO: 9.

15. The plant according to claim 9 or 10 to 13 wherein said modified polypeptide comprises a deletion or substitution of amino acid residue 432 of SEQ ID NO: 10 wherein said modified polypeptide is at least 90% identical to the amino acid sequence set forth in SEQ ID NO: 10.

16. The plant according to claim 9 or 10 wherein said modified polypeptide comprises a deletion or substitution of amino acid residue 431 of SEQ ID NO: 11 wherein said modified polypeptide is at least 90% identical to the amino acid sequence set forth

in SEQ ID NO: 11.

17. The plant according to claim 9 or 10 wherein said modified polypeptide comprises a substitution or deletion at amino acid arginine 431 of the amino acid sequence set forth in SEQ ID NO: 9.

5 18. The plant according to claim 9 or 10 wherein said modified polypeptide comprises a substitution or deletion at amino acid arginine 432 of the amino acid sequence set forth in SEQ ID NO: 10.

10 19. The plant according claim 9 or 10 wherein said modified polypeptide comprises a substitution or deletion at amino acid arginine 431 of the amino acid sequence set forth in SEQ ID NO: 11.

20. The plant according to claim 9 or 10 wherein said modification is the substitution of arginine for tryptophan in at position 431 of the amino acid sequences set forth in SEQ ID NO: 9.

15 21. The plant according to claim 9 or 10 wherein said modification is the substitution of arginine for tryptophan in at position 432 of the amino acid sequences set forth in SEQ ID NO: 10.

22. The plant according to claim 9 or 10 wherein said modification is the substitution of arginine for tryptophan in at position 431 of the amino acid sequences set forth in SEQ ID NO: 11.

20 23. The plant according to claim 9 or 10 wherein said modified polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 27.

24. The plant according to claim 9 or 10 wherein said modified polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 28.

25 25. The plant according to claim 9 or 10 wherein said modified polypeptide comprises the amino acid sequence set forth in SEQ ID NO: 29.

26. The plant according to claim 9 or 10 wherein said modified polypeptide is encoded by a nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 30.

30 27. The plant according to claim 9 or 10 wherein said modified polypeptide is encoded by a nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 31.

28. The plant according to claim 9 or 10 wherein said modified polypeptide is encoded by a nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 32.

5 29. The plant according to any one of claims 1 to 28 wherein said plant is heterozygous for said modified gene.

30. The plant according to any one of claims 1 to 28 wherein said plant is homozygous for said modified gene.

10 31. The plant according to any one of claims 1 to 30 wherein said plant comprises one or more genes comprising a nucleotide sequence selected from the group consisting of:

- i) a nucleotide sequence as represented by the sequence in SEQ ID NO: 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26;
- 15 ii) a nucleotide sequence wherein said sequence is degenerate as a result of the genetic code to the nucleotide sequence defined in (i);
- 20 iii) a nucleic acid molecule the complementary strand of which hybridizes under stringent hybridization conditions to the sequence in SEQ ID NO: 17, 18, 19, 20, 21, 22, 23, 24, 25 or 26 wherein said nucleic acid molecule encodes polypeptides involved in the biosynthesis of *Papaver* noscapine or intermediates in the biosynthesis of noscapine.

25 32. The plant according to claim 31 wherein said plant comprises enhanced levels of noscapine or intermediates in the synthesis of noscapine.

33. The plant according to claim 32 wherein noscapine comprises at least 50% of the benzyloisoquinoline alkaloid content of said modified plant.

30 34. The plant according to claim 33 wherein said plant comprises a noscapine content that is at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% of the of the benzyloisoquinoline alkaloid content of said modified plant.

35. The plant according to claim 33 or 34 wherein said plant comprises a benzyloisoquinoline content that comprises substantially noscapine.

36. The plant according to any one of claims 1 to 35 wherein said *Papaver* plant is *Papaver somniferum*.

5 37. A *Papaver* plant modified by transformation or transfection with an expression vector adapted to express an antisense, siRNA or shRNA designed with reference to a nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33 wherein noscapine comprises at least 50% of the benzyloquinoline alkaloid content of said modified plant.

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38. The plant according to claim 37 wherein said modified plant comprises a noscapine content that is at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% of the of the benzyloquinoline alkaloid content of said modified plant.

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39. The plant according to claim 37 or 38 wherein said modified plant comprises a benzyloquinoline alkaloid content that comprises substantially noscapine.

20 40. A *Papaver* capsule obtained from the modified plant according to any one of claims 1 to 39.

41. A *Papaver* straw obtained from the modified plant according to any one of claims 1 to 39.

25 42. A *Papaver* seed obtained from the modified plant according to any one of claims 1 to 39.

43. A modified polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 27.

30 44. A modified polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 28.

45. A modified polypeptide comprising the amino acid sequence set forth in SEQ ID NO: 29.

35 46. A nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 30.

47. A nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 31.

5 48. A nucleic acid molecule comprising a nucleotide sequence set forth in SEQ ID NO: 32.

49. A method to obtain a *Papaver* plant comprising enhanced or elevated noscapine, or intermediates in the synthesis of noscapine, comprising the steps of:

- 10 i) mutagenesis of wild-type seed from a *Papaver* plant that comprises a gene encoding a functional cytochrome P450-oxidoreductase catalysing the conversion of (*S*)-reticuline to (*R*)-reticuline;
- ii) cultivation of the seed in i) to produce first and subsequent generations of plants;
- 15 iii) obtaining seed from the first generation plant and subsequent generations of plants;
- iv) determining if the seed from said first and subsequent generations of plants has altered nucleotide sequence of said encoding a cytochrome P450-oxidoreductase;
- 20 v) obtaining a sample and analysing the nucleic acid sequence of a nucleic acid molecule selected from the group consisting of:
- i. a nucleic acid molecule comprising a nucleotide sequence as represented in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33;
- 25 ii. a nucleic acid molecule that hybridises to a complementary strand of said nucleic acid molecule in a) under stringent hybridisation conditions and that encodes a non-functional cytochrome P450-oxidoreductase; and optionally
- vi) comparing the nucleotide sequence of the nucleic acid molecule in said sample to a nucleotide sequence of a nucleic acid molecule of the original wild-type plant.
- 30

50. The method according to claim 49 wherein said plants have an altered expression of said cytochrome P450-oxidoreductase.

51. The method according to claim 49 or 50 wherein said modified nucleic acid molecule is at least 55%, 60%, 65%, 70%, 75%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% identical to the nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33 over the full nucleotide sequence.

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52. The method according to any one of claims 49 to 51 wherein said nucleic acid molecule is analysed by a method comprising the steps of:

- i) extracting nucleic acid from said mutated plants;
- ii) amplification of a part of said nucleic acid molecule by a polymerase chain reaction;
- iii) forming a preparation comprising the amplified nucleic acid and nucleic acid extracted from wild-type seed to form heteroduplex nucleic acid;
- iv) incubating said preparation with a single stranded nuclease that cuts at a region of heteroduplex nucleic acid to identify the mismatch in said heteroduplex; and
- v) determining the site of the mismatch in said nucleic acid heteroduplex.

53. The method according to any one of claims 49 to 52 wherein said modified plant is analysed for noscapine content and optionally compared to the original unmodified plant.

54. A plant obtained by the method according to any one of claims 49 to 53.

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55. The use of a gene encoding a cytochrome P450-oxidoreductase catalysing the conversion of (*S*)-reticuline to (*R*)-reticuline in the identification of *Papaver* cultivars that have enhanced levels of noscapine or intermediates in noscapine synthesis.

56. Use according to claim 55 wherein said gene comprises a nucleotide sequence selected from the group consisting of:

- i) a nucleic acid molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 1, 2 or 3;
- ii) a nucleic acid molecule molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 4 or 33;
- iii) a nucleic acid molecule molecule comprising a nucleotide sequence as set forth in SEQ ID NO: 5;

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- iv) a nucleic acid molecule comprising a nucleic acid sequence as set forth in SEQ ID NO: 6, 7 or 8;
- v) a nucleic acid molecule the complementary strand of which hybridizes under stringent hybridization conditions to the nucleotide sequence set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33 and which encodes a polypeptide that has reduced or abrogated expression or activity of a cytochrome P450-oxidoreductase catalysing the conversion of (*S*)-reticuline to (*R*)-reticuline compared to a wild-type *Papaver* plant comprising a gene encoding a functional cytochrome P450-oxidoreductase.

57. A method to identify a *Papaver* plant comprising a modified cytochrome P450-oxidoreductase gene comprising the steps:

- i) obtaining a sample of plant material from a plant to be tested;
- ii) extracting genomic nucleic acid from said plant material; and
- iii) analysing the extracted genomic nucleic acid encoding a variant nucleotide sequence of the nucleotide sequences set forth in SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33; and optionally,
- iv) comparing the sequence to a nucleotide sequence comprising SEQ ID NO: 1, 2, 3, 4, 5, 6, 7, 8 or 33.

58. A method according to claim 57 wherein said analysis comprises the steps:

- i) preparing a reaction mixture comprising:
 - a) genomic DNA to be analysed,
 - b) a first allele specific forward oligonucleotide primer comprising a nucleotide sequence complementary to said cytochrome P450-oxidoreductase gene nucleotide sequence and a nucleotide sequence complementary to a nucleotide sequence of a first fluorescent reporter cassette,
 - c) a second allele specific forward oligonucleotide primer comprising a nucleotide sequence complementary to said cytochrome P450-oxidoreductase gene an a nucleotide sequence complementary to a nucleotide sequence of a second fluorescent reporter cassette wherein the nucleotide at the 3'-end of said second oligonucleotide primer sequence is complimentary to a polymorphic nucleotide sequence variant of said cytochrome P450-oxidoreductase gene, and wherein the nucleotide sequence of said first and second oligonucleotide primers are complementary to the same upstream nucleotide sequence of said cytochrome P450-oxidoreductase gene;

d) a reverse oligonucleotide primer comprising a nucleotide sequence complementary to a nucleotide sequence downstream of said cytochrome P450-oxidoreductase gene nucleotide sequence; and

e) two reporter cassettes comprising double stranded nucleic acid
5 comprising:

a first double stranded nucleic acid wherein one strand comprises a fluorescent label and is complementary to said first allele specific primer and a second strand comprising a quencher label that quenches said fluorescent label; and

a second double stranded nucleic acid wherein one strand
10 comprises a fluorescent label different from the fluorescent label of i) and is complementary to said second allele specific primer and a second strand comprising a quencher label that quenches said second fluorescent label;

ii) heating said reaction mixture to denature genomic nucleic acid and said double stranded reporters and providing conditions to anneal primers to their respective
15 complementary nucleotide sequence, primer extending said annealed oligonucleotide primers and polymerase chain amplification of said mixture; and

iii) detecting fluorescence emission.

59. A method according to claims 58 wherein said modified and wild type cytochrome
20 P450-oxidoreductase gene nucleic acid sequence is as set forth SEQ ID NO: 6.

60. A method according to claims 58 or 59 wherein said first (forward) primer
comprise (SEQ ID NO: 35) CCAGGCAATCATCAAAGAATCAATGT, said second
(forward) primer comprises (SEQ ID NO: 34) CCAGGCAATCATCAAAGAATCAATGC
25 and said reverse primer comprises (SEQ ID NO 36)CACGGGGCTGGCTGGATACAA.

61. The method or use according to any one of claims 58 to 60 wherein said use or
method includes analysis of the sequence or expression of a further gene selected from
the group consisting of:

30 i) a nucleotide sequence as represented by the sequence in SEQ ID
NO: 17, 18, 19 20, 21, 22, 23, 24, 25 or 26;

ii) a nucleotide sequence wherein said sequence is degenerate as a
result of the genetic code to the nucleotide sequence defined in (i);

35 iii) a nucleic acid molecule the complementary strand of which
hybridizes under stringent hybridization conditions to the sequence
in SEQ ID NO: 17, 18, 19 20, 21, 22, 23, 24, 25 or 26 wherein said
nucleic acid molecule encodes polypeptides involved in the

biosynthesis of *Papaver* noscapine or intermediates in the biosynthesis of noscapine.

62. A method for the extraction of noscapine from a modified plant or capsule or
5 straw according to the invention comprising the steps:

- i) harvesting a modified plant, capsule or straw according any
one of claims 1 to 39 and forming a reaction mixture of
particulate plant material;
- 10 ii) solvent extracting said reaction mixture to provide an
alkaloid enriched fraction; and
- iii) concentrating said enriched alkaloid fraction to provide an
enriched noscapine fraction.

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Figure 1

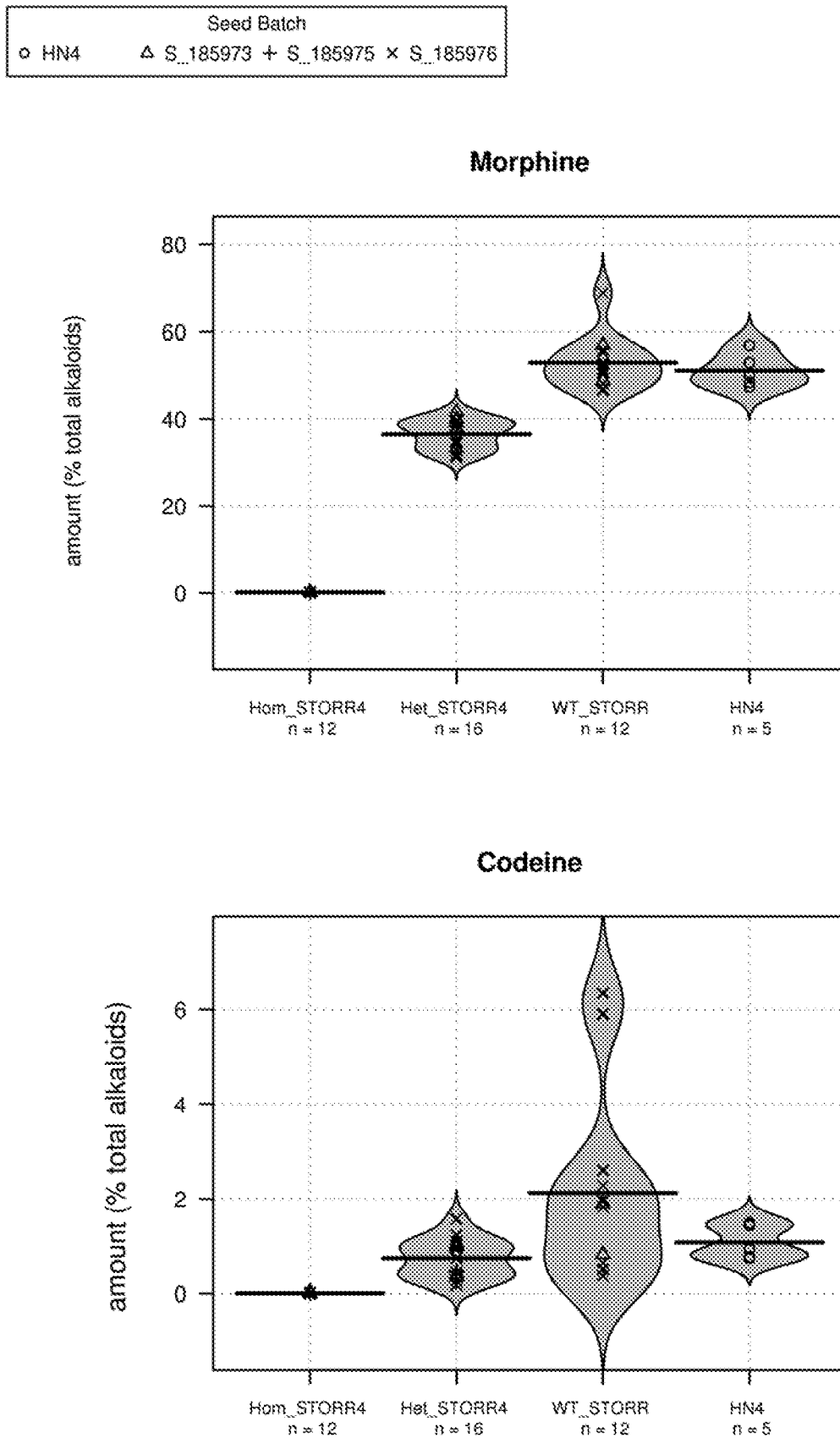


Figure 1 continued

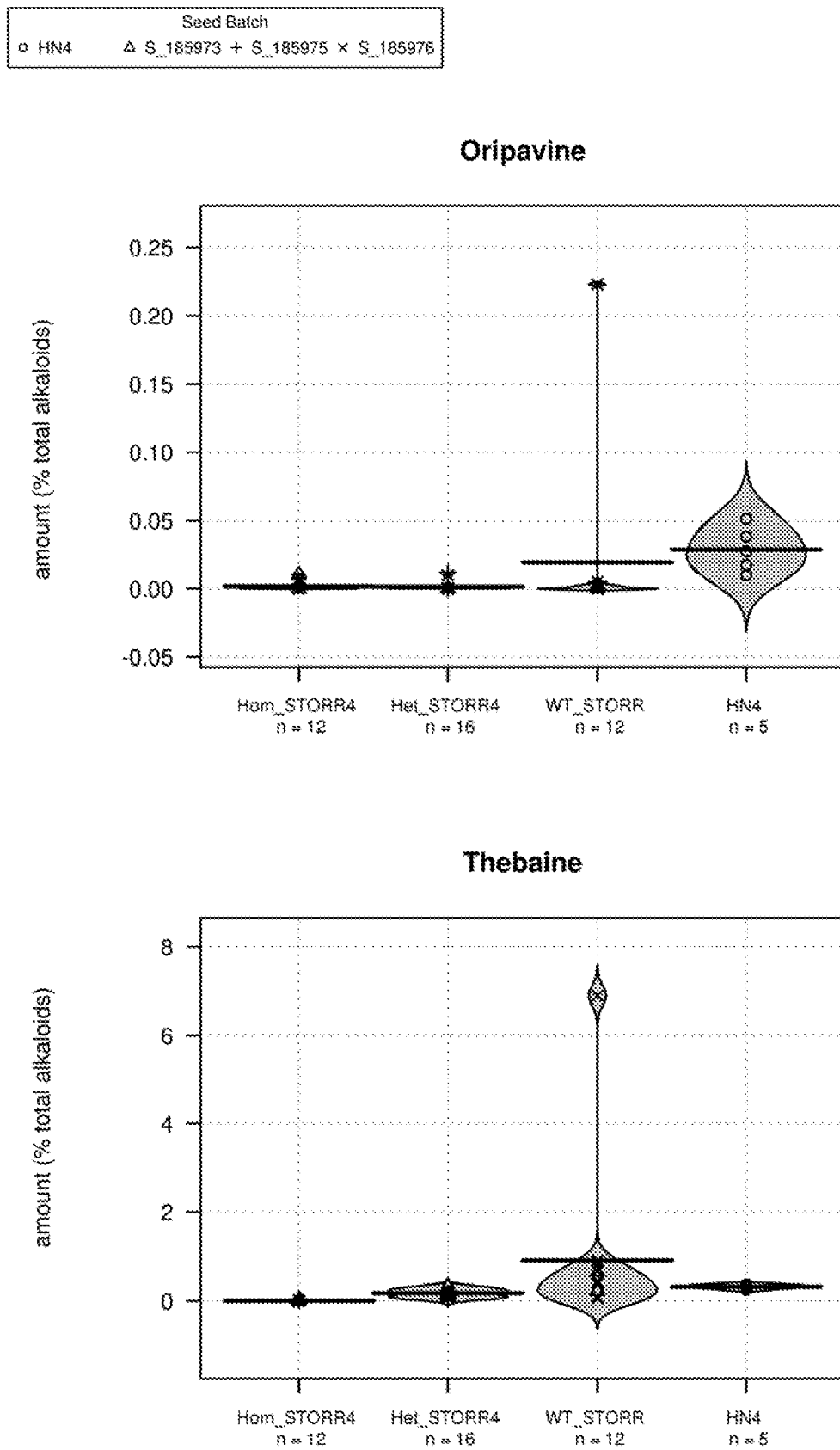


Figure 1 continued

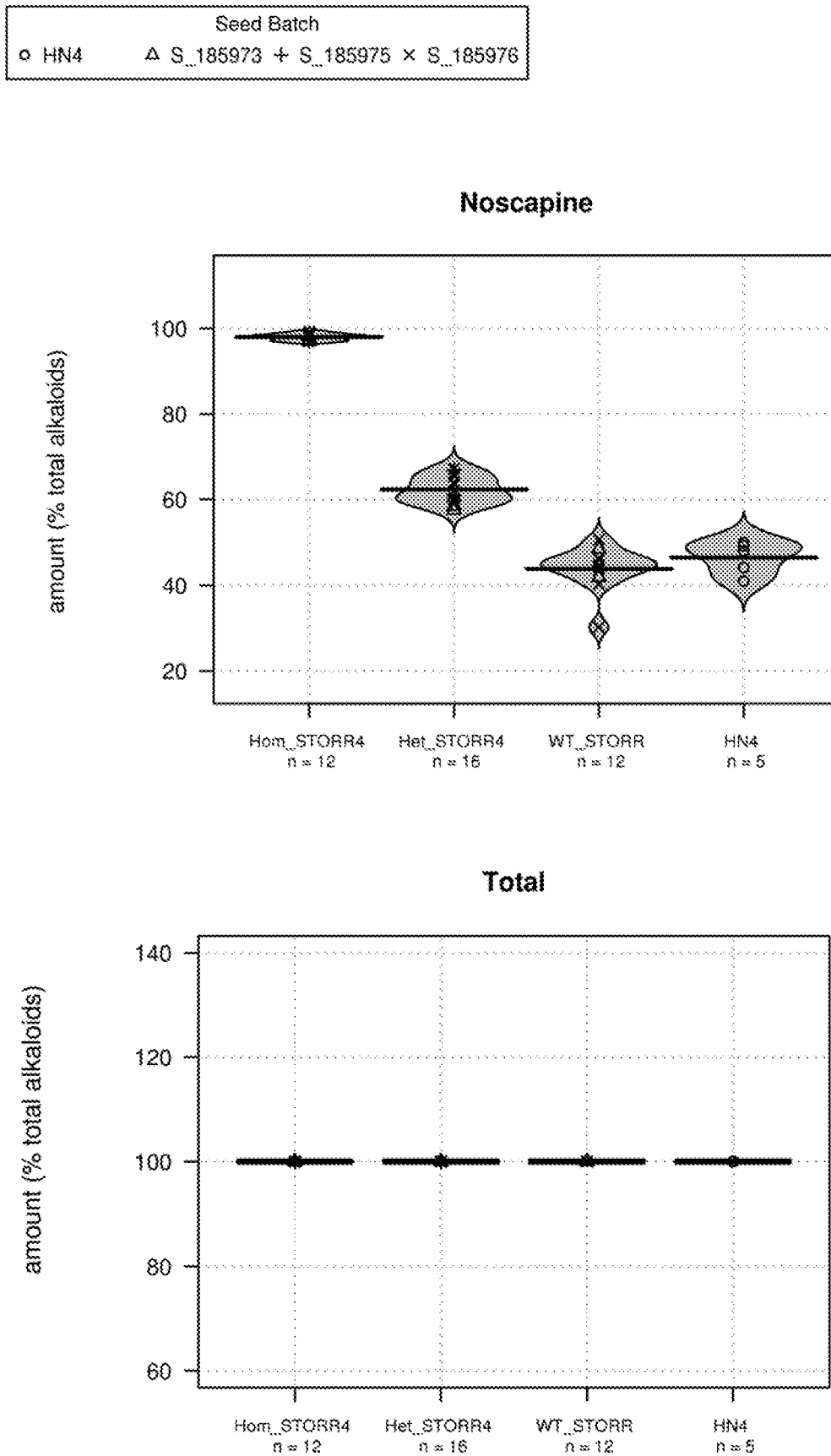


Figure 2

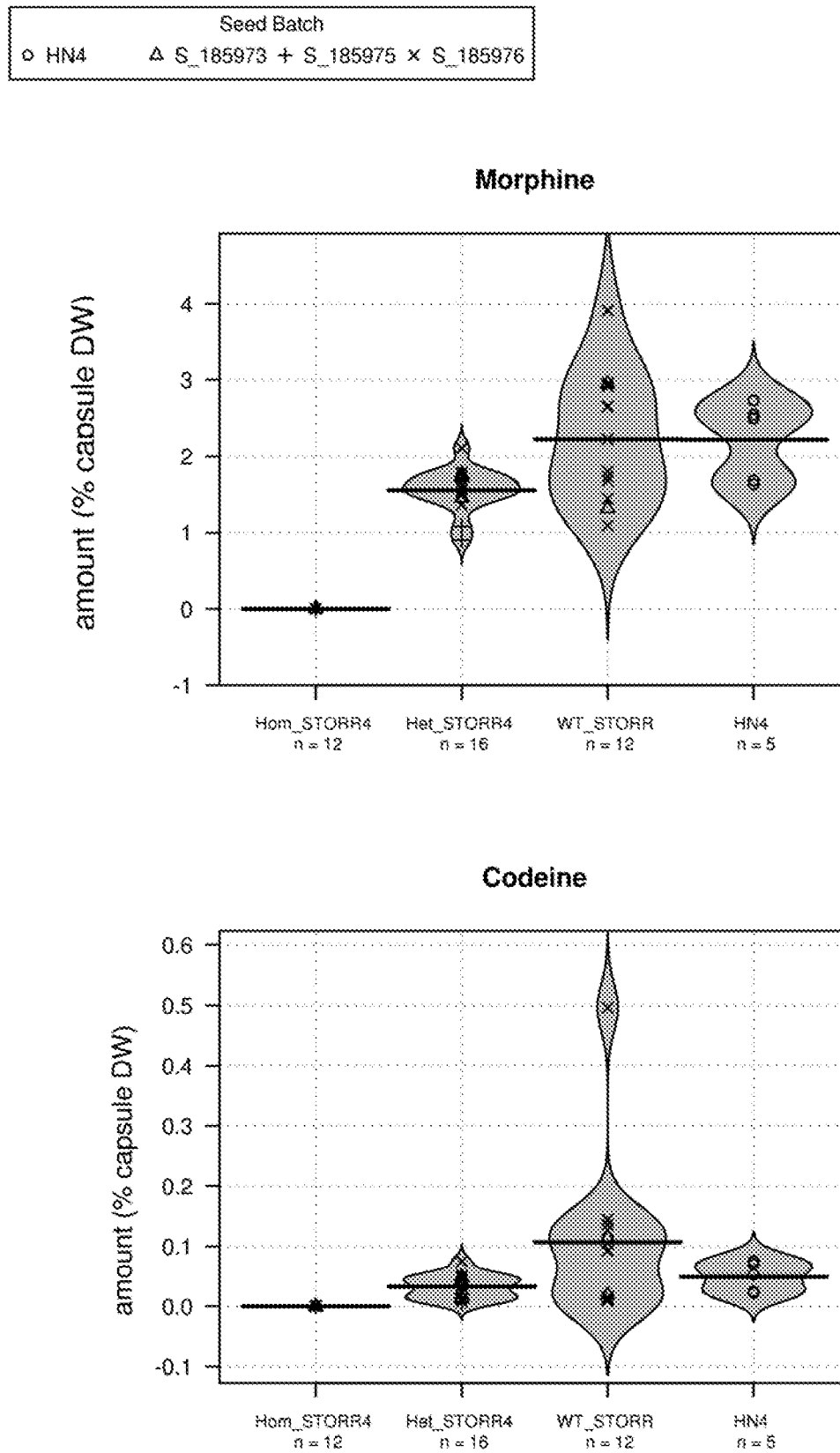


Figure 2 continued

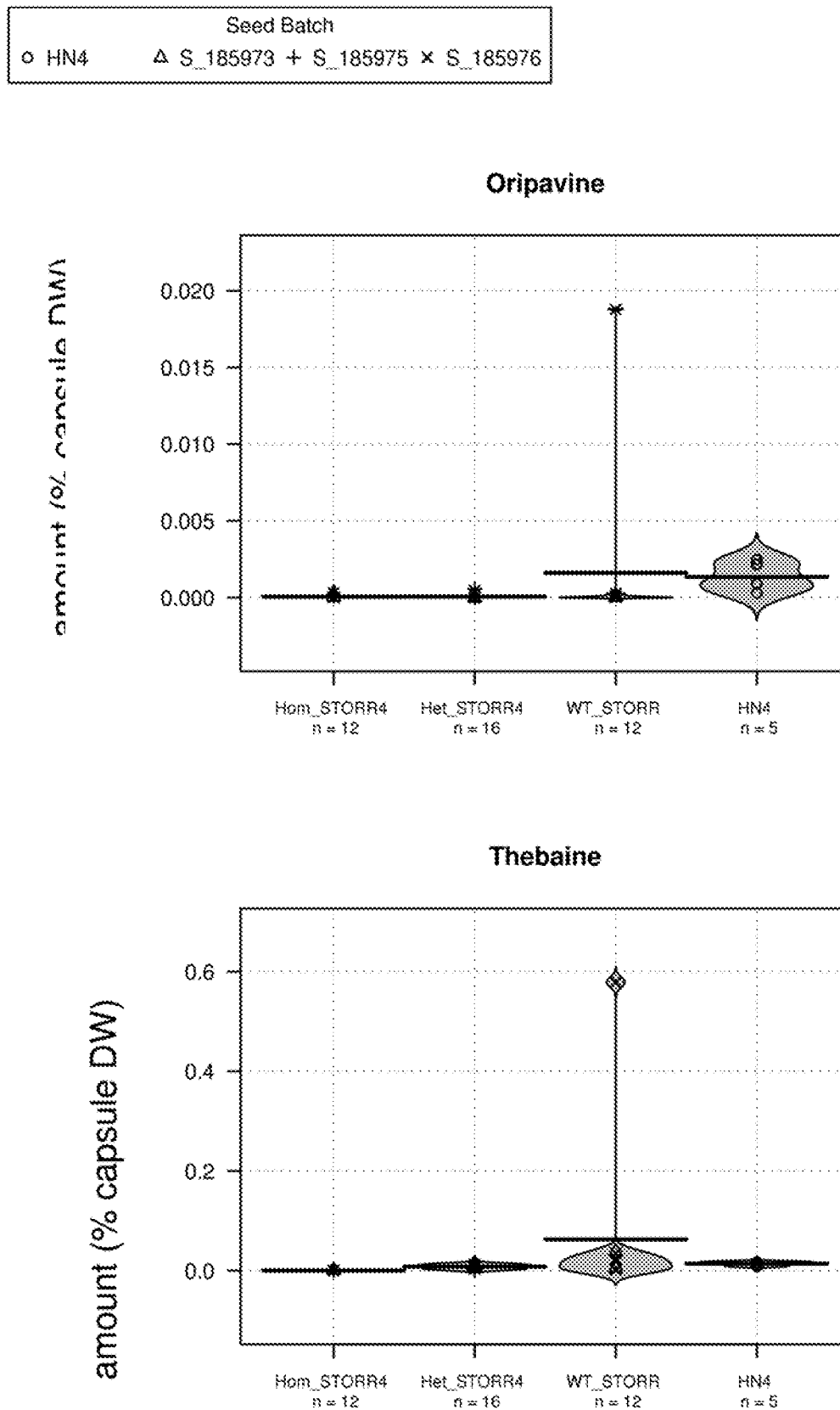


Figure 2 continued

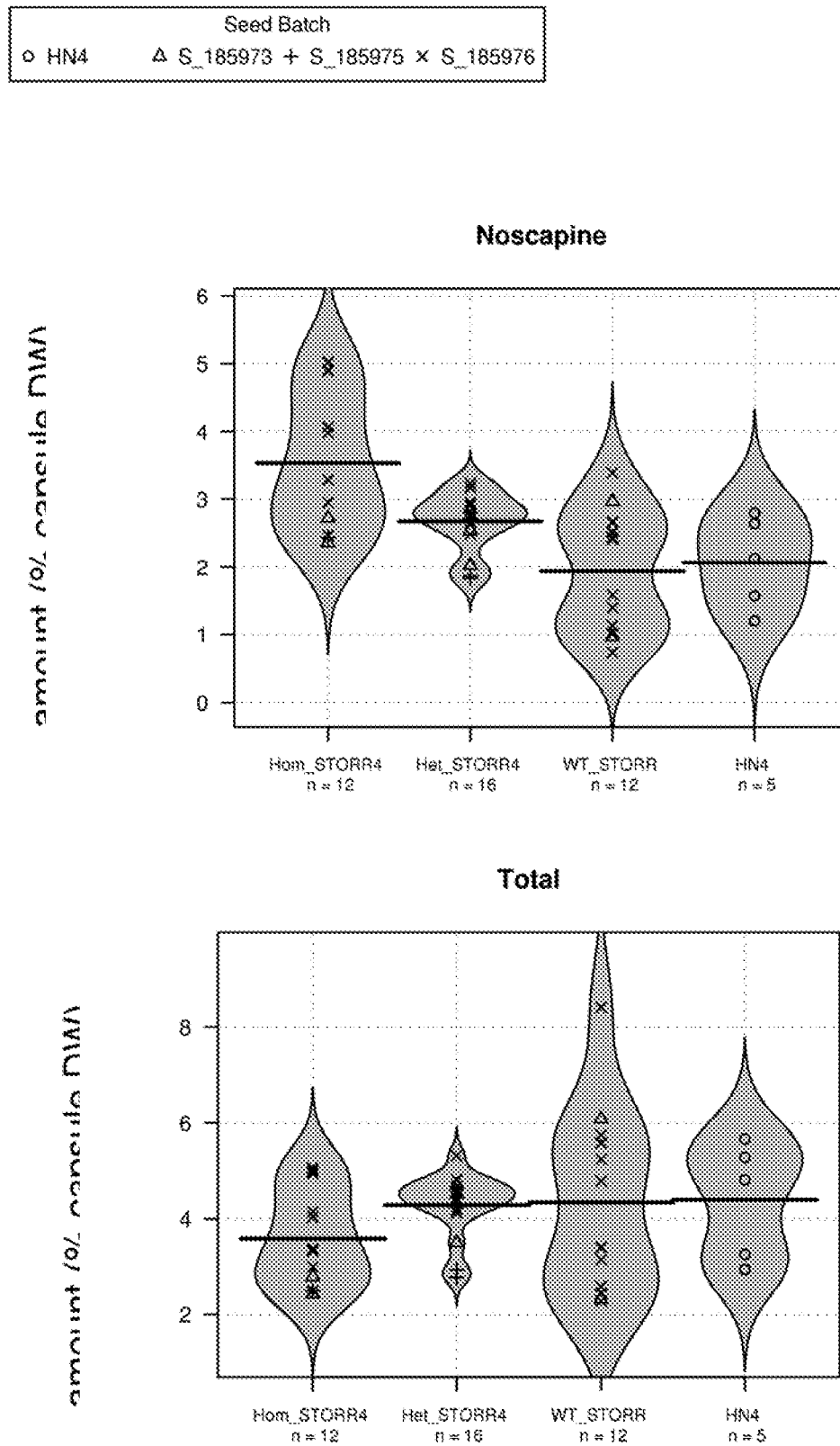
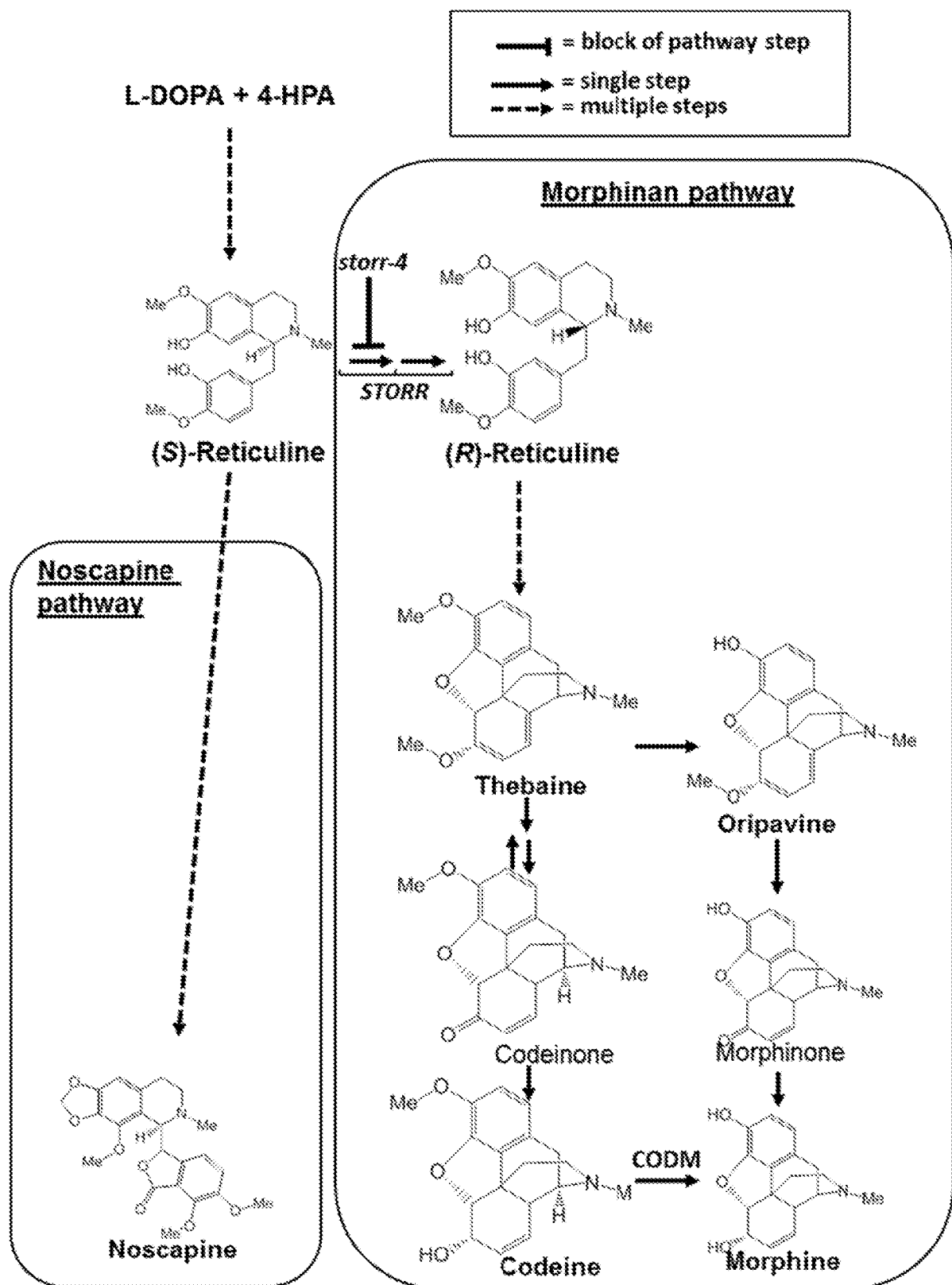


Figure 3



INTERNATIONAL SEARCH REPORT

International application No

PCT/GB2016/051887

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/02 C12N15/82

ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

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

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

EPO-Internal, WPI Data, BIOSIS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P	WO 2015/173590 A1 (UNIVERSITY OF YORK [GB]; SUN PHARMACEUTICAL IND AUSTRALIA PTY LTD) 19 November 2015 (2015-11-19) cited in the application claims 1, 22, 36, 63, 69; figures 1,3; table 3; sequences 21, 24, 27 -----	1-62
X,P	T. WINZER ET AL: "Morphinan biosynthesis in opium poppy requires a P450-oxidoreductase fusion protein", SCIENCE, vol. 349, no. 6245, 17 July 2015 (2015-07-17), pages 309-312, XP055298478, US ISSN: 0036-8075, DOI: 10.1126/science.aab1852 page 309, column 2 - column 3; figure 1 ----- -/--	1-62



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

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

"&" document member of the same patent family

Date of the actual completion of the international search

31 August 2016

Date of mailing of the international search report

12/09/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
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Fax: (+31-70) 340-3016

Authorized officer

Krüger, Julia

INTERNATIONAL SEARCH REPORT

International application No
PCT/GB2016/051887

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2015/081437 A1 (EPIMERON INC [CA]) 11 June 2015 (2015-06-11) cited in the application claims 15,20,41; figures 1-3; examples 1, 5; sequences 322, 323, 332, 337 -----	1-62
X	WO 2015/021561 A1 (EPIMERON INC [CA]) 19 February 2015 (2015-02-19) sequence 89 -----	46-48
A	FUMIHIKO SATO: "Improved Production of Plant Isoquinoline Alkaloids by Metabolic Engineering", ADVANCES IN BOTANICAL RESEARCH, LONDON, GB, vol. 68, 1 January 2013 (2013-01-01), pages 163-181, XP009191456, ISSN: 0065-2296 figure 6.2 -----	1-62

INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB2016/051887

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: **49-53 (partially)**
because they relate to subject matter not required to be searched by this Authority, namely:
Rule 39.1(ii) PCT - Essentially biological process for the production of plants
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/GB2016/051887

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 2015173590	A1	19-11-2015	NONE

WO 2015081437	A1	11-06-2015	AU 2014360597 A1 09-06-2016
			CA 2932448 A1 11-06-2015
			KR 20160093042 A 05-08-2016
			WO 2015081437 A1 11-06-2015

WO 2015021561	A1	19-02-2015	AU 2014306345 A1 24-03-2016
			CA 2921468 A1 19-02-2015
			EP 3033347 A1 22-06-2016
			US 2016201101 A1 14-07-2016
			WO 2015021561 A1 19-02-2015
