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(54) Title: COMPOSITIONS AND METHODS FOR ALTERING BIOSYNTHESIS OF TAXANES AND TAXANE-RELATED COMPOUNDS

(57) Abstract: Isolated nucleic acid and amino acid sequences for phenylalanine aminomutase enzyme and methods for purifying this enzyme are provided. Methods for altering biosynthesis of compounds in plant cell cultures are also provided. In particular, methods for altering production of taxanes and taxane-related compounds are provided.
COMPOSITIONS AND METHODS FOR ALTERING BIOSYNTHESIS OF
TAXANES AND TAXANE-RELATED COMPOUNDS

Field of the Invention

This application claims a benefit of priority from U.S. Application Serial No. 60/355,144 filed February 8, 2002, the entire disclosure of which is herein incorporated by reference.

The present invention provides isolated nucleic acid and deduced amino acid sequences for phenylalanine aminomutase enzyme as well as methods for purification of this enzyme. Also provided are methods of using the phenylalanine aminomutase gene of this enzyme to alter biosynthesis of compounds in plant cell cultures. For example, transferring the phenylalanine aminomutase gene to Taxus plant cells alters biosynthesis of taxanes and taxane-related compounds by these cells. The invention also relates to methods for altering the production of taxanes and taxane-related compounds in vivo by supplying to the cell β-phenylalanine alone or in combination with benzoic acid or a salt thereof in the production medium.

Background of the Invention

Paclitaxel is a useful therapeutic agent for various cancers including, but not limited to, ovarian cancer, breast cancer, and lung cancer. This taxane-type diterpene was first identified after isolation from Taxus brevifolia NUTT, a plant belonging to the genus Taxus, family Taxaceae. While paclitaxel can be found in all parts of the plant body, the bark has been found to have the highest concentration. At present, paclitaxel is collected from a natural or cultivated plant body. However, plants belonging to the genus Taxus grow very slowly, taking more than 10 years to grow to a height of 20 cm above the ground. Further, stripping of the bark generally results in tree death. Thus, isolating from natural sources the large amounts of paclitaxel needed for therapeutic uses is extremely difficult, if not impossible.

Attempts have been made to chemically synthesize paclitaxel. However, paclitaxel is a large, structurally complex molecule and large scale synthesis from simple, available chemicals is currently not a viable commercial alternative.
Semi-synthetic production via chemical attachment of a side chain to the agriculturally produced paclitaxel precursor, 10-deacetyl baccatin, has also been proposed (U.S. Patent 4,924,011; U.S. Patent 5,015,744). 10-Deacetyl baccatin is obtained from the needles of trees of the *Taxus* species. However, obtaining this precursor is by no means trivial and 10-deacetyl baccatin content of the needles may not be as high as initially reported. Thus, semi-synthetic production is expensive and also unlikely to be able to supply the necessary amount of paclitaxel required for its chemotherapeutic applications.

The most promising means for producing sufficient amounts of paclitaxel necessary for therapeutic applications is through plant cell cultures.

A variety of methods for producing paclitaxel and other taxanes or taxane-related compounds via cultured plant cells have been described.

U.S. Patent 5,019,504 describes a method for producing paclitaxel and derivatives thereof via cultured cells of *Taxus brevifolia*. However, the yield from this method ranges between 1 to 3 mg/L and is insufficient for industrial applications.

U.S. Patent 5,015,744 describes a method for production of baccatin III via plant cell culture, which can then be used in semi-synthetic production of paclitaxel.

U.S. Patent 5,407,816 and WO 93/17121 describe a method for producing paclitaxel and paclitaxel-like compounds from *Taxus* cells inoculated on a nutrient medium. In this method, paclitaxel is produced in an amount of at least 10-fold greater than that produced by native *Taxus* cells. Using this method, *Taxus chinensis* cells produced a yield of 153 mg/L of paclitaxel and taxanes. However, the requirements of the nutrient media are quite complicated and growth conditions are quite limited, thus rendering industrial applicability of this method also questionable.

Methods for increasing paclitaxel production via addition of stimulators to plant cell culture medium have also been proposed. For example, U.S. Patent 5,637,484 describes a method for increasing paclitaxel production by addition of jasmonate and Ag-containing compounds to the culture medium. Addition of methyl jasmonate to increase production of paclitaxel in *Taxus* cell cultures was also disclosed by Yukimune et al. (Nature Biotechnology 1996 14:1129-1132) and Mirjalili and Linden (Biotechnol. Prog. 1996 12:110-118). WO 97/44476 describes methods for producing paclitaxel, baccatin III and other paclitaxel-like compounds in
high yield from *Taxus* cells cultured with enhancement agents such as silver ion or complex, jasmonic acid, auxin-related growth regulators, and inhibitors of the phenylpropanoid pathway such as 3,4-methylenedioxy-6-nitrocinnamic acid. U.S. Patent 5,871,979 describes a method for producing paclitaxel in high yields from semi-continuous cultures of *Taxus* genus plant wherein the plant cells are inoculated on a medium containing sugar. U.S. Patent 6,248,572 also describes methods for producing paclitaxel in large amounts by culturing *Taxus* genus plant cells in a culture medium containing sugar alone or sugar in combination with AgNO₃. EP 0 727 492 A2 describes a method for producing taxane-type diterpenes in a plant cell or tissue by culturing the cell or tissue in the presence of a coronatine, a bacterium which produces a coronatine, a culture solution or a culture extract of such bacterium, cyclic polysaccharides, fatty acids or an imino or amino derivative of jasmonic acid. Furmanowa et al. (Biotechnology Letters 2000 22:1449-1452) describe methods for increasing paclitaxel production in *Taxus cuspidate* cell culture via addition of vanadyl sulfate, phenylalanine, or chitosan and methods for increasing baccatin III production in *Taxus media* cell culture via addition of aminobenzoic acid. Additionally, studies performed to reveal compounds that may enhance paclitaxel production *in vivo* suggest that β-phenylalanine may increase paclitaxel production; however, in the production study only a mixture of α and β-phenylalanine is fed. β-phenylalanine alone was not used (WO 97/44476).

**Summary of the Invention**

An object of the present invention is to provide methods for purifying phenylalanine aminomutase from plant cells. In a preferred embodiment, the phenylalanine aminomutase is purified from plant cells of a *Taxus sp*.

Another object of the present invention is to provide purified phenylalanine aminomutase.

Another object of the present invention is to provide isolated nucleic acid sequences encoding phenylalanine aminomutase enzyme.

Another object of the present invention is to provide vectors comprising a nucleic acid encoding a phenylalanine aminomutase enzyme and host cells comprising these vectors which express a phenylalanine aminomutase enzyme.
Another object of the present invention is to provide methods for altering biosynthesis of compounds by plant cell cultures via genetic transformation of phenylalanine aminomutase genes. In one embodiment of the present invention, phenylalanine aminomutase gene is genetically transformed into plant cells, preferably Taxus plant cells, to alter biosynthesis of taxanes and taxane-related compounds. In this embodiment, a vector comprising a nucleic acid sequence encoding phenylalanine aminomutase may be transformed into the cells so that expression of phenylalanine aminomutase is increased.

Another object of the present invention is to provide methods for altering levels of taxanes and taxane-related compounds in plant cell cultures which comprise culturing the plant cells with β-phenylalanine (3-amino-3-phenylpropionic acid). In a preferred embodiment, this method further comprises adding benzoic acid or a salt thereof to the plant cell cultures.

Another object of the present invention is to provide methods for altering the ratio of paclitaxel to baccatin III in plant cell cultures, which comprises culturing the plant cells with β-phenylalanine and/or benzoic acid or a salt thereof.

Yet another object of the present invention is to provide methods of using the purified phenylalanine aminomutase and/or isolated nucleic acid sequences encoding phenylalanine aminomutase enzyme to identify phenylalanine aminomutase genes in other species, to identify mutations in phenylalanine genes which alter the biocatalytic properties of the protein encoded by these genes, and to isolate genomic sequences adjacent to the phenylalanine aminomutase gene in a plant cell.

**Brief Description of the Figures**

Figure 1 provides a proposed biosynthetic scheme for paclitaxel. The chemical names of the numbered structures are as follows: 1, Geranylgeranyl diphosphate; 2, Taxadiene; 3, Taxa-4(20),11-diene-2-α-9,10β-13α-tetrahydroxy-5α-acetate; 4, Benzoyl CoA; 5, 9-10β-13α-Trihydroxy-5α-acetate-2α-benzoate-taxa-4(20),11 diene; 6, α-Phenylalanine; 7, β-Phenylalanine; 8, Phenylisoserine; 9, Baccatin III; 10, benzoic acid; 11, Debenzoyl Paclitaxel; 12, Paclitaxel.

Figure 2 provides a nucleic acid sequence (SEQ ID NO:1) encoding phenylalanine aminomutase which begins at nucleotide 9 and ends at nucleotide 2069.
Figure 3 provides the deduced amino acid sequence (SEQ ID NO:2) of phenylalanine aminomutase.

Figure 4 provides a nucleic acid sequence of a phenylalanine aminomutase (PAM) genomic polymerase chain reaction (PCR) product (SEQ ID NO:3) from *T. chinensis*. The starting methionine codon (ATG) begins at nucleotide 63, the stop codon (TAG) begins at nucleotide 2293 and the entire fragment is 2411 nucleotides in length. The genomic fragment contains an intron that begins at nucleotide 1154 and ends at nucleotide 1322.

Figure 5 provides a nucleic acid sequence of a phenylalanine aminomutase genomic PCR product (SEQ ID NO:4) from *T. media*. The starting methionine codon (ATG) begins at nucleotide 1, the stop codon (TAG) begins at nucleotide 2231 and the entire fragment is 2233 nucleotides in length. The genomic fragment contains an intron that begins at nucleotide 1091 and ends at nucleotide 1260.

Figure 6 provides a nucleic acid sequence of a phenylalanine aminomutase genomic PCR product (SEQ ID NO:5) from *T. canadensis*. The starting methionine codon (ATG) begins at nucleotide 1, the stop codon (TAG) begins at nucleotide 2231 and the entire fragment is 2235 nucleotides in length. The genomic fragment contains an intron that begins at nucleotide 1091 and ends at nucleotide 1260.

Figure 7 is a bargraph showing the effects of DL-β-phenylalanine (DL-3-amino-3-phenylpropionic acid) on paclitaxel and baccatin III production in batch cultivation of *T. chinensis* cells. Each value represents the average of four samples. Figure 7A shows production of paclitaxel in the presence of 0 (open bar), 1 mM (grayed bar), and 5 mM (filled bar) of DL-β-phenylalanine in plant cell cultures after 1, 2, 3, 4, and 5 weeks. Figure 7B shows production of baccatin III in the presence of 0 (open bar), 1 mM (grayed bar), and 5 mM (filled bar) of DL-β-phenylalanine in plant cell cultures after 1, 2, 3, 4, and 5 weeks.

Figure 8 is a bargraph showing the effects of DL-β-phenylalanine (DL-3-amino-3-phenylpropionic acid) and benzoic acid on paclitaxel and baccatin III production in fed-batch cultivation of *T. chinensis* cells. Each value represents the average of six samples. Figure 8A shows production of paclitaxel in control cells (open bar), cells exposed to DL-β-phenylalanine (grayed bar), and cells exposed to
DL-β-phenylalanine and benzoic acid (filled bar) after 2, 3, 4, and 5 weeks. Figure 8B shows production of baccatin III in control cells (open bar), cells exposed to β-phenylalanine (grayed bar), and cells exposed to DL-β-phenylalanine and benzoic acid (filled bar) after 2, 3, 4, and 5 weeks.

Figure 9 provides a comparison of the deduced amino acid sequence of phenylalanine aminomutase cDNA and phenylalanine ammonia-lyase from *Pinus taeda*. Identical amino acid residues are highlighted.

**Detailed Description of the Invention**

Paclitaxel is a highly complex diterpene alkaloid consisting of a C13 side chain derived from phenylalanine and a highly modified diterpene (baccatin III). Biochemical evidence derived from feeding studies of various potential intermediates to the C13 side chain suggests the following biosynthetic scheme (also see Figure 1). The first committed step is the conversion of α-phenylalanine to β-phenylalanine (3-amino-3-phenylpropionic acid). β-phenylalanine is then hydroxylated to form phenylisoserine, which is transferred to baccatin III at the C13 hydroxyl group (Fleming et al. J. Am. Chem. Soc. 1994 116: 4137-4138). The resulting debenzoylpaclitaxel is benzyolated to form paclitaxel (Fleming et al. J. Am. Chem. Soc. 1994 116: 4137-4138). β-phenylalanine also has been shown to be the source of the benzoic acid moiety transferred to the C13 side chain as the final enzymatic step in the synthesis of paclitaxel (Fleming et al. J. Am. Chem. Soc. 1994 116: 4137-4138).

The baccatin III biosynthetic pathway begins with the ubiquitous primary metabolite geranylgeranyl diphosphate and, following 13 to 15 enzymatic steps, ends with the transacylation of 10-deacetyl baccatin III to baccatin III (Fleming et al. J. Am. Chem. Soc. 1994 116: 4137-4138). As shown in Figure 1, baccatin III is benzyolated at the C2 position, thus indicating that the biosynthesis of baccatin III requires a source of benzoic acid. Feeding studies using benzoic acid and β-phenylalanine indicate benzoic acid is derived from β-phenylalanine to be incorporated into the paclitaxel C13 side chain, thus suggesting that two pools of benzoic acid exist within the *Taxus* cell, one for the C13 side chain derived from β-phenylalanine and another for baccatin III derived from the traditional phenylalanine to cinammic acid pathway.
(Bjorklund and Leete, Phytochemistry 1992 31:3883). The presence of two distinct pools of benzoic acid are indicative of spatial separation between the baccatin III biosynthetic pathway and the C13 side chain/paclitaxel biosynthetic pathway.

The conversion of α-phenylalanine to β-phenylalanine by phenylalanine aminomutase being the first committed step in paclitaxel biosynthesis and β-phenylalanine being identified as the first intermediate in the side chain biosynthesis indicates that manipulation of either β-phenylalanine or phenylalanine aminomutase will have a direct impact on paclitaxel biosynthesis. The present invention enables the manipulation of taxane compositions and levels by providing a method to purify phenylalanine aminomutase to homogeneity, by isolating the phenylalanine aminomutase cDNA, and by demonstrating the addition of β-phenylalanine and/or benzoic acid to alter paclitaxel yield and ratios of paclitaxel to baccatin III. Thus, the present invention provides compositions and methods for altering the biosynthesis of taxanes such as paclitaxel and taxane-related compounds such as baccatin III and baccatin VI. In addition, the compositions of the present invention can be used in accordance with well known techniques to identify phenylalanine aminomutase genes in other species. Mutants in the phenylalanine aminomutase gene affording enzymes whose biocatalytic properties have been altered can also be generated using well known techniques. Also, common techniques using the phenylalanine aminomutase gene sequence can be employed to isolate genomic sequences adjacent to the gene, which contain the cis-acting elements that regulate gene expression.

By “phenylalanine aminomutase”, it is meant an enzyme capable of catalyzing the transfer of an amino group from a 2-position of L-α-phenylalanine to form 3-amino-3-phenylpropionic acid (β-phenylalanine).

In one aspect of the present invention, a method of purifying phenylalanine aminomutase from plant cell cultures to homogeneity is provided. Also provided is the purified phenylalanine aminomutase enzyme.

By “pure” or “purified” as used herein, it is meant that the phenylalanine aminomutase is equal to, or more preferably greater than, 95% homogeneous as determined by SDS-PAGE.

In this purification method, crude extracts from plant cells are first prepared. In a preferred embodiment, the crude extracts are prepared via suspension of frozen
plant cells in potassium phosphate buffer containing dithiothreitol (DTT) extracts are treated to remove phenolic compounds preferably via addit X resin (Sigma Aldrich Corp., St. Louis, MO) and polyvinylpyrrolidone (PVP) resin (Sigma Aldrich Corp., St. Louis, MO). The extracts are then stirred at 4°C for 1 hour followed by centrifugation, preferably at 10,000 x g for 2 hours, to remove and phenolic compounds. The supernatant is then concentrated, preferably approximately 50 ml, and the concentrated fraction is dialyzed by adding at 200 ml of the potassium phosphate DTT containing buffer. The dialyzed fraction is concentrated by ultrafiltration to remove endogenous β-phenylalanine. All final volume of the crude extracts after the above procedure is about 40 ml contains 100-150 mg of protein.

Protein is then precipitated from the crude extract preferably via two separate aliquots of solid ammonium sulfate. Ammonia sulfate fraction gives excellent yield and reasonable purification. In this procedure, a freshly solid ammonium sulfate is added to 30% saturation while stirring. The solution is centrifuged, preferably at 10,000 x g for 60 minutes, and the pellet is dissolved. Additional solid ammonium sulfate is then added to 50% saturation while stirring. The solution is again centrifuged at 10,000 x g for 60 minutes to obtain the The phenylalanine aminomutase enzyme is then purified to homogeneity with additional steps resulting in more than 150-fold purification.

In the first of these additional steps, the pellet from the second ammonium sulfate precipitation is dissolved in Tris-HCl buffer (pH 7.4) at 0.1 mM DTT and 1 mM MgCl₂. The solution is then applied to an affinity chromatography column such as Reactive Green 19 (Sigma Aldrich Cc. 1 MO) chromatography column (2 x 10 cm) comprising a dye attached to equilibrated with the same buffer from which the flow-through and an ml of the buffer wash are collected. This affinity chromatography step utilizes another closely related enzyme, phenylalanine ammonia-lyase. Phenylalanine ammonia-lyase utilizes the same substrate and has a similar molecular structure but presence of Mg₂⁺ is also essential to this step because phenylalanine ammonia-lyase only binds to the column in the presence of Mg₂⁺.
Next, the flow-through and wash fractions are combined and dialyzed by ultrafiltration to change the buffer to a Tris-HCl buffer at pH 9.0 containing 1 mM DTT and 1 mM MgCl₂ in a final volume of 7-8 ml.

This final volume of 7-8 ml is then applied onto a second affinity chromatography column, preferably a 10 ml (2 x 10 cm) column containing Heparin-Agarose Type I (Sigma Aldrich Corp., St. Louis, MO), equilibrated with the Tris-HCl buffer at pH 9.0 containing 1 mM DTT and 1 mM MgCl₂. The column is first washed with approximately 30 ml of the buffer. The enzyme is then eluted with approximately 20 ml of the buffer containing 0.1 M NaCl. The eluant can then be further concentrated. This final step gives a relatively low yield due mainly to the weak binding between phenylalanine aminomutase and Heparin-agarose. Typically binding will not occur if the buffer pH is below 9.0. Accordingly, it is preferred that the buffer has a pH of at least 9.0.

The results of a typical purification of phenylalanine aminomutase from plant cell cultures are summarized in Table 1.

**Table 1. Purification of Phenylalanine Aminomutase (PAM)**

<table>
<thead>
<tr>
<th>Step</th>
<th>Total Protein (mg)</th>
<th>Total activity (µg/h)</th>
<th>Specific activity (µg/h/mg)</th>
<th>Yield (%)</th>
<th>Purification (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>123.61</td>
<td>178.0</td>
<td>1.44</td>
<td>100</td>
<td>1</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>39.60</td>
<td>161.5</td>
<td>4.08</td>
<td>90.8</td>
<td>2.8</td>
</tr>
<tr>
<td>Reactive Green 19</td>
<td>2.46</td>
<td>158.8</td>
<td>64.54</td>
<td>89.2</td>
<td>44.8</td>
</tr>
<tr>
<td>Heparin-agarose</td>
<td>0.065</td>
<td>14.8</td>
<td>227.33</td>
<td>8.3</td>
<td>157.9</td>
</tr>
</tbody>
</table>

SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) was performed to separate the proteins from each step and to demonstrate that the phenylalanine aminomutase protein was purified to homogeneity.

Characteristics of the purified phenylalanine aminomutase were then determined. The denatured molecular weight of phenylalanine aminomutase was estimated to be 80kDa by SDS-PAGE, and the native molecular weight was determined to be 162kDa. These results indicate that phenylalanine aminomutase is a homodimeric protein. Unlike lysine aminomutase, activity of purified phenylalanine
aminomutase was not dependent upon any cofactors. Neither pyridoxyl-5-phosphate (PLP) nor S-adenosyl-L-methionine (SAM) affected the enzyme activity and the enzyme was fully functional without any cofactors. The study of substrate specificity indicated L-α-phenylalanine to be the only substrate for this enzyme. Neither D-α-phenylalanine nor any other amino acids examined were substrates for the purified phenylalanine aminomutase.

Kinetic constants for the purified phenylalanine aminomutase were also measured by standard first order kinetic model. The Km value for L-α-phenylalanine was 1.1 mM while Vmax value was 303.1 μg/minute/mg protein. Purified phenylalanine aminomutase was relatively stable at both 4°C and room temperature.

The pH optimum of phenylalanine aminomutase was determined with sodium acetate, potassium phosphate and Tris-HCl at different pHs. The optimum pH for phenylalanine aminomutase was found to occur between 7.5 and 8.0 with either 50 mM potassium phosphate or 50 mM Tris-HCl buffer.

Sequencing of the purified phenylaminomutase was also performed. More specifically, N-terminal sequencing was performed on the whole protein as well as reverse HPLC-purified tryptic fragments using an Enhanced Hewlett-Packard G1005A amino acid sequencer (Hewlett-Packard, Palo Alto, CA). Each sample was analyzed for 15-20 cycles and the cycles with determinable amino acid residues are reported below in Table 2.

Uppercase letters represent highest confidence, lowercase represent reasonable confidence, lowercase letters in parentheses represent lower confidence assignments.

Table 2: Amino acid sequence of PAM peptides

<table>
<thead>
<tr>
<th>HPLC Fractions</th>
<th>Amino Acid Sequence</th>
<th>Peptide Name</th>
<th>Location in PAM</th>
<th>SEQ ID NO:</th>
</tr>
</thead>
<tbody>
<tr>
<td>106-108:</td>
<td>LLNSNVSp(m)(m)</td>
<td>PAM4</td>
<td>158</td>
<td>6</td>
</tr>
<tr>
<td>112-114a:</td>
<td>EYYSIDK</td>
<td>PAM5</td>
<td>307</td>
<td>7</td>
</tr>
<tr>
<td>112-114b:</td>
<td>LAEFEK</td>
<td>PAM6</td>
<td>581</td>
<td>8</td>
</tr>
<tr>
<td>119-122:</td>
<td>LsDRLENEMTAVR</td>
<td>PAM1</td>
<td>588</td>
<td>9</td>
</tr>
<tr>
<td>127-129a:</td>
<td>tCAs(s)VDELPatatr</td>
<td>PAM7</td>
<td>117</td>
<td>10</td>
</tr>
<tr>
<td>127-129b:</td>
<td>(g)atr</td>
<td>PAM8</td>
<td>Not found</td>
<td>11</td>
</tr>
<tr>
<td>151-154:</td>
<td>VGLR</td>
<td>PAM2</td>
<td>216</td>
<td>12</td>
</tr>
<tr>
<td>158-161:</td>
<td>LNtFTYGcXGir</td>
<td>PAM3</td>
<td>137</td>
<td>13</td>
</tr>
<tr>
<td>N-terminal</td>
<td>(f)FAVEAR(s)(h)V</td>
<td></td>
<td></td>
<td>14</td>
</tr>
</tbody>
</table>
In another aspect of the present invention, isolated nucleic acid sequences encoding phenylalanine aminomutase and polypeptides encoded thereby are provided. The cDNA encoding phenylalanine aminomutase is depicted in Figure 2, SEQ ID NO:1. The starting methionine codon (ATG) begins at nucleotide 9, the stop codon (TAG) begins at nucleotide 2136 and the entire cDNA is 2277 nucleotides. The deduced amino acid sequence for a phenylalanine aminomutase polypeptide encoded by this nucleic acid sequence is depicted in Figure 3, SEQ ID NO:2. The appearance of eight of the nine peptide sequences from the purified phenylalanine aminomutase (shown in Table 2) in the deduced amino acid sequence of phenylalanine aminomutase confirms that the correct cDNA was isolated.

Searches performed using the Basic Logic Alignment Search Tool (BLAST®, NCBI, Bethesda, MD; Altschul et al. J. Mol. Biol. 215(3):4-3-410 (1990)) indicate phenylalanine aminomutase to be related to phenylalanine ammonia-lyases. For example, a BLAST® search showed that the amino acid sequence of phenylalanine ammonia-lyase deduced from the cDNA isolated from *Pinus taeda* (Genbank Accession number AAA84889) is 43% identical and 68% similar to the phenylalanine aminomutase cDNA. Figure 9 provides a comparison of these sequences that reveals at least four highly conserved amino acid regions believed to be functionally significant as well as useful in designing degenerate nucleotide PCR primers for isolating phenylalanine aminomutase from other plant species. The four regions are located at numbers 79-87, 423-428, 453-460 and 477-482 of the phenylalanine aminomutase amino acid sequence shown in Figure 9.

Nucleic acid sequences of the genomic PCR fragment encoding phenylalanine aminomutase from three different Taxus species, namely *T. chinensis*, *T. media* and *T. canadensis*, are depicted in Figure 4 (SEQ ID NO:3), Figure 5 (SEQ ID NO:4) and Figure 6 (SEQ ID NO:5), respectively. In the nucleic acid sequence of SEQ ID NO:3, the starting methionine codon (ATG) begins at nucleotide 63, the stop codon (TAG) begins at nucleotide 2293 and the entire fragment is 2411 nucleotides in length. The genomic fragment contains an intron that begins at nucleotide 1154 and ends at nucleotide 1312. In the nucleic acid sequence of SEQ ID NO:4, the starting methionine codon (ATG) begins at nucleotide 1, the stop codon (TAG) begins at nucleotide 2231 and the entire fragment is 2233 nucleotides in length. The genomic
fragment contains an intron that begins at nucleotide 1091 and ends at nucleotide 1260. In the nucleic acid sequence of SEQ ID NO:5, the starting methionine codon (ATG) begins at nucleotide 1, the stop codon (TAG) begins at nucleotide 2231 and the entire fragment is 2235 nucleotides in length. The genomic fragment contains an intron that begins at nucleotide 1091 and ends at nucleotide 1260.

The isolation and functional expression of a phenylalanine aminomutase cDNA was accomplished using a reverse genetics approach. Degenerate primers as depicted in Table 3 were designed from four of the nine peptides sequences generated from the purified phenylalanine aminomutase protein (see Table 2).

Table 3: Degenerate Primers to selected phenylalanine aminomutase peptides

<table>
<thead>
<tr>
<th>Peptide Name</th>
<th>Amino Acids ID</th>
<th>SEQ ID NO:</th>
<th>Primer Sequence</th>
<th>SEQ NO:</th>
</tr>
</thead>
<tbody>
<tr>
<td>PAM1</td>
<td>LENEMT</td>
<td>15</td>
<td>YTN GAR AAY GAR ATG AC</td>
<td>16</td>
</tr>
<tr>
<td>PAM1.2</td>
<td>LENEMTAV</td>
<td>17</td>
<td>YTN GAR AAY GAR ATG ACI GCI GT</td>
<td>18</td>
</tr>
<tr>
<td>PAM3</td>
<td>LNSFTYG</td>
<td>19</td>
<td>YTI AAY WBI TTI CAN TAY GG</td>
<td>20</td>
</tr>
<tr>
<td>PAM3.1</td>
<td>LNSFTYG</td>
<td>21</td>
<td>YTI AAY WBI TTY CAN TAY GG</td>
<td>22</td>
</tr>
<tr>
<td>PAM4</td>
<td>LLNSNV</td>
<td>23</td>
<td>YTI YTI AAY WBN AAY GT</td>
<td>24</td>
</tr>
<tr>
<td>PAM4.1</td>
<td>LLNSNV</td>
<td>25</td>
<td>YTI YTI AAY WBI AAY GTN CC</td>
<td>26</td>
</tr>
<tr>
<td>PAM5</td>
<td>EYYSID</td>
<td>27</td>
<td>GAM TAY TAY WBN AYH GA</td>
<td>28</td>
</tr>
<tr>
<td>PAM5.1</td>
<td>EYYSIDK</td>
<td>7</td>
<td>GAM TAY TAY WBN ATH GAY AA</td>
<td>29</td>
</tr>
</tbody>
</table>

Following the redesign of the degenerate primers based on four of the eight peptide sequences, two DNA fragments (approximately 800 and 600 bps in length) were synthesized in a 3’ RACE PCR reaction. The reaction consisted of the induced vector cDNA library as a template, the degenerate primer PAM 1.2 (designed to the internal peptide, LSDRLENEMTAVR (SEQ ID NO:10) and the M13F primer (5’ TGACCGGCAGCAAATG3’ (SEQ ID NO:30) as the anchor. Both fragments were sequenced and shown to be 100% identical through to the poly A addition site of the 600 bp fragment with the 800 bp fragment extending 200 bp further on the 3’ end, thus suggesting that the fragments are examples of the same gene using different poly(A) addition sites.

To clone the 5’ end of the cDNA, a RACE (Rapid Amplification of cDNA Ends) library was synthesized using a MARATHON™ cDNA amplification kit (Clontech, Palo Alto, CA). The library was constructed using mRNA isolated from T.
*chinensis* cells grown in production medium for 6 days. A 5' RACE, gene specific primer (5' TGCATCGAACACTTCTTGACGTCCTCTCT 3'; SEQ ID NO:31) was designed to the 600 bp PAM 3' RACE fragment. The resulting PCR amplicon was 2.2 kb in length and was subcloned into PCR-Script™ (Stratagene, La Jolla, CA).

The full length cDNA was isolated with a 5' specific primer pam93mut,
TCCTTGCTCTCATATTATGGGTTTGC (SEQ ID NO:32), that created a NdeI restriction site at the starting ATG and a 3' specific primer pam2350mutc,
GGGATCTTTTTAAAAACAAAAATAATTAGGGTT (SEQ ID NO:33) that created a BamHI site and included 227 nucleotide of the 3' untranslated region. The resulting full length phenylalanine aminomutase cDNA was subcloned into PCR-Script™ (Stratagene, La Jolla, CA) and sequenced. This sequence is depicted in Figure 2. A NdeI BamHI restriction digest phenylalanine aminomutase DNA fragment was subcloned into the pBMS2000 *E. coli* protein expression vector (described in U.S. Patent 6,068,991, the teachings of which are herein incorporated by reference in their entirety) and then transformed into *E. coli* strain Epicurian coli® XL1-Blue (Stratagene, La Jolla, CA). Following expression and enzymatic assay, the functional enzyme synthesized an average of 588 ng β-phenylalanine per mg of *E. coli* protein. This functional expression demonstrates conclusively that the cDNA to phenylalanine aminomutase has been amplified and cloned.

The genomic DNA as depicted in Figure 4, SEQ ID NO:3, was generated using PCR containing gene specific primers PAM5F2,
TCAGTTTTATCTCGCTCAAGT (SEQ ID NO:34), and PAM3R,
TACAGTCGCTCTGCGGAAT (SEQ ID NO:35), designed to the 5' and 3' untranslated regions of the phenylalanine aminomutase cDNA and genomic DNA isolated from *Taxus chinensis* cell line P97-1 cell cultures. The genomic PCR product was sequenced directly from the reaction. Therefore the sequence is considered a consensus phenylalanine aminomutase sequence since the product contains a small percentage of sequence heterogeneity caused by slight differences found between even allelic genes. The cloned cDNA and the genomic PCR product are 99.8% identical on the nucleotide level and 99.7% identical on the amino acid level (see Table 4).
The genomic PCR products from two other *Taxus* species, *T. media* and *T. canadensis* were synthesized and sequenced in a similar manner. In order to ensure that the PCR reactions would generate specific products, the 5' primer pam63, ATGGGGTTTGCCGTTGGAATCG (SEQ ID NO:36), and the 3' primer pam2137c, CTAGACGCCGTTGCGCA (SEQ ID NO:37), were designed to start and end with the beginning and ending of the coding region of phenylalanine aminomutase. The *T. media* sequence is depicted in Figure 5, SEQ ID NO:4, and the *T. canadensis* sequence is depicted in Figure 6, SEQ ID NO:5. Both sequences contain an intron beginning at nucleotide 1092 and ending at nucleotide 1260.

All three phenylalanine aminomutase sequences are highly conserved. The *T. chinensis* is 98.6% identical to *T. media* and 98.7% identical to *T. canadensis* on the nucleotide level. The differences that result in changes in amino acids are presented in Table 4.

**Table 4: Amino Acid Differences Identified in Phenylalanine Aminomutase**

<table>
<thead>
<tr>
<th>cDNA and genomic PCR fragments</th>
<th>Sequences</th>
<th>Location</th>
<th>Sequences</th>
<th>Location</th>
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<tr>
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<td></td>
<td>I II III IV</td>
<td></td>
</tr>
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<td>473</td>
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<td>C C C S</td>
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<tr>
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<tr>
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<td>V V L L</td>
<td>239</td>
<td>E E D D</td>
<td>644</td>
</tr>
</tbody>
</table>

I= PAM *T. chinensis* cDNA  
II= PAM *T. chinensis* genomic  
III= PAM *T. media* genomic  
IV= PAM *T. canadensis* genomic

Due to degeneracy of the genetic code, nucleic acid sequences of the present invention may also comprise different nucleic acid sequences to those depicted in Figures 2, 4 and 5 encoding the same phenylalanine aminomutase enzyme. Nucleic acid sequences of the present invention are also inclusive of polynucleotides which hybridize under stringent conditions to the above-described nucleic acid sequences. As used herein, the term "stringent conditions" means at least 60%, more preferably at
least 80%, homology at hybridization conditions of 60°C at 2xSSC buffer. More preferred are isolated nucleic acid molecules capable of hybridizing to the nucleic acid sequence set forth in Figure 2, 4 or 5, or to the complementary sequence of the nucleic acid sequence set forth in Figure 2, 4 or 5, under hybridization conditions of 3X SSC at 65°C for 16 hours, and which are capable of remaining hybridized to the nucleic acid sequence set forth in Figure 2, 4 or 5, or to the complementary sequence of the nucleic acid sequence set forth in Figure 2, 4 or 5, under wash conditions of 0.5X SSC, 55°C for 30 minutes. Nucleic acid sequences of the present invention may also comprise fragments, and derivative or variant nucleic acid sequences encoding derivatives, variants or active fragments of this enzyme. The term “variant” is inclusive of naturally occurring variants such as allelic variants, as well as mutants prepared by well-known mutagenesis techniques. With respect to variant or derivative nucleic acid sequences, differences are generally limited so that the polypeptide encoded by the nucleic acid sequence exhibits similar activity to the phenylalanine aminomutase enzyme. By “similar activity” for purposes of the present invention, it is meant that the variant enzyme is still capable of catalyzing the transfer of an amino group from the 2-position of L-α-phenylalanine to form 3-amino-3-phenylpropionic acid. Thus, changes in the nucleic acid sequences of a variant or derivative of the present invention may be silent. That is, they may not alter the amino acids encoded by the nucleic acid sequences. Alternatively, changes in the nucleic acid sequence may alter the amino acid sequence as compared to that of the phenylalanine aminomutase enzyme. Such changes may comprise amino acid substitutions, additions, deletions, fusions and truncations as compared to the amino acid sequence of the phenylalanine aminomutase enzyme. By “fragment” it is meant a nucleic acid sequence or polypeptide encoded thereby which comprises less nucleotides or amino acids than depicted in SEQ ID NO: 1, 3, 4 or 5, or 2, respectively. Preferred fragments, variants and derivatives of the present invention, when referring to the nucleic acid sequences, are those that encode polypeptides that retain essentially the same biological function as the phenylalanine aminomutase enzyme. Preferred fragments, variants and derivatives of the present invention, when referring to polypeptides, are those, which retain essentially the same biological function as the phenylalanine aminomutase enzyme. Such preferred fragments can
be identified via assays measuring enzymatic catalyzation of the transfer of an amino
group from a 2-position of L-α-phenylalanine to form 3-amino-3-phenylpropionic
acid.

In addition to being useful in the production of phenylalanine aminomutase
enzymes, the nucleic acid sequences of the present invention are also useful in
isolation of genomic sequences adjacent to the phenylalanine aminomutase enzyme,
as hybridization probes to isolate phenylalanine aminomutase genes or genes similar
thereto in other species, and in the identification of mutations that alter the
biocatalytic properties of enzymes encoded thereby.

For example, regulatory genomic sequences adjacent to a phenylalanine
aminomutase gene can be identified using well known techniques to identify an
isolated nucleic acid sequence of the present invention in a gene and to isolate a
genomic sequence adjacent thereto. These sequences contain the cis-acting elements
that regulate gene expression. Thus, isolation of such sequences may be useful in
identifying modulators of expression of the phenylalanine aminomutase gene.

Nucleic acid sequences of the present invention, particularly fragments thereof
can also serve as hybridization probes useful in the identification and isolation of
phenylalanine aminomutase genes or genes similar thereto in other species.
Production and use of such probes is performed routinely in accordance with
 teachings provided herein and in accordance with well known techniques.

The nucleic acid sequences of the present invention can also be used to
identify mutant phenylalanine aminomutase genes encoding proteins with biocatalytic
properties altered as compared to a wild-type phenylalanine aminomutase enzyme. In
this aspect of the present invention, an isolated nucleic acid sequence encoding a wild
type phenylalanine aminomutase such as the nucleic acid sequence depicted in Figure
2, 4 or 5 is mutated in accordance with well known, standard techniques. A
polypeptide encoded by the mutant isolated nucleic acid sequence is then expressed
and one or more biocatalytic properties of the expressed polypeptide are assessed.
Examples of biocatalytic properties of the polypeptide which can be determined
include, but are not limited to substrate specificity, Km, turnover rate, pH optimum,
temperature optimum and solvent environment. One or more biocatalytic properties
of the expressed polypeptide are then compared with the same biocatalytic property or
properties of a wild-type phenylalanine aminomutase to identify any mutations to the nucleic acid sequences resulting in polypeptides with biocatalytic activities different from wild-type phenylalanine aminomutase.

Various examples of plants cells are known wherein increased gene expression to increase a biosynthetic enzyme level results in an overall increase in a particular secondary metabolite. For example, alfalfa plants respond to wounding by synthesizing large amounts of an isoflavonoid phytoalexin. Since this biosynthetic pathway requires 8 to 12 enzymes, it is similar to paclitaxel whose pathway requires 12 to 14 enzymes. By increasing expression of methyltransferase, significant increases in phytoalexin were demonstrated (He, X.Z. and Dixon, R.A. The Plant Cell 2000 12(9):1689-1702). In addition, an increase in yield of monoterpenes occurs in mint when a reductoisomerase from the biosynthetic pathway is genetically overexpressed (Mahmoud, S.S. and Croteau, R.B. Proc. Natl Acad. Sci. USA 2001 98:8915-8920). Interestingly, Mahmoud and Croteau also show that the composition of the monoterpenes may be altered when a gene is genetically manipulated so that underexpression occurs. Overexpression of phenylalanine aminomutase is expected to increase paclitaxel titer and increase the paclitaxel to baccatin III ratio, a desired outcome in downstream processing, in similar fashion. In contrast, a significant decrease in phenylalanine aminomutase expression is expected to reduce paclitaxel levels and increase baccatins including, but not limited to, baccatin III and baccatin VI.

In embodiments wherein a nucleic acid sequence encoding the phenylalanine aminomutase enzyme is added to the plant cells, it is preferred that this be performed via genetic transformation with a vector comprising the nucleic acid sequence. The enzyme can be overexpressed in host cells transformed with a vector comprising a nucleic acid sequence of the present invention. For example, Example 4 of the instant application sets forth a method for transforming E. coli with a vector comprising a nucleic acid sequence of the present invention and expressing phenylalanine aminomutase in this host cell. However, the substrate for phenylalanine aminomutase, L-α-phenylalanine, is ubiquitous in all living creatures. Accordingly, the phenylalanine aminomutase gene may be expressed in almost any yeast, bacterial or mammalian host cell.
Thus, additional aspects of the present invention relate to vectors comprising a nucleic acid sequence encoding phenylalanine aminomutase as well as host cells comprising these vectors and expressing phenylalanine aminomutase.

Another aspect of the present invention relates to methods for altering production of taxanes and taxane-related compounds by biosynthesis, which comprises supplementing production medium with β-phenylalanine and/or benzoic acid or a salt thereof. Further, supplementing production medium with β-phenylalanine and/or benzoic acid or a salt thereof increases the ratio of taxanes produced as compared to taxane-related compounds.

Enhanced productivity of paclitaxel was observed in batch cultivation of *T. chinensis* cells upon addition of 1 mM DL-β-phenylalanine. Further, in only the second week of cultivation, baccatin III titer in the control showed a marked difference from the 5 mM DL-β-phenylalanine batch. Results from these experiments are depicted in Figure 7. In the presence of 5 mM DL-β-phenylalanine, a 40% increase of paclitaxel (see Figure 7A) and an 80% reduction of baccatin III were observed at the end of the second week (see Figure 7B). However, the increase in paclitaxel production decreased in subsequent weeks and was no longer significantly increased as compared to the control at the end of the fifth week. These results indicate that 5 mM DL-β-phenylalanine enables rapid conversion of baccatin III to paclitaxel in a short period. Cells cultured in medium supplemented with 1 mM DL-β-phenylalanine consistently showed enhancement of paclitaxel production and produced 26% more paclitaxel than the controls (192 units/L vs. control 152 units/L) at the end of the fifth week.

Enhanced paclitaxel production was also observed in fed-batch cultivation *T. chinensis* cells with feeding of DL-β-phenylalanine and benzoic acid. Results from these experiments are depicted in Figure 8. In the presence of 2.2 mM DL-β-phenylalanine, the cells produced 29% more paclitaxel (171 units/L vs. control 132 units/L) than the control at the end of the fifth week (see Figure 8A). Feeding with a mixture of DL-β-phenylalanine and benzoic acid further enhanced paclitaxel productivity during fed-batch cultivation to 43% (189 units/L vs. control 132 units/L) at the end of the fifth week (see Figure 8A). While a decrease in baccatin III titers was not observed in any of the fed-batch cultivations, the ratio of paclitaxel to
baccatin III of cells cultivated in medium supplemented with DL-β-phenylalanine alone (2.9) or in combination with benzoic acid (3.6) was higher than controls (2.2) (see Figure 8B).

These results indicate that both β-phenylalanine and benzoic acid enhanced paclitaxel production in *T. chinensis* cell cultures. Accordingly, supplementing the production medium of plant cell cultures with β-phenylalanine alone or benzoic acid or a salt thereof alone, or more preferably a combination of β-phenylalanine and benzoic acid or a salt thereof, provides a useful means for enhancing production of taxanes such as paclitaxel.

The following nonlimiting examples are provided to further illustrate the present invention.

**EXAMPLES**

**Example 1**

*Phenylalanine aminomutase protein isolation and characterization*

*Enzyme Assay and HPLC Method*

The standard PAM activity assay mixture contains 5mM L-α-phenylalanine, 1 to 10 μg enzyme in 50 mM Tris-HCl buffer (pH 7.5) in a final volume of 0.5 ml. Enzyme reactions were carried out at 28°C, 150 rpm for 18 hours and terminated by addition of 50 μl ethanol to precipitate proteins. After centrifugation for 5 minutes, the supernatants were analyzed by a reverse phase HPLC method. The formation of β-phenylalanine was quantified based on peak area compared to the β-phenylalanine standard curve. Enzyme activity is expressed as μg β-phenylalanine formed per hour per mg protein. HPLC analysis used a YMC Ph column (4.6 x 150mm; Waters, Milford, MA), and a flow rate of 1.2 ml/minutes. Absorbance was monitored at 210 nm. The method employed two solvents A, water; and B, CH₃CN and a gradient consisting 0% to 12% B for 6 minutes, 12% to 90% in 1 minute and returning to 0% in 1 minute. Retention times of L-α-phenylalanine and β-phenylalanine are 4.3 minutes and 3.5 minutes, respectively.
**Protein Assay**

Protein concentrations were determined using a propositional protein binding dye method and microassay kit purchased from Bio-Rad Laboratories (Hercules, CA).

**Enzyme Purification**

To prepare the crude extracts, 100 grams (wet weight) of frozen plant cells were suspended in 500 ml of cold 50 mM potassium phosphate buffer (pH 7.5) containing 1 mM dithiothreitol (DTT). After addition of 50 grams of the nonionic polymeric adsorbent XAD-4 resin (Sigma Aldrich Corp., St. Louis, MO) and 50 g PVP, the extracts were stirred at 4°C for 2 hours, then centrifuged at 10,000 x g for 2 hours. The supernatant was concentrated to approximately 50 ml by an ultrafiltration concentrator with a PM-30 membrane (Amicon, Inc. Beverly, MA), and the concentrated fraction was further dialyzed by addition of at least 200 ml of the buffer and concentrated by ultrafiltration to remove endogenous β-phenylalanine. Typically, the final volume of the crude extracts after above procedures is about 40-50 ml containing 100-150 mg of protein.

Solid ammonium sulfate was added to the crude extracts to 30% saturation while stirring. The solution was centrifuged at 10,000 x g for 60 minutes and the pellet was discarded. Additional solid ammonium sulfate was added to 50% saturation while stirring. The solution was then centrifuged at 10,000 x g for 60 minutes to obtain the pellet.

The pellet from 50% ammonium sulfate precipitation was dissolved in 15 ml of 10 mM Tris-HCl buffer (pH 7.0) containing 1 mM DTT and 1 mM MgCl₂. The dissolved pellet was then applied onto a 10 ml Reactive Green 19 (Sigma Aldrich Corp., St. Louis, MO) chromatography column (2 x 10 cm) comprising this green dye attached to dextran equilibrated with 10 mM Tris-HCl buffer (pH 7.0) containing 1 mM DTT and 1 mM MgCl₂, and the flow-through and an additional 15 ml of the buffer wash were collected. The flow-through and wash fractions were combined and dialyzed by ultrafiltration to change the buffer to 10 mM Tris-HCl buffer (pH 9.0) containing 1 mM DTT and 1 mM MgCl₂ in final volume of 7 to 8 ml.

The active fraction was then applied onto a 10 ml Heparin-Agarose Type I (Sigma Aldrich Corp., St. Louis, MO) column (2 x 10 cm) equilibrated with 10 mM
Tris-HCl buffer (pH 9.0) containing 1 mM DTT and 1 mM MgCl₂. The column was washed with 30 ml of the buffer and the enzyme was eluted with 20 ml of the buffer containing 0.1 M NaCl. The eluant was then concentrated to small volume.

**SDS-PAGE**

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with the 10% ready gels from Bio-Rad (Hercules, CA). The gels were stained with Coomassie blue. Broad range protein standards from Bio-Rad (Hercules, CA) were used to estimate denatured molecular weight of the enzyme.

**Determination of Native Molecular Weight**

Size exclusion chromatography was performed using a 100 ml column of Sephacryl S-200 HR (Sigma Aldrich Corp., St. Louis, MO) gel filtration column (2.5 x 35 cm) equilibrated with 10 mM potassium phosphate buffer (pH 7.0) containing 1 mM DTT. Enzyme samples and 0.25 ml of molecular weight standards (670 kDa, 158 kDa, 44 kDa, 17 kDa and 1.35 kDa) from Bio-Rad (Hercules, CA) were applied onto the column. The column was eluted with the buffer at 1 ml/minute by collecting every 4 mls as a fraction monitored at 280 nm. The native molecular weight of the enzyme was calculated by comparing the elution volume to the standard curve.

**Other Determinations**

Purified enzyme preparations were used to determine the properties of the enzyme including cofactor requirements, substrate specificity, kinetic constants, thermostability and pH optima. For the determination of cofactor requirements, 1 mM pyrodoxal 5'-phosphate (PLP) or S-adenosyl-L-methionine (SAM) was included in the standard reaction mixtures and the formation of β-phenylalanine was analyzed by HPLC. To determine substrate specificity, a variety of amino acids in both L- and D- including, but not limited to lysine, tyrosine, tryptophan, and histidine, were used at 5 mM as substitutes for L-α-phenylalanine in the standard reaction mixtures. For the determination of kinetic constants, L-α-phenylalanine concentration in the reaction mixture was varied in the range of 0.156 mM to 15 mM for measuring enzyme activity. Thermostability and pH optima were conducted by incubating the
enzyme reaction mixtures at different temperatures or in different buffers with varying pHs.

**Phenylalanine Aminomutase Peptide Mapping**

Purified phenylalanine aminomutase protein was run on a SDS-PAGE gel, blotted onto PVDF (polyvinylidene difluoride) membrane (Bio-Rad, Hercules, CA), and the approximately 80 kDa band excised. All protein analysis effort was done in accordance with Hewlett-Packard (Palo Alto, CA) sequencing protocols (Miller C. G., Adsorptive Biphasic Column Technology for Protein Sequence Analysis and Protein Chemical Modification. Methods: A Companion to Methods in Enzymology (Academic Press), Vol. 6, No. 3, Sep 1994, pp. 315-333). The PVDF-bound sample (approximately 4 µg) and PVDF control sample were subjected to alkylation using a Hewlett-Packard Protein Chemistry station. The PVDF strips were treated at 50°C under an argon atmosphere with 10 mM dithiothreitol in 6 M guanidine at pH 8.3 followed by alkylation with 2 M acrylamide dissolved in the same solvent. Strips were then placed into a solution containing 100 ng of modified porcine trypsin (Promega, Madison, WI), 1% hydrogenated Triton X-100, 10% acetonitrile, 20 mM ammonium bicarbonate, and 5 mM ammonium carbonate (pH 8). Following a 4 hour incubation at 40°C, an additional 100 ng of trypsin was added to each mixture and the incubations continued for a total of 12 hours. The solutions were then loaded onto Perisorb RP-18 columns (EM Science, Hawthorne, NY), which were subsequently washed with 1 ml of 2% trifluoroacetic acid (TFA) prior to the HPLC analysis.

**Example 2**

**Isolation of the phenylalanine aminomutase cDNA**

**RNA Isolation**

RNA was extracted from *Taxus* cells grown in either growth media (controls) or production media (induced) for 6 days. Total RNA isolation was performed using a RNA isolation kit from Qiagen, Inc. (Valencia, CA) according to the manufacturer’s instructions with two modifications. The extraction buffer volume was increased two-fold to maintain the weight of tissue to buffer volume ratio suggested by the manufacturer. A 10% ethanol precipitation as taught by Levinsohn et al. (Plant Mol.
Bio. Rep. 1994 12(1):20-25) was incorporated to remove contaminating polysaccharides. Poly A RNA was isolated using latex beads from Qiagen Inc. (Valencia, CA) according the manufacturer’s instructions.

**Vector cDNA Library Construction, Phage DNA Isolation and RACE**

**Rapid Amplification of cDNA Ends** cDNA library construction

The vector cDNA was synthesized with 5 μg of induced mRNA using a cDNA synthesis kit from Stratagene (La Jolla, CA) following the manufacturer’s instructions. Bacteriophage DNA containing the Taxus cDNA was isolated from contaminating E. coli DNA using a protocol described by Ausubel et al. (Current protocols of molecular biology. Current protocols 1987 Vol. 1: 1.13.7). The RACE library was constructed with 1 μg of induced poly A RNA using the MARATHON™ cDNA amplification kit (Clontech, Palo Alto, CA) according to the manufacturer’s protocol.

**PCR protocols and DNA sequencing**

PCR reactions were performed using Taq DNA polymerase (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. PCR reaction using the RACE library was performed with the ADVANTAGE™ 2 PCR kit (Clontech, Palo Alto, CA) in accordance with the manufacturer’s instructions. DNA sequencing was performed using the ABI PRISM® Big Dye™ terminator cycle sequencing kit (Applied Biosystem, Foster City, CA) according to the manufacturer’s instructions.

**Example 3**

**Paclitaxel biosynthesis enhancement by supplementing production medium with DL-β-phenylalanine and benzoic acid**

Vegetative cells of *T. chinensis* cell cultures were inoculated into production medium at cell density of 200 grams/L. The following two cultivation procedures were used.

The first cultivation procedure was a batch cultivation using 1 or 5 mM DL-β-phenylalanine treatments in TAXOL production medium, which contains Medium N plus α-naphthaleneacetic acid, 3,4-(methylenedioxy)cinnamic acid, methyl jasmonate, and silver thiosulfate (WO97/44476). Production medium without the addition of
DL-β-phenylalanine served as a control. Samples were collected weekly for analysis via high pressure liquid chromatography (HPLC) for five weeks.

The second cultivation procedure was a fed-batch cultivation, which initially used a batch medium containing 1.2X strength Medium N lacking glutamine plus 3,4-(methylenedioxy)cinnamic acid and silver thiosulfate (WO97/44476). Methyl jasmonate, α-naphthalene acetic acid, glutamine, and DL-β-phenylalanine were then fed from day 3 to day 35. Feedings were performed every three to four-days with a feeding rate equivalent to 5 mL/L/day. The total amount of DL-β-phenylalanine fed was 2.2 mmol/L during the 5 week production period. The fed-batch cultivation using a feeding solution lacking DL-β-phenylalanine was used as control. A second fed-batch cultivation treatment using DL-β-phenylalanine and benzoic acid was also performed. The total amount of both DL-β-phenylalanine and benzoic acid fed to the broth was 2.2 mmol/L during the 5-week production period. Samples were taken for HPLC assay at the end of weeks 2, 3, 4, and 5.

Example 4
Expression of phenylalanine aminomutase in E.coli Expression

To express phenylalanine aminomutase in E. coli, the cDNA (SEQ ID NO:1) was subcloned into pBMS2000 protein expression vector and transformed into Epicurian coli® XL-1 blue cells (Stratagene, La Jolla, CA). Following confirmation of proper cloning via digestion with Nde I and BamH I, the plasmid was named pPAM2000 and the protein expression of the phenylalanine aminomutase cDNA was begun.

Cultures of XL1-Blue cells containing either pPAM2000 or pBMS2000 were grown overnight and used to inoculate fresh cultures of LB media and grown at 30°C. When the cultures reach an optical density of 0.5-1.0, they were induced to synthesize the phenylalanine aminomutase protein by the addition of 100 μM IPTG (isopropylthio-β-galactoside). The bacteria were collected by centrifugation at 5000 x g for 10 minutes, washed in assay buffer (50 mM Tris pH 7.5), and resuspended in assay buffer. The bacteria were lysed by sonication (2 x 20 pulses on ice). The enzyme was assayed by addition of L-α-phenylalanine. Samples were incubated at 18°C overnight and the reactions were terminated by addition of ethanol. The
samples were clarified by centrifugation at 16,000g for 10 minutes. The supernatant was transferred to HPLC vials and analyzed for L-β-phenylalanine production. The HPLC method used a μBONDApak™ C18 column (Millipore, Milford, MA) and a reverse phase water (A) to acetonitrile (B) gradient of 0 to 20% B in one minute, 20 to 30% B in 6 minutes and 30 to 90% B in one minute. L-α-phenylalanine eluted at 5.2 minutes and L-β-phenylalanine eluted at 4.9 minutes. In two experiments, the recombinant PAM produced 694 and 483 ng of β-phenylalanine/mg E. coli protein and the pBMS2000 containing E. coli extracts failed to synthesize any detectable amounts of β-phenylalanine.
What is Claimed is:

1. A method for purifying phenylalanine aminomutase from plant cells comprising:

(a) preparing a crude extract from the plant cells;
(b) precipitating proteins from the crude extract;
(c) separating phenylalanine aminomutase from other precipitated proteins via
   (i) subjecting a solution comprising the precipitated proteins and a buffer at pH 7.0 to affinity chromatography in the presence of Mg\(^{2+}\);
   (ii) dialyzing flow-through and wash fractions from step (i) by ultrafiltration to change the buffer to a buffer with a pH of at least 9.0; and
   (iii) applying the dialyzed and ultrafiltrated fraction obtained in step (ii) to affinity chromatography; and
(d) eluting purified phenylalanine aminomutase obtained via the affinity chromatography in step (iii) using a buffer containing 0.1M NaCl.

2. A purified phenylalanine aminomutase enzyme.

3. The purified phenylalanine aminomutase enzyme of claim 2 which is isolated from a *Taxus* species.

4. The purified phenylalanine aminomutase enzyme of claim 2 comprising SEQ ID NO:2.

5. An isolated nucleic acid sequence encoding a phenylalanine aminomutase enzyme.

6. The isolated nucleic acid sequence of claim 5 comprising SEQ ID NO:1, 3, 4, or 5 or a fragment or variant thereof.
7. The isolated nucleic acid sequence of claim 5 wherein the phenylalanine aminomutase enzyme comprises SEQ ID NO:2 or a fragment or variant thereof.

8. A vector comprising the nucleic acid sequence of claim 5.

9. A host cell expressing the vector of claim 8.

10. A method of isolating regulatory genomic sequences adjacent to a phenylalanine aminomutase gene comprising:
(a) identifying the isolated nucleic acid sequence of claim 5 in a gene; and
(b) isolating genomic sequences adjacent thereto.

11. A method of identifying a phenylalanine aminomutase gene in a species comprising contacting DNA or RNA from the species with a hybridization probe which hybridizes to the isolated nucleic acid sequence of claim 5.

12. A method of identifying a mutant phenylalanine aminomutase gene encoding a protein with biocatalytic properties altered as compared to a wild-type phenylalanine aminomutase comprising:
(a) mutating the isolated nucleic acid sequence of claim 5;
(b) expressing a polypeptide encoded by the mutant isolated nucleic acid sequence; and
(c) comparing a biocatalytic property of the expressed polypeptide with the same biocatalytic property of a wild-type phenylalanine aminomutase.

13. A method for altering biosynthesis of a compound by a plant cell culture comprising altering levels or activity of phenylalanine aminomutase in the plant cell culture.
14. The method of claim 13 wherein levels of phenylalanine aminomutase are increased by transforming the plant cell culture with a vector comprising a nucleic acid sequence for phenylalanine aminomutase.

15. The method of claim 13 wherein the plant cell culture comprises a Taxus species and the compound biosynthesized comprises a taxane or taxane-related compound.

16. A method for altering levels of a taxane or taxane-related compound produced by a plant cell culture comprising culturing the plant cells with β-phenylalanine.

17. The method of claim 16 further comprising culturing the plant cells with benzoic acid or a salt thereof.

18. The method of claim 16 or 17 wherein the level of taxanes produced by the plant cell culture is increased.

19. The method of claim 18 wherein the taxane is paclitaxel.

20. A method for altering the ratio of paclitaxel to baccatin III in plant cell cultures comprising culturing the plant cells with β-phenylalanine.

21. The method of claim 20 further comprising culturing the plant cells with benzoic acid or a salt thereof.
FIG. 1
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**FIG. 4.**
FIG. 5

1 ATGGGTTTGC CCCTGGAATC GCTTGTCTAC GTAAGAGATA TATTTGGGCT GTACCAACAG
61 TTTCAAGGGG TGAAGGAAAT TACATGAGAC GTAGCAACCC CCACTCAAGGT GGGCATTGTC
121 CGCGGCTGCG CCCGGGAGCC TGGAGGTAGG GTGGGATTGG AGGGCGGAGA ATCGAGAGGC
181 CAGTGTGGAAA CTGGCTCGTG TGGGGGCAAA CGAGGAACAG AGAGATGAGC AGGCCCTGCC
241 GGGTGCACCA GGGGTTGCGG GGGTGGCTCG AGGCGGAGGA CCAACACTG GAGCGGAGTG
301 CAAAGTGGGCC TCAATGCGTC CCTGGCTCGGC GGGTTTGTTA CTAAGAAGAT CAGCTGCTCC
361 GTCGACAGAG CCCTCAGGCG CCCTCAGGCG GCCCTGCGAC TGGCCAGAGC TAGGCGGCGG
421 ACCTATGGAAT GTGGCGGCGAT CGCTGGGGAG GTCAATGGAAC GCTTCTCAAC
481 AGCACTGTGC CTCCCTAAAAT GAGATGCGGG GTAATGCGAG GAGGTAAGGC AGCCCTAC
541 GGCTGCTGGCT ACATGGGCGG GCTGCTGATC GGGAACACTA CGTATTCGCG TGGCATAAGG
601 GACGAATGGCG AGGTTGCCGT CCGCCGCATT TGGAGGCGGG CGCCCTCGCC CCCACTCAAG
661 ATCCAGGCGGA AAGGAAGGCT CGGGCTCGCT AAGCAGCCCT CCTGCAGCGC CGGGGCCTCC
721 TTCCACCAGCT CAAGTTGCTGA ATCTGCGCAG TGGCTCTGATG TGGAGAAGCT TTGGCGGAGT
781 TCTGCGGAGG TGATCTTTTG AAGGAGAAGG TGCCGAGCGT CGCTGTCCCA TAAAGTGAAG
841 CCGACCCCGG GTGGGAGATC TGGGAGAAGG GCCCTGATCG CAGGAGCGCC TGTCAGCCCG
901 GTTGGCGGAC GTGGGAGGAA GTATGTTATG AGGAGAAGG TGGGAGGGAAG GAGAGACAGG
961 CTAATGGCGG AATTTTTTTA TCTGAGACTA TTGTGCGGTT TCTGCTGCTT CTCTGGGCCG
1021 GCCACCCACTA CAGTGCGGAG GCAGGCGGAC TGCAGGATGA AACAGTGCGA GTGCCGACGC
1081 GCCAAATGCA GTTAAATGAC ATCTGCGCAG AGGAGAAGG TGGGAGGGAAG GAGAGACAGG
1141 CCAAAAATCTC TGAAACAAAA TGAGAAGGTA GTGGAGGCAAG GGAGGAGGTC CTTTTTAAAA
1201 ATTGACATTG TTTTTTTTAT ACATGACACT ATCTGCGGTT TCTGCTGCTT CTTTGGGCAAG
1261 GCCCTCCTCAT GTGGGAGGAT CACGGGAGCA GAGGAATCTTT TTTGGGAGAG GCACAGGGGG
1321 GATGGGGATT GGAAATATTG GGGGTTGGGG GCCAGATTCT GCCAGAATTC GTTGGGAGGAC
1381 GTTAGAAGGC ACGGGTACGT CCGGCGACCT TTCTGGGAGG CCGGCAGCTG GCCGGAGCTA
1441 GCCCGCTCAAG GGGCTGCAAC TGGCAAGATT GAGATGCGGG GAGGAGGATG GAGGAGGATG
1501 GAAATGCTGCG GGGGAGGAAT CGGGAGGAGA GGAGAATACG GGAGGAGGATG GAGGAGGATG
1561 GGGCGTCGAC TGGGCCGACA AAGCGAGAGG GCCAGGGGCA GTGGGAGGATG GAGGAGGATG
1621 CTGGAGAATTG AAGCAATTTG GTTGGGAGGAC GCCAGGAGAG GCCAGGAGAG GCCAGGAGAG
1681 AGGAAACTGG TGGAGAAGGT TTTCCTCACT TGGCAAGAGA TGGGAGGAGG GCCAGGAGAG
1741 AAAGGAAGTT GAGATGCGG TTTTGGGAGA TGAGAAGGTT TGGGAGGAGG GCCAGGAGAG
1801 CAGGAGACCC AGGCTCCTCC TCTGGTTGAG CTAGGAAACAA TCTGGTGGGG ATGGGCATCT
1861 TGGCTGACC AAAAAAGACG GAGTTGGAGC GAGACCTTTG GTGACATCGC GGGCGAGTTT
1921 CGGAATCGGG GTGGGAGGAC GGGGGAGGGA GAGATGCGGG GAGGAGGATG GAGGAGGATG
1981 AAAAGAAGAG AGTAAATGGA TGGGAGGAAT TGGGAGGAGA GAGGAGGATG GAGGAGGATG
2041 ATCTGCGGTT GCTTTATCTT TGGTGGGGAG GAGGAGGATG GAGGAGGATG GAGGAGGATG
2101 AAGGAGACAG TGCCGCGGAA GGAGGAGGATG CAGATGGTGA GTGAGAAGTCC AGGGTGACAA
2161 ATTAAGGATT CTTGCTTGCTG ACTGCGGTCG AAGGTTTTTT GGGCAACCAA ATGGGATGGG
2221 CRAGGGCGTC TAG
FIG. 6

1. ATGGGCTTTTG
   CCTGGGAAATC
   GCGTTCTTAC
   GTAAGGGATA
   TATTGGGCTC
   GAATCAACAG

61. TTCCACAGAGG
   TGAGAAATAAT
   TACAGTGAAC
   GGTACAGACC
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   GGCACAAGTC

121. GCGGCGTCGG
   CCCCCAGCCA
   TGACGTGAAG
   GGTGCTTGGG
   ATGGGAGACA
   GTCGAGACCC

181. CCGCGCTGGAA
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   GCCACGGCCC
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241. GGGCCTCGACA
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301. CAGAGACTGCG
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   GGCTGGTTTA
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   GCCTCCCTCC

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   GCGTACCAGC
   AGGCGGATGC
   TGGCTCTCCG
   TTATGCTTTT

421. ACCTATGGAT
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   CGGGTGAGAG
   GCAAGAGAAG
   GCTGCTCAGC
   AGCTCTCACG

481. AGCAATTCTCT
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   GCTCTCCGAG
   GAGTCTGAGA
   GGCCTGTGGG
   AGACCTACGC

541. CCGGCTCGCCT
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   GGTGCTGATG
   GGAAGCCTTA
   GGTTATACGG
   TGCGCATAGC

601. GACGAATCGCG
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   GGGCGGGCCG
   TTGGGAGGG
   TGGGCGTGC
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661. CTCAGGCGCC
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   GGGCGGTCGT
   AAGGGCACTT
   CTTGCTGCCG
   GCGTGCCGCC

721. TCCACCGCTCA
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   TGGCTGATAG
   CGAACACCTG
   TGGGGAAGTG

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   GAACTAGGAG

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   TGGCTGATGG
   GGCCTGGTGG
   GATGGACCGC

901. TTTCAGGACC
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   TCCGCAATGG
   ATAACCGCCTA
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   TGGGGCGCTC
   TTTTATTAAA
   CCTTTTAAAG

1201. ATGAACATATA
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   TTTGCGTGGT
   TTGGCTGATC
   AATTTTTCAG

1261. GGGTCTCGCTT
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   TCCAGGAATA
   CGCGGAGCAG
   TTTGACTAACG
   ATGGACAGCG

1321. CATGCGCATCG
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   GGAACACTCT
   GTGCGCTGAT
   TACAGGAGAC
   TGAATGACGA

1381. ATTATACAGC
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   CGGGGACCTC
   ATGGGATGG
   CCGGCACTGA
   GCGTGGACTA

1441. CGCGCTCGAG
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   TCGACTGCC
   GGGCCATACG
   TCGACGGTCC
   GATCCCTGGC

1501. GATCGCTCGTG
   AGGCAGCCTG
   TCAAGACGC
   GAGACAGCAC
   AACCAGGAGA
   GCACTGTCCG

1561. GGGCGCTCTAC
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   AGGCGGCTAG
   GCCGGCTGAT
   ATGTTATAGA
   ATGTAGCTCG

1621. CTGGCAATTGA
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   GCCAGCAGCT
   GCAGCTGGCG
   GACGGCTAGA
   AAACGATGTC

1681. AAAAGTCTGGG
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   TGAGACGAGC
   TGGCGCTCCC
   TCAATAGCAC

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   TAGGCAAGGC
   GGGCGCCTGTT
   TACAGATACC
   TGGAAATCCC

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   TCTGGTGATG
   AAGCAGAATC
   TGGCAGTCGG
   TCGGAGATTT

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   GGGACTCTTG
   TGGAGCGGCG
   TGGCGAGATT

1921. CGAGAAAGCG
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   CGAGATGGAC
   GTTCATGGGG
   TTGTGACAGA

1981. AAGAAGAAGG
   CATAAAACCTG
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   CGAGCCCTCC
   TGAGAAATCC
   AGGGTCCCAA

2041. ATTCCTCTTCTCT
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   GGTGGGCGGG
   CGACGCTGAC
   AGCAGCGTGG
   GACGGGCTAG

2101. AAGGAGAGCC
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   AGGCGATGGA
   GAAAGGGCTG
   TATGGACATT
   CGGAGGACGC

2161. AATTAAGGTTG
   CCTCTGCTGC
   ACTGCGCTGCA
   AGGGTTTCTC
   GSCCAACCAA
   AATGGTGCCG

2221. CAACGGGGTC
   TAGAC
FIG. 8

B. Baccatin III

A. Taxol

Production period

Units/mL

200 150 100 50 0

2nd Wk.

3rd Wk.

4th Wk.

5th Wk.