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(54) Title: A METHOD OF REGULATING LIGNIN CONTENT IN THE PLANT OF *HEVEA BRASILIENSIS*

(57) Abstract: A method of reducing production of lignin content in the plant of *Hevea brasiliensis* comprises the step of down-regulating expression of at least one nucleic acid template containing sequence SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4 in at least one cell.



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## A METHOD OF REGULATING LIGNIN CONTENT IN THE PLANT OF *HEVEA BRASILIENSIS*

### Field Of Invention

- 5 The present invention relates to a method in regulating lignin content in the plant of *Hevea brasiliensis* and a transgenic *Hevea brasiliensis* plant acquired thereof. More specifically, the lignin content in the plant of *Hevea brasiliensis* can be regulated via modulating expression of the lignification-associated enzymes

### 10 Background Of The Invention

- Secondary xylem of trees, from which pulp is derived, is composed of cellulose (b-1, 4-glucan), lignin (phenolic polymer), and hemicelluloses (heterogeneous polysaccharides) in approximate proportions of 2:1:11. During tree growth, cellulose microfibrils give cell walls tensile strength, and lignin encasing the cellulose microfibrils imparts rigidity to cell walls. Lignin provides the essential mechanical reinforcement for growth of the tree, while plant overloaded with lignin substantially limits the use of plant material for fiber, chemical, and energy production. Particularly, in plants like *Hevea brasiliensis* that accumulation of too much lignin may affect quantity of the latex can be harvested thereof. Thus, strategies for lignin down regulation are of considerable interest. As a result, there has been long-standing incentive to develop healthy trees that accumulate less lignin and/or more extractable lignin to facilitate pulping. Significant progress has been made in recent years toward an understanding of lignin biosynthesis through characterization of lignin biosynthetic pathway enzymes and genes from both herbaceous and tree crops. For example, United States patent application no. 5451514 discloses a method to genetically suppress cinnamyl alcohol dehydrogenase production resulting in formation of modified lignin more extractable after subjected to alkaline treatment. Similarly, in United States patent application no. 6479732, Kajita et al. provide a method to modify lignin structure in a plant through modulating 4-coumarate coenzyme A ligase expression genetically. Further, transgenic tobacco plants are described in United States patent application no. 5850060% and 5952486 respectively. These tobacco plants particularly contains cDNAs templates, of enzymes involved in lignin
- 15  
20  
25  
30

production, derived from *Pinus radiata* and *Eucalyptus grandis*. These applications claim the transgenic plants show relatively lower lignin content compared to wild type tobacco plants. Other approaches to attain similar object can be found in International Patent Publication no. WO9423044. It describes a method of reducing lignin content  
5 in plants by transfecting the plant cells with a vector comprising transcribable antisense gene for caffeic acid 3-O-methyltransferase that transcription of the antisense gene in turn lead to down regulation of the caffeic acid 3-O-methyltransferase. Likewise, Canadian patent application no. 2404104 offers a method applicable to forage legume cell that the method transfecting the legume cell  
10 with open reading frame of caffeoyl CoA 3-O-methyltransferase enzyme.

Though the above mentioned methods have achieved the desired result at various degree of success, the proposed methods may not be applicable on plants of different species besides the preferred types. Considering the lignin synthesis pathway and  
15 genetic makeup of each species are potentially varied one and other, genetic approaches for regulating lignin production in a plant is much preferable to be species-specific to yield better results.

### Summary Of The Invention

20 The present invention aims to provide a method capable of reducing or modulating lignin content in the plant of *Hevea brasiliensis*. The disclosed method is highly lignin-specific with less inference towards other biosynthesis pathways. In more particular, the disclosed method only genetically intervenes monolignol synthesis pathway responsible for lignin production in opposed to methods offered in most of  
25 the prior arts.

Another object of the present invention is to disclose a transgenic plant of *Hevea brasiliensis* with reduced lignin content. Preferably, the transgenic *Hevea brasiliensis* is rendered with reduced lignin content by suppressing expression of the  
30 lignin-producing enzymes in the plant.

Still another object of the present invention is to provide isolated polynucleotides having specific nucleotide sequence to facilitate performing of the disclosed method and acquiring of the transgenic *Hevea brasiliensis* plant.

- 5 At least one of the preceding objects is met, in whole or in part, by the present invention, in which one of the embodiment of the present invention involves a method of reducing production of lignin content in the plant of *Hevea brasiliensis* comprising the step of down-regulating expression of at least one nucleic acid template containing sequence SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID  
10 No.4 in at least one cell.

In another aspect, the down-regulating step of the disclosed method can be conducted by introducing a dsRNA having a strand containing at least 60% sequential nucleic acids complementary to the nucleic acid template in the cell.

- 15 Still, in another aspect, the down-regulating step can be achieved by transforming the cell with a recombinant gene construct encoding open reading frame containing nucleic acid sequence having at least 70% sequential nucleotides identical to SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4 and expressing the nucleic acid  
20 sequence of the DNA construct via expression control of a lignification-associated promoter.

- Preferably, a transgenic plant of *Hevea brasiliensis* with reduced lignin content is another embodiment disclosed in the present invention. The transgenic plant is  
25 characterized in that expression of at least one nucleic acid template containing sequence SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4 in at least one cell is down-regulated. More preferably, in one embodiment, the condition of reduced-lignin content in the transgenic plant is realized via the setting forth methods.

- 30 Further embodiment of the disclosed invention includes an isolated polynucleotide encoding lignification-associated enzyme of the plant *Hevea brasiliensis* comprising

nucleotide sequence as set forth in SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4 and a recombinant gene construct containing the set forth polynucleotide.

In further aspect, the present invention also provide isolated polypeptide containing amino acids sequence or fragments thereof encoded by the mentioned polynucleotide.

### Brief Description Of The Drawings

- Figure 1 shows a general biosynthetic pathway of lignin in woody angiosperms;
- 10 Figure 2 shows the polynucleotide sequence SEQ ID No.1 encoding the enzyme cinnamoyl A reductase 1 (CCR1) of the plant *Hevea brasiliensis* ;
- Figure 3 shows the polynucleotide sequence SEQ ID No.2 encoding the enzyme cinnamoyl A reductase 2 (CCR2) of the plant *Hevea brasiliensis* ;
- 15 Figure 4 shows the polynucleotide sequence SEQ ID No.3 encoding the enzyme cinnamyl alcohol dehydrogenase 1 (CAD1);
- Figure 5 shows the polynucleotide sequence SEQ ID No.4 encoding the enzyme cinnamyl alcohol dehydrogenase 2 (CAD2);
- 20 Figure 6 shows the polypeptides sequence SEQ ID No.5 encoded by the SEQ ID No.1 in figure 2;
- 25 Figure 7 shows the polypeptides sequence SEQ ID No.6 encoded by the SEQ ID No.2 in figure 3;
- Figure 8 shows the polypeptides sequence SEQ ID No.7 encoded by the SEQ ID No.3 in figure 4; and
- 30 Figure 9 shows the polypeptides sequence SEQ ID No.8 encoded by the SEQ

**Detailed Description Of The Invention**

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The embodiment describes herein is not intended as limitations on the scope of the invention.

The term “gene” is defined as the genomic sequence of the plant *Hevea brasiliensis* particularly polynucleotide sequences encoding polypeptide sequence of the enzyme CCR1, CCR2, CAD1 and/or CAD2.

The term “polynucleotide”, as used herein, is a nucleic acid containing a sequence that is greater than about 100 nucleotides in length.

The term “oligonucleotide”, as used herein, is a short polynucleotide or a portion of polynucleotide which preferably comprises from about 8 to 35 nucleotides. In respect to the embodiment of the present invention, nucleotides contained within the oligonucleotides can be analogs or derivatives of naturally occurring nucleotides.

The abbreviation used throughout the specification to refer to nucleic acids comprising nucleotide sequences are the conventional one-letter abbreviations. Thus, when included in a nucleic acid, the naturally occurring encoding nucleotides are abbreviated as follows: adenine (A), guanine (G), cytosine (C), thymine (T) and uracil (U). Also, unless otherwise specified, nucleic acid sequences that are presented as a series of one-letter abbreviations are presented in the 5'→3' direction.

The term “wild-type”, as used herein, refers to a gene or gene product that has characteristics of that gene or gene product that is most frequently observed in population and is, thus, arbitrarily designated the “normal” or “wild-type” form of the gene. In contrast, the term “variance” or “mutant”, as used herein refers to gene or gene product that displays modifications in sequence and/or functional properties

As used herein, the term “complementary” and derivatives thereof are used in reference to pairing of nucleic acids by the well known rules that A pairs with T or U and C pairs with G. Complement can be “partial” or “complete”. In partial  
5 complement, only some of the nucleic acid bases are matched according to the base pairing rules; while in complete or total complement, all the bases are matched according to the pairing rule. The degree of complement between the nucleic acid strands may have significant effects on the efficiency and strength of hybridization between nucleic acid strands as well known in the art. This may be of particular in  
10 detection method that depends upon binding between nucleic acids.

According to one of the preferred embodiment, the present invention includes a method of reducing production of lignin content in the plant of *Hevea brasiliensis* comprising the step of down-regulating expression of at least one nucleic acid  
15 template containing sequence SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4 in at least one cell. These sequences encode for the enzyme cinnamoyl A reductase 1 (CCR1), cinnamoyl A reductase 2 (CCR2), cinnamyl alcohol dehydrogenase 1 (CAD1) and cinnamyl alcohol dehydrogenase 2 (CAD2) respectively are shown in the figure 2 to 5. Formation of lignin in the plant of *Hevea*  
20 *brasiliensis* is performed through a multi-stage synthetic pathway that each stage in the pathway is catalyzed by a specific enzyme type. The CCR1, CCR2, CAD1 and CAD2 are known to be responsible in synthesizing guaiacyl monolignol and syringyl monolignol which can be turned into free radicals to constitute core structure of lignin in the *Hevea brasiliensis* upon polymerization. Thus, guaiacyl monolignol and  
25 syringyl monolignol are deemed as the specific precursors to be employed in *Hevea brasiliensis* for producing lignin. It was found by the inventors of the present invention that prohibiting formation of guaiacyl monolignol and syringyl monolignol is an effective approach to significantly reduce lignin content in *Hevea brasiliensis*.  
To prevent formation of monolignol, the present invention, as setting forth, discloses  
30 a method to genetically intervene production of the enzymes converting cinnamoyl-Co A to monolignol in the plant cells therefore capable of modulating the lignin

In one of the preferred embodiment, the down-regulating step is conducted by transforming the cell with a recombinant gene construct encoding open reading frame containing nucleic acid sequence having at least 70% sequential nucleotides identical to SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4 and expressing the nucleic acid sequence of the DNA construct via expression control of a lignification-associated promoter. The open reading frame containing nucleic acid sequence having at least 70% sequential nucleotides identical to SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4 allows generation of an antisense mRNA capable of hybridizing onto the complementary mRNA having nucleotide sequence of SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4 preventing transcription of the hybridized mRNA to produce the encoded enzyme thereof. More preferably, the expression of the antisense mRNA in the present invention is initiated by the lignification-associated promoter located at upper stream of the open reading frame.

Using the lignification-associated promoter in the present invention renders the expression of the antisense mRNA corresponding to the expression of the CCR1, CCR2, CAD1 and/or CAD2 mRNA in the transfected cell upon receipt of the signal to generate lignin in the *Hevea brasiliensis*. The lignification-associated promoter serves as a regulatory mechanism to control expression of the antisense mRNA; particularly only in the specific cell types being signaled by the plant to produce lignin. Such approach renders the disclosed method only generate the antisense mRNA specifically in the lignin producing cells and not having any effect in other transfected non-lignin producing cell types. More preferably, the disclosed method may selectively transform only the lignin-producing cells with the recombinant gene construct. More specifically, xylem tissue, which gives rise to lignin accumulation in the plant, is transfected with the recombinant gene construct. With only the targeted cells are transfected, other pathways in the genetically modified *Hevea brasiliensis* is less likely disrupted.

Another approach to silence expression the CCR1, CCR2, CAD1 and/or CAD2 in the present invention can be performed through RNA interference that the down-

least 60% sequential nucleic acids complementary to the nucleic acid template in the cell. This complementary strand of the introduced dsRNA coupled with the RNA-induced silencing complexes in the transfected cells to initiate degradation of the mRNA of the CCR1, CCR2, CAD1 and/or CAD2 therefore prohibits subsequent  
5 creation of the CCR1, CCR2, CAD1 and/or CAD2 enzymes in the affected cells. To carry out this embodiment, a sense and antisense RNA molecule either fully or partially complementary to the SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4 is expressed in the transfected cells resulting in inhibition of the expression of the correspondin endogenous mRNA encoding CCR1, CCR2, CAD1 or CAD2  
10 respectively. Apart from RNA interference, other embodiments of the disclosed method for prohibiting expression of the CCR1, CCR2, CAD1 or CAD2 gene may involve hairpin RNA interference as well.

Further embodiment of the present invention is a transgenic plant of *Hevea brasiliensis* with reduced lignin content characterized in that expression of at least  
15 one nucleic acid template containing sequence SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4 in at least one cell is down-regulated. As in the foregoing, the nucleic acid template containing sequence SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4 encodes polypeptides sequence of the enzyme CCR1, CCR2,  
20 CAD1 or CAD2 respectively. Down-regulated expression of these nucleic templates, preferably in the form of mRNA, reduces availability of these enzymes in the transgenic *Hevea brasiliensis* to be presented in synthesizing lignin. Consequently, less lignin is produced in the plant owing to absence or reduced amount of these enzymes in the transgenic *Hevea brasiliensis*. Referring the description above

25 As described above, the down-regulation of SEQ ID No.1, SEQ ID No.2, SEQ ID No. 3 and SEQ ID No.4 in the transgenic plant can be performed via expression of complementary antisense mRNA in the targeted cells. The targeted cells are transfected with a recombinant gene construct encoding open reading frame  
30 containing nucleic acid sequence having at least 70% sequential nucelotides identical to SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4 and expressing the

associated promoter. The lignification-associated promoter serves as a regulatory mechanism to control expression of the antisense mRNA of the polynucleotides of EQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4. Upon receiving signal to produce lignin, the similar signal shall simultaneously activate lignification-associated promoter to express the antisense mRNA to act on the complementary endogenous sense mRNA encoding polypeptides sequence of the CCR1, CCR2, CAD1 and/or CAD2. More preferably, the disclosed transgenic *Hevea brasiliensis* may only have the selected lignin-producing cells transfect the recombinant gene construct. Preferably, at least part of xylem tissue of the transgenic *Hevea brasiliensis* is transfected with the recombinant gene construct to achieve significant lignin content reduced in the transgenic plant. With only the targeted cells are transfected, other pathways in the genetically modified *Hevea brasiliensis* of the present invention is less likely disrupted.

Likewise, RNA interference may be employed in the present invention as another approach in the disclosed transgenic plant to down-regulate expression SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and/or SEQ ID No.4 in the disclosed transgenic plant that a dsRNA having a strand containing at least 60% sequential nucleic acids complementary to the nucleic acid template is introduced into the cells through any known methods in the field. The dsRNA for carrying out the RNA interference in the transgenic plant may range from as short as 20bp to few hundred bp. The RNA strand of the introduced dsRNA having complementary sequence towards the mRNA of SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and/or SEQ ID No.4 functions as the guide strand to couple with and lead the RNA-induced silencing complexes in the transfected cells to degrade the complementary the mRNA with SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and/or SEQ ID No.4 2 therefore prohibits subsequent transcription of the CCR1, CCR2, CAD1 and/or CAD2 enzymes from the mRNA. To carry out this embodiment, a sense and antisense RNA molecule either fully or partially complementary to the SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4 is expressed in the transfected cells resulting in inhibition of the expression of the correspondin endogenous mRNA encoding CCR1, CCR2, CAD1 or CAD2

Pursuant to another preferred embodiment, the present invention also discloses an isolated polynucleotide encoding lignification-associated enzyme of the plant *Hevea brasiliensis* comprising nucleotide sequence as set forth in SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4. The isolated polynucleotide of SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4 encode for the polypeptides sequence of the enzyme CCR1, CCR2, CAD1 or CAD2 respectively for the plant of *Hevea brasiliensis*. The disclosed isolated polynucleotides of the present invention permits genetic intervention to be performed onto the plants of *Hevea brasiliensis* to significantly reduce lignin production via the method described above. These disclosed polynucleotides sequences are specifically for the plant of *Hevea brasiliensis* that such specificity is important to ensure achievement of the mentioned object in decreasing lignin content. Moreover, another embodiment of the present invention includes also isolated polypeptide containing full or part of amino acids sequence encoded by the above mentioned polynucleotide of SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4. These polypeptides can be synthesized chemically or biologically using methods known in the arts.

Still another embodiment of the present invention is a recombinant gene construct containing polynucleotide having sequence of SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4. Preferably, the recombinant gene construct is a vector for delivering the genetic material needed to modulate the lignin content in the *Hevea brasiliensis*.

The following example is intended to further illustrate the invention, without any intent for the invention to be limited to the specific embodiments described therein.

### Example 1

Total RNA was isolated from the young/tender leaves of 8 months old *Hevea brasiliensis* cultivar RRIM 600 using QIAGEN- RNeasy Mini Kit according to the manufacturer's instructions. The quality or the integrity of the RNA was checked by

agarose gel electrophoresis and was quantified using Thermo Scientific Nano Drop 2000 as per standard procedures taking. cDNA first strand was synthesised using SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) following the manufacturer's instructions. The gene was amplified from the cDNA by PCR using the gene specific primers for cinnamoyl A reductase and cinnamyl alcohol dehydrogenase respectively. The PCR reaction (50 µL) contained 1µL of cDNA, 20 pmoles of each primers, 5 µL of 10X Pfu Buffer, 5 µL of 2.5 mM dNTP mix and 1.0 unit of PfuTurbo® DNA polymerase (Stratagene). PCR was carried out in Veriti™ Thermal Cycler (Applied Biosystems) using the following conditions. Initial denaturation for 5 min at 95°C followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 54-55°C for 30 sec and extension at 72°C for 1 to 2.0 min depending on the length of the targeted gene, with a final extension at 72°C for 7 min. The PCR product was analysed by 1% agarose gel using 1X TAE buffer and the amplicon was eluted from the gel using GENECLEAN® TURBO Gel band elution kit (MP Biomedicals) following the manufacturer's instructions. The purified PCR product was ligated into pCR® 4 Blunt TOPO® Vector (Invitrogen) and transformed into One Shot® Mach1™-T1R Chemically Competent *E. coli* cells (Invitrogen). Plasmids were isolated from putative colonies using QIAprep Spin® Miniprep Kit (Qiagen) following the manufacturer's instructions. The presence of the insert was checked by digesting with EcoRI (NEB) and positive plasmids were subjected to sequencing.

**Primers amplified CCR gene from *Hevea brasiliensis*.**

**CCR GENE 1**

**FP CCR1 5' ATGTCAATCCAAAACAAAGAGCC 3'**

**RP CCR1 5' TGATGTTGACATTTTGTGCTTTAG 3'**

**CCR GENE 2**

**FP CCR2 5' ATGAGTGGAGAAGGAAGGGTGG 3'**

**RP CCR2 5' GAAATTAAGAAAGCCTCTCTC 3'**

**Primers amplified CAD gene from *Hevea brasiliensis*.**

**CAD GENE 1**

**FP CAD1 5' ATGGCGAAATCATCAGAAGCACAAC 3'**

**RP CAD1 5' TAACTGAGACAAGGAAGTTCC 3'**

**CAD GENE 2**

**FP CAD2 5' ATGGTAGCTAAATTGCCAGAGAAAG 3'**

**RP CAD 2 5' TTGCTGATATAAGGAGTTTCC 3'**

**Example 2****Analysis of the sequence**

The nucleotide sequence and the aminoacid sequences were analysed by BLASTN and BLASTP programmes respectively.

5

It is to be understood that the present invention may be embodied in other specific forms and is not limited to the sole embodiment described above. However modification and equivalents of the disclosed concepts such as those which readily  
10 occur to one skilled in the art are intended to be included within the scope of the claims which are appended thereto.

## Claims

1. A method of reducing production of lignin content in the plant of *Hevea brasiliensis* comprising the step of down-regulating expression of at least one nucleic acid template containing sequence SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4 in at least one cell.  
5
2. A method of claim 1, wherein the down-regulating step is conducted by introducing a dsRNA having a strand containing at least 60% sequential nucleic acids complementary to the nucleic acid template in the cell.  
10
3. A method of claim 1, wherein the down-regulating step is conducted by transforming the cell with a recombinant gene construct encoding open reading frame containing nucleic acid sequence having at least 70% sequential nucleotides identical to SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4 and expressing the nucleic acid sequence of the DNA construct via expression control of a lignification-associated promoter.  
15
4. A transgenic plant of *Hevea brasiliensis* with reduced lignin content characterized in that expression of at least one nucleic acid template containing sequence SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4 in at least one cell is down-regulated.  
20
5. A transgenic plant of claim 4, wherein the down-regulation of SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4 is performed by introducing a dsRNA having a strand containing at least 60% sequential nucleic acids complementary to the nucleic acid template.  
25
6. A transgenic plant of claim 4, wherein the down-regulation of SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 and SEQ ID No.4 is performed by transforming the cell with a recombinant gene construct encoding open reading frame  
30

identical to SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4 and expressing the nucleic acid sequence of the DNA construct via expression control of a lignification-associated promoter.

- 5        7. An isolated polynucleotide encoding lignification-associated enzyme of the plant *Hevea brasiliensis* comprising nucleotide sequence as set forth in SEQ ID No.1, SEQ ID No.2, SEQ ID No.3 or SEQ ID No.4.
- 10       8. An isolated polypeptide containing amino acids sequence encoded by the polynucleotide claimed in claim 7.
9. A recombinant gene construct containing polynucleotide of claim 7.
- 15       10. A recombinant gene construct of claim 9 is a vector.

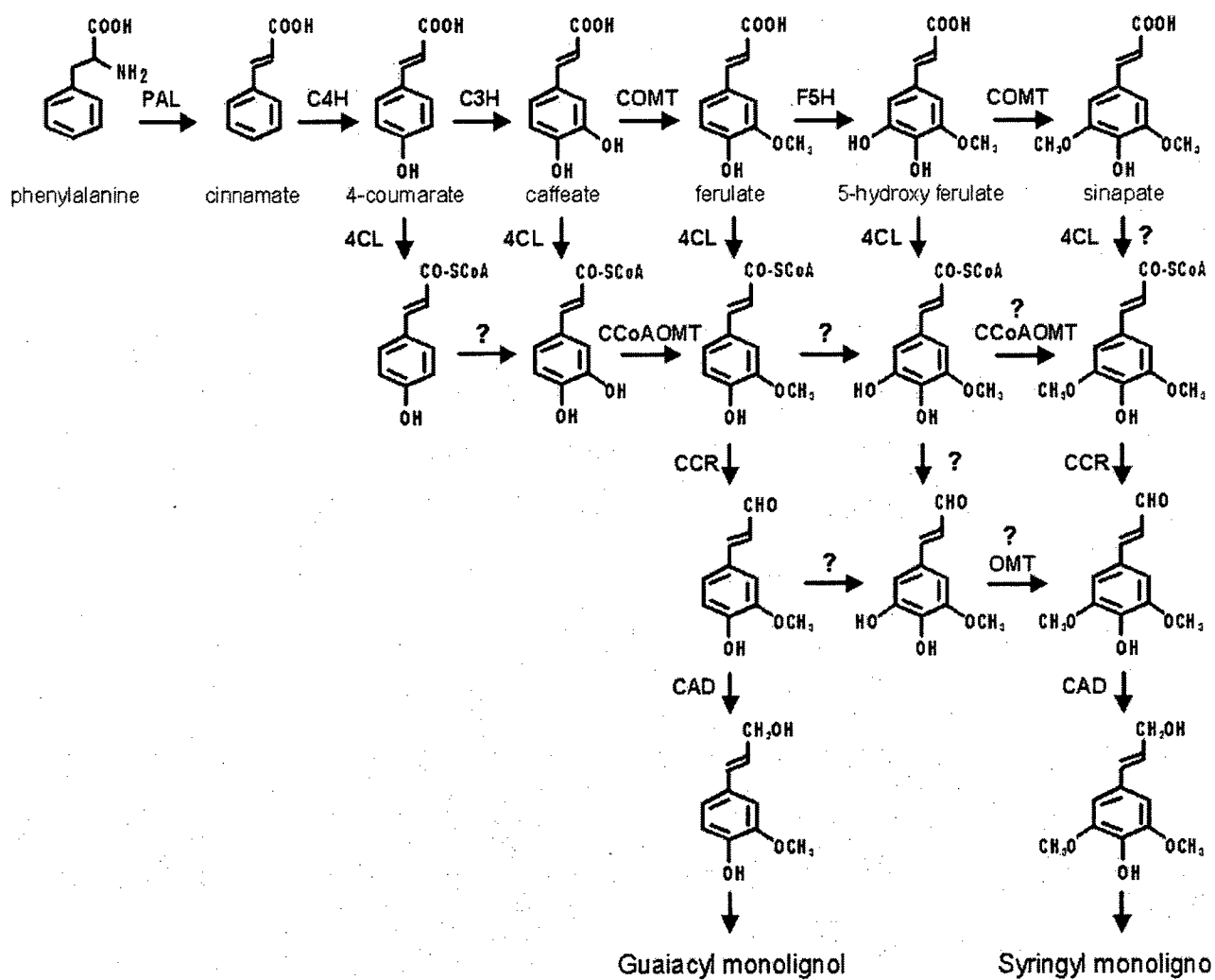


Figure 1

SEQ ID No. 1

ATGTCAATCCAAAACAAAGAGCCAGTCTGCGTCACTGGAGCCAATGGCTTCATT  
GGATCCTACCTGGTCCGGACCCTCATAACCCATGGCTACACCACCATCCACGCA  
TCCATCTACCCCTCCTCCGATGCGTCCACCTCTTCCACCTCCTCCCTTCCGCC  
GCCTACTCTAACGTCCAACCTTCGTATCTTCGAGGCCGACGTTCTTGACTACGAC  
GCCGTTGCCAAGGCCGTTGAGGGCTGTCGGGGGGTTTTCCACGTGGCCTCTCC  
CTGTACGCTAGAGGACCCTAAGGACCCGCAGATGGAGCTTGTATTGCCTGCGG  
TGCAGGGCACGATTAATGTGCTGCAGGCTGCCAAGAAGTTCGGTGTGAGGCGC  
GTGGTGGTCACGTCGTCTATTTGGCGTTGGTTCCTAATCCTAATTGGCCGGCC  
GGTAAAGTGTGTTGATGAGTCGTCTGGACTGACATCGATTACTGCAAGTCTCGT  
CAGAAGTGGTATCCAGTGTCAAAGACACTGGCAGAGAAAGAAGCGTGGGACTT  
TGCAAAGAAGCATGGAATGAATCTGGTGACAATCCATCCTGCTACATGTCTAGG  
CCCCTGTTGCAGCCTAGTTTGAATGCTAGCTGTGCTGTGTTGAAGCAATTATTG  
GAGGGATCAGAAGACACTCAGGAATATCACTGGTTGGGAGCTGTGCATGTCAAA  
GATGTGGCTGAGTCACAAATTTTGGTGTGTTGAGACTCCTACTGCTTCTGGTAGAT  
ATCTCTGCACAAATGGTATTTATCAATTTGGTGATTTTGCAGAGAGGGTTTCCAA  
GCTCTTCCCTGAGTTTCCTGTTACAGGTTTGTGGGGAAACTCAACCGGGCCT  
GACAACTTGCAAAGATGCTGCCAAGAGATTAATTGAGATGGGGCTAGTTTTAC  
TCCAGTTGAAGATGCCATCCAGGACACGGTGGAAAGTCTAAAGGCTAAAGGATT  
CCTAAAGCACAAAATGTCAACATCATAG

Figure 2

SEQ ID No. 2

ATGAGTGGAGAAGGAAGGGTGGTGTGTGTAAGTGGAGGATCAGGTTATATAGC  
TTCATGGCTGGTCAAGCTCTTACTCCAGCGAGGTTACACAGTTAAAGCTACTGTT  
CTTGATCCCAATGATCCTAAGCAAACAGAACATTTGCTTGCACTCGATGGAGCTA  
AAGAGAGACTTCAGTTGTTCAAGGCAAATTTAGTGGAAGAAGGAACTTTTGATTC  
AGCAGTTGATGGATGTGAAGGTGTATTTACATACAGCTTCTCCTGTAATTCTTTCA  
ACAGATGACCCACAGAATGTATTGCTTGAGCCTGCTGTTAAGGGAACACTAAAC  
GTTCTTAAATCGTGTTGAAAGTCCCTTTTATCAAGAGAGTGGTTGTAACCTTCTT  
CTATCGCAACAGTACTGTTCAATGGAACACCTCTGACCCCTGATGTGGTAGTTG  
ATGAGACTTGGTTTTCTGATCCTGCTTTCTGCAAGGAAAATAAGCATTGGTATAG  
GCTATCAAAAACCTTAGCAGAGGATGTTGCTTGGAATTTGCAAATGAAAATGGA  
ATTGACTTGGTTACAATACATCCAGGGTTTGTGATTGGTCCTGTCTTCCAGCCAA  
CTCTGAATCTCACTATGAAGATTATTTTGAACATACATAACTGGTCAAACATTTCCA  
AATGAGACTTTAGATCTGTTGATGTTAGAGATGTTGCAAATGCACATATTCTAGC  
ATTTGAAAGATCTTCAGCCAGTGGCAGATACTGTTTAGTTGGACAAGTAGCACA  
CTTGTCTGAGTTCTTTAAGATTCTGCGCCAACTACCCGACACTACCACTTCCT  
GAAAAATGTGTAGATGACAAGCCTTTTGTGCCAAAATATGAGGTATCTAAAGAGA  
AGGCAAAAGACTTGGGTGTGGACTTCACTCCTTTGGAGGTTACTGCTAAAAACA  
TCATTGAAAGCTTGAAAGAGAGAGGCTTTCTTAATTTCTGA

Figure 3

SEQ ID No. 3

ATGGCGAAATCATCAGAAGCACAAACACCCACAGAAGGTTTTTGGTTGGGCTGCA  
AAAGATACGTCTGGGATCCTTTCTCCTTTCCATTTCTCTCGAAGGGGAAAATGGTG  
ATGAAGATGTGAGTATAAAAATCCTGTACTGTGGTGTGTGCCACTCTGACTTGCA  
TTCTTGCAAGGAATGAATGGGGTAATGCCCATACCCTCTGGTTCCAGGGCATGA  
AATTGTTGGTACAGTTACAAAAATTGGGAGCAATGTGAAGAAATTTAAAGTGGGT  
GATAGGGTTGGTGTGGTGTATGGTGGGATCTTGCAAGTCATGCGAGTACTGC  
AAACAGGACTTGGAATAATTATGCCCTAAACACATATTGACTTACAACCTCCATTG  
ACAATGATGGGACAAGAAGCTTATGGTGGTATGCTGATATGATTGTTGTTGACCA  
AACTTTGTGGTTCACTTTCTGACGGCTTACCTTCTGATGCGGGTGCACCGCT  
ATTATGTGCTGGGATCACAGTGTACAGCCCAATGAAATATTATGGAATGACTGAA  
CCAGGCAAGCATTGGAATAGTGGGGCTTGGCGGGCTTGGTCATGTTGCTGT  
GAGGATTGCTAAAGCTTTTGGGTTGAAAGTTACTGTCATTAGTTCCTCACCAAGG  
AAGGAGAGTGAAGCAATTAATAGACTTGGTGCTGATGCATTCCTTGTTAGCAAT  
GACGCTGGAAAAATGAAGGGAGCTATTGGTACTATGGATTTTCATCATTGATACA  
GTGTCAGCAGTTCATGCCCTGGCACCCTTCTTAGTCTGCTGAAGGCAAATGGA  
AAGCTGATCACTTTGGGCTTGCCAAATAAGCCCCTGGAGCTGCCTATTTTCTCT  
CTAGTTTTGGGAAGGAAGCTTGTTGGAGGAAGTGACATAGGAGGGATGAAAGA  
GACACAGGAGATGCTGGACTTCTGTGCAAAGCACAATATAACTGCAGATGTTGA  
GGTGATCAGAATGGATGAAATCAACACAGCCATGGATAGGCTCGCCAAATCTGA  
TGTCAGTACCGGTTTGTGATTGATGTGGGAAGTTCCTTGTCTCAGTTATGA

Figure 4

SEQ ID No. 4

ATGGTAGCTAAATTGCCAGAGAAAGAGCATCCCAAACAGTCATTCCGGATGGGCA  
GCAAGAGACCAATCTGGCGTTCTCTCTCCCTTCAAATTCTCCAGGAGGGCAACG  
GGAGAGAAAGACGTTTTCTTTAAAGTCCTGTATTGTGGGATGTGTCACTCGGAC  
ATTCACATGGTCAAGAATGAATGGGGCTCTTCTACTTACCCTCTCGTCCCTGGG  
CATGAGATTGTGGGAGTGGTGACAGAGGTGGGGAGCAAGGTAGAAAAATTCAA  
GGTGGGAGATAAAGTGGGTGTAGGCTGCATGGTTGGATCATGCCACTCCTGCG  
ATAACTGCACAAACAATCTTGAAAATTACTGCCCACAAATGATACTTACTTATGGT  
GCCAAGTACTACGATGGAACCACCACTTATGGCGGCTATTCCGACAACATGGTT  
GCCGATGAGCACTTCATTGTTCTGATACAGATAACCTGCCTCTTGATGCTACTG  
CTCCTCTCCTTTGTGCTGGGATCACAGTGTACAGCCCCTTGAAATATTATGGACT  
CGACAAACCTGGCTTGCATGTAGGCGTAGTTGGCCTCGGTGGGCTTGGTCATA  
TGGCGGTGAAATTTGCCAAGGCTATGGGAGCTAAGGTGACTGTGATCAGCACC  
TCACCTAACAAGCAGCAGGAGGCTATTGAGCGTCTTGGTGCAGATTCATTTTTG  
GTCGGTCGTGACCAAGATCAAATGCAGGTTGCAATGGGCACAATGGATGGTATA  
ATTGACACAGTGTCTGCAATGCACCCTCTTTTGCCTTTGCTTGGTTTATTGAAGT  
CGCATGGAAAGTTGATTTTGGTTGGTGCCCCAGAGAAGCCACTTGAGCTACCAG  
CCTTTCCTTTGCTAATGGGGAGGAAGATGGTGGGTGGTAGTTGCATTGGGGGA  
ATGAAAGAGACACAAGAAATGGTTGATTTGCGAGCCAAACACGGCATAACATCG  
GACATTGAAGTTATCCCAATTGACTATGTGAACACTGCTATTGAACGCATGCTGA  
AAGCTGATGTTAGGTACCGATTTGTGATTGATATTGGCAACACAATCAAATCAAG  
CCATTGA

Figure 5

SEQ ID No. 5

MSIQNKEPVCVTGANGFIGSYLVRTLITHGYTTIHASIYPS  
SDASHLFHLLPSAAYSNNVQLRIFEADVLDYDAVAKAVEGC  
RGVFHVASPCTLEDPKDPQMELVLPVAVQGTINVLQAAKNF  
GVRRVVVTSSISALVPNPWPAGKVFDESSWTDIDYCKSR  
QKWYPVSKTLAEKEAWDFAKKHGMNLVTIHPATCLGPLL  
QPSLNASCAVLKQLLEGSEDTQEYHWLGAVHVKDVAESQ  
ILLFETPTASGRYLCTNGIYQFGDFAERVSKLFPEFPVHRF  
VGETQPGLTTCKDAAKRLIEMGLVFTPVEDAIQDTVESLK  
AKGFLKHKMSTS Stop

Figure 6

SEQ ID No. 6

EHLLALDGAKEERLQLFKANLVEEGTFDSAVDGCEGV  
FHTASPVILSTDDPQNVLLEPAVKGTNLNVLKSCSKVP  
FIKRVVVTSSIATVLFNGTPLTPDVVVDETWFSDPAF  
CKENKHWYRLSKTLAEDVAWKFANENGIDLVTIHPGF  
VIGPVFQPTLNLTMKIILNYITGQTFFNETLRSVDVRD  
VANAHLAFERSSASGRYCLVGQVAHLSEFFKILRQH  
YPTLPLPEKCVDDKPFVPKYEVSKAKAKDLGVDFTPL  
EVTAKNIIESLKERGFLNF Stop

Figure 7

SEQ ID No. 6

MAKSSEAQHPQKVFGWAAKDTSGILSPFHFSRRENG  
DEDVSIKILYCGVCHSDLHSCRNEWGNAHYPLVPGH  
EIVGTVTKIGSNVKKFKVGD RVGVGMVGSCKSCEY  
CKQDLENYCPKHILT YNSIDNDGTRTYGGYADMIVVD  
QNFVVHFPDGLPSDAGAPLLCAGITVYSPMKYYGMT  
EPGKHLGIVGLGGLGHVAVRIAKAFGLKVTVISSSPR  
KESEAINRLGADAF LVSNDAGKMKGAIGTMDFIIDTV  
SAVHALAPLLSLLKANGKLITLGLPNKPLELPFPLVL  
GRKLVGGSDIGGMKETQEMLDFCAKHNITADVEVIR  
MDEINTAMDRLAKSDVKYRFVIDVGSSLSQLS

Figure 8

SEQ ID No. 6

MVAKLPEKEHPKQSFGWAARDQSGVLSPFKFSRRAT  
GEKDVSFKVLYCGMCHSDIHMVKNEWGSSTYPLVPG  
HEIVGVVTEVGSKVEKFKVGDKVGVGCMVGSCCHSCD  
NCTNNLENYCPQMILTYGAKYYDGTTTTYGGYSDNMV  
ADEHFIVRIPDNLPLDATAPLLCAGITVYSPLKYYGLD  
KPGLHVGVVGLGGLGHMAVKFAKAMGAKVTVISTSP  
NKQQEAIERLGADSFLVGRDQDQMQVAMGTMDGIID  
TVSAMHPLLPLLGLLKSHGKLILVGAPEKPLELPAFPL  
LMGRKMVGGS CIGGMKETQEMVDFAAKHGITS DIEVI  
PIDYVNTAIERMLKADVRYRFVIDIGNTIKSSHS

Figure 9

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/MY2012/000011

## A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl.

C12N 9/02 (2006.01)

C07K 14/415 (2006.01)

C12N 15/53 (2006.01)

A01H 1/00 (2006.01)

C12N 15/29 (2006.01)

C12N 15/82 (2006.01)

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)

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)

GENOMEQUEST (SEQ ID NO: 1-8),

EPODOC, WPI, CAPlus (lignin, monolignol, cinnamoyl CoA reductase - CCR, cinnamyl alcohol dehydrogenase -CAD, *Hevea*, rubber tree)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                                                                                                                                                                                                   | Relevant to claim No. |
|-----------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | US6855870 B2 (CAHOON ET AL., MO [US]) February 15 2005<br>See SEQ ID Nos: 3, 11, 12 and Example 3                                                                                                                                                                                                                    | 1-10                  |
| X         | Chabannes <i>et al.</i> Strong decrease in lignin content without significant alteration of plant development is induced by simultaneous down-regulation of cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) in tobacco plants, The Plant Journal (2001), 28 (3), 257-270<br>See pages 257-258 | 1-6                   |



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                                                                | "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                                              |
| "E"                                      | earlier application or patent but published on or after the international filing date                                                                               | "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                                                                     |
| "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) | "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 |
| "O"                                      | document referring to an oral disclosure, use, exhibition or other means                                                                                            | "&" | document member of the same patent family                                                                                                                                                                                                    |
| "P"                                      | document published prior to the international filing date but later than the priority date claimed                                                                  |     |                                                                                                                                                                                                                                              |

|                                                                                                                                                                                       |                                                                                                                                                   |
|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------|
| Date of the actual completion of the international search<br>7 June 2012                                                                                                              | Date of mailing of the international search report<br>12 June 2012                                                                                |
| Name and mailing address of the ISA/AU<br>AUSTRALIAN PATENT OFFICE<br>PO BOX 200, WODEN ACT 2606, AUSTRALIA<br>E-mail address: pct@ipaustalia.gov.au<br>Facsimile No. +61 2 6283 7999 | Authorized officer<br><b>Rebecca Hinton</b><br>AUSTRALIAN PATENT OFFICE<br>(ISO 9001 Quality Certified Service)<br>Telephone No : +61 2 6283 2194 |

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/MY2012/000011

**Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)**

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing filed or furnished:
  - a. (means)  
☐ on paper  
☐ in electronic form
  - b. (time)  
☐ in the international application as filed  
☐ together with the international application in electronic form  
☐ subsequently to this Authority for the purposes of search
2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.
3. Additional comments:  
The sequences provided in the drawing section were utilized for the purposes of carrying out a search.

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/MY2012/000011

**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.:  
because they relate to subject matter not required to be searched by this Authority, namely:
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:

See Supplemental Box I

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 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

International application No.

PCT/MY2012/000011

C (Continuation).

DOCUMENTS CONSIDERED TO BE RELEVANT

| Category* | Citation of document, with indication, where appropriate, of the relevant passages                                                  | Relevant to claim No. |
|-----------|-------------------------------------------------------------------------------------------------------------------------------------|-----------------------|
| X         | GenBank Accession No. XM_002314017 12 September 2006<br>Sequence shares 77.2% identity with SEQ ID NO:2 of the instant application  | 7-10                  |
| X         | GenBank Accession No. XM_002300175 12 September 2006<br>Sequence shares 83.59% identity with SEQ ID NO:3 of the instant application | 7-10                  |
| X         | GenBank Accession No. XM_002309234 12 September 2006<br>Sequence shares 83.03% identity with SEQ ID NO:4 of the instant application | 7-10                  |

**Supplemental Box I**

(To be used when the space in any of Boxes I to IV is not sufficient)

**Continuation of Box No III:**

This International Application does not comply with the requirements of unity of invention because it does not relate to one invention or to a group of inventions so linked as to form a single general inventive concept.

This Authority has found that there are different inventions based on the following features that separate the claims into two distinct groups:

- Claims 1-10 (in part) are directed to a method of reducing lignin content through down-regulation of cinnamoyl CoA reductase (CCR)1/CCR2, a transgenic plant with downregulated expression of CCR1/CCR2, and an isolated polynucleotide/polypeptide or recombinant gene construct encoding CCR1/CCR2. The feature of SEQUENCE ID NO:1 and 2 encoding CCR1/CCR2 is specific to this group of claims.
- Claims 1-10 (in part) are directed to a method of reducing lignin content through down-regulation of cinnamyl-alcohol dehydrogenase (CAD)1/CAD2, a transgenic plant with downregulated expression of CAD1/CAD2, and an isolated polynucleotide/polypeptide or recombinant gene construct encoding CAD1/CAD2. The feature of SEQUENCE ID NO:1 and 2 encoding CAD1/CAD2 is specific to this group of claims.

PCT Rule 13.2, first sentence, states that unity of invention is only fulfilled when there is a technical relationship among the claimed inventions involving one or more of the same or corresponding special technical features. PCT Rule 13.2, second sentence, defines a special technical feature as a feature which makes a contribution over the prior art.

When there is no special technical feature common to all the claimed inventions there is no unity of invention.

In the above groups of claims, the identified features may have the potential to make a contribution over the prior art but are not common to all the claimed inventions and therefore cannot provide the required technical relationship. Therefore there is no special technical feature common to all the claimed inventions and the requirements for unity of invention are consequently not satisfied *a priori*.

**INTERNATIONAL SEARCH REPORT**

Information on patent family members

International application No.

**PCT/MY2012/000011**

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

| Patent Document Cited in<br>Search Report                                                                                 |         | Patent Family Member |         |    |            |
|---------------------------------------------------------------------------------------------------------------------------|---------|----------------------|---------|----|------------|
| US                                                                                                                        | 6855870 | US                   | 6552249 | US | 2003159170 |
| Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001. |         |                      |         |    |            |
| END OF ANNEX                                                                                                              |         |                      |         |    |            |