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Genetic engineering of wood color in plants

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The invention relates to genetically engineering the wood color of woody plants by incorporation of the lignin pathway gene O-methyltransferase into the genome of the plants.
GENETIC ENGINEERING OF WOOD COLOR IN PLANTS

RELATED APPLICATION

This application claims the benefit of prior filed, copending provisional application Ser. No. 60/007,727 filed Nov. 30, 1995 entitled “GENETIC MODIFICATION OF ANGIOSPERM PULPWOOD SPECIES”.

FIELD OF THE INVENTION

The invention relates to genetically modifying the wood color of woody plants, and more particularly, to genetically modifying the wood color of woody plants through the genetic manipulation of a lignin pathway gene such as O-methyltransferase.

BACKGROUND OF THE INVENTION

Genetic engineering of forest tree species to conform to desired traits has shifted the emphasis in forest tree improvement away from the traditional breeding programs during the past decade. Although research on genetic engineering of forest trees has been vigorous, the progress has been slow due to very little progress has been reported regarding the genetic engineering of color in plant species. The ability to genetically alter the color of plants would be of great value to industries such as the furniture industry to make furniture from genetically modified wood or to the paper industry. Accordingly, there is a need for such genetic color modification of plant species.

Further, there is a need for improving the efficiency of pulping of wood. Considerable monetary and environmental costs are incurred by the paper industry in removing lignin from cellulose during the production of wood pulp and paper.

SUMMARY OF THE INVENTION

The invention provides a method to genetically alter the wood color of woody plants using the lignin pathway gene O-methyltransferase. The genetically altered color creates unique grain patterns in wood. Due to the genetic modification using a lignin pathway gene, the genetically altered woody plant also has an altered lignin structure making wood color of woody plants using the lignin pathway gene such as O-methyltransferase.

The color of woody plant species can be modified by transformation with a lignin pathway gene and specifically the lignin pathway gene that codes for the enzyme O-methyltransferase (OMT).

The O-methyltransferase enzyme of gymnosperms and angiosperms differ in substrate specificity for caffeic acid, with gymnosperms being monospecific for caffeic acid and angiosperms being bispecific, catalyzing the methylation of both caffeic acid and 5-hydroxyferulic acid. Gymnosperm lignin also termed guaiacyl lignin is composed mainly of one precursor (coniferyl alcohol) whereas angiosperm lignin also termed guaiacyl-syringyl lignin is formed from the polymerization of two main precursors (coniferyl alcohol and sinapyl alcohol). The ratio of syringyl to guaiacyl units is directly related to the efficiency of Kraft delignification, with higher syringyl quantities improving the efficiency. Softwoods largely synthesize coniferyl alcohol and form a lignin which is virtually completely made up of guaiacyl units. Hardwoods synthesize both coniferyl and sinapyl alcohols forming less condensed lignin of guaiacyl/syringyl mixtures in various proportions. The ratio of syringyl to guaiacyl units is directly related to the efficiency of Kraft pulping, as the lignin found in angiospermous trees is less condensed than the lignin in gymnospermous trees, and is therefore more easily separated from the wood’s cellulose in the pulping process. The sinapyl alcohol precursor of syringyl lignin is absent in softwoods, due to a deficiency of two key enzymes in the phenylpropanoid pathway; bi-specific O-methyltransferase and ferulic acid 5-hydroxylase.

The OMT enzyme has been studied in many plants some of which include Japanese black pine, shoots of bamboo, ginkgo, poplar, tobacco, spinach beet, soybean, parley, alfalfa root nodules, eucalyptus and aspen.

Generally, the wood color of woody plant species can be altered by genetic transformation with a homologous OMT gene in the sense orientation. The description of the invention below refers to aspen (Populus tremuloides) when necessary for the sake of example. However, it should be noted that the invention is not limited to the modification of the wood color in aspen. The method of the present invention is capable of being practiced for other woody plant species using an homologous OMT gene.

A. OMT Gene

The present invention utilizes a homologous OMT gene to genetically alter the wood color of woody plants. The invention as described below utilizes a cDNA clone of the OMT gene. However, it should be noted that genomic DNA can also be utilized in the present invention.

PURIFIED AND ISOLATED OMT DNA can be obtained using a cDNA cloning method such as set forth below and in Bugos et al., Plant Mol. Bio. 17:1203–1215 (1991) which is incorporated herein by reference. A cDNA clone encoding OMT can also be utilized in the present invention.

FIG. 1 is a diagram of construct pFOMT1 which contains a 1.1 kb bi-OMT cDNA sense fragment with the whole coding region between 35S enhancer-promoter/AMV RNA4 and NOS terminator, and FIG. 2 is a diagram of construct pFOMT2 which contains a 2.7 kb genomic bi-OMT full-length DNA in the sense orientation between 35S promoter and NOS terminator.

Before one embodiment of the invention is explained in detail, it is to be understood that the invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the drawings. The invention is capable of other embodiments and of being practiced or being carried out in various ways. Also, it is to be understood that the phraseology and terminology used herein is for the purpose of description and should not be regarded as limiting.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The method to genetically alter the wood color of woody plants is capable of being practiced for other woody plant species using an homologous OMT gene.
The differentiating xylem of the species is obtained. Total RNA is extracted from the developing secondary xylem. See for example Logemann et al., Anal. Biochem. 163:16–20 (1987). 5M guanidine hydrochloride is used in order to reduce starch gelatinization. The RNA is further purified by precipitation with 2.5M LiCl. See for example Okita et al., Plant Physiol. 69:834–839 (1982). Poly(A)+ RNA is isolated using Hybond-mAP paper. From the poly(A)+ RNA, double-stranded cDNA is prepared using a library construction system from Invitrogen Corporation, San Diego, Calif. See for example Sambrook et al., Molecular Cloning, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989). The double stranded cDNA is ligated to linkers and then cloned into a vector, for example to EcoRI-NotI linkers and then into the EcoRI site of λgt11 (Stratagene Cloning Systems, La Jolla, Calif.).

The vector is then used to transform or transfect a host cell. With λgt11, the insert-containing lambda vectors are packaged with lambda proteins and infected into Escherichia coli such as strain Y1090. See for example Mirenkorf et al., Methods in Enzymol. 152:458–569 (1987).

The cDNA library thus prepared can be screened in any suitable manner. In a preferred embodiment, the host cells are transformed or transfected in a manner allowing the host cell to express the polypeptide of the DNA inserted into the vector. This can be done by utilizing a vector having DNA sequences flanking the insertion area with one or more codons preferred for expression in E. coli cells. In such a case, the host cells themselves, or extracts of the host cells, can be screened with antibodies against the OMT enzyme.

The OMT enzyme used to prepare antibodies is purified from xylem using a combination of purification techniques as set forth in Bugos et al., Phytochemistry 31:5:1495–1498 (1992). The OMT enzyme is isolated from differentiating xylem and is then purified such as 180-fold by a process using DEAE-cellulose chromatography, HPLC gel filtration and affinity chromatography on S-adenosyl-L-homocysteine agarose. Using denaturing polyacrylamide gel electrophoresis (SDS-PAGE), one protein band with a molecular weight of 45,000 daltons is observed. The purified OMT enzyme catalyzes the methylation of both 5-hydroxyferulic acid and caffeic acid, with an activity ratio of 3:1:1. S-adenosyl-L-homocysteine is an effective inhibitor of the enzyme.

Using the purified enzyme, rabbit antibodies to the OMT enzyme can be produced in a conventional manner. Bugos et al., Phytochemistry 31:5:1495–1498 (1992). The cDNA can then be screened with antibodies against the OMT enzyme. Clones can be detected by the antibodies as expressing OMT polypeptides. The DNA from the clones can then be isolated. The clones have an insert DNA of about 1.5 kb. After the putative, positive λgt11 clones are plaque purified. Insert DNA of a clone is excised with NotI and sub-cloned into Bluescript II. See for example Sambrook et al., Molecular Cloning, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor (1989).

The nucleotide sequence can then be determined by the dideoxynucleotide method, for example Sanger et al., Proc. Natl. Acad. Sci. 74:5463–5467 (1977), using a T7 Sequencing Kit for the entire length of the clone in both directions. Subclones are prepared by excision with EcoRI, BamHI, XhoI and HinClI and sequenced using primers complementary to sequences bordering the multiple cloning site of Bluescript II (Stratagene Cloning Systems, La Jolla, Calif.). Synthetic oligonucleotide primers are used to verify overlap regions of restriction sites. An open-reading frame of 1095 bp encodes a polypeptide of 365 amino acid residues with a predicted molecular weight of 39,802 daltons which corresponds well with the size of the bispecific OMT subunit.

The nucleotide sequence of bispecific aspen OMT cDNA clone is set forth as SEQ ID NO:1. The nucleotide sequence of SEQ ID NO:1 is numbered beginning with the 5' end of the cDNA clone. SEQ ID NO:1 includes the amino acid sequence, in standard three letter designations, directly beneath the corresponding codons.

The deduced amino-acid sequence for aspen bispecific OMT is set forth as SEQ ID NO:2. The first methionine of the open reading frame of the amino acid sequence is designated as the first amino acid of the putative polypeptide.

Three internal peptides of purified aspen bispecific OMT sequenced by automated Edman degradation are identical to portions of SEQ ID NO:1. Since the amino terminus of aspen bispecific OMT is blocked, as determined by the automated Edman degradation, these three internal peptides were deduced by digesting the purified enzyme with Staphylococcus aureus endopeptidase Glu-C. The peptides were isolated by reverse-phase HPLC, and were sequenced by automated Edman degradation.

The poly nucleotide code for the OMT enzyme was expressed as a protein in E. coli, as the Bluescript II vector has codons preferred for expression in E. coli cells. The OMT expressed from the Bluescript II vector in E. coli was found to have bispecific activities in approximately the same ratio as that of the natural enzyme. This expressed protein was also recognized by the antibodies for bispecific OMT enzyme.

The antibody for aspen bispecific OMT was also used to select an OMT clone from an alfalfa cDNA library, which was prepared from RNAs induced by a fungal elicitor, and 85% of the alfalfa OMT's predicted amino acid residues were found to be identical to that of the aspen bispecific OMT. This demonstrates a substantial amount of duplication in amino acid sequences encoding plant O-methyltransferases from diverse plant species.

The OMT gene has been isolated and sequenced in the following plant species: aspen, hybrid aspen, hybrid poplar, alfalfa, tobacco, prunus, zinnia and eucalyptus.

Comparisons have been made between the nucleotide level between the nucleotide sequence of aspen OMT cDNA and that of other plant species. The percentage identity of OMT of various plant species is set forth below with accession numbers referring to the Gene Bank:

Hybrid aspen (P.Kitakamiensis) OMT genomic DNA, accession #D49710, 97% identity;

Hybrid aspen (P.Kitaekamienis) OMT genomic DNA, accession #D49711, 89% identity (exons);

Prunus (Prunus amygdalus) OMT cDNA, accession #X83217, 81% identity;

Eucalyptus (Eucalyptus gunnii) OMT cDNA, accession #X74814, 74% identity;

Alfalfa (Medicago sativa) OMT cDNA, accession #M63853, 77% identity;

Tobacco (Nicotiana tabacum) OMTla cDNA, accession #X74452, 74% identity;

Tobacco (Nicotiana tabacum) OMTlb cDNA, accession #X74453, 75% identity;

Zinnia (Zinnia elegans) OMT cDNA, accession #U19911, 71% identity; and

Chrysosplenium americanum OMT cDNA, accession #U16703, 75% identity.
B. Transformation and Regeneration

Several methods for gene transformation of plant species with the OMT gene are available such as the use of a transformation vector, agroinfection, electroporation, particle bombardment with a gene gun or microinjection. Preferably, a binary vector construct such as those set forth in FIGS. 1 and 2 is mobilized into a strain of Agrobacterium species. Preferably, Agrobacterium such as _Agrobacterium tumefaciens_ strain C58 is used as the DNA delivery system due to its efficiency and low cost. See Koncz, C. et al., Mol. Gen. Genet. 204:383-396 (1986). The vectors are mobilized in _Agrobacterium tumefaciens_ using the freeze-thaw method of Holstein et al., Mol. Gen. Genet. 163:181-187 (1978). The vectors are described in Tsai et al., Plant Cell Reports 14:94-97 (1994) which is hereby incorporated by reference. The constructs pFOMT1 and pFOMT2 are also available from Michigan Technological University, Houghton, Mich.

Explants of young leaves from cuttings of aspen are obtained by cutting leaf disks from the young leaves along the midrib of the leaves using a cork borer that is 7 mm in diameter. The explants are surface sterilized in 20% commercial bleach for 10 minutes followed by rinsing three times with sterile double-distilled water.

All of the culture media used in this method includes the basal medium of woody plant medium (WPM) as described in Lloyd et al., Proc. Int. Plant Prop. Soc. 30:421-437 (1980) and supplemented with 2% sucrose. 650 mg/L calcium gluconate and 500 mg/L MES are added as pH buffers as described in De Block, Plant Physiol. 93:1110-1116 (1990). All culture media is adjusted to pH 5.5 prior to the addition of 0.075% Difco Bacto Agar and then autoclaved at 121° C. and 15 psi for 20 minutes. Filter sterilized antibiotics are added to all culture media after autoclaving. All culture media are maintained at 23±1° C. in a growth chamber with 16 hour photoperiods (160 μmol/m²/s) except for callus induction (as will be described later) which is maintained in the dark.

The sterilized explants are then inoculated with the mobilized vector with an overnight-grown agrobacterial suspension containing 20 μM acetosyringone. After cocultivation, the explants are washed in sterile distilled water containing 300 mg/L cefotaxime to decontaminate. The explants are blotted dry with sterile Whatman No. 1 filter paper and transferred onto callus induction medium containing 40 mg/L kanamycin for selection of transformed cells. The callus induction medium is the basal medium with the addition of 6-benzyladenine (BA) and 2,4-dichlorophenoxyacetic acid (2,4-D) at concentrations of 0.5 mg/L and 1 mg/L, respectively, to induce callus. Cefotaxime at 300 mg/L is added to kill Agrobacterium.

The kanamycin-resistant explants are then subcultured on fresh callus induction media every two weeks. Callus formation occurs after approximately four weeks. Formed callus are separated from the explant and subcultured periodically for further proliferation.

When the callus clumps reach approximately 3 mm in diameter, the callus clumps are transferred to shoot regeneration medium. The shoot regeneration medium is the basal medium containing 100 mg/L kanamycin, 0.5 mg/L thidiazuron (TDZ) as a plant growth regulator and cefotaxime at 300 mg/L to kill Agrobacterium. Shoots were regenerated about four weeks after callus is transferred to regeneration medium.

Accordingly, as soon as the shoots are regenerated, they are immediately transferred to hormone-free elongation medium containing 100 mg/L kanamycin and, whenever necessary, cefotaxime (300 mg/L), to promote elongation. Green and healthy shoots elongated to 2-3 cm in length are excised and planted separately in a hormone-free rooting medium containing 100 mg/L kanamycin. The efficient uptake of kanamycin by shoots during their rooting stage provides the most effective selection for positive transformants.

Transgenic plants are then transplanted into soil medium of vermiculite:peatmoss:perlite at 1:1:1 and grown in the greenhouse.


C. Color Alteration and Lignin Structure

The results of the transformation can be confirmed with conventional PCR and Southern analysis.

The present invention alters the natural wood color of woody plants. In aspen, the natural white/yellow color of the wood is altered to a brownish-red. The appearance of the wood color in aspen is achievable in both a solid and spotted appearance and is stable over time. Furthermore, the altered color of the wood appears in plants that are vegetatively propagated from the original transgenic plant. It should also be noted that with the present invention, the alteration of the natural color in the woody plants is not linked to any threshold increase or decrease in OMT activity.

The transformation of woody plants with a homologous OMT gene also alters the structure of lignin since the OMT gene is a part of the lignin synthesis pathway. For example, in aspen, due to cosuppression, the syringyl units decrease thus altering the structure of the lignin. The altered lignin will aid in the more efficient pulping of the wood of the transgenic plants.
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   (B) TYPE: amino acid
   (D) TOPOLOGY: linear

(11) MOLECULE TYPE:
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(111) HYPOTHETICAL: no

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| Ala Lys Ala Gly Pro Gly Ala Phe Leu Ser Thr Ser Glu Ile Ala Ser 50 55 60 | |
| His Leu Pro Thr Lys Asn Pro Asp Ala Pro Val Met Leu Asp Arg Ile 65 70 75 80 | |

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-continued
We claim:

1. A method for altering the wood color of a woody plant comprising incorporating into the genome of the woody plant a nucleotide sequence encoding the endogenous full-length enzyme O-methyltransferase in the sense orientation such that when the nucleotide sequence is expressed in the woody plant, the wood color of the woody plant is altered from the natural color.

2. A method for altering the natural wood color of a woody plant as set forth in claim 1 wherein the color of the altered wood is reddish-brown.

3. A method for altering the natural wood color of a woody plant as set forth in claim 1 wherein the nucleotide sequence is incorporated in the genome of the woody plant by transformation.

4. A method for altering the natural wood color of a woody plant as set forth in claim 3 wherein the transformation includes the use of an Agrobacterium transfer vector.

5. A method for altering the natural wood color of a woody plant as set forth in claim 1 wherein the nucleotide sequence is a cloned cDNA sequence of O-methyltransferase.

6. A method for altering the natural wood color of a woody plant as set forth in claim 1 wherein the nucleotide sequence includes a gene promoter sequence.

7. A method for altering the natural wood color of a woody plant as set forth in claim 1 wherein when the nucleotide sequence is expressed in the woody plant, the structure of the lignin of the woody plant is altered.

8. A method for altering the natural wood color of a woody plant as set forth in claim 1 wherein the gene promoter sequence includes CaMV35S.

9. A method for altering the natural wood color of a woody plant as set forth in claim 1 wherein the woody plant is of the genus Populus.

10. A woody plant having the color of its wood altered through the incorporation into the genome of the woody plant a nucleotide sequence encoding the endogenous full-length enzyme O-methyltransferase in the sense orientation.

11. A woody plant as set forth in claim 10 wherein the color of the altered wood is reddish-brown.
12. A woody plant as set forth in claim 10 wherein the nucleotide sequence is incorporated in the genome of the woody plant by transformation.

13. A woody plant as set forth in claim 12 wherein the transformation includes the use of an Agrobacterium transfer vector.

14. A woody plant as set forth in claim 10 wherein the nucleotide sequence is derived from cloned cDNA of O-methyltransferase.

15. A woody plant as set forth in claim 10 wherein the nucleotide sequence includes a gene promoter sequence.

16. A woody plant as set forth in claim 15 wherein the gene promoter sequence includes CaMV35S.

17. A woody plant as set forth in claim 10 wherein when the nucleotide sequence is expressed in the woody plant, the structure of the lignin of the woody plant is altered.

18. A woody plant as set forth in claim 10 wherein the woody plant is of the genus Populus.

19. A recombinant DNA comprising a gene promoter sequence, a gene terminator, and an interposed region comprising a nucleotide sequence encoding the endogenous full-length enzyme O-methyltransferase in the sense orientation such that when the nucleotide sequence is expressed in the woody plant, the wood color of the woody plant is altered from its natural color.

20. A recombinant DNA as set forth in claim 19 wherein the gene promoter sequence includes CaMV35S.

21. A recombinant DNA as set forth in claim 19 and further when the nucleotide sequence is expressed in the woody plant, the structure of the lignin of the woody plant is altered.

22. A method for altering the wood color of a plant of the genus Populus comprising incorporating into the genome of the plant through transformation a nucleotide sequence encoding the endogenous full-length enzyme O-methyltransferase in the sense orientation such that when the nucleotide sequence is expressed in the plant, the wood color of the plant is altered from its natural color.

23. A method for altering the wood color of a plant of the genus Populus as set forth in claim 22 wherein the color of the altered wood is reddish-brown.

24. A method for altering the wood color of a plant of the genus Populus as set forth in claim 22 wherein the transformation includes the use of an Agrobacterium transfer vector.

25. A method for altering the wood color of a plant of the genus Populus as set forth in claim 22 wherein the nucleotide sequence includes a cloned cDNA sequence of O-methyltransferase.

26. A method for altering the wood color of a plant of the genus Populus as set forth in claim 22 wherein the nucleotide sequence includes a gene promoter sequence.

27. A method for altering the wood color of a plant of the genus Populus as set forth in claim 26 wherein the gene promoter sequence includes CaMV35S.

28. A method for altering the wood color of a plant of the genus Populus as set forth in claim 22 wherein when the nucleotide sequence is expressed in the woody plant, the structure of the lignin of the plant is altered.

29. A woody plant of the genus Populus having the natural color of its wood altered through the incorporation into the genome of the woody plant a nucleotide sequence encoding the endogenous full-length enzyme O-methyltransferase in the sense orientation.

30. A woody plant of the genus Populus as set forth in claim 29 wherein the nucleotide sequence includes a CaMV35S gene promoter sequence.