Genetic engineering of syringyl-enriched lignin in plants

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ABSTRACT

The present invention relates to a novel DNA sequence, which encodes a previously unidentified lignin biosynthetic pathway enzyme, sinapyl alcohol dehydrogenase (SAD) that regulates the biosynthesis of syringyl lignin in plants. Also provided are methods for incorporating this novel SAD gene sequence or substantially similar sequences into a plant genome for genetic engineering of syringyl-enriched lignin in plants.

17 Claims, 10 Drawing Sheets
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FIG. 2A  SAD cDNA sequence

1  TTTTTTTTTT TTTCCTTAGCC TTCCCTTCGG ACGATATTTCA CTTATCTGAA
51  GCAAGGACACATA GTCCTGAGCTC ACCGAGAAAGAA CAAACCCCGTG TGAAGGCCCTT
101  CGGGTTGGGGCT CTTAGGGAATC AAATCTGGTCA TCTTTTTCCTG TACAACCTTCT
151  CCAAGGAGGGCC AACTGGTGAAGAAGGATGTGA GGTCAAGGTGCTGACTGCTG
201  GGGATATGGCC ATTCCTGACCT TCACAGTATC AAGAATGACTG GGGCCTTCTC
251  CATGTACCCT TTTGTTTCTCG GGCACTGAAAT TGGGGGGAAAT GTGACAGAAG
301  TTCCAGAGCAA GGTGGAAAAAG GTTAATGTGG GAGCAAAAGGT GGGCGTGCGGA
351  TGCTTGCTTTG GTGCAATGCTA CTCCCTGTGAG AGTTGTGCAC ATGATCCTGAA
401  AAATTACTGT CCAAAATAGTA TCCTGACATA CGCCTCCATA TACCATGACG
451  GAACCATCAG TTAGGGTGCGC TACCTGACAT CACATTGTGCG TAAAGGAACGC
501  TACATCATATC GATTTCCCGAG TAGCATGCGG GCTGAGCTTCT CTTGGAAGACT
551  CTTTGGTACC GGGATACAGA GTGATAGCTC TTGGGAATAT TTTGGACTACT
601  ATGAAACCGGC TAAGCATATC GGTTATGGTAC AGCTTGGTAC ACTTGGTACG
651  GTGGCTGTACA AATTTGGCCAA CGCCTTTGGA TCTAAAGTAG CAGTAATGAC
701  TACCTCCCTC TCCACAAGAGG AGGAGGCTTTG GAAGAACTCTC GGGCGAGACT
751  CATTTTTGCTG TAGTGCTGTAC CAAGAGCAA TGCAGCTGAC CGCAGGAACA
801  TTAGATGGCCA TCACAGTATAC AGTTTCTGCA GTTCACCCCG TTTGCGCTTT
851  GTTTGGACAGT TGGAATGCTC AGCGGAACTC TAGCTTGGTAC GGTGACCGGG
901  AAAAGCCTCT TGGCTACCT GCCTTTCTCT GTATGGCTGG AAGGAAGATA
951  GTTGCCGGGA GTTTGATTTG AGCGATGAGA AACACAGGAAGAATGATGAGTGA
1001 TTTCAGCGCA AAAAAACACA TCACAGCAGA TAGCGAGCTT ATTTCACCGG
1051 ACTATCTTAAC TACCGGCATA GACGTGTGGG CTAAAAACGA TGTCAGATAC
1101 CGATTTGGTA CTTGACGGTGAA CATATTGCTTG GCAAGCTACGA AGCCCCTAAGG
1151 AGAGACATGC ATGTTCCTGAA ACCCTTATAPA AAACTCAGTA ACATGTTGTTG
1201 ATTTCATGAA AAAAAAGATT ACTTTTGCGA TTTTTCTTTA ATCAAGCAAG
1251 TGGTTTCGCAA AACTTAAACAT CGGCAATTACC CTGGCGAGCTA CGGAAACGCC
1301 TTAGAATTTG TTTGAATGTT GTTTTCATTA GGTTGATACC ATGCTCTGCA
1351 TTCTTTGGTA AGATCCATTG ATAGTTTGCGT GAATTGCATG AACTAAATACT
1401 ATGTTTGGCGG CTCTCTTCTCA AAAAAAAAAA AAAAAAAAAA AAAAA
FIG. 2B  SAD protein sequence

1  MSKSPEEEHP  VKAFCWAWARD  QSGHLSPPNF  SRRATGEEEDV  RFKVLYCGIC
51  HSDLHSIKND  WGFSMYPLVP  GHEIVGEVTE  VGSKVKKVN V  GDKVCGGCLV
101  GACHSCESCA  NDLENYCPKM  ILTYASIYHD  GTITYGGYSD  HMVANERYII
151  RPPDNMPLDG  GAPLLCAGIT  VYSPLKYFGL  DEPGKHIGIV  GLGGLGHVAV
201  KFAKAFGSKV  TVISTSPSSK  EEALKNFGAD  SFLVSRDQE Q  MQAAAGTLDG
251  IIDTVSAVHP  LLPLFGLLKS  HGKLILVGAP  EKPLELPAFS  LIAGRKIVAG
301  SGIGGMKETQ  EMIDFAAKHN  ITADIEVIST  DYLNTAIERL  AKNDVRYRFV
351  IDVGNLAAAT  KP*
Phylogenetic Analysis of Aspen SAD and Plant CADs.

An unweighted pair-group method using arithmetic averages was used for phylogenetic tree analysis of aspen SAD (PtSAD) and other full-length plant CAD protein sequences available in the Gen-Bank database.
Figure 4 Molecular Characterization of Aspen PtCAD and PtSAD

(A) and (B) Genomic DNA gel blot analysis. Aspen Genomic DNA (10μg/lane) was digested with restriction enzymes and hybridized with 32P-labeled full-length PtCAD (A) and PtSAD (B) cDNAs.

(C) and (D) RNA gel blot analysis of PtCAD and PtSAD tissue-specific expression patterns. Total RNA (10μg/lane) from each organ or tissue type was hybridized with 32P-labeled full-length PtCAD (C) and PtSAD (D) cDNAs.

(E) and (F) Protein gel blot analysis of anti-PtCAD and anti-PtSAD antibody specificity and tissue-specific expression of PtCAD and PtSAD. Immunoblots of E. coli-expressed and affinity-purified PtCAD and PtSAD recombinant proteins (25 ng/lane) and plant protein extracts (10μg/lane) with anti-PtCAD (E) and anti-PtSAD (F) antibodies.
Figure 5. HPLC-UV/MS Analysis of Recombinant PtCAD and PtSAD Reactions.

(A) HPLC-MS (selected ion monitoring, 70 V; mass-to-charge ratio [m/z], 179.0) chromatogram showing the PtCAD reduction (see Methods) of coniferaldehyde (retention time [Rt] = 13.58 min) into coniferyl alcohol (Rt = 7.79 min). The inset shows the negative ion electrospray mass spectrum (scanning mode at 70 V) of coniferyl alcohol with properties (UV [HPLC mobile phase] λmax I, 262 nm, λmax II, 294 nm; MS [150 V] mass-to-charge ratio [%], 179.1 [100%], 164 [38%], 161 [25%]) identical to the authentic standard. MW, molecular weight.

(B) HPLC-MS (selected monitoring, 70 V; mass-to-charge ratio [m/z], 209.0) chromatogram showing the PtSAD-mediated sinapaldehyde (Rt=12.09 min) reduction into sinapyl alcohol (Rt = 7.03 min). The inset shows the negative ion electrospray mass spectrum of sinapyl alcohol with properties (UV[HPLC mobile phase] λmax I, 222 nm, λmax II, 274 nm; MS [150 V] mass-to-charge ratio [%], 209.1 [100%], 194 [41%], 176 [11%]) identical to the authentic standard. MW, molecular weight.

(C) and (D) HPLC-MS (selected monitoring, 70 V; mass-to-charge ratio [m/z], 179.0 and 209.0) chromatograms of PtCAD and PtSAD reactions with a mixture of equal molar coniferaldehyde (Rt=13.58 min) and sinapaldehyde (Rt=12.09 min). Coniferyl alcohol (Rt=7.79 min) is the exclusive product of the PtCAD reaction (C), and sinapyl alcohol (Rt=7.03 min) is the only product of the PtSAD reaction (D).

O-Coumaric acid was the internal standard (I.S.) in all reactions.
Figure 6. Inhibition Kinetics of PtCAD and PtSAD.

Lineweaver-Burk plots of $1/v$ versus $1/[S]$ in the presence of different levels of inhibitor concentrations as indicated. The insets show re-plots of apparent $K_m'$ versus the corresponding inhibitor concentration, used to calculate the $K_i$.

(A) Competitive inhibition effects of coniferaldehyde on PtCAD reduction of sinapaldehyde in mixed substrate assays.

(B) Competitive inhibition effects of coniferaldehyde on PtSAD reduction of coniferaldehyde in mixed substrate assays.
Figure 7. Detection of Guaiacyl and Syringyl Lignins in Aspen Stem.

Cross/Bevan histochemical analysis of transverse sections of stem internodes showing the exclusive presence of guaiacyl lignin in primary xylem tissues ([A] to [C]), and the deposition of guaiacyl-syringyl lignin in secondary growth tissues ([E] to [G] and [I]). The differential deposition of these lignins along the stem was confirmed by thioacidolysis analysis of the stem lignin ([D] and [H]).

(A) Internode 3.
(B) Internode 4.
(C) A magnified section of the image in (B).
(D) and (H) Gas chromatograms of trithioethylated monomeric lignin products after thioacidolysis, demonstrating the exclusive presence of guaiacyl lignin in internodes 1 to 4 ([D]) and the presence of guaiacyl and syringyl lignins in internodes 5 to 20 ([H]). Typical erythro (a) and threo (t) isomers (1:1 ratio) of guaiacyl and syringyl monomers were present. The internal standard (I.S.) was hexacosane.
(E) Internode 8. The primary xylem is the only vascular tissue having the pure guaiacyl lignin.
(F) Internode 10. The primary xylem is the only vascular tissue having the pure guaiacyl lignin.
(G) Internode 8 revealing the sequential deposition of guaiacyl followed by syringyl lignins in secondary xylem elements. Note the deposition of only the guaiacyl lignin in metaxylem vessels.
(H) Internode 6 showing the onset of syringyl lignin deposition in primary phloem fibers.

Dsxf, developing secondary xylem fibers; Dsxv, developing secondary xylem vessels; Mxv, metaxytem vessels; Ppc, protophloem parenchyma cells; Ppf, primary phloem fibers; Px, primary xylem; Pxv, protoxylem vessels; Rp, ray parenchyma cells; Sx, secondary xylem. Bars in (A), (B), (E), and (F) = 100 µm; bars in (C), (G), and (I) = 30 µm.
Figure 8. Immunolocalization of PtCAD, PtCAD5H, and PtSAD Proteins in Aspen Stem.

Light micrographs of stem transverse sections showing localizations of PtCAD ([A], [D], [F], and [H]) PtCAD5H ([C]) and PtSAD ([B], [E], [G], [I]).

(A) to (C) Internode 3. PtCAD was localized exclusively to primary xylem elements (a), whereas PtSAD (B) and PtCAD5H (C) were not detected in these primary xylem elements but were abundant in protophloem parenchyma cells and the medullary sheath.

(D) and (E) Primary phloem fibers in internode 8.

(F) and (G) Internode 8. Note the strong PtCAD signals in developing secondary xylem vessels (F), but PtSAD signals were nearly absent from these cells (G).

(H) and (I) Internode 12. The appearance of PtSAD (I) lagged behind that of PtCAD (H) in fusiform initials.

Ap, axial parenchyma cells; Ffi, fusiform initials; Ms, medullary sheath. Other abbreviations are as given in Figure 7.

Bars in (A) to (C), (H), and (I) = 50 μm; bars in (D) to (G) = 30 μm.
Figure 9. Immunoblot Detection of CAD and SAD Proteins in Various Plants.

CAD was detected by immunoblotting in developing xylem of all plants analyzed (top), but SAD was found only in angiosperm species (bottom). Seventy-five nanograms of recombinant protein per lane was used, and other lanes were loaded with 10 μg of plant xylem crude protein extracts.
GENETIC ENGINEERING OF SYRINGYL-ENRICHED LIGNIN IN PLANTS

CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims the benefit of U.S. Provisional Application No. 60/230,086, filed on Sep. 5, 2000, and is incorporated herein by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with United States Government support awarded by the Energy Biosciences Program, and the United States Department of Agriculture, research grant numbers USDA 99-35103-7986, USDA 01-03749, and DOE DE-FG02-01ER15179. The United States Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

The invention relates to a novel DNA sequence, which encodes a previously unidentified lignin biosynthetic pathway enzyme, sinapyl alcohol dehydrogenase (SAD), that regulates the biosynthesis of syringyl lignin in plants. Methods for incorporating this novel SAD gene sequence or sequences similar to this SAD gene into plant genome for genetic engineering of syringyl-enriched lignin in plants are also provided.

Lignin, a complex phenolic polymer, is a major part of the supportive structure of most woody plants including angiosperm and gymnosperm trees which, in turn, are the principal sources of fiber for making paper and cellulose products. Lignin generally constitutes about 25% of the dry weight of the wood, making it the second most abundant organic compound on earth after cellulose. Lignin provides rigidity to wood for which it is well suited due, in part, to its resistance to biochemical degradation.

Despite its importance to plant growth and structure, lignin is nonetheless problematic to post-harvest, cellulose-based wood/crop processing for fiber, chemical, and energy production because it must be removed or degraded from cellulose at great expense. Certain structural constituents of lignin, such as the guaiacyl moiety, promote monomer cross-linkages that increase lignin resistance to degradation (Sarkanen, 1971; Chang and Sarkanen, 1973; Chiang and Funaoka, 1990). In angiosperms, lignin is composed of a mixture of guaiacyl and syringyl monolignols, and can be degraded at considerably less energy and chemical cost than gymnosperm lignin, which consists almost entirely of guaiacyl moieties (Freudenberg, 1965). It has been estimated that, if syringyl lignin through genetic engineering, could be incorporated into gymnosperm guaiacyl lignin or into angiosperms to increase the syringyl lignin content, the annual saving in processing of such genetically engineered plants as opposed to their wild types would be in the range of $8 to $10 billion in the U.S. alone. Consequently, there has been long-standing incentive to understand the biosynthesis of syringyl monolignol to genetically engineer plants to contain more syringyl lignin, thus, facilitating wood/crop processing (Trotter, 1990; Bugos et al., 1991; Boudet et al., 1995; Hu et al., 1999).

Although it has been known that syringyl lignin is derived from guaiacyl lignin, only a partial syringyl monolignol pathway and its genes encoding the enzymes that catalyze the steps of the pathway have been uncovered. This partial syringyl monolignol pathway, which diverges from guaiacyl pathway at coniferaldehyde, is mediated by genes encoding the enzymes coniferyl alcohol 5-hydroxylase (CAD5H) (Osakabe et al., 1999) and S-adenosyl-L-methionine (SAM)-dependent 5-hydroxyconiferaldehyde O-methyltransferase (AldOMT) (Li et al., 2000), respectively, for the formation of sinapaldehyde (see, FIG. 1). However, sinapaldehyde must be enzymatically converted into sinapyl alcohol, the syringyl monolignol, for the biosynthesis of syringyl lignin in plants (see, FIG. 1). The gene encoding such an enzyme, i.e., a sinapyl alcohol dehydrogenase (SAD), has never been identified nor cloned and represents a missing gene that is indispensable for genetic engineering of syringyl lignin in plants. Thus, there is a need for the identification and isolation of this key gene (SAD) for genetic augmentation of syringyl monolignol biosynthesis in plants and for the methods for such genetic manipulation comprising the use of this key gene.

BRIEF SUMMARY OF THE INVENTION

The present invention provides an isolated complete DNA sequence encoding a novel enzyme, sinapyl alcohol dehydrogenase, SAD, that is obligatory to and plays a dominant role in the biosynthesis of syringyl lignin in plants. SAD catalyzes the conversion of sinapaldehyde to sinapyl alcohol, the syringyl monolignol. The production of SAD, and hence, the production of syringyl monolignol may be increased by supplying extra copies of the SAD gene, or decreased by insertion of the SAD gene or a portion thereof into the genome of a plant, in antisense orientation so that the amount of SAD for catalyzing the sinapyl alcohol is reduced, if so desired.

In one aspect, the invention provides whole gymnosperm plants containing genes which increase production of syringyl lignin and repress production of guaiacyl lignin. Thus the invention addresses the problem of providing gymnosperm species which are easier to delignify in pulping processes.

In another aspect, the invention provides a method for making an expression cassette insertable into a gymnosperm cell for the purpose of inducing formation of syringyl lignin in a gymnosperm plant derived from the cell.

In an additional aspect, the present invention provides a method for modifying genes involved in lignin biosynthesis in gymnosperm species so that production of syringyl lignin is increased while production of guaiacyl lignin is suppressed.

The invention advantageously identifies, isolates, and/or clones those genes in angiosperms responsible for production of syringyl lignin. The invention also advantageously provides for identification and isolation of a polynucleotide encoding a sinapyl alcohol dehydrogenase (SAD) from an angiosperm species and for the use of such polynucleotide to alter the lignin biosynthesis in a gymnosperm.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 is a schematic representation of plant monolignol pathways for production of coniferyl alcohol and sinapyl alcohol;

FIG. 2 illustrates a SAD polynucleotide DNA sequence (SEQ ID NO: 1) and its corresponding SAD amino acid sequence (SEQ ID NO: 2), FIGS. 2A and 2B respectively; FIG. 3 is a phylogenetic analysis of Aspen SAD and plant CADs;

FIG. 4 illustrates molecular characterization of Aspen PICAD and PSAD;
The present invention relates to a gene that encodes sinapyl alcohol dehydrogenase (SAD), the last key enzyme in syringyl monolignol biosynthesis and provides an isolated complete DNA sequence encoding the novel enzyme, SAD. The invention further provides a method of altering the lignin composition, i.e., producing syringyl-enriched lignin in plants, by transforming the plants with the SAD gene wherein the gene is expressed and causes an increased syringyl content of the lignin polymer.

The present invention is of particular value to the paper and pulp industries because lignin containing higher syringyl monomer content is more susceptible to chemical delignification. Currently, vast amounts of energy and time are consumed in the delignification process. Woody plants transformed with an active SAD gene offer a significant advantage in the delignification process over conventional paper feedstocks. Similarly, modification of the lignin composition in grasses by the insertion and expression of a heterologous SAD gene offers a unique method for increasing the digestibility of grasses and is of significant potential economic benefit to the farm and agricultural industries.

The invention provides a gene and a DNA construct useful for the transformation of plant tissue to alter the lignin monomer composition. Plants suitable for transformation in accordance with the present invention include plants that naturally lack syringyl lignin or those that accumulate lignin with a high guaiacyl-syringyl ratio. Plants also suitable for transformation in accordance with the present invention include plants whose lignin could be modified using antisense transformation constructs that reduce the syringyl contents of the transgenic plant’s lignin if such an alternation is desirable. Specifically, suitable plants include, but are not limited to gymnosperms, angiosperms, grasses, legumes, forage crops and the like.

The terms used in this specification generally have their ordinary meanings in the art, within the context of the invention and in the specific context where each term is used. Certain terms are discussed below, or elsewhere in the specification, to provide additional guidance to the person of skill in the art in describing the compositions and methods of the invention and how to make and use them. It will be appreciated that the same thing can be said in more than one way. Consequently, alternative language and synonyms may be used for any one or more of the terms discussed herein, nor is any special significance to be placed upon whether or not a term is elaborated or discussed herein. Synonyms for certain terms are provided. A recital of one or more synonyms does not exclude the use of other synonyms. The use of examples anywhere in this specification, including examples of any terms discussed herein, is illustrative only, and in no way limits the scope and meaning of the invention or of any exemplified term. Likewise, the invention is not limited to the preferred embodiments.

DETAILED DESCRIPTION OF THE INVENTION

FIG. 5 illustrates HPLC-UV/MS analysis of recombinant PtCAD and PtSAD reactions;
FIG. 6 illustrates inhibition kinetics of PtCAD and PtSAD;
FIG. 7 illustrates the location of guaiacyl and syringyl lignins in Aspen stem;
FIG. 8 illustrates the immunolocalization of PtCAD and PtSAD proteins in Aspen stem;
FIG. 9 illustrates the immuno-reactivity of CAD and SAD proteins in various plants.

As used herein, “gene” refers to a nucleic acid fragment that expresses a specific protein including the regulatory sequences preceding (5' noncoding) and following (3' noncoding) the coding region. “Native” gene refers to the gene as found in nature with its own regulatory sequences. “Non-coding region” refers to that portion of the gene that does not directly encode a polypeptide. The boundaries of the non-coding region are located before the start codon and after the stop codon. The non-coding region includes the untranslatable regions of the genomic DNA.

“Endogenous gene” refers to the native gene normally found in its natural location in the genome.

“Transgene” refers to a gene that is introduced by gene transfer into the host organism.

“Coding sequence” refers to that portion of the gene that contains the information for encoding a polypeptide. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxyl) terminus. A coding sequence can include, for example, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA, and even synthetic DNA sequences.

“Promoter” (or promoter sequence) refers to a DNA sequence, in a given gene, which sequence controls the expression of the coding sequence by providing the recognition site for RNA polymerase and other factors required for proper transcription. Most genes have regions of DNA sequence that are promoter sequences which regulate gene expression. Promoter regions are typically found in the 5' flanking DNA sequence upstream from the coding sequence in both prokaryotic and eukaryotic cells. A promoter sequence provides for regulation of transcription of the downstream gene sequence and typically includes from about 50 to about 2000 nucleotide base pairs. Promoter sequences also contain regulatory sequences such as enhancer sequences that can influence the level of gene expression. Some isolated promoter sequences can provide for gene expression of heterologous DNAs, that is DNA different from the natural homologous DNA. Promoter sequences are also known to be strong or weak or inducible. A strong promoter provides for a high level of gene expression, whereas a weak promoter provides for a very low level of gene expression. An inducible promoter is a promoter that provides for turning on and off of gene expression in response to an exogenously added agent or to an environmental or developmental stimulus. An isolated promoter sequence that is a strong promoter for heterologous DNAs is advantageous because it provides for a sufficient level of gene expression to allow for easy detection and selection of transformed cells, and provides for a high level of gene expression when desired. A promoter may also contain DNA sequences that are involved in the binding of protein factors which control the effectiveness of transcription initiation in response to physiological or developmental conditions.

“Regulatory sequence(s)” refers to nucleotide sequences located upstream (5'), within, and/or downstream (3') of a coding sequence, which control the transcription and/or expression of the coding sequences in conjunction with the protein biosynthetic apparatus of the cell. Regulatory sequences include promoters, translation leader sequences, transcription termination sequences and polyadenylation sequences.

“Encoding” and “coding” refer to the process by which a gene, through the mechanisms of transcription and translation, provides the information to a cell from which a series of amino acids can be assembled into a specific amino...
acid sequences to produce an active enzyme. It is understood that the process of encoding a specific amino acid sequence includes DNA sequences that may involve base changes that do not cause a change in the encoded amino acid, or which involve base changes which may alter one or more amino acids, but do not affect the functional properties of the protein encoded by the DNA sequence. It is therefore understood that the invention encompasses more than the specific exemplary sequences. Modifications to the sequences, such as deletions, insertions or substitutions in the sequence which produce silent changes that do not substantially affect the functional properties of the resulting protein molecule are also contemplated. For example, alterations in the sequence which reflect the degeneracy of the genetic code, or which result in the production of a chemically equivalent amino acid at a given site, are contemplated. Thus, a codon for the amino acid alanine, a hydrophobic amino acid, may be substituted by a codon encoding another less hydrophobic residue, such as glycine, or a more hydrophobic residue, such as valine, leucine or isoleucine. Similarly, changes which result in substitution of one negatively charged residue for another, such as aspartic acid for glutamic acid, or one positively charged residue for another, such as lysine for arginine, can also be expected to produce a biologically equivalent product. Nucleotide changes which result in alteration of the N-terminal and C-terminal portions of the protein molecule would also not be expected to alter the activity of the protein. In some cases, it may in fact be desirable to make mutants of the sequence to study the effect of retention of biological activity of the protein. Each of these proposed modifications is well within the routine skill in the art, as is the determination of retention of biological activity in the encoded products. Moreover, the skilled artisan recognizes that sequences encompassed by this invention are also defined by their ability to hybridize, under stringent condition, with the sequences exemplified herein.

“Expression” is meant to refer to the production of a protein product encoded by a gene. “Overexpression” refers to the production of a gene product in transgenic organisms that exceed levels of production in normal or non-transformed organisms.

“Functional portion” or “functional fragment” or “functional equivalent” of an enzyme is that portion, fragment, or equivalent which contains the active site for binding one or more reactants or is capable of improving or regulating the rate of reaction. The active site may be made up of separate portions present on one or more polypeptide chains and will generally exhibit high substrate specificity.

“Enzyme encoded by a nucleotide sequence” includes enzymes encoded by a nucleotide sequence which includes partial isolated DNA sequences.

“Transformation” refers to the transfer of a foreign gene into the genome of a host organism and its genetically stable inheritance.

“% identity” refers to the percentage of the nucleotides/amino acids of one polynucleotide/polypeptide that are identical to the nucleotides/amino acids of another sequence of polynucleotide/polypeptide as identified by a program such as GAP from Genetics Computer Group Wisconsin (GCC) package (version 9.0) (Madison, Wis.). GAP uses the algorithm of Needleman and Wunsch (J. Mol. Biol. 48: 443–453, 1970) to find the alignment of two complete sequences that maximizes the number of matches and minimizes the number of gaps. When parameters required to run the above algorithm are not specified, the default values offered by the program are contemplated.

“Substantial homology” or “substantial similarity” refers to a 70% or more similarity or 70% homology wherein “% similarity” or “% homology” between two polypeptide sequences is a function of the number of similar positions shared by two sequences on the basis of the scoring matrix used divided by the number of positions compared and then multiplied by 100. This comparison is made when two sequences are aligned (by introducing gaps if needed) to determine maximum homology. The PowerBlast program, implemented by the National Center for Biotechnology Information, can be used to compute optimal, gapped alignments. GAP program from Genetics Computer Group Wisconsin package (version 9.0) (Madison, Wis.) can also be used.

“Lignin monomer composition” refers to the relative ratios of guaiacyl monomer and syringyl monomer found in lignified plant tissue.

“Plant” includes whole plants and portions of plants, including plant organs (e.g., roots, stems, leaves, etc).

“Angiosperm” refers to plants that produce seeds encased in an ovary. A specific example of an angiosperm is Liquidambar styraciflua (L.) [sweetgum].

“Gymnosperm” refers to plants that produce naked seeds, i.e., seeds that are not encased in an ovary. A specific example of a gymnosperm is Pinus taeda (L.) [lobolly pine].

“Isolated” and/or “purified” in reference to a nucleic acid molecule or polypeptide are meant to refer to in vitro isolation of a nucleic acid or polypeptide molecule from its natural cellular environment, and from association with other components of the cell so that it can be sequenced, replicated and/or expressed.

“Vector” is a recombinant nucleic acid construct, such as a plasmid, phage, genome, virus genome, cosmid, or artificial chromosome, to which a polynucleotide of the present invention may be attached. In a specific embodiment, the vector may bring about the replication of the attached segment, e.g., in the case of a cloning vector.

“Sinapyl alcohol dehydrogenase” or “SAD” refers to the enzyme in the plant phenylpropanoid biosynthetic pathway, which catalyzes the conversion of sinapaldehyde to sinapyl alcohol and permits the production of syringyl lignin. In the illustrated embodiment of the present invention, the SAD DNA sequence (FIG. 2A) was identified from quaking aspen, Populus tremuloides. It is understood that this sequence can be used as a probe to clone its equivalent (i.e., with substantial homology or substantial identity is defined herein) from any plant species by techniques (EST, PCR, RT-PCR, anti-SAD antibodies, anti-SAD polypeptide antibodies, etc.) well known in the art. SAD DNA sequence is defined herein as any DNA sequences that encode enzymes capable of catalyzing specifically the conversion of sinapaldehyde into sinapyl alcohol according to the enzyme assay methods described herein.

The Phenyl Propanoid Biosynthetic Pathway

Reference is made to FIG. 1 which shows different steps in the biosynthetic pathways from 4-coumarate (1) to guaiacyl (coniferyl alcohol (6)) and syringyl (sinapyl alcohol (9)) monolignols for the formation of guaiacyl-syringyl lignin together with the enzymes responsible for catalyzing each step. The enzymes indicated for each of the reaction steps are: 4-coumaric acid 3-hydroxylase (C3H) which converts 4-coumarate (1) to caffeate (2); 4-coumarate-CoA ligase (4CL) converts caffeate (2) to caffeoyl-CoA (3) which in turn is converted to feruloyl-CoA (4) by caffeoyl-CoA O-methyltransferase (CCoAOMT); cinnamoyl-CoA reductase (CCR) converts feruloyl-CoA (4) to coniferaldehyde...
DNA Constructs

According to the present invention, there is provided a DNA construct which is a plant DNA having a promoter sequence, a coding region and a terminator sequence. The coding region encodes the SAD enzyme essential to lignin biosynthesis. The coding region is suitably a minimum size of 50 bases. The gene promoter is positioned at the 5'-end of the coding region and a gene termination sequence that is located at the 3'-end of the transgene for signaling the end of the transcription of the transgene.

The DNA construct in accordance with the present invention is suitably incorporated into the genome of a plant by transformation to alter lignin biosynthesis, e.g., to provide syringyl-enriched lignin. The DNA construct may include clones provided herein, namely PtXAD (later renamed PtSAD), described hereinafter, and variants thereof such as are permitted by the degeneracy of the genetic code or the functional equivalents thereof.

The DNA constructs of the present invention may be inserted into plants to regulate production of the SAD enzyme. Depending on the nature of the construct, the production of the protein may be increased or reduced, either throughout or at particular stages in the life of the plant. For example, the orientation of the DNA coding sequence, promoter, and termination sequence can serve to either suppress lignin formation or amplify lignin formation. For the down-regulation of lignin synthesis, the DNA is in the antisense orientation. For the amplification of lignin biosynthesis, the DNA is in the sense orientation, thus to provide one or more additional copies of the DNA in the plant genome. In this case, the DNA is suitably a full-length cDNA copy. It is also possible to target expression of the gene to specific cell types of the plants, such as the epidermis, the xylem, the roots, etc. Constructs in accordance with the present invention may be used to transform cells of both monocotyledons and dicotyledons plants in various ways known in the art. In many cases, such plant cells may be cultured to regenerate whole plants which subsequently reproduce to give successive generations of genetically modified plants. Examples of plants that are suitably genetically modified in accordance with the present invention, include but are not limited to, trees such as aspen, poplar, pine and eucalyptus.

Promoters and Termination Sequences

Various gene promoter sequences are well known in the art and can be used in the DNA constructs of present invention. The promoter in a construct of the present invention can provide for expression of the linked DNA segment. The promoter can also be inducible so that gene expression

<table>
<thead>
<tr>
<th>TABLE 1</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Comparison of all CAD-like protein sequences available in the database.</strong></td>
</tr>
<tr>
<td><strong>Values indicate % amino acid sequence identity.</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td>1</td>
</tr>
</tbody>
</table>

can be turned on or off by an exogenously added agent. It may also be preferable to combine the desired DNA segment with a promoter that provides tissue specific expression or developmentally regulated gene expression in plants.

The promoter may be selected from promoters known to operate in plants, e.g., CaMV 35S, GPA1, GPA2 and endogenous plant promoters controlling expression of the SAD enzyme, i.e., the endogenous promoter of the SAD gene. Use of a constitutive promoter such as the CaMV 35S promoter (Odell et al. 1985), or CaMV 19S (Lawton et al., 1987) can be used to drive the expression of the transgenes in all tissue types in a target plant. Other promoters are nos (Ebert et al. 1987), Adh (Walker et al., 1987), sucrose synthase (Yang et al., 1990), t-tubulin, ubiquitin, actin (Wang et al., 1992), cab (Sullivan et al., 1989), PEPCase (Hudsprong et al., 1989) or those associated with the R gene complex (Chandler et al., 1989). On the other hand, use of a tissue specific promoter permits functions to be controlled more selectively. The use of a tissue-specific promoter has the advantage that the SAD enzyme is only produced in the tissue in which its action is required. Suitably, tissue-specific promoters, such as those that confine the expression of the transgenes in developing xylem where lignification occurs, may be used in the inventive DNA constructs.

A DNA segment can be combined with the promoter by standard methods as described in Sambrook et al., 2nd ed. (1982). Briefly, a plasmid containing a promoter such as the CaMV 35S promoter can be constructed as described in Jefferson (1987) or obtained from Clontech Lab, Palo Alto, Calif. (e.g., pBI121 or pBI221). Typically, these plasmids are constructed to provide for multiple cloning sites having specificity for different restriction enzymes downstream from the promoter. The DNA segment can be subcloned downstream from the promoter using restriction enzymes to ensure that the DNA is inserted in proper orientation with respect to the promoter so that the DNA can be expressed.

The gene termination sequence is located 3' to the DNA sequence to be transcribed. Various gene termination sequences known in the art may be used in the present inventive constructs. These include nopaline synthase (NOS) gene termination sequence (see, e.g., references cited in co-pending, commonly-owned PCT Application “Method to Introduce Multiple Genes into Plants”, Ser. No. PCT/US00/27704, filed Oct. 6, 2000).

Marker Genes

A marker gene may also be incorporated into the inventive DNA constructs to aid in the selection of plant tissues with positive integration of the transgene. “Marker genes” are genes that impart a distinct phenotype to cells expressing the marker gene and thus, allow such transformed cells to be distinguished from cells that do not have the marker. Many examples of suitable marker genes are known in the art and can be employed in the practice of the invention, such as neomycin phosphotransferase II (NPT II) gene that confers resistance to kanamycin or hygromycin antibiotics which would kill the non-transformed plant tissues containing no NPT II gene (Bevan et al., 1983). Numerous other exemplary marker genes are described in co-pending, commonly owned PCT Application “Method to Introduce Multiple Genes into Plants”, Ser. No. PCT/US00/27704, filed Oct. 6, 2000, incorporated herein by reference.

Transformation

Transformation of tissues or cells from plants, for instance, trees, with the inventive DNA construct and the subsequent production of transgenic plants can be achieved by a variety of techniques known in the art. For example, Agrobacterium- and microprojectile-mediated techniques for transferring a DNA construct into host plant tissues are particularly suitable for tree species (Tsai et al., 1994; Ellis et al. 1993; and others described in co-pending, commonly owned PCT Application, PCT/US00/27704, filed Oct. 6, 2000 “Method to introduce multiple genes into plants”, incorporated herein by reference). After transformation, transgenic plant tissues resistant to, e.g., antibiotics, such as kanamycin, can be selected and cultured to regenerate whole plants, using techniques also well known in the art (Tsai et al., 1994; Ellis et al. 1993; and others described in co-pending, commonly owned PCT Application, PCT/US00/27704 (supra)).

Transformation and regeneration protocols are readily adaptable to many plant species. Many transformation and regeneration protocols have been published for plant species. (See, co-pending, commonly-owned PCT Application Ser. No. PCT/US00/27704 (supra)).

DNA Clones

A guaiacyl pathway gene encoding coniferyl alcohol dehydrogenase (CAD, FIG. 1) from Populus tremuloides (GenBank #AF217957) was first cloned. This cDNA was designated as PtCAD (GenBank #AF217957). E. coli-expressed recombinant PtCAD protein was characterized for its catalytic activity with coniferaldehyde and sinapaldehyde. Because monolignol biosynthetic pathway intermediates are present together in lignifying tissues (Osakabe et al., 1999; Li et al. 2000), the PtCAD enzyme activity with a mixture of coniferaldehyde and sinapaldehyde was assayed. As shown in FIG. 5 (a) and (c), the reaction mixture was separated by HPLC after the enzyme reaction. Only coniferyl alcohol was formed from coniferaldehyde, as corroborated by mass spectrometry-based chemical structural analysis, and PtCAD had no reaction with sinapaldehyde. Thus, PtCAD has a specific catalytic reaction function with coniferaldehyde for the biosynthesis of guaiacyl monolignol, coniferyl alcohol (FIG. 1). CAD's substrate specificity for coniferaldehyde also confirms that there is a need for another enzyme, SAD of the present invention, in the biosynthesis of syringyl monolignol, sinapyl alcohol, as illustrated in FIG. 1.

A differential cloning strategy was designed to isolate candidate SAD genes based on their discernible sequences from that of CAD, as described above. Low- and high-stringency differential screening (further described in Example 2 herein below) of 1.8×10⁶ pfu from the Populus tremuloides xylem cDNA library using PtCAD cDNA as a probe resulted in the isolation of two groups of positive clones, based on sequence analysis, i.e., Group I sequences and Group II sequences. Group I sequences of 12 clones were identical to PtCAD, and the sequences of group II cDNAs were identical to each other but different from PtCAD. Two of the 8 clones in group II were full-length cDNAs and designated as PtSAD. PtSAD cDNA (SEQ ID NO: 1) is 1,446-bp long, encoding an ORF of 362 amino acids (SEQ ID NO: 2) with a calculated MW of 38,991 and a pI of 6.69. Co-factor and zinc binding sequences identified in common alcohol dehydrogenases (ADHs) (Jornvall et al., 1987; O’Malley et al., 1992; Galliano et al., 1993) were found in PtSAD cDNA. Zn1 binding motif and the structural Zn2 consensus were located at amino acid residues 71 to 85 and residues 91 to 117, respectively. An NADP-binding site was identified at residues 191 to 196.

Furthermore, PtSAD exhibited only about 50% amino acid sequence identity to all the other known full-length monolignol CADDs (see, Table 1 supra). However, it showed insignificantly low amino acid sequence identity (10-40%) to those ADHs considered to be associated with pathogen-
defense functions (Jornvall et al., 1987; Brill et al., 1999). These sequence characteristics confirm that PtSAD belongs to a novel class of ADHs, discernible from the commonly known monolignol CADs. This is fully supported by the phylogenetic analysis of PtSAD and available full-length monolignol CAD protein sequences showing that these monolignol CADs (FIG. 3) form a cluster distinctly separated from PtSAD. This cluster of monolignol CADs is guaiacyl-specific in view of the extensive amino acid sequence similarity (80 to 98%) of these genes with the guaiacyl-specific PICAD and with CADs from loblolly pine and spruce, the single-copy gene involved in the biosynthesis of guaiacyl monolignol in gymnosperms (O'Malley et al., 1992; Galliano et al., 1993; MacKay et al. 1995). Thus, the phylogenetic analysis may reflect an ancient divergence of the guaiacyl CAD phylogenetic group into a more syringyl-specialized one, to which PtSAD belongs. Repeated screening of independent sets of pfu from Populus tremuloides xylem cDNA libraries with either PtCAD or PtSAD cDNA probes always resulted in the isolation of clones identical to either PtCAD or PtSAD, indicating the two predominant monolignol ADHs in lignifying xylem, CAD and SAD.

To demonstrate the biochemical function of PtSAD gene, PtSAD cDNA was expressed in E. coli to produce its recombinant protein. When coniferaldehyde alone was incubated with PtSAD recombinant protein, HPLC-MS analysis of the enzyme reaction production demonstrated that PtSAD had a specific activity of 786 mmol/min/mg protein with coniferaldehyde. However, PtSAD had a specific activity of 694 mmol/min/mg protein with sinapaldehyde, indicating that the catalytic efficiency of PtSAD with sinapaldehyde is 9 times higher than with coniferaldehyde. Thus, PtSAD is specific for sinapaldehyde and SAD sinapyl alcohol dehydrogenase catalyzes the conversion of sinapaldehyde to sinapyl alcohol.

Proof of the sinapaldehyde-specific nature of PtSAD came from the PtSAD recombinant protein reaction with a mixture of coniferaldehyde and sinapaldehyde. HPLC-MS analysis (FIG. 5) of the reaction of PtSAD with a mixture of coniferaldehyde and sinapaldehyde demonstrated conclusively that sinapyl alcohol is the exclusive product from sinapaldehyde and that in an in vivo situation PtSAD would not react with coniferaldehyde.

Thus, taken together, CAD is guaiacyl-specific for the formation of guaiacyl monolignol, coniferyl alcohol, and SAD catalyzes the exclusive biosynthesis of syringyl monolignol, sinapyl alcohol. Furthermore, these results suggest discrete roles in plants for CAD and SAD proteins in coordinating cell-specific biosynthesis of guaiacyl and syringyl lignins.

A cDNA encoding this SAD enzyme has now been cloned for the first time. It was concluded that the presence of SAD enzyme is essential for the biosynthesis of syringyl lignin in plants, and that the incorporation of an SAD gene into transgenic plants is a viable mechanism for successful engineering of syringyl lignin in plants.

The invention will be further described by the following non-limiting examples.

EXAMPLE 1

Isolation of SAD cDNA from aspen Populus tremuloides

An aspen xylem cDNA library was constructed in Sgt22A vector according to manufacturer’s protocols (GIBCO BRL). The aspen CAD cDNA (GenBank accession number AF217957) was used as a probe to differentially screen the cDNA library under high and low stringency hybridization conditions. About 6,000 pfu from the cDNA library were lifted onto 4 individual nylon membranes. Two such blotted membranes were hybridized with 32P-labeled CAD cDNA probe under low stringency conditions (50°C) and the other two under high stringency (65°C). In this way, a total of 18,000 pfu were screened. High density hybridization signals were detected on membranes probed under either high or low stringency conditions. However, low density signal were detected only on membranes probed under low stringency condition. The high density signals on high stringency membrane were perfectly aligned with the high density signals on low stringency membrane, allowing a differential isolation of positive clones with low density signal. The high density clones were confirmed to be aspen CAD cDNA. The positives with low density signal were further screened until a single clone was isolated. The purified Sgt22A clones were then subcloned into pBluescript SK plasmid vector through NotI and EcoRI cloning sites. The sequencing results indicated that the isolated cDNA clone is 1,425-bp long (SEQ ID NO: 1) and encodes a 362 amino acid protein (SEQ ID NO: 2) which shows a 53% sequence identity with aspen CAD protein. This cDNA clone was first designated as PtXAD and then renamed as PtSAD after its biochemical function was confirmed.

Sequences of clones that hybridized with the probe only under low stringency conditions were identical to each other but distinct from PtCAD. Two of these low stringency probe-hybridizing clones were found to be full-length cDNAs; they were designated PtSAD and sequenced (AB1310; Perkin-Elmer) in both directions (GenBank accession number AF273256).

EXAMPLE 2

Cloning of a Novel Alcohol Dehydrogenase Gene, PtSAD, from Aspen

To test the hypothesis of distinct CAD and SAD genes in angiosperms, CAD cDNA, PtCAD, was cloned from developing xylem of aspen and used to screen for related sequences in the same species. Low- and high-stringency differential screening of 2.4x104 plaque-forming units from an aspen xylem cDNA library (Wu et al., 2000) resulted in the isolation of two groups of positive clones. Group I contained 12 cDNAs with sequences identical to PtCAD. Sequences of the eight cDNAs constituting group II were identical to each other but differed from PtCAD. Two of the eight clones in group II were full-length cDNAs and were tentatively named PtSAD.

The open reading frame of PtSAD was 1086 bp, encoding a 39-kD protein with a pI of 6.69. The deduced amino acid sequence of PtSAD was 53% identical to that of PtCAD and ~50% identical to that of other angiosperm monolignol CADs, but it exhibited insignificant identity (10 to 40%) with the sequences of alcohol dehydrogenases (ADHs) associated with pathogen defense (Brill et al., 1999). PtCAD, on the other hand, showed extensive amino acid sequence identity with CADs from Populus trichocarpa X Populus deltoids (97%) (PtCAD; Van Doornseleare et al., 1995), Eucalyptus gunnii (81%) (pEuCAD2; Grima-Pettenati et al., 1993), tobacco (82%) (pICAD14; Knight et al., 1992), lucern (79%) (MsCad2, Brill et al., 1999), and other reported angiosperms (~80%) (Brill et al., 1999). Therefore, PtSAD belongs to a novel class of ADHs.

Cofactor and zinc binding sequences conserved in ADHs (Jornvall et al., 1987) were present in PtSAD (FIG. 1). The
Zn1 binding motif and structural Zn2 consensus regions (Jornvall et al., 1987; MacKay et al., 1995) were located at amino acid residues 71 to 85 and 91 to 117, respectively. A NADP binding site (Jornvall et al., 1987) was identified at residues 191 to 196. Repeated screening of the aspen xylem cDNA library with either PtCAD or PtSAD cDNA probes always resulted in the isolation of clones identical to either PtCAD or PtSAD, indicating that they are the two predominant monolignol-related ADHs in lignifying xylem.

Phylogenetic analysis of PtSAD and available full-length monolignol CAD protein sequences showed that gymnosperm and angiosperm CADs form a cluster that does not include PtSAD (FIG. 2). The angiosperm monolignol CADs in this cluster share ~70% amino acid sequence identity with gymnosperm CADs but ~50% with PtSAD, suggesting that all of these putative angiosperm monolignol CADs may be guaiacyl specific. These results also may reflect a divergence of the guaiacyl CAD phylogenetic group into a more syringyl-specialized group, to which PtSAD belongs.

EXAMPLE 3
DNA and RNA Gel Blot Analysis

Aspen genomic DNA and total RNA from various aspen tissues were isolated as described (Li et al., 1997; Hu et al., 1998). To determine whether there are other PtCAD and PtSAD related sequences in aspen, a gel blot analysis was done with aspen genomic DNA digested by various restriction enzymes and hybridized with either PtCAD (FIG. 4A) or PtSAD (FIG. 4B) full-length cDNA probes. DNA and RNA gel blot hybridizations were performed under high stringency conditions (Hu et al., 1998). Probes were PtCAD or PtSAD cDNA labeled with α-32P-dATP (Amersham) using the DECA prime labeling system (Ambion, Austin, Tex.).

There was a strong single band in each lane, but a weak single band also was detected in each lane, perhaps evidence of a distantly related sequence. Together with our cDNA screening results, we interpret these data to indicate that PtCAD and PtSAD likely are the predominant members of a small gene family in aspen.

DNA gel blot analysis also clearly demonstrated that PtCAD and PtSAD did not cross-hybridize with each other. Thus, using the same hybridization conditions and PtCAD and PtSAD full-length cDNA probes, RNA gel blot analysis was conducted to investigate the tissue-specific expression of PtCAD and PtSAD in aspen. The greatest PtCAD expression was found in tissue types containing a large amount of lignifying xylem, but its expression was lower in phloem-enriched tissues (internodes 1 to 3; FIG. 4C). Strong expression of PtSAD was detected in tissues undergoing rapid lignification xylem, but its expression was lower in phloem-syringyl-specialized group, to which PtSAD belongs.
The basic enzyme reaction mixture contained 50 mM sodium phosphate buffer, 5 mM β-mercaptoethanol, 500 μM NADPH or NADP, purified recombinant protein (boiled protein was used as a control), and phenolic substrate in a final volume of 500 μL. For the substrate specificity test, 500 μM aldehyde substrate and 1 μg of purified recombinant PtCAD or PtSAD protein (~25 pmol) were used. To characterize the enzyme pH optima, the substrate and recombinant PtCAD or PtSAD protein concentrations described above were used in sodium phosphate buffers, pH 5 to 8.5. All reactions were for 10 minutes at 30°C.

For normal and inhibition kinetic analyses, the reaction time was 4 min and pH was 8.0 for PtCAD (1.2 μg of purified recombinant protein) and 7.0 for PtSAD (0.1 μg). For kinetics, varying concentrations (0.5 to 200 μM) of p-coumaraldehyde, caffealdehyde, coniferaldehyde, 5-hydroxyconiferaldehyde, or sinapaldehyde were used to measure K_m, V_max, and the enzyme turnover number, k_cat.

For inhibition kinetics, the PtCAD-mediated reduction of sinapaldehyde (1 to 200 μM) was assayed in the presence of 1 to 5 μM coniferaldehyde, and the PtSAD-catalyzed reduction of coniferaldehyde (1 to 200 μM) was assayed in the presence of 1 to 5 μM sinapaldehyde. All reactions were terminated by the addition of 10 μL of 6 N HCl (to bring the pH to 2) and 500 μg of internal standard o-coumaric acid and analyzed by HPLC-UV/MS.

An aliquot of 100 μL of reaction mixture was injected directly onto a Supelcosil LC-ABZ column (15 cm×4.6 mm×5 μm; Supelco, Bellefonte, Pa.) with automatic sample injection and separated isocratically with a Hewlett-Packard (HP) 1100 liquid chromatography system at 4°C and a flow rate of 0.25 mL/min. The gradient program was 20% acetonitrile in 10 mM formic acid, pH 2.5, for 12 min, 20 to 100% acetonitrile from 12 to 16 min, and hold at 100% acetonitrile for 5 to 10 min; detection was with an HP 1100 diode array detector and an HP 1100 liquid chromatography-MS detector system with an atmospheric pressure ionization-electrospray source in negative ion mode. The reaction products were identified and confirmed by comparing the ion fragmentation patterns of the product and the authentic standard in MS scanning mode at 70 V. The product quantity and K_m, V_max, k_cat, and apparent inhibition constant (K_i) values (mean±SE) were determined as described (Osakabe et al., 1999; Li et al., 2000). The results of the inhibition kinetics for both the CAD enzyme (i.e., Table 2) and the SAD enzyme (i.e., Table 3) using a variety of substrates are illustrated below.

### Table 2

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_m (μM)</th>
<th>V_max (nmol.min⁻¹.μg⁻¹)</th>
<th>k_cat (min⁻¹)</th>
<th>V_max/K_m (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Coumaraldehyde</td>
<td>6.2 ± 0.14</td>
<td>0.17 ± 0.04</td>
<td>6.8 ± 0.22</td>
<td>30.3 ± 0.08</td>
</tr>
<tr>
<td>Caffealdehyde</td>
<td>3.5 ± 0.10</td>
<td>0.30 ± 0.04</td>
<td>8.0 ± 0.30</td>
<td>10.0 ± 0.03</td>
</tr>
<tr>
<td>Coniferaldehyde</td>
<td>2.3 ± 0.15</td>
<td>0.21 ± 0.03</td>
<td>8.0 ± 0.15</td>
<td>10.0 ± 0.03</td>
</tr>
<tr>
<td>5-Hydroxyconiferaldehyde</td>
<td>15.6 ± 0.12</td>
<td>0.17 ± 0.04</td>
<td>6.8 ± 0.22</td>
<td>30.3 ± 0.08</td>
</tr>
<tr>
<td>Sinapaldehyde</td>
<td>1.1 ± 0.10</td>
<td>0.10 ± 0.04</td>
<td>8.0 ± 0.30</td>
<td>10.0 ± 0.03</td>
</tr>
</tbody>
</table>

k_cat enzyme turnover number
Values are means ± SE for three independent assays.

### Table 3

<table>
<thead>
<tr>
<th>Substrate</th>
<th>K_m (μM)</th>
<th>V_max (nmol.min⁻¹.μg⁻¹)</th>
<th>k_cat (min⁻¹)</th>
<th>V_max/K_m (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-Coumaryl alcohol</td>
<td>15.6 ± 0.12</td>
<td>0.17 ± 0.04</td>
<td>6.8 ± 0.22</td>
<td>30.3 ± 0.08</td>
</tr>
<tr>
<td>Caffealdehyde</td>
<td>1.1 ± 0.10</td>
<td>0.10 ± 0.04</td>
<td>8.0 ± 0.30</td>
<td>10.0 ± 0.03</td>
</tr>
<tr>
<td>Coniferaldehyde</td>
<td>2.3 ± 0.15</td>
<td>0.21 ± 0.03</td>
<td>8.0 ± 0.15</td>
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</tr>
<tr>
<td>Sinapaldehyde</td>
<td>1.1 ± 0.10</td>
<td>0.10 ± 0.04</td>
<td>8.0 ± 0.30</td>
<td>10.0 ± 0.03</td>
</tr>
</tbody>
</table>

k_cat enzyme turnover number
Values are means ± SE for three independent assays.

### Example 5

**Chemical Synthesis and Thioacidolysis Analysis of Aspen Stem Monolignol composition**

All aldehydes and their alcohol derivatives were obtained from Sigma/Aldrich, except the following: p-coumaraldehyde, p-coumaryl alcohol, caffealdehyde, caf- feyl alcohol, 5-hydroxyconiferaldehyde, and 5-hydroxyconiferyl alcohol were prepared chemically from their corresponding benzaldehyde derivatives as described (Osakabe et al., 1999; Li et al., 2000). The structural identities of these compounds were confirmed by 1H-NMR.

### Example 6

**Histochemical Lignin Analysis, Immunolocalization, and Microscopy**

For histochemical localization of lignin, fresh hand-cut sections (~20 μm thick) from stem internodes of 4-month-old, greenhouse-grown aspen plants (clone 271) were incubated immediately in freshly prepared saturated chlorinated water for 10 min at 4°C. After three washes with water, the sections were incubated in 4% sodium sulfite at room temperature for 15 minutes.
Polymerization was performed under UV light (365 nm) for 40 hr at -20°C. In a UVC2 CRYO Chamber (PELCO, Redding, Calif.). Sections (3 μm thick) were prepared with a Leica (wetzlar, Germany) RM 2155 microtome and mounted on Superfast/plus (Fisher) slides. Slides were rinsed with acetone to remove butyl methyl methacrylate from the sections, which were rehydrated in an ethanol series and block first with 0.1 M hydroxyxaminum chloride for 5 min and then with 1% BSA for 30 min at room temperature. After incubation with anti-PtCAD, anti-PtCAld5H (Osakabe et al., 1999), anti-PtAldOMT (Li et al., 2000), or anti-PtSAD antibodies (in 1:500 dilution) for 2 hr at 37°C, slides were washed in PBS containing 0.1% BSA and incubated with goat anti-rabbit antibody conjugated with alkaline phosphatase (1:100; Boehringer Mannheim) for 1.5 hr at 37°C. After washing in PBS, slides incubated with anti-PtSAD, anti-PtCAld5H, or anti-PtAldOMT antibodies were reacted at pH 9.5 with a mixture of dimethylformamide and nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate, and those incubated with anti-PtCAD antibodies were treated with Fast Red TR/Naphthol AS-MX (Sigma); both treatments were for 20 to 30 min at room temperature. Pre-immune serum was used as the control. The slides then were mounted in 50% glycerol and observed with a Nikon Eclipse 400 microscope, and images were taken using a Sony (Tokyo, Japan) DKC-5000 digital photo camera.

EXAMPLE 7

Histochemical and Chemical Detection of Guaiacyl and Syringyl Lignin Distributions in Aspen Stem Vascular Tissues

The in situ relationship between PtCAD/PtSAD and guaiacyl syringyl lignin biosynthesis was identified by analyzing the distribution of guaiacyl and syringyl lignins in vascular systems of the aspen stem. Guaiacyl lignin can be distinguished chromogenically from guaiacyl lignin in situ by Cross/Bevan or Maule color reaction (Nakano and Meshitsuka, 1992). The lignin-based chromophore-forming mechanisms in these two methods are similar. The chlorination of the syringyl nucleus leads to a pink (lignifying cells) or red (lignified cells) color, whereas the guaiacyl nucleus produces a light (lignifying cells) to dark (lignified cells) brown color (Bland, 1966; Wardrop, 1981). The Cross/Bevan method was used in these experiments because of its mild reaction conditions, circumventing the problem of thin tissue section destruction that often occurs during Maule color reactions.

In the primary vascular tissues, lignin was observed only in xylem and was of the guaiacyl type, as revealed by the brown staining of protoxylem and metaxylem vessel elements between stem internodes 1 and 4 (FIGS. 7A and 7B). This was further confirmed by thioacidolysis analysis of stem lignin, which demonstrated the exclusive detection of guaiacyl monomers (FIG. 7D). The primary xylem remained as the only stem tissue containing pure guaiacyl lignin (FIGS. 7E to 7G). Guaiacyl-syringyl lignin appeared during the differentiation of secondary vascular systems, as indicated by the chemical analyses of stem internodes 5 and beyond (FIG. 7H). However, the deposition of syringyl lignin in the secondary xylem followed that of guaiacyl lignin, as manifested by the color change from bright light brown to pink and then red in developing, partially lignified, and extensively lignified secondary xylem elements, respectively (FIG. 7G). This is consistent with the reported sequential deposition of guaiacyl followed by syringyl lignins in xylem cells of angiosperms (Terashima et al., 1986; Saka and Goring, 1988).

Aggregated protophloem parenchyma cells, the precursors of primary phloem fibers (Esau, 1965), were present in primary growth tissues (FIGS. 7A to 7C), but these cells were not stained for lignin, likely because of their lack of secondary wall thickening. They also failed to stain for guaiacyl lignin once lignification and secondary thickening began (FIG. 7I). Instead, syringyl-positive pink (FIG. 7I) to red (FIGS. 7E and 7F) coloration prevailed in these cells as they differentiated into fibers. Indeed, phloem fibers are known for their enrichment in syringyl lignin, but they do accumulate guaiacyl-syringyl lignin (Grand et al., 1982). Together, these observations indicate that in direct contrast to the lignification sequence in secondary xylem elements, the biosynthesis of syringyl lignin precedes and overwhelms that of guaiacyl lignin in primary phloem fibers.

Immunolocalization was used to verify whether PtCAD is associated with guaiacyl lignin-synthesizing primary xylem and whether the distribution of PtCAD and PtSAD is in line with the guaiacyl and syringyl lignin deposition patterns in phloem and xylem elements. The distribution of another syringyl pathway protein, PtCAld5H, also was analyzed.

EXAMPLE 8

Immunolocalization of PtCAD, PtCAld5H, and PtSAD in Aspen Stem Internodes

Conditions similar to those present during protein gel blot analyses, by which the specificities of PtCAD and PtSAD antibodies were verified (FIGS. 4E and 4F), were applied to cellular immunolocalization. PtSAD and PtCAld5H were visualized in tissue sections after the anti-rabbit IgG-alkaline phosphatase reaction with nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate substrate. PtCAD signals were visualized with Fast Red TR substrate. Serial sections were analyzed. Pre-immune serum used at the same protein concentration as the anti-PtCAD, anti-PtSAD, or anti-PtCAld5H antiserum gave no immunolabeling signal (data not shown). At the third internode, PtCAD was detected almost exclusively in developing metaxylem vessels (FIG. 8A). PtSAD was not detected in metaxylem vessels but was most conspicuous in protophloem parenchyma cells and in the parenchymatous storage tissue, the medullary sheath (FIG. 8B).

The cellular distribution of PtCAld5H (FIG. 8C) conformed with that of PtSAD. At the third internode, the lignification and secondary wall thickening had begun in metaxylem vessels but not in protophloem parenchyma cells (FIGS. 7A to 7C). Consistently, no lignin color reaction was observed in protophloem parenchyma cells (FIG. 7A), despite the detection in these cells of PtSAD and PtCAld5H (FIGS. 8B and 8C). However, at internode 6, both syringyl lignin deposition (FIG. 7I) and PtSAD signals (data not shown) were observed in these cells undergoing differen-
transformation into primary phloem fibers. At the eighth internode, PtSAD signals diminished in these differentiating fiber cells (FIG. 8E), signifying the near completion of syringyl monolignol biosynthesis in these cells (FIG. 7E).

At this stage, PtCAD became more conspicuous than PtSAD in these maturing fibers (FIG. 8D), indicative of an active biosynthesis of guaiacyl monolignol. As the primary phloem continued its centripetal course of differentiation, new protophloem parenchyma cells appeared adjacent to the maturing fibers toward the center of the stem. These new primary phloem fiber precursors (Esau, 1965) were labeled with PtSAD (FIG. 8E) but not yet with PtCAD (FIG. 8D). At internode 12, PtSAD signals disappeared in primary phloem fibers (FIG. 8I), suggesting the completion of syringyl monolignol biosynthesis in these cells. However, PtCAD signals remained strong in these maturing fibers (FIG. 8H). At internode 15, neither PtCAD nor PtSAD was detected in these fibers that became fully lignified (data not shown). These results agree with those of histochemical lignin localization indicating that the biosynthesis of syringyl lignin precedes that of guaiacyl lignin in primary phloem fibers.

However, these procambium-derived primary phloem elements and the secondary xylem exhibited contrasting lignification sequences. PtCAD appeared in xylem fusiform initials before PtSAD (FIGS. 8H and 8I), consistent with chemical and histochemical evidence that the biosynthesis of syringyl lignin lags behind that of guaiacyl lignin in the secondary xylem. Furthermore, in the differentiating secondary xylem, PtCAD signals were most conspicuous in maturing vessels (FIGS. 8I and 8H) but also were strong in developing fiber and ray cells. PtSAD signals were strongest in syringyl lignin-enriched radial and axial ray cells (FIG. 8G) (Wardrop and Dadswell, 1952; Musha and Goring, 1975) and were conspicuous in maturing fiber cells but were nearly absent from developing vessels (FIGS. 8G and 8I). These protein distribution patterns in the secondary xylem were sustained through older internodes (data not shown). These results and histochemical observations consistently demonstrated that PtCAD is associated with cells specializing in guaiacyl lignin synthesis and that PtSAD and PtCAD5H are associated with vascular elements containing enriched syringyl lignin.

**EXAMPLE 9**

Production of syringyl-enriched lignin in transformed plants

The following genetic transformations illustrate production of syringyl-enriched lignin in plants. A. To produce syringyl-enriched lignin in angiosperm plants, angiosperm plants are genetically transformed with sense SAD gene driven by any appropriate promoter and via any appropriate genetic transformation system.

B. To produce syringyl-enriched lignin in gymnosperm plants, gymnosperm plants are genetically transformed with sense SAD genes driven by any appropriate promoter and via any appropriate genetic transformation system.

The invention has now been described and exemplified with some specificity, those skilled in the art will appreciate the various modifications, including variation, additions and omissions, that may be made in what has been described. Accordingly, it is intended that these modifications also be encompassed by the present invention and that the scope of the present invention be limited solely by the broadest interpretation that lawfully can be accorded the appended claims.

**References**


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Ala Val His Pro Leu Leu Pro Leu Phe Gly Leu Lys Ser His Gly 260 265 270
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What is claimed is:

1. An isolated polynucleotide encoding a polypeptide comprising the amino acid sequence of SEQ ID NO: 2, wherein the polypeptide catalyzes the conversion of sinapaldehyde to sinapyl alcohol.

2. The polynucleotide of claim 1, wherein the polypeptide exhibits greater substrate specificity for sinapaldehyde than for coniferaldehyde.

3. The polynucleotide of claim 1, wherein the polypeptide has a lower $k_m$ for sinapaldehyde than for coniferaldehyde.

4. The polynucleotide of claim 1, comprising a sequence selected from the group consisting of nucleotides 60-1145 of SEQ ID NO: 1 and the full length complement thereof.

5. The polynucleotide of claim 4, wherein the polynucleotide comprises a sequence selected from the group consisting of SEQ ID NO: 1 and the full length complement of SEQ ID NO: 1.

6. A DNA construct comprising the polynucleotide sequence of claim 1, operably linked to a promoter.

7. A plant cell comprising the construct of claim 6.

8. A plant comprising the cell of claim 7.

9. The plant of claim 8, wherein the plant exhibits increased expression of a polypeptide comprising the amino acid sequence of SEQ ID NO: 2 and having the ability to catalyze the conversion of sinapaldehyde to sinapyl alcohol, relative to a control plant lacking the construct.

10. The plant of claim 8, wherein the plant exhibits increased expression of a polypeptide comprising SEQ ID NO: 2, relative to a control plant lacking the construct.

11. The plant of claim 8, wherein the plant is a tree.

12. The tree of claim 11, wherein the tree is selected from the group consisting of aspen, poplar, pine, and eucalyptus.

13. The tree of claim 11, wherein the tree is an angiosperm.

14. The tree of claim 11, wherein the tree is a gymnosperm.

15. The DNA construct of claim 6, wherein the promoter is selected from the group consisting of a constitutive promoter and a tissue-specific promoter.

16. The DNA construct of claim 15, wherein the promoter is xylem tissue-specific.

17. A method of increasing expression of sinapyl alcohol dehydrogenase in a plant cell comprising incorporating the construct of claim 6 into the genome of the plant cell wherein the polynucleotide encoding the polypeptide of SEQ ID NO: 2 is expressed.

* * *
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 11,
Lines 29 and 31, delete “mmol/min/mg”, and insert -- nmol/min/mg --.

Signed and Sealed this

Thirtieth Day of August, 2005

JON W. DUDAS

Director of the United States Patent and Trademark Office
It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Beginning at Column 1, line 11, delete the following paragraph:

“STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH OR DEVELOPMENT

This invention was made with United States Government support awarded by the Energy Biosciences Program, and the United States Department of Agriculture, research grant numbers USDA 99-35103-7986, USDA 01-03749, and DOE DE-FGO2-01ER15179. The United States Government has certain rights in this invention.”

Signed and Sealed this

Twelfth Day of December, 2006

JON W. DUDAS
Director of the United States Patent and Trademark Office