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Methods for simultaneous control of lignin content and composition, and cellulose content in plants

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Chiang et al.

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(45) **Date of Patent:** **Feb. 15, 2005**

(54) **METHODS FOR SIMULTANEOUS CONTROL OF LIGNIN CONTENT AND COMPOSITION, AND CELLULOSE CONTENT IN PLANTS**

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(73) Assignee: **Board of Control of Michigan Technological University**, Houghton, MI (US)

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(51) **Int. Cl.**⁷ **C12N 5/04**; C12N 15/82; C12N 15/29; A01H 5/00

(52) **U.S. Cl.** **800/278**; 800/290; 800/286; 800/298; 800/319; 800/287; 800/284; 800/303; 800/289; 435/468; 435/419

(58) **Field of Search** 800/290, 287, 800/278, 286, 298, 284, 303, 319, 289; 536/23.1, 23.6; 435/468, 419

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(57) **ABSTRACT**

The present invention relates to a method of concurrently introducing multiple genes into plants and trees is provided. The method includes simultaneous transformation of plants with multiple genes from the phenylpropanoid pathways including 4CL, CALD5H, AldOMT, SAD and CAD genes and combinations thereof to produce various lines of transgenic plants displaying altered agronomic traits. The agronomic traits of the plants are regulated by the orientation of the specific genes and the selected gene combinations, which are incorporated into the plant genome.

30 Claims, 25 Drawing Sheets

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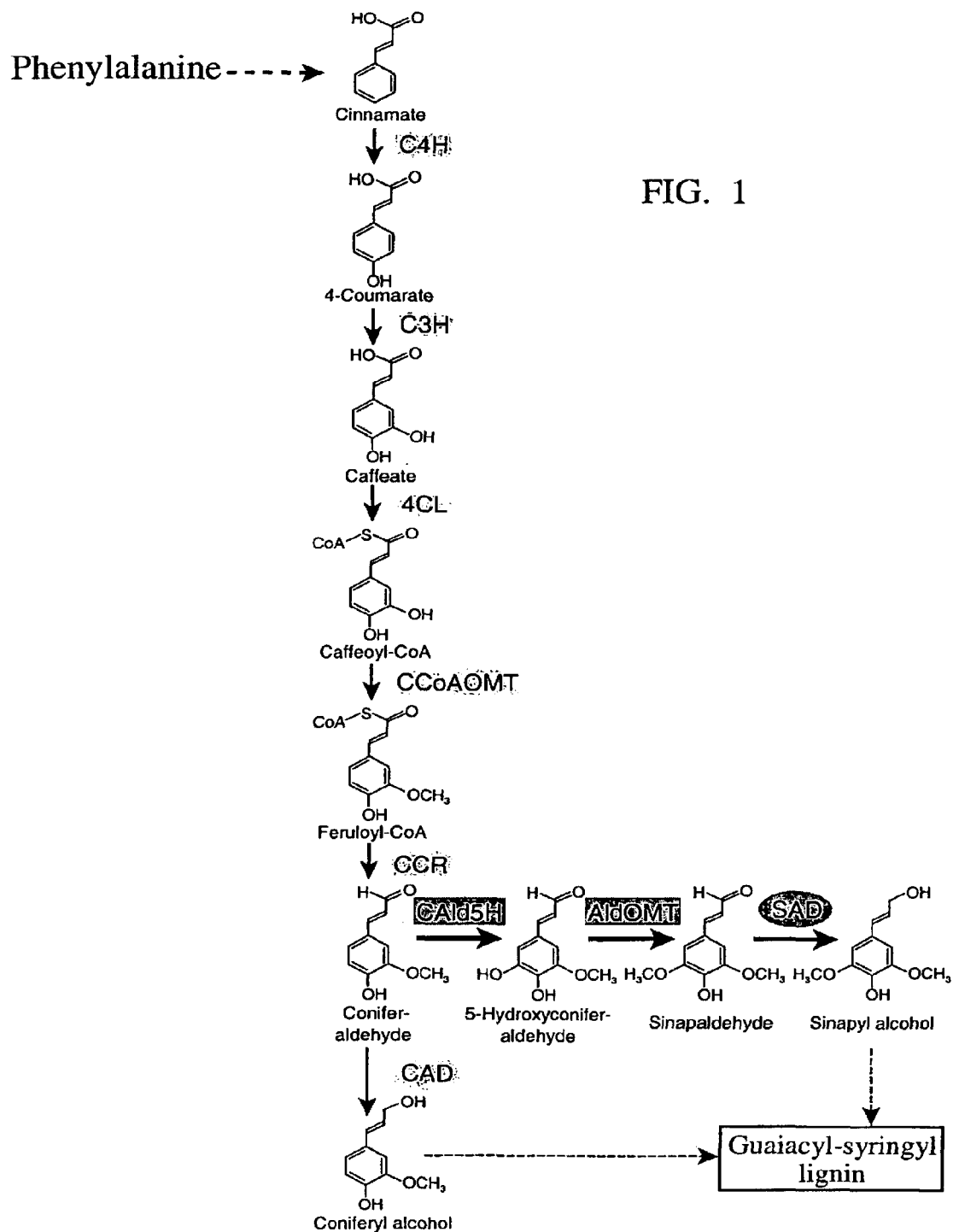


FIG. 2A SAD cDNA sequence

1 TTTTTTTTTT TTCCTAGCC TTCCTTCTCG ACGATATTC TCTATCTGAA
51 GCAAGCACCA TGTCCAAGTC ACCAGAAGAA GAACACCCTG TGAAGGCCTT
101 CGGGTGGGCT GCTAGGGATC AATCTGGTCA TCTTTCTCCC TTCAACTTCT
151 CCAGGAGGGC AACTGGTGAA GAGGATGTGA GGTTC AAGGT GCTGTACTGC
201 GGGATATGCC ATTCTGACCT TCACAGTATC AAGAATGACT GGGGCTTCTC
251 CATGTACCCT TTGGTTCCTG GGCATGAAAT TGTGGGGGAA GTGACAGAAG
301 TTGGGAGCAA GGTGAAAAAG GTTAATGTGG GAGACAAAGT GGGCGTGGGA
351 TGCTTGGTTG GTGCATGTCA CTCCTGTGAG AGTTGTGCCA ATGATCTTGA
401 AAATTACTGT CCAAAAATGA TCCTGACATA CGCCTCCATC TACCATGACG
451 GAACCATCAC TTACGGTGGC TACTCAGATC ACATGGTCGC TAACGAACGC
501 TACATCATTG GATCCCCGA TAACATGCCG CTTGACGGTG GCGCTCCTCT
551 CCTTTGTGCC GGGATTACAG TGTATAGTCC CTTGAAATAT TTTGGACTAG
601 ATGAACCCGG TAAGCATATC GGTATCGTTG GCTTAGGTGG ACTTGGTCAC
651 GTGGCTGTCA AATTTGCCAA GGCCTTTGGA TCTAAAGTGA CAGTAATTAG
701 TACCTCCCCT TCCAAGAAGG AGGAGGCTTT GAAGAACTTC GGTGCAGACT
751 CATTTTTGGT TAGTCGTGAC CAAGAGCAAA TGCAGGCTGC CGCAGGAACA
801 TTAGATGGCA TCATCGATAC AGTTTCTGCA GTTCACCCCC TTTTGCCATT
851 GTTTGGACTG TTGAAGTCTC ACGGGAAGCT TATCTTGGTG GGTGCACCGG
901 AAAAGCCTCT TGAGCTACCT GCCTTTTCTT TGATTGCTGG AAGGAAGATA
951 GTTGCCGGGA GTGGTATTGG AGGCATGAAG GAGACACAAG AGATGATTGA
1001 TTTTGCAGCA AAACACAACA TCACAGCAGA TATCGAAGTT ATTTCAACGG
1051 ACTATCTTAA TACGGCGATA GAACGTTTGG CTAAAAACGA TGTCAGATAC
1101 CGATTCTGCA TTGACGTTGG CAATACTTTG GCAGCTACGA AGCCCTAAGG
1151 AGAAGATCCC ATGTTCTCGA ACCCTTTATA AAATCTGATA ACATGTGTTG
1201 ATTTTCATGAA TAAATAGATT ATCTTTGGGA TTTTCTTTA ATAAACGAAG
1251 TGTTCTCGAA AACTTAACAT CGGCAATACC CTGGCAGCTA CGAGAAACGC
1301 TTTAGAATTG TTTGTAAGTT TGTTCATTA GGGTGATACC ATGCTCTCGA
1351 GTCCTTTGTA AGATCCATTT ATAGTTGCGT GAATGCTATG AACAAATAAT
1401 ATGTTTGC GG CTTCTCTTCA AAAAAAAAAA AAAAAAAAAA AAAAAA

FIG. 2B SAD protein sequence

1 MSKSPEEEHP VKAFGWAARD QSGHLSPPNF SRRATGEEDV RFKVLYCGIC
51 HSDLHSIKND WGFSMYPLVP GHEIVGEVTE VGSKVKKVNV GDKVGVGCLV
101 GACHSCESCA NDLENYCPKM ILTYASIIYHD GTITYGGYSD HMVANERYII
151 RFPDNMPLDG GAPLLCAGIT VYSPLKYFGL DEPGKHIGIV GLGGLGHVAV
201 KFAKAFGSKV TVISTSPSKK EEALKNFGAD SFLVSRDQEQ MQAAAGTLDG
251 IIDTVSAVHP LLPLFGLLKS HGKLILVGAP EKPLELPAFS LIAGRKIVAG
301 SGIGGMKETQ EMIDFAAKHN ITADIEVIST DYLNTAIERL AKNDVRYRFV
351 IDVGNTLAAT KP*

FIG. 3A Aspen (*P. tremuloides*) PtCald5H cDNA sequence

```
1 TAAAGTCTTG TGGATTACAC AAAATACAGA CTGAAAACAT CCATAGGCAC
51 CAACACATAA ACCATCCATG GATTCTCTTG TCCAATCTTT GCAAGCTTCA
101 CCCATGTCTC TCTTCTTGAT CGTTATCTCT TCACTCTTCT TCTTCGGTCT
151 CCTCTCTCGC CTTCGCCGAA GATTGCCATA TCCACCAGGG CCTAAAGGGT
201 TGCCACTTGT AGGTAGCATG CACATGATGG ACCAAATAAC TCACCGTGGG
251 TTAGCTAAAC TAGCTAAGCA ATATGGTGGG CTCTTTCATA TGCGCATGGG
301 GTACTTGCAT ATGGTCACTG TTTCATCTCC TGAAATAGCT CGCCAAGTTC
351 TGCAGGTCCA GGACAACATT TTCTCCAACA GACCAGCCAA CATAGCCATA
401 AGTTACTTAA CCTATGATCG TGCAGATATG GCCTTTGCCC ACTACGGTCC
451 TTTCTGGCGA CAGATGCGTA AGCTCTGCGT CATGAAGCTT TTTAGCCGGA
501 AAAGGGCTGA ATCATGGGAG TCTGTGAGAG ATGAGGTGGA CTCAATGCTT
551 AAGACAGTTG AAGCCAATAT AGGCAAGCCT GTGAATCTTG GGGAATTGAT
601 TTTTACGTTG ACCATGAACA TCACCTACAG AGCAGCTTTC GGGGCTAAAA
651 ATGAAGGACA GGATGAGTTC ATCAAGATTT TGCAGGAGTT CTCTAAGCTT
701 TTTGGAGCAT TCAACATGTC TGATTTTATT CCCTGGCTGG GCTGGATTGA
751 CCCCCAAGGG CTCAGCGCTA GACTTGTCAA GGCTCGCAAG GCTCTTGATA
801 GATTCATCGA CTCTATCATC GATGATCATA TCCAGAAAAG AAAACAGAAT
851 AAGTTCTCTG AAGATGCTGA AACCGATATG GTCGATGACA TGCTAGCCTT
901 TTATGGTGAA GAAGCAAGGA AAGTAGATGA ATCAGATGAT TTACAAAAG
951 CCATCAGCCT TACTAAAGAC AACATCAAAG CCATAATCAT GGATGTGATG
1001 TTTGGTGGGA CAGAGACGGT GGCCTCGGCA ATAGAGTGGG TCATGGCGGA
1051 GCTAATGAAG AGTCCAGAGG ATCAAAAAG AGTCCAGCAA GAGCTCGCAG
1101 AGGTGGTGGG TTTAGAGCGG CGCGTGGAGG AAAGTGATAT TGACAAACTT
1151 ACGTTCTTGA AATGCGCCCT CAAAGAAACC TTAAGGATGC ACCACCAAT
1201 CCCACTTCTC TTACATGAAA CTTCTGAGGA TGCTGAGGTT GCTGGTTATT
1251 TCATTCCAAA GCAAACAAGG GTGATGATCA ATGCTTATGC TATTGGGAGA
1301 GACAAGAATT CATGGGAAGA TCCTGATGCT TTTAAGCCTT CAAGTTTTTT
1351 GAAACCAGGG GTGCCTGATT TTAAAGGGAA TCACTTTGAG TTTATTCCTT
1401 TCGGGTCTGG TCGGAGGTCT TGCCCCGGTA TGCAGCTTGG GTTATACACA
1451 CTTGATTTGG CTGTTGCTCA CTTGCTTCAT TGTTTTACAT GGGAATTGCC
1501 TGATGGCATG AAACCGAGTG AACTTGACAT GACTGATATG TTTGGACTCA
1551 CCGCGCCAAG AGCAACTCGA CTCGTTGCCG TTCCGAGCAA GCGTGTGCTC
1601 TGTCTCTCT AAGGAAGGGA AAAAGGTAAG GGATGGAAT GAATGGGATT
1651 CCCTTCTTTC GTGGATTCTA TACAGAATTG AGGCCATGGT GACAAAGGGT
1701 CAATTTGCAG GTTTTTTTTT TTATATATAT ATATATATAA TTGGGTAAA
1751 AAAAAAAAAA AAAA
```

FIG. 3B Aspen (*P. tremuloides*) PtCAld5H protein sequence

1 MDSLQVSLQA SPMSLFLIVI SSLFFFGLLS RLRRRLPYPP GPKGLPLVGS
51 MHMMDQITHR GLAKLAKQYG GLFHMRMGYL HMVTVSSPEI ARQVLQVQDN
101 IFSNRPANIA ISYLTYDRAD MAFAHYGPFW RQMRKLCVMK LFSRKRAESW
151 ESVRDEVDSM LKTVEANIGK PVNLGELIFT LTMNITYRAA FGAKNEGQDE
201 FIKILQEFSK LFGAFNMSDF IPWLGWIDPQ GLSARLVKAR KALDRFIDSI
251 IDDHIQKRKQ NKFSEDAETD MVDDMLAFYG EEARKVDES DDLQKAISLTK
301 DNIKAIIMDV MFGGTETVAS AIEWVMAELM KSPEDQKRVQ QELAEVVGLE
351 RRVEESDIDK LTFLKCALKE TLRMHPIPL LLHETSEDAE VAGYFIPKQT
401 RVMINAYAIG RDKNSWEDPD AFKPSRFLKP GVPDFKGNHF EFIPFGSGRR
451 SCPGMQLGLY TLDLAVAHLL HCFTWELPDG MKPSELDMTD MFGLTAPRAT
501 RLVAVPSKRV LCPL*

FIG. 4A Aspen (*P. tremuloides*) PtAldOMT cDNA sequence

GenBank accession number: X62096

```
1   tcacttcctt tccttacacc ttcttcaacc ttttgtttcc ttgtagaatt
51  caatctcgat caagatgggt tcaacagggt aaactcagat gactccaact
101 caggtatcag atgaagaggc acacctcttt gccatgcaac tagccagtgc
151 ttcagttcta ccaatgatcc tcaaaacagc cattgaactc gaccttcttg
201 aatcatggc  taaagctggc cctggtgctt tcttgtccac atctgagata
251 gcttctcacc tccctacca  aaaccctgat ggcctgtca  tgtagaccg
301 tatectgcgc ctctgggcta gctactccat tcttacctgc tctctgaaag
351 atcttcctga tgggaagggt gagagactgt atggcctcgc tctgtttgt
401 aaattcttga ccaagaacga ggacgggtgc tctgtcagcc ctctctgtct
451 catgaaccag gacaaggctc tcatggaaag ctggtattat ttgaaagatg
501 caattcttga tggaggaatt ccatttaaca aggcctatgg gatgactgca
551 tttgaataac atggcacgga tccaagattc aacaaggctc tcaacaaggg
601 aatgtctgac cactctacca ttaccatgaa gaagattctt gagacctaca
651 aaggctttga aggctcacg  tcttgggtgg atgttgggtg tgggactgga
701 gccgtcgta  acaccatcgt ctctaaatac ccttcaatca agggcattaa
751 cttcgatctg cccacgtea  ttgaggatgc cccatcttat cccggagtgg
801 agcatgttgg tggcgacatg tttgttagtg tgcccaaagc agatgccgtt
851 ttcatagaag ggatatgcca tgattggagc gacgccact  gcttaaaatt
901 cttgaagaat tgctatgacg cgttgccgga aaacggcaag gtgatacttg
951 ttgagtgcac tcttcccgtg gtcctgaca  caagccttgc caccaaggga
1001 gtcgtgcacg ttgatgtcat catgctggcg cacaaccccg gtgggaaaga
1051 gaggaccgag aaggaatttg agggcttagc taaggagct  ggctccaag
1101 gttttgaagt aatgtgctgt gcattcaaca cacatgtcat tgaattccgc
1151 aagaaggcct aaggccatg  tccaagctcc aagttacttg gggttttgca
1201 gacaacgttg ctgctgtctc tgcgtttgat gtttctgatt gctttttttt
1251 atacgaggag tagctatctc ttatgaaaca tgtaaggata agattgcggt
1301 ttgtatgcct gattttctca aataacttca ctgcctccct caaaattctt
1351 aatacatgtg aaaagatttc ctattggcct tctgcttcaa acagtaaaga
1401 cttctgtaac ggaaaagaaa gcaattcatg atgtatgtat cttgcaagat
1451 tatgagtatt gttctaagca ttaagtgatt gttcaaaaaa aaaaaaaaaa
1501 aaa
```

FIG. 4B Aspen (*P. tremuloides*) PtAldOMT protein sequence

GenBank accession number: X62096

```
1  MGSTGETQMT PTQVSDEEAH LFAMQLASAS VLP MILKTAI ELDLLEIMAK
51  AGPGAFLSTS EIASHLPTKN PDAPVMLDRI LRL LASYSIL TCSLKDLPDG
101 KVERLYGLAP VCKFLT K NED GVSVSPLCLM NQDKVLMESW YYLKDAILDG
151 GIPFNKAYGM TAFEYHG TDP RFNKVFNKGM SDHSTITMKK ILETYKGFEG
201 LTSLVDVGGG TGAVVNTIVS KYPSIKGINF DLP HVIEDAP SYPGVEHVGG
251 DMFVSVPKAD AVFMKWICH D WSDAHCLKFL KNCYDALPEN GKVILVECIL
301 PVAPDTSLAT KGVVHVDVIM LAHNPGGKER TEKEFEGLAK GAGFQGF EVM
351 CCAFNTHVIE FRKKA
```

FIG. 5A 4CL polynucleotide DNA sequence

ccctcgcgaa actccgaaaa cagagagcac ctaaaactca ccattctctcc ctctgcatct	60
ttagcccgca atggacgcca ca atg aat cca caa gaa ttc atc ttt cgc tca	112
aaa tta cca gac atc tac atc ccg aaa aac ctt ccc ctg cat tca tac	160
gtt ctt gag aac ttg tct aaa cat tca tca aaa cct tgc ctg ata aat	208
ggc gcg aat gga gat gtc tac acc tat gct gat gtt gag ctc aca gca	256
aga aga gtt gct tct ggt ctg aac aag att ggt att caa caa ggt gac	304
gtg atc atg ctc ttc cta cca agt tca cct gaa ttc gtg ctt gct ttc	352
cta ggc gct tca cac aga ggt gcc atg atc act gct gcc aat cct ttc	400
tcc acc cct gca gag cta gca aaa cat gcc aag gcc tcg aga gca aag	448
ctt ctg ata aca cag gct tgt tac tac gag aag gtt aaa gat ttt gcc	496
cga gaa agt gat gtt aag gtc atg tgc gtg gac tct gcc ccg gac ggt	544
gct tca ctt ttc aga gct cac aca cag gca gac gaa aat gaa gtg cct	592
cag gtc gac att agt cct gat gat gtc gta gca ttg cct tat tca tca	640
ggg act aca ggg ttg cca aaa ggg gtc atg tta acg cac aaa ggg cta	688
ata acc agt gtg gct caa cag gta gat gga gac aat cct aac ctg tat	736
ttt cac agt gaa gat gtg att ctg tgt gtg ctt cct atg ttc cat atc	784
tat gct ctg aat tca atg atg ctc tgt ggt ctg aga gtt ggt gcc tcg	832
att ttg ata atg cca aag ttt gag att ggt tct ttg ctg gga ttg att	880
gag aag tac aag gta tct ata gca cca gtt gtt cca cct gtg atg atg	928
gca att gct aag tca cct gat ctt gac aag cat gac ctg tct tct ttg	976
agg atg ata aaa tct gga ggg gct cca ttg ggc aag gaa ctt gaa gat	1024
act gtc aga gct aag ttt cct cag gct aga ctt ggt cag gga tat gga	1072
atg acc gag gca gga cct gtt cta gca atg tgc ttg gca ttt gcc aag	1120
gaa cca ttc gac ata aaa cca ggt gca tgt gga act gta gtc agg aat	1168
gca gag atg aag att gtt gac cca gaa aca ggg gtc tct cta ccg agg	1216
aac cag cct ggt gag atc tgc atc cgg ggt gat cag atc atg aaa gga	1264
tat ctt aat gac ccc gag gca acc tca aga aca ata gac aaa gaa gga	1312
tgg ctg cac aca ggc gat atc ggc tac att gat gat gat gat gag ctt	1360
ttc atc gtt gac aga ttg aag gaa ttg atc aag tat aaa ggg ttt cag	1408
gtt gct cct act gaa ctc gaa gct ttg tta ata gcc cat cca gag ata	1456
tcc gat gct gct gta gta gga ttg aaa gat gag gat gcg gga gaa gtt	1504
cct gtt gca ttt gta gtg aaa tca gaa aag tct cag gcc acc gaa gat	1552
gaa att aag cag tat att tca aaa cag gtg atc ttc tac aag aga ata	1600
aaa cga gtt ttc ttc att gaa gca att ccc aag gca cca tca ggc aag	1648
atc ctg agg aag aat ctg aaa gag aag ttg cca ggc ata taactgaaga	1697
tgttactgaa catttaaccc tctgtcttat ttctttaata cttgcaatc attgtagtgt	1757
tgaaccaagc atgcttggaa aagacacgta cccaacgtaa gacagttact gttcctagta	1817
tacaagctct ttaatgttcg ttttgaactt gggaaaacat aagttctcct gtcgccatat	1877
ggagtaattc aattgaatat tttggtttct ttaatgat	1915

FIG. 6A Aspen (*P. tremuloides*) PtCAD protein sequence

GenBank accession number: AF217957

```
1  MGSLETERKI VGWAATDSTG HLA PYT YSLR DTG PEDVLIK VISCGICHTD
51  IHQIKNDLGM SHYPMVPGHE VVGEVVEVGS DVTKFKAGDV VGVGVIVGSC
101 KNCHPCKSEL EQYCNKKIWS YNDVYTDGKP TQGGFAESMV VDQKFVVRIP
151 DGMSPEQAAP LLCAGLTVYS PLKHFGLKQS GLRGGILGLG GVGHMGVKIA
201 KAMGHHVTVI SSSDKKREEA MEHLGADEYL VSSDVESMQK AADQLDYIID
251 TVPVVHPLEP YLSLLKLDGK LILMGVINTP LQFVSPMVML GRKSITGSFI
301 GSMKETEEML EFCKEKGLAS MIEVIKMDYI NTA FERLEKN DVRYRFVVDV
351 AGSKLIP*
```

FIG. 6B Aspen (*P. tremuloides*) PtCAD cDNA sequence

GenBank accession number: AF217957

```
1  AAACTCCATC CCTCTCTCTT AGCCTCGTTG TTTCAAGAAA ATGGGTAGCC
51  TTGAAACAGA GAGAAAAATT GTAGGATGGG CAGCAACAGA CTCAACTGGG
101 CATCTCGCTC CTTACACCTA TAGTCTCAGA GATACGGGGC CAGAAGATGT
151 TCTTATCAAG GTTATCAGCT GTGGAATTTG CCATACCGAT ATCCACCAAA
201 TCAAAAATGA TCTTGGCATG TCACACTATC CTATGGTCCC TGGCCATGAA
251 GTGGTTGGTG AGGTTGTTGA GGTGGGATCA GATGTGACAA AGTTCAAAGC
301 TGGAGATGTT GTTGGTGTGG GAGTCATCGT TGGAAAGCTGC AAGAATTGTC
351 ATCCATGCAA ATCAGAGCTT GAGCAATACT GCAACAAGAA AATCTGGTCT
401 TACAATGATG TCTACACTGA TGGCAAACCC ACCCAAGGAG GCTTTGCTGA
451 ATCCATGGTT GTCGATCAAA AGTTTGTGGT GAGAATTCCT GATGGGATGT
501 CACCAGAACA AGCAGCGCCG CTGTTGTGCG CTGGATTGAC AGTTTACAGC
551 CCACTCAAAC ACTTTGGACT GAAACAGAGT GGGCTAAGAG GAGGGATTTT
601 AGGACTTGGA GGAGTAGGGC ACATGGGGGT GAAGATAGCA AAGGCAATGG
651 GACACCATGT AACTGTGATT AGTTCTTCTG ACAAGAAGCG GGAGGAGGCT
701 ATGGAACATC TTGGTGCTGA TGAATACCTG GTCAGCTCGG ATGTGGAAAG
751 CATGCAAAAA GCTGCTGATC AACTTGACTA TATCATCGAT ACTGTGCCTG
801 TGGTTCACCC TCTCGAGCCT TACCTTTCTC TATTGAAACT TGATGGCAAG
851 CTGATCTTGA TGGGTGTTAT TAATACCCCA TTGCAGTTTG TTTCGCCAAT
901 GGTTATGCTT GGGAGAAAGT CGATCACCGG GAGCTTCATA GGGAGCATGA
951 AGGAGACAGA GGAGATGCTT GAGTTCCTGCA AGGAAAAGGG ATTGCCTCC
1001 ATGATTGAAG TGATCAAAAT GGATTATATC AACACAGCAT TCGAGAGGCT
1051 TGAGAAAAAT GATGTGAGAT ATAGATTCGT TGTCGATGTT GCTGGTAGCA
1101 AGCTTATTCC CTGAACGACA ATACCATTCA TATTCGAAAA AACGCGATAT
1151 ACATTGATAC CTGTTTCAGA CTTGACTTTA TTTTCGAGTG ATGTGTTTTG
1201 TGGTCAAAT GTGACAGTTT GTCTTTGCTT TTAAAATAAA GAAAAAGTTG
1251 AGTTGTTTTT TTATTTTCAT TAATGGGCAT GCGTTACCTT GTAATTGAAT
1301 GCGCTGCATC TGGTGTATCTG TCCCATAAAC TAATCTCTTG TGGCAATGAA
1351 AGATGACGAA CTTTCTGAAA AAAAAAAAAA AAAAAAAAAA AAAAA
```

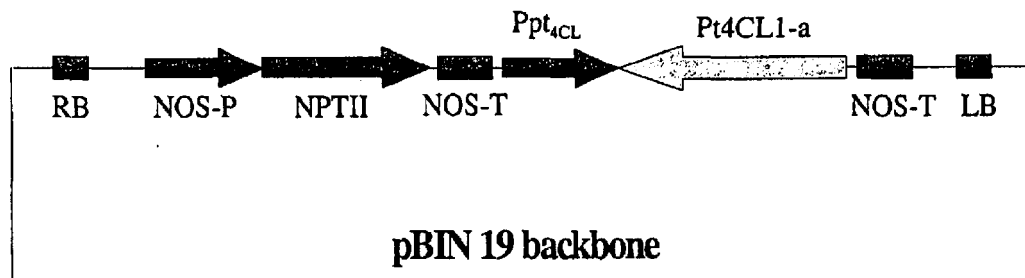


Fig. 7. pBKP_{pt_{4CL}} Pt4CL1-a construct

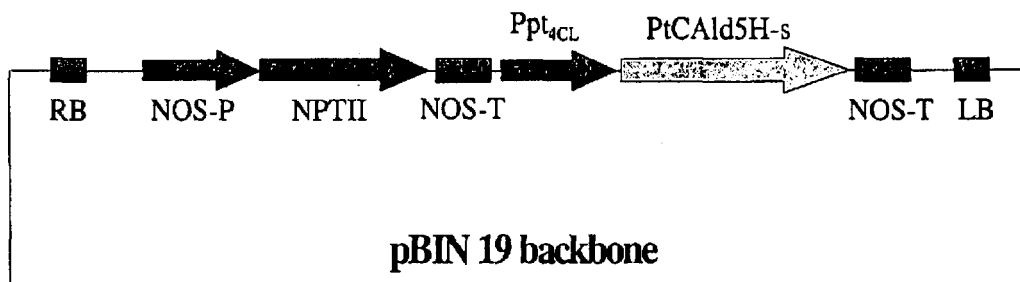


Fig. 8. pBKP_{pt_{4CL}} PtCA1d5H-s construct

FIG. 9-1 The alignment of plant AldOMT protein sequences

	1				50
1	~~~~~	~~~~~MG	STG..ETQMT	PTQVSDEEAH	LFAMQLASAS
2	~~~~~	~~~~~MG	STG..ETQMT	PTQVSDEEAH	LFAMQLASAS
3	~~~~~	~~~~~MG	STG..ETQMT	PTQVSDEEAN	LFAMQLASAS
4	~~~~~	~~~~~MG	STG..ETQMT	PTHVSDEEAN	LFAMQLASAS
5	~~~~~	~~~~~MG	STG..ETQIT	PTHSDEEAN	LFAMQLASAS
6	~~~~~	~~~~~MG	STG.SETQMT	PTQVSDEEAN	LFAMQLASAS
7	~~~~~	~~~~~MG	STGNAETQLT	PTHVSDEEAN	LFAMQLASAS
8	~~~~~	~~~~~MG	STSETKMSPS	EAAAAEEEF	VFAMQLTSAS
9	~~~~~	~~~~~MG	ST..AETQLT	PVQVTDDEAA	LFAMQLASAS
10	~~~~~	~~~~~MG	ST..SESQSN	SLTHTEDEAF	LFAMQLCSAS
11	MESTLAFNSG	SNSMNQSFSS	SAEFNSPVPE	TIPKSEEDTF	VFATLLTSAS
	51				100
1	VLPMILKTAI	ELDLLEIMAK	A...GPGAFI	STSEIASHLP	TKNPDAPVML
2	VLPMILKTAI	ELDLLEIMAK	A...GPGAFI	STSEIASHLP	TKNPDAPVML
3	VLPMVLKAAI	ELDLLEIMAK	A...GPGVFL	SPTDIASQLP	TKNPDAPVML
4	VLPMVLKAAI	ELDLLEIMAK	A...GPGSFL	SPSDLASQLP	TKNPEAPVML
5	VLPMILKSAL	ELDLLEIIAK	A...GPGAQI	SPIEIASQLP	TTNPDAPVML
6	VLPMVLKAAI	ELDLLEIMAK	A...GPGAFI	SPGEVAAQLP	TQNPEAPVML
7	VLPMVLKAAI	ELDVLEIMAK	SIPHGSGAYI	SPAEDIAAQLP	TTNPDAPVML
8	VLPMVLKSAI	ELDVLEIMAK	A...GPGAHI	STSDIASKLP	TKNPDAAVML
9	VLPMALKSAL	ELDLLEIMAKNGSPM	SPTDIASKLP	TKNPEAPVML
10	VLPMVLKSAV	ELDLLEIMAK	A...GPGAAI	SPSELAQLS	TQNPEAPVML
11	VLPMALKSAL	ELDLLEIIAK	A...GPGAFV	STSEIAAKIT	KRNPKAPVML
	101				150
1	DRILRLLASY	SILTCCLKDL	PDGKVERLYG	LAPVCKFLTK	NEDGVSVSPL
2	DRILRLLASY	SILTCCLKDH	PDGKVERLYG	LAPVCKFLTK	NEDGVSVSPL
3	DRMLRLLASY	SILTYSRLTL	ADGKVERLYG	LGPVCKFLTK	NEEGVSIAPL
4	DRMLRLLASY	SILTCCLKDL	PDGKVERLYC	LGPVCKFLTK	NEDGVSIAAL
5	DRMLRLLASY	IILTCVTRTQ	QDGKVQRLYG	LATVAKYLVK	NEDGVSISAL
6	DRIFRLLASY	SVLTCTLRNL	PDGKVERLYG	LAPVCKFLVK	NEDGVSIAAL
7	DRVLRLLASY	SVVTCCLKREL	PDGKVERLYG	LAPVCKFLTK	NEDGVS LAPL
8	DRMLRLLASY	SVLTCLKDL	PDGKIERLYG	LAPVCKFLTR	NDDGVSIAAL
9	DRILRLLASY	SVLTCLKDL	SGDGVERIYG	LGPVCKYLT	NEDGVSIAAL
10	DRMLRLLASY	SVLNCTLRNL	PDSSVERLYS	LAPVCKYLT	NADGVS LAPL
11	DRILRLLASY	DVVKCLKDL	PDGGVERLYG	LGPVCKYFTT	NEDGVS LAPL
	151				200
1	CLMNQDKVLM	ES.WYCLKDA	ILDGGIPFNK	AYGMTAFEYH	GTDPRFNKVF
2	CLMNQDKVLM	ES.WYCLKDA	ILDGGIPFNK	AYGMTAFEYH	GTDPRFNKVF
3	CLMNQDKVLL	ES.WYHLKDA	VLEGGIPFNK	AYGMTAFEYH	GTDPRFNKVF
4	CLMNQDKVLV	ES.WYHLKDA	VLDGGIPFNK	AYGMTAFDYH	GTDPRFNKVF
5	NLMNQDKVLM	ES.WYHLKDA	VLDGGIPFNK	AYGMTAFEYH	GTDPRFNKVF
6	NLMNQDKILM	ES.WYCLKDA	VLEGGIPFNK	AYGMTAFEYH	GTDPRFNKIF

FIG. 9-2

7	CLMNQDKVLM	ES.WYYLKDA	ILDGGIPFNK	AYGMTAFEYH	GTDPRFNKVF
8	SLMNQDKVLM	ES.WYHLTEA	VLEGGIPFNK	AYGMTAFEYH	GTDPRFNTVF
9	CLMNQDKVLM	ES.WYHLKDA	ILDGGIPFNK	AYGMSAFEYH	GTDPRFNKVF
10	LLMNQDKVLM	ES.WYHLKDA	VLDGGIPFNK	AYGMTAFEYH	GTDPRFNKVF
11	LLMNQDKVPM	QSKRYHLKDA	VLDGGIPFNK	AYGMTDFEYH	GTEPRFNKVF

	201				250
1	NKGMSDHSTI	TMKKILETYK	GFEGLTSLVD	VGGGTGAVVN	TIVSKYPSIK
2	NKGMSDHSTI	TMKKILETYK	GFEGLTSLVD	VGGGTGAVVN	TIVSKYPSIK
3	NRGMADHSTI	TMKKILETYK	GFEGLTSVVD	VGGGTGAVLN	MIVSKYPSIK
4	NKGMSDHSTI	TMKKILETYK	GFEGLSIVD	VGGGTGAVVN	MIVSKYPSIK
5	NKGMSDHSTI	TMKKILETYT	GFEGLSLVD	VGGGTGAVIN	TIVSKYPTIK
6	NRGMSDHSTI	TMKKILETYK	GFEGLETVVD	VGGGTGAVLS	MIVAKYPSMK
7	NRGMSDHSTI	TMKKIFEMYT	GFEALNTIVD	VGGGTGAVLS	MIVAKYPSIK
8	NNGMSNHSTI	TMKKILETYK	GFEGLSVVD	VGGGTGAHLN	MIIAKYPMIK
9	NNGMSNHSTI	TMKKILETYK	GFEGLTSLVD	VGGGIGATLK	MIVSKYPNLK
10	NRGMSDHSTM	SMKKILEDYK	GFEGLSIVD	VGGGTGATVN	MIVSKYPSIK
11	NNGVSGHPTI	TMKKILEAYK	GFEGLSIVD	VGGGTGATLN	MIISKYPTIK

motif I

	251				300
1	GINFDLPHVI	EDAPSYPGVE	HVGGDMFVSV	PKADAVFMKW	ICHDWSDAHC
2	GINFDLPHVI	EDAPSYPGVE	HVGGDMFVSV	PKADAVFMKW	ICHDWSDAHC
3	GINFDLPHVI	EDAPQYPGVE	HVGGDMFVSV	PKGDAIFMKW	ICHDWSDEHC
4	GINFDLPHVI	EDAPQYPGVQ	HVGGDMFVSV	PKGNAIFMKW	ICHDWSDEHC
5	GINFDLPHVI	EDAPSYPGVE	HVGGDMFVSI	PKADAVFMKW	ICHDWSDEHC
6	GINFDLPHVI	EDAPPLPGVK	HVGGDMFVSV	PKGDAIFMKW	ICHDWSDDHC
7	GINFDLPHVI	EDAPIYPGVE	HVGGDMFVSV	PKGDAIFMKW	ICHDWSDEHC
8	GINFDLPHVI	EEAPSYPGVE	HVGGDMFVSV	PKGDAIFMKW	ICHDWSDEHC
9	GINFNLPHVI	EDAPSHPGIE	HVGGDMFVSV	PKGDAIFMKW	ICHDWSDEHC
10	GINFDLPHVI	GDAPTYPGVE	HVGGDMFASV	PKADAI FMKW	ICHDWSDEHC
11	GINFDLPHVI	DDAPSYPGVE	HVGGDMFVSV	PKGDAIFMKW	MCYEWDDAHC

motif II

	301				350
1	LKFLKNCYDA	LPENGKVILV	ECILPVAPDT	SLATKGVVHV	DVIMLAHNPG
2	LKFLKNCYDA	LPENGKVILV	ECILPVAPDT	SLATKGVVHI	DVIMLAHNPG
3	LKFLKNCYAA	LPDNGKVILG	ECILPVAPDS	SLATKGVVHI	DVIMLAHNPG
4	IKFLKNCYAA	LPDDGKVILA	ECILPVAPDT	SLATKGVVHM	DVIMLAHNPG
5	LKFLKNCYEA	LPDNGKVIVA	ECILPVAPDS	SLATKGVVHI	DVIMLAHNPG
6	AKFLKNCYDA	LPNIGKVIVA	ECVLPVYPDT	SLATKNVIHI	DCIMLAHNPG
7	LKFLKNCYAA	LPEHGKVIVA	ECILPLSPDP	SLATKGVVIHI	DAIMLAHNPG
8	LKFLKKCYEA	LPTNGKVILA	ECILPVAPDA	SLPTKAVVHI	DVIMLAHNPG
9	VKFLKNCYES	LPEDGKVILA	ECILPETPDS	SLSTKQVVHV	DCIMLAHNPG

FIG. 9-3

10 LKFLKNCYEA LPANGKVIIA ECILPEAPDT SLATKNTVHV DIVMLAHNPG
 11 LKFLENCYQA LPDNGKVIVA ECILPVVPDT SLATKSAVHI DVIMLAYNTG

motif III

	351		389
1	GKERTEKEFE	GLAKGAGFQG	FEVMCCAFNT HVIEFRKKA
2	GKERTEKEFE	GLAKGAGFQG	FEVMCCAFNT HVIELRKN~
3	GKERTEQEFQ	ALAKGAGFQG	FNVACSAFNT YVIEFLKKN
4	GKERTEQEFE	ALAKGSGFQG	IRVCCDAFNT YVIEFLKKI
5	GKERTQKEFE	DLAKGAGFQG	FKVHCNAFNT YIMEFLKKV
6	GKERTQKEFE	TLAKGAGFQG	FQVMCCAFGT HVMEFLKTA
7	GKERTEKEFE	ALAIGAGFKG	FKVACCAFNT YVMEFLKTA
8	GKERTEKEFE	ALAKGAGFEG	FRVALCAYNT WIIEFLKKI
9	GKERTEKEFE	ALAKASGFKG	IKVVCDAFGV NLIELLKKL
10	GKERTEKEFE	ALAKGAGFTG	FARLVALTTL GSWNSTSN~
11	GKARTEKEFE	ALAKGAGFQG	FKVVCCAFNS WIMEFCKTA

Plant AldOMTs from

- | | |
|-------------------------|------------------------------|
| 1) Aspen, X62096 | 7) Clarkia breweri, AF006009 |
| 2) Poplar, M73431 | 8) Sweetgum, AF139533 |
| 3) Almond, X83217 | 9) Arabidopsis, U70424 |
| 4) Strawberry, AF220491 | 10) Tobacco, X74452 |
| 5) Alfalfa, M63853 | 11) Vitis vinifera, AF239740 |
| 6) Eucalyptus, X74814 | |

FIG. 10-1 The alignment of full length plant CAD protein sequences available in the GenBank database

	1				50
1	MGSLE.TEKT	VTGYAARDSS	GHLSPYTYNL	RKKGPEDVIV	KVIYCGICHS
2	MGSLE.SEKT	VTGYAARDSS	GHLSPYTYNL	RKKGPEDVIV	KVIYCGICHS
3	MGSLE.SEKT	VTGYAARDSS	GHLSPYTYNL	RKKGPEDVIV	KVIYCGICHS
4	MGSLE.SERT	VTGYAARDSS	GHLSPYTYTL	RNKGPELVIV	RVIYCGICHS
5	MGSL.ASERK	VVGWAARDAT	GHLSPYSYTL	RNTGPEDVVV	KVLYCGICHT
6	MGSL.ASERK	VVGWAARDAT	GHLSPYSYTL	RNTGPEDVVV	KVLYCGICHT
7	MGSL.ASERK	VVGWAARDAT	GHLAPYTYTL	RSTGPEDVVV	KVLYCGICHT
8	MGSIEAAERT	TVGLAAKDPS	GILTPYTYTL	RNTGPDDVVI	KIHVYCGVCHS
9	MGSIEAAERT	TVGLAAKDPS	GILTPYTYTL	RNTGPDDVVI	KIHVYCGVCHS
10	MGSLEK.ERT	TTGWAARDPS	GVLSPYTYSL	RNTGPEDLYI	KVLSCGICHS
11	MGSLEK.ERT	TTGWAARDPS	GVLSPYTYSL	RNTGPEDLYI	KVLSCGICHS
12	MGGLEV.EKT	TIGWAARDPS	GVLSPYTYTL	RNTGPEDVEV	KVLYCGLCHT
13	MGSLDV.EKS	AIGWAARDPS	GLLSPYTYTL	RNTGPEDVQV	KVLYCGLCHS
14	MGSLET.ERK	IVGWAATDST	GHLAPYTYSL	RDTGPEDVLI	KVISCVICHT
15	MGSLET.ERK	IVGWAATDST	GHLAPYTYSL	RDTGPEDVFI	KVISCVICHT
16	MGSLEA.ERK	TTGWAARDPS	GVLSPYTYTL	RETGPEDVFI	KI IYCGICHT
	51				100
1	DLVQMRNEMG	MSHYPMVPGH	EVVGIVTEIG	SEVKKFKVGE	HVGVCIVGS
2	DLVQMRNEMG	MSHYPMVPGH	EVVGIVTEIG	SEVKKFKVGE	HVGVCIVGS
3	DLVQMRNEMG	MSHYPMVPGH	EVVGIVTEIG	SEVKKFKVGE	HVGVCIVGS
4	DLVQMHNEMG	MSNYPMVPGH	EVVGVVTEIG	SEVKKFKVGE	HVGVCIVGS
5	DIHQAKNHLG	ASKYPMVPGH	EVVGEVVEVG	PEVAKYGVGD	VVGVGIVGC
6	DIHQAKNHLG	ASKYPMVPGH	EVVGEVVEVG	PEVAKYGVGD	VVGVGIVGC
7	DIHQAKNHLG	ASKYPMVPGH	EVVGEVVEVG	PEVTKYGVGD	VVGVGIVGC
8	DLHQIKNDLG	MSNYPMVPGH	EVVGEVLEVG	SNVTRFKVGE	IVGVGLLVGC
9	DLHQIKNDLG	MSNYPMVPGH	EVVGEVLEVG	SNVTRFKVGE	IVGVGLLVGC
10	DIHQIKNDLG	MSHYPMVPGH	EVVGEVLEVG	SEVTKYRVGD	RVGTGIVVGC
11	DIHQIKNDLG	MSHYPMVPGH	EVVGEVLEVG	SEVTKYRVGD	RVGTGIVVGC
12	DLHQVKNDLG	MSNYPLVPGH	EVVGEVVEVG	PDVSKFKVGD	TVGVGLLVGS
13	DLHQVKNDLG	MSNYPLVPGH	EVVGVVVEVG	ADVSKFKVGD	TVGVGLLVGS
14	DIHQIKNDLG	MSHYPMVPGH	EVVGEVVEVG	SDVTKFKAGD	VVGVGIVGS
15	DIHQIKNDLG	MSHYPMVPGH	EVVGEVVEVG	SDVTRFKVGD	VVGVGIVGS
16	DIHQIKNDLG	ASNYPMVPGH	EVVGEVVEVG	SDVTKFKVGD	CVGDGTIVGC
			Zn1		
Zn2					
	101				150
1	CRSCGNCNQS	MEQYCSKRIW	TYNDVNHDGT	PTQGGFASSM	VVDQMFVVRI
2	CRSCGNCNQS	MEQYCSKRIW	TYNDVNHDGT	PTQGGFASSM	VVDQMFVVRI
3	CRSCGNCNQS	MEQYCSKRIW	TYNDVNHDGT	PTQGGFASSM	VVDQMFVVRI
4	CRSCSNCNGS	MEQYCSKRIW	TYNDVNHDGT	PTQGGFASSM	VVDQMFVVRI
5	CRECSPCKAN	VEQYCNKKIW	SYNDVYTDGR	PTQGGFASTM	VVDQKFVVKI
6	CRECSPCKAN	VEQYCNKKIW	SYNDVYTDGR	PTQGGFASTM	VVDQKFVVKI
7	CRECKPCKAN	VEQYCNKKIW	SYNDVYTDGR	PTQGGFASTM	VVDQKFVVKI
8	CKSCRACDSE	IEQYCNKKIW	SYNDVYTDGK	ITQGGFAEST	VVEQKFVVKI
9	CKSCRACDSE	IEQYCNKKIW	SYNDVYTDGK	ITQGGFAEST	VVEQKFVVKI

FIG. 10-2

10	CRSCSPCNSD	QEQYCNKKIW	NYNDVYTDGK	PTQGGFAGEI	VVGERFVVKI
11	CRSCSPCNSD	QEQYCNKKIW	NYNDVYTDGK	PTQGGFAGEI	VVGERFVVKI
12	CRNCGPCKRD	IEQYCNKKIW	NCNDVYTDGK	PTQGGFAKSM	VVDQKFVVKI
13	CRNCGPCKRE	IEQYCNKKIW	NCNDVYTDGK	PTQGGFANSM	VVDQNFVVKI
14	CKNCHPCKSE	LEQYCNKKIW	SYNDVYTDGK	PTQGGFAESM	VVDQKFVVRI
15	CKNCHPCKSE	IEQYCNKKIW	SYNDVYTDGK	PTQGGFAESM	VVHQKFVVRI
16	<u>CKTCRPCKAD</u>	<u>VEQYCNKKIW</u>	SYNDVYTDGK	PTQGGFSGHM	VVDQKFVVKI

Zn²

	151				200
1	PENLPLEQAA	PLLCAGTVVF	SPMKHFAMTE	.PGKKCGILG	LGGVGHMGVK
2	PENLPLEQAA	PLLCAGTVVF	SPMKHFAMTE	.PGKKCGILG	LGGVGHMGVK
3	PENLPLEQAA	PLLCAGTVVF	SPMKHFAMTE	.PGKKCGILG	LGGVGHMGVK
4	PENLPLEQAA	PLLCAGTVVY	SPMKHFGMTE	.PGKKCGILG	LGGVGHMGVK
5	PAGLAPEQAA	PLLCAGTVVY	SPLKHFGL.T	TPGLRGGILG	LGGVGHMGVK
6	PAGLAPEQAA	PLLCAGTVVY	SPLKHFGL.T	NPGLRGGILG	LGGVGHMGVK
7	PAGLAPEQAA	PLLCAGTVVY	SPLKAFGL.T	TPGLRGAILG	LGGVGHMGVK
8	PEGLAPEQVA	PLLCAGTVVY	SPLSHFGLK.	TPGLRGGILG	LGGVGHMGVK
9	PEGLAPEQVA	PLLCAGTVVY	SPLSHFGLK.	TPGLRGGILG	LGGVGHMGVK
10	PDGLESEQAA	PLMCAGTVVY	SPLVRFGLKQ	.SGLRGGILG	LGGVGHMGVK
11	PDGLESEQAA	PLMCAGTVVY	SPLVRFGLKQ	.SGLRGGILG	LGGVGHMGVK
12	PEGMAPEQAA	PLLCAGITVY	SPLNHFGFKQ	.SGLRGGILG	LGGVGHMGVK
13	PEGMAPEQAA	PLLCAGITVY	SPFNHFGFNQ	.SGFRGGILG	LGGVGHMGVK
14	PDGMSPEQAA	PLLCAGLTVY	SPLKHFGLKQ	.SGLRGGILG	LGGVGHMGVK
15	PDGMSPEQAA	PLLCAGLTVY	SPLKHFGLKQ	.SGLRGGILG	LGGVGHMGVK
16	PDGMAPEQAA	PLLCAGTVVY	SPLTHFGLKE	ISGLRGGILG	<u>LGGVGHMGVK</u>

NADP

	201				250
1	IAKAFGLHVT	VISSSDKKKE	EAMEVLGADA	YLVSKDTEKM	MEAAESLDYI
2	IAKAFGLHVT	VISSSDKKKE	EAMEVLGADA	YLVSKDTEKM	MEAAESLDYI
3	IAKAFGLHVT	VISSSDKKKE	EAMEVLGADA	YLVSKDTEKM	MEAAESLDYI
4	IAKAFGLHVT	VISSSDKKKE	EALEVLGADA	YLVSKDAEKM	QEAAESLDYI
5	VAKAMGHHVT	VISSSSKKRA	EAMDHLGADA	YLVSSDAAAM	GPAADSLDYI
6	VAKAMGHHVT	VISSSSKKRA	EAMDHLGADA	YLVSSDAAAM	AAAADSLDYI
7	VAKAMGHHVT	VISSSSKKRA	EAMDHLGADA	YLVSSDAAAM	AAAADSLDYI
8	VAKALGHHVT	VISSSDKKKK	EALDHLGADN	YLVSSDTVGM	QEAAADSLDYI
9	VAKALGHHVT	VISSSDKKKK	EALDHLGADN	YLVSSDTVGM	QEAAADSLDYI
10	IAKAMGHHVT	VISSSDKKRT	EALEHLGADA	YLVSSDENG	KEATDSLDYI
11	IAKAMGHHVT	VISSSDKKRT	EALEHLGADA	YLVSSDENG	KEATDSLDYI
12	IAKAMGHHVT	VISSSNKKRQ	EALEHLGADD	YLVSSDSDKM	QEASDSLDYI
13	IAKAMGHHVT	VISSSNKKRQ	EALEHLGADD	YLVSSDSDKM	QEASDSLDYI
14	IAKAMGHHVT	VISSSDKKRE	EAMEHLGADE	YLVSSDVESM	QKAADQLDYI
15	IAKAMGHHVT	VISSSDKKRE	EAMEHLGADE	YLVSSDVESM	QKAADQLDYI
16	LAKAMGHHVT	VISSSDKKKE	EAI DHLGADA	YLVSSDATQM	QEAAADSLDYI

FIG. 10-3

251				300	
1	MDTIPVAHPL	EPYLALLKTN	GKLVMLGVVP	EPLHFVTPLL	ILGRRSIAGS
2	MDTIPVAHPL	EPYLALLKTN	GKLVMLGVVP	EPLHFVTPLL	ILGRRSIAGS
3	MDTIPVAHPL	EPYLALLKTN	GKLVMLGVVP	EPLHFVTPLL	ILGRRSIAGS
4	MDTIPVAHPL	EPYLALLKTN	GKLVMLGVVP	EPLHFVTPLL	ILGRRSIAGS
5	IDTVPVHHPL	EPYLALLKLD	GKLVLLGVIG	EPLSEFVSPMV	MLGRKAITGS
6	IDTVPVHHPL	EPYLALLKLD	GKLVLLGVIG	EPLSEFVSPMV	MLGRKAITGS
7	IDTVPVHHPL	EPYLALLKLD	GKLVLLGVIG	EPLSEFVSPMV	MLGRKAITGS
8	IDTVPVGHPL	EPYLSLLKID	GKLILMGVIN	TPLQFVTPMV	MLGRKSITGS
9	IDTVPVGHPL	EPYLSLLKID	GKLILMGVIN	TPLQFVTPMV	MLGRKSITGS
10	FDTIPVVHPL	EPYLALLKLD	GKLILTVGIN	APLQFISPMV	MLGRKSITGS
11	FDTIPVVHPL	EPYLALLKLD	GKLILTVGIN	APLQFISPMV	MLGRKSITGS
12	IDTVPVGHPL	EPYLSLLKID	GKLILMGVIN	TPLQFISPMV	MLGRKSITGS
13	IDTVPVGHPL	ELYLSLLKID	GKLILIGVIN	TPLQFISPMV	MLGRKSITGS
14	IDTVPVVHPL	EPYLSLLKLD	GKLILMGVIN	TPLQFVSPMV	MLGRKSITGS
15	IDTVPVVHPL	EPYLSLLKLD	GKLILMGVIN	APLQFVTPMV	MLGRKSITGS
16	IDTVPVFHPL	EPYLSLLKLD	GKLILMGVIN	TPLQFISPMV	MLGRKAITGS
	301			350	
1	FIGSMEETQE	TLDFCAEKKV	SSMIEVVGLD	YINTAMERLE	KNDVRYRFVV
2	FIGSMEETQE	TLDFCAEKKV	SSMIEVVGLD	YINTAMKRLE	KNDVRYRFVV
3	FIGGMEETQE	TLDFCAEKKV	SSMIEVVGLD	YINTAMERLE	KNDVRYRFVV
4	FIGSMEETQE	TLDFCAEKKV	SSMIEVVGLD	YINTAMERLV	KNDVRYRFVV
5	FIGSIDETA	VLQFCVDKGL	TSQIEVVKMG	YVNEALERLE	RNDVRYRFVV
6	FIGSIDETA	VLQFCVDKGL	TSQIEVVKMG	YVNEALERLE	RNDVRYRFVV
7	FIGSIDETA	VLQFCVDKGL	TSQIEVVKMG	YVNEALDRLE	RNDVRYRFVV
8	FVGSVKETEE	MLEFWKEKGL	TSMIEIVTMD	YINKAFERLE	KNDVRYRFVV
9	FVGSVKETEE	MLEFWKEKGL	TSMIEIVTMD	YINKAFERLE	KNDVRYRFVV
10	FIGSMKETEE	MLEFCKEKGL	TSQIEVIKMD	YVNTALERLE	KNDVRYRFVV
11	FIGSMKETEE	MLEFCKEKGL	TSQIEVIKMD	YVNTALERLE	KNDVRYRFVV
12	FIGSMKETEE	MLDFCKEKGV	TSQIEIVKMD	YINTAMERLE	KNDVRYRFVV
13	FIGSMKETEE	MLDFCNEKGI	TSTIEVVKMD	YINTAFERLE	KNDVRYRFVV
14	FIGSMKETEE	MLEFCKEKGL	ASMIEVIKMD	YINTAFERLE	KNDVRYRFVV
15	FIGSMKETEE	MLEFCKEKGV	ASMIEVIKMD	YINTAFERLE	KNDVRYRFVV
16	FIGSMKETEE	MLDFCNEKGI	TSTIEVVKMD	YINTAFERLE	KNDVRYRFVV
	351		370		
1	DVAGSKLDN*	~~~~~			
2	DVAASKLDN*	~~~~~			
3	DVAGSELDN*	~~~~~			
4	DVAASNLDK*	~~~~~			
5	DVAGSNVEAE	AAAADAASN*			
6	DVAGSNVEAE	AAAADAASN*			
7	DVAGSNV . . E	EVAADAPSN*			
8	DVKGSKFEE*	~~~~~			
9	DVKGSKFEE*	~~~~~			
10	DVVGSKLD*~	~~~~~			

FIG. 10-4

11 DVVGSKLD*~ ~~~~~
12 DVIGSKLDQ* ~~~~~
13 DVAGSKLDQ* ~~~~~
14 DVAGSKLIP* ~~~~~
15 DVAGSKLIH* ~~~~~
16 DVAGSKLDQE T*~~~~~

Full length plant CADs from

- | | |
|--------------------------|--------------------------|
| 1) Radiata pine, U62394 | 9) Lucerne, Z19573 |
| 2) Loblolly pine, Z37992 | 10) Eucalyptus, AF038561 |
| 3) Loblolly pine, Z37991 | 11) Eucalyptus, X65631 |
| 4) Norway spruce, X72675 | 12) Tobacco, X62343 |
| 5) Maize, aj005702 | 13) Tobacco, X62344 |
| 6) Maize, Y13733 | 14) Aspen, AF217957 |
| 7) Sugarcane, AJ231135 | 15) Cottonwood, Z19568 |
| 8) Lucerne, AF083332 | 16) Udo, D13991 |

FIG. 11-1 The alignment of full length plant CAld5H protein sequences

1) Aspen; 2) Poplar, AJ010324; 3) Sweetgum, AF139532; 4) Arabidopsis, U38416. *, Heme-binding signature

	1				50					
1	~MDSL	VQSLQ	AS..PMSLFL	IVISSLFFFG	LLSRLRRRLP	YPPGPKGLPL				
2	~MDSL	LQSLQ	TL..PMSFFL	IIISSIFFLG	LISRLRRRSP	YPPGPKGFPL				
3	MDSL	LHEALQ	PL..PMTLFF	I.IPLLLLLL	LVSRLRQRLP	YPPGPKGLPV				
4	MESSIS	QTL	KLSDPTTSLV	IVVSLFIFIS	FITR.RRRPP	YPPGPRGWPI				
	51				100					
1	VGSMH	MMDQI	THRGLAKLAK	QYGGLFHMRM	GYLHMVTVSS	PEIARQVLQV				
2	IGSMH	LMDQL	TDRGLAKLAK	QYGGLFHMRM	GYLHMVAGSS	PEVARQVLQV				
3	IGNML	MMDQL	THRGLAKLAK	QYGGLFHLKM	GFLHMVAVST	PDMARQVLQV				
4	IGNML	MMDQL	THRGLANLAK	KYGGLCHLRM	GFLHMYAVSS	PEVARQVLQV				
	101				150					
1	QDNIF	SNRPA	NIAISYLT	YD	RADMAFAHYG	PFWRQMRKLC	VMKLF	SRKRA		
2	QDNMF	SNRPA	NIAISYLT	YD	RADMAFAHYG	PFWRQMRKLC	VMKLF	SRKRA		
3	QDNIF	SNRPA	TIAISYLT	YD	RADMAFAHYG	PFWRQMRKLC	VMKLF	SRKRA		
4	QDSVF	SNRPA	TIAISYLT	YD	RADMAFAHYG	PFWRQMRKVC	VMKV	FSRKRA		
	151				200					
1	ESWES	VRDEV	DSMLKTVEAN	IGKPVNLGEL	IFTLTMNITY	RAAFGA.KNE				
2	ESWES	VRDEV	DSMVKTVESN	IGKPVNVGEL	IFTLTMNITY	RAAFGA.KNE				
3	ESWES	VRDEV	DSAVRVVASN	IGSTVNI	GEL	VFALTKNITY	RAAFGTISHE			
4	ESWAS	VRDEV	DKMVR	SVSCN	VGKPINVGEQ	IFALTRNITY	RAAFGSACEK			
	201				250					
1	GQDEF	IKILQ	EFSKLF	GAFN	MSDFIPWL	GW	IDPQGLSARL	VKARKALDRF		
2	GQDEF	IKILQ	EFSKLF	GAFN	ISDFIPWL	GW	IDPQGLTARL	VKARKALDKF		
3	DQDEF	VAILQ	EFSQLF	GAFN	IADFIPWL	KW	V.PQGINVRL	NKARGALDGF		
4	GQDEF	FIRILQ	EFSKLF	GAFN	VADFIPYFGW		IDPQGINKRL	VKARNDL	DGF	
	251				300					
1	IDSII	DDHIQ	KRKQNK	FSED	...AETDMVD	DMLAFY	GEEA	RKVDESDDLQ		
2	IDHII	DDHIQ	KRKQNN	YSEE	...AETDMVD	DMLTFY	SEET	.KVNESDDLQ		
3	IDKII	DDHIQ	KGSKN	..SEE	...VDTDMVD	DL	LAFY	GEEA	.KVSESDDLQ	
4	IDDI	I	DEHMK	KKENQNAVDD	GDVVDTDMVD	DL	LAFY	SEEA	KLVSETADLQ	
	301				350					
1	KAISL	TKDNI	KAIIMD	V	MFG	GTETV	VASAIE	WVMAELMKSP	EDQKR	VQOEL
2	NAIKL	TRDNI	KAIIMD	V	MFG	GTETV	VASAIE	WAMAEL	LKSP	EDIKRVQOEL
3	NSIKL	TKDNI	KA	IMD	V	MFG	GTETV	VASAIE	WAMTELMKSP	EDLKKVQOEL
4	NSIKL	TRDNI	KAIIMD	V	MFG	GTETV	VASAIE	WALTELLRSP	EDLKR	VQOEL

FIG. 11-2

351 400
1 AEVVGLERRV EESDIDKLTf LKCALKETLR MHPPiPLLLH ETSEDAEVAG
2 ADVVGLERRV EESDFDKLTf FKCTLKETLR LHPPiPLLLH ETSEDAEVAG
3 AVVVGLDRRV EEKDFEKLTy LKCVLKEVLR LHPPiPLLLH ETAEDAevGG
4 AEVVGLDRRV EESDIEKLTy LKCTLKETLR MHPPiPLLLH ETAEDTSIDG

401 450
1 YFIPKQTRVM INAYAIGRDk NSWEDPDAFK PSRFLKPGVP DFKGNHFEFI
2 YYVPKKTRVM INAYAIGRDk NSWEDPDSFK PSRFLEPGVP DFKGNHFEFI
3 YYIPAKSRVM INACAIGRDk NSWADPDTFR PSRFLKDGVP DFKGNHFEFI
4 FFIPKKSVM INAFAIGRDp TSWTDPDTFR PSRFLEPGVP DFKGSNFEFI

451 500
1 PFGSGRRSCP GMQLGLyTLd LAVAhLLHCF TWELPDGMKP SELDMTDMFG
2 PFGSGRRSCP GMQLGLyALd LAVAhLLHCF TWELPDGMKP SELDMTDMFG
3 PFGSGRRSCP GMQLGLyALe TTVAHLLHCF TWELPDGMKP SELEMNDVFG
4 PFGSGRRSCP GMQLGLyALd LAVAhILHCF TWKLPDGMKP SELDMNDVFG
***** *

501 523
1 LTAPRATRLV AVPSKRVLCp L*
2 LTAPRATRLV AVPRKRVVCP L~~
3 LTAPRAIRLT AVPSRLLCP LY*
4 LTAPKATRLF AVPTTRLICA L~~

FIG. 12-1 PLANT 4CL AMINO ACID SEQUENCE ALIGNMENTS

(1) 1:-----MNPQ-EFIFRSKLPDIYIPKNLPLHSYVLENLSKHSSKPCLI 41
(2) 1:-----MDAIMNSQEEFIFRSKLPDIYIPKNLPLHSYVLENLSKYSSKPCLI 46
(3) 1:-----MGDCVAPKEDLIFRSKLPDIYIPKHLPLHTYCFENISKVGDKSCLI 46
(4) 1:-----MPMDTETKQSGDLIFRSKLPDIYIPKHLPLHSYCFENLSEFNRSRPLI 48
(5) 1:-----M-AVQTPQHNIIVYRSKLPDIHIPNHLPLHSYIFQNKSHLTSKPCII 45
(6) 1:-----MPMDTETKQSGDLIFRSKLPDIYIPKHLPLHSYCFENLSEFNRSRPLI 48
(7) 1:-----MEKDTKH-GDIFRSKLPDIYIPNHLPLHSYCFENISEFSSSRPLI 45
(8) 1:MGSME-Q-QQPES-AAPATEASPEIIFRSKLQDIAITNTLPLHRYCFERLPEVAARPLI 57
(9) 1:MITLAPSLDTPKTDQNQVSDPQTSHVFKSKLPDIPISNHLPLHSYCFQNLQFAHRPLI 60
(10) 1:MAPQE-Q-AVSQVMEKQSNNNNSDVIIFRSKLPDIYIPNHLPLHSDYIFQNISEFATKPCLI 58
(11) 1:----A-N-GI-K----KV-E----HLYRSKLPDIEISDHLPLHSYCFERVAEFADRPLI 44
(12) 1:M---A-N-GI-K----KV-E----HLYRSKLPDIEISDHLPLHSYCFERVAEFADRPLI 45
(13) 1:-----
(14) 1:-----
(15) 1:-----LI 2
(16) 1:-----PCLI 4

(1) 42:NG-ANGDVITYADVELTARRVA-SGLNKIGIQQGDVIMLFLPSSPEFVLAFLGASHRGAM 99
(2) 47:NG-ANGDVITYADVELTARRVA-SGLNKIGIQQGDVIMLFLPSSPEFVLAFLGASHRGAI 104
(3) 47:NG-ATGETFTYSQVELLSRKVA-SGLNKLGIQQGDTIMILLPNSPEYFFAFLGASVRGAI 104
(4) 49:DG-ANDRIYTYAEVELTSRKVA-VGLNKLGIQQKDTIMILLPNCPEFVFAFIGASYLGAI 106
(5) 46:NG-TTGDIIHTYAKFKLTARKVA-SGLNKLGIQKQDVFMILLPNTSEFVFAFLGASFCGAM 103
(6) 49:DG-ANDRIYTYAEVELTSRKVA-VGLNKLGIQQKDTIMILLPNCPEFVFAFIGASYLGAI 106
(7) 46:NG-ANKQIYTYADVELSSRKVA-AGLHKQGIQQKDTIMILLPNSPEFVFAFIGASYLGAI 103
(8) 58:DGATGGVLTADVDRLSRRLAAALRRAPLGLRRGVVMSLLRNSPEFVLSFFAASRVGAA 117
(9) 61:VG-PASKTFTYADTHLISSKIA-AGLSNLGILKGDVVMILLQNSADVFVFLAISMGAV 118
(10) 59:NGPTGHVYTYSDVHVISRQIAANFHK--LGVNQNDVVMILLPNCPEFVLSFLAASFRGAT 116
(11) 45:DG-ATDRTYCFSEVELISRKVA-AGLAKLGLQQGVVMLLLPNCIEFAFVFMGASVRGAI 102
(12) 46:DG-ATDRTYCFSEVELISRKVA-AGLAKLGLQQGVVMLLLPNCIEFAFVFMGASVRGAI 103
(13) 1:-----A-----K-----A- 3
(14) 1:-----
(15) 3:DG-STNKTYNFAEVELISRKVA-AGLAKLGLKKGQVVMILLQNCIEFAFVFMGASVLGAV 60
(16) 5:DG-ATGKTHCFAEVELISRKVA-AGLVNGLLQQGVVMLLLQNCVEFAFVFMGAALRGAI 62

(1) 100:ITAANPFSTPAELAKHAKASRAKLLITQACYYEKVK--DFARESDVKVMCVDs-APD-GA 155
(2) 105:VTAANPFSTPAELAKHAKPRTKLLITQACYVDKVK--DFARESDVKVMCVDs-APD-GC 160
(3) 105:STMANPFSTSAEVIKQLKASQAKLIITQACYVDKVK--DYAAEKNIQIICID-DAP-QDC 160
(4) 107:STMANPLFTPAEVVKQAKASAKIVITQACFAGKVK--DYAIENDLKVICVD-SVP-EGC 162
(5) 104:MTAANPFSTPAEIAKQAKASKAKLIITFACYVDKVK--DLSCD-EVKLMCIDSPPDSSC 160
(6) 107:STMANPLFTPAEVVKQAKASAKIITQACFAGKVK--DYAIENDLKVICVD-SAP-EGC 162
(7) 104:STMANPLFTAEEVVKQVKASGAKIIVTQACHVNKVK--DYALENNVKIICID-SAP-EGC 159
(8) 118:VTTANPMSTPHEIESQLAAAGATVVITESMAADKL-PSHSHGALTVV-LID-E--R-RDG 171
(9) 119:ATTANPFYTAPEIFKQFTVSKAKLIITQAMYVDKLRNHDGAKLGEDFKVVTVDDPP-ENC 177
(10) 117:ATAANPFSTPAEIAKQAKASNTKLIITEARYVDKIKPLQNDGIVVIVCIDDNESVPIPEG 176
(11) 103:VTTANPFYKPGEIAKQAKAAGARIIVTLAAYVEKL-A-D-LQ-SHDVLVITIDGAPKEGC 158
(12) 104:VTTANPFYKPGEIAKQAKAAGARIIVTLAAYVEKL-A-D-LQ-SHDVLVITIDGAPKEGC 159
(13) 4:---A-----G-----ARIIVTQAAYVDKL-A-D-LQ-SDDMIVIAIDGAPKEGC 40
(14) 1:-----KPGEIAKQAKAAGARIIVTQAAYVEKL-A-D-LQ-NDDVIVITIDAAPKDCG 48
(15) 61:VTTANPFYKPGEIAKQAKAADARIIVTQAAYVDKL-A-D-LQ-SEDVIVISIDGAPKEGC 116
(16) 63:VTTANPFYKPGEIAKQAKAAGARIIVTQAAYVEKL-A-D-LQ-SDDVIVITIDGAPKDCG 118

(1) 156:SLFRAHTQADENEVPQV-----DISPDDVVALPYSSGTTGLPKGVMLTHKGLITSVA 207
(2) 161:LHFSELTQADENEVPQV-----DFSPDDVVALPYSSGTTGLPKGVMLTHKGLITSVA 212
(3) 161:LHFSKLMEADESEMPEV-----VINSDDVVALPYSSGTTGLPKGVMLTHKGLVTSVA 212
(4) 163:VHFSELIQSDEHEIPDV-----KIQPDVVALPYSSGTTGLPKGVMLTHKGLVTSVA 214
(5) 161:LHFSELTQSDENDVPDV-----DISPDDVVALPYSSGTTGLPKGVMLTHKGLVTSVS 212

FIG. 12-2

(6) 163:VHFSELIQSDEHEIPDV-----KIQPDDVVALPYSSGTTGLPKGVMLTHKGLVTSVA 214
(7) 160:LHFSVLTQADEHDIPEV-----EIQPDDVVALPYSSGTTGLPKGVMLTHKGLVTSVA 211
(8) 172:CLHFWDLMSEDEASPLAGDEDEKVFDPDDVVALPYSSGTTGLPKGVMLTHRSLSVTSVA 231
(9) 178:LHFSVLSEANESDVPEV-----EIHPPDDAVAMPFSSGTTGLPKGVMLTHKSLTTSVA 229
(10) 177:CLRF-TEL-TQSTTEA-SEVIDSVEI-SPDDVVALPYSSGTTGLPKGVMLTHKGLVTSVA 232
(11) 159:QHISVLTEADETQCPAV-----KIHPDDVVALPYSSGTTGLPKGVMLTHKGLVSSVA 210
(12) 160:QHISVLTEADETQCPAV-----KIHPDDVVALPYSSGTTGLPKGVMLTHKGLVSSVA 211
(13) 41:QHISILTEADETQCPSV-----EIHPPDDVVALPYSSGTTGLPKGVMLTHKSQVSSVA 92
(14) 49:QHISVLTEADETQCPSV-----EIQPDDVVALPYSSGTTGLPKGVMLTHKGLVSSVA 100
(15) 117:QHISVLTEADETQCPSV-----EIHPPDDVVALPYSSGTTGLPKGVMLTHKSLVSSVA 168
(16) 119:KDISVLTEADGTQCPSV-----EIQPDDVVALPYSSGTTGLPKGVMLTHKGLVSSVA 170

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(1) 208:QQVDGDNPNLYFHSEDVILCVLPMFHIYALNSMMLCGLRVGASILIMPKEFIGSLLGLIE 267
(2) 213:QQVDGDNPNLYFHSEDVILCVLPMFHIYALNSIMLCGLRVGASILIMPKFDIGTLLGLIE 272
(3) 213:QQVDGDNPNLYMHSEDVMICILPLFHIYSLNAVLCCGLRAGVTILIMQKFDIVPFLELIQ 272
(4) 215:QQVDGENANLYMHSDVLMCVLPLFHIYSLNSVLLCALRVGAAILIMQKFDIAQFLELIP 274
(5) 213:QQVDGENPNLYYSSDDVLCVPLFHIYSLNSVLLCGLRAGAAILLMQKFEIVSLELMQ 272
(6) 215:QQVDGENANLYMHSDVLMCVLPLFHIYSLNSVLLCALRVGAAILIMQKFDIAQFLELIP 274
(7) 212:QQVDGENRNLYIHSEDVLLCVLPLFHIYSLNSVLLCGLRVGAAILIMQKFDIVPFLELIQ 271
(8) 232:QQVDGENPNIGLHAGDVILCALPMFHIYSLNTIMMCGLRVGAAILVMMRRFDLAAMMDLVE 291
(9) 230:QQVDGENPNLYLTTEDEVLLCVLPLFHIYSLNSVLLCALRAGSAVLLMQKFEIGTLLLELIQ 289
(10) 233:QQVDGENPNLYFHSDVILCVLPMFHIYALNSIMLCGLRVGAAILIMPKEFINLLELIQ 292
(11) 211:QQVDGENPNLYFHSDVILCVLPLFHIYSLNSVLLCGLRAGAAATLIMQKFNLTTCLELIQ 270
(12) 212:QQVDGENPNLYFHSDVILCVLPLFHIYSLNSVLLCGLRAGAAATLIMQKFNLTTCLELIQ 271
(13) 93:QQVDGENPNLYFHSEDVILCVLPLFHIYSLNSVLLCGLRAGAAATLIMQKFNLTALLELIQ 152
(14) 101:QQVDGENPNLYFHSDVILCVLPLFHIYSLNSVLLCGLRAGAAATLIMQKFNMTSFLLELIQ 160
(15) 169:QQVDGENPNLYFHSEDVILCVLPLFHIYSLNSVLLCGLRAGAAATLIMQKFNLTTCLELIQ 228
(16) 171:QQVDGENPNLYFHSEDVVMCVLPLFHIYSLNSVLLCGLRAGAAATLIMQKFNMTSFLLELIQ 230

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(1) 268:KYKVSIAVPPVPMMAIAKSPDLKHDLSLRLMIKSGGAPLGKELEDTVRAKFPQARLQ 327
(2) 273:KYKVSIAVPPVPMMAIAKSPDFDKHDLSSLRLMIKSGGAPLGKELEDTVRAKFPQARLQ 332
(3) 273:KYKVTIGPFVPPIVLAIKSPVVDKYDLSVVRTVMGGAAPLGKELEDAVRAKFPNAKLGQ 332
(4) 275:KHKVTIGPFVPPIVLAIKSPVVDNYDLSVVRTVMGGAAPLGKELEDAVRAKFPNAKLGQ 334
(5) 273:KHRVSVAPIVPPVTLAIKSPVVDKDYDLSIRVLKSGGAPLGKELEDTVRAKFPNVTLGQ 332
(6) 275:KHKVTIGPFVPPIVLAIKSPVVDHNYDLSVVRTVMGGAAPLGKELEDAVRAKFPNAKLGQ 334
(7) 272:NYKVTIGPFVPPIVLAIKSPVVDYDLSVVRTVMGGAAPLGKELEDTVRAKFPNAKLGQ 331
(8) 292:RHRVTIAPLVPPIVVAVAKSEAAAARDLSSVRMVLGGAAPMGKDIEDAFMAKLPGAVLGQ 351
(9) 290:RHRVSVAMVPPVPLVLAALAKNPMVADFLLSIRVLVSGAAPLGKELEEAALRNMPQAVLGQ 349
(10) 293:RCKVTVAPMVPPIVLAIKSPVVDTEKYDLSIRVVKSGAAPLGKELEDAVNAKFPNAKLGQ 352
(11) 271:KYKVTVAPIVPPIVLDITKSPIVSQYDVSSVRIIMSGAAPLGKELEDALRERFPKAI FGQ 330
(12) 272:KYKVTVAPIVPPIVLDITKSPIVSQYDVSSVRIIMSGAAPLGKELEDALRERFPKAI FGQ 331
(13) 153:RYKVTVAPIVPPIVLEISKNPISVQYDVPSVRIIMSGAAPLGKELEDALRERFPKAI FGQ 212
(14) 161:RYKVTVAPIVPPIVLDITKSPIISQYDVSSVRIIMSGAAPLGKELEDALRDRFPQAI FGQ 220
(15) 229:RYKVTVAPIVPPIVLDITKNPISVQYDVSSVRIIMSGAAPLGKELEDALRERFPKAI FGQ 288
(16) 231:RYKVTVAPIVPPVLEITKSPIVSQYDISSVRIIVSGGAPLGKELEDAIRDRLPHAI FGQ 290

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(1) 328:GYGMTEAGPVLAMCLAFAKEPFDIKPGACGTVVRNAEMKIVDPETGVSLPRNQPGEICIR 387
(2) 333:GYGMTEAGPVLAMCLAFAKEPFDIKPGACGTVVRNAEMKIVDPETGASLRRNQPGEICIR 392
(3) 333:GYGMTEAGPVLAMCLAFAKEPEIKSGACGTVVRNAEMKIVDPETNASLPRNQPGEICIR 392
(4) 335:GYGMTEAGPVLAMCLAFAKEPFDIKSGACGTVVRNAEMKIVDPDTGCSLPRNQPGEICIR 394
(5) 333:GYGMTEAGPVLTMCLAFAKEPFEVKGCGGTVVRNAELKIVDPETGASLPRNHGPGEICIR 392
(6) 335:GYGMTEAGTVLTMCLAFAKEPFDIKSGACGTVVRNAEMKIVDPDTGCSLPRNHGPGEICIR 394
(7) 332:GYGMTEAGPVLAMCLAFAKEPFEIKSGACGTVVRNAEMKIVDPETGNSLPRNQSGEICIR 391
(8) 352:GYGMTEAGPVLMSCLAFAKEPFEVKGSGACGTVVRNAELKIDPDTGKSLGRNLRGEGEICIR 411
(9) 350:GYGMTEAGPVLMSCLGFAKQPFQTKSGSCGTVVRNAELKVVDPETGRSLGYNQPGEICIR 409

FIG. 12-3

(10) 353:GYGMTEAGPVLAMSLGFAKEPPFPVKSGACGTVVRNAEMKIVDPDTGDSLRSRNPGEICIR 412
(11) 331:GYGMTEAGPVLAMNLAFAKNPPFPVKSGCGTVVRNAQIKILDTEGSLPHNQAGEICIR 390
(12) 332:GYGMTEAGPVLAMNLAFAKNPPFPVKSGCGTVVRNAQIKILDTEGSLPHNQAGEICIR 391
(13) 213:GYGMTEAGPVL----- 223
(14) 221:GYGMTEAGPV----- 230
(15) 289:GYGMTEAGPVLAMNLAFAKEPPFPVKSGSC----- 317
(16) 291:GYGMTEAGPVLAMNLAFAKEPPFPVKSGS----- 318
***** *

(1) 388:GDQIMKGYLNDPEATSRTIDKEGWLHTGDIGYIDDDDELFI VDR LKELIKYKGFQVAPTE 447
(2) 393:GDQIMKGYLNDPEATSRTIDKEGWLHTGDIGYIDDDDELFI VDR LKELIKYKGFQVAPAE 452
(3) 393:GDQIMKGYLNDPESTRTTIDEEGWLHTGDIGFIDDDDELFI VDR LKEI IKYKGFQVAPAE 452
(4) 395:GDQIMKGYLNDPEATARTIEKEGWLHTGDIGFIDDDDELFI VDR LKELIKYKGFQVAPAE 454
(5) 393:GHQIMKGYLNDPEATRTTIDKQGWLHTGDIGFIDDDDELFI VDR LKELIKYKGFQVAPAE 452
(6) 395:GDQIMKGYLNDPEATARTIEEEGWLHTGDIGFIDDDDELFI VDR LKELIKYKGFQVAPAE 454
(7) 392:GDQIMKGYLNDPEATARTIDKEGWLYTGDIGYIDDDDELFI VDR LKELIKYKGFQVAPAE 451
(8) 412:GQQIMKGYLNNPEATKNTIDAEGWLHTGDIGYVDDDEIFIVDR LKEI IKYRGFQVAPAE 471
(9) 410:GQQIMKGYLNDAAATASTIDSEGWLHTGDVGVYDDDEIFIVDR VKELIKYKGFQVPPAE 469
(10) 413:GHQIMKGYLNNPAATAETIDKDGWLHTGDIGLIDDDDELFI VDR LKELIKYKGFQVAPAE 472
(11) 391:GPEIMKGYINDPESTAATIDEEGWLHTGDVEYIDDDDEIFIVDR VKELIKYKGFQVAPAE 450
(12) 392:GPEIMKGYINDPESTAATIDEEGWLHTGDVGYIDDDDEIFIVDR VKELIKYKGFQVAPAE 451
(13) 224:-----
(14) 231:-----
(15) 318:-----
(16) 319:-----

(1) 448:LEALLIAHPEISDAAVVGLKDEDAGEVPVAFVVKSEKSQATEDEIKQYISKQVIFYKRIK 507
(2) 453:LEALLLAHPQISDAAVVGMKDEDAGEVPVAFVVKSEKSQATEDEIKQYISKQVIFYKRIK 512
(3) 453:LEALLLTHPTISDAAVVPMIDEKAGEVPVAFVVRTNGFTTTEELKQFVSKQVIFYKRIK 512
(4) 455:LEALLINHPDISDAAVVPMIDEQAGEVPVAFVVRNSGSTITEDEVKDFISKQVIFYKRIK 514
(5) 453:LEALLVTHPNISDAAVVPMKDDAAGEVPVAFVVPKSGSQITEDEIKQFISKQVIFYKRIK 512
(6) 455:LEALLINHPDISDAAVVPMIDEQAGEVPVAFVVRNSGSTITEDEVKDFISKQVIFYKRIK 514
(7) 452:LEALLLNHPFSDAAVVPKDEQAEEVPVAFVVRSSGSTITEDEVKDFISKQVIFYKRIK 511
(8) 472:LEALLNTHPSIADA AVVGLK---FGEIPVAFVAKTEGSELSEDDVKQFVAKEVIYYKKIR 528
(9) 470:LEGLLVSHPSIADA AVVPQKDVAAGEVPVAFVVRNSGFDLTEEAVKEFIAKQVIFYKRLH 529
(10) 473:LEALLIGHPDI TDVAVVAMKEEAAGEVPVAFVVKSKDSELSEDDVKQFVSKQVIFYKRIN 532
(11) 451:LEALLVAHPSIADA AVVPQKHEEAGEVPVAFVVKKS-S-EISEQEIKEFVAKQVIFYKRIH 508
(12) 452:LEALLVAHPSIADA AVVPQKHEEAGEVPVAFVVKKS-S-EISEQEIKEFVAKQVIFYKRIH 509
(13) 224:-----
(14) 231:-----
(15) 318:-----
(16) 319:-----

(1) 508:RVFFIEAIPKAPSGKILRKNLKEKL-PGI----- 535
(2) 513:RVFFIEAIPKAPSGKILRKNLRETL-PGI----- 540
(3) 513:RVFFVDAIPKSPSGKILRKDLRARIASGDLPK--- 544
(4) 515:RVFFVETVPKSPSGKILRKDLRARLAAGISN--- 545
(5) 513:RVFFIEAIPKSPSGKILRKELRAKLAAGFAN--- 543
(6) 515:RVFFVETVPKSPSGKILRKDLRARLAAGISN--- 545
(7) 512:RVFFVDAVPKSPSGKILRKDLRAKLAAGLPN--- 542
(8) 529:EVFFVDKIPKAPSGKILRKELRKQLQHLQEQEALTN 563
(9) 530:KVYFVHAI PKSPSGKILRKDLRAKLETAATQTP-- 562
(10) 533:KVFFTESIPKAPSGKILRKDLRAKLANGL----- 561
(11) 509:RVYFVDAIPKSPSGKILRKDLRSRLAAK----- 536
(12) 510:RVYFVDAIPKSPSGKILRKDLRSRLAAK----- 537

FIG. 12-4

(13) 224:-----
(14) 231:-----
(15) 318:-----
(16) 319:-----

- | | |
|----------------------------|--------------------------|
| 1: aspen AF041049 | 9: soybean x69955 |
| 2: Hybrid populus AF283552 | 10: Ara AF106084 |
| 3: Parsley X13324 | 11: PinusteadaU12012 |
| 4: potato M62755 | 12: Pinus teada U12013 |
| 5: Rubus idaeus AF239687 | 13: Larix AF144513 |
| 6: solanum AF150686 | 14: PseudolarixAF144528 |
| 7: Tobacco D43773 | 15: Pseudotsuga AF144511 |
| 8: rice x52623 | 16: Tsuga AF144526 |

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METHODS FOR SIMULTANEOUS CONTROL OF LIGNIN CONTENT AND COMPOSITION, AND CELLULOSE CONTENT 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, United States Department of Energy, 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 provides a method of introducing two or more genes, involved in lignin biosynthesis, into plant cells. The method of the invention employs either an *Agrobacterium*-mediated or other appropriate plant gene delivery system by which multiple genes together with a single selectable marker gene are simultaneously transferred and inserted into the genome of plants with high frequencies.

The ability to introduce foreign genes into plants is a prerequisite for engineering agronomic traits in plants. Many systems have been developed for introducing a foreign gene into plant cells, which involve mainly either *Agrobacterium*- or microprojectile bombardment-mediated transformation (Christou, 1996). The principle of all these systems involves the insertion of a target gene into the host plant genome together with a selectable marker gene encoding either antibiotic or herbicide resistance to aid in the selection of transgenic cells from non-transgenic cells. These systems generally are only effective for introducing a single target gene into the host plant.

To alter agronomic traits, which generally are polygenic in nature, multiple genes involved in complex biosynthetic pathways must be introduced and expressed in plant cells. In this context, the traditional single-gene transfer systems are essentially useless for the following two reasons: 1) it is impractical to introduce multiple genes by repetitive insertion of single genes into transgenic plants due to the time and effort required for recovery of the transgenic tissues; in particular, a repetitive single-gene approach is highly impractical for plant species such as trees which, depending upon the species, require two to three years for transgenic tissue selection and regeneration into a tree; and 2) the presence of a selectable marker gene in a transgenic line precludes the use of the same marker gene in subsequent transformations of plant material from that line. Moreover, the number of available marker genes is limited, and many plant species are recalcitrant to regeneration unless appropriate antibiotic or herbicide selection is used.

Chen et al. (1998) recently reported the genetic transformation of rice with multiple genes by cobombardment of several gene constructs into embryogenic suspension tissues. However, particle bombardment-mediated gene transfer into embryogenic tissues is highly species-dependent, and regeneration of whole plants from embryogenic cells cannot be achieved for a variety of plant species (Horsch et al., 1985).

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In contrast, *Agrobacterium*-mediated gene transfer and whole plant regeneration through organogenesis is a simple process and a less species-dependent system than bombardment-mediated transformation and regeneration via embryogenesis. However, the introduction of more than one gene in a single plasmid vector via *Agrobacterium* may be technically troublesome and limited by the number or the size of the target genes (Chen et al., 1998). For example, Tricoli et al. (1995) reported the transfer of three target genes to squash via *Agrobacterium*-mediated gene transfer. A binary plasmid vector containing the three target genes was incorporated into an *Agrobacterium* strain, which was subsequently used to infect the leaf tissue of squash. As only one line was recovered from numerous infected squash tissues that contained all of the target genes, the use of a single binary vector with a number of genes appears to be a highly inefficient method to produce transgenic plants with multiple gene transfers. Therefore, it was commonly accepted that transfer of multiple genes via *Agrobacterium*-mediated transformation was impractical (Ebinuma et al., 1997), until success of multiple gene transfer via *Agrobacterium* was first reported in co-pending, commonly owned PCT application, PCT/US/0027704, filed Oct. 6, 2000, entitled "Method of Introducing a Plurality of Genes into Plants" by Chiang et al, incorporated herein by reference. However, homologous tissue-specific preparation of transgenic trees to specifically alter lignin content, increase S/G (syringyl:guaiacyl) lignin ratio and increase cellulose quantity, as compared to an untransformed plant was unsuccessful.

Yet, the altering of lignin content and composition in plants has been a goal of genetically engineered traits in plants. 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 cellulosic 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 (G) 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 (G) and syringyl (S) 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 could be genetically 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 \$6 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).

Depending on the use for the plant, genetic engineering of certain traits has been attempted. For some plants, as indi-

cated above, there has been a long-standing incentive to genetically modify lignin and cellulose to decrease lignin and increase cellulose contents. For example, it has been demonstrated that the digestibility of forage crops by ruminants is inversely proportional to lignin content in plants (Buxton and Roussel, 1988, *Crop. Sci.*, 28, 553–558; Jung and Vogel, 1986, *J. Anim., Sci.*, 62, 1703–1712). Therefore, decreased lignin and high cellulose plants are desirable in forage crops to increase their digestibility by ruminants, thereby providing the animal with more nutrients per unit of forage.

In other plants, genetically increasing the S/G ratio of the lignin has been sought. As noted above, lignin in angiosperms is composed of guaiacyl (G) and syringyl (S) monomeric units, whereas gymnosperm lignin consists entirely of G units. The structural characteristics of G units in gymnosperm lignin promote monomer cross-linkages that increase lignin resistance to chemical extraction during wood pulp production. However, the S units present in angiosperm lignin prevent such chemical resistant cross-links. Therefore, without exception, chemical extraction of G lignin in pulping of gymnosperms is more difficult and requires more chemicals, longer reaction times and higher energy levels than the extraction of G-S lignin during pulping of angiosperms (Sarkanen, K. V., 1971, in *Lignins: Occurrence, Formation, Structure and Reaction*, Sarkanen, K. V. & Ludwig, C. H., eds., Wiley-Interscience, New York; Chang, H. M. and Sarkanen, K. V., 1973, *TAPPI*, 56:132–136). As a rule, the reaction rate of extracting lignin during wood pulping is directly proportional to the quantity of the S unit in lignin (Chang, H. M. and Sarkanen, K. V., 1973, *TAPPI*, 56:132–136). Hence, altering lignin into more reactive G-S type in gymnosperms and into high S/G ratio in angiosperms would represent a pivotal opportunity to enhance current pulping and bleaching efficiency and to provide better, more economical, and more environmentally sound utilization of wood.

Recent results have indicated that high S/G ratio may also add further mechanical advantages to plants, balancing the likely loss of sturdiness of plants with severe lignin reduction (Li et al., 2001, *Plant Cell*, 13:1567–1585). Moreover, a high S/G lignin ratio would also improve the digestibility of forage crops by ruminants (Buxton and Roussel, 1988, *Crop. Sci.*, 28, 553–558; Jung and Vogel, 1986, *J. Anim., Sci.*, 62, 1703–1712).

In some applications, both a high lignin content and high S/G ratio have been sought (i.e., combining these two traits in plants). For example, it has been demonstrated that when lignin is extracted out from wood during chemical pulping, lignin in the pulping liquor is normally used as a fuel source to provide energy to the pulping and bleaching operations. This lignin-associated energy source, which is not necessary for pulp mills using purchased fuel for energy, is essential to some pulp mills which depend upon internal sources, such as extracted lignin, to be self-sufficient in energy. Therefore, for this purpose, it may be desirable to increase lignin content in pulpwood species, and at the same time to increase the S/G ratio in these species to facilitate the extraction of more lignin to be used as fuel.

Additionally, for grain production and other non-related purposes, increased lignin content and/or S/G lignin ratio are desirable to provide extra sturdiness in plants to prevent the loss of socially and economically important food crops due to dislodging and due to damage to the aerial parts of the plant.

The plant monolignol biosynthetic pathway is set forth in FIG. 1 and will be explained in more detail hereinbelow. The

key lignin control sites in the monolignol biosynthetic pathway are mediated by genes encoding the enzymes 4-coumarate-CoA ligase (4CL) (Lee et al., 1997), coniferyl aldehyde 5-hydroxylase (CAlD5H) (Osakabe et al., 1999) and S-adenosyl-L-methionine (SAM)-dependent 5-hydroxyconifer aldehyde O-methyltransferase (AldOMT) (Li et al., 2000), respectively, for the formation of sinapaldehyde (see, FIG. 1). Further, coniferyl alcohol dehydrogenase (CAD) (MacKay et al., 1997) catalyzes the reaction including the substrate coniferyl aldehyde to coniferyl alcohol. It has recently been discovered that sinapyl alcohol dehydrogenase (SAD) enzymatically converts sinapaldehyde into sinapyl alcohol, the syringyl monolignol, for the biosynthesis of syringyl lignin in plants (see, FIG. 1). See, concurrently filed, commonly owned U.S. non-provisional application entitled “Genetic Engineering of Syringyl-Enriched Lignin in Plants,” incorporated herein by reference. It should be noted that the gene encoding the enzyme sinapyl alcohol dehydrogenase (SAD) represents the last gene that is indispensable for genetic engineering of syringyl lignin in plants.

A summary of the conserved regions contained within the coding sequence of each of the above listed proteins is described below. Because SAD is a recently discovered enzyme in Aspen, sequence alignments with other representative species were unable to be performed.

The protein sequence alignments of plant AldOMTs are shown in FIG. 9. All AldOMTs have three conserved sequence motifs (I, II, and III) which are the binding sites of S-adenosyl-L-methionine (SAM), the co-substrate or methyl donor for the OMT reaction (Ibrahim, 1997, *Trends Plant Sci.*, 2:249–250; Li et al., 1997, *Proc. Natl. Acad. Sci. USA*, 94:5461–5466; Joshi and Chiang, 1998, *Plant Mol. Biol.*, 37:663–674). These signature sequence motifs and the high sequence homology of these proteins to PtAldOMT attest to their function as an AldOMT specific for converting 5-hydroxyconifer aldehyde into sinapaldehyde (Li et al., 2000, *J. Biol. Chem.*, 275:6537–6545), the content of which is incorporated by reference, herein, in its entirety. This AldOMT, like CAlD5H, also operates at the aldehyde level of the plant monolignol biosynthetic pathway.

The protein sequence alignments of plant CADs are shown in FIG. 10. It was recently proven that CADs are actually guaiacyl monolignol pathway specific (Li et al., 2001, *Plant Cell*, 13:1567–1585). Based on high sequence homology, the alignment program picked up CADs from angiosperms as well as gymnosperms (radiata pine, loblolly pine and spruce) which have only G-lignin. All CADs have the Zn1 binding motif and structural Zn2 consensus region, as well as a NADP binding site (Jornvall et al., 1987, *Eur. J. Biochem.*, 167:195–201; MacKay et al., 1995, *Mol. Gen. Genet.*, 247:537–545). All these sequence characteristics and high sequence homology to PtCAD attest to these CAD function as a G-monolignol specific CAD (Li et al., 2001, *Plant Cell*, 13:1567–1585).

The protein sequence alignments of plant CAlD5Hs are shown in FIG. 11. Although, there are different types of 5-hydroxylases, i.e., F5H, CAlD5H is the sole enzyme catalyzing specifically the conversion of conifer aldehyde into 5-hydroxyconifer aldehyde. All full-length CAlD5Hs have the proline-rich region located from amino acid 40 to 45 which is believed to be involved in the process of correct folding of microsomal P450s and is also important in heme incorporation into P450s (Yamazaki et al. 1993, *J. Biochem.* 114:652–657). Also they all have the heme-binding domain (PFGXGXXXCXG) that is conserved in all P450 proteins (Nelson et al. 1996, *Pharmacogenetics*, 6:1–41). These signature sequences and the high sequence homology of these

proteins to PtCald5H their function as a 5-hydroxylase that is specific for converting coniferaldehyde into 5-hydroxyconiferaldehyde (Osakabe et al., 1999, Proc. Natl. Acad. Sci. USA, 96:8955-8960).

The protein sequence alignment of plant 4CLs are shown in FIG. 12. In general, 4CL catalyzes the activation of the hydroxycinnamic acids to their corresponding hydroxycinnamoyl-CoA esters. 4CL has the highest activity with p-coumaric acid. 4CL cDNA sequences have been reported from a number of representative angiosperms and gymnosperms, revealing two highly conserved regions, a putative AMP-binding region (SSGTTGLPKGV), and a catalytic motif (GEICIRG). The amino acid sequences of 4CL from plants contain a total of five conserved Cys residues.

Despite recognition of these key enzymes in lignin biosynthesis, there continues to be a need to develop an improved method to simultaneously control the lignin quantity, lignin compositions, and cellulose contents in plants by introducing multiple genes into plant cells.

BRIEF SUMMARY OF THE INVENTION

The invention provides a method of introducing two or more genes involved in lignin biosynthesis present in one or more independent vectors into plant cells. The method of the invention suitably employs an *Agrobacterium*-mediated or another gene delivery system by which multiple genes together with a single selectable marker gene are simultaneously transferred and inserted into the genome of plants with high frequencies.

If an *Agrobacterium*-mediated gene delivery system is used, each gene of interest is present in a binary vector that has been introduced into *Agrobacterium* to yield an isolated *Agrobacterium* strain comprising the binary vector. Moreover, more than one gene of interest may be present in each binary vector. Plant materials comprising plant cells, e.g., plant seed, plant parts or plant tissue including explant materials such as leaf discs, from a target plant species are suitably inoculated with at least two, preferably at least three, and more preferably at least four or more, of the isolated *Agrobacterium* strains, each containing a different gene of interest. A mixture of the strains is suitably contacted with plant cells. At least one of the binary vectors in the isolated *Agrobacterium* strains contains a marker gene, and any marker gene encoding a trait for selecting transformed cells from non-transformed cells may be used. Transformed plant cells are regenerated to yield a transgenic plant, the genome of which is augmented with DNA from at least two, preferably at least three, and more preferably at least four, and even more preferably at least five of the binary vectors.

The method of the invention is thus applicable to all plant species that are susceptible to the transfer of genetic information by *Agrobacterium* or other gene delivery system. Suitable plant species useful in the method of the invention include agriculture and forage crops, as well as monocots. In particular, plant species useful in the method of the invention include trees, e.g., angiosperms and gymnosperms, and more suitably a forest tree, but are not limited to the tree.

The method of the invention is suitably employed to enhance a desired agronomic trait by altering the expression of two or more genes. Such traits include alterations in lignin biosynthesis (e.g., reduction, augmentation and/or structural changes), cellulose biosynthesis (e.g., augmentation, reduction, and/or quality including high degree of polymerization and crystallinity), growth, wood quality (e.g., high density, low juvenile wood, high mature wood, low reaction

wood, desirable fiber angle), stress resistance (e.g., cold-, heat-, and salt-tolerance, pathogen-, insect- and other disease-resistance, herbicide-resistance), sterility, high grain yield (for forage and food crops), and increased nutrient level.

Thus, the present invention advantageously provides gymnosperm and angiosperm plants with decreased lignin content, increased syringyl/guaiacyl (S/G) lignin ratio and increased cellulose content in which a single trait or multiple traits are changed.

In another aspect, the invention provides gymnosperm plants with syringyl enriched lignin and/or increased lignin content and/or increased syringyl/guaiacyl (S/G) lignin ratio.

Similarly, the present invention also provides angiosperm plants with increased lignin content.

Other advantages and a fuller appreciation of specific attributes and variations of the invention will be gained upon an examination of the following detailed description of exemplary embodiments and the like in conjunction with the appended claims.

BRIEF DESCRIPTION OF THE DRAWING(S)

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

FIG. 2 is the SAD polynucleotide DNA sequence (SEQ ID NO: 1) and the SAD amino acid sequence (SEQ ID NO: 2) respectively FIGS. 2A and 2B;

FIG. 3 is the CALd5H polynucleotide DNA sequence (SEQ ID NO: 3) and the CALd5H amino acid sequence (SEQ ID NO: 4) respectively FIGS. 3A and 3B;

FIG. 4 is the AldOMT polynucleotide DNA sequence (SEQ ID NO: 5) and the AldOMT amino acid sequence (SEQ ID NO: 6) respectively FIGS. 4A and 4B;

FIG. 5 is the 4CL polynucleotide DNA sequence (SEQ ID NO: 7) and the 4CL amino acid sequence (SEQ ID NO: 10) respectively FIGS. 5A and 5B;

FIG. 6 is the CAD polynucleotide DNA sequence (SEQ ID NO: 8) and the CAD amino acid sequence (SEQ ID NO: 9) respectively FIGS. 6A and 6B;

FIG. 7 is a map of the DNA construct, pBKPpt_{4CL} Pt4CL1-a, positioned in a plant transformation binary vector.

FIG. 8 is a map of the DNA construct, pBKPpt_{4CL} PtCald5H-s, positioned in a plant transformation binary vector.

FIG. 9 is the protein sequence alignment of AldOMTs for representative species of plants.

FIG. 10 is the protein sequence alignment of CADs for representative species of plants.

FIG. 11 is the protein sequence alignment of CALd5Hs for representative species of plants.

FIG. 12 is the protein sequence alignment of 4CLs for representative species of plants.

It is expressly understood that the figures of the drawing are for the purposes of illustration and description only and are not intended as a definition of the limits of the invention.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method and DNA constructs useful for the transformation of plant tissue for the

alteration of lignin monomer composition, increased syringyl/guaiacyl (S/G) lignin ratio and increased cellulose content and transgenic plants resulting from such transformations. 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. Woody plants transformed with the DNA constructs provided herein 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 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.

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 or coding sequence (See, below). "Native" gene refers to the gene as found in nature with its own regulatory sequences.

"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" or "Coding Region" 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 sequence 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 gene 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 equivalents” of an enzyme is that portion, fragment or equivalent section 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 (GCG) 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.) [loblolly pine].

As used herein, the terms “isolated and/or purified” with reference to a nucleic acid molecule or polypeptide 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, such as nucleic acid or polypeptide, so that it can be sequenced, replicated and/or expressed.

An “isolated” strain of *Agrobacterium* refers to cells derived from a clone of *Agrobacterium* that is transformed in vitro with an isolated binary vector.

A “vector” is a recombinant nucleic acid construct, such as plasmid, phage genome, virus genome, cosmid, or artificial chromosome to which a polynucleotide in accordance with the 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”, coniferyl alcohol dehydrogenase or “CAD”, coniferaldehyde 5-hydroxylase or “Cald5H”, 5-hydroxyconiferaldehyde O-methyltransferase or “AldOMT”, and 4-coumarate-CoA ligase or “4CL” refer to enzymes in the plant phenylpropanoid biosynthetic pathway. In the illustrated embodiments of the present invention, the DNA sequences encoding these enzymes were identified from quaking aspen *Populus tremuloides*. It is understood that each sequence can be used as a probe to clone its equivalent from any plant species by techniques (EST, PCR, RT-PCR, antibodies, etc.) well known in the art.

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 (5); coniferyl alcohol dehydrogenase (CAD) converts coniferaldehyde (5) to the guaiacyl monolignol coniferyl alcohol (6); at coniferaldehyde (5), the pathway splits wherein coniferaldehyde (5) can also be converted to 5-hydroxyconiferaldehyde (7) by coniferaldehyde 5-hydroxylase (Cald5H); 5-hydroxyconiferaldehyde O-methyltransferase (AldOMT) converts 5-hydroxyconiferaldehyde (7) to sinapaldehyde (8) which, in turn, is converted to the syringyl monolignol, sinapyl alcohol (9) by sinapyl alcohol dehydrogenase (SAD).

DNA Constructs

According to the present invention, a DNA construct is provided which is a plant DNA having a promoter sequence, a coding region and a terminator sequence. The coding region encodes a combination of enzymes essential to lignin biosynthesis, specifically, SAD, CAD, Cald5OMT, and 4CL protein sequences, substantially similar sequences, or functional fragments thereof. The coding region is suitably a minimum size of 50 bases. The gene promoter is positioned at the 5'-end of a transgene (e.g., 4CL alone or together with SAD, Cald5H, and AldOMT, and combinations thereof, or 4CL and CAD alone, or together with Cald5H, SAD, and AldOMT, and combinations thereof, as described hereinafter) for controlling the transgene expression, 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 can be incorporated into the genome of a plant by transformation to alter lignin biosynthesis, increase syringyl/guaiacyl (S/G) lignin ratio and increase cellulose content. The DNA construct may include clones of Cald5H, SAD, AldOMT, CAD, and 4CL, and variants thereof such as are permitted by the degeneracy of the genetic code and the functional equivalents thereof.

The DNA constructs of the present invention may be inserted into plants to regulate production the following

enzymes: CAld5H, SAD, AldOMT, CAD, and 4CL. Depending on the nature of the construct, the production of the protein may be increased or decreased, either throughout or at particular stages in the life of the plant, relative to a similar control plant that does not incorporate the construct into its genome. 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 an 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 the constructs in accordance with the present invention suitably provides for expression of the linked DNA segment. The promoter can also be inducible so that gene expression 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., CaMV35S, GPAL2, GPAL3 and endogenous plant promoter controlling expression of the enzyme of interest. Use of a constitutive promoter such as the CaMV35S 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), Δ -tubulin, ubiquitin, actin (Wang et al., 1992), cab (Sullivan et al., 1989), PEPCase (Hudspeth et al., 1989) or those associate 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 desired protein is only produced in the tissue in which its action is required. Suitably, tissue-specific promoters, such as those would 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, PCT/US/0027704, filed Oct. 6, 2000, entitled "Method of Introducing a Plurality of Genes into Plants," incorporated herein by reference.)

Marker Genes

A marker gene may also be incorporated into the inventive DNA constructs to aid 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 to 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 used in the method, in accordance with the present invention are listed in Table 1 of co-pending, commonly owned of PCT/US/0027704, filed Oct. 6, 2000, entitled "Method of Introducing a Plurality of Genes into Plants," incorporated herein by reference.

Therefore, it will be understood that the following discussion is exemplary rather than exhaustive. In light of the techniques disclosed herein and the general recombinant techniques which are known in the art, the present invention renders possible the introduction of any gene, including marker genes, into a recipient cell to generate a transformed plant.

Optional Sequences in the Expression Cassette

The expression cassette containing DNA sequences in accordance with the present invention can also optionally contain other DNA sequences. Transcription enhancers or duplications of enhancers can be used to increase expression from a particular promoter. One may wish to obtain novel tissue-specific promoter sequences for use in accordance with the present invention. To achieve this, one may first isolate cDNA clones from the tissue concerned and identify those clones which are expressed specifically in that tissue, for example, using Northern blotting. Ideally, one would like to identify a gene that is not present in a high copy number, but which gene product is relatively abundant in specific tissues. The promoter and control elements of corresponding genomic clones may then be localized using the techniques of molecular biology known to those of skill in the art.

Expression of some genes in transgenic plants will occur only under specified conditions. It is known that a large number of genes exist that respond to the environment. In some embodiments of the present invention expression of a DNA segment in a transgenic plant will occur only in a certain time period during the development of the plant. Developmental timing is frequently correlated with tissue specific gene expression.

As the DNA sequence inserted between the transcription initiation site and the start of the coding sequence, i.e., the untranslated leader sequence, can influence gene expression, one can also employ a particular leader sequence. Preferred leader sequence include those which comprise sequences selected to direct optimum expression of the attached gene, i.e., to include a preferred consensus leader sequence which can increase or maintain mRNA stability and prevent inappropriate initiation of translation (Joshi, 1987). Such

sequences are known to those of skill in the art. Sequences that are derived from genes that are highly expressed in plants will be most preferred.

Additionally, expression cassettes can be constructed and employed to target the gene product of the DNA segment to an intracellular compartment within plant cells or to direct a protein to the extracellular environment. This can generally be achieved by joining a DNA sequence encoding a transit or signal peptide sequence to the coding sequence of the DNA segment. Also, the DNA segment can be directed to a particular organelle, such as the chloroplast rather than to the cytoplasm.

Alternatively, the DNA fragment coding for the transit peptide may be chemically synthesized either wholly or in part from the known sequences of transit peptides such as those listed above. The description of the optional sequences in the expression cassette, is commonly owned, co-pending PCT/US/0027704, filed Oct. 6, 2000, entitled "Method of Introducing a Plurality of Genes into Plants," incorporated herein by reference.

Transformation

Transformation of cells from plants, e.g., trees, and the subsequent production of transgenic plants using e.g., *Agrobacterium*-mediated transformation procedures known in the art, and further described herein, is one example of a method for introducing a foreign gene into plants. Although, the method of the invention can be performed by other modes of transformation, *Agrobacterium*-mediated transformation procedures are cited as examples, herein. For example, transgenic plants may be produced by the following steps: (i) culturing *Agrobacterium* in low-pH induction medium at low temperature and preconditioning, i.e., coculturing bacteria with wounded tobacco leaf extract in order to induce a high level of expression of the *Agrobacterium* vir genes whose products are involved in the T-DNA transfer; (ii) coculturing desired plant tissue explants, including zygotic and/or somatic embryo tissues derived from cultured explants, with the incited *Agrobacterium*; (iii) selecting transformed callus tissue on a medium containing antibiotics; and (iv) converting the embryos into platelets.

Any non-tumorigenic *A. tumefaciens* strain harboring a disarmed Ti plasmid may be used in the method in accordance with the invention. Any *Agrobacterium* system may be used. For example, Ti plasmid/binary vector system or a cointegrative vector system with one Ti plasmid may be used. Also, any marker gene or polynucleotide conferring the ability to select transformed cells, callus, embryos or plants and any other gene, such as for example a gene conferring resistance to a disease, or one improving lignin content or structure or cellulose content, may also be used. A person of ordinary skill in the art can determine which markers and genes are used depending on particular needs.

To increase the infectivity of the bacteria, *Agrobacterium* is cultured in low-pH induction medium, i.e., any bacterium culture media with a pH value adjusted to from 4.5 to 6.0, most preferably about 5.2, and at low temperature such as for example about 19–30° C., preferably about 21–26° C. The conditions of low-pH and low temperature are among the well-defined critical factors for inducing virulence activity in *Agrobacterium* (e.g., Altmorbe et al., 1989; Fullner et al., 1996; Fullner and Nester, 1996).

The bacteria is preconditioned by coculturing with wounded tobacco leaf extract (prepared according to methods known generally in the art) to induce a high level of expression of the *Agrobacterium* vir genes. Prior to inoculation of plant somatic embryos, *Agrobacterium* cells can be treated with a tobacco extract prepared from wounded leaf

tissues of tobacco plants grown in vitro. To achieve optimal stimulation of the expression of *Agrobacterium* vir genes by wound-induced metabolites and other cellular factors, tobacco leaves can be wounded and pre-cultured overnight.

Culturing of bacteria in low pH medium and at low temperature can be used to further enhance the bacteria vir gene expression and infectivity. Preconditioning with tobacco extract and the vir genes involved in the T-DNA transfer process are generally known in the art.

Agrobacterium treated as described above is then cocultured with a plant tissue explant, such as for example, zygotic and/or somatic embryo tissue. Non-zygotic (i.e., somatic) or zygotic tissues can be used. Any plant tissue may be used as a source of explants. For example, cotyledons from seeds, young leaf tissue, root tissues, parts of stems including nodal explants, and tissues from primary somatic embryos such as the root axis may be used. Generally, young tissues are a preferred source of explants.

The above-described transformation and regeneration protocol is readily adaptable to other plant species. Other published transformation and regeneration protocols for plant species include Danekar et al., 1987; McGranahan et al., 1988; McGranahan et al., 1990; Chen, Ph.D. Thesis, 1991; Sullivan et al., 1993; Huang et al., 1991; Wilde et al., 1992; Minocha et al., 1986; Parsons et al., 1986; Fillatti et al., 1987; Pythoud et al., 1987; De Block, 1990; Brasileiro et al., 1991; Brasileiro et al., 1992; Howe et al., 1991; Klopfenstein et al., 1991; Leple et al., 1992; and Nilsson et al., 1992.

Characterization

To confirm the presence of the DNA segment(s) or "transgene(s)" in the regenerated plants, a variety of assays may be performed. Such assays include, for example, "molecular biological" assays well known to those of skill in the art, such as Southern and Northern blotting and PCR; "biochemical" assays, such as detecting the presence of a protein product, e.g., by immunological means (ELISAs and Western blots) or by enzymatic function; plant part assays, such as leaf or root assays; and also, by analyzing the phenotype of the whole regenerated plant.

1. DNA Integration, RNA Expression and Inheritance

Genomic DNA may be isolated from callus cell lines or any plant parts to determine the presence of the DNA segment through the use of techniques well known to those skilled in the art. Note that intact sequences will not always be present, presumably due to rearrangement or deletion of sequences in the cell.

The presence of DNA elements introduced through the methods of this invention may be determined by polymerase chain reaction (PCR). Using this technique, discreet fragments of DNA are amplified and detected by gel electrophoresis. This type of analysis permits one to determine whether a DNA segment is present in a stable transformant, but does not prove integration of the introduced DNA segment into the host cell genome. In addition, it is not possible using PCR techniques to determine whether transformants have exogenous genes introduced into different sites in the genome, i.e., whether transformants are of independent origin. It is contemplated that using PCR techniques it would be possible to clone fragments of the host genomic DNA adjacent to an introduced DNA segment.

Positive proof of DNA integration into the host genome and the independent identities of transformants may be determined using the technique of Southern hybridization. Using this technique, specific DNA sequences that were introduced into the host genome and flanking host DNA sequences can be identified. Hence the Southern hybridiza-

tion pattern of a given transformant serves as an identifying characteristic of that transformant. In addition, it is possible through Southern hybridization to demonstrate the presence of introduced DNA segments in high molecular weight DNA, i.e., confirm that the introduced DNA segment has been integrated into the host cell genome. The technique of Southern hybridization provides information that is obtained using PCR, e.g., the presence of a DNA segment, but also demonstrates integration into the genome and characterizes each individual transformant.

It is contemplated that by using the techniques of dot or slot blot hybridization which are modifications of Southern hybridization techniques, one could obtain the same information that is derived from PCR, e.g., the presence of a DNA segment.

Both PCR and Southern hybridization techniques can be used to demonstrate transmission of a DNA segment to progeny. In most instances the characteristic Southern hybridization pattern for a given transformant will segregate in progeny as one or more Mendelian genes (Spencer et al., 1992; Laursen et al., 1994) indicating stable inheritance of the gene.

Whereas DNA analysis techniques may be conducted using DNA isolated from any part of a plant, RNA may only be expressed in particular cells or tissue types, and hence, it will be necessary to prepare RNA for analysis from these tissues. PCR techniques may also be used for detection and quantitation of RNA produced from introduced DNA segments. In this application of PCR, it is first necessary to reverse transcribe RNA into DNA, using enzymes such as reverse transcriptase, and then through the use of conventional PCR techniques amplify the DNA. In most instances, PCR techniques, while useful, will not demonstrate integrity of the RNA product. Further information about the nature of the RNA product may be obtained by Northern blotting. This technique will demonstrate the presence of an RNA species and give information about the integrity of that RNA. The presence or absence of an RNA species can also be determined using dot or slot blot Northern hybridizations. These techniques are modifications of Northern blotting and demonstrate only the presence or absence of an RNA species.

2. Gene Expression

While Southern blotting and PCR may be used to detect the DNA segment in question, they do not provide information as to whether the DNA segment is being expressed. Expression may be evaluated by specifically identifying the protein products of the introduced DNA segments or evaluating the phenotypic changes brought about by their expression.

Assays for the production and identification of specific proteins may make use of physical-chemical, structural, functional, or other properties of the proteins. Unique physical-chemical or structural properties allow the proteins to be separated and identified by electrophoretic procedures, such as native or denaturing gel electrophoresis or isoelectric focussing, or by chromatographic techniques such as ion exchange or gel exclusion chromatography. The unique structures of individual proteins also offer opportunities for use of specific antibodies to detect their presence in formats such as an ELISA assay. Combinations of approaches may be employed with even greater specificity such as western blotting in which antibodies are used to locate individual gene products that have been separated by electrophoretic techniques. Additional techniques may be employed to absolutely confirm the identity of the product of interest such as evaluation by amino acid sequencing following purification. Although these are among the most commonly employed, other procedures may be additionally used.

Assay procedures may also be used to identify the expression of proteins by their functionality, especially the ability of enzymes to catalyze specific chemical reactions involving specific substrates and products. These reactions may be followed by providing and quantifying the loss of substrates or the generation of products of the reactions by physical or chemical procedures. Examples are as varied as the enzyme to be analyzed and may include assays for PAT enzymatic activity by following production of radiolabelled acetylated phosphinothricin from phosphinothricin.

Very frequently the expression of a gene product is determined by evaluating the phenotypic results of its expression. These assays also may take many forms including but not limited to analyzing changes in the chemical composition, morphology, or physiological properties of the plant. Chemical composition may be altered by expression of DNA segments encoding enzymes or storage proteins which change amino acid composition and may be detected by amino acid analysis, or by enzymes which change starch quantity which may be analyzed by near infrared reflectance spectrometry. Morphological changes may include greater stature or thicker stalks. Most often changes in response of plants or plant parts to imposed treatments are evaluated under carefully controlled conditions termed bioassays.

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

EXAMPLE 1

Preparation of Transgenic Aspen

Construction of Binary Vectors

pBKPpt_{4CL} Pt4CL1-a: Aspen 4CL1 xylem specific promoter (Pp_{4CL}, 1.1 kb, GenBank AF041051) was prepared and linked to aspen 4CL1 cDNA (Pt4CL1, GenBank AF041049) which was orientated in the antisense direction. Then the cassette containing aspen 4CL1 promoter and antisense aspen 4CL1 cDNA was positioned in a plant transformation binary vector, as shown in FIG. 1. (pBKPpt_{4CL} Pt4CL1-a construct)

pBKPpt_{4cl} PtCald5H-s: From pBKPpt_{4CL} Pt4CL-a construct, the antisense Pt4CL1 was replaced with PtCald5H cDNA in a sense orientation, yielding a pBKPpt_{4CL} PtCald5H-s transformation binary construct, as shown in FIG. 8.

Also, Example 1 of PCT application PCT/US/0027704, filed Oct. 6, 2000, entitled "Method of Introducing a Plurality of Genes into Plants," incorporated herein by reference, describes a number of other gene constructs for preparing transgenic plants. The plants are transformed with a genes from the phenylpropanoid pathway (i.e., 4CL, AEOMT, CoAOMT, and CAld5H) using an operably linked to either a homologous or a heterologous and either a constitutive or tissue-specific promoter

Incorporation of Binary Vector into *Agrobacterium*

According to the protocol described in Tsai et al. (1994, Plant Cell Reports, 14:94-97) *Agrobacterium* C58/pMP90 strain was grown in LB with selection of gentamicin at 28° C. overnight. Cells were harvested by centrifugation at 10,000 rpm for 10 minutes at 4° C. The cell pellet was washed with 0.5 volume of ice-cold 20 mM CaCl₂, and centrifuged again. The cells were then resuspended in 0.1 volume of ice-cold 20 mM CaCl₂ in a sample tube. About 1 μg of binary vector DNA was added to 200 μL of the cell suspension and mixed by pipetting. The sample tube was chilled in liquid N₂ for 5 minutes and thawed at 37° C. in a water bath for 5 minutes. One mL of LB medium was added and the mixture was incubated at 28° C. for 3 hours with gentle shaking. Twenty μL of the cells were spread onto a LB

plate containing 25 $\mu\text{g}/\text{mL}$ gentamicin and 50 $\mu\text{g}/\text{mL}$ kanamycin and incubated at 28° C. for 2 days. PCR (amplification conditions, cycling parameters and primers are described below) was used to verify the presence of DNA from the vector in the transformed colonies.

Simultaneous Transformation of Aspen with Multiple Genes Via Engineered *Agrobacterium* Strains

For simultaneous transformation of multiple genes, pBKPt_{4cl} Pt4CL-a and pBKPt_{4cl} PtCald5H *Agrobacterium* clones were cultured in LB medium at 28° C. overnight separately. The *Agrobacterium* strains were subcultured individually by a 100-fold dilution into 50 mL of LB (pH 5.4) containing 50 $\mu\text{g}/\text{mL}$ kanamycin, 25 $\mu\text{g}/\text{mL}$ gentamycin and 20 μM acetosyringone (in DMSO), and grown overnight at 28° C. with shaking. An equal volume of the same density of individually cultured *Agrobacterium* strains was then mixed. Leaves excised from sterile tobacco plants were cut into pieces with a size of about 5 mm² and the leaf discs were then immersed in the *Agrobacterium* mixture for 5 minutes.

After removing excess *Agrobacterium* cells, the treated leaf discs were placed on callus induction medium (WPM:Woody Plant Medium, BA: 6-benzyladenine+2,4-D: 2,4-dichlorophenoxyacetic acid; Tsai et al. 1994, Plant Cell Reports, 14:94-97) and cultured for 2 days. Then, the pre-cultured leaf discs were rinsed with sterile water several times to remove the *Agrobacterium* cells and washed in 1 mg/mL claforan and 1 mg/mL ticarcillin with shaking for 3 hours to kill *Agrobacterium*. After briefly blot-drying, the pre-cultured and washed leaf discs were cultured on callus induction medium containing 50 $\mu\text{g}/\text{mL}$ kanamycin and 300 $\mu\text{g}/\text{mL}$ claforan for selection of transformed cells. After 2 to 3 subcultures (10 days/subculture), the calli grown on the leaf discs were excised and transferred onto shoot induction medium (WPM+TDZ: N-phenyl-N'-1,2,3-thiadiazol-5-yl-urea) containing 50 $\mu\text{g}/\text{mL}$ kanamycin and 300 $\mu\text{g}/\text{mL}$ claforan for regenerating shoots. After shoots were grown to about 0.5 cm high, they excised and planted to rooting media (WPM with kanamycin and claforan). Whole plants about 7 cm high were transplanted into soil and maintained in a greenhouse for subsequent molecular characterization.

Genomic DNA Isolation

Genomic DNA was isolated according to Hu et al. (1998). About 100 mg of young leaves were collected from each plant growing in the greenhouse and ground in liquid N₂ to fine powder for DNA isolation using QIAGEN plant DNA isolation kit (Valencia, Calif.). Specifically, the powdered tissue was added to extract buffer containing 2% hexadecyltrimethylammonium bromide (CTAB), 100 mM Tris-HCl, pH 8.0, 20 mM EDTA, 1.4 M NaCl and 30 mM β -mercaptoethanol at 5 mL/g tissue. The extraction mixture was incubated in a tube at 60° C. for 1 hour with occasional shaking. One volume of chloroform-isoamyl alcohol (24:1) was added and mixed gently. The two phases were separated by centrifugation at 10,000 \times g for 10 minutes. The aqueous phase was transferred to a new tube and extracted with chloroform in the presence of 1% CTAB and 0.7 M NaCl. The DNA was precipitated by addition of 2/3 volume of isopropanol (-20° C.) and kept at -20° C. for 20 minutes. Following the centrifugation at 10,000 \times g for 10 minutes, the pelleted DNA was washed with 70% ethanol-10 mM ammonia acetate. Then the pellet was dissolved in 2 mL TE buffer (10 mM Tris-HCl/0.1 mM EDTA, pH 8) and treated with 2 μg RNase A at 37° C. for 20 minutes. The DNA was precipitated by addition of 2 mL of 5 M ammonia acetate

and 10 mL of 95% ethanol at -20° C. for 20 minutes. After centrifugation, the pellet was washed with 70% ethanol. After a brief drying, genomic DNA was dissolved in TE buffer.

PCR Verification of Foreign Gene Insertion in Host Plant Genome

PCR was used to verify the integration of the gene constructs in the genome of transgenic plants. Two specific primers were synthesized for each construct and used to PCR-amplify the corresponding construct in genome of transgenic Aspen. For the PBKPPt_{4CL} Pt4CL1-a construct, two specific primers were synthesized that amplify a 4CL cDNA fragment. Pt4CL1 promoter sense primer (5'CAGGAATGCTCTGCACTCTG3') (SEQ ID NO:11) and Pt4CL1 sense primer (5'ATGAATCCACAAGAATTCAT3') (SEQ ID NO:12) at the translation start region. Primers for PCR verification of pBKPt_{4CL} PtCald5H-s construct are Pt4CL1 promoter sense primer (5'CAGGAATGCTCTGCACTCTG3') (SEQ ID NO:13) and PtCald5H antisense primer (5'TTAGAGAGGACAGAGCACACG3') (SEQ ID NO:14) at translation stop region.

The PCR reaction mixture contained 100 ng genomic DNA of transformed aspen, and 0.2 μM of each primer, 100 μM of each deoxyribonucleotide triphosphate, 1 \times PCR buffer and 2.5 Units of Taq DNA polymerase (Promega Madison, Wis.) in a total volume of 50 μL . The cycling parameters were as follows: 94° C. for 1 minute, 56° C. for 1 minute (for 4CL and CALD5H or can vary between cDNA templates used) according to different gene checked) and 72° C. for 2 minute, for 40 cycles, with 5 minutes at 72° C. extension. The PCR products were electrophoresized on a 1% agarose gel.

EXAMPLE 2

Preparation of Other Transgenic Plants

It is important to recognize that there is a substantial percentage of sequence homology among the plant genes involved in the lignin biosynthetic pathway, discussed herein. This substantial sequence homology allows the method in accordance with the invention disclosed herein to be applicable to all plants that possess the requisite genes involved in the lignin biosynthetic pathway. To demonstrate the substantial sequence homology among plant genes, the percentage sequence homology is set forth in tabular form, for example, CALD5H genes (Table 1), AldOMT genes (Table 2), CAD genes (Table 3), and 4CL genes (See FIG. 12). Therefore, it is possible to alter lignin monomer composition, increase S/G lignin ratio, and increase cellulose content in all plants by using the method in accordance with the invention, described herein.

TABLE 1

	Protein sequence homology (%) of plant Coniferyl Aldehyde 5-hydroxylase (CALD5H) from 1) Aspen; 2) Poplar, AJ010324; 3) Sweetgum, AF139532; 4) Arabidopsis (Ferulic Acid 5-hydroxylase, F5H)			
	1	2	3	4
1				
2	99			
3	84	84		
4	81	83	83	

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TABLE 2

Protein sequence homology (%) of plant AldOMTs from 1) Aspen, X62096; 2) Poplar, M73431; 3) Almond, X83217; 4) Strawberry, AF220491; 5) Alfalfa, M63853; 6) Eucalyptus, X74814; 7) Clarkia breweri, AF006009; 8) Sweetgum, AF139533; 9) Arabidopsis, U70424; 10) Tobacco, X74452; 11) Vitis vinifera, AF239740

	1	2	3	4	5	6	7	8	9	10	11
1											
2	99										
3	92	92									
4	91	90	94								
5	90	90	89	89							
6	89	89	89	87	87						
7	88	88	89	88	87	90					
8	88	87	88	87	86	85	83				
9	84	84	85	86	82	82	83				
10	83	83	83	82	81	82	80	83	77		
11	80	80	78	77	78	77	78	80	76	77	

TABLE 3

Protein sequence homology (%) of plant CADs from 1) Aspen, AF217957; 2) Cottonwood, Z19568 and 3) Udo, D13991; 4) Tobacco, X62343; 5) Tobacco, X62344; 6) Eucalyptus, AF038561; 7) Eucalyptus, X65631; 8) Lucerne, AF083332; 9) Lucerne, Z19573; 10) Maize, AJ005702; 11) Maize, Y13733; 12) Sugarcane, AJ231135; 13) Radiata pine, U62394; 14) Loblolly pine, Z37992; 15) Loblolly pine, Z37991; 16) Norway spruce, X72675.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1																
2	97															
3	85	84														
4	82	82	84													
5	80	80	81	94												
6	81	81	82	80	78											
7	81	80	81	80	78	80										
8	79	79	80	80	79	79	79									
9	79	80	80	79	78	78	79	99								
10	78	77	79	76	74	76	77	73	73							
11	78	78	79	77	74	76	76	73	72	99						
12	77	76	78	74	73	75	74	73	73	95	96					
13	70	71	69	70	70	69	68	67	68	67	68	68				
14	69	70	69	69	69	69	68	68	68	67	67	67	99			
15	69	70	68	69	69	68	68	67	67	67	67	67	99	95		
16	69	69	70	70	69	68	68	68	67	69	69	67	95	95	94	

To further demonstrate the versatility of this invention in transferring a variety of foreign genes and the applicability of this invention to plants other than the herbaceous species, different binary vectors were constructed and transferred into aspen (*Populus tremuloides*) tree. Two binary vectors, each containing a cDNA sequence and a neomycin phosphotransferase (NPT II) cDNA encoding kanamycin resistance, were constructed. Each vector was then individually mobilized into *Agrobacterium* strain C58 to create two isolated (engineered) *Agrobacterium* strains. It should be noted that about 50 transgenic tobacco plants were generated by the same technique harboring 4 different sets of foreign genes, as described in the PCT application PCTUS0027704 filed Oct. 6, 2000, entitled "Method of Introducing a Plurality of Genes into Plants," incorporated herein by reference.

Table 4 summarizes the numerical results from simultaneous manipulating xylem-specific expression of 4CL and CAld5H in transgenic aspen. After DNA constructs were

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incorporated into plant cells by *Agrobacterium* mediated transformation, as set forth by the method in accordance with the invention and after PCR confirmation of transgene integration, 14 positive transgenic trees were randomly selected, representing three different transgenic groups, i.e., Groups I, II and III. Group I (plant #21, 22, 23, 25, and 37) consists of those with the integration of only antisense Pt4CL1 cDNA (Table 4). Group II plants (# 32, 84, 93, and 94) harbored only sense PtCAld5H cDNA, whereas Group III plants (#71, 72, 74, and 141) contained both antisense Pt4CL1 and sense PtCAld5H transgenes. These transgenic trees were then further analyzed for their lignin and cellulose contents and lignin S/G ratio (Table 4). It is clear that, when compared with the control, untransformed aspen, transgenic plants (#21, 22, 23, 25, and 37) engineered for the suppression of 4CL gene with antisense Pt4CL1 transgene had drastic reductions in their lignin content, with significant increases in their cellulose content. Transgenic plants (#32, 84, 93, 94, and 108) engineered for the overexpression of

CAld5H with sense PtCA1b5H transgene had pronounced increases in their S/G ratio, but their lignin and cellulose contents remained essentially unaffected. When engineered for the simultaneous suppression of 4CL gene and overexpression of CAld5H gene, transgenic plants (#71, 72, 74, and 141) all exhibited low lignin content, high S/G ratio and elevated cellulose quantity. In summary, these results show that multiple genes carried by individual *Agrobacterium* strains can be integrated simultaneously into the plant genome.

Moreover, it was demonstrated as shown herein below, that transgenic plants with a nearly 30% increase in cellulose content and over 50% lignin quantity reduction, accompanied with a significant augmentation of the S/G ratio, can be easily produced. It is conceivable that more genes can also be efficiently transferred at one time. Only one suitable marker gene is required for this system, although a number of marker genes can also be employed.

TABLE 4

<u>Simultaneous manipulating xylem-specific expression of 4CL and CAld5H in transgenic aspen.</u>															
Plant #	Control	21	22	23	25	37	32	84	93	94	108	71	72	74	141
<u>Gene integrated</u>															
4CL-a		Y	Y	Y	Y	Y						Y	Y	Y	Y
CAld5H-s							Y	Y	Y	Y	Y	Y	Y	Y	Y
Lignin content (%)	22.4	16.0	15.3	14.4	13.1	14.9	22.4	21.6	21.1	20.7	19.7	13.2	13.7	12.4	10.7
Lignin S/G ratio	2.2	2.1	2.0	2.2	2.3	2.1	4.8	4.0	5.5	4.9	3.0	3.3	3.6	3.4	2.7
Cellulose content (%)	41.4	43.1	ND	ND	47.3	ND	40.0	ND	44.7	ND	ND	ND	49.2	ND	53.3

ND: not determined

EXAMPLE 3

Production of Commercially Desirable Agronomic Traits in Transformed Plants

The following genetic transformations illustrate the production of commercially desirable agronomic traits in plants.

Gymnosperms

- A. To produce syringyl-enriched lignin in gymnosperm plants, gymnosperm plants are genetically transformed with SAD, CAld5H, and AldOMT genes in the sense orientation driven by any appropriate promoter and via any appropriate genetic transformation system allows. These three genes can be transferred into the host plant either simultaneously (in one or individual constructs) or sequentially (in individual constructs) in any order.
- B. To produce decreased lignin content, increased syringyl/guaiacyl (S/G) lignin ratio and increased cellulose quantity in gymnosperm plants, gymnosperm plants are genetically transformed with SAD, CAld5H and AldOMT genes in the sense orientation and 4CL gene in either sense or antisense orientation driven by any appropriate promoter and via any appropriate genetic transformation system. These four genes can be transferred into the host plant either simultaneously (in one or individual constructs) or sequentially (in individual constructs) in any order.
- C. To produce decreased lignin content, increased syringyl/guaiacyl (S/G) lignin ratio and increased cellulose quantity in gymnosperm plants, gymnosperm plants are genetically transformed with SAD, CAld5H and AldOMT genes in the sense orientation and 4CL and CAD genes in either sense or antisense orientation driven by any appropriate promoter and via any appropriate genetic transformation system. These five genes can be transferred into the host plant either simultaneously (in one or individual constructs) or sequentially (in individual constructs) in any order.
- D. To produce increased lignin content in gymnosperm plants, gymnosperm plants are genetically transformed with 4CL gene in the sense orientation driven by any appropriate promoter and via any appropriate genetic transformation system.
- E. To produce increased lignin content and increased syringyl/guaiacyl (S/G) lignin ratio in gymnosperm plants, gymnosperm plants are genetically transformed with SAD, CAld5H, AldOMT, and 4CL genes in the sense orientation driven by any appropriate promoter and via any appropriate genetic transformation system. These four genes can be transferred into the host plant either simultaneously (in one or individual constructs) or sequentially (in individual constructs) in any order.

- F. To produce increased lignin content, increased syringyl/guaiacyl (S/G) lignin ratio in gymnosperm plants, gymnosperm plants are genetically transformed with SAD, CAld5H, AldOMT, and 4CL genes in the sense orientation and CAD gene in the antisense orientation driven by any appropriate promoter and via any appropriate genetic transformation system. These four genes can be transferred into the host plant either simultaneously (in one or individual constructs) or sequentially (in individual constructs) in any order.

Angiosperms

- A. To produce increased S/G lignin ratio in angiosperm plants, angiosperm plants are genetically transformed with either CAld5H, AldOMT, or SAD genes in sense orientation driven by any appropriate promoter and via any appropriate genetic transformation system. These three genes can be transferred into the host plant either simultaneously (in one or individual constructs) or sequentially (in individual constructs) in any order.
- B. To produce decreased lignin content, increased S/G lignin ratio and increased cellulose quantity in angiosperm plants, angiosperm plants are genetically transformed with either SAD, CAld5H, or AldOMT genes in the sense orientation and 4CL gene in the sense or antisense orientation driven by any appropriate promoter and via any appropriate genetic transformation system. These four genes can be transferred into the host plant either simultaneously (in one or individual constructs) or sequentially (in individual constructs) in any order.
- C. To produce decreased lignin content, increased S/G lignin ratio and increased cellulose quantity in angiosperm plants, angiosperm plants are genetically transformed with either SAD, CAld5H, or AldOMT genes in the sense orientation and 4CL and CAD genes in the sense or antisense orientation driven by any appropriate promoter and via any appropriate genetic transformation system. These five genes can be transferred into the host plant either simultaneously (in one or individual constructs) or sequentially (in individual constructs) in any order.
- D. To produce increased lignin content in angiosperm plants, angiosperm plants are genetically transformed with 4CL gene in the sense orientation driven by any appropriate promoter and via any appropriate genetic transformation system.
- E. To produce increased lignin content and increased S/G ratio in angiosperm plants, angiosperm plants are genetically transformed with 4CL in the sense orientation and either SAD, CAld5H, or AldOMT genes also

in the sense orientation driven by any appropriate promoter and via any appropriate genetic transformation system. These four genes can be transferred into the host plant either simultaneously (in one or individual constructs) or sequentially (in individual constructs) in any order.

F. To produce increased lignin content and increased S/G ratio in angiosperm plants, angiosperm plants are genetically transformed with 4CL in the sense orientation and either SAD, CAld5H, or AldOMT genes also in the sense orientation and CAD in the antisense orientation driven by any appropriate promoter and via any appropriate genetic transformation system. These four genes can be transferred into the host plant either simultaneously (in one or individual constructs) or sequentially (in individual constructs) in any order.

All publications, patents and patent applications cited herein are incorporated herein by reference. While in the foregoing specification, this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details herein may be varied considerably without departing from the basic principles of the invention. Accordingly, it is intended that the present invention be solely limited by the broadest interpretation that can be accorded the appended claims.

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<212> TYPE: DNA

<213> ORGANISM: aspen populus tremuloides

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<221> NAME/KEY: misc.feature

<223> OTHER INFORMATION: CAD; GenBank accession number: AF217957

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<212> TYPE: PRT

<213> ORGANISM: aspen populus tremuloides

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Gly Pro Glu Asp Val Leu Ile Lys Val Ile Ser Cys Gly Ile Cys His
35          40          45
Thr Asp Ile His Gln Ile Lys Asn Asp Leu Gly Met Ser His Tyr Pro
50          55          60
Met Val Pro Gly His Glu Val Val Gly Glu Val Val Glu Val Gly Ser
65          70          75          80
Asp Val Thr Lys Phe Lys Ala Gly Asp Val Val Gly Val Gly Val Ile
85          90          95
Val Gly Ser Cys Lys Asn Cys His Pro Cys Lys Ser Glu Leu Glu Gln
100         105         110
Tyr Cys Asn Lys Lys Ile Trp Ser Tyr Asn Asp Val Tyr Thr Asp Gly
115        120        125
Lys Pro Thr Gln Gly Gly Phe Ala Glu Ser Met Val Val Asp Gln Lys
130        135        140
Phe Val Val Arg Ile Pro Asp Gly Met Ser Pro Glu Gln Ala Ala Pro
145        150        155        160
Leu Leu Cys Ala Gly Leu Thr Val Tyr Ser Pro Leu Lys His Phe Gly
165        170        175
Leu Lys Gln Ser Gly Leu Arg Gly Gly Ile Leu Gly Leu Gly Gly Val
180        185        190

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 Ile Leu Cys Val Leu Pro Met Phe His Ile Tyr Ala Leu Asn Ser Met
 225 230 235 240
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 245 250 255
 Phe Glu Ile Gly Ser Leu Leu Gly Leu Ile Glu Lys Tyr Lys Val Ser
 260 265 270
 Ile Ala Pro Val Val Pro Pro Val Met Met Ala Ile Ala Lys Ser Pro
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 Asp Leu Asp Lys His Asp Leu Ser Ser Leu Arg Met Ile Lys Ser Gly
 290 295 300
 Gly Ala Pro Leu Gly Lys Glu Leu Glu Asp Thr Val Arg Ala Lys Phe
 305 310 315 320
 Pro Gln Ala Arg Leu Gly Gln Gly Tyr Gly Met Thr Glu Ala Gly Pro
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 Val Leu Ala Met Cys Leu Ala Phe Ala Lys Glu Pro Phe Asp Ile Lys
 340 345 350
 Pro Gly Ala Cys Gly Thr Val Val Arg Asn Ala Glu Met Lys Ile Val
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 Asp Pro Glu Thr Gly Val Ser Leu Pro Arg Asn Gln Pro Gly Glu Ile
 370 375 380
 Cys Ile Arg Gly Asp Gln Ile Met Lys Gly Tyr Leu Asn Asp Pro Glu
 385 390 395 400
 Ala Thr Ser Arg Thr Ile Asp Lys Glu Gly Trp Leu His Thr Gly Asp
 405 410 415
 Ile Gly Tyr Ile Asp Asp Asp Asp Glu Leu Phe Ile Val Asp Arg Leu
 420 425 430
 Lys Glu Leu Ile Lys Tyr Lys Gly Phe Gln Val Ala Pro Thr Glu Leu
 435 440 445
 Glu Ala Leu Leu Ile Ala His Pro Glu Ile Ser Asp Ala Ala Val Val
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 Gly Leu Lys Asp Glu Asp Ala Gly Glu Val Pro Val Ala Phe Val Val
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 Ser Lys Gln Val Ile Phe Tyr Lys Arg Ile Lys Arg Val Phe Phe Ile
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 <221> NAME/KEY: misc_feature
 <223> OTHER INFORMATION: Pt4CL1 promoter sense primer
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<210> SEQ ID NO 13
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What is claimed is:

1. A method of producing a transgenic plant comprising: (a) introducing into a plant cell at least one polynucleotide sequence encoding at least two of 4CL antisense RNA, aspen CAld5H, aspen AldOMT and SEQ ID NO:2; and (b) regenerating the transformed cell to produce a transgenic plant.

2. The method of claim 1, wherein the polynucleotide sequence comprises a sequence encoding 4CL antisense RNA and sequences encoding CAld5H, AldOMT, and SEQ ID NO:2.

3. The method of claim 2 wherein the transgenic plant comprises decreased lignin content, increased syringyl/guaiacyl (S/G) lignin ratio, or increased cellulose content compared to a non-transformed plant.

4. The method of claim 2 wherein the plant is an angiosperm or a gymnosperm.

5. The method of claim 1 wherein the lignin content of the transgenic plant is decreased relative to the lignin content of a non-transformed plant.

6. The method of claim 1 wherein the transgenic plant comprises an increased syringyl/guaiacyl (S/G) lignin ratio relative to a non-transformed plant.

7. The method of claim 1 wherein the transgenic plant comprises increased cellulose content relative to a non-transformed plant.

8. The method of claim 1 wherein the plant is an angiosperm.

9. The method of claim 8 wherein the angiosperm is a *Populus tremuloides*.

10. The method of claim 1 wherein the plant is a gymnosperm.

11. The method of claim 1, wherein the polynucleotide comprises 4CL in an antisense orientation; and wherein the

35 transgenic plant comprises a characteristic selected from the group consisting of altered lignin content, increased syringyl/guaiacyl (S/G) lignin ratio, altered cellulose content and combinations thereof compared to a non-transformed control plant.

40 12. The method of claim 11 wherein the transgenic plant comprises decreased lignin content.

13. The method of claim 11 wherein the transgenic plant comprises increased syringyl/guaiacyl (S/G) lignin ratio.

45 14. The method of claim 11 wherein the transgenic plant comprises increased cellulose content.

15. The method of claim 11 wherein the plant is an angiosperm.

16. The method of claim 15 wherein the angiosperm is a *Populus tremuloides*.

50 17. The method of claim 11 wherein the plant is a gymnosperm.

18. The method of claim 1 wherein each polynucleotide coding sequence is operably linked to a promoter sequence functional in the plant and a termination sequence; and wherein the plant comprises a characteristic selected from the group consisting of altered lignin content, increased syringyl/guaiacyl (S/G) lignin ratio, altered cellulose content, altered agronomic traits, and combinations thereof compared to a control plant that is not transformed with the polynucleotide sequence.

60 19. The method of claim 18 wherein the promoter is tissue-specific.

20. The method of claim 18 wherein the plant is an angiosperm.

65 21. The method of claim 20 wherein the angiosperm is a *Populus tremuloides*.

22. The method of claim 18 wherein the plant is a gymnosperm.

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23. The method of claim 18 wherein the plant comprises an altered agronomic trait selected from the group consisting of growth, wood quality, stress resistance, sterility, grain yield or nutritional value.

24. The method of claim 1, wherein the polynucleotide sequence encodes 4CL antisense RNA and CAld5H.

25. The method of claim 24, wherein the transgenic plant comprises decreased lignin content, increased syringyl/guaiacyl (S/G) lignin ratio, or increased cellulose content compared to a non-transformed plant.

26. The method of claim 24, wherein the CAld5H comprises SEQ ID NO:4.

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27. The method of claim 1, wherein the CAld5H comprises SEQ ID NO:4.

28. The method of claim 27, wherein the polynucleotide sequence comprises SEQ ID NO:3.

29. The method of claim 1, wherein the AldOMT comprises SEQ ID NO:6.

30. The method of claim 29, wherein the polynucleotide sequence comprises SEQ ID NO:5.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 6,855,864 B2
APPLICATION NO. : 09/947027
DATED : February 15, 2005
INVENTOR(S) : Vincent Lee C. Chiang et al.

Page 1 of 1

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, United States Department of Energy, 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.”

Signed and Sealed this

Twelfth Day of December, 2006

A handwritten signature in black ink on a light gray dotted background. The signature reads "Jon W. Dudas" in a cursive style.

JON W. DUDAS

Director of the United States Patent and Trademark Office