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Genetic engineering of plants through manipulation of lignin biosynthesis

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ABSTRACT

The invention pertains to the genetically down regulating a lignin pathway p-coumarate Co-enzyme A ligase (CCL) in trees.
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FIG. 1

PHENYLALANINE  CINNAMATE  PARA-COUMARATE  CAFFEATE  FERULATE  S-HYDROXYFERULATE  SINAPATE

PAL  →  C4H  →  C3H  →  OMT  →  F5H  →  OMT  →  4CL  →  4CL  →  4CL  →  4CL  →  4CL

PHENYLALANINE  CINNAMIC ACID  PARA-COUMARIC ACID  CAFFEIC ACID  FERULIC ACID  S-HYDROXYFERULIC ACID  SINAPIC ACID

4CL  →  CCoA-3H  →  CCoA-OMT  →  CCoA-OMT  →  CCoA-OMT

PARA-COUMAROYL-CoA  CAFFEOYL-CoA  FERULOYL-CoA  5-HYDROXYFERULOYL-CoA  SINAPOLYL-CoA

CCR  →  O₃C-H  →  O₃C-H  →  O₃C-H  →  O₃C-H  →  O₃C-H

PARA-COUMERALDEHYDE  CONIFERALDEHYDE  5-HYDROXYCONIFERALDEHYDE  SINAPALDEHYDE

CAD  →  OMT  →  CAD  →  CAD  →  CAD

PARA-COUMARYL  CONIFERYL ALCOHOL  SINAPYL ALCOHOL
FIG. 2
Pt4CL1g-4

FIG. 3

2 Kb

Pt4CL1 gene 4 Kb

2.3 Kb

(IN pGEM7Z)

Xba I Sph I EcoRI Xho I

Sac I

Xh

K

EE S E S

X S E S

X
1 Kb

E: Eco R I
K: Kpn I
S: Sal I
X: Xba I
Xh: Xho I
FIG. 5

**SEQEUCING STRATEGY. ARROWS INDICATE THE LENGTH AND DIRECTION OF DNA STRANDS SEQUENCED.**

1 Kb Pt4CL1 PROMOTER

**p7Z-4XS**

PtCCL1g-4

2 Kb Pt4CL1 gene 4 Kb

E: Eco R I
X: Xba I
S: Sal I

(IN pGEM7Z)

Sph I
EcoR I
Xho I

Hind III
BamH I
FIG. 6

PtCCL2g-11

1.2 Kb Pt4CL2 gene 3.9 Kb

(IN pSK+)

EcoR I

Hind III Nsi I Sph I

Sequencing strategy. Arrows indicate the length and direction of DNA strands sequenced.

1.2 Kb Pt4CL2 promoter

FIG. 6
FIG. 7

Hind III  BamHI

1 Kb Pt4CL1 PROMOTER

Hind III Pst I  Xba I Sme I
Sph I  Sal I  BamHI

RB

NOS-pro  NPT II  NOS-ter

PBI101

ATG

BETA-GLUCURONIDASE

TGA

NOS-ter

LB
FIG. 8

[Diagram showing genetic constructs and restriction enzyme sites, including Hind III, BamHI, Pst I, Xba I, Sma I, Sph I, Sal I, and BamHI, with NOS-pro, NPT II, NOS-ter, BETA-GLUCURONIDASE, ATG, TGA, RB, and LB.]
GENETIC ENGINEERING OF PLANTS THROUGH MANIPULATION OF LIGNIN BIOSYNTHESIS

BACKGROUND OF THE INVENTION

The invention relates to genetically modifying trees through manipulation of the lignin biosynthesis pathway, and more particularly, to genetically modifying trees through the down regulation of p-coumarate Co-enzyme A ligase (CCL) to achieve faster growth, and/or altered lignin content, and/or altered lignin structure, and/or altered cellulose content and/or disease resistance of the trees and to the use of promoters of the CCL genes to drive gene expression specifically in xylem tissue or specifically in epidermal tissues.

SUMMARY OF THE INVENTION

The invention provides a method to genetically alter trees through the down regulation of p-coumarate Co-enzyme A ligase (CCL). Such down regulation of CCL results in faster growth, and/or reduced lignin content, and/or altered lignin structure, and/or altered cellulose content and/or disease resistance. The invention also provides for genetically engineered trees which have been altered to down regulate p-coumarate Co-enzyme A ligase (CCL) to achieve faster growth, and/or reduced lignin content, and/or altered lignin structure, and/or increased cellulose content and/or increased disease resistance. The invention also provides for genetically altering trees to reduce their lignin content.

It is one object of the present invention to down regulate p-coumarate Co-enzyme A ligase (CCL) in trees.

It is another object of the present invention to provide a method to genetically alter trees to grow faster.

It is another object of the present invention to provide a method to genetically alter the growth of trees through manipulation the lignin pathway p-coumarate Co-enzyme A ligase.

It is another object of the present invention to provide a method to genetically alter trees to reduce their lignin content.

It is another object of the present invention to provide a method to genetically alter the lignin content of trees through manipulation of a lignin pathway enzyme.

It is another object of the present invention to genetically engineer trees which have reduced lignin content through manipulation of lignin pathway p-coumarate Co-enzyme A ligase.

It is another object of the present invention to provide a method to genetically alter trees to change their lignin content.

It is another object of the present invention to provide genetically altered trees with a reduced lignin content.

It is another object of the present invention to provide genetically altered trees with an accelerated growth characteristic.

It is another object of the present invention to provide transgenic trees with an accelerated growth characteristic which have been genetically altered by down regulating lignin pathway p-coumarate Co-enzyme A ligase.

It is another object of the present invention to provide a method to genetically alter trees to reduce their lignin content.

It is another object of the present invention to provide a method to genetically alter the lignin content of trees through manipulation of a lignin pathway enzyme.

It is another object of the present invention to genetically engineer trees which have reduced lignin content through manipulation of lignin pathway p-coumarate Co-enzyme A ligase.

It is another object of the present invention to provide genetically altered trees with a reduced lignin content.

It is another object of the present invention to provide genetically altered trees with an accelerated growth characteristic.

It is another object of the present invention to provide transgenic trees with an accelerated growth characteristic which have been genetically altered by down regulating the p-coumarate Co-enzyme A ligase (CCL).

It is another object of the present invention to provide a method to genetically alter trees to change their lignin content.

It is another object of the present invention to provide genetically altered trees with a reduced lignin content.

It is another object of the present invention to provide genetically altered trees with an accelerated growth characteristic.

It is another object of the present invention to provide transgenic trees with an accelerated growth characteristic which have been genetically altered by down regulating the p-coumarate Co-enzyme A ligase (CCL).
3 structure through manipulation of lignin pathway p-coumarate Co-enzyme A ligase.

It is another object of the present invention to provide trees with altered lignin structure.

It is another object of the present invention to provide a method to increase the cellulose content in trees.

It is another object of the present invention to provide a method to increase the cellulose content of trees through the manipulation of a lignin pathway enzyme.

It is another object of the present invention to provide trees with increased cellulose content.

It is another object of the present invention to provide transgenic trees having increased cellulose content from the down regulation of CCL.

It is another object of the present invention to provide a method to genetically alter trees to increase their disease resistance.

It is another object of the present invention to provide a method to genetically alter trees to be more disease resistant through manipulation of the lignin pathway p-coumarate Co-enzyme A ligase.

It is another object of the present invention to genetically alter trees to increase their disease resistance to fungal pathogens.

It is another object of the present invention to provide trees with increased disease resistance.

It is another object of the present invention to provide transgenic trees with increased disease resistance through down regulation of the lignin pathway p-coumarate Co-enzyme A ligase.

It is another object of the present invention to provide a method using a promoter of a CCL gene to target gene expression in specific plant tissue.

It is another object of the present invention to provide a method using a promoter of a CCL gene to target gene expression specifically in plant xylem.

It is another object of the present invention to provide a method using a promoter of the CCL gene to target gene expression specifically in the epidermal tissues of plants.

It is another object of the present invention to provide a CCL gene promoter that targets gene expression specifically in the xylem of plants.

It is another object of the present invention to provide a CCL gene promoter that targets gene expression specifically in the epidermal tissues of plants.

Other features and advantages of the invention will become apparent to those of ordinary skill in the art upon review of the following drawing, detailed description and claims.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 is a schematic of a phenylpropanoid pathway; Fig. 2 is a diagram of Agrobacterium T-DNA construct pACCL1; Fig. 3 is a restriction map of genomic clone PtCCL1g-4; Fig. 4 is a restriction map of genomic clone PtCCL2g-11; Fig. 5 is a restriction map of subcloned PtCCL1 gene promoter p7Z4XS; Fig. 6 is a restriction map of subcloned PtCCL2 gene promoter p8K11HE; Fig. 7 is an Agrobacterium T-DNA construct of PtCCL1 promoter and GUS fusion gene, PtCCL1p-GUS; and Fig. 8 is an Agrobacterium T-DNA construct of PtCCL2 promoter and GUS fusion gene, PtCCL2p-GUS.

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

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

The invention pertains to genetically down regulating a lignin pathway p-coumarate Co-enzyme A ligase (CCL). Trees which have been genetically transformed to down regulate CCL will hereafter be called transgenic trees. Such down regulation can result in faster growing trees. Such down regulation can result in a reduction in the lignin content of the trees and/or altered lignin structure. Such down regulation can result in increased cellulose content. Such down regulation can result in increased tree disease resistance. Further, by using a specific promoter of CCL, targeted tissue gene expression can be achieved in either the xylem or the epidermal tissues of the plant.

A. CCL

Lignin is synthesized by the oxidative coupling of three monolignols (coumaryl, coniferyl and sinapyl alcohols) formed via the phenylpropanoid pathway as shown in FIG. 1. Reactions in the phenylpropanoid pathway include the deamination of phenylalanine to cinnamic acid followed by hydroxylations, methylations and activation of substituted cinnamic acids to coenzyme A (CoA) esters. The CoA esters are then reduced to form monolignols which are secreted from cells to form lignin.

The products of the phenylpropanoid pathway are not only required for the synthesis of lignin but also required for the synthesis of a wide range of aromatic compounds including flavonoids, phytoalexins, stilbenes and suberin.

In angiosperms (hardwoods), lignin is composed of both coniferyl and sinapyl alcohol and is classified as guaiacyl-syringyl lignin. Grasses synthesize a third precursor (p-coumaryl alcohol) which is polymerized along with coniferyl and sinapyl alcohol. In gymnosperms (softwoods), lignin is composed of mainly coniferyl alcohol and is classified as guaiacyl lignin.

In the phenylpropanoid pathway, CCL activates a number of cinnamic acid derivatives, including p-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid and sinapic acid. The resulting products, CoA esters, serve as substrates for entry into various branch pathways, such as lignin, flavonoids, phytoalexins, stilbenes and suberin. The esterification reactions catalyzed by CCL require high energy and the reactions are not likely to occur without CCL. CCL is important in making a continuous flow of the lignin biosynthesis pathway. CCL is also important because it is located at the branching points of the phenylpropanoid metabolism.

CCL is suggested to play a pivotal role in regulating carbon flow into specific branch pathways of the phenylpropanoid metabolism in response to stages of development and environmental stress.

The basic properties of CCL are quite uniform. CCL depends on ATP as a cosubstrate and requires Mg" as a cofactor. The optimal pH for CCL ranges from pH 7.0 to 8.5 and the molecular weight of CCL isoforms from various plant species ranges from 40 kD to 75 kD. Most CCLs have high affinity with substituted cinnamic acids. CCL has the highest activity with p-coumaric acid.
CCL cDNA sequences have been reported for parsley, potato, soybean, loblolly pine, *Arabidopsis*, *Lithospermum* and tobacco. CCL genes have been isolated and sequenced for parsley, rice, potato and loblolly pine. The analysis of CCL cDNAs and genes indicates that CCL is encoded by multiple divergent genes in rice, soybean, and *Lithospermum*, very similar genes in parsley, potato, tobacco and loblolly pine, and a single gene in *Arabidopsis*. CCL promoters have been isolated and sequenced for parsley, rice and potato.

Alignment of deduced amino acid sequences of cloned plant CCL sequences reveals two highly conserved regions. The first conserved region (SSGTTLPKGV) (SEQ ID NO:7) proposed to designate a putative AMP-binding region, is very rich in Gly, Ser and Thr and is followed by a conserved Lys. The second conserved region (GEICIRG), (SEQ ID NO:8) contains one common Cys residue. The amino acid sequences of CCL from plants contain a total of five conserved Cys residues.

The CCL genes of parsley, potato and rice contain five exons and four introns. The CCL genes also share the same exon-intron splice junction sites, but have different lengths of introns. The genomic sequences of loblolly pine CCL are composed of four exons and three introns. It has been found that two similar CCL genes of the same species may differ slightly in length of intron as shown in two parsley genes (PC4CL1 and PC4CL2) and in two loblolly pine genes (LP4CL1 and LP4CL2).

By Northern blot analysis, it has been shown that CCL is expressed in leaf, shoot tip, stem, root, flower and cell culture. Two similar CCL cDNAs in parsley, potato and tobacco have been shown to be expressed at similar level in response to the environmental stress and during different developmental stages. Two distinct CCL cDNAs in soybean and *Lithospermum* have shown different expression levels when pathogens or chemicals were applied to the cell cultures. It appears that the expression of the CCL genes is developmentally regulated and inducible by many environmental stresses at the transcription level.

Genetic transformation with a CCL sequence can result in several significant effects. The description of the invention hereafter refers to aspen, and in particular quaking aspen (*Populus tremulaeoides* Michx) when necessary for the sake of example. However, it should be noted that the invention is not limited to genetic transformation of aspen. The method of the present invention is capable of being practiced for other trees, including for example, other angiosperms, other gymnosperm forest tree species, etc.

Preferably, the CCL down regulation is accomplished through transformation with a homologous CCL sequence in an antisense orientation. However, it should be noted that a heterologous antisense CCL sequence could be utilized and incorporated into a tree species to down regulate CCL if the heterologous CCL gene sequence has a high nucleotide sequence homology, approximately higher than 70%, to the endogenous CCL gene sequence of that tree species.

In addition, trees transformed with a sense CCL sequence could also cause a sequence homology-based co-suppression of the expression of the transgene and endogenous CCL gene, thereby achieving down regulation of CCL in these trees.

B. Isolation of CCL cDNAs

The present invention utilizes a homologous CCL sequence to genetically alter trees. The preferred embodiment of the invention as further described below utilizes a CCL DNA clone of the quaking aspen CCL gene.

Two aspen (*Populus tremulaeoides* Michx) cDNAs encoding two distinct CCL isoforms, PtCCL1 and PtCCL2 have been cloned. PtCCL1 cDNA is lignin pathway-specific and is different from PtCCL2 cDNA, which is involved in flavonoid synthesis. The cloning of PtCCL1 and PtCCL2 cDNAs and the identification of their biochemical functions will be discussed in more length below. PtCCL1 and PtCCL2 genomic clones including their 5'-end regulatory promoter sequences were also isolated. The promoter of PtCCL1 (PtCCL1p) directs xylem tissue-specific gene expression in a plant, whereas the promoter of PtCCL2 (PtCCL2p) drives the expression of genes specifically in epidermal tissues of stem and leaf of a plant. These tissue specific promoters will be discussed in more length in Section I below.

Two CCL cDNAs, PtCCL1 and PtCCL2, have been isolated from quaking aspen using either a conventional cDNA library screening method or a PCR-based cDNA cloning method. It should be noted that the methods described below are set forth as an example and should not be considered limiting. These CCL cDNA clones are available from Michigan Technological University, Institute of Wood Research, Houghton, Mich.

Young leaves and shoot tips are collected from greenhouse-grown quaking aspen (*Populus tremulaeoides* Michx). Differentiating xylem and sclerenchyma are collected from three to four year old quaking aspen. The bark is peeled from the tree exposing the developing secondary xylem on the woody stem and the sclerenchyma on the inner side of the bark. Developing secondary xylem and sclerenchyma are scraped from the stem and bark with a razor blade and immediately frozen in liquid nitrogen until use.

Total RNA is isolated from the young leaves, shoot tips, xylem and sclerenchyma following the method of Bugos RC et al. (1995), RNA Isolation from Plant Tissue Recalcitrant to Extraction in Guanidine, Biotechniques 19(5):734–737. Poly(A)+RNA is purified from total RNA using Poly(A)+ mRNA Isolation Kit from Tel-test B, Inc. A unidirectional Lambda gt22 expression cDNA library was constructed from the xylem mRNA using Superscript S System from Life Technologies, Inc. and Gigapack Packaging Extracts from Stratagene. The PtCCL1 cDNA was obtained by screening the cDNA library with a 32P-labeled parsley 4CL cDNA probe. The parsley 4CL cDNA (pc4CL2) has Genbank accession number X13325, (SEQ ID NO:15).

The PtCCL2 cDNA was obtained by RT-PCR. The reverse transcription of total RNA isolated from shoot tips was carried our using the Superscript II reverse transcriptase from Life Technologies. Two sense primers (R1S, 5'-TTGGATCCGIGACIGICIGTTICCIAARGG-3') (SEQ ID NO:11), and H1S, 5'-TTGGATCCGIGACIGICICARGTIGAYGG-3' (SEQ ID NO:10) are designed around the first consensys AMP-binding region of CCL that was previously discussed. One antisense primer (R2 A, 5'-ATGTCGACCICGDATRCADATYTCICC-3') (SEQ ID NO:11) is designed based on the sequence of the putative catalytic motif GECIGIR (SEQ ID NO:8). One fifth of the reverse transcription reaction (4 Tl) is used as the template in a 50 Tl PCR reaction containing 1x reaction buffer, 200 TM each deoxyribonucleotide triphosphate, 2 TM each R1S and oligo-dT (20 mer) primers, and 2.5 units of Taq DNA polymerase. The PCR reaction mixture was denatured at 940 C for 5 minutes followed by 30 cycles of 940 C/45 seconds, 500 C/1 minute, 720 C/90 seconds and is ended with a 5 minute extension at 720 C. 2 Tl of the PCR amplification products are used for a second run PCR re-amplification using primers H1S and R2A. A 0.6 kb PCR fragment is cloned using the TA Cloning Kit from Invitrogen and used...
as a probe to screen an aspen genomic library to obtain the PtCCL2 genomic clone. Two primers (2A, 5'-TCTGCTCTAGATGTCGGACGGA-3' (SEQ ID NO:12) and 2B, 5'-TATGATCTTAGCATGTCGGTGGCC-3' (SEQ ID NO:13)) are designed based on the genomic sequence of PtCCL2 at around the deduced transcription start site and stop codon for the cloning of PtCCL2 cDNA by RT-PCR as determined using—Taq Cycle Sequencing Kit from Amer- sham.

The PtCCL1 cDNA has an open reading frame of 1620 bp which encodes a polypeptide of 540 amino acid residues with a predicted molecular weight of 59 kd and pl of 5.8. The nucleotide sequence of the aspen CCL cDNA clone PtCCL1 is set forth as SEQ ID NO:1. The deduced amino acid sequence for the aspen CCL1 protein is set forth as SEQ ID NO:2.

The PtCCL2 cDNA has an open reading frame of 1713 bp which encodes a polypeptide of 571 amino acid residues with a predicted molecular weight of 61.8 kd and pl of 5.1. The nucleotide sequence of the aspen CCL cDNA clone PtCCL2 is set forth as SEQ ID NO:3. The deduced amino acid sequence for the aspen CCL2 protein is set forth as SEQ ID NO:4.

The aspen PtCCL1 cDNA shares a 59–74% identity at the nucleotide level and 59–81% identity at the amino acid level with other reported CCL cDNAs and genes, whereas the PtCCL2 cDNA shares a 60–73% identity at the nucleotide level and 57–74% at the amino acid level with other CCL cDNAs and genes as set forth in the following table.

**TABLE 1**

<table>
<thead>
<tr>
<th>cDNA*</th>
<th>DNA Identity % PtCCL1</th>
<th>DNA Identity % PtCCL2</th>
<th>Amino Acid Identity % PtCCL1</th>
<th>Amino Acid Identity % PtCCL2</th>
</tr>
</thead>
<tbody>
<tr>
<td>PtCCL1</td>
<td>62</td>
<td>62</td>
<td>63</td>
<td>63</td>
</tr>
<tr>
<td>LE4CL1</td>
<td>69</td>
<td>62</td>
<td>71</td>
<td>71</td>
</tr>
<tr>
<td>LE4CL2</td>
<td>62</td>
<td>71</td>
<td>59</td>
<td>72</td>
</tr>
<tr>
<td>GM14</td>
<td>74</td>
<td>67</td>
<td>81</td>
<td>69</td>
</tr>
<tr>
<td>GM16</td>
<td>62</td>
<td>73</td>
<td>65</td>
<td>73</td>
</tr>
<tr>
<td>NT4CL1</td>
<td>67</td>
<td>62</td>
<td>75</td>
<td>74</td>
</tr>
<tr>
<td>NT4CL2</td>
<td>66</td>
<td>63</td>
<td>75</td>
<td>66</td>
</tr>
<tr>
<td>PC4CL1</td>
<td>66</td>
<td>64</td>
<td>71</td>
<td>64</td>
</tr>
<tr>
<td>PC4CL2</td>
<td>66</td>
<td>63</td>
<td>72</td>
<td>64</td>
</tr>
<tr>
<td>ST4CL1</td>
<td>67</td>
<td>63</td>
<td>75</td>
<td>64</td>
</tr>
<tr>
<td>AT3CL</td>
<td>66</td>
<td>63</td>
<td>70</td>
<td>61</td>
</tr>
<tr>
<td>LP4CL</td>
<td>61</td>
<td>64</td>
<td>63</td>
<td>67</td>
</tr>
<tr>
<td>OS4CL1</td>
<td>59</td>
<td>60</td>
<td>59</td>
<td>57</td>
</tr>
</tbody>
</table>

*PtCCL1: aspen CCL
PtCCL2: aspen CCL
LE4CL1 and LE4CL2: Lithospermum erythrorhizon CCL
GM14 and GM16: soybean CCL
NT4CL1 and NT4CL2: tobacco CCL
PC4CL1 and PC4CL2: poplar CCL
ST4CL1: potato CCL
AT3CL: A. thaliana CCL
LP4CL: loblolly pine CCL
OS4CL1: rice CCL

The results of sequence analysis, phylogenetic tree and genomic Southern blot analysis indicate that PtCCL1 and PtCCL2 cDNAs encode two distinct CCLs that belong to two divergent gene families in aspen. The deduced amino acid sequence for the PtCCL2 protein contains a longer N-terminal sequence than the PtCCL1 protein but shows profound similarity in the central and C-terminal portions of protein to the PtCCL1 protein.

PtCCL1 and PtCCL2 cDNAs display distinct tissue-specific expression patterns. The PtCCL1 sequence is expressed highly in the secondary developing xylem and in the 6th to 10th internodes whereas the PtCCL2 sequence is expressed in the shoot tip and leaves. These tissue-specific expression patterns were investigated by fusing promoters of PtCCL1 and PtCCL2 genes to a Gus reporter gene. The tissue specific promoters for PtCCL1 and PtCCL2 will be discussed in more length in Section I below.

The substrate specificity of PtCCL1 and PtCCL2 is also different from each other as determined using recombinant proteins produced in *E. coli*. PtCCL1 utilized p-coumaric acid, caffeic acid, ferulic acid and 5-hydroxyferulic acid as substrates. PtCCL2 showed activity to p-coumaric acid, caffeic acid and ferulic acid but not to 5-hydroxyferulic acid.

Specifically, PtCCL1 and PtCCL2 were used to construct expression vectors for *E. coli* expression. The substrate specificity of PtCCL1 and PtCCL2 were tested using fusion proteins produced in *E. coli*. Two plasmids, pQE/CCL1 and pQE/CCL2, were constructed in which the coding regions of PtCCL1 and PtCCL2, respectively were fused to N-terminal His tags in expression plasmids pQE-31 and pQE-32 (Qiagen, Chatsworth, Calif.). The recombinant proteins of PtCCL1 and PtCCL2 produced by *E. coli* are approximately 59 kd and 63 kd, respectively.

The two recombinant proteins were tested for their activity in utilizing cinnamic acid derivatives. PtCCL1 recombinant protein showed 100, 81, 18 and 0% relative activity to p-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid and sinapic acid, respectively. PtCCL2 recombinant protein exhibited 100, 14, 27, 0 and 0% relative activity to p-coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid and sinapic acid, respectively.

Neither recombinant protein showed detectable activity to sinapic acid.

The results of the tissue-specific expression pattern and substrate specificity suggests that in addition to the general function of CCL, PtCCL1 apparently is more related to lignin synthesis in the xylem tissue and PtCCL2 apparently is more likely involved in flavonoid synthesis and UV protection.

It should be noted that the isolation and characterization of the PtCCL1 and PtCCL2 cDNA clones is described in Kawaoka A, Chiang VL (1995), The Molecular Cloning and Expression of Syringyl- and Guaiacyl-Specific Hydroxycinnamate: CoA Ligases from Aspen (*Populus tremuloides*), Proceedings of the 6th International Conference on Biotechnology in the Pulp and Paper Industry, Vienna, Austria; and in Hu, Wen-Jing, Isolation and Characterization of p-coumarate Co-enzyme A ligase cDNAs and Genes from Quaking Aspen (*Populus tremuloides*) Michx., Ph.D Dissertation, Michigan Technological University, Houghton, Mich. (1997), which are both herein incorporated by reference.

C. Transformation and Regeneration
Several methods for gene transformation of plant species with the CCL sequence are available such as the use of a transformation vector, agroinfection, electroinjection, particle bombardment with a gene gun or microinjection.

Preferably, a CCL cDNA clone is positioned in a binary expression vector in an antisense orientation under the control of double cauliflower mosaic virus 35S promoter. The vector is then preferably mobilized into a strain of *Agrobacterium* species such as *tumefaciens* strain C58/ pMP90 and is used as the DNA delivery system due to its efficiency and low cost.
For example, with reference to FIG. 2, the binary expression pACCL1 used for plant transformations is shown. Specifically, the pACCL1 cDNA is inserted in an antisense orientation into Pac I and BamHI sites between the double CaMV 35S:AMV RNA4 and the 3′ terminator sequence of the nopaline synthase gene in a binary cloning vector pACCL1 (FIG. 2). The binary vector containing hygromycin phosphotransferase (HPT) gene is modified from pBin 19.

The gene construct pACCL1 is available from Michigan Technological University, Institute of Wood Research, Houghton, Mich.

The binary vector construct is mobilized in Agrobacterium tumefaciens using the freeze-thaw method of Holsters et al., Mol. Gen. Genet. 163:181–187 (1978). For the freeze-thaw method, 1.5 ml of overnight cultures Agrobacterium tumefaciens strain C58/pMP90 is pelleted at 5000g for 3 minutes at 40 C and suspended in 1 ml of ice cold 20 mM CaCl2. To the suspension is added 10 II binary vector DNA (from an alkaline lysis minipreparation) and mixed by pipetting. The microcentrifuge tube is then frozen in liquid nitrogen for 5 minutes and thawed at 370 C for 5 minutes. After being cooled on ice, 1 ml of LB is added and the mixture is incubated at 280 C for 2 hours with gentle shaking. 200 II of the cells is spread onto LB plates containing gentamycin and kanamycin and incubated at 280 C for 2 days. Colonies grown on the selection plates are randomly picked or miniprep and restriction enzyme digestion analysis is used to verify the integration.

The resulting binary vector containing Agrobacterium strain is used to transform quaking aspen according to Tsai et al., Agrobacterium-Mediated Transformation of Quaking Aspen (Populus tremuloides) and Regeneration of Transgenic Plants, Plant Cell Rep. 14:9497 as set forth below.

Explants of young leaves from cuttings of aspen are obtained by cutting leaf disks of approximately 7 mm square from the young leaves along the midrib of the leaves. The explants are surface sterilized in 20% commercial bleach for 10 minutes followed by rinsing 3 times with sterile double-distilled water.


D. Phenotype Changes

The results of the transformation can be confirmed with conventional PCR and Southern analysis. For example, transferring CCL cDNA in an antisense orientation down regulates CCL in the tree. Expression of the CCL has been found to be blocked up to 96 percent in some transgenic trees.

After acclimation, the transgenic aspen display an unusual phenotype, including big curly leaves, thick diameters, longer internodes, more young leaves in the shoot tip and a red pigmentation in the petioles extending into midvein leaves. Red coloration of the developing secondary xylem tissues is observed after peeling of the bark in the transgenic plants.

E. Accelerated Growth

Down regulation of CCL alters growth of the transgenic trees. For example, transformation with an antisense CCL sequence accelerates the growth of the tree. Enhanced growth is markedly noticeable at all ages. In particular, the transgenic trees show enhanced growth in the form of thicker stems and enlarged leaves as compared to control trees. These characteristics are retained in the vegetative propagules of these transgenic trees. Table 2 sets forth exemplary data with respect to several lines of transgenic
11

quaking aspen grown in the greenhouse after eight months. Volume represents the overall quantitative growth of the tree.

**TABLE 2**

<table>
<thead>
<tr>
<th>PLANT #</th>
<th>HEIGHT (cm)</th>
<th>DIAMETER (cm)*</th>
<th>VOLUME (cm³)*</th>
<th>AVERAGE LENGTH OF INTERNODE (cm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control 1</td>
<td>247.7</td>
<td>1.08</td>
<td>78.6</td>
<td>2.6</td>
</tr>
<tr>
<td>Control 2</td>
<td>250.2</td>
<td>1.01</td>
<td>66.8</td>
<td>0.8</td>
</tr>
<tr>
<td>11-1</td>
<td>394.8</td>
<td>2.15</td>
<td>105.5</td>
<td>3.3</td>
</tr>
<tr>
<td>11-2</td>
<td>346.9</td>
<td>2.01</td>
<td>66.4</td>
<td>3.4</td>
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<tr>
<td>11-3</td>
<td>241.3</td>
<td>0.84</td>
<td>44.6</td>
<td>3.2</td>
</tr>
<tr>
<td>11-4</td>
<td>288.3</td>
<td>0.94</td>
<td>66.7</td>
<td>3.4</td>
</tr>
<tr>
<td>11-5</td>
<td>246.4</td>
<td>0.92</td>
<td>54.6</td>
<td>3.3</td>
</tr>
<tr>
<td>11-7</td>
<td>226.7</td>
<td>1.13</td>
<td>75.7</td>
<td>3.4</td>
</tr>
<tr>
<td>11-8</td>
<td>289.6</td>
<td>1.36</td>
<td>102.0</td>
<td>3.3</td>
</tr>
<tr>
<td>11-9</td>
<td>287.0</td>
<td>1.76</td>
<td>232.6</td>
<td>4.3</td>
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<tr>
<td>11-10</td>
<td>252.7</td>
<td>0.83</td>
<td>45.6</td>
<td>3.3</td>
</tr>
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<td>247.7</td>
<td>0.86</td>
<td>48.0</td>
<td>3.5</td>
</tr>
<tr>
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<td>247.7</td>
<td>1.11</td>
<td>78.4</td>
<td>2.7</td>
</tr>
<tr>
<td>12-2</td>
<td>199.4</td>
<td>0.96</td>
<td>48.1</td>
<td>2.5</td>
</tr>
<tr>
<td>12-6</td>
<td>294.6</td>
<td>0.92</td>
<td>65.2</td>
<td>3.2</td>
</tr>
<tr>
<td>16-1</td>
<td>227.3</td>
<td>0.95</td>
<td>53.7</td>
<td>2.8</td>
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<tr>
<td>16-2</td>
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<td>68.5</td>
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</tr>
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<td>16-3</td>
<td>265.4</td>
<td>1.09</td>
<td>82.5</td>
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<tr>
<td>17-2</td>
<td>243.8</td>
<td>0.89</td>
<td>50.5</td>
<td>2.6</td>
</tr>
</tbody>
</table>

* at 10 cm above ground

The averages for height, diameter, volume and average length between internodes for the control plants are as follows:

- Height: 248.95 cm
- Diameter: 1.045 cm
- Volume: 71.2 cm³
- Average length of internodes: 2.7 cm

With respect to height alone, for those transgenic plants (11-1, 11-4, 11-8, 11-9, 12-6, 16-2, 16-3) having a statistically larger height than the control plants, the average height was 286.83 cm as compared to the control plant average height of 248.95 cm.

With respect to diameter alone, for those transgenic plants (11-1, 11-7, 11-8, 11-9) having a statistically larger diameter than the control plants, the average diameter was 1.30 cm as compared to the control plant average diameter of 1.045 cm.

With respect to volume alone, for those transgenic plants (11-1, 11-8, 11-9, 12-1, 16-3) having a statistically larger volume than the control plants, the average volume was 120.2 cm³ as compared to the control plant average volume of 71.2 cm³.

With respect to average length of internodes alone, for those transgenic plants (11-1, 11-2, 11-3, 11-4, 11-5, 11-7, 11-8, 11-9, 11-10, 12-6, 16-2, 16-3) having a statistically larger average length of internodes than the control plants, the average average length of internodes was 3.39 cm as compared to the control plant average average length of internodes of 2.70 cm.

As demonstrated in Table 2, while there are variations in growth among the transgenic trees, the average length of the internodes for the transgenic trees is consistently and significantly higher than that of the control plants. Variations in the growth of the transgenic trees is normal and to be expected. Preferably, a transgenic tree with a particular growth rate is selected and this tree is vegetatively propagated to produce an unlimited number of clones that all exhibit the identical growth rate.

F. Lignin

Down regulation of lignin pathway CCL results in production of trees with reduced lignin content.

The following table shows the reduction of lignin content and CCL enzyme activity in several transgenic aspen which have been transformed with an homologous antisense CCL sequence.

**TABLE 3**

<table>
<thead>
<tr>
<th>Transgenic Plant #</th>
<th>Wood Weight (%)</th>
<th>% Lignin Reduction</th>
<th>CCL Enzyme Activity (%)</th>
<th>% CCL Enzyme Activity Reduction</th>
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</thead>
<tbody>
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<td>control</td>
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<td>0.0</td>
<td>868</td>
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<td>10.3</td>
<td>515</td>
<td>45</td>
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<td>11-3</td>
<td>20.9</td>
<td>5.3</td>
<td>922</td>
<td>6</td>
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<td>7.9</td>
<td>1032</td>
<td>-19</td>
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<td>691</td>
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<td>0.0</td>
<td>925</td>
<td>1</td>
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</tbody>
</table>

*activity is expressed as pkat/(mg protein) using p-coumaric acid as the substrate

Lignin content was determined according to Chiang and Funaoka (1990) Holzforschung 44:147–155. CCL enzyme activity was determined according to Ranjeva et al. (1976), Biochimie 58:1255–1262.

The data in Table 3 demonstrates a correlation between down regulation of CCL and reduction in lignin content.

Transgenic trees with reduced lignin content have an altered phenotype in that the stem is more elastic to the touch and the leaves are typically curlier.

It should also be noted that for those transgenic trees (12-1, 12-2, 12-3 and 16-1) with the approximately 40% reduction in lignin content and the corresponding approximately 95% reduction in CCL enzyme levels, all of those transgenic trees had a consistent deep red coloration in the wood of the plant. Accordingly, the deep red color can be used as an identifier of reduced lignin content.

Down regulation of lignin pathway CCL also results in production of trees with an altered lignin structure. Based upon thioacidolysis (Rolando et al. (1992) Thioacidolysis, Methods in Lignin Chemistry, Springer-Verlag, Berlin, pp 334–349) of plants 12-3 and 16-1, coniferyl alcohol and sinapyl alcohol lignin units are significantly reduced in these two trees as compared to the control tree, as shown in the following table.
The alteration of the frequency of the structural units in lignin of these transgenic trees is evident that the overall structure of lignin in these plants has been genetically altered.

G. Cellulose Content

Down regulation of lignin pathway CCL results in increased cellulose content of the transgenic plants. Analysis of control and transgenic aspen for carbohydrate content demonstrate a higher cellulose content in the transgenic trees than the control trees. Particularly, the transgenic trees that have over 40% lignin reduction have about 10–15% higher cellulose content than the control. Data is set forth in the following tables for trees that were transformed with homoelogous CCL in an antisense orientation:

**TABLE 4**

<table>
<thead>
<tr>
<th>Plant #</th>
<th>Coniferyl Alcohol Units*</th>
<th>Sinapyl Alcohol Units*</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>733</td>
<td>1700</td>
</tr>
<tr>
<td>12-3</td>
<td>283</td>
<td>592</td>
</tr>
<tr>
<td>16-1</td>
<td>247</td>
<td>445</td>
</tr>
</tbody>
</table>

*Micro-mole/g of lignin

The procedure for carbohydrate analysis utilized is as follows. About 100 mg of powdery woody tissue with sizes that pass a 80-mesh screen was hydrolyzed with 1 mL of 72% (W/W) H2SO4 for 1 hr at 300°C. Samples were then diluted to 4% (W/W) H2SO4 with distilled water, fucose was added as an internal standard, and a secondary hydrolysis was performed for 1 hr at 121°C. After secondary hydrolysis, the sugar contents of the hydrolysates are determined by anion exchange high performance liquid chromatography using pulsed amperometric detection. Sugar contents are expressed as % of the weight of the woody tissue used. The above procedures are similar to those in a publication by R.C. Pettersen and V.H. Schwandt, 1991, J. Wood Chem. & Technol. 11:495–501.

**TABLE 5**

<table>
<thead>
<tr>
<th>Plant #</th>
<th>Glucan</th>
<th>Arabinan</th>
<th>Rhamnan</th>
<th>Xylan</th>
<th>Mannan</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>44.23%</td>
<td>0.47%</td>
<td>0.79%</td>
<td>0.37%</td>
<td>17.39%</td>
</tr>
<tr>
<td>12-2</td>
<td>49.05%</td>
<td>0.36%</td>
<td>1.05%</td>
<td>0.38%</td>
<td>15.34%</td>
</tr>
<tr>
<td>11-9</td>
<td>48.95%</td>
<td>0.40%</td>
<td>0.80%</td>
<td>0.37%</td>
<td>17.12%</td>
</tr>
<tr>
<td>11-10</td>
<td>47.49%</td>
<td>0.43%</td>
<td>0.99%</td>
<td>0.40%</td>
<td>16.24%</td>
</tr>
<tr>
<td>12-3</td>
<td>50.83%</td>
<td>0.55%</td>
<td>1.24%</td>
<td>0.48%</td>
<td>17.25%</td>
</tr>
<tr>
<td>16-1</td>
<td>48.14%</td>
<td>0.56%</td>
<td>1.07%</td>
<td>0.48%</td>
<td>19.14%</td>
</tr>
<tr>
<td>16-2</td>
<td>46.55%</td>
<td>0.34%</td>
<td>0.82%</td>
<td>0.37%</td>
<td>16.75%</td>
</tr>
</tbody>
</table>

Comparison of Lignin and Cellulose (glucan) Contents in Transgenic and Control Aspen

<table>
<thead>
<tr>
<th>Plant #</th>
<th>Content % on wood</th>
<th>% reduction</th>
<th>Content % on wood</th>
<th>% increase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>21.4</td>
<td>0</td>
<td>44.23</td>
<td>0</td>
</tr>
<tr>
<td>11-2</td>
<td>19.2</td>
<td>10.3</td>
<td>40.05</td>
<td>11.0</td>
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<tr>
<td>11-9</td>
<td>20.4</td>
<td>4.7</td>
<td>45.95</td>
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<td>47.49</td>
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<td>48.14</td>
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<td>20.6</td>
<td>3.6</td>
<td>46.55</td>
<td>5.2</td>
</tr>
</tbody>
</table>

**TABLE 6**

The procedure for carbohydrate analysis utilized is as follows. About 100 mg of powdery woody tissue with sizes that pass a 80-mesh screen was hydrolyzed with 1 mL of 72% (W/W) H2SO4 for 1 hr at 300°C. Samples were then diluted to 4% (W/W) H2SO4 with distilled water, fucose was added as an internal standard, and a secondary hydrolysis was performed for 1 hr at 121°C. After secondary hydrolysis, the sugar contents of the hydrolysates are determined by anion exchange high performance liquid chromatography using pulsed amperometric detection. Sugar contents are expressed as % of the weight of the woody tissue used. The above procedures are similar to those in a publication by R.C. Pettersen and V.H. Schwandt, 1991, J. Wood Chem. & Technol. 11:495–501.

**H. Increased Disease Resistance**

Down regulation of lignin pathway CCL results in production of trees with increased disease resistance, and in particular, with increased fungal pathogen resistance. In particular, greenhouse transgenic aspen plants showed a disease resistance to fungi such as those which induce leaf-blight disease.

**I. Promoters**

Two distinct genes encoding CCL and their promoters were cloned. The promoter of PtCCL1 can drive gene expression specifically in xylem tissue and the promoter for PtCCL2 confers gene expression exclusively in the epidermal tissues. These promoters can be used to manipulate gene expression to engineer traits of interest in specific tissues of target plants. The significance of the promoters is the application of the xylem-specific promoter to direct the expression of any relevant genes specifically in the xylem for engineering lignin content, lignin structure, enhanced tree growth, cellulose content and other value-added wood qualities, etc. The importance of the epidermis-specific promoter is its ability to drive the expression of any relevant genes specifically in epidermal tissues for engineering disease-, UV light-, cold-, heat-, drought-, and other stress resistance traits in trees.

Specifically, the promoters of the PtCCL1 and PtCCL2 were conventionally isolated as follows. An aspen genomic library was screened with PtCCL1 cDNA and PtCCL2 partial cDNA fragment to isolate genomic clones of PtCCL1 and PtCCL2. Eleven and seven positive genomic clones were identified for PtCCL1 and PtCCL2 gene, respectively. Among 11 positive clones for PtCCL1, PtCCL1g-4 contained a full length coding sequence and at least 2 kb 5' flanking regions. The restriction map of PtCCL1g-4 is set forth at FIG. 3.

With respect to PtCCL2, restriction map analysis was performed on DNA of positive genomic clone PtCCL2g-11.

The restriction map of PtCCL2g-11 is set forth at FIG. 4.

Approximately a 2.3 kb 5' flanking region of PtCCL1 was digested from PtCCL1g-4 using Xba I and Sac I sites and cloned into pGEM7Z Xba I and Sac I sites. The subcloned PtCCL1 promoter was named p7Z-4XS and the restriction map of p7Z-4XS is set forth at FIG. 5. The 5' unilateral deletion of p7Z-4XS was generated for DNA sequencing by exonuclease III/S1 nuclease digestion using Erase-a-Base System (Promega, Madison, Wis.). The deletion series was sequenced using a primer on pGEM7Z vector.

A 1.6 kb Hind III and EcoR I fragment containing a 1.2 kb 5' flanking region of PtCCL2 and 0.4 kb coding region of PtCCL2g-11 were subcloned in pBluescript II SK+ Hind III and EcoR I sites. The restriction map of the resulting clone, pSK-11HE, was determined by digesting the plasmid with several restriction enzymes, as in set forth at FIG. 6. In order to determine the sequence of the PtCCL2 promoter, pSK-11HE was further digested into small fragments according to the restriction map and subcloned into vectors with suitable cloning sites. The DNA sequence was determined using M13 universal primer and reverse primer on the vector.

The DNA sequences of the two promoters was determined and analyzed using qTaq cycle sequencing Kit (USB, Cleveland, Ohio), and GENETYX-MAC 7.3 sequence analysis software from Software Development Co., Ltd. The nucleotide sequence of promoter region of PtCCL1 is set forth as SEQ ID NO:5 and the nucleotide sequence of the promoter region of PtCCL2 is set forth as SEQ ID NO:6.
The promoter gene constructs PtCCL1p and PtCCL2p are available from Michigan Technological University, Institute of Wood Research, Houghton, Mich.

Tissue-specific expression can be achieved by conventionally fusing the promoters of PtCCL1 or PtCCL2 to a gene of interest and transferred to a plant species via Agrobacterium. For the sake of example, the promoters of PtCCL1 and PtCCL2 were fused to a GUS reporter gene as detailed below. However, it should be noted that genes other than the GUS reporter gene can be fused to these promoters for tissue specific expression.

In order to construct PtCCL1 promoter-GUS binary vector, a 1 Kb fragment covering 5'-flanking region and 117 bp coding region of PtCCL1 was subcloned into pGEM7Z Sph I and EcoR I sites for constructing promoter-GUS binary vector. In this 1 kb DNA fragment, it is found that one Xho I site locates at 486 bases proximal to the translation start site and the EcoR I site at 117 bases downstream the translation site. This 0.6 Kb fragment was subcloned into pGEM7Z Xho I and EcoR I sites and used as a template in PCR amplification.

In order to construct a promoter-GUS transcriptional fusion, a BamH I site was introduced in front of the translation start site of PtCCL1 by PCR. PCR amplification was performed using p7Z-4XE as the template, M13 universal primer on pGEM7Z vector as 5' end primer and PtCCLlp-1 primer containing a BamH I site at the end is complementary to a sequence upstream of the translation start site of PtCCL1 and to incorporate BamH I site at the end. Amplification was performed using p7Z1 SE as a template, M13 universal primer as the 5' end primer and PtCCL2p-3 as the 3' end primer. A PCR reaction was carried out and the amplified PCR product was cloned and sequenced to check the fidelity of the PCR amplification. The 0.2 Kb Sph I-BamH I DNA fragment with correct sequence was fused to pSK-1HE linearized with Sph I and BamH I. The resulting plasmid was named pSK-1HE. The promoter of PtCCL2 was then excised from pSK-1HE with Hind III and BamH I and ligated to pBI101 Hind III and BamH I site to make PtCCL2p-GUS transcriptional fusion binary vector as shown in FIG. 8.

The PtCCL1p-GUS and PtCCL2p-GUS constructs are then mobilized into Agrobacterium tumefaciens strain C58/pMP90 by freeze and thaw method as explained previously.

Leaf disk transformation of tobacco with these two Agrobacterium constructs is conducted according to the method of Horsch R. B. (1988) Leaf Disk Transformation, Plant Molecular Biology Manual, A5:1-9. Histochemical GUS staining of promoter-GUS transgenic tobacco plants demonstrated that the PtCCL1 promoter restricted GUS expression in xylem tissue whereas PtCCL2 promoter regulated GUS expression in epidermal cells.
<table>
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<th>360</th>
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 290   295   300
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Thr Ala Asn Thr Ile Asp Val G1y Trp Leu His Thr Gly Asp Ile
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450 455 460
Glu Ile Ile Lys Phe Lys Gly Phe Gln Val Pro Ala G1u Glu
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485 490 495
Gln Lys Asp G1u Val Ala Gly Val Pro Val Ala Phe Val Val Arg
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Lys G1u Val Phe Tyr Lys Leu His Lys Phe Phe Val His
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tgcggtgt tgacggc gactttttttattattata ttcgacc  240
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LOCATION: (11)
OTHER INFORMATION: n represents inosine
FEATURE:
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OTHER INFORMATION: n represents inosine
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SEQ ID NO 11
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SEQUENCE: 11
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SEQ ID NO 12
LENGTH: 7
TYPE: PRT
ORGANISM: Populus tremuloides Michx. (aspen) unknown

SEQUENCE: 12
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We claim:
2. A polynucleotide construct comprising the promoter of claim 1 operably connected to a polynucleotide coding sequence.
3. The construct of claim 2, wherein the coding sequence encodes a p-coumarate Co-enzyme A amino acid sequence.
4. A method of expressing a polynucleotide coding sequence in the epidermis of a plant, comprising:
   (a) introducing the DNA construct of claim 2 into a plant cell; and
   (b) regenerating a transgenic plant from the plant cell, wherein the polynucleotide coding sequence is expressed in the epidermis of the plant.
5. The method of claim 4, wherein the polynucleotide coding sequence confers a trait selected from the group consisting of: altered flavanoid content, altered disease resistance, altered stress resistance, altered growth and combinations thereof, relative to a control plant.

* * * * *