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COMT1 Gene fiber-specific promoter elements from poplar

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(54) **COMT1 GENE FIBER-SPECIFIC PROMOTER ELEMENTS FROM POPLAR**

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(58) **Field of Classification Search**
None
See application file for complete search history.

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(57) **ABSTRACT**

Polynucleotide constructs contain fiber-specific elements which are used to target expression of polynucleotides and polypeptides to the vascular fibers of a plant. The constructs can be contained within a vector. Transgenic plants transformed with the fiber-specific elements can be made which have expression of a polynucleotide or polypeptide directed to the plant fibers.

21 Claims, 7 Drawing Sheets

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-473                                                        -414
TAAGTTCAGTAAATATAATCGGGTGAATATCTCATCATGTAATTAAATATCTTAATCTC
       YACT (- strand, YACT box)
                NGATT (- strand, ARR element)
                      GTGA (+ strand, GTGA motif)
                           GATA (- strand, GATA box)
                                        AATTAAA(AT rich)
                                               GATA (-, GATA box)
                                                     NGATT (-, ARR)
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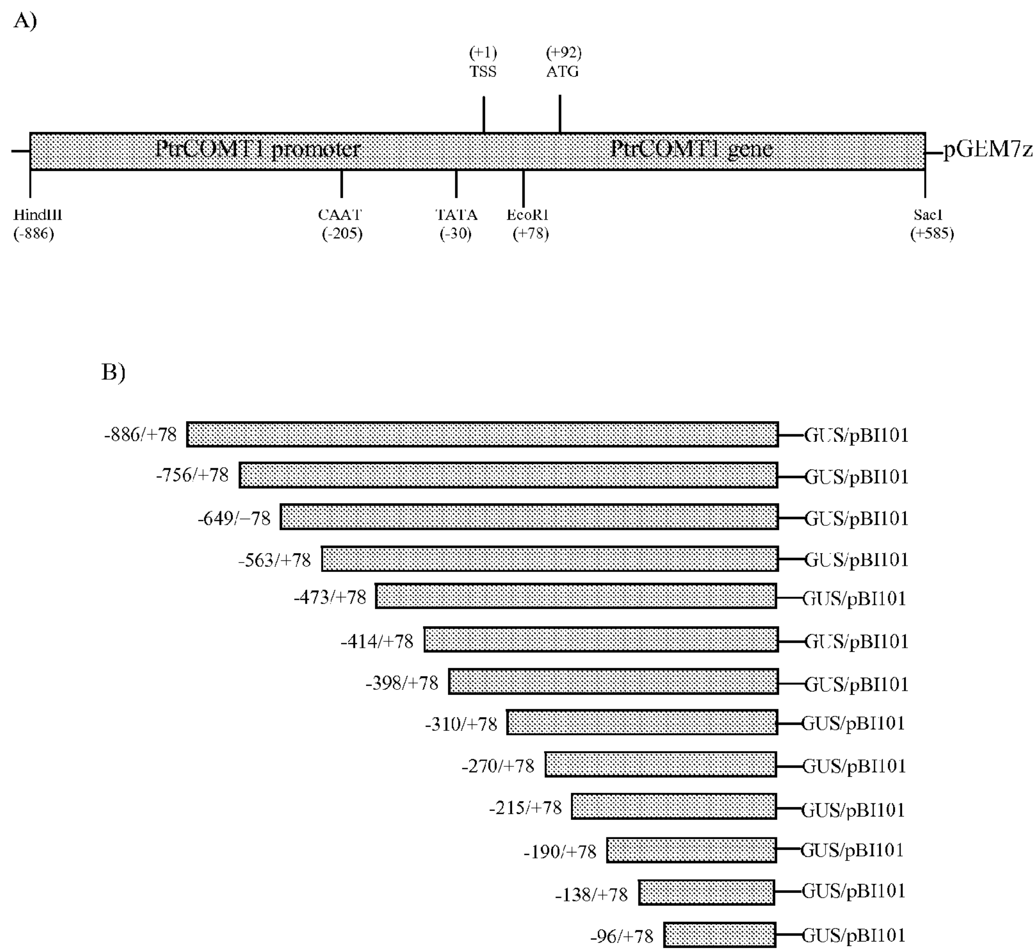
-473                                                      -414
TAAGTTCAGTAAATATAATCGGGTGAATATCTCATCATGTAATTAAATATCTTAATCTC
       YACT (- strand, YACT box)
                NGATT (- strand, ARR element)
                      GTGA (+ strand, GTGA motif)
                           GATA (- strand, GATA box)
                                        AATTAAA(AT rich)
                                               GATA (-, GATA box)
                                                     NGATT (-, ARR)
```

FIG. 1


A)
(+1)
TSS
(+92)
ATG
PtrCOMT1 promoter
PtrCOMT1 gene
pGEM7z
HindIII
(-886)
CAAT
(-205)
TATA
(-30)
EcoR1
(+78)
Sac1
(+585)
B)
-886/+78
-756/+78
-649/–78
-563/+78
-473/+78
-414/+78
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-270/+78
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-190/+78
-138/+78
-96/+78
GUS/pBI101

FIG. 2

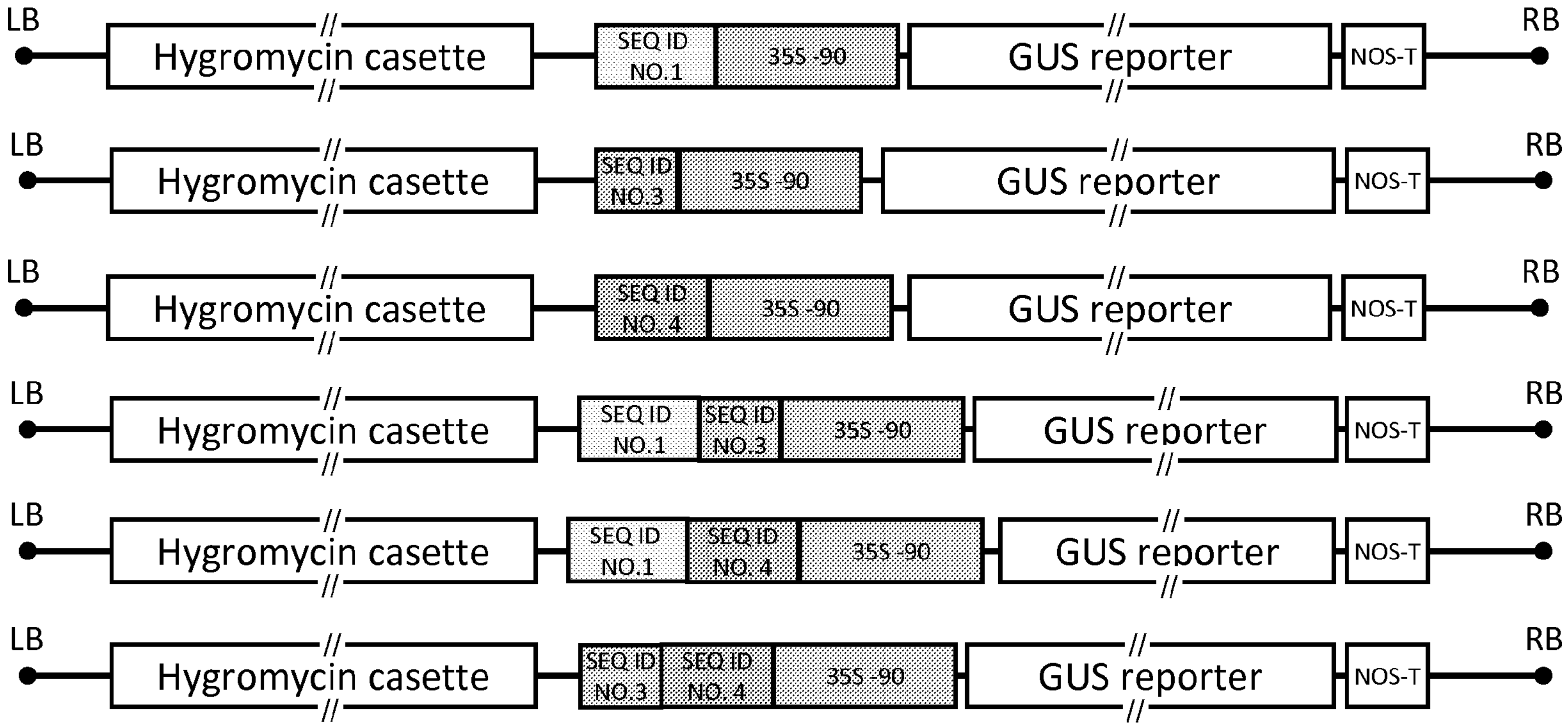

LB
Hygromycin casette
SEQ ID NO.1
35S -90
GUS reporter
NOS-T
RB
LB
Hygromycin casette
SEQ ID NO.3
35S -90
GUS reporter
NOS-T
RB
LB
Hygromycin casette
SEQ ID NO. 4
35S -90
GUS reporter
NOS-T
RB
LB
Hygromycin casette
SEQ ID NO.1
SEQ ID NO.3
35S -90
GUS reporter
NOS-T
RB
LB
Hygromycin casette
SEQ ID NO.1
SEQ ID NO. 4
35S -90
GUS reporter
NOS-T
RB
LB
Hygromycin casette
SEQ ID NO.3
SEQ ID NO. 4
35S -90
GUS reporter
NOS-T
RB

FIG. 3

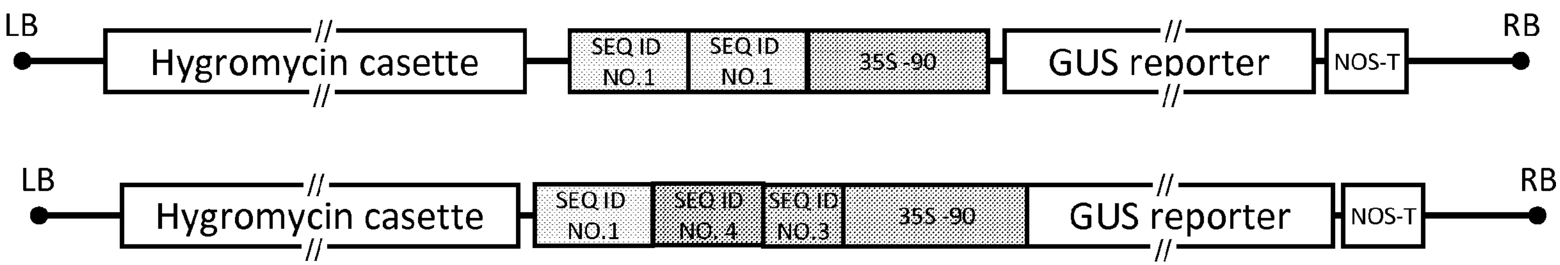
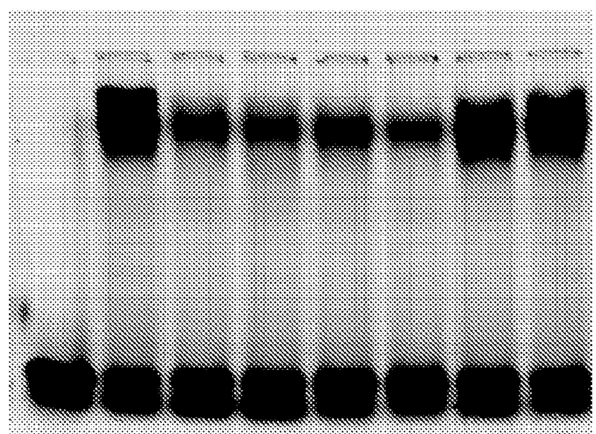

LB
Hygromycin casette
SEQ ID NO.1
SEQ ID NO.1
35S -90
GUS reporter
NOS-T
RB
LB
Hygromycin casette
SEQ ID NO.1
SEQ ID NO.4
SEQ ID NO.3
35S -90
GUS reporter
NOS-T
RB

FIG. 4

A

```
      WT   AGTTCAGTAAATATAATCGGGTGAATATCTCATCA (Positions 3-34
(inclusive) of SEQ ID NO. 1)
     Mut2 AGTTCAGTAAATATccTaGGGTGAATgctTCATCA (SEQ ID NO:7)
     Mut3 AGTTCAGTAAATATccTaGGGTGAATATCTCATCA (SEQ ID NO:8)
     Mut4 AGTTCAGTAAATATAATCGGGTGAATgctTCATCA (SEQ ID NO:9)
     Mut5 AGTTCAGTAAATATAATCGGtcctATgctTCATCA (SEQ ID NO:10)
     Mut6 AGTTCAGTAAATATccTacGtcctATgctTCATCA (SEQ ID NO:11)
     Mut7 AGTTCttcAAATATccTacGtcctATgctTCATCA (SEQ ID NO:12)
```

B



Comt1-471 probe	+	+	+	+	+	+	+	+
xylem	-	+	+	+	+	+	+	+
Mutant competitor	-	-	+	+	+	+	+	+

FIG. 5

A

```
WT-450    TGAATATCTCATCATGTAATTAAATATCTTAATC (Positions 24-
57 (inclusive)of SEQ ID NO: 1)
Mut 3     TGAATgcCTCATCATGTAAccAcATgcCTTAATC (SEQ ID NO:13)
Mut 4     TGAATgcCTCATCATGTAATTAAATgcCTTAATC (SEQ ID NO:14)
Mut 5     TGAATgcCTCATCATGTAAccAcATATCTTAATC (SEQ ID NO:15)
Mut 6     TGAATATCTCATCATGTAAccAcATATCTTAATC (SEQ ID NO:16)
Mut 7     TGAATATCTCATCATGTAATTAAATgcCTTAATC (SEQ ID NO:17)
Mut 8     TGAATgcCTCATCATGTAATTAAATATCTTAATC (SEQ ID NO:18)
Mut 9     TGccTgctTCATCATGTAATTAAATATCTTAATC (SEQ ID NO:19)
Mut 10    TGccTgctTCATCATGTAAccAtAgcTgTTAATC (SEQ ID NO:20)
Mut 11    TGccTgctTCtttcTGTAATTAAATATCTTAATC (SEQ ID NO:21)
Mut 12    TGccTgctTCATCATccttTTAAATATCTTAATC (SEQ ID NO:22)
```

B

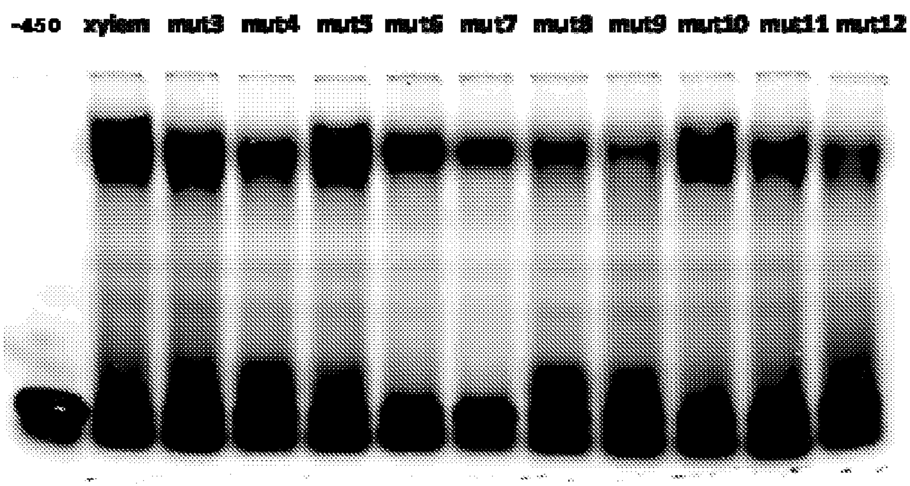
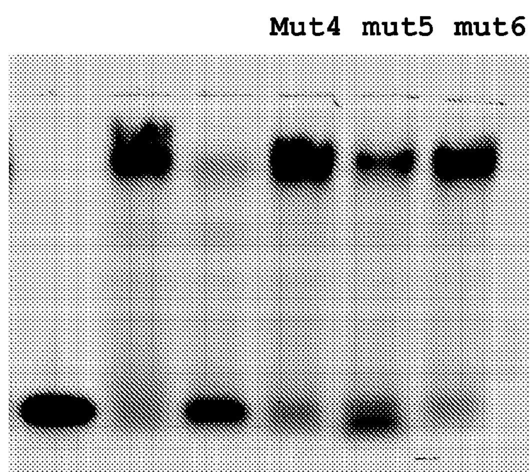


FIG. 6

A

```
WT -422 TTAATCTCCATTATTTCTTAATTTTTTTTA  (Positions 465-
493 (inclusive) of SEQ ID NO. 5)
Mut4    TTcgTaTCCATTAgTgCTTAATTTTTTTTA  (SEQ ID NO:23)
Mut5    TTcgTaTCCATTATTTCTTAATTTTTTTTA  (SEQ ID NO:24)
Mut6    TTAATCTCCATTAgTgCTTAATTTTTTTTA  (SEQ ID NO:25)
```

B



COMT1-422 Probe	+	+	+	+	+	+
xylem	-	+	+	+	+	+
WT competitor	-	-	+	-	-	-
Mut competitor	-	-	-	+	+	+

FIG. 7

COMT1 GENE FIBER-SPECIFIC PROMOTER ELEMENTS FROM POPLAR

CROSS-REFERENCE TO RELATED APPLICATIONS

This application is a national stage filing under 35 U.S.C. 371 of International Application No. PCT/US2009/041956, filed on Apr. 28, 2009, which claims the benefit of priority to U.S. Provisional Patent Application Ser. No. 61/048,435, filed on Apr. 28, 2008, each of which is incorporated herein by reference in its entirety.

STATEMENT REGARDING FEDERAL SPONSORED RESEARCH OR DEVELOPMENT

This invention was supported in part by grant nos. OR22072-121 and EPA82947901-129. The United States government has certain rights in this invention.

BACKGROUND

Manipulation and control of the amounts and types of cellulose and lignin synthesized and deposited in plants and trees is of interest in the forestry, paper and biofuels industries. Tree species synthesize large quantities of lignin, particularly in and around the vascular tissues. Manipulating lignin and/or cellulose in plants and trees can prove beneficial by providing trees and plants with improved disease resistance, increased strength for use in construction, increased biomass usable as fuel or biofuel, improved digestibility (such as for forage crops), as well as having qualitative and quantitative variation in cellulose and/or lignin for paper processing. However, progress in this area has been impeded by difficulties in regulating gene expression in transgenic plants in tissue- or cell-type specific manners.

SUMMARY

In one embodiment, the invention provides a nucleic acid construct containing a fiber-specific element having at least 15 consecutive base pairs of SEQ ID NO: 1, or a reverse complement of at least 15 consecutive base pairs of SEQ ID NO: 1, operably connected to a promoter sequence not natively associated with SEQ ID NO: 1.

In another embodiment, the invention provides a nucleic acid construct containing two or more fiber-specific elements that each have at least 9 consecutive base pairs of SEQ ID NO: 1, or a reverse complement of at least 9 consecutive base pairs of SEQ ID NO: 1, operably connected to a promoter sequence not natively associated with SEQ ID NO: 1.

In a further embodiment, the invention provides a nucleic acid construct containing at least two fiber-specific elements operably connected to a promoter sequence. Each of fiber specific elements contain at least 9 consecutive base pairs of SEQ ID NO: 1, or a reverse complement of at least 9 consecutive base pairs of SEQ ID NO: 1, and are from partially or completely overlapping regions of SEQ ID NO: 1, or are the same.

In another embodiment, the invention provides a method of directing expression of a polypeptide to the fibers of a plant by transforming the plant with constructs containing fiber-specific elements of the invention.

Other aspects of the invention will become apparent by consideration of the detailed description and accompanying drawings.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. **1** is a schematic representation of the 59 base-pair sequence of SEQ ID NO: 1 which identifies elements within SEQ ID NO: 1 that may direct transcription of a polynucleotide to the fibers of a plant.

FIG. **2** is a schematic representation showing promoter deletion: GUS fusion constructs

FIG. **3** is a schematic representation showing constructs containing fiber-specific elements operably connected to a promoter element and a polynucleotide encoding a GUS reporter polypeptide.

FIG. **4** is a schematic representation showing constructs containing fiber-specific elements operably connected to a promoter element and a polynucleotide encoding a GUS reporter polypeptide.

FIG. **5**A is a schematic representation showing constructs used to make probes for use in an Electrophoretic Mobility Shift Assay. FIG. **5**B is a photograph showing the results of an Electrophoretic Mobility Shift Assay

FIG. **6**A is a schematic representation showing constructs used to make probes for use in an Electrophoretic Mobility Shift Assay. FIG. **6**B is a photograph showing the results of an Electrophoretic Mobility Shift Assay

FIG. **7**A is a schematic representation showing constructs used to make probes for use in an Electrophoretic Mobility Shift Assay. FIG. **7**B is a photograph showing the results of an Electrophoretic Mobility Shift Assay

DETAILED DESCRIPTION

Manipulation of the amounts and types of cellulose and lignin in plants is of importance in forestry, agriculture and paper processing. Preferably, expression of genes affecting biochemical pathways involved in the metabolism of lignin and cellulose is regulated with respect to particular tissues or regions of the plant, such as the vascular tissue and the plant fibers.

Specificity of expression in the plant fibers is particularly desirable for manipulating enzymes involved in lignin and cellulose biosynthesis. The fiber-specific elements of the instant invention can be used to express nucleotide sequences in vascular tissue and plant fibers to modify the content and composition of cellulose, thereby affecting plant growth and biomass characteristics. The biosynthesis of lignin and composition of lignin in the plant fibers may also be manipulated to produce plants or trees adapted for a particular end-use.

In one embodiment, the present invention provides a nucleotide construct that can be used to direct expression of a polypeptide to the fibers of a plant. The nucleotide construct contains a fiber-specific element and a promoter sequence not natively associated with the fiber-specific element. The construct can be used to develop other constructs including sequences encoding polypeptides ("coding sequences") that one wishes to specifically express in plant fibers. The coding sequence is operably linked to the promoter sequence to allow fiber-specific expression in plants into which the constructs are delivered. Optionally, the constructs may include features useful in gene cloning, including, but not limited to, unique restriction sites, multiple cloning sites, selectable markers, origins of replication, etc.

As described in the Examples below, a 59 base sequence (SEQ ID NO: 1), found upstream of the coding sequence of a *Populus tremuloides* caffeic acid O-methyltransferase I gene (PtrCOMT1), was discovered to direct fiber-specific expression of a GUS coding sequence.

SEQ ID NO: 1 corresponds to nucleotides of positions −473 to −413 of the positive DNA strand upstream of the transcription start site (TSS) of the PtrCOMPT1 gene, with position 1 of SEQ ID NO: 1 corresponding to position −473 upstream from the transcription start site. The transcription start site is 92 nucleotides upstream of the ATG translation start site, and begins at position 888 of SEQ ID NO. 5. SEQ ID NO: 1 is included within a 978 base sequence (SEQ ID NO:5) located upstream of the translation start site (ATG) of the PtrCOMT1 coding sequence (SEQ ID NO: 6), with position 1 of SEQ ID NO: 5 corresponding to position −886 of the sequence upstream from the transcription start site (TSS) of PtrCOMT1.

It is envisioned that subsequences of the sequence of SEQ ID NO:1 would be sufficient to serve as a fiber-specific element, provided that the subsequence have the ability to function with a promoter sequence to allow fiber-specific expression of a coding sequence or other polynucleotide operably linked to the promoter.

As used herein, a fiber-specific element is an element that, when associated with a promoter sequence, increases or causes fiber-specific expression of a coding sequence operably linked to the promoter sequence, relative to the expression of the coding sequence linked to a promoter sequence not associated with the element.

Fiber-specific expression means that expression of polynucleotides occurs predominantly in the plant fibers. Fiber-specific expression may be determined by operably connecting the promoter sequence and the fiber-specific element to a reporter sequence, such as a sequence encoding GUS (β-glucuronidase), and evaluating expression of the reporter sequence or polypeptide in the fibers, and other regions of the plant. One of skill in the art will appreciate that fiber-specific expression does not exclude the possibility that the reporter sequence or polypeptide may be expressed at relatively low levels in non-fiber parts of the plant.

As used herein, the fibers of a plant, or plant fibers, refers to one or more cells or cell types comprising the vascular tissue of the plant, including, for example, the xylem libriform fibers, xylem fiber tracheids and phloem fibers of angiosperms, and tracheids of gymnosperms. Constructs of the invention may be used to direct transcription of a polynucleotide in one or more of these cell types.

Suitably, a fiber specific element may comprise consecutive base pairs of SEQ ID NO:1, e.g., at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16, at least 17, at least 18, at least 19, at least 20, at least 25, at least 30, at least 35, at least 40, at least 45, or at least 50 consecutive base pairs of SEQ ID NO: 1, or of a reverse complement of SEQ ID NO: 1.

In one embodiment, the fiber-specific element may be designed to contain one or more particular non-contiguous subsequences of SEQ ID NO: 1 and/or its reverse complement (designated the negative strand herein). These subsequences may work alone or in concert to target transcription and/or expression to the plant fibers.

Subsequences potentially suitable for use in a fiber-specific element are depicted in FIG. **1** and include a GTGA motif, (+ strand, positions 23-26 inclusive of SEQ ID NO: 1), one or more AT rich regions (+/− strand, positions 41-47, 10-19, 26-30, 40-50, and 52-56 (each inclusive) of SEQ ID NO: 1), YACT box (− strand, positions 8-11 inclusive of SEQ ID NO: 1), an *Arabidopsis* response regulator element (NGATT, where N is any nucleotide) (− strand, positions 17-21 and 54-58 inclusive of SEQ ID NO: 1), GTGA motif (− strand, positions 23-26 inclusive of SEQ ID NO: 1), one or more GATA boxes (− strand, positions 28-31 and 48-51 inclusive of SEQ ID NO: 1). Constructs of the invention may contain at least one, at least two, at least three, at least four, at least five, at least six or at least seven of these elements, and/or the reverse complement of the subsequences identified above, in any combination effective to direct fiber-specific expression. For example, the construct may contain, in the forward and/or reverse complement form, a GATA box, an *Arabidopsis* response regulator element and the AT-rich element, such as the element from positions 41-46 of SEQ ID NO: 1.

A construct according to the present invention may contain more than one fiber-specific element, for example, it may contain at least two, at least three, at least four, at least five, at least six, at least seven, at least eight, at least nine, at least ten, at least fifteen, or at least twenty, or more fiber-specific elements, which may include the same sequence, or non-identical overlapping or non-overlapping sequences within SEQ ID NO: 1, and/or the reverse complement of SEQ ID NO: 1. In one embodiment, the fiber-specific elements are present as tandem repeats. The inclusion of repeated sequences suitably enhances the specificity of expression of a nucleotide sequence, such as a coding sequence, to the plant fibers.

In addition to the fiber-specific elements of SEQ ID NO: 1, the constructs may include other sequences that enhance or alter transcription or expression of a polynucleotide. For example, the constructs may include at least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, or at least 15 consecutive base pairs of SEQ ID NO: 2, SEQ ID NO: 3, and/or SEQ ID NO: 4, or a reverse complement of SEQ ID NO: 2, SEQ ID NO: 3, and/or SEQ ID NO: 4. SEQ ID NO: 2 is from −414 to −398 base-pairs upstream of the transcription start site (TSS) of the PtrCOMT1 coding sequence. SEQ ID NO: 3 is from −310 to −270 base-pairs upstream of the TSS of the PtrCOMT1 coding sequence. SEQ ID NO: 4 is from −270 to −215 base-pairs upstream of the TSS of the PtrCOMT1 coding sequence. These elements of SEQ ID NO: 2-4 may be used alone or in combination with each other, and/or with the fiber-specific elements of SEQ ID NO: 1.

The constructs also contain a promoter sequence that is not natively associated with the fiber-specific element and which is operably connected to fiber-specific element, such that when the construct is introduced into a plant, transcription will occur specifically in plant fibers.

As used herein, a "promoter sequence" is intended to mean a nucleic acid that binds RNA polymerase, either directly or via transcription factors, and facilitates transcription of DNA to generate an mRNA molecule from a nucleic acid molecule that is operably linked to the promoter.

A promoter sequence not natively associated with the fiber-specific element may include any promoter sequence other than the promoter of PtrCOMT1. Suitable promoter sequences include, without limitation, the CaMV 35S minimal promoter, the NOS promoter from *Agrobacterium*, mannopine synthetase promoter, and sequences upstream of polynucleotides encoding enzymes of the cellulose synthesis pathway or phenylpropanoid pathway.

Other suitable promoter sequences not natively associated with the fiber-specific element may include modified PtrCOMT1 promoter sequences, i.e., promoter sequences that have been reconstituted to contain one or more sub-regions and/or altered regions of the ptrCOMPT1 sequence.

In some embodiments, the promoter sequence may include the native PtrCOMT1 sequence if the fiber-specific elements are repeated or are manipulated to be in a different position or orientation than is found in the native PtrCOMT1 sequence. This includes, for example, constructs in which two or more of the fiber-specific elements are from partially or completely overlapping regions of SEQ ID NO: 1, or are the same.

The one or more fiber-specific elements do not need to be directly connected to each other, to the promoter sequence, or to the polynucleotide to be transcribed, and the construct may contain nucleotides intervening between these sequences, while still being capable of directing expression of a polynucleotide to the plant fibers.

A construct according to the present invention may contain a particular desired polynucleotide to be transcribed that is operably connected to the promoter sequence. As used herein, "operably connected" with respect to the promoter sequence and the desired polynucleotide means that the promoter sequence can facilitate transcription of the desired nucleotide sequence to produce an RNA molecule under appropriate conditions. The RNA generated may code for a protein or polypeptide or may code for an RNA interfering, or antisense molecule. When the nucleotide sequence is a coding sequence, the polypeptide is suitably expressed.

The coding sequence or other polynucleotide to be transcribed may be any one where expression in the plant fibers is desirable. In one embodiment, the nucleotide sequence to be transcribed encodes a polypeptide that is an enzyme of the phenylpropanoid pathway, an enzyme in the G-lignin pathway, an enzyme in the S-lignin pathway, a cellulose synthase, a sucrose synthase, a cellulase, a transcription factor, an enzyme in phytohormone biosynthesis or a microtubule component. The polynucleotide may encode a polypeptide that regulates the synthesis of lignin or cellulose. In one embodiment, expression of the polypeptide in the plant fibers may be altered by varying external or environmental conditions.

Examples of polynucleotides that may be used to manipulate lignin content or composition in the plant fibers include those encoding one or more of cinnamyl alcohol dehydrogenase (CAD), cinnamate 4-hydroxylase (C4H), coumarate 3-hydroxylase (C3H), phenolase (PNL), caffeoyl-CoA O-methyl transferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), phenylalanine ammonia-lyase (PAL), 4-coumarate:CoA ligase (4CL), peroxidase (PDX) coniferin β-glucosidase (CBG), hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT), and caffeic acid 3-O-methyltransferase (COMT).

When constructs are operably connected to DNA or RNA that encodes antisense RNA or interfering RNA, which corresponds to the coding sequence of a polypeptide of interest, a decreased amount of the polypeptide of interest may result. Polypeptides targeted for suppression include enzymes involved in lignin, cellulose, sucrose, phytohormone or microtubule metabolism as discussed above. The use of RNAi to inhibit gene expression in plants is specifically described in WO 99/61631, which is herein incorporated by reference in its entirety.

The present invention also provides vectors comprising the nucleic acid constructs. Numerous vectors have been described in the literature, many of which are commercially available. Suitable vectors include, for example, Ti-plasmids derived from the *A. tumefaciens*, and plasmids capable of replication in a bacterial host, such as *E. coli*. Additionally, vectors and constructs may include an origin of replication (replicons) for a particular host cell. Various prokaryotic replicons are known to those skilled in the art, and function to direct autonomous replication and maintenance of a recombinant molecule in a prokaryotic host cell.

A plasmid vector suitable for the introduction of nucleic acid of the current invention in monocots may contain, for example, in addition to the fiber-specific element and promoter region, an intron that provides a splice site to facilitate expression of the coding sequence (such as the Hsp70 intron; PCT Publication WO 93/19189); and a 3' polyadenylation sequence such as the nopaline synthase 3' sequence (NOS 3; Fraley et al. (1983) Proc Natl Acad Sci USA 80: 4803-4807). This expression cassette may be assembled on high copy replicons suitable for the production of large quantities of DNA.

An *Agrobacterium*-based plant transformation vector for use in transformation of dicotyledonous plants is plasmid vector pMON530 (Rogers et al. (1987) In Methods in Enzymology. Edited by R. Wu and L. Grossman. p 253-277. San Diego: Academic Press). Plasmid pMON530 is a derivative of pMON505 prepared by transferring the 2.3 kb StuI-HindIII fragment of pMON316 into pMON526. Another useful Ti plasmid cassette vector is pMON17227, described in PCT Publication WO 92/04449 (herein incorporated by reference in its entirety) and contains a sequence encoding an enzyme conferring glyphosate resistance fused to the *Arabidopsis* EPSPS chloroplast transit peptide.

Vectors and constructs of the invention may include a selectable marker so that transformed cells can be easily identified and selected from non-transformed cells. Examples of such markers include, but are not limited to, a neomycin phosphotransferase coding sequence, which confers kanamycin resistance; a bar coding sequence, which confers bialaphos resistance; a mutant EPSP synthase coding sequence, which confers glyphosate resistance; a nitrilase coding sequence, which confers resistance to bromoxynil; a mutant acetolactate synthase coding sequence, which confers imidazolinone or sulphonylurea resistance; and a methotrexate resistant DHFR coding sequence. Other selectable markers include, but are not limited to, those conferring resistance to hygromycin, tetracycline and ampicillin.

Various sequences used in the construct can be made by any suitable means, including, for example, joining synthesized oligonucleotides, joining fragments generated by PCR, or using cloning techniques.

The invention also provides host cells which comprise the vectors of the current invention. As used herein, a host cell refers to the cell in which the coding product is ultimately expressed. Accordingly, a host cell can be an individual cell, a cell culture or cells as part of an organism.

The invention further provides a method for generating plants in which the transcription of polynucleotides and/or expression of polypeptides is targeted or directed to the fibers of a plant. In one embodiment, the invention provides methods of directing expression of a polypeptide to the fibers of a plant by transforming the plant with vectors and/or constructs of the invention, such that expression of the polypeptide is targeted to the plant fibers.

Transformation of a plant may be carried out by introducing into a plant cell or plant vectors and/or constructs of the invention, to form a transformed or transgenic plant. If a plant cell is used, the plant may be subsequently regenerated from the plant cell. Methods for transforming plants and regenerating plants from plant cells are known. Suitable methods for transforming plants and trees include, without limitation, those disclosed Tsai C-J, Podila G K, Chiang V L (1994), Plant Cell Reports 14: 94-97; Han, K.-H., Meilan, R., Ma, C., and Strauss, S. H. (2000) Plant Cell Reports 19:315-320; and Meilan, R. and Ma, C. (2006) In: Methods in Molecular Biology, vol. 344:143-151; Kan Wang, Editor, *Agrobacterium* Protocols, 2/e, volume 2, Humana Press Inc., Totowa, N.J., and in U.S. Pat. No. 5,922,928, herein incorporated by reference in its entirety.

Plants transformed with vectors and/or constructs containing the fiber-specific elements are also provided. Any plant into which the constructs of the invention can be introduced and expression targeted to the plant fibers may be used. Suitable plants include, but are not limited to, woody plants, trees, crop plants and biofuel plants such as alfalfa, cotton, maize, rice, tobacco, grasses (such as switchgrass), aspen, poplar, cottonwood, pines (such as loblolly pine), sweetgum, eucalyptus, fir, maple, oak, willow and acacia plants. A "woody plant" is herein defined as a perennial plant whose stem comprises woody tissue. Examples of woody plants may include trees, shrubs or vines.

In one embodiment, plants transformed with a vector or construct of the invention, show expression or suppression of a polypeptide in one or more of the fibers in xylem and phloem of normal wood, tension wood (TW) and opposite wood (OW) of the plant, relative to a similar plant that has not been transformed with a vector or construct of the invention. A transformed or transgenic plant suitably produces altered (increased or decreased) amounts or ratios of lignin or cellulose, or produces lignin or cellulose of a different structure or type (such as S lignin, G lignin) compared with plants not expressing the polynucleotides.

It is to be understood that the invention is not limited in its application to the details of construction and the arrangement of components set forth in the following description or illustrated in the following drawings. The invention is capable of other embodiments and of being practiced or of being carried out in various ways.

EXAMPLES

Example 1

PtrCOMT1 Promoter Deletion:GUS Fusion Constructs and Aspen Transformation

Promoter deletion GUS fusion constructs were generated from a 1.5 Kb PtrCOMT1 promoter fragment cloned into the pGEM-7Z vector backbone and were used to transform aspen leaf discs. Thirteen PtrCOMT1 promoter deletion::GUS fusion constructs (depicted in FIG. **2**) were transformed into *Agrobacterium tumefaciens* strain C58/pM90 by the freeze and thaw method. Positive transformants were confirmed using PCR and transferred to greenhouse pots for further analysis.

The thirteen 5'-unidirectional promoter deletion::GUS fusions were analyzed in transgenic aspen to examine PtrCOMT1 promoter activity during stem development. Under control of the −886/+78 fragment (hereafter referred to as the 'full-length' promoter), GUS activity was localized to the metaxylem and cambial zone, with faint staining in the cortex of internode 3. No staining was observed in the primary phloem. At internode 5, promoter activity was restricted to the vessels and developing xylem fibers surrounding the vessels. GUS staining was also observed in pith cells adjacent to the medullary sheath, but was absent in phloem and cortex. In stem internodes undergoing secondary thickening, GUS signal was observed in xylem and phloem fibers, visible at the 13th internode. GUS staining in xylem was absent in vessels and in newly formed fibers of the expanding zone, but preferentially localized to older, thick-walled fiber cells that also stained red with the Maule reagent, indicative of S-lignin deposition. These results suggest the involvement of PtrCOMT1 in S lignin biosynthesis.

Deletion of the promoter to −756 did not appear to affect its activity, but removal of an additional 107 by (to −649) abolished GUS staining Further deletion to 563 and −473 restored GUS staining in xylem and phloem fibers, similar to the pattern observed with the full length promoter. Deletion of an additional 59 by (−414) resulted in GUS staining in thin-walled vessels and fibers of the expanding xylem, cambial region and xylary rays, and a near loss of activity in phloem fibers. Deletion to −398 abolished GUS staining, but deletion to −310 restored weak staining in xylem and phloem fibers. The −270 fragment exhibited detectable promoter activity in our study, directing GUS in primary xylem, cambial zone, cortex and pith at young (third) internode. At older internodes, GUS staining was observed primarily in phloem fibers, and weakly in cortex, ray parenchyma and pith. Taken together, these results indicate that the PtrCOMT1 promoter has elements for both activation and repression of transcription.

Deletion from −756 to −649 resulted in loss of PtrCOMT1 promoter activity. Apart from WI, this region also contains a MYC binding site that partially overlaps an I-box. A 30 by oligo (−715 to −687) containing both MYC and I-box interacted strongly with xylem nuclear extracts but weakly with phloem nuclear proteins. Specificity of the binding was confirmed by competition with unlabeled oligos containing either wildtype or mutated MYC and I-box sequences. Labeled oligos containing another I-box element (−246 to −275) showed specific interaction with xylem-derived nuclear extracts. A xylem-specific gel retardation pattern was also detected using probes bearing the MYB1 element, a suspected negative regulator between −398 and −310. Deletion of this region restored GUS activity to the remaining 3' end of the fragment. Competition with a 100-fold molar excess of unlabeled wildtype oligo greatly reduced the binding signal, whereas competition with a similar amount of mutant oligo did not have an effect. EMSA experiments involving another MYB-binding site, MYB2, performed using the same amount of xylem nuclear extracts, showed an interaction. Finally, a DPBF-containing oligo also interacted strongly with xylem, but not phloem nuclear proteins, and the specificity of the interaction was validated by competition experiments. A GT-1 containing oligo (−149 and −120) showed weak interaction with xylem.

The PtrCOMT1 promoter directed GUS activity primarily to thick-walled fiber cells of xylem and phloem in aspen stems. Activities were also noted in non-lignifying cells of young internodes, including cortex, phloem, ray parenchyma and pith. The expression of PtrCOMT1 in thick-walled xylem fibers and its absence in newer, thin-walled fibers suggests a role for PtrCOMT1 in S lignin synthesis and a delay in S lignin deposition, relative to G lignin, during early stem development in angiosperms. COMT1 promoters were responsive to mechanical stress, with their activities becoming restricted to the tension wood side, and being expressed in all cell types. This pattern of expression suggests their likely involvement in synthesis of stress-induced phenylpropanoids, such as lignans in tension wood.

Promoter deletion analysis revealed that the minimum 3' fragment required to sustain the magnitude, tissue-specificity and gravitational responsiveness of PtrCOMT1 expression was −473, although a nominal level of activity could still be seen with the −270 promoter. Deletion of −473 to −414 (SEQ ID NO: 1) led to an unusual pattern of cambium and expanding xylem localized activity, and an ambiguous response to mechanical bending. Progressive deletion beyond −414 abolished, and then restored PtrCOMT1 activity (e.g., −398 and 310). The unusual activity pattern of −414 suggests that the −414 fragment (SEQ ID NO: 1) harbors evolutionary conserved core element(s) for regulating lignin biosynthesis. It is envisaged that the I-box between −270 and 215 confers basal PtrCOMT1 expression in xylem, and is bending-responsive.

A slight enhancement of GUS signal in xylem fiber cells with deletion −310 was observed. Two regions, −398/−310 and −649/−573, appeared to reduce PtrCOMT1 expression. A tissue-specific element may be present between −414 and −398 for directing expression in expanding xylem.

The region between −473 and −414 was found to contain one or more dominant fiber-specific elements. The TF-complex may hinder TF interaction with the adjacent, expanding xylem-specific element at −398/−414, by virtue of their proximity. Its deletion would relieve the hindrance, thus enabling expression in expanding xylem as seen with −414. The region between −756 and −649 contains a MYC box, an I-box, a WI box and a GATA-box, all of which were confirmed to interact specifically with xylem-derived nuclear proteins. Putative cis elements located upstream of −756 or downstream of −215 did not appear to affect PtrCOMT1 expression, although downstream MYC (−195 and −88) and MYB2 (−115 and −61) elements also exhibited binding with xylem nuclear extracts. These elements may confer bending-induced activity in pith, as seen with −270.

PtrCOMT1 promoter activity was mainly found in thick-walled xylem fibers and was responsive to mechanical stress with its activity becoming restricted to the tension wood side. The minimum promoter sequence capable of sustaining PtrCOMT1 tissue-specific and gravistimulated expression was −473, although basal level of activity could be seen with the −270 fragment.

Example 2

Electrophoretic Mobility Shift Assays (EMSA)

EMSA was performed using nuclear protein prepared from xylem, phloem and leaf tissues. Binding reactions were carried out for 30 minutes at room temperature. Each 25 μl binding reaction contained 1 pmol/μl of oligonucleotide probe with the promoter target sequence labeled with the infrared fluorophore IRDye700 (Licor biosciences, Lincoln, Nebr.), 10 μg nuclear proteins in 1× binding buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM DTT, 60% glycerol, 2 μg poly (dI·dC) and 0.5 mM PMSF. For competition experiments, unlabeled oligonucleotides were added with a 50-100-fold molar excess ratio relative of the probe. The reaction mixture was electrophoresed at 4° C. on a 4% native polyacrylamide gel run at 50V for 2 hours in Tris-glycine buffer (25 mM Tris-HCl, 250 mM glycine and 1 mM EDTA, pH 8.5). After electrophoresis, the gel was analyzed using the Odyssey infrared imaging scanner (Licor Inc.)

In a first competition experiment, a probe containing the polynucleotide from position 2 to position 34 of SEQ ID NO. 1 (inclusive) was generated, and the sequence was mutated to provide six additional probes, as shown in FIG. **5**A (SEQ ID NOs: 7-12). Results of the competition assay are shown in FIG. **5**B. Competition was abolished when SEQ ID NO: 11 and 12 were used.

In a second competition experiment, a probe from position 24 to position 57 of SEQ ID NO. 1 (inclusive) was generated, and the sequence was mutated to provide ten additional probes, as shown in FIG. **6**A (SEQ ID NOs: 13-22). Results of the competition assay are shown in FIG. **6**B. Competition was abolished when probes containing SEQ ID NOs: 13, 15 and 20 were used.

In a third competition experiment, a probe from position 465 to position 493 of SEQ ID NO. 5 (inclusive) was generated, and the sequence was mutated to provide three additional probes, as shown in FIG. **7**A (SEQ ID NOs: 23-25). Results of the competition assay are shown in FIG. **7**B. Competition was least effective when the probe containing SEQ ID NO: 23 was used.

The region from and surrounding position 478 to position 482 (inclusive) of SEQ ID NO. 5 (TTTCT), and/or the reverse complement, may be important for targeting to "stem cell" type (meristemic) cells such as cambia and newly formed xylem cells.

Example 3

Transformation of Plants with Constructs Containing Fiber-specific Elements

Constructs were formed having a hygromyacin cassette, the minimal 35S promoter and a polynucleotide encoding the GUS reporter polypeptide. The construct contained various combinations of SEQ ID NOs. 1, 3 and 4, as shown in FIGS. **3** and **4**. The constructs shown in FIG. **3** were used to transform aspen plants. Fiber-specific expression of the GUS reporter polynucleotide in the fibers of the aspen plants is expected.

It is specifically contemplated that any embodiment of any method or composition of the invention may be used with any other method or composition of the invention. As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to a composition containing "a conjugate" includes a mixture of two or more conjugates. It should also be noted that the term "or" is generally employed in its sense including "and/or" unless the content clearly dictates otherwise.

It also is specifically understood that any numerical value recited herein includes all values from the lower value to the upper value, i.e., all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application. For example, if a range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1% to 3%, etc., are expressly enumerated in this specification.

Various features and advantages of the invention are set forth in the following claims.

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What is claimed is:

1. A nucleic acid construct comprising a first fiber-specific element having at least 15 consecutive base pairs of SEQ ID NO: 1 or a reverse complement of at least 15 consecutive base pairs of SEQ ID NO: 1 operably connected to a promoter sequence not natively associated with the first fiber-specific element.

2. The construct of claim **1**, further comprising a polynucleotide encoding a polypeptide operably connected to the promoter sequence.

3. The construct of claim **2**, wherein the polypeptide is selected from an enzyme in the G-lignin pathway, an enzyme in the S-lignin pathway, a cellulose synthase, a sucrose synthase, a cellulase, a transcription factor, an enzyme in phytohormone biosynthesis and a microtubule component.

4. The construct of claim **1**, further comprising a second fiber-specific element having at least 5 consecutive base pairs of SEQ ID NO: 1.

5. The construct of claim **4**, wherein the first and second fiber-specific elements are from non-overlapping regions of SEQ ID NO: 1.

6. The construct of claim **4**, wherein the first and second fiber-specific elements are from partially or completely overlapping regions of SEQ ID NO: 1, or are the same.

7. The construct of claim **1**, wherein the construct comprises SEQ ID NO: 1.

8. The nucleic acid construct of claim **1**, wherein the first fiber-specific element comprises a GATA box, an *Arabidopsis* response regulator element (NGATT), a GTGA box, an AT-rich element of at least 5 base-pairs, or a combination thereof.

9. The nucleic acid construct of claim **1**, wherein the first fiber-specific element comprises a GATA box, a *Arabidopsis* response regulator element (NGATT), a GTGA element, and an AT-rich element of at least 5 base pairs.

10. The nucleic acid construct of claim **1**, further comprising a second fiber-specific element having at least 9 consecutive base pairs of SEQ ID NO: 1 or a reverse complement of at least 9 consecutive base pairs of SEQ ID NO: 1.

11. The construct of claim **10**, wherein the first and second fiber-specific elements are from non-overlapping regions of SEQ ID NO: 1.

12. The construct of claim **10**, further comprising a third fiber-specific element having at least 5 consecutive base pairs of at least one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4, or the reverse complements of at least 5 consecutive base pairs of at least one of SEQ ID NO: 1, SEQ ID NO: 2, SEQ ID NO: 3, or SEQ ID NO: 4.

13. The construct of claim **10**, wherein the first or second fiber-specific element is repeated.

14. The construct of claim **10**, further comprising a polynucleotide encoding a polypeptide operably connected to the promoter sequence.

15. The construct of claim **10**, wherein the polypeptide is selected from an enzyme in the G-lignin pathway, an enzyme in the S-lignin pathway, a cellulose synthase, a sucrose synthase, a cellulase, a transcription factor, an enzyme in phytohormone biosynthesis and a microtubule component.

16. A nucleic acid construct comprising a first fiber-specific element having at least 9 consecutive base pairs of SEQ ID NO: 1 or a reverse complement of at least 9 consecutive base pairs of SEQ ID NO: 1, a second fiber-specific element having at least 9 consecutive base pairs of SEQ ID NO: 1 or a reverse complement of at least 9 consecutive base pairs of SEQ ID NO: 1 and a third fiber-specific element having at least 9 consecutive base pairs of SEQ ID NO: 1 or a reverse complement of at least 9 consecutive base pairs of SEQ ID NO: 1 operably connected to a promoter sequence, the first, and second and third fiber-specific elements being from partially or completely overlapping regions of SEQ ID NO: 1, being the same, or a combination thereof.

17. The construct of claim **16**, further comprising a polynucleotide encoding a polypeptide operably connected to the promoter sequence.

18. A method of directing expression of a polynucleotide to the fibers of a plant comprising transforming the plant with the construct of claim **2**.

19. The method of claim **18**, wherein the plant is a tree.

20. The method of claim **18**, wherein the polynucleotide encodes a polypeptide selected from an enzyme in the G-lignin pathway, an enzyme in the S-lignin pathway, a cellulose synthase, a sucrose synthase, a cellulose, a transcription factor, a phytohormone and a microtubule component.

21. A plant produced by the method of claim **18**.

* * * * *

UNITED STATES PATENT AND TRADEMARK OFFICE

CERTIFICATE OF CORRECTION

PATENT NO. : 8,536,406 B2
APPLICATION NO. : 12/990001
DATED : September 17, 2013
INVENTOR(S) : Tsai et al.

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

On the Title Page:

The first or sole Notice should read --

Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 277 days.

Signed and Sealed this
Fifteenth Day of September, 2015

Michelle K. Lee

Michelle K. Lee
Director of the United States Patent and Trademark Office