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(54) Title: NEW TRANSGENIC PLANTS AND METHOD FOR THEIR PRODUCTION

(57) Abstract: A transgenic plant exhibiting an improved biomass related property, such as an altered lignin content, lignin composition or extractability of lignin, or an altered fibre length, or a combination thereof, said alteration determined by comparison to the corresponding property of the wild-type of the same species, can be achieved by modifying the expression of a pectin methyl esterase (PME) gene in at least one cell of said plant. The down-regulation of PttPMEI (GenBank Accession No. AJ277547) has been shown to result in a fibre length increase of about 5 % and the up- regulation of said gene correspondingly a reduction of about 7 %. Transgenic plants exhibiting altered biomass related properties and methods for their production are disclosed.

New transgenic plants and method for their production

Field of the invention

The present invention concerns transgenic plants and biomass exhibiting improved properties, and in particular properties of economic importance, one being altered lignin content or altered lignin composition, another being altered fibre length, both compared to the corresponding properties of the wild type of the same species. The invention also encompasses seeds and cells of said transgenic plants and other types of propagating material, as well as methods for their production.

Background

Plants and plant derived biomass is a source of numerous valuable raw materials. The primary objective of plant genetic engineering and molecular breeding is to improve quality and yield with respect to these raw materials. A major disadvantage with traditional plant breeding is the length of time necessary to isolate and introduce new phenotypes. This is especially true for trees where the generation time is long. However, gene technology makes it possible to significantly reduce the time needed to test and ultimately improve selected properties.

When considering trees and wood, as one example, it becomes obvious that the constantly growing demand for wood products and wood derived products constitutes a problem of global proportions. It is estimated that the maximum sustainable rate of harvesting from the world's forests has already been reached, or even exceeded. This is a strong argument for an increase in emphasis on plantation wood production worldwide. The rapid growth rates of *Populus* species, including their many hybrids, indicate that they are a valuable resource for the production of biomass, for use both as a raw material and as fuel.

Control of cell wall development leading to increase cell size, and in particular to increase fibre length is one area of great interest. Wood formation and fibre development in plants is an extremely important example in economic terms. Wood is the most significant and renewable source of natural fibres. Fibre utility properties depend on fibre cell length. For example, long fibres make stronger paper, increase

pulp yield and decrease consumption of alkali. Therefore several genetic approaches have been taken to select "long fibre" varieties.

Fibres develop from fusiform initials located within a meristem tissue called vascular cambium situated between xylem and phloem. Unlike other meristematic cells, the fusiform initials are highly elongated and they divide longitudinally whereby a new cell wall is formed along the cell axis. Thus, following cell division, the developing fibre is already 0.5-2.0 mm long. Further increase in length occurs by intrusive tip growth that involves a localised cell wall deposition of new wall material at a tip while the rest of the cell wall is not growing any more. Intrusive tip growth requires the dissolution of the middle lamella where tip penetration is taking place and is limited by the strength of the adhesion between the adjacent cells. This process determines the final fibre length, which could be several times longer than the length of fusiform initial.

The properties of the middle lamella limit intrusive growth. Middle lamellae of developing wood cells are rich in pectins, in particular homogalacturonans. The homogalact-urans are synthesised and secreted to walls in methylesterified form and they can be demethylated *in muro* by a wall residing pectin methyl esterase (PME). PME is a ubiquitous enzyme encoded by a multigene family. In *Arabidopsis*, there are 58 genes encoding PME (Henrissat *et al.*, 2001) while poplar EST databases contain at least 22 different sequences. Poplar wood forming tissues are rich in several isoforms of PME enzyme (Guglielmino *et al.*, 1997; Micheli *et al.*, 2000). The isoforms exhibit seasonal and tissue type variation suggesting that the regulation of gene expression and functions might be the reason for the multiplicity of PME genes.

Another important area of great interest is the chemical composition of the biomass, in particular the lignin content in wood and other fibrous plants. Lignin is a general term for a group of polymeric substances, known to occur together with cellulose in all higher plants. Lignin contributes to the mechanical strength of the plant, which is a prerequisite for all vertically growing plants, and trees in particular. In wood fibres, where the cellulose microfibrils are embedded in lignin, it accounts for about 25 – 30 % (w/w).

In the production of paper pulp, the lignin is partially decomposed and separated from the fibres during the cooking of chemical pulp. The extent of lignin removal is determined as the kappa number. This denotes the quantity of potassium

permanganate consumed by one gram of pulp (dry weight) under specific conditions, according to standardised procedures (SCAN-C.1). The kappa number is expressed as the number of millilitres of a 20 mmol/l solution which is consumed. It is frequently desired to remove as much lignin as possible, as a high lignin content negatively influences the colour and storage stability of the paper. Pre-harvest reduction of lignin would advantageous for the optimisation of the pulping process, e.g. making it possible to significantly reduce the time, energy and chemicals used in delignification and bleaching.

It is also conceived that the lignin composition could be altered, thus improving lignin extractability, or altering the properties of the plant.

Further, lignin is resistant to degradation by microorganisms and resistant to digestion in the gastro-intestinal tract of mammals. This makes it interesting to reduce the lignin content also in fibrous plants intended for food or feed applications.

Genes responsible for the regulation of biomass and fibre properties, such as fibre length, are not well known. A transgenic approach has been used to increase fibre length by altering gibberellin hormone levels. These transgenic plants were shown to have increased biomass and altered wood development. High gibberellin content in developing wood obtained by ectopic expression of GA 20- oxidase resulted in increased fibre length (Eriksson *et al.*, 2000).

Altering the expression of genes involved in the development of cell walls in potato has revealed that the suppression of pectin methyl esterase (PME) activity caused more rapid elongation of the stem (Pilling *et al.*, 2000). In contrast, the inhibition of a specific PME isoform resulted in a decreased stem elongation and increased leaf blade expansion in width (Pilling *et al.,* 2004).

F. Wen *et al.*, The Plant Cell, 11, 1129-1140, June 1999, showed that pectin methylesterase gene expression had an influence on root development in a model, where pea (*Pisum sativum*) root border cells were studied.

Several PME isoforms have been detected and shown to be encoded by a multigene family. The PME proteins encode pre-pro-proteins that have peptide motifs considered to be signatures of PMEs. The pre-region is required for the protein targeting to the endoplasmic reticulum, the pro-PME is secreted to the apoplasm via the Golgi-network (Micheli 2001).

Genetic engineering approaches to the evaluation and eventually the modification of lignin biosynthesis have been attempted by many. Reference is here made to a review article (M. Baucher *et al.*, Critical Reviews in Biochemistry and Molecular Biology, 38:305-350, 2003). There is also one indication, however inconclusive, that the repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees (Wen-Jing Hu *et al.*, Nature Biotechnology, Vol. 17, Aug 1999, 808-812).

Approaches to modulate the expression of pectin methyl esterase have been tried in fruits (US 5,659121, apples, pears; WO 02/16613, pears), and vegetables (Mishra *et al.*, The Plant Journal, 1998, Vol. 15, No. 5, p. 583-592; and Tieman *et al.*, The Plant Cell, June 1992, Vol. 4, p. 667-679; both tomato; Pilling *et al.*, supra, 2004, potato). Studies have also been performed in tobacco plants, a popular experimental model (Gaffe *et al.,* Plant Physiology, 1997, Vol. 114, p. 1547-1556) where increased PME expression was achieved. The studies in fruits (fruit-bearing trees) obviously focused on the ripening of the fruits.

WO 03/098186 (Wilkins) has investigated cotton fibre properties and mentions several genes, including one that encodes pectin methyl esterase (PME).

To the best knowledge of the inventors, there is no conclusive evidence of the role of PME in lignin synthesis.

It remains a problem to make available transgenic fibrous plants exhibiting improved properties such as lignin content and fibre length, and to find effective and repeatable approaches to regulate these properties in fibrous plants.

Summary of the invention

It has surprisingly been shown that transgenic fibrous plants, in particular transgenic woody plants, exhibiting exhibiting improved properties with regard to biomass related properties of economic importance, such as lignin content, lignin composition, and fibre length, compared to the same property of the corresponding wild-type of the same species, can be achieved by modifying the expression of a pectin methyl esterase (PME) gene in at least one cell of said plant.

It has in particular been shown that an altered lignin content and / or composition compared to the lignin content / composition of the corresponding wild-type of the same species can be achieved by modifying the expression of a pectin methyl esterase (PME) gene in at least one cell of said plant. The down-regulation of PttPME1 (GenBank Accession No. AJ277547) has been shown to result in a 10 % reduction of the lignin content. The altered lignin content is also reflected in significantly reduced kappa numbers after pulping batches of wood, from transgenic trees according to the invention. Transgenic plants exhibiting altered properties, such as reduced lignin content are disclosed, as well as methods for their production. The invention is further defined in the attached claims, incorporated herein by reference.

Description of drawings

The invention will be described in closer detail in the following description, examples, and attached drawings, in which

Figure 1 shows the characterisation of *PttPME1*. (A) PttPME1 deduced peptide. The signal peptide is marked by lower case letters. Two hypothetical glycosylation sites are indicated. Post-translational cleavage site releases mature 33.7 kDa enzyme. (B) *PttPME1* expression in mature stem tissues of active and dormant poplar.

Figure 2 shows the characterisation of transgenic lines with *PttPME1* cDNA in a sense (lines 1A, 2B, 3B, 5, 7, 8, and 10B) and in antisense (line AS) orientation. (A) Expression of *PttPME1* in wood forming tissues. Data are obtained by a quantitative RT-PCR that detects only sense transcripts. Numbers indicate % of expression relative to WT. Bars represent standard error (SE), n=3. (B) Transgene integration pattern. Genomic DNA was digested with *Hind III* and probed with the transgene-specific fragment. Number of bands corresponds to the number of inserts.

Figure 3 illustrates the PME activity in the wall bound and in soluble protein fraction extracted from wood forming tissues of transgenic lines up- (line 2B) or down-regulated (lines 5 and 8) for*PttPME1* expression (A) and the relationship between PME activities and the PttPME1 expression levels in the transgenic lines and the WT (B). Percent of activity relative to WT is indicated. Bars = SE, n = 6 - 12, means accompanied by the same letter are not significantly different (Duncan multi-range test, $P \leq 0.05$).

Figure 4 shows results of isoelectric focussing of PME activity in the wall bound protein fraction extracted from cambium/phloem and from developing xylem tissues of transgenic lines up- (2B) and down- (5) regulated for *PttPME1* expression (A) and the corresponding overall PME activity (B). Samples were calibrated to 200 μg of dried wall material.

Figure 5 shows results of the analysis of water- and CDTA-extractable pectin in transgenic lines up- (line 2B) or down-regulated (lines 5 and 8) for *PttPME1* expression. Galacturonic acid content (A) and methanol released from each extract upon saponification (B). Bars=SE, n = means accompanied by the same letter are not significantly different (Duncan multirange test, P≤0.05). (C) Immuno dot blot of pectins extracted from wood forming tissues using CDTA and probed with the monoclonal antibody Pam1, specific to at least 30 consecutive units of non-methylesterified homogalacturonan. (D) The relationship between Pam1 signal and PttPME1 expression level.

Figure 6 presents the immunolocalization of pectin epitopes with different patterns of methylesterification in the wood forming tissues of the WT and PttPME1-transgenic lines.

Figure 7 illustrates the intrusive growth in the fibres in transgenic lines up- (lines 2B and 7) or down-regulated (lines 5 and 8) for*PttPME1* expression (A) and the relationship between fiber length and the expression of PttPME1 (B). Bars=SE, n=approx 1000, means accompanied by the same letter are not significantly different (Duncan multirange test, P≤0.05).

Figure 8 comprises two graphs showing plant longitudinal growth characteristics of transgenic lines showing most prominent changes in PME activity. Stem elongation (A) and the rate of leaf formation (B). Bars represent standard errors. (Duncan multirange test, P≤0.05).

Figure 9 shows the plant radial growth characteristics of transgenic lines. Radial width of xylem, phloem, phloem fibres and cortex was measured in lines showing most prominent PME activity changes. Bars represent standard errors.

Figure 10 shows different kappa numbers plotted vs. H-factor for different *PttPME1* experimental lines, batch 1. As can be seen, the line 2B (overexpressing *PttPME1*)

seems to delignify significantly slower compared to the wild type and line 5 (down-regulated for *PttPME1* expression). Mature aspen data (control) are from reference X

Figure 11 shows the kappa numbers reached after pulping of batch 2. All cooks were run in triplicate. The figures in brackets show the origin of the sample; (1-2) indicates that the sample was taken from the upper part of the stem, and (3-4) indicates that the sample was taken from the lower part of the stem.

Figure 12 shows the lignin content in PttPME1-modified trees (wood) after pulping of batch 3, determined as Klason lignin. (1-2) –upper part, (3-4) – lower part of stem.

Figure 13 illustrates the amount of principal carbohydrate components in the wood of PttPME1-modified trees from batch 2, indicating that the line 5 trees from all three batches contained increased amounts of cellulose and decreased amounts of xylose compared to the wild type.

Figure 14 illustrates the amount of principal carbohydrate components in batch 2 pulp from PttPME1 modified lines and the WT. Labels as in Fig 11.

Figure 15 illustrates the amount of principal carbohydrate components in PME1 batch 3 wood; and

Figure 16 illustrates the amount of principal carbohydrate components in PME1 batch 3 pulp.

Description of the invention

Definitions

Before the present invention is described, it is to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting, since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

The following terms will be used:

"Complementary" in the context of this description refers to the capacity for precise pairing between two nucleotides.

Further, in the context of the present invention, "hybridization" refers to hydrogen bonding, which may be Watson-Crick, Hoogsteen or reverse Hoogsteen hydrogen

bonding, between complementary nucleoside or nucleotide bases. Thus complementarity and hybridisation are terms used to indicate a sufficient degree of complementarity or precise paring such that stable and specific binding occurs between the oligonucleotide and the DNA or RNA target.

An antisense compound is specifically hybridisable when binding of the compound to the target DNA or RNA molecule interferes with the normal function of the target DNA or RNA to cause a loss of utility, and there is a sufficient degree of complementarity to avoid non-specific binding of the antisense compound to non-specific target sequences under conditions in which specific binding is desired.

The phrase "hybridisation under stringent conditions" refers to criteria regarding temperature and buffers well known to those skilled in the art.

In this context the term "functionally homologous" means sequences sharing perhaps a lower structural homology with the disclosed sequence, but exhibiting homologous function *in vivo*, in either the healthy or the diseased organism, e.g. coding the same or highly similar proteins with similar cellular functions.

In this context the term "functionally inserted" or "operationally inserted" denotes that a sequence has been inserted in a host genome in such orientation, location and with such promoters and/or enhancers, where applicable, that the correct expression of said sequence occurs.

"Modulation" as used in this context means either an increase (stimulation) or a decrease (inhibition) in the expression of a gene, as well as the increase or decrease of enzyme level and/or enzyme activity.

The term "altered lignin content" is here defined as being one or more of the following: a detectable change in the chemical composition of the lignin; a detectable change in the association of the lignin to the cellulose fibres; a detectable change in the amount of lignin present in the biomass (% w/w); or a change in the kappa number for chemical pulp based on transgenic biomass, all compared to the corresponding features in an un-altered plant or in the wild type.

The term "altered lignin composition" refers to changes in the chemical and/or physical composition of the lignin, such as the reciprocal concentrations of the polymeric substances generally referred to as "lignin", as well as their individual properties and their reciprocal properties, such as adhesion etc.

Objects of the invention

It is one object of the present invention to provide transgenic plants, seeds and plant cells, exhibiting improved growth parameters and in particular improved parameters of economic value. Further objects, or remaining problems in the prior art, and their corresponding solutions will become evident to a skilled person upon study of the description, examples and claims. The above objects are fulfilled through a transgenic plant according to the attached claims, in which gene levels are modulated to alter one or several of fibre length, lignin content, lignin composition, lignin extractability, or combinations thereof..

The present inventors have surprisingly shown that ectopic up- and down regulation of a pectin methyl esterase (PME) gene in a plant, here exemplified by a temperate zone deciduous tree, results in significant changes in fibre length and intrusive tip growth, as well as significant changes in the lignin content and/or composition of the plant. The results obtained by the present inventors show that genetic modification of PME levels in trees can be used to modify traits that are extremely important for the plant fibre, forest, pulp and paper industries.

The present invention relates to transgenic plants exhibiting an altered fibre length and/or an altered lignin content, or lignin composition, preferably a reduced lignin content or improved extractability of lignin.

According to one embodiment of the invention, an increased fibre length is obtained by down-regulation of the expression of a pectin methyl esterase (PME) gene, such as the PttPME1 gene (GenBank Accession No. AJ277547), in at least one cell of said plant. The increase in fibre length takes place in the cambial and differentiating xylem tissues and the increased fibre length of said transgenic plant is significantly longer than the fibre length of a non-transgenic plant of the same species.

According to another embodiment of the invention, a reduced fibre length is obtained by up-regulation of the expression of a pectin methyl esterase (PME) gene, such as the PttPME1 gene, in at least one cell of said plant.

According to another embodiment of the invention, a reduced lignin content is obtained by down-regulation of the expression of a pectin methyl esterase (PME) gene, such as the PttPME1 gene (GenBank Accession No. AJ277547), in at least one cell of said plant.

A transgenic plant of the present invention is primarily a plant that has fibres that elongate through intrusive tip growth, which means that an increased fibre length leads to an increased intrusive tip growth.

As far as the wood fibers are concerned, the intrusive tip growth is observed in the wood fibers of dicotyledonous woody species (Larson, 1994; Mellerowicz *et al.,* 2001; Wenham and Cusick, 1975; Esau, 1965). In conifers, there is only a small increase of fiber length during fiber differentiation and the fiber length is directly dependent on the length of fusiform initials (Larson, 1994). However, since the latter depends on the intrusive tip growth, the present invention is also valid for conifers. It is also valid for other types of fibers that grow by intrusive growth. These include in particular phloem fibers found in sisal, abaca, jute, flax, ramie, hemp, kenaf, bagasse, and fibers of wheat and bamboo (Esau, 1965; McDougal *et al.*, 1993, Carpita and McCann, 2000).

According to a preferred embodiment of the present invention, the plant is a woody plant, more preferably a hardwood plant. The hardwood plant is selected from the group consisting of acacia, eucalyptus, hornbeam, beech, mahogany, walnut, oak, ash, willow, hickory, birch, chestnut, poplar, aspen, alder, maple and sycamore. Rapid growing plants are particularly preferred, e.g. plants belonging to the *Populus* or *Salicaceae* groups, including variants thereof. Presently, the effect has been shown in an experimental model using plants belonging to the *Populus* group, but it is held that the results are equally applicable to other fibrous plants.

According to another preferred embodiment of the present invention, the plant is a conifer. The plant is preferably selected from the group consisting of cypress, Douglas fir, fir, sequoia, hemlock, cedar, juniper, larch, pine, redwood, spruce and yew, most preferably one of fir and pine.

According to another preferred embodiment of the present invention, the plant is selected from the group consisting of bamboo and rubber plants.

The present invention also relates to transgenic plants having a nucleotide sequence effective as an antisense sequence in relation to a PME gene, e.g. the PttPME1 gene or a functionally homologous sequence thereof functionally inserted into the plant genome. A functionally homologous sequence is a non-identical sequence which, when operationally introduced in an organism, produces the same or similar effect as

the first sequence, to which it is functionally homologous. A functionally homologous sequence frequently exhibits sequence homology. In this context, a sequence homology of at least 70 %, more preferably at least 80 %, and most preferably at least 90 % is intended. A person skilled in the art is able to determine, using routine techniques, whether a sequence codes for a polypeptide with PME activity, that is hether it is functionally homologous. Suitable PME sequences for down-regulation also include fragments and mutations of said sequence.

The invention further relates to seeds, cells and propagating material of the transgenic plants according to any of the above embodiments.

Another object of the present invention is to provide a method for modifying fibre length and for the production and proliferation of said transgenic plants. Thus, the present invention further makes available a method for the regulation of the fibre properties and intrusive tip growth in the cambial and developing wood tissue of a plant.

The method according to one embodiment comprises the step of down-regulating the expression of a PME gene, such as the PttPME1 gene, in said plant in order to increase the fibre length. The length of the fibres of the transgenic plant is at least 1 % longer, preferably at least 5 % and most preferably at least 10 % longer than the fibre length of a non-transgenic plant of the same species. While an increase of 5 % was observed in the experiments performed so far, it is contemplated by the present inventors that a larger increase will be seen in other species.

The method according to one embodiment comprises the step of up-regulating the expression of a PME gene, such as the PttPME1 gene, in said plant in order to reduce the fibre length. The length of the fibres of the transgenic plant is at least 1 % shorter, preferably at least 5 %, more preferably at least 7 % shorter, and most preferably at least 10 % shorter than the fibre length of a non-transgenic plant of the same species. While a reduction of 7 % was observed in the experiments performed so far, it is contemplated by the present inventors that a larger reduction will be seen in other species.

The method according to another embodiment comprises the step of modulating the expression of a pectin methyl esterase (PME) gene, such as the PttPME1 gene

(GenBank Accession No. AJ277547), in at least one cell of said plant in order to alter the lignin content or alter the lignin composition.

The method according to a preferred embodiment comprises the step of down-regulation of the expression of a pectin methyl esterase (PME) gene, such as the PttPME1 gene (GenBank Accession No. AJ277547), in at least one cell of said plant in order to reduce the lignin content or alter the lignin composition.

The method of the present invention relates primarily to the modification of plants having fibres that elongate through intrusive tip growth, which means that an increased intrusive growth leads to an increased fiber length. Such fibres are wood fibers and for example sisal, abaca, jute, flax, ramie, hemp, and kenaf fibres.

As discussed above, relating the transgenic plants of the present invention, the intrusive tip growth is of highest importance for the elongation of wood fibers of dicotyledonous woody species. This is applicable also to the method according to the invention, which therefore is also valid for conifers as well as other types of fibers that grow by intrusive growth. These include in particular phloem fibers found in sisal, abaca, jute, flax, ramie, hemp, kenaf, bagasse, wheat and bamboo (Esau, 1965; McDougal *et al.*, 1993).

Specifically, the method involves using enzymes acting on cell adhesion to modify intrusive growth. For example, pectin methyl esterase (PME) activity can be down regulated using an antisense construct, co-suppression of a sense construct of the PME sequence, gene silencing, RNAi, overexpression of a PME inhibitor or other methods known to a person skilled in the art.

Examples

Materials & Methods

General

Plant material

Hybrid aspen (*Populus tremula L. x Populus tremuloides Michx*), clone T89 (WT), subsequently denoted as poplar, was grown in a greenhouse under a photoperiod of 18 h and a temperature of 22°C/15°C (day/night). To induce cambial dormancy, trees were placed in an unheated greenhouse at the end of July and exposed to the

photoperiod (Northern Sweden) until mid-winter when they reached the quiescent stage of dormancy.

Cloning of PttPME1 cDNA

Two degenerate primers (Pr1.1: 5'-AMTGGAACARTCGATTTCATYTTCGG-3' and Pr2.2: 5'-GAATATTCCTTCCAHGGMCGACCAARATAC-3') were used to amplify a 200 bp PME fragment from the genomic DNA. A λt22a cDNA library, prepared from the cambial region (Sterky *et al.*, 1998), was screened under high stringency conditions using the 200 bp fragment. Positive cDNA inserts were cloned in pBluescript SK (Stratagene) and sequenced on both strands.

RNA extraction

Lowermost internodes of 2 m tall plants were harvested at the active or dormant stage of the cambial growth. Cambium/phloem, developing xylem and cortex tissues were dissected and ground in the liquid nitrogen. Total RNA was extracted with hot CTAB method (Fang *et al.,* 1992) and purified with the RNeasy Plant Mini Kit columns (Qiagen).

PttPME1 expression analysis by dot blot

Serial dilutions of gene-specific 3' end fragment of *PttPME1* cDNA were denatured in 0.4 N NaOH and spotted onto a Nylon positively-charged membrane (Amersham) using the Vacuum Minifold I (Schleicher and Schuell).

The single strand cDNA probe was prepared as described by Micheli *et al.* (1998), purified on Sephadex G50 (Nick column, Pharmacia) and added to hybridization buffer (Church and Gilbert, 1984) at a concentration of 8.8 10^6 cpm/ml per membrane. Hybridization was done under high stringency conditions. Autoradiographs were scanned using Photoshop (Adobe Systems, Inc. MacApp, Mountainview, CA, USA) and the images were analysed with NIH Image 1.57 (Wayne Rasband, NIH, Bethesda, MA., USA).

Generation of transgenic poplar

PttPME1 cDNA either full length (sense) or its 3' gene specific fragment (antisense) was cloned into a binary vector pBI121 (Clontech). The vector was transferred to poplar via *Agrobacterium tumefaciens* strain GV3101 (pMP90RK - Koncz and Schell,

1986). Kanamycin-resistant lines were clonally propagated on the Murashige and Skoog medium before planting in the greenhouse.

Southern blot analysis

Total genomic DNA was extracted from apical portions of the shoot, using a modified CTAB protocol (Fang *et al.*, 1992). The DNA was digested with Hind III to completion and electrophoresed on a 0.7% agarose gel. The DNA was transferred to Hybond-N+ nylon membrane (Amersham Pharmacia Biotech) under alkaline conditions (Current Protocols). The membrane was probed at high stringency with a NPTII gene fragment. The radioactivity was analyzed on Phosphor Imaging System (GS-525, Molecular Imager, Bio-Rad, Solna Sweden).

Quantitative RT-PCR

Total RNA was treated with DNase and reverse transcribed using random hexamer primers (50 ng/μl) and the MLV reverse transcriptase (BRL). The first strand cDNA was used as a template in the polymerase chain reaction using the primer set (5'-ATT TCA TTT TCG GCA ATG CT –3' and 5'-GCG CCA CGA AGA GAA TAC AT-3'), which gives the 0.516 kb product specific for the sense mRNA. The PCR was performed using 32 cycles of 94°C for 30 sec, 62°C for 30sec and 72°C for 30sec. The quantification of the ratio between the initial mRNA content and 18S rRNA was done using the Quantum RNA kit (Ambion) using 18S specific primers.

PME activity assays

Water-soluble and ionically bound, 1M NaCl extractable proteins were isolated from cambium/phloem and xylem fractions according to Richard *et al.* (1994) and Micheli *et al.* (2000). PME activity was measured spectrophotometrically using Pharmacia LKB Biochrom 4060 UV-Visible Spectrophotometer by monitoring pH changes resulting from demethylation of citrus pectin (methylated at 89% from Sigma) with methyl red indicator (Richard *et al.*, 1994). A calibration curve was obtained by adding 0 to 100 nEq of H^+ to the reaction solution. Starting pH was 6.1. Readings were taken at 3 timepoints within a linear range of the reaction rate. The PME activity was expressed per mg of ground material determined by turbidimetry (Micheli *et al.*, 2000) or by mg of total protein determined by DC Protein Assay (Bio-Rad).

Isoelectric focussing of PME

Cell wall proteins extracted with 1NaCl were fractionated by IEF on ultrathin polyacrylamide slab gels containing 10% (v/v) pharmalytes (pH range from 3-10; Amersham Biosciences). Before loading, samples were desalted and calibrated to 20, 50, 100 or 200 µg of lyophilized cell wall material from which proteins were eluted. Zymogram of the PMEs was made according to Micheli *et al.* (2000). Apparent pI was determined by reference to pI markers (Bio-Rad, Hercules, CA).

Pectin analysis

Pectin was extracted from liquid N_2 ground cambium/phloem and xylem tissues, using either water or a buffer containing a chelating agent (50mM Tris-HCl, pH 7,2, 50 mM CDTA and 25 mM DTT). Uronic acid content and methylation were determined according to Kim and Carpita (1992).

For immunoblotting (Willats and Knox, 1999), pectins were extracted with the buffer containing CDTA, as above, except that the temperature of the buffer was increased to 95° C to increase the efficiency of extraction. Equal amounts of uronic acid s were dot-blotted onto nitrocellulose in a 5 x dilution series and probed with Pam1 antibody, followed by a secondary anti-His monoclonal antibody and a tertiary anti-mouse antibody coupled to peroxidase (both from Sigma). The peroxidase product was detected with Amersham ECL Plus reagents and quantified in fluorescence mode using 415 nm excitation and 455 nm detection, with the Tayphoon scanner 9400 (Amersham).

Pectin immunolocalization

Stem internodes 15-20 were free-hand sectioned and processed as described by Willats *et al.* All monoclonal antibodies were gifts from Dr. William G. T. Willats and Dr. J. Paul Knox. Negative controls were done without primary antibodies or without all antibodies (autofluorescence control). The sections were examined by confocal laser microscopy (Zeiss LSM 510). Sections were excited with 488 nm light and the FITC signal was detected between 505 and 530 nm. The chlorophyll autofluorescence signal was detected above 650 nm was superimposed onto the transmitted light signal for anatomical detail. All samples that were compared were scanned at identical FITC detection settings.

Wood cell measurements

Wood of internode 40 was macerated in acetic acid-peroxide cocktail (Berlyn and Miksche, 1976) until single cells were obtained. Cells were stained with Toluidine blue O and examined in Axioplan 2 (Zeiss). Extended focus images were captured by an AxioVision camera (Zeiss) and cells (about 1000 per line) were measured directly on the screen.

Statistical analysis

Data were analysed by GLM procedure followed by a Duncan multiple range test (SAS Inc.)

Investigation of lignin content

Samples

The first PME sample (hereafter referred to as batch 1) consisted of three different aspen samples (lines) – 2B, 5 and wt. Each line was represented by three individual trees: wt(T89) 4,5 and 8; 2B 2, 3 and 4; 5 1, 2 and 3, see appendix. The samples were further divided into 6-8 fractions based on nodes (height). The samples were transported fresh under cool conditions debarked and chipped on arrival and kept in the fridge before pulping. In each line all stems with approximately the same nodes were pooled into four fractions. Later eight additional wt trees (T89) were received in dried state. These had been debarked and dried before they were sent to us. It was found that this type of samples could not be stored because microorganisms will rapidly attack them. Later samples where therefore immediately air dried.

The second batch of PME trees was not divided into as many pieces as the first batch. Instead the stems were cut into four segments each, designated 1-4 counting from the top, see appendix. A third batch (batch 3) was also received. This material consisted of thin stem samples all coming from the lower part of the stem (segments 3-4).

Pulping

The initial pulping experiments of PME trees (batch 1) were run at a liquor-to-wood ratio of 6 using 20% effective alkali and 35% sulfidity. In each experiment 50 g wood was charged. The temperature was raised 1°C/min. from 70°C to 160°C. The cooks were run to 700 or 900 H-factors. After treatment the samples were carefully washed

and disintegrated in deionised water. The samples were dried and stored before further use.

Batch 2 samples were pulped in triplicate in order to gain statistically accurate values. The liquor-to-wood ration was 6 and 10 g sample was charged. 20% effective alkali and 35% sulfidity was used. A temperature profile, 2°C/min. from 70°C to 160°C, to a H-factor of 700 was used. After treatment the samples were carefully washed and disintegrated in deionised water. The samples were dried and stored before further use.

A further lowering of sample size was made when batch 3 was pulped. In this case 4 g was used in each experiment. This lowering of sample size made it necessary to mill the whole material before samples was removed for pulping. The samples were milled in a Wiley mill equipped with a course screen (Ø6mm). Finely divided wood will absorb more water and a higher liquor-to-wood ratio of 20 was therefore used. All experiments were run in triplicate using 0.4 M NaOH and 0.22 M NaHS. Because of the higher liquor-to-wood ratio used a slightly altered temperature profile was used, 70°C/10 min-155°C/2°C/min to a H-factor of 450. After treatment the samples were carefully washed and disintegrated in deionised water. The samples were dried and stored before further use.

Analysis

The wood samples were analysed with regard to lignin content and carbohydrate composition as described in the method AH 23-18. The method is based on full hydrolysis of samples followed by derivatization of liberated monomers and gas chromatography. Lignin will form a solid residue that is gravimetrically determined.

Kappa number was determined according to the well-known standard SCAN-C.1 and pulp carbohydrate composition according to the corresponding standard AH 23-9. The method according to AH 23-9 is based on enzymatic digestion of sample follower by capillary zone electrophoresis of derivatized monomers.

Results

General

Cloning and expression of PttPME1

Screening of a cDNA library of cambial region tissues (Sterky *et al.*, 1998) resulted in an isolation of a 2149 bp - *PttPME1* cDNA (GenBank accession number AJ277547). The 1739 bp ORF encoded a preprotein of 62.9 kDa that showed a high similarity to most plant PME precursors. It carried a signal peptide with a predicted cleavage site at position Ala^{46} (Von Heijne, 1986), and a potential proteolytic cleavage site in the vicinity of PRO^{267}(Micheli, 2001) that would release a mature protein of 34 kDa with an IEP of 9. Two potential N-linked glycosylation sites, specified by the sequence Asn-X-Ser/Thr, were found in the N-terminal region (Figure 1A).

To investigate the role of *PttPME1* in wood formation, its expression was analysed in the stem tissues at the active and dormant stage (Figure 1B). The transcripts were found primarily in the cambium/phloem and developing xylem, less in the cortex in the active stage. No expression was detected in dormant tissues.

Generation of transgenic poplars

Function of *PttPME1* in wood fibre elongation was further studied in transgenic lines of poplars generated using a constitutive 35S promoter and either a full length *PttPME1* cDNA in sense orientation or a 3' fragment of *PttPME1* cDNA in an antisense orientation. The 3' fragment was unique to *PttPME1* gene as indicated by a high-stringency Southern blot hybridization (data now shown). Seven sense and one antisense lines were grown and when secondary growth was developed, *PttPME1* expression was investigated in the wood forming tissues by the quantitative RT-PCR. Two lines exhibited increase in the *PttPME1* transcript level in cambium/phloem tissues up to 170% WT (Figure 2A). Most sense lines had a reduced transcript level down to 50% of WT .

Reduction in *PttPME1* transcript levels in the sense lines suggested a co-suppression that frequently occurs when multiple copies of a transgene are inserted. To examine the transgene insertion pattern, a Southern blot analysis of the transgenic lines was performed using a combination of a probe and a restriction enzyme that revealed the number of T-DNA insertions. Indeed, the lines that were

the most suppressed contained multiple insertions (Figure 2B). The analysis also showed that all the lines were resulting from independent transformation events. The present inventors selected two suppressed lines (5 and 8) and one over expressing line (2B) for the functional characterisation of *PttPME1*.

PME activity in transgenic lines

To determine if changes in the transcript level of *PttPME1* gene affected the overall enzyme activity in wood forming tissues, the present inventors examined the PME activity in wall bound proteins extracted with 1M NaCl and in soluble proteins obtained from cambium/phloem tissues of selected transgenic lines. Regardless of line, wall bound proteins contained two orders of magnitude more activity than the soluble proteins (Figure 3 AB). Both protein fractions were affected by transgene expression. Line 2B had between 130% and 170% of WT activity in wall bound and soluble fraction, respectively, while the suppressed lines generally exhibited a reduced PME activity. The level of suppression was particularly severe in line 5 reaching only 10% and 60% of the WT level in the wall bound and soluble fraction, respectively. A good correlation between the PttPME1 expression level and the PME activity (Figure 2B) indicates that the PttPME1 encoded enzyme has a PME activity.

Patterns of PME isoenzymes in transgenic lines

Since there are several isoenzymes of PME in the vascular cambium region of poplar (Guglielmino *et al.*, 1997, Micheli *et al.*, 2000) and the identity of corresponding genes is unknown, the present inventors were interested in isoenzyme patterns in transgenic lines with modified *PttPME1* transcript levels.

In WT plants, quantitative changes occurred between tissues. Overall activity per mg of dried tissue was nine-fold higher in the cambium/phloem than in developing xylem (Figure 4B). This increase was accounted for by an increase in neutral and a basic isoforms of pI 7.2 and 8.5 (Figure 4A) as previously observed (Micheli *et al.*, 2000). In contrast, two acidic isoforms of pI 4.0 and 5.2 were more abundantly produced in the xylem. Isoforms of pI 4.4 and 5.4 appeared to be ubiquitous throughout the stem.

Over-expression of *PttPME1* in line 2B lead to an increase of global PME activity per mg d.w. in both the cambium/phloem and the xylem (Figure 4B) as observed earlier per mg protein (Figure 3AB). Surprisingly, this enhancement can be related to an over-production of not one but three PME isoforms (pI 5.4, 7.2 and 8.5) in the

cambium/phloem, and two isoforms (pI 5.4 and 7.2) in the xylem, while the most acidic isoforms were not affected by the transgene (Figure 4A). Most increase in PME activity can be accounted for by an increase in the neutral isoform of pI 7.2.

Line 5 suppressed in *PttPME1* showed a dramatic decrease in global PME activity per mg d.w., both in the cambium/phloem and in developing xylem (Figure 4B) as observed earlier per mg protein (Figure 3). However, not all PME isoforms were affected. The most striking difference was observed in the isoform of pI 7.2, which disappeared almost completely in the transgenic line in both tissues accounting for most reduction of the global PME activity observed. A lesser decrease occurred in the basic isoform with pI 8.5 in the xylem and the acidic isoforms with pI 4.0 and 5.4 in the phloem.

Effects of modified expression of PttPME1 on pectins

To examine if modified PME activity in transgenic lines affected the amount of pectins in the wood forming tissues, pectins were extracted either with water or with CDTA and the polygalacturonic acid (PGA) content was measured in the presence of the cell wall material to quantify 'total' amount of accessible pectin. In the WT, the CDTA extract contained about 1.5 times more pectin compared to the water extract (Figure 5A). The down-regulated lines (5 and 8) had a reduced PGA content in the CDTA fraction while the up-regulated line 2B had a similar PGA content as the WT.

Next, the present inventor examined if modified *PttPME1* expression induced the expected changes in the degree of methylesterification of homogalacturonan by quantifying methanol released form the water and CDTA fractions by saponification. The overexpressing line 2B had significantly reduced methanol level compared to WT in the water fraction, in the cambium/phloem tissues. The down-regulated lines 5 and 8 had significantly increased methanol levels in the CDTA fraction, while only line 5 was affected in the water fraction.

Molar content of methanol released from the cambium/phloem tissues in the CDTA fraction exceeded that of galacturonic acid, indicating that the methanol was released not only from PGA but also from other sources as well.

To analyze specifically pectin methylesterification in the transgenic lines we used the Pam1 antibody that reacts with at least 30 contiguous non-methylesterified HG units (Clausen *et al*, 2003). UAs extracted with CDTA were dot blotted onto nitrocellulose

and probed with Pam1. The overall results indicate that the Pam1 epitope was more abundant in line 2B and less abundant lines 5 and 8, compared to WT (Fig. 5 C, D). This result supports the conclusion that the methylesterification pattern was affected in the transgenic lines as predicted from the *PttPME1* expression levels.

Monoclonal antibodies specific for various HG epitopes were also used to investigate the *in situ* patterns of pectin methylesterification in the transgenic lines. The Jim5 antibody recognizes HG with a range of methylesterification levels from few to 70% and it cannot bind to the completely de-methylesterified HG (Clausen *et al*, 2003, Willats *et al*, 2000). Jim5 epitopes were abundantly present in the cell walls of the cambial meristem, in differentiating, radially expanding xylem elements and in the phloem of the WT (Fig. 6 B). PttPME1 overexpression reduced the presence of Jim5 epitopes while PME its suppression resulted in their substantial increase (Fig. 6 A-C). The Jim7 antibody recognizes HG with a wide range of methylesterification (36) but the binding sites of Jim7 and Jim5 are not necessarily overlapping (35Clausen *et al*, 2003). In the WT, Jim7 epitopes were present in the same cell types as the Jim5 epitopes, but were less abundant. Both, up- and down-regulation of PME increased Jim7 labeling (Fig. 6 D-F). In the WT, the Pam1 epitope was detected in expanding phloem and xylem elements, and only at cell junctions where cell separation occurs (arrows in Fig. 6 H). The labeling was markedly enhanced in PME overexpressing line 2B and reduced in line 5 (Fig. 6 G-I), consistent with the expected mode of PME action, i.e. the block-wise removal of methyl ester groups of HG (Micheli, 2001). The LM7 epitope consists of stretches of sparsely methylesterified HG with four or more consecutive de-esterified HG units (Willats *et al*, 2001, Clausen *et al*, 2003). This epitope was seen only in the PME down-regulated line in the intercellular spaces formed during xylem radial cell expansion (arrowheads in Fig. 6L), indicating that the PttPME1 enzyme, when present, removes sparsely distributed methyl ester groups in the radial expansion zone. Taken together, the immunolocalizations using a set of pectin specific antibodies add further evidence to the idea that the pattern of methylestrification was altered in the transgenic lines, and demonstrate that these changes took place in the tissue where intrusive tip growth takes place.

Intrusive tip growth

The impact of the changed HG methylation pattern on the intrusive tip growth was estimated by measuring the length of fibers and vessel elements in macerated wood

samples because the length of wood cells depends on the intrusive growth within the meristem (for both fibers and vessel elements) and in the radial expansion zone (for fibers only). In addition to lines 2B, 5, and 8, we analyzed also line 7 that was overexpressing *PttPME1* at 170% of the WT (Fig. 2A). Fiber length was significantly affected by *PttPME1* expression level and a negative correlation between fiber length and the level of *PttPME1* expression was found (Fig. 7A, B). A similar negative effect of *PttPME1* expression on the length of vessel elements was observed, but was less prominent indicating that PME plays a less important role in vessel element elongation.

The results show that a decrease in the endogenous level of the biologically active PME cause increased pectin methylation, decreased pectin content and an increase in fibre length. This was demonstrated by expressing *PttPME1* cDNA (GenBank accession number AJ277547) in the sense orientation where multiple inserts caused co-suppression of PME.

Phenotypic characterization of transgenic lines

To make sure that the transgene did not induce non-desirable effects on plant growth, the stem elongation and the rate of leaf formation was followed for 6 weeks during intensive growth of the selected lines. Generally, the transgenic lines with modified PttPME1 expression level did not exhibit major changes in the height growth or the rate of leaf formation but small statistically significant effects on growth were evident for most affected lines (Figures 8AB). Line 2B had significantly reduced stem elongation compared to WT while all transgenic lines regardless of the transgene expression level had slightly reduced leaf formation rate (Duncan test, $P \leq 0.05$). Anatomical inspection of stem cross sections did not reveal any effects of transgene expression on xylem anatomy or on the rate of xylem formation (Figure 9).

The present data suggests that the most abundant neutral isoform present during cambial growth is encoded by the *PttPME1* gene. A severe reduction of the *PttPME1* expression in the transgenic line 5 results in an almost complete disappearance of the isoform (Figure 4A). In contrast, the down regulation of *PttPME1* gene results in severe decrease of total PME activity in the wood forming tissues (Figures 3AB and 4B). Co-variation of *PttPME1* transcript and other less abundant PME isoforms in transgenic lines suggests that these isoforms might represent post translation modifications of the *PttPME1* protein.

Importance of PME in regulation of methylesterification of HG

When *PttPME1* cDNA was expressed under 35S CMV promoter in sense orientation, the transformation rate was low compared to rates typically observed with other constructs and only two up-regulated lines were obtained while most sense lines were co-suppressed. This suggests that the up-regulation of the gene, leading to an excessive homogalacturonan demethylation, is unfavourable for plant growth and development.

Altered reactivity of CDTA-extracted pectin with Pam1 antibody, recognizing non-methylesterifed HG (Willats *et al*, 2001), demonstrated the expected changes in pectin methylesterification in the transgenic lines (Fig. 5 C, D). This was further supported by changes in the methanol released from the water and CDTA pectin extracts upon saponification (Fig. 5 B) even though the method was overestimating HG methylesterification level. Altogether, the data indicate that the *PttPME1* encoded enzyme exhibits PME activity *in vivo*. Increase in the Pam1 epitope in the overexpressing line (Fig. 5 C and 6), along with the appearance of LM7 epitope and a higher abundance of Jim 5 epitope when *PttPME1* was suppressed (Fig. 6) are consistent with the enzyme exhibiting a block-wise PME activity in wood forming tissues. Other studies using transgenic plants with suppressed PME activity showed either similar (Tieman *et al*, 1992) or opposite (Pilling *et al.*, 2004) effects on HG methylesterification as compared to our results (Fig. 5) and the reasons behind these discrepancies are not clear.

Immunolocalization of pectin epitopes in the stem of WT and PME-modified poplar lines brought new information about localized changes in pectin methylesterification and the PME action. In the WT, the Pam1 epitope was abundant in the intracellular spaces formed during cell expansion but it was rarely present in the cambium (Fig. 6H) in spite of the high PME activity in this meristem (Micheli *et al.* 2000). Similarly, the ectopic overexpression of *PttPME1* in line 2B increased Pam1 epitope only in the radial expansion zone but not in the cambium (Fig. 6G). Therefore, the pectin in the cambium must be somehow protected against de-methylesterification involving at least 30 HG units. The Jim5 antibody, on the other hand, detected epitopes in the cambium that decreased in abundance as a result of PttPME1 activity (Fig. 6 A-C). This would happen if, for example, PttPME1 would remove methyl ester groups from stretches of HG shorter than 30 units in the cambium.

Jim5 specificity only partially overlaps with that of LM7, and the cambium must contain a high abundance of Jim5 epitopes other than the epitope of LM7. The Jim7 antibody has a large spectrum of specificities to methylesterified HG (Willats *et al.* 2000, Clausen *et al*, 2003) and it labeled more epitopes both in line 2B and 5 compared to WT (Fig. 6 D-F). These results cannot be understood until the entire spectra of Jim antibodies are known. Nevertheless, the immunolocalization data demonstrated that the altered PME activity in the transgenic poplars resulted in altered abundance of specific HG epitopes in the cambial meristem and expanding xylem cells, *i.e.* in tissues where the intrusive tip growth occurs.

PME suppression in poplar wood-forming tissues resulted in a significantly lower amount of CDTA-extractable HG (Fig. 5 A). This suggests that the degree and pattern of pectin methylation affect pectin metabolism and turnover. Similar correlation between HG methylesterification and the amount of HG were observed in other studies with modified PME expression (Tieman *et al*, 1992, Pilling *et al.* 2004). It is possible that the susceptibility of HG to native pectin/pectate lyase and polygalacturonase be affected by its methylesterification pattern as known for fungal enzymes (Limberg *et al*, 2000, van Alebeek *et al*, 2002). The lower amount of HG in the walls of suppressed lines may also be caused by the inability to sequester methylesterified pectin into the wall-immobilized, Ca^{2+}-bound fraction corresponding to the CDTA-extractable pectin (Jarvis, 1984). If PttPME1 exhibits transacylation activity as was reported for some PME isoforms in pea (Hou and Chang, 1997), the reduction in *PttPME1* expression would result also in reduced pectin cross linking within the cell wall leading also to facilitated pectin endocytosis and turnover.

Role of methylesterification in pectin metabolism in wood

The present inventors were able to increase the methylation levels in wood forming tissues by suppressing the PttPME1 expression (Figures 4, 5, 6). This could have several consequences for pectin metabolism and properties of cell walls in these tissues. Firstly, the susceptibility of homogalacturonan to the enzymatic attack by pectate lyase and polygalacturonase would have been modified. The fact that the levels of PGA were significantly decreased in the suppressed lines would suggest that increased methylation facilitated the degradation of PGA by these enzymes. In addition, the cation binding abilities of methylesterified homogalarcuronan would be

less, resulting in overall decrease in wall bound calcium that would in turn decrease wall rigidity and affect swelling and water binding.

Novel role of PME in methylation-controlled intrusive tip growth

Correlative evidence supports the regulatory role of PME in the process of intrusive tip growth. Using an antibody to a neutral isoform of PME, the enzyme was immunolocalized to cell junctions in developing wood (Guglielmino *et al.*, 1997) where the penetration of intrusively growing fibre tips occurs. Conclusive evidence comes from the analysis of intrusive growth in transgenic line with modified PttPME1 expression levels. Intrusive growth of fibres was enhanced when PttPME1 expression and PME activity was suppressed in wood forming tissues and it was decreased when the gene expression and enzyme activity was up-regulated (Figures 2-7). The mechanism of such a control could be by affecting the homogalacturan methylation (Figure 5), which in turn could affect pectin amount, swelling and ion binding properties, as discussed above. These changes are likely to impact on pectin cross-linking that would mediate cell-to-cell adhesion. Effects of methylesterification of homogalacturonan on the cellular adhesion were also seen in other tissues. Similarly, highly acetylated pectins were associated with low adhesion phenotype. Recently, the boron-mediated cross-linking and the presence of arabinosyl chains in Rhamnogalacturonan II and I, respectively, were proved essential for maintaining the cell-to-cell adhesion. Also the homogalacturonan content was demonstrated to play a role by analysis of the *quasimodo 1* mutant defective in a glucosyltransferase essential for pectin biosynthesis. It is therefore likely, that the intrusive tip growth could be controlled also pectin properties other than methylation. The enzymes involved in the modification of these properties are therefore a biotechnological target for increasing the length of wood fibres as well as other fibres that elongate via intrusive growth including sisal, abaca, jute, flax, ramie, hemp and kenaf.

Pulping studies and lignin content

The PME samples have a genetically altered production of the pectin methylation enzyme. Five different lines have been tested: wild type (T89), 2B (up-regulated), 5S (down-regulated), 7S () and 9S (). Three different sample batches have been evaluated on different scales. Batch 1 was run pulped using 50 g wood chips. This is still a rather large amount of material when screening of many different constructs is

to be made. It is also still not possible to perform a full bleaching/sheet property evaluation on this scale. The results from this experiment show that there exist differences between the samples, figure 9. Average reached kappa number for wt – 16.6, 2B – 20.1 and 5s – 14.0. The error in these measurements is estimated to be 0.5-1.0 kappa number units. Based on these data, it was observed that the down regulated line 5S contained less lignin compared to the wild type (T89). The up-regulated trees (2B) reached significantly higher levels.

The second batch (batch 2) of PME1 trees was pulped on a smaller scale than batch 1. In each run 10 g wood was charged. However because each cook was made in triplicate 30 g was used for each line. See Figure 10.

A third independently grown trial, batch 3, consisting of four lines, wt (T89), 5 (S5), 5 (S7) and 2B was pulped at an even smaller scale than batch 1 and 2. Each pulping experiment was run using 4 g air-dried sample. Debarked and cleaned tree material was milled before pulp samples were taken. This procedure was adopted in order to ensure even samples when the sample size was decreased. When finely divided wood is pulped it is necessary to increase the liquor-to- wood ration because the material will adsorb relatively larger amounts of water. When increased amounts of water is used the chemical charge need to be adjusted as well and both lower OH- concentration and HS- concentration was used in these cooks. The pulping temperature was also decreased in order to minimize loss of carbohydrates. The result of triplicate cooking experiments confirm the earlier observed results with regard to lignin content of produced pulp, see figure 11. However while 2B contained significantly higher amounts of lignin in batch 1 and 2 the difference was not as large in the batch 3 experiments, figure 11.

The difference in lignin content between batch 2 and 3 is probably explained by differences growth conditions and time of harvesting.

Chemical composition of wood samples and pulp

A key property of pulp fibers is their carbohydrate composition. The amount of cellulose plays a central role and is the principal load bearing component if the fibres. This strength will also be manifest in paper properties. The hemicelluloses also play a central role and are thought to be important for the formation of e.g. fibre-fibre bonds. The chemical analysis of batch 2 and 3 wood show that 5S samples, having

down-regulated pectin methyl esterase 1 activity, contained increased amounts of cellulose and decreased amounts of xylose as compared to wild type (T89) trees.

Chemical analysis of pulp produced from the PME1 trees showed that the differences observed in the wood was conserved in the pulp so that the line 5 trees (S5) from all three batches studied contained increased amounts of cellulose and decreased amounts of xylose as compared to the wild type. See Figures 12 and 13.

For the up-regulated trees 2B there was some differences observed between batch 2 and 3.

For the up-regulated 2B trees the effect on chemical composition was not as clear as for the 5s samples. The batch 2 material contained significantly decreased amounts of glucose and increased amount of xylose. In the batch 3 samples this was not observed and in fact the cellulose content in these do seem to be increased as compared to the wild type. The difference is however small, less than a percentage unit. Also the xylan content seems to be decreased in batch 3 2B samples. The batch 3 material also contained a third modified sample, 7 (S7), that exhibited properties close to what was observed for the 2B samples from batch 2 - higher lignin content both in wood and pulp and decreased cellulose and increased xylose content.

Although the invention has been described with regard to its preferred embodiments, which constitute the best mode presently known to the inventors, it should be understood that various changes and modifications as would be obvious to one having the ordinary skill in this art may be made without departing from the scope of the invention which is set forth in the claims appended hereto.

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Claims

1. A transgenic plant exhibiting an altered property with respect to the biomass, said property being one of altered lignin content, lignin composition, or extractability of lignin, or altered fibre length, or a combination thereof, said altered property determined by comparison with the corresponding property of the wild type of the same species, **characterized in** that the expression of a pectin methyl esterase (PME) gene or the PME enzyme level or activity is modulated in at least one cell of said plant.
2. A transgenic plant according to claim 1, wherein said altered property is altered lignin content or composition.
3. A transgenic plant according to claim 2, wherein said altered property is reduced lignin content and the expression of a pectin methyl esterase (PME) gene is down-regulated in at least one cell of said plant.
4. A transgenic plant according to claim 2, wherein said altered property includes reduced lignin content and increased cellulose content, and the expression of a pectin methyl esterase (PME) gene is down-regulated in at least one cell of said plant.
5. The transgenic plant according to claim 1, wherein the fibre length is increased through the down-regulation of a PME gene and the increase in fibre length takes place in the cambial tissue of said plant.
6. The transgenic plant according to claim 1, wherein the fibre length is reduced through the up-regulation of a PME gene.
7. The transgenic plant according to any one of claims 1 - 6, wherein the plant is a plant in which the fibres elongate through intrusive tip growth.
8. The transgenic plant according to claim 7, wherein said plant belongs to a woody species.
9. The transgenic plant according to claim 7, wherein said plant is a hardwood plant.
10. The transgenic plant according to claim 7, wherein said plant is selected from the group consisting of acacia, eucalyptus, hornbeam, beech, mahogany,

walnut, oak, ash, willow, hickory, birch, chestnut, poplar, aspen, alder, maple and sycamore.

11. The transgenic plant according to claim 7, wherein said plant is selected from the *Eucalyptus, Populus* or *Salicaceae* groups, including variants thereof.

12. The transgenic plant according to claim 7, wherein said plant belongs to an *Eucalyptus* species, including variants thereof.

13. The transgenic plant according to claim 7, wherein said plant is selected from the *Populus* or *Salicaceae* groups, including variants thereof.

14. The transgenic plant according to claim 7, wherein said plant is a conifer.

15. The transgenic plant according to claim 7, wherein said plant is selected from the group consisting of cypress, Douglas fir, fir, sequoia, hemlock, cedar, juniper, larch, pine, redwood, spruce and yew, most preferably one of fir and pine.

16. The transgenic plant according to claim 7, wherein said plant is selected from the group consisting of bamboo and rubber plants.

17. The transgenic plant according to claim 7, wherein the fibres are chosen among sisal, abaca, jute, flax, ramie, hemp and kenaf fibres.

18. The transgenic plant according to any one of claims 1 –17, wherein the PME gene is PttPME1 (GenBank Accession No. AJ277547).

19. Seeds of a transgenic plant according to any one of the preceding claims.

20. Cells of a transgenic plant according to any one of the preceding claims, said cells exhibiting modulated expression of a pectin methyl esterase (PME) gene or altered PME enzyme level or activity compared to the wild type of said plant.

21. Propagating material of a transgenic plant according to any one of the preceding claims.

22. A method for producing a transgenic plant exhibiting an altered property with respect to the biomass, said property being one of altered lignin content, lignin composition, or extractability of lignin, or altered fibre length, said altered property determined by comparison with the corresponding property of the

wild type of the same species, **characterized in** that the expression of a pectin methyl esterase (PME) gene or the PME enzyme level or activity is modulated in at least one cell of said plant.

23. The method according to claim 22, wherein said altered property is altered lignin content or composition.

24. The method according to claim 22, wherein said altered property is reduced lignin content and the expression of a pectin methyl esterase (PME) gene is down-regulated in at least one cell of said plant.

25. The method according to claim 22, wherein said altered property includes reduced lignin content and increased cellulose content, and the expression of a pectin methyl esterase (PME) gene is down-regulated in at least one cell of said plant.

26. The method according to claim 22, wherein the fibre length is increased through the down-regulation of a PME gene and the increase in fibre length takes place in the cambial tissue of said plant.

27. The method according to claim 22, wherein the fibre length is reduced through the up-regulation of a PME gene.

28. The method according to any one of claims 22 - 27, wherein the plant is a plant in which the fibres elongate through intrusive tip growth.

29. The method according to claim 28, wherein said plant belongs to a woody species.

30. The method according to claim 28, wherein said plant is a hardwood plant.

31. The method according to claim 28, wherein said plant is selected from the group consisting of acacia, eucalyptus, hornbeam, beech, mahogany, walnut, oak, ash, willow, hickory, birch, chestnut, poplar, aspen, alder, maple and sycamore.

32. The method according to claim 28, wherein said plant is selected from the *Eucalyptus, Populus* or *Salicaceae* groups, including variants thereof.

33. The method according to claim 28, wherein said plant belongs to an *Eucalyptus* species.

34. The method according to claim 28, wherein said plant is selected from the Populus or Salicaceae groups, including variants thereof.

35. The method according to claim 28, wherein said plant is a conifer.

36. The method according to claim 28, wherein said plant is selected from the group consisting of cypress, Douglas fir, fir, sequoia, hemlock, cedar, juniper, larch, pine, redwood, spruce and yew, most preferably one of fir and pine.

37. The method according to claim 28, wherein said plant is selected from the group consisting of bamboo and rubber plants.

38. The method according to claim 28, wherein the fibres are chosen among sisal, abaca, jute, flax, ramie, hemp and kenaf fibres.

39. The method according to any one of claim 22-38, wherein the PME gene is PttPME1 (GenBank Accession No. AJ277547).

40. The method according to claim 39, wherein the PME gene is down-regulated using an antisense construct.

41. The method according to claim 39, wherein the PME gene is down-regulated via co-suppression of a sense construct of the PME sequence.

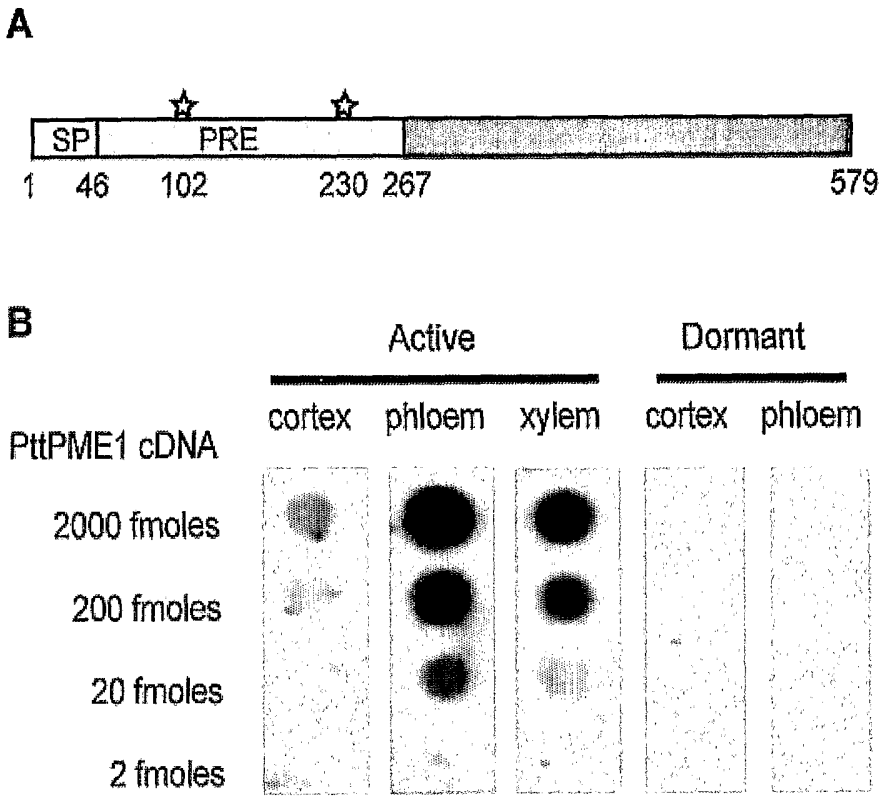

A
SP
PRE
1
46
102
230
267
579
B
Active
Dormant
cortex
phloem
xylem
cortex
phloem
PttPME1 cDNA
2000 fmoles
200 fmoles
20 fmoles
2 fmoles

Fig 1

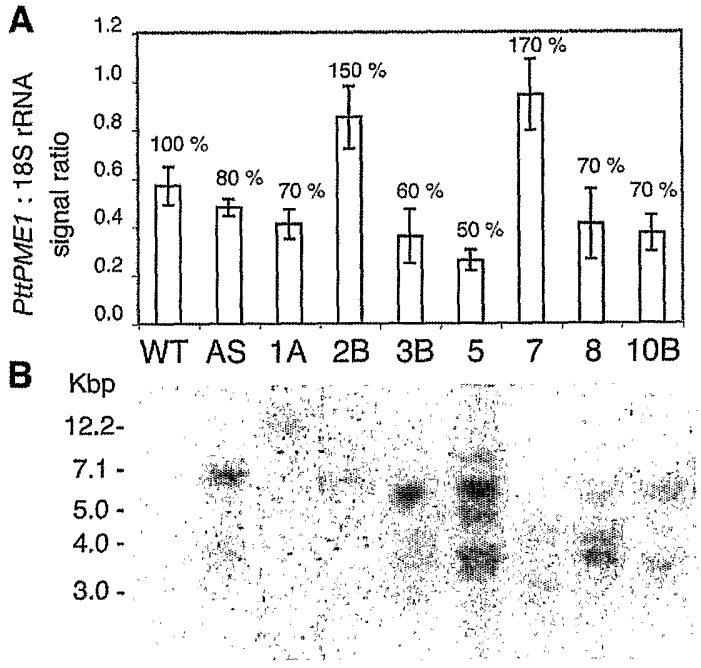

A
PttPME1 : 18S rRNA signal ratio
1.2
1.0
0.8
0.6
0.4
0.2
0.0
100 %
80 %
70 %
150 %
60 %
50 %
170 %
70 %
70 %
WT
AS
1A
2B
3B
5
7
8
10B
B
Kbp
12.2-
7.1 -
5.0 -
4.0 -
3.0 -

Fig. 2

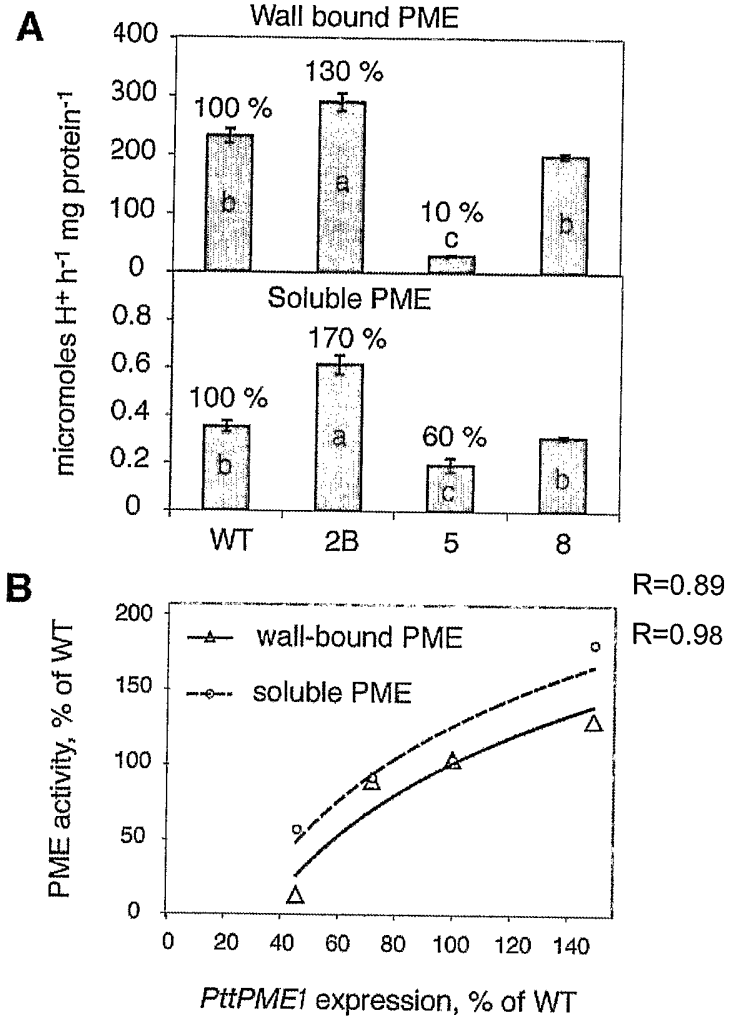

A
Wall bound PME
400
300
200
100
0
micromoles H+ h-1 mg protein-1
100 %
130 %
10 %
b
a
c
b
Soluble PME
0.8
0.6
0.4
0.2
0
170 %
100 %
60 %
b
a
c
b
WT
2B
5
8
B
R=0.89
R=0.98
200
150
100
50
0
wall-bound PME
soluble PME
PME activity, % of WT
0
20
40
60
80
100
120
140
PttPME1 expression, % of WT

Fig. 3

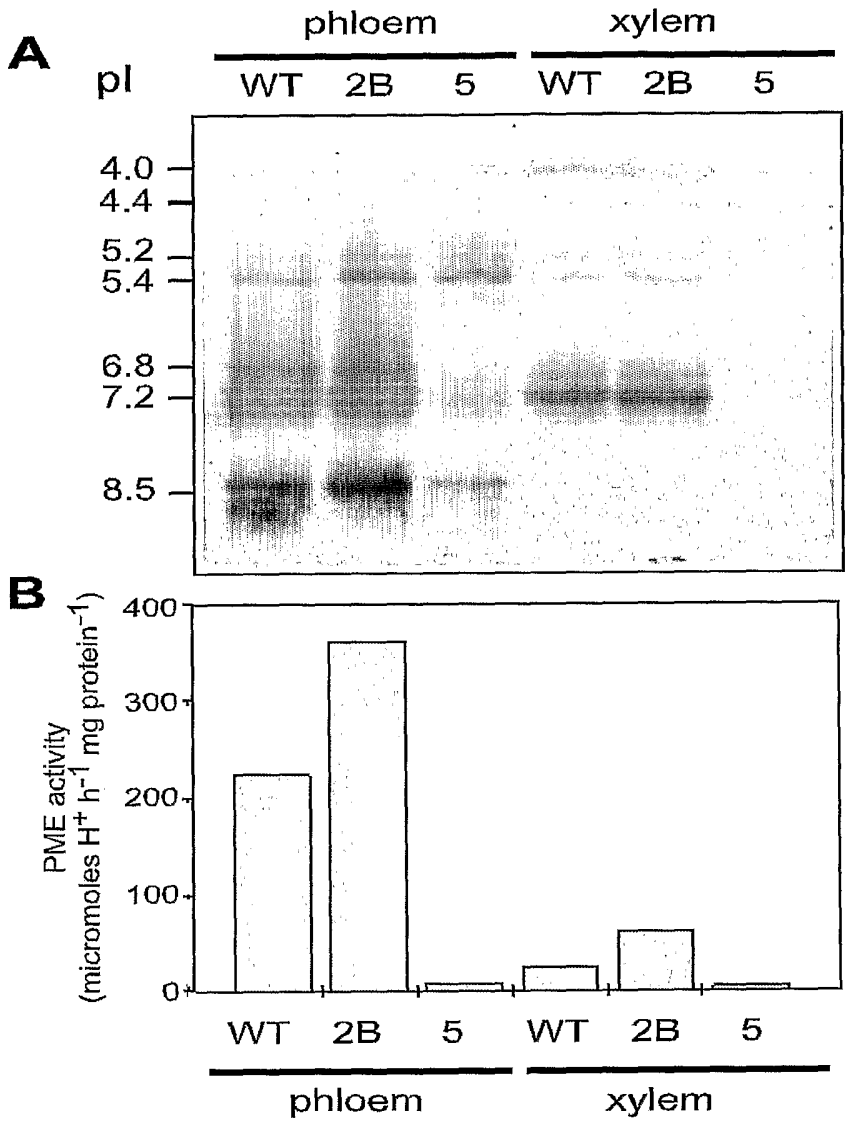

A
phloem
xylem
pI
WT 2B 5 WT 2B 5
4.0
4.4
5.2
5.4
6.8
7.2
8.5
B
PME activity
(micromoles H+ h-1 mg protein-1)
400
300
200
100
0
WT 2B 5 WT 2B 5
phloem
xylem

Fig. 4

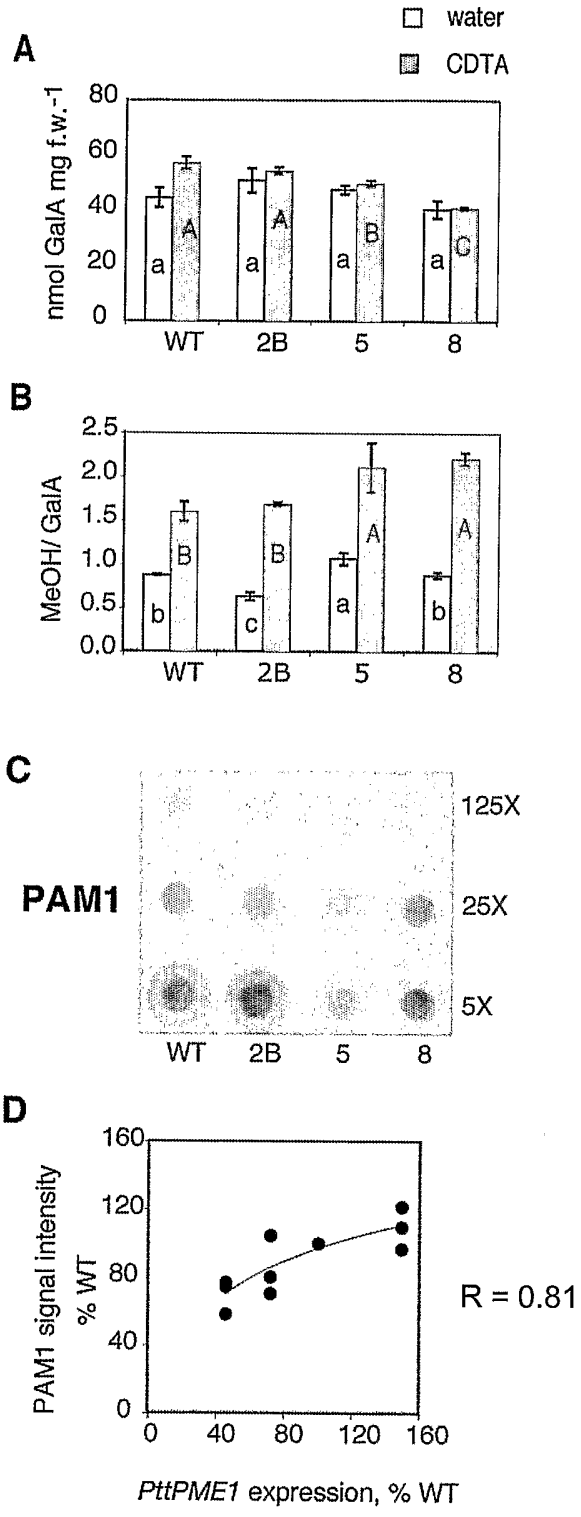

A
water
CDTA
nmol GalA mg f.w.-1
80
60
40
20
0
a
A
a
A
a
B
a
C
WT
2B
5
8
B
MeOH/ GalA
2.5
2.0
1.5
1.0
0.5
0.0
b
B
c
B
a
A
b
A
WT
2B
5
8
C
PAM1
125X
25X
5X
WT
2B
5
8
D
PAM1 signal intensity
% WT
160
120
80
40
0
0
40
80
120
160
R = 0.81
PttPME1 expression, % WT

Fig. 5

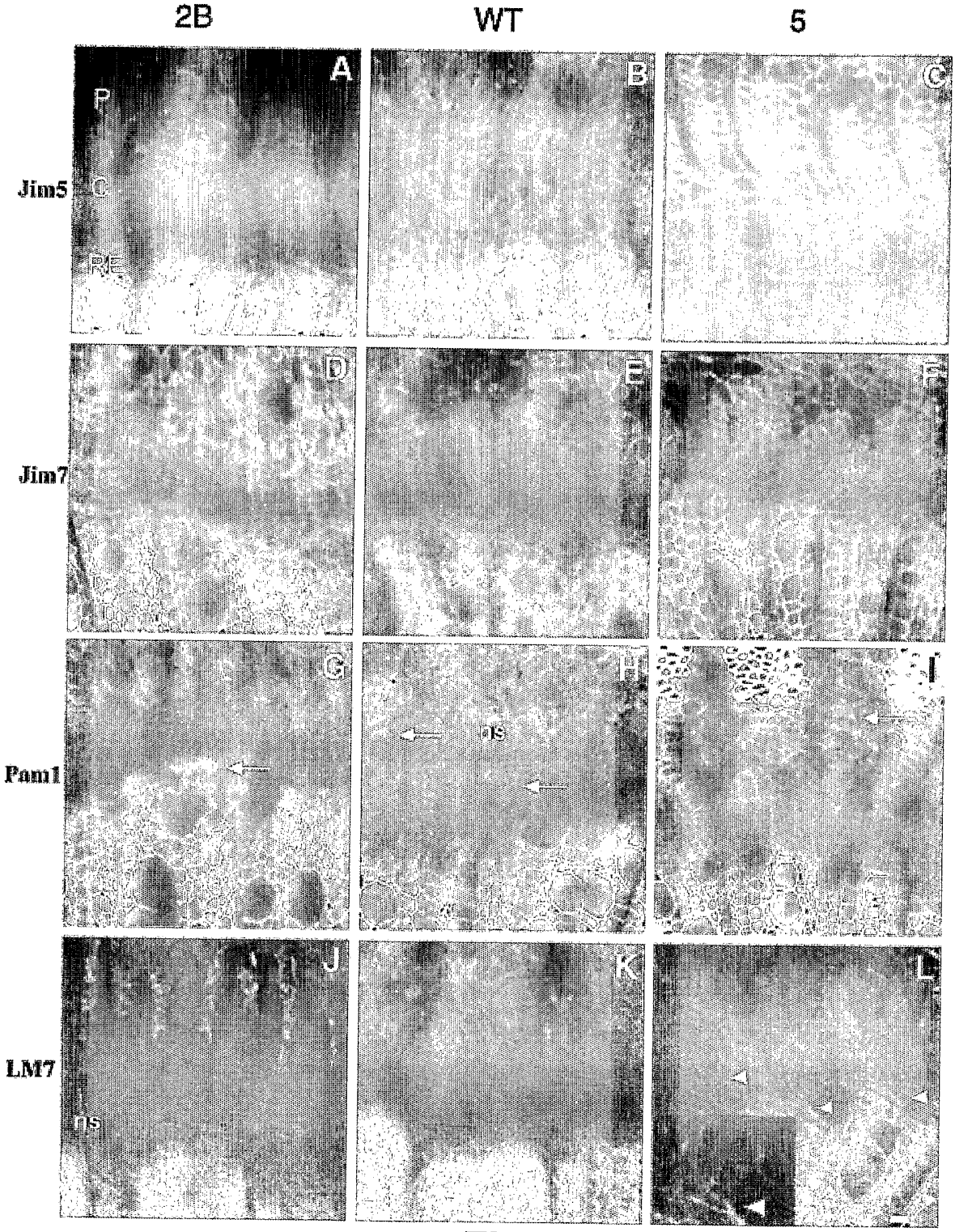


Fig. 6

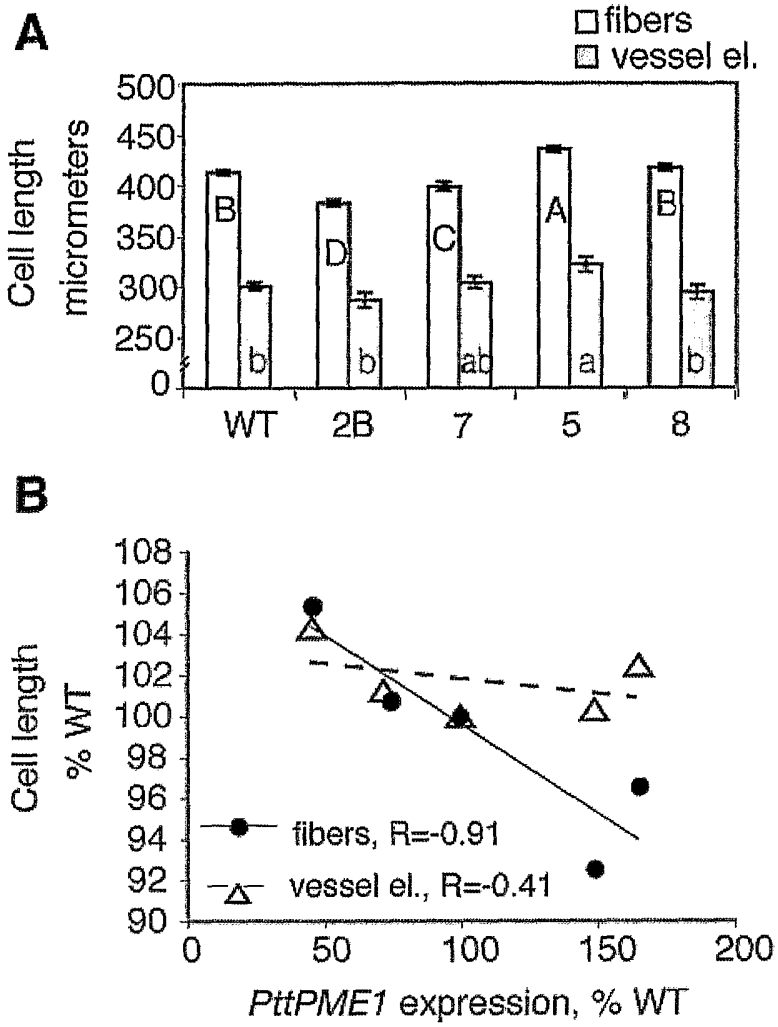

A
fibers
vessel el.
Cell length micrometers
500
450
400
350
300
250
0
B
D
C
A
B
b
b
ab
a
b
WT
2B
7
5
8
B
Cell length % WT
108
106
104
102
100
98
96
94
92
90
fibers, R=-0.91
vessel el., R=-0.41
0
50
100
150
200
PttPME1 expression, % WT

Fig. 7

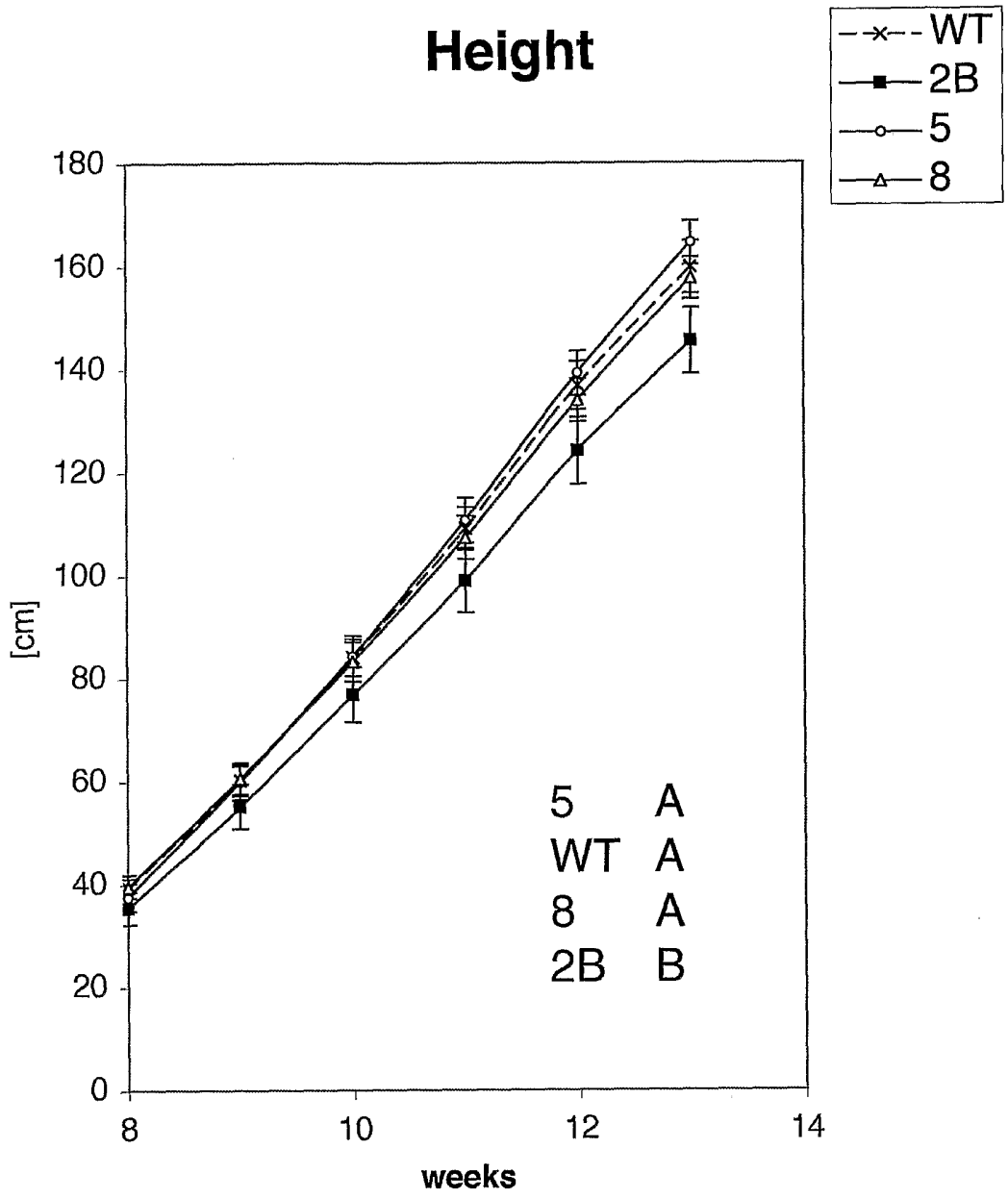

Height
WT
2B
5
8
180
160
140
120
100
80
60
40
20
0
[cm]
8
10
12
14
weeks
5 A
WT A
8 A
2B B

Fig. 8A

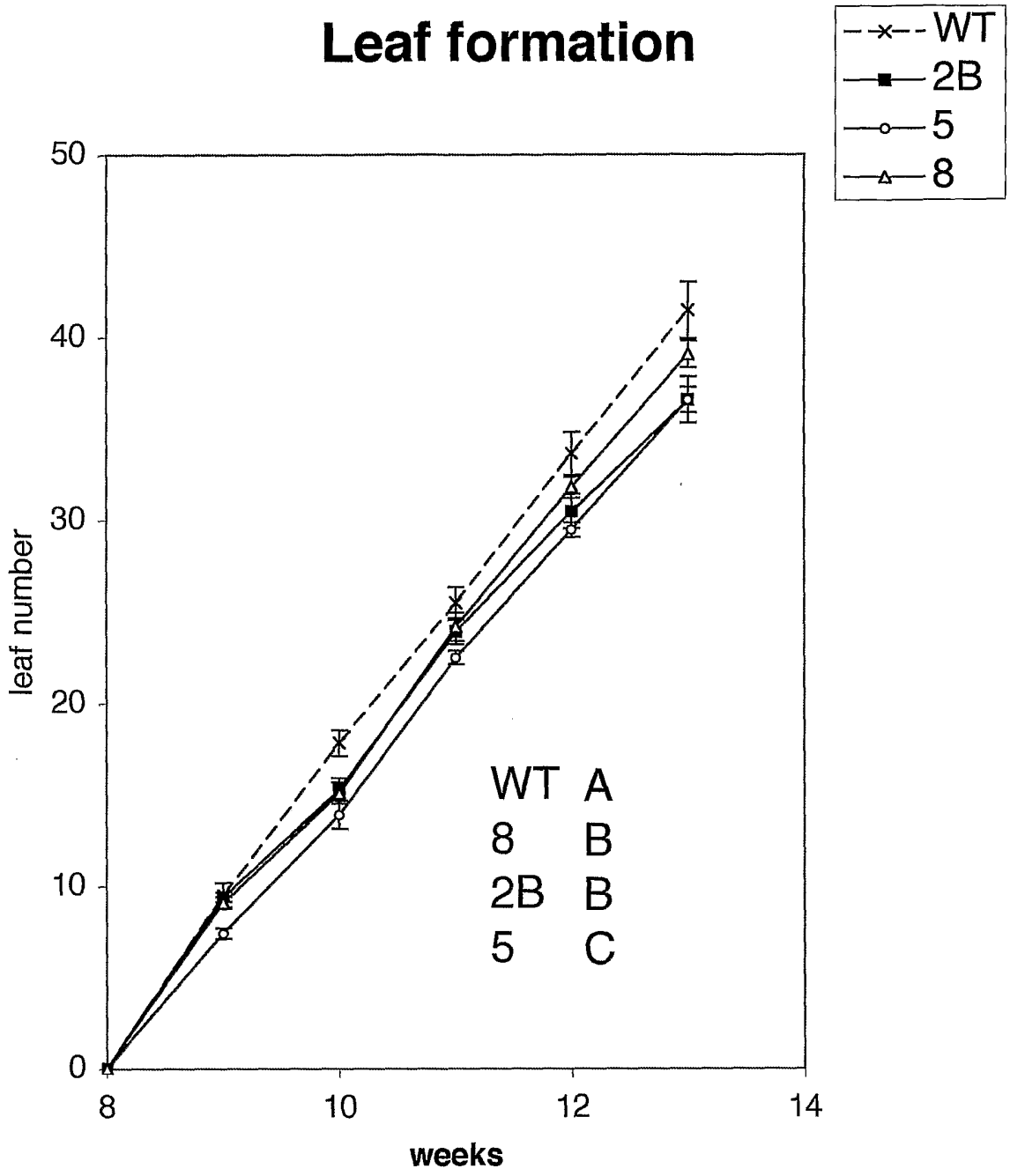

Leaf formation
WT
2B
5
8
50
40
30
20
10
0
leaf number
8
10
12
14
weeks
WT A
8 B
2B B
5 C

Fig. 8B

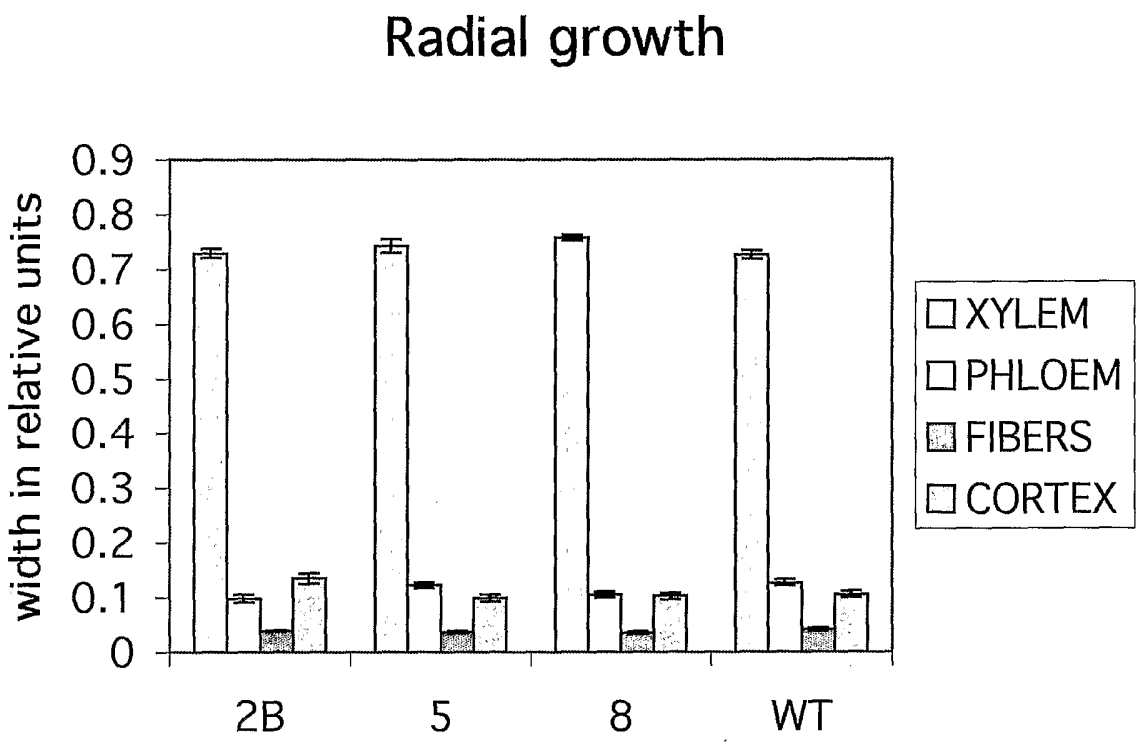

Radial growth
width in relative units
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
2B
5
8
WT
XYLEM
PHLOEM
FIBERS
CORTEX

Fig. 9

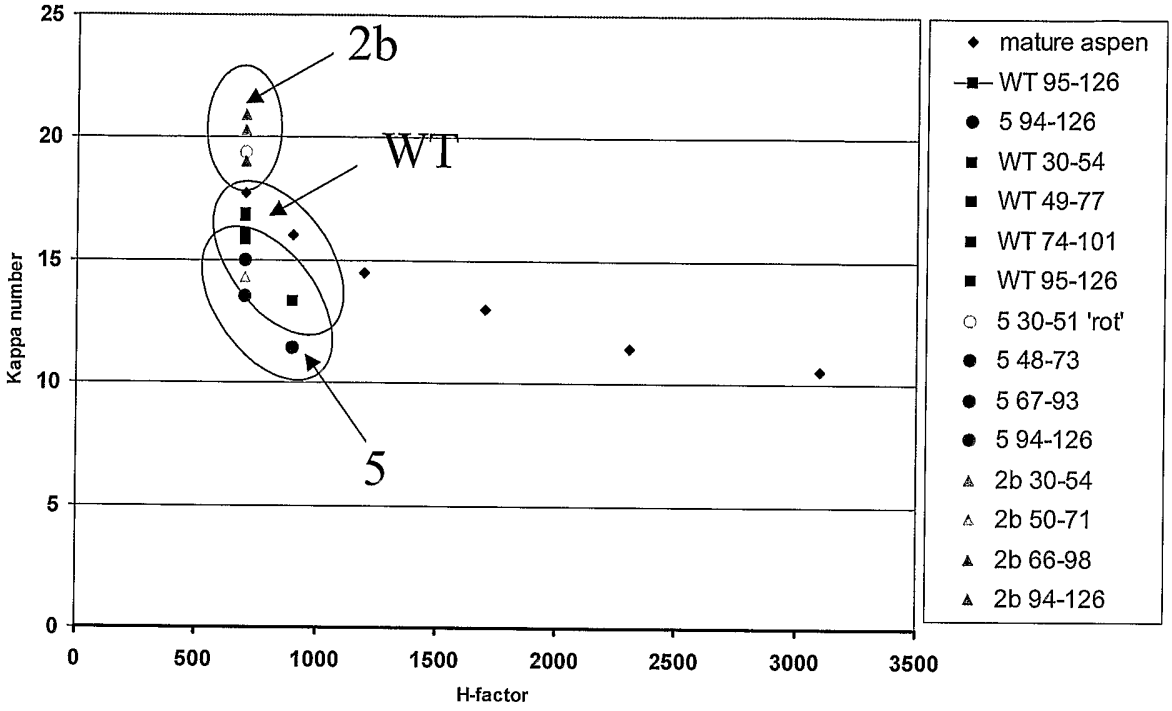
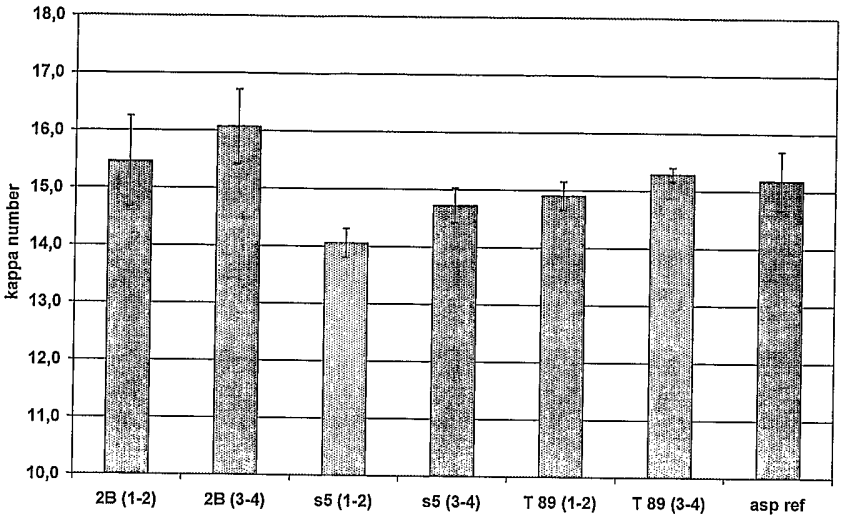
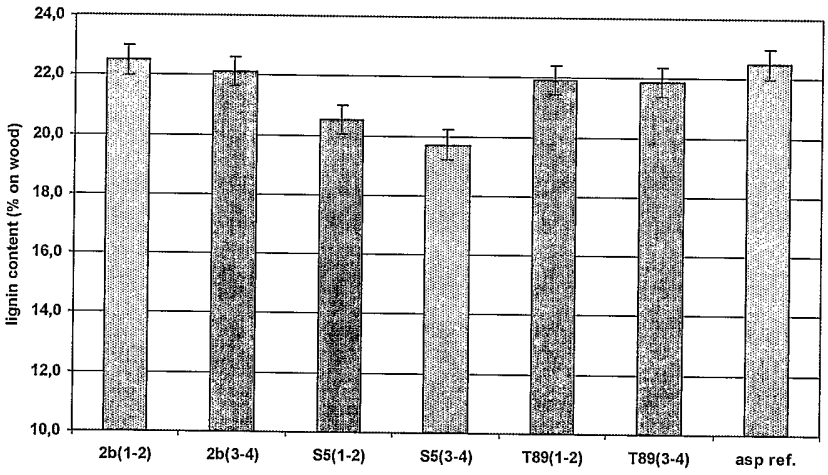

25
20
15
10
5
0
Kappa number
0
500
1000
1500
2000
2500
3000
3500
H-factor
2b
WT
5
mature aspen
WT 95-126
5 94-126
WT 30-54
WT 49-77
WT 74-101
WT 95-126
5 30-51 'rot'
5 48-73
5 67-93
5 94-126
2b 30-54
2b 50-71
2b 66-98
2b 94-126

Fig. 10

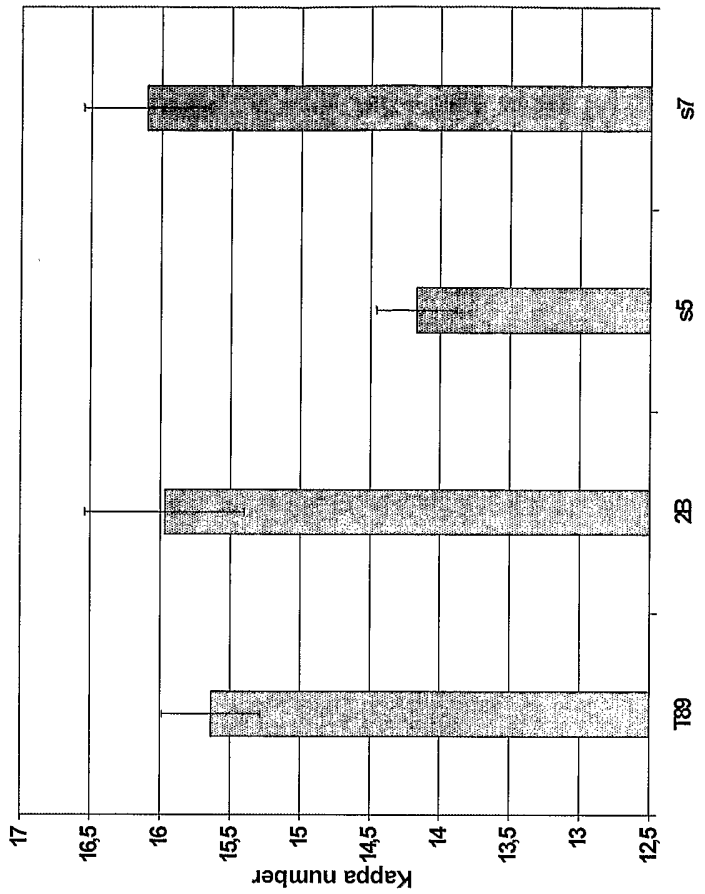

18,0
17,0
16,0
15,0
14,0
13,0
12,0
11,0
10,0
kappa number
2B (1-2)
2B (3-4)
s5 (1-2)
s5 (3-4)
T 89 (1-2)
T 89 (3-4)
asp ref

(a)


24,0
22,0
20,0
18,0
16,0
14,0
12,0
10,0
lignin content (% on wood)
2b(1-2)
2b(3-4)
S5(1-2)
S5(3-4)
T89(1-2)
T89(3-4)
asp ref.

(b)

Fig. 11


Kappa number
17
16,5
16
15,5
15
14,5
14
13,5
13
12,5
T89
2B
S5
S7

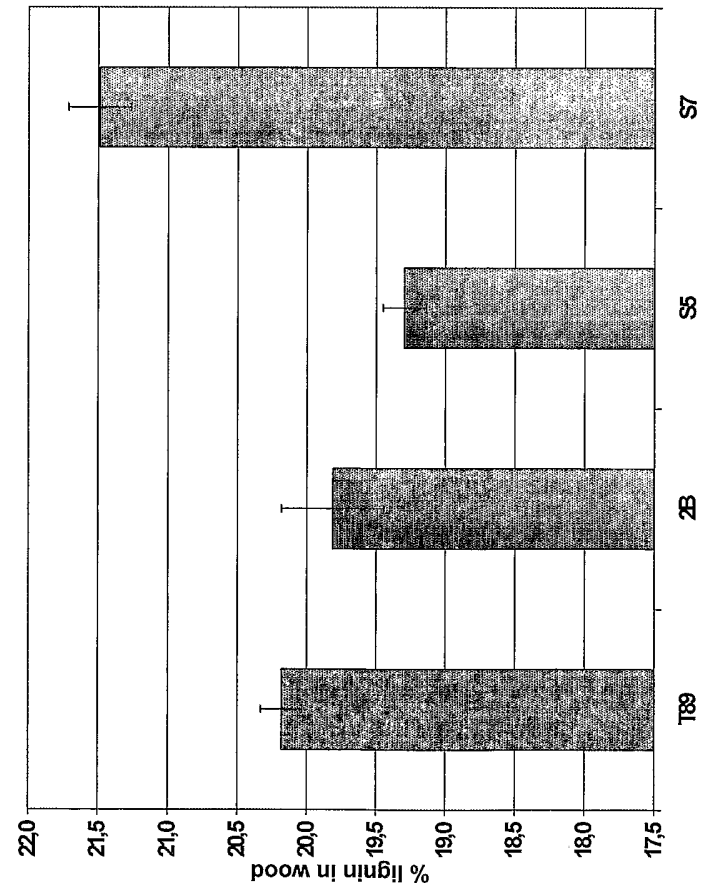
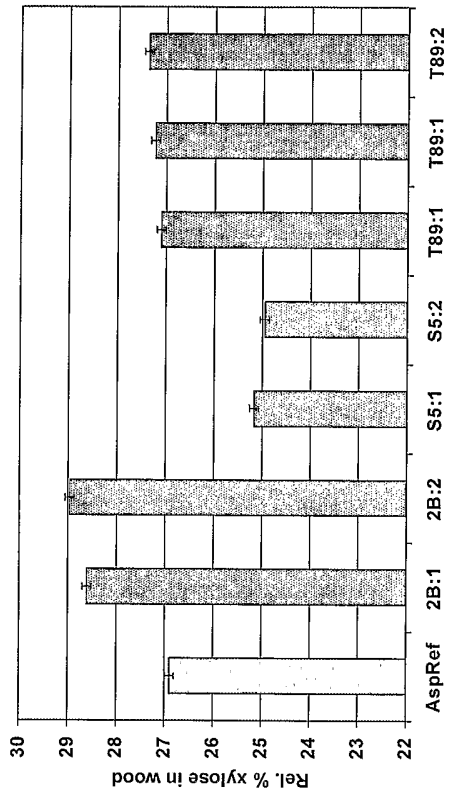
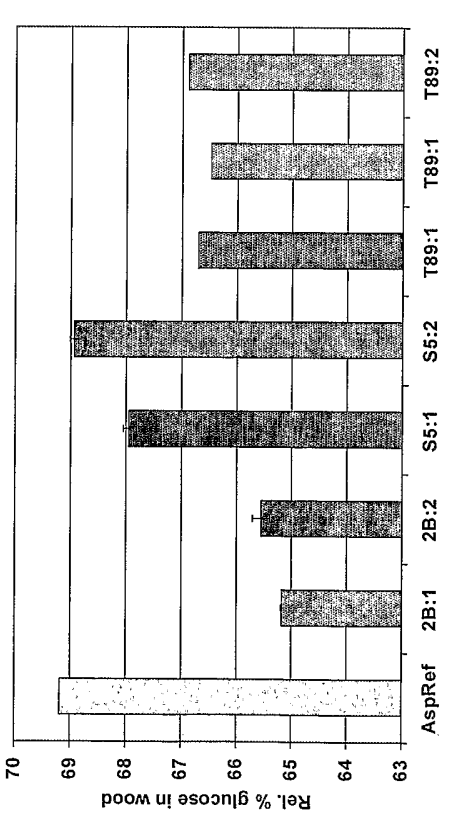
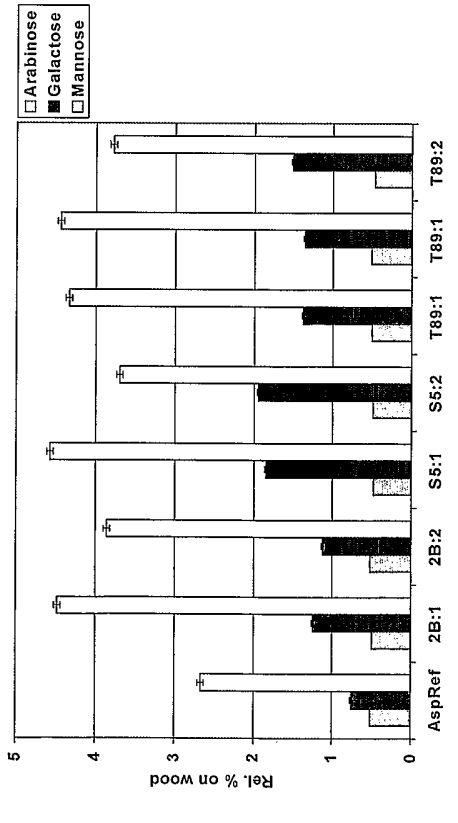

% lignin in wood
22,0
21,5
21,0
20,5
20,0
19,5
19,0
18,5
18,0
17,5
T89
2B
S5
S7

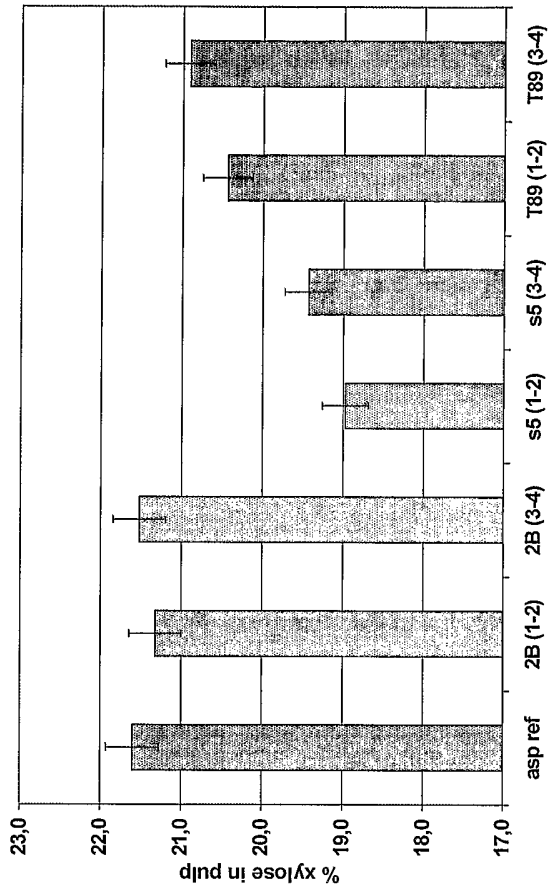
Fig. 12

Fig. 13


Rel. % xylose in wood
30
29
28
27
26
25
24
23
22
AspRef
2B:1
2B:2
S5:1
S5:2
T89:1
T89:1
T89:2


Rel. % glucose in wood
70
69
68
67
66
65
64
63
AspRef
2B:1
2B:2
S5:1
S5:2
T89:1
T89:1
T89:2


Arabinose
Galactose
Mannose
Rel. % on wood
5
4
3
2
1
0
AspRef
2B:1
2B:2
S5:1
S5:2
T89:1
T89:1
T89:2


% xylose in pulp
23,0
22,0
21,0
20,0
19,0
18,0
17,0
asp ref
2B (1-2)
2B (3-4)
s5 (1-2)
s5 (3-4)
T89 (1-2)
T89 (3-4)

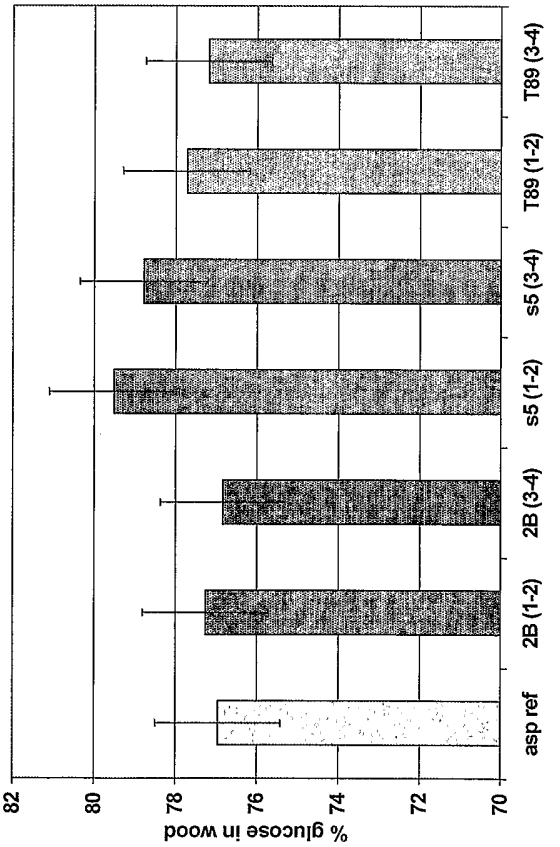
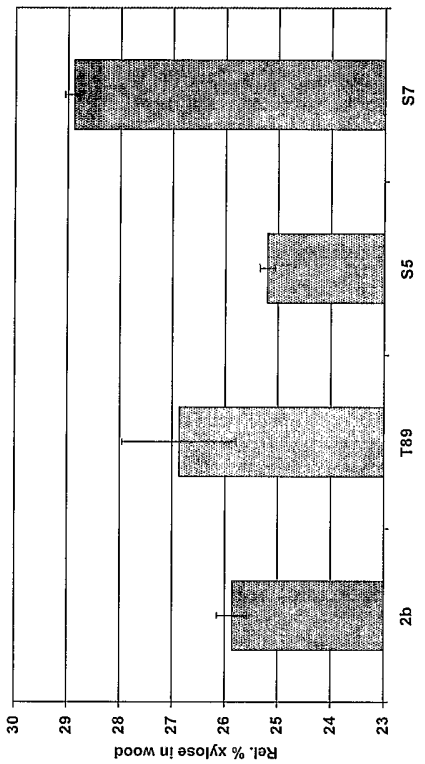
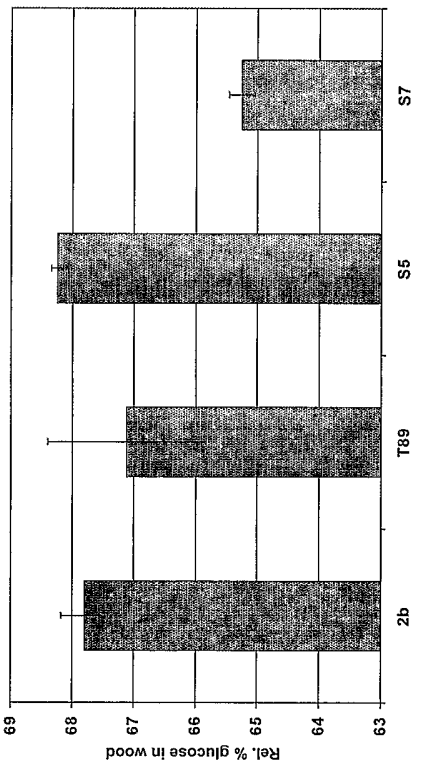

% glucose in wood
82
80
78
76
74
72
70
asp ref
2B (1-2)
2B (3-4)
s5 (1-2)
s5 (3-4)
T89 (1-2)
T89 (3-4)

Fig. 14


Rel. % xylose in wood
30
29
28
27
26
25
24
23
2b
T89
S5
S7


Rel. % glucose in wood
69
68
67
66
65
64
63
2b
T89
S5
S7

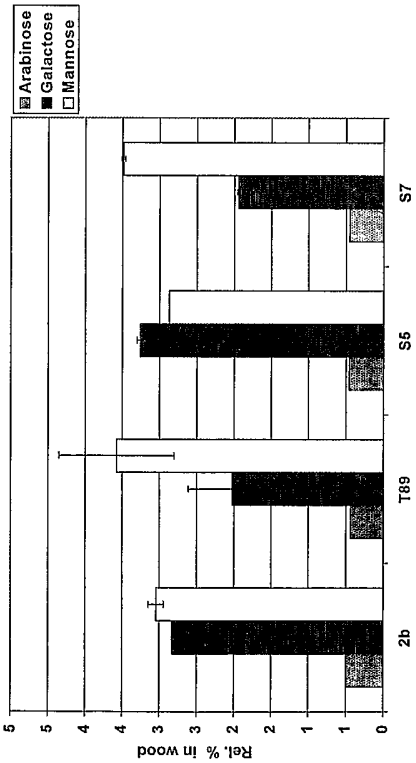
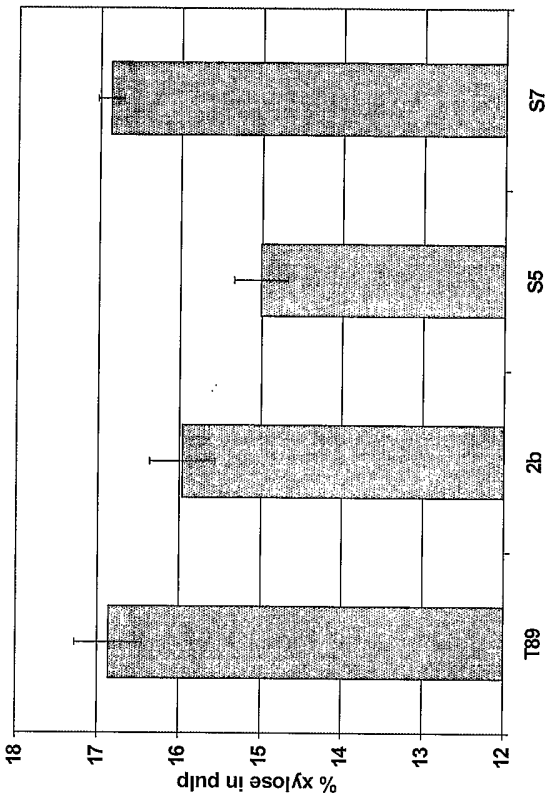

Arabinose
Galactose
Mannose
Rel. % in wood
5
5
4
4
3
2
2
1
1
0
2b
T89
S5
S7

Fig. 15


% xylose in pulp
18
17
16
15
14
13
12
T89
2b
S5
S7

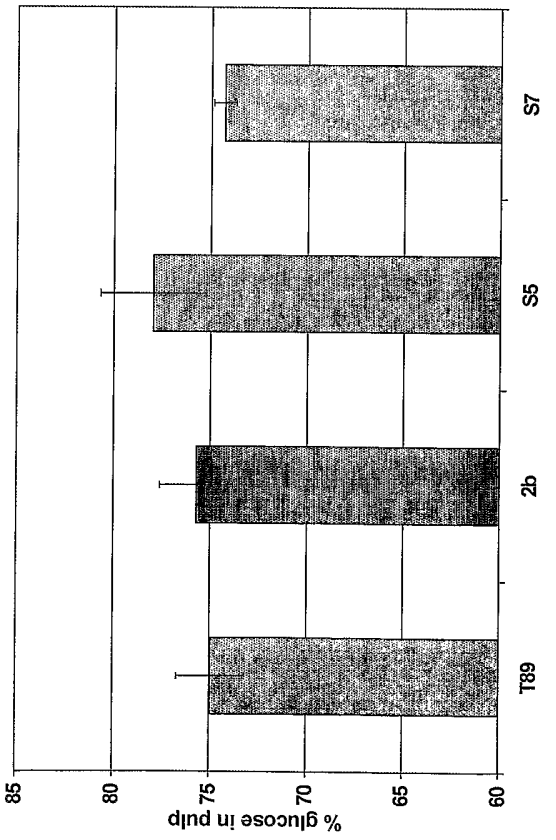

% glucose in pulp
85
80
75
70
65
60
T89
2b
S5
S7

Fig 16

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2005/001983

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

.../...

1. ☒ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☒ No protest accompanied the payment of additional search fees.

Box III

1: Claims 1 (partly), 2-4, 7-22 (partly), 23-25 and 28-41 (partly) directed to a transgenic plant exhibiting altered lignin content, lignin composition or extractability of lignin characterized in that the expression of a pectin methyl esterase (PME) gene or the PME enzyme level or activity is modulated in at least one cell of said plant. Method for producing said transgenic plant.

2: Claims 1 (partly), 5-6, 7-22 (partly), 26-27 and 28-41 (partly) directed to a transgenic plant exhibiting fibre length characterized in that the expression of a pectin methyl esterase (PME) gene or the PME enzyme level or activity is modulated in at least one cell of said plant. Method for producing said transgenic plant.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2005/001983

A. CLASSIFICATION OF SUBJECT MATTER

IPC: see extra sheet
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: A01H, C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-INTERNAL, WPI DATA, PAJ, BIOSIS, MEDLINE, EMBASE, CHEM ABS DATA, SEQUENCE SEARCH (EBI)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 03098186 A2 (THE REGENTS OF THE UNIVERSITY OF CALIFORNIA), 27 November 2003 (27.11.2003), abstract, claims; paragraphs (0007); (0009); (0013); (0021); (0041)-(0044); (0050); (0051); (0057);-(0058); (0085)-(0091); (0067)-(0073)	1-17,19-38
Y		18,39-41
A		1-41
	--	
X	WO 0216613 A2 (INSTITUTO DE CIENCIA APLICADA E TECNOLOGIA (ICAT)), 28 February 2002 (28.02.2002), page 4, line 12 - line 19; page 9, line 17 - line 27; page 10, line 7 - line 8, page 8, line 6 - line 9; abstract	1-10,19-30
	--	

[X] Further documents are listed in the continuation of Box C. [X] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
10 March 2006	06 -04- 2006
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Terese Sandström/Els Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2005/001983

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 20030229922 A1 (LEONARD N. BLOKSBERG), 11 December 2003 (11.12.2003), paragraphs (0014); (0015); (0019); (0020); (0031); table 1	1-15,19-36
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X	WEN, FUSHI ET AL, "Effect of Pectin Methylesterase Gene Expression on Pea Root Development", The Plant Cell, June 1999, vol. 11, page 1129 - page 1140, abstract	1-6,22-25
A		1-41
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X	PILLING, JENS ET AL, "Expression of a Petunia inflata pectin methyl esterase in Solanum tuberosum L. enhances stem elongation and modifies cation distribution", Planta, 2000, vol. 210, page 391 - page 399, abstract	1-2,6,22-23, 27
A		1-41
	--	
Y	Database "Nucleotide", (Online), accession no. AJ277547, 3 August 2000, retrieved from database host NCBI	18,39-41
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A	MELLEROWICZ, EWA J. et al "Unravelling cell wall formation in the woody dicot stem", Plant Molecular Biology, 2001, vol. 47, page 239 - page 274, page 259, column 1, paragraph 3 - page 263, column 2, paragraph 3	1-4,7-25, 28-41
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A	WEN-JING HU et al, "Repression of lignin biosynthesis promotes cellulose accumulation and growth in transgenic trees", Nature Biotechnology, August 1999, vol. 17, pages 808-812	1-4,7-25, 28-41
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/SE2005/001983

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 0166777 A1 (SWETREE GENOMICS AB), 13 Sept 2001 (13.09.2001) --	1,5-22,26-41
A	MARIA ISRAELSSON et al, "Changes in gene expression in the wood-forming tissue of transgenic hybrid aspen with increased secondary growth", Plant Molecular Biology, 2003, vol. 52, pages 893-903 -- -------	1,5-22,26-41

International patent classification (IPC)

C12N 15/82 (2006.01)
A01H 5/00 (2006.01)
A01H 5/04 (2006.01)
A01H 7/00 (2006.01)
C12N 9/18 (2006.01)

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Paper copies can be ordered at a cost of 50 SEK per copy from PRV InterPat (telephone number 08-782 28 85).

Cited literature, if any, will be enclosed in paper form.

INTERNATIONAL SEARCH REPORT
Information on patent family members

31/12/2005

International application No.
PCT/SE2005/001983

WO	03098186	A2	27/11/2003	AU	2003234587	A	02/12/2003
				US	20030221218	A	27/11/2003
				US	20040006794	A	08/01/2004
WO	0216613	A2	28/02/2002	AU	8273101	A	04/03/2002
				BR	0113366	A	29/07/2003
				EP	1322770	A	02/07/2003
				IL	154455	D	00/00/0000
				PT	102511	A	28/02/2002
				US	20040049809	A	11/03/2004
US	20030229922	A1	11/12/2003	AU	777500	B	21/10/2004
				AU	1083700	A	01/05/2000
				BR	9914437	A	16/10/2001
				CA	2345046	A	20/04/2000
				CN	1344325	A,T	10/04/2002
				EP	1123404	A	16/08/2001
				JP	2002527056	T	27/08/2002
				NZ	511049	A	25/06/2004
				WO	0022092	A	20/04/2000
				ZA	200102535	A	01/10/2001
WO	0166777	A1	13/09/2001	AU	4131701	A	17/09/2001
				BR	0108987	A	03/06/2003
				CA	2400986	A	13/09/2001
				CN	1416468	A,T	07/05/2003
				EE	200100565	A	17/02/2003
				EP	1180297	A	20/02/2002
				EP	1261726	A	04/12/2002
				JP	2003525618	T	02/09/2003
				NO	20015022	A	18/12/2001
				NZ	521066	A	26/11/2004
				SE	0000751	D	00/00/0000
				US	20030172402	A	11/09/2003
				ZA	200206789	A	23/11/2004