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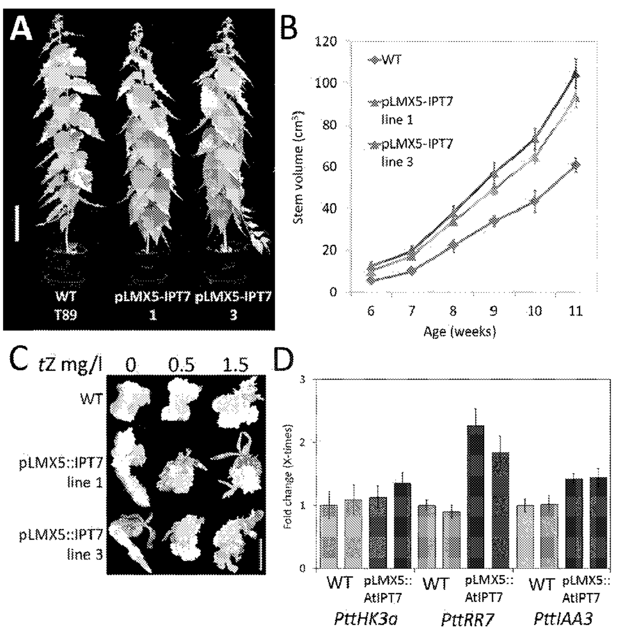
(54) Title: A METHOD FOR IMPROVING STEM VOLUME GROWTH AND BIOMASS PRODUCTION IN TREES



Figs. 5A - 5D

(57) Abstract: The present invention relates to a genetic construct comprising a nucleic acid sequence encoding cytokinin biosynthetic isopentenyl-transferase enzyme (IPT) operable linked to a promoter allowing expression of said nucleic acid sequence in cambial cells. The invention relates also a method for producing a transgenic plant capable of increased biomass production and/or increased stem volume growth compared to wild type plant and a method for improving the production of biomass and/or increased stem volume growth in trees, as well as to a tree that over expresses an endogenous or exogenous nucleic acid sequence encoding IPT in cambial cells and a wood product obtainable from the transgenic tree.

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A METHOD FOR IMPROVING STEM VOLUME GROWTH AND BIOMASS PRODUCTION IN TREES

Field of the invention

The present invention relates to a method for producing a transgenic plant capable of increased stem volume growth and/or biomass production and also to a method for improving the stem volume growth and/or the production of biomass in trees. The invention relates also to a genetically modified tree, a wood product derived from said tree, genetic constructs and vectors and a tree expressing said genetic constructs and vectors.

Background of the invention

Activity of vascular cambium, the lateral meristem of woody plant species, gives rise to the secondary vascular tissues. Cambial meristem forms a thin cylinder along a tree trunk (or a root or branch), and it produces new vascular tissues both inwards and outwards. These tissues, secondary xylem and phloem, form the bulk of lateral growth in plant organs. The conducting vascular cells in both of them acquire their final functional form gradually, through a multi-step differentiation process. The developing xylem cells will undergo expansion, secondary cell wall formation, programmed cell death and final lignification. Similarly, functional phloem cells will be formed through the succession of several developmental steps, including the differentiation of sieve elements and companion cells. These multi-step differentiation programs form two oppositely oriented developmental gradients across the cambial region; the further apart a phloem or xylem cell is from the meristematic middle, the more advanced it is in its differentiation process. Remarkably, the core of cambial meristem; the actively dividing cells, retain their meristematic nature and remain undifferentiated into either form of vascular tissues. Periclinal cell divisions both renew the population of meristematic cells and provide nascent material for vascular tissue differentiation programs, whereas anticlinal divisions enable the creation of novel cell files and expansion of the cambial circle.

The scale of secondary development is highly different in tree species; they display an extreme and economically highly valuable capacity for wood production during their long lifespan. Potentially as an adaptation for the massive secondary growth, the wood of most trees also contains an extensive lateral transport system, the vascular ray network. Other novel challenges for the cambial function of perennial tree species are presented by the annual activity-dormancy cycle. To ensure their survival, trees must adapt their cambial activity to the yearly cycle of cold and warm (or wet and dry) seasons. They must be able to activate their cambial meristem in the spring and deactivate it into a dormant resting stage during the autumn.

It would be highly valuable for the economy of wood production, if the growth of trees could be improved and if, in particular, the stem volume could be enhanced.

Cytokinin signaling has been shown to be required for cambial function. Transgenic Populus trees with impaired cytokinin signaling displayed compromised radial growth caused by a decreased number of cell divisions in the vascular cambium (Nieminen et al., 2008). In addition, genes encoding cytokinin receptors and cytokinin primary response genes were abundant in the cambial region of Populus stem (Nieminen et al., 2008).

Although it is known that cytokinin signaling is connected to tree biomass production, the picture is complicated, since there are at least some 100 cambium enriched and cytokinin regulated genes with several functions. It is not known which of these genes are needed for radial growth of stem cells (Tuskan et al., 2006).

To add further complexity the hormonal regulation of cambium, studies in other tissues have revealed a highly interconnected network between cytokinin and auxin signaling (El-Showk et al., 2013). Cytokinin can affect both auxin bio-synthesis and transport. Interestingly, this regulation appears to be highly complex, as there have been several reports about both positive and negative effects of cytokinin on auxin biosynthesis. Similar results have been obtained about the effect of cytokinin on auxin transport, where this hormone has been reported to both up- and downregulate auxin transporter levels. Most probably these diverse results reflect fine-tuned regulation patterns; cytokinin may have different effect on different auxin biosynthetic enzymes and transporters, most probably on a tissue-specific manner. On top of that, also auxin is known to have a similarly complex role in the regulation of cytokinin biosynthesis and signaling.

International patent publication WO 2006/034286 describes compositions and methods which employ isopentenyl transferase (IPT) polypeptides and poly-nucleotides that are involved in modulating plant development. In the methods described expression of the IPT maintains or improves for example the stress tolerance of the plant, maintains or increases the size of the plant, maintains seed set, or increases shoot growth.

Although some attempts have been made in the prior art to improve plant growth, there is still a need for methods and constructs which could be used to improve tree growth, in particular to improve stem volume growth and biomass production.

Summary of the invention

One object of the present invention is to provide a solution to the problems encountered in the prior art. Specifically, the present invention aims to provide a solution how to improve the growth of trees. Furthermore, the present invention aims to increase the stem volume growth and production of biomass in trees.

In particular, it is one object of the present invention to provide a solution, which improves radial growth in trees.

To achieve these objects the invention is characterized by the features that are enlisted in the independent claims. Other claims represent the preferred embodiments of the invention.

The invention is based on the finding that it is possible to enhance the cell division in the cambial cells by enhancing the cytokine signaling in cambial cells. More specifically, it is possible to enhance the cell division in cambial cells by allowing expression of specific genes encoding cytokinin biosynthetic isopentenyl-transferase enzyme in cambial cells.

It has now been surprisingly found that by enhanced expression of cytokinin biosynthetic isopentenyl-transferase enzyme in cambial cells results in enhanced stem volume growth and/or increased biomass production.

Hence, in one aspect, the present invention provides a genetic construct comprising a first nucleic acid sequence (effector) encoding cytokinin biosynthetic isopentenyl-transferase enzyme operable linked to a second nucleic acid sequence (promoter) allowing expression of said first nucleic acid sequence in cambial cells as defined in claim 1.

The present invention provides in another aspect a vector comprising the genetic construct as defined in claim 7.

Hence, in a third aspect, the present invention provides a tree which overexpresses an endogenous nucleic acid sequence, or expresses an exogenous nucleic acid sequence, encoding cytokinin biosynthetic isopentenyl-transferase enzyme in cambial cells as defined in claim 8.

In a fourth aspect, the present invention provides a wood product obtainable from the tree as defined in claim 16.

In a fifth aspect, the present invention provides a method for producing a transgenic plant capable of increased biomass production and/or increased stem volume growth compared to wild type plant as defined in claim 17.

In a sixth aspect, the present invention provides a method for improving the production of biomass and/or increased stem volume growth in trees as defined in claim 18.

Brief description of the Figures

Fig. 1. Phylogenetic tree indicating the average distance of various IPTs, AtIPT5 being the closest Arabidopsis ortholog for the AtIPT7.

Fig. 2. Conserved domains within IPTs: domains A, B and C from different origin and the corresponding domains A’, B’ and C’ in *Arabidopsis thaliana*. x means any amino acid, x in parentheses (x) means an amino acid not required. Brackets denote any one of the amino acid residues in brackets [ ]

Fig. 3. Comparison of the amino acid sequences of AtIPT7 and AtIPT5 orthologs and the consensus sequence with over 50% similar identity (capital letters indicate amino acids with 100% identical amino acids, whereas lower-case letters indicate identical amino acids in 50-90% of the compared sequences).

Fig. 4. Part of the transformation vector inserted into the plant genome (ca. 8200bp). The construct map shows the different sites, together with their origin, estimated size and function.

Fig. 5A. Phenotypes of WT and pLMX5-IPT7 line 1 and 3 *Populus* trees in the age of three months.

Fig. 5B. The trunk volume of the transgenic pLMX5-IPT7 *Populus* lines 1 and 3 as compared to the WT.

Fig. 5C. Cytokinin responsiveness assay of the WT and pLMX5-IPT7 lines.

Fig. 5D. Expression of a cytokinin receptor (*PttHK3a*), a cytokinin signaling primary response gene (type-A RR *PttRR7*) and an auxin signaling marker gene (*PttIAA3*) in the WT and pLMX5-IPT7 line 1 stem.

Fig. 6. Cambial anatomy, hormonal content and hormonal signaling profiles of WT (A) and transgenic *Populus* line pLMX5::IPT7 line 1 stem (B). Four fractions (A-D) were collected for the hormonal analysis (A, B).

Detailed description of the invention

The present invention provides transgenic trees having increased stem volume growth and/or biomass production. Genetic constructs and vectors are described useful in producing said transgenic trees as well as methods used in producing these trees.

The present invention provides a genetic construct comprising a first nucleic acid sequence (effector) encoding cytokinin biosynthetic isopentenyl-transferase enzyme operable linked to a second nucleic acid sequence (promoter) allowing expression of said first nucleic acid sequence in cambial cells.

By “a first nucleic acid sequence” is meant here an effector gene, which encodes cytokinin biosynthetic isopentenyl-transferase enzyme. The first nucleic acid sequence is selected from the group of

(a) a nucleic acid sequence comprising SEQ ID NO:1;

(b) a nucleic acid sequence encoding SEQ ID NO:2;

(c) a nucleic acid sequence encoding an amino acid sequence comprising a conserved domain area A, B and/or C having an amino acid sequence selected from the group of SEQ ID NO:3, 4 and 5;

(d) a nucleic acid sequence encoding an amino acid sequence comprising an area D having at least 80% identity, preferably at least 85% identity, more preferably at least 90% identity, still more preferably at least 95% identity to amino acid sequence SEQ ID NO:6 (i.e. amino acids 40-141 of SEQ ID NO:2; see third line in Figure 3);

(e) a nucleic acid sequence encoding an amino acid sequence showing at least 80% identity, preferably at least 85% identity, more preferably at least 90% identity, still more preferably at least 95% identity to SEQ ID NO: 2; and

(f) a nucleic acid sequence encoding an enzyme belonging to enzyme class EC 2.5.1.27.

The invention encompasses also embodiments where the first nucleic acid sequence encodes an amino acid sequence comprising a conserved domain area A’, B’ and/or C’ having an amino acid sequence of domain A’, B’ and/or C’ of *Arabidopsis thaliana* as shown in Figure 2.

Genes encoding cytokinin biosynthetic isopentenyl-transferase enzyme (IPTs) are found in several plant genera and species both in angiosperms and in gymnosperms. When the amino acid sequences of the IPTs have been compared, close homologies have been found in specific domains in different plant genera, see WO 2006/034286. It is therefore possible to find IPTs from different plant genera and species which function in a similar manner as the genes herein described.

Sequence analysis by Kakimoto (2001) of Arabidopsis IPTs AtIPT1-9, in comparison with IPTs from *Agrobacterium tumefaciens*, *Pseudomonas syringae* and *Pantoea agglomerans*, revealed three consensus patterns: domain A (SEQ ID NO:3), domain B (SEQ ID NO:4) and domain C (SEQ ID NO:5). The consensus patterns are shown in Figure 2, where x denotes any amino acid residue, (x) means an amino acid residue not required, brackets denote any one of the amino acid residues in brackets [ ]. The corresponding domain areas A', B' and C' of 9 different IPTs of *Arabidopsis thaliana* are also shown in Figure 2.

Similar conserved domains (shadowed) are present also in the closest AtIPT7 orthologs identified from *Populus trichocarpa* (PtIPT7a eugene3.00041149; PtIPT7b eugene3.00080280; PtIPT5a fgenesh4_pg.C_LG_X000229; PtIPT5b_fgenesh4_pg.C_LG_VIII001825) and Eucalyptus grandis (Eucgr.B01146; Eucgr.G00473; Eucgr.G01887; Eucgr.H03602) genomes as shown in Fig. 3.

In the phylogenetic average distance tree AtIPT7 and AtIPT5 have been shown to form a clade together. AtIPT5 appears to be closest Arabidopsis ortholog for the AtIPT7. Between the AtIPT7 orthologs, the consensus sequence, called here consensus area D, with over 50% similar identity is shown in Figure 3 (capital letters indicate amino acids with 100% identical amino acids, whereas lowercase letters indicate identical amino acids in 50-90% of the compared sequences). In *A. thaliana* IPT7 amino acids 40-141 correspond the conserved sequence, third line in Figure 3 (in Sequence listing SEQ ID NO: 6).

Methods of alignment of nucleic amino acid sequences are well known for a person skilled in the art, for example Smith-Waterman algorithm (modified for speed enhancements) to calculate the local alignment of two sequences. Blast is the most useful tool for identity determination: Basic Local Alignment Search Tool, or BLAST, is an algorithm for comparing primary biological sequence information, such as the amino acid sequences of different proteins or the nucleotides of DNA sequences. A BLAST search enables a researcher to compare a query sequence with a library or database of sequences, and identify library sequences that resemble the query sequence above a certain threshold. Different types of BLASTs are available according to the query sequences. The BLAST program was designed by Stephen Altschul (Altschul, 1990).

The gene encoding cytokinin biosynthetic isopentenyl-transferase enzyme can be selected among genes encoding different IPTs, preferably from the group of genes encoding IPTs, which belong to enzyme class EC 2.5.1.27.

More preferably the gene encoding cytokinin biosynthetic isopentenyl-transferase enzyme comprise a conserved domain area or areas A, B and/or C having an amino acid sequence or sequences selected from the group of SEQ ID NO: 3, 4 and 5.

Still more preferably the gene encoding cytokinin biosynthetic isopentenyl-transferase enzyme comprise a conserved domain area or areas A', B' and/or C' having an amino acid sequence or sequences of the corresponding domain areas A', B' and/or C' shown in Figure 2 of 9 IPTs of *Arabidopsis thaliana*.

Still and still more preferably the gene encoding cytokinin biosynthetic isopentenyl-transferase enzyme comprise an area D having at least 80% identity, preferably at least 85% identity, more preferably at least 90% identity, still more preferably at least 95% identity, more and more preferably at least 98% identity, still more preferably at least 99% identity, most preferably 100% identity to amino acid sequence SEQ ID NO:6, (i.e. with the corresponding area in SEQ ID NO:2).

The other areas of the gene encoding cytokinin biosynthetic isopentenyl-transferase enzyme can vary in broader range than the area encoding conserved domain A, B and /or C or A', B' and/or C' and/or area D. The identity % in these areas can be less than 80%, less than 75%, less than 70%, less than 60%, or even less than 50%.

In preferred embodiments of the invention a gene encoding cytokinin biosynthetic isopentenyl-transferase enzyme encodes an amino acid sequence showing at least 80% identity, preferably at least 85% identity, more preferably at least 90% identity, still more preferably at least 95%, more and more preferably at least 98%, still more preferably at least 99%, most preferably at least 100% identity to SEQ ID NO: 2.

A gene encoding cytokinin biosynthetic isopentenyl-transferase enzyme can be selected among genes encoding different IPTs, preferably the gene encodes IPT 7 or IPT 5, more preferably IPT7.

A gene encoding cytokinin biosynthetic isopentenyl-transferase enzyme may be derived from any plant genera or species expressing a functional cytokinin biosynthetic isopentenyl-transferase enzyme. Typically the plant is an angiosperm, preferably an *Arabidopsis*, a *Betula*, a *Populus* or a *Eucalyptus* plant.

The effector gene AT3G23630, *Arabidopsis thaliana* isopentenyltransferase *7 (AtIPT7)* is from *Arabidopsis*, the gene sequence, and functional analysis of a highly orthologous *Arabidopsis* IPT, AtIPT4, protein has been published by Kakimoto 2001.

The present invention has been exemplified by using *Arabidopsis* cytokinin biosynthetic isopentenyl-transferase enzyme IPT7 encoding gene (gene AT3G23630) SEQ ID NO: 1. Said gene encodes amino acid sequence SEQ ID NO: 2. When the amino acid sequence SEQ ID NO: 2 has been compared with IPTs from other sources, it has been found that close homologies can be found in domain area A, domain area B, and/or in domain area C or between different IPTs in *Arabidopsis thaliana*, it has been found that close homologies can be found in domain area A', domain area B', and/or in domain area C' (see Figures 2 and 3). The identity % of these areas between amino acid sequences from different origin is at least 80%, preferably at least 85%, more preferably at least 90%, still more preferably at least 95% even more preferably at least 97%, more and more preferably at least 98%, more and more preferably at least 99%, most preferably 100% identity.

In the present invention it is therefore possible to use genes functioning in similar manner as IPT7 gene from *Arabidopsis*, from several other plant genera and species and/or from different IPTs. It is also possible to use nucleic acid sequences comprising substitutions, insertions, deletions or other modifications compared to SEQ ID NO:1, provided that the nucleic acid sequence encodes cytokinin biosynthetic isopentenyl-transferase enzyme, preferably belonging to enzyme class EC 2.5.1.27. More preferably the enzyme belongs to IPT7.

Nucleic acid sequences encoding cytokinin biosynthetic isopentenyl-transferase enzymes and which are used in the genetic constructs as described herein are typically sequences isolated from their origin, for example *A. thaliana* IPT7 is used in a genetic construct introduced to *Populus* cells to grow a transgenic *Populus* tree. However, it is also possible to enhance the expression of endogenous nucleic acid sequences encoding IPTs.

The genetic construct according to this disclosure comprises a second nucleic acid sequence, which is a promoter allowing expression of cytokinin bio-synthetic isopentenyl-transferase enzyme in meristematic cells of a plant. Preferably the promoter allows expression in cambial cells and apical cells, more preferably specifically in cambial cells.

By “a promoter” is meant a DNA region binding RNA polymerase and directing the enzyme to the appropriate transcription initiation site for a particular poly-nucleotide sequence. A promoter may additionally comprise other recognition sequences referred to as upstream promoter elements, which influence the transcription initiation rate.

An example of a promoter allowing expression in meristematic cells in cambium and in apical cells is birch meristem promoter pBpCRE1. The promoter is preferably defined by SEQ ID NO: 7 (GenBank EU583454, Nieminen et al. 2008).

Another example of a promoter allowing expression in meristematic cells is a promoter allowing expression specifically in cambial cells. Such specifically in cambial cells expressing promoter is *Populus* cambial specific promoter pLM5, preferably defined by SEQ ID NO: 8 (pLM5 promoter is described also in WO2004097024A1 as SEQIDNO4 LMX5 A055P19U).

In the genetic construct the first nucleic acid sequence (effector) is operable linked to the second nucleic acid sequence (promoter). By “operable linked “ is meant that two genetic elements are linked by a functional linkage, for example an effector gene is operable linked to a promoter allowing expression of the effector gene.

A genetic construct can contain also a selectable marker for the selection of cells comprising the introduced genetic construct. Selectable markers are for example antibiotic resistances ampicilline, carbenicilline and hygromysin B resistance.

In the present disclosure the linking of promoter and effector has been exemplified by promoter pLMX5, which has been operably linked to the effector gene by inserting it into the close proximity of the effector gene in the Gateway 2nd box cloning site (Fig.4)

The following Gateway cloning primers have been used:

IPT7_Fwd GW primer:

ACAAAAAAGCAGGCTTAATGAAGTTCTCAATCTCA (SEQ ID NO: 9)

IPT7_REV GW primer:

TACAAGAAAGCTGGGTATCATATCATATTGTGGG (SEQ ID NO: 10)

When the LMX5::AtIPT7 construct (SEQ ID NO:11) has been introduced into trees, transgenic trees with the LMX5::AtIPT7 construct display ectopic over-expression of *Arabidopsis thaliana* adenosine phosphate-isopentenyltransfer-ase 7(IPT; EC 2.5.1.27), expressed in the cambial zone through the LMX5 promoter (described in Love et al. 2009).In the transgenic trees, cytokinin signaling has been stimulated by increasing the amount of cytokinin plant hormone in the cambial zone. Adenosine phosphate-isopentenyltransferase 7 (AtIPT7) enzyme from *Arabidopsis thaliana* catalyzes the first (rate-limiting) reaction in the biosynthesis pathway of isoprene cytokinins. AtIPT7 is expressed at the vascular tissue in *Arabidopsis* (Sakakibara, 2006).

In Figure 4 are presented the following regions:

- LB Left Border: *Agrobacterium tumefaciens*; 25 bp; recognition site for the virulence genes in the Ti-plasmidin; start of the insert (part of the plasmid transferred into the plant genome). Start position the 1st bp.ColE1 (replicon): part of *Escherichia coli* pBR322 plasmid; 615 bp; amplification of the bacte-rial cultures (not expressed in the transgenic plants);
- *β-lactamase*(*bla*)-gene: part of *E. coli* pBR322 plasmid; 861 bp; gene gives an ampicilline/carbenicilline–resistance in bacterial cultures (not expressed in the transgenic plants);

- *pLMX5*: hybrid aspen (*Populus tremula*×*P. tremuloides*); 1807 bp; promotor used for the overexpression of the *adenosine phosphate-isopentenyltransferase* 7 *(AtIPT7)* enzyme gene. Start position the 3000th bp.
- *att*B1: synthetic (Invitrogen-company); 19 bp; recombination site in the Gateway-technique;
- Gene coding for *Adenosine phosphate-isopentenyltransferase* 7 *(AtIPT7)*enzyme: A. thaliana; 990 bp. Start position 4858th bp.
- *att*B:; synthetic (Invitrogen-company); 17 bp; recombination site in the Gateway-technique;
- *pAnos*(non-coding 3'region of the *nopaline synthase*-gene): *A.tumefaciens*; ca. 200 bp[(1)]; polyadenylation signal (signal for the end of the transcription) for the *ERF*-genes;
- *pnos*, *A.tumefaciens*; ca. 200 bp[(1)]; promotor for *hygromycinphosphotransferase* (*hpt*)-gene expression;
- *hpt*; *E. coli*; 1000 bp[(1)]; gene gives a resistance for the hygromysin B used for the selection;
- *pAg4* (T_L-DNA:n gene 4): *A. tumefaciens*; about 200 bp[(1)]; polyadenyation signal for the*hpt*-gene
- RB ('Right border'): *Agrobacterium tumefaciens*; 25 bp; recognition region of the virulence genes in the Ti-plasmid; end of the plant genome insert. [(1)] In the original reference articles (Walden et al., 1990; Koncz et al., 1994) the size of the site is not defined, and it cannot be deduced from other sources.
- Backbone vector similar to *Agrobacterium* binary gap repair vector pGAP-Hyg, (complete sequence: Sequence ID: gb|EU933993.1, length: 7942) and to pBR322.

To introduce the genetic construct into a plant a vector is usually needed. Suitable vector is for example bacterium Agrobacterium tumefaciens.

There are also other systems available for introducing genetic material to plants. Such systems do not necessarily need vector. It is possible for example to introduce genetic material to angiosperm and gymnosperm species through sexual reproduction between trees and by particle bombardment (DNA covered gold particles are shot into cells).

The present invention provides a tree, which overexpresses an endogenous nucleic acid sequence encoding cytokinin biosynthetic isopentenyl-transferase enzyme, or expresses an exogenous nucleic acid sequence encoding cytokinin biosynthetic isopentenyl-transferase enzyme.

As described herein the effector gene needs to be expressed in cambial cells. This is possible by using a promoter allowing expression in meristematic cells generally. However, it is of disadvantage, if the cell division is enhanced in any meristematic cells. If for example the leaves of a tree are grown very large or tight that may of disadvantage, although the stem volume is increased at the same time. According to the present disclosure a promoter allowing expression in cambial cells and apical cells is preferably used, since the overall growth of the tree is not huge, only the stem volume growth and growth of the height. Most preferably a promoter is used, allowing expression specifically in cambial cells. In this case the stem volume growth is increased, but not the overall growth of the tree and not either the height of the tree is increased. All the comparisons are meant to be made to a wild type tree of the same species, age and growth conditions.

The effector gene can be introduced to a tree by using the genetic construct as described herein. Alternatively, the expression of an effector gene being endogenous to a tree can be improved. For example in *Populus* the expression of *Populus* IPT 7 can be improved.

Expression of the gene can be enhanced through ectopic overexpression, by driving the endogenous gene as through an alternative promoter, driving a higher expression level than the endogenous promoter. This can be done by introducing a novel copy of the endogenous gene, under the chosen promoter, into the genome. Alternatively, expression of the endogenous gene can be enhanced through activation tagging, where enhancer elements are introduced into plant genome, where they are able to enhance transcription of genes in their proximity. In the future, enhanced expression of the endogenous gene may also be attained through genome editing, e.g. with engineered nucleases, which can be used to delete silencor elements repressing expression of the desired genes.

A transgenic tree produced as described herein expresses at least 40%, preferably at least 44%, more preferably at least 46%, still more preferably at least 50%, more and more preferably at least 60% higher levels of cytokinin signaling in cambial cells during cambial development compared to a WT tree.

Furthermore, in a transgenic tree produced as described herein the stem volume growth in said tree is at least 35% higher, preferably at least 38%, more preferably at least 40%, still more preferably at least 45%, more and more preferably at least 50% higher compared to wild type (WT) tree.

In one aspect of the invention, the tree expressing an effector gene in cambial cells belongs preferably to angiosperms. The tree is an annual tree or a perennial tree, preferably a perennial tree. The tree belongs to genera *Betula*, *Populus* or *Eucalyptus*. Preferably the tree belongs to genus *Populus. The Populus is selected from the group of Populus* species *P. tremula, P. alba, P. tremuloides, P. canescens, P. deltoids, P. fremontii, P. nigra, P. Canadensis, P. inopina and Populus tremula x tremuloides.* The function of the construct has been tested and confirmed in the hybrid aspen, *Populus tremula x tremuloides.*

In second aspect of the invention the tree expressing an effector gene in cambial cells belongs to gymnosperms. The tree is preferably spruce or pine.

The present invention encompasses also various wood products obtainable from the transgenic trees of the invention. Such wood products are for example trunks, branches, roots and seeds.

The present invention provides also a method for producing a transgenic plant capable of increased biomass production and/or increased stem volume growth compared to wild type plant. The method comprises the steps of

-introducing a nucleic acid sequence encoding cytokinin biosynthetic isopentenyl-transferase enzyme operationally linked to a promoter allowing expression in cambial cells, to a tree cell,

-cultivating said cell to form a cell culture,

-regenerating the cell culture to a plant, in which the nucleic acid sequence encoding cytokinin biosynthetic isopentenyl-transferase enzyme is expressed in cambial cells during cambial development.

Agrobacterium based transformation methods for angiosperm trees have been published by e.g. Häggman et al. 2003, Seppänen et al. 2004 and Nilsson et al. 1992. In general the method comprises that plants explants (leaf discs, stem segments, etc.) are incubated in an *Agrobacterium* culture, after which they are co-cultured with *Agrobacterium* bacteria on a solid culture medium. To end the co-culture, Agrobacterium bacteria are removed by washing. Plants explants are grown on a callus production medium supplemented by an antibiotic to limit the callus production to transgenic cells harbouring the antibiotic resistance gene. The forming callus tissues are transferred onto a regeneration medium for shoot production. The regenerated shoots are transferred onto a root induction medium. After the roots are formed, the plantlets can be grown in soil.

The present invention provides also a method for improving the production of biomass and/or increased stem volume growth in trees. The method comprises the steps of

-introducing a nucleic acid sequence encoding cytokinin biosynthetic isopentenyl-transferase enzyme operationally linked to promoter allowing expression in cambial cells, to a tree cell,

-cultivating said cell to form a cell culture,

-regenerating the cell culture to a plant, in which the gene encoding cytokinin biosynthetic isopentenyl-transferase enzyme is expressed in cambial cells during cambial development,

-allowing said plant to grow to an adult tree having enhanced radial growth compared to wild type tree.

In *Agrobacterium* mediated transformation plant explants are co-cultured with *Agrobacterium* bacteria containing the desired transgene. *Agrobacterium* bacteria will transform plant cells in the explants through the integration of transgenic DNA into the plant genome. Placed on selectable rooting and shooting media, transgenic plants will be regenerated from the transformed cells.

In particle (microprojectile) bombardment method particles of gold or tungsten are coated with DNA and shot into plant cells. Inserted DNA will integrate into the plant genome.

In electroporation method transient holes are formed in plant protoplast membranes using electric shock; this allows transgene DNA to enter plant protoplasts.

In viral transformation (transduction) method the desired transgene is packaged into a suitable plant virus, and the plant is infected by this virus. The transgenic material will integrate into the plant genome.

By “increased biomass production” is meant here the additional amount of biomass (stem dry weight mass) of transgenic trees compared to wild type trees at the same age.

In this description stem dry mass of WT trees was measured at the age of 16 weeks (average of 3 trees) and was 35±2 (STDEV) g, whereas the stem of pLMX5-IPT7 trees (3 trees) was 51±8 g.

By “increased stem volume growth” is meant here the additional amount of stem volume in transgenic trees compared to wild type trees at the same age.

In this description stem volume was measured once per week, 3 measurements points (10 cm above soil level, middle tree, 2 cm below apex), volume was calculated by formula of fructa (sum of basal to middle and middle to apex).

$$V = \frac{\pi h}{3}(r^2 + rR + R^2)$$

wherein V = volume

h = height

r= radius of upper part

R= radius of lower part

http://www.mathwords.com/f/frustum.htm.

Transgenic IPT7 overexpressing trees had more stem volume compared to WT trees (Fig. 5). The stem volume growth in transgenic trees was in average 53% higher, and at least 38% higher, if standard errors were taken into account.

Transgenic trees expressed in average 83% and at least 44%, if standard errors were taken into account, higher levels of cytokinin signaling in cambial cells during cambial development compared to WT trees.

The present invention encompasses also applications where the transgenic tree is sterile tree not capable of flower, pollus or seed development. Methods used to produce sterile trees are known for a person skilled in the art.

Sterile clones of hybrids between two related species with different chromosome numbers (tetraploid crossed with diploid to make a sterile triploid for example) can be selected for transformation. Transgenic trees can be clonally propagated and tested for their sterility (for abolished, aborted or sterile flower, pollen or seed development).

To exemplify the present invention the engineering of transgenic trees displaying an elevated cytokinin signaling level is described herein. Of these trees the status and pattern of auxin and cytokinin distribution and signaling were analyzed.

The concentration of auxin and cytokinin profiles across the cambial meristem in *Populus* stem was characterized. Furthermore, to correlate the cytokinin hormonal profiling with cytokinin signaling, an extensive analysis of the expression profiles of cytokinin biosynthetic and signaling genes across the *Populus* cambial zone was made.

To better understand the interaction between two major hormonal pathways, cytokinin and auxin, in the regulation of cambial cell divisions, their concentration levels across the cambial zone of *Populus trichocarpa* stem were analyzed. Stem cryosections representing phloem, conducting phloem, developing phloem, cambium, developing xylem and xylem tissues (Fig. 6) were analyzed.

To verify the tissue identity of analyzed cryosections, marker genes for various tissue types were included in the analysis. *PtSUC2* was used as a phloem cell marker, *PtANT*, as a marker for dividing cambial cells, and *PtCOMT2* for phloem fibers and xylem cells. The markers correlated well with the identity of the tissues determined through microscopy during the cryo-sectioning.

The present invention is based on a detailed analysis of cytokinin function in the regulation of cambial development in a tree stem. In a manner similar to auxin, also cytokinin hormone has a concentration gradient across the cambial zone. The cytokinin concentration peak coincides with the high expression domain of biosynthetic and signaling genes of this hormone.

With the exception of PtCKI1 genes, expression of all components of the cytokinin biosynthesis and signal transduction pathway in the Populus cambium was detected. Either the effective expression level of the CKI1 genes is very low, below the detection limit of the expression analysis, or they are not required for cambial development during the active growth of Populus trees.

The expression of all components of cytokinin signaling confirms the importance of this hormonal signaling pathway for the activity of vascular cambium.

Interestingly, the cambial distribution profile of cytokinin is distinct, but partially overlapping with, the concentration profiles of auxin. The high auxin concentration is restricted at the domain of actively dividing, undifferentiated cambial cells; whereas the high cytokinin concentration has a larger domain extending from the undifferented cambium to the developing phloem.

In this disclosure has been shown that biomass accumulation in tree stem can be enhanced by stimulating cytokinin signaling in the transgenic *Populus* trees. These trees displayed enhanced cytokinin responsiveness together with an elevated level of cytokinin signaling. The cambial cell division activity of the transgenic trees was increased as compared to the WT trees, and respectively the radial growth of the stem was accelerated. As these trees were of WT height, the stimulatory effect of cytokinin on the radial growth was independent of the apical growth rate. Furthermore, this stimulative action of cytokinin appeared to take place through crosstalk between CK and auxin: an elevated CK concentration and signaling increased the level and widened the domain of auxin concentration and signaling in the cambial region. Potentially the partially overlapping domains of auxin and cytokinin action have specific functions in the regulation of different developmental processes taking place across the cambial zone. Cross-talk between auxin and cytokinin at the middle of the cambium may define the stem cell niche for the maintenance of an actively dividing cell pool. Respectively, possibly the high cytokinin to auxin ratio at the phloem side of the cambial zone contributes to the determination of the phloem identity of the developing vascular cells.

The invention is illustrated by the following non-limiting examples. The invention is applicable to other genes, genetic constructs and plants than those illustrated in examples. It should be understood, however, that the embodiments given in the description above and in the examples are for illustrative purposes only, and that various changes and modifications are possible within the scope of invention.

Examples

Example 1

Engineering of transgenic *Populus* trees with stimulated cambial cytokinin signaling

To study the effect of cytokinin signaling on stem growth, transgenic *Populus* (*P. tremula x tremuloides*) trees were engineered to display elevated cytokinin signaling during cambial development. To stimulate biosynthesis, AtIPT7 gene from *Arabidopsis* encoding a cytokinin biosynthetic isopentenyltransferase was used. The AtIPT7 was expressed under the cambial specific PttLMX5 promoter (Love et al. 2009), which shows a high expression in the cambial and developing xylem cells.

Several separate transgenic lines with the LMX5::IPT7 construct were obtained showing a detectable AtIPT7 expression. No AtIPT7 expression was detected in the untransformed lines. Two lines (AtIPT7 1 and 3) with a high transgene expression level were selected for further analyses.

Example 2

Accelerated radial growth of the tree trunk in the transgenic lines

To evaluate the effect of AtIPT7 activity on tree development, growth dynamics of the transgenic trees was studied under greenhouse conditions (Fig. 5A). The apical growth rate of the pLMX5::AtIPT7 lines was similar to wild-type plants; the transgenic plants had the same height as the controls at the same age (Fig. 5A). In contrast, the diameter of the stem was increased in the transgenic trees as compared to the WT trees. Respectively the stem volume, which was counted as the additive volume of internodes without the leaves, was larger than that of the WT trees (Fig. 5B).

Fig 5A shows the phenotypes of WT and pLMX5-IPT7 line 1 and 3 *Populus* trees in the age of three months. All trees had similar height.

Fig 5B shows the trunk volume of the transgenic pLMX5-IPT7 *Populus* lines 1 and 3 as compared to the WT. The total stem volume of the transgenic lines was increased as compared to the WT. Values are averages (±SD) from five individual trees per each line.

Example 3

Enhanced cytokinin responsiveness of the transgenic lines

To evaluate the effect of cambial AtIPT7 expression on cytokinin signaling, cytokinin responsiveness of the transgenic trees was tested. In the classic cytokinin responsiveness assay (Skoog & Miller 1957), a low cytokinin-to-auxin ratio induces root regeneration from plant segments and a high cytokinin-to-auxin ratio promotes instead shoot regeneration. In this assay, shoot segments were cut from greenhouse grown transgenic and WT lines, and then grown in *in vitro* conditions on a medium with a varying concentration of trans-Zeatin (tZ).

In the assay, a majority of the stem segments from the IPT7 lines produced shoots even in the 0.5 mg/l tZ concentration, whereas only a few WT samples were able to do so (Fig. 5C). This result indicates that the transgenic lines display an elevated basal level of cytokinin signaling, as even a moderate concentration of applied cytokinin can induce shoot production; a typical cytokinin response phenotype. Additionally, the transgenic lines produced roots on the medium with 0 mg/l tZ. As auxin, together with cytokinin, promotes root formation, the result indicates that these lines may have had both more cytokinin and auxin than the control trees.

Fig 5C depicts **c**ytokinin responsiveness assay of the WT and pLMX5-IPT7 lines. Stem segments were grown on a medium with 0.5 mg/L auxin (IAA) and 0, 0.5 or 1.5 mg/L cytokinin t-zeatin. Transgenic lines regenerated shoots already in low cytokinin concentrations (0.5 mg/L), whereas WT required a higher (1.5 mg/L) concentration of this hormone.

Example 4

Elevated cambial cytokinin signaling levels in the transgenic trees

The status of cambial cytokinin signaling in the transgenic trees was studied. The expression levels of two cytokinin marker genes were analyzed. Two marker genes were used to evaluate the cytokinin signaling level: a cytokinin receptor PttHK3a and a type-A response regulator PttRR7. The level of auxin signaling was studied through an auxin signaling marker gene (PttIAA3). The PttRR7 represents a primary response gene of cytokinin phosphorelay: expression of A-type response regulator genes is upregulated by cytokinin signaling: the expression level of this gene reflects the level of cytokinin response taking place in the analyzed trees. In the IPT7-lines the expression of cytokinin receptor PttHK3a was essentially the same as in the WT trees whereas the expression levels of PttRR7 and PttIAA3 were elevated (Fig. 5D).

Fig 5D depicts the expression of a cytokinin receptor (*PttHK3a*), a cytokinin signaling primary response gene (type-A RR *PttRR7*) and an auxin signaling marker gene (*PttIAA3*) in the WT and pLMX5-IPT7 line 1 stem. The expression levels of *PttRR7* and *PttIAA3* were elevated in the pLMX5-IPT7 line as compared to WT, whereas the expression of *PttHK3a* was not affected. Two individual trees per line were analyzed by qRT-PCR (error bars = SD).

This result shows that the level of cytokinin and auxin signaling was successfully elevated through an elevated CK concentration, whereas the capacity for cytokinin perception had not been modified.

Example 5

Increased number of cambial cell divisions in the transgenic trees

To study the effect of elevated cytokinin signaling on the vascular architecture, the cambial anatomy of transgenic trees was analyzed. Meristematic undifferentiated cambial cells were defined in the cross-sections as the small and flat, thin-walled cells localized in the cambial cell files between the differentiating xylem and phloem cells. The first differentiating xylem and phloem cells were defined as having a larger and more round size. In the IPT7-trees, the vascular cambium contained more meristematic cells in the cambial cell files than the WT trees (24 vs 15) (Fig. 6A-B). Based on the increased cell number, it can be concluded that the cambial cell files were undergoing additional cell divisions, as compared to the WT.

Furthermore, it was studied if; in addition of stimulating the cell division rate, the elevated cytokinin signaling level also affected the morphology of the produced xylem cells. To find this out, the dimensions of the xylem cells, vessels and fibers was analyzed, in macerated stem samples. As compared to the WT trees, the length and width of the xylem cells in the IPT7-trees was not significantly different.

Example 6

Elevated cytokinin signaling affects cambial auxin signaling domain

Next it was studied how the hormonal regulation of cambial meristem reacts to an elevated cytokinin concentration. To study this, the cambial hormone signaling dynamics was profiled through a hormone concentration and marker gene expression studies. The concentrations of bioactive iP and tZ were almost 30% higher in the transgenic trees, whereas the IAA and cZOG concentrations were doubled. Notably, the cytokinin distribution profiles were generally similar between the WT and transgenic line, whereas the shape of auxin distribution was different. Transgenic tree had a wider domain of high auxin concentration: the IAA level was higher in developing xylem and xylem cells than in the WT.

To connect the hormonal profiles with signaling pattern, the expression pattern for auxin and cytokinin signaling marker genes was characterized across the cambial zone. PttRR7 was used as a marker for cytokinin and PttIAA3 for auxin signaling. Similar to the hormone concentration study, cryo-sections representing phloem, developing phloem, cambium, developing xylem and xylem, were analyzed. The PttSUC2 and PttCOMT were used as marker genes for phloem and phloem fibers and xylem cells, respectively, to confirm the identity of the sections. Two wild-type trees and two IPT7 trees were analyzed. In both transgenic and WT trees, the RR7 expression peaks in the developing phloem tissue, where also the phloem marker PtSUC2 has high expression (Fig. 6). Cambium, where the phloem and xylem markers have low expression levels, displays high IAA3 and rising RR7 expression levels. Developing xylem tissue has high IAA3 and a rising COMT expression, whereas in maturing xylem only the xylem marker expression is high.

When the WT and transgenic trees were compared, it can be seen that the IPT7-trees have a wider domain of high auxin signaling. The cambium, which is a domain of high RR7 and IAA3 expression, and developing xylem, a domain of high IAA3 and moderate RR7 expression, tissues are larger in the transgenic lines as compared to the WT tree (Fig. 6). This widened domain of auxin signaling corresponds with the increase in the number of meristematic cells.

Cambial anatomy, hormonal content and hormonal signaling profiles of WT (A) and transgenic *Populus* line pLMX5::IPT7 line 1 stem (B) are shown in Fig. 6. In the IPT7-trees, the vascular cambium contained more meristematic cells in the cambial cell files than in the WT trees (24 vs 15). Four fractions (A-D) were collected for the hormonal analysis. In WT, the 4th fraction represents fully developed xylem cells, whereas in pLMX5::IPT7 it still contains developing xylem cells, indicating that the meristematic cambial zone is wider in the pLMX5::IPT7 stem than in WT.

The hormonal profiles of auxin (IAA) and bioactive cytokinins (iP and tZ) together with a cytokinin storage form (cZOG) were analyzed in four cambial fractions (A-D). The concentrations of bioactive cytokinins iP and tZ were almost 30% higher in the transgenic trees, whereas the IAA and cZOG concentrations were doubled. Notably, cytokinin distribution profiles were generally similar between the WT and transgenic line, whereas the shape of auxin distribution was different. Transgenic tree had a wider domain of high auxin concentration (highlighted by grey shading).

To correlate the hormonal profiles with signaling domains, expression patterns of marker genes were analyzed by qRT-PCR in fourteen cryosections from phloem (1) into the xylem (14) tissues. The letters under the graph indicate the position of the four hormone analysis fractions (A-D). PttSUC2 was used as a phloem marker, a cytokinin primary response gene PttRR7 as a CK signaling marker, PttIAA3 as an auxin signaling marker, and PttCOMT2 as a phloem fiber and xylem identity marker. Based on the PttRR7 expression, the cytokinin signaling level was elevated in the pLMX5::IPT7 tree. The width of high cytokinin concentration domain (fractions 3-7 in both WT and pLMX5::IPT7) was instead not affected. In contrast, the cambial domain with high auxin marker gene expression and decreasing cytokinin marker gene expression (WT fractions 5-7 vs pLMX5::IPT7 fractions 5-11) was wider in the transgenic line than in the WT tree (3 vs 7 fractions). The level of transgene AtIPT7 expression was below detection limit in WT, whereas had a high expression at the cambial zone of the pLMX5::IPT7 tree.

References

Altschul SF; Gish W; Miller W; Myers EW; Lipman DJ. 1990. Basic local alignment search tool. National Center for Biotechnology Information, National Library of Medicine, National Institutes of Health, Bethesda, MD 20894. *J Mol Biol 215: 403-10 (1990)*

El-Showk S, Ruonala R, Helariutta Y. 2013. Crossing paths: cytokinin signalling and crosstalk. Development. Apr;140(7):1373-83. doi: 10.1242/dev.086371.

Häggman H, Frey AD, Aronen T, Ryynänen L, Julkunen-Tiitto R, Tiimonen H, Pihakaski-Maunsbach K, Jokipii S, Chen X, Kallio PT. 2003. Expression of *Vitreoscilla* hemoglobin in hybrid aspen (*Populus tremula* x *tremuloides*). Plant Biotechnology Journal 1: 287-300.

Kakimoto T. Identification of plant cytokinin biosynthetic enzymes as dimethylallyl diphosphate:ATP/ADP isopentenyltransferases. Plant Cell Physiol. 2001 Jul;42(7):677-85.

Koncz C, Martini N, Szabadosz L, Hrouda M, Bachmair A, Schell J (1994) in *Plant Molecular Biology Manual*, edsGelvin SB, Schilperoort RA, Verma DPS (Kluwer, Dordrecht, The Netherlands), Vol B2, pp 1–22.

Love J, Björklund S, Vahala J, Hertzberg M, Kangasjärvi J, Sundberg B. 2009. Ethylene is an endogenous stimulator of cell division in the cambial meristem of Populus. Proceedings of the National Academy of Sciences, USA 106: 5984–5989.

Nieminen K, Immanen J, Laxell M, Kauppinen L, Tarkowski P, Dolezal K, et al. Cytokinin signalling regulates cambial development in poplar. Proc Natl Acad Sci USA 2008;105:20032–7.

Nilsson et al. 1992. Spatial pattern of cauliflower mosaic virus 35S promoter-luciferase expression in transgenic hybrid aspen trees monitored by enzymatic assay and non-destructive imaging. Transgenic Research 1: 209-220.

Sakakibara (2006).Cytokinins: activity, biosynthesis, and translocation. Annual Review of Plant Biology 57:431-449.

Seppänen SK, Syrjälä L, von Weissenberg K, Teeri TH, Paajanen L, Pappinen A. 2004. Antifungal activity of stilbenes in in vitro bioassays and in transgenic Populus expressing a gene encoding pinosylvin synthase. Plant Cell Reporter 22: 584-93.

Skoog F., Miller C.O. (1957). Chemical regulation of growth and organ formation in plant tissue cultures in vitro. Symp. Soc. Exp. Biol. 11: 118–131.

Tuskan et al. (2006).Science. 313(5793):1596-604.

Walden R, Koncz C, Schell J (1990) The use of gene vectors in plant molecular biology. Methods in Molecular and Cellular Biology 4: 175–194.

Claims

1. A genetic construct comprising a first nucleic acid sequence (effector) encoding cytokinin biosynthetic isopentenyl-transferase enzyme operable linked to a second nucleic acid sequence (promoter) allowing expression of said first nucleic acid sequence in cambial cells, said first nucleic acid sequence being selected from the group of

   a) a nucleic acid sequence comprising SEQ ID NO:1;

   b) a nucleic acid sequence encoding SEQ ID NO:2;

   c) a nucleic acid sequence encoding an amino acid sequence comprising a conserved domain area A, B and/or C having an amino acid sequence selected from the group of SEQ ID NO:3, 4 and 5,

   d) a nucleic acid sequence encoding an amino acid sequence comprising an area D having at least 80% identity to amino acid sequence SEQ ID NO:6;

   e) a nucleic acid sequence encoding an amino acid sequence showing at least 80% identity to SEQ ID NO: 2; and

   f) a nucleic acid sequence encoding an enzyme belonging to enzyme class EC 2.5.1.27.

2. The genetic construct according to claim 1, wherein said first nucleic acid sequence encodes adenosine phosphate-isopentenyltransferase enzyme 7, IPT7.

3. The genetic construct according to claim 1 or 2, wherein said first nucleic acid sequence is derived from *Arabidopsis*.

4. The genetic construct according to any one of claims 1 to 3, wherein said second nucleic acid sequence is birch meristem promoter pBpCRE1, preferably defined by SEQ ID NO: 7.

5. The genetic construct according to any one of claims 1 to 4, wherein said second nucleic acid sequence is a cambial specific promoter.

6. The genetic construct according to claim 5, wherein said promoter is *Populus* cambial specific promoter pLM5, preferably defined by SEQ ID NO:8.

7. A vector comprising the genetic construct according to any one of claims 1 to 6.

8. A tree, **characterized** in that it

   (a) overexpresses an endogenous nucleic acid sequence, or

   (b) expresses an exogenous nucleic acid sequence

   encoding cytokinin biosynthetic isopentenyl-transferase enzyme in cambial cells.

9. The tree according to claim 8, wherein said tree expresses the genetic construct according to any one of claims 1 to 6 or the vector of claim 7.

10. The tree according to claim 8 or 9, wherein said tree expresses at least 40%, preferably at least 50% higher levels of cytokinin signaling in cambial cells during cambial development compared to WT tree.

11. The tree according to any one of claims 8 to 10, wherein the stem volume growth in said tree is at least 35% higher, preferably at least 40% higher compared to WT tree.

12. The tree according to any one of claims 8 to 11, wherein said tree belongs to angiosperms.

13. The tree according to claim 12, wherein said tree is selected from the group of genera *Betula*, *Populus* and *Eucalyptus*, preferably *Populus.*

14. The tree according to any one of claims 8 to 13, wherein said tree belongs to gymnosperms.

15. The tree according to claim 14, wherein said tree is selected from the group of spruce and pine.

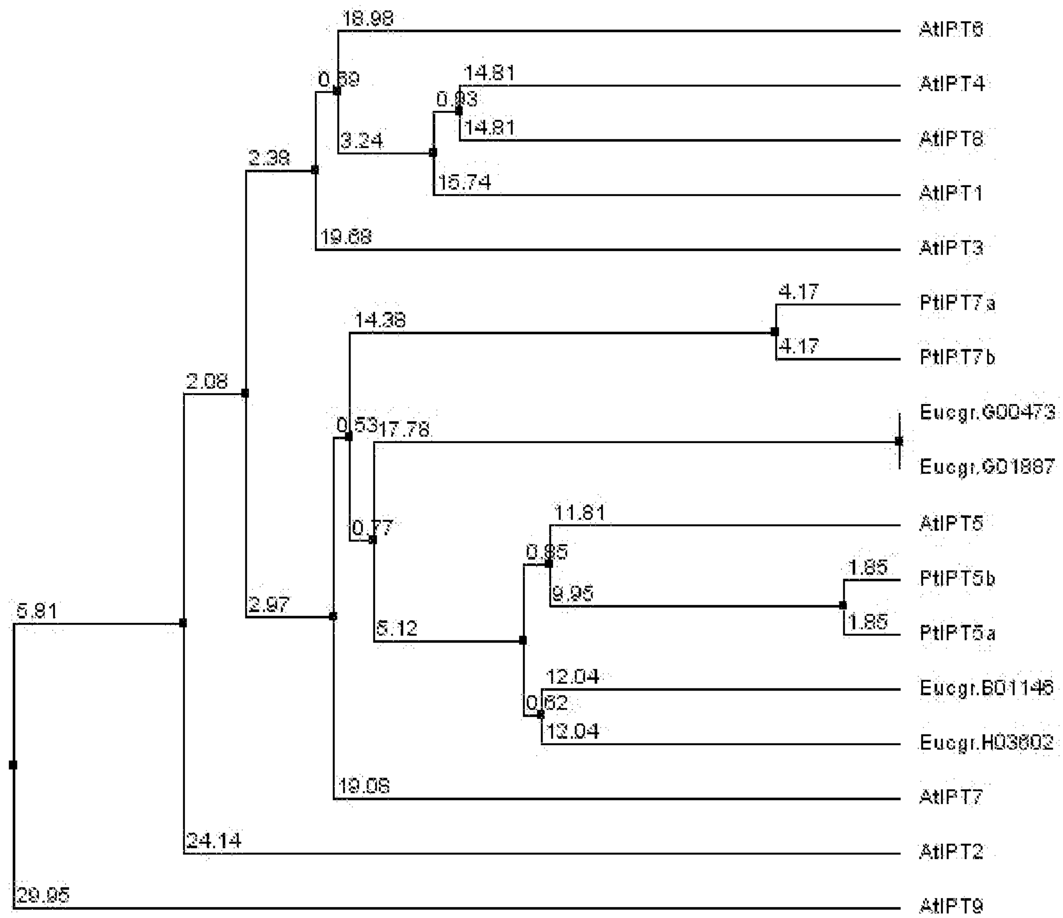
16. A wood product obtainable from the tree as defined in any one of claims 8 to 15.

17. A method for producing a transgenic plant capable of increased biomass production and/or increased stem volume growth compared to wild type plant, which comprises the steps of

- introducing a nucleic acid sequence encoding cytokinin biosynthetic isopentenyl-transferase enzyme operationally linked to a promoter allowing expression in cambial cells, to a tree cell,
- cultivating said cell to form a cell culture,
- regenerating the cell culture to a plant, in which the nucleic acid sequence encoding cytokinin biosynthetic isopentenyl-transferase enzyme is expressed in cambial cells during cambial development.

18. A method for improving the production of biomass and/or increased stem volume growth in trees, which comprises the steps of

- introducing a nucleic acid sequence encoding cytokinin biosynthetic isopentenyl-transferase enzyme operationally linked to promoter allowing expression in cambial cells, to a tree cell,
- cultivating said cell to form a cell culture,
- regenerating the cell culture to a plant, in which the gene encoding cytokinin biosynthetic isopentenyl-transferase enzyme is expressed in cambial cells during cambial development,
- allowing said plant to grow to an adult tree having enhanced radial growth compared to wild type tree.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AtIPT6
0.69
14.81
AtIPT4
0.93
14.81
AtIPT8
3.24
2.39
15.74
AtIPT1
19.68
AtIPT3
4.17
PtIPT7a
14.38
4.17
PtIPT7b
2.08
0.53
17.78
Eucgr.G00473
Eucgr.G01887
11.81
AtIPT5
0.77
0.85
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PtIPT5b
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5.81
2.97
1.85
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5.12
12.04
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0.62
12.04
Eucgr.H03602
19.08
AtIPT7
24.14
AtIPT2
29.95
AtIPT9

Fig. 1

domain A	GxTxxGK[ST] (SEQ ID NO:3)
domain A’	G[ATP]TG[STA]GKS
domain B	[VLI]xxxxxxx[VLI][VLI]xxDxxQ (SEQ ID NO:4)
domain B’	[LI]Ax[RH](x)[FL]xxEI[IV][NS][SA]D[KAS][IMV]Q
domain C	[VLI][VLI]xGG[ST] (SEQ ID NO:5)
domain C’	[IV][IVL][AVT]GG[ST]

Fig.2

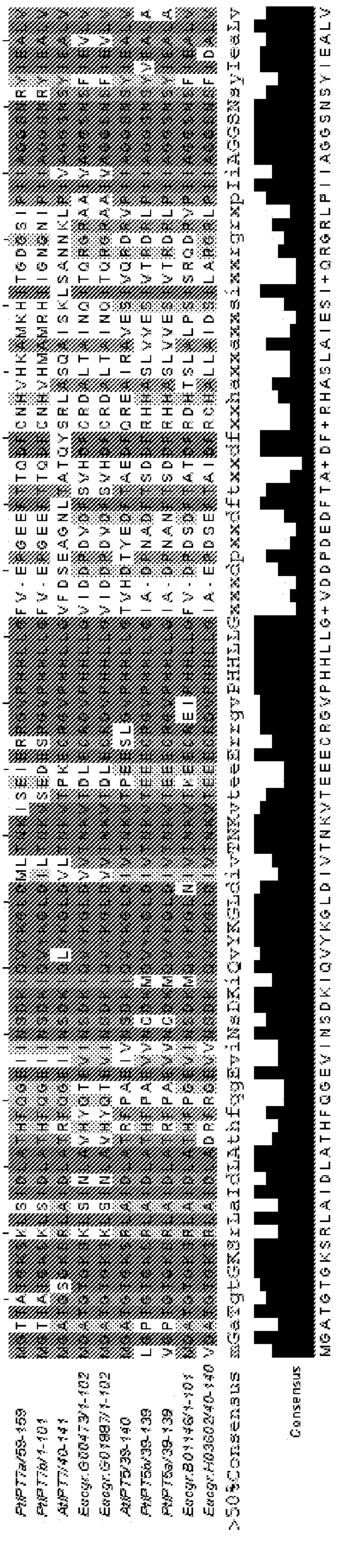


Fig. 3

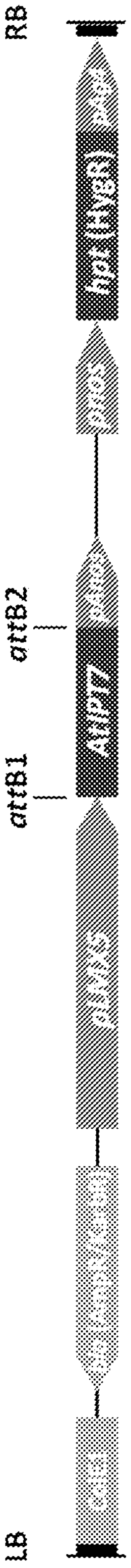
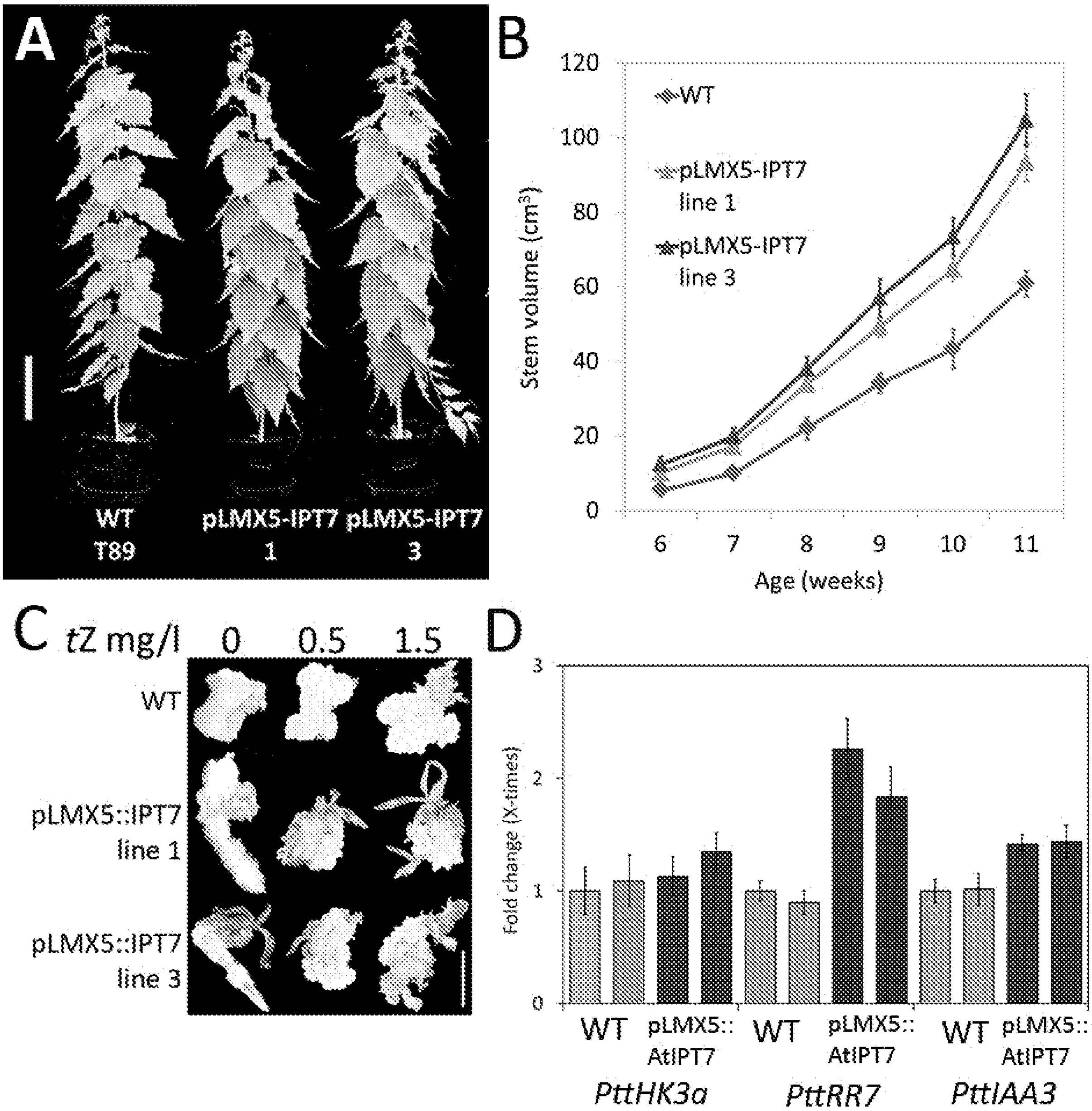


Fig. 4


A
WT
T89
pLMX5-IPT7
1
pLMX5-IPT7
3
B
Stem volume (cm³)
Age (weeks)
WT
pLMX5-IPT7
line 1
pLMX5-IPT7
line 3
C
tZ mg/l
0
0.5
1.5
WT
pLMX5::IPT7
line 1
pLMX5::IPT7
line 3
D
Fold change (X-times)
WT
pLMX5::
AtIPT7
PttHK3a
PttRR7
PttIAA3

Figs. 5A - 5D

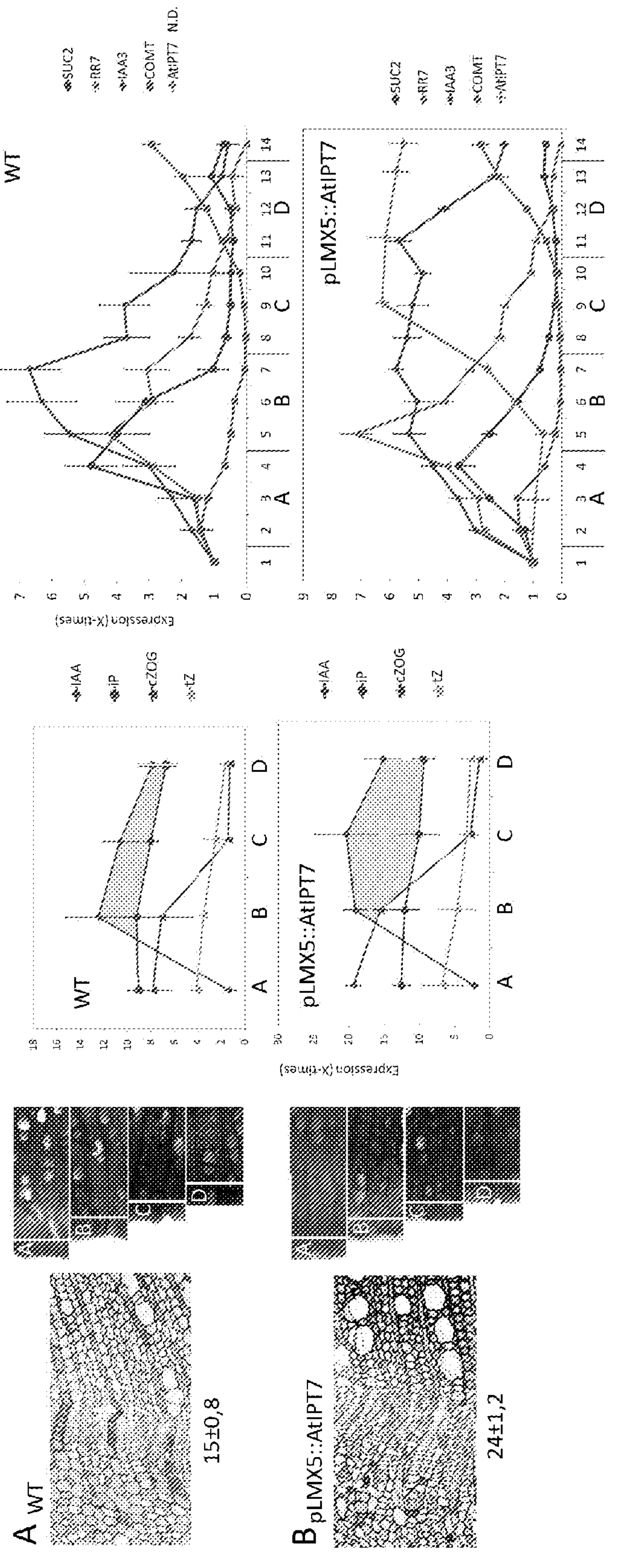

A WT
15±0,8
B pLMX5::AtIPT7
24±1,2
WT
pLMX5::AtIPT7
Expression (X-times)
IAA
iP
cZOG
tZ
SUC2
RR7
IAA3
COMT
AtIPT7 N.D.
AtIPT7

Fig. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/FI2014/051057

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, PAJ, WPI data, BIOSIS, CHEM ABS Data, COMPENDEX, EMBASE, INSPEC, MEDLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Matsumoto-Kitano M. et al. "Cytokinins are central regulators of cambial activity", 2008, In: PNAS, Vol. 105, pp. 20027-20031; page 20029, column 2, paragraph [0003]; page 20030, column 1, line 6 - line 10; page 20030, column 2, paragraph [0002]; figure s8; Abstract	1-3, 7-18
Y	--	4-6
X	Elo. A. et al. "Stem cell function during plant vascular development", 2009, In: Seminars in Cell & Developmental Biology, Vol. 20, pp. 1097-1106; page 1098, column 1, paragraph [0002]; page 1102, paragraph [0042]; Abstract	1-3, 7-18
Y	--	4-6

☒ Further documents are listed in the continuation of Box C. ☒ 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
31-03-2015	01-04-2015
Name and mailing address of the ISA/SE Patent- och registreringsverket Box 5055 S-102 42 STOCKHOLM Facsimile No. + 46 8 666 02 86	Authorized officer Linus Plym Forshell Telephone No. + 46 8 782 25 00

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Nieminen K. et al. "Cytokinin signaling regulates cambial development in poplar", 2008, IN: PNAS, Vol. 105, pp. 20032-20037; page 20033, column 2, line 1 - line 8; page 20034, column 1, paragraph [0002]; page 20037, column 2; Abstract, page 20037, section "constructs" --	4
Y	WO 2004097024 A1 (SWETREE TECHNOLOGIES AB ET AL), 11 November 2004 (2004-11-11); page 1, line 3 - page 1, line 6; figure 1d; claims 1-6; SEQIDNO4 LMX5 A055P19U --	5-6
X	EP 1384776 A1 (SUNTORY LTD ET AL), 28 January 2004 (2004-01-28); paragraphs [0015], [0042]; claims 3-12 --	1-3, 7
Y	EP 1522586 A1 (SUMITOMO CHEMICAL CO), 13 April 2005 (2005-04-13); paragraphs [0006], [0030], [0033], [0037]; RN: 849382-58-7 & 849381-46-0, 2004-10-08 [online] [retrieved on 2015-03-18] Retrieved from: STN, Database Registry, SEQ ID NO:1 show 100% homology (990/990 bp) to sequence 17 of this document. SEQ ID NO:2 show 100% homology (329/329 aa) to sequence 18 of this document and SEQ ID NO:6 is 100% homologous to parts of (102/102 aa) sequence 18 in this document. --	1-3, 7
Y	US 20050183159 A1 (CLARK DAVID G ET AL), 18 August 2005 (2005-08-18); paragraphs [0003]-[0007], [0028]; RN: 340063-16-3, 2005-01-26 [online][retrieved on 2015-03-18] Retrieved from: STN, Database Registry, SEQ ID NO:1 show 99% homology (989/990 bp) to sequence 1 of this document. --	1-2, 7
Y	US 20060064786 A1 (BRUGIERE NORBERT), 23 March 2006 (2006-03-23); paragraph [0035]; claims 6, 37-39; Abstract; RN: 878821-76-2, 2005-09-16 [online][retrieved on 2015-03-18]Retrieved from: STN, Database Registry, SEQ ID NO:2 show 100% homology (329/329 aa) to sequence 37 of this document and SEQ ID NO:6 is 100% homologous to parts of (102/102 aa) sequence 37 of this document. --	1-2, 7

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 2004074442 A2 (MONSANTO TECHNOLOGY LLC ET AL), 2 September 2004 (2004-09-02); claims 22-23; Abstract; RN: 742225-86-1, 2004-02-13 [online][retrieved on 2015-03-18]Retrieved from: STN, Database Registry, SEQ ID NO:2 show 100% homology (329/329 aa) to sequence 35 of this document and SEQ ID NO:6 is 100% homologous to parts of (102/102 aa) sequence 35 of this document. --	1-2, 7
A	EP 1033405 A2 (CERES INC), 6 September 2000 (2000-09-06); paragraph [0003]; RN: 302660-97-5 & 282742-36-3, 2000-02-25 [online][retrieved on 2015-03-18] Retrieved from: STN, Database Registry, SEQ ID NO:1 show 100% homology (990/990 bp) to clone Ceres_2153076 and 99% homology (988/990 bp) to clone Ceres_1396251 of this document. SEQ ID NO:2 show 100% homology (329/329 aa) to clone MDB19 of this document and SEQ ID NO:6 is 100% (102/102 aa) homologous to parts of clone Ceres_ 1396253 in this document. --	1-2, 7
A	WO 0210210 A2 (BAYER AG ET AL), 7 February 2002 (2002-02-07); Abstract; RN: 395137-18-5, 2001-08-28 [online][retrieved on 2015-03-18] Retrieved from: STN, Database Registry, SEQ ID NO:2 show 100% homology (329/329 aa) to sequence 1616 of this document and SEQ ID NO:6 is 100% homologous to parts of (102/102 aa) sequence 1616 of this document. --	1-2, 7
A	Dello Ioio R. et al. "A PHABULOSA/Cytokinin Feedback Loop Controls Root Growth in Arabidopsis", 2012, In:Current Biology, Vol. 22, pp. 1699-1704; Summary --	1-18
A	WO 2007090143 A2 (PIONEER HI BRED INT ET AL), 9 August 2007 (2007-08-09); page 3, line 19 - page 4, line 10 -- --------	1-18

WO	2004097024	A1	11/11/2004	AU	2004235272	A1	11/11/2004
				BR	PI0409871	A	16/05/2006
				CA	2522929	A1	11/11/2004
				CN	1780915	A	31/05/2006
				EP	1618200	A1	25/01/2006
				JP	2007528701	A	18/10/2007
				KR	20060013380	A	09/02/2006
				US	20070180580	A1	02/08/2007
				ZA	200508665	A	26/07/2006
EP	1384776	A1	28/01/2004	AT	441708	T	15/09/2009
				AU	2002237573	B2	28/02/2008
				CA	2441791	A1	19/09/2002
				CN	1496401	B	26/05/2010
				DE	60233563	D1	15/10/2009
				JP	4334228	B2	30/09/2009
				NZ	528152	A	30/06/2008
				US	20040177403	A1	09/09/2004
				US	7807867	B2	05/10/2010
				US	20060236431	A1	19/10/2006
				WO	02072818	A1	19/09/2002
EP	1522586	A1	13/04/2005	AT	370240	T	15/09/2007
				DE	602004008180	T2	30/04/2008
				SG	111316	A1	30/05/2005
				US	20050164370	A1	28/07/2005
US	20050183159	A1	18/08/2005	US	7253340	B2	07/08/2007
US	20060064786	A1	23/03/2006	AR	051633	A1	31/01/2007
				AU	2005286853	A1	30/03/2006
				BR	PI0515424	A	22/07/2008
				CA	2794442	A1	30/03/2006
				CA	2580761	C	22/01/2013
				CN	103074310	A	01/05/2013
				EP	1799832	A2	27/06/2007
				MX	2007003201	A	31/08/2008
				MX	307394	B	14/02/2013
				NZ	553864	A	27/11/2009
				US	20090165177	A1	25/06/2009
				US	8916749	B2	23/12/2014
				WO	2006034286	A3	26/10/2006
WO	2004074442	A2	02/09/2004	AR	043185	A1	20/07/2005
				US	20060212970	A1	21/09/2006
				US	7799970	B2	21/09/2010
EP	1033405	A2	06/09/2000	NONE			

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/FI2014/051057

WO	0210210 A2	07/02/2002	AU	8984301	A	13/02/2002
WO	2007090143 A2	09/08/2007	AR	059268	A1	19/03/2008
			AU	2007211057	A1	09/08/2007
			BR	PI0706791	A2	05/04/2011
			CA	2641226	C	28/05/2013
			CN	101415821	A	22/04/2009
			EP	1984492	A2	29/10/2008
			NZ	570108	A	31/03/2011
			US	7553951	B2	30/06/2009
			US	8569577	B2	29/10/2013
			US	7893236	B2	22/02/2011
			US	20110113513	A1	12/05/2011
			US	20090229018	A1	10/09/2009
			US	20070245425	A1	18/10/2007
			ZA	200806624	A	28/10/2009

Continuation of: second sheet

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