Bundesministerium
für Bildung
und Forschung

Veröffentlichung der Ergebnisse von Forschungsvorhaben im BMBF-Programm

Forschungsvorhaben: Plant-KBBE III – Verbundvorhaben: ‚Verbesserung der Holzeigenschaften von Eukalyptus und Pappel zur Gewinnung von Bioenergie (TREE_FOR_JOULES) (Teilprojekt A)'

Förderkennzeichen: 0315914A

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I. Kurze Darstellung zu

1 Aufgabenstellung

The efforts to reduce the dependence on non-renewable fossil fuels and to mitigate climate change are leading to an increasing interest in sustainable bio-energy production. The use of ligno-cellulosic biomass from forest plantations as second-generation renewable bio-energy feedstock is gaining more and more attention, as trees are non-food crops, which can be grown under relatively poor soil conditions. Fast-growing tree species such as poplar and eucalyptus grown as short-rotation coppice are easy to establish and produce high yields of lignocellulosic biomass.

Regrettably, the structure and composition of the lignified secondary cell wall, which mainly consists of cellulose (about 45%), hemicelluloses and lignin (each about 25%), render woody feedstock particularly recalcitrant to degradation for e.g. bioethanol production (cellulose is the most valuable component for biofuel-production, while lignins impair the accessibility and are considered as a barrier for the fermentation process).

For this reason, improved genetic material is needed to use trees as energy crops in an efficient manner. The project “TreeForJoules” therefore aims to uncover processes/genes regulating relevant wood cell wall properties in poplar and eucalyptus. This knowledge will be invaluable for breeding fast-growing “elite” trees for improved down-stream processing and efficient conversion into biofuels. “TreeForJoules” general aims are to:

- identify and characterize the regulatory candidate genes (i.e. transcription factors and miRNAs) that control wood properties relevant to bioenergy;
- delineate and characterize genomic regions in eucalyptus and poplar that control wood properties of interest through comparative genetic and physical mapping, and comparative QTL mapping;
- develop highly efficient methods for wood property measurements, which can be used for the analysis of tree lines produced in the project;
- test relevant samples for their bio-ethanol and bio-oil potential.

The specific roles of the Johann Heinrich von Thünen Institute are to:

- perform a transcriptome analysis of developing xylem to identify candidate genes controlling wood properties in *Populus tremula*;
- overexpress / downregulate these candidate genes (CG) by genetic transformation of poplar;
- analyse bioenergy potentials, saccharification, and bio-oil of CG-transgenic poplar;
- micro-phenotype transgenic wood zones through microscopic and microspectrophotometric investigation methods.

2 Voraussetzungen, unter denen das Vorhaben durchgeführt wurde

The work was conducted within a consortium of in total 13 partners from France, Spain and Portugal. During the project, P1 requested support in respect of transformation 5 of eucalypt genes into poplar as the original aim to transform *Eucalyptus* could not be reached due to difficulties in eucalypt transformation. The prerequisites to perform the project were adequate to fulfil all deliverables and milestones.

3 Planung und Ablauf des Vorhabens

See project proposal

4 Wissenschaftlicher und technischer Stand

See project proposal

5 Zusammenarbeit mit anderen Stellen

The work was conducted within a consortium of in total 13 partners from France, Spain and Portugal. Main cooperation was established with P1 (Université Paul Sabatier, Jacqueline Grima-Pettenati), P2 (INRA, Jean-Charles Leple), P4 (FCBA, Luc Harvengt), P6 (Biopos, FI Biopos e.V.), P8 (IBET, Jorge Pinto Paiva), P9 (IICT, José Carlos Rodrigues), and P11 (Universidad de Málaga, Francisco Cantón and Fernando Gallardo Alba).

The work of P5, Johann Heinrich von Thünen Institute, was shared between two partners, P5a (Thünen Institute of Forest Genetics) and P5b, c, d (Thünen Institute of Wood Research; Wood anatomy, wood chemistry [saccharification, biooil]).

II. Eingehende Darstellung

1 der Verwendung der Zuwendung und der erzielten Ergebnisse

Task 1.1 *In silico* identification of CGs from compendia of expression data

Subtask 1.1.1 Development of new transcriptomic resources

The Thünen-Institute of Forest Genetics (P5a) performed a transcriptome sequencing analysis of developing wood in *Populus tremula* x *Populus tremuloides* by Illumina High-Seq. 2000 (GenXPro, Frankfurt; 100bp reads of a non-normalized mRNA-libary; 50 bp reads of a miRNA library). Bioinformatic analysis was done and transcriptome databases (transcription factors, miRNA) have been built.
In total, 19,139 different *P. trichocarpa* transcript identifiers could be assigned, including 707 transcription factors/transcription regulators identified to date. The miRNA was prepared from total RNA by size-fractionation using gel filtration. In total, 191,214 different miRNA tags were generated by Illumina sequencing with HiSeq 2000 sequencer.

Task 1.4 Functional characterization of a limited number of selected CGs

Networking activities:

Five constructs comprising candidate genes for wood properties from *Eucalyptus grandis*, produced by the working group of Jacqueline Grima-Pettenati (P1, University of Toulouse, France), were transformed into poplar by the Thünen Institute of Forest Genetics (P5a). The plants are under selection.

Identification of candidate genes

The 707 transcription factors identified so far by xylem transcriptome sequencing were analysed for their potential as candidate genes for wood properties important for bioenergy. Criteria for the selection of candidate genes were discussed in telephone conferences and personal meetings with the different partners of the project.

Candidate genes were chosen based on relative transcript levels in the transcriptome sequencing approach, *P. trichocarpa* xylem eFP expression data (http://www.bar.utoronto.ca/efppop/cgi-bin/efpWeb.cgi), and on different expression levels between tension and opposite wood which show differences in their relative composition concerning lignin and cellulose (data by P2).

The NAC, LIM, KNOX and MYB transcription factors, which have been shown to regulate the monolignolpathway were considered in particular.

Characterisation of candidate genes

Up to now, the Thünen Institute of Forest Genetics (former vTI, P5a) has chosen 13 candidate genes probably regulating wood properties for testing via stable transformation (knockdown and over-expression lines). Constructs comprising nine overexpression constructs, three RNAi-constructs and one amiRNA-construct have been produced and transformed into *Populus*.

Altogether, 23 transformation approaches (+ 6 for Jacqueline Grima-Pettenati, P1, University of Toulouse, France) have been carried out up to now. Regenerated plants are under selection for all constructs. Independent genetic lines with propagated individuals exist for eleven of the 18 constructs (one to 20 lines per construct up to now, more plants are still under selection). Transgenic lines of 10 (11) constructs have successfully been PCR-tested for transgene presence with gene- and T-DNA specific primers. The construct copy- number has been analyzed by Southern Blot experiments for transgenic lines representing five different constructs. RT-PCR experiments for construct expression analysis have been started.

Transgenic lines representing 10 (11) constructs have been transferred to the greenhouse. Four of these transgenic lines representing two different constructs show apparent phenotypic aberrations, including dwarfing or reduced growth and/or bended stems and stronger and irregular leaf serration or brown spotted and crumpled leaves (Fig. 1). Transgenic lines representing five different constructs were old enough for relevant wood production, and have been harvested for wood property analysis.

Fig. 1: Phenotypic changes within the transgenic poplar lines produced during the project. A and D: wildtype. B and C: phenotypic changes in plants overexpressing the transcription factor Potri 018G068700.1, a NAM (no apical meristem)-like protein, with high levels of xylem expression and a higher expression rate in tension versus opposite wood. E and F: phenotypic changes in plants overexpressing the transcription factor Potri. 003G195300.1, an alcohol dehydrogenase /Myb/SANT-like Transcription factor, with high levels of xylem expression and a detectable difference in expression rates in tension versus opposite wood.

Task 2.2 Bioenergy potentials, saccharification, and bio-oil

Networking activities:

For chemical analyses, 127 transgenic samples were received from three different partner organizations comprising 50 poplar hybrids (*P. deltoids x P. trichocarpa*), 24 eucalyptus hybrids (*E. urophylla x E. grandis*), and 38 poplars (*P. tremula, P. tremuloides, P. x canescens, P. alba*).

Lignin content and sugar composition

The samples were subjected to a two-step acid hydrolysis for determination of the Klason lignin content. Klason lignin contents of the poplar hybrids varied between 19.4 and 22.5 wt%, the eucalyptus species varied between 23.7 and 33.1 wt% and the poplars varied between 15.8 and 25.5 wt%. Monomeric sugars resembling the carbohydrate composition of the cell wall were also determined by acid hydrolysis with subsequent borate anion exchange chromatography.

Micro steam-treatment

A micro steam-treatment system was constructed (Fig. 2) and a suitable methods was developed and optimized (210 °C for 15 min, see Fig. 3) to give comparable results with a procedure at laboratory scale. The samples were further enzymatically hydrolyzed and yielded approximately 65wt% of fermentable sugars.

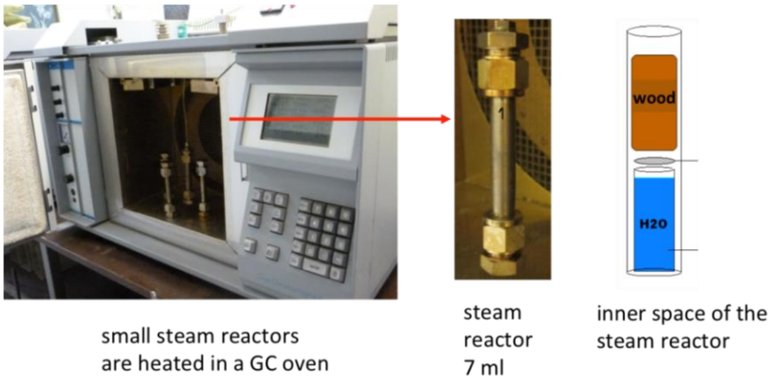

Fig. 2: Equipment for micro-steam treatment

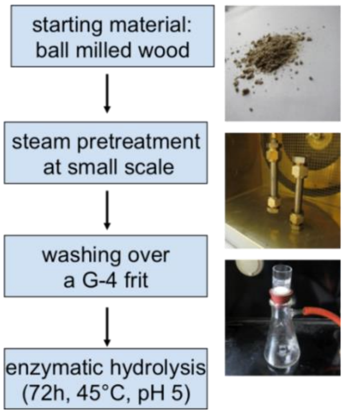

Fig. 3: Preparation steps for micro-steam treatment

Analytical Pyrolysis-Gas Chromatography/Mass Spectroscopy

For the estimation of bio-oil potential all samples have been subjected to analytical pyrolysis combined with GC/MS. From each sample 4 repetitive runs were performed. A typical chromatogram showing all volatile products is presented in Fig. .

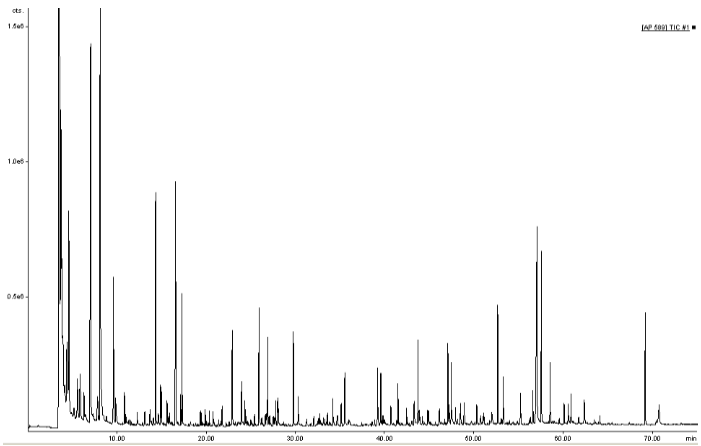

Fig. 4: Typical chromatogram of volatile components from poplar wood

The resulting data from pyrolysis have been chemometrically evaluated by multivariate data treatment. (see Fig.). The calculations include a multistep procedure consisting of (1) peak alignment, (2) transformation (3) principal component analysis and (4) partial least square regression analysis. Preliminary results show that samples with different gene constructs could be discriminated based on some lignin and carbohydrate peaks (see Fig. 6). Further evaluation and data interpretation is still ongoing.

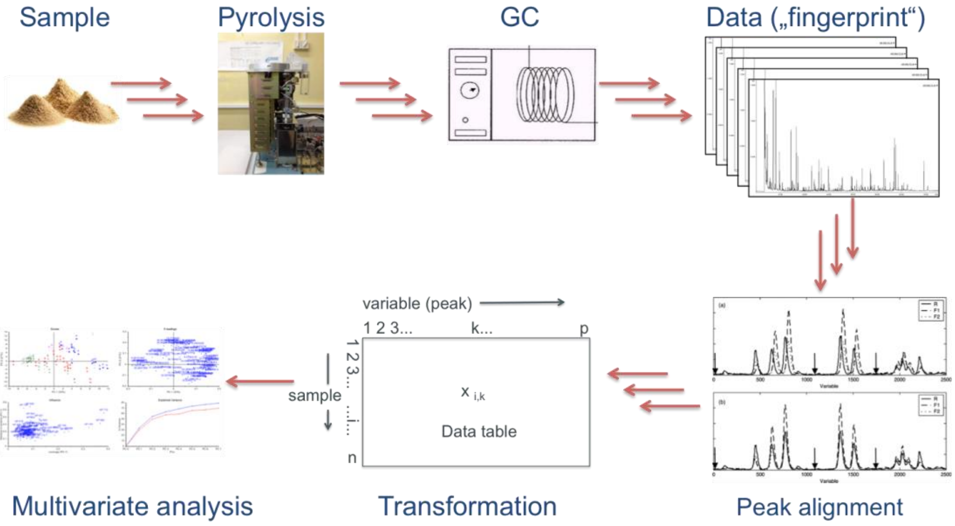

Fig. 5: Typical workflow from sample to multivariate data analysis

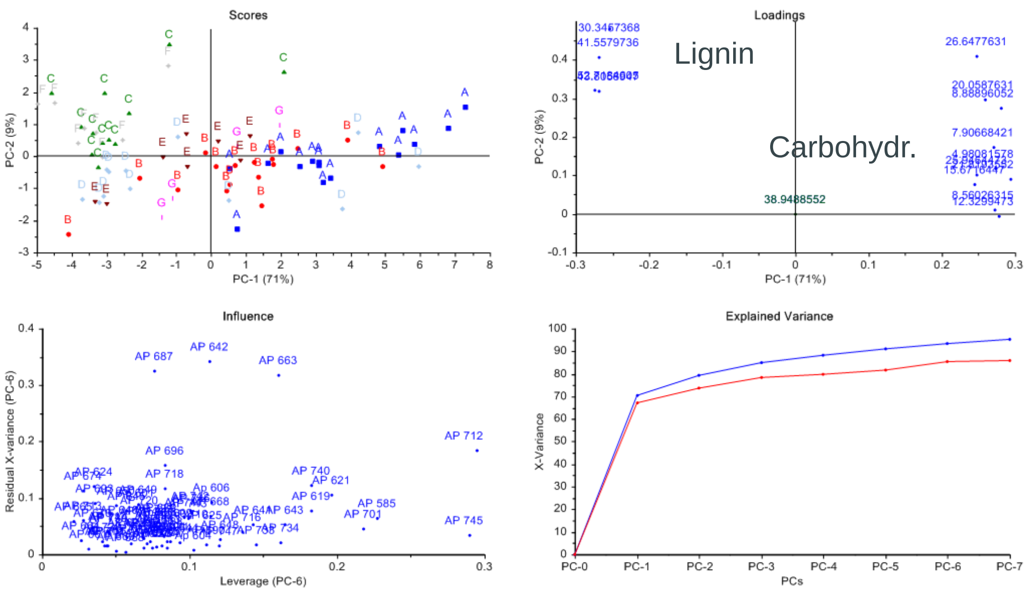

Fig. 6: Principal component analysis (PCA) of based on selected lignin and carbohydrate-derived components

Preparative Mini-Pyrolysis

In order to obtain mass balances and investigate products from bio-oil production, a mini pyrolyzer was used (see Figure 6a). The whole system could be weight before and after the experiment. Approximately 100 mg were pyrolyzed, so that oil yields and char yields could be determined gravimetrically. In addition, the biooil composition could be analyzed by gas chromatography.

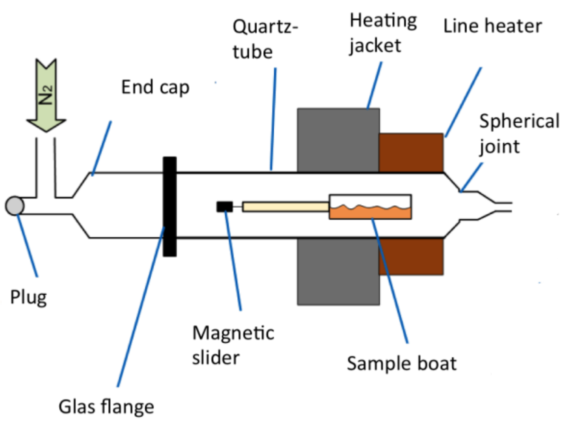

Figure 6a Mini pyrolyzer for bio-oil production

Yields of biooil and char are presented in Figure 6b. In comparison to standard beech wood, the oil yields are slightly lower (2-5 % absolute) and could be caused by the lower lignin content. Figure 6c demonstrates great differences in biooil yield (30-45 wt.%) from INRA samples. Samples from the wild type yields typically more biooil which can be related to the lignin content.

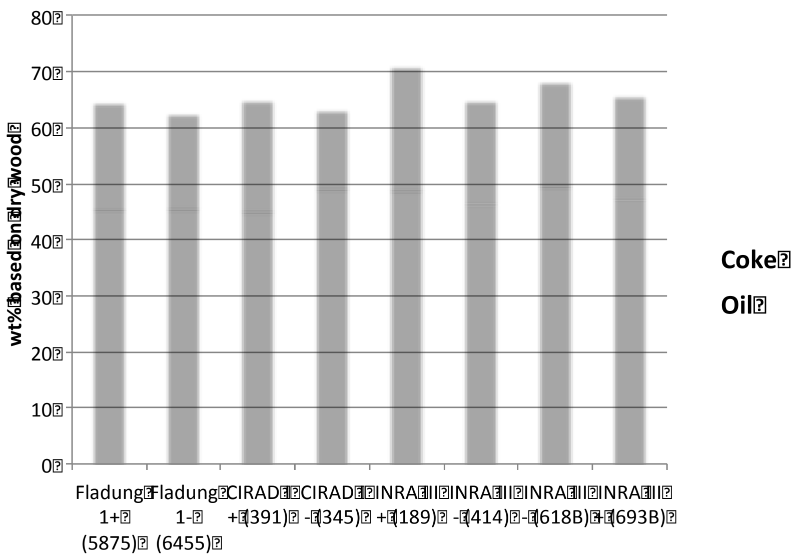

Figure 6b Yields of biooil and char of genetically modified samples

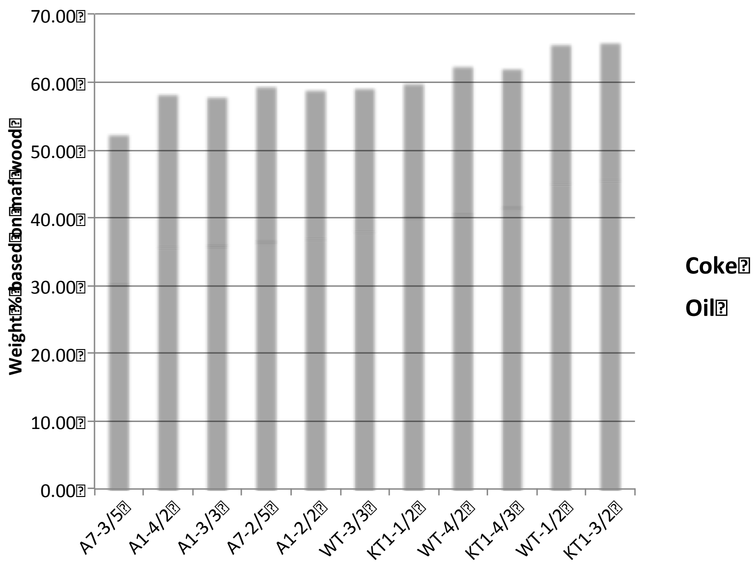

Figure 6c Variation of biooil yields from INRA samples

Task 2.3 Micro-phenotyping of transgenic wood zones through microscopic and microspectrophotometric investigation methods

Networking activities:

For microphenotyping, samples from INRA Orleans, CIRAD Montpellier and the Thünen Institute of Forest Genetics were taken from various transgenic poplar trees (*Populus tremula x tremuloides*) and one series of eucalypt trees (*Eucalyptus urograndis*).

Structural and topochemical analyses

Beside symptomless xylem samples, slightly brownish discolored xylem portions were prepared for micro-phenotyping. Briefly, for transmission electron microscopy (TEM) the samples taken with razor blades from stem portions were prepared as follows: after fixation in an aldehyde solution, samples were washed, de-hydrated in acetone, and finally embedded in Spurr's epoxy resin. Ultrathin sections (0.1 µm thick) were cut with a diamond knife and analysed with a Philips CM12 transmission electron microscope at accelerating voltages of 60 or 80 kV.

A parallel set of samples with same origin was prepared for cellular UV-microspectrophotometry (UMSP) in the same way to analyze lignin topochemistry. However, 1 µm semithin sections were prepared. Lignin top-ochemistry was analyzed in the scanning mode at a constant wavelength of 278 nm (absorbance maximum of hardwood lignin).

It was shown by cellular UV-microspectralphotometry that walls of the various cell types in hardwoods had a heterogeneous lignin distribution. At a wavelength of 278nm, i.e., maximum absorbance for hardwood lignin, most lignin was found in the cell corner regions with values between 0.25 and somewhat above 0.3 (Fig. 13). On the other hand relatively low absorbance was recorded for the secondary wall layers with val-ues mostly below 0.1. When comparing the secondary walls of vessels with secondary walls of fibres, more lignin was found in vessel walls (Figs. 7 and 10). In some cases, no lignin was found in inner secondary wall portions; most probably, these wall portions could be anatomically identified as so called gelatinous layers

(Fig. 8). Gelatinous layers or G-layers are composed of cellulose only, without any lignin deposited. Those fibres are formed in wood under tension stress, therefore belonging to reaction wood tissue. Varying lignin contents in cell corner regions have been regularly observed in many hardwoods. According to their fine structure, those differences became evident after staining with potassium permanganate showing either intensely stained regions or empty appearing regions (Figs. 9 and 11). According to Fig. 13, the different transgenic lines often had different lignin levels. E.g. some xylem regions showed a relatively high lignin content in their fibre secondary walls as well as in their cell corner regions (Fig. 12).

Some genetically modified poplar trees with homogeneous lignin distribution in fibres of adjacent growth rings and without tension wood showed similar absorbance values like control trees. Scans of xylem portions without tension wood fibres revealed a homogeneous lignin distribution in cell walls throughout the outermost two growth rings.

Electron microscopy added information on the cell wall architecture of topochemically varying xylem fibres. Wall thicknesses corresponded to those visualized by UV-microscopy. We observed rather thin walls with a narrow S2 layer as well as thick walls with broadened S2 layer. The formation of G-layers which were deposited onto the innermost secondary wall layer was also well demonstrated and found in several transgenic lines. Also poplar trees without genetic modifications partly showed tension wood zones. Potassium permanganate staining gave no evidence that G-layers of these samples contained any phenolic constituents which might also affect UV-absorbance behavior.

Chemical analyses of Klason lignin contents vary more or less distinct between transgenic lines. As considered above, wall thicknesses as well as amount of tension wood fibres with reduced lignin amounts in their walls can also play a certain role for the determined variations in lignin contents.

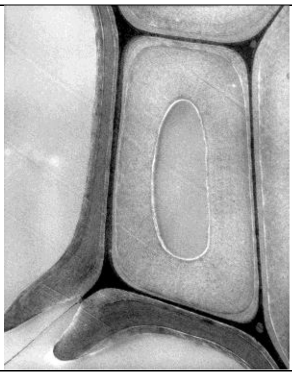

Fig. 7: Electron micrograph of poplar with thick walled fibres (right) and thin walled, densely stained vessel wall (left); vessel walls contain more lignin than fibre walls

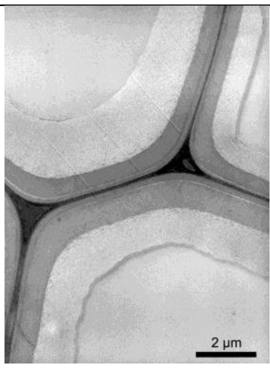

Fig 8: Electron micrograph of poplar fibres with secondary cell walls consisting of outer S1 and S2 layer and an inner gelatinous layer

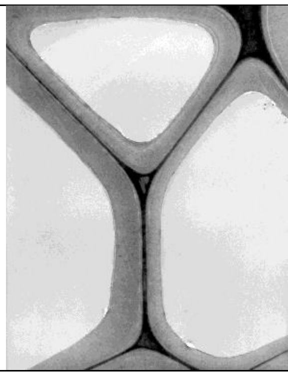

Fig. 9: Electron micrograph of poplar fibres showing cell corner with varying ultrastructure; empty appearing cell corner regions probably contain less lignin

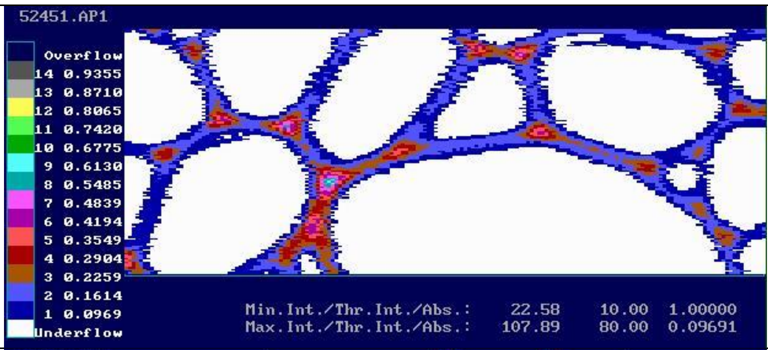

Fig. 10: UV micrograph of poplar xylem with fibres (top) and a vessel (bottom) taken at a wavelength of 278 nm. Secondary walls lower absorbance than cell corner regions.

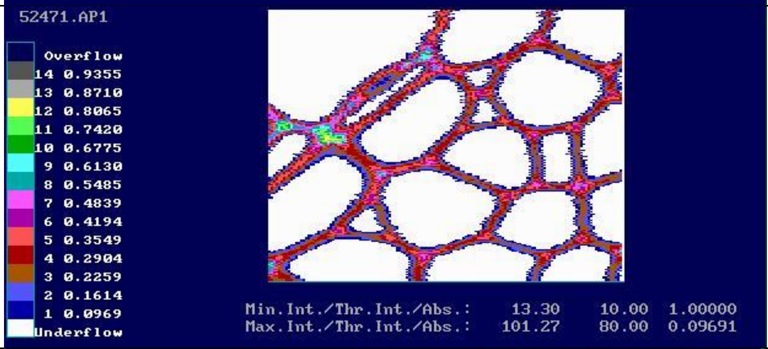

Fig. 11: UV micrograph of poplar xylem with fibres (top) and a vessel (bottom) taken at a wavelength of 278 nm. Fiibres with a relatively high absorbance of their secondary walls.

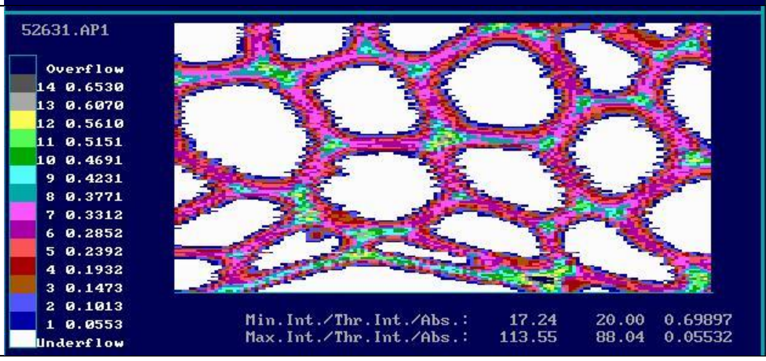

Fig. 12: UV micrograph of poplar xylem with fibres (top) and a vessel (bottom) taken at a wavelength of 278 nm. Thick-walled poplar fibres with very high absorbances in both secondary wall and cell corner regions.

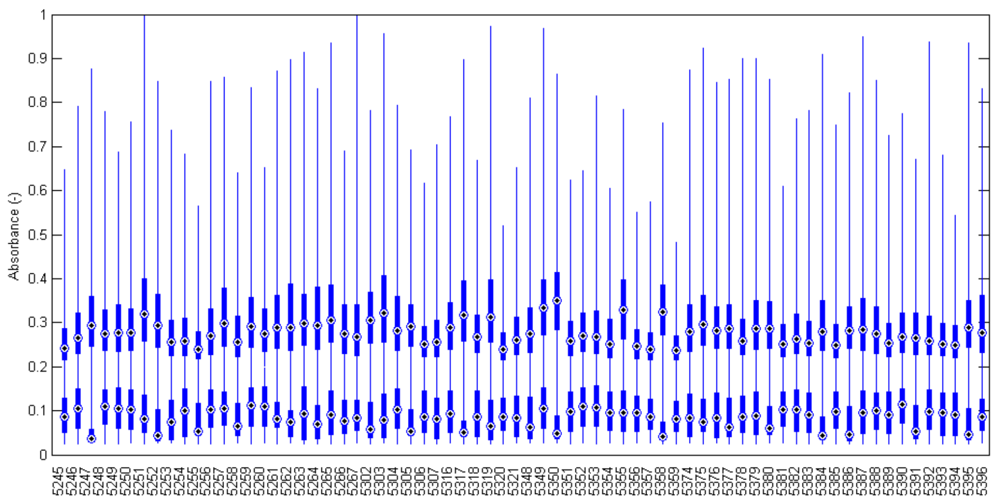

Fig. 13: Absorbance values of poplar and eucalypt xylem samples at 278 nm wavelength. It could be shown that cell corner regions generally had higher absorbance values than secondary walls. Variations between transgenic lines of both poplar and eucalypt were in some cases quite evident.

In conclusion, when comparing structural, topochemical and chemical analyses it could not clearly be stated that reduced or increased lignin contents are alone the results of modified fibre walls with more or less lignin. The formation of tension wood with their lignin free G-layers as well as the formation of narrow or broad secondary wall layers in fibres cannot be attributed to a certain transgenic poplar line. This is due to a rather inhomogeneous xylem tissue in the investigated trees. It is recommended to exactly determine the amount of tension wood -which we did not perform in the present study- to exclude this as a major structural feature affecting chemical composition of trees.

Task 4.2 TREEFORJOULES Bioinformatic network

The Thünen Institute has contributed to the bioinformatic network led by P1 (University of Toulouse, France), assisted by GenoToul Bioinfo and a Bioinformatic Committee, with analyses of RNAseq data.

2 Vergleich des Stands des Vorhabens mit der ursprünglichen (bzw. mit Zustimmung des Zuwendungsgebers geänderten) Arbeits-, Zeit- und Ausgabenplanung.

Status of deliverables

For the first 18 months, P5 is involved in the deliverables D1, D3, D5, D12, D14 and D16. D1 (due to month 6 of the project) D3, and D12 (both due to month 12) are already completed. For D5 (due to month 15), all constructs are already designed and ordered. Out of these, 4 constructs will be available soon. Transformation of *Populus* (D6) has nearly been completed. Transformed lines are being regenerated into plants, plants of some lines have already been transferred to greenhouse. From oldest transgenic plants (12-16 months), analyses of wood properties (D14 [saccharification potential], D16 [bio-oil potential]) have already been started, in part are completed. The same holds true for deliverables D17 (key cell-wall constituents) and D19 (lignin distribution and cellulosic compounds). However, transgenic plants transferred to greenhouse during the last six months still need to be analysed. Thus, a slight delay of D14, D16, D17 and D19 due to low age of transgenic plants grown in the greenhouse should be noted. An extension of the project until December 2014 is therefore needed. An application for a cost-neutral extension was made.

Status of budget

The P5 budget is in the frame of the approved amount. Additional money is required but will not be approved.

3 Haben sich die Aussichten für die Erreichung der Ziele des Vorhabens innerhalb des angegebenen Ausgabenzeitraums gegenüber dem ursprünglichen Antrag geändert (Begründung)?

In principle no, only the age of the transgenic trees to be analysed for wood properties was not well estimated.

4 Sind inzwischen von dritter Seite Ergebnisse bekannt geworden, die für die Durchführung des Vorhabens relevant sind? (Darstellung der aktuellen Informationsrecherchen nach Nr. 2.1 BNBest-BMBF 98)

No

5 Sind oder werden Änderungen in der Zielsetzung notwendig?

No

6 Fortschreibung des Verwertungsplans:

6.1 Erfindungen/Schutzrechtsanmeldungen und erteilte Schutzrechte

Keine

6.2 Wirtschaftliche Erfolgsaussichten nach Projektende (mit Zeithorizont) - z.B. auch funktionale/wirtschaftliche Vorteile gegenüber Konkurrenzlösungen, Nutzen für verschiedene Anwendergruppen/-industrien am Standort Deutschland, Umsetzungs- und Transferstrategien

Jeder Partner im Projekt TREEFORJOULES hat die Möglichkeit, Technologien direkt in die Praxis umzusetzen (die industriellen Partner betreiben jeweils ein eigenes Züchtungsprogramm). Eine rasche Vermarktung der Ergebnisse aus TREEFORJOULES wird jedoch bevorzugt von den industriellen Partnern erfolgen. Allerdings sind wirtschaftliche Erfolgsaussichten zurzeit noch nicht absehbar.

6.3 Wissenschaftliche und/oder technische Erfolgsaussichten nach Projektende (mit Zeithorizont) im Hinblick auf öffentliche Aufgaben, Datenbanken, Netzwerke, Transferstellen etc.

Das Ziel von TREEFORJOULES ist, neues Wissen zu generieren bzw. neue Gene/Loci zu entdecken, die für Pappel und Eukalyptus wichtige Merkmale mit Relevanz zu Bioenergieproduktion, und Interaktionen zwischen Genprodukten, Metaboliten und Zellwandpolymere sowie zwischen Umweltfaktoren und Baumwachstum und –entwicklung steuern. Dieses Wissen wird die Grundlage für neue Selektionsstrategien in der Forstpflanzenzüchtung ggf. in Kombination mit gentechnischen Methoden darstellen. Somit besteht eine sehr hohe Wahrscheinlichkeit, dass im Laufe des Projekts verwertbares geistiges Eigentum entwickelt werden wird, was auch auf andere verholzende Pflanzen übertragbar ist. Sicher ist auch, dass das während des Projekts TREEFORJOULES entwickelte geistige Eigentum in Form von entdeckten Genen und induzierten Zellwandmodifikationen besonders für die Industrie verwertbare Anwendungen haben wird. Datenbanken und Netzwerke befinden sich zurzeit im Aufbau. Ergebnisse sollen nach Fertigstellung der Datenbanken *peau-a-peau* in diese einfließen.

6.4 Wissenschaftliche und wirtschaftliche Anschlussfähigkeit für eine mögliche notwendige nächste Phase bzw. die nächsten innovatorischen Schritte zur erfolgreichen Umsetzung der Ergebnisse.

TREEFORJOULES hat die Möglichkeit, Technologien direkt in die Praxis umzusetzen (die industriellen Partner betreiben jeweils ein eigenes Züchtungsprogramm). Eine rasche Vermarktung der Ergebnisse aus TREEFORJOULES wird jedoch bevorzugt von den industriellen Partnern erfolgen. Es ist zu erwarten, dass dieses einen positiven Einfluss auf die Erscheinung der im Projekt involvierten europäischen Firmen hat. Zum Schutz des in dem Projekt entwickelten geistigen Eigentums, das für international tätige Firmen von Interesse ist, sollen weitere Wege für die internationale Verwertung beschritten werden, so z.B. über „kommerzielle Vereinbarungen“ und/oder Technologielizenzen. Eine Verbundvereinbarung wurde von allen Projektpartnern unterzeichnet.

<u>Vorträge und Poster seit Projektbeginn</u>

Vortrag: “Transcript profiling of selected CGs.” Kick-off "Tree for Joules", Toulouse, 12.04.2011

Vortrag: “TREEFORJOULES, a Plant KBBE project to improve eucalypt and poplar wood properties for bioenergy”. 26th New Phytologist symposium: Bioenergy Trees, 17−19th May 2011

Vortrag: “Transcriptome analysis of developing wood in *Populus*”. Verbundtreffen "Tree for Joules", Lissabon, Portugal, 11.-14.10.2011.

Vortrag: “General aspects of discoloration of woody tissue”. INRA Orléans, France, October 2011.

Poster: “TreeForJoules- Verbesserung der Holzeigenschaften von Eukalyptus und Pappel für die Bioenergiegewinnung”. Symposium „Züchtung und Ertragsleistung schnellwachsender Baumarten im Kurzumtrieb - Erkenntnisse aus drei Jahren FastWOOD, ProLoc und Weidenzüchtung“, Hannoversch

Münden, 21.-22.9.2011.

Poster: “TreeForJoules- Verbesserung der Holzeigenschaften von Eukalyptus und Pappel für die Bioenergiegewinnung”. 3. Symposium Energiepflanzen, Berlin, 02.-03.11.2011.

Poster: “TreeForJoules- Improving eucalyptus and poplar wood properties for bioenergy”. Statusseminar Plant2030, Potsdam, 06.-08.03.2012.

Vortrag: TREEFORJOULES Improving eucalypt and poplar wood properties for bioenergy Plant-KBBE conference 2012, CRAG, Universitat Autonoma de Barcelona (Spain), March 19-20th, 2012

Vortrag: Results of RNAseq of Poplar xylem: selection of CGs. Verbundtreffen "Tree for Joules", Malaga, 11.04.2012.

Vortrag: “TreeForJoules- Improving eucalyptus and poplar wood properties for bioenergy”. Fastwood project meeting, Großhansdorf, 07.11.2012.

Vortrag: Lignin distribution in secondary xylem cell walls of genetically modified poplar trees, Québec, Canada, 15.09.2015 (accepted)

Poster: “TreeForJoules- Verbesserung der Holzeigenschaften von Eukalyptus und Pappel für die Bioenergiegewinnung”. Internationaler Agrarholz-Kongress, Berlin, 19.-20. 02.2013

Poster: “TreeForJoules- Improving eucalyptus and poplar wood properties for bioenergy”. Plant 2030 status seminar, Potsdam, 6.-8.03.2013

Poster: “TreeForJoules- Improving eucalyptus and poplar wood properties for bioenergy”. IUFRO Tree Biotechnology, Asheville, NC, USA, 26.05.2013-01.06.2013

Poster: “TreeForJoules- Verbesserung der Holzeigenschaften von Eukalyptus und Pappel für die Bioenergiegewinnung”. 4. Symposium Energiepflanzen, Berlin, 22-23.10.2013

Poster: “TreeForJoules- Improving eucalyptus and poplar wood properties for bioenergy”. COST Action FP0905 Final Conference, Rome, Italy, 4.-5.3. 2014