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Impact of RAV1-engineering on poplar biomass production: a short-rotation coppice field trial
Alicia Moreno-Cortés; José Manuel Ramos-Sánchez; Tamara Hernández-Verdeja; Pablo González-Melendi; Ana Alves; Rita Simões; José Carlos Rodrigues; Mercedes Guijarro; Isabel Canellas; Hortensia Sixto; Isabel Allona
Biotechnology for Biofuels

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IMPACT OF RAV1-ENGINEERING ON POPLAR BIOMASS PRODUCTION: A SHORT-ROTATION COPPICE FIELD TRIAL

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ABSTRACT

Background: Early branching or syllepsis has been positively correlated with high biomass yields in short-rotation coppice (SRC) poplar plantations, which could represent an important lignocellulosic feedstock for the production of second-generation bioenergy. In prior work, we generated hybrid poplars overexpressing the chestnut gene *RELATED TO ABI3/VP1 1* (*CsRAV1*), which featured c. 80% more sylleptic branches than non-modified trees in growth chambers. Given the high plasticity of syllepsis, we established a field trial to monitor the performance of these trees under outdoor conditions and a SRC management.

Results: We examined two CsRAV1-overexpression poplar events for their ability to maintain syllepsis and their potential to enhance biomass production. Two poplar events with reduced expression of the *CsRAV1* homologous poplar genes *PtaRAV1* and *PtaRAV2* were also included in the trial. Under our culture conditions, CsRAV1-overexpression poplars continued developing syllepsis over two cultivation cycles. Biomass production increased on completion of the first cycle for one of the overexpression events, showing unaltered structural, chemical or combustion wood properties. On completion of the second cycle, aerial growth of both overexpression events was dampened.

Conclusions: These findings support the potential application of CsRAV1-overexpression to increase syllepsis in commercial elite trees without changing other traits. Yet, improvements on biomass yielding will depend on the

achievement of the optimal conditions enabling sustainability of an increased aboveground growth.

KEYWORDS

poplar, tree biotechnology, RAV1, sylleptic branchiness, lignocellulosic biomass, field trial, short rotation coppice (SRC)

BACKGROUND

Lignocellulosic biomass production is met with the challenge to enhance yields and improve physical and chemical traits to become a sustainable, carbon-neutral renewable energy source [1,2]. Energy produced from lignocellulosic crops will help alleviate our current high dependency on fossil fuels and reduce greenhouse gas emissions responsible for global warming. A further benefit is that such crops do not directly compete with food demand [3,4]. This has sparked a recent interest in short-rotation coppice (SRC) cultivation of fast-growing species such as poplar for the production of lignocellulosic biomass [5]. Coppicing promotes the resprout of multiple shoots, which increases final biomass, and enables multiple harvests from the original rootstock [6,7]. Growth and development related traits are fundamental components of productivity. In poplar, numerous studies have investigated the relative contribution of several of these traits to productivity and their degree of reliability as productivity determinants in field conditions, particularly when poplars are cultivated as SRC [8-10]. Recent advances have been made in the identification of putative loci underlying phenotypic variation of growth and developmental related traits. These works exploded natural genetic variation

66 by means of genome-wide association studies (GWAS), from populations of *Populus*
67 species growing in common gardens [11,12], even as SRC [13].

68 Among those traits, early or sylleptic branching have been reported to be positively
69 correlated with high biomass yields [14-18]. Trees growing in temperate and boreal
70 regions need to go through a stage of winter dormancy to develop so-called
71 proleptic branches from axillary meristems formed the preceding year. Some poplar
72 species produce early or sylleptic branches without undergoing a dormant period
73 [19]. Syllepsis adds leaf area per se, but also leaves on sylleptic branches are larger
74 and often grow faster than those on the main axis [20]. This additional leaf area
75 helps to rapidly close the canopy, increasing light interception and suppressing
76 weed growth, which is especially important for the establishment of a SRC
77 plantation and biomass production [21,22]. However, early branching is a highly
78 plastic trait, strongly affected by the availability of resources and environmental
79 cues [15,17,18]. Actually, sylleptic branches often show a shorter lifespan than
80 proleptics but, in this short time, they play an important role in the carbon balance,
81 providing a quick return for a relatively small resources investment [14]. These
82 features make syllepsis a valuable productivity-related trait with the potential for
83 the development of new high-yielding SRC genotypes [22]. Although in poplar
84 syllepsis shows much genetic variation and high heritability [15,20], available data
85 regarding the specific loci and mechanisms controlling syllepsis are still limited. It is
86 well established that auxins play a key role in apical dominance and syllepsis in
87 poplar [23,24]. Hence, genes related to auxins or to hormones affecting auxin
88 signals are targets to optimize branching for biomass production via the release of

89 axillary buds from paradormancy [25,26]. However, experiences in the field with
90 engineered trees for any of these genes and their impact on biomass yield have not
91 been carried out so far.

92 In prior work, we generated hybrid poplars overexpressing the chestnut gene
93 *RELATED TO ABI3/VP1 1* (*CsRAV1*) homolog to *TEMPRANILLO 1* and *TEMPRANILLO 2*
94 from Arabidopsis [27]. These trees featured c. 80% more sylleptic branches than
95 non-modified or *PtaRAV1* and *PtaRAV2* downregulated trees in growth chambers,
96 under controlled conditions [28]. Tree performance in a greenhouse in terms of
97 syllepsis or any other trait may significantly differ from the situation outdoors,
98 where trees may show greater phenotypic variation [15,29]. Therefore, field trials
99 to monitor tree performance under natural conditions over several years are
100 needed to select the best events or individuals [30]. So far, reports of field trials on
101 genetically engineered trees are scarce and, with several exceptions, have mostly
102 pursued lignin modification [31-34]. Here we report a field trial, in which we
103 examined two poplar transgenic events overexpressing CsRAV1 (hereinafter
104 referred to as CsRAV1-overexpression or CsRAV1 OX events). These trangenics were
105 tested for their ability to maintain this trait under field conditions, their wood
106 properties and their potential to enhance biomass production under SRC. The trial
107 was run for four years, during which two cultivation cycles were conducted.
108 Transgenic poplars showing a reduced expression of endogenous *PtaRAV1* and
109 *PtaRAV2* (hereinafter referred to as PtaRAV1&2-knockdown or PtaRAV1&2 KD
110 events) were also included in the trial.

METHODS

Field trial design, establishment and management

A field trial was designed to test the growth performance of transgenic *Populus tremula x P. alba* INRA clone 717 1B hybrid poplars. The trees included were the wild-type genotype as control (WT), events #37 and #60 of transformed trees carrying the *35S::3xHA:CsRAV1* cassette (hereafter referred to as CsRAV1-overexpression or CsRAV1 OX events), and events #1 and #22 of transformed trees carrying the *35S::PtaRAV1-hpiRNA* cassette (hereafter referred to as PtaRAV1&2-knockdown or PtaRAV1&2 KD events). CsRAV1-overexpression events #37 and #60 were selected on the basis of their high branch syllepsis of c. 80% shown when growing under controlled environmental conditions. The criterion for the selection of PtaRAV1&2-knockdown events #1 and #22 was their *PtaRAV1* and *PtaRAV2* transcript abundances, lower than in the wild-type genotype [35]. *In vitro*-rooted cuttings were initially potted in March 2012 and grown in the greenhouse as previously described [35]. The field trial was established in July 2012 in an experimental plot in Madrid (Spain) after obtaining a permit for the release of genetically modified higher plants from the Spanish authorities (notification numbers B/ES/12/30 and B/ES/12/34). At that time, plants were four-months old and had reached a height of c. 2 m. After planting, one WT individual died and five PtaRAV1&2-knockdown #22 lost their shoot tips, so they were excluded from the statistical analysis of sylleptic branching the first year. The trial design included 30 individual trees per genotype distributed in 3 blocks of 10 trees each. The

experimental plot area was 204 m^2, and the plantation density was 10000 trees/ha. Trees were planted in 12x17 rows with spacings of 2 x 0.5 m. To avoid edge effects, an additional row around the trial was planted using the genotype I-214 (*P. x canadensis* Moench.). A protective fence (mesh size 4 cm) was installed around the plot to prevent access of *Leporidae*. The trial was run for two cultivation cycles during 4 years: a first cycle from 2012 to 2013, and a second cycle from 2014-2015. Given the flowering time of this hybrid poplar of around 4-5 years, the trees did not flower during the trial.

Each year from June to September the plot was drip-irrigated. At the beginning of each growing season, a complex fertilizer (N21:P8:K11) was applied at a dose of 25 g per tree. Weed spreading was avoided using an anti-weeds cover in the plantation. No herbicides were used. For pest and disease control, the following treatments were applied: 0.04% deltamethrin against *Gypsonoma aceriana* Dupn. (May 2013), 0.06% imidacloprid against *Myzus persicae* (August 2013) and 0.1% abamectin against *Tetranychus urticae* (August 2014).

Production of antibodies against the poplar RAV1 protein

Polyclonal antibodies were raised against the poplar RAV1 protein using as antigen the epitope NH_2-CIDRQYSKKQRIVGAL-COOH, which is located at the C-terminal end of the PtRAV1 protein from *P. trichocarpa*. Antibodies were produced in rabbit and purified by Pineda Antikörper-Service (Berlin, Germany). The monospecific IgG fraction (in Tris-HCl buffer pH 7.5, 0.5 M NaCl, 1 mg/ml bovine serum albumin, 0.02% sodium azide) was 1:1 diluted with glycerol and stored at -20 °C.

Protein extraction from stem tissues and Western immunoblotting

Basal branches were sampled in December 2012 and June 2013 to assess the expression of the transgenes in the field. About 250 mg of ground stem material were resuspended in 800 µl Laemli sample buffer (61.9 mM Tris-HCl, 8 M urea, pH 6.8, 2% SDS), 5% β-mercaptoethanol and 1X protease inhibitor mix for plant cell and tissue extracts (Sigma-Aldrich Co. LLC., Saint Louis, MO, USA). Tissue suspensions were vortexed for 1 min and sonicated in a water bath for 2 min, twice, and clarified by centrifugation for 15 min at 12000 *g* and room temperature. Proteins were precipitated overnight at 4 °C with 0.5 volumes of 50% trichloroacetic acid, and the following day were washed twice with 1 ml of cold acetone. Air-dried protein pellets were resuspended in 250 µl Laemli sample buffer, 5% β-mercaptoethanol. Samples were quantified with a nanodrop at 280 nm to ensure equal loading.

Proteins were separated on 10% sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and blotted onto 0.45 µm polyvinylidene difluoride membranes (Amersham™ Hybond™, GE Healthcare Life Sciences, Little Chalfont, UK). Immunoblottings were conducted as described previously (Berrocal-Lobo *et al.*, 2011) using a 1:1000 dilution of anti-haemagglutinin (anti-HA) (High Affinity clone 3F1C; Roche Diagnostics, Indianapolis, IN, USA) or 1:500 of anti-PtRAV1 antibodies. Secondary hybridations were run using a 1:100000 dilution of horseradish peroxidase (HRP)-linked goat anti-rabbit IgG (Sigma-Aldrich Co. LLC.). MagicMark™ XP Western Protein Standard (Thermo Fisher Scientific/Life Technologies/Invitrogen) was used as a molecular weight marker. Target proteins

were detected using the Immobilon Western Chemiluminescent HRP Susbstrate (Merck Millipore, Billerica, MA, USA). To confirm equal loadings per lane, membranes were stained with Ponceau S.

Growth-related and biomass measurements

Growth-related measurements for all trees in the trial were taken every year during dormancy periods (December 2012, 2013, 2014 and 2015). Heights (cm) of main stems and dominant shoots were measured using a pole. Diameters (mm) were measured over the bark at 130 cm above the ground using a digital caliper. Biomass yields were determined by recording the fresh weights of total above-ground biomass (stems and branches) per tree (kg) after the first (December, 2013) and the second (December, 2015) cuttings. Dry weights were estimated by subtracting from the fresh weights a moisture content estimated by subsampling a tree from each block and genotype and oven-drying it to constant weight at 100 °C.

Wood chemistry and high calorific value

After coppicing, 2 cm-thick main stem cross-sections taken at 100, 150 and 200 cm above the ground were sampled from WT, CsRAV1 OX#60 and PtaRAV1&2 KD#1 trees (4 trees per genotype, *n*=4). Once the bark and pith were removed from the xylem, the disks were oven-dried for 48 h at 60°C. Samples were ground in an ultra centrifugal mill (RETSCH GmbH, Haan, Germany) until passing through a 0.75 mm sieve. Milled samples were sequentially extracted with dichloromethane (6 h), 95% ethanol (16 h) and distilled water (16 h). Extractions were run in a 125 ml Soxhlet apparatus on eleven batches of six samples (1.5 g per sample) keeping individuals

separate in filter bags (ANKON Technology, Macedon, NY, USA). Extractive contents were determined by assessing weight loss after each step [36]. Klason lignin contents were determined in extractive-free samples following the procedure described by [37]. For analytical pyrolysis, about 30 mg of extracted samples were further milled in a vibratory ball mill (RETSCH GmbH) for 5 min, and stored in a desiccator. Pyrolysis analyses were performed using Pyroprobe 1000 (CDS Analytical Inc, Oxford, PA, USA) with a coil filament probe connected to a gas chromatograph Agilent/HP7820 (Agilent Technologies Inc, Santa Clara, CA, USA) equipped with a flame ionization detector. Pyrolysis runs were conducted at 600 °C for 5 s on 75-82 µg of extractive-free ball-milled samples, and the resulting products separated on a 60 m DB-1701 column (Agilent Technologies Inc). The syringyl/guaiacyl ratio (S/G) was calculated with Chemstation Software (Agilent Technologies, Palo Alto, USA) as the ratio of the sum of the areas of the S peaks divided by the sum of the area of G peaks. Details about the conditions and quantification procedures have been published elsewhere [36,38,39].

The high calorific value of the wood was established using the method outlined in International Standard ISO 1716. Three trees per genotype WT, CsRAV1 OX#60 and PtaRAV1&2 KD#1 were randomly selected. A representative wood sample per tree was ground in a mill (IKA®-Werke GmbH & CO. KG, Staufen, Germany) to a particle size of 0.5 mm. Pellets of about 1 g were prepared from the ground material using a hand press, oven-dried at 100±5 °C for 24 h and then weighed. Measurements were made using an adiabatic bomb calorimeter with a platinum resistance sensor PT-

100 (IKA®-Werke GmbH & CO. KG). High calorific values were expressed as the average of measurements made in three pellets per tree.

Histochemistry

Fifth internodes of several branches were collected in spring 2013 and fixed under vacuum in a solution of 4% formaldehyde (freshly prepared from paraformaldehyde) in phosphate-buffered saline (PBS: 137 mM NaCl, 0.27 mM KCl, 1 mM phosphate buffer, pH 7.4), kept overnight at 4°C and then stored in a solution of 0.1% formaldehyde in PBS at 4 °C until further use. 50 µm-thick sections were cut on a Vibratome 1000 Plus (The Vibratome Company, St. Louis, MO, USA) under water. The sections were either stained with calcofluor white to visualize cellulose or left untreated to detect lignin autofluorescence. Stacks of sections were collected on a confocal microscope Leica TCS SP8 (Leica Microsystems, Wetzlar, Germany) under the excitation line of 405 nm. Xylem areas were identified on the inner sides of the cambium cell layers, along with sclerenchyma-supporting tissue and cortex.

Statistical analysis

A fixed-effect one-way ANOVA was used to assess differences in variables among genotypes. The linear model was:

$$y_{ijk} = \mu + \beta_i + \delta_j + \varepsilon_{ijk}$$

where y_{ijk} is the response of kth plant in the jth block of the ith event; µ is the overall mean; β_i is the ith event effect; δ_j is the jth block effect and ε_{ijk} is the experimental error, $\varepsilon_{ijk} \sim N(0, \sigma^2)$.

All statistical analyses were carried out with R. The Shapiro-Wilk test was used to check the normality of the data and Levene test [40] to check the homocedasticity. Normality was tested both for variable and residual distributions. When any of these assumptions was violated, the Kruskal-Wallis test [41] was used to analyse the data. To identify differences among genotypes, we used the Tukey HSD *post-hoc* test for ANOVA analyses and pairwise comparisons using the Wilcoxon test for Kruskal-Wallis analyses. The particular test used on each variable (trait) is detailed in Supplementary data Table S2.

RESULTS

Sylleptic branching and genetic modifications are retained over cultivation cycles

The present field trial was established in July 2012 in an experimental plot in Madrid (Spain), and included 30 trees per genotype distributed in 3 blocks (Figure 1a). That year, during the remaining growing season, the occurrence of sylleptic branches in CsRAV1-overexpression poplars was evident (Figure 1b). In December 2012, average densities of sylleptic branches (i.e. number of branches per unit of stem length) in CsRAV1-overexpressors were about 50% (event #60) and 75% (event #37) higher than in wild-type (WT) trees ($p > 0.05$) (Figure 1c; see Additional file 1: Figure S1a and Additional file 4: Table S1). During the next growing season of this first cultivation cycle, axillary buds on the new growth of the main stem or on lateral branches, both sylleptics and proleptics, did not burst in any of the five genotypes on trial. A major concern about the sustainability of genetically modified crops is related to the potential instability of the introduced genetic modification over time,

involving silencing mechanisms that could disable the desired trait [32]. To test whether the introduced genetic modifications persisted over time, during 2013 basal branches were sampled to analyze the stability of those transgenes in the field. The transgenic fusion protein 3xHA:CsRAV1 was detected in both CsRAV1 OX events, whereas the endogenous target protein PtaRAV1 was detected in all transgenic and WT trees, showing a similar abundance in CsRAV1-overexpressors and WT trees, and very reduced levels in PtaRAV1&2 KD events #1 and #22 relative to the WT (Figure 1d). It indicated that the genetic modifications introduced in these poplars, CsRAV1 overexpression and PtaRAV1 downregulation, continued functioning after several months of growing in the field, and that both events tested per modification behaved similarly at the molecular level.

After coppicing in December 2013, trees grew as multi-trunk individuals with multiple shoots resprouting from the remaining 10 cm-long stumps. As in the first cultivation cycle, sylleptic branches developed during the first but not the second growing season of the cycle. So, at the end of 2014, we calculated densities of sylleptic branches growing along dominant shoots (i.e. the highest and thickest shoot resprouted from each tree stump). Average densities of sylleptics on dominant shoots in both CsRAV1 OXs were about 9% (event #37) and 55% (event #60) higher than in WT trees. Conversely, PtaRAV1&2 KDs developed some 10% (event #1) and 18% (event #22) less sylleptics than WT trees ($p > 0.05$) (Figure 2a; see Additional file 1: Figure S1a and Additional file 4: Table S1). CsRAV1 OX and PtaRAV1&2 KD events showed a greater and a slightly lower degree of syllepsis, respectively, relative to WT trees. This tendency, which persisted up until the

completion of the field trial 4 years after its establishment, suggested that those genetic modifications were working over all that time.

Shoots growing from each coppiced tree stump were also counted. Data were collected in December 2015, on completion of the second cultivation cycle, and they revealed that CsRAV1 OX and PtaRAV1&2 KD events tended to develop slightly fewer (c. 5%) and more (c. 5%) shoots, respectively, relative to WT trees ($p > 0.05$) (Figure 2b; see Additional file 1: Figure S1a and Additional file 4: Table S1).

Genetically modified trees maintained the same structural, chemical composition and combustion wood properties as the WT poplars

Besides transgene stability over time, another major concern about transgenesis is pleiotropy and non-desirable side effects caused by the introduced genetic change. The assayed transgenics in this field trial showed an unaltered overall health condition respect to the WT trees. Closer inspection was made of those traits concerning the quality of the produced wood. Individuals of CsRAV1 OX#60, WT and PtaRAV1&2 KD#1 tree genotypes were randomly selected to compare anatomy, chemical composition and combustion properties of their woods. Calcofluor white staining and lignin autofluorescence of branch sections (fifth internodes) showed a similar overall structure and organization, as well as similar cellulose and lignin contents of the transgenic and WT woods (Figure 3a). Chemical analyses confirmed that there were no significant differences among these genotypes in wood extractives ($p > 0.05$), Klason lignin contents ($p > 0.05$) and syringyl/guaiacyl (S/G) subunit ratios ($p > 0.05$) (Figure 3b; see Additional file 4: Table S1). We further

309 determined wood high calorific values for these genotypes, and in accordance with
310 the ascertained data for wood composition, found that the transgenic and WT
311 woods produced the same amount of heat by combustion ($p > 0.05$) (Figure 3c; see
312 Additional file 4: Table S1). Thus, it is reasonable to predict that any modification of
313 the RAV1 gene expression in a commercial elite poplar clone is not likely to affect
314 the structure and composition of its wood, nor the bioenergy properties of its
315 biomass.

316 **RAV1-engineering impacts differentially on growth and aerial biomass yield over**
317 **cultivation cycles**

318 On completion of the first cultivation cycle in December 2013 (Figure 4a), event
319 CsRAV1 OX#60 showed an average diameter of its main stem about 6% thicker and
320 an average aerial biomass yield about 9% greater than in WT trees. Conversely,
321 event PtaRAV1&2 KD#1 displayed an average diameter of its main stem that was
322 some 6% thinner and an average aerial biomass yield about 11% lower than in WT
323 trees (stem diameter $p < 0.01$; aerial biomass yield $p < 0.05$) (Table 1 and Figure 4b;
324 see Additional file 2: Figure S2a, Additional file 3: Figure S3 and Additional file 4:
325 Table S1). However, significance relied solely when comparing means from CsRAV1
326 OX#60 and PtaRAV1&2 KD#1 genotypes (stem diameter $p < 0.05$; aerial biomass
327 yield $p < 0.05$). Therefore, these results obtained over the course of a first
328 cultivation cycle (before coppicing) stand up for the viability of RAV1-engineering to
329 improve aerial biomass yields of high-density poplar plantations of trees growing as
330 single-trunk individuals.

On completion of the second cultivation cycle in December 2015, shoot growth and aerial biomass yields data from the CsRAV1 OX events revealed that despite having developed sylleptic branches, dominant shoots from both CsRAV1-overexpressors were smaller than in WT trees, showing reduced average diameters ($p < 0.001$) and heights ($p < 0.001$). Diameters were reduced about 15% (event #60 $p < 0.05$) and 18% (event #37 $p < 0.01$); heights were reduced about 11% (event #60 $p < 0.05$) and 14% (event #37 $p < 0.01$) (Table 1; see Additional file 2: Figure S2b and Additional file 4: Table S1). As a result, these transgenics yielded an average aerial biomass that was some 25% less than in WT trees ($p > 0.05$). Unexpectedly, growth performance of PtaRAV1&2-knockdown events was slightly altered, leading them to yield about 10% (event #1) and 17% (event #22) less aerial biomass than WT trees ($p > 0.05$) (Figure 4c; see Additional file 3: Figure S3 and Additional file 4: Table S1). Their dominant shoots displayed reduced average diameters and heights of about 5% for both traits ($p > 0.05$) (Table 1; see Additional file 3: Figure S2b and Additional file 4: Table S1).

DISCUSSION

Cultivation of poplar and other fast-growing woody species as SRC is an increasingly widespread practice for the production of lignocellulosic biomass as carbon-neutral renewable energy source. Productivity and sustainability of forest and SRC plantations depends on the cultivars used but also and very importantly on the interactions of their productivity determinants with the environmental conditions over time. In this work we established a field trial to test the sustainability of the

353 increased syllepsic branchiness of CsRAV1-overexpression hybrid poplars over
354 subsequent cultivation cycles and outdoors, where those interactions are much
355 more complex than in a greenhouse and therefore plants may show a greater
356 phenotypic variation, making unpredictable the outcome of such experimental
357 approach.

358 On completion of the first cultivation cycle in December 2013, aerial biomass yields
359 and stem growth data from events CsRAV1 OX#60 and PtaRAV1&2 KD#1 were
360 consistent with those reported in other studies, in which sylleptics were noted to
361 contribute to the thickening of stems by allocating to a greater portion of
362 photosynthates than proleptics, and hence to enhance the aboveground biomass
363 yield [14-18]. Inversely, aerial biomass yields and shoot growth data gathered on
364 completion of the second cultivation cycle in December 2015 from the CsRAV1-
365 overexpressors pointed to what has been reported for the relationship between
366 syllepsis and stem growth and its dependency on the environmental conditions
367 [15]. Despite having developed sylleptic branches, dominant shoots from both
368 events CsRAV1 OX were smaller than in WT trees. Also, shoot resprouting after
369 coppicing was reduced in these events, suggesting that the available nutrient
370 resources were mainly invested in the production of sylleptic branches. A recent
371 study by [42] has enabled the identification in willow of a resprouting locus *SxMAX4*
372 (*MORE AXILLARY GROWTH 4*) mapping within a quantitative trait locus for coppicing
373 response.

We concluded that over the course of the two cultivation cycles CsRAV1-overexpression led to an enhanced development of sylleptic branching in the field. These facts confirmed that local geoclimate factors and the chosen culture conditions of planting density, watering and fertilization regimes were adequate to allow for and sustain syllepsis in CsRAV1-overexpression poplars, at least during the first growing seasons of each cultivation cycle as single- and multiple-trunk individuals (first and second cultivation cycles, respectively). Yet, on the basis of the aerial biomass yields and shoot growth data on completion of the second cultivation cycle, we speculate that after coppicing and resprouting of multiple shoots, CsRAV1-overexpression trees, which displayed a larger light interception area and a carbon gain provided by leaves on sylleptics, might have suffered a carbon (C)/nitrogen (N) imbalance [43]. In effect, available N for those events could have been ultimately insufficient, impairing their aboveground growth [44,45,46]. In line with this hypothesis, this phenomenon did not occur before coppicing when trees put out a single main stem. As a solution to this problem, increasing the N supply appears unfeasible in the long term, given the increasing costs of N fertilizers and their adverse effects on the environment. More sustainable solutions would be the addition of endophytes to the soil to improve nitrogen fixation [47] along with biotechnology-driven solutions to enhance N utilization. In effect, the latter have proved successful in a broad range of crop plants [48,49], including poplar trees grown in the field [31]. Another option could be to target *RAV1* expression in commercial varieties bred for high-efficiency N use returning good yields under SRC management.

It is worth noting that average amounts of aerial biomass obtained from the hybrid poplars used in this trial, widely used in basic research, were far from those reported for commercial poplar varieties bred to produce good yields [22], so the viability of RAV1-engineering will depend on the genetic transformation of these commercial elite trees. In addition, disparity of results between events of the same transgenic line (CsRAV1-overexpression line or PtaRAV1&2-knockdown line) points out the necessity and importance of selecting the best performing events in the field.

CONCLUSIONS

In summary, syllepsis and growth measurements while growing as single trunk individuals as well as wood structure and composition analyses showed that, apart from early branching, no other traits were altered in our CsRAV1-overexpression trees. These findings support the potential application of this genetic modification to increase syllepsis in commercial elite trees without changing other traits. Thus, RAV1-engineering or marker-assisted breeding based on this gene followed by the selection of the best performing events or individuals could certainly improve early branching and eventually lignocellulosic biomass production of poplar SRC. Yet, improvements on biomass yielding will depend on the achievement of the optimal conditions enabling sustainability of an increased aboveground growth. The use of tree biotechnology has the potential to develop forest plantations highly productive and sustainable, which in turn will help conserve natural forests and mitigate the

418 effects of climate change. Indeed, few other options can match the potential of
419 forestry in this respect [50].

420 **LIST OF ABBREVIATIONS**

421 co: cortex; DNA: deoxyribonucleic acid; G: guaiacyl; GWAS: genome-wide
422 association study; HA: haemagglutinin; hpiRNA: hairpin RNA interference; IgG:
423 Immunoglobulin G; KD: knockdown; OX: overexpression; RNA: ribonucleic acid; S:
424 syringyl; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; SRC:
425 short rotation coppice; WT: wild-type; xy: xylem.

426 **DECLARATIONS**

427 **Ethics approval and consent to participate**

428 Not applicable

429 **Consent for publication**

430 Not applicable

431 **Availability of data and materials**

432 All datasets generated and analyzed during the current study are available from the
433 corresponding author on reasonable request. All the analyses made are included in
434 this published article and its supplementary information files.

435 **Competing interests**

436 The authors declare that they have no competing interests

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Authors' contributions

A. M-C., and I.A. planned and designed the research, A. M-C., T. H-V. and P. G-M. performed experiments, H.S. and I.C. design, conducted and analyzed fieldwork, M.G., A.A., R.S. and JC.R. designed, conducted and analyzed wood chemistry analysis, A.M.C and JM.R-S. analyzed data, A.M-C. and I.A. wrote the manuscript.

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FIGURES, TABLES, ADDITIONAL FILES

Figures

Figure 1. Field trial establishment, syllepsis of RAV1-engineered poplars and RAV1-protein abundances during the first cultivation cycle. (a) Image of the field trial once established (July, 2012). (b) Sylleptic branches on the apical segment of the main stem in the representative event CsRAV1 OX#60 (white arrows), as opposed to wild-type (WT), and event PtaRAV1&2 KD#1 (November, 2012); bar=10 cm. (c) Densities of sylleptic branches on the main stem of WT and CsRAV1-overexpression and PtaRAV1&2-knockdown transgenic poplars at the end of the establishment year (December, 2012). Bars represent average values ±SE (CsRAV1 OX#60 *n*=30, CsRAV1 OX#37 *n*=30, WT *n*=29, PtaRAV1&2 KD#22 *n*=25, PtaRAV1&2 KD#1 *n*=30). (d) Upper panel: Western blot of the chestnut transgenic protein CsRAV1 tagged to 3xHA in both CsRAV1-overexpression events tested and the WT. Lower panel: Western blot of the poplar endogenous protein PtaRAV1 in all four transgenics and the WT as control. Membranes were stained with Ponceau to ensure equal sample loading.

Figure 2. Sylleptic branching and shoot resprouting phenotypes of RAV1-engineered poplars during the second cultivation cycle. (a) Densities of sylleptic branches on the dominant shoots of wild-type (WT) and CsRAV1-overexpression and PtaRAV1&2-knockdown transgenics. Measurements were made in December 2014 at the end of the first growing season after the first coppicing. (a) Shoot number growing from the remaining 10 cm-long stumps of WT and events CsRAV1 OX and PtaRAV1&2 KD. Scoring was made before a second harvest in December 2015. Bars represent

619 average values ±SE (CsRAV1 OX#60 *n*=30, CsRAV1 OX#37 *n*=30, WT *n*=29,
620 PtaRAV1&2 KD#22 *n*=30, PtaRAV1&2 KD#1 *n*=30).

621 Figure 3. Wood structure and chemical wood composition of the RAV1-engineered
622 poplars. (a) Wood histochemistry analyses of branch cross sections (5^{th} internode)
623 obtained from wild-type (WT) trees and representative events 3xHA:CsRAV1 OX#60
624 and PtaRAV1&2 KD#1, sampled after coppicing in December 2013. Left column:
625 cellulose detection by calcofluor white staining. Right column: detection of lignin
626 autofluorescence. co: cortex; xy: xylem; *: sclerenchyma; bar=100 µm. (b) Xylem
627 composition of WT trees and representative events CsRAV1 OX#60 and PtaRAV1&2
628 KD#1 after coppicing in December 2013, including total extractives, Klason lignin
629 content and S/G ratio. Bars represent average values ±SD (CsRAV1 OX#60 *n*=4, WT
630 *n*=4, PtaRAV1&2 KD#1 *n*=4). (c) High calorific values of coppiced biomass obtained
631 from WT trees and events CsRAV1 OX#60 and PtaRAV1&2 KD#1. Bars represent
632 average values ±SD (CsRAV1 OX#60 *n*=3, WT *n*=3, PtaRAV1&2 KD#1 *n*=3).

633 Figure 4. Aboveground biomass yields of the RAV1-engineered poplars after two
634 cultivation cycles. (a) Picture of the field trial after coppicing in December 2013,
635 showing the 10 cm-long stumps. Dry aerial biomass yields of wild-type (WT) and
636 CsRAV1-overexpression and PtaRAV1&2-knockdown transgenics, after (b) the first
637 coppicing in December 2013, and (c) the second coppicing in December 2015. Bars
638 represent average values ±SE (CsRAV1 OX#60 *n*=30, CsRAV1 OX#37 *n*=30, WT *n*=29,
639 PtaRAV1&2 KD#22 *n*=30, PtaRAV1&2 KD#1 *n*=30). Letters represent significant
640 differences between genotypes ($p < 0.05$).

641 **Tables**

642 Table 1. Summary of growth-related data recorded from RAV1-engineered poplars
643 over the course of the field trial.

	CsRAV1 OX#60	CsRAV1 OX#37	wild-type	PtaRAV1&2 KD#22	PtaRAV1&2 KD#22
First rotation					
Year 2012					
Stem height (cm)	321.3 ± 5.0 a	313.8 ± 3.7 ab	318.1 ± 4.0 a	295.2 ± 5.6 b	313.9 ± 4.6 ab
Stem diameter (mm)	12.9 ± 0.4 a	12.8 ± 0.3 a	11.9 ± 0.3 ab	10.7 ± 0.4 b	11.8 ± 0.3 ab
Year 2013					
Stem heigh (cm)	506.8 ± 11.1 ns	484.8 ± 9.2 ns	498.8 ± 12.7 ns	496.1 ± 11.5 ns	475.7 ± 15.4 ns
Stem diameter (mm)	24.8 ± 0.6 a	23.1 ± 0.5 ab	23.3 ± 0.6 ab	22.4 ± 0.6 ab	21.5 ± 0.8 b
Second rotation					
2014					
Dominant shoot height (cm)	537.9 ± 11.9 bc	515.6 ± 11.5 b	602.3 ± 10.1 a	565.4 ± 9.8 ac	574.8 ± 9.1 ac
Dominant shoot diameter (mm)	21.1 ± 0.9 a	20.6 ± 0.8 a	26.7 ± 0.8 b	24.6 ± 1.0 b	26.4 ± 0.8 b
2015					
Dominant shoot height (cm)	704.1 ± 20.0 a	679.5 ± 20.5 a	793.5 ± 16.9 b	728.2 ± 21.0 ab	779.7 ± 15.5 b

Dominant shoot diameter (mm)	31.2 ± 1.3	a	30.0 ± 1.2	a	36.6 ± 1.3	b	33.3 ± 1.3	ab	35.9 ± 1.0	b

Average values for heights and diameters of the main stem and the dominant shoot ±SE (CsRAV1 OX#60 *n*=30, CsRAV1 OX#37 *n*=30, WT *n*=29, PtaRAV1&2 KD#22 *n*=30, PtaRAV1&2 KD#1 *n*=30) of wild-type (WT) and CsRAV1-overexpression and PtaRAV1&2-knockdown transgenics. Measurements were made at the end of every year. Letters a, b and c represent significant differences between genotypes ($p < 0.05$); ns: no significance.

ADDITIONAL FILES

Additional file 1. Containing supplementary figure S1; supplementary figure legend is contained within the file. (.pdf)

Figure S1. Syllepsis and shoot resprouting performance of the RAV1-engineered poplars in the field. Scatterplots showing the distribution of individual values per block (a) for densities of sylleptic branches on the main stem (first cultivation cycle, upper graph) and on the dominant shoot (second cultivation cycle, lower graph); and (b) for the number of shoots resprouting from the remaining 10 cm-long stumps. Counting of sylleptic branches was made in December 2012 and 2014, and shoots in December 2015, respectively. Horizontal lines represent median values per block.

Additional file 2. Containing supplementary figure S2; supplementary figure legend is contained within the file. (.pdf)

663 Figure S2. Growth-related characteristics of the RAV1-engineered poplars in the
664 field. Scatterplots showing the distributions of individual values per block (a) for
665 heights and diameters (a) of the main stem (first cultivation cycle, years 2012 and
666 2013) and (b) of the dominant shoot (second cultivation cycle, years 2014 and 2015)
667 of wild-type (WT) and CsRAV1-overexpressing and PtaRAV1&2-knockdown
668 transgenic poplars. Horizontal lines represent median values per block.

669 Additional file 3. Containing supplementary figure S3; supplementary figure legend
670 is contained within the file. (.pdf)

671 Figure S3. Aboveground biomass yields of the RAV1-engineered poplars after two
672 cultivation cycles. Scatterplots showing the distributions of individual values per
673 block, for the aerial biomass production of wild-type (WT) and CsRAV1-
674 overexpression and PtaRAV1&2-knockdown transgenics. Trees were coppiced in
675 December 2013 (first cultivation cycle, upper graph) and December 2015 (second
676 cultivation cycle, lower graph). Horizontal lines represent median values per block.

677 Additional file 4. Containing supplementary table S1; showing the statistical tests
678 used to analyze all traits measured over the course of the field trial. (.pdf)

679 Table S1. Statistical tests used to analyze all traits measured over the course of the
680 field trial. Differences among genotypes were identified using *post-hoc* Tukey HSD
681 test for ANOVA analyses, and pairwise comparisons with the Wilcoxon test for
682 Kruskal-Wallis analyses.