

A systems biology view of wood formation in *Eucalyptus grandis* trees submitted to different potassium and water regimes

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Summary

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- Wood production in fast-growing *Eucalyptus grandis* trees is highly dependent on both potassium (K) fertilization and water availability but the molecular processes underlying wood formation in response to the combined effects of these two limiting factors remain unknown.
- *E. grandis* trees were submitted to four combinations of K-fertilization and water supply. Weighted gene co-expression network analysis and MixOmics-based co-regulation networks were used to integrate xylem transcriptome, metabolome and complex wood traits. Functional characterization of a candidate gene was performed in transgenic *E. grandis* hairy roots.
- This integrated network-based approach enabled us to identify meaningful biological processes and regulators impacted by K-fertilization and/or water limitation. It revealed that modules of co-regulated genes and metabolites strongly correlated to wood complex traits are in the heart of a complex trade-off between biomass production and stress responses. Nested in these modules, potential new cell-wall regulators were identified, as further confirmed by the functional characterization of *EgMYB137*.
- These findings provide new insights into the regulatory mechanisms of wood formation under stressful conditions, pointing out both known and new regulators co-opted by K-fertilization and/or water limitation that may potentially promote adaptive wood traits.

Introduction

Wood, or secondary xylem, is formed by the activity of an internal meristem called the vascular cambium, through a complex differentiation process leading to highly specialized xylem cells characterized by thick, lignified secondary cell walls (SCWs; Plomion *et al.*, 2001). Besides being a renewable source of material for humans, providing raw material for timber, paper, energy, second-generation biofuels as well as for added-value biomaterials (Mizrachi *et al.*, 2012), wood represents a major carbon sink important for climate regulation (Bonan, 2008). The physico-chemical properties of wood, which depend mainly on the chemical composition and the structure of SCWs, determine wood industrial end-uses (Mansfield, 2009). In xylem, SCWs are made of *c.* 75% of polysaccharides (cellulose and hemicelluloses) and 25% of lignin, a complex hydrophobic phenolic polymer.

The fast-growing eucalypts, characterized by their outstanding growth performance, short rotation time and wide adaptability, are the most planted trees worldwide (Hinchee *et al.*, 2009). Eucalypt plantations are mostly established in tropical and

subtropical regions where the main environmental constraints are soil nutrient starvation and drought episodes (Gonçalves *et al.*, 1997). In highly weathered tropical soils, potassium (K) is a major limiting factor for tree growth (Laclau *et al.*, 2009; Wright, 2011; Epron *et al.*, 2012). Besides its essential role in a variety of cellular processes (reviewed by Sardans & Peñuelas, 2015), K is considered as a key osmolyte for wood formation (Fromm, 2010). K fertilization in K-deficient soils can lead to a dramatic increase in wood biomass production in tree species such as pine (Smethurst *et al.*, 2007), poplar (Arend *et al.*, 2004; Wind *et al.*, 2004) and eucalypts (Laclau *et al.*, 2009). In these conditions, a higher cambial activity is correlated with a peak of K in the cambial zone (Kuhn *et al.*, 1997; Arend *et al.*, 2002; Langer *et al.*, 2004; Wind *et al.*, 2004; Fromm, 2010). K also influences xylem structure, probably through its role in turgor-driven elongation of new xylem cells. It has an impact on the deposition dynamics of SCWs as K-starved trees show earlier initiation of SCW deposition than controls (Dunisch *et al.*, 1998; Langer *et al.*, 2002).

In a context of climate change, the intensity and frequency of future drought episodes are likely to increase in tropical and

subtropical regions, affecting the productivity of *Eucalyptus* plantations (Hawkins & Sutton, 2012; IPCC, 2013). Potassium contributes to plant resistance to abiotic stresses, including drought, by improving processes such as osmotic adjustment, stomatal aperture control, photosynthesis activation, reactive oxygen species (ROS) detoxification and phloem loading of photoassimilates (reviewed by Wang *et al.*, 2013). Some studies have suggested that K modulates xylem hydraulic conductance, possibly by interacting with pectins in intervessel pits, enhancing tree resistance to drought (Nardini *et al.*, 2010; Jansen *et al.*, 2011). Recently, using a large-scale through-fall exclusion experiment, Battie-Laclau *et al.* (2016) showed that K fertilization improved water use efficiency and biomass production in *Eucalyptus*. However, during drought episodes, they noticed that the beneficial effect of K may not be sufficient to counterbalance the detrimental effect of increased water demand caused by higher growth rates of K-fertilized trees (Battie-Laclau *et al.*, 2014a,b). Tree responses to drought in interaction with K-fertilization start to be well documented in organs such as leaves and roots (Cakmak, 2005; Anschütz *et al.*, 2014), but xylem has received much less attention. Most studies performed in this tissue have focused on drought-induced structural modifications and their impact on water transport properties (Arend & Fromm, 2007; Zwieniecki & Secchi, 2015). The changes occurring at the transcriptomic and metabolomic levels in xylem in response to drought and K-fertilization remain unstudied.

Wood formation is a highly regulated process mainly controlled at the transcriptional level, and the expression of SCW-associated genes is tightly spatiotemporally regulated (Hertzberg *et al.*, 2001; Demura & Fukuda, 2007) through a complex hierarchical regulatory network (reviewed by Hussey *et al.*, 2013; Yang & Wang, 2016). Recent studies have highlighted the importance of a dynamic cross-talk between the regulation of SCWs during development and in response to stresses, which is probably necessary to promote adaptation to environmental changes (Zinkgraf *et al.*, 2017). For instance, changes in the thickness, composition and/or structure of SCWs have been observed in response to several stresses such as nitrogen excess or depletion (Camargo *et al.*, 2014; Euring *et al.*, 2014), mechanical stress (Mellerowicz & Gorshkova, 2012) or low temperature (Poyet *et al.*, 2018). Transcription factors (TFs) involved in the regulation of SCW formation as well as SCW biosynthesis genes were reported to be induced by environmental constraints such as high salinity or iron deprivation in *Arabidopsis* (Taylor-Teeple *et al.*, 2015) or cold stress in *Eucalyptus* (Poyet *et al.*, 2018). Some of these TFs were shown to be involved in stress tolerance (Ramírez *et al.*, 2011; Guo *et al.*, 2017).

To gain a comprehensive view of a complex process such as the regulation of wood formation in response to environmental cues, systems biology approaches seem the most well adapted (Cramer *et al.*, 2011). Indeed, the integration of transcriptomic and metabolomic approaches has provided very valuable insights into tension wood formation (Andersson-Gunnerås *et al.*, 2006) and the lignin biosynthetic pathway (Vanholme *et al.*, 2012; Chen *et al.*, 2014) as well as the primary wall transition to SCWs (Li *et al.*, 2016a,b). Taylor-Teeple *et al.* (2015) constructed

co-regulation networks inferred from protein–DNA interactions to refine the SCW regulatory network under abiotic stresses in *Arabidopsis* and highlighted new key regulators. In poplar, co-expression networks were used to identify cell-type-specific TFs involved in the regulation of SCW biosynthesis in xylem (Shi *et al.*, 2017), and modules of genes involved in xylem formation under abiotic stresses (Cai *et al.*, 2014; Zinkgraf *et al.*, 2017; Wildhagen *et al.*, 2018). Mizrahi *et al.* (2017) applied a network-based data integration method to reveal the pathways underlying biomass and bioenergy-related traits using a segregating *Eucalyptus* hybrid population. Very recently, Wang *et al.* (2018) developed a multi-omics integrative analysis of lignin biosynthesis in poplar to quantify the effects of the expression of monolignol biosynthetic pathway genes on wood properties.

To gain an integrated view of the regulation of wood formation in response to a combination of K and water regimes, we collected differentiating xylem from 4-yr-old *Eucalyptus grandis* trees grown in four contrasting treatments. We performed both transcriptomic and metabolomic profiling to detect differentially expressed genes (DEGs) and differentially accumulated mass signatures (DAMs), respectively. We also evaluated trunk growth, xylem structure and saccharification yield as a proxy for changes in SCW composition. We then constructed WGCNA (weighted gene co-expression network analysis) and MixOmics-based co-regulation networks to correlate changes in transcriptome, metabolome and wood traits. This systems biology approach enabled us to identify biological processes and meaningful subset of genes impacted by K-fertilization and/or water limitation. Among the latter, TFs highly connected to SCW biosynthetic genes and wood traits were highlighted as potentially involved in SCW regulation. We functionally characterized the most promising candidate and confirmed its ability to regulate SCW deposition in *E. grandis*.

Materials and Methods

Plant material and sampling

We used the experimental field trial set up in 2010 using an *E. grandis* commercial clone (Suzano Company, São Paulo, Brazil) at the Itatinga Experimental Station of the University of São Paulo (23°020'S, 48°380'W, for a detailed description see Battie-laclau *et al.* (2014a)). Our study focused on four conditions combining K-fertilization and water availability: [+W–K] (no K-fertilization and no throughfall exclusion); [–W–K] (no K-fertilization and 37% of throughfall excluded); [+W+K] (0.45 mol K m^{–2} applied as KCl and no throughfall exclusion); and [–W+K] (0.45 mol K m^{–2} applied as KCl and 37% throughfall exclusion). For each condition, phenotyping and xylem sampling were performed on four independent trees (Supporting Information Fig. S1).

Tree circumference (CBH) was measured at breast height (BH; 1.3 m above ground) every 15 d. Trunk biomass was quantified after cutting down the trees as described by Battie-Laclau *et al.* (2016). An allometric equation allowing calculation of trunk biomass from CBH (González-García *et al.*, 2016) was applied to

predict biomass production per day (kg of dry matter d⁻¹ per tree) for each tree, over the 3 months preceding sampling. Daily average gross primary productivity (GPP, gC m⁻² d⁻¹) was estimated using the model established by Christina *et al.* (2015). The proportion of carbon allocated to trunk secondary growth (%GPP_{stem}) was obtained for each tree by dividing the trunk biomass increment (kgDM d⁻¹ per tree) by the simulated tree gross primary productivity (gC d⁻¹ per tree).

Wood samplings were performed on four independent trees per condition (at BH) in May 2014, during a severe dry season (Fig. S2). Wood disks were collected for wood micro-density measurements, and micro-core samples (length: 15 mm; diameter: 2 mm) were fixed and stored in 80% ethanol for histology analyses. Developing xylem (including cambium) was collected by scraping after removing the bark and immediately frozen in liquid nitrogen for transcriptomic and metabolomic analyses. Metabolome and wood properties were analyzed on the four biological replicates for each condition and three replicates were used to perform transcriptomic analyses (Fig. S3).

RNA extraction and analyses of the transcriptome

RNA extraction and integrity controls were performed as previously described (Ployet *et al.*, 2018). Three sequencing libraries per treatment were generated and sequenced (Illumina, San Diego, CA, USA; 150 bp paired-end reads) by the Genotoul GeT platform (<https://get.genotoul.fr/en/>; France) using the HiSeq3000 (Illumina). An average of 46 million reads per library was generated, and > 90% were mapped to the *E. grandis* reference genome (v.1.1, <https://phytozome.jgi.doe.gov/>) using TOPHAT2 (Kim *et al.*, 2013). Sequencing data were registered at the NCBI SRA database (PRJNA514408). For a subset of selected genes, RNAseq expression levels were confirmed by real-time quantitative PCR (Fig. S4; Table S1) as described by Ployet *et al.* (2018).

Metabolite extraction and biochemical analyses

Soluble metabolites were extracted from four independent biological replicates and analyzed according to the methods described by De Vos *et al.* (2007) and Hoffmann *et al.* (2017) for GC-MS and LC-MS, respectively, including minor changes as detailed in Method S2. Biochemical analyses of SCWs were done in triplicate from extractive-free xylem residue (EXR) obtained as described by Ployet *et al.* (2018) and detailed in Method S1. Saccharification was estimated with or without alkali pretreatment, mainly as described by Van Acker *et al.* (2016). Reducing sugar concentration was assessed with dinitrosalicylic acid (DNS) reagent using a 10 µl aliquot of the supernatant after 6, 24 and 48 h of reaction. Enzyme activity was assessed at 0.25 filter paper units (FPU) ml⁻¹ in our conditions.

Vector construction and plant transformation

The coding region (1050 bp) of *EgMYB137* (*Eucgr.K02806*), and a fragment of 2 kb upstream of the start codon (promoter)

were amplified from *E. grandis* xylem cDNA and genomic DNA, respectively, using specific primers (Table S1). The DNA fragments were then cloned into Gateway-adapted binary vectors pGWAY-0 and pGWAY-1, for overexpression and promoter activity analyses respectively and transformed into *Eucalyptus* hairy roots as described by Plasencia *et al.* (2016). *Eucalyptus* composite plants harboring transgenic hairy roots were grown in OIL DRI substrate US-Special Substrate (Type III/R; Damolin, Fur, Denmark) in 200 ml pots for a period of 5 months (16 h photoperiod, 25/22°C). Fluorescent roots expressing DsRed were collected on seven to nine independent lines for further analyses. Histochemical GUS assays were performed as described by Plasencia *et al.* (2016).

Microscopy and histochemistry

Wood density was measured as described by Dobner *et al.* (2018). Average density (g cm⁻³) was measured by X-ray radiography every 40 µm along the first 4 mm of differentiating xylem zone using a QTRS-01 × Tree Ring Analyzer according to the manufacturer's procedure (Quintek Measurement Systems, Knoxville, TN, USA). For xylem structure analysis, transverse sections were made from micro-core samples (three biological replicates per condition) and transgenic roots (six *p35S:EgMYB137* lines and four controls), and embedded in LR White resin (London Resin Company Ltd, Reading, UK). Semi-automatic image analyses of semi-thin sections (1 µm) were performed as previously described (Ployet *et al.*, 2018) to estimate SCW thickness (Method S1). Average vessel diameter was measured by image analyses on > 400 vessels (diameter cut-off > 35 µm) using IMAGEJ software (v.1.5).

Statistical analyses and data integration

For transcriptomic analyses, we combined CUFFDIFF v.2.2.1 (Trapnell *et al.*, 2012) and DESEQ2 R package v.1.10 (Love *et al.*, 2014) to detect a total of 5573 DEGs (fold change ≥ 2, adjusted *P*-value (false discovery rate, FDR) < 0.01, Table S1). For metabolomic analyses, 2250 mass signatures were obtained by LC-MS (850) and GC-MS (1400). Mass signatures detected in fewer than two technical repetitions and two conditions were discarded. We identified 516 DAMs, 231 and 285 for LC-MS and GC-MS respectively (fold change ≥ 2, FDR < 0.01, Table S2). Missing values were substituted by random forest imputation (Gromski *et al.*, 2014), using the MISSFOREST R package (Stekhoven & Buhlmann, 2012). In total, 6089 DEGs and DAMs were used to perform data mining. Discrimination of the different treatments and biological replicates was done using partial least squares – discriminant analysis (PLS-DA), from the R package MIXOMICS (Lê Cao *et al.*, 2016).

We used the BiNGO package (CYTOSCAPE software, Maere *et al.*, 2005) and G:PROFILER (Reimand *et al.*, 2016) to perform gene ontology enrichment based on *Arabidopsis thaliana* Gene Ontology (GO) classification on the first blast hit of *Eucalyptus* DEGs in the *Arabidopsis* genome (PHYTOZOME v.12). Two networks of GO enrichment were obtained for genes regulated by

either water availability (+W treatments vs –W treatments) or K supply (+K treatments vs –K treatments). They were merged and manually curated to remove redundant GO terms (Fig. 2).

Network analyses based on Pearson pairwise correlations, calculated from three values per condition, were performed as described previously (Ployet *et al.*, 2018). We used WGCNA (Langfelder & Horvath, 2008) to detect modules (softPower = 10, deepSplit = 3 and minModuleSize = 100) and to compute correlations between average module profiles and phenotypic variables. Correlations between standardized profiles of DAMs and DEGs of modules M1–6 were further investigated using MIXDIABLO (Data Integration Analysis for Biomarker discovery using a Latent component method for Omics studies, Singh *et al.*, 2016). In the sparse PLS-DA (sPLS-DA), a full design was chosen in which all datasets are connected, all variables were kept and the number of components set to three. Correlation values (Table S3) were used to graphically represent networks using CYTOSCAPE software. For WGCNA, significant Pearson correlations were used ($FDR < 5.10 \times 10^{-5}$) to select 4305 nodes. For MIXDIABLO, a correlation threshold of 0.8 within the MIXDIABLO similarity matrix was chosen to optimize modularity and allow comparison with the WGCNA network (Fig. S5).

Results

Remodeling of the xylem transcriptome and metabolome in response to K-supply and water availability

Analyses of the transcriptome and the metabolome of differentiating xylem tissues collected from field-grown *E. grandis* trees submitted to four different treatments combining rainfall water availability and K fertilization (Fig. 1a,b) revealed a total of 5573 DEGs and 516 DAMs (Fig. 1c,d, Tables S1, S2), respectively. The four treatments were clearly separated by the first three principal components (PC) of the PLS-DA, explaining 82% and 76% of the total variance of the DEG expression profiles and of the abundance profiles of DAMs, respectively (Figs 1c,d, S3). The first component (PC1) explained the highest percentage of variance (61% for DEGs and 39% for DAMs) clearly separating K-fertilized [+K] from nonfertilized [–K] samples. Indeed, most of the DEGs (>60%, Fig. 1c) and a large proportion of the DAMs (42%, Fig. 1d) were exclusively regulated by K-fertilization. In sharp contrast, 4% of the DEGs and 12% of the DAMs were regulated by water availability only. Interestingly, a substantial percentage of the DAMs (45%) and of the DEGs (34.7%) was regulated by both water and K. The vast majority of the DEGs (90%) and DAMs (79%) regulated by water were also regulated by K whereas only 36% of the DEGs and 52% of the DAMs regulated by K were also regulated by water.

Using a GO enrichment approach, we found that 159 biological processes (Fig. 2; Table S1) were significantly enriched among the DEGs. We made a hierarchical classification of these GO categories from the most general to the most specialized level (level 9, 45 GOs) and represented by green, blue and gray dots, the categories enriched in K-regulated, W-regulated and [K and/or W]-regulated DEGs, respectively. Considering level 9-GO categories,

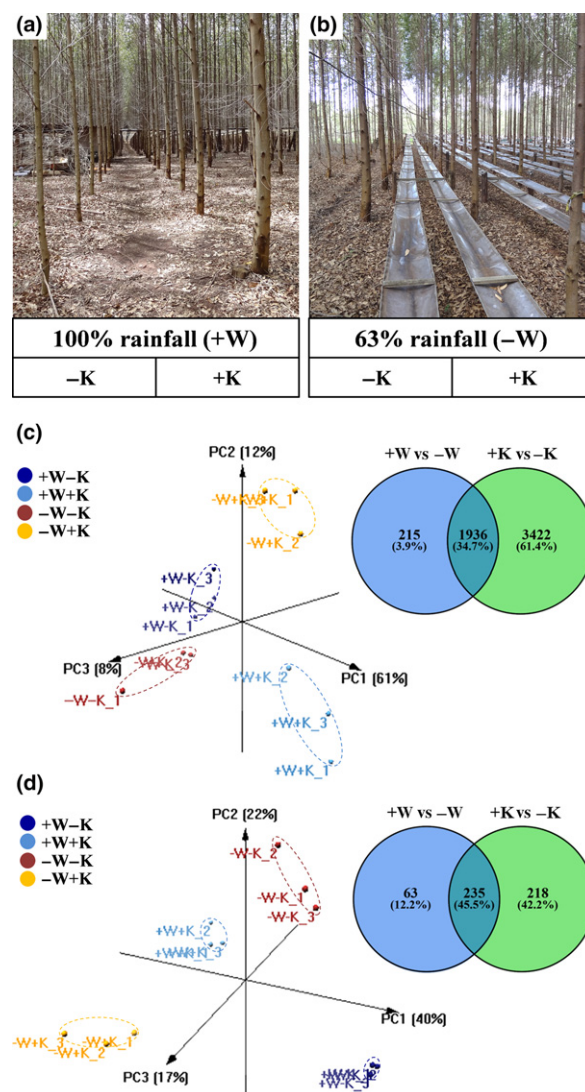


Fig. 1 Water exclusion and K-fertilization induce changes in xylem transcriptome and metabolome. (a, b) Field pictures of the 4-yr-old *Eucalyptus grandis* trees submitted to four treatments: [+W–K], [–W–K], [+W+K] and [–W+K]. In conditions [+W] and [–W], trees received respectively 100% and 63% of rainfall water (Battie-Laclau *et al.*, 2014b). In conditions [+K] and [–K], trees were supplemented or not with K. (c) PLS-DA analyses of 5573 genes (DEGs) differentially expressed (fold change ≥ 2 or ≤ 0.5 and $P_{FDR} < 0.01$, $n = 3$) in response to water exclusion and K fertilization (Supporting Information Table S1). The first three principal components (PC) separate the four treatments and explain 82% of the total variability. PC1 (61% of the total variability) explains the separation between [+K] and [–K] treatments. PC2 and PC3 (respectively 12% and 8% of the total variability) explain the separation between [+W] and [–W] treatments. The Venn diagram describes the proportion of DEGs regulated by water exclusion (+W vs –W), potassium supply (+K vs –K) or both. (d) PLS-DA analyses of 516 mass signatures (DAMs) differentially accumulated ($P_{FDR} < 0.05$, $n = 4$, peak intensity fold change ≥ 2 or ≤ 0.5) in response to water exclusion and K fertilization (Table S2). The first three principal components (PC) explain 76% of the total variability and separate the four treatments. PC1 (39% of the total variability) explains the separation between [+K] and [–K] treatments. PC2 and PC3 (21% and 16% of the total variability respectively) explain the separation between [+W] and [–W] treatments. The Venn diagram describes the proportion of DAMs regulated by water exclusion, potassium supply or both.

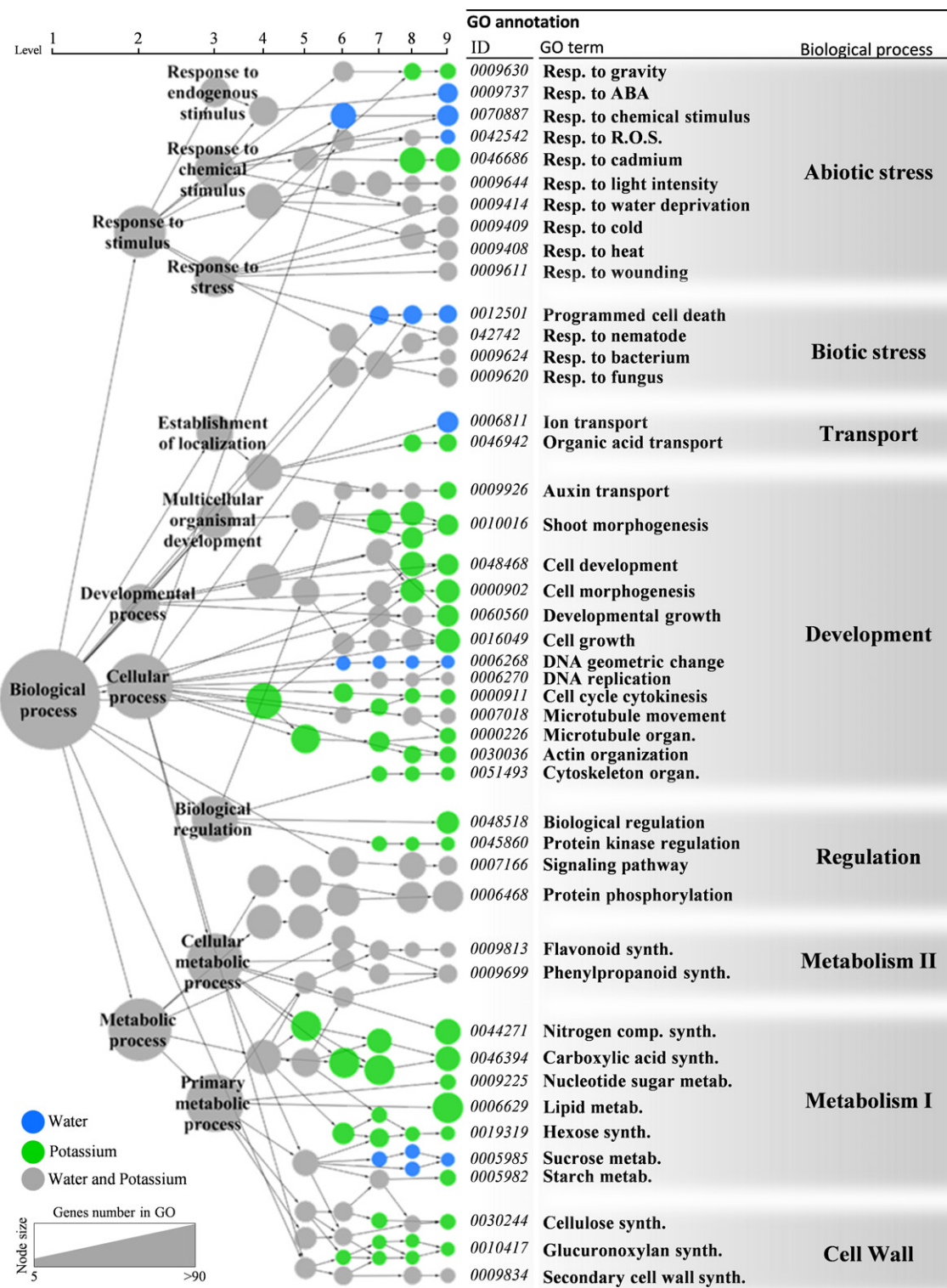


Fig. 2 The changes in transcriptome occur in a broad range of biological processes. Gene Ontology (GO) enrichment analysis on differentially expressed genes (DEGs), in response to K fertilization (5358 DEGs, 96%) or water exclusion (2151 DEGs, 38.5%), in *Eucalyptus grandis* trees. The network was created from BiNGO (Maere *et al.*, 2005) results, manually reorganized and curated to remove noninformative GO terms. A total of 159 significantly enriched GO terms ($P_{FDR} < 0.01$) were kept. Node size is proportional to the number of genes found within GO categories. We made a hierarchical classification of these categories from the most general (level 1) to the most specialized level (level 9). A complete list of GO terms of levels 1–9 is provided in Supporting Information Table S1. The 45 level 9 GOs are linked to development (13 GOs), biotic and abiotic stress responses (4 and 10 GOs respectively), primary metabolism (7 GOs), biological regulation (4 GOs), cell wall biosynthesis (3 GOs), secondary metabolism (2 GOs) and transport (2 GOs). Green dots represent the GOs enriched in DEGs regulated only by K (23 GOs), blue the GOs enriched in DEGs regulated only by W (7 GOs) and gray the GOs enriched in DEGs regulated by W and K (15 GOs).

23 were enriched in K-regulated DEGs, mainly related to development, primary metabolism, cell wall formation, regulatory processes, transport and abiotic stresses. The 15 GOs enriched in K- and/or W-regulated DEGs were related to secondary metabolism, biotic and abiotic stress responses and biological regulation. Only seven GOs were enriched in W-regulated DEGs and included abiotic stress responses and transport.

Integration of transcriptomic and metabolomic data through correlation network analyses

To gain insights into the regulation of xylem transcriptome and metabolome, we analyzed the correlations between 6089 variables representing 5573 DEGs and 516 DAMs (Table S3). We performed a correlations network analysis based on Pearson pairwise correlations to detect gene–gene or gene–metabolite profiles similarities. We selected the most robust correlations (FDR-adjusted

$P < 5.10 \times 10^{-5}$) to build a network in which 4305 nodes (representing either DEGs or DAMs) were connected by 170 629 edges whose lengths were proportional to the absolute values of the corresponding correlations (Fig. 3). We obtained a dense, strongly correlated core network linked to six small subnetworks (Fig. 3). Sixty per cent of the 155 DAMs were spread across the network and correlated to DEGs but the remaining 40% were clustered in a specific subnetwork poorly connected with DEGs (i.e. only six DAMs were connected to DEGs).

Using the WGCNA method, we detected 12 modules containing highly correlated variables and therefore potentially involved in the same biological process (Fig. 3). For each module, we performed a GO enrichment analysis (Table S1) and represented by heatmap DEG profiles in the four treatments (Fig. 3). The expression profiles of the genes in all modules, except module 12, were more dependent on K-fertilization than on water status (Fig. S6).

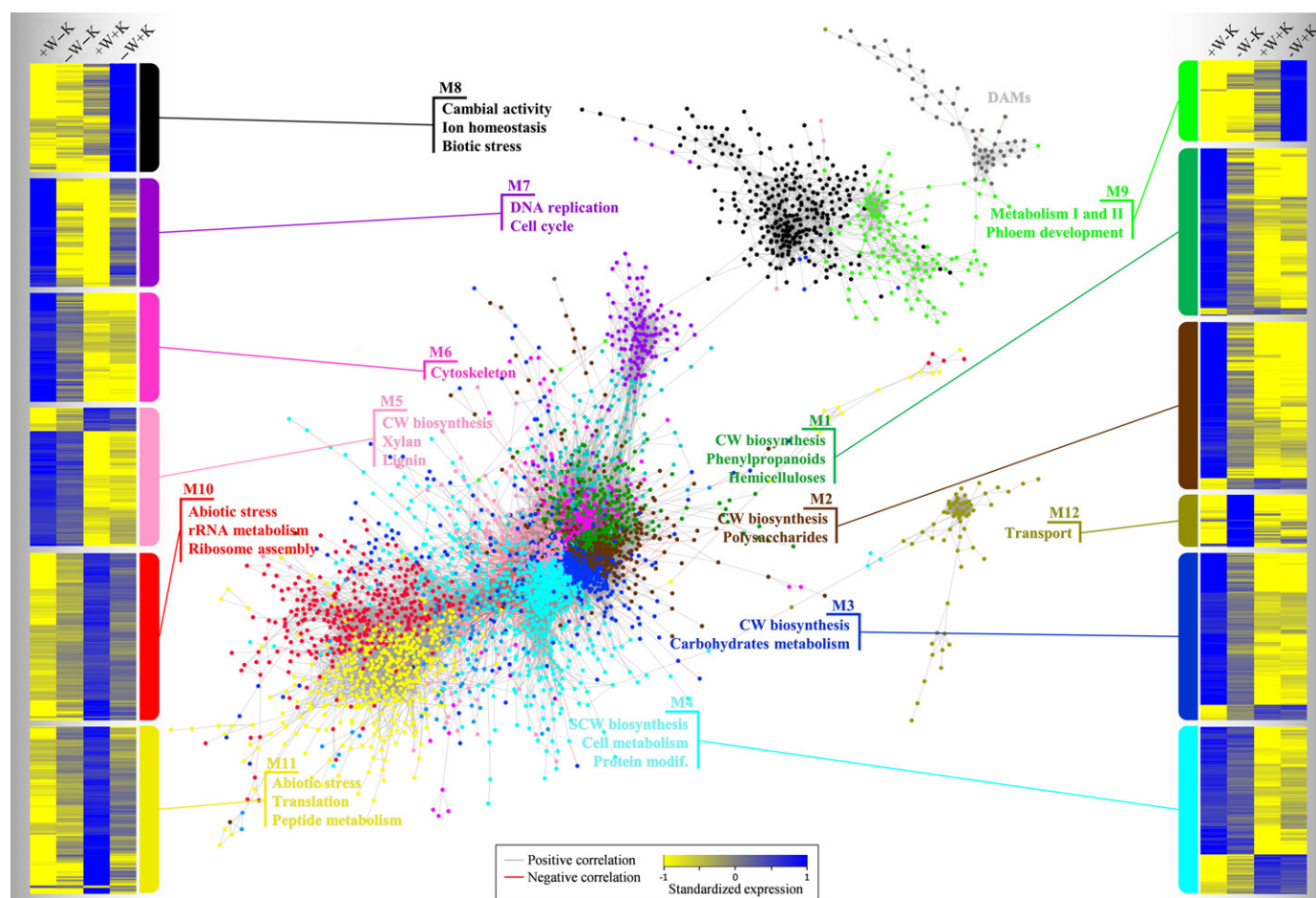


Fig. 3 Co-regulation network highlighting modules of highly correlated genes involved in biological processes associated with cell wall formation. Weighted co-expression network obtained by computing Pearson correlations between standardized profiles of 5573 DEGs and 516 DAMs. Edge lengths are proportional to correlations. The network was visualized using CYTOSCAPE software (force directed layout), and is composed of 170 629 significant correlations ($P_{\text{FDR}} < 5 \times 10^{-5}$) between 4305 nodes. Gray and red edges represent positive and negative correlations respectively. Only 2135 correlations were negative (Supporting Information Table S3). Two small isolated subnetworks (< 10 variables) were not considered in the analyses. Twelve modules, detected by WGCNA analyses (Langfelder & Horvath, 2008), were represented by different node colors. For each module, gene expression profiles in the four treatments (Table S1) were represented by heatmaps. [+K], [–K], with and without potassium; [–W], [+W], with and without water exclusion. We performed a Gene Ontology (GO) enrichment approach using functional annotation of the first BLAST hit of *Eucalyptus grandis* genes in the *A. thaliana* genome (Table S4) to detect biological processes associated with DEGs enclosed in each module.

Modules M1–M5 located in the core of the network were enriched in cell-wall-related genes, especially in genes involved in the synthesis and in the regulation of the three main SCW polymers (cellulose, xylan, lignin) (Fig. 3, Table S4). Most of these genes (80%) were downregulated in response to K-fertilization while the remaining 20%, mostly involved in cell primary metabolism, were upregulated and thus negatively correlated with the first ones (Table S2). This included *EgrNAC12*, a putative ortholog of the drought inducible *ANAC002* in *Arabidopsis* (Table S4). Among the K-downregulated genes, 109 TFs were identified including regulators acting in the early phase of xylem differentiation such as *AtWRKY12*, *AtWRKY13*, *AtIFL1/REV*, *ATHB8* and *ATHB15* (Table S4). Twenty-three TFs were orthologs of the regulators belonging to the SCW hierarchical transcriptional network in *Arabidopsis* (Table S4) such as *EgrNAC61* and *EgrNAC26*, orthologs of first-level master switches *AtSND1* and *AtVND6*, respectively. The second-level positive (*EgMYB2*) and negative (*EgMYB1*) master switches functionally characterized in *Eucalyptus* were also present (Goicoechea *et al.*, 2005; Legay *et al.*, 2007). Fifty-two DAMs (33%) were correlated with the M1–M5 modules with M2 presenting the highest number of connected DAMs.

Nested in the core of the network, module M6 was highly connected with M1–M5 and significantly enriched with genes involved in cytoskeleton remodeling. No DAMs were linked to M6. Noteworthy, the Rac-like small GTPase *EgROP1* (Table S4), previously shown to be involved in the control of cell morphology and SCW formation in *Eucalyptus* xylem (Foucart *et al.*, 2009), was among the most connected genes. M6 also contained orthologs of genes involved in vesicle trafficking of CW polymers and CW-modifying enzymes, through interaction with microtubules such as *AtAAA1*, *AtRABA5a*, *AtRABA4a* and *AtROPGAP3* (Table S4).

Modules M10 and M11 were enriched in genes related to cell activity (RNA and protein metabolism) and to abiotic stress responses such as genes encoding heat shock proteins, ROS detoxification enzymes, secondary metabolism enzymes, stachyose and polyamine biosynthesis enzymes (Table S4). These modules contained orthologs of most of the key components of the ABA signaling pathway (Table S4), including receptors (*AtRCAR1/AtPYL9*, *AtRCAR3/AtPYL8*), receptor-associated kinase (*AtSnRK2.6/AtOST1*) and kinase's direct substrate (*AtAREB3*). ABA-inducible TFs, such as *ATHB7*, *ANAC081* and *ANAC102* (Table S4), were also present, as well as *AtRAE1* (ABA signaling) and *AtXERICO* (ABA metabolism). All these genes were strongly upregulated in response to K-fertilization and especially when water was available [+W+K]. M10 and M11 DEGs were connected with 19 DAMs.

M7 and M12 were enriched in genes strongly regulated by water in addition to regulated by K (Fig. S6). Most of the genes of M7, related to 'DNA replication and cell cycle', were downregulated by water exclusion in K-deficient trees, but upregulated by water exclusion in K-fertilized trees. Among these DEGs, 29 out of 126 belong to cell division machinery, chromosome segregation and cytokinesis (Table S4) including cyclin-dependent kinases, such as *Eucgr.A00867*, a putative ortholog of *AtCDKB2;2* required for meristem integrity in *Arabidopsis*.

EgrAP2-11, the only detected TF in M7, is a putative ortholog of *AtANT*, associated with cell division regulation in *Arabidopsis* shoot meristem (Table S4). M12 was enriched in genes related to transport of molecules that were upregulated in [–W–K] treatment. Nine encode proteins similar to transporters essential for xylem development or sap transport in *Arabidopsis* such as possible orthologs of *AtABCG11* and *AtABCB15* required for vascular development, and a close ortholog of *AtWAT1*, a tonoplast-localized transporter involved in SCW biosynthesis of xylem fibers in *Arabidopsis* (Table S4). Only one DAM was connected with M12 DEGs.

Modules M8 and M9 both contained genes upregulated by water exclusion in K-fertilized trees (Fig. S6). M8 enclosed genes related to cambial activity, such as several components of the *CLAVATA* peptide signaling pathways, as well as genes close to *AtMOL1* receptor and its ligand *AtCLE44/TDIF* (Table S4). Among the putative TFs detected in M8, we found orthologs of *AtDof5.6* and *AtARF4* which are involved in the formation of interfascicular cambium and vascular patterning in *Arabidopsis* roots, and in promoting cambium activity respectively (Table S4). Orthologs of genes related to K-homeostasis (*AtSKOR* and *AtZIFL2*) and to calcium sensing and transport (*AtSOS3*, *AtCAX1*, *AtCAX3* and *AtCAX7*) were also found in M8. The M9 module was enriched in genes related to phloem development (Table S4), such as putative orthologs of *AtSEOR1* required for phloem filament formation and orthologs of *AtAPL* required for phloem identity in *Arabidopsis*. Sixteen DAMs were associated with M8 and M9.

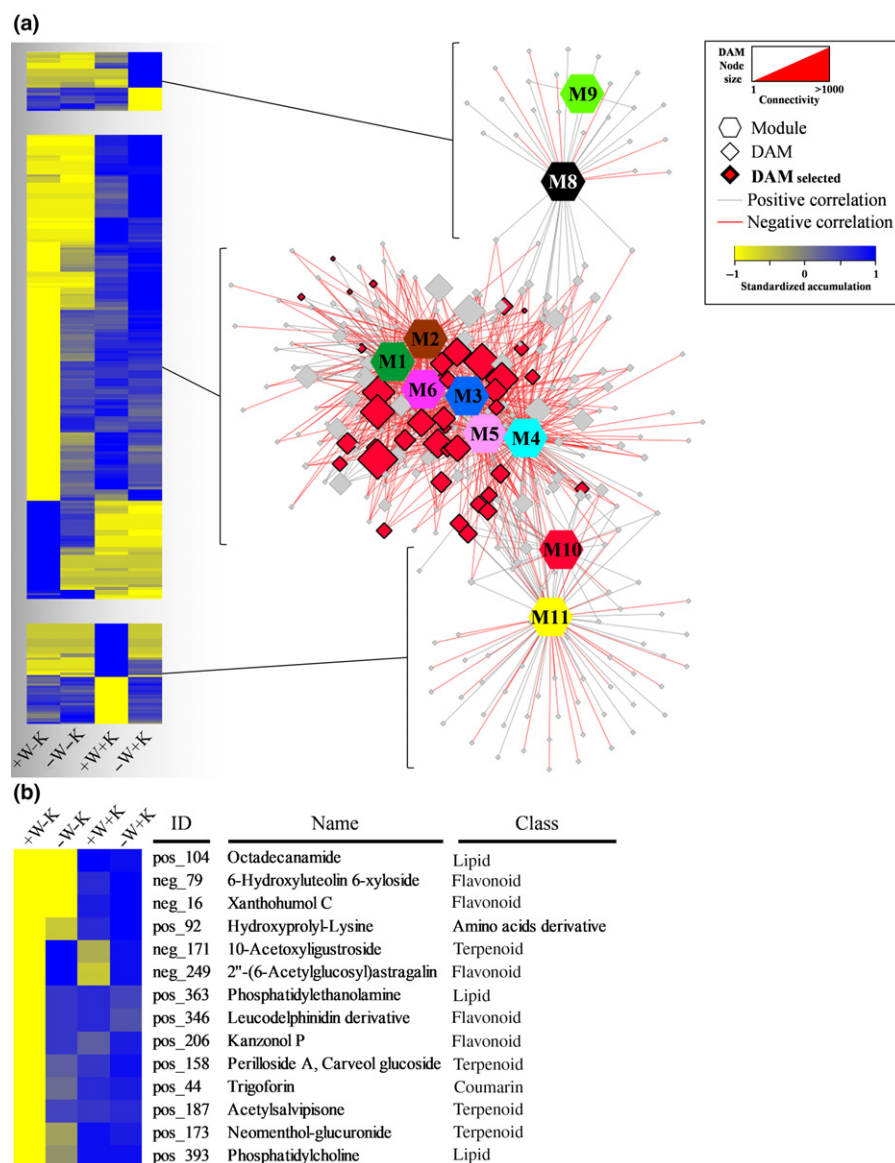
Correlations between identified DAMs and network modules

In an attempt to optimize the integration of the metabolomic and transcriptomic datasets, we decided to use the MIXDIABLO method of the MIXOMICS R package, a method designed for testing correlations between heterogeneous 'omics' datasets. As shown in Fig. 4(a), where the correlations between DAMs and modules are represented, the number of highly connected DAMs (282) was substantially increased as compared to the first method where only 93 were correlated with DEGs (Fig. 3). None of the DAMs was connected to the M7 and M12 modules, in agreement with the first correlation analysis. The vast majority of the highly connected DAMs (213, 76% of the DAMs) were associated with modules M1–M6 related to cell wall formation and cytoskeleton. Most of these DAMs accumulated in [+K] conditions and were negatively correlated with expression profiles of SCW-related genes. Among the most connected DAMs (red diamonds, Fig. 4a), 37 were selected for LC-MS-MS analyses and 14 provided robust identification. They belong mostly to the flavonoid, coumarin and terpen categories, but also to lipid and amino acid derivatives (Fig. 4b). Twelve were clearly accumulated in [+K] conditions and two in [–W] conditions.

Correlations between wood properties and network modules

The quantification of phenotypic traits related to wood formation in the four treatments (Fig. 5) revealed that saccharification

Fig. 4 DAMs–module correlation network using MixOmics integrative approach. (a) Network representing correlations (edges, > 0.8) detected between DAMs, DEGs and the average profiles of the 12 modules. Module profiles were calculated from the average expression profiles of DEGs within each module and we used the same color code as in Fig. 3. Diamond nodes represent mass signatures with size proportional to their connectivity. Poorly connected nodes were removed for graphical representation (connectivity < 3). Red diamonds represent DAMs selected for identification by LC-MS-MS (all connected to modules M1–M6). Edges are proportional to the absolute value of the correlation (Supporting Information Table S3). DAM–DAM correlations were removed to improve graphical representation. DAM accumulation profiles in the four treatments are represented by a heat map. [+K], [–K], *Eucalyptus grandis* trees with and without potassium; [–W], [+W], *Eucalyptus grandis* trees with and without water exclusion. (b) Heat map representing accumulation profiles of the 14 identified DAMs. Most of them are negatively correlated with modules M1–M6.



yield, vessel density and vessel diameter were almost exclusively regulated by K-fertilization whereas stem biomass increments and wood density were affected by both water exclusion and K-fertilization. Saccharification yield and vessel density decreased significantly (–14% and –16% respectively) in K-deficient trees relative to K-fertilized trees. By contrast, vessel diameter increased significantly in K-fertilized trees and slightly more when water was excluded (+15%, Fig. 5). Stem biomass increments were the highest in K-fertilized trees but were significantly reduced by water exclusion. Wood density tended to increase in response to water exclusion, but only significantly for K-fertilized trees (Fig. 5). The lowest carbon allocation for trunk growth (GPP_{stem}) was found in [–W–K] treatment whereas unexpectedly the highest value was found in the [–W+K] treatment.

We then used the WGCNA method to compute the correlations between the module profiles (mean profiles of all the variables contained in a module) of M1–M12 and the phenotypic traits. In the network represented in Fig. 5, the

phenotypic traits were correlated with the 12 module profiles as well as with 2184 variables contained in these modules (2148 DEGs and 36 DAMs). The core-network modules M1–M6 were positively correlated with vessel density and saccharification yield (Fig. 5). By contrast, these modules, except M2, were negatively correlated with vessel diameter (Fig. 5). The correlations obtained between traits and module profiles reflected well those between traits and variables (Fig. S7; Table S3). Indeed, most of the 1554 variables in modules M1–M6 were positively correlated (1187 variables) with saccharification and vessel density, whereas 720 variables were negatively correlated with vessel diameter. Stem biomass increments were positively correlated with modules M10 and M11 but negatively with modules M4 and M5. Wood density was positively correlated with modules M8 and M9 that were negatively correlated with saccharification yield. Module M8 was also positively correlated with vessel diameter. GPP_{stem} was positively correlated with module M7 and negatively with M12.

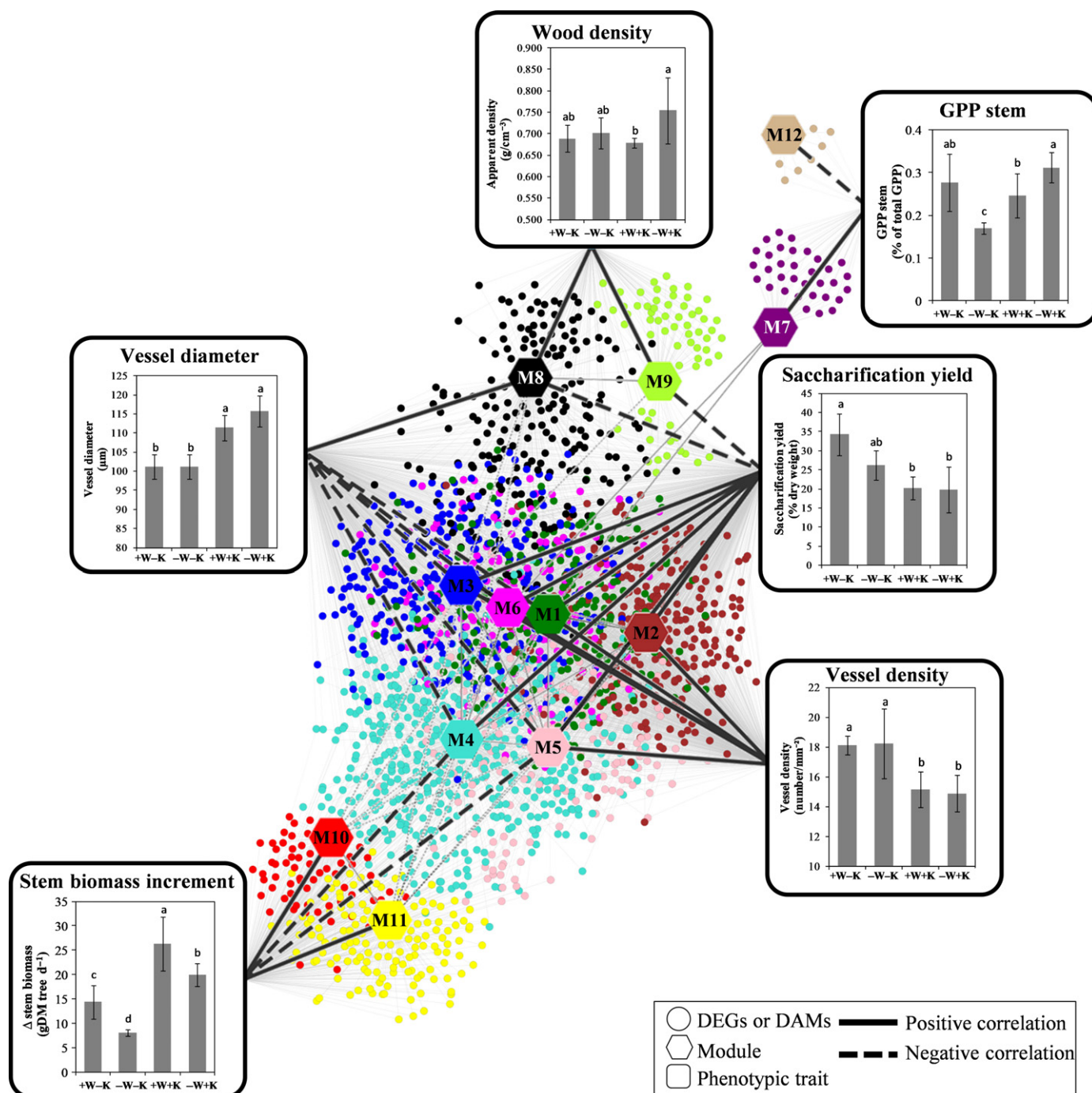


Fig. 5 Cell-wall-related modules are correlated with wood phenotypic traits. Average stem biomass increment (Δ stem biomass), average gross primary productivity allocated to stem growth (GPP_{stem}), saccharification yield, wood density, vessel size and vessel density were measured on developing xylems of *Eucalyptus grandis* trees submitted to potassium supply (+K and -K) and water exclusion (+W and -W). The phenotypic trait profiles are correlated to the average profile of the 12 modules (Fig. 3) and 2184 variables (36 DAMs and 2148 DEGs) presenting the highest correlation values (<0.9) with at least one phenotypic trait ($P < 0.05$, Supporting Information Table S3). Edges (5726) are proportional to the absolute value of correlations. Positive and negative correlations are represented respectively by solid and dashed lines. DEGs, differentially expressed genes; DAMs, differentially accumulated metabolites. Dot colors correspond to module colors in Fig. 3. Letters represent significant differences between treatments (ANOVA Tukey's honest significant difference test, $n = 4$) and error bars represent $\pm$ SD.

Identification of TFs related to wood formation in response to water exclusion and K-fertilization

With the aim of identifying new TFs involved in wood formation and regulated by K-fertilization and/or W status, we extracted

from modules M1–M6 (Figs 3, 4) a subnetwork containing 113 genes known to be involved in SCW biosynthesis (Fig. 6). All the genes present in this subnetwork were tightly co-expressed and strongly downregulated by K-fertilization. Their expression profiles were positively correlated to saccharification yield and vessel

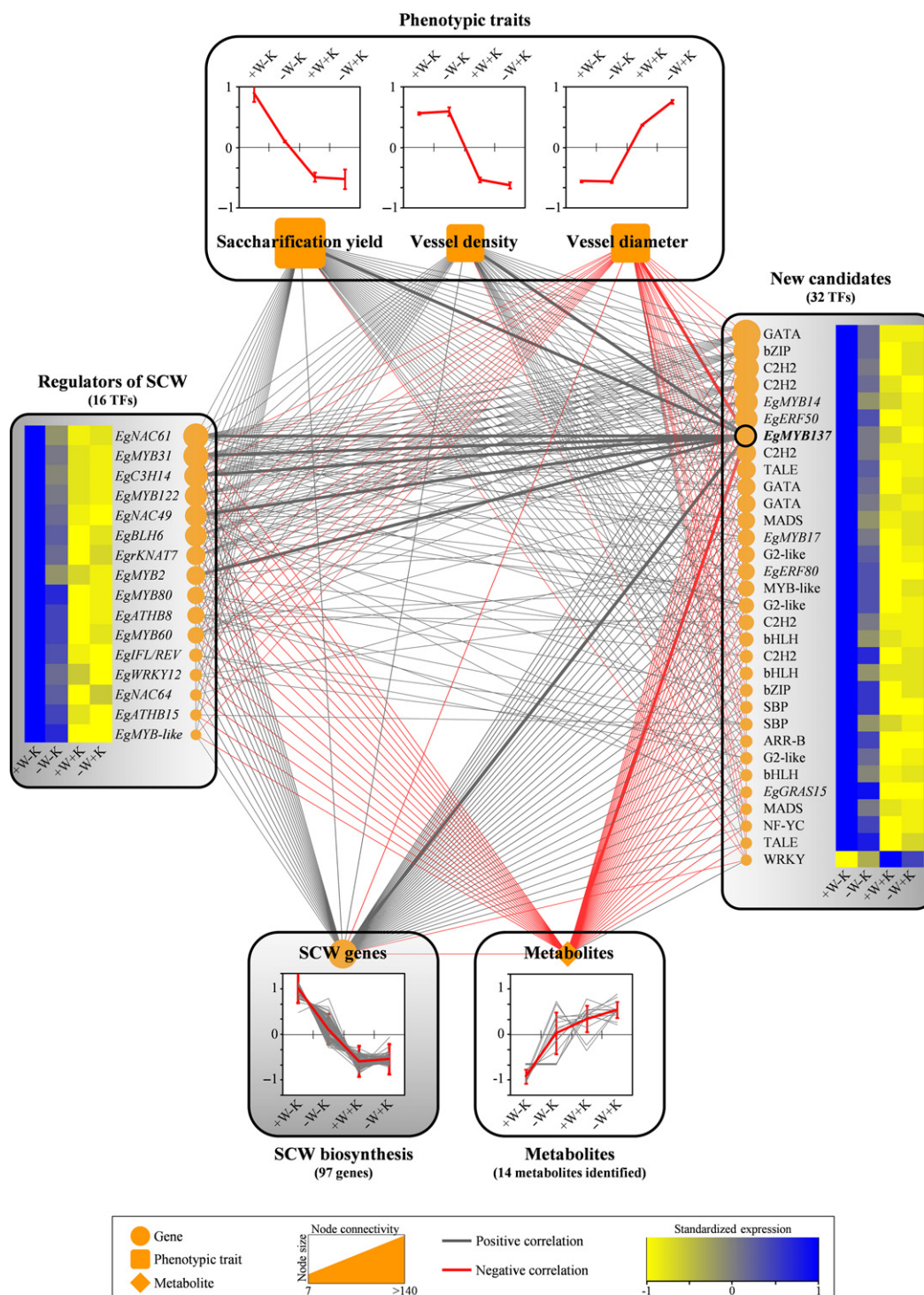


Fig. 6 Transcription factors highly co-regulated with secondary cell wall biosynthesis in stress conditions. This subnetwork of 162 nodes was extracted from modules M1–M6 (Fig. 3) and included significant correlations (edges, $P_{\text{FDR}} < 5.10^{-5}$) between 145 DEGs. In addition to DEG–DEG correlations we added the correlations with the 14 identified DAMs (DEG–DAM correlations from MixOmics analyses, Fig. 4), and the correlations with saccharification yield, vessel density and vessel size (DEG–phenotypic traits correlations from WGCNA analyses, Fig. 5). Among the DEGs, 113 were related to SCW formation: 97 were related to CW or SCW biosynthesis (22 related to cellulose, 56 to hemicelluloses, 19 to lignin, bottom panel) and 16 were putative orthologs of TFs involved in SCW formation (left panel). In the right panel, 32 uncharacterized TFs represent new candidates potentially involved in SCW remodeling. Only the most connected TFs (connectivity > 4) correlated with at least one phenotypic trait were kept (nine were not represented). They were all correlated positively with saccharification yield and vessel density, and negatively with vessel diameter and identified DAM accumulation profiles. The edges connected to the best candidate *EgMYB137* (Supporting Information Table S5) are represented in bold. Node size is proportional to the degree of connectivity (Fig. 3; Table S3). Letters represent significant differences between treatments (ANOVA Tukey's honest significant difference test, $n = 4$) and error bars represent SD. Normalized DEG and DAM profiles are represented by heatmaps and graphs in the four treatments: [+K], [–K], *Eucalyptus grandis* trees with and without potassium; [–W], [+W], *Eucalyptus grandis* trees with and without water exclusion.

density but negatively correlated with vessel diameter. Among the SCW-associated DEGs, 65 were correlated with wood phenotypic traits and with identified DAMs (Fig. 6; Table S3). Sixteen of them were orthologs of TFs known to regulate SCW biosynthesis (Table S4) while 32 TFs were not yet functionally characterized in *Arabidopsis* nor *Populus*. We examined their connectivity in networks, their expression profiles in EucGenIE (<https://eucgenie.org/>) and their presence in previous data integration studies in *Arabidopsis*, *Populus* and *Eucalyptus* (Fig. 6; Table S5). *EgMYB137* obtained the best score in the ranking of the 32 TFs. It was correlated with 22 SCW biosynthesis genes and five SCW-TFs which were the closest orthologs of *AtSND1*, *AtNST1*, *AtMYB46*, *AtMYB83* and *AtC3H14* (Table S4). Of note, we found that this *EgMYB137*'s subnetwork was conserved in both *Populus trichocarpa* and *A. thaliana* (Fig. S8).

EgMYB137 is a regulator of xylem formation in *Eucalyptus*

To functionally characterize this promising new candidate, we investigated its spatial expression using *E. grandis* transgenic hairy roots transformed with a promoter–GUS fusion and overexpressed its coding sequence under control of the CamV35S promoter. Histochemical analyses revealed that the *EgMYB137* promoter drives GUS activity in the vascular cylinder of young roots (Fig. 7a) and in parenchyma cells surrounding developing vessels close to the cambial zone in older roots (Fig. 7b–d). Cross-sections of transgenic roots ongoing secondary growth revealed an enhanced deposition of SCW in xylem (Fig. 7e,f). The SCW of fibers of the *p35S:EgMYB137* lines were significantly thicker (+12%) as compared to control lines. By contrast,

SCWs of vessels were significantly thinner in *p35S:EgMYB137* (−4%) as compared to control lines (Fig. 7g). Vessel density was significantly reduced (−35%) in *p35S:EgMYB137* as compared to control lines (Fig. 7h) but no significant change in vessel or

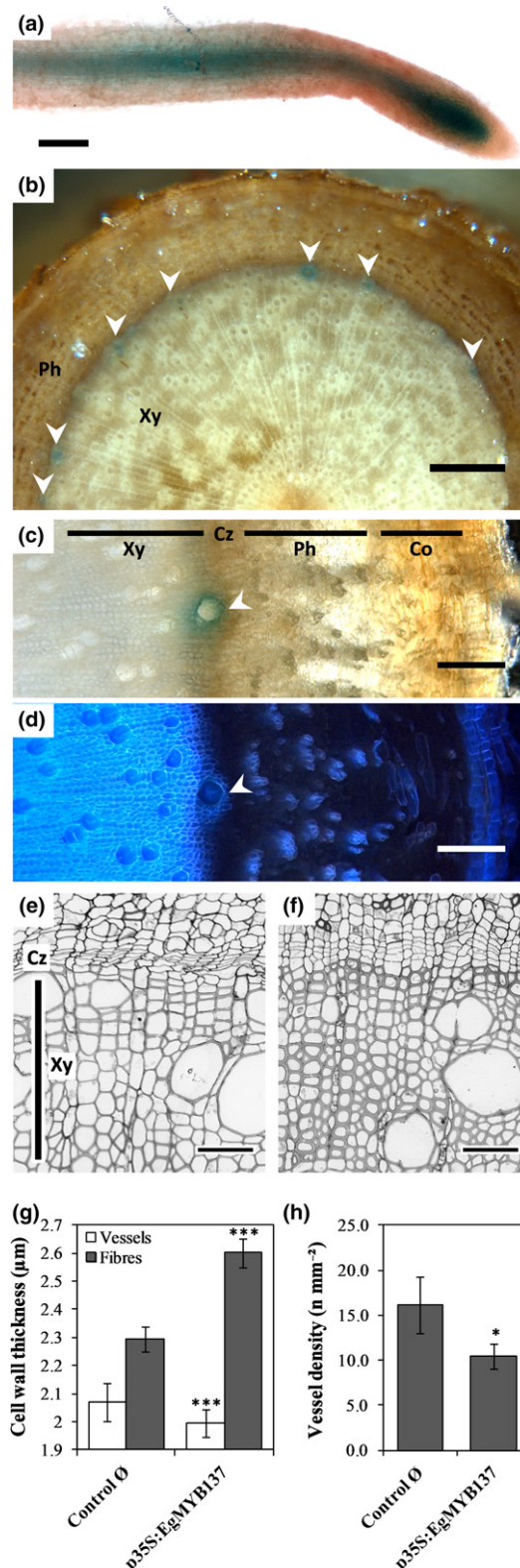


Fig. 7 *EgMYB137* is involved in xylem formation in *Eucalyptus* roots. GUS expression driven by the *EgMYB137* promoter was observed in the main root of *Eucalyptus grandis* transgenic lines, in (a) vascular tissues (*in vitro*, young root tip), (b) xylem vessels (differentiation zone) and (c) axial parenchyma cells surrounding the youngest vessels immediately below the cambium (5-month-old roots). Observations were made in brightfield mode on free-hand cut sections. White arrowheads represent vessels. Co, cortex; Cz, cambial zone; Ph, phloem; Xy, xylem. (d) Five-month-old *Eucalyptus* roots observed under epifluorescence microscopy. Highest autofluorescence intensity is observed in the cell wall (CW) of vessels and axial parenchyma cells immediately below the cambium (white arrowhead). (e) Brightfield observations of semithin section of 5-month-old control root (empty vector) stained with toluidine blue. Cz, cambial zone; Xy, xylem. (f) Brightfield observations of semithin section of 5-month-old *p35S:EgMYB137* roots, showing an increase in fiber CW thickness. (g) Changes in CW thickness of vessels and fibers in *p35S:EgMYB137* root xylem. Vessel SCWs were significantly thinner in *p35S:EgMYB137* lines ($1.99 \pm 0.04 \mu\text{m}$) as compared to control lines ($2.07 \pm 0.06 \mu\text{m}$). Fiber SCWs were significantly thicker in *p35S:EgMYB137* lines ($2.60 \pm 0.05 \mu\text{m}$) as compared to control lines ($2.29 \pm 0.04 \mu\text{m}$). ***, $P < 0.0001$ (Student's *t*-test). (h) Decrease in vessel density in *p35S:EgMYB137* root xylem ($10.4 \pm 1.4 \text{ mm}^{-2}$) as compared to control ($16.1 \pm 3.1 \text{ mm}^{-2}$). *, $P < 0.05$ (Student's *t*-test). Error bars represent SD ($n = 6$ for transgenic lines, $n = 4$ for controls). Vessel CW thickness was measured on > 70 vessels and > 1200 fibers. Vessel density and lumen diameter were measured on > 440 vessels and > 1200 fibers. Scale bars, (a) 200 μm ; (b) 500 μm ; (c–d) 150 μm ; (e–f) 50 μm .

fiber lumen diameter was observed (data not shown). We further investigated the consequences of *EgMYB137* overexpression on saccharification yield (Table 1). With or without pretreatment, saccharification yield was reduced by *c.* 16% in *35S::EgMYB137* lines as compared to control lines.

Discussion

K-fertilization and water availability trigger interconnected regulations in xylem

We developed a systems biology approach that enabled us to get a comprehensive view of the regulations involved in wood formation of field-grown *E. grandis* trees submitted to four treatments combining K-fertilization and rainfall water availability. In our conditions, K-fertilization was responsible for the highest proportion of DEGs and DAMs, the highest proportion of variance explained by PLS-DA and the highest proportion of enriched GO categories. Most of these GO categories were related to development, primary metabolism and cell wall biosynthesis. Most of the genes of the coexpression network (modules M1–M12) were strongly regulated by K-fertilization with no or minor impact of water exclusion. These findings, demonstrating the prevailing impact of K on the remodeling of the transcriptome and the metabolome, are in line with our previous physiological studies on 3-yr-old trees showing that regardless water supply, K-fertilization increased dramatically gross primary productivity and above-ground biomass production (Battie-Laclau *et al.*, 2014a,b; Christina *et al.*, 2015). Similarly, we observed an increase in stem biomass increments in K-fertilized trees.

In response to water exclusion, enriched GO categories were mainly related to stress response and molecule transport but a high proportion of the stress-related genes were also differentially regulated by K-supply. The separation between [–W] and [+W] treatments by PLS-DA was highly dependent on K-fertilization, suggesting that tree responses to water shortage is highly dependent on K-availability. By contrast, genes in modules M10 and M11, involved in abiotic stress response and containing numerous genes of the ABA signaling pathway, were highly upregulated in either [–W+K] or [+W+K] treatments, suggesting that K-fertilized trees were stressed independently of water availability. This finding is consistent with previous physiological studies showing that at the end of the dry season, K-fertilized trees reached a higher level of water stress than K-deficient trees (Battie-Laclau *et al.*, 2014a,b, 2016). This drought-induced effect of K-fertilization was linked to the enhancement of tree growth rate causing a faster decrease in soil water stocks during dry periods (Christina *et al.*, 2018). This was particularly true in our study because of the exceptionally low amount of rainfall during the months preceding sampling (Fig. S2). Trees grown in [–W+K] underwent continuous drought episodes and developed adaptive traits to survive (Battie-Laclau *et al.*, 2014a,b, 2016). In comparison, trees grown in [+W+K] were less acclimated to severe drought events. In agreement with this hypothesis, [–W+K] trees exhibited a higher GPP_{stem} than [+W+K] and the highest expression level of stress responsive genes (modules M10 and M11),

Table 1 Over-expression of *EgMYB137* in *Eucalyptus grandis* roots changed saccharification yield.

	<i>p35S::EgMYB137</i>	Control Ø
Saccharification yield without pretreatment (%DW)	16.0 ± 1.8*	19.1 ± 2.2
Saccharification yield with pretreatment (%DW)	53.6 ± 5.8**	63.6 ± 6.8

Analyses of saccharification yield were performed in triplicate for nine lines of *p35S::EgMYB137* and six lines of controls (Ø, empty vectors). Values were significantly lower in *p35S::EgMYB137* lines compared to controls, in both pretreated and nonpretreated samples (± represents SD; *, $P < 0.05$; **, $P < 0.001$; Student's *t* test). Values are given as the percentage of dry weight (DW) of extracted xylem residue (EXR).

such as the drought-responsive TF *EgDREB2.5* (Fig. S4), was observed in [+W+K] treatment relative to [–W+K]. DEGs involved in secondary metabolism (module M9) and most of the (LC-MS) DAMs were also strongly induced in K-fertilized trees. Among the latter, the 14 DAMs identified could be related to drought stress response as signaling lipids (Hou *et al.*, 2016) or antioxidant compounds such as flavonoids, terpenoids and coumarins (Karabourniotis *et al.*, 2014).

SCW formation in the heart of the xylem response to K-fertilization and water supply

The SCW modules (M1–M6) constitute the core of the network and the vast majority of the SCW-associated genes were drastically downregulated in response to K-fertilization, suggesting a reduction or a delayed SCW deposition in developing xylem cells. These six modules were strongly correlated with saccharification yield, which reflects the accessibility of cell wall polysaccharides to enzymatic degradation, and is a crucial parameter for second-generation biofuel production (Van Acker *et al.*, 2014). A subset of genes in M1–M6, associated with primary metabolism, were negatively correlated with the SCW-associated genes and upregulated in K-fertilized trees. Among them, we found *EgrNAC12*, a putative ortholog of *ANAC002*, an ABA-inducible key TF that is strongly upregulated in response to cellular carbon depletion in *Arabidopsis* and responsive to stress including drought (Garapati *et al.*, 2015). Given that K-fertilization triggers drought stress signaling in our conditions, one possible hypothesis could be that K-fertilized trees develop strategies to mitigate drought stress such as stomatal closure to reduce transpiration, resulting in a reduction of carbon fixation by photosynthesis. Prolonged episodes of drought are known to lead to lower carbon uptake and consequently to carbon starvation for all tree organs (Mitchell *et al.*, 2014). K-fertilized trees could therefore modify carbon allocation at the detriment of SCW biosynthesis, as suggested by the repression of genes related to nucleotide-sugars (e.g. UDP-galactose transporters), often included within SCW modules (Li *et al.*, 2016b). Similarly, the accumulation of 80% of DAMs, negatively correlated with SCW-DEGs, could be at least partially a consequence of the repression of a phenylpropanoid pathway branch (Vanholme *et al.*, 2012).

Another hypothesis not exclusive of the first is that as in poplar, SCW deposition could be delayed in K-fertilized trees as a result of a stimulation of cambial activity (Langer *et al.*, 2002). In support of this hypothesis, we detected two groups of modules (M8–9 and M10–11) associated with cambial activity, cell activity and metabolism that were induced in response to K-fertilization. The expression profiles of these genes, which were also positively correlated with trunk biomass increments and wood density, pointed to a positive effect of K-fertilization on cambial activity and cell metabolism. This agrees with the essential role of K in maintaining cambial activity (Fromm, 2010). According to Langer *et al.* (2002), the osmotic role of K is also essential for vessel expansion. Modules M1–M6 were correlated negatively with vessel diameters and positively with vessel density, which increased and decreased respectively in response to K-fertilization. Besides enhancing transport efficiency, wider vessels can increase the vulnerability of xylem to cavitation, making trees more sensitive to drought episodes although other parameters such as vessel SCW composition and structure also influence xylem cavitation (Hacke *et al.*, 2001).

Most systems biology studies performed on woody tissues have focused on SCW, and only a few uncovered genes involved in cambial activity (Zinkgraf *et al.*, 2017; Ployet *et al.*, 2018). In the present study, the fine sampling of immature cells between bark and differentiating xylem allowed the identification of 18 TFs as promising candidates for the control of cambial activity in response to K-fertilization and water exclusion.

Integrative approach linked meaningful genes to complex traits

Within the core SCW modules (M1–M6), 56 TFs correlated to SCW genes and wood traits. These known and potential SCW-related TFs, downregulated in response to K fertilization and to a lesser extent by water shortage, probably play a role in the adaptation of trees to these environmental cues through modification of SCW properties and/or xylem anatomy. For instance, the strong downregulation of the SCW positive master switches such as *EgMYB2* suggests that SCW deposition in fiber cells is dramatically reduced in K-fertilized trees. Similar profiles were observed for a suite of TFs (*EgMYB31*, *EgMYB60*, *EgMYB80*, *EgMYB122*, *Eucgr.F02796*, *Eucgr.D01219* and *Eucgr.D01935*) which are the orthologs of TFs regulating SCW deposition in *Arabidopsis* *AtMYB83*, *AtMYB103*, *AtMYB69*, *AtMYB43*, *AtC3H14*, *AtWRKY12* and *AtKNAT7* respectively. TFs described in the literature as positive and negative regulators of SCW deposition (Hussey *et al.*, 2013) were both poorly expressed in K-treatments, suggesting that xylem differentiation is slowed down, to the benefit of cambial cell proliferation. In fully elongated immature xylem cells of *Arabidopsis*, SCW deposition is activated by either *AtSND1* and *AtNST1/2* in fibers or *AtVND6* and *AtVND7* in vessels (Zhong *et al.*, 2006; Mitsuda *et al.*, 2007; Yamaguchi *et al.*, 2010). SCW modules contain *EgNAC61* and *EgNAC49*, the closest orthologs of *AtSND1* and *AtNST1/2*, but no putative orthologs of the VND (vascular NAC domain) TFs. The absence of significant downregulation of VND master

switches suggests that the deposition of SCW in vessels is moderately impacted by fertilization, possibly to preserve the integrity of vessel SCWs, essential for water transport. Similarly, we observed an opposite pattern of SCW deposition in fibers and vessels of *35S::MYB137* transgenic roots (Fig. 7). *EgMYB137* appeared as the best candidate from a list of 32 TFs of unknown function, tightly correlated with a large number of known SCW biosynthesis genes as well as with wood properties (saccharification yield and vessel properties). To our knowledge, these TFs highly expressed in xylem, or their close orthologs, have never been characterized although they have been detected in other systems biology studies (Table S5). Some of them were coexpressed with SCW-related genes or direct targets of SCW regulators in *Arabidopsis* and *Eucalyptus* (Table S5). In poplar, putative orthologs for 22 of them were clustered in wood-related modules together with SCW genes (Table S5). *EgMYB137*, a member of the large R2R3MYB family (Soler *et al.*, 2015), showed the highest ranking among all candidates (Table S5).

The highly connected xylem-specific TF *EgMYB137* can regulate SCW deposition

Functional characterization of *EgMYB137* strongly supports that this TF regulates SCW formation as inferred from the network where it was correlated with saccharification yield, vessel density and vessel diameter: the *EgMYB137* promoter is specifically active in developing vessels and surrounding axial parenchyma; and in *EgMYB137*-overexpressing lines, cell wall thickness increases whereas vessel density and saccharification markedly decrease. Based on the positive correlations between *EgMYB137* expression profiles and both saccharification and vessel density, it was unexpected to observe a decrease of both in the transgenic overexpressing hairy roots. This apparent discrepancy might reflect the complex mode of regulation of some TFs, which can have a dual function depending on their expression level (dose-dependent effect) and the abundance of additional regulator proteins. This was clearly illustrated for *SND2* a well-known SCW TF, whose overexpression could increase SW deposition within a limited range of overexpression levels (Hussey *et al.*, 2011). Above a certain level, *AtSND2* overexpression had a negative effect on interfascicular fiber SCW deposition in *Arabidopsis* (Hussey *et al.*, 2011). More investigations are required to define the mode of action of *EgMYB137*, although our data clearly demonstrated that it has an important role in the regulation of wood formation. These result also strongly support SCW-regulatory roles for the 31 other TFs that deserve to be functionally characterized and further highlight the powerfulness of the network approach developed here.

In conclusion, our network-based approach revealed that co-regulated genes and metabolite modules related to wood formation are at the heart of a complex trade-off between secondary growth and stress response. Nested in these modules, new uncharacterized TFs were highlighted, potentially involved in SCW remodeling in stress conditions, leading to adaptive wood traits. We demonstrated that the most promising of them, the TF *EgMYB137*, was involved in the regulation of xylem structure

and composition. Together, this study represents an original attempt to correlate molecular regulations and complex wood traits in xylem.

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Author contribution

RP, MTVL, MC, BF, MTF, J-PL, CAL, GC, JG-P and FM contributed to manuscript redaction. RP, HSC, BF, MD and FM contributed to statistics, bioinformatics and data analyses. RP and GC performed xylem sampling. RP, MM, GC and FM contributed to RNA extraction and wood phenotypic traits analyses. RP, MTVL, TRC and BF contributed to extraction and metabolomics analyses. RP, FM and MM contributed to characterization of transgenic plants.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information section at the end of the article.

Fig. S1 Description of biological replicates and experimental methodology.

Fig. S2 Monthly measurements of rainfall amount on the experimental system from 2010 to 2018.

Fig. S3 Principal component analyses of metabolomic data illustrating the reproducibility of the four independent replicates.

Fig. S4 Comparison of expression profiles obtained by real-time quantitative PCR and RNA sequencing for a subset of selected genes.

Fig. S5 MIXOMICS network threshold selection.

Fig. S6 Venn diagrams describing the effect of water availability and potassium supply on DEGs and DAMs repartition within each of the 12 modules detected by WGCNA analyses.

Fig. S7 Heatmaps representing correlation values between average gene module profiles and wood phenotypic traits.

Fig. S8 Conservation of the EgMYB137 subnetwork in *Eucalyptus grandis*, *Populus trichocarpa* and *Arabidopsis thaliana*.

Methods S1 Biomass estimation, biochemical analyses, microscopy and histochemistry.

Methods S2 GC and LC-MS analyses.

Table S1 Transcriptomic data and Gene Ontology enrichment analyses.

Table S2 Metabolomic data analyses and identification of DAMs.

Table S3 WGCNA network construction and analyses.

Table S4 Expert annotation of DEGs related to SCW formation based on a comprehensive survey of the literature and detailed matching of the closest orthologs in the *Arabidopsis* genome.

Table S5 Identification of transcription factors (TFs) potentially involved in SCW remodeling in response to water exclusion and K-supply.

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