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Plant Biotechnology Journal

Plant Biotechnology Journal (2016) **14**, pp. 1381–1393 doi: 10.1111/pbi.12502

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Received 7 August 2015;
revised 2 October 2015;
accepted 17 October 2015.
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Keywords: *Agrobacterium rhizogenes*, *Eucalyptus*, hairy roots, xylem, secondary cell wall, lignin.

Summary

Eucalyptus are of tremendous economic importance being the most planted hardwoods worldwide for pulp and paper, timber and bioenergy. The recent release of the *Eucalyptus grandis* genome sequence pointed out many new candidate genes potentially involved in secondary growth, wood formation or lineage-specific biosynthetic pathways. Their functional characterization is, however, hindered by the tedious, time-consuming and inefficient transformation systems available hitherto for eucalypts. To overcome this limitation, we developed a fast, reliable and efficient protocol to obtain and easily detect co-transformed *E. grandis* hairy roots using fluorescent markers, with an average efficiency of 62%. We set up conditions both to cultivate excised roots *in vitro* and to harden composite plants and verified that hairy root morphology and vascular system anatomy were similar to wild-type ones. We further demonstrated that co-transformed hairy roots are suitable for medium-throughput functional studies enabling, for instance, protein subcellular localization, gene expression patterns through RT-qPCR and promoter expression, as well as the modulation of endogenous gene expression. Down-regulation of the *Eucalyptus cinnamoyl-CoA reductase1* (*EgCCR1*) gene, encoding a key enzyme in lignin biosynthesis, led to transgenic roots with reduced lignin levels and thinner cell walls. This gene was used as a proof of concept to demonstrate that the function of genes involved in secondary cell wall biosynthesis and wood formation can be elucidated in transgenic hairy roots using histochemical, transcriptomic and biochemical approaches. The method described here is timely because it will accelerate gene mining of the genome for both basic research and industry purposes.

Introduction

Because of their rapid growth rate, broad adaptability to diverse edaphoclimatic conditions and their multipurpose wood properties, *Eucalyptus* species are among the leading sources of woody biomass worldwide (Myburg *et al.*, 2007; Paiva *et al.*, 2011). Their considerable economic importance has recently increased because traditional interests in pulp and paper production have been extended to the emergent areas of biofuels and biomaterials. Wood is formed of secondary cell walls (SCWs) mainly constituted of cellulose, hemicelluloses and lignins. The composition and structure of the SCWs, relying among interactions between these biopolymers, are major determinants of industrial processing efficiencies (Holladay *et al.*, 2007).

The huge economic importance of *Eucalyptus* wood has been a driving force to delineate the lignin pathway in this genus. More than 20 years ago, the gene encoding the cinnamyl alcohol dehydrogenase (CAD), which catalyses the last step in monolignol biosynthesis, was cloned in *Eucalyptus* right after that from tobacco among all plant species (Grima-Pettenati *et al.*, 1993). The gene encoding cinnamoyl-CoA reductase (CCR), which catalyses the penultimate step in monolignol biosynthesis, was first cloned in *Eucalyptus* (Lacombe *et al.*, 1997) and subsequently used to clone its orthologs in other plant species such as poplar (Leplé *et al.*, 1998), maize (Pichon *et al.*, 1998) and *Arabidopsis* (Lauvergeat *et al.*, 2001). Other SCW-related genes, including transcription factors, have been cloned in *Eucalyptus* but, due to the lack of an efficient stable transformation system, their functional characterization had been achieved mainly using heterologous systems such as poplar (Feuillet *et al.*, 1995; Hawkins *et al.*, 1997; Lauvergeat *et al.*, 2002; Legay *et al.*, 2010; Šamaj *et al.*, 1998), tobacco (Goicoechea *et al.*, 2005; Lacombe *et al.*, 2000) and *Arabidopsis* (Baghdady *et al.*, 2006; Creux *et al.*, 2008; Foucart *et al.*, 2009; Hussey *et al.*, 2011; Legay *et al.*, 2010).

The recent availability of the *Eucalyptus grandis* genome (Myburg *et al.*, 2014) has allowed genome-wide characterization of many gene families, notably those involved in the lignin biosynthetic pathway (Carocha *et al.*, 2015) as well as transcription factor families containing members known to regulate SCW formation such as the R2R3-MYB (Soler *et al.*, 2015), NAC (Hussey *et al.*, 2015), ARF (Yu *et al.*, 2014) and Aux/IAA (Yu *et al.*, 2015) among others. These studies have underscored

many new candidates potentially regulating wood formation that need to be functionally characterized. Although stable transformation protocols have been established for several *Eucalyptus* species (Girijashankar, 2011; de la Torre *et al.*, 2014; Tournier *et al.*, 2003, and references therein), they are not suitable for medium-/high-throughput functional characterization of genes because they are tedious, time-consuming and present low efficiencies. For these reasons, only very few functional studies have been performed in transgenic *Eucalyptus* (reviewed in Girijashankar, 2011).

To overcome these limitations, we developed an alternative stable transformation system using *Agrobacterium rhizogenes* that allow rapid *in vivo* analysis of transgenes. *A. rhizogenes,* a soilborne Gram-negative bacterium discovered more than 80 years ago (Riker *et al.*, 1930), induces the production of numerous secondary functional roots called 'hairy roots' upon wounding and infection of many plants. This is the consequence of the stable integration and expression into the host cell of a T-DNA encoding *root locus* (*rol*) genes carried on the root-inducing (Ri) plasmid (Chandra, 2012; Chilton *et al.*, 1982). If in addition to the Ri plasmid, *A. rhizogenes* harbours a binary vector, transgenic roots co-transformed with both the T-DNA from the Ri plasmid and the T-DNA from the binary vector can be obtained. *A. rhizogenes*-mediated root transformation has indeed been applied for a wide variety of purposes, ranging from studying nodulation, mycorrhization, interaction of plants with nematodes or pathogens, to the production of secondary metabolites, phytoremediation and rooting of recalcitrant species (reviewed in Christey, 2001; Georgiev *et al.*, 2012; Guillon *et al.*, 2006). Transformed hairy roots can be *in vitro* cultured in axenic conditions to produce secondary metabolites or recombinant proteins among other applications, but the development of composite plants (wild-type shoots with transgenic roots) has been a key milestone for functional characterization of genes (Hansen *et al.*, 1989). However, while hairy roots have been established in more than 100 species, the proportion of woody species transformed using this system is very low in comparison with annual plants (Christey, 2001). Besides, none of these studies fully explored the hairy root anatomy at the vascular tissue level, and whether the hairy roots are suitable to elucidate the function of genes involved in xylem or SCW formation is still an open question, particularly important for woody species.

In this article, we describe a fast, efficient and reproducible *A. rhizogenes*-mediated transformation protocol for *E. grandis* that allows the production of transgenic roots easily detectable by fluorescent markers. We show that *Eucalyptus* hairy roots are suitable for medium-throughput functional characterization of genes, enabling among others, protein subcellular localization, spatial and temporal patterns of gene expression and down-regulation of endogenous genes. Last but not least, we demonstrate taking CCR down-regulation as a proof of concept that the function of genes involved in SCW biosynthesis and wood formation can be elucidated using histochemical, transcriptomic and biochemical approaches.

Results

Optimization of *A. rhizogenes*-mediated transformation and generation of *E. grandis* composite plants

We first tested the hypervirulent *A. rhizogenes* strain A4RS previously shown to be the most effective in infecting other trees (Alpizar *et al.*, 2006; Bosselut *et al.*, 2011; Diouf *et al.*, 1995; Gherbi *et al.*, 2008) as well as in another *Eucalyptus* species, *E. camaldulensis* (Balasubramanian *et al.*, 2011). We compared four protocols combining three types of *E. grandis* explants and three infection methods: (i) stabbing the hypocotyl or (ii) cutting and inoculating the base of the hypocotyl of 14-day-old seedlings, (iii) cutting and inoculating the radicle apex of 3-day-old germinating seeds and (iv) stabbing the stem of an *in vitro* clonal line. The screening of the co-transformed hairy roots co-expressing both the T-DNA from the Ri plasmid and the T-DNA from a binary vector was performed using fluorescent markers (GFP or DsRed). The development time course of *E. grandis* transgenic hairy roots is described in Figure 1A. GFP or DsRed fluorescence was easily detected in calli neo-formed at wounding sites 7 days after infection and in emerging roots appearing in average 1–2 weeks later (Figures 1A and S1). Twenty-one days after infection was found to be the optimal time to calculate co-transformation efficiencies (Figure S1).

Co-transformation efficiencies were variable depending on the plant material and the infection protocol (Table 1). The efficiency of the hypocotyl stabbing protocol of 14-day-old *E. grandis* seedlings (62% in average, reaching up to 75%) was found to be by far the highest.

Having selected the best inoculation system and starting plant material, we tested two other *A. rhizogenes* strains bearing pA4-type Ri plasmids (A4 and ARqua1), known to elicit a limited number of Ri T-DNA-transformed roots with growth and morphology comparable to normal roots (Boisson-Dernier *et al.*, 2001; Chabaud *et al.*, 2006; Imanishi *et al.*, 2011; Quandt *et al.*, 1993). However, the infection of hypocotyls with ARqua1 led to transformation efficiency of only 4%, whereas no transformed hairy roots at all were obtained with A4 (Table 1). As it usually happens due to the inherent recalcitrance of woody plants, only the most virulent strain (A4RS) gave satisfactory co-transformation efficiencies (Alpizar *et al.*, 2006). Therefore, all subsequent experiments were performed using the A4RS strain and the hypocotyl stabbing infection strategy following the protocol steps described in Figure 2.

One month after infection, the transgenic hairy roots were ramified and reached a size between 4 and 8 cm allowing excision of a lateral root (2 cm long in average) for subsequent *in vitro* cultivation on solid media. The remaining fluorescent roots with the aerial part of the *in vitro*-grown composite plants were hardened for subsequent sampling and analyses (Figure 2f, g). Before hardening, we removed by cutting the nontransformed roots. All the composite plants survived the acclimation period and grew similarly to the nontransformed eucalypt plants. Although the length, thickness and branching of the composite *E. grandis* hairy roots were comparable to those of wild-type plant roots, one obvious difference was the presence of a high number of short and thin roots developing in the upper part of the hairy root system (Figure 2i). The fluorescence of either the GFP or the DsRed reporters was still easily detectable after 1.5 month (Figure 1B) and even after 1 year of growth. Notably, transgenic roots could restart growing after one to three sampling events when the older part (approximately one-third) of the root system was left on the composite plant and still express stably the fluorescent reporter gene.

Construction of expression vectors for gene functional characterization and promoter activity analysis

Whereas the hairy roots expressing GFP were easily detectable under a fluorescent stereomicroscope, those expressing the

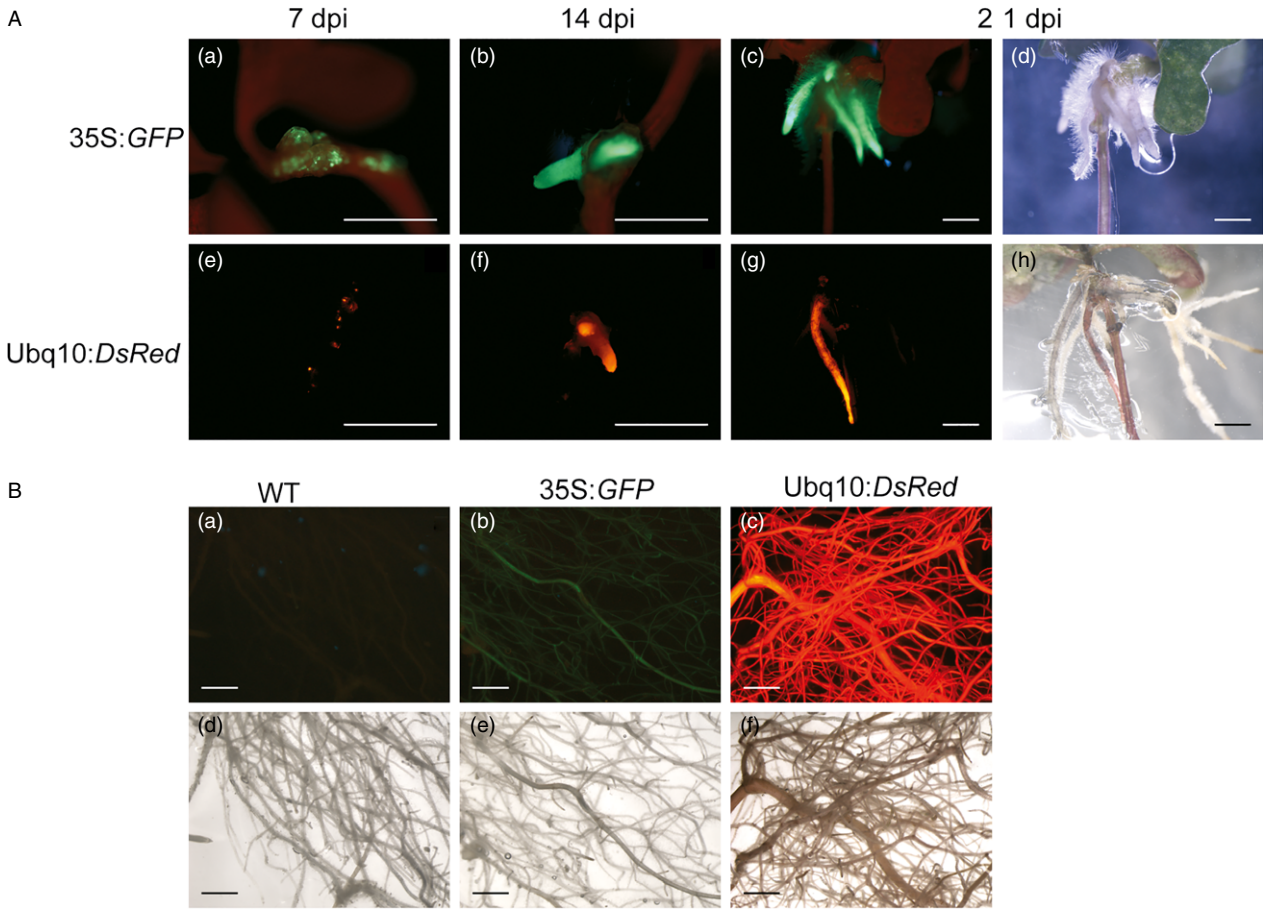

Figure 1 Developmental time course of *Eucalyptus grandis* hairy roots expressing either the GFP or the DsRed fluorescent markers. Panel A: stereomicrographs of roots emerging from infected sites on hypocotyls at 7 (a, e), 14 (b, f) or 21 (c, d, g, h) days after infection (dpi). GFP-transformed calli or roots appeared in green, whereas autofluorescence appeared in red (a, b, c). DsRed-transformed calli or roots appeared in red without noticeable autofluorescence (e, f, g). Panel B: stereomicrographs of hairy roots from wild-type (a, d) and composite plants overexpressing GFP (b, e) or DsRED (c, f) hardened for 1.5 month (75 dpi). Bright field was used to show root morphology and to localize untransformed roots (Panel A: d, h; Panel B: d–f). Scale bars = 2 mm.

DsRed were even easier to detect because there is no overlap between this fluorochrome and the natural autofluorescence of eucalyptus roots (Figure 1B). For this reason, the Gateway-adapted binary vectors that we constructed for modulating gene expression (pGWAY-0, Figure S2) and/or enabling promoter studies (pGWAY-1, Figure S2) both contained the DsRed reporter gene. Using these new vectors, transformation efficiency dropped to 17.8% in average, which is lower than that obtained with the pHKN29 vector (62% in average). Estrada-Navarrete *et al.* (2007) also reported that co-transformation efficiencies vary with different binary vectors. Both vector backbone and size are parameters that can affect co-transformation efficiency. The pGWAY-0 and PGWAY-1 are bigger plasmids (17.5 kb) derived from a pBIN19 backbone, whereas the smaller pHKN29 (9 kb) is derived from a pCAMBIA 1300 backbone.

In vitro hairy root cultures and subcellular localization of proteins

In order to determine the best culture conditions for excised transgenic hairy roots, we compared their growth curves in three solid media up to 2 months after excision (Figure S3). In the MER medium, the roots length increased slightly more than in the M medium and substantially more than in the MS medium, in which hairy roots virtually did not grow. Moreover, to demonstrate that *E. grandis* hairy roots can be used to study fluorescent-tagged proteins, we also used *in vitro*-cultured roots transformed with a vector expressing an *E. grandis* histone linker (H1) fused to the Cyan Fluorescent Protein (CFP). As shown in Figure 3, in all root cells, the H1 protein was localized in the nucleus, consistent with its well-known subcellular localization.

Xylem structure from hairy roots

To evaluate whether transgenic hairy roots could be used as a proxy to explore the function of genes involved in xylem SCW formation, we compared the radial patterning and xylem anatomy of hairy roots relative to wild-type roots. The observations under light microscopy and under UV light (exciting the natural lignin autofluorescence) showed that the development of the xylem was similar between hairy roots and nontransformed roots when sampling at the same distance from the apex (Figure 4). At 5 cm from the apex, primary xylem with four- to five-branch star pattern was observed in both types of roots (Figure 4a–d), whereas at 10 cm xylem exhibited a nearly circular shape, implicating that secondary growth started to occur with vascular cambium derived from the procambium producing

Table 1 Mean efficiency of *in vitro* co-transformation of *Eucalyptus grandis* explants with *Agrobacterium rhizogenes*

	E. grandis				
A. rhizogenes strain	Age (days)	Material	Infection method	Number of analysed plants	Co-transformed roots (%)*
A4RS	14	Seedlings	Hypocotyl stabbing	96	62.2
A4RS	14	Seedlings	Cut hypocotyl base	65	29.8
A4RS	3	Seedlings	Cut radicle	30	12.5
A4RS	21	*In vitro*-cultured clonal line	Stem stabbing	30	10.0
A4	14	Seedlings	Hypocotyl stabbing	30	0.0
ARqua1	14	Seedlings	Hypocotyl stabbing	30	4.0

*Co-transformed root efficiency calculated at 21 dpi as the percentage of plants presenting at least one fluorescent root over the total number of infected plants.

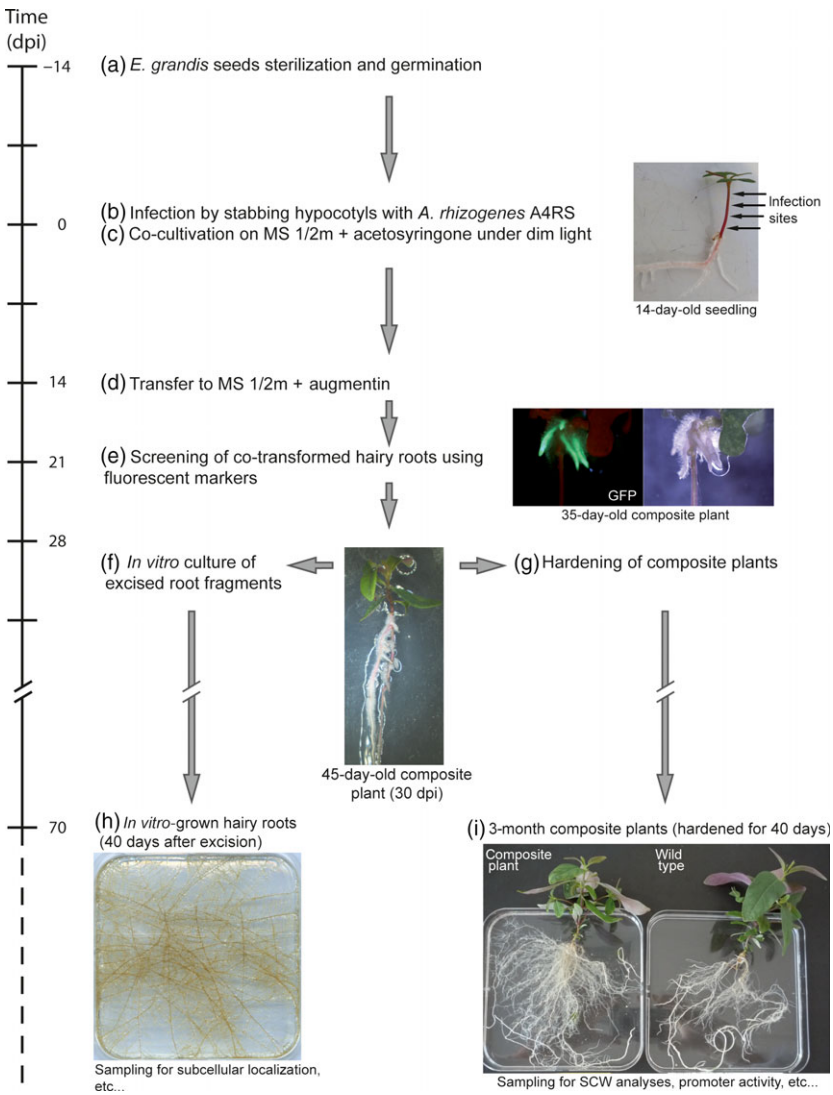

Figure 2 Sequential steps of the transformation of 14-day-old *Eucalyptus grandis* seedlings by A4RS harbouring either a GFP- or DsRed-based binary vector. (a) *E. grandis* seeds were sterilized and germinated in 1/4 strength MS medium. (b) 14-day-old seedlings were infected by stabbing the hypocotyl with a needle swabbed with *Agrobacterium rhizogenes*. (c) Infected plants were co-cultivated with agrobacteria for 14 days on MS medium with 1/2 strength macroelements (MS 1/2m) supplemented with acetosyringone under dim light. (d) Plants were transferred to MS 1/2m medium supplemented with augmentin. (e) The hairy roots generated were examined at 21 days after infection (dpi) under a stereo fluorescence microscope. (f) Co-transformed roots were excised to be cultivated *in vitro* on MER media. (g) Before hardening, nontransformed roots were removed and the resulting composite plants were placed in pots and cultivated in a phytotron. (i) After 40 days of hardening, roots were sampled for secondary cell wall and promoter activity analyses, whereas *in vitro*-grown excised roots (h) could be used for other purposes such as subcellular localization of proteins.

secondary xylem (Figure 4e–h). At 20 cm, secondary xylem and secondary phloem were clearly distinguishable (Figure 4i–l). In all stages, endodermis also fluoresced under UV light due to the presence of suberin. Both primary and secondary xylem developed in a similar way between transformed and nontransformed roots.

Tissue and cell expression pattern of the *EgCCR1* and *EgCAD2* promoters in hairy roots

To validate that hairy roots were suitable to study the promoter activities of genes involved in xylem SCW formation, we produced independent transgenic hairy root lines

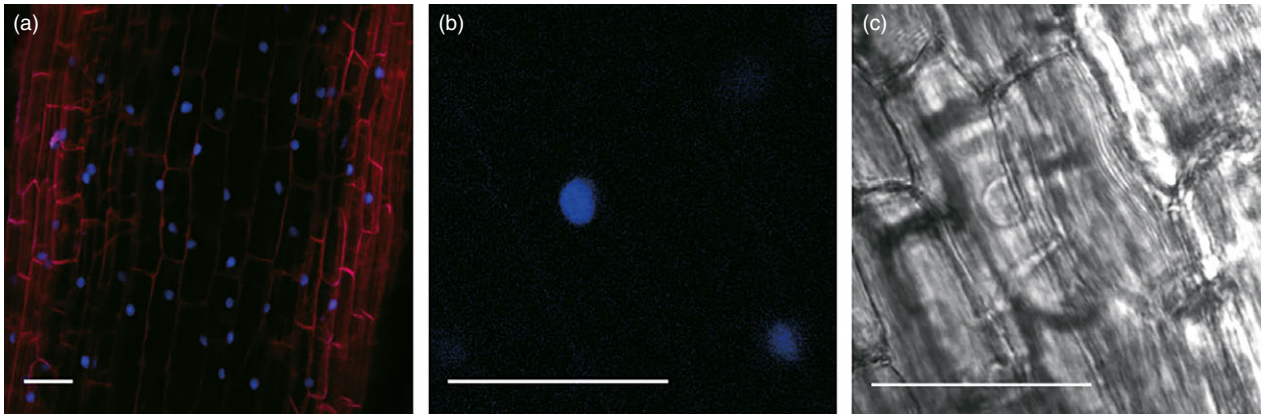

Figure 3 Nuclear localization of histone linker (H1) fused to CFP in *Eucalyptus grandis* root cells. Confocal images of *E. grandis* hairy root cells expressing H1-CFP fusion protein. All nuclei fluoresced in blue and the cell walls in red (a). Detail of fluorescent nuclei under CFP emission (b) and bright field (c). Scale bars = 30 μm.

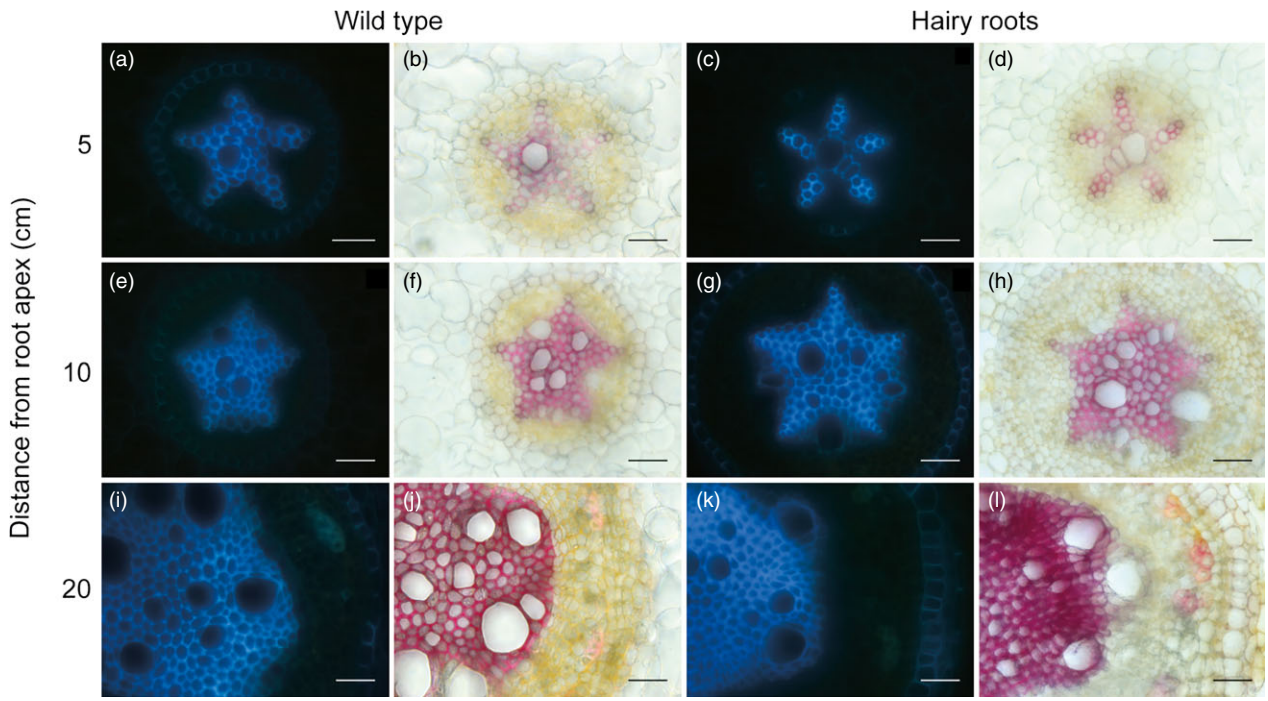

Figure 4 Comparison of xylem development and lignified secondary cell walls between transgenic and wild-type roots. Transversal root sections made at 5 (a–d), 10 (e–h) and 20 cm (i–l) from the root apex for wild-type (a, b, e, f, i, j) and hairy (c, d, g, h, k, l) roots. Lignified cell walls are visualized in blue by UV autofluorescence (a, c, e, g, i, k) and in red by using phloroglucinol–HCl (b, d, f, h, j, l). Scale bars = 30 μm.

expressing the reporter gene GUS under the control of the *EgCCR1* or the *EgCAD2* promoter. Both promoters conferred a preferential expression in the vascular cylinder of hairy roots (Figure 5a–c, i–j). A strong GUS activity was also observed in root tips (Figure 5b) and at the emergence of lateral roots (Figure 5c). As observed in cross-sections performed at 15 cm from the root in apex of composite plants (Figure 5e), GUS staining was observed in xylem lignifying cells still having a cytoplasm, which possibly are developing vessels prior to autolysis or fibres. The *EgCCR1* promoter was also expressed in paratracheal parenchyma cells located in vicinity of mature vessel elements that have already undergone autolysis and are not stained in blue. The paratracheal parenchyma cells are supposed to contribute to postmortem lignification of vessels (Baghdady *et al.*, 2006; Pesquet *et al.*, 2013). In older roots exhibiting clear secondary growth (Figure 5f–g), GUS activity was also shown in cambial cells and epidermis (Figure 5k).

EgCCR1 silencing in hairy roots

To further confirm that hairy roots were suitable to study the function of genes involved in xylem SCW formation, we performed, as a proof of concept, down-regulation of cinnamoyl-CoA reductase 1 (*EgCCR1*). We transformed the roots

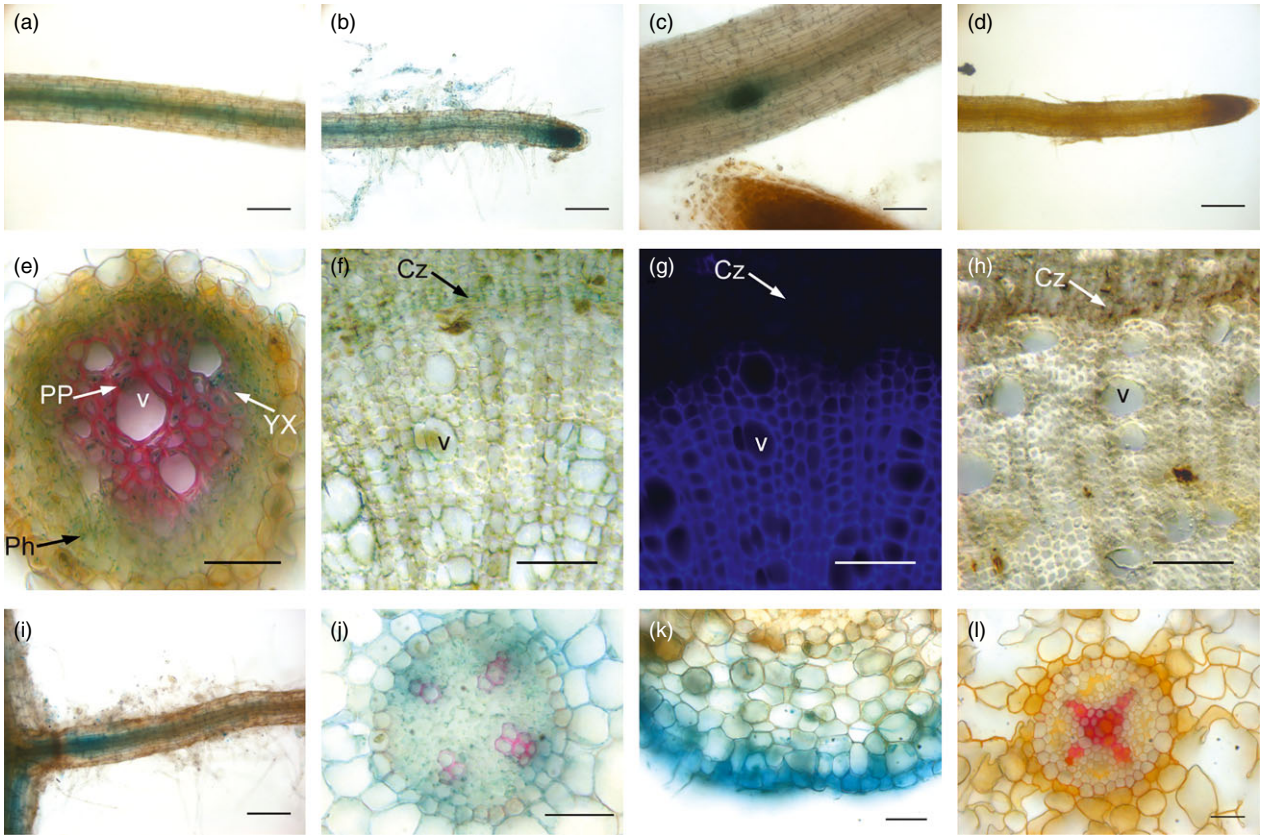

Figure 5 Histochemical localization of GUS activity in *Eucalyptus grandis* hairy roots transformed with the *EgCCR1* and *EgCAD2* promoters. Roots from hardened composite plants (3 months old) showing GUS activity for *EgCCR1* (a–c, e–g) and *EgCAD2* (i–k) promoters. Control roots are also shown (d, h, l). GUS activity is observed in the vascular cylinder (a, b, c, i), the root tip (b) and at the emergence of a lateral root (c). Transversal cross-sections were performed at 2 (j, l), 15 (e, k) and 30 cm (f–h) from the root apex. Lignified cell walls are visualized in red using phloroglucinol–HCl performed after GUS staining (e, j, l) or, in the case of roots at 30 cm (f), are alternatively visualized under UV light (g). GUS activity is observed in secondary xylem cells (e, f), cells from the cambial zone (f) and cells from epidermis (k). No GUS activity is observed in control roots (d, h, l). YX: young xylem cells; PP: paratracheal parenchyma cell; Ph: phloem; Cz: cambial zone; v: vessel. Scale bars = 100 μm (a–d, f–i) and 30 μm (e, j–l).

with an antisense *EgCCR1* construct cloned into the pGWAY-0 vector containing a DsRed marker. Four empty vectors and 10 antisense *EgCCR1* (*CCR*as) composite plants were hardened and analysed. Some of the *CCR*as hairy roots grew slower and showed a reduced size as compared to transgenic roots transformed with the empty vector, consistent with the reduced growth and size of the strongly *EgCCR1* down-regulated tobacco plants (Piquemal *et al.*, 1998).

Steady-state transcript levels of endogenous and antisense *EgCCR1* RNAs were analysed by RT-qPCR in the younger parts (0–5 cm from the apex) of the co-transformed roots. All the transformants tested expressed the antisense *EgCCR1* transgene albeit to different levels (Figure S4). Three of nine *CCR*as lines (5-1, 13-2 and 3-1) showed a strong silencing of endogenous EgCCR1 mRNA with residual transcript levels lower than 17% of those of control lines. Four *CCR*as lines (16-1, 5-2, 3-2 and 16-3) expressed intermediate levels comprised between 53% and 28% of that of control lines (Figure 6a), whereas two lines (9-2 and 8-3) did not show endogenous *EgCCR1* silencing. This variability in the extent of down-regulation of a target gene is frequently observed with the antisense strategy (Piquemal *et al.*, 1998). Transcript-level analyses were not performed for line *CCR*as 5-3 because of the low quality of the RNA obtained, which could not be resampled and extracted a second time because the plant died after the first sampling. This is likely due to its very strong *CCR*as phenotype, as shown below.

Transcript levels of five lignin (*EgPAL9, EgF5H1, EgF5H2, EgCAD2* and *EgCCR1*), two cellulose (*EgCesA1* and *EgCesA3*) and two hemicellulose (*EgGUX1* and *EgIRX7*) biosynthetic genes were quantified in roots with either a strong or an intermediate level of *EgCCR1* silencing. All these genes are related to SCW biosynthesis (Carocha *et al.*, 2015; Myburg *et al.*, 2014) and, with the exception of *EgF5H1*, were expressed in young roots (Figure 6b). Among the analysed lignin-related genes, all but *EgF5H2* showed a significant transcript-level reduction in *CCR*as roots, meaning that lignin biosynthesis was also repressed at transcriptional level. Moreover, some of the cellulose and hemicellulose biosynthetic genes analysed were also repressed. Transcriptomic analyses performed in *CCR*as poplar plants also pointed out a reduced hemicellulose biosynthesis (Leplé *et al.*, 2007).

Histochemical observations of sections performed at 5 cm from root apex displaying a strong *EgCCR1* down-regulation showed that xylem cell walls reacted faintly to phloroglucinol staining as compared to roots transformed with an empty vector (Figure 7a,b), which appeared strongly stained in red. These observations were consistently made on several silenced lines further supporting a strong reduction in the lignin content as a consequence of *EgCCR1* silencing. These roots observed under

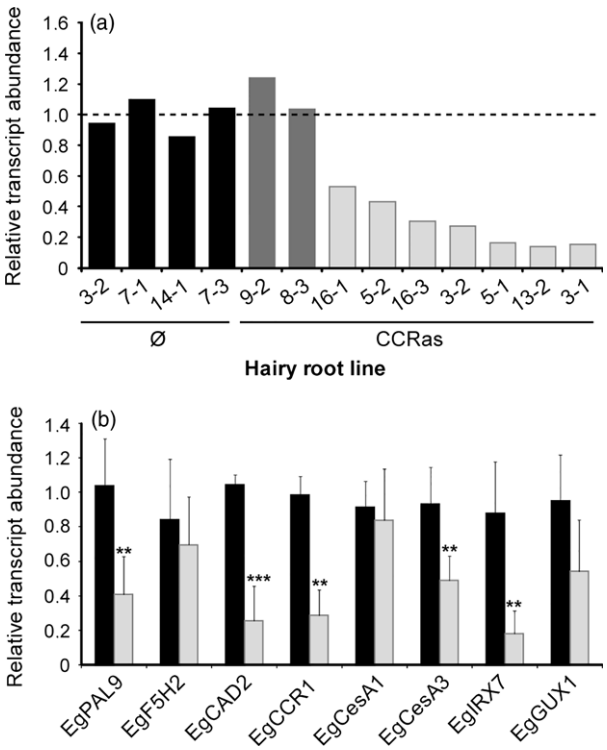

Figure 6 Relative transcript levels of *EgCCR1* and secondary cell wall (SCW)-related genes in antisense *EgCCR1* (*CCR*as) hairy root lines. (a) Mean relative transcript levels of *EgCCR1* analysed by RT-qPCR. Black bars: control roots transformed with an empty pGWAY-1 vector (Ø); dark grey bars: nonsilenced *CCR*as lines (9-2; 8-3), light grey bars: *CCR*as lines exhibiting different degrees of silencing (16-1; 5-2; 16-3; 3-2; 5-1; 13-2; 3-1). (b) Transcript levels of several SCW-related biosynthetic genes were assessed in control (black bars) and silenced *CCR*as roots (light grey bars). For each gene, mean relative transcript levels (±SD) for the four controls and for the seven silenced *CCR*as lines are shown. $^{**}P < 0.01$, $^{***}P < 0.001$. *EgPAL9: phenylalanine ammonia lyase 9, EgF5H2: ferulate 5-hydroxylase 2, EgCCR1: cinnamoyl-CoA reductase 1, EgCAD2: cinnamyl alcohol dehydrogenase 2, EgCesA: cellulose synthase, EgIRX7: glucuronoxylan glucuronosyltransferase, EgGUX1: glucuronyltransferase.*

scanning electron microscopy (SEM) showed thinner cell walls and irregular shapes for both xylem fibres and vessels (Figure 7c–f). The structure of the SCW was strongly altered resulting in collapsed vessels.

Because of the low amount of available plant material, lignin evaluation was performed by thioacidolysis, a sensitive degradation method that specifically gives rise to *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monomers from H, G and S lignin units only involved in labile ether bonds (Méchin *et al.*, 2014). The lignin-derived monomers were recovered from extractive-free samples prepared from the youngest parts of roots exhibiting only primary xylem as well as from more developed roots provided with secondary xylem. For each sample type, we analysed nonsilenced, medium and strongly silenced lines. The total thioacidolysis yield, expressed in μmoles (H+G+S) per gram of extract-free sample, gives an estimate of the amount of parent lignin structures. Consistent with their higher proportion of lignified cell walls, the older parts of the hairy roots gave rise to higher thioacidolysis yields that, in control lines, is 2.8-fold higher than those from younger ones (Figure 8a,b). Differences between *CCR*as lines and controls were more obvious for older root fragments. When we normalized data together for the two kinds of roots used, thioacidolysis yields were significantly reduced in silenced lines ($P < 0.05$, Figure 8c). Interestingly, we also analysed by thioacidolysis samples from line *CCR*as 5-3 although we could not determine the extent of *EgCCR1* down-regulation as mentioned above. This line showed not only a strong phenotype (stunted roots), but also a lower thioacidolysis yield likely indicative of a lower lignin content. In addition, the percentage of G thioacidolysis monomers was reduced by 31% leading to a higher S/G ratio (Table S1), and more importantly, the marker compound for CCR deficiency (G-CHR-CHR_2, with R = Set) (Ralph *et al.*, 2008) was clearly observed on the corresponding GC-MS trace (Figure S5).

Discussion

Here, we describe an efficient, versatile and rapid method to obtain and screen *Eucalyptus* transgenic hairy roots enabling medium-throughput functional gene characterization and gene function hypothesis testing. This homologous transformation system enables gene silencing, as shown for the *EgCCR1* gene as a case study, and compensates the lack of mutant collections. Besides, it is worth noting that for the first time the *EgCCR1* gene, cloned initially in *Eucalyptus* in 1997 (Lacombe *et al.*, 1997) and studied since in many plant species (Leplé *et al.*, 2007; Mir Derikvand *et al.*, 2008; Piquemal *et al.*, 1998), is functionally characterized in a *Eucalyptus* homolog system.

The down-regulation of *EgCCR1* using an antisense construct led to a decrease in lignin content, an alteration of SCW structure and the presence of collapsed vessels as found in other species (Goujon *et al.*, 2003; Jones *et al.*, 2001; Leplé *et al.*, 2007; Piquemal *et al.*, 1998). Nearly all the lignin-related genes were down-regulated similarly to what was found in *CCR*as tobacco plants (Dauwe *et al.*, 2007), with the exception of the *EgF5H2* gene. Because ferulic acid is known to accumulate in *CCR*as plants (Mir Derikvand *et al.*, 2008), one can hypothesize that *EgF5H2* could be involved in detoxification processes via metabolizing ferulic acid into sinapic acid as suggested by Leplé *et al.* (2007) and Meyermans *et al.* (2000).

We further showed that the co-transformed hairy roots are a versatile system suitable for many applications, such as protein subcellular localization and analysis of promoter activity pattern. With that tool, we demonstrated that both the *EgCCR1* and the *EgCAD2* promoters conferred a preferential expression in the vascular cylinder of hairy roots. This result is in agreement with previous studies performed with the same promoters in heterologous transformation systems such as poplar (Feuillet *et al.*, 1995; Hawkins *et al.*, 1997; Lauvergeat *et al.*, 2002; Šamaj *et al.*, 1998), grapevine (Lauvergeat *et al.*, 2002), tobacco (Lacombe *et al.*, 2000; Lauvergeat *et al.*, 2002) and *Arabidopsis* (Baghdady *et al.*, 2006), all showing vascular preferential expression in both stems and roots. Consistent also with these previous studies, a strong GUS activity was observed in root tips and at the emergence of lateral roots. GUS activity was also detected in cambial cells and epidermis of older roots as previously described in poplar (Hawkins *et al.*, 1997; Lauvergeat *et al.*, 2002; Šamaj *et al.*, 1998). These results confirm that the fine spatial and temporal dissection of these *Eucalyptus* promoters was accurately studied in heterologous plant systems, in line with the highly conserved network regulating SCW biosynthesis and deposition (Zhong *et al.*, 2010). However, the expression patterns driven by the *Eucalyptus cellulose synthase* (*CesA*) promoters showed

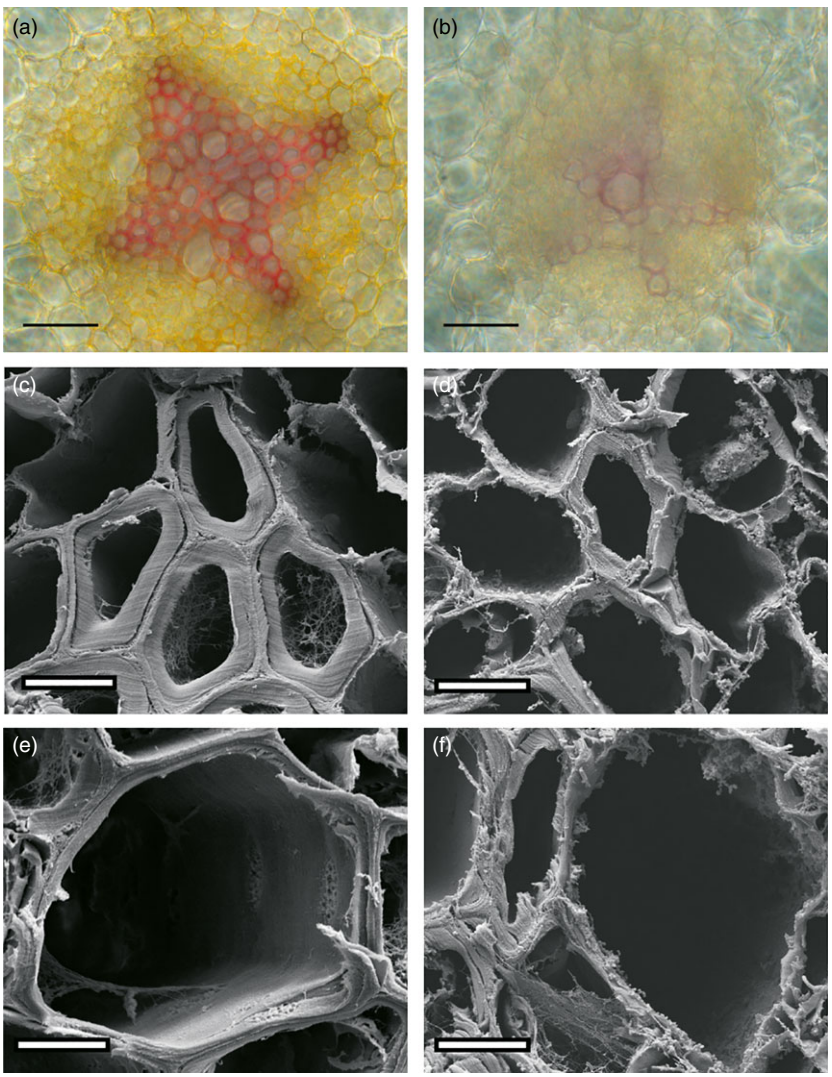

Figure 7 Transversal sections of *EgCCR1*-down-regulated hairy roots. Sections were made at 5 cm from the root apex both for control roots transformed with pGWAY-0 empty vector (a, c, e) and for the *EgCCR1*-down-regulated hairy roots (b, d, f). (a, b) Phloroglucinol staining; scale bars = 30 μm. (c–f) Scanning electron microscopy images of xylem fibres (c, d) and vessels (e, f); scale bars = 5 μm.

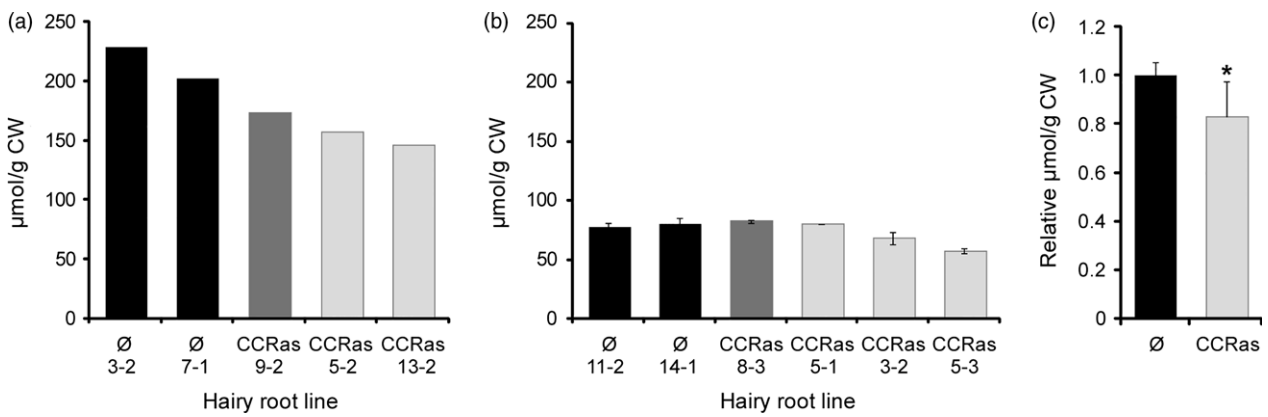

Figure 8 Thioacidolysis lignin yields in *CCR*as roots. Thioacidolysis lignin yields, expressed in μmoles per gram of extract-free sample, for each individual line using older roots fragments (a) and young roots fragments (b). Normalized thioacidolysis lignin yields (mean values ± SD) are also shown for control (4 lines) and down-regulated *CCR*as (5 lines) roots (c). Black bars: control roots transformed with pGWAY-0 empty vector (Ø); dark grey bars, nonsilenced *CCR*as roots; light grey bars, silenced *CCR*as roots. *$P < 0.05$.

differences between homologous (*Eucalyptus*) and heterologous (poplar) transformations (Creux *et al.*, 2013), further supporting the importance of homologous transformation systems such as the one presented here.

Traditional *Agrobacterium tumefaciens*-mediated stable transformation and regeneration systems of *Eucalyptus* are long, tedious and low-efficient procedures (Girijashankar, 2011; de la Torre *et al.*, 2014, and references therein). One major advantage

to use *A. rhizogenes*-mediated transformation is the time needed to obtain material suitable for functional analyses, which is considerably reduced. One month after infection, composite plants are ready to be hardened and root fragments can also be excised to be cultivated *in vitro*. Transgenic roots from hardened composite plants developed secondary xylem similar to that of untransformed roots. In addition, after harvesting, hairy roots can restart growing from the remaining upper part (at least 15 cm), providing biological replicates. Roots can also be excised from *in vitro* composite plants and grown on solid medium to perform analysis in a more controlled environment. In addition, it is in theory possible to regenerate the whole plant from a transformed hairy root, as previously performed, for instance, for *Allocasuarina verticillata* (Phelep *et al.*, 1991).

The *Eucalyptus* hairy root system developed in this study has also several advantages as compared to another alternative method called 'induced somatic sector analysis' or 'ISSA', previously developed by Spokevicius *et al.* (2005) and Van Beveren *et al.* (2006) to overcome the low-efficiency and time-consuming *A. tumefaciens*-mediated transformation and regeneration of *Eucalyptus* plants. This method is based on *A. tumefaciens* transformation but restricted to wood sectors of stem from actively growing trees, where the transgene is transferred into actively dividing cambial, xylem, phloem and ray initial cells. Being successfully applied in the analysis of transgenes in woody stem tissues of *Pinus, Populus* and *Eucalyptus*, ISSA can only be used to functionally characterize genes involved in the formation of vascular tissues and is particularly useful for promoter analyses (Creux *et al.*, 2013; Hussey *et al.*, 2011). In contrast, the hairy root system can be used for a vast panel of genes, thus being a more versatile system, with the only limitation that for obtaining the most accurate results, genes of interest should be expressed in roots. Another important advantage of the transgenic hairy root system in comparison with ISSA is the quantity of transgenic material obtained. ISSA allows obtaining very limited regions of transformed cells surrounded by untransformed cells, thus making difficult the harvesting and analysis of transgenic wood. In contrast, transgenic hairy roots are homogeneously transformed and easily detected with fluorescence and provide sufficient quantities of material for molecular biology, histochemical and biochemical approaches, as illustrated for the characterization of *EgCCR1* down-regulated hairy roots.

To the best of our knowledge, less than a handful of studies were dedicated to *Eucalyptus* hairy roots. With the aim of improving the rooting capacities of some *Eucalyptus* clones, the early work of MacRae and Van Staden (1993) had evaluated their susceptibility to several *A. rhizogenes* strains. Later on, a protocol for the generation of composite plants of *E. camaldulensis* using a GFP-based screening was briefly described (Balasubramanian *et al.*, 2011), but no functional gene characterization was achieved. In that study, 68% of plants showed root induction, but only 36% of those were co-transformed, resulting in a co-transformation efficiency of 25%. Using the same GFP-based vector (PHKN29), we obtained much higher co-transformation efficiencies (62%), while using the larger pGWAY vectors, the efficiencies were of the same order of magnitude of that obtained by Balasubramanian *et al.* (2011) with the small pHKN29 vector. A recent study reported gene silencing in *E. urograndis* (*E. grandis* × *E. urophylla*) hairy roots of STOP1, a transcription factor regulating aluminium and proton tolerance in plants (Sawaki *et al.*, 2014), but the *A. rhizogenes* transformation method used was not detailed and surprisingly only referred to that of tobacco (Sawaki *et al.*, 2013).

In conclusion, we demonstrated here the powerfulness of the hairy roots as a homologous, versatile, rapid and efficient transformation system enabling medium-throughput functional characterization of genes, especially those involved in xylem and SCW formation, which are of particular importance in woody plants. The development of such a tool that can be used easily and in routine is timely, because it will accelerate gene mining of the recently sequenced *Eucalyptus* genome for both basic research and industry purposes. Moreover, the composite plants as well as the excised roots open a whole range of new applications such as, for instance, the study of interactions with root pathogens and mycorrhizae.

Experimental procedures

Plant material

Commercial *E. grandis* seeds (W. Hill ex Maiden, cultivar LCF A001) purchased at Instituto de Pesquisas e Estudos Florestais (IPEF, Piracicaba, Brazil) were surface-sterilized by 15-min treatment in a 1% sodium hypochlorite solution containing Twin-20. Germination was carried out on ¼ strength Murashige and Skoog medium (MS medium; Sigma-Aldrich, St. Louis, MO) solidified with Phytagel 5.5 g/L (Sigma-Aldrich) at 25 °C in the dark for 3 days. To obtain 0.5- to 1-cm-long radicle, germination was performed at 25 °C for 3 days in dark. We inverted the plates to allow the growth of the radicle towards the exterior of the media. To obtain *in vitro* plantlets around 8 mm long with the hypocotyls fully expanded and the first two leaves just appearing, we germinated seeds inside plates at normal position at 25 °C for 3 days in the dark followed by 11 more days in light (12 μmol/m^2/s, 8- to 16-h photoperiod, 50% humidity). We also used an in-house *E. grandis* line obtained by *in vitro* clonal propagation of one seedling, grown in M media (Tournier *et al.*, 2003) for 3 weeks in conditions described above.

Agrobacterium rhizogenes strains

Three strains of *A. rhizogenes* were tested: (i) the A4 agropine mannopine-type strain originally isolated from roses exhibiting hairy root symptoms (Moore *et al.*, 1979), (ii) the A4RS strain, an A4-derived strain selected as resistant to the antibiotics rifampicin and spectinomycin (Jouanin *et al.*, 1986), and (iii) the low virulence ARqua1 strain, a Smr derivative of *A. rhizogenes* strain A4T (Boisson-Dernier *et al.*, 2001; Quandt *et al.*, 1993).

The binary vectors were introduced into *A. rhizogenes* strains by electroporation (Nagel *et al.*, 1990) and transformants selected on 50 mg/L kanamycin. Prior to infection, ARqua1, A4 and A4RS strains were grown for 72 h at 28 °C in LB, 2xYT and AG (Franche *et al.*, 1997) solid media, respectively, supplemented with acetosyringone 100 μM and kanamycin 50 mg/L. In the case of A4RS strains, all media used were also supplemented with spectinomycin 100 mg/L.

Binary vector construction

To optimize the transformation protocol, we used pCAMBIA-based binary vectors expressing either the GFP (pHKN29, Kumagai and Kouchi, 2003) or the DsRed fluorescent proteins (pCAMBIA 2200-derived vector, Fliegmann *et al.*, 2013), allowing easy detection of co-transformed hairy roots.

We constructed two Gateway-based binary vectors pGWAY-0 and pGWAY-1 (Figure S3) to overexpress genes of interest under

the 35S CaMV promoter and to investigate promoter activities, respectively. The construction of the vectors is described in Figure S3 legend. The *EgCCR1* cDNA (Lacombe *et al.*, 1997), the *EgCAD2* promoter (Feuillet *et al.*, 1995), the *EgCCR1* promoter (Gago *et al.*, 2011) and the histone linker protein (Eucgr.I02364) were amplified using primers listed in Table S2 and cloned into binary vectors as described in Appendix S1.

Induction of hairy roots and generation of composite *E. grandis* plants

We tested four different transformation protocols using at least 30 plants for each. We removed the tip of the emerging radicle of 3-day-old seedlings and infected the wound surface as previously described (Boisson-Dernier *et al.*, 2001). We also inoculated 14-day-old seedlings by cutting and infecting the base of hypocotyls based on the work of (MacRae and Van Staden, 1993). We finally used the hypocotyl-/stem-stabbing infection protocol as reported (Balasubramanian *et al.*, 2011) using an infected 0.45-mm-thick needle to inoculate 14-day-old seedlings and 3-week-old clonal lines of *in vitro*-grown *E. grandis* plantlets.

In all cases, co-culture was performed after infection for 14 days in MS with ½ strength of macroelements supplemented with 30 g/L of sucrose and 100 μM acetosyringone, at 22–20 °C, 40% humidity and under dim light (7 μmol/m^2/s, 8- to 16-h photoperiod conditions). Plants were further transferred on the same medium without acetosyringone and supplemented with 300 mg/L augmentin (amoxicillin/clavulanic acid) to prevent agrobacteria growth (leamkhang and Chatchawankanphanich, 2005). Plants were grown in controlled conditions (12 μmol/m^2/s, 8- to 16-h photoperiod, 40% humidity), and the screening for co-transformed hairy roots was performed 21 days after infection (21 dpi). GFP or DsRed fluorescence indicating co-transformed roots was detected using a stereomicroscope. Plants with at least one co-transformed root (containing both Ri and a binary vectors) were considered as positive. To calculate transformation efficiencies, about two to five independent transformation experiments were performed.

Hardening of *Eucalyptus* composite plants

In order to obtain roots containing enough xylem, 45-day-old composite plants were then cultivated in 200-mL pots on Oil-Dri US-Special Substrate (Type III/R; Damolin, Fur, Denmark) in a phytotron (130 μmol/m^2/s, 8- to 16-h photoperiod, 25–22 °C, 80% humidity). Plants were currently watered with tap water and once a week using MS with ½ strength of macroelements solution. After 4–8 weeks, GFP or DsRed fluorescence was verified again. Fluorescent roots were then sampled for RNA extraction (segments of the first five cm from root apex) and for biochemical analyses (many segments from 0 to 10 cm from root apex for young material, or a single thick segment 10 cm long from 20 to 30 cm from the main root for old material). Samples were immediately frozen with liquid nitrogen and further kept at −80 °C until analysis. Root segments (at least 20 cm from the root apex) were fixed with ethanol 80% for histology analyses or directly placed into GUS buffer (see below) for promoter activity analyses. After sampling, aerial part was partially pruned to prevent imbalance between aerial and root parts, and plants were maintained in the phytotron.

Hairy root cultures

Fluorescent root tips at least 2 cm long were excised from *in vitro* composite plants at 30 dpi and grown with augmentin on solid M media (Bécard and Fortin, 1988) containing 3% Phytagel and subsequently subcultured in order to obtain clonal lines. After at least two transfers in M media, three media all supplemented with sucrose 30 g/L were tested to optimize root growth: (i) M media (Bécard and Fortin, 1988), (ii) MS media with ½ strength of macroelements and (iii) MER media (modified M media by increasing KNO_3 to a final concentration of 2.5 mM and by adding NaH_2PO_4 to 0.9 mM). Elongation rate of roots was studied by placing ten 2-cm tips of lateral roots belonging to four different transformation events on each medium for 60 days in Petri dishes. The linear elongation of each individual root was measured every 10 days.

RNA extraction and reverse transcription quantitative polymerase chain reaction (RT-qPCR)

We tested several segments of the roots and several protocols of RNA extraction detailed in Appendix S2. The best RNA yield and quality were obtained using 0- to 5-cm roots grinded by Fastprep (MP Biomedical, Santa Ana, CA) extracted with modifications of the protocol of Muoki *et al.* (2012) detailed in Appendix S2.

Remaining traces of DNA were removed with Turbo DNA-free DNase I (Ambion, Austin, TX). RNA quality and quantity was checked with a Nanodrop ND-1000 Spectrophotometer (Thermo Fisher Scientific, Waltham, MA). First-strand cDNA synthesis was performed with 1 μg of total RNA using High Capacity cDNA RT Kit (Applied Biosystems, Foster City, CA). RT-qPCR was performed in technical triplicate using ABI 7900HT fast real-time PCR system (Applied Biosystems) with the Power SYBR Green PCR Master Mix (Applied Biosystems). Relative transcript abundance was calculated using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) with the housekeeping genes *PP2A-1* and *PP2A-3* to normalize data (Cassan-Wang *et al.*, 2012) and the mean of the control samples to standardize results.

EgCCR1 transcript level was assessed using primers annealing at the 3′UTR region, which do not amplify the *EgCCR1 antisense* messenger, and primers hybridizing against the CDS region of the *EgCCR1* gene, thus quantifying both the *EgCCR1* gene and the *EgCCR1 antisense* mRNA levels (Table S3). We also measured relative transcript abundance for some genes involved in lignin, cellulose and hemicellulose biosynthesis, selected according to their expression pattern in Eucalyptus (Carocha *et al.*, 2015; Myburg *et al.*, 2014). Genes selected were *EgPAL9, EgF5H1, EgF5H2* and *EgCAD2* involved in the lignin biosynthetic pathway, *EgCesA1* and *EgCesA3* involved in cellulose biosynthesis, and *EgFRA1/IRX7* and *EgGUX1* involved in hemicellulose biosynthesis. All primers are listed in Table S3.

Biochemical lignin analyses

Young root samples from (0–10 cm from root apex) and old root samples (single thick segment 10 cm long from 20 to 30 cm from the main root) were ground to a fine powder using a ball mill (MM400; Retsch, Haan, Germany). These samples were subjected to exhaustive water and then ethanol extraction in a Soxhlet apparatus. The recovered extract-free samples were dried at 40 °C overnight before analysis by thioacidolysis as previously described (Méchin *et al.*, 2014).

Histochemical GUS assay

Histochemical GUS assay was based on the methods described by Hawkins *et al.* (1997). Briefly, transformed root sections were incubated at 37 °C for 12 h in 100 mM sodium phosphate (pH = 7.0), 10 mM EDTA, 0.5 mM $K_3[Fe(CN)_6]$, 0.5 mM K_4[Fe

$(CN)_6$] and 1 mM 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc; Euromedex, Souffel Weyersheim, France). Samples were further fixed using 3.7% formaldehyde, 5% acetic acid and 50% ethanol buffer.

Microscopy and cell imaging

GFP or DsRed fluorescence indicating co-transformed roots was detected using a stereomicroscope Axiozoom V16 (Zeiss, Marly le Roi, France) equipped with a colour CCD camera (ICC5; Zeiss) and with filter sets for GFP (525/50 nm) and for DsRed (607/80 nm). CFP fluorescence was analysed with a confocal laser scanning microscope (TCS SP2-SE; Leica, Wetzlar, Germany).

Transverse sections (50 μm thick) of roots embedded in 5% low gelling point agarose (Sigma-Aldrich) were obtained using a vibratome (VT 100S; Leica) and observed using an inverted microscope (DM IRBE; Leica) equipped with a CDD colour camera (DFC300 FX; Leica), or using the stereomicroscope. Lignified SCWs were visualized either in red by phloroglucinol–HCl staining or in blue due to autofluorescence under UV light.

Sections were also dehydrated in an ethanol series, submitted to a critical point dry with CO_2 as a transitional fluid using a CPD300 unit (Leica) and, once dried, coated with nickel (5 nm) in a EM MED020 coating system (Leica) and analysed using a scanning electron microscope (ESEM Quanta 250 FEG; FEI, Mérignac, France) at 5 KV.

Acknowledgements

The authors are grateful to C. Rosenberg for kindly providing a pCAMBIA-derived vector as well as for useful advice while starting this work, F. Carvalho-Niebel and the endomycorrhizal symbiosis and cell signalling team (LRSV) for their precious advice, L. Marchesi for her help with transformation, R. Ployet for providing primers for *GUX1* gene. We also thank the TREEFOR-JOULES project (ANR-2010-KBBE-007-01), the CNRS, the Ministry of Education, Research and Technology, France (fellowship to A.P.) and the Toulouse III University (UPS) for their financial support.

The authors declare that they have no conflict of interest.

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Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Co-transformation efficiency time course of *E. grandis* explants using *A. rhizogenes* strains A4RS transformed either with pHKN29 or with pGWAY vectors.
Figure S2 Maps of pGWAY-0 and pGWAY-1 destination vectors.
Figure S3 Linear growth of excised *E. grandis* hairy roots.
Figure S4 Relative transcript level of *EgCCR*1 gene (dark grey) and *EgCCR1* antisense mRNA + *EgCCR1* mRNA (light grey) for each line of controls (4 lines) and *CCR*as (7 silenced lines and 2 nonsilenced lines) hairy roots.
Figure S5 Partial GC-MS chromatograms reconstructed at *m/z* 269 showing the guaiacyl (G) thioacidolysis monomers released from 10-cm segments of young roots of silenced *CCR*as 5-3 (a) and control lines (b).
Table S1 Mean values ± SD for H, G and S monomers percentage and for S/G ratio from young roots of control (Ø, empty pGWAY-0 vector) or *CCR*as lines.
Table S2 Primer sequences used for cloning.
Table S3 Primer sequences of genes used for RT-qPCR analyses.
Appendix S1 Details on binary vector construction.
Appendix S2 RNA extraction optimisation and detailed RNA extraction protocol.

