Coordinated transcriptional regulation of two key genes in the lignin branch pathway - CAD and CCR - is mediated through MYB- binding sites

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Abstract

Background: Cinnamoyl CoA reductase (CCR) and cinnamyl alcohol dehydrogenase (CAD) catalyze the final steps in the biosynthesis of monolignols, the monomeric units of the phenolic lignin polymers which confer rigidity, imperviousness and resistance to biodegradation to cell walls. We have previously shown that the Eucalyptus gunnii CCR and CAD2 promoters direct similar expression patterns in vascular tissues suggesting that monolignol production is controlled, at least in part, by the coordinated transcriptional regulation of these two genes. Although consensus motifs for MYB transcription factors occur in most gene promoters of the whole phenylpropanoid pathway, functional evidence for their contribution to promoter activity has only been demonstrated for a few of them. Here, in the lignin-specific branch, we studied the functional role of MYB elements as well as other cis-elements identified in the regulatory regions of EgCAD2 and EgCCR promoters, in the transcriptional activity of these gene promoters.

Results: By using promoter deletion analysis and in vivo footprinting, we identified an 80 bp regulatory region in the Eucalyptus gunnii EgCAD2 promoter that contains two MYB elements, each arranged in a distinct module with newly identified cis-elements. A directed mutagenesis approach was used to introduce block mutations in all putative cis-elements of the EgCAD2 promoter and in those of the 50 bp regulatory region previously delineated in the EgCCR promoter. We showed that the conserved MYB elements in EgCAD2 and EgCCR promoters are crucial both for the formation of DNA-protein complexes in EMSA experiments and for the transcriptional activation of EgCAD2 and EgCCR promoters in vascular tissues in planta. In addition, a new regulatory cis-element that modulates the balance between two DNA-protein complexes in vitro was found to be important for EgCAD2 expression in the cambial zone.

Conclusions: Our assignment of functional roles to the identified cis-elements clearly demonstrates the importance of MYB cis-elements in the transcriptional regulation of two genes of the lignin-specific pathway and support the hypothesis that MYB elements serve as a common means for the coordinated regulation of genes in the entire lignin biosynthetic pathway.

Background

Vascular cambium is a cylindrical secondary meristem that produces both secondary phloem and secondary xylem (i.e. wood in trees). The most characteristic components of secondary cell walls are lignins, complex phenolic polymers, which play fundamental roles in mechanical support, water and solute conductive properties and disease resistance in higher plants [1]. The biosynthesis of the lignin polymers derives from the general phenylpropanoid pathway which provides precursors for several branch pathways leading to the elaboration of a wide range of compounds involved in various aspects of plant development and defence [2]. In the lignin-specific branch pathway, the conversion of hydroxycinnamoyl CoA esters into cinnamyl alcohols (or monolignols), the monomeric units that are incorporated into the lignin heteropolymer, is catalyzed by cinnamoyl CoA reductase.
(CCR; EC 1.2.1.44) and cinnamyl alcohol dehydrogenase (CAD; EC 1.1.1.195) successively.

The spatial and temporal expression of the *Eucalyptus gunnii* CCR and CAD2 genes was investigated by fusing the gene promoters to the *uidA* reporter gene coding for β-glucuronidase (GUS) and expressing these constructs in transgenic woody and herbaceous plants [3-6]. Both *EgCAD2* and *EgCCR* gene promoters have been reported to direct GUS activity in the vascular tissues of all organs in all the plant species examined. Similar expression patterns have been reported for the *Arabidopsis thaliana AtCAD-C* and *AtCAD-D* genes, which encode proteins that are closely related to the *EgCAD2* enzyme [7]. The GUS activities were found to be consistent with tissue and cell locations of the CCR and CAD2 transcripts and proteins obtained by the use of *in situ* hybridization and immunolocalization [8,9]. These observations showed that the two genes have the same expression pattern, suggesting that the control of monolignol production is, at least in part, achieved through their coordinated transcriptional regulation.

Deletion studies of the *EgCCR* and *EgCAD2* promoters showed that the *EgCCR* promoter deleted to -119 bp upstream of the transcription start site retained its ability to direct GUS expression in a pattern similar to that obtained with the full-length promoter whereas the promoter deleted to -70 bp was inactive [5]. In the *EgCAD2* promoter, a region necessary for GUS expression in cambium and secondary xylem was mapped between -340 and -124 bp upstream of the transcription start site [6]. These results indicate that expression of the *EgCCR* and *EgCAD2* genes is under the control of a promoter region (50 bp in *EgCCR* and 216 bp in *EgCAD2*) proximal to the transcription start site. As revealed by *in silico* studies, both regulatory regions contain putative binding sites for MYB transcription factors.

Plant MYB proteins belong to one of the largest families of transcription factors. According to their predicted sequences and structural features, they have been classified into several subgroups [10-12]. The R2R3 two-repeat MYB family occurs specifically in plant lineages and its members have been postulated to participate in the regulation of a wide range of developmental and metabolic processes, notably the phenylpropanoid biosynthetic pathway [13-15]. However, despite the conservation of MYB consensus motifs (previously called AC-elements) in most phenylpropanoid gene promoters, functional evidence for their contribution to the promoter activity has only been directly proven for a few of them (reviewed in [16]). Although the involvement of MYB proteins in the monolignol-specific branch pathway has been suggested (reviewed in [17]), the functional role of MYB cis-elements and the molecular mechanism by which they participate in promoter activities have not yet been investigated.

The main goal of this study was to define the functional role of putative cis-elements in the transcriptional activities of *EgCAD2* and *EgCCR* promoters. Firstly, we restricted the regulatory region of the *EgCAD2* promoter to an 80 bp region necessary for expression both in secondary xylem and in cambium. This 80 bp region contains two MYB sites and two unreferenced sites that are revealed by *in vivo* footprinting. A straightforward approach to investigate the functional role of promoter elements is to introduce block mutations into the putative cis-elements in the context of the shortest active promoter. Thus, by using *EgCCR* and *EgCAD2* short promoter versions carrying single or combined mutations in their putative cis-elements, we clearly identified the MYB elements in the *EgCCR* and *EgCAD2* promoters as being crucial not only for the formation of protein-DNA complexes *in vitro* either with recombinant MYB protein or with cellular protein extracts, but also for the transcriptional regulation of *EgCCR* and *EgCAD2* genes *in planta*. In addition, the experimental data showed that a newly identified cis-element is required for *EgCAD2* expression in the cambial zone.

**Results**

An 80 bp *EgCAD2* cis-regulatory region is involved in *EgCAD2* expression in xylem tissues and in dividing cells

To further localize the cis-elements in the 216 bp region ([−340/−124]) involved in the transcriptional regulation of *EgCAD2* [6], we introduced several truncated versions of the promoter fused to the GUS reporter gene into tobacco by *Agrobacterium*-mediated transformation. Tobacco, a model herbaceous plant which undergoes secondary thickening, was previously shown to be a suitable model plant for studying the *EgCAD2* promoter from trees [6] and also phenylpropanoid promoters from herbaceous or woody plants (see for example [18-20]). The shortest tested 5′-deletion showing a similar vascular expression pattern to the full-length (−2500 bp) promoter was deletion −203 (Fig. 1a). Stem cross-sections showed GUS expression in cells connected to lignification, such as differentiating xylem and parenchyma cells of the primary and secondary xylem (rays), but also in cells apparently unconcerned by lignification, such as the dividing cells of the vascular cambium and the cells of the external and internal phloems, especially the companion cells (Fig. 1b). GUS expression in xylem and cambium was lost upon deletion of the promoter to −124 which retained GUS activity only in the internal phloem of tobacco stem [6], delimiting a shorter (80 bp) regulatory region [−203/−124] involved in promoter activity in xylem and vascular cambium.
Fine mapping of cis-elements in the EgCAD2 promoter by in vivo DMS footprinting

To precisely map the cis-elements in the EgCAD2 promoter interacting with trans-acting factors, we used in vivo dimethyl sulphate (DMS) footprinting, a technique allowing location of protein-DNA interactions with single nucleotide resolution [21]. The chemical agent DMS methylates the unprotected guanine residues rendering them susceptible to cleavage by piperidine.

The footprints were examined over a broad region [-348/-90] encompassing the regulatory region (Fig. 2a). In exponentially-dividing cells that highly expressed EgCAD2 (Day 8, see these data in additional file 1), several guanine residues of the promoter sequence showed differences in the footprint relative to the control (protein-free DNA), whereas in stationary phase cells with low expression of EgCAD2 (Day 15) the footprint was almost identical to the control (Fig. 2b). The protected (P) or hypersensitive (H) sites, corresponding to lower or higher intensity bands respectively relative to the control, are indicated on the promoter sequence (Fig. 2c). Two protected areas were identified, each composed of two adjacent guanines (positions -166, -167 and positions -146, -147), surrounded by hypersensitive residues (positions -141, -142, -155, -187). Hypersensitivity to DMS methylation can indeed result from alterations in the local DNA topology induced by protein binding [21]. The protected guanines fall within two identical sequences (CTGGTT), which we call BSa (binding site a) and BSb (Fig. 2c), and which have no homology with consensus binding sequences in databases. Just downstream of both BSa and BSb, however, are sequences that correspond to MBSIIIG, one of the plant MYB transcription factor binding sites (consensus motif G(G/T)T(A/T)GGT(A/G); [22]). These elements are named MYBa (-163 -156 in reverse orientation) and MYBb (-146 -139 in direct orientation). All the footprints are included within the 80 bp regulatory region delineated above. No other footprint was observed within the investigated [-348/-90] broad promoter region (Fig. 2a), indicating the absence of additional interaction sites upstream and downstream of the 80 bp regulatory region.

When surveying the CAD2 regulatory region in several Eucalyptus species, a few base substitutions were found in BSa and BSb sequences whereas the MYB sites were perfectly conserved, arguing for their possible functional importance (see additional file 2). It is worthy of note that similar conservation across Eucalyptus species was also found for the unique MYB site in the CCR regulatory region (additional file 2).

These results suggest that four relatively close putative cis-elements in the regulatory region [-203/-129] of the EgCAD2 promoter could interact with several transcrip-
tion factors in cells that are transcriptionally active for the EgCAD2 gene.

Effect of mutations on in vitro and in vivo binding of EgMYB2 to the EgCAD2 and EgCCR promoters

To investigate the ability of MYB sites of EgCAD2 and EgCCR promoters to interact in vitro and in vivo with MYB factor, site-directed mutagenesis was performed to disrupt these putative regulatory sites individually or in combination. Electrophoretic mobility gel assays (EMSA) and transactivation experiments were carried out in the presence of the Eucalyptus protein EgMYB2, a R2R3 MYB factor, that was hypothesized to positively regulate the transcription of genes belonging to the monolignol pathway [23].

EMSAs were performed using recombinant EgMYB2 and DNA probes corresponding to [-203/-129] EgCAD2 and [-119/-70] EgCCR regulatory fragments with mutated MYB sites (Fig. 3a). The specificity of the DNA-protein complex was verified by competition with a 100-fold molar excess of unlabelled specific competitor (corresponding to the respective EgCAD2 and EgCCR regulatory fragments, lanes 3 and 9) whereas an unlabelled non-specific DNA (unrelated DNA fragment, lanes 2 and 8)
did not affect the formation of the complexes. Mutation of the MYBa and MYBb sites in \textit{EgCAD2} and mutation of the MYB site in \textit{EgCCR} abolished the binding of recombinant \textit{EgMYB2} to the regulatory fragments. This loss of binding occurred even when a single MYB site remained intact in the \textit{EgCAD2} promoter, indicating that both MYB sites are required for maximal binding of \textit{EgMYB2}.

Transactivation experiments were performed by co-transfecting both the \textit{EgMYB2} cDNA expressed under the control of the \textit{CaMV 35S} promoter (effector construct) and wild-type or mutated -203 \textit{EgCAD2} or -119 \textit{EgCCR} promoters-GUS fusions (reporter constructs) into leaf mesophyll cells (Fig. 3b). When the wild-type -203 \textit{EgCAD2} promoter was co-transfected with the \textit{EgMYB2} construct, GUS activity was induced 3.4-fold when compared to the control construct (plasmid without \textit{EgMYB2} cDNA). Interestingly, the endogenous activity of the MYBa-mutated promoter was notably higher than that of the wild-type promoter, suggesting that the MYBa element might bind a repressor, at least in leaf mesophyll cells. Single or combined mutations of the MYBa and MYBb elements reduced the GUS expression driven by \textit{EgMYB2} to the same level as their respective controls, thereby reflecting the loss of activation by \textit{EgMYB2}. Again, this effect was observed even with a single mutated MYB site, indicating that both MYB sites are required for transactivation of the \textit{EgCAD2} promoter by \textit{EgMYB2}. Transactivation of the -119 \textit{EgCCR} wild-type promoter by \textit{EgMYB2} by about 2-fold was also abolished upon mutation of the MYB site (Fig. 3b).

Together, these \textit{in vitro} and \textit{in vivo} data indicate that, in the experimental conditions used, the three MYB \textit{cis}-elements are able to directly interact with \textit{EgMYB2} transcription factor and are involved in the transcriptional activation of \textit{EgCAD2} and \textit{EgCCR} genes mediated by \textit{EgMYB2}.

**Effect of mutations in \textit{cis}-elements on the protein binding activities of the \textit{EgCAD2} and \textit{EgCCR} regulatory regions**

To investigate the role of MYB and BS putative \textit{cis}-elements of \textit{EgCAD2} and \textit{EgCCR} promoters in the binding of transcription factors to the regulatory regions, EMSAs were performed using the regulatory fragments carrying several combinations of block mutations and tobacco protein extracts (Fig. 4). For both wild-type protein fragments, a large fast-migrating band was observed and another slower-migrating complex (called \textit{H} or \textit{H1}, for higher) was also detected when the protein extracts were used immediately after extraction (Fig. 4, lanes 1, 4 and 12, 15). The large band was composed of two closely migrating complexes already described for \textit{EgCCR} (LMC1 and LMC2, [5]) and named \textit{L1} and \textit{L2} for \textit{EgCAD2}.

Competition with a 100-fold molar excess of the corresponding unlabelled specific competitor DNA fragments blocked the formation of all the complexes (Fig. 4, lanes 3 and 14) whereas a similar amount of an unlabelled non-specific DNA had no effect (Fig. 4, lanes 2 and 13), indicating the specificity of the interactions.

Whereas mutation of the MYBa site in \textit{EgCAD2} had no major effect on the EMSA result (Fig. 4, lane 5), mutation of the MYBb site prevented the formation of both the slower migrating complex (\textit{H}) and the lowest part of the faster migrating large band (\textit{L2}) (Fig. 4, lane 6). Single or double mutations in the BS elements of \textit{EgCAD2} somewhat perturbed the pattern of the complexes with a notable decrease in \textit{H} and a concomitant increase in \textit{L2} (Fig. 4, lanes 9-11), especially when BSb was mutated (Fig. 4, lanes 9, 10). This concomitant increase in \textit{L2} was lost with the additional mutation in MYBb (Fig. 4, lane 8). Thus, when combined mutations including MYBb were tested (i.e. MYBa + MYBb, lane 7 or BSb + MYBb, lane 8), similar results were obtained as with the mutation of MYBb alone, \textit{i.e} loss of the \textit{H} and \textit{L2} complexes. Taken together, these data suggest that MYBb is a key \textit{cis}-ele-
ment involved in the formation of the H and L2 complexes and that BSb, and to a lesser extent BSa, apparently influence the ratio between the H and L2 complexes. Concerning the EgCCR promoter, only the mutation of the MYB element led to an overall decrease in all the complexes (Fig. 5, lanes 16, 18).

**Effect of mutations in cis-elements on vascular expression of EgCAD2 and EgCCR promoters**

To investigate the role of each cis-element on tissue-specific expression from EgCAD2 and EgCCR promoters, transgenic tobacco plants expressing GUS under the control of the wild-type or mutated -203 EgCAD2 or -119 bp EgCCR promoters were generated.

The BSa- or MYBa-mutated EgCAD2 promoters (see additional file 3) drove a GUS expression pattern similar to the wild-type -203 EgCAD2 promoter (Fig. 5a). In contrast, mutation of MYBb resulted in a considerable global diminution of GUS staining in all vascular tissues, suggesting that MYBb could be bound by an activator factor (Fig. 5a). This decrease in GUS staining was particularly pronounced in the xylem rays and in the parenchyma cells surrounding the primary xylem. Although with a lower intensity, the cambial-phloem zone still appeared as a continuous ring like with the wild-type promoter (Fig. 5a). This GUS expression pattern was also observed with the MYBa-MYBb double mutant (see additional file 3), confirming that MYBa does not play a crucial role in the promoter activity under these experimental conditions.

Upon mutation of the BSb cis-element, a substantial decrease in GUS activity was observed in the vascular cambium and, to a lesser extent, in the immediately derived parenchymatous cells, ie the xylem rays on the inner side and the parenchyma cells between phloem islands on the outer side (Fig. 5a). Thus, the cambial-phloem zone resulted in a spotted pattern with the spots showing GUS activity restricted to the external phloem area. The internal phloem still displayed GUS staining (Fig. 5a).

The double mutation BSb-MYBb reflected the combined actions of the single mutations with an overall reduction of GUS activity.

GUS activity driven by the wild-type -119 EgCCR promoter construct showed a vascular pattern similar to that driven by the -203 EgCAD2 promoter and to that reported in [5] using the full-length EgCCR promoter (Fig. 5b). This staining pattern was not affected by mutation of the BS1 cis-element. In contrast, GUS activity in plants carrying the single MYB mutant construct or the BS1-MYB double mutant construct notably diminished in all vascular tissues. As already observed for EgCAD2, GUS expression remained evident in the internal phloem of stem (Fig. 5b).

These observations were further corroborated by fluorimetric measurement of GUS activities (Fig. 5c). EgCAD2 MYBb and BSb single mutants showed a strong reduction of GUS activity (76% and 50%, respectively) when compared to the wild-type -203 promoter. Similarly, mutation of the MYB site in the EgCCR promoter resulted in a marked decrease of GUS activity (63%) when compared to the wild-type -119 promoter (Fig. 5c).

Taken together, these data provide evidence for a functional module comprising BSb-MYBb in the EgCAD2 promoter: MYBb appears to bind a general activator factor in all cell types whereas BSb might bind a protein specific to the cambium dividing cells. In the EgCCR promoter, the unique MYB cis-element might fulfill these dual functions.

**Discussion**

**Contribution of the EgCAD2 and EgCCR cis-elements to the protein binding and to transcriptional activities of the promoters**

Here, we delineated a short regulatory region [-203/-129] in the EgCAD2 promoter which is required for expression both in xylem tissues and in dividing undifferentiated cells either cultivated in vitro or already present in planta embedded within other tissues such as the cambial cells. Four cis-elements potentially involved in the regulation of EgCAD2 expression were identified within this region. They are arranged in two modules, each comprising an identical repeat of an unreferenced protein binding site (BS) with the sequence CTGGTT and an MBIIIG consensus site for MYB proteins. A simpler organisation exists in the [-119/-70] regulatory region of the EgCCR promoter with two putative cis-elements: a G-rich box (BS1) and a MBIIIG MYB consensus site [5].

Our results with EMSAs and transgenic plants with mutated cis-elements revealed the importance of the MYBb cis-element in EgCAD2 and of the MYB cis-element in EgCCR for the formation of the high and low mobility complexes and for the transcriptional activation of the EgCAD2 and EgCCR promoters in vascular tissues.

It is intriguing that neither EMSAs nor the transgenic plant approaches provided clear evidence of a functional role for MYBa in EgCAD2 promoter activity. This does not exclude its participation in other promoter functions, such as the response to external stimuli [6], or mechanisms specific to woody plant species [4,6], or expression in other parts of the plant, as does, for example, the ACIII/MYB element of the bean PAL2 promoter, which drives expression specifically in flowers [19]. Moreover, our observation in transactivation assays that the endogenous activity of the MYBa-mutated promoter in the absence of EgMYB2 was notably higher than the wild-type promoter activity, suggests that the MYBa element might bind a repressor, at least in leaf mesophyll, a tissue...
Figure 5 GUS expression driven by *EgCAD2* and *EgCCR* promoters containing mutated cis-elements. (A, B) Histochemical analyses of GUS activity, driven by the wild-type or the -203 *EgCAD2* and -119 *EgCCR* promoters mutated in the indicated cis-elements, in stem cross-sections of 8-week-old transgenic tobacco plants. (A) Upper panels, general views of the vascular tissues; lower panels, enlargements of the cambial zone, bars represent 200 μm and 50 μm, respectively. CZ, cambial zone; EP, external phloem; IP, internal phloem; PX, primary xylem; RP, xylem ray parenchyma. (B) Upper panels, general views of stem cross-sections; lower panels, enlargements of the vascular tissues. Bars represent 200 μm. (C) Fluorimetric assays of GUS activity in stems. Histograms represent mean values and standard deviations, expressed as % of the respective wild-type promoter-GUS activities. 7-10 independent transformants were examined for each construct. Activities driven by mutated *EgCAD2* and *EgCCR* promoter are statistically different relative to the control (WT -203 *EgCAD2* or WT -119 *EgCCR*), for MYBb, BSb and MYB Student’s *P* = 9 10^{-7}, 9 10^{-4} and 0.06, respectively.
where *EgCAD2* and *EgCCR* promoters are poorly expressed. Such repressors of lignin biosynthesis genes have already been demonstrated in the MYB family; some of them have broad tissue expression patterns whereas others are tissue-specific or are preferentially expressed in particular tissues [24-27].

Mutation of the BSa site in the *EgCAD2* promoter or of the G-rich BS1 site in the *EgCCR* promoter had no major effect on complex formation *in vitro* or on promoter activity in transgenic plants. In contrast, mutation of BSb in *EgCAD2* drastically reduced the formation of the H complex in EMSAs and altered the expression pattern in transgenic plants, leading to decreased promoter activity in cambial cells and in the immediately derived parenchyma cells.

Ongoing studies show that the proteins of the slow-migrating H complex are found in the phosphorylated fraction of tobacco nucleus proteins whereas those of the faster-migrating L2 complex are found in the unphosphorylated one (data not shown). We believe that the H and L2 complexes which both bind the MYBb element of *EgCAD2* could differ from each other by the addition of one or more protein(s) which can be subjected to phosphorylation. BSb that is located close to MYBb in *EgCAD2* could be involved in the formation of the phosphorylated complexes since its disruption affects the ratio between H and L2. The possible involvement of the BSb site both in phosphorylated transcriptional complexes and in *EgCAD2* expression in cambial cells (dividing cells) is consistent with the crucial and widely known role of phosphorylation mechanisms in metabolism connected to cell division [28].

The similar protein binding activities of the *EgCAD2* and *EgCCR* regulatory fragments in EMSAs and the similar expression patterns driven by the promoters in vascular tissues suggest that the transcriptional mechanisms necessary for their coordinated expression may be mediated by their common MYB cis-elements.

**Functional architecture of the promoters**

The complex organisation of the *EgCAD2* promoter with its cis-elements arranged in two similar modules (BS-MYB) suggests that redundancy and mechanisms of cooperation or competition between cis-elements and trans-acting factors might be involved in the regulation of the promoter activity. In particular, EMSAs with recombinant EgMYB2 and transactivation assays clearly demonstrated that the two MYB sites of the *EgCAD2* promoter cooperated for binding and activation. When studying *in silico* the distribution of MYB sites in the proximal 500 bp of the *Arabidopsis* phenylpropanoid gene promoters (core, monolignol and flavonoid pathways), it appears that two copies of MYB elements and, more specifically, MBSIIG or MBSII sites (as defined by [22]) are found in most promoters, generally separated by 50 to 100 bp (see additional file 4, [15,16]). Although the *CCR* gene involved in lignin biosynthesis in *Arabidopsis* follows this rule, intriguingly the promoter of the *CCR* gene in *Eucalyptus* contains a single MYB element (see additional file 4). The two nearby MBSIIG of bean PAL2 and PAL3 genes (core phenylpropanoid pathway) were demonstrated to be crucial for the transcriptional regulation of these genes *in planta* [19,29], suggesting a functional significance for this promoter architecture. Furthermore, as in our study, cooperative binding was also reported for the two MYB elements of the bean PAL2 promoter in the presence of the pine PtMYB1 protein [30].

Combinatorial interactions between the transcription factors binding to MYB elements and those binding to other closely located cis-elements are crucial for transcriptional regulation of the flavonoid branch pathway genes [31-33]. Other putative cis-elements that could cooperate with MYB elements were detected in the regulatory regions of *EgCAD2* and *EgCCR* by using the PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/signalscan.html). GT-1 elements, putative targets for trihelix-related transcription factors [35], appear to be well conserved in the regulatory regions of *CAD2* and *CCR* in various *Eucalyptus* species (see additional file 2). Interestingly, the hypersensitive site at -187 revealed in this study by *in vivo* footprinting falls within a WRKY transcription factor binding site [36] that is conserved in the *CAD2* regulatory regions; however it is more variable in *CCR*, especially in the invariant core which is essential for WRKY protein binding. In addition, the *CAD2* regulatory region contains a conserved motif similar to the TERE cis-element that is involved in secondary wall formation during differentiation of tracheary elements [37]; this motif is, however, not found in the *EgCCR* regulatory region. Presently, we cannot exclude the involvement of these putative cis-elements in *CAD* or *CCR* expression. However, the strong conservation of the MYB elements together with their key role in promoter activity, as demonstrated in this paper, indicates that the coordinated developmental regulation of *EgCAD2* and *EgCCR* expression is mediated through MYB transcription factors.

**MYB candidates for transcriptional regulation of *EgCAD2* and *EgCCR* genes**

*EgCAD2* and *EgCCR* genes which, as demonstrated in this study, required functional MYB binding sites in their promoters, exhibit expression in cell types consistent with their function in monolignol biosynthesis. Their expression was indeed found (i) in xylem tissues undergoing lignification and in parenchyma cells surrounding and feeding the lignified tissues with monolignols and (ii) in
the vascular cambium in which small monolignol oligomers may be components of a signal transduction pathway leading to the cell division [38].

MYB transcription factors responsible for EgCAD2 and EgCCR transcriptional activation should be found in the cell types exhibiting CAD2 and CCR expression and the interplay between various MYB factors or other transcription factors might contribute to the spatio-temporal expression patterns of EgCAD2 and EgCCR genes within specific cell types.

Particular MYB transcription factors have been detected in the cambium [39] and in xylem rays [40], and might contribute to EgCAD2 and EgCCR promoter activities in such parenchymatous tissues. Other MYB factors appear more specifically expressed in relation to secondary wall formation in cells that synthesize lignins. The Eucalyptus EgMYB2 and the pine (Pinus taeda) PtMYB1 and PtMYB4 transcription factors are preferentially expressed in secondary xylem. When overexpressed, these transcription factors affect the expression of monolignol biosynthesis genes and the amount or composition of lignins [23,41,42]. In Arabidopsis, the transcriptional network regulating secondary wall synthesis has been investigated [43]. AtMYB46, a close homologue of EgMYB2 and PtMYB4, appears to be a key switch in mediating the biosynthesis of the three major components of secondary walls (i.e. cellulose, hemicellulose/xylan and lignin) through the control of downstream, pathway-specific MYB and other transcription factors [44]. Recently, other Arabidopsis MYB factors, more closely related to PtMYB1, were shown to directly regulate the promoters of lignin biosynthesis genes [43,45]. At the present time, the role of EgMYB2 in the regulation of EgCAD2 and EgCCR gene expression must be clarified in order to determine whether EgMYB2 activates the transcription of these genes by direct binding on their promoters in vivo or by an upstream control as suggested for AtMYB46.

A complex picture of the transcriptional regulatory network controlling lignin biosynthesis genes is therefore emerging, with various MYB factors acting at different levels. As in the flavonoid branch pathway [31], the MYB factors controlling the monolignol pathway are likely to cooperate within multiprotein complexes with other protein partners that remain to be characterised.

Conclusions
The overall goal of this project was to characterize the functional role of putative cis-elements from promoters of genes involved in the monolignol biosynthetic pathway. In this study, we first demonstrated that the MYB cis-elements found in the delineated regulatory regions of EgCAD2 and EgCCR promoters are able to be bound by MYB factors to form specific DNA-protein complexes in vitro. MYB cis-elements play a key role in the vascular expression of these two genes and support the hypothesis that MYB elements serve as a common means for the coordinated regulation of genes in the entire lignin biosynthetic pathway. Moreover, a novel functional cis-element was identified and shown to be involved, possibly in combination with the MYB cis-element, in the transcriptional regulation of EgCAD2 in cambial cells. A comprehensive understanding of the functional cis-elements involved in the transcriptional regulation of EgCAD2 and EgCCR is the first step to dissect the regulatory network controlling lignin biosynthesis. Future studies will aim at the identification of the transcription factors that bind these cis-elements and at the mechanisms by which these transcription factors interplay with each other to provide the final spatio-temporal regulation of the lignin pathway.

Methods

Plant material and transformation
Tobacco plants (Nicotiana tabacum cv. Samsun NN) were grown in vitro and in the greenhouse as described previously [46]. Tobacco leaf disk transformation with Agrobacterium was performed as described by [6]. Tobacco cells (Nicotiana tabacum cv Bright Yellow 2, BY2) were grown at 25°C and transformed with Agrobacterium as described by [47]. Eucalyptus cell suspensions were grown as in [48].

Nucleic acids methods
Recombinant DNA methods were as recommended in [49]. DNA sequencing was performed with an ABI Prism 3700 DNA sequencer, using the ABI PRISM Dye terminator Cycle Sequencing Ready Reaction Kit (Applied Biosystems). The gene-specific primers used in this work and referred to hereafter are reported in additional file 5.

In vivo footprinting
In vivo DMS treatment of Eucalyptus suspension-cultured cells was performed as described in [50]. Briefly, cells were treated with 0.5% dimethylsulfate (DMS) for 2 min and the reaction was stopped by 10-fold dilution with ice-cold water. As a control, genomic DNA extracted from Eucalyptus cells was treated in vitro under the same conditions. Methylated DNA was extracted using the DNeasy Plant Mini Kit (Qiagen) according to the manufacturer’s instructions. In vivo and in vitro methylated DNAs were then cleaved with 1 M piperidine and recovered by lyophilisation and ethanol precipitation.

Methylated and cleaved DNA was used for ligation-mediated PCR (LMPCR) as described in [50]. Double-stranded, blunt-ended molecules were generated by primer extension from the EgCAD2-specific oligonucleotide 1 (see additional file 5). Ligation of the unidirectional common linker was performed as originally
described by [21]. PCR amplification (15 cycles) was then performed by using DynazymeII DNA polymerase (Finnzymes) with *EgCAD2* oligonucleotide 2 and common linker (additional file 5). Following amplification, *EgCAD2*-specific PCR products were labelled by extension from an *EgCAD2*-specific 32P-end-labelled oligonucleotide 3 (additional file 5). DNA was ethanol-precipitated and electrophoresed on a 6% sequencing polyacrylamide gel. The gels were dried and autoradiographed.

**Generation of *EgCAD2* and *EgCCR* promoter-GUS fusion constructs**

5'-deletions in the *EgCAD2* promoter were obtained by PCR amplification of the pOGUS-*EgCAD2* plasmid containing the full-length 2.5 kb *EgCAD2* promoter [51] with primers that introduced an EcoRI site at positions -301, -247, -203 and a Ncol site at the ATG start codon (additional file 5). After re-cloning the PCR fragments into the pOGUS vector, the promoter-GUS cassettes were released by EcoRI and PstI digestion, and cloned into the pBluescriptSKM13+ vector to generate a XbaI site necessary for cloning into the pBin19 binary plasmid.

Site-directed mutagenesis was performed using the QuikChange Site-Directed Mutagenesis Kit (Stratagene) on *EgCAD2* or *EgCCR* promoters in the pOGUS vector [5,51]. Two single-stranded complementary mutagenic oligonucleotides were used for each mutation (additional file 5). Double mutants were obtained from single mutants by using the same primers as for the single mutations. The PCR reactions were performed in 25 μl with 25 ng plasmid template, 0.2 mM dNTP, 1 unit Pfu Turbo DNA polymerase (Stratagene) and 62.5 ng of each mutagenic oligonucleotide. The parental strand was removed by digestion with DpnI. Mutated plasmid DNA was introduced into *E. coli* by electroporation. Mutant plasmids were obtained by digestion with EcoR1-NcoI fragment and cloning into the pOGUS vector.

**Electrophoretic mobility shift assay (EMSA)**

The [-203/-129] *EgCAD2* and [-119/-70] *EgCCR* promoter regulatory fragments used as probes for EMSA were obtained by amplification of pCambiaZ containing the wild-type or mutated cis-elements. Primers (4 pmol) common to the wild-type or mutated promoters were phosphorylated with 4 pmol of g[ 32P]-ATP (6000 Ci/mmol; Amersham) and T4 polynucleotide kinase (Promega) in 8 μl at 30°C for 30 min. The regulatory fragments were amplified by Pfu DNA polymerase with the radiolabelled common primer and variable second primers, depending on the introduced mutation (additional file 5). The labelled fragments were purified on a 4.8% polyacrylamide gel and eluted in water (100 μl) overnight at 4°C. The radioactivity incorporated was measured by Cerenkov counting.

Total protein extracts were prepared from exponentially growing 4-day-old tobacco BY2 cells by grinding in extraction buffer (50 mM Tris-HCl, pH 8, 500 mM NaCl, 10 mM MgCl, 10% glycerol, 7 mM β-mercaptoethanol, 1 mM EDTA, 0.5 mM PMSF). The BY2 cell protein extract was clarified by centrifugation for 30 min at 35000 g.

Binding reactions were performed at room temperature for 30 min in 25 μl binding buffer containing 25 mM Tris-HCl, pH 8, 250 mM NaCl, 10% glycerol, 7 mM β-mercaptoethanol, 2 μg poly (ddIdC): poly(ddIdC), 75 000 cpm of labelled probe and protein extracts. Free and bound DNAs were separated on 4.8% polyacrylamide gels in 0.5x TBE, 2.5% glycerol. Gels were then fixed, dried and autoradiographed.

**GUS fluorimetric and histochemical tests**

GUS fluorimetric tests were carried out as described previously by using 4-methylumbelliferyl-β-D-glucuronide as substrate [6]. Measurement was done with a fluorometer (BIO-TEK FL600 Microplate Fluorescence Reader). Protein concentrations were determined by the Bradford method (Bio-Rad).

Histochemical localization of GUS activity was performed as described by [6] after pre-fixation in 0.3% formaldehyde in 10 mM MES, pH5.6, 0.3 M mannitol. General views were acquired with a binocular microscope (Leica MZ16) equipped with a camera (DC500, Leica). Transverse sections (300 μm) were cut on a vibratome with vibrating blade Leica VT1000S, mounted on glass slides and observed under an inverted microscope (Leitz DMIRBE, Leica Microsystems, Wetzlar, Germany). Images were acquired using a CCD camera (Color Coolview, Photonic Science, Millham, UK).

**Expression of GST-*EgMYB2* in *E. coli***

The *E. coli* strain BL21 containing the plasmid pGEX-5X-1-*EgMYB2* cDNA was described in [23]. Induction of the GST-*EgMYB2* fusion protein was performed by adding isopropyl β-D-thiogalactoside (Sigma) to a final concentration of 0.1 mM. After growth at 28°C for 5 h, cells were lysed by two passages through a French press at 12000 p.s.i. (French Pressure Cell, Thermo Scientific, Waltham, MA, USA) in 20 mM potassium phosphate buffer, pH 7.5, 140 mM NaCl, 0.5 mM PMSF and 10 mM β-mercaptoethanol. The fusion protein was purified by FPLC (ÄKTA™ FPLC™ chromatographic system, Amersham...
Biosciences) on prepacked 1 ml GSTrap™ FF columns (Amersham biosciences) following the supplier’s instructions. Proteins were eluted with 50 mM Tris-HCl pH 8 containing 10 mM reduced glutathione protein (Sigma) and were then concentrated and dialysed by centrifugation on a Vivaspin concentrator (10000 MWCO, molecular weight cut-off, Vivascience) with 50 mM Tris-HCl, pH 8, 140 mM NaCl, 7 mM β-mercaptoethanol, 20% glycerol. Protein concentration was determined with the Bradford reagent (Bio-Rad).

Co-transfection experiments
Co-transfection experiments were performed essentially according to the method of [52]. Effector constructs (pJRI binary vector with or without EgMYB2 cDNA under the control of the cauliflower mosaic virus (CaMV) 35S promoter) were as in [23]. Reporter constructs (pCambiaZ binary vector containing wild-type or mutated EgCAD2 promoter-GUS or EgCCR promoter-GUS fusions) are described above. Agrobacterium strains GV3101:pMP90 containing effector or reporter constructs were co-infiltrated into leaves of GV3101:pMP90 containing effector or reporter constructs and were then concentrated and dialysed by centrifugation on a Vivaspin concentrator (10000 MWCO, molecular weight cut-off, Vivascience) following the supplier’s instructions. Proteins were eluted with 50 mM Tris-HCl, pH 8 (Amersham biosciences) following the supplier's instructions.

Additional material
Additional file 2: Eucalyptus CAD2 gene expression in suspension-cultured cells.
Additional file 3: Sequence analysis of the regulatory regions of the CAD2 and CCR promoters from several Eucalyptus species.
Additional file 4: GUS expression driven by EgCAD2 promoters containing mutated BSa, MYBa or MYBa-MYBb sites.
Additional file 5: cis-elements within the 500 bp proximal promoter regions of phenylpropanoid biosynthesis genes.
Additional file 6: 8 cis-gene family expression in transgenic poplar.

Authors’ contributions
AR and PR performed the experiments. AR helped to draft the manuscript. YM participated in the histological analysis. JGP initiated and coordinated the study, supervised the assays, analyzed the data, discussed the results and wrote the paper. All authors read and approved the final manuscript.

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