Plant Mol Biol (2006) 62:809–823
DOI 10.1007/s11103-006-9058-2

Down-regulation of the maize and Arabidopsis thaliana caffeic acid *O*-methyl-transferase genes by two new maize R2R3-MYB transcription factors

Silvia Fornalé · Fathi-Mohamed Sonbol · Tamara Maes · Montserrat Capellades · Pere Puigdomènech · Joan Rigau · David Caparrós-Ruiz

Received: 26 January 2006 / Accepted: 13 July 2006 / Published online: 29 August 2006

Abstract The maize (*Zea mays* L.) caffeic acid *O*-methyl-transferase (COMT) is a key enzyme in the biosynthesis of lignin. In this work we have characterized the involvement of COMT in the lignification process through the study of the molecular mechanisms involved in its regulation. The examination of the maize *COMT* gene promoter revealed a putative ACIII box, typically recognized by R2R3-MYB transcription factors. We used the sequence of known R2R3-MYB factors to isolate five maize R2R3-MYB factors (ZmMYB2, ZmMYB8, ZmMYB31, ZmMYB39, and ZmMYB42) and study their possible roles as regulators of the maize *COMT* gene. The factors ZmMYB8, ZmMY31, and ZmMYB42 belong to the subgroup 4 of the R2R3-MYB family along with other factors associated with lignin biosynthesis repression. In addition, the induction pattern of *ZmMYB31* and *ZmMYB42* gene expression on wounding is that expected for repressors of the maize *COMT* gene. *Arabidopsis thaliana* plants over-expressing ZmMYB31 and ZmMYB42 down-regulate both the *A. thaliana* and the maize *COMT* genes. Furthermore, the over-expression of *ZmMYB31* and *ZmMYB42* also affect the expression of other genes of the lignin pathway and produces a decrease in lignin content of the transgenic plants.

Keywords *Arabidopsis thaliana* · COMT · Lignification · Maize · R2R3-MYB factors

S. Fornalé · F.-M. Sonbol · T. Maes · M. Capellades · P. Puigdomènech · J. Rigau (✉) · D. Caparrós-Ruiz
Departament de Genètica Molecular, Laboratori de Genètica Molecular Vegetal, CSIC-IRTA, Jordi Girona 18-26, 08034 Barcelona, Spain
e-mail: jrlgmr@ibmb.csic.es

Present Address:
T. Maes
Oryzon Genomics, Parc Científic de la Universitat de Barcelona, Josep Samitier 1-5, 08028 Barcelona, Spain

Introduction

Lignification is a complex process common to all vascular plants. Lignin is mainly composed of three subunits, *p*-hydroxyphenyl (H), guaiacyl (G), and syringyl (S), whose proportions in the final polymer differ depending on the plant species (Lewis and Yamamoto 1990). The accumulation of lignins in plant cell walls increases the strength and stiffness of fibers, improves the efficiency of water transport through the vascular system, and protects plants from pathogen attack (Whetten et al. 1998; Mellerowicz et al. 2001; Boerjan et al. 2003; Boudet et al. 2003).

Most of the studies on lignin biosynthesis performed so far have been done using dicot plants as a model, with many transgenic plants down-regulating and/or over-expressing one of the genes involved in lignification. An exhaustive compilation of the results obtained by studying these transgenes has been published (Anterola and Lewis 2002). In contrast, much less is known about lignification in *Zea mays*. In fact, only the characterization of some spontaneous maize mutants having a brown midrib (*bm*) pigmentation in lignifying tissues have been associated with lignin metabolism. Among all the *bm* mutants, the most characterized are *bm1* and *bm3*, defective in cinnamyl

alcohol dehydrogenase (CAD) activity and caffeic acid *O*-methyl-transferase (COMT) activity, respectively (Halpin et al. 1998; Vignols et al. 1995; Morrow et al. 1997). Indeed, it has been demonstrated that the lack of COMT activity in the *bm3* mutant is associated with a disruption of the *COMT* gene (Vignols et al. 1995).

Over the last few years, the regulation of some genes of the lignin biosynthetic pathway has begun to be unraveled by the isolation and characterization of R2R3-MYB factors. Although several R2R3-MYB factors belonging to different subgroups have been described as regulators of lignification, such as the *Pinus taeda* PtMYB4, poplar PttMYB21a (Patzlaff et al. 2003a; Karpinska et al. 2004), and *Eucalyptus gunnii* EgMYB2 (Goicoechea et al. 2005), at present, a clear role in the repression of lignin biosynthesis has only been attributed to R2R3-MYB factors belonging to the subgroup 4. In fact, the first MYB factors that were associated with down-regulation of lignification are the *Antirrhinum majus* AmMYB308 and AmMYB330 (Tamagnone et al., 1998a). These two factors are able to down-regulate *4-coumarate:CoA ligase* (*4CL1*), *cinnamate-4-hydroxylase* (*C4H*), and *CAD* when over-expressed in tobacco plants. The later characterization of another subgroup 4 factor from *A. thaliana*, AtMYB4, revealed that, in the knock-out *Atmyb4* plant there is an increase in *C4H* gene expression, as well as a decrease in *caffeoyl-CoA O-methyl-transferase* (*CCoAOMT*) gene expression (Jin et al. 2000). Recently, a subgroup 4 R2R3-MYB factor from *A. thaliana*, AtMYB32, has been proposed to repress the *A. thaliana COMT* gene as the *Atmyb32* mutant slightly increases the expression of *AtCOMT* (Preston et al. 2004).

Maize is an important crop and forage plant, whose economical and nutritional value is affected by its lignin content, and many studies have been undertaken to improve its digestibility by reducing the accumulation of this polymer (Boudet et al. 2003; Boerjan et al. 2003). Different enzymes of the lignin biosynthesis pathway have been already described in maize but COMT (EC 2.1.1.6) is probably the best known enzyme associated with lignification in this species. Maize COMT has been characterized as an enzyme encoded by a single gene, which is mainly expressed in lignifying tissues and specifically methylates caffeic acid in vitro (Collazo et al. 1992). As for many genes involved in lignification, *COMT* gene expression is also regulated by wounding (Capellades et al. 1996). A similar *bm3* phenotype has been obtained by partial down-regulation of COMT in transgenic maize plants (Piquemal et al. 2002).

At present, virtually nothing is known concerning the molecular mechanisms involved in the regulation of lignin genes in maize, in which only five maize R2R3-MYB factors, C1 and PL (Paz-Ares et al. 1987; Marocco et al. 1989; Cone et al. 1993; Pilu et al. 2003), P (Grotewold et al. 1991, 1994), Zm1 and Zm38 (Franken et al. 1989; Marocco et al. 1989) have been previously characterized as transcription factors involved in the regulation of flavonoid biosynthesis. For this reason and to gain more information on the regulation of lignin biosynthesis in maize, we used *COMT* as a target gene to identify factors acting as repressors of maize lignification. Previous work has shown that a 2 kb region of the maize *COMT* gene promoter drives the expression of the GUS gene with the same specificity in maize and tobacco, suggesting that the molecular mechanisms governing the *COMT* gene expression are at least partially conserved between such two evolutionary distant species (Capellades et al. 1996).

The detailed examination of the maize *COMT* gene promoter revealed a putative ACIII box, 200 bp upstream from the transcription initiation site, suggesting a possible involvement of MYB factors in the regulation of maize *COMT* gene expression. Several R2R3-MYB factors, such as PtMYB1 (Patzlaff et al. 2003a), PtMYB4 (Patzlaff et al. 2003b), and EgMYB2 (Goicoechea et al. 2005) have already been associated with the regulation of lignin biosynthesis through their in vitro interaction with ACI, ACII, and ACIII *cis*-elements. Therefore, using degenerated primers from the subgroup 4 R2R3-MYB factors and screening a cDNA library, we isolated five new maize R2R3-MYB transcription factors belonging to three different subgroups of the family. We generated *A. thaliana* lines over-expressing these factors to study their possible involvement in the regulation of *COMT* gene. We observed that only the ZmMYB31 and ZmMYB42 subgroup 4 factors down-regulate both the *A. thaliana* and the maize *COMT* genes. Thus, we report for the first time the identification of two repressors of the maize *COMT* gene through the isolation and identification of a new set of maize R2R3-MYB transcription factors. In addition, we showed that ZmMYB31 and ZmMYB42 regulate other genes of the lignin pathway and affect the lignin content of the *A. thaliana* transgenic plants.

Materials and methods

Plant material

Zea mays L. (W64A inbred line) plants were grown under greenhouse conditions (28°C day and 22°C night with 70% humidity). Plants received a 16/8 h photoperiod of natural and supplemental light (high-pressure sodium lamps 250 μE m^{-2} s^{-1}) and samples of six different plants, were collected at different growth stages (6–10 and 20 days). For wounding assays, maize leaves of 10-day-old plants were wounded by transversal incisions with a scalpel blade and harvested at different times after treatment (ranging from 0.5 h to 24 h). Each sample was constituted by six leaves taken from three different plants. Plant tissues were immediately frozen in liquid nitrogen and stored at –80°C until analyzed.

Arabidopsis thaliana (ecotype C24, WS, and Columbia) plants were grown under standard greenhouse conditions (25°C day and 22°C night with 50% humidity) with a 16/8 h photoperiod as mentioned above. pCOMT::GFP homozygous *A. thaliana* plants was used to pollinate homozygous *A. thaliana* plants over-expressing ZmMYB2, ZmMYB8, ZmMYB31, ZmMYB39 and ZmMYB42 factors. Hemizygous seeds from these crosses were then germinated in vitro on MS medium (Murashige and Skoog 1962), supplemented with 3 g l^{-1} sucrose for ten days, and analyzed for green florescence.

Rosette leaves of homozygous pCOMT::GFP adult plants were wounded by transversal incisions with a scalpel blade and harvested at 6 h for green florescence analyses.

Isolation of five maize R2R3-MYB transcription factors

The isolation of maize R2R3-MYB cDNA was done using degenerated primers based on the sequence of *A. majus AmMYB308* and *AmMYB330* cDNAs (Tamagnone et al. 1998a). The sequences of the degenerated primers designed for this work were:

Fw1: 5′-ccNtgYtgYgaRaaRgcNcaYacNaa-3′; Fw2: 5′-gaYctNaaRcgNggNaaYttYac-3′.
Rv1: 5′-ggNatHgaYccNacNacNca-3′; Rv2: 5′-ggNatHgaYccNcaRacNca-3′.

These degenerated primers were used to amplify the corresponding cDNA region of maize R2R3-MYB factors from the elongation region of a maize root cDNA library described by Vignols et al. (1999) in which lignification is widely active (Vignols et al., 1999; de Obeso et al. 2003; Caparrós-Ruiz et al. 2006). Briefly, total RNA was isolated from 9-day old tip-less roots and Poly(A)$^+$ RNA isolated from total RNA, using the PolyATrack kit (Promega). For the synthesis of double-stranded cDNA, 5 μg of Poly(A)$^+$ RNA (λZAPII-cDNA synthesis kit from Stratagene) was used. Finally, ligation and packaging of cDNA (Gigapack, Stratagene) was performed according to the manufacturer's instructions.

The amplified cDNA fragments were used as a probe to screen the previous cDNA library for full-length R2R3-MYB cDNA clones. Seventy clones were excised in vivo to generate pBluescript phagemids containing the cDNA inserts and sequenced. Five different maize R2R3-MYB cDNA clones resulting from this cDNA screening were obtained and named *ZmMYB2*, *ZmMYB8*, *ZmMYB31*, *ZmMYB39* and *ZmMYB42*. *ZmMYB2* cDNA was isolated as a partial clone, so its 5′end was obtained using the 5′RACE amplification kit (Invitrogen) according to the manufacture's instructions.

The primers used for 5'RACE amplification were:

ZmMYB2-RACE-1: 5′-agcagcttcttgcggatgt-3′; ZmMYB2-RACE-2: 5′-gagaggatcttcgtcgtg-3′.

pCOMT::GFP expression construct, ZmMYB2, ZmMYB8, ZmMYB31, ZmMYB39 and ZmMYB42 over-expression constructs, and *A. thaliana* transformation

The GFP cDNA was obtained from the pBIN19-35S-SGFP-TYG-nos vector (Chiu et al. 1996), and the GFP and nos-terminator digested with *BamH*I and *EcoR*I restriction enzymes. This fragment was cloned into a pUC18 vector containing the 2 kb maize *COMT* gene promoter (Capellades et al. 1996). The maize *COMT* gene promoter fused to GFP and the nos-terminator was then digested with *Hind*III and *EcoR*I, cloned into the pGREENII-0029 vector (Hellens et al. 2000a) and the plasmid transferred into *Agrobacterium tumefaciens* (C58pJIC strain) (Hellens et al. 2000b) by heat shock (Hofgen and Willmitzer 1988). Finally, *A. thaliana* (C24 inbred line) was transformed with *A. tumefaciens* carrying the pCOMT::GFP construct, by floral dip (Clough and Bent 1998).

The cDNA sequence of *ZmMYB2, ZmMYB8, ZmMYB31, ZmMYB39 and ZmMYB42* was placed in the sense orientation between the double CaMV 35S

promoter sequence and the pA35S transcription terminator using the vector described by Reichel et al. (1996). These five constructs were cloned into the pCAMBIA1300 vector in the *Hind*III site. Finally, they were transferred into *A. tumefaciens* (C5851 strain) by heat shock and used for *A. thaliana* plant transformation by floral dip (Clough and Bent 1998).

Isolation of total RNA, RT-PCR and PCR

Total RNA was extracted with Trizol Reagent according to the manufacturer's instructions (Invitrogen). Approximately 4 μg of total RNA were reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. First-strand cDNA was generated using an oligo$(dT)_{15}$ primer, and 2 μl of the first-strand cDNA used as a template in subsequent PCR reactions. Gene-specific primers were used to amplify *ZmMYB2*, *ZmMYB8*, *ZmMYB31*, *ZmMYB39*, and *ZmMYB42*, and ''no-RT'' PCR assays were performed to confirm the absence of genomic DNA contamination. For each assay, several numbers of cycles were tested to ensure that the amplification was in the exponential range. The identity of the amplified bands was further confirmed by DNA sequencing with the following primers:

Fw-ZmMYB2: 5′-ctccgctggaccacctacct-3′; Rv-ZmMYB2: 5′-gctgcaccacatcatcagtt-3′.
Fw-ZmMYB8: 5′-ggccatatccgctagctaca-3′; Rv-ZmMYB8: 5′-actggaacacgcacatcaag-3′.
Fw-ZmMYB31: 5′-gaccaaggaggaggacgag-3′; Rv-ZmMYB31: 5′-tcatcatccatgtgaggtgtg-3′.
Fw-ZmMYB39: 5′-gccacggccacagcaactgg-3′; Rv-ZmMYB39: 5′-aaccgtctaggtccgtttcc-3′.
Fw-ZmMYB42: 5′-cttgaggtccagctcctcgt-3′; Rv-ZmMYB42: 5′-ctactggaacacgcacatcc-3′.

The sequences of the primers used to amplify *ZmCOMT* (AY323283), *ZmActin* (BT016731), and *ZmUbiquitin* (U29159) were:

Fw-ZmCOMT: 5′-aagtggctcacccccaacgag-3′; Rv-ZmCOMT: 5′-gaacacgccctgcgccttgc-3′.
Fw-ZmActin: 5′-ccaaggccaacagagagaaa-3′; Rv-ZmActin: 5′-tgaccatcaggcatctcgta-3′.
Fw-ZmUbiq: 5′-taagctgccgatgtgcctgcgtcg-3′; Rv-ZmUbiq: 5′-ctgaaagacagaacataatgagcacaggc-3′.

The gene-specific primers used for the analysis of *A. thaliana* lignin biosynthetic genes had been derived from the ''lignin toolbox'' previously described (Raes et al. 2003).

The sequences of the primers used to amplify *AtCOMT* (AY081565), *At4CL1* (AY099747), *AtC4H* (AY065145), *AtCCoAOMT1* (AY143979), *AtCAD6* (AY113919) and *AtActin* (AY096397) were:

Fw-AtCOMT: 5′-cgtcgcagacaactttgatg-3′; Rv-AtCOMT: 5′-tgatctcccacatgtcatcg-3′.
Fw-At4CL1: 5′-ctccggtgtctggatcaact-3′; Rv-At4CL1: 5′-gaaatctggtgctgctcctc-3′.
Fw-AtC4H: 5′-gcaagctgaattgtccacct-3′; Rv-AtC4H: 5′-cacatccttgaagctgagca-3′
Fw-AtCCoAOMT1: 5′-catcatcgaccaatggagaa-3′; Rv-AtCCoAOMT: 5′-tcgatcaaacgcttgtggta-3′
Fw-AtCAD6: 5′-cgagtctctcaaacgcagtg-3′; Rv-AtCAD6: 5′-gttaggtggagtcggtcaca-3′
Fw-AtActin: 5′-catctgttggaaggtgctga-3′; Rv-AtActin: 5′-actaccgcagaacgggaaat-3′.

The sequence of the primers used to amplify pCOMT::GFP construct was:

Fw-pCOMT: 5′-ggtgtggtggttggtgagcc-3′; Rv-GFP: 5′-tc acgaactccagcaggacc -3′.

Sequence alignment, phylogenetic tree, and image analysis

For the alignment of the maize R2R3-MYB sequences the ClustalW program (http://www.ebi.ac.uk/clustalw/) was used (Higgins et al. 1994). Poorly aligned positions and divergent regions were eliminated by using Gblocks 0.91b following the given options for a less stringency (Castresana 2000). To construct the phylogenetic trees the PHYML (PHYlogenies by Maximum Likelihood) online execution program (http://www.atgc.lirmm.fr/phyml/) was used (Guindon et al. 2005), using the JTT model of amino acid substitution with four substitution rate categories and an estimated proportion of invariable sites.

Confocal Laser Scanning Microscopy (CLSM) was done using a Leica TCS SP confocal laser scanning microscope (Heidelberg, Germany) fitted to/with spectrophotometers for emission band wavelength selection was used with an argon ion laser emitting at 488 nm to excite GFP. During scanning, we used a triple-dichroic beam splitter (TD 488/543/633). For visualization of GFP, the emission window was set at 495–540 nm. Serial optical slices of apical stems of wild type and homozygous pCOMT::GFP adult plants were taken each 1.5 μm. Confocal image stacks were combined as *x–y* projection images. The

transmitted laser light of the Argon laser emitting at 488 nm was collected on a photomultiplier tub (PMTT).

Green florescence was measured using a Leica Stereo-microscope (Leica, Wetzlar, Germany). Photos were taken with a Leica DC 200 camera and further analyzed with the Leica Confocal Lite Software program (Leica, Wetzlar, Germany). An average of 8 roots was analyzed for each experiment. The green florescence was quantified by densitometry using the Quantity One 4.1.1 program (BIO-RAD). Each determination was repeated three times and the results were expressed as percentage of relative signal intensity ±SD.

Lignin measurement

Lignin quantitative assay was performed by derivatization with thioglycolic acid (Whitmore 1978) from alcohol-insoluble residues (AIRs) of *A. thaliana* stem cell material. Samples of wild type and *A. thaliana* plants over-expressing ZmMYB31 and ZmMYB42 were homogenized in 20 volumes of absolute MeOH at top speed for 1 min. The homogenate was vacuum filtered over Whatman GF/C and rinsed with MeOH. The resulting residue was then transferred to a glass beaker and dried at 60°C during 24 h and the AIR obtained was used for lignin determination. To 25 mg of AIR 2.5 ml of 2 N HCl and 0.25 ml of thioglycolic acid were added in glass–screw–cap tubes and the sealed tubes were placed at 100°C in a water bath. After 4 h of incubation, the tubes were cooled and the content was transferred to polypropylene centrifuge tubes. After 10 min centrifugation at 30,000 × *g* at RT, the resulting pellet was washed with 2.5 ml of H_2O and resuspended in 2.5 ml of 0.5 N NaOH. Lignin thioglycolate was extracted by an 18 h gentle agitation at 25°C. After a 10 min centrifugation at 30,000 × *g* at RT, the supernatant was transferred to glass tubes and 0.5 ml of concentrated HCl was added. The lignin thioglycolic acid (LTGA) was precipitated 4 h at 4°C and after a 10 min centrifugation at top speed, the resulting pellet was dissolved in 5 ml of 0.5N NaOH and the A_{280} was measured. Each sample was constituted by four different plants and each determination was repeated three times.

The sequences designated *ZmMYB2*, *ZmMYB8*, *ZmMYB31*, *ZmMYB39*, and *ZmMYB42* have been submitted to the Nucleotide Sequence Database (AM156904, AM156905, AM156906, AM156907, and AM156908, respectively).

Results

The maize COMT gene promoter directs GFP expression in vascular tissues in *A. thaliana*

It has previously been shown that the maize *COMT* gene promoter is able to preserve its expression specificity in transgenic tobacco plants (Capellades et al. 1996). Our results showed that, on fusing 2 kb of the *COMT* gene promoter fragment to GFP, this specificity of expression is maintained in *A. thaliana*, as GFP was detected only in the vascular tissues of plant undergoing lignification such as stems and roots (Fig. 1A and B), and is absent in non-lignifying tissues such as the root apical meristem (Fig. 1B). In addition, the maize *COMT* gene promoter is also specifically induced by wounding only in vascular tissues (Fig. 1C). These results indicate that the molecular mechanisms involved in the maize *COMT* gene promoter regulation are at least partially conserved in *A. thaliana* and maize.

Isolation of five maize R2R3-MYB transcription factors: structure and comparison of proteins

The maize *COMT* gene promoter contains a putative ACIII box approximately 200 bp upstream from the transcription initiation site (Fig. 1D), typically recognized by R2R3-MYB transcription factors. Therefore, using degenerated primers based on the *A. majus* AmMYB308 and AmMYB330 sequences (Tamagnone et al. 1998a), we amplified a set of cDNA fragments using a cDNA library obtained from the elongation zone of the maize root in which lignification is widely active as previously described (Vignols et al. 1999). Once identified by sequencing, the interesting clones (*ZmMYB2*, *ZmMYB8*, *ZmMYB31*, *ZmMYB39*, and *ZmMYB42*) were used as a probe to obtain their respective full-length sequences by the screening of the same maize cDNA library.

The presumably full-length cDNA sequences of *ZmMYB2*, *ZmMYB8*, *ZmMYB31*, *ZmMYB39*, and *ZmMYB42* are 1,339, 1,089, 1,264, 1,078, and 1,129 bp, respectively, with the start codon of the deduced proteins at position 69, 185, 141, 152, and 103 bp. The deduced ZmMYB2, ZmMYB8, ZmMYB31, ZmMYB39, and ZmMYB42 proteins have 339, 226, 275, 256, and 260 amino acid residues respectively.

Sequence alignment of the five ZmMYB factors shows a highly conserved N-terminal region, constituted by R2 and R3 domains**,** while the C-terminal domain of each ZmMYB is more divergent, both in sequence and length (Fig. 2A). Protein sequence

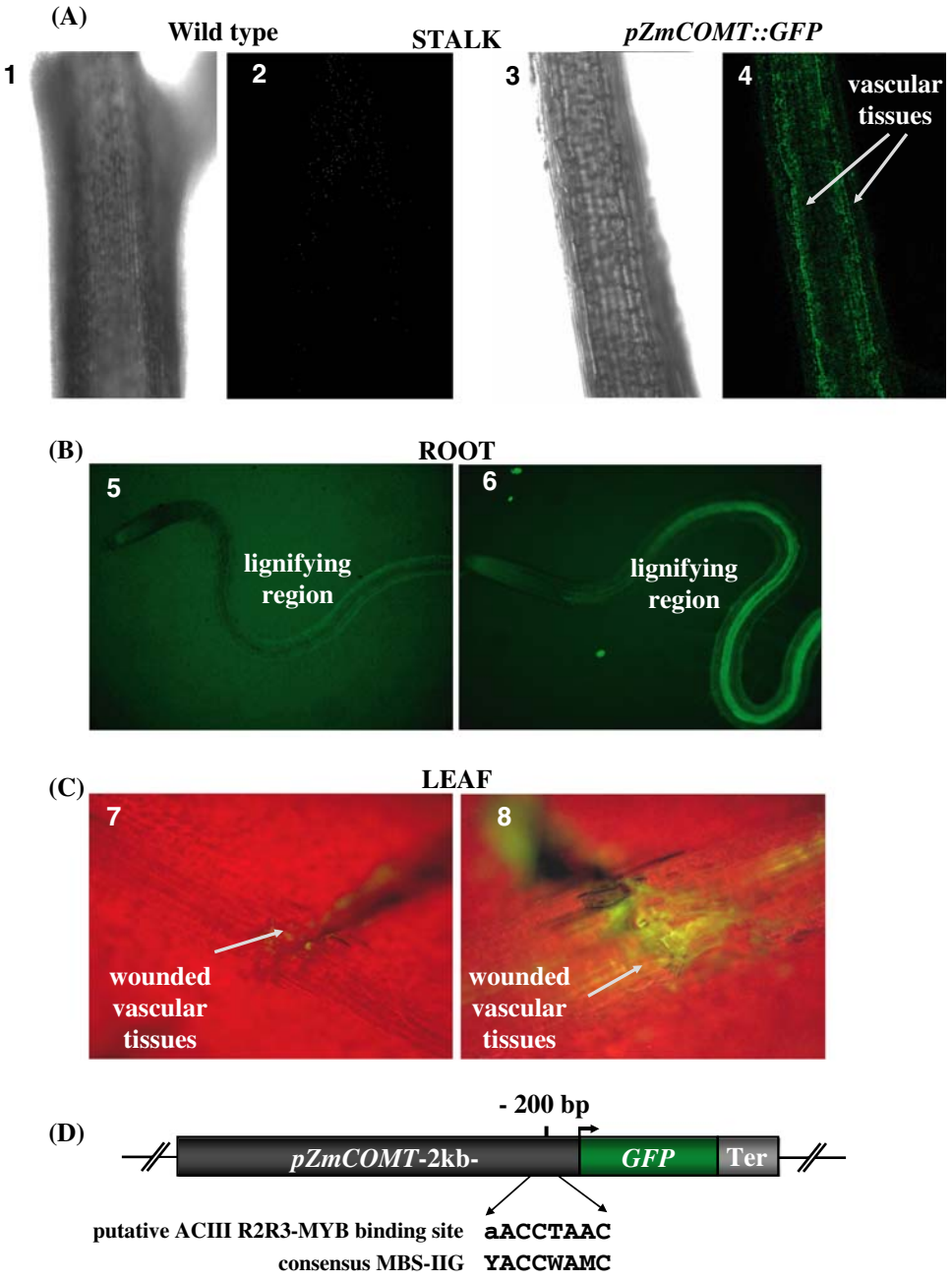


Fig. 1 (**A**) GFP fluorescence pattern in stems of *A. thaliana* wild type and *A. thaliana* carrying the pZmCOMT::GFP construct visualized using confocal microscopy. (1) Transmission image of a wild type stem. (2) GFP detection of an intermediate wild type stem section. (3) Transmission image of the stem *A. thaliana* carrying the pZmCOMT::GFP construct. (4) GFP detection of the corresponding intermediate transgenic stem section. (**B**) GFP fluorescence pattern in roots of *A. thaliana* wild type (5) and *A. thaliana* carrying the pZmCOMT::GFP construct (6). (**C**) GFP fluorescence pattern in wounded leaves of *A. thaliana* wild type (7) and *A. thaliana* carrying the pZmCOMT::GFP construct (8). (**D**) Schematic representation of the pZmCOMT::GFP construction used in this study. The GFP sequence was fused to the 2 kb maize *COMT* promoter and the nos-terminator, as described in materials and methods. The sequence and position of the ACIII R2R3-MYB binding site together with its MYB-Binding Site type IIG (MBS-IIG) consensus sequence (Romero et al. 1998) are indicated and found 200 bp upstream from the putative transcription initiation site (indicated with an arrow). The lower case letter refers to an Adenine that does not follow the MBS-IIG consensus sequence. Y refers to Pyrimidines; W refers to Adenine or Thymine; M refers to Adenine or Cytosine

analysis showed that, among the 22 subgroups in which the R2R3-MYB family is clustered (Kranz et al. 1998), ZmMYB8, ZmMYB31, and ZmMYB42 have the typical motifs of the subgroup 4, while ZmMYB39 and ZmMYB2 are closely related to subgroup 2 and subgroup 9, respectively. We therefore produced a

phylogenetic tree of the three subgroups to identify the most closely related R2R3-MYB factors (Fig. 2B). The phylogenetic analysis shows that ZmMYB31 and ZmMYB42 are closely related to well known lignin repressors, such as AmMYB308 and AmMYB330, AtMYB4, and AtMYB32 (Tamagnone et al. 1998a; Jin et al. 2000; Preston et al. 2004), while ZmMYB8 is phylogenetically distant from these factors. At present, in subgroup four only one maize R2R3-MYB factor, Zm38, has been reported (Marocco et al. 1989) and has been already characterized as a transcription factor involved in the regulation of the anthocyanin biosynthesis pathway (Franken et al. 1989).

On the other hand, while ZmMYB39 is closely related to other monocot R2R3-MYB factors, whose function has not been yet attributed, ZmMYB2 is closely related to R2R3-MYB factors that have been related to nitrogen metabolism such as LjMYB101 and LjMYB103 (Miyake et al. 2003).

Analysis of *ZmMYB2*, *ZmMYB8*, *ZmMYB31*, *ZmMYB39*, and *ZmMYB42* gene expression

To gain more information on their expression pattern within the maize plant, we performed RT-PCR analysis of samples from different organs (apical and basal root, leaf sheath and leaf blade) and at different growth stages (6, 10 and 20 days) in which lignification is active. The results obtained (Fig. 3) show that *ZmMYB8* and *ZmMYB42* are mainly expressed in the aerial part of the maize plant, although the expression pattern of *ZmMYB8* is restricted to the leaf sheath. On the other hand, *ZmMYB31* is expressed both in the root and in the aerial part of the plant. According to these results, the analysis of maize databases indicates the existence of few ESTs corresponding to *ZmMYB8*, *ZmMYB42*, while 20 ESTs corresponding to *ZmMYB31* were found.

On the other hand, *ZmMYB2* (subgroup 9) and *ZmMYB39* (subgroup 2) transcripts accumulate mainly in roots. In particular, *ZmMYB39* is highly expressed during the early growth stages of the plant. At present very few ESTs can be detected on maize databases for *ZmMYB2* and *ZmMYB39* and the only one EST identified arising from ZmMYB39 was obtained from a maize root cDNA library.

Effect of wounding on *ZmMYB2*, *ZmMYB8*, *ZmMYB31*, *ZmMYB39*, and *ZmMYB42* gene expression

A typical feature of most of the genes involved in lignification is their up-regulation by wounding, as has been shown in the case of the maize *COMT* gene (Capellades et al. 1996). On this basis, we analyzed the effect of this abiotic stress on the expression pattern of the five R2R3-MYB factors.

RT-PCR assays show that *ZmMYB2* is the only gene whose expression is repressed by wounding, whereas the expression of *ZmMYB39* is quickly induced. Within the R2R3-MYB factors belonging to subgroup 4, *ZmMYB8* is not affected by wounding, while *ZmMYB31* and *ZmMYB42* are both induced. The induction pattern of *ZmMYB31* and *ZmMYB42* by wounding is compatible with a role as repressors of *COMT*, since the induction of these two factors begins when the activation of *COMT* gene ends (Fig. 4)

ZmMYB31 and *ZmMYB42* down-regulate the *A. thaliana COMT* gene

Wounding assays in maize led us to consider the implication of ZmMYB31 and ZmMYB42 in the repression of the *COMT* gene. We produced transgenic *A. thaliana* lines over-expressing each of the five R2R3-MYB factors to study their effect on the *A. thaliana COMT* gene expression, as this gene contains a putative MYB-binding site type-I (MBS-I) (CTGTTA) approximately 340 bp upstream from the transcription initiation site (Romero et al. 1998).

In agreement with wounding assays, RT-PCR assays showed that only those plants over-expressing ZmMYB31 and ZmMYB42 show the down-regulation of the endogenous *COMT* gene (Fig. 5), while no effect was observed in the case of plants over-expressing ZmMYB2, ZmMYB8 or ZmMYB39. These findings led us to propose ZmMYB31 and ZmMYB42 as repressors of the *COMT* gene, but not ZmMYB8, although it belongs to subgroup 4.

ZmMYB31 and ZmMYB42 down-regulate the maize *COMT* gene

The results obtained from analyzing the transgenic *A. thaliana* plants indicate that two maize R2R3-MYB factors (ZmMYB31 and ZmMYB42) may act as repressors of the *A. thaliana COMT* gene. To discard non-specific effects due to the evolutionary distance between these two species, we examined whether these factors are also able to repress the maize *COMT* gene, by crossing pollen of *A. thaliana* plants expressing GFP under the control of the maize *COMT* gene promoter with *A. thaliana* plants over-expressing the subgroup 4 factors (ZmMYB31, ZmMYB42 and ZmMYB8).

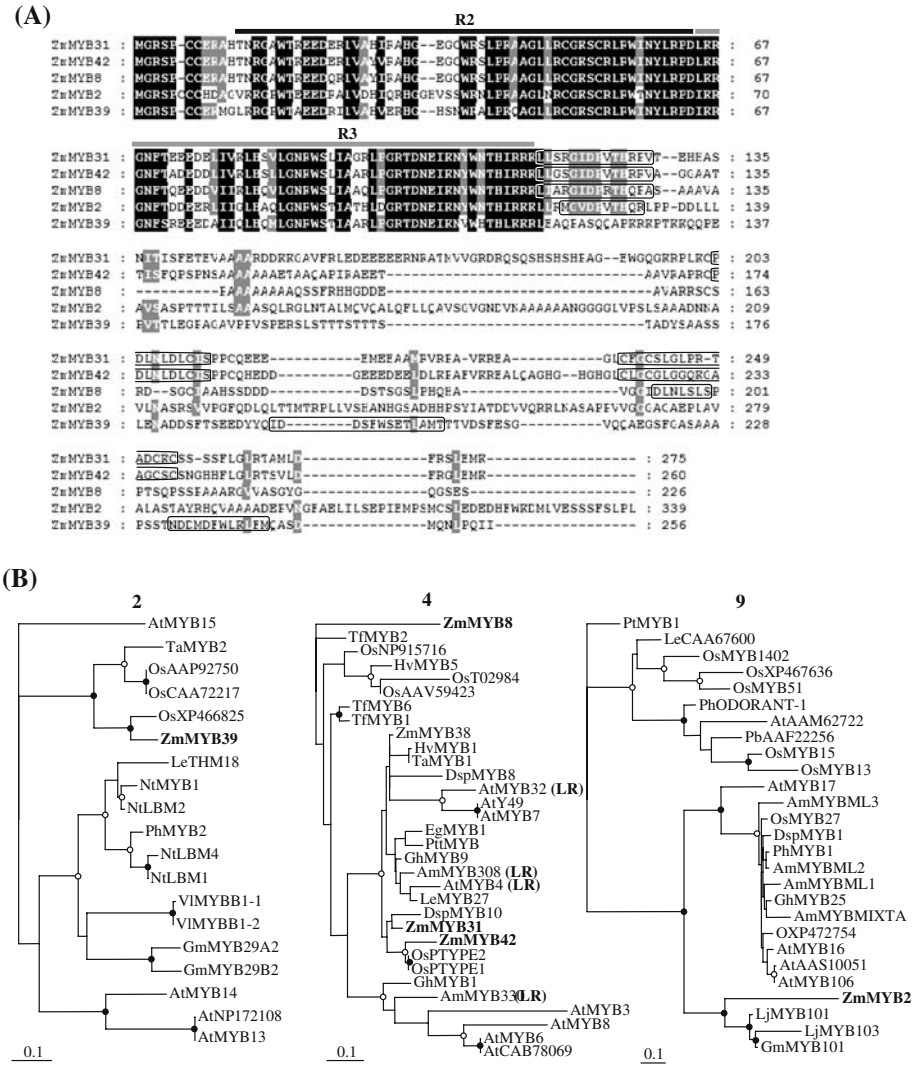


Fig. 2 (**A**) Alignment of the predicted amino acids sequences of ZmMYB2 (subgroup 9), ZmMYB8, ZmMYB31, ZmMYB42 (subgroup 4) and ZmMYB39 (subgroup 2). Residues highlighted in black or grey are identical or homologous in five or four sequences, respectively. R2 and R3 domains are indicated by a black and grey bar, respectively. Boxes refer to protein motifs characteristics of subgroup 2 (IDeSFWxE/Dxlstd and E/NddMdFwynvfi), subgroup 4 (LlsrGIDPxT/SHRxI/L, pdLNLD/ELxiG/S and $CX_{1-2}CX_{7-12}CX_2C$) and subgroup 9 (MGiDPvTHkp). (**B**) Phylogenetic relationships between the five maize MYB factors and the protein belonging to the corresponding subgroup 2, 4 and 9 (according to Kranz et al. 1998) using the PHYML online package program, as described in materials and methods. ZmMYB2, ZmMYB8, ZmMYB31, ZmMYB39 and ZmMYB42 are in bold. White and black circles indicate branches supported at a bootstrap proportion of >60% and ≥90%, respectively. The scale bar represents 0.1 substitutions per position. **LR** indicates R2R3-MYB factors characterized as repressors of lignin biosynthesis. Accession numbers for subgroup 2 sequences: AtMYB15 (AK176693), TaMYB2 (AY615199), OsAAP92750 (AAP92750), OsCAA72217 (CAA72217), OsXP466825 (XP_466825), LeTHM18 (CAA66952), NtMYB1 (AAB41101), NtLBM2 (BAA88222), PhMYB2 (CAA78387), NtLBM4 (BAA88224), NtLBM1 (BAA88221), VlMYBB1-1 (AB073016), VlMYBB2-1 (AB073017), GmMYB29A2 (AB029161), GmMTB29B2 (AB029165), AtMYB14 (AY519575), AtNP172108 (NP_172108), AtMYB13 (AY519550). Accession numbers for subgroup 4 sequences: TfMYB2 (AAS19476), OsNP91576 (NP_91576), HvMYB5 (CAA50221), OsT02984 (T02984), OsAAV59423 (AAV59423), TfMYB6 (AAS19480), TfMYB1 (AAS19475), HvMYB1 (CAA50224), TaMYB1 (AAT37167), DspMYB8 (AAO49417), AtMYB32 (NM_119665), AtY49 (CAA62033), AtMYB7 (U26937), EgMYB1 (CAE09058), PttMYB (CAD98762), GhMYB9 (AAK19619), AmMYB308 (JQ0960), AtMYB4 (AY519615), LeMYB27 (CAA64614), DspMYB10 (AAO49419), OsPTYPE2 (XP_483665), OsPTYPE1 (AAL84628), GhMYB1 (AAN28270), AmMYB330 (JQ0957), AtMYB3 (AF062859), AtMYB8 (NP849749), AtMYB6 (AL161515), AtCAB78069 (CAB78069). Accession numbers for subgroup 9 sequences: PtMYB1 (AAQ62541), LeCAA67600 (CAA67600), OsXP467636 (XP_467636), OsMYB1402 (Y11351), OsMYB51 (XP_482497), AtAAM62722 (AAM62722), PbAAF22256 (AAF22256), PhODORANT-1 (AAV98200), OsMYB15 (CAC85052), OsMYB13 (XP_483654), AtMYB17 (CAB71055), PhMYB1 (CAA78386), AmMYBML3 (AAU13905), AmMYBML2 (AAV70655), DspMYB1 (AAO49410), OsMYB27 (XP_482547), OsXP472754 (XP_472754), AtMYB16 (AF370613), AtAAS10051 (AAS10051), AtMYB106 (AF249309), AmMYBML1 (CAB43399), GhMYB25 (AAK19616), AmMYBMIXTA (X79108), LcMYB101 (BAC75671), LcMYB103 (BAC75673), GmMYB101 (BAC75674)

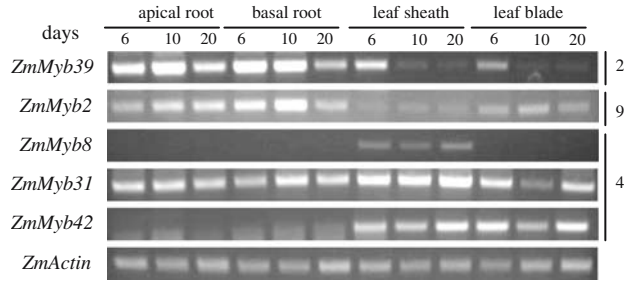


Fig. 3 Relative expression levels of *ZmMYB2*, *ZmMYB8*, *ZmMYB31*, *ZmMYB39* and *ZmMYB42* in different organs (apical root, basal root, leaf sheath and leaf blade) of maize plants at different developmental stages. Plants were harvested at 6-10 and 20-days and transcript accumulation was determined by RT-PCR. 6 individual plants were used for each sample. Numbers on the right of the figure refer to the subgroup of each maize R2R3-MYB factor. Gel band identity was confirmed by direct sequencing. Maize actin was used as loading control

The presence of the pCOMT::GFP construct was confirmed by PCR assays (Fig. 6A), and the expression of GFP was analyzed in the double transgenic plants (Fig. 6B). The results show that ZmMYB31 and ZmMYB42 produce a strong decrease of the GFP expression that is reduced by 3.7- and 2.6-fold, respectively, confirming that these two factors act as repressors of the maize *COMT* gene. No alteration of the GFP expression is observed with the ZmMYB8 factor.

Phenotype of ZmMYB8, ZmMYB31 and ZmMYB42 plants

Previous studies have shown that the over-expression of R2R3-MYB factors acting as repressors of genes involved in the lignin pathway produce alterations of the leaf morphology, with the appearance of white lesions on the older leaves, and reduction of the growth rate when over-expressed in tobacco and *A. thaliana* (Tamagnone et al. 1998a; Jin et al. 2000). Plants over-expressing ZmMYB31 show similar morphological alterations (Fig. 7A and B), while the ones over-expressing ZmMYB42 display a more attenuate phenotype, in which only the reduction of the growth rate and leaf curvature is observed (Fig. 7A and B) without the occurrence of white lesions. No phenotypic alterations were observed in plants over-expressing ZmMYB8.

One common trait between tobacco plants over-expressing the AmMYB308 and AmMYB330 and *A. thaliana* plants over-expressing AtMYB4 is the down-regulation of *4CL1* gene expression. We investigated whether ZmMYB31 and ZmMYB42 also regulate this structural gene of lignin biosynthesis. The results show that both maize R2R3-MYB factors negatively regulate the *At4CL1* gene (Fig. 7C). As expected from the lack of any morphological alteration, ZmMYB8 does not affect *At4CL1* gene expression.

ZmMYB31 and ZmMYB42 over-expression affects lignification in *A. thaliana*

To better address the role of ZmMYB31 and ZmMYB42 in the regulation of lignification in *A. thaliana* transgenic plants, we analyzed by RT-PCR the expression of *C4H*, *CCoAOMT1* and *CAD6*. These three structural genes were differently affected by the subgroup 4 R2R3-MYB factors AmMYB308 and AmMYB330 (Tamagnone et al. 1998a), AtMYB4 (Jin et al. 2000) and AtMYB32 (Preston et al. 2004). Our results show that ZmMYB31 slightly increases the *AtCAD6* gene expression and does not regulate *AtC4H* and *AtCCoAOMT1* genes expression (Fig. 8A). In contrast, ZmMYB42 is able to down-regulate *AtC4H* and *AtCAD6* but does not regulate *AtCCoAOMT1* (Fig. 8A).

To elucidate the impact of ZmMYB31 and ZmMYB42 on lignin accumulation, we measured the lignin content of the entire transgenic *A. thaliana* mature stems. As shown in figure 8B, significant differences between control and transgenic plants were detected. Plants over-expressing ZmMYB31 and ZmMYB42 showed a 3.7- and 1.6-fold reduction of the lignin thioglycolic acid (LTGA) respectively, measured from 25 mg of alcohol insoluble residue (AIR) in respect to wild-type. This reduction was more evident if

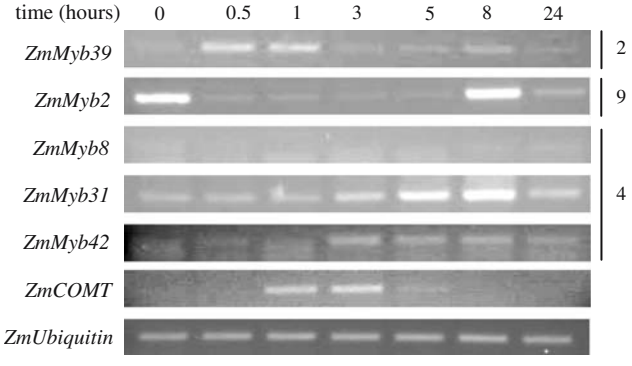


Fig. 4 Effect of wounding on the expression levels of *ZmCOMT*, *ZmMYB2*, *ZmMYB8*, *ZmMYB31*, *ZmMYB39* and *ZmMYB42* genes in maize leaves. Maize leaves of 10-days old plants were wounded by transversal incisions and samples of 6 leaves, taken from three individual plants, were collected at different time intervals after treatment (0 h, 30 min, 1, 3, 5, 8 and 24 h). Transcript accumulation was determined by RT-PCR. Numbers on the right of the figure refer to the subgroup of each maize R2R3-MYB factor. Gel band identity was confirmed by direct sequencing. Maize ubiquitin was used as loading control

referred to fresh weight, since for ZmMYB31 and ZmMYB42 an 8- and 2.1-fold decrease was observed respectively.

Discussion

In this work we report the down-regulation of the maize and *A. thaliana COMT* gene by two new maize R2R3-MYB transcription factors, ZmMYB31 and ZmMYB42. We showed that the specific *COMT* gene expression in maize lignifying tissues is maintained when the gene is expressed in *A. thaliana* plants (Fig. 1A and B), and that it is specifically induced in wounded vascular tissues with the same pattern of expression as in the maize plant (Fig. 1C). This indicates that at least some of the factors involved in the regulation of the *COMT* gene are conserved in two evolutionary distant species such as maize and *A. thaliana*.

The detailed examination of the maize *COMT* gene promoter revealed a putative ACIII box, approximately 200 bp upstream from the transcription initiation site (Fig. 1D). The presence of AC elements in the promoters of several genes of the lignin pathway (Bugos et al. 1991; Hauffe et al. 1993; Ye et al. 1994; Sablowski et al. 1994; Feuillet et al. 1995; Douglas 1996; Raes et al. 2003) has focused a great deal of attention on the isolation and characterization of transcription factors belonging to the R2R3-MYB family. To date, the subgroup 4 clusters all the R2R3-MYB factors characterized as repressors of lignin biosynthesis (Tamagnone et al. 1998a; Jin et al. 2000; Preston et al. 2004). Based on the sequence of these well-characterized factors, we searched for maize subgroup 4 R2R3-MYB factors using degenerated primers, isolating five R2R3-MYB factors to study their possible implication in the regulation of the maize *COMT* gene. Bioinformatics analyses revealed that only three of them, *ZmMYB8*, *ZmMYB31*, and *ZmMYB42*, belong to subgroup 4 (Fig. 2).

The well-known induction of the *COMT* gene expression by wounding (Capellades et al. 1996) was used as a parameter to associate this set of factors with the regulation of *COMT*. We found that *ZmMYB31*, *ZmMYB39* and *ZmMYB42* are induced by wounding, but only the induction pattern of *ZmMYB31* and *ZmMYB42* is compatible with a role as repressors of *COMT*, since the induction of these two factors begins when the activation of *COMT* ends (Fig. 4).

Computational analyses indicated that the *A. thaliana COMT* gene promoter does not contain the typical ACI, ACII or ACIII MYB-binding sites (Raes et al. 2003) belonging to the type-IIG MYB-binding site (MBS-IIG, Romero et al. 1998). However, the *A. thaliana COMT* gene promoter contains a putative MBS-I *cis*-element (CNGTTR, Romero et al. 1998) located approximately 340 bp upstream from the transcription initiation site, suggesting that this gene could be regulated by R2R3-MYB factors. Accordingly, the over-expression of ZmMYB31 and ZmMYB42 in *A. thaliana* indicates that these two factors, which are evolutionarily closely related, repress the expression of the endogenous *COMT* gene (Fig. 5). Instead ZmMYB8, evolutionarily far from ZmMYB31 and ZmMYB42, does not affect the expression of the *A. thaliana COMT* gene.

ZmMYB31 and ZmMYB42 are also phylogenetically related to AmMYB308, AmMYB330, AtMYB4,

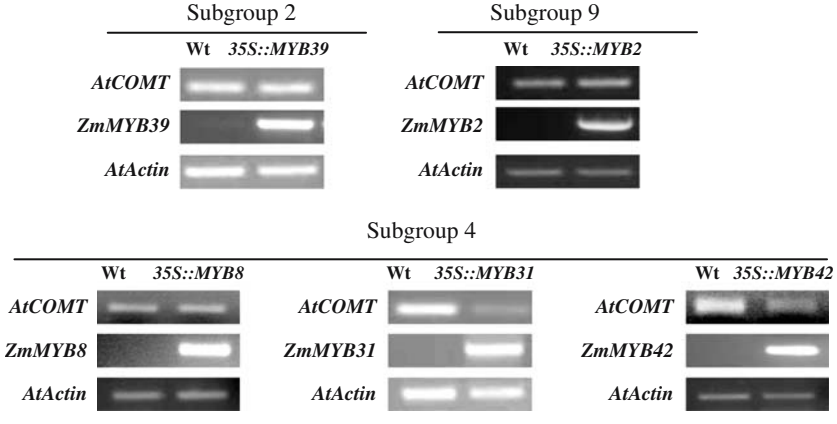


Fig. 5 Relative expression levels of *AtCOMT* in *A. thaliana* plants over-expressing *ZmMYB2*, *ZmMYB8*, *ZmMYB31*, *ZmMYB39* and *ZmMYB42* under the control of the 35S CaMV promoter. Two week-old transgenic plants (20 plants for each sample) were harvested, and the accumulation of transcripts corresponding to *AtCOMT*, the maize *MYB* factors and *AtActin* (used as loading control) were determined by RT-PCR

and AtMYB32 all of them already characterized as R2R3-MYB factors acting as repressors of lignin biosynthesis (Tamagnone et al. 1998a; Jin et al. 2000; Preston et al. 2004).

No putative function of ZmMYB39 can be proposed based on evolutionary analysis, but ZmMYB2 clusters with R2R3-MYB factors that are related to nitrogen metabolism such as LjMYB101 and LjMYB103 (Miyake et al. 2003) and this, together with the preferential expression of *ZmMYB2* in the maize root, suggests a possible role of this factor in the nitrogen metabolism pathway.

The repression of *ZmMYB2* by wounding, accompanied by the induction of the maize *COMT* gene suggested its possible role in the regulation of this structural gene (Fig. 4), but this hypothesis was discarded due to the lack of effect of ZmMYB2 on *COMT* gene regulation when over-expressed in *A. thaliana* plants.

The repression of the maize *COMT* gene expression by the maize R2R3-MYB factors belonging to subgroup 4 was confirmed by the reduction of GFP expression in plants over-expressing ZmMYB31 and ZmMYB42 (Fig. 6). However, the repression of the maize *COMT* gene expression by ZmMYB31 is stronger than the one produced by ZmMYB42, suggesting that the latter factor may participate in the regulation of lignin biosynthesis but with a minor role compared to ZmMYB31. In contrast, ZmMYB8 did not reduce GFP fluorescence, confirming that this maize factor does not repress the maize *COMT* gene.

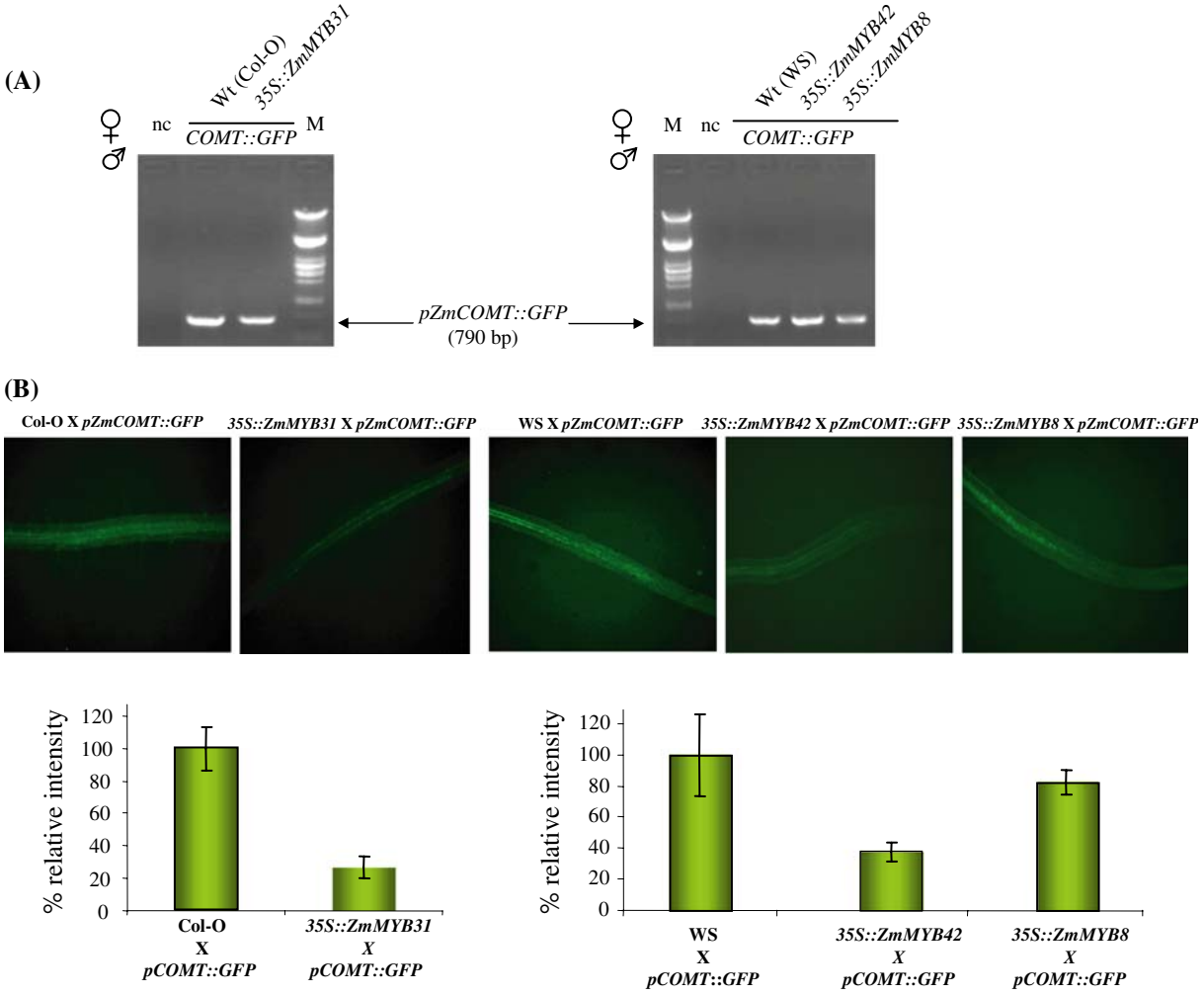


Fig. 6 Effect of the maize MYB factors belonging to subgroup 4 on the expression of the maize COMT in transgenic *A. thaliana* plants. Plants over-expressing ZmMYB8, ZmMYB31 and ZmMYB42 were pollinated with pollen from pZmCOMT::GFP homozygous plants, as described in Materials and Methods. (**A**) The presence of the reporter gene construction was confirmed by PCR assays. nc: control plants not crossed with pCOMT::GFP plants. (**B**) The double transgenic plants were analyzed for green fluorescence. The level of green fluorescence was subjected to densitometric analysis expressed as percentage of relative signal intensity

The over-expression of ZmMYB31 produces a reduction of growth rate and the appearance of white lesions on the mature leaves. These lesions suggest a reduction of the levels of the hydroxycinnamic acids derivatives (Jin et al. 2000; Tamagnone et al. 1998b) which is a common trait in plants over-expressing the *A. majus* AmMYB330 and AmMYB308 (Tamagnone et al. 1998a) and AtMYB4 (Jin et al. 2000). On the contrary, the *A. thaliana* plants over-expressing ZmMYB42 present a weaker phenotype characterized only by the reduction of the growth rate and by leaf curvature, whereas the over-expression of ZmMYB8 does not lead to visible alterations.

Interestingly, a second common feature of the transgenic lines over-expressing AmMYB308, AmMYB330 and AtMYB4 is the down-regulation of *4CL1* gene expression. This common trait was also observed in plants over-expressing ZmMYB31 and ZmMYB42 (Fig. 7), reinforcing the idea that these factors affect lignin metabolism, even if the over-expression of ZmMYB42 does not produce white lesions in *A. thaliana* leaves.

On the other hand, the lack of effect of ZmMYB8 on the expression of *4CL1* (Fig. 7) as well as on both the *A. thaliana* and the maize *COMT* (Fig. 6) and the lack of typical phenotype alterations, such as the appearance of leaf white lesions, indicate that ZmMYB8 is not involved in the regulation of lignin biosynthesis.

In addition to *4CL1*, previously described subgroup 4 R2R3-MYB factors are able to regulate other genes of the lignin pathway. In fact, AmMYB308 and AmMYB330 down-regulate *C4H* and *CAD* when over-expressed in tobacco plants (Tamagnone et al.

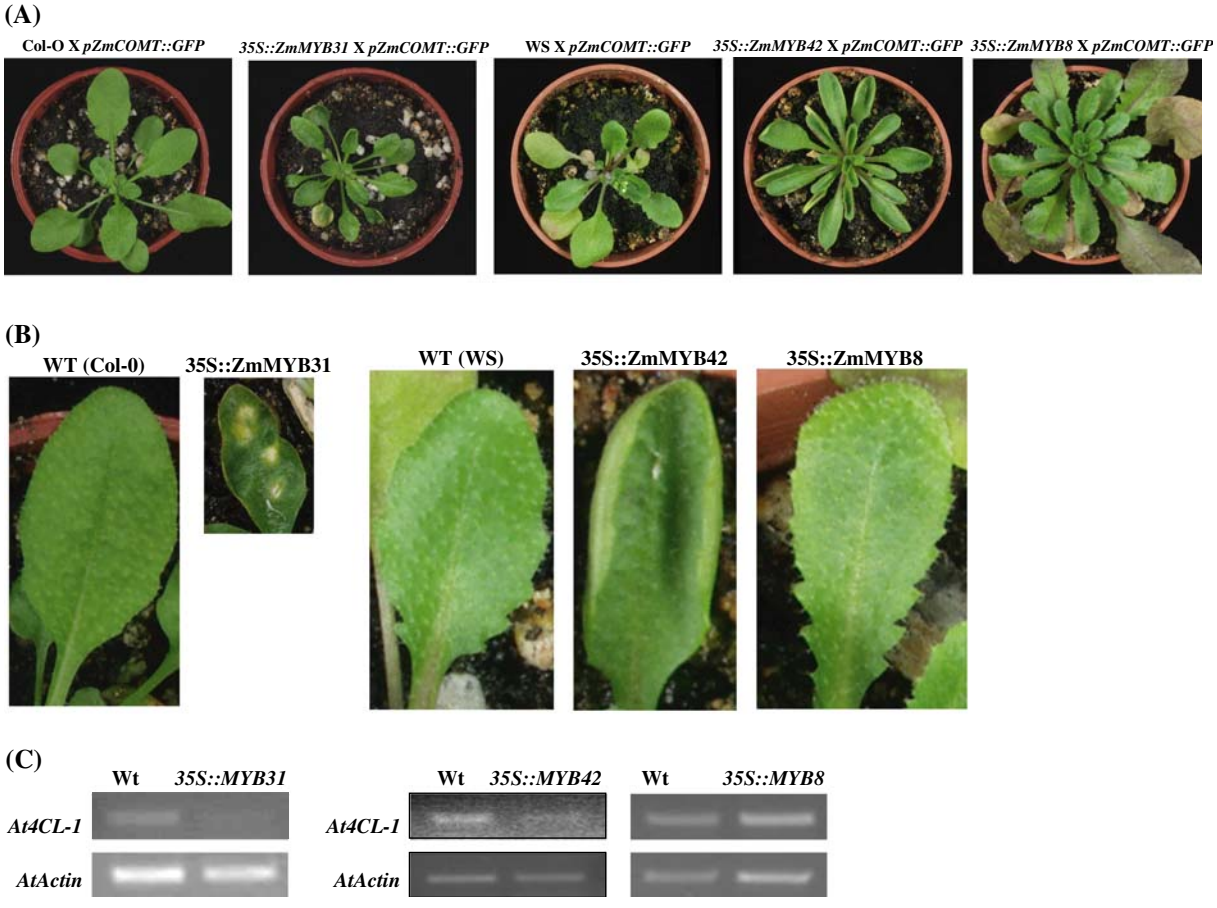
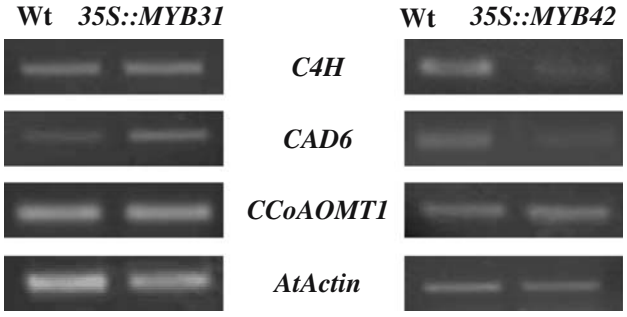


Fig. 7 Phenotypic effects on *Arabidopsis thaliana* plants over-expressing ZmMYB8, ZmMYB31, and ZmMYB42. (**A**) Transgenic *Arabidopsis thaliana* plantlets. (**B**) Mature leaves of wild type (Wt) and transgenic *Arabidopsis thaliana* plants. The relative leaf size is maintained in the picture. (**C**) Relative expression levels of *At4CL1* in *A. thaliana* plants over-expressing *ZmMYB8*, *ZmMYB31*, and *ZmMYB42*, under the control of the 35S CaMV promoter. Two week-old transgenic plants (20 plants for each sample) were harvested and the accumulation of transcripts corresponding to *At4CL1*, the maize *MYB* factors and *AtActin* (used as loading control) were determined by RT-PCR

(A)



(B)

	Wt	*35S::MYB31*	Wt	*35S::MYB42*
AIR (mg/gFW)	228.8	88.6	98.3	70.9
Relative lignin content (referred to AIR)	100%	26.7% ± 0.7	100%	63.1% ± 0.7
Relative lignin content (referred to FW)	100%	12.5% ± 0.7	100%	47.5% ± 0.4

Fig. 8 Effect of ZmMYB31 and ZmMYB42 on *AtC4H*, *AtCAD6*, *AtCCoAOMT1* gene expression in transgenic *A. thaliana* plants and on lignin accumulation. (**A**) Relative expression levels of *AtC4H*, *AtCAD6*, *AtCCoAOMT1* in *A. thaliana* plants over-expressing *ZmMYB31* and *ZmMYB42*, under the control of the 35S CaMV promoter. Two week-old transgenic plants (20 plants for each sample) were harvested and the accumulation of transcripts corresponding to *AtC4H*, *AtCAD6*, *AtCCoAOMT1*, and *AtActin* (used as loading control) were determined by RT-PCR. (**B**) Lignin measurement in transgenic plants over-expressing ZmMYB31 and ZmMYB42 and wild type plants. Results are expressed as A_{280} nm absorbance of LTGA in 0.5 N NaOH. FW refers to Fresh Weight and AIR refers to Alcohol Insoluble Residues

1998a) while the knock-out *Atmyb4* plant shows an increase in *C4H* gene expression, as well as a decrease in *CCoAOMT* gene expression (Jin et al. 2000).

To gain more information on the role of ZmMYB31 and ZmMYB42 in the regulation of lignin synthesis we analyzed their effect on the expression of these three structural genes. The results obtained reinforce the idea that both ZmMYB31 and ZmMYB42 negatively regulate lignification by acting on different targets. In fact, ZmMYB31 does not affect the expression of *C4H* and *CCoAOMT* but slightly enhances the expression of *CAD* gene. On the other hand, the repression pattern of ZmMYB42 resembles the one of AmMYB308 and AmMYB330 factors (Tamagnone et al. 1998a). In fact, ZmMYB42 down-regulates the expression of both *C4H* and *CAD* but does not affect *CCoAOMT* gene expression (Fig. 8).

The over-expression of both ZmMYB31 and ZmMYB42 produces a decrease in the lignin content of the transgenic *A. thaliana* plants even if this reduction is more severe in the case of ZmMYB31. This different effect could be due to their characteristic regulation pattern over several genes of the lignin pathway. Thus, the more drastic impact of ZmMYB31 on lignin accumulation is in line with the stronger inhibition of *COMT* gene expression by this factor in respect to ZmMYB42.

In summary, this is the first identification of two subgroup 4 R2R3-MYB transcription factors, ZmMYB31 and ZmMYB42 that repress the maize *COMT* gene and this represents the first characterization of R2R3-MYB transcription factors associated with the regulation of lignin biosynthesis in maize. The results presented in this work indicate a major implication of ZmMYB31 in the regulation of this pathway in respect to ZmMYB42. The finding that ZmMYB31 and ZmMYB42 are able to down-regulate both the maize and *A. thaliana COMT* genes reinforces the idea of evolutionary conserved molecular mechanisms involved in the regulation of *COMT* gene expression in plants. In addition to *AtCOMT*, ZmMYB31 and ZmMYB42 differentially regulate other structural genes of the lignin pathway, such as *At4CL1*, *AtC4H*, *At4CCoAOMT1* and *At4CAD6* resulting in both cases in a reduction of the total lignin content of the transgenic *A. thaliana* plants.

Acknowledgments We are indebted to Dr. Sheen (MGH, USA) for kindly providing us with the synthetic GFP (sGFP) as well as the John Innes Center (Norwich, UK) for providing us

with the pGREENII-0029 vector. We want to thank Dr. Piulachs (IBMB-CSIC) for her advice and comments on the phylogenetic analyses performed in this work and Dr. Pons for the confocal microscopy analyses. We are also indebted to the sequencing and greenhouse teams of IBMB-CSIC, as well as Dr. Burgess for the English correction of this manuscript. This work was funded by the Spanish ''Ministerio de Ciencia y Tecnología'' (BIO2001-1140). S-F was financed by a post-doctoral grant from the Autonomous Government of Catalonia (2003PIV-A-00033). F.M-S was financed by the European Project INCO-II (ICA4-CT-2000-30017) and by the ''Consorci CSIC-IRTA'' laboratory. T-M was financed by the European Project MapMaize (PL 962312). D-C.R was financed by the Spanish ''Ministerio de Ciencia y Tecnología'' (''Ramón y Cajal'' Program). This work was carried out within the framework of the ''Centre de Referència de Biotecnologia'' (CERBA) from the Autonomous Government of Catalonia.

References

Anterola AM, Lewis NG (2002) Trends in lignin modifications: a comprehensive analysis of the effects of genetics manipulations/mutations on lignification and vascular integrity. Phytochemistry 61:221–294

Boerjan W, Ralph J, Baucher M (2003) Lignin biosynthesis. Annu Rev Plant Biol 54:519–546

Boudet AM, Kajita S, Grima-Pettenati J, Goffner D (2003) Lignins and lignocellulosics: a better control of synthesis for new and improved uses. Trends Plant Sci 8:576–581

Bugos RC, Chiang VL, Campbell WH (1991) cDNA cloning, sequence analysis and seasonal expression of lignin-bispecific caffeic acid/5-hydroxyferulic acid *O*-methyltransferase of aspen. Plant Mol Biol 17:1203–1215

Caparrós-Ruiz D, Fornalé S, Civardi L, Puigdomènech P, Rigau J (2006) Isolation and characterisation of a family of laccases in maize. Plant Sci 171:217–225

Capellades M, Torres MA, Bastisch I, Stiefel V, Vignols F, Bruce WB, Peterson D, Puigdomenech P, Rigau J (1996) The maize caffeic acid *O*-methyltransferase gene promoter is active in transgenic tobacco and maize plant tissues. Plant Mol Biol 31:307–332

Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. Mol Biol Evol 17:540–552

Chiu W, Niwa Y, Zeng W, Hirano T, Kobayashi H, Sheen J (1996) Engineered GFP as a vital reporter in plants. Curr Biol 6:325–330

Clough SJ, Bent AF (1998) Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J 16:735–743

Collazo P, Montoliu L, Puigdomènech P, Rigau J (1992) Structure and expression of the lignin *O*-methyltransferase gene from *Zea mays* L. Plant Mol Biol 20:857–867

Cone KC, Cocciolone SM, Burr FA, Burr B (1993) Maize anthocyanin regulatory gene pI is a duplicate of c1 that functions in the plant. Plant Cell 5:1795–1805

de Obeso M, Caparrós-Ruiz D, Vignols F, Puigdomènech P, Rigau J (2003) Characterisation of maize peroxidases having differential patterns of mRNA accumulation in relation to lignifying tissues. Gene 309:23–33

Douglas CJ (1996) Phenylpropanoid metabolism and lignin biosynthesis: from weeds to trees. Trends Plant Sci 1:171–178

Feuillet C, Lauvergeat V, Deswarte C, Pilate G, Boudet A, Grima-Pettenati J (1995) Tissue- and cell-specific expression of a cinnamyl alcohol dehydrogenase promoter in transgenic poplar plants. Plant Mol Biol 27:651–667

Franken P, Schrell S, Peterson PA, Saedler H, Wienand U (1989) Molecular analysis of protein domain function encoded by the myb-homologous maize genes C1, Zm 1 and Zm 38. Plant J 6:21–30

Goicoechea M, Lacombe E, Legay S, Mihaljevic S, Rech P, Jauneau A, Lapierre C, Pollet B, Verhaegen D, Chaubet-Gigot N, Grima-Pettenati G (2005) EgMYB2, a new transcriptional activator from Eucalyptus xylem, regulates secondary cell wall formation and lignin biosynthesis. Plant J 43:553–567

Grotewold E, Athma P, Peterson T (1991) Alternatively spliced products of the maize P gene encode proteins with homology to the DNA-binding domain of myb-like transcription factors. Proc Natl Acad Sci USA 88:4587–4591

Grotewold E, Drummond BJ, Bowen B, Peterson T (1994) The myb-homologous P gene controls phlobaphene pigmentation in maize floral organs by directly activating a flavonoid biosynthetic gene subset. Cell 76:543–553

Guindon S, Lethiec F, Duroux P, Gascuel O (2005) PHYML Online-a web server for fast maximum likelihood-based phylogenetic inference. Nucleic Acids Res 33(Web Server issue):W557–W559

Halpin C, Holt K, Chojecki J, Olivier D, Chabbert B, Monties B, Edwards K, Barakate A, Foxon GA (1998) Brown-midrib maize (bm1): a mutation affecting cinnamyl alcohol dehydrogenase gene. Plant J 14:545–553

Hauffe KD, Lee SP, Subramaniam R, Douglas CJ (1993) Combinatorial interactions between positive and negative *cis*-acting elements control spatial patterns of 4CL-1 expression in transgenic tobacco. Plant J 4:235–253

Hellens RP, Edwards EA, Leyland NR, Bean S, Mullineaux PM (2000a) pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol Biol 42:819–832

Hellens R, Mullineaux P, Klee H (2000b) A guide to *Agrobacterium* binary Ti vectors. Trends Plant Sci 5:446–451

Higgins D, Thompson J, Gibson T, Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTALW: improving the sensibility of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acid Res 22:4673–4680

Hofgen R, Willmitzer L (1988) Storage of competent cells for Agrobacterium transformation. Nucleic Acids Res 16:9877

Jin H, Cominelli E, Bailey P, Parr A, Mehrtens F, Jones J, Tonelli C, Weisshaar B, Martin C (2000) Transcriptional repression by *AtMYB4* controls production of UV-protecting sunscreens in *Arabidopsis*. Plant J 26:205–216

Karpinska B, Karlsson M, Srivastava M, Stenberg A, Schrader J, Sterky F, Bhalerao R, Wingsle G (2004) MYB transcription factors are differentially expressed and regulated during secondary vascular tissue development in hybrid aspen. Plant Mol Bio l56:255–270

Kranz HD, Denekamp M, Greco R, Jin H, Leyva A, Meissner RC, Petroni K, Urzainqui A, Bevan M, Martin C, Smeekens S, Tonelli C, Paz-Ares J, Weisshaar B (1998) Towards functional characterisation of the members of the R2R3-MYB gene family from *Arabidopsis thaliana*. Plant J 16:263–76

Lewis NG, Yamamoto E (1990) Lignin: occurrence, biogenesis and biodegradation. Annu Rev Plant Physiol Plant Mol Biol 41:455–496

Marocco A, Wissenbach M, Becker D, Paz-Ares J, Saedler H, Salamini F, Rohde W (1989) Multiple genes are transcribed in Hordeum vulgare and Zea mays that carry the DNA binding domain of the myb oncoproteins. Mol Gen Genet 216:183–187

Mellerowicz EJ, Baucher M, Sundberg B, Boerjan W (2001) Unravelling cell wall formation in the woody dicot stem. Plant Mol Biol 47:239–274

Miyake K, Ito T, Senda M, Ishikawa R, Harada T, Niizeki M, Akada S (2003) Isolation of a subfamily of genes for R2R3-MYB transcription factors showing up-regulated expression under nitrogen nutrient-limited conditions. Plant Mol Biol 53:237–245

Morrow MP, Mascia P, Self KP, Altschuler M (1997) Molecular characterization of a brown midrib deletion mutation in maize. Mol Breed 3:351–357

Murashige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant 15:473–497

Patzlaff A, Newman LJ, Dubos C, Whetten RW, Smith C, McInnis S, Bevan MW, Sederoff RR, Campbell MM (2003a) Characterisation of Pt MYB1, an R2R3-MYB from pine xylem. Plant Mol Biol 53:597–608

Patzlaff A, McInnis S, Courtenay A, Surman C, Newman LJ, Smith C, Bevan MW, Mansfield S, Whetten RW, Sederoff RR, Campbell MM (2003b) Characterisation of a pine MYB that regulates lignification. Plant J 36:743–754

Paz-Ares J, Ghosal D, Wienand U, Peterson PA, Saedler H (1987) The regulatory c1 locus of Zea mays encodes a protein with homology to myb proto-oncogene products and with structural similarities to transcriptional activators. EMBO J 6:3553–3558

Piquemal J, Chamayou S, Nadaud I, Beckert M, Barriere Y, Mila I, Lapierre C, Rigau J, Puigdomènech P, Jauneau A, Digonnet C, Boudet AM, Goffner D, Pichon M (2002) Down-regulation of caffeic acid o-methyltransferase in maize revisited using a transgenic approach. Plant Physiol 130:1675–1685

Pilu R, Piazza P, Petroni K, Ronchi A, Martin C, Tonelli C (2003) pl-bol3, a complex allele of the anthocyanin regulatory pl1 locus that arose in a naturally occurring maize population. Plant J 36:510–521

Preston J, Wheeler J, Heazlewood J, Li SF, Parish RW (2004) AtMYB32 is required for normal pollen development in Arabidopsis thaliana. Plant J 40:979–995

Raes J, Rohde A, Christensen JH, Van de Peer Y, Boerjan W (2003) Genome-wide characterization of the lignification toolbox in Arabidopsis. Plant Physiol 133:1051–1071

Reichel C, Mathur J, Eckes P, Langenkemper K, Koncz C, Schell J, Reiss B, Maas C (1996) Enhanced green fluorescence by the expression of an *Aequorea Victoria* green fluorescent protein mutant in mono- and dicotyledonous plant cells. Proc Natl Acad Sci USA 93:5888–5893

Romero I, Fuertes A, Benito MJ, Malpica JM, Leyva A, Paz-Ares J (1998) More than 80 R2R3-MYB regulatory genes in the genome of *Arabidopsis thaliana*. Plant J 14:273–284

Sablowski RW, Moyano E, Culianez-Macia FA, Schuch W, Martin C, Bevan M (1994) A flower-specific Myb protein activates transcription of phenylpropanoid biosynthetic genes. EMBO J 13:128–37

Tamagnone L, Merida A, Parr A, Mackay S, Culianez-Macia FA, Roberts K, Martin C (1998a) The AmMYB308 and AmMYB330 transcription factors from antirrhinum regulate phenylpropanoid and lignin biosynthesis in transgenic tobacco. Plant Cell 10:135–54

Tamagnone L, Merida A, Stacey N, Plaskitt K, Parr A, Chang CF, Lynn D, Dow JM, Roberts K, Martin C (1998b) Inhibition of phenolic acid metabolism results in precocious cell death and altered cell morphology in leaves of transgenic tobacco plants. Plant Cell 10:1801–16

Vignols F, Rigau J, Torres MA, Capellades M, Puigdomenech P (1995) The brown midrib3 (bm3) mutation in maize occurs in the gene encoding caffeic acid O-methyltransferase. Plant Cell 7:407–416

Vignols F, José-Estanyol M, Caparrós-Ruiz D, Rigau J, Puigdomènech P (1999) Involvement of a maize proline-rich protein in secondary cell wall formation as deduced from its specific mRNA localisation. Plant Mol Biol 39:945–952

Whetten RW, MacKay JJ, Sederoff RR (1998) Recent advances in understanding lignin biosynthesis. Annu Rev Plant Physiol Plant Mol Biol 49:585–609

Whitmore FW (1978) Lignin-carbohydrate complex formed in isolated cell walls of callus. Phytochemistry 17:421–425

Ye ZH, Kneusel RE, Matern U, Varner JE (1994) An alternative methylation pathway in lignin biosynthesis in Zinnia. Plant Cell 6:1427–1439