Plant Mol Biol (2009) 70:283–296
DOI 10.1007/s11103-009-9473-2

The maize *Zm*MYB42 represses the phenylpropanoid pathway and affects the cell wall structure, composition and degradability in *Arabidopsis thaliana*

Fathi-Mohamed Sonbol · Silvia Fornalé · Montserrat Capellades · Antonio Encina · Sonia Touriño · Josep-Lluís Torres · Pere Rovira · Katia Ruel · Pere Puigdomènech · Joan Rigau · David Caparrós-Ruiz

Received: 4 December 2008 / Accepted: 12 February 2009 / Published online: 24 February 2009

Abstract The involvement of the maize *Zm*MYB42 R2R3-MYB factor in the phenylpropanoid pathway and cell wall structure and composition was investigated by overexpression in *Arabidopsis thaliana*. *Zm*MYB42 down-regulates several genes of the lignin pathway and this effect reduces the lignin content in all lignified tissues. In addition, *Zm*MYB42 plants generate a lignin polymer with a decreased S to G ratio through the enrichment in H and G subunits and depletion in S subunits. This transcription factor also regulates other genes involved in the synthesis of sinapate esters and flavonoids. Furthermore, *Zm*MYB42 affects the cell wall structure and degradability, and its polysaccharide composition. Together, these results suggest that *Zm*MYB42 may be part of the regulatory network controlling the phenylpropanoid biosynthetic pathway.

Keywords *Arabidopsis thaliana* · Maize · Phenylpropanoids · Lignin regulation · R2R3-MYB factors

Introduction

Lignin is, after cellulose the most abundant component of biomass (Boerjan et al. 2003). This polymer is synthesised through the phenylpropanoid pathway, a metabolic grid that synthesises other secondary metabolites such as flavonoids and sinapate esters (Fig. 1). Lignin is deposited in the secondary cell wall of vascular plants and its presence increases the efficiency of water transport, the stiffness of mechanical tissues and constitutes by itself a physical barrier against microbial attacks. However, the covalent interaction of lignin with cell wall polysaccharides makes

Fathi-Mohamed Sonbol and Silvia Fornalé contributed equally to this work.

Electronic supplementary material The online version of this article (doi:10.1007/s11103-009-9473-2) contains supplementary material, which is available to authorized users.

F.-M. Sonbol · S. Fornalé · M. Capellades · P. Puigdomènech · J. Rigau (✉) · D. Caparrós-Ruiz
Consorci CSIC-IRTA-UAB, Centre de Recerca en AgriGenomica (CRAG), Jordi Girona 18-26, 08034 Barcelona, Spain
e-mail: rigau@ibmb.csic.es

A. Encina
Departamento de Ingeniería y Ciencias Agrarias, Universidad de León, 24071 León, Spain

S. Touriño · J.-L. Torres
Institute for Chemical and Environmental Research, IIQAB-CSIC, Barcelona, Spain

P. Rovira
Departament de Biologia Vegetal, Facultat de Biologia, Universitat de Barcelona, 08028 Barcelona, Spain

K. Ruel
Centre de Recherche sur les Macromolecules Vegetales, UPR-CNRS-5301, 38041 Grenoble Cedex 09, France

Present Address:
F.-M. Sonbol
Department of Biology, Faculty of Dentistry, Sinai University, P.O. Box 7, El Massaid-El Arish, North Sinai, Egypt

Present Address:
P. Rovira
Centre Tecnològic Forestal Catalunya, 25280 Solsona, Spain

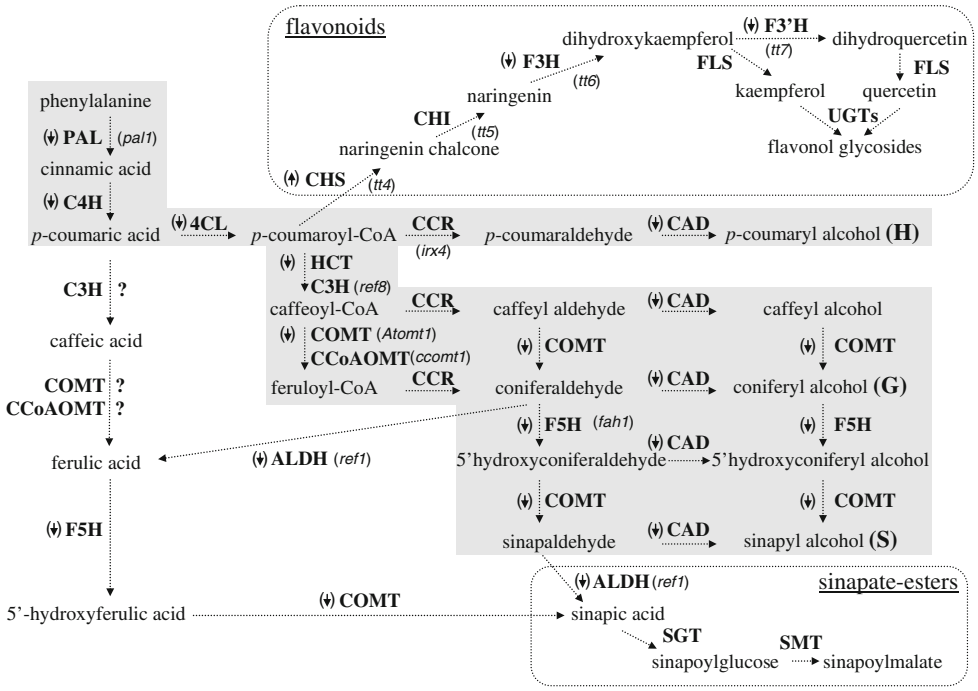


Fig. 1 The phenylpropanoid biosynthetic pathway. Monolignol biosynthesis: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; C3H, 4-coumarate 3-hydroxylase; COMT, caffeic acid *o*-methyltransferase; CCoAOMT, caffeoyl-CoA o-methyltransferase; F5H, ferulate-5-hydroxylase. Flavonoid biosynthesis: CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; UGTs, UDP sugar glycosyltransferases. Sinapate-ester biosynthesis: ALDH, aldehyde dehydrogenase; SGT, sinapate UDP-glucose sinapoyltransferase; SMT, sinapoylglucose malate sinapoyltransferase. Question mark refers to enzymatic steps not fully characterised. *Small arrows* in brackets indicate the genes whose expression is repressed by *Zm*MYB42 in *A. thaliana* (with the exception of *CHS* gene which is induced). *A. thaliana* mutants for genes involved in phenylpropanoid synthesis are indicated in brackets

this polymer undesired for biotechnological applications (Torney et al. 2007; Li et al. 2008; Sticklen 2008; Vanholme et al. 2008).

During the last years, a step forward to better understand how lignification takes place has been achieved through the identification of transcription factors regulating lignin biosynthesis. Several proteins belonging to families such as MYB, MADS-box, bHLH, KNOX, and LIM have been shown to be involved in the regulation of lignification (Tamagnone et al. 1998a; Jin et al. 2000; Kawaoka and Ebinuma 2001; Mele et al. 2003; Arnaud et al. 2007; Zhong and Ye 2007; Bomal et al. 2008).

Plant transcription factors containing the MYB domain have been involved in several physiological and biochemical processes (Martin and Paz-Ares 1997; Stracke et al. 2001; Bonke et al. 2003; Liang et al. 2005; Carlsbecker and Helariutta 2005) and belong to a large multigene family composed by 126 members in *A. thaliana* (Yanhui et al. 2006) distributed into 22 subgroups (Stracke et al. 2001). In the case of monocot plants, the maize genome is expected to encode more than 200 MYB proteins (Rabinowicz et al. 1999) while 183 MYB genes have been identified in the rice genome (Jia et al. 2004; Yanhui et al. 2006). The *Antirrhinum majus Am*MYB308 and *Am*MYB330 were the first R2R3-MYB factors associated with the down-regulation of lignification (Tamagnone et al. 1998a) and they were shown to down-regulate three structural genes of the lignin pathway (*4CL1*, *C4H* and *CAD*) when heterologously overexpressed in tobacco plants. Later on Jin et al. (2000) described a knock-out *Atmyb4* plant displaying an increase of the *C4H* gene expression and a decrease in *CCoAOMT* gene expression. In both cases, the overexpression of *AmMYB308*, *AmMYB330*, and *AtMYB4* in tobacco and *A. thaliana* respectively, affected plant growth and development (Tamagnone et al. 1998a, b; Jin et al. 2000). More recently, the *At*MYB32 factor has been proposed to repress the *A. thaliana COMT* gene as the *Atmyb32* mutant slightly increases the expression of *AtCOMT* (Preston et al. 2004). Similarly, the *Eucalyptus gunnii Eg*MYB1 factor has been also proposed as a repressor of lignification (Legay et al. 2007). Interestingly, all these repressors belong to the subgroup 4 of the R2R3-MYB transcription factors.

Many R2R3-MYB proteins belonging to other subgroups have been described as regulators of lignification, such as *Pinus taeda Pt*MYB1 and *Pt*MYB4 (Patzlaff et al. 2003a, b), poplar *Ptt*MYB21a (Karpinska et al. 2004),

*At*MYB61 (Newman et al. 2004), *Eg*MYB2 (Goicoechea et al. 2005), *Vitis vinifera Vv*MYB5a (Deluc et al. 2006) and tobacco *Nt*MYBJS1 (Gális et al. 2006). These factors have been associated with the regulation of lignin biosynthesis through their *in vitro* interaction with ACI, ACII, and ACIII *cis*-elements typically recognised by these transcription factors (Romero et al. 1998).

In maize, few members of the R2R3-MYB family have been characterised so far. This is the case for 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), *Zm*1 and *Zm*38 (Franken et al. 1989; Marocco et al. 1989), all of which are involved in the regulation of flavonoid biosynthesis. Another R2R3-MYB factor has been cloned and proposed to participate in maize heterosis (Ju et al. 2006) while other maize factors, such as *Zm*MYB-IF35, have been associated with secondary metabolic pathways (Heine et al. 2007). Recently five new maize R2R3-MYB factors, *Zm*MYB2, *Zm*MYB8, *Zm*MYB31, *Zm*MYB39, and *Zm*MYB42, have been identified and we have shown that two of them, *Zm*MYB31 and *Zm*MYB42, act as repressors of lignin biosynthesis (Fornalé et al. 2006).

In this work, we have investigated the role of *Zm*MYB42 in the biosynthesis of the lignin polymer and studied its role in the regulation of other branches of the phenylpropanoid pathway, such as the biosynthesis of sinapate esters and flavonoids. In addition, we have also investigated the effect of *Zm*MYB42 on cell wall structure, polysaccharides content and composition, and cell wall degradability.

Material and methods

Plant material

Arabidopsis thaliana (ecotype WS) plants were grown under standard greenhouse conditions (25°C day and 22°C night with 60% humidity) and a 16/8 hr photoperiod. For the *in vitro* culture, plants were grown in solid MS medium (Murashige and Skoog 1962) supplemented with 1% sucrose in Petri dishes and kept in growth chamber at 22 ± 2°C with a 16 h light period. The transgenic plants used in this work have been already published (Fornalé et al. 2006) and several independent transgenic lines were studied.

Isolation of total RNA, RT-PCR and PCR

Total RNA was extracted with Trizol Reagent (Invitrogen) and 4 µg of total RNA were reverse-transcribed using M-MLV Reverse Transcriptase (Invitrogen). 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 *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 gene-specific primers used in this work are reported in the Supplementary Table S1 online.

Histology

Cross-sections (150 µm thick) of the basal part of inflorescence stems were obtained using a vibratome (Vibratome Series 1000, TPI Inc., St. Louis, MO, USA) and stained as follow. For the Wiesner staining, sections were incubated for 1 min in 2% Phloroglucinol (w/v) in 95% ethanol, then in 50% HCl previous to the observation under light microscope. For the Maüle staining, sections were stained for 10 min in 0.5% $KMnO_4$ (w/v) solution. After a brief wash in distilled water, samples were incubated for 5 min in 10% HCl, washed with distilled water and mounted onto microscope slides using a concentrated NH_4OH solution for observation under light microscopy.

Lignin auto-florescence was detected using UV-excitation under DAPI-filter and the thickness measurement of lignified tissues was performed using the ImageJ 1.38x program (Rasband 2007).

Assay of Klason lignin

Lignin content from *Arabidopsis thaliana* mature stems was quantitatively measured using the Klason method (Kirk and Obst 1988). Briefly, plant material was extracted four times in methanol and vacuum dried. 100 mg of the samples were hydrolysed in 2 ml of 72% (v/v) H_2SO_4 at 30°C for 1 h. The hydrolysate was diluted with 56 ml of water and autoclaved for 1 h. The sample solution was filtered through a fritted glass-crucible and lignin was measured and expressed as mg of Klason lignin per gram of cell wall residue.

Analysis of lignin monomer composition

The analysis was carried out by the CuO oxidation method (Kögel and Bochter 1985; Heddges and Mann 1979). Briefly, mature stems from 30 wt and *Zm*MYB42 plants respectively were collected and immediately frozen and grounded in liquid nitrogen. After extraction in 20 volumes of methanol, the extract-free samples were dried and aliquots of 50 mg of dried plant material were placed in a sealable Pyrex tube, together with 100 mg of CuO, 200 mg of Fe_2SO_4 and 10 ml of 2 M NaOH were added and a stream of N_2 was bubbled through the solution. The tube was sealed under a N_2 stream and placed at 170°C for 2 h, with occasional shaking. Tubes were cooled to room temperature and their content was transferred to polypropylene

tubes and centrifuged at 3,000 rpm. The pellet was washed with 20 ml of distilled water, centrifuged, and the new supernatant pooled with the previous one. The extract was then acidified with HCl to pH 1, centrifuged again and the pellet washed with 0.1 M HCl. The acidified extract was then filtered through a Waters sep-pack cartridge (Waters) and dried under a N_2 stream, and eluted, first with 2 ml ethyl acetate, then with 1 ml acetonitrile. Eluates were collected in Pyrex vials, dried under N_2 stream and re-dissolved with 1 ml methanol for the HPLC analysis.

The separation and quantitation of the phenolic units was performed with a chromatograph under the following conditions. Injected volume: 20 µl. Column: Teknokroma Tracer Extrasil ODS2 column of 5 mm, 25 × 0.46 cm. Eluents: (a) 0.05% H_3PO_4, (b) acetonitrile. Flux: 1 ml min^{-1}. Temperature: 40°C. Gradient: 10% A at the start, increasing slowly up to 17% in 40 min. The column was washed with 100% acetonitrile between two samples, following the sequence: 17% to 100% in 10 min, 100% during 5 min, dropping to 10% in 10 min, 5 minutes in 10% A to recover a total equilibrium. Phenolic compounds were detected at 280 nm.

Organic extraction of soluble phenolics and high-performance liquid chromatograph with diode array detection (HPLC/DAD)

Leaves or stems (ca. 500 mg) from wt and *Zm*MYB42 plants were frozen in liquid N_2 and extracted as previously reported (Pérez-Jiménez and Saura-Calixto 2008). Samples were analysed by HPLC/DAD on a Hitachi (San Jose, CA, USA) Lachrom Elite HPLC system equipped with a quaternary pump, temperature control unit and photo-diode array UV detector (DAD) fitted with a Kromasil C-18 (Tecknokroma, Madrid, Spain) column (25 × 0.4 cm i.d., 100 Å, 5 μ particle size). Acquisition was made using EZChrom Elite version 3.1.3 from Scientific Software Inc (Pleasanton, CA, USA). Loads: 50 µl. Elution with a binary system of solvents [A] 0.1% (v/v) aqueous TFA, [B] 0.05% TFA in CH_3CN under gradient conditions percentage [B] from 0 to 8 over 5 min; 8 to 10 over 10 min and 10 to 50 over 30 min followed by washing 100% CH_3CN for 10 min and re-equilibration of the column to the initial gradient conditions.

High-performance liquid chromatography–electrospray mass spectrometry (HPLC/ESI/MS)

Analyses were performed on a HPLC/MS system, consisting of a LC200 pump, PE Nelson 1050S integrator (Perkin Elmer, USA) fitted with a reversed phase Phenomenex (Torrance, CA, USA) Luna C18 (2) column (150 × 2.0 mm i.d, 5 µm particle size) coupled to a API3000 triple quadrupole mass spectrometer PE Sciex (Concord, Canada) with a turbo ion spray source that was used to obtain the MS/MS data. HPLC elution conditions: [A] 0,1% aqueous formic acid, [B] CH_3CN gradient from 5 to 23 over 30 min followed washing and equilibration of the column to the initial conditions. The flow rate was 0.4 ml min^{-1}. Ionisation (negative mode) conditions: capillary voltage −3500 V, nebulizer gas (N_2) 10 (arbitrary units), curtain gas (N_2) 12 (arbitrary units), focusing potential −200 V, entrance potential 10 V, drying gas (N_2) heated to 400°C. Analysis of the ions was carried out using full scan (FS) data acquisition from m/z 100 to 800 μ in profile mode and using a cycle time of 2 s with a step size of 0.1 μ and a pause between each of 2 ms. To confirm the identity of some of the compounds, neutral loss (NL) experiments by tandem mass spectrometry (HPLC/ESI/MS/MS) were performed.

HPLC/DAD/MS analysis of sinapate esters

The HPLC/DAD profile after injection of the polyphenolic extract from leaves included a major peak corresponding to sinapoylmalate as ascertained by UV spectrophotometry and LC/ESI/MS. The UV spectrum (190–600 nm) of this peak on HPLC-DAD was compatible with sinapate. Two major MS signals recorded in the negative mode, namely the molecular ion (*m/z* 339.2, MW sinapoylmalate 340) and a fragment corresponding to the sinapate moiety (*m/z* 223.1) revealed the structure of this major phenolic.

Auxin transport assay

Auxin transport was measured according to Besseau et al. (2007). A 2.5-cm segment of flowering stems was excised 5 cm above the base of the stem. Segments were placed in a 1.5-ml microcentrifuge tube and the apical ends submerged in 30 µl of MES buffer (5 mM MES and 1% sucrose, pH 5.5) containing 1 µM indole-3-acetic acid and 66 nM of tritiated indole-3-acetic acid. After 4 h of incubation in the dark, segments were removed and the last 5 mm of the non-submerged ends were excised and placed in 2.5 ml of liquid scintillation cocktail (Optiphase-high-safe 2, Perkin-Elmer). Samples were slowly shaken overnight before measuring radioactivity in a scintillation counter (Beckman LS6000 SC).

Cell wall analysis

Dried plant material (1 g) was added to Poly-Prep tubes and extracted with 10 volumes of 70% EtOH for 5 days at room temperature with wheel-shaking, then washed 6 times with 70% EtOH, 6 times with acetone, and air-dried to obtain the alcohol insoluble residue (AIR). AIR

was then de-starched, treated with acidified phenol and washed with organic solvents to obtain the cell wall residue as previously described (Encina et al. 2002).

Neutral sugar analysis was performed according to Albersheim et al. (1967). Dried cell walls were hydrolysed with 2 M TFA (trifluoroacetic acid) for 1 h at 121°C and the resulting sugars were derivatised to alditol acetates and analysed by gas chromatography (GC) on a Supelco SP-2330 column. Uronic acid contents were determined by the m-hydroxybiphenyl method (Blumenkrantz and Asboe-Hansen 1973), with galacturonic acid as a standard. Cellulose was quantified in crude cell walls by the Updegraff method (Updegraff 1969) with the hydrolytic conditions described by Saeman et al. (1963) and quantification of the glucose released by the anthrone method (Dische 1962) with glucose as a standard.

For the cell wall degradability assays, cell walls were hydrolysed (20 mg/1.5 ml) in a mixture of Cellulase R10 (1%); Macerozyme R-10 (0.5%) and purified Driselase (0.1%) dissolved in sodium acetate 20 mM (pH 4.8). Aliquots were taken at 6, 48 and 72 h, clarified by centrifugation and assayed for total sugars (Dubois et al. 1956).

UV treatment

UV-B treatment was performed according to Jin et al. (2000). Plants were grown for 10 days onto solid MS medium. The lids of Petri dishes were removed and plants were irradiated for 10 min with a short wave transilluminator. After exposure, plants were grown for one week more and then photographed.

Measurement of soluble phenolics

Total phenolic quantitation was performed according to Cliff et al. (2007). Stems were extracted with 10% EtOH (0.1 mg FW/µl). A 10 µl aliquot of each sample was added to 10 µl of 0.1% HCl in 95% ethanol and 182 µl of 2% HCl. Each sample was vortexed and allowed to stand for 15 min. Absorbance was measured at 280, 360, and 520 nm using a Shimadzu UV-1630 spectrophotometer. Phenolic content was determined from standard curves obtained using dilutions of gallic acid, rutin and cyanidin chloride at 280, 360, and 520 nm, respectively.

Electron microscopy assays

Hand-cut transverse sections of the basal region of stems of 5-week-old plants were processed for TEM as previously described (Day et al. 2005). Wall polysaccharides of ultra-thin transverse sections (50 nm) were PATAg (Periodic acid-thio carbohydrazide silver proteinate) stained according to Ruel et al. (1977). Observations were performed at 80 kV with a Philips CM 200 Cryo-electron microscope.

Results

Phenotype of *A. thaliana* plants overexpressing *Zm*MYB42

Transgenic plants overexpressing *Zm*MYB42 show several phenotypic alterations (Fig. 2a) at the macroscopic level: they are dwarf with smaller leaves that present a severe adaxial curvature and an altered vascular network characterised by a reduced number of tertiary veins. Previous studies have shown that the overexpression of R2R3-MYB factors acting as repressors of lignification produces alterations of the leaf morphology, with the appearance of white lesions on the older leaves, and reduction of the growth rate when overexpressed in tobacco and *A. thaliana* (Tamagnone et al. 1998a; Jin et al. 2000). In the case of *Zm*MYB42, transgenic plants do not present these white lesions.

*Zm*MYB42 affects lignin biosynthesis

The lignification pattern in different tissues of wt and *Zm*MYB42 plants was analysed by Wiesner staining (Figs. 2b, 3). In stem cross-sections of wild type and transgenic plants, xylem vessels (X) and interfascicular fibres (IF) regions are stained with a red colour, indicative of lignin deposition. However, transgenic stems retain less colour intensity than wild type (Fig. 2b).

Transgenic stems are thinner than the wild type ones (about 65%) and while lignified tissues contain 5-6 cell layers in wild type, transgenic plants display a reduction of approximately 3 cell layers and do not develop secondary xylem at least in the interfascicular region (Fig. 2b). Despite the reduction of lignified layers, the thickness of vascular bundles is not affected in transgenic plants when compared to the stem size (Fig. 2c). In contrast, the thickness of the area of interfascicular fibres decreases by about 30% (Fig. 2c).

Mature siliques of plants expressing *Zm*MYB42 show reduced colour intensity both in the replum and pedicel, indicative of reduced lignin content (Fig. 3). Both the lignified valve marginal cell and endocarp b regions of the silique are also reduced in the transgenic lines (Fig. 3).

We determined the absolute lignin content by the Klason method. This result shows that *Zm*MYB42 plants have 60% reduction of total lignin content compared to wt plants (Fig. 4a). We also analysed the lignin monomer composition by staining stem cross sections with the Maüle reagent

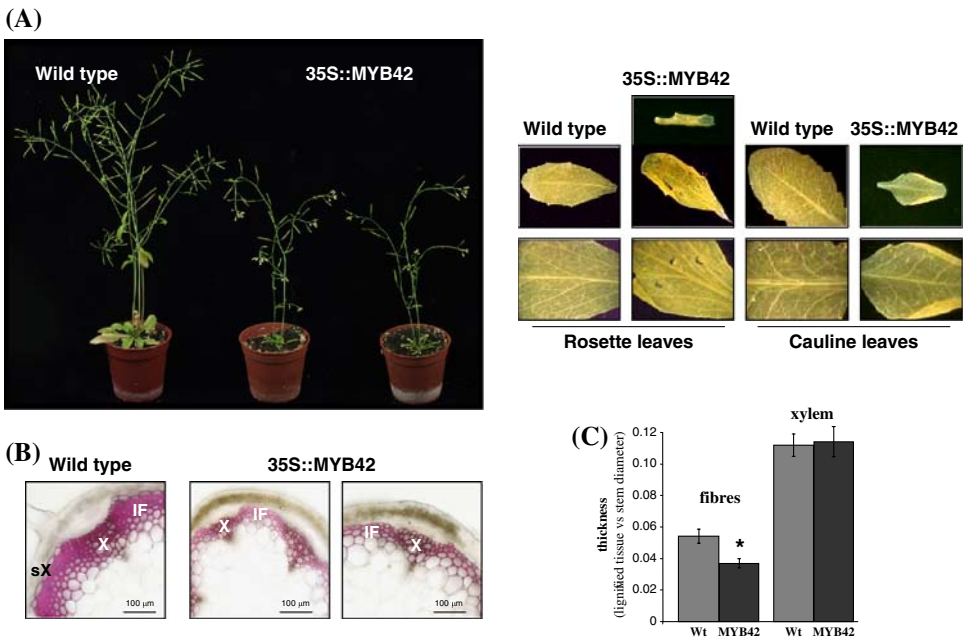


Fig. 2 *Zm*MYB42 affects *Arabidopsis* growth. **a** Phenotype of wt and two independent transgenic plants and leaves at the end of the inflorescence stage. **b** Wiesner staining of cross sections of lignified stalks. (X, xylem; sX, secondary xylem; IF, interfascicular fibres). **c** Quantification of thickness of xylem and fibres in wt and transgenic plants. Values are means of $n = 10$ independent measurements. (*) indicates significant differences ($P \leq 0.01$). The cell thickness was measured using the ImageJ 1.38x program (Rasband 2007)

that stains the S subunits of the lignin polymer specifically. Wild type plants exhibit two staining patterns (Fig. 3): dark red staining is detected in the IF region, indicative of S-enriched lignin, while the fascicular region stain slightly yellow-red, reflecting the deposition of G-enriched lignin. The transgenic *Zm*MYB42 stem show the same pattern,

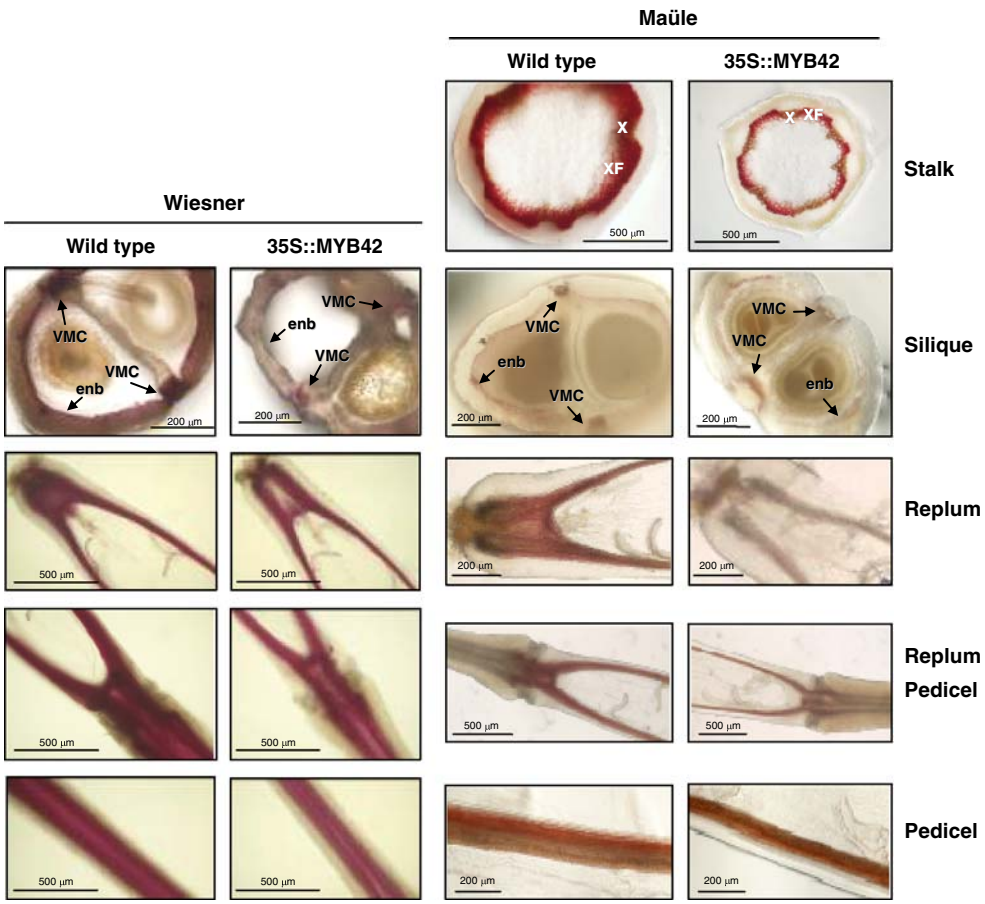


Fig. 3 Histochemical analyses of *Zm*MYB42 transgenic plants. Wiesner (*left*) and Maüle (*right*) staining of cross sections of lignified tissues (VMC, valve marginal cell; enb, endocarp-b)

even though the staining intensity is strongly reduced, in accordance with the reduced lignin content indicated by the Wiesner staining. However, the bright yellow staining of the fascicular region indicates a decrease of S-lignin in this tissue compared to the wild type plants.

A similar result was obtained with mature siliques: besides the general reduction of the stained tissues, the weaker red staining of transgenic siliques indicates a reduction of S-lignin in the lines expressing *Zm*MYB42.

To quantify the effect of *Zm*MYB42 on lignin monomer composition further, we performed HPLC analysis of the phenolic compounds obtained from cell walls of the control and transgenic plants (Fig. 4a). We observed a huge increase in *P*-hydroxybenzaldehyde accounting for a 4-fold increase in the H subunits in the lignin of *Zm*MYB42 expressing plants and a strong reduction of syringic acid, accounting for a 50% reduction of S subunits, even when this method overestimates the S subunits (Lewis and Yamamoto 1990; Heddges and Mann 1979). We observed a 70% increase in G subunits due to an increased accumulation of vanillin. All these changes lead to a 3.4-fold reduction of the S/G ratio of transgenic plants compared to control plants. Thus, the over-expression of *Zm*MYB42 leads to substantial differences in lignin composition compared to the control samples, in line with the histological staining obtained with the Maüle reagent. Interestingly, the expression of this maize transcription factor in *A. thaliana* leads to the production of a lignin polymer with a final composition more similar to that of maize.

In addition to the major lignin-derived compounds, levels of *P*-coumaric and ferulic acid were analysed. While the endogenous levels of *P*-coumaric acid are not affected in *Zm*MYB42 plants, ferulic acid increases 2-fold in transgenic plants compared to wt plants (Fig. 4b).

To further understand the function of *Zm*MYB42 we analysed the expression of all the genes involved in lignification (Fig. 4b). Our analyses showed that the accumulation of *PAL1* mRNA (in addition to *C4H*, and *4CL1;* Fornalé et al. 2006), is reduced in *Zm*MYB42 transgenic lines. This means that *Zm*MYB42 represses the expression of the three genes of the general phenylpropanoid metabolism which is in line with the reduction of the total lignin content in transgenic plants. In addition, the repression pattern of *HCT* and *F5H1* (in addition to *COMT1* and *CAD6;* Fornalé et al. 2006), is in line with the

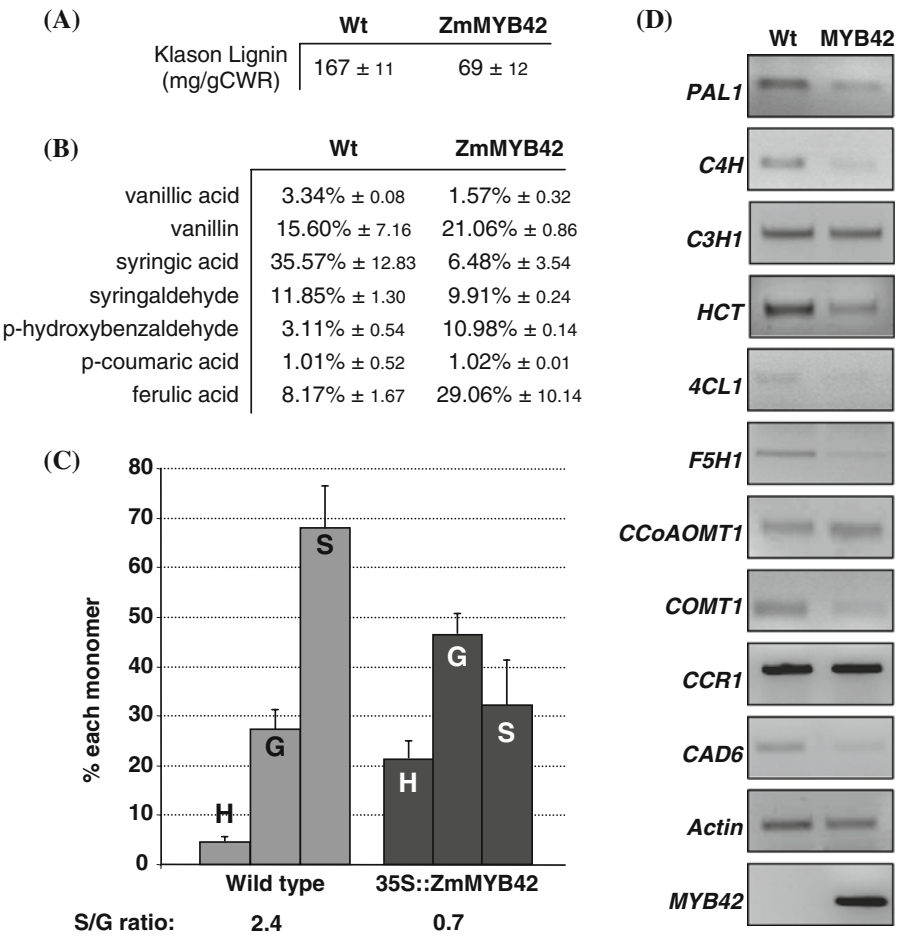


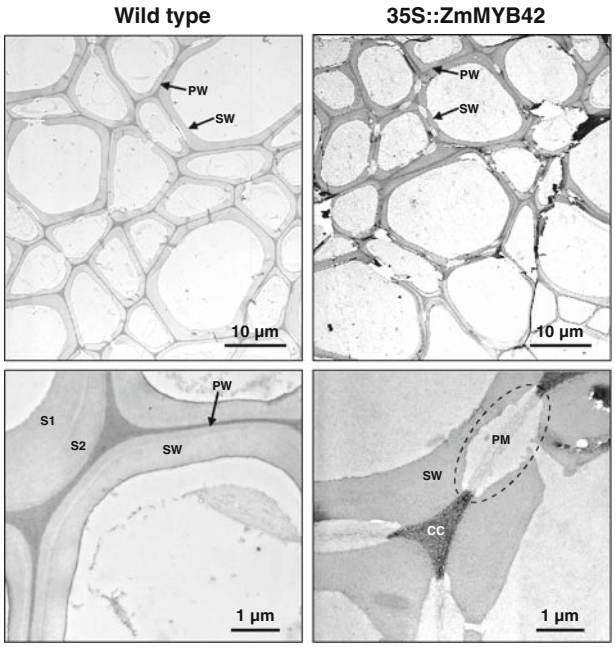
Fig. 4 *Zm*MYB42 alters lignin content and composition. **a** Quantitative lignin content determination from wt and *Zm*MYB42 mature stems by the Klason method. **b** HPLC analysis of lignin composition from wt and *Zm*MYB42 plants determined by the CuO oxidation method. Results are shown as relative amounts of each compound. **c** Histogram reporting the lignin composition of *Zm*MYB42 and wt plants. H refers to *P*-hydroxybenzaldehyde; G refers to the sum of vanillin and vanillic acid; S refers to the sum of syringaldehyde and syringic acid. Data correspond to the mean value ± SD of three independent assays. **d** Relative expression levels of all the monolignol biosynthetic genes

alteration in the final lignin composition of the transgenic plants. On the other hand, the expression of *C3H*, *CCoAOMT* and *CCR* genes is not affected by *Zm*MYB42.

*Zm*MYB42 affects cell wall structure, polysaccharide content, composition and degradability

The huge reduction of lignin and its altered S/G ratio led us to investigate whether these changes affected the structure of the cell wall of *Zm*MYB42 plants. Therefore, we analysed by TEM (transmission electron microscopy) the cell wall structure of wild type and transgenic plants (Fig. 5a). The modifications induced by *Zm*MYB42 essentially affect the interfascicular fibres. In *Zm*MYB42 plants, the secondary cell wall thickenings are interrupted by a greater number of wide "pits" in which the primary wall is clearly visible and sometimes extended between two cell corners. In addition, the interrupted secondary wall of the transgenic plants exhibits a weaker delimitation between S1 and S2 sub-layers. It is worth noting that the reactivity to PATAg of *Zm*MYB42 fibre walls appears enhanced. This may be explained by an enhanced accessibility of polysaccharides to the periodic acid treatment due to the reduced lignin content.

As lignin interacts with polysaccharides within the cell wall and putatively constitutes a competing carbon sink, we also investigated whether *Zm*MYB42 affected the cell wall polysaccharide content and composition by GC analysis. The cell wall yield does not vary between wild type (0.59 ± 0.02) and plants overexpressing *Zm*MYB42 (0.60 ± 0.02). As shown in Fig. 5b, while cellulose content is not significatively affected by the overexpression of *Zm*MYB42, a broad alteration in cell wall composition is detected. In particular, a general increase



(B)

µg mg^{-1} dry cell wall		Wild type	35S::ZmMYB42	
	Cellulose	366.90 ± 38.2	354.21 ± 24.6	
Hemicelluloses	Fucose	0.32 ± 0.0	1.81 ± 0.3	*
	Arabinose	4.63 ± 0.1	25.82 ± 2.6	*
	Xylose	248.58 ± 0.5	219.27 ± 6.2	*
	Glucose	5.36 ± 0.2	6.33 ± 3.0	
	Mannose	2.59 ± 0.1	2.49 ± 0.6	
Pectins	Uronic acids	151.32 ± 9.7	233.73 ± 26.4	*
	Rhamnose	3.78 ± 0.1	9.50 ± 3.6	*
	Galactose	2.99 ± 0.2	13.05 ± 1.4	*
	Matrix poly.	419.50 ± 37.9	512.00 ± 83.7	
	Total sugars	786.40 ± 65.2	866.21 ± 103.4	

(C)

carbohydrates released (mg g^{-1} dry cell wall)	Wild type	35S::ZmMYB42
6h	249.7 ± 11.5	406.2 ± 35.5
48h	270.9 ± 16.8	475.5 ± 27.9
72h	326.6 ± 14.9	545.2 ± 26.9
degradability ratio	1	1.68

Fig. 5 *Zm*MYB42 affects cell wall structure and composition. **a** Transmission electron microscopy after PATAg staining of interfascicular fibres of wt and transgenic plants (PM, Pit-Membrane; PW, primary wall; SW, secondary wall; S1 and S2, secondary cell wall sub-layers; CC, cell corner). Broken line circle indicates the appearance of wide "pits" in transgenic cell walls. **b** Sugar analyses of cell walls from stems of wt and transgenic plants. Data are expressed as mean value of six independent assays ± SD. (*) indicate significant differences by Student's t test ($P < 0.05$). **c** Resistance of cell walls to digestion with polysaccharide hydrolases. A time-course of cell wall degradability was performed using stems of wt and transgenic plants. Data correspond to the mean value ± SD of three independent assays

of polysaccharides habitually present in the primary cell wall (arabinose, galactose, uronic acids, rhamnose and fucose) is observed while a decrease of xylose, a secondary cell wall polysaccharide, is also observed. However, these changes do not lead to significant changes in the amounts of total sugars in transgenic cell walls with respect to wild type plants.

The observed changes in lignin, cell wall structure and polysaccharides in the cell wall prompted us to determine whether the transgenic cell walls were more susceptible to enzymatic degradability. Our results indicate that *Zm*MYB42 plants have cell walls more degradable when treated with the cellulase-macerozyme-driselase enzymatic cocktail (Fig. 5c).

*Zm*MYB42 plants have reduced levels of sinapoylmalate

Similarly to what observed in the case of the *fah1-2* and the *ref8* mutants (Franke et al. 2002), *Zm*MYB42 plants show a red colour when visualised under UV-A, suggesting a reduction of sinapoylmalate (Fig. 6a). We therefore analysed by HPLC/DAD/MS extracts of 4-week-old rosette leaves and the results obtained confirmed that transgenic plants show a strong reduction of this compound (Fig. 6b).

As sinapoylmalate plays a central role as a UV-protectant in *A. thaliana*, we investigated whether *Zm*MYB42 plants were more sensitive to the UV-B radiation. Thus, we treated 10-days old wild type and transgenic plants with 10 min UV-B light and we analysed their response. After 7 days, transgenic plants show yellowing leaves, indicative of a severe and extensive damage compared to the wild type plants (Fig. 6c). The expression analysis of the main genes involved in sinapoylmalate biosynthesis indicated that *Zm*MYB42 represses the *ALDH* gene (Fig. 6d). As *At*MYB4 has been shown to affect the synthesis of sinapoylmalate (Jin et al. 2000; Hemm et al. 2001) we also investigated whether *Zm*MYB42 could affect the expression of this transcription factor. Our results show that *AtMYB4* gene expression is reduced in *Zm*MYB42 plants (Fig. 6d).

*Zm*MYB42 represses flavonol biosynthesis

The observation of stem cross-sections of *Zm*MYB42 plants under UV light indicates an altered fluorescence, indicative of possible changes in flavonoid accumulation compared to wild type plants (Fig. 7a).

As *Zm*MYB42 represses the expression of genes of the core phenylpropanoid pathway, we investigated whether this factor could regulate other branches of this complex network, such as the one leading to the synthesis of flavonoids (Fig. 1).

Thus, we performed a quantitative analysis of the main phenolics from 2-month old plants, which revealed that *Zm*MYB42 plants present more than a 66% reduction of the total phenolic content compared to the wild type plants. This decrease is mainly due to the huge reduction of flavonols (about 50% of the wild type), while the anthocyanins level remains practically unchanged (Fig. 7b). The HPLC/DAD/MS analysis indicated that the reduced flavonol content is mainly due to a decrease in the accumulation of flavonols belonging to the kaempferol family (Fig. 7c).

As previous studies demonstrated that flavonoids are negative regulators of auxin transport (Jacobs and Rubery 1988), we investigated whether this reduction of flavonols affected the transport of these hormones using a radiochemical method. The results obtained indicate that auxin transport in *Zm*MYB42 plants is not affected compared to wild type plants (Fig. 7d).

Finally, we performed RT-PCR assays to study the effect of *Zm*MYB42 on the main genes of the flavonoid pathway (Fig. 1). Our results indicated that *Zm*MYB42 down-regulates the *F3H* and *F3′H* gene expression. In contrast, the mRNA accumulation of the *CHS* gene is increased compared to wild type plants (Fig. 7e).

Discussion

In a previous studies, we reported the isolation of *Zm*MYB42, a new R2R3-MYB factor belonging to the subgroup 4 that down-regulates the maize and the *A. thaliana COMT* genes and we showed that transgenic plants overexpressing this factor contain only half of the total lignin content (Fornalé et al. 2006). Here we characterised the effects of *Zm*MYB42 in relation to lignin biosynthesis and also to other branches of the phenylpropanoid pathway. *Zm*MYB42 plants are dwarf and their leaves are severely curved with a reduction of their vascular network. The reduced growth may be caused by the difficulty of plants in producing vascular and mechanical tissues. Similar phenotypes appear in mutants with reduced lignin content (e.g. *ref8* mutant; Franke et al. 2002).

The strong reduction of lignin content was observed in all lignified tissues when analysed by phloroglucinol staining. In addition, we showed that in transgenic plants, there is a strong reduction of the S/G ratio of the lignin polymer, due to both a decrease of the S subunits and an increase of the G subunits. Indeed, the appearance of high levels of H subunits makes the final lignin polymer of the *Zm*MYB42 *A. thaliana* plants more similar to that typically produced by maize.

The impact on lignin content and composition caused by *Zm*MYB42 relies on the downregulation of the phenylpropanoid pathway (Fig. 1); the general phenylpropanoid

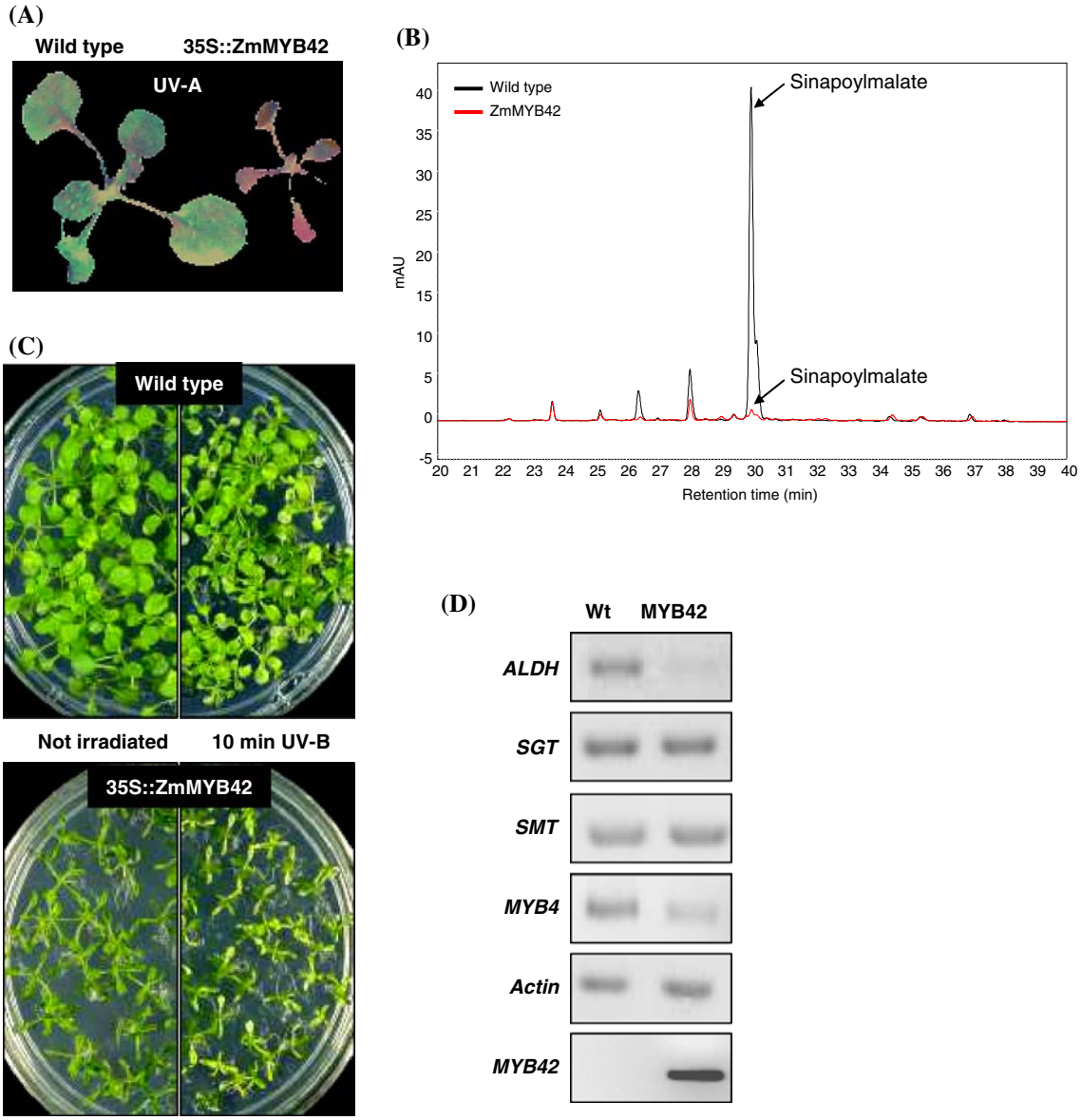


Fig. 6 *Zm*MYB42 represses sinapoylmalate biosynthesis. **a** Phenotype of wt and transgenic plantlets exposed to UV light. **b** HPLC-DAD analysis of sinapoylmalate content in leaves of wt and transgenic plants. **c** Phenotype of 20-days old wt and transgenic plantlets submitted to 10 min UV irradiation. **d** Relative expression of the genes involved in sinapoylmalate synthesis and of *AtMYB4* gene

genes (*PAL*, *C4H*, and *4CL*), several genes involved in the conversion from esters to aldehydes and alcohols (*HCT* and *CAD*) and genes belonging to the branches leading to the production of the three main monolignols (*F5H* and *COMT*). In contrast, *C3H*, *CCoAOMT* and *CCR* genes are not regulated by this transcription factor (Figs. 1, 4). Together, and in accordance with previous observations (Ralph et al. 2008) these results show once more the high level of flexibility of the phenylpropanoid pathway.

Several reports have shown that reduction of CAD (Sibout et al. 2003), F5H (Marita et al. 1999) and COMT (Goujon et al. 2003; Do et al. 2007) results in a decrease of the S units in the final lignin polymer. In agreement with these results, the repression of *F5H*, *COMT* and *CAD* genes produces a lignin polymer strongly depleted in S units. In addition, the increased levels of ferulic acid are not expected if this compound is produced by COMT, as this gene is repressed in *Zm*MYB42 plants. Thus, this finding is in line with other works indicating that COMT does not catalyse the *in vivo* methylation of caffeic acid to produce ferulic acid (Do et al. 2007). Together, the overall pattern of repression of the lignin genes accounts for the reduced capacity of transgenic plants to produce S units.

Cell walls of transgenic fibres show an altered structure characterised by a higher number of pit-membranes and a reduced thickness of secondary cell walls. The increase of

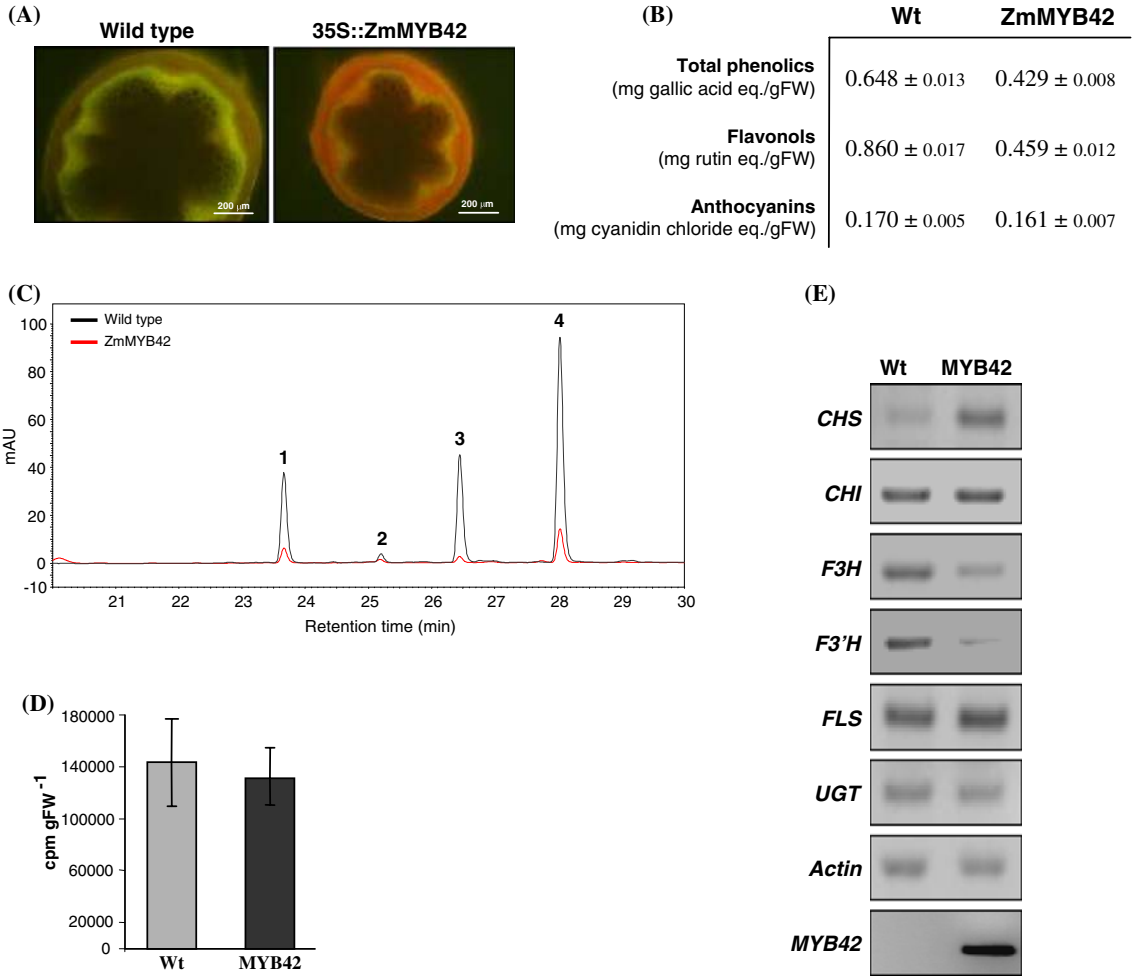


Fig. 7 *Zm*MYB42 represses flavonols biosynthesis. **a** Sections of wt and transgenic stalks visualised under UV microscope at the end of the inflorescence stage. **b** Quantification of phenolic compounds from wt and transgenic plants (eq means equivalents). **c** HPLC-DAD analysis of flavonols content in stalks of wt and transgenic plants. (a: Kaempferol 3-*O*-rhamnosyl-glucoside 7-*O*-rhamnoside (*m/z* 739.3, MW 740); b: Kaempferol 3-*O*-glucosyl-glucoside 7-*O*-rhamnoside (*m/z* 755.1, MW 756); c: Kaempferol 3-*O*-glucoside 7-*O*-rhamnoside (*m/z* 593.4, MW 594); d: Kaempferol 3-*O*-rhamnoside 7-*O*-rhamnoside (*m/z* 577.2, MW 578)). The structures were confirmed by neutral loss experiments (MS-MS). The losses detected were rhamnoside moieties (146 mass units) from each of the four flavonol glycosides and an additional glucoside moiety (162 mass units) from kaempferol 3-*O*-glucoside-7-*O*-rhamnoside. **d** Auxin transport determination in wt and transgenic plants. Data correspond to the mean value ± SD of three independent assays. **e** Relative expression of the main genes involved in flavonol synthesis

PATAg staining in transgenic plants also suggests that the reduction of the lignin content leads to a looser interaction between cellulose microfibrils which favours the enhancement of the size of silver grain deposits. Similar loosening of microfibrils association was previously observed in relation to a decrease in syringyl units, suggesting the important role of non-condensed substructures in the secondary wall cohesion (Ruel et al. 2001).

In addition to lignin content and composition, and cell wall structure, *Zm*MYB42 also affects the cell wall composition. Our results show that the majority of primary cell wall type monosaccharides are increased while xylose, present in the secondary cell wall, is decreased. Therefore, cell wall compositional analysis indicates an overall enrichment in matrix polysaccharides that are characteristics of the primary cell wall. Based on these results it seems that because of the reduction in lignin, *Zm*MYB42 stems retain primary cell wall enriched tissues.

In agreement with the reduction of total lignin content and the strong reduction of the S/G ratio, transgenic cell walls are more degradable. Histological studies, together with the reduction of the S subunits of the lignin polymer and the electron microscopy data all indicate that *Zm*MYB42 mainly affects the development of the mechanical tissues, which are normally enriched in S subunits (Mellerowicz et al. 2001).

Plants having a reduction of *C4H* (Jin et al. 2000), *F5H* (in the *fah1* mutant; Ruegger et al. 1999), *C3H* (in the *ref8*

mutant; Franke et al. 2002) and *COMT* gene expression (in the *Atomt1* mutant; Goujon et al. 2003; Do et al. 2007) show a reduction in the synthesis of sinapoylmalate and display red fluorescence when visualised under UV light. Accordingly, *Zm*MYB42 represses *C4H*, *F5H*, and *COMT* gene expression, and thus transgenic plants have a strong reduction in sinapoylmalate as demonstrated by HPLC/DAD/MS, the observation under UV light and the increased sensitivity to UV-B irradiation. In addition, *Zm*MYB42 also represses the expression of *ALDH* gene, which is the first specific enzyme for the synthesis of sinapoylmalate (Fig. 1). This result is in line with what observed in the Arabidopsis *ref1* mutant in which the mutation of the *ALDH* gene results in a strong reduction of the sinapate esters (Nair et al. 2004).

The involvement of *C4H* repression in the synthesis of sinapoylmalate was described through the study of *AtMYB4* gene (Jin et al. 2000), a repressor of sinapoylmalate synthesis. In *Zm*MYB42 plants, the expression of *AtMYB4* gene is downregulated. This behaviour could be the consequence of a homeostatic response by which the transgenic plants tend to counteract the effect produced by the overexpression of *Zm*MYB42.

As mentioned above, *Zm*MYB42 also represses the synthesis of *PAL1*, *C4H*, and *4CL* genes. These genes are involved in the core-phenylpropanoid pathway and catalyse the synthesis of hydroxycinnamic acid intermediates, which are precursors for the route-specific pathways leading to the synthesis of a wide range of secondary metabolites such as flavonoids (Fig. 1). Transgenic plants overexpressing *Zm*MYB42 have stems in which the cortex region appears orange under UV light, suggesting an alteration in the synthesis of some flavonoids. A deeper characterisation revealed that transgenic plants have decreased levels of total soluble phenolics and in particular, a strong reduction of flavonols belonging to the family of kaempferol. Although *Zm*MYB42 induces the expression of *CHS* gene, the entry point to flavonoid biosynthesis, no increased levels of naringenin chalcone were detected (data not shown). However, *Zm*MYB42 represses the expression of *F3H* and *F3′H* genes, which could explain the reduced levels of flavonols in transgenic plants. Therefore, in addition to the strong reduction of sinapoylmalate, the fact that transgenic plants are more sensitive to UV irradiation could be, at least partially, also caused by the reduction of the flavonol content as described in the case of the *transparent testa 4* (*tt4*) mutant (Li et al. 1993).

It has been reported that flavonoids are negative regulators of the auxin transport (Brown et al. 2001; Besseau et al. 2007). In our case, although *Zm*MYB42 have reduced levels of flavonoids, the auxin transport is not induced in transgenic plants, suggesting that a 50% reduction of flavonols is not sufficient to increase the levels of auxin transport.

Together, these results show that *Zm*MYB42 is a general repressor of the phenylpropanoid pathway affecting cell wall structure and composition when overexpressed in *A. thaliana*. The reduction of the total lignin content, the strong reduction of its S/G ratio, and the increased cell wall degradability, makes this factor a good candidate to control lignin metabolism in maize for biotechnological applications.

Acknowledgements This work received financial support from the CONSOLIDER-INGENIO programme (CSD2007-00036) from the Spanish Ministerio de Ciencia e Innovación. F.M. Sonbol was financed by the European Project INCO-II (ICA4-CT-2000-30017), by the CRAG laboratory, and by the "Instituto de Cooperación con el Mundo Árabe, Agencia Española de Cooperación Internacional". S. Fornale was financed by a post-doctoral grant from the "Generalitat de Catalunya" (2003PIV-A-00033) and by an I3P contract from the "Consejo Superior de Investigaciones Científicas". D. Caparrós-Ruiz was financed by the Spanish "Ministerio de Educación y Ciencia" ("Ramón y Cajal" Program). This work was carried out within the framework of the "Xarxa de Referència de Biotecnologia" (XarBa) from the Autonomous Government of Catalonia. We are indebted to Dr. Jean-Paul Joseleau (CERMAV-CNRS Grenoble) and Dr. Cathie R. Martin (John Innes Center) for critical reading of the manuscript. We also thank the sequencing and greenhouse teams of IBMB-CSIC as well as Dr. R.S. Joshi for the English revision of the manuscript.

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