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ZmMYB31 directly represses maize lignin genes and redirects the phenylpropanoid metabolic flux

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SUMMARY

Few regulators of phenylpropanoids have been identified in monocots having potential as biofuel crops. Here we demonstrate the role of the maize (*Zea mays*) R2R3-MYB factor ZmMYB31 in the control of the phenylpropanoid pathway. We determined its *in vitro* consensus DNA-binding sequence as $ACC^{T}/_{A}ACC$, and chromatin immunoprecipitation (ChIP) established that it interacts with two lignin gene promoters *in vivo*. To explore the potential of ZmMYB31 as a regulator of phenylpropanoids in other plants, its role in the regulation of the phenylpropanoid pathway was further investigated in *Arabidopsis thaliana*. ZmMYB31 downregulates several genes involved in the synthesis of monolignols and transgenic plants are dwarf and show a significantly reduced lignin content with unaltered polymer composition. We demonstrate that these changes increase cell wall degradability of the transgenic plants. In addition, ZmMYB31 represses the synthesis of sinapoylmalate, resulting in plants that are more sensitive to UV irradiation, and induces several stress-related proteins. Our results suggest that, as an indirect effect of repression of lignin biosynthesis, transgenic plants redirect carbon flux towards the biosynthesis of anthocyanins. Thus, ZmMYB31 can be considered a good candidate for the manipulation of lignin biosynthesis in biotechnological applications.

Keywords: R2R3-MYB, lignin, cell wall, flavonoids, phenylpropanoids, maize.

INTRODUCTION

Lignin is a complex and heterogeneous polymer that constitutes one of the major components of the secondary walls of xylem cells and fibres (Mellerowicz *et al.*, 2001). Lignification confers mechanical support, optimises the transport of water and solutes along the vascular system and protects plants against pathogens (Boerjan *et al.*, 2003). Lignin is nature's second most abundant product, and is formed by the oxidative polymerisation of three main constituents, the monolignols *p*-coumaryl, coniferyl and sinapyl alcohols. They are synthesised through the phenylpropanoid pathway and differ in their degree of methoxylation. Once incorporated in the lignin polymer, these precursors are known as *p*-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) subunits, respectively (Anterola and Lewis, 2002; Boerjan *et al.*, 2003).

Lignins, together with anthocyanins, flavonols and proanthocyanidins, constitute the main group of plant phenylpropanoids. These metabolites play important functions in plant growth and adaptation to environmental perturbations, and several studies characterising R2R3-MYB factors have highlighted a general impact of these

transcription factors on various parts of the phenylpropanoid pathway (Tamagnone *et al.*, 1998a; Borevitz *et al.*, 2000; Preston *et al.*, 2004; Deluc *et al.*, 2006).

Currently there is great interest in modifying the content and/or the composition of lignin, since it constitutes a negative value for agro-industrial purposes such as forage stocks or second-generation bioethanol production (Boudet and Grima-Pettenati, 1996; Boudet *et al.*, 2003; Reddy *et al.*, 2005; Torney *et al.*, 2007; Vanholme *et al.*, 2008). Over the last decade, many efforts have been geared towards the characterisation of the genes involved in the monolignol biosynthetic pathway (Raes *et al.*, 2003; Guillaumie *et al.*, 2007).

Emerging evidence indicates that lignin biosynthesis is highly regulated by the action of different transcription factors, including members of the R2R3-MYB family (Patzlaff *et al.*, 2003a,b; Goicoechea *et al.*, 2005; Demura and Fukuda, 2007; Zhong and Ye, 2009) which comprises many regulators of the phenylpropanoid pathway (Grotewold *et al.*, 1994; Sablowski *et al.*, 1994; Deluc *et al.*, 2006). R2R3-MYBs are one of the largest families of transcription factors in angiosperms, with 126 members in *Arabidopsis thaliana* (Stracke *et al.*, 2001) and at least 125 and 173 in rice and maize, respectively (Yilmaz *et al.*, 2009). R2R3-MYB family members are classified into 22 subgroups, based on the presence of distinctive motifs outside of the conserved MYB domains (Kranz *et al.*, 1998).

While many R2R3-MYB factors act as transcriptional activators of lignin synthesis (Patzlaff *et al.*, 2003a,b; Karpinska *et al.*, 2004; Goicoechea *et al.*, 2005), at present only MYB factors belonging to subgroup 4 have been shown to act as repressors of the monolignol biosynthetic genes. This is the case for snapdragon (*Antirrhinum majus*) AmMYB308, AmMYB330 (Tamagnone *et al.*, 1998a), Arabidopsis AtMYB4 (Jin *et al.*, 2000) and AtMYB32 (Preston *et al.*, 2004) and eucalyptus (*Eucalyptus gunnii*) EgMYB1 (Legay *et al.*, 2007). However, no *in vivo* direct interaction of any of these R2R3-MYB factors with the repressed lignin genes has so far been demonstrated.

We previously described the isolation of the maize (*Zea mays*) R2R3-MYB factors ZmMYB31 and ZmMYB42 as members of subgroup 4. We showed that, when expressed in *A. thaliana*, they function as repressors of both the maize and *A. thaliana COMT* (caffeic acid *O*-methyltransferase) gene, suggesting that the regulation of this lignin biosynthetic gene might be controlled by molecular mechanisms evolutionarily conserved between monocots and dicots. In addition, both factors repress the *A. thaliana 4CL1* (4-coumarate-CoA ligase) gene and produce a strong reduction in the lignin content (Fornalé *et al.*, 2006). Subsequently we showed that, in addition to repression of lignin biosynthesis, ZmMYB42 also reduces the S/G ratio of the polymer, potentially increasing the availability of cell wall polysaccharides. ZmMYB42 negatively regulates other branches of the phenylpropanoid pathway, reducing the accumulation of flavonols and sinapoylmalate (Sonbol *et al.*, 2009).

In maize, few members of the R2R3-MYB family have so far been characterised. C1 and PL (Paz-Ares *et al.*, 1987; Marocco *et al.*, 1989; Cone *et al.*, 1993; Pilu *et al.*, 2003), P1 (Grotewold *et al.*, 1991, 1994), Zm1 and Zm38 (Franken *et al.*, 1989; Marocco *et al.*, 1989) participate in the regulation of flavonoid biosynthesis. In contrast, ZmMYB-IF35 (ZmMYB40) functions as a positive regulator of phenylpropanoid biosynthesis (Dias and Grotewold, 2003; Heine *et al.*, 2007).

The aim of the present study was to apply an *in vivo* approach to demonstrate a direct interaction of ZmMYB31 with lignin gene promoters in maize and to characterise the role of this factor within the phenylpropanoid pathway and its effect on cell wall polysaccharide composition by its over-expression in *A. thaliana*. Our results show that ZmMYB31 binds *in vivo* to the maize *ZmCOMT* and *ZmF5H* gene promoters, resulting in the repression of lignin biosynthetic gene expression. Despite the strong reduction in lignin synthesis when over-expressed in *A. thaliana*, ZmMYB31 does not affect the composition of the lignin polymer, nor does it alter the polysaccharide composition and content of the cell walls, but it does significantly increase the degradability of cell walls. ZmMYB31 affects the biosynthesis of flavonoids and increases the accumulation of anthocyanins. In addition, it negatively affects the accumulation of sinapoylmalate, making plants more sensitive to UV radiation. Finally, the over-expression of *ZmMYB31* in *A. thaliana* induces the expression of several stress-responsive proteins.

RESULTS

Identification of a consensus DNA-binding site for the maize ZmMYB31 transcription factor by SELEX: interaction of ZmMYB31 with the maize *COMT* gene promoter *in vitro*

Systematic evolution of ligands by exponential enrichment (SELEX) was used to investigate whether ZmMYB31 recognises DNA, and, if so, to define its DNA-binding sequence consensus (see the detailed procedure in Appendix S1 in Supporting information). By analyzing 52 unique sequences derived from SELEX (Table S1), a core binding motif for the ZmMYB31 DNA-binding domain was established to be $ACC^{T}/_{A}ACC$ (Figure 1a). This consensus sequence is very similar to that of P1, namely $CC^{T}/_{A}ACC$ (Grotewold *et al.*, 1994), despite P1 and ZmMYB31 being in different subgroups. The maize *COMT* gene promoter contains AC-II (−51 to −45, all distances with respect to the transcription start site, TSS) and AC-III (−200 to −194) MYB-binding sites that could be putatively recognised by ZmMYB31. To test this possibility, we performed an electrophoretic mobility shift assay (EMSA) using three DNA fragments spanning regions −67 to −31, −216 to −180 and −1422 to −1417 as probes

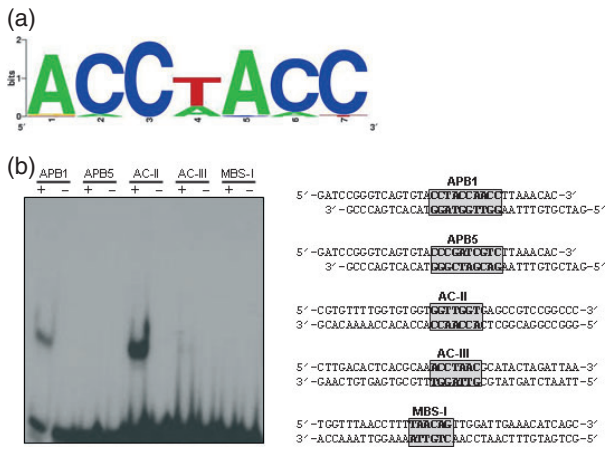

Figure 1. Identification of the ZmMYB31 binding site and interaction with different R2R3-MYB DNA sequences.
(a) Identification of the consensus DNA-binding site of ZmMYB31 performed by systematic evolution of ligands by exponential enrichment (SELEX).
(b) ZmMYB31 interacts with AC-II and APB1 DNA-binding motifs *in vitro*. DNA-binding reactions were performed essentially as previously described (Grotewold *et al.*, 1994). The synthetic oligonucleotides used were AC-II, AC-III (present in the maize *COMT* gene promoter), MBS-I (present in the maize *CAD* gene promoter), APB1 (present in the maize *A1* gene promoter) and APB5 (a mutated version of APB1 in which the DNA–protein interaction is abolished).

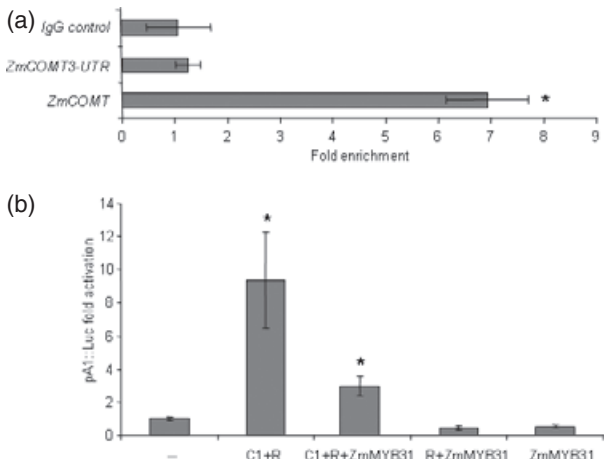

Figure 2. The *ZmCOMT* gene is a direct target of ZmMYB31.
(a) Validation that the *ZmCOMT* target is chromatin immunoprecipitated using anti-ZmMYB31 in young leaf sheath tissues. Quantitative PCR analysis of chromatin immunoprecipitation (ChIP) DNA enrichment performed with purified ZmMYB31-specific antibody or pre-immune serum control (IgG control). Enrichment of the *ZmCOMT* promoter region or 3′ untranslated region (UTR) is relative to enrichment of the ZmActin. Statistical analysis of differences between samples was performed using a Student's *t*-test. *Significant at $P < 0.05$.
(b) Transient expression of pA1::Luc by 35S::R and 35S::C1 in the absence or presence of ZmMYB31 after co-bombardment of cultured Black Mexican Sweet (BMS) maize cells. 35S::*Renilla* was used as a normalisation control in all bombardments. Each treatment was done in four replicates. The fold activation corresponds to the ratio of each particular treatment and the treatment with pA1::Luc without activator. The average values are shown, and the error bars indicate the standard deviation of the samples. Statistical analysis of differences between samples was performed using a Student's *t*-test. *Significant at $P < 0.03$.

which contain AC-II and AC-III elements (see the detailed procedure in Appendix S1). The result shows that ZmMYB31 binds to AC-II, but not to AC-III (Figure 1b), in strong agreement with the consensus sequences derived from SELEX experiments (Figure 1b).

We previously showed that the over-expression of ZmMYB31 downregulates the *A. thaliana COMT* gene (Fornalé *et al.*, 2006). However, the *AtCOMT* promoter only contains a MBS-I element, as a putative MYB-binding site. In agreement with the SELEX results, EMSA experiments using as a probe the MBS-I present in the *ZmCAD* gene promoter show that ZmMYB31 does not interact with this putative MYB DNA-binding element (Figure 1b).

We also show that ZmMYB31 interacts with the high-affinity P1-binding site (APB1) present in the flavonoid *A1* gene promoter, but not with a mutant (APB5) site (Figure 1b). These results demonstrate that ZmMYB31 binds to similar DNA sequences as other plant R2R3-MYB factors, and identify the ZmMYB31-binding sites in the *ZmCOMT* promoter.

ZmMYB31 interacts with the maize *COMT* and *A1* gene promoters *in vivo*

Specific antibodies against ZmMYB31 (αMYB31) were developed and employed in chromatin immunoprecipitation (ChIP) experiments (see the detailed procedure in Appendix S1). A schematic representation of the amplified regions of *ZmCOMT* and *ZmActin* genes is shown in Figure S1. A significant enrichment of the maize *COMT* gene promoter was detected (relative to *actin*) with αMYB31, whereas no enrichment was observed of the *COMT* 3′ untranslated region (UTR) (Figure 2a), demonstrating that ZmMYB31 binds to an element within this promoter during early maize sheath development *in vivo*.

The maize *A1* gene promoter is also a target of the MYB factors C1/PL1 (Sainz *et al.*, 1997), which activate *A1* gene expression through interaction with the bHLH proteins R/B (Goff *et al.*, 1992; Grotewold *et al.*, 1994). While C1/PL + R/B are likely to require a WD40 protein for robust flavonoid gene activation (Selinger and Chandler, 1999; Walker *et al.*, 1999), we have previously shown that in Black Mexican Sweet (BMS) maize cells, R + C1 efficiently activate transcription of all flavonoid biosynthetic genes (Grotewold *et al.*, 1998), suggesting that the corresponding maize WD40 protein PAC1 (Carey *et al.*, 2004) is expressed there. Therefore, we performed transient expression assays in maize BMS cells to determine if ZmMYB31 played any role in the regulation of *A1*, by fusing the *A1* promoter to the luciferase reporter (pA1::Luc). Our results show that, in the presence of ZmMYB31, the level of pA1::Luc activation by C1 + R is reduced threefold ($P < 0.03$) by ZmMYB31 (Figure 2b),

indicating that ZmMYB31 is acting as a repressor of *A1* gene transcription. In addition, ChIP assays using αMYB31 demonstrate that ZmMYB31 interacts with the maize *A1* gene promoter *in vivo* (Figure S2).

ZmMYB31 affects the total lignin content and downregulates several genes involved in monolignol biosynthesis when over-expressed in *A. thaliana*

Arabidopsis plants constitutively expressing ZmMYB31 (p35S::ZmMYB31) present severe phenotypic alterations at the macroscopic level: They grow to only about one-third of the size of control plants, show a proportional reduction of leaf, stalk and flower size and have delayed flowering (Figure 3a). Similar to what was observed for *AmMYB308*, *AmMYB330* and *AtMYB4* over-expression, p35S::ZmMYB31 plants also show white lesions on mature leaves with an upward cupping that becomes more evident in the older leaves (Figure 3a).

To obtain information about the lignin content and composition, cross-sections of the basal part of the inflorescence stem of wild-type and transgenic *A. thaliana* plants were treated with phloroglucinol and Maüle reagent (Figure 3b). In both cases, the intensity of the staining is reproducibly lower in the transgenic plants, indicating a strong reduction of the overall lignin content. In addition, a decrease in the cell wall thickness of both vessels and fibres and a reduced number of lignified cell layers (three in the transgenic plants versus five in the wild-type plants) is observed in p35S::ZmMYB31 plants, which also display an altered vascular anatomy (Figure 3b). In fact, the transgenic plants show an approximately three-fold increase in the size of their vessels (Figure 3b), but have a significantly reduced number (approximately 28 xylem vessels in the wild type versus 14–16 in the transgenic plants) (Figure 3b, bottom).

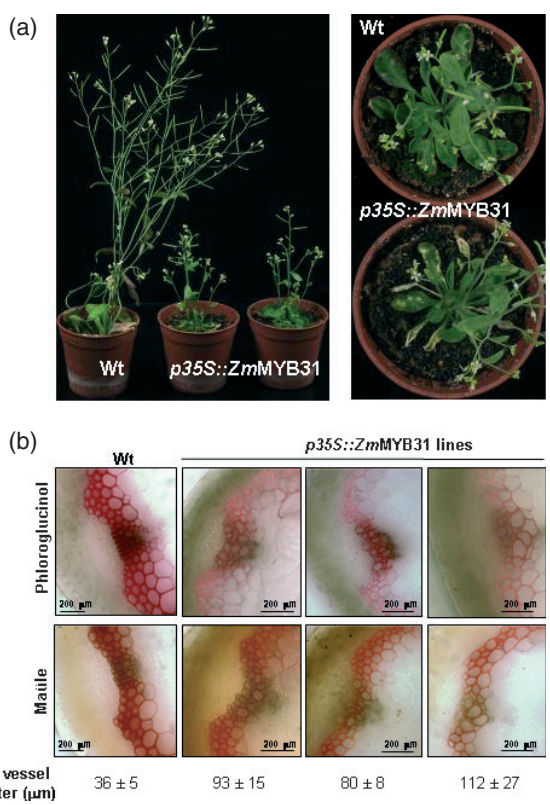

Figure 3. ZmMYB31 over-expression affects *Arabidopsis thaliana* growth.
(a) Phenotype of wild type (Wt) and two independent ZmMYB31 transgenic plants at the end of the inflorescence stage.
(b) Wiesner (top) and Maule (bottom) stainings of cross-sections of lignified stalks of Wt and three independent ZmMYB31 transgenic plants. Quantification of xylem vessel diameter was done using the ImageJ 1.38x program (http://rsb.info.nih.gov/ij/).

According to the results of the Maüle staining, which provides a qualitative assessment of the S and G subunit composition of lignin, ZmMYB31 did not produce a visible alteration of the S/G ratio (Figure 3b). To quantify these alterations, we determined the absolute lignin content by the Klason method. p35S::ZmMYB31 plants have a 70% reduction in total lignin content compared with wild-type plants (Table 1). The HPLC analyses of the cell wall phenolics obtained by the CuO oxidation method indicated that ZmMYB31 does not lead to substantial differences in terms of lignin composition (S/G ratio) with respect to control samples (Table 1), in agreement with the histological observations (Figure 3b). Nevertheless, a fourfold increase in H subunits was detected in the lignin of transgenic plants (Table 1). These H subunits represent a minor fraction of the *A. thaliana* lignin polymer while they constitute a major fraction of the maize lignin polymer.

In addition to the major lignin-derived compounds, the levels of *p*-coumaric and ferulic acids were analysed. While

Table 1 ZmMYB31 over-expression in *A. thaliana* alters total lignin content. Quantitative lignin content determination by the Klason method and HPLC analysis of lignin composition from wild-type and 35S::ZmMYB31 plants determined by the CuO oxidation method. Results are shown as relative amounts of each compound. Data correspond to the mean value ± SD of three independent assays

	Wild type	35S::ZmMYB31
Klason lignin (mg per gCWR)	117 ± 16	34 ± 14
Vanillic acid	1.93 ± 0.17%	3.25 ± 0.84%
Vanillin	56.13 ± 0.01%	54.46 ± 1.72%
Syringic acid	1.79 ± 0.18%	2.05 ± 0.14%
Syringaldehyde	39.24 ± 0.22%	36.92 ± 2.34%
p-Hydroxybenzaldehyde	0.62 ± 0.05%	2.03 ± 0.08%
p-Coumaric acid	0.05 ± 0.05%	0.12 ± 0.08%
Ferulic acid	0.85 ± 0.16%	3.21 ± 0.72%
H	0.6%	2.5%
G	58.4%	58.0%
S	41.0%	39.5%
S/G	0.70	0.68

H, *p*-hydroxybenzaldehyde; G, vanillin and vanillic acid; S, syringaldehyde and syringic acid, gCWR, grams of cell wall residue.

the endogenous levels of *p*-coumaric acid are not affected in p35S::ZmMYB31 plants, ferulic acid increases fourfold in transgenic plants compared with wild-type plants (Table 1).

ZmMYB31 downregulates four structural genes of the lignin pathway (Figure 4b): the *4CL-1* gene, belonging to the early steps of phenylpropanoid metabolism (Figure 4a), and *C3H*, *F5H* and *COMT* that belong to the different branches leading to the synthesis of the monolignols. The level of repression of these genes was further quantified by quantitative (q)RT-PCR. These results show that the Arabidopsis *C3H*, *4CL1*, *F5H* and *COMT* genes are weakly repressed (Figure 4c).

ZmMYB31 also interacts with the maize *F5H* gene promoter *in vivo*

Based on the repression pattern observed in the p35S::ZmMYB31 plants, we investigated by ChIP assays whether ZmMYB31 can interact *in vivo* with *C3H*, *4CL* and *F5H* genes in maize. A schematic representation of the amplified regions of these genes is shown in Figure S1. Although all these genes present at least one putative MYB31 DNA-binding motif in their promoters (Figure 5a), only a significant enrichment of the maize *F5H* gene promoter was detected with αMYB31, demonstrating that ZmMYB31 binds to this promoter in young maize leaf sheath tissues *in vivo* (Figure 5b). In contrast, no significant enrichment of the maize *4CL1* (and *4CL2* and *4CL3*) and *C3H* was observed, indicating that ZmMYB31 either does not interact directly with these genes' promoters in maize leaf sheath tissue (Figure 5b) or that the correct timing or conditions for binding were not met in our studies.

ZmMYB31 increases cell wall degradability without altering cell wall polysaccharides in *A. thaliana*

As lignin interacts with cell wall polysaccharides and putatively constitutes a competing carbon sink, we investigated whether the 70% reduction of lignin in p35S::ZmMYB31 plants affects the content and composition of cell wall polysaccharides. Therefore, we performed a GC analysis of the cell wall polysaccharides of flowering stems. Cell wall accounted for 49 and 51% of dry matter in 35S::ZmMYB31 and wild-type plants, respectively. The results indicate that no significant changes occur in the sugar composition and monosaccharide yield of transgenic cell walls compared with control plants (Table 2). Nevertheless, the large decrease in lignin content prompted us to determine whether the transgenic cell walls were more susceptible to enzymatic degradation. The results obtained indicate that

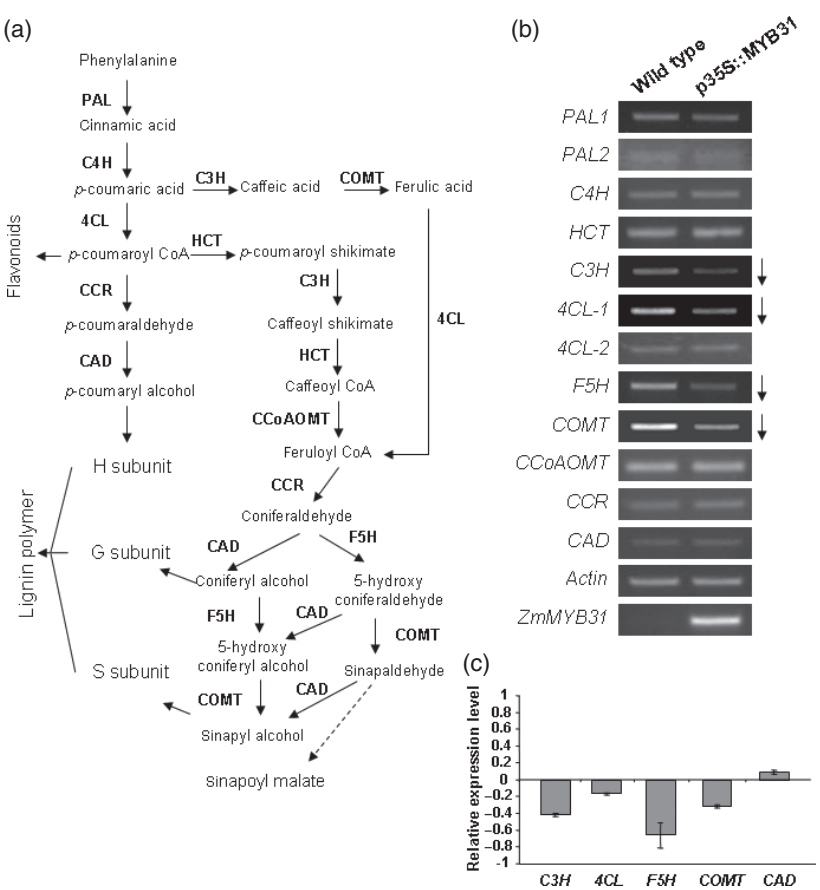

Figure 4. ZmMYB31 over-expression affects monolignol biosynthesis in *Arabidopsis thaliana*.
(a) The monolignol biosynthetic pathway. PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate-CoA ligase; CCR, cinnamoyl-CoA reductase; CAD, cinnamyl alcohol dehydrogenase; HCT, hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase; C3H, 4-coumarate 3-hydroxylase; COMT, caffeic acid *o*-methyltransferase; CCoAOMT, caffeoyl-CoA *o*-methyltransferase; F5H, ferulate-5-hydroxylase.
(b) Relative expression levels of all the monolignol biosynthetic genes.
(c) Quantification of the *A. thaliana C3H*, *4CL1*, *COMT* and *F5H* genes expression by quantitative RT-PCR.

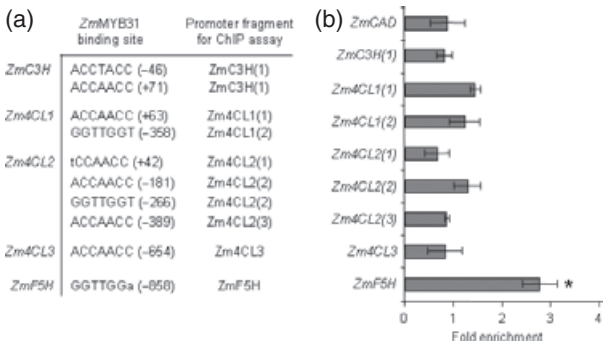

Figure 5. The ZmF5H gene is a direct target of ZmMYB31. (a) ZmMYB31 DNA-binding sequences present in maize lignin gene promoters. In brackets, distance from the putative transcription start site.
(b) Quantitative PCR analysis of chromatin immunoprecipitation (ChIP) DNA enrichment performed with purified ZmMYB31-specific antibodies using *ZmC3H*, *ZmF5H*, *Zm4CL1*, *Zm4CL2* and *Zm4CL3* as target genes. Enrichment of the gene promoter regions is relative to enrichment of the ZmActin. Statistical analysis of differences between samples was performed using a Student's *t*-test. *Significant at $P < 0.05$.

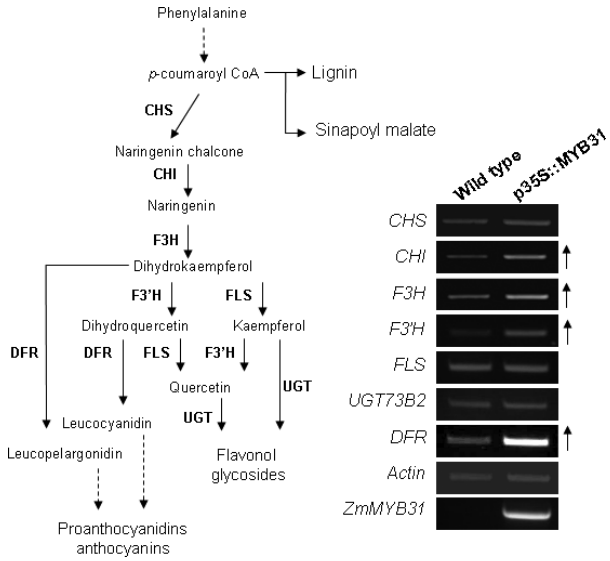

Figure 6. ZmMYB31 over-expression affects flavonoid biosynthesis in *Arabidopsis thaliana*.
(a) The flavonoid biosynthetic pathway. CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; FLS, flavonol synthase; UGTs, UDP sugar glycosyltransferases; DFR, dihydroflavonol reductase.
(b) Relative expression of the main genes involved in flavonol synthesis.

Table 2 ZmMYB31 over-expression in *Arabidopsis thaliana* does not affect cell wall structure and composition. Sugar analyses of cell walls from stems of wild-type and transgenic plants. Data are expressed as the mean value of six independent assays ± SD and resistance of cell walls to digestion with polysaccharide hydrolases. Data correspond to the mean value ± SD of three independent assays

(μg mg^{-1} dry cell wall)	Wild type	35S::ZmMYB31
Hemicelluloses		
Cellulose	451.2 ± 75.5	391.1 ± 74.4
Fucose	0.6 ± 0.1	0.9 ± 0.2
Arabinose	8.4 ± 2.1	9.3 ± 2.4
Xylose	181.8 ± 9.3	182.6 ± 9.8
Glucose	16.9 ± 8.6	14.1 ± 7.0
Mannose	14.3 ± 3.7	12.9 ± 3.3
Pectins		
Uronic acids	167.1 ± 9.72	173.8 ± 2.9
Rhamnose	5.6 ± 1.1	6.8 ± 2.1
Galactose	22.1 ± 1.2	21.2 ± 0.6
Carbohydrate released (mg g^{-1} cell wall)	572.1 ± 24.8	654.8 ± 11.5
Degradability ratio	1	1.14

p35S::ZmMYB31 plants display a 14% increase in cell wall degradability when treated with the cellulase–macerozyme–driselase enzymatic cocktail (Table 2).

ZmMYB31 induces anthocyanin production in *A. thaliana*

Since ZmMYB31 negatively regulates the *4CL-1* gene, the first committed step in phenylpropanoid biosynthesis, we investigated whether ZmMYB31 could also affect the biosynthesis of flavonoids in 14-day-old transgenic and wild-type plants. Results from RT-PCR experiments indicate that ZmMYB31 over-expression increases the expression of *CHI*, *F3H*, *F3′H* and *DFR* (Figure 6). This general up-regulation is in accordance with the 40% increase in the total soluble phenolics, and this result is mainly due to the large increase in the anthocyanin level, which almost doubled the levels present in wild-type plants, while flavonol levels remained practically unchanged (Figure 7a).

These results were further confirmed by HPLC–UV–electrospray ionisation (ESI)–tandem mass spectrometry (MS/MS) analyses of the main flavonoids (see the detailed procedure in Appendix S1), which indicate that p35S::ZmMYB31 plants produce higher levels of the main *A. thaliana* anthocyanins (Figure 7b).

ZmMYB31 represses sinapoylmalate biosynthesis in *A. thaliana*

Previous works showed that reduced expression of the *A. thaliana F5H* and *COMT* genes is accompanied by a decrease in the accumulation of sinapoylmalate, and this reduction confers a red fluorescence when plants are observed under UV light (Ruegger *et al.*, 1999; Franke *et al.*, 2002; Goujon *et al.*, 2003; Sonbol *et al.*, 2009). p35S::ZmMYB31 plants also present a repression of both *F5H* and *COMT* genes (Figure 4b) and display a clear red UV-fluorescence (Figure 8a), indicating a reduced accumulation of sinapoylmalate that was confirmed by HPLC–UV–ESI–MS/MS (see detailed procedure in Appendix S1) (Figure 8b).

As it was well established that a reduction in the endogenous sinapoylmalate levels increases the sensitivity of *A. thaliana* towards UV radiation (Jin *et al.*, 2000; Sonbol

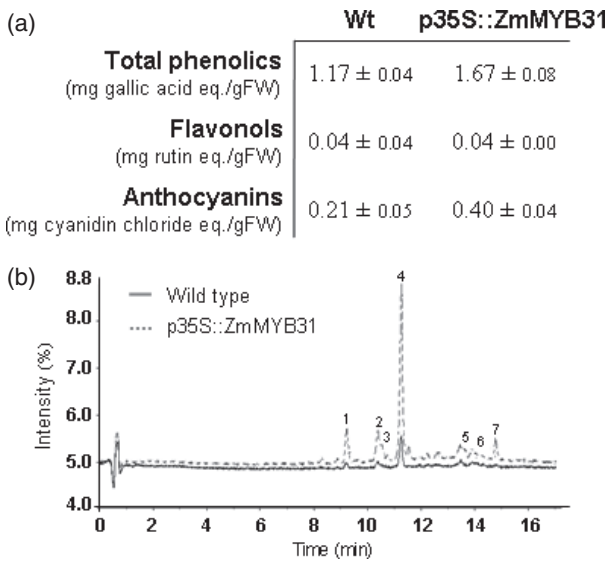

Figure 7. ZmMYB31 over-expression induces anthocyanin biosynthesis in *Arabidopsis thaliana*.
(a) Quantification of phenolic compounds from wild-type (Wt) and transgenic plants (eq means equivalents).
(b) A HPLC–diode array detection (DAD) analysis of anthocyanin content in stalks of Wt and transgenic plants (broken line). Peak identification: (1) cyanidin-3-*O*-[2″-*O*-(6‴-*O*-(sinapoyl)xylosyl)6″-*O*-(*p*-*O*-(glucosyl)-*p*-coumaroyl) glucoside]5-*O*-(6⁗-*O*-malonyl)glucoside; (2) cyanidin-3-*O*-[2″-*O*-(2‴-*O*-(sinapoyl)xylosyl)6″-*O*-(*p*-*O*-(glucosyl)-*p*-coumaroyl)glucoside]5-*O*-glucoside; (3) cyanidin-3-*O*-[2″-*O*-(xylosyl)-6″-*O*-(*p*-*O*-(glucosyl)-*p*-coumaroyl)glucoside]5-*O*-(6‴-O-malonyl)glucoside; (4) cyanidin-3-*O*-[2″-*O*-(6‴-*O*-(sinapoyl)xylosyl) 6″-*O*-(*p*-*O*-(glucosyl)-*p*-coumaroyl)glucoside]5-*O*-(6⁗-*O*-malonyl)glucoside; (5) cyanidin-3-*O*-[2″-*O*-(2‴-*O*-(sinapoyl)xylosyl)6″-*O*-(*p*-coumaroyl)glucoside] 5-*O*-glucoside; (6) cyanidin-3-*O*-[2″-*O*-(xylosyl)-6″-*O*-(-*p*-coumaroyl)glucoside]5-*O*-(6⁗-*O*-malonyl)glucoside; (7) cyanidin-3-*O*-[2″-*O*-(2‴-*O*-(sinapoyl) xylosyl)6″-*O*-(*p*-*O*-coumaroyl)glucoside]5-*O*-(6⁗-*O*-malonyl)glucoside.

et al., 2009), we treated wild-type and transgenic plants with UV-B light. The results obtained clearly indicate that ZmMYB31 affects the sensitivity of transgenic plants towards UV-B radiation, which resulted in no cell death in wild-type plants but extensive damage in the p35S::ZmMYB31 transgenic ones (Figure 8c).

Finally, we performed an expression analysis of the main genes of the sinapoylmalate pathway (Figure 7d) in 3-week-old transgenic and wild-type plants. Our results show that ZmMYB31 represses *SMT* and induces accumulation of *SGT* mRNA (Figure 8d).

ZmMYB31 induces the synthesis of stress-related proteins

Lignin is a critical component of vascular plants and its strong reduction in ZmMYB31 plants may produce alteration in several metabolic pathways. In fact, it has been shown that lignin downregulation in tobacco plants induces the activation of oxidative-stress pathways (Dauwe *et al.*, 2007). Hence, to gain further information on the alterations induced by the over-expression of *ZmMYB31*, we compared the proteome of transgenic and wild-type roots (see the detailed procedure in Appendix S1). On the whole, the intensity of 30 spots was consistently changed compared with the relative controls. Twenty of these spots were picked, and the identity of the 19 proteins was determined by matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS). Nine of them correspond to proteins induced and 11 to proteins repressed in ZmMYB31, compared with the wild-type plants (Table S2). The results obtained support the role of ZmMYB31 as a repressor of lignin synthesis, because one of the repressed proteins corresponds to AtCOMT. The proteins whose expression was significantly increased by ZmMYB31 are related to cell wall metabolism (glucanases, chitinase) and stress responses (osmotin, annexin, glutathione *S*-transferase).

DISCUSSION

The maize ZmMYB31 transcription factor belongs to subgroup 4 of the R2R3-MYB family and downregulates the maize *COMT* gene when over-expressed in *A. thaliana* (Fornalé *et al.*, 2006). In this work, using SELEX assays, we defined the consensus DNA-binding site of ZmMYB31 and showed that it corresponds to the canonical AC-II element ($CC^T/_CA^C/_AC$) recognised by R2R3-MYB factors. Accordingly, ZmMYB31 interacts with the AC-II element of the maize *COMT* gene promoter *in vitro* and ChIP assays demonstrated that ZmMYB31 interacts with the lignin *ZmCOMT* and *ZmF5H* genes and flavonoid *ZmA1* gene promoters *in vivo*. Indeed, ZmMYB31 represses *ZmA1* gene expression, perhaps by competing with the C1 + R activators for the same DNA-binding element.

ZmMYB31 downregulates the *A. thaliana COMT* (Fornalé *et al.*, 2006) and *F5H* genes. The *A. thaliana F5H* gene promoter contains an ACCTACC motif 82 bp from the ATG, suggesting that ZmMYB31 is directly regulating its expression. For the *AtCOMT* gene, gel shift assays show that ZmMYB31 does not bind to the MYB type-I *cis*-element (the only MYB-binding motif present in the *AtCOMT* gene promoter). Therefore, these results suggest an indirect effect of ZmMYB31 on the expression of the *AtCOMT* gene and show an evolutionary divergence between the regulation of the maize and the *A. thaliana COMT* genes by R2R3-MYB factors.

ZmMYB31 also downregulates the *A. thaliana* C*3H and 4CL1* genes in transgenic plants. As no direct interaction is found in maize, the weak repression of *A. thaliana C3H* and *4CL1* gene expression could be attributed to the high levels of ZmMYB31 expression in the transgenics. Alternatively, changes in phenolic metabolism, rather than ZmMYB31 itself, might affect the steady-state mRNA levels for these genes. Similar results were observed with the AtMYB4 factor; while *C4H* is the only gene affected in the mutant *atmyb4* plants, the over-expression of this R2R3-MYB factor also affects the expression of *4CL1*, *4CL3* and *CHS* (Jin *et al.*, 2000).

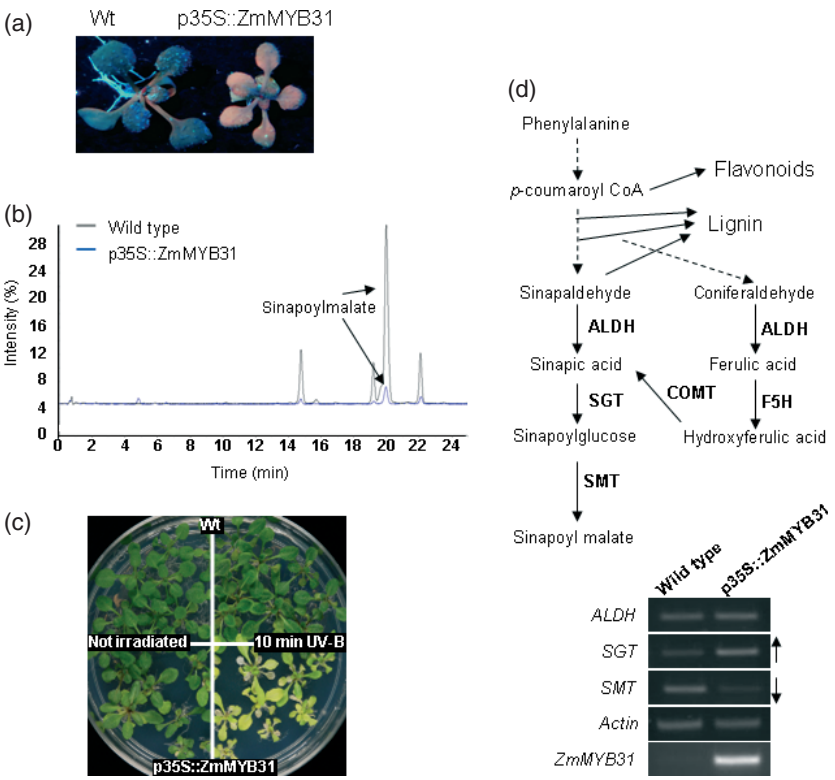

Figure 8. ZmMYB31 over-expression represses sinapoylmalate biosynthesis in *Arabidopsis thaliana*.
(a) Phenotype of wild-type (Wt) and transgenic plantlets exposed to UV light.
(b) A HPLC–diode array detection (DAD) analysis of sinapoylmalate content in leaves of Wt and transgenic plants.
(c) Phenotype of 20-day-old Wt and transgenic plantlets submitted to 10-min UV irradiation.
(d) Relative expression of the genes involved in sinapoylmalate synthesis.

The repression of this set of genes in transgenic plant leads to a 70% reduction in the total lignin content and resulted in severe phenotypic effects. It has already been reported that the downregulation of C3H, 4CL1, F5H or COMT enzymes in *A. thaliana* affects the final lignin composition (Chapple *et al.*, 1992; Lee *et al.*, 1997; Meyer *et al.*, 1998; Franke *et al.*, 2002; Goujon *et al.*, 2003). The *A. thaliana* mutant *ref8* (lacking C3H activity) has a strong reduction in lignin content and a block in the synthesis of the S and G subunits, causing the deposition of an unusual lignin highly enriched in H subunits (Franke *et al.*, 2002). In the case of the *4CL* gene, *A. thaliana*, having 20% enzymatic activity, displays a significant reduction in the lignin content and an increased S/G ratio (Lee *et al.*, 1997), while the characterisation of *fah1* (F5H) and *Atcomt1* (COMT) mutants revealed their crucial roles in the synthesis of the S subunits (Chapple *et al.*, 1992; Meyer *et al.*, 1998; Goujon *et al.*, 2003). Analogously, the synthesis of a lignin greatly depleted in S subunits has been also detected in transgenic tobacco and poplar plants with suppressed COMT activities (Atanassova *et al.*, 1995; Van Doorsselaere *et al.*, 1995). We recently described that the over-expression of ZmMYB42 in *A. thaliana* strongly reduces the S/G ratio of the lignin polymer and also downregulates *4CL*, *COMT* and *F5H* genes (Sonbol *et al.*, 2009). ZmMYB31 downregulates *C3H*, *4CL*, *COMT* and *F5H* genes, but the resulting S/G composition of the lignin polymer is not modified. The strong reduction in lignin content and the absence of significant changes in the composition of the final polymer are likely to depend on the general repression produced by ZmMYB31 on each of the branches leading to the synthesis of the main monolignols. In this context, the fourfold increase in H subunits observed in the transgenic plants could be the result of a preferential repression of the branches leading to the biosynthesis of the G and S monolignols.

The strong repression of lignin synthesis led us to hypothesise on the occurrence of changes in the content and/or composition of the cell wall polysaccharides in p35S::ZmMYB31 plants, but the results obtained indicate that, despite the 70% reduction of lignin content, the polysaccharide matrix of the transgenic walls remains unchanged. Nevertheless, a 14% increase of the enzymatic degradability of the transgenic cell walls was observed indicating that access to the cellulose matrix by the enzymes had been increased slightly.

In the case of ZmMYB42, transgenic plants over-expressing this factor have a 60% reduction in lignin content, a strong decrease in the S/G ratio and a 68% increase in the enzymatic degradability of the cell walls (Sonbol *et al.*, 2009). This fivefold increase in cell wall degradability compared with ZmMYB31 plants (even when ZmMYB42 plants have twice the amount of lignin as ZmMYB31 plants) suggests that the combined effect on lignin content and composition is a more efficient way to increase the degradability of the cell walls than just reducing the lignin content only.

Several studies demonstrated that a reduced *COMT* (Goujon *et al.*, 2003) and *F5H* (Meyer *et al.*, 1998; Ruegger *et al.*, 1999) in *A. thaliana* decreased the level of sinapoylmalate, and these two genes are downregulated by ZmMYB31. Accordingly, ZmMYB31 plants accumulate reduced levels of this compound and are much more sensitive to UV irradiation, despite the huge increase in anthocyanin. This result is in accordance with previous studies (Landry *et al.*, 1995), which showed that sinapate esters are more important than flavonoids in preventing UV-B injury in *A. thaliana*, as demonstrated by the greater sensitivity of the F5H-deficient *fah1* (defective in sinapate esters) over the CHI-deficient *tt5* mutant (defective in flavonoid synthesis). The increase in anthocyanin levels in p35S::ZmMYB31 plants indicates that the reduction in lignin synthesis redirects the metabolic flux along the phenylpropanoid pathway towards other branches, as previously reported (Dauwe *et al.*, 2007; Mir Derikvand *et al.*, 2008).

It has been shown that the growth reduction associated with repressed lignin biosynthesis in *A. thaliana* is independent of flavonoids (Li *et al.*, 2010). Thus, the dwarf phenotype of p35S::ZmMYB31 seems to be mainly caused by the strong reduction of the total lignin content plants, rather than the change in flavonoids, similarly to what observed in the case of *A. thaliana* plants over-expressing ZmMYB42 (Sonbol *et al.*, 2009).

Previous works suggested that the appearance of white lesions in the leaf of tobacco plants over-expressing the R2R3MYB factors AmMYB308 and AtMYB4 could be related to the inhibition of phenolic acid metabolism (Tamagnone *et al.*, 1998a,b; Jin *et al.*, 2000). The presence of these lesions in the leaves of plants over-expressing ZmMYB31 suggests that this phenotype is likely to be linked to other factors, because these plants accumulate higher levels of total phenolics than the wild-type plants. ZmMYB31 plants present upward leaf curling and a similar phenotype has already been reported in plants with reduced levels of UV-protectant after exposure to UV radiation (Landry *et al.*, 1995; Sonbol *et al.*, 2009).

Lignin is fundamental to the structural integrity of the cell wall and for the strength of the stem of the vascular plants (Chabannes *et al.*, 2001), and it is likely that any alteration in its content affects the general metabolism of the plant. Proteomic studies revealed that ZmMYB31 induces a protein pattern that resembles the one expected in a typical stress response. In fact, plants react against environmental stresses and pathogen infection with a variety of biochemical and physiological adaptations including cell wall lignification, the synthesis of phytoalexins, the enhanced expression of genes encoding pathogenesis-related (PR) proteins and hydrolytic enzymes (Hwang *et al.*, 2007). Accordingly, ZmMYB31 induces the accumulation of osmotin, a PR protein whose expression can be activated by microbial infections and by a variety of abiotic stress factors (Zhu *et al.*, 1995; Velazhahan *et al.*, 1999), of an endochitinase and of β-glucanases.

ZmMYB31 plants accumulate higher levels of an annexin, a multifunctional membrane protein which participates in diverse physiological processes (Clark and Roux, 1995) including the response to abiotic stresses (Kovács *et al.*, 1998; Gorecka *et al.*, 2007). Glutathione *S*-transferases (GSTs) are also induced in ZmMYB31 plants. These proteins, among others, play an important role in protecting plants against oxidative damage (reviewed by Frova, 2006) and in flavonoid metabolism (Loyall *et al.*, 2000; Smith *et al.*, 2003) and their expression is induced by many biotic and abiotic stresses (Marrs, 1996; Dixon *et al.*, 1998; Wagner *et al.*, 2002).

Among the proteins whose expression is downregulated by ZmMYB31, a remarkable finding is the identification of the *A. thaliana* COMT protein, in line with the role of ZmMYB31 as a repressor of this lignin gene (Fornalé *et al.*, 2006). ZmMYB31 downregulates an aconitase: this class of proteins is involved in the resistance to oxidative stress; it has been reported that *A. thaliana* aconitase knockout plants are more tolerant to oxidative stress (Moeder *et al.*, 2007).

Altogether, it is likely that the reduction in lignin content by ZmMYB31 triggers signalling pathways leading to the activation of stress genes; this is in accordance with a previous work reporting a wound-like response, characterised by the induction of reactive oxygen species (ROS) scavengers and of detoxifying enzymes such as GST, in transgenic CCR-downregulated poplar plants displaying a 50% reduction of lignin (Leplé *et al.*, 2007). Furthermore, the activation of oxidative stress pathways in CCR-downregulated tobacco plants has been reported (Dauwe *et al.*, 2007). Nevertheless, in our case, it cannot be excluded that the stress response observed in ZmMYB31 plants could also depend on their increased UV sensitivity.

Thus, ZmMYB31 seems to play an important role in carbon partitioning along the phenylpropanoid pathway. Its over-expression greatly reduces the synthesis of lignin and represses the accumulation of sinapoylmalate, redirecting the metabolic flux toward flavonoid biosynthesis. The great reduction in lignin content could account for the dwarf phenotype of the transgenic plants. Finally, the alterations induced by the over-expression of *ZmMYB31* induce a general stress response with the accumulation of stress-related proteins.

The combined information arising from the characterisation of ZmMYB42 (Sonbol *et al.*, 2009) and ZmMYB31 shows that these two factors play non-redundant functions in the regulation of the phenylpropanoid pathway, even when they are phylogenetically closely related.

Taking all this together, in this work we report a direct interaction of a R2R3-MYB transcription factor with the *ZmCOMT* and *ZmF5H* lignin gene promoters *in vivo*. The functional characterisation of ZmMYB31 in *A. thaliana*

indicates that this factor strongly represses lignin synthesis without affecting the S/G ratio and affects the expression of several stress-related proteins. Transgenic plants display enhanced cell wall degradability that could have significant consequences for the manipulation of the lignin polymer for different biotechnological applications.

EXPERIMENTAL PROCEDURES

Plant material, UV-B treatment and measurement of soluble phenolics

Maize plants corresponding to the B73 inbred line, and Arabidopsis (Columbia) plants were grown under standard condition in the greenhouse and on MS plates (16 h light).

The UV-B treatment was performed according to Jin *et al.* (2000). Total phenolic quantification was performed as described previously (Sonbol *et al.*, 2009). Phenolic content was determined from standard curves obtained using dilutions of gallic acid, rutin and cyanidin chloride at 280, 360 and 520 nm, respectively.

Histology

Cross-sections (150 μm thick) in the basal part of inflorescence stems were obtained using a Vibratome (Vibratome Series 1000, http://www.vibratome.com). Sections were observed either under UV-excitation [4′6-diamidino-2-phenylindole (DAPI) filter] or under bright-field after phloroglucinol and Maüle staining that were performed as already described (Sonbol *et al.*, 2009). Images were taken using a Leica Stereo-microscope equipped with a Leica DC 200 camera and further analysed with the Leica Confocal Lite Software program (Leica, http://www.leica.com/).

Isolation of total RNA, RT-PCR and PCR and qRT-PCR

Total RNA was extracted with TRIzol reagent according to the manufacturer's instructions (Invitrogen, http://www.invitrogen.com/). The RT-PCR was performed using oligo dT and a no-RT negative control. Gene-specific primers were used to analyse the transcript levels of the genes belonging to the *A. thaliana* 'lignin toolbox' (Raes *et al.*, 2003). All the gene-specific primers used in this work are listed in Table S3.

Quantitative RT-PCR assays were run for *At4CL1*, *AtF5H*, *AtC3H*, *AtCOMT* and *AtCAD* and *AtActin* genes. Assays were performed from two aliquots of RNA preparations from root tissues and from two separate extractions, totalling four biological replicates and three technical (qRT-PCR) replicates for each of them. Quantitative RT-PCR reactions were performed using the Light Cycler® 480 (Roche, http://www.roche.com/) and LC480 SYBR®Green I Master (Roche). Gene-specific primers were designed using the Primer Express software (Applied Biosystems, http://www.appliedbiosystems.com/). The PCR conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles of a denaturation step at 95°C for 10 sec and an annealing/extension step at 60°C for 30 sec.

Cell wall analyses and degradability assay

Lignin content (Klason method), lignin composition and cell wall polysaccharide assays from *A. thaliana* mature stems were measured and performed as already described (Sonbol *et al.*, 2009).

For the cell wall degradability assays, cell walls were hydrolysed (20 mg per 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).

Systematic evolution of ligands by exponential enrichment (SELEX)

The SELEX procedure was performed as previously described (Grotewold *et al.*, 1994). After the last (seventh) round of selection, the DNA from the shifted protein–DNA complex was extracted, PCR amplified and cloned into a pCR®-2.1-TOPO vector (Invitrogen) and then sequenced. The sequences extracted from sequencing were analyzed by both manual inspection and using a Gibbs Motif Sampler (http://bayesweb.wadsworth.org/gibbs/gibbs.html) and displayed with Weblogo (http://weblogo.berkeley.edu/logo.cgi).

Chromatin immunoprecipitation (ChIP) experiments

The ChIP experiments were performed as described previously (Morohashi *et al.*, 2007; Ferreyra *et al.*, 2010). Immunoprecipitation was performed overnight at 4°C with either 2 μl of IgG, or 5 μl of purified αMYB31 and the reverse cross-linked DNA was then purified. For enrichment tests on ChIPed material, primers for *ZmCOMT*, *ZmC3H*, *Zm4CL1*, *Zm4CL2*, *Zm4CL3*, *ZmF5H*, *ZmCAD* and *actin* for a negative control were used in PCR reactions. Enrichment was assessed by quantitative (real-time) PCR (QPCR), performed using iQ® SYBR Green supermix (Bio-Rad, http://www.bio-rad.com/) in a 20 μl QPCR reaction according to the manufacturer's protocols. The PCR amplification was performed on a Bio-Rad CFX96 real-time PCR detection system (Applied Biosystems) with an initial 95°C, 3-min denaturation followed by 40 cycles of 95°C, 10 sec; 60°C, 15 sec; 72°C, 10 sec, followed by a melting curve determination. In all experiments, no-template controls and input samples were used for every primer set. Target enrichment was measured by dividing the change in $C_{\Delta}T$ values (input minus ChIP) for the *ZmMYB31* promoter region by the change in $C_{\Delta}T$ values for the *ZmActin* gene. Non-specific precipitation and enrichment were determined using anti-IgG antiserum. The maize *actin1* gene UTR (bp 10–129 of GenBank accession number J01238) was used as the negative control in these experiments.

Transient expression experiments in maize cells

All plant expression vectors (p35S::ZmMYB31, p35S::C1, p35S::R) include the cauliflower mosaic virus 35S promoter, the tobacco mosaic virus Ω′ leader and the maize first Adh1-S intron in the 5′ UTR, and the potato proteinase II (pinII) termination signal. The full length of ZmMYB31 was generated by PCR and then cloned into the DP471 (p35S::R) vector as a *Bam*HI fragment. Previously described plasmids include p35S::C1, p35S::R and pA1::Luc (Grotewold *et al.*, 1994; Sainz *et al.*, 1997). p35S::BAR (pPHP611) was used for normalising the concentration of p35S sequences delivered in each bombardment (Grotewold *et al.*, 1998). Microprojectile bombardment of maize BMS suspension cells and transient expression assays for luciferase were performed as previously described (Sainz *et al.*, 1997). For transient expression assays of firefly luciferase and *Renilla* luciferase (*Renilla*), the Dual-Luciferase Reporter Assay System (Promega, http://www.promega.com/) was used. For each microprojectile preparation, the mass of DNA was adjusted to 10 μg with p35S::BAR to equalise the amount of 35S promoter in each bombardment. One microgram of each regulator and 3 μg of reporter plasmid (pA1::Luc) were used in each bombardment. Each treatment was done in at least four replicates.

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SUPPORTING INFORMATION

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

Figure S1. Schematic representation of the gene promoters used for chromatin immunoprecipitation (ChIP) assays.

Figure S2. The *ZmA1* gene is a direct target of ZmMYB31.

Table S1. Systematic evolution of ligands by exponential enrichment (SELEX) sequences.

Table S2. Sequence and accession number of the gene-specific primers used in this work.

Table S3. ZmMYB31 over-expression in *Arabidopsis thaliana* induces the synthesis of stress-related proteins.

Appendix S1. Experimental procedures.

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