The Heterologous Expression of the Chrysanthemum R2R3-MYB Transcription Factor CmMYB1 Alters Lignin Composition and Represses Flavonoid Synthesis in Arabidopsis thaliana

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Abstract

Plant R2R3-MYB transcription factor genes are widely distributed in higher plants and play important roles in the regulation of many secondary metabolites at the transcriptional level. In this study, a chrysanthemum subgroup 4 R2R3-MYB transcription factor gene, designated CmMYB1, was isolated through screening chrysanthemum EST (expressed sequence tag) libraries and using rapid application of cDNA ends (RACE) methods and functionally characterized. CmMYB1 is expressed in the root, stem, leaf and flowers, but most strongly in the stem and most weakly in the root. Its heterologous expression in Arabidopsis thaliana reduced the lignin content and altered the lignin composition. The heterologous expression also repressed the flavonoids content in A. thaliana. Together, these results suggested that CmMYB1 is a negative regulator of genes involved in the lignin pathway and flavonoid pathway, it may be a promising gene for controlling lignin and flavonoids profiles in plants.

Introduction

Transcription factors play critical roles in regulating plant development and response to environmental stress. The structure of the DNA binding sequences of transcription factors has allowed the recognition of several distinct gene families [1]. In plants, MYB family is one of the most abundant transcription factor classes [2]. Among approximately 1500 transcription factor genes identified in the Arabidopsis thaliana genome, 130 belong to the MYB members [3,4]. The MYB DNA binding domain consists of one to three imperfect 50–53 residue repeats [5], and the number of these repeats present has been used to categorize the family into the subgroups R2R3-MYB, R2R3-MYB and a MYB-related group; of these, R2R3-MYB is the most commonly occurring type [6,7]. Based on variation within certain conserved C-terminal motifs, the R2R3-MYBs have been classified into 22 sub-groups [8], with members within each sub-group predicted to share similar or identical functions [9].

The complex polyphenol molecule lignin is deposited in the secondary cell walls of all vascular plants and is synthesized through the lignin biosynthesis pathway, a major branch of the phenylpropanoid pathway [10]. Its accumulation improves the efficiency of water transport, increases the stiffness of mechanical tissues, and forms a physical barrier against pathogens and wounding [10–12]. The lignin synthesis is heavily regulated by R2R3-MYB transcription factors [13,14]. Several R2R3-MYB transcription factors belonging to different subgroups such as Arabidopsis AtMYBPAP1 [15], Populus trichocarpa PtMYB1 and PtMYB4 [16,17], poplar PthMYB21a [18], Eucalyptus gunnii EgmMYB2 [19] and Vitis vinifera VvMYB5a [20] have already been shown to either positively or negatively control the lignin biosynthesis through their in vivo interaction with ACI, ACH, and ACIII cis-elements. So far, all the repressors of lignin synthesis appear to be confined to subgroup 4. For example, the Antirrhinum majus AmMYB306 and AmMYB330 were the first R2R3-MYB factors associated with the down-regulation of lignification. When either of the MYB proteins was heterologously expressed in tobacco, the vascular tissue lignin content was markedly reduced, and transcript abundance of the genes encoding 4-coumarateCoA ligase (4CL1), cinnamate-4-hydroxylase (CAD1) and cinnamyl alcohol dehydrogenase (CAD) was reduced [21]. Similarly, CAH expression was enhanced in the A. thaliana knockout mutant Amyb4 while that of CCoAOMT (caffeoyl-CoA O-methyltransferase) was reduced [22]; meanwhile in the Amyb32 mutant, the gene encoding caffeic acid O-methyl-
transferase (COMT) was up-regulated [23]. The two *Zea mays* subgroup 4 R2R3-MYB factors *ZmMYB31* and *ZmMYB42* also act as repressors of lignin synthesis [24,25], and the heterologous expression of *ZmMYB42* in *A. thaliana* appears to reduce lignin content, alter lignin composition and repress flavonoids biosynthesis [26]. Similarly, the over-expression of *ZmMYB31* significantly reduces lignin content with alter polymer composition, and enhances *CHI, F3H, F3’H* and *DFR* gene expression in *A. thaliana* [25]. Finally, *EgMYB1* expression reduces lignin synthesis in both transgenic *A. thaliana* and *P. trichocarpa* [27,28].

Chrysanthemum (*Chrysanthemum morifolium*) is a commercially important ornamental plant worldwide. Here, we describe the isolation of the subgroup 4 R2R3-MYB transcription factor *CmMYB1*. Its participation in the regulation of lignin synthesis and flavonoid synthesis was demonstrated by its heterologous expression in *A. thaliana*. A series of gene expression experiments demonstrated that several genes in the lignin synthesis pathway and flavonoid biosynthesis were down-regulated by the presence of the *CmMYB1* transgene, leading to a reduction in the lignin content, lignin compositions as well as a decrease in the flavonoids content in the transgenic plants.

**Materials and Methods**

**Plant Materials and Plant Growing Conditions**

The chrysanthemum variety ‘Zhongshanzigui’ was obtained from the Chrysanthemum Germplasm Resource Preserving Centre, Nanjing Agricultural University, China. Plants were grown in a 1:1 mixture of garden soil and vermiculite without any additional fertilizer, and were maintained in a greenhouse under standard growing conditions. Young plants were watered daily, and fertilized weekly with half strength Hoagland’s nutrient solution. The expression profile of *CmMYB1* was determined in the root, stem, young leaf and ray floret of each of three chrysanthemum plants, using RNA extracted from snap-frozen fresh plant material. *A. thaliana* ecotype Col-0 plants were grown in a 1:1:1 mixture of perlite:vermiculite:soilrite in a growth chamber set to deliver a 16 h photoperiod at 23°C during the light period (80–100 μmol m⁻² s⁻¹ illumination) and 18°C during the dark period. The plants were watered every 4 days. To determine the expression level of various lignin synthetic genes, RNA was extracted from root, stem, young leaf, and ray floret of each of the three chrysanthemum plants and from the three biological replicates.

![Figure 1](image1.png) **Figure 1.** The deduced peptide sequence of *CmMYB1* (marked in bold) and related MYBs. a. Peptide alignment. R2 and R3 MYB DNA binding domains are shown underlined. C1 motif: LLsrGIDPX[T/S]HRX[I/L]. C2 motif: pdLNL[D/E]LXi[G/S]. C4 motif: GYDFLG[L/M]X 4–7LX[Y/F][R/S]XLEMK. Zing finger motif: CX1–2CX7–12CX1–2C; b. The phylogeny of *CmMYB1* and related MYBs. Bootstrap values of each branch of the derived tree are given, and the scale bar represents 0.02 substitutions per site. The genes encoding the amino acid sequences and their GenBank accession numbers are: *VvMYB4a* (XP_002278222), *GhMYB9* (AAK19619), *ZmMYB31* (NP_001105949), *AtMYB4* (AAS10085), *AmMYB308* (P81393), *GmMYB48* (ABH02823), *TaMYB1* (AA37167), *Os09g0538400* (NP_001063796), *OsMYB1* (AA23337), *AtMYB32* (NP_195225), *PgMYB5* (ABO1221), *HvMYB1* (P20026), *HvMYB5* (CAAS01221), *TMYB1* (AA19475), *ZmMYB42* (NP_001106009), *AmMYB30* (P81393), *AtMYB8* (NP_192684), *EgMYB1* (CAE09055), *GhMYB1* (AAN22270), *TMYB2* (AA19476). *CmMYB1* is in bold. LR: R2R3-MYB transcription factors characterized as repressors of lignin synthesis. doi:10.1371/journal.pone.0065680.g001

![Figure 2](image2.png) **Figure 2.** The expression of *CmMYB1* in various chrysanthemum tissues. Values shown are means ± SE, as calculated from three biological replicates. doi:10.1371/journal.pone.0065680.g002
The CmMYB1 peptide sequence was aligned with that of its presumed homologues using ClustalW [31]. A neighbour-joining based phylogenetic tree was constructed using MEGAS [32].

Quantitative Real-time PCR (qRT-PCR)

CmMYB1 expression profiles were inferred from qRT-PCR outputs. Total RNA was extracted using the RNAiso reagent (TaKaRa, Japan), treated with DNaseI to remove any contaminating genomic DNA and converted into cDNA using SuperScript III reverse transcriptase (Invitrogen, USA). The qRT-PCR used SYBR® Green I (TOYOBO, Japan). The primer pair MYB1-RT-F/-R (table S1 in file S1) was applied to amplify a 178 bp fragment in the 3′ region of the gene, avoiding the more well-conserved segments of the gene. A portion of the chrysanthemum GAPDH sequence (DK941612), amplified with primers CmGAPDH-F/-R, was used as a reference. Each 25 µl qRT-PCR contained 10 µl SYBR Green PCR master mix, 0.2 µM of each primer and 10 ng cDNA, and the amplification regime consisted of an initial denaturation of 95 °C/60 s, followed by 40 cycles of 95 °C/15 s, 60 °C/15 s, 72 °C/45 s. The resulting data are given as means ± SE of three biological replicates. Relative expression levels were calculated based on the 2^(-ΔΔCT) method [33,34]. The expression levels of genes involved in the synthesis of lignin, cellulose and xylan, and flavonoid were also derived by qRT-PCR.

Vector Construction and A. thaliana Transformation

The CmMYB1 coding sequence was amplified using a forward primer (MYB1-1301-F) incorporating the sequence of the adaptor primer. Then a nested PCR was employed, using the gene-specific primer pair GSP3′-1/-2 and the adaptor primer (dIT-AP). For the 5′ RACE reaction, the nested PCR was based on the 5′ RACE adaptor primer (Abridged Anchor Primer, AAP), the Abridged Universal Amplification Primer (AUAP) provided with the 5′ RACE System kit v2.0 (Invitrogen) and the gene-specific primers (GSP5′-1, GSP5′-2 and GSP5′-3). PCR products were purified using a Biospin Gel Extraction kit (Bio Flux) and cloned into the pMD19-T easy plasmid (TaKaRa) for DNA sequencing. Finally, a pair of gene-specific primers (Full-F/Full-R), designed from the putative 5′ and 3′ UTR sequences, was used to amplify the complete CmMYB1 open reading frame. The sequences of all the above primers are given in table S1 in file S1. Chrysanthemum genomic DNA was isolated from young leaves using a CTAB method [30], and the genomic sequence of CmMYB1 was amplified using the primer pair Full-F/Full-R. The resulting product was purified using a Biospin Gel Extraction kit and cloned into pMD19-T easy for sequencing.

Figure 3. Lignin synthesis in transgenic and wild type A. thaliana plants. a. RT-PCR demonstrates the heterologous expression of CmMYB1 in the transgenic lines. The AtUBQ sequence was used as an internal control; b. The histochemical detection of lignin in wild type Col-0 (WT) and transgenic A. thaliana plants expressing CmMYB1 (35S::CmMYB1-1 and 35S::CmMYB1-2). Lignified tissues are stained red with phloroglucinol-HCl. The image shown is representative of ten observations. Co: cortex; F: interfascicular fibres; X: xylem; M: medular parenchyma.

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extracted from snap-frozen intact plants of three week old whole plants Col-0 and transgenic A. thaliana plants.

Full-length cDNA Isolation and Sequencing

Total RNA was isolated from chrysanthemum leaves using the RNAiso reagent (TaKaRa, Japan), following the manufacturer’s instructions. The cDNA first strand was synthesized from 1 µg total RNA using SuperScript III reverse transcriptase (Invitrogen, USA), according to the manufacturer’s instructions. A gene-specific primer pair (F/R) was designed to amplify a fragment of CmMYB1 based on the sequence of a chrysanthemum EST (DK942906) [29], and RACE PCR was then used to obtain the full length cDNA. For the 3′ RACE reaction, the first strand was synthesized using an oligo (dT) primer incorporating the sequence of the adaptor primer. Then a nested PCR was employed, using the gene-specific primer pair GSP3′-1/-2 and the adaptor primer (dIT-AP). For the 5′ RACE reaction, the nested PCR was based on the 5′ RACE adaptor primer (Abridged Anchor Primer, AAP), the Abridged Universal Amplification Primer (AUAP) provided with the 5′ RACE System kit v2.0 (Invitrogen) and the gene-specific primers (GSP5′-1, GSP5′-2 and GSP5′-3). PCR products were purified using a Biospin Gel Extraction kit (Bio Flux) and cloned into the pMD19-T easy plasmid (TaKaRa) for DNA sequencing. Finally, a pair of gene-specific primers (Full-F/Full-R), designed from the putative 5′ and 3′ UTR sequences, was used to amplify the complete CmMYB1 open reading frame. The sequences of all the above primers are given in table S1 in file S1. Chrysanthemum genomic DNA was isolated from young leaves using a CTAB method [30], and the genomic sequence of CmMYB1 was amplified using the primer pair Full-F/Full-R. The resulting product was purified using a Biospin Gel Extraction kit and cloned into pMD19-T easy for sequencing.

Sequence Alignment and Phylogenetic Analysis of CmMYB1

The CmMYB1 peptide sequence was aligned with that of its presumed homologues using ClustalW [31]. A neighbour-joining based phylogenetic tree was constructed using MEGAS [32].
pCAMBIA1301 to generate a 35S::CmMYB1 construct, which was introduced into Col-0 via an Agrobacterium tumefaciens EHA105-mediated oral dip method [37]. Transformed progeny were selected by germination on a standard medium containing 50 μg mL⁻¹ hygromycin, and confirmed by subsequent RT-PCR analysis.

**Lignin Analysis**

Stem cross-sections of ten 35S::CmMYB1 transgenic A. thaliana plants and ten wild type plants were prepared using a scalpel blade, respectively. Sections of thickness 70 μm were mounted on a microscope slide and kept moist by the addition of distilled water. After removal of all excess water, a few drops of 6 mol.L⁻¹ HCl were placed on the sections and left for 3 min. Thereafter, a few drops of 5% (w/v) phloroglucinol-HCl were added and the sample covered with a cover slip for 10 min [38]. Phloroglucinol-HCl reacts with the hydroxycinnamaldehyde and benzaldehyde groups of lignin, and the intensity of the red stain generated by this reaction roughly reflects total lignin content [39]. The samples were inspected by light microscopy.

Total lignin content was determined by the spectrophotometric acetyl bromide lignin method with modifications [40]. The samples were determined for three biological replicates, each with 60 mg of drying stem. The cell walls of samples were isolated and then extracted in 100 μL acetyl bromide (25% v/v acetyl bromide in glacial acetic acid) at 50°C for 2 h, and cooled on ice to room temperature. Subsequently, add 400 μL 2 mol.L⁻¹ NaOH and 70 μL of freshly prepared 0.5 mol.L⁻¹ hydroxylamine hydrochloride to the cooled samples, vortex volumetric flasks. Fill up volumetric flask exactly to the 2 ml with glacial acetic acid, cap

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**Figure 4.** Expression profiles of genes involved in lignin synthesis in wild type and transgenic A. thaliana plants heterologously expressing CmMYB1. Values shown are means ± SE, as calculated from three biological replicates. doi:10.1371/journal.pone.0065680.g004

**Figure 5.** Heterologously expressing CmMYB1 does affect the expression of secondary wall–associated cellulose synthase genes (CesA4, CesA7, and CesA8) and xylan biosynthetic gene IRX9, but does not induce another xylan biosynthetic gene IRX8. Values shown are means ± SE, as calculated from three biological replicates. doi:10.1371/journal.pone.0065680.g005
were terminated by adding 500 µL of 40% H2O2 and 50% acetic acid to the hydrolyzate. After being cooled in room temperature, the hydrolysis mixture was then washed with water and acetone and added to 72% sulfuric acid to hydrolyze completely. The glucose content was read at 625 nm.

**Results**

**Isolation of CmMYB1, a new Subgroup 4 R2R3-MYB Factor from chrysanthemum**

The full length cDNA CmMYB1 sequence (JF795917) was isolated by RT-PCR and RACE based on an EST created by Chen [29]. It consisted of 1,237 nucleotides, of which 846 bp represented an open reading frame encoding 281 residues. The predicted gene product is a protein of molecular mass 31.7 kDa and a pI of 8.77, containing one putative DNA binding site, one localized between residues 9 and 61, and a pI of 8.77, containing one predicted gene product is a protein of molecular mass 31.7 kDa and a pI of 8.77, containing one DNA binding site, one localized between residues 9 and 61, and a pI of 8.77, containing one DNA binding site, one localized between residues 9 and 61, and one between residues 62 and 116. Four conserved regions lie at its C-terminus, namely LLsrGIDPX[T/S]HRX[I/L],

| Table 1. Lignin content in the stems of wild type and heterologous expression of CmMYB1 on A. thaliana. |
|-----------------|-----------------|-----------------|
| **Wild type**   | **35S::CmMYB1-1** | **35S::CmMYB1-2** |
| Lignin content(mg/g) | 217.7±0.42 | 193.6±0.91* | 171.0±0.27** |
| H*(mg/g)         | 0.049±0.01 | 0.48±0.34 | 0.24±0.01 |
| G*(mg/g)         | 1.45±0.82 | 1.88±0.76* | 2.06±0.67** |
| S*(mg/g)         | 2.27±0.05 | 1.27±0.16** | 1.85±0.79* |
| S/G              | 1.57       | 0.67       | 0.90       |

* refers to p-hydroxybenzaldehyde; 
^ refers to the sum of vanillic acid and vanillin; 
\(^\) refers to the sum of syringic acid and syringaldehyde. Values shown are means ± SE, as calculated from three biological replicates. * and **, significant differences (respectively, P<0.05 and P<0.01). 

**Cellulose Content Analysis**

Method for measuring the content of cellulose is essentially described by Updegraff [42]. Briefly, 60 mg dried cell wall was added to 1 ml of Updegraff reagent (Acetic acid: nitric acid: water, 8:1:2 v/v) and heated at 100°C for 30 min. The pellets were washed with water and acetone and added to 72% sulfuric acid to hydrolyze completely. The glucose content was read at 625 nm.

**Flavonoids Analysis by HPLC**

HPLC analysis was performed using method of Burbulis [43] with minor revision. For flavonoids analysis, 100 mg fresh mature stems were harvested, and then extracted using 500 µL methanol with thoroughly vortexing and centrifuged for 10 min at 10,000 rpm. The supernatant was hydrolyzed with 2 mol.L-1 HCl at 70°C for 40 min. After being cooled in room temperature, the hydrolysis mixture was then washed with water and acetone and added to 72% sulfuric acid to hydrolyze completely. The glucose content was read at 625 nm.

**Table 2. Cellulose content in the stems of wild type and heterologous expression of CmMYB1 on A. thaliana.**

| Plant                  | Cellulose content(mg/g dry cell wall) |
|------------------------|--------------------------------------|
| Wild type              | 285.77±11.92                         |
| 35S::CmMYB1-1          | 264.36±8.43                          |
| 35S::CmMYB1-2          | 230.58±14.39                         |

Cellulose content from stems of wild type and heterologous expression of CmMYB1 (35S::CmMYB1-1, 35S::CmMYB1-2) on A. thaliana. Values shown are means ± SE, as calculated from three biological replicates. 

The Effect of Heterologous Expression of CmMYB1 on A. thaliana

CmMYB1 was introduced under the control of the cauliflower mosaic virus 35S promoter into binary vector pCAMBIA1301 containing the hygromycin B and gusA as selectable markers. Transformation was performed using floral dip method [37]. Two independent transgenic A. thaliana lines (35S::CmMYB1-1, 35S::CmMYB1-2) which showed higher levels of transgene expression were selected from several transgenic lines for further experiments (Fig. 3a). There was no observable phenotypic difference between wild type and transgenic plants grown under long days (16 h light and 8 h dark). The phloroglucinol staining difference between wild type and transgenic plants grown under long days (16 h light and 8 h dark). The phloroglucinol staining pattern was uniform in the transgenic leaves (Fig. 3a), in contrast to the wild type which had a more intense reaction in the leaf margins (Fig. 3a). The expression level of a set of key lignin synthesis genes (AtPAL1, AtC4H, At4CL1, AtHCT, AtCOMT1, AtCCoAOMT1, AtACR1, AtF5H, AtCOMT1 and AtCAD6) was monitored in the transgenic lines using qRT-PCR, respectively. This experiment showed that the expression of AtCOMT1 and AtCAD6 had been depressed to 10% of the wild type level and that of AtC4H, At4CL1, AtHCT, AtACR1, AtF5H to between 25% and 50%. However, the level

CmMYB1 Affects Lignin Biosynthesis

The expression level of a set of key lignin synthesis genes (AtFEL1, AtC4H, At4CL1, AtHCT, AtCOMT1, AtCAD6) was monitored in the transgenic lines using qRT-PCR, respectively. This experiment showed that the expression of AtCOMT1 and AtCAD6 had been depressed to 10% of the wild type level and that of AtC4H, At4CL1, AtHCT, AtACR1, AtF5H to between 25% and 50%. However, the level...
of AtPAL1, AtC3H, AtC3H1 and AtCcaO1MTH1 expression appeared to have been less affected (Fig. 4).

The expression level of genes involved in the biosynthesis of cellulose was also affected by heterologous expression of CmMYB1. qRT-PCR analysis showed that the expression of three secondary wall–associated cellulose synthase genes (CesA4, CesA7, and CesA8) [46,47] had been decreased. But that of xylan biosynthetic genes IRX9 [48] had been less down-regulated. The level of another gene of xylan biosynthetic IRX9 expression, however, was increased (Fig. 5).

To better determine the changes of lignin in two independent transgenic A. thaliana (35S::CmMYB1-1, 35S::CmMYB1-2), we assayed the content of acetyl bromide lignin in stems. The analysis revealed that the acetyl bromide lignin content decreased in stems of both the two transgenic plants. The lignin content of transgenic plant CmMYB1-1 and CmMYB1-2 had been decreased by 10% and 20% of the wild type plant, respectively (Table 1). We also analysed the lignin monomer composition. An increase in H subunits was observed in two independent transgenic A. thaliana plants. The content of G subunits increased significantly by 40% of the wild type while the content of S subunits decreased significantly by 40% of the wild type (Table 1).

### Table 3. Flavonoids content in the stems of wild type and heterologous expression of CmMYB1 on A. thaliana.

| Plant          | Quercetin (mg/g fresh wt) | Kaempferol (mg/g fresh wt) | Isorhamnetin (mg/g fresh wt) |
|---------------|---------------------------|----------------------------|-----------------------------|
| Wild type     | 1.094±0.082               | 0.732±0.0                  | 0.212±0.030                 |
| 35S::CmMYB1-1 | 0.391±0.012**             | 0.295±0.038**              | 0.101±0.004**               |
| 35S::CmMYB1-2 | 0.558±0.031**             | 0.387±0.010**              | 0.099±0.006**               |

Flavonoids content was performed by HPLC from stems of wild type and heterologous expression of CmMYB1 (35S::CmMYB1-1, 35S::CmMYB1-2). Kaempferol, quercetin and isorhamnetin were detected at 254 nm. Values shown are means ± SE, as calculated from three biological replicates.

**Significant differences (P<0.01).**

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Cellulose is another important secondary wall component. We also performed analysis of cellulose content, however, no significant reduction in cellulose was observed (Table 2).

### CmMYB1 Suppresses Flavonoid Biosynthesis

We performed qRT-PCR assays to determine the effect of CmMYB1 on flavonoids biosynthesis. The analysis indicated that the expression of CHS, CHI, FLS1 and DFR was suppressed, while F3H and F3′H increased slightly in transgenic plants compared to that of the wild type plants (Fig. 6).

Flavonoids content in wild type and two independent transgenic plants of A. thaliana (35S::CmMYB1-1, 35S::CmMYB1-2) were determined via HPLC analysis. The contents of quercetin, kaempferol and isorhamnetin in transgenic plant CmMYB1-1 and CmMYB1-2 decreased by 60% and 50% of the wild type plant, respectively, which inferred that CmMYB1 might suppress the flavonoid synthesis (Table 3).

### Discussion

MYB genes are a particularly abundant class of plant transcription factors, and the R2R3-MYB subfamily is prominent in higher plant species. Here we have described the isolation of the chrysanthemum R2R3-MYB transcription factor CmMYB1. Its sequence alignment and phylogeny strongly indicated that it belongs to R2R3-MYB subgroup 4, which includes a number of members involved in the repression of lignin synthesis [21,22,24,28]. It includes the C2 motif known to confer transcriptional repressor activity [45], which supports the idea that CmMYB1 functions as a negative regulator in chrysanthemum. CmMYB1 was ubiquitously expressed in the plant, although most strongly in the stem, which implies that it may play a role in the lignifying tissue of chrysanthemum.

A number of studies have shown that the over-expression of R2R3-MYB factors known to act as repressors of lignin synthesis produce alternations of the leaf morphology, with the appearance of white lesions on mature leaves and a reduction in growth rate when heterologous-expressed in tobacco and over-expressed in Arabidopsis [21,22]. However, in the present study the heterologous expression of CmMYB1 in A. thaliana did not produce any...
observed phenotype, which is different from previous researches. To determine whether CmMYB1 gene was related to the synthesis of lignin, we employed a direct histochemical staining method to detect the lignin content in stem, where the color intensity generated in the reaction with phloroglucinol was enhanced with the increase of lignin content. By this method, the strong reduction of lignin content was observed in inflorescence stems of CmMYB1 transgenic A. thaliana plants. We also employed the acetyl bromide lignin method to determine the lignin content in stems. By this quantitative method, we could confirm the lignin content of transgenic A. thaliana plants was decreased (Table 1). In addition, CmMYB1 overexpression in A. thaliana plants altered the lignin composition, showing higher content of H and G subunits while lower content of S subunits characterized by decreased S/G ratio of the lignin. Lignin synthesis was known to interact with cellulose and other secondary cell wall synthesis [26], however, cellulose synthesis was less affected in CmMYB1 transgenic plants (Table 2).

PAL is the first enzyme of the phenylpropanoid pathway. Overexpression of PtMYB4 in tobacco plants could reduce the expression level of PAL [17]. 4CL genes play key roles in the metabolic pathway of lignin synthesis, catalyzing the reaction for lignin synthesis. Most vascular plants (including A. thaliana) possess three distinct 4CL forms [49]. In A. thaliana, At4CL1 and At4CL2 are largely responsible for controlling the branching of the growing lignin molecule, while At4CL3 controls flavonoid branching. A common feature between tobacco heterologous expressing Am- MYB308 or AmMYB330 and A. thaliana over-expressing AtMYB4 is the down-regulation of 4CL expression, as also displayed in the transgenic A. thaliana lines expressing CmMYB1 (Fig. 4); this commonality indicates that CmMYB1 is involved in lignin synthesis. CAD is another key gene in lignin synthesis of A. thaliana stems [50], involved in the final step of the reduction reaction of lignin monomer. The lignin content in the stem of double mutant A. thaliana transgenic plants altered the lignin content in stems [50], involved in the final step of the reduction reaction of lignin monomer. The lignin content in the stem of double mutant plant (cad-c, cad-d) was reduced by 40%, compared to that of normal species, so the stems of A. thaliana double mutant were soft and easy to lodge. Therefore, heterologous expression of CmMYB1 on A. thaliana reduced the expression of key enzymes ACAD and affected lignin synthesis (Fig. 4). In addition to 4CL and CAD, subgroup 4 R2R3-MYB factors have also been reported to regulate other key genes in the lignin pathways. AmMYB308 and AmMYB330 both negatively regulate the expression of C4H and CAD when heterologously over-expressed in tobacco [21], and AmMYB4 is proved to be function as a repressor, particularly of C4H [22]. On the other hand, ZnMYB31 marginally enhances CAD expression and decreases COMT expression, but does not affect the expression of either C4H or COMT, while ZnMYB42 down-regulates the expression of C4H, CAD and COMT but does not affect the expression of COMT [24]. Here, similar to the behavior of both AmMYB308 and ZnMYB42, the heterologous expression of CmMYB1 suppressed the expression of C4H, COMT and CAD, while that of COMT was unaffected (Fig. 4). It has been reported that the down-regulated expression of C4H, 4CL, F3H or COMT could alter the final lignin composition [51,52]. CmMYB1 involved in the expression of lignin biosynthetic genes suggests the process that A. thaliana biosynthetic pathways lead to the biosynthesis of cellulose, xylan, and lignin may be cogitated by pathway-specific transcription factors. C4H genes play an important part in cellulose biosynthesis [46,47] and REX and REX9 genes involved in xylan biosynthesis [40]. These results suggest that CmMYB1 is a transcription factor influencing cellulose biosynthesis, but appeared to have been largely unaffected in xylan biosynthesis (Fig. 5).

CmMYB1 seems to play an important role in regulating flavonoid pathway which is another branch of phenylpropanoid pathway. ZnMYB1, a R2R3-MYB transcription factor, is a functional regulator of flavonol synthesis [53]. ZnMYB42 repress the flavonol biosynthesis [26]. AmMYB4 of R2R3-MYB factor also affected the expression level of 4CL3 and CHS [22]. Overexpression of CmMYB1 in A. thaliana reduced the expression level of 4CL3, CHS, CHI, FLS and DFR. The contents of quercetin, kaempferol andisorhamnetin contents decreased significantly in consistency with the suppression of genes expression in two independent transgenic A. thaliana. Overall, the data indicate that CmMYB1 negatively regulates flavonoids synthesis.

**Supporting Information**

File S1 Supporting information file containing the following files. Table S1. Primer sequences used in this study. Table S2. Primer sequences used in qRT-PCR.

(DOC)

**Author Contributions**

Conceived and designed the experiments: LZ HS SC JJ GZ AS FC. Performed the experiments: LZ HS. Analyzed the data: LZ HS SC JJ AS. Contributed reagents/materials/analysis tools: LZ HS SC JJ AS FC. Wrote the paper: LZ HS SC JJ FC.

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