Overexpression of *CmMYB15* provides chrysanthemum resistance to aphids by regulating the biosynthesis of lignin

Cong An¹, Liping Sheng¹, Xinping Du¹, Yinjie Wang¹, Yi Zhang¹, Aiping Song¹, Jiafu Jiang¹, Zhiyong Guan¹, Weimin Fang¹, Fadi Chen¹ and Sumei Chen¹

**Abstract**

MYB transcription factors are widely involved in the development of and physiological processes in plants. Here, we isolated the chrysanthemum R2R3-MYB family transcription factor *CmMYB15*, a homologous gene of *AtMYB15*. It was demonstrated that *CmMYB15* expression was induced by aphids and that *CmMYB15* could bind to AC elements, which usually exist in the promoter of lignin biosynthesis genes. Overexpression of *CmMYB15* in chrysanthemum enhanced the resistance of aphids. Additionally, the content of lignin and the expression of several lignin biosynthesis genes increased. In summary, the results indicate that *CmMYB15* regulates lignin biosynthesis genes that enhance the resistance of chrysanthemum to aphids.

**Introduction**

Members of the MYB family, one of the largest transcription factor families in plants, have a common conserved N-terminal MYB domain consisting of imperfect amino acid repeats of ~52 amino acids¹. These members are generally divided into four classes, 1R-, R2R3-, 3R- and 4R-MYB proteins, according to the number of adjacent repeats, with the R2R3-MYB subfamily comprising one of the largest families of transcription factors in plants². MYBs have been shown to be key factors involved in vital regulatory networks that regulate physiological and biochemical processes, for instance, plant development, responses to biotic and abiotic stress³, and primary and secondary metabolism, such as flavonoid biosynthesis and phenylpropanoid metabolism⁴,⁵.

The lignin biosynthetic pathway is among the important branches of the phenylpropanoid metabolic pathway, as lignin is the second most abundant plant biopolymer. Lignin is a major secondary cell wall component that provides mechanical support for plants and is also a chemical and physical barrier that provides defense against biotic and abiotic stress⁶. The precursors of lignin, which are phenolic compounds, can participate in plant-pathogen interaction pathways, such as that of the interaction between grapevine and *Erysiphe necator*⁷. A relatively high accumulation of lignin increases the strength of birch leaves, which reduces vulnerability to the autumnal moth, *Epirrita autumnata*⁸. Lignin enhances resistance to cell wall-degrading enzymes (CWDEs), which are produced by fungal necrotrophs, therefore preventing the diffusion of pathogen-produced toxins⁹. Enzymes participating in lignin biosynthesis include phenylalanine ammonia lyase (PAL), 4-hydroxycinnamoyl-CoA ligase (4CL), caffeoyl shikimate esterase (CSE), p-coumarate 3-hydroxylase (C3H), caffeoyl-CoA O-methyltransferase (CCoAOMT), cinnamoyl-CoA reductase (CCR), ferulate 5-hydroxylase (F5H), caffeic acid/5-hydroxyferulic acid O-methyltransferase (COMT), and cinnamyl alcohol dehydrogenase (CAD)¹⁰,¹¹. Analysis of promoter sequences showed that most of these genes contain one or more AC-
rich elements in their promoters, except for F5H\(^{12}\). Transcription factors such as MYB proteins regulate the expression of these genes by binding to AC elements. \(\textit{AtMYB46}\) activates specific pathways of secondary cell wall biosynthesis and simultaneously upregulates \(\textit{AtMYB85}\), a downstream secondary cell wall-associated transcription factor\(^{13}\). \(\textit{AtMYB58}\) and \(\textit{AtMYB63}\) specifically activate lignin biosynthetic genes, which leads to the ectopic deposition of lignin in cells that are normally un lignified\(^9\). Similarly, \(\textit{PtoMYB216}\), a MYB-encoding gene in \(\textit{Populus tomentosa}\), was also found to activate lignin upstream biosynthetic genes\(^{14}\). On the other hand, \(\textit{Eriobotrya japonica EjMYB1}\), a transcriptional activator, and \(\textit{EjMYB2}\), a repressor, have an antagonistic relationship in terms of lignification, as the activator and repressor compete with each other for interaction with AC elements\(^{15}\). \(\textit{PdMYB221}\) negatively regulates secondary cell wall formation by binding to the AC elements in the promoter of \(\textit{PdCESA8}\), \(\textit{PdGT47C}\), and \(\textit{PdCOMT2}\), leading to decreased lignin production\(^{16}\). Previous studies have indicated that several members of the MYB family regulate the synthesis of lignin. However, if other members of the MYB family are involved in lignin biosynthesis, their regulatory mechanism remains to be elucidated.

Aphids are major agricultural pests that directly cause damage to plants by depleting them of nutrients, and aphids are also vectors for viral pathogens\(^{17}\). \(\textit{Chrysanthemum} (\textit{Chrysanthemum morifolium})\) is a valuable ornamental species that is vulnerable to \(\textit{ Macrosiphoniella sanborni}\). Aphid infestations hinder not only vegetative growth but also the quality and yield of chrysanthemum flowers. Our previous studies showed that chrysanthemum has evolved a number of strategies to cope with aphid infestation, such as increased defensive enzyme activity\(^{18}\) and gene expression reprogramming\(^{19,20}\). We previously found that \(\textit{CmMYB19}\), a chrysanthemum R2R3-MYB gene, improved the resistance of chrysanthemum to aphids by promoting the biosynthesis of lignin\(^{21}\).

In the present study, we isolated chrysanthemum \(\textit{CmMYB15}\), a gene homologous to \(\textit{AtMYB15}\), and found that it played a role in lignin biosynthesis and aphid resistance by regulating several downstream genes different from \(\textit{CmMYB19}\). This study will lay a foundation for the understanding of the physical defense mechanisms of chrysanthemum when challenged by aphids.

**Results**

**Isolation and sequence analyses of \(\textit{CmMYB15}\)**

\(\textit{CmMYB15}\) was isolated from ‘Nannong Xunzhang’ chrysanthemum. The \(\textit{CmMYB15}\) (KT763374) gene consists of 890 bp, with a 756 bp open reading frame (ORF) encoding a 251 amino acid polypeptide. \(\textit{CmMYB15}\) belongs to the R2R3-MYB subfamily, as it has two conserved MYB domains; an R2MYB domain between aa 16–61 and an R3MYB domain between aa 69–112 (Fig. 1a). Phylogenetic analysis indicated that this gene has 32.73% similarity with \(\textit{AtMYB15}\) (Fig. 1b).

**\(\textit{CmMYB15}\) is localized to the cell nucleus**

To determine the subcellular localization of \(\textit{CmMYB15}\), 2 × 35S::\(\textit{CmMYB15}\)-GFP and a negative control, 2 × 35S::GFP, were separately transformed into onion epidermal cells. GFP signals were detected in the nucleus of the cells transformed with 2 × 35S::\(\textit{CmMYB15}\)-GFP, indicating that \(\textit{CmMYB15}\) was nuclear localized. The GFP signal was detected in both the cytoplasm and nucleus of the cells transformed with 2 × 35S::GFP (positive control) (Fig. 2).

**Transcriptional activation of \(\textit{CmMYB15}\) and the binding ability of \(\textit{CmMYB15}\) with AC elements**

Yeast harboring pGBK7-\(\textit{CmMYB15}\), or a pCL1 positive control, grew well on SD/His-/Ade medium and turned blue on the SD/His-/Ade medium with X-α-gal. The negative control transformed with pGBK7 was unable to grow on the SD/His-/Ade medium (Fig. 3a), indicating that \(\textit{CmMYB15}\) has transcriptional activation activity. A yeast one-hybrid assay showed that cells transformed with pHI5i-AC-I, pHI5i-AC-II or pHI5i-AC-III and its corresponding mutated form pHI5i-mAC grew well on SD/His-/Ura medium but not on medium with 25 mM 3-amino-1,2,4-triazole (3-AT) (Fig. 3b). Bait Y1H cells (those harboring AC elements or a mAC element) transformed with pGADT7-\(\textit{CmMYB15}\) could grow on SD/His-/Ura medium and on medium supplemented with 25 mM 3-AT (Fig. 3b), suggesting that \(\textit{CmMYB15}\) could bind all three types of AC elements.

**The gene \(\textit{CmMYB15}\) is highly expressed in leaves and stems and is induced by aphid infestation**

The expression of \(\textit{CmMYB15}\) was detected in the root, stem and leaf tissues of ‘Nannong Xunzhang’. \(\textit{CmMYB15}\) had the lowest abundance in the roots, while it was highly expressed in the leaves and stems. The expression levels in the stems and leaves were 8.63 times and 15.79 times the level in the roots, respectively (Fig. 4a). In response to aphid infestation, the expression level of \(\textit{CmMYB15}\) in the aphid-infested plants was 6.84 times greater than that in the control plants at 3 h. During 3 h to 12 h after aphid infestation, the expression level of \(\textit{CmMYB15}\) remained significantly higher than that of the control. The expression level of \(\textit{CmMYB15}\) was affected by mock puncture except at −2 h, 12, and 24 h; however, the changes in the expression level of \(\textit{CmMYB15}\) in response to puncture were much less drastic than those in response to infestation by aphid (Fig. 4b).
Overexpression of CmMYB15 inhibited the population of aphids on chrysanthemum

Putative CmMYB15 overexpression lines were identified by PCR using HptII primers (Fig. 5a), and the transcriptional level of CmMYB15 was measured by qRT-PCR using CmMYB15-specific primers (Fig. 5b). Two independent transgenic lines, CmMYB15-ox3 and CmMYB15-ox4, which presented a higher gene expression level of CmMYB15, were selected for additional aphid resistance assays. The aphids were more widely distributed on the WT plants than on transgenic lines at 21 days after infestation (Fig. 5c). At 11 days after infestation, the number of aphids began to increase rapidly in the control group (WT) plants and reached an average of 270.83 aphids per plant, while the number of aphids on the CmMYB15-ox3 and CmMYB15-ox4 plants were, on average, 153.45 and 236.80 per plant on day 21 (Fig. 5d). CmMYB15-overexpressing plants had a lower average multiplication rate (MR) of 30.69 for CmMYB15-ox3 and 47.36 for CmMYB15-ox4 in comparison to the WT MR of 54.17. Compared to the WT plants, the overexpression lines had higher average inhibition rates (IRs). The IRs for CmMYB15-ox3 and CmMYB15-ox4 in comparison to WT were 43.34% and 12.57%, respectively (Table 1).

Overexpression of CmMYB15 enhanced the lignin content and expression of lignin biosynthesis genes

The acetyl bromide procedure was used to analyze the lignin content of two CmMYB15 overexpression lines. The lignin content of CmMYB15-ox3 and CmMYB15-ox4 was 6.56% and 6.27% respectively, while the lignin content of the WT was 5.97% (Fig. 6a).

The expression levels of genes in the lignin biosynthesis pathway, including CmPAL1, Cm4CL1, CmC3H1, CmCSE, CmCoAMOT1, CmCCRI, CmF5H1, CmCOMT, and CmCAD6, were also quantified. The expression levels of Cm4CL1, CmCCRI, CmF5H1, and CmCAD6 were higher in the overexpression plants than in the WT plants; among these, compared to the other genes analyzed, the expression of CmCCRI and CmCAD6 increased significantly (Fig. 6b).
Discussion

CmMYB15 is a MYB transcription factor

MYB transcription factors, which are widely distributed in plants, play an important role in regulating a variety of processes in plants\(^1\). MYBs act as transcription activators or repressors to regulate the transcription of downstream genes. Of all the divisions of plant MYB proteins, including 4R-MYB, 3R-MYB, R2R3-MYB and MYB-related types, R2R3-MYB proteins account for the majority\(^2\). Most R2R3-MYB and 3R-MYB subfamilies in
Arabidopsis are divided into 16 groups with different functions, such as involvement in stress responses, primary and secondary metabolism, lignin biosynthesis or wall deposition, cell wall thickening, cell differentiation or embryogenesis, the cell cycle, anther development, stomatal development and meristem formation\textsuperscript{23,25}. Previously, only a few MYB transcription factors in chrysanthemum had been isolated and analyzed. Among them, chrysanthemum CmMYB1 is a repressor that reduces the lignin content and represses flavonoid synthesis in Arabidopsis\textsuperscript{26}. Chrysanthemum CmMYB2 enhances the salinity and drought tolerance by improving sensitivity to abscisic acid, but it weakened the freezing tolerance of Arabidopsis by suppressing the expression of CBF1/DEB1, which activated cold-responsive genes\textsuperscript{38,39}. On the other hand, the Fagopyrum tataricum MYB gene FtMYB15 enhances the synthesis of anthocyanins and proanthocyanidins, which help resist abiotic stress\textsuperscript{40}. Similarly, SIMYB75 in tomato also contributes to the accumulation of anthocyanins and participates in several stresses responses\textsuperscript{41}.

At present, the MYBs involved in the response to biotic stresses such as pathogen attack and pest feeding have received increased amounts of attention. By promoting the biosynthesis of salicylic acid, AtMYB96 induces pathogen resistance in Arabidopsis\textsuperscript{42}. The role of MYBs in insect defense has been recognized; for example, AtMYB102 is expressed locally at the feeding sites of Pieris rapae in damaged leaves, which then activates the expression of defense-related genes and cell wall modification genes\textsuperscript{43}. Overexpression of AtMYB12 in Nicotiana tabacum enhances the expression of phenylpropanoid pathway genes and the accumulation of flavonols, especially rutin, consequently leading to insect resistance\textsuperscript{31}. In the present study, CmMYB15 was significantly induced by aphid infestation. CmMYB15-overexpressing plants exhibited a lower average multiplication rate and higher average inhibition ratio of aphids, in which CmMYB15-ox3 lines, which presented a relatively high expression of the CmMYB15 gene, showed a higher IR than CmMYB15-ox4 lines, which presented relatively low CmMYB15 expression. These results indicated that CmMYB15 contributed to the resistance of chrysanthemum to aphids.

**CmMYB15 enhanced aphid resistance by increasing lignin synthesis**

When faced with phytophagous pests, plants can enhance their physical structures and alter their secondary metabolites to hinder the invasion of pests or inhibit pest growth and reproduction. Research in rice (Oryza
sativa) has demonstrated that three MYB genes, MYB30, MYB55, and MYB110, which are induced by pathogen infestation, are involved in plant primary immunity (known as a microbe-associated molecular pattern) by specifically inducing monolignol pathway genes\textsuperscript{44}. In Arabidopsis, recent research has demonstrated that AtMYB102 promotes ethylene (ET) biosynthesis by upregulating ET biosynthesis pathway genes and increases the susceptibility of plants to aphids\textsuperscript{45}. Phloem-based defense (PBD) regulated by ethylene (ET) and MYB transcription factors is a mechanism that has been studied substantially in plants with specific regard to how plants are resistant to insects such as aphids. Sieve-specific phloem proteins (PP) can plug sieve pores and prevent aphids feeding on the phloem, which is a physical plant defense mechanism\textsuperscript{46}. In wheat (\textit{Triticum aestivum}), TaMYB19, TaMYB29, and TaMYB44 act as coregulators of PBD, which is activated by the infestation of English grain aphids via crosstalk with the ethylene signaling pathway\textsuperscript{46}. Three Arabidopsis mutants, \textit{atmyb15}, \textit{atmyb38}, and \textit{atmyb44}, are susceptible to aphid infestation due to the disruption of ethylene signaling\textsuperscript{47}, with \textit{AtMYB44} playing a dominant role in activating EIN2-affected defense, an ethylene signaling regulation defense mechanism, to control resistance to the green peach aphid \textit{Myzus persicae} Sulzer. This defense requires ethylene-

**Fig. 5 The aphid resistance of wild-type chrysanthemum and CmMYB15-overexpressing chrysanthemum.**

\textbf{a} Identification of CmMYB15-overexpressing chrysanthemum plants by PCR, in which the plasmid pMDC43-2 × 35 S: CmMYB15 acted as a positive control and wild-type (WT) acted as a negative control. -ox1, -ox2, -ox3 and -ox4 represent transgenic plants overexpressing CmMYB15, \textbf{b} transcription level of CmMYB15 in wild-type and CmMYB15-overexpressing chrysanthemum plants, \textbf{c} differential proliferation of aphids between CmMYB15-overexpressing and nontransgenic plants, \textbf{d} average number of aphids on CmMYB15-overexpressing and wild-type plants measured at 3–21 days after aphid infestation.
induced resistance involving the PBD response\textsuperscript{48–50}. However, the mechanism of \textit{AtMYB15} in aphid resistance has not been further studied. Recently, it was determined that \textit{AtMYB15} regulates the biosynthesis of G-lignin to promote defense-induced lignification in Arabidopsis in response to pathogens, with \textit{AtMYB15} interfering with flg22, the bioactive epitope of bacterial flagellin\textsuperscript{8}. Our previous study showed that \textit{CmMYB19}, an aphid stress response gene, promoted lignin synthesis and resistance to aphids\textsuperscript{21}. Here, we found that \textit{CmMYB15}, a member of the MYB family, also enhanced aphid resistance and lignin synthesis. However, different sets of lignin genes were induced downstream in \textit{CmMYB19-} and \textit{CmMYB15-}overexpressing plants. \textit{CmC4H1} and \textit{CmCCR1} were induced in both \textit{CmMYB19} and \textit{CmMYB15} overexpression plants, while \textit{CmF5H1} and \textit{CmCAD6} were induced only in the \textit{CmMYB15} overexpression plants. \textit{CmPAL1}, \textit{CmC4H}, \textit{CmHCT} and \textit{CmCCoAOMT1} were enhanced by overexpression of \textit{CmMYB19}\textsuperscript{21}; however, the expression level of \textit{CmMYB19} was relatively little affected by \textit{CmMYB15} (Fig. S1), suggesting that the regulatory mechanisms of lignin biosynthesis of \textit{CmMYB15} and \textit{CmMYB19} were different from each other to some extent.

We suppose that \textit{CmMYB15} might mainly regulate the synthesis of lignin monomers and that \textit{CmMYB19} might contribute to the very upstream steps of lignin biosynthesis and the assembly of lignin monomers; this speculation should be validated in our ongoing research.

Yeast assays showed that \textit{CmMYB15} was able to bind to the AC elements in promoter regions of some lignin pathway genes, suggesting a role for \textit{CmMYB15} in the activation of lignin pathway genes. However, whether the elevation of genes involved in lignin biosynthesis is directly or indirectly regulated by \textit{CmMYB15} is not currently known. In conclusion, increasing \textit{CmMYB15} expression could lead to changes in lignin content, thereby enhancing the aphid resistance of chrysanthemum, which provides much promise for improving physical defense to aphids.

**Materials and methods**

**Plant materials**

‘Nannong Xunzhang’ (aphid resistant) and ‘Jinba’ (aphid susceptible) chrysanthemum plants were obtained from the Chrysanthemum Germplasm Resource Preserving Centre (Nanjing Agricultural University, China). The plants were grown under conditions of 25 °C and 100 μmol m\textsuperscript{-2} s\textsuperscript{-1}, with a 16/8 h light/dark photoperiod.

**Gene isolation and sequence analysis**

Total RNA from ‘Nannong Xunzhang’ chrysanthemum was isolated using RNAiso Plus Reagent (TaKaRa, Japan). First-strand cDNA was synthesized by M-MLV reverse transcriptase (TaKaRa, Japan). The cDNA fragments of \textit{CmMYB15} were amplified using Phusion High-Fidelity DNA Polymerase (ThermoFisher, USA) with genespecific primers (Supplementary Table S1) based on the transcriptome data of ‘Nannong Xunzhang’\textsuperscript{20}. 3’-RACE (3’-random amplification of cDNA ends) and 5’-RACE were then performed to obtain the full-length cDNA as described previously\textsuperscript{21}. The ORF of \textit{CmMYB15} was

### Table 1  Comparison of aphid populations on wild-type plants and transgenic chrysanthemum plants overexpressing \textit{CmMYB15} after infestation

| Plants         | Multiplication rate (MR) of the aphid population | Inhibition rate (IR) of the aphid population % |
|----------------|-----------------------------------------------|---------------------------------------------|
| WT             | 54.17 ± 2.286\textsuperscript{a}              | 0                                           |
| CmMYB15-ox3    | 30.69 ± 1.422\textsuperscript{c}              | 43.34                                       |
| CmMYB15-ox4    | 47.36 ± 1.724\textsuperscript{b}              | 12.57                                       |

Multiplication rate (MR) of the aphid population was calculated by \(N_{t}/N_{0}\), in which \(N_{t}\) represents the average number of aphids on WT plants or transgenic plants on the 21st day after aphid infestation. The inhibition rate (IR) of aphids on the transgenic plants was given by the formula \((N_{0}/N_{t})\times 100\), in which \(N_{0}\) and \(N_{t}\) represents the number of aphids on the WT and on the \textit{CmMYB15}-overexpressing plants at 21 days after aphid infestation, respectively\textsuperscript{22}. The different letters (a, b, c) represent significantly different aphid multiplications between the wild-type and \textit{CmMYB15}-overexpressing plants.
amplified using the gene-specific primers CmMYB15-F/R (Supplementary Table S1).

The homologous sequences of CmMYB15 were retrieved and analyzed using online BLAST tools from the NCBI (National Center for Biotechnology Information, USA). The sequences of MYB proteins were aligned using DNAMAN and Muscle software. A phylogenetic tree of CmMYB15 with its homologs in Arabidopsis was constructed using MEGA 7.0 with the maximum likelihood method (bootstraps = 1000).

Subcellular localization of CmMYB15

The ORF of CmMYB15 was ligated into the vector pENTR1A using Kpn I and Xho I restriction enzymes to generate a pENTR1A-CmMYB15 vector. A pMDC43-CmMYB15 vector driven by the 2×35S promoter was then constructed via recombination of pENTR1A-CmMYB15 with pMDC43-GFP using LR Clonase II enzyme mix (Invitrogen, USA). By particle bombardment (PDS-1000; Bio-Rad, USA), the plasmid DNA of pMDC43-CmMYB15 was transformed into onion epidermal cells. The GFP signal of the transformed cells was detected by laser scanning confocal microscopy (LSCM, Leica, Germany) after an overnight dark incubation at 22°C on Murashige and Skoog (MS) medium.

Yeast one-hybrid assay for the transcriptional activation activity and binding of AC cis-elements by CmMYB15

To detect the transcriptional activity of CmMYB15, pGBK7-CmMYB15 was constructed via recombination using pENTR1A-CmMYB15 as the donor vector and pGBK7 as the destination vector with the aid of LR Clonase II enzyme mix (Invitrogen, USA). pGBK7-CmMYB15, pGBK7 (an empty vector, as the negative control) and a positive control (pCL1) were transformed separately into Y2H competent cells (Clontech, USA) containing the GAL4/UAS system. The transformed cells were cultured as described previously.

To identify whether CmMYB15 could bind AC elements, a yeast one-hybrid assay was performed using the Matchmaker Gold Yeast One-Hybrid Library Screening System (Clontech, USA) according to the manufacturer’s protocol. Three forms of AC elements, i.e., AC-I (ACCAACCCC-3′), AC-II (ACCTACCTCC-3′), and AC-III (AAATATTCC-3′), are available thus far. Therefore, the fragments containing three tandem repeat AC elements, AC-I (5′-TCCACCTACCTCCAAACCTATCCACCTCC-3′), AC-II (5′-TCCACCAACCCCTTACACCCAACCC-3′), and AC-III (5′-TCCACCTACCTCCACCAACCTTACACCAACCC-3′), respectively, and their mutated form, mA (5′-TCCAAATTTTCCAAATATTTTCCAAATATCC-3′), were assembled into a pHISi vector at the EcoRI and Xba I restriction enzyme cutting sites. The plasmids were transformed into a Y1H Gold line after linearization by an Apa I restriction enzyme. The binding assay was performed as described previously.

Gene expression analysis by quantitative real-time PCR (qRT-PCR)

To analyze the expression profiles of CmMYB15 in different tissues, the roots, the fourth internode of the stem and the third fully expanded leaf counted from the apex were harvested from ‘Nannong Xunzhang’.

To dissect the expression profiles of CmMYB15 in response to aphid infestation, aphid infestation was performed as described by Xia et al. The time point when aphids were infested was defined as −2 h; thus, 0 h refers to 2 h after infestation. To mimic piercing by an aphid, a puncture treatment was performed on the third leaf of another group of plants as described previously. A group of untreated plants served as the control (CK). All the treated leaves were covered by transparent and ventilated petri dishes. For RNA isolation, leaves from each treatment were harvested from three individual plants at −2, 0, 3, 6, 9, 12, and 24 h. The aphids were removed when the leaves were harvested. Each test included three biological replicates.

qRT-PCR was performed using SYBR Premix Ex Taq II (TaKaRa, Japan) according to the manufacturer’s instructions. The gene Elongation Factor 1α (CmEF1α, KF305681) was used as the reference gene. The qRT-PCR data were analyzed using the 2−ΔΔCT method.

Overexpression of CmMYB15 in ‘Jinba’ chrysanthemum

To identify the function of CmMYB15, pMDC43-2×35S::CmMYB15 was transformed into Agrobacterium tumefaciens strain EHA105 using the freeze-thaw method. Information on the in vitro regeneration of ‘Nannong Xunzhang’ chrysanthemum is not available; thus, a CmMYB15-overexpressing ‘Jinba’ chrysanthemum was produced by Agrobacterium-mediated transformation as described previously. Hygromycin-resistant shoots were rooted on MS medium containing hygromycin B, and the putative transgenic plants were identified by PCR using HptII primer pairs (Supplementary Table S1). The expression levels of CmMYB15 in the putative overexpression lines and wild-type (WT) plants were measured using the primer pair CmMYB15-qF/qR (Supplementary Table S1).

The resistance of CmMYB15-overexpressing chrysanthemum plants to aphid

Two independent overexpression lines were used for an aphid resistance assay according to the methods of Hu et al. Transgenic plants and WT ‘Jinba’ plants at the 6–8-leaf stage were used for aphid resistance assays. The infestation of aphids was performed according to the method of
Li et al. Each infestation assay contained ten plants with three biological replications. The number of aphids was counted every 2 days. The average multiplication rate (MR) and average IR of the aphid population were calculated based on the aphid data counted over 21 days.

The determination of lignin content and expression levels of lignin biosynthesis genes in CmMYB15-overexpressing plants

The fourth internode of the overexpressing lines and WT 'Jinba' plants at the 14-to-16-leaf stage was sampled, dried and ground. The lignin content was analyzed using the acetyl bromide procedure. The samples were prepared following the description by Foster et al. The absorbance at 280 nm was determined using a microplate reader. The lignin content was then determined using the formula %ABSL = [abs (absorbance) × (2 ml × 100%)]/[15.69 × 0.539 cm × weight], where abs (absorbance) represents the average absorbance of 3 reads.

The fourth internode of the overexpressing lines and 'Jinba' plants at the 14-to-16-leaf stage was harvested. The expression of lignin biosynthesis genes, including CmPAL1, CmC4CL1, CmC3H1, CmCSE, CmCCoAMOTI, CmCCR1, CmF5H1, CmCOMT, and CmCAD6, was measured by qRT-PCR as mentioned above using the primers listed in Supplementary Table S1.

Statistical analysis

Tukey’s multiple range test (P<0.05) and one-way analysis of variance were performed to determine the significance of the data. SPSS v19.0 was used for all statistical analyses.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (Grant No. 31672192), the Program for Key Research and Development, Jiangsu, China (Grant No. BE2017318), the National Key Research and Development Program of China (2018YFD1000402), and the Fundamental Research Funds for the Central Universities (KQN201812).

Author contributions

CA, LS, and SC conceived and designed the experiments. CA, LS, YW, and YZ analyzed the data. A.S., JJ, ZG, WF, and FC contributed the reagents/plant materials/analysis tools. CA and SC wrote the manuscript. All authors read and approved the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest.

Supplementary Information

accompanies this paper at (https://doi.org/10.1038/s41438-019-0166-y).

Received: 17 February 2019 Revised: 10 May 2019 Accepted: 17 May 2019 Published online: 11 July 2019

References

1. Ambawat, S., Sharma, P., Yadav, N. R. & Yadav, R. C. MYB transcription factor genes as regulators for plant responses: an overview. Physiol. Mol. Biol. Plants 19, 307–321 (2013).
2. Baldoni, E., Genga, A. & Cornicelli, E. Plant MYB transcription factors: their role in drought response mechanisms. Int. J. Mol. Sci. 16, 15881–15891 (2015).
3. Dubos, C. et al. MYB transcription factors in Arabidopsis. Trends Plant Sci. 15, 573–581 (2010).
4. Huang, W. et al. Isolation and molecular characterization of thirteen R2R3-MYB transcription factors from Eupatorium sagittatum. Int. J. Mol. Sci. 14, 594–610 (2012).
5. Du, H., Feng, B.-R., Yang, S.-S., Huang, Y.-B. & Tang, Y.-X. The R2R3-MYB transcription factor gene family in maize. PLoS ONE 7, e37463 (2012).
6. Chezem, W. R., Memon, A., Li, F.-S., Weng, J.-K. & Clay, N. K. SG2-type R2R3-MYB transcription factor MYB15 controls defense-induced lignification and basal immunity in Arabidopsis. Plant Cell 10.1093/pcimb/cpt020 (2017).
7. Weng, K. et al. Transcription profile of Eutypa lethocerus-infected Vitis pseudotabidula provides insights into grapevine resistance to powdery mildew. Plant Dis. 109, 1409–1415 (2015).
8. Ossipov, V., Haukojo, E., Ossipova, S., Hanhimaki, S. & Pihlaja, K. Phenolic and phenolic-related factors as determinants of suitability of mountain birch leaves to an herbivorous insect. Biochem. Syst. Ecol. 29, 223–240 (2001).
9. Bellincampi, D., Cervone, F. & Lionetti, V. Plant cell wall dynamics and wall-related susceptibility in plant-pathogen interactions. Front. Plant Sci. 5, 228 (2014).
10. Zhou, J., Lee, C., Zhong, R. & Ye, Z.-H. MYB88 and MYB86 are transcriptional activators of the lignin biosynthetic pathway during secondary cell wall formation in Arabidopsis. Plant Cell 21, 248–266 (2009).
11. Vanholme, R. et al. Caffeoyl shikimate esterase (CSE) is an enzyme in the lignin biosynthetic pathway in Arabidopsis. Science 341, 1103–1106 (2013).
12. Raes, J., Rohde, A., Christensen, J. H., Van de Peer, Y. & Boerjan, W. Genome-wide characterization of the lignification toolbox in Arabidopsis. Plant Physiol. 133, 1051–1071 (2003).
13. Zhong, R., Richardson, E. A. & Ye, Z.-H. The MYB46 transcription factor is a direct target of SND1 and regulates secondary wall biosynthesis in Arabidopsis. Plant Cell 19, 2776–2792 (2007).
14. Tian, Q. et al. Functional characterization of the poplar R2R3-MYB transcription factor PopMYB216 involved in the regulation of lignin biosynthesis during wood formation. PLoS ONE 8, e76369 (2013).
15. Xu, Q. et al. Activator-and repressor-type MYB transcription factors are involved in chilling injury induced fleshy lignification in loquat via their interactions with the phenylpropanoid pathway. J. Exp. Bot. 65, 4349–4359 (2014).
16. Tang, X. et al. Poplar PdMYB221 is involved in the direct and indirect regulation of secondary wall biosynthesis during wood formation. Sci. Rep. 5, 12240 (2015).
17. Goggin, F. L. Plant-aplhi interactions: molecular and ecological perspectives. Curr. Opin. Plant Biol. 10, 399–408 (2007).
18. He, J. et al. Chrysanthemum leaf epidermal surface morphology and antioxidant and defense enzyme activity in response to aphid infestation. J. Plant Physiol. 168, 687–693 (2011).
19. Xia, X. et al. MicroRNA expression profile during aphid feeding in chrysanthemum (Chrysanthemum monilifolium). PLoS ONE 10, e0143720 (2015).
20. Xia, X. et al. Gene expression profiles responses to aphid feeding in chrysanthemum (Chrysanthemum monilifolium). BMC Genom. 15, 1050 (2014).
21. Wang, Y. et al. CmMYB19 over-expression improves aphid tolerance in Chrysanthemum by promoting lignin synthesis. Int. J. Mol. Sci. 18, 619 (2017).
22. Deng, Y. et al. Production and characterisation of the intergeneric hybrids between Dendranthera monilifolia and Artemisia vulgaris exhibiting enhanced resistance to chrysanthemum aphid (Macrosiphoniellavornae). Planta 231, 693–703 (2010).
23. Foster, C. E., Martin, T. M. & Pauly, M. Comprehensive compositional analysis of plant cell walls (lignocellulosic biomass) part I: lignin. J. Visualized Exp. 37, e1745 (2010).
24. Wilkins, O., Nahal, H., Foong, J., Provat, N. J. & Campbell, M. M. Expansion and diversification of the Populus R2R3-MYB family of transcription factors. Plant Physiol. 149, 981–993 (2009).
25. Wang, S. et al. Genome-wide identification and characterization of the R2R3-MYB transcription factor superfamily in eggplant (Solanum melongena L.). Agric. Gene. 2, 38–52 (2016).

26. Zhu, L. et al. The heterologous expression of the chrysanthemum R2R3-MYB transcription factor CrMYB1 alters lignin composition and represses flavonoid synthesis in Arabidopsis thaliana. PLoS ONE 8, e65680 (2013).

27. Shan, H. et al. Heterologous expression of the chrysanthemum R2R3-MYB transcription factor CrMYB2 enhances drought and salinity tolerance, increases hypersensitivity to ABA and delays flowering in Arabidopsis thaliana. Mol. Biotechnol. 51, 160–173 (2012).

28. Liu, X.-f. et al. The identification of a MYB transcription factor controlling anthocyanin biosynthesis regulation in Chrysanthemum flowers. Sci. Hort. 194, 278–285 (2015).

29. Chen, N. et al. Identification of 30 MYB transcription factor genes and analysis of their expression during abiotic stress in peanut (Arachis hypogaea L.). Gene 533, 332–345 (2014).

30. Roy, S. Function of MYB domain transcription factors in abiotic stress and epigenetic control of stress response in plant genome. Plant Signal. Behav. 11, e1117723 (2016).

31. Misra, P. et al. Modulation of transcriptome and metabolome of tobacco by a Chrysanthemum R2R3-MYB transcription factor superfamily in eggplant (Solanum melongena L.). Front. Plant Sci. 3, 51–60 (2012).

32. Xie, Z., Li, D., Wang, L., Sack, F. D. & Groteveld, E. Role of the stomatal development regulators FLP/MYB88 in abiotic stress responses. Plant J. 64, 731–739 (2010).

33. Denekamp, M. & Smeekens, S. C. Integration of wounding and osmotic stress signals determines the expression of the AtMYB102 transcription factor gene. Plant Physiol. 167, 1461–1465 (2013).

34. Ding, Z. et al. Transgenic expression of MYB15 confers enhanced sensitivity to abscisic acid and improved drought tolerance in Arabidopsis thaliana. J. Genet. Genom. 36, 17–29 (2009).

35. Agarwal, M. et al. A R2R3 type MYB transcription factor is involved in the cold regulation of CBF genes and in acquired freezing tolerance. J. Biol. Chem. 281, 37636–37645 (2006).

36. Xu, R. et al. Salt-induced transcription factor MYB74 is regulated by the RNA-directed DNA methylation pathway in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 110, 12260–12265 (2013).

37. Liu, R. et al. Thirty-seven transcription factor genes differentially respond to a harpin protein and affected defences in Arabidopsis. Mol. Plant–Microbe Interact. 24, 377–389 (2011).

38. Will, T. & van Bel, A. J. Induction as well as suppression: How aphid saliva may affect defensive responses. Insect Biochem. Mol. Biol. 38, 153–160 (2008).

39. Li, F. et al. Chrysanthemum Cm HSFA 4 gene positively regulates salt stress tolerance in transgenic chrysanthemum. Plant Biotechnol. J. 16, 1311–1321 (2018).

40. Luo, X. et al. An R2R3-MYB transcription factor AtMYB15 involved in the regulation of chrysanthemum anthocyanin and proanthocyanidins from Tartary Buckwheat. J. Plant Growth Regul. 37, 76–84 (2018).

41. Jia, W. et al. SIMYB75, an MYB-type transcription factor, promotes anthocyanin accumulation and enhances volatile aroma production in tomato fruits. Hortic. Res. 6, 22 (2019).

42. Seo, P. J. & Park, C. M. MYB96-mediated abscisic acid signals induce pathogen resistance response by promoting salicylic acid biosynthesis in Arabidopsis. New Phytol. 186, 471–483 (2010).

43. De Vos, M. et al. The Arabidopsis thaliana transcription factor AtMYB102 functions in defense against the insect herbivore Pieris rapae. Plant Signal. Behav. 1, 305–311 (2006).

44. Kishi-Kaboshi, M., Sano, S., Takahashi, A. & Hirochika, H. The MAMP-responsive MYB transcription factor MYB30, MYB85 and MYB110 activate the HCAA synthesis pathway and enhance immunity in rice. Plant Cell Physiol. 59, 903–915 (2018).

45. Zhu, L., Guo, J., Ma, Z., Wang, J. & Zhou, C. Arabidopsis transcription factor MYB102 increases plant susceptibility to aphids by substantial activation of ethylene biosynthesis. Biomolecules 8, 39 (2018).

46. Zhu, Y. et al. Three MYB genes co-regulate the phloem-based defence against English grain aphid in wheat. J. Exp. Bot. 68, 4135–4169 (2017).

47. Li, R. et al. Thirty-seven transcription factor genes differentially respond to a harpin protein and affect resistance to the green peach aphid in Arabidopsis. J. Biosci. 35, 435–450 (2010).

48. Lu, B. et al. AtMYB 44 regulates resistance to the green peach aphid and diamondback moth by activating EIN2-affect ed defences in Arabidopsis. Plant Biol. 15, 841–850 (2013).

49. Liu, R. et al. Transcription factor AtMYB44 regulates induced expression of the ETHYLENE INSENSITIVE2 gene in Arabidopsis responding to a harpin protein. Mol. Plant–Microbe Interact. 24, 377–389 (2011).

50. Will, T. & van Bel, A. J. Induction as well as suppression: How aphid saliva may affect defensive responses. Insect Biochem. Mol. Biol. 38, 153–160 (2008).

51. von Amir, A. Subcellular localization of GUS and GFP-tagged proteins in onion epidermal cells. Cold Spring Harb. Protoc. 2007, e65689 (2007).

52. Ma, D. & Constabel, C. P. MYB repressors as regulators of phenylpropanoid metabolism in plants. Trends Plant Sci. 24, 275–289 (2019).

53. Li, F. et al. Chrysanthemum Cm HSFA 4 gene positively regulates salt stress tolerance in transgenic chrysanthemum. Plant Biotechnol. J. 16, 1311–1321 (2018).

54. Livak, K. J. & Schmittgen, T. D. Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods. 25, 402–408 (2001).

55. Li, P. et al. The over-expression of a chrysanthemum WRKY transcription factor enhances aphid resistance. Plant Physiol. Biochem. 95, 26–34 (2015).

56. Hu, H. et al. Modification of chrysanthemum odour and taste with chrysanthemol synthase induces strong dual resistance against cotton aphids. Plant Biotechnol. J. 16, 1434–1445 (2018).