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The R2R3-MYB transcription factor MtMYB134 orchestrates flavonol biosynthesis in *Medicago truncatula*

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Abstract

Key message **Our results provide insights into the flavonol biosynthesis regulation of *M. truncatula*. The R2R3-MYB transcription factor MtMYB134 emerged as tool to improve the flavonol biosynthesis.**

Abstract Flavonols are plant specialized metabolites with vital roles in plant development and defense and are known as diet compound beneficial to human health. In leguminous plants, the regulatory proteins involved in flavonol biosynthesis are not well characterized. Using a homology-based approach, three R2R3-MYB transcription factor encoding genes have been identified in the *Medicago truncatula* reference genome sequence. The gene encoding a protein with highest similarity to known flavonol regulators, *MtMYB134*, was chosen for further experiments and was characterized as a functional flavonol regulator from *M. truncatula*. *MtMYB134* expression levels are correlated with the expression of *MtFLS2*, encoding a key enzyme of flavonol biosynthesis, and with flavonol metabolite content. MtMYB134 was shown to activate the promoters of the *A. thaliana* flavonol biosynthesis genes *AtCHS* and *AtFLS1* in Arabidopsis protoplasts in a transactivation assay and to interact with the Medicago promoters of *MtCHS2* and *MtFLS2* in yeast 1-hybrid assays. To ascertain the functional aspect of the identified transcription factor, we developed a sextuple mutant, which is defective in anthocyanin and flavonol biosynthesis. Ectopic expression of *MtMYB134* in a multiple *myb A. thaliana* mutant restored flavonol biosynthesis. Furthermore, overexpression of *MtMYB134* in hairy roots of *M. truncatula* enhanced the biosynthesis of various flavonol derivatives. Taken together, our results provide insight into the understanding of flavonol biosynthesis regulation in *M. truncatula* and provides MtMYB134 as tool for genetic manipulation to improve flavonol synthesis.

Keywords Metabolic engineering · Legume · Gene expression analysis · Hairy root transformation · Flavonol biosynthesis

Introduction

Flavonoids constitute a group of plant specialized metabolites, synthesized through the phenylpropanoid pathway. Diverse enzymatic activities lead to the biosynthesis of different types of flavonoids such as flavonols, anthocyanins, flavones, isoflavones and proanthocyanidin. The manifold functions of flavonoids in plants and their potential beneficial effects on human health prompted the researchers to study their biosynthesis as well as to design strategies to manipulate the flavonoid biosynthesis through genetic tools (Ververidis et al. 2007). While manipulation of structural genes showed limited impact over biosynthesis of a particular class of flavonoids, manipulation by regulatory genes proved to have far reaching consequences on the flavonoid biosynthesis and thereby having biotechnological applications (Butelli et al. 2008; Misra et al. 2010). Studies in various plant species, mainly *Petunia hybrida*, *Antirrhinum majus, Zea mays* and *Arabidopsis thaliana,* have concluded differential regulation of this pathway through similar sets of regulatory proteins. At least two roughly discrete sets of coordinately regulating units for the early biosynthesis genes (EBGs; leading to the biosynthesis of dihydroflavonols,

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flavonols and flavones) and the late biosynthesis genes (LBGs; leading to the biosynthesis of anthocyanin and proanthocyanidins) exist in higher plants (Quattrocchio et al. 2006; Lepiniec et al. 2006; Gonzalez et al. 2008). The main difference between the two regulating units is, that EBGs are predominantly regulated by co-factor independent R2R3-MYBs. In contrast, the LBGs are regulated by a transcription factor complex mainly composed of R2R3-MYBs, bHLHs and a WD40-repeat protein (summarized in Xu et al. 2015). Both regulating units have in common that R2R3-MYB transcription factors are involved.

Plant R2R3-MYB transcription factor coding genes comprise one of the largest known gene families. These factors are involved in the regulation of a variety of cellular processes in plants including control of specialized metabolism, cell differentiation and morphogenesis (Bar et al. 2016; Wang et al. 2019). While only 126 MYBs of the R2R3-type have been described in *A. thaliana* (Stracke et al. 2001), 155 and 285 R2R3-MYB encoding genes have been identified in the *M. truncatula* and *M. acuminata* genomes, respectively (Wei et al. 2017; Pucker et al. 2020).

In the view of various important role of flavonols for both, plant biology and human health, P-like R2R3-MYB regulatory proteins have been explored for the enhancement of flavonols in different model systems like tobacco (Pandey et al. 2014a, 2015a) and tomato (Pandey et al. 2015b; Zhang et al. 2015). Since overexpression of the flavonol-specific regulator *AtMYB12* significantly enhanced flavonol content in the heterologous plants, tobacco and tomato, this transcription factor has also been utilized for the metabolic channeling of substrate flux towards the biosynthesis of phytoestrogen genistein (Pandey et al. 2014b).

In maize, the R2R3-MYB protein ZmP alone is sufficient to activate the flavonoid pathway genes involved in the synthesis of deoxy flavonoids and red phlobaphene pigments (Lepiniec et al. 2006). In *A. thaliana*, tissue specific expression of functionally redundant MYBs (*AtMYB12*, *AtMYB111* and *AtMYB11*) lead to the transcription of flavonol biosynthesis target genes (*CHS*, *CHI*, *F3H* and *FLS*) without requiring a bHLH co-factor interaction (Mehrtens et al. 2005; Stracke et al. 2007, 2010a). Furthermore, seedlings of a loss-of-function triple mutant *myb12 myb111 myb11* are devoid of flavonol pigments while anthocyanin biosynthesis is not affected, which indicated the existence of a separate set of regulators for the anthocyanin and flavonol branch. In *Vitis vinifera* (grapevine), a flavonol specific regulator VvMYBF1 has been identified, which like AtMYB12, specifically targets the *FLS* promoter and a few EBGs. Moreover, it could complement the root flavonol deficient phenotype of *A. thaliana myb12* mutant (Czemmel et al. 2009).

Despite their important role for plants as well as nutraceutical value, the flavonol-specific transcription factors have not yet been studied in the model leguminous plant *M. truncatula*; in contrast to the later flavonoid pathway, where regulatory proteins involved in anthocyanin and proanthocyanidin biosynthesis have been reported (Peel et al. 2009; Verdiera et al. 2012; Li et al. 2016). The release of the *M. truncatula* reference genome sequence (Young et al. 2011) provides a useful genetic resource for functional genomics of this species. In the present study, we identified the *M. truncatula* R2R3-MYB MtMYB134 as a flavonol specific activator. Using gene expression studies, expression-metabolite correlations, transient transactivation assays in Arabidopsis protoplasts, yeast-1-hybrid studies, Arabidopsis mutant complementation and *M. truncatula* hairy root expression, we describe the molecular mechanism underlying transcriptional regulation of the flavonol biosynthesis in *M. truncatula*.

Materials and methods

Plant materials

Medicago truncatula cultivars R108 and A17 were grown in the fields of National Institute of Plant Genome Research (NIPGR), New Delhi, India and used for gene expression and metabolite accumulation studies. For hairy root transformation, A17 seedlings were grown on MS plates in a growth chamber. Tissue cultures were grown at 22 ± 2 °C with 16 h light/8 h dark cycles. A *myb* sextuple mutant was generated by crossing a triple *myb* mutant (*myb12 myb111 myb11,* Stracke et al. 2007) with the triple *myb* mutant (*myb75 myb90 myb114,* Appelhagen et al. 2011). The *myb* sextuple mutant was identified in the F2 generation by PCR analysis and characterized in terms of metabolite accumulation. Seedlings of this regulatory mutant were not able to accumulate flavonols or anthocyanins. The *A. thaliana* mutant, wild type (Col-0) plants and *MtMYB134* overexpressing lines were grown with a photoperiod 16 h light and 8 h dark at 22 °C in Plant Growth Chamber (AR-41L3; Percival). The *A. thaliana* suspension cell culture At7 (Trezzini et al. 1993) is derived from hypocotyl of the reference accession Columbia (Col) and handled as described in Stracke et al. (2016).

Identification and expression analysis of flavonol biosynthesis genes of *M. truncatula*

Exploratory gene trees were generated via MAFFT v.7.229b (Katoh and Standley 2013), phynx (Brown et al. 2017), and FastTree2 (Price et al. 2010) based on Python scripts (Yang et al. 2015) which were previously combined into a pipeline and optimized for usage on a high-performance compute cluster. Well characterized bait sequences retrieved from the NCBI database served as baits. Candidate genes were inferred from the resulting phylogenetic trees. Microarray

expression data were retrieved from the *M. truncatula* gene expression atlas (https://mtgea.noble.org/v3/). A customized Python script was deployed to generate gene expression heatmaps for selected samples and candidate genes based on the package seaborn (https://seaborn.pydata.org/generated/seaborn.heatmap.html).

Identification and cloning of flavonol specific transcription factor from *M. truncatula*

The functionally characterized flavonol specific transcription factors from various plants were subjected to a tblastn search against the *M. truncatula* genome sequence (GCF_000219495.3) for the identification of candidate R2R3-MYB transcription factor putatively involved in the regulation of flavonol biosynthesis. The first strand cDNA of *M. truncatula* seedlings R108 was used as template for the PCR amplification of full-length *MtMYB134* coding sequence using a set of oligonucleotides, designed on the basis of sequence information available in *M. truncatula* genome database (Table S1). The PCR product was analyzed on 1% agarose gel in TBE buffer. The amplicon of expected size was excised from the gel and purified by using Amersham gel extraction column (GE Healthcare, USA) as per the manufacturer's recommendation. The amplicon was cloned into the Gateway cloning vector pDONR^{TM}zeo and transformed in *E. coli* TOP10. Integrity of plasmid DNA was proven by Sanger sequencing at the sequencing core facility of NIPGR, New Delhi, India.

Phylogenetic analysis

MtMYB peptide sequences were aligned to a previously described set of landmark MYBs (Stracke et al. 2014) via MAFFT v.7.299b (Katoh and Standley 2013). The alignment was trimmed via phyx (Brown et al. 2017) and finally subjected to MEGA X (Kumar et al. 2018) for the construction of a Neighbor joining phylogenetic tree with 1000 bootstrapping rounds.

Co-transfection analysis using protoplast of At7 cell lines

To prepare the effector constructs for At7 protoplast co-transfection analysis, the *MtMYB134* CDS was cloned into pBTDest vector (Baudry et al. 2004). The co-transfection analysis using protoplast of cultured At7 cell lines has been described previously (Stracke et al. 2016). The reporter constructs of Arabidopsis flavonoid biosynthesis enzymes promoters were also described previously (Mehrtens et al. 2005). The transfection assay was performed with a total of 25 µg of premixed plasmid DNA having 10 µg of reporter construct, 10 µg of effector construct and 5 µg of LUC plasmid as transfection control and standardization plasmid (Sprenger-Haussels and Weisshaar 2000). Briefly, transfected protoplasts were incubated for 20 h at 26 °C in the dark followed by harvesting the protoplast for LUC, GUS and Bradford assay. Specific GUS activity is given in pmol 4-methylumbelliferone (4-MU) mg^{-1} of protein min^{-1}. Standardized GUS activity was calculated by multiplication of the specific GUS activity value with a correction factor derived from the ratio of the specific LUC activity in the given sample to the mean specific LUC activity (describing the transformation efficiency) of a set of six experiments.

Yeast 1-hybrid assay

The Matchmaker™ Gold Yeast 1-hybrid library screening system (Clontech) with the Y1H gold yeast strain was used for this purpose. Promoter fragments of the major flavonol biosynthesis genes *MtCHS2, MtF3H4* and *MtFLS2* were amplified and cloned into pABAi vector (Cat. No. 630491, Takara Bio USA, Inc.). The pABAi plasmid carrying promoter fragments were linearized and transformed into Y1H Gold strain according to the Matchmaker™ manual. The CDS corresponding to the *MtMYB134* was cloned into pGADT7-GW vector to make effector constructs (Lu et al. 2010). Effector construct plasmids and empty pGADT7-GW vector control were transformed into Y1H strains with genome-integrated reporter constructs. Screening for interactions was performed by checking growth on Aureobasidin supplemented media. Yeast growth was checked under different concentration of aureobasidin (AbA) to determine MIC (minimum inhibitory concentration) values.

Complementation analysis in Arabidopsis

For Arabidopsis complementation analysis, *MtMYB134* CDS was cloned from the gateway entry plasmid into the T-DNA binary destination vector pLEELA (Jakoby et al. 2004) harbouring double enhancer 35S (2×35S) promoter and BASTA resistance for selection of transgenic lines. *MtMYB134* in pLEELA was used for floral dipping (Clough and Bent 1998) in the *myb* sextuple mutant (*myb11 myb12 myb111 myb75 myb90 myb114*) via *Agrobacterium tumefaciens* (GV3101::pM90RK) (Koncz and Schell 1986).

Hairy root transformation of *M. trucatula*

For hairy root transformation, we utilized *MtMYB134* CDS in pSITE-4NB vector) and empty vector control (Chakrabarty et al. 2007; Singh et al. 2020). The *M. truncatula* accession A17 was used for hairy root transformation with the *Agrobacterium rhizogenes* ARqua1 strain as described previously (Dernier et al. 2001). Sterilized seeds were kept in dark till germination. Seedlings with approx. 1 cm long

roots were cut at the middle of the root, and the cut end was coated with ARqua1 culture by gently scraping. Finally, the seedlings were kept and placed on Fahraeus Media and incubated at 22 °C with 14–10 h light–dark photoperiods for 12–14 days. The transformed hairy roots were identified under a stereomicroscope, showing RFP fluorescence using a red filter/RFP filter.

Gene expression analysis

Total RNA was isolated from different organs of *M. truncatula* plants using the Spectrum Plant Total RNA kit (Sigma-Aldrich) and subsequently treated with RNase-free DNase (Fermentas life sciences). Total RNA was subjected to reverse transcription to generate first-strand cDNA using oligo (dT) primers (MBI Fermentas). RT-PCR analysis of a set of selected genes was carried out using 2 × PCR Master mix (ABI). The list of selected genes and the sequences of oligonucleotides used in the study are provided in Table S1. The PCR mix contained 1 µl of diluted cDNA (corresponding to 10 ng total RNA), 10 µl of 2 × SYBR Green PCR Master Mix (Applied Biosystems, USA) and 200 nM of each gene-specific primer in a final volume of 20 µl. PCRs with no template controls were also performed for each primer pair. Expression of different genes involved in the flavonoid biosynthesis was studied through 7500 Fast Real time PCR System (Applied Biosystems). *MtACTIN2* was used as a reference control in the analysis (Li et al. 2016) and the expression levels are given as relative fold change ($2^{-\Delta\Delta CT}$) as compared to the lowest expressing tissue (set as 1). All PCRs were performed under the following conditions: 20 s at 95 °C, 3 s at 95 °C, and 40 cycles of 30 s at 60 °C in 96-well optical reaction plates (Applied Biosystems, USA). The specificity of amplicons was verified by melting curve analysis (60–95 °C) after 40 cycles. Three technical replicates were analyzed for each cDNA (three biological replicates).

High-performance thin layer chromatography and DPBA staining for detection of flavonol glycosides

Approximately 300 mg of whole *A. thaliana* seedlings were extracted with 80% methanol. Samples were homogenized in a Geno/Grinder® (SPEX Sample Prep P-2010). Homogenized plant materials were incubated at 70 °C for 15 min followed by centrifugation. Supernatants were transferred to a fresh reaction tube and vacuum-dried in a SpeedVac at 65 °C. The dried pellets are redissolved in a smaller volume of 80% methanol and used for HPTLC analysis as described previously (Stracke et al. 2010a). The flavonol glycosides were stained by spraying solutions (w/v) of 1% DPBA and 5% PEG 4000 and detected under UV light (365 nm). The kaempferol and quercetin derivatives provide greenish and orange fluorescence, respectively. Apart from green and orange fluorescence, the blue color spots represent sinapate derivatives (Stracke et al. 2010a).

LC–MS analysis for quantification of flavonols

Targeted analysis of flavonols was carried out as described previously (Pandey et al. 2016; Sharma et al. 2016). Plant flavonols were extracted with 80% methanol at room temperature under brief agitation followed by centrifugation. Supernatants were transferred to a fresh reaction tube and vacuum-dried in a SpeedVac at 65 °C. The dried pellets were dissolved in 80% methanol.

The LC–MS analysis was performed using an UPLC system (Exion LC Sciex) coupled to a triple quadrupole system (QTRAP6500 + ; ABSciex) using an electrospray ionization. The voltage was set at 5500 V for positive ionization. The values of gas 1 and gas 2 (70 psi), curtain gas (40 psi), collision-assisted dissociation (medium), and temperature of the source (650 °C) were used. The mass spectrometer was used in multiple reaction monitoring mode (MRM) for qualitative and quantitative analysis. Analytical standards were purchased from Merck. Identification and quantitative analysis were carried out using Analyst software (version 1.5.2).

Visualization of anthocyanins

Seeds of different Arabidopsis lines were placed on a filter paper soaked with 3 µg/ml of the bleaching herbicide norflurazon (Supelco) along with 4% sucrose for induction of anthocyanin accumulation. After 2 days at 4 °C, the plates were transferred to a plant growth chamber (AR-41L3; Percival) with 16 h of light per day at 22 °C. After 5 days, images of the seedlings were captured using a stereomicroscope.

Results

Identification of candidate flavonoid biosynthesis enzyme-coding genes

Since systematic information regarding individual enzymes and their homologs involved in flavonoid biosynthesis were not available for *M. truncatula*, we screened the reference genome sequence (GCF_000219495.3) for the candidate genes. Phylogenetic trees, based on deduced amino acid sequence similarity to protein sequences of representative flavonoid biosynthesis enzymes, led to the identification of *M. truncatula* candidate genes involved in the enzymatic steps in flavonoid biosynthesis (Fig. S1 to S8). We identified

ten candidates for CHS (MtCHS1-MtCHS10), four candidates for CHI (MtCHI1-MtCHI4), F3H (MtF3H1-MtF3H4) and F3′H (MtF3′H1-MtF3′H4), three candidates for FLS (MtFLS1-MtFLS3), two candidates for F3′5′H (MtF3′5′H1-MtF3′5′H2) and DFR (MtDFR1-MtDFR2), and single candidates for ANS (MtANS), ANR (MtANR) and LAR (MtLAR). The expression data of the candidate genes in different organs was extracted from the *M. truncatula* gene expression atlas (https://mtgea.noble.org/v3/) and is given in Fig. 1.

Identification of flavonol-specific transcription factors from *M. truncatula*

In an earlier study, in silico analysis led to the identification of putative R2R3-MYB-type flavonol specific regulators in *M. truncatula* (Wei et al. 2017). The putative R2R3 MYB served as basis for the identification of corresponding regulators in the *M. truncatula* genome through sequence similarity. Three homologous R2R3-MYB proteins MtMYB30, MtMYB77 and MtMYB134 were identified as candidate flavonol specific regulators (Fig. 2). MtMYB30, MtMYB77, and MtMYB134 encode proteins of 332 amino acids (aa), 261 aa, and 348 aa, respectively. The phylogenetic analysis indicated, that all three candidate proteins cluster in the same

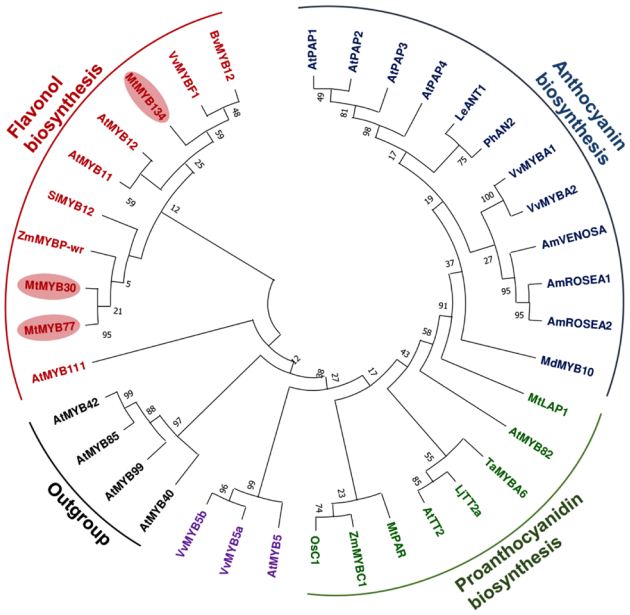


Fig. 2 Identification of candidate Medicago R2R3-MYB flavonol regulators. The depicted R2R3-MYB proteins were classified into three major groups as flavonol-, anthocyanin- and proanthocyanidin biosynthesis specific regulators using landmark flavonoid specific R2R3-MYBs from different plant species. The phylogenetic tree was constructed using MEGA X. MtMYBs are highlighted by red ovals

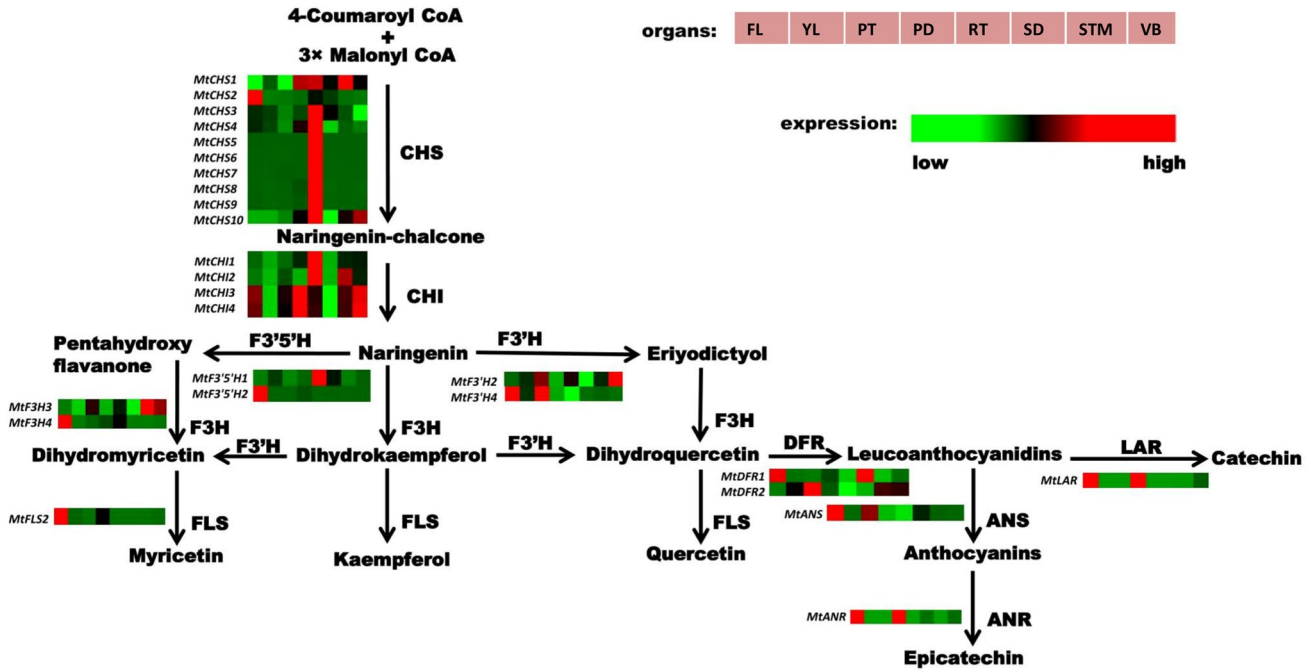
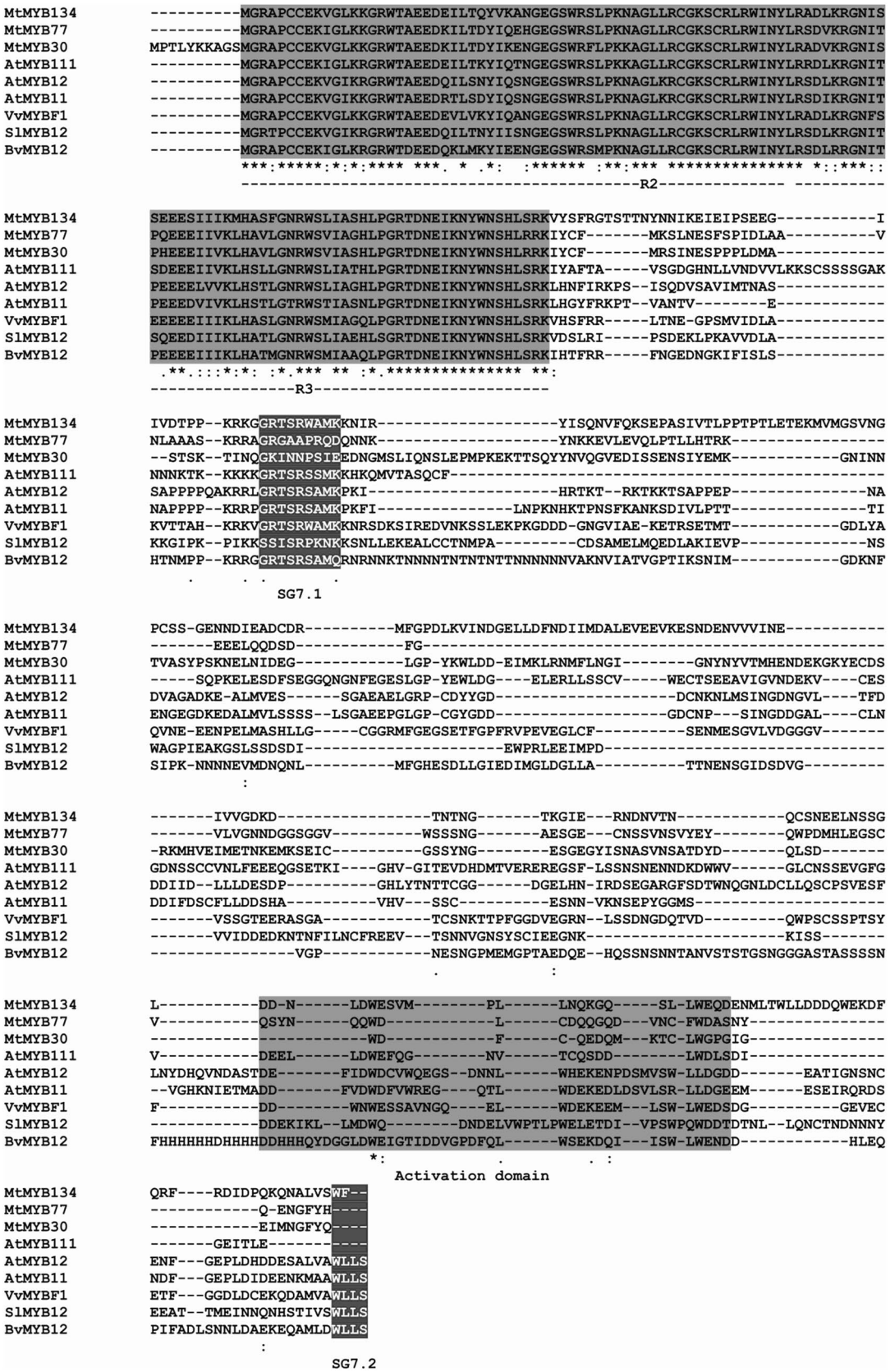


Fig. 1 Schematic representation and expression analysis of general flavonoid biosynthesis pathway in *M. truncatula*. Each column from left to right represents gene expression in different organs, FL (Flower), YL (Young leaves), PT (Petiole), PD (Pod), SD (Seed), STM (Stem) and VB (Vegetative Bud) and individual genes of *M. truncatula* genome are displayed in rows. *CHS* chalcone synthase, *CHI* chalcone isomerase, *F3H* flavanone 3-hydroxylase, *F3′H* flavonoid 3′-hydroxylase, *F3′5′H* flavonoid 3′5′-hydroxylase, *FLS* flavonol synthase, *DFR* dihydroflavonol reductase, *ANS* anthocyanin synthase, *ANR* anthocyanin reductase, *LAR* leucoanthocyanidin reductase. Given expression values are derived from the *M. truncatula* gene expression atlas. Color bar scale represents the range of expression data after normalization (X-m/σ)


MtMYB134
MtMYB77
MtMYB30
AtMYB111
AtMYB12
AtMYB11
VvMYBF1
SlMYB12
BvMYB12
R2
R3
SG7.1
Activation domain
SG7.2

◂**Fig. 3** Multiple sequence alignment of candidate flavonol specific R2R3-MYB proteins from *M. truncatula* with known flavonol regulators. The R2 and R3 repeats of the MYB domain, SG7 defining motifs and the putative activation domain are marked according to previously characterized flavonol specific R2R3-MYBs

clade together with functionally proven flavonol specific regulatory proteins from diverse plant species.

Structural analogy of candidate MtMYB proteins with SG7 R2R3-MYB transcription factors

Comprehensive bioinformatic analysis disclosed high aa sequence conservation (more than 40% overall sequence similarity) of MtMYB30, MtMYB77 and MtMYB134 to landmark R2R3-MYBs having been implicated flavonol-specific activators of flavonoid biosynthesis. This especially includes the *V. vinifera* flavonol regulator VvMYBF1 and the *A. thaliana* PRODUCTION OF FLAVONOL GLYCOSIDES (PFG) family constituted of AtMYB12/AtPFG1, AtMYB11/AtPFG2 and AtMYB111/AtPFG3. Similarity is highest in the N-terminal R2R3-MYB domain (Fig. 3). Further analysis of the deduced amino acid sequences of MtMYB30, MtMYB77 and MtMYB134 revealed that only MtMYB134 contains one of the motifs which have been described to be characteristic for flavonol regulators. The subgroup S7 motif (GRTxRSxMK, Stracke et al. 2001) was found to be present in the C-terminus of MtMYB134 with one amino acid substitution (GRTSRWAMK) while it was absent in MtMYB30 and MtMYB77. The C-terminal SG7-2 motif [(W/x)(L/x)LS, Czemmel et al. 2009] was found to be partially conserved in MtMYB134, while it was absent in MtMYB30 and MtMYB77 (Fig. 3). The putative activation domains (AD) of the identified proteins were mapped according to the identified AD of AtMYB12 (Stracke et al. 2017). MtMYB134 showed highest similarity of the three MtMYBs to the AtMYB12 AD sequence. These results made us choosing MtMYB134 as the most promising candidate flavonol specific regulator from *M. truncatula,* and we focused the further work on this factor.

MtMYB134 is differentially expressed

Tissue specific RT-qPCR based expression analysis suggested higher transcript abundance of *MtMYB134* in seeds as compared to other tissues (Fig. 4a). Differential expression was found for the *MtMYB134* gene in whole seedlings, seedling roots, seedling shoots, young leaves and stem (Fig. 4a). To correlate the expression of *MtMYB134* with its putative target genes, we have analyzed the expression of selected Medicago candidate flavonol biosynthesis enzymes which showed highest similarity to known flavonoid biosynthesis enzymes of other leguminous plants. The expression of *MtCHS2, MtCHI1, MtF3H4, MtF3'H2* and *MtFLS2* was analyzed in the same tissues (Fig. 4b). Similar to *MtMYB134*, higher transcript abundance was noticed in seed tissue (SD). Despite lowest expression of *MtMYB134* in roots, the expression of *MtCHS2* and *MtCHI1* was higher in this organ.

Flavonols are differentially accumulated in various organs of *M. truncatula*

Targeted metabolite profiling by LC–MS was performed for quantitative estimation of various flavonol aglycones and glycones in different vegetative and reproductive tissues of *M. truncatula*. We quantified one of the most prominent flavonol glycosides, rutin, a glycosylated form of quercetin along with aglycone forms of kaempferol and quercetin. We found that young leaves accumulated the highest amount of kaempferol and rutin. Quercetin content was found to be elevated in young leaves, but mature seeds have the highest quercetin content. We noticed least accumulation of all these flavonols in roots. All other tissues showed varying level of different flavonols (Fig. 5).

These metabolite data can be correlated to the differential expression of *MtMYB134* along with flavonol biosynthesis genes. Higher expression of *MtMYB134* was observed in mature seeds with the root tissue having least expression (Fig. 4a). Higher amount of quercetin and rutin was observed in mature seeds which can be correlated to higher transcript abundance of *MtF3H4, MtF3'H2, MtFLS2* and *MtMYB134* (Figs. 4a, b, 5). Flavonol content was also higher in young leaf tissue despite lower expression of *MtCHS2*, *MtCHI1* and *MtF3H4*.

Assessment of the regulatory specificities of MtMYB134

To analyze the transactivation potential of MtMYB134 and to test its functionality, we utilized a PEG-mediated co-transfection based transient expression system using protoplasts of the *A. thaliana* At7 cell line. This system allows co-transfection of effector and reporter constructs for quantification of GUS activity, which after normalization, was taken as a measure of promoter activation. The effector construct harbors MtMYB134 ORF fused with *uidA* ORF (GUS). The responsiveness of the effector construct was tested with the promoter (reporter construct) of structural genes involved in the flavonol biosynthesis in *A. thaliana*. Apart from these reporters, we utilized *AtDFR* promoter to test whether the identified regulatory protein activates anthocyanin biosynthesis. The co-transfection analysis suggested, that the candidate flavonol regulator has the potential to transactivate exclusively *AtCHS* and *AtFLS1* promoters at different levels (Fig. 6). As expected, the identified regulatory protein revealed no transactivation potential with the

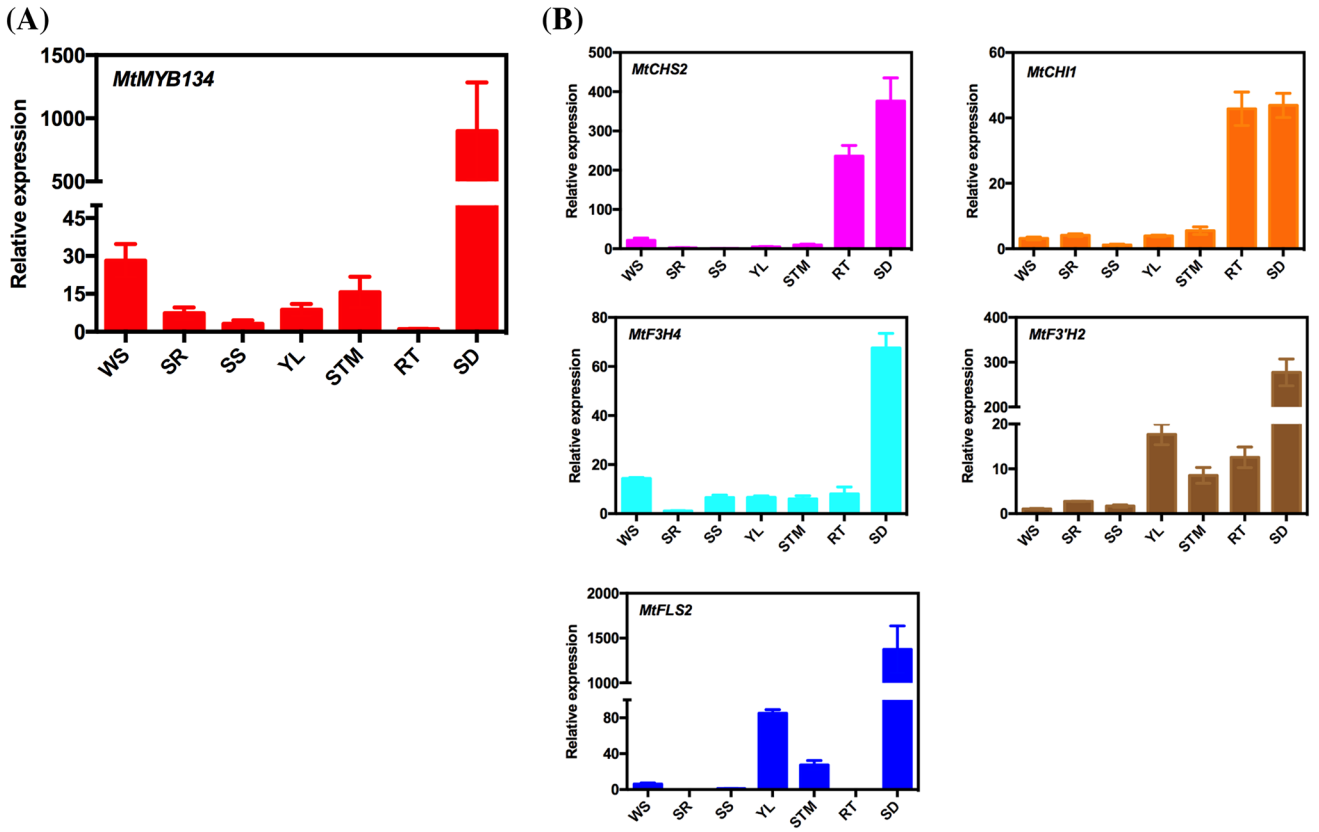


Fig. 4 Tissue specific expression of *MtMYB134* and structural candidate flavonol biosynthesis genes. Quantitative real time expression analysis of *MtMYB134* (**a**) and structural flavonol biosynthesis genes (**b**) was carried out in WS (whole seedling), SR (seedling root), SS (seedling shoot), YL (young leaves), STM (stem), RT (root) and SD (seed). The graphs show values ± SD of three technical replicates

AtDFR promoter. As known from other flavonol specific regulators, MtMYB134 does not require any known co-factor for transactivation in this assay. These results indicate, that MtMYB134 has the potential to transactivate *AtCHS* and *AtFLS* through binding of the promoters in a heterologous system.

MtMYB134 complements the flavonol-deficient phenotype of an *A. thaliana myb mutant*

To further validate the *in planta* function of *MtMYB134* as a flavonol regulator, we over-expressed *MtMYB134* under the control of double CaMV35S promoter in a flavonol-deficient regulatory multiple *myb* mutant of *A. thaliana*. The transgenic *A. thaliana* seedlings were analysed by high performance thin layer chromatography (HPTLC) fingerprint for their ability to reconstitute flavonol glycoside accumulation. The HPTLC analysis showed clear differences between WT (Col-0), mutant line, and three *MtMYB134* complemented lines. The expression of *MtMYB134* lead to the accumulation of various quercetin and kaempferol derivatives (Fig. 7a) in the multiple *myb* mutant. For quantitative estimation of flavonols, we performed LC–MS analysis of Arabidopsis seedlings which revealed that the content of quercetin, kaempferol and rutin was almost comparable in WT and complemented lines, while in mutants no flavonols could be detect (Fig. 7b). Taken together, the complementation analysis suggested that *MtMYB134* functions as flavonol regulator *in planta*.

We also analyzed anthocyanin pigmentation in the *MtMYB134* complemented seedlings. Visual inspection of sucrose-induced anthocyanin pigmentation in norflurazon-bleached seedlings showed no indication of accumulation of anthocyanin pigments (Fig. 7c), suggesting that *MtMYB134* do not have the potential to activate anthocyanin biosynthesis in Arabidopsis.

Ectopic expression of *MtMYB134* in *M. truncatula* hairy roots

To analyze MtMYB134 function in a homologous system and to validate its possible application towards pathway engineering of flavonol biosynthesis, we used hairy root transformation in *M. truncatula* with a *35S::MtMYB134-RFP* construct. The transformed hairy root (HR) lines were screened for the presence of RFP fluorescence, indicating

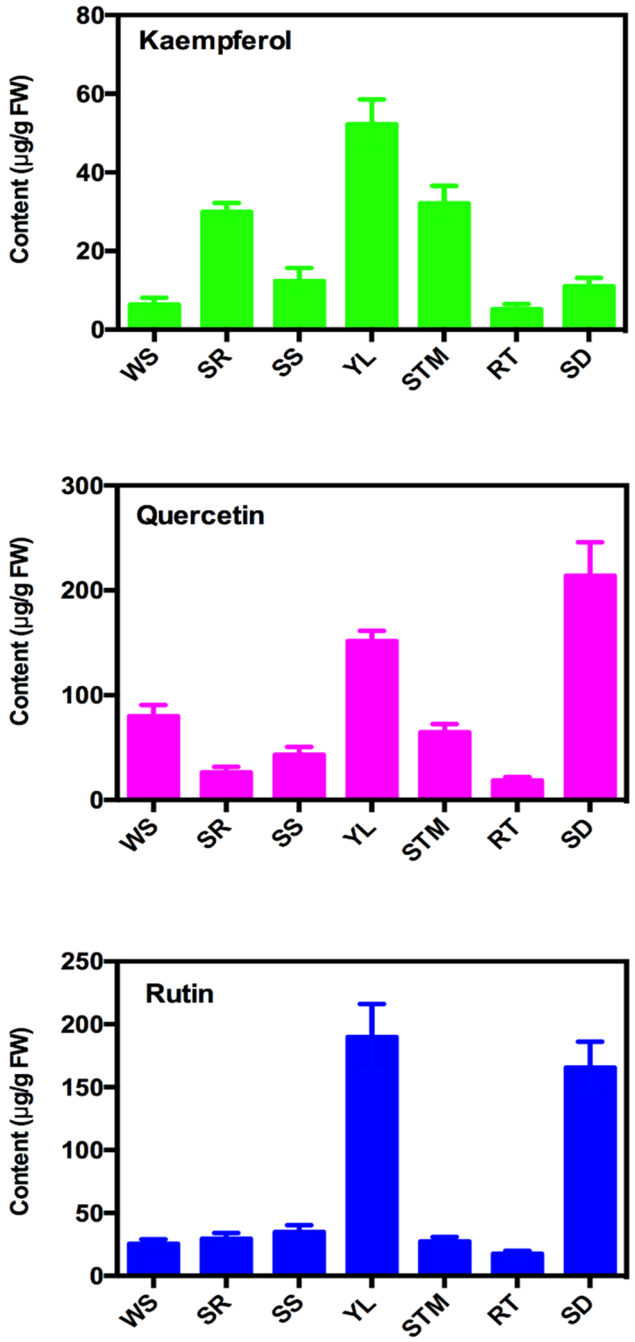


Fig. 5 Targeted LC–MS analysis of various flavonols in *M. truncatula* organs. Quantitative estimation of flavonols in *M. truncatula* organs. LC–MS analysis was carried out using methanolic extracts of different tissues (as described in Fig. 4). Compounds were quantified by developing calibration and multiple reaction monitoring (MRM) of authentic standards of quercetin, kaempferol and rutin. The graph shows values ± SD of three biological and six technical replicates from each sample

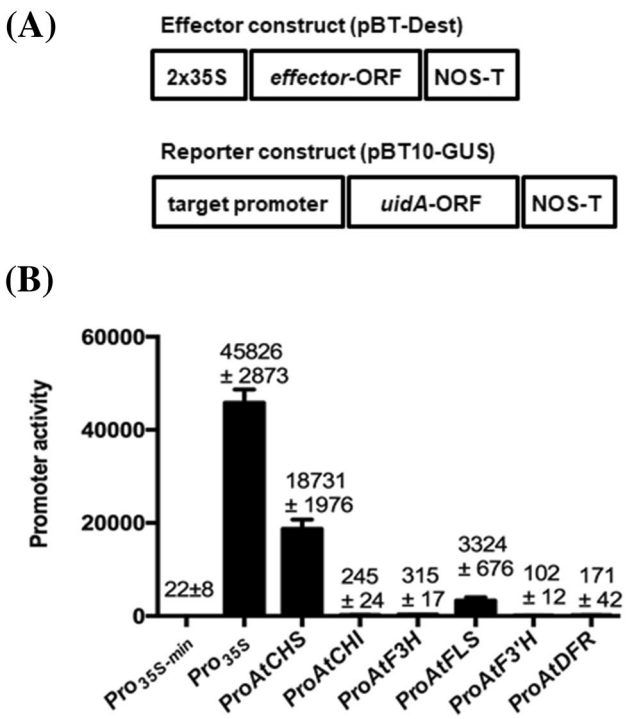


Fig. 6 Co-transfection analysis in At7 protoplasts indicate in vivo regulatory potential of MtMYB134 on *A. thaliana CHS* and *FLS1* promoters. Schematic representation of the effector and reporter constructs used. Fragments of Arabidopsis flavonoid biosynthesis gene promoters (reporters) were assayed for their responsiveness to the effector MtMYB134 expressed under the control of the CaMV 35S promoter (**a**). Results from co-transfection experiments in At7 protoplasts. The figure shows promoter activity (mean GUS activity) resulting from the influence of the effector protein on different reporters (**b**)

transformants (Fig. 8a). Expression of *MtMYB134* was substantially higher in *MtMYB134-RFP* overexpressing hairy roots as compared to the *35S::RFP* empty vector (EV) transformed roots (Fig. 8b). We have also scored the expression of flavonol biosynthesis genes in EV and *MtMYB134-RFP* overexpressing HR. RT-qPCR analysis suggested that the transcript levels of *MtCHS2*, *MtCHI1*, *MtF3H4*, *MtF3'H2* and *MtFLS2* were higher in *MtMYB134-RFP* as compared to transformed HR (Fig. 8c). In particularly, the *MtFLS2* transcript was significantly higher (more than 25-fold) in *MtMYB134-RFP* line. To further validate, whether the higher expression of targeted genes led to increased biosynthesis of flavonols, we performed targeted LC–MS analysis to quantify various flavonols in EV and *MtMYB134-RFP* overexpressing HR. Our LC–MS analysis suggested higher content of kaempferol (two fold), quercetin (four fold) and rutin (six fold) in *MtMYB134-RFP* overexpressing lines (Fig. 8d).

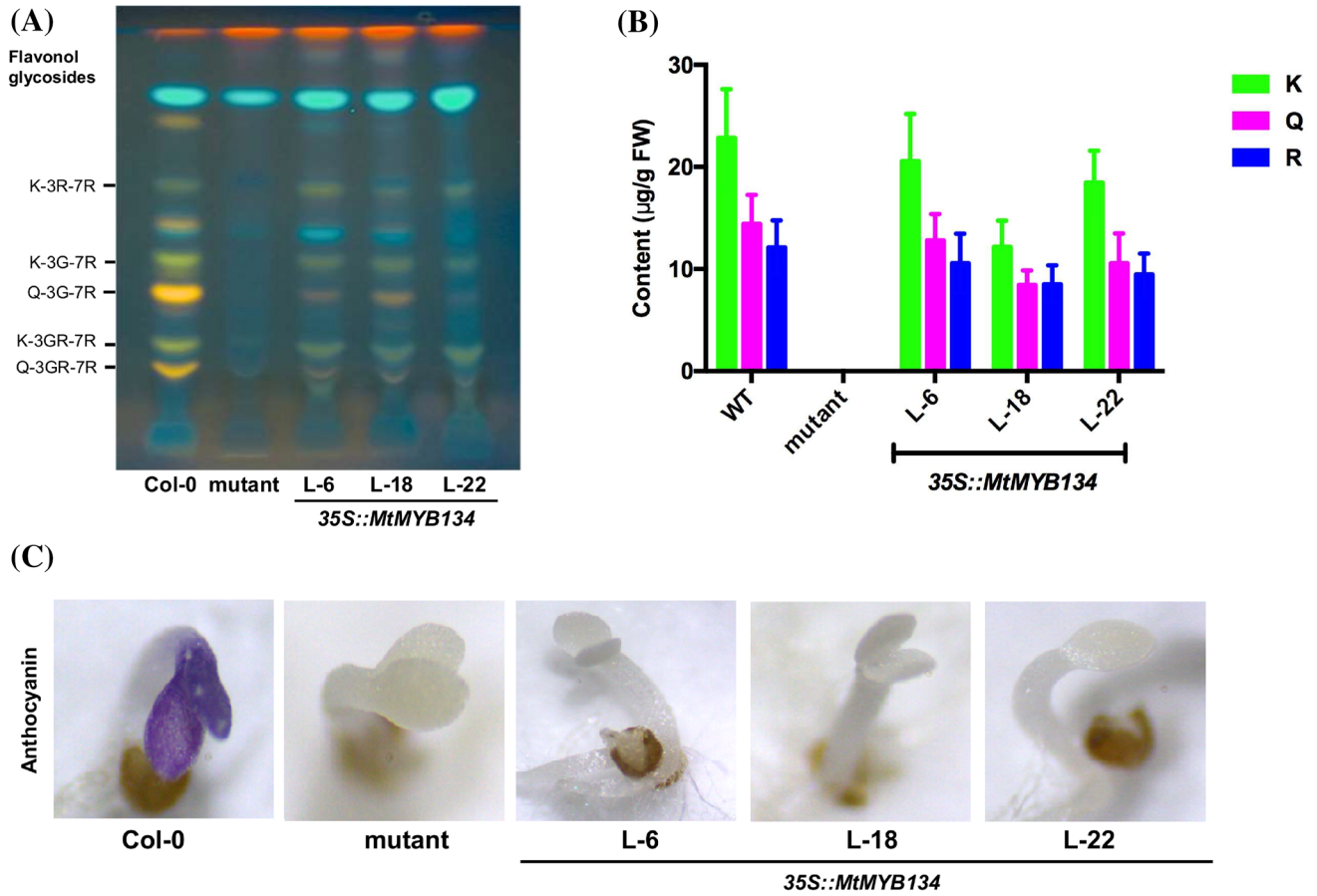


Fig. 7 *MtMYB134* restores the flavonol-deficient phenotype of a regulatory multiple *A. thaliana myb* mutant. HPTLC of methanolic extracts of T2 seedlings of independent transgenic *A. thaliana* lines (L-6, L-18, L-22) containing T-DNA insertions with a *35S:MtMYB134* construct in the flavonol- and anthocyanin-deficient multiple *myb* mutant background. Flavonol glycosides were detected by DPBA staining and visualisation under UV illumination. Green fluorescence indicates different derivatives of kaempferol and orange fluorescence indicate the presence of quercetin derivatives. K3R7R, kaempferol-3-o-rhamnoside-7-o-rhamnoside; K3G7R, kaempferol-3-o-glucoside-7-o-rhamnoside; Q3G7R, quercetin-3-o-glucoside-7-o-rhamnoside; K3GR7R, kaempferol-3-o-glucorhamnosid-7-Orhamnoside; Q3GR7R, quercetin-3-o-glucorhamnosid-7-o-rhamnoside (**a**). Quantification of various flavonols in WT, mutant and complementation lines (**b**). Phenotypic characterization of WT, mutant and *MtMYB134* complementation lines of norflurazon-bleached, sucrose-induced seedlings, showing accumulation of purple anthocyanin pigments only in WT (**c**)

MtMYB134 binds to *MtCHS2* and *MtFLS2* promoters

To check whether MtMYB134 can bind to the selected promoters of *M. truncatula* flavonol biosynthesis genes, we performed Y1H assays. The reporter constructs with *proMtCHS2-1141*, *proMtF3H4-1113* and *proMtFLS2-503,* respectively were integrated into the genome of *S. cerevisiae* Y1H Gold strain and tested for their interaction with MtMYB134 (more precisely MtMYB134-Gal4AD). We observed, that *proMtCHS2* + MtMYB134 and *proMtFLS2* + MtMYB134 showed growth on 100 ng/ml AbA supplemented leucine dropout media while the corresponding empty vector controls did not. No yeast growth was detected with *proMtF3H4* + MtMYB134 combination. These results indicated that MtMYB134 could bind to the promoters of *MtCHS2* and *MtFLS2* in vivo (Fig. 9a and b).

Discussion

Flavonols, besides offering several health benefits to humans, play variety of functions in plant biology. In leguminous plants, flavonoids have been implicated in root nodule formation (Wasson et al. 2006) and disease resistance (Chen et al. 2019; Long et al. 2019). In addition, flavonoid biosynthesis is an important agronomic trait in forage legumes (Zhao et al. 2010). Not least because of the presumed health-promoting effect of flavonoids, it is desirable to understand molecular mechanism of flavonoid biosynthesis and its regulation in leguminous plants. As there is a gap in our knowledge in this area, particularly in flavonol biosynthesis, we aimed to fill it. To this end, we, herein, have functionally characterized the SG7 R2R3-MYB transcription factor MtMYB134 as a flavonol specific regulator in

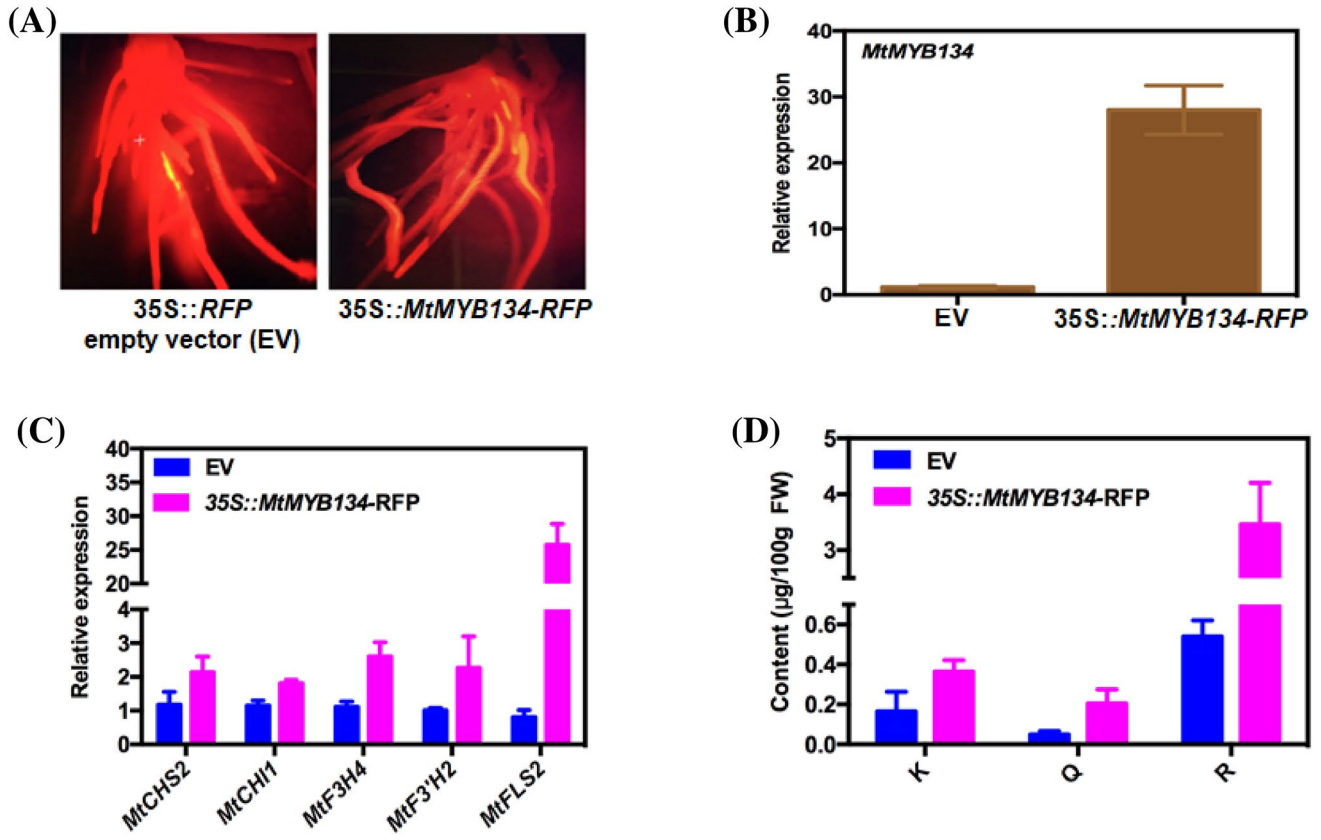


Fig. 8 *M. truncatula* hairy roots, overexpressing *MtMYB134*, show enhanced expression of structural flavonol biosynthesis genes and enhanced flavonol accumulation. RFP detection as a transformation marker in empty vector (EV, Left) and *MtMYB134-RFP* overexpressing line (Right) (**a**). Expression of *MtMYB134* in EV and *MtMYB134-RFP* transformed hairy roots (**b**). Relative amount of structural flavonol biosynthesis gene transcripts in EV and *MtMYB134-RFP* transformed hairy roots, determined by RT-qPCR (**c**). Quantitative targeted LC–MS analysis in EV and *MtMYB134-RFP* transformed hairy roots showing flavonol content. The graph shows values ± SD of three technical replicates, each derived from several hairy roots (**d**)

M. truncatula. The regulatory specificities of MtMYB134 have been studied in detail which demonstrated its potential application in the genetic manipulation of flavonol biosynthesis in the leguminous plants.

Using a homology-based approach, three *M. truncatula* candidate R2R3-MYB transcription factors, potentially involved in the transcriptional regulation of flavonol biosynthesis, were identified. However, a detailed analysis of their amino acid sequences, suggested that only one candidate, namely MtMYB134, retained the characteristic SG7 motif of flavonol activators. Therefore, MtMYB134 was selected to study its role in the transcriptional regulation of flavonol biosynthesis in *M. truncatula*. As the P-like MYBs (belonging to the SG7 of R2R3-MYBs) operate in a bHLH independent manner to transactivate their target genes (Mehrtens et al. 2005; Stracke et al. 2007), the absence of a bHLH binding motif (Zimmermann et al. 2004) in MtMYB134 supported our candidate selection. In our venture to study the transcriptional regulation potential of MtMYB134, we aimed to determine structural target genes, and thus identified candidate genes encoding the enzymes needed for flavonol aglycon biosynthesis in *M. truncatula*. Since various flavonoid metabolites were detected in *M. truncatula* before (Li et al. 2016), all structural genes need to be represented by at least one functional copy in the genome. Our finding of multiple isoforms for several enzymes with differing expression domains (Fig. 1) indicated possible sub-functionalization of these gene copies in *M. truncatula*. It is possible that various copies are dedicated to specific purposes such as fine-tuning of flavonol biosynthesis under different spatial and temporal cues, as reported for *Freesia hybrida* (Shan et al. 2020). Here, two copies of flavonol synthase, named *FhFLS1* and *FhFLS2*, are spatiotemporally regulated in different tissues and developmental stages by two different clades of MYB transcription factors. *FhFLS1* is expressed in early flower buds and calyxes and is regulated by four SG7 R2R3-MYB proteins, whereas *FhFLS2* expressed in late developing flowers is regulated by the non-SG7 R2R3-MYB, FhMYB21L2 (Shan et al. 2020). AtMYB21 and AtMYB24 could also activate flavonol biosynthesis in pollen and anther by directly

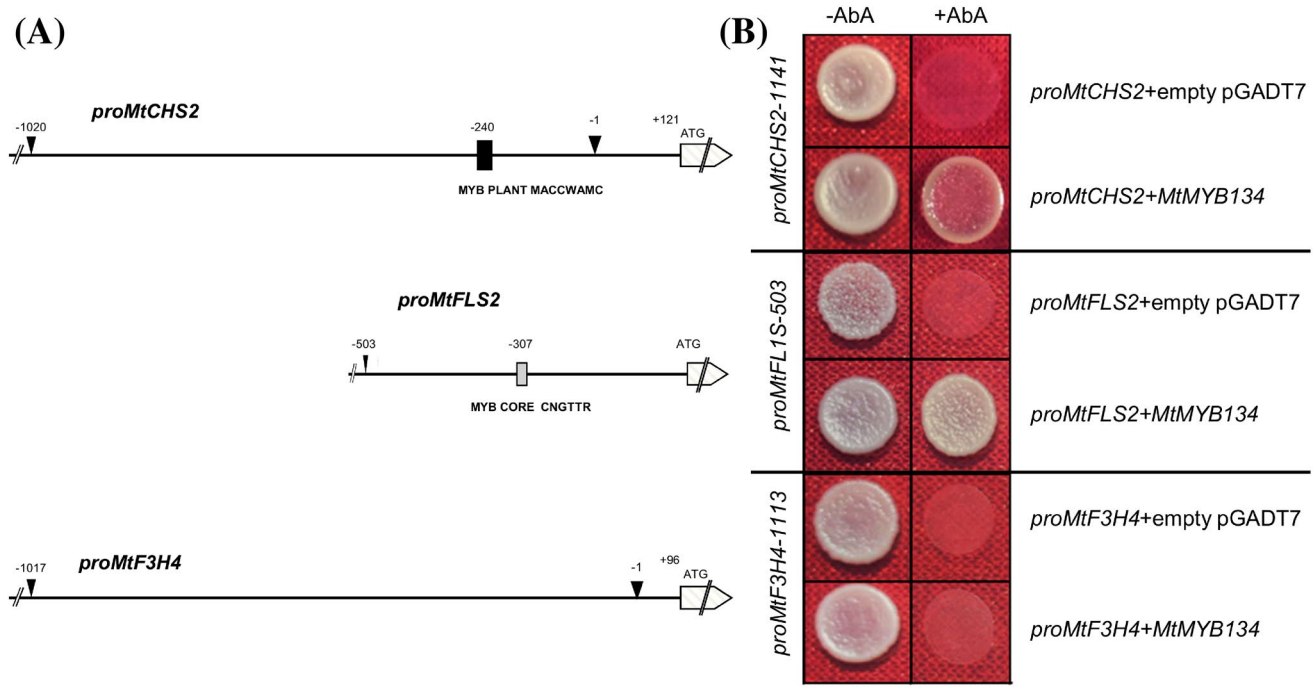


Fig. 9 Yeast 1-hybrid assay indicates the interaction of MtMYB134 with the promoters of *MtCHS2* and *MtFLS2*. Schematic presentation of the *M. truncatula* promoters *proCHS2* (1141 bp fragment), *proFLS2* (503 bp fragment) and *proF3H4* (1113 bp fragment). Putative MYB binding sites MYBPLANT and MYBCORE are presented as black and grey labelled boxes, respectively (**a**). Physical interaction between MtMYB134 and *proMtCHS2*, *proMtFLS2* and *proMtF3H4* was checked on leucine dropout media supplemented with appropriate concentration of AbA. pGADT7 vector transformed strains are taken as negative control. MIC value for *proMtCHS2* and *proMtFLS2* was 100 ng/ml AbA, where it was 950 ng/ml for *proMtF3H4* (**b**)

activating *AtFLS* in Arabidopsis (Shan et al. 2020). Such sub-functionalization is also suggested by the observation of qualitative and quantitative differences in flavonol accumulation in various organs as previously reported for other plant species (Stracke et al. 2007, 2010a; Alseekh et al. 2020), suggesting different functional roles of specific flavonol derivatives.

The expression pattern of *MtMYB134* could be correlated to flavonol accumulation and expression of structural genes in different organs of *M. truncatula* (Figs. 4, 5), providing another clue regarding its involvement in the transcriptional regulation of flavonol biosynthesis. Particularly in seed tissue, we found a strong correlation among the flavonol metabolite, expression level of biosynthesis genes like *MtCHS2*, *MtCHI1*, *MtF3H4*, *MtF3'H2*, *MtFLS2*, and regulatory gene *MtMYB134*. The expression level of *MtMYB134* and the above mentioned structural genes were highest in seed tissue, which can be correlated to the high rutin and quercetin content in seed, although kaempferol content was much higher. This can be due to higher expression of *MtF3'H2* which converts kaempferol into quercetin. Similar cases were observed in shoot and root seedling where the flavonol content and the expression level of associated genes was very less. It is also worth mentioning that transcribed mRNA has to be translated to functional protein, and therefore correlation can not be based on the expression level only. Although we noticed positive correlation among the transcript level of *MtMYB134*, flavonol content and expression of structural flavonol biosynthesis genes, in some cases, the correlation was not apparent. For example, the transcript level of *MtMYB134* along with *MtCHS2*, *MtCHI1* and *MtF3H4* was shown to be relatively low in young leaves, despite the high level of flavonols in that tissue. These results suggest that additional factors, besides the regulation offered by MtMYB134, might play a role in the regulation of flavonol biosynthesis here. For instance, the different paralogs of the structural genes might encode enzymes differing in their activity. It may be due to tissue specific catalysis of flavonols by different paralogs of these biosynthesis enzymes as their paralogs are also identified in reference genome (Fig. 1). It would be interesting to study the impact of *MtMYB134* over qualitative and quantitative profile of flavonoids other than flavonols. In addition, other transcriptional regulators could be involved in the regulation of flavonol biosynthesis, as known from Arabidopsis HY5, promoting flavonol biosynthesis by binding to the CHS promoter under UV-B light conditions (Lee et al. 2007; Stracke et al. 2010b; Job et al. 2018). In *Zea mays,* it has been shown that the same R2R3-MYB-type transcription factors can regulate different specialized metabolite pathways. C1/PL1

(together with the bHLH-type factors R/B) are able to regulate the accumulation of both, anthocyanins and flavones, in different tissues (Falcone Ferreyra et al. 2012, 2015; Righini et al, 2018). In *Malus crabapple*, McMYB12 was shown to regulate the accumulation of anthocyanins and proanthocyanidins (Tian et al. 2017). These findings show that, some MYB-type transcription factors have the capacity to regulate structural genes of multiple branches of flavonoid biosynthesis. In line with these findings, we cannot exclude that MtMYB134 is also involved in the regulation of the formation of other flavonoids, besides flavonols, in *M. truncatula*. It is also possible that some other regulators might be acting at post transcriptional and post translational level. In Arabidopsis, MicroRNA858 negatively regulates flavonol biosynthesis by cleaving the transcript of MYB12, MYB11, and MYB111 (Sharma et al. 2016). A small peptide miPEP858a encoded by miR858a positively regulates the expression of pri-miRNA858a leading to down-regulation of flavonol biosynthesis (Sharma et al. 2020). Therefore, in future, it will be interesting to study the role of such factors in the regulation of flavonol biosynthesis in *M. truncatula.*

Results of the transactivation assay in Arabidopsis protoplasts provided clear evidence that MtMYB134 transcriptionally activates the promoters of *AtCHS* and *AtFLS1 in planta* (Fig. 6). However, the promoters of *AtCHI*, *AtF3H, AtF3'H* and *AtDFR* could not be activated by MtMYB134 in this assay. Although the mechanism of regulation of flavonoid biosynthesis, in general, is conserved in land plants, there are species specific differences in the binding affinity of transcription factors and the *cis*-elements in the promoter they bind to, which result from the course of evolution. In maize, for example, ZmP activates *ZmDFR* promoter but not *ZmF3H* promoter, while it could activate *AtF3H* promoter but not *AtDFR* (Mehrtens et al. 2005). Both AtMYB12 and ZmP share high similarity in sequence structure and function but the transactivation activity of ZmP towards *AtFLS1* promoter is lower than that of AtMYB12 (Mehrtens et al. 2005) In view of that, it is likely that MtMYB134 might have weaker affinity towards the heterologous promoters of *AtCHI* and *AtF3H*. In future, studies using *MtCHI* and *MtF3H* promoters in the transactivation assay might provide clear insights in that regard. Another possibility could be the involvement of other tissue specific regulatory factors, which in concert with MtMYB134 might be involved in the regulation of *AtCHI* and *AtF3H*. Since we have used At7 cell line for the transactivation assay, the availability of such additional cell and development specific factors might be a limiting factor. The results from the heterologous Arabidopsis system prompted us to study the role of *MtMYB134* in *M. truncatula* itself, using the hairy root system, which has been extensively utilized for the functional characterization of genes in different plant species (Peel et al. 2009; Verdiera et al. 2012; Brear et al. 2020). *MtMYB134* overexpressing *M. truncatula* HR clearly displayed enhanced flavonol content as compared to EV control lines. Furthermore, the expression of the structural genes involved in the flavonol biosynthesis (*MtCHS2*, *MtCHI1*, *MtF3H4*, *MtF3'H2* and *MtFLS2*) was higher in *MtMYB134* overexpressing HR. These results are in agreement with the fact that MtMYB134 is a transcriptional activator of flavonol biosynthesis.

The promoter regions of *MtCHS2*, *MtFLS2* and *MtF3H4* were reported to harbor MYB recognition sites (MRS), characteristic *cis*-acting elements in the promoter regions of genes involved in the flavonol biosynthesis (Hartmann et al. 2005; Czemmel et al. 2009). The yeast 1-hybrid assay demonstrated that MtMYB134 is able to interact with the promoters of *MtCHS2* and *MtFLS2*. However, in accordance with our transactivation assay, MtMYB134 did not display binding to the *MtF3H4* promoter. It could be speculated that some other factors along with MtMYB134 might work in the regulation of *MtF3H4*. We could not detect a potential MRS within the used *MtF3H4* promoter fragment, however, it is not possible to deduce its functionality solely from the presence of a conserved *cis*-acting element within a promoter (Moyano et al. 1996). Even the detection of the binding of an activator to a conserved promoter element does not necessarily lead to activation of the transcription. For example, two MYB factors AmMYB305 and AmMYB340 from *A. majus,* when expressed in yeast, they could bind the promoters of *AmCHS*, *AmDFR* and *AmLDOX* but they could not activate their expression (Moyano et al. 1996). Further, it would be interesting to see if the similar regulatory protein can also activate *MtPAL, MtC4H* and *Mt4CL* (Zhang et al. 2016; He et al. 2020).

Complementation of *MtMYB134* in flavonol deficient multiple *myb* mutant restored specifically flavonol glycosides without affecting anthocyanin biosynthesis (Fig. 7) which revealed the conserved regulatory mechanism throughout the land plant lineage and reflects the conservation in structure and function of this regulatory protein and *cis* element present in their target promoters.

Legumes form the root nodule, for symbiotic nitrogen fixation (Hartman et al. 2017). The rhizobacterium, called *Sinorhizobium meliloti*, colonizes in the root nodule of *M. truncatula* where flavones and flavonols play a prominent role in nodule organogenesis by inhibiting auxin transport (Wasson et al. 2006). Wasson et al (2006) showed that the silencing of CHS resulted in increased auxin transport as compared to control plant roots and inhibition of root nodule formation. The supplementation of naringenin, the flavonoids, precursor resulted in reversal of the phenotype. In a later study, it was observed that flavones might play a major role in *nod* gene induction while flavonols act as inhibitor of auxin transport (Zhang et al. 2009). Flavonols have also been reported to alter PIN2 polar localization, where PIN2 polarity is dependent on flavonol induced

PINOID activity in Arabidopsis (Kuhn et al. 2017). In another study, supplementation of flavonol to *cre1* mutant, which is unable to form nodule, restored nodulation efficiency in *cre1* mutant and auxin transport control in response to rhizobia (Ng et al. 2015). Particularly, kaempferol 3-o-rhamnoside-7-o-rhamnoside has been reported as the endogenous inhibitor of polar auxin transport in Arabidopsis shoots (Yin et al. 2014). Altogether, identification of flavonol specific regulator, MtMYB134 should be helpful in detailed studies on understanding the role of flavonols in regulation of root nodule formation in legumes.

The genetic manipulation of flavonol biosynthesis has potential to develop pathogen resistant and neutraceutially enriched leguminous plants (Skadhauge et al. 1997; Irmisch et al. 2019; Chen et al. 2019). Besides, flavonoid content affects the quality of the forage legume and therefore, the discovery of flavonol specific regulator in the present work should provide fresh avenues for the development of improved forage crops. The leguminous taxa comprise important plants, which are consumed by humans. Flavonol biosynthesis and its regulation need to be explored further in *M. truncatula* for identification of regulatory modules, which may include small RNAs, other stress and hormone associated transcription factors, proteolytic regulators etc. Thus, the characterization of a flavonol specific regulator will help to understand basic biology of the role of flavonols in the root nodule formations in forage legume and metabolic engineering of flavonol biosynthesis for animal health with reference to animal husbandry.

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Author contributions AP conceived the idea and designed the research. JN, RR and BP conducted experiments. JN and AP interpreted the data. JN, RS and AP wrote the manuscript. All authors read and approved the final manuscript.

Declarations

Conflict of interest The authors declare no conflict of interest.

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