Coexpression Analysis Identified PcMYB25 as a Patchoulol Synthase Gene Activator to Enhance Patchouli Alcohol Biosynthesis in Pogostemon Cablin

Xuanxuan Zhou
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Xilin Wang
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Huiling Huang
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Daidi Wu
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Xiaobing Wang
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Baiyang Zhong
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Junren Li
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Xiuzhen Chen
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Ruoting Zhan
Guangzhou University of Traditional Chinese Medicine: Guangzhou University of Chinese Medicine
Likai Chen (chenlk@gzucm.edu.cn)
Guangzhou University of Chinese Medicine https://orcid.org/0000-0001-7273-575X

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Coexpression analysis identified PcMYB25 as a patchoulol synthase gene activator to enhance patchouli alcohol biosynthesis in *Pogostemon cablin*

Xuanxuan Zhou[1], Xilin Wang[1], Huiling Huang[1], Daidi Wu[1], Xiaobing Wang[1], Baiyang Zhong[1], Junren Li[1], Xiuzhen Chen[1], Ruoting Zhan[1,2,*], Likai Chen[1,2,*]

[1] *Research Center of Chinese Herbal Resource Science and Engineering, Guangzhou University of Chinese Medicine; Key Laboratory of Chinese Medicinal Resource from Lingnan (Guangzhou University of Chinese Medicine), Ministry of Education; Joint Laboratory of National Engineering Research Center for the Pharmaceutics of Traditional Chinese Medicines; School of Pharmaceutical Sciences, Guangzhou University of Chinese Medicine, Guangzhou,510006, P. R. China*

[2] *Ministry of Education, Maoming Branch*, *Guangdong Laboratory for Lingnan Modern Agriculture*, *Guangdong*, *China*

Authors' email:

Xuanxuan zhou, 15521166528@163.com
Xilin Wang, 524996384@qq.com
Huiling Huang, hhl1107456909@126.com
Daidi Wu, w13726752703@163.com
Xiaobing Wang, 13570332896@163.com
Baiyang Zhong, 1642774256@qq.com
Junren Li, leejunren@163.com
Xiuzhen Chen, xiuzhenchan@163.com

[*]Corresponding authors: chenlk@gzucm.edu.cn (Likai Chen) and zhanrt@gzucm.edu.cn (Ruoting Zhan)

Abstract

Background

Patchouli alcohol is an effective component of the medicinal plant patchouli. Similar to other secondary metabolites, its synthesis is likely also regulated by transcription factors. Although the biosynthetic pathway of patchouli alcohol has been characterized, the regulatory mechanism of patchouli alcohol biosynthesis has not been fully revealed.

Results

This study combined the transcriptome data of patchouli leaves treated with different hormones and WGCNA to establish a coexpression network. The modules correlated to patchouli alcohol content were identified, and *PcMYB25* played a crucial role in regulating patchouli alcohol biosynthesis. The overexpression of *PcMYB25* can promote the expression of patchouli alcohol synthase (PTS), thereby increasing the content of patchouli alcohol.

Conclusions

This is the first report that *MYB25* regulates the secondary metabolism of patchouli. These experimental results lay the foundation for further analysis of the regulatory mechanism of patchouli alcohol synthesis.

Key words:

WGCNA, patchouli alcohol, transcription factor, MYB

Background

Pogostemon cablin (Blanco) Benth. is a kind of herbaceous plant that belongs to Labiatae and is distributed in Southern and Southeast Asia (Swamy and Sinniah 2016). The oil extracted from patchouli has been widely used for medical treatment, fragrances, and cosmetics. Patchouli oil is a special volatile oil, which contains more than 20 kinds of sesquiterpenes (Deguerry et al. 2006). Patchouli alcohol is the main component in the volatile oil of patchouli (Yan et al. 2021). It has been reported that the content of patchouli alcohol (PA) accounts for

55.7% of the oil extracted from the aerial parts of the plant (Lee et al. 2020). Patchouli alcohol is synthesized throughout the entire plant, and patchouli alcohol is mainly synthesized via the MVA pathway (Bouvier et al. 2005; Liao et al. 2016). The process of patchouli alcohol biosynthesis in patchouli includes three steps; first, isopentenyl pyrophosphate (IPP) and dimethylallylpyrophosphate (DMAPP) are mainly synthesized through the mevalonate acid-dependent (MVA) pathway. The second step involves the synthesis of farnesyl diphosphate (FPP), which is catalyzed by farnesyl pyrophosphate synthase (FPPS). In the third step, PTS functions as an all-purpose sesquiterpene synthase and produces at least 13 sesquiterpenes (Deguerry et al. 2006).

In clinical medicine, patchouli alcohol has analgesic, anti-inflammatory, antidepressant, and anticancer effects (Lee et al. 2020). It is reported that patchouli alcohol can also be used as a food additive to prevent metabolic diseases, such as atherosclerosis. Because of patchouli alcohol's great application and commercial value, increasing the yield of patchouli alcohol by genetic engineering has become a hot research area. Mitsui et al. expressed the fusion proteins of FPPS and PTS in yeast, ultimately yielding 8.42 mg/L/d (Mitsui et al. 2020). However, the biosynthesis of terpenoids is a complicated process and is affected by many rate-limiting enzymes. When only a few genes are modified, the anticipated effects are not always achieved. Additionally, transcription factors can simultaneously regulate multiple genes involved in terpenoid metabolism to achieve the accumulation of metabolites. For example, ORCA3 is a jasmonate-responsive transcription factor from *Catharanthus roseus*; the overexpression of ORCA3 upregulates the expression levels of the key enzyme genes AS, TDC, DXS, CPR, G10H, SLS, STR, SGD, and D4H in the biosynthetic pathway of terpene indole alkaloids, resulting in an increase of the production of TIA (Sun et al. 2020). The AP2/ERF family transcription factor AaORA in *Artemisia annua* is a positive regulator of genes such as *AaADS*, *AaCYP71AV1*, *AaDBR2*, and *AaERF1*. It can positively regulate the expression levels of these genes to increase the content of artemisinin. After *AaORA* overexpression, the content of artemisinin and dihydroartemisinic acid increased 40%~53% and 22%~35%, respectively (Lu et al. 2013; Shen et al. 2016).

In nature, the growth and development of plants are affected by many factors, such as temperature, humidity, and salinity. When sensing environmental changes, plants respond

through hormone changes (Buchner et al. 2017; Jan et al. 2019; Verma et al. 2016). Altering concentration for various hormones could activate or inhibit activities of specific transcription factors, which in turn affects the synthesis and accumulation of secondary metabolites. Patchouli alcohol is a natural sesquiterpene; as a secondary metabolite, its synthesis is also affected by hormones and other signaling molecules. Wang et al. overexpressed PatJaz6 in patchouli, which led to the downregulation of PTS and a reduction in the content of patchouli alcohol in its leaves, indicating that PatJaz6 is a negative regulator of patchouli synthesis (Wang et al. 2019). Chen et al. showed through dual-luciferase experiments that the PatSWC4 transcription factor can bind to the promoter of *PatPTS* to increase its transcription activity. Yeast two-hybrid (Y2H) results indicated that PatSWC4 and PatJAZ4 proteins can interact, and suggested that PatSWC4 plays a part in the JA response network (Chen et al. 2020).

A good deal of research has been applied to reveal the regulatory network of patchouli alcohol biosynthesis for increasing its production. The rapid development of transcriptome sequencing technology and the continuous reduction in costs have led to a large amount of data support for seeking the functional genes. However, traditional comparative analysis is costly, and the data usually cannot be fully used. Weighted gene co-expression correlation network analysis (WGCNA) is a systematic biological analysis method that can correlate phenotypic trait data with specific genes. Currently, WGCNA plays an important role in the analysis of plant secondary metabolism. Lu et al. used WGCNA in combination with correlation analyses to screen out six transcription factors in chrysanthemum. It is speculated that these six transcription factors may affect the synthesis and accumulation of carotenoids in chrysanthemum by regulating genes involved in metabolism and plastid development (Lu et al. 2019). El-Sharkawy constructed a co-expression network for the differentially expressed genes in two kinds of apples and found 34 genes positively related to anthocyanin metabolism, and 12 of these genes in the anthocyanin metabolism pathway. It can be seen that WGCNA can be used to search for many functionally important genes (El-Sharkawy et al. 2015). However, there are few reports try to identify transcription factors related to patchouli alcohol metabolism by WGCNA.

In this study, based on transcriptome sequencing data, the patchouli alcohol content is used as phenotypic data to link with gene modules according to WGCNA. A R2R3 MYB

transcription factor gene, MYB25, was identified from these modules. Transient overexpression analysis confirmed that MYB25 is a positive regulator in the accumulation of patchouli alcohol. This study was the first that identified a transcription factor that positively regulates patchouli alcohol synthesis, laying the foundation for further research on the patchouli alcohol synthesis regulatory network in the future.

Methods

Plant materials and treatment

Pogostemon cablin (Blanco) Benth. was collected from Yangjiang, Guangdong, China (21.87° N,111.98° E), and grew in a growth chamber in the Research Center of Chinese Herbal Resource Science and Engineering, Guangzhou University of Chinese Medicine in Guangzhou University of Chinese Medicine (23.03° N, 113.23° E). The temperature and humidity of the growth chamber was set to 26℃/75% during the day and 21℃/65% at night. Light/dark period was set to 16/8 h

Six-leaf stage patchouli plants were used for hormone treatment. Patchouli plants with similar growth state were divided randomly into control (0.5% ethanol) and five phytohormone-treatment groups (MeJA, SA, ETH, ABA, and MeJA/ETH). Control ethanol and various hormones (dissolved in 0.5% ethanol) were applied on patchouli leaves by spraying. After spraying, the different treatment groups were enclosed by see-through plastic containers to prevent hormones from dissipating. Eight hours after spraying, leaves collected from each treatment group were frozen immediately in liquid nitrogen, and store at -80°C, used for RNA-Seq and extraction of patchouli alcohol. Concentrations used for various hormones are 300 M (MeJA, SA, and ABA) or 500 M (ETH).

Identification of DEGs and the co-expression network

The transcriptome sequencing, sequencing data assembly, splicing, expression measurement, differentially expressed gene screening, WGCNA, and visual analysis of the samples were all performed using the free online Majorbio Cloud Platform (www.majorbio.com).

Sequence alignment and phylogenetic tree construction of MYB25-related

sequences

The MYB25-related sequences from other species were identified from the NCBI database using PcMYB25 as a bait. Sequence alignment was conducted using DNAMAN with default parameters and a phylogenetic tree was constructed using MEGA 7.0.

Transient overexpression in patchouli leaves

Patchouli plants with six leaves were used for transient overexpression by agrobacteria-mediated transformation. In order to overexpress PcMYB25 in the patchouli leaves, the overexpression vector pJLTRBO (source should be indicated) was used. The 969-bp *PcMYB25* ORF was constructed between the *Not*I and *Pac*I sites. The resulting pJLTRBO-PcMYB25 and an empty pJLTRBO vector (a negative control) were transformed separately into *Agrobacterium* GV3101 (pSoup p19) (Weidi Biothchnology, Shanghai). Then the transformed agrobacteria were plated on Luria-Bertani plates containing 50 μg/mL kanamycin and 25 μg/mL rifampicin for 48 h for plasmid selection.

The method of agrobacteria-mediated transient expression has been described previously(Wang et al. 2019b). The agrobacteria were cultured in Luria-Bertani medium supplemented with 50 μg/mL kanamycin and 25 μg/mL rifampicin for 24 h until the OD600 reached 0.6-0.8, then the agrobacteria were harvested by centrifugation and resuspended in the induction medium (10 mM MES, pH 5.7, 10 mM MgCl2, 200 μM acetosyringone) at an OD600 of 1.0. Cells were incubated at room temperature for 2-4 h. Two to three leaves per plant were infiltrated on the abaxial sides using 1 mL needleless syringe. After infection, the plants were incubated at 25℃ for 3-5 days. Then the patchouli leaves were collected and stored at -80 °C for RNA extraction and patchouli alcohol content detection.

RNA extraction and quantitative reverse-transcription PCR (RT-qPCR)

Total RNA was extracted from patchouli leaves using the Favor PrepTM Plant Total RNA Mini Kit (Favor Biothch, China). Then HiScript III RT Super Mix for qPCR (+gDNA wiper) (Vazyme Biotech, China) was used for reverse transcription. For each sample, a 200 mg RNA was used for reverse transcription. AceQ Universal SYBR qPCR Master Mix (Vazyme Biotech, China) was used for qPCR. The internal reference gene 18S rRNA was used to normalize the relative expression for tested genes. The values shown are averages of three biological replicates. The primers used are listed in Additional file 1: Fig. S6.

PA extraction and quantification

The method of PA extraction and quantification used has been described previously (Wang et al. 2019b). Patchouli leaves were ground in liquid nitrogen and 200 mg of powder, after adding 1.5 mL hexane, was extracted by ultrasonic at 60 Hz for 30 min. Then, the mixture was placed in a 56 °C water bath for 1 h. After a short centrifugation, the supernatant was filtered with a 0.22 μm organic microporous membrane, then the filtrate was taken as the test solution for GC-MS detection.

Agilent 7890B/5977A GC-MS and HP5-ms capillary column (30 m × 250 μm × 0.25 μm) was used for the separation and detection of PA. the procedure was set with an initial temperature of 50℃ and kept for 2 min, then the temperature is increased to 130℃ at a rate of 20℃/min, and the temperature is increased to 150℃ at 2℃/min, and maintained for 5 min at 150 ℃. After that, the temperature increased to 230℃ at rate of 20℃/min. In addition, the patchouli alcohol purchased from Feiyu (Nantong, China) was used as a standard.

Results

PA content altered in patchouli leaves treated with various hormones

The biosynthesis of PA mainly occurs in the leaves of patchouli and the content of secondary metabolites is often affected by exogenous plant hormones (Chen et al. 2014; Santner and Estelle 2009; Verma et al. 2019). The six-leaf-stage patchouli leaves were treated with 0.5% ethanol (control) or various hormones (MeJA, SA, ETH, ABA, and MeJA/ETH (MandE)). Among different hormone treatments, the highest PA content was found with the MeJA, and the lowest PA content was observed with MandE. The PA content observed with the MeJA showed significantly difference from that observed with the SA, ETH, and MandE (Additional file 1: Fig. S1).

RNA-sequencing, assembly of sequences and functional annotation

To find the critical transcription factors involved in regulation of the biosynthetic pathway of patchouli alcohol, patchouli leaves in triplicate were sprayed by 0.5% ethanol (control), MeJA, ETH, ABA, SA, and MandE, respectively. A total of 154,779 unigenes were assembled, with a size of 223,580,816 bp and an N50 of 1058. These data indicate that the quality of RNA sequencing is high enough to be used for further analysis. To access the expression pattern of

the patchouli transcriptomes for various hormone treatments, the assembled transcriptome sequences were annotated and compared with six databases: NR, Swiss-Prot, Pfam, COG, GO and KEGG. Of total 154,779 unigenes annotated: 60,039, 55,123, and 43,196 unigenes were annotated from NR, Swiss-Prot, and Pfam, respectively. We used GO, COG, and KEGG databases to classify unigenes. In order to understand the function and classification of unigenes in a global view, GO functional annotations were performed. Of 43,477 unigenes annotated to this database, three main categories were grouped: biological process (BP), cellular component (CC), and molecular function (MF) (Additional file 1: Fig. S2). For biological process, organic substance metabolic process (GO:0071704), primary metabolic process (GO:0044238), cellular metabolic process (GO:0044237) were the most enriched items. For cellular component, unigenes related to membrane part (GO:0044425), cell part (GO:0044464), intrinsic component of membrane (GO:0031224) are the most abundant items. For molecular function category, organic cyclic compound binding (GO:0097159), heterocyclic compound binding (GO:1901363), and ion binding (GO:0043167) were the most highly represented GO terms. Of 8,162 unigenes annotated with COG, functional groups belong to 24 categories (Additional file 1: Fig. S3): 758 unigenes belong to Translation, ribosomal structure and biogenesis (the J category). Moreover, 35,368 unigenes were mapped into the KEGG pathways. The most representative pathways were "translation", "Carbohydrate metabolism" and "Folding, sorting and degradation" (Additional file 1: Fig. S4).

Identification of differentially expressed genes (DEGs)

To find the potential transcription factors involved in regulation of the PA biosynthetic pathway, we identified DEGs among the five hormone treatment groups by comparing MandE, MeJA, SA, ABA, and ETH with control; and by comparing MandE with ETH or MeJA. Here, DESeq2 was used for differential expression analysis, and genes with $p<0.05$ and $FC\geq2$ or $FC\leq0.5$ (genes up-regulated twice or down-regulated more than two times) were considered DEGs. Here, we found a total of 14,419 DEGs. The numbers of up- and down-regulated DEGs of all comparisons were shown in Additional file 1: Fig. S5. Among them, compared with leaf, MandE has the most differentially expressed genes after treatment, with a total of 9563 unigenes. Among them, there are 5086 up-regulated genes and 4477 down-regulated genes. Compared

with control, SA has the least up-regulated and down-regulated unigenes (77 and 54). GO and KEGG enrichment analysis were performed on DEGs to predict their biological functions. The GO enrichment analysis divided 14,419 differentially expressed unigenes into three categories: cellular component, molecular function, biological process (Fig. 1A). Among the cell components, membrane (19.60%) and membrane part (18.98%) accounted for the highest proportions. Among molecular functions, catalysis and binding account for the highest proportions, at 30% and 26.87%. The most enriched items are membrane (19.60%) and membrane part (18.98%) for cellular component category; catalysis (30%) and binding (26.87%) for molecular function category; and metabolic process (17.22%) and cellular process (16.57%) for biological process category. The results reflected that more DEGs are involved in metabolic processes or cellular processes and have catalytic and binding functions. By KEGG pathway analysis, it was found that 151 unigenes participated in plant hormone signal transduction, 46 unigenes participated in terpenoid backbone biosynthesis, and 34 unigenes participated in sesquiterpene and triterpenoid biosynthesis (Fig. 1B).

Construction of the WGCNA and the identification of hub genes

The DEGs were evaluated using WGCNA. Here, genes whose expression level is less than one and whose coefficient of variation is less than 0.1 are filtered. Then, the soft power is adjusted to 12, and the module are identified. Here, the processed genes are divided into 10 modules (Fig. 2A). Association of the PA content (trait) with 10 modules was analyzed to identified key modules and potentially involved transcription factors (Fig. 2B). Among these 10 modules, four modules, MEmagenta (r^2=-0.643, *p*-value=0.004), MEgreen (r^2=-0.591, *p*-value=0.0098), MEpink (r^2=0.544, *p*-value=0.0196), and MEblack (r^2=-0.525, *p*-value=0.0253), were regarded as the key modules associated with the patchouli alcohol content ($P<0.05$) (Fig. 2B). Notably, MEmagenta (r^2=-0.643, *p*-value=0.004) has the strongest correction to patchouli alcohol among these 10 modules, but there was not any transcription factor being found in this module. Therefore, we focus on analysis of MEgreen, which is significantly related to the content of patchouli alcohol (r^2=-0.591, *p*-value=0.0098). A total of 358 genes are included in this module, including 8 structural genes related to patchouli alcohol synthesis (Additional file: fig: S 9). In addition, 14 out of the 358 genes have been identified as transcription factors (Additional file: fig: S8). Especially, we found a key transcription factor,

TRINITY_DN73869_c4_g1 (kME=0.89181), name here as *PcMYB25*. As a crucial gene, *PcMYB25* processes top 30 degree in MEgreen module (degree=44.12) (Additional file: fig: S7). Moreover, the gene expression correlation analysis network diagram shows that *PcMYB25* is closely associated with terpenoid metabolism-related genes (Fig. 2C). In addition, according to previous reports, MYB is widely involved in the process of plant secondary metabolism (Dubos et al. 2010). Thus, we speculate that *PcMYB25* may play an essential role in patchouli alcohol biosynthesis.

Overexpression of PcMYB25 increased the PcPTS expression level and patchouli alcohol production

To reveal the function of *PcMYB25* in patchouli, the pJLTRBO vector was used to overexpress *PcMYB25* in the leaves of patchouli, which is mediated by agrobacteria (Fig. 3A). The efficiency of this overexpression was evaluated by RT-qPCR four days after the injection. Compared with that of the control group, the expression level in the experimental groups increased by 2.55-fold (Fig. 3B). The transcript levels of some genes in the upstream patchouli alcohol biosynthetic pathway, such as *AACT* and *PMK*, were significantly increased; on the contrary, the HMGR level was considerably reduced. Notably, the expression level of *PcPTS* was significantly increased, by 309%, compared with that of the control group (Fig. 4). Interestingly, the PA content was increased by 85% in patchouli leaves overexpressing PcMYB25, which is in accordance with the upregulation of *PcPTS* (Fig. 3C and Fig. 3D).

***PcMYB25* codes a R2R3 MYB protein**

We cloned the full-length coding sequence of *PcMYB25* from patchouli cDNAs. After sequence analysis, the ORF including the stop codon of PcMYB25 was determined to be 969 bp in length and encodes 322 aa. Amino acid sequence alignment confirms that PcMYB25 shares high similarities with the PcMYB25-related proteins retrieved from *Rehmannia glutinosa*, *Salvia splendens*, and *Actinidia rufa*. PcMYB25 harbors the conserved R2 and R3 domains in N-terminus, typical of a R2R3-MYB transcription factor. To help predicting potential functions for PcMYB25, a phylogenetic tree was constructed by MEGA 7 (Fig. 5B). Results showed that the closest MYB proteins were from *Rehmannia glutinosa*, *Salvia splendens* and *Salvia miltiorrhiza*. PcMYB25 also displays high similarity (95%) to the R2-R3

297 conserved domains with *Arabidopsis* AtMYB25 (Fig. 5B).

298

299 ## Discussion

300 ### Changes of patchouli under different hormone treatments

301 Plants are usually subjected to various adversity stresses during their growth. These adverse
302 stresses usually lead to changes in plant hormone levels, thereby affecting the synthesis and
303 accumulation of secondary metabolites (Santner and Estelle 2009). Like most secondary
304 metabolites, the synthesis of patchouli alcohol has previously been found to be associated with
305 phytohormone. Tang et al used MeJA, ABA, SA to treat the leaves of patchouli, it was found
306 that the expression of HMGS, PMV and MVK on the MVA pathway have increased (Tang et
307 al. 2019). In comparison with control, content of secondary metabolites including PA were
308 found to be significantly different in patchouli leaves treated with MeJA, ETH, or MandE. After
309 treatments, 254, 229, and 400, respectively, differentially expressed proteins were identified.
310 These differentially expressed proteins are mainly involved in photosynthesis, secondary
311 metabolites, carbohydrate and energy metabolism (Li et al. 2019). These previous studies have
312 shown expression levels or content of genes, proteins and secondary metabolites changed when
313 patchouli leaves were treated with exogenous hormones. To determine the effect of various
314 hormones on PA content, PA content of patchouli leaves treated with various hormones was
315 determined. The highest content of PA was found with MeJA-treated leaves, and the lowest
316 content was associated with MandE-treated. The PA content of the MeJA-treated leaves was
317 significantly different from that of the SA-, ETH-, and MandE-treated.

318 RNA-Seq is a powerful tool to identify the transcriptional regulation mechanism of
319 secondary metabolism. This study provides a transcriptome survey to understand the functions
320 of DEGs. DEGs identified are mainly enriched in metabolic and cellular processes, and are
321 mainly involved in catalysis and binding. The enriched signal pathways include plant hormone
322 signal transduction, MAPK signaling pathway, and alpha-linoenic acid metabolism pathways.

323 ### Co-expression facilitates the identification of PA-related transcription factors

324 The MYB proteins belong to a large, functionally diverse family of transcription factors in all
325 eukaryotes, and participate in cell proliferation (Yusenko et al. 2020), differentiation (Jakoby
326 et al. 2008), apoptosis (Srivastava et al. 2015), abiotic and biotic stress (Kim et al. 2013; Shen

et al. 2017) and secondary metabolism (Matías-Hernández et al. 2017; Paz Ares et al. 1987). The N-termini of the MYB transcription factors contain 1-3 conserved DNA binding domains: R1, R2, R3, and/or R4. Based on the presence of specific DNA binding domains, MYB transcription factors can be divided into four categories: R1-MYBs, R2R3-MYBs, R1R2R3-MYBs, and R4-MYBs. According to previous reports, R2R3-MYBs are transcription regulators for synthesizing secondary metabolites in plants. For example, PpMYB140 works as a transcriptional repressor to directly inhibit the expression of anthocyanin-related genes and prevent anthocyanin over accumulation in bananas (Ni et al. 2021). *CaMYB48* encodes an R2R3 transcription factor to activate the promoters of *AT3* and *KasIa*, leading to increases in Cap and DhCap (Sun et al. 2020). Using WGCNA, we identified a R2R3-MYB, PcMYB25, which is most similar to an R2R3-MYB protein from *Rehmannia glutinosa*. Available studies on PcMYB25-related transcription factors indicated roles on plant cell growth and cotton fiber formation. Wang et al. identified a MYB factor from *Rehmannia glutinos*a as a MYB25 for the first time, however, functions of this MYB had not been illuminated in detail (Wang et al. 2015). An Arabidopsis T-DNA-inserted MYB25-null mutant exhibited deformed pollen cell wall, two-celled pollen, and misarranged male germ unit, indicating a role of AtMYB25 in *Arabidopsis* pollen development (Reňák et al. 2012). An *Arabidopsis* T-DNA-inserted MYB25-null mutant exhibited deformed pollen cell wall, two-celled pollen, and misarranged male germ unit, indicating a role of AtMYB25 in *Arabidopsis* pollen development (Walford et al. 2011). Our work on PcMYB25, however, is the first study showing involvement of a MYB25 in secondary metabolism. By WGCNA, we identified a MEgreen module, which shows a tight correlation between gene expression patterns and PA content. Numerous genes from this module were related to terpene synthesis. Prior to this study, the co-expression network had not been used to identify genes and transcription factors related to PA biosynthesis. Although, genes involved in the PA biosynthetic pathway have been identified, the regulatory network response has not been fully elucidated. Identifying more critical genes from this module and their interplays will be a topic in the future research to further examine regulatory network for PA biosynthesis.

In the combined correlation between the expression level of *PcMYB25* and patchouli alcohol content, the Pearson correlation coefficient was 0.926. Similarly, the correlation coefficients of *PcMYB25* and *PTS* expression was 0.897. Thus, we speculate that *PcMYB25* might be a key

activator to enhance biosynthesis of patchouli alcohol in its leaves. In addition, after the overexpression of *PcMYB25*, the PA content in plant leaves was increased by 85%. After testing the gene expression in the patchouli alcohol synthetic pathway, it was found that *PcMYB25* had a negligible effect on the upstream genes of the pathway, but it specifically increased the expression of *PTS*, which was 4.09-fold greater than the original expression level. However, module and trait correlation heat map show a negative correction (r2=-0.591, *p-value*=0.0098) for green module with PA (Fig. 2B). A similar situation exists in Pan's research (Pan et al. 2020). Pan et al. performed a WGCNA of all genes of Y12-4 (cold-tolerance genotypes). Modules-trait relation showed that *LTG5* in the module greenyellow (r2=0.35, p-value=0.2) is a key gene in regulating cold tolerance at the germination stage. Overexpression of *LTG5* can increase the germination rate of rice at low temperature. However, there are *COLD1* (Ma et al. 2015), *CTB4a* (Zhang et al. 2017), *LTG1* (Lu et al. 2014), *ctb1* (Saito et al. 2010), *ICE1* (Chinnusamy et al. 2007) in the blue module (r2=-0.98, *p-value*=$2e^{-10}$), *qLTG3–1* (Fujino and Iwata 2011) in the brown module (r2=-0.92, *p-value*=$2e^{-0.6}$). These genes can improve the tolerance of rice at low temperature conditions as well.

In addition, constructing a co-expression network, the correlation between gene expression level and trait is considered, but the influence of gene interaction on trait is not considered. Modules clustering heat map showed that, genes in the green module highly expressed in MandE treatment group, lowly expressed in ETH treatment group, and expressed irregularly in other treatment groups. However, the PA content was the lowest under the MandE treatment, so the WGCNA analysis showed that the module was negatively correlated with the gene. In addition, the module is negatively correlated with genes, but there may be differences of genes in green module, which could be the reason of the positive correlation between the *PcMYB25* and PA.

Conclusion

In this study, through WGCNA, we identified that the MEgreen module was related to patchouli alcohol biosynthesis. This module provides candidate genes related to patchouli alcohol, and the hub transcription factor *PcMYB25* was able to upregulate *PTS* to increase the content of patchouli alcohol. This is the first time that MYB25 has been reported as a transcriptional activator to regulate the biosynthesis of secondary metabolism. This discovery

lays the foundation for further research on the transcriptional regulatory network of patchouli alcohol biosynthesis in patchouli and provides mechanical insights into the evolution of plant secondary metabolism.

Abbreviations

AACT: acetoacetyl-CoA thiolase
ABA: abscisic acid
AS: anthranilic acid.
BP: biological process
Cap: capsaicinoid
CC: cellular component
COG: Clusters of Orthologous Groups of Proteins
CPR: cytochrome P450 reductase
D4H: desacetoxyvindoline 4-hydroxylase
DEG: differentially expressed genes
DMAPP: dimethylallylpyrophosphate
DXS: 1-deoxy-D-xylulose 5-phosphate synthase
ETH: ethrel
FPP: synthesis farnesyl diphosphate
FPPS: farnesyl pyrophosphate synthase
G10H: geraniol 10hydroxylase
GC-MS: gas chromatography-mass spectrometry
GO: Gene Ontology
HMGR: 3-hydroxy-3-methylglutaryl-coenzyme A reductase
HMGS: hydroxy-3-methylglutaryl-coenzyme A synthase
IPP: isopentenyl pyrophosphate
IPPI: isopentenyl diphosphate
JA: methyl jasmonate
KEGG: Kyoto Encyclopedia of Genes and Genomes
MandE: MeJA with ETH
MeJA: methyl jasmonate
MF: molecular function
MVA pathway: mevalonic acid pathway
MVA: mevalonate acid
MVD: mevalonate diphosphate decarboxylase
MVK: mevalonate kinase
NR: non-redundant protein sequence database
PA: patchouli alcohol
PMK: phosphomevalonate kinase
PTS: patchouli alcohol synthase
RT-qPCR: quantitative real-time PCR
SA: salicylic acid

SGD: strictosidine beta-glucosidase
SLS: secologanin synthase
STR: strictosidine synthase
Swiss-Prot: Swiss-Prot Protein Sequence Database
TDC: tryptophan decarboxylase
TIA: terpenoid indole alkaloids
WGCNA: Weighted gene co-expression correlation network analysis
Y2H: yeast two-hybrid assay

Declarations

Authors' contributions

RTZ and LKC designed the study; XXZ and XLW performed experiments; HLH and DDW analyzed the data; XBW, JRL, and XZC helped the field works; XXZ wrote the manuscript. LKC edited the manuscript and provided guidance during this experimentation. All authors read and approved the final manuscript.

Competing interests

The authors declare that they have no conflicts of interest.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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Figure legends

Fig.1 A. GO function classification of DEGs. The ordinate on the left of the figure represents the secondary classification of GO, the abscissa represents the number of unigenes included in the

secondary classification, and the three colors on the right represent the three major branches of GO (BP, CC, MF). B. KEGG classification of DEGs. The ordinate shows the names of the KEGG metabolic pathways and the abscissa indicates the number of unigenes.

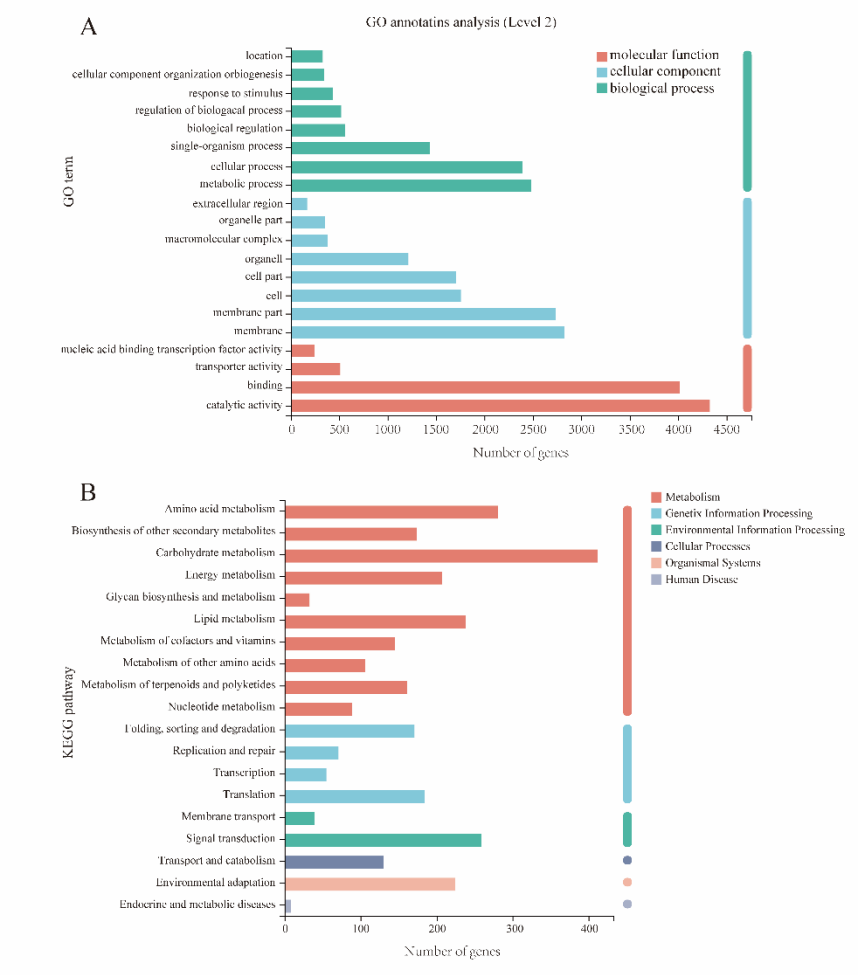
Fig. 2 Network analysis dendrogram shows the modules identified by Weighted Gene Co-expression Network Analysis (WGCNA). A. Tree diagram with color annotations. Each color represents a module. B. Module and phenotype correlation heat map. Module-patchouli alcohol weight correlation and corresponding *P-value*. The left panel shows 10 modules. The color scale on the right shows the correlation of module characteristics from -1 (blue) to 1 (red). C. Network analysis of *PcMYB25*, genes related with terpenoid and other genes in MEgreen module. Red pot represents *PcMYB25*，orange spots represent terpenoid related genes, blue spots represent other 358 genes in MEgreen.

Fig. 3 Transient overexpress analysis of PcMYB25 in patchouli leaves. A. PcMYB25 was cloned into overexpression vector pJLTRBO to form pJLTRBO-PcMYB25 construct with the restriction enzyme site PacI and NotI. B. The expression level of PcMYB25 in control (control group) and OX (overexpression group) analyzed by RT-qPCR. C. Chromatograms for PA content in control and PcMYB25-overexpressed leaves in comparison with standard. D. The content of patchouli alcohol detected in control and OX. Asterisks indicate a significant difference from the control (Student's t-test; *** $p < 0.001$, * $p < 0.05$). FW, fresh weight.

Fig. 4 The expression level of genes on the patchoulol synthesis pathway after transient overexpression of *PcMYB25.* The expression level of genes on MVA pathway was determined by RT-qPCR, and control represents an empty vector control. Using Pc18S as the internal reference gene, the relative expression level of the gene was calculated according to the 2-△△Ct method. Asterisks represent significant differences (*P<0.05, **P<0.01).

Fig. 5 Phylogenetic analysis of *PcMYB25*. A. Sequence analysis of PcMYB25 and other MYB transcription factors. In order to compare the MYB sequence between different plant species, PcMYB25 was used as a bait gene, and MYB transcription factors with similar sequences in *Rehmannia glutinosa*, *Salvia miltiorrhiza*, *Actinidia violacea* and *Arabidopsis thaliana* were identified from NCBI database. B. Phylogenetic tree of MYB transcription factors. Some MYB protein share similar sequences for phylogenetic analysis. A phylogenetic tree was constructed by

MEGA7.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Nucleotide metabolism
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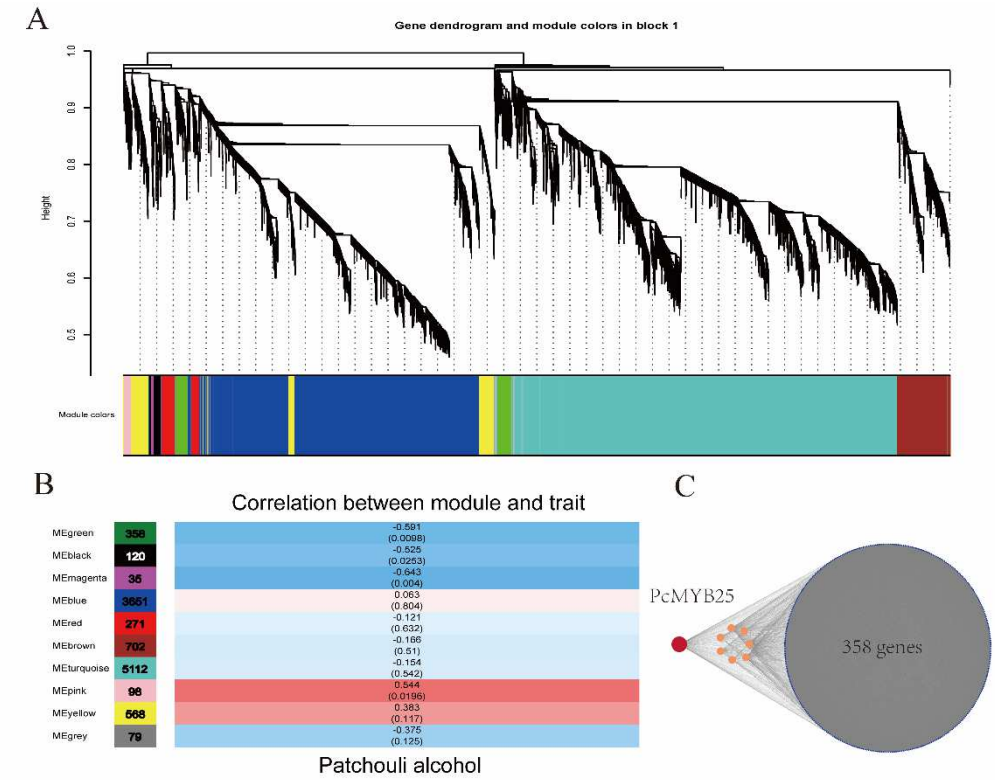

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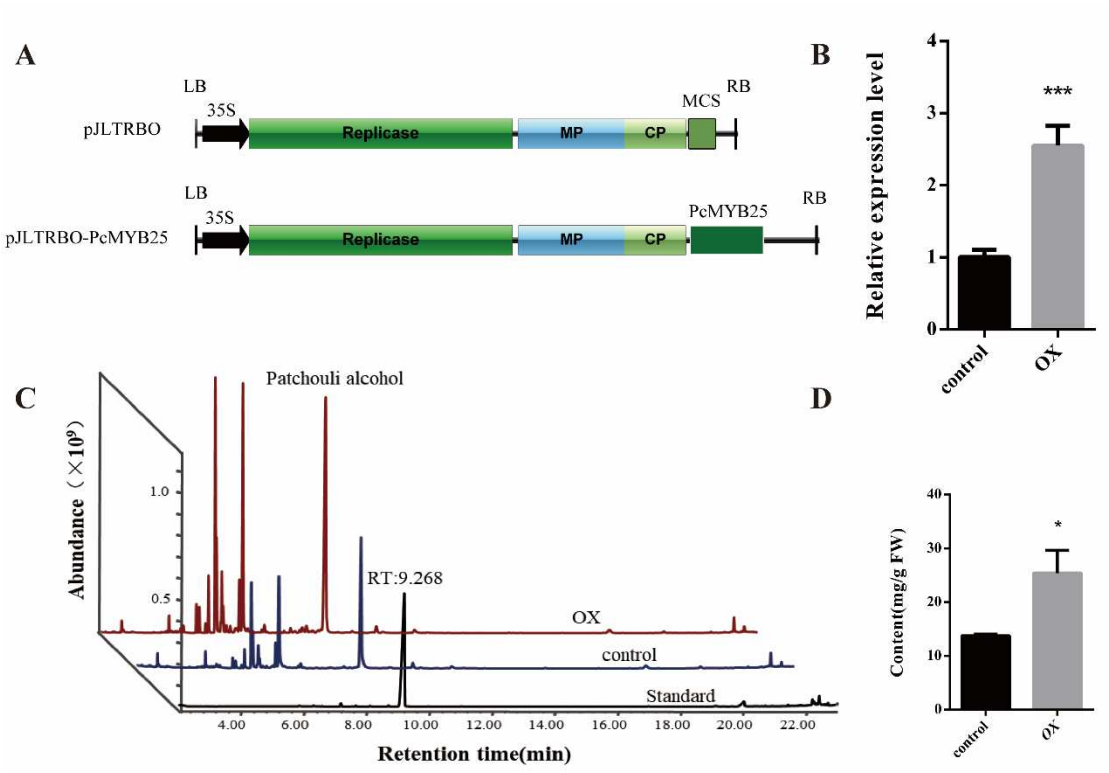

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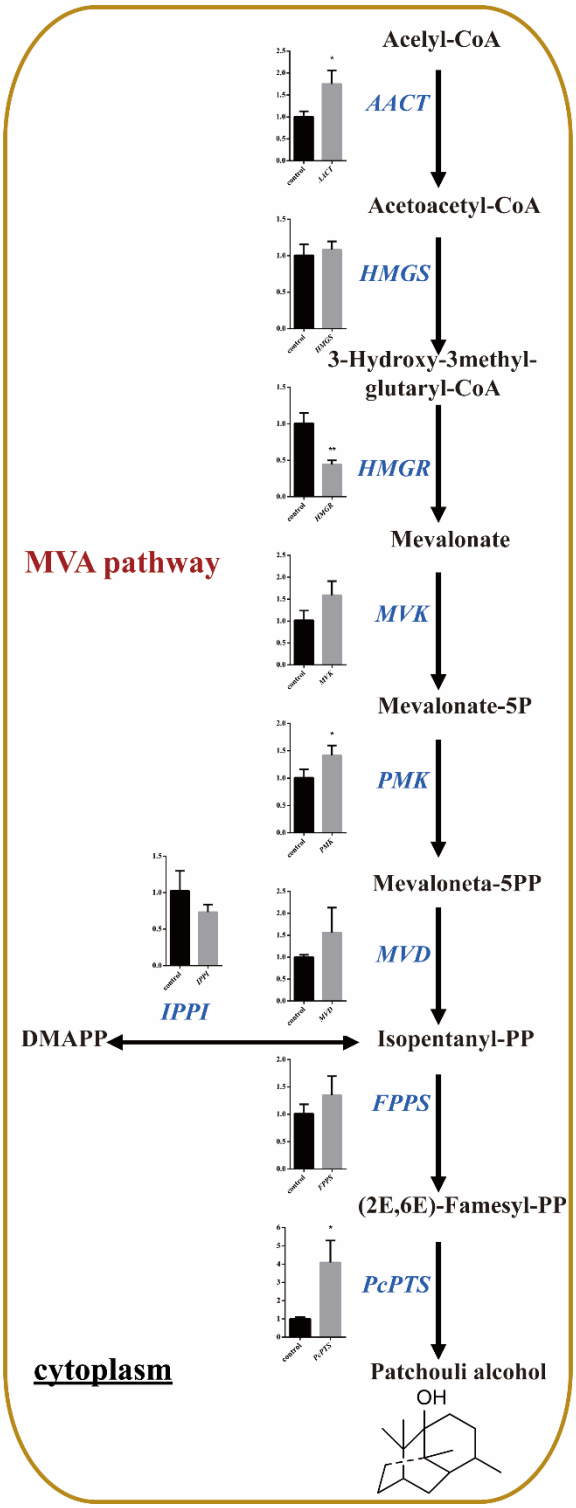

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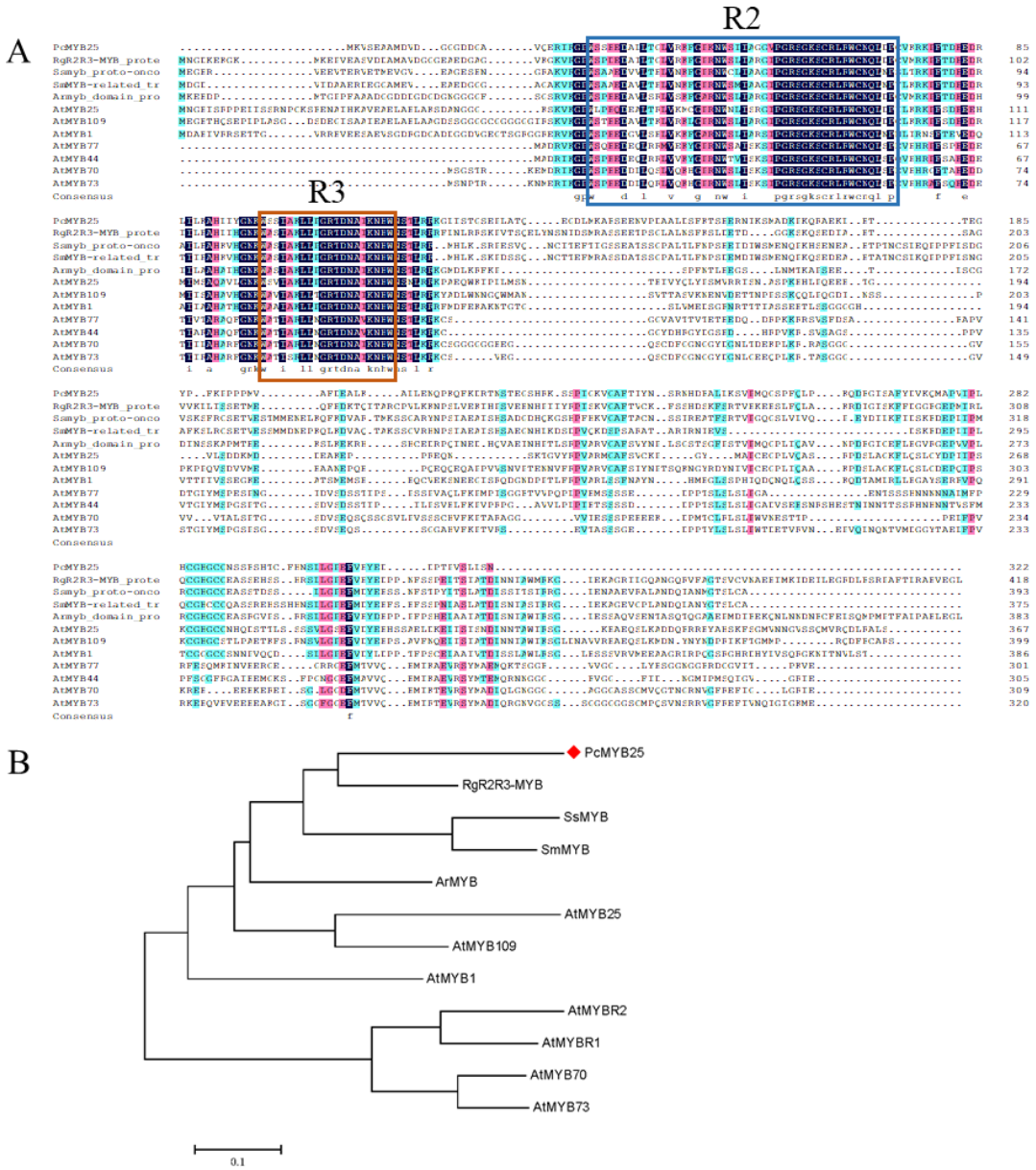

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