NtMYB12a acts downstream of sucrose to inhibit fatty acid accumulation by targeting lipoxygenase and lipase genes in tobacco

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Abstract

MYB12 promotes flavonol biosynthesis in plants by targeting several early biosynthesis genes (EBGs) of this pathway. The transcriptions of these EBGs are also induced by sucrose signal. However, whether MYB12 is activated by sucrose signal and the other roles of MYB12 in regulating plant metabolism are poorly understood. In this study, two NtMYB12 genes were cloned from Nicotiana tabacum. Both NtMYB12a and NtMYB12b are involved in regulating flavonoids biosynthesis in tobacco. NtMYB12a is further shown to inhibit the accumulation of fatty acid (FA) in tobacco leaves and seeds. Posttranslational activation and chromatin immunoprecipitation assays demonstrate that NtMYB12a directly promotes the transcriptions of NtLOX6, NtLOX5, NtSFAR4, and NtGDSL2, which encode lipoxygenase (LOX) or lipase enzymes catalyzing the degradation of FA. NtLOX6 and NtLOX5 are shown to prevent the accumulation of FA in the mature seeds, and significantly reduced the percentage of polyunsaturated fatty acids (PUFAs) in tobacco. Sucrose stimulates the transcription of NtMYB12a, and loss function of NtMYB12a partially suppresses the decrease of FA content in tobacco seedlings caused by sucrose treatment. The regulation of sucrose on the expression of NtLOX6 and NtGDSL2 genes is mediated by NtMYB12a, but those of NtLOX5 and NtSFAR4 genes are independent of sucrose.

Nicotiana tabacum

1 INTRODUCTION

Flavonoids are important polyphenolic secondary metabolites in plants, and the biosynthetic process of flavonoids has been well studied in Arabidopsis by using the series of *transparent testa* (*tt*) mutants (Buer, Imin, & Djordjevic, 2010). The content and distribution of flavonoids in Arabidopsis are precisely regulated by multiple transcription factors, especially the well-known MBW (MYB-bHLH-WD40) protein complex (Baudry et al., 2004; Gonzalez, Zhao, Leavitt, & Lloyd, 2008; Xu, Dubos, & Lepiniec, 2015; Xu et al., 2014). Interestingly, several transcription factors involved in flavonoids biosynthesis also participate in regulating FA metabolism. TRANSPARENT TESTA2 (TT2) is proved to regulate embryonic FA biosynthesis by targeting *FUSCA3*during the early developmental stage of Arabidopsis seeds (Z. Wang et al., 2014). TT8 inhibits seed FA accumulation by targeting several seed development regulators in Arabidopsis (Chen et al., 2014). Overexpression of the *SiTTG1* gene from *Setaria italica* can induce the transcription of genes involved in accumulation of seed FA in developing seeds of Arabidopsis *ttg1-13* plants (K. Liu et al., 2017).

Many *tt*mutants in Arabidopsis are deficient in flavonoids biosynthesis, but show higher contents of FA than WT plants (Z. Wang et al., 2014), indicating a close relationship between these two metabolites.

FA and FA-derived complex lipids are the main energy reserves in many higher plant seeds. The biosynthesis of FA is a complex physiological and biochemical process, involving the co-expression of many enzymes, and the participation of several cell structures, such as plastid, cytoplasm and endoplasmic reticulum. Seed oil is mainly stored in the form of triacylglycerol (TAG), which is formed by three FA molecules connected to the skeleton of one glycerol molecule (Baud & Lepiniec, 2009). The degradation of seed oil is initiated with the breaking of TAG into FA and glycerin, which might be realized by three ways, including lipase hydrolysis, acyl CoA diesterylglycerol acyltransferase (DAGAT) pathway (Zou et al., 1999), and lipoxygenase pathway (Bannenberg, Martinez, Hamberg, & Castresana, 2009). Several GDSL type lipases have been shown to stimulate FA degradation in Arabidopsis (Chen, Du, et al., 2012; Huang, Lai, Chen, Chan, & Shaw, 2015). The free polyunsaturated fatty acids (PUFAs) including linolenic acid and linoleic acid are the preferred substrate for LOX enzymes to generate oxidation products like aldehydes, alcohols, ketones, acids (B. Li et al., 2016; Sjovall, Virtalaine, Lapvetelainen, & Kallio, 2000). Wheat germ (WG) contains large amount of unsaturated lipids, which makes them sensitive to rancidity during storage due to the presence of lipase (LA) and LOX (Kumar & Krishna, 2015; B. Li et al., 2016). About 95% of the total FA esters in tobacco are of chain length 16 carbons or 18 carbons, including palmitate, stearate, oleate, linoleate, and linolenate (16:0, 18:0, 18:1, 18:2, and 18:3, respectively) (Chu & Tso, 1968). The PUFA 18:2 and 18:3 are the major FA in tobacco. For instance, the relative amount of linolenic acid (18:3) in tobacco leaves is about 30% at early developing stages, but increases to 60 % at maturity stage, while the percentage of other FAs (18:2, 18:1, 18:0, and 16:0) decrease progressively with leaf development. There is a rapid increase of FA content in tobacco flowers developed into seedpods, and the linoleic acid (18:2) comprises 75 % of tobacco seed oil (Chu & Tso, 1968).

MYB12 is a flavonol-specific regulator, which in parallel activates the transcriptions of several EBGs, including *CHS* (*Chalcone synthase*), *CHI* (*Chalcone isomerase*), *F3H*(*Flavanone 3-hydroxylase*), and *FLS* (*Flavonol synthase*) in Arabidopsis (Mehrtens, Kranz, Bednarek, & Weisshaar, 2005). MYB12 does not need a bHLH or a WD protein as partner, but shares significant structural and functional similarity with MYB11 and MYB111 (Stracke et al., 2007). In Arabidopsis, MYB12, MYB11, and MYB111 form the subgroup 7 of the R2R3-MYB family, but show differential spatial activity. For instance, flavonol biosynthesis in the roots is mainly controlled by MYB12, while in cotyledons the flavonol biosynthesis is primarily controlled by MYB111 (Stracke et al., 2007). *MYB12* gene has been cloned in many plant species, including grape (Czemmel et al., 2009), tomato (Ballester et al., 2010), apple (N. Wang et al., 2017), buckwheat (Matsui et al., 2018), and pear (Zhai et al., 2019). Interestingly, AtMYB12 activates the biosynthesis of both flavonol and caffeoyl quinic acid in tomato fruit (Luo et al., 2008), though it has been well characterized as a flavonol-specific regulator in Arabidopsis. Moreover, when overexpressed in tobacco, AtMYB12 not only promotes the accumulation of flavonol, but also regulates the transcription of genes involved in many pathways, such as amino acid metabolism, carbohydrate and lipid metabolism, auxin response, and defense response (Misra et al., 2010). Subsequent research confirms that over-expressed *AtMYB12* enhances the tolerance of transgenic Arabidopsis plants to the salt and drought stresses (F. Wang et al., 2016). However, the exact role of MYB12 in other pathways still needs to be studied.

Tobacco is often used as a model plant to study the function of MYB12 from other plant species. One *NtMYB12* gene has been identified from tobacco so far, and was shown to positively regulate flavonol biosynthesis, as well as to enhance plant tolerance to low Pi stress (Song et al., 2019). However, whether there are duplicated *NtMYB12* genes in allotetraploid tobacco genome, and whether the duplicated *NtMYB12* genes have functional differentiation in tobacco have not been reported so far. Thus, we identified two duplicated *NtMYB12* genes from tobacco genome, and verified their regulation on flavonoids biosynthesis. We further found that sucrose induced the transcription of *NtMYB12a* gene, which directly targets *NtSFAR4* , *NtLOX5* , *NtLOX6* , and*NtGDSL2* genes to stimulate the FA degradation in tobacco. Consequently, our results provide evidence for revealing the multiple roles of MYB12 in plants.

2 MATERIALS AND METHODS

2.1 Plant materials and growth conditions

The Honghua Dajinyua cultivar (*N. tabacum*) kept by our lab was used for transgenic manipulation, gene expression analysis, and detection of flavonoids content in the present study. Tobacco seeds were soaked and sterilized as described previously (Z. Wang et al., 2018; Z. Wang et al., 2020), and then germinated on MS medium. The sterile seedlings were either used for transgenic manipulation, or transplanted into pots for normal growth (28°C/16h light, 23°C/8h darkness) until the flowering stage. Various tissues were collected from the plants for RNA extraction or flavonoids content detection.

2.2 Gene cloning and bioinformatics

The coding sequences and amino acid sequences of Arabidopsis AtMYB12 (At2g47460), AtMYB11 (At3g62610), and AtMYB111 (At5g49330) were used as query sequences to make blast in the NCBI database. Six homologous proteins were identified (XP_016480310.1, XP_016482665.1, XP_016508470.1, XP_016487451.1, XP_016444804.1, and XP_009790561.1) in the *N. tabacum* database, and the full length gene sequences and coding sequences were amplified and sequenced with gene-specific primers (Supplementary Table S1). Exon/intron structure of each gene was determined by aligning the CDS and genomic DNA sequences with Clustal X (version 1.83). Multiple sequence alignment of the NtMYB proteins was performed using the DNAMAN (version 6.0) software with default gap penalties (Jeanmougin, Thompson, Gouy, Higgins, & Gibson, 1998). The phylogenetic tree was built up using the MEGA 5.0 software (Tamura et al., 2011) and the following protein accessions in GenBank: AtMYB90 (AAG42002), AtMYB75 (AAG42001), AtMYB113 (NP_176811), PhAN2 (AAF66727,*Petunia hybrid*), MdMYB10 (XP_028963317.1, *Malus domestica*), VvMYBA1 (BAD18977.1, *Vitis vinifera*), VvMYBA2 (BAD18978.1), MdMYB11 (AAZ20431.1), MdMYB12 (XP_008337875.1), GhMYB38 (AAK19618.1, *Gossypium hirsutum*), VvMYBPA2 (ACK56131.1), AtMYB123 (Q9FJA2), MdMYB9 (NP_001280749.1), PmMBF1 (AAA82943.1, *Picea mariana*), MdMYB22 (AAZ20438.1), SlMYB12 (ACB46530, *Solanum lycopersicum*), ZmP (P27898, *Zea maize*), GhMYB1 (CAD87007.1,*Gerbera hybrid cultivar*), LjMYB12 (BAF74782.1, *Lotus japonicas*), and VvMYBF1 (FJ948477).

2.3 Vector construction and tobacco transformation

For RNAi vector construction, the different CDS fragments of*NtMYB12a* and *MtMYB12b* genes were amplified with gene specific primers containing attB adaptor, the correct fragments were then cloned into pHellsgate 2 vectors via BP reaction. The complete open reading frame of *NtMYB12a* gene without the stop codon was amplified and digested with the endonucleases Kpn I and Spe I (Supplementary Figure S6a), and then cloned into the pCAMBIA1300 vector to obtain the fusion NtMYB12a-GFP, under the control of the CaMV35S promoter (Supplementary Figure S6b). Similarly, the *NtMYB12a* CDS sequence was also amplified and digested with the endonucleases XhoI and ApaI, and then cloned into pGreen-35S-GR (GLUCOCORTICOID RECEPTOR) to obtain an in frame fusion of NtMYB12a-GR (Chen et al., 2014). For Cas9/sgRNA vector construction, 20 nucleotides located in the first exon of*NtMYB12a* gene was selected and used for sgRNA generation via overlapping PCR method as described previously (Xie et al., 2017). The sgRNA was then subcloned into the pORE-Cas9 binary vector. The predicted promoter fragment (2 kb) of *NtMYB12a* gene was amplified and digested with Hind III and BamH I, and then cloned into pBI121 to construct the$Pro_{NtMYB12a}$*: GUS* vector. All the primers used in the vector constructions are listed in Supplementary Table S3.

The recombinant plasmids were transformed into *Agrobacterium tumefaciens* strain GV3101, which was used for the transgenic manipulation via leaf disks method (Palmgren, Mattson, & Okkels, 1993). The positive transgenic plants were selected with hygromycin or basta, confirmed by DNA and RNA analyses (Supplementary Figure S6c), and then self-pollinated twice to generate T_2 transgenic progeny. To detect the mutations generated by Cas9/sgRNA, an about 250 bp fragment of *NtMYB12a* gene containing the target sites was amplified and used for Hi-TOM sequencing analysis as described in previous study (Q. Liu et al., 2019). The individual plants with >90% mutations were chosen for the subsequent studies (Supplementary

Figure S6d).

2.4 Subcellular localization of NtMYB12a-GFP fusion protein

The subcellular localization of NtMYB12a was analyzed in tobacco protoplasts as described previously (Yoo, Cho, & Sheen, 2007). The recombinant *35S:NtMYB12a-GFP* and 3*5S: GFP* plasmid was introduced into tobacco protoplasts obtained from leaf tissues using polyethylene glycol-mediated transformation. The fluorescent signal was captured 16-20 h after transformation with a confocal laser scanning microscope (ZEISS LSM 700, Germany). The nuclear localized marker protein (NLS) was as described in the previous reference (Shcherbo et al., 2007).

2.5 Flavonoids measurement

Flavonoids contents in tobacco tissues were determined as described in our previous studies (Z. Wang et al., 2018; Z. Wang et al., 2020). In brief, fresh tobacco tissues were frozen in liquid nitrogen, and then freeze-dried till constant weight. The tissues were grinded to powder, and suspended with 1.5 ml 80% ethanol (with 0.012 g/L vitexin as internal standard). The mix was ultrasonicated for 30 min, and then centrifuged (14,000 rpm) for 10 min. A 0.22 μm membrane was adapted to filter the supernatant, which was used for flavonoids content determination by HPLC-UV. The experimental parameters are as follows: chromatographic column-ACQUITY UPLC®BEH Phenyl (1.7 μm 2.1mm×150 mm, Waters); injection volume-1μl; gas temperature-350°C; flow-0.3 mL/min; wave length-230 nm, 260nm, 360 nm, and 570 nm; mobile phase-A 100% water, B 100% acetonitrile; gradient elution-8 min 85% A+15% B, 5 min 58% A+42% B, 0.01 min 100% B, 3 min 95% A+5% B, 18 min stop.

2.6 RNA-seq and data analyses

Total RNA from different tobacco tissues was extracted with Trizol reagent (Invitrogen), and the Agilent Bio-Analyzer 2100 was adapted to determine the RNA integrity. Qualified RNA samples were then used for the sequencing library construction with an Illumina TruSeq RNA Sample PreKit, and the sequencing program was performed on the Illumina Hiseq 2500 sequencing platform by Beijing Annoroad Co. Ltd. Data analysis was performed as described in our previous study (Z. Wang et al., 2020). Briefly, the quality of the mRNA-Seq reads was evaluated by the FastQC method (http:// www.bioinformatics.bbsrc.ac.uk/projects/fastqc/). The adaptor sequence and the low quality (<20) bases at the 5' and 3' ends were removed by Trimmomatic (v0.30) (Bolger, Lohse, & Usadel, 2014). The clean reads were mapped to the reference genome of *N. tabacum*(*ftp://ftp.solgenomics.net/genomes/Nicotiana_tabacum/*) (Edwards et al., 2017) with HISAT2 software (v2.1.0) (Kim, Langmead, & Salzberg, 2015). Gene expression levels were calculated with Cufflinks (v2.2.1) (Trapnell et al., 2010), and Cuffdiff (Trapnell et al., 2013) was used to identify the DEGs (differentially expressed genes) with log2 ratios [?]2 or [?]-2 (FDR<0.05) . All the DEGs are listed in Supplementary Table S4 and Table S5.

2.7 FA measurement

The freeze-dried tobacco seeds or leaves were suspended with methanol solution containing 1M HCl, and heated at 80degC for 2 h. 2 mL of 0.9% (w/v) NaCl and 2 mL hexane were added to the supernatant to extract the FA methyl esters, and the organic phase with methyl heptadecanoate as an internal standard was used for the following analysis. Gas chromatography was then performed on a GC–2014 gas chromatograph system (Shimadzu,*http://shimadzu.com/an/gc/gc2014.html*) as described previously (Chen, Wang, et al., 2012; Siloto et al., 2006; Z. Wang et al., 2014). Each FA species was identified by the characteristic retention times, and their concentrations were normalized against the internal control.

2.8 qRT-PCR

Total RNA samples were isolated with a SuperPure Plantpoly RNA Kit (Gene Answer) following the manufacturer's instructions. RNase-free DNAse I (New England Biolabs) was used to remove any trace genomic DNA. First-strand complementary DNA (cDNA) was synthesized with Reverse Transcriptase M-MLV (Takara) and oligo (dT) 12-18 as a primer. The PCR reactions were performed with SYBR Green kit (Roche)

on a LightCycler$^{(r)}$ 96 SW 1.1 cycler (Roche). The*NtL25* gene was chosen as the internal reference control (Schmidt & Delaney, 2010), and the realtive expression levels of the target genes were calculated as described previously (Livak & Schmittgen, 2001). All the gene-specific primers for qRT-PCR were listed in Supplemental Table S3.

2.9 GR induction

Tobacco seeds harboring NtMYB12a-GR fusion protein were surface sterilized with 6% of sodium hypochlorite, and germinated on MS medium. 15 day seedlings were transferred to new mediums containing DMSO (control), DEX (10 μM), CYC (5 μM), and DEX+CYC, respectively. The seedlings were collected at 1 h, 4 h, and 8 h after treatment, and used for RNA extraction and gene expression level analyses.

2.10 ChIP assay

The ChIP assays were performed as described in our previous study (Z. Wang et al., 2020). Briefly, 15 day tobacco seedlings on MS medium were harvested, and immediately fixed in formaldehyde solution (1.0%) under vacuum infiltration for 10 min. Glycine (final concentration 0.125 M) was added to the solution under vacuum infiltration for an additional 5 minutes to stop the cross-linking reaction. The tissues were then grinded to powders in liquid nitrogen, and suspended with lysis buffer provided by the EpiQuik Chromatin Immunoprecipitation (ChIP) Kit (Epigentek). The pellet nuclei was centrifuged, and then sheared by sonication to break the DNA into 200-1000 bp fragments. The NtMYB12a-GFP fusion protein was pulled down in the strip wells with Anti-GFP antibody (Abcam, ab290). DNA fragments were released from the fusion protein, and collected for the qRT-PCR analyses. The enrichment of the genomic fragment in two tubulin beta chain genes (*NtTUBB* , XP_016456097.1 and NP_001312648.1) was used as a negative control. The primers used for fragment amplification are listed in Supplementary Table S3.

2.11 Gus staining

Tobacco seedlings harboring the *ProNtMYB12a* : *Gus* vector were transformed to MS medium containing 0, 50, 88 (Control), 150, and 200 mM sucrose for 7 days. Gus staining was performed with a GUS Staining Kit (Coolaber, SL7160) following the manufacturer's instructions. The seedlings were immersed in the staining buffer provided by the kit under vacuum infiltration for 30 min, and then kept at 37°C overnight. The green seedlings were decolorized with 95% and 75% ethanol successively. The photographs were taken using an OLYMPUS SZ 61 stereomicroscope.

2.12 Statistical analysis

Statistical analysis was performed as described in our previous study (Z. Wang et al., 2014). A completely randomized block design with at least three biological replicates was applied for each experiment. Win-Excel was used to collect and classify the data, which was further analyzed using the SPSS statistical package (version 8.0). Tukey's test was performed, and the P value (0.05) was used to indicate significance.

3. RESULTS

3.1 Isolation of *NtMYB12* genes from *N. tabacum*genome

Six *MYB12* homologous genes were cloned and sequenced from*N. tabacum* genome, and their sequence information was shown in Supplementary Table S1. Phylogenetic tree was constructed with 24 known R2R3-MYB TFs, which has been clustered into PA-related subgroup (subgroup 5/SG5), anthocyanin-related subgroup (SG6), and flavonol-related subgroup (SG7) (N. Wang et al., 2017). All the 6 NtMYB proteins belonged to SG7, and were divided into 3 pairs (Figure 1a). The two members within each pair shared highest sequence identities (Supplementary Table S2). One pair genes close to *AtMYB111* were named *NtMYB111a/NtMYB111b* , while the other two pairs were named *NtMYB12a/NtMYB12b* and*NtMYB11a/NtMYB11b* , respectively. Sequence alignment showed that the R2-R3 domains were highly conserved among the 6 NtMYB proteins at the N -terminus, but their C terminal sequences were diverse (Figure 1b). NtMYB11b, NtMYB111a, and NtMYB111b contained the typical SG7 motif ([K/R][R/x][R/K]xGRT[S/x][R/G]xx[M/x]K), while NtMYB12a, NtMYB12b, and NtMYB11a contained

both SG7 motif and SG7-2 motif ([W/x][L/x]LS) (Figure 1b), indicating their putative function in regulating flavonol biosynthesis. Gene structure analyses showed that *NtMYB12a* gene shared similar exon/intron organization with *NtMYB12b* gene, while those within other two pairs were diverse (Figure 1c). Therefore, *NtMYB12a* and *NtMYB12b* were a pair of homologous genes with high similarity, and their respective functions needs to be clarified.

3.2 Functional divergence of *NtMYB12* genes in regulating flavonoid biosynthesis

Both *NtMYB12a* and *NtMYB12b* showed high expression levels in tobacco leaves, buds, flowers, and capsules (Figure 2a). In the developing tobacco leaves, the expression levels of *NtMYB12a* and*NtMYB12b* genes decreased gradually from the seedling stage to the early senescence stage, but slightly increased in the late senescence stage (Figure 2b). *NtMYB11a* was mainly expressed in the roots, buds, and flowers, and *NtMYB111b* showed high expression levels in axillary buds, capsules, and seeds (Figure 2a). The transcription of*NtMYB11b* and *NtMYB111a* genes could hardly be detected in all the tissues examined. Since both *NtMYB12a* and *NtMYB12b*genes showed high transcriptional activity, their subcellular localization was then investigated in tobacco protoplast. As shown in Figure 2c, the location of NtMYB12a-GFP and NtMYB12b-GFP are complete match with that of the NLS marker protein, indicating NtMYB12a and NtMYB12b are nuclear proteins.

To explore the roles of NtMYB12a and NtMYB12b in regulating flavonoid biosynthesis, five independent *NtMYB12a-RNAi* and*NtMYB12b-RNAi* lines were generated with gene specific CDS fragments (Figure 3a), respectively. The *NtMYB12a* gene was specifically silenced in the *NtMYB12a-RNAi* lines, while the*NtMYB12b* gene was specifically silenced in the*NtMYB12b-RNAi* lines (Figure 3b). Silencing of *NtMYB12a*reduced the flavonol content by about 31%-76% in the leaves of five RNAi lines, while silencing of *NtMYB12b* reduced the flavonol content by about 51%-86% (Figure 3c).The flavonol contents in three*NtMYB12a-RNAi* lines were significant higher than those of the five *NtMYB12b-RNAi* lines (Figure 3c), indicating a weak role of NtMYB12a in regulating flavonol biosynthesis. The contents of anthocyanin in four *NtMYB12b-RNAi* lines showed no significant differences with that in the WT plants (Figure 3d). However, the anthocyanin contents of the five *NtMYB12a-RNAi* lines were all significant lower than that of the WT plants (Figure 3d). Moreover, the expression levels of *NtCHS* , *NtCHI* , *NtF3H* , and*NtFLS* genes were all significantly down-regulated in the five*NtMYB12b-RNAi* lines, but were not significantly affected in some*NtMYB12a-RNAi* lines (Supplemental Figure S1).

NtMYB12b seems to be a flavonol-specific regulator in tobacco, but NtMYB12a might also be involved in regulating anthocyanin biosynthesis. To fully characterize the role of NtMYB12a in regulating flavonoid biosynthesis, the *NtMYB12a* over-expression (OE) and*ntmyb12a* mutant plants were generated, respectively. The flowers of *NtMYB12a* OE plants were dark red, while the color of the mutant plants turned light red (Figure 4a). In accordance with this, the anthocyanin contents in the flowers of two *NtMYB12a* OE lines were significant higher than that in WT flowers, and the flowers of the three mutant lines possessed higher anthocyanin contents than the WT plants (Figure 4b). The anthocyanin contents also significantly increased in the leaves of two *NtMYB12a* OE lines, but significantly decreased in the leaves of two *ntmyb12a* mutant lines (Figure 4c). Moreover, the flavonol contents of the flowers and leaves were significant higher in the *NtMYB12a* OE lines than those in the WT plants, but significant lower in the *ntmyb12a* mutant lines (Figure 4d). These results indicate NtMYB12a might possess new functions in tobacco besides promoting flavonol biosynthesis.

3.3 NtMYB12a prevents the accumulation of FA in tobacco

To explore the potential new functions of NtMYB12a, the transcriptomes of *NtMYB12a-OE* and WT plants were detected by RNA-seq (Supplemental Figure S2a). There were 1,152 DEGs between the OE and WT plants, among which the expression levels of 692 genes were up-regulated, and those of 453 genes were down-regulated in the*NtMYB12a-OE* plants (Supplemental Figure S2b). Gene annotation showed that these DEGs were mainly involved in plant-pathogen interaction, starch and sucrose metabolism, plant hormone signal transduction, flavonoid biosynthesis, lipid metabolism, and so on (Supplemental Figure S2c). It is

noteworthy that the lipid-related DEGs mainly encoded the GDSL esterase lipase, lipooxygenase (LOX) and FAH1 (Table 1), which could catalyze the dehydration of FA as described in the previous studies (Chauvin, Caldelari, Wolfender, & Farmer, 2013; Nagano et al., 2012; Nalam, Keereetaweep, & Shah, 2013).

The relative expression levels of *NtLOX6* , *NtLOX5* ,*NtFAH1* , *NtSFAR4* , *NtGDSL1* , *NtGDSL2* ,*NtGDSL3* , and *NtGDSL4* genes significantly increased by more than 2 folds in the *NtMYB12a-OE* plants compared to those in the WT plants, but the transcripts of these genes significantly decreased in the RNAi and mutant plants (Figure 5a & 5b). When compared to that in the WT plants, the FA contents in the leaves of three independent*NtMYB12a-OE* lines decreased by 31%-48%, while those in the leaves of three independent RNAi lines increased by 2.3 to 3.2 fold, and those in the mutant plants also increase by 1.5 to 1.7 folds (Figure 5c). The percentage of PUFAs in the leaves of WT plants was about 71%. However, the percentage of PUFAs in the *NtMYB12a-OE* lines was about 43%-59%, and the percentage of PUFAs was more than 81% in all the RNAi and mutant plants (Figure 5d).

In the developing seedpods of WT plants, the transcripts of*NtSFAR4* , *NtLOX5* , *NtLOX6* , *NtFAH1* , and*NtGDSL1* genes could be detected at 10 DAF, then increased to higher levels at 20 DAF, but finally decreased to low levels at 30 DAF (Figure 6a). The *NtGDSL2* , *NtGDSL3* , and *NtGDSL4* genes showed low expression levels during the whole developmental process of WT seedpods. Generally, the expression levels of these eight genes were all up-regulated in the *NtMYB12a-OE* plants at each time point, especially at 30 DAF, when their expression levels were more than five folds of those in the WT plants (Figure 6a). By contrast, the expression levels of these eight genes in the developing seedpods of*NtMYB12a-RNAi* and mutant plants were lower than those of the WT plants at each time point (Figure 6a). There were no significant changes occurred to the phenotype and weight of the mature seeds of the*NtMYB12a* transgenic and mutant plants (Figure 6b & 6c). However, when compared to those in the WT plants, the FA contents in the mature seeds of three independent *NtMYB12a-OE* plants significantly decreased by 29.6% to 42.3%, while the contents in the three RNAi lines increased by 55.9% to 78.1%, and the FA contents in the mutant lines even increased by 85.6% to 106.3% (Figure 6d). Taken together, our results indicate that NtMYB12a could induce the transcription of*LOX* and lipase genes, and then prevent the FA accumulation in tobacco leaves and seeds.

3.4 NtMYB12a directly targets two *LOX* and two lipase genes

The 35S:NtMYB12a-GR seedlings were treated with DEX, CYC, and DEX+CYC, respectively. The *NtCHS* , *NtCHI* , *NtF3H* , and*NtFLS* genes, which are known direct targets of MYB12, were selected as the marker genes to show the effects of treatments. The transcriptions of these four genes were significantly induces by DEX treatment owing to the release of NtMYB12a-GR fusion protein from the nuclear membrane, but significantly repressed by CYC, which can inhibit protein synthesis in plants (Supplemental Figure S3). Moreover, the transcriptions of these four marker genes were also significantly induced under DEX+CYC treatment, indicating the regulation of NtMYB12a on the expression of these four genes requires no intermediate protein synthesis. Similarly, when compared to those in the mock seedlings, the expression levels of *NtSFAR4* , *NtLOX5* , *NtLOX6* , and*NtGDSL2* genes were significantly lower in the seedlings treated with CYC, but significantly higher in the seedlings treated with DEX or DEX+CYC at all the processing time points (Figure 7a). Meanwhile, the transcription of *NtFAH1* , *NtGDSL1* , *NtGDSL3* , and*NtGDSL4* genes were also induced by DEX, and repressed by CYC, but showed no significant difference between the mock and DEX+CYC treatment (Supplemental Figure S4a).

The putative binding sites of MYB12 were searched in the promoter regions of the eight lipid-related DEGs (Figure 7b, Supplemental Figure S4b), and ChIP-qPCR was then performed to verify the direct targets of NtMYB12a in tobacco. As shown in Figure 7c, three fragments containing the binding site of MYB12 in the promoter regions of *NtSFAR4* and*NtGDSL2* genes were significantly enriched after immunoprecipitation, while two fragments in the promoter regions of*NtLOX5* and *NtLOX6* genes were also significantly enriched, respectively. However, the fragments with the binding site of MYB12 in the promoter regions of *NtGDSL1* , *NtGDSL3* , *NtGDSL4* , and *NtFAH1* genes were not significantly enriched (Supplemental Figure S4c). These results indicate that *NtSFAR4* , *NtLOX5* ,*NtLOX6* , and *NtGDSL2* are the direct targets of NtMYB12a.

3.5 NtLOX5 and NtLOX6 inhibit the accumulation of FA in tobacco seeds

Among the four direct targets of NtMYB12a, the homologous gene of*NtSFAR4* has been shown to be involved in FA degradation in Arabidopsis (Huang et al., 2015). The*NtLOX5* and *NtLOX6* genes encode 9-LOX and 13-LOX proteins (Figure 8a), respectively. To determine whether they affect FA content in tobacco, the overexpression and RNAi plants of these two genes were generated. As shown in Figure 8b & 8c, the transcription levels of*NtLOX5* and *NtLOX6* genes significantly increased in their OE lines, but significantly decreased in their RNAi lines, respectively. The FA contents in the mature seeds of two independent *NtLOX5-OE*lines were significantly lower than those in the WT seeds, but the FA contents in the seeds of three *NtLOX5-RNAi* lines were all significantly higher than those in the WT seeds (Figure 8d). The percentage of PUFA (18:2 and 18:3) in the WT seeds was about 77%, which significantly decreased to 67%-71% in the three independent*NtLOX5-OE* lines, but significantly increased to 81% and 84% in two independent *NtLOX5-RNAi* lines (Figure 8e). Similarly, the FA contents in the mature seeds of three independent *NtLOX6-OE* lines were significantly lower than those in the WT seeds, but the FA contents in the seeds of three *NtLOX6-RNAi* lines were all significantly higher than those in the WT seeds (Figure 8f). The percentage of PUFA significantly decreased to 69%-72% in the three independent*NtLOX6-OE* lines, but significantly increased to 83% and 84% in two *NtLOX6-RNAi* lines (Figure 8g). Taken together, the activation of NtLOX5 and NtLOX6 could significantly inhibit the accumulation of FA in tobacco seeds.

3.6 Sucrose enhances the effect of NtMYB12a on FA metabolism

The transcriptome analysis showed that many DEGs in the*NtMYB12a-OE* plants were involved in the starch and sucrose metabolism (Supplemental Figure S2c). To further test whether sucrose affects the activity of NtMYB12a, the expression pattern of*NtMYB12a* gene under various sucrose concentration was firstly analyzed with the $Pro_{NtMYB12a}$*:GUS* seedlings. The GUS signal was mainly detected in the shoot apical meristem (SAM) of the seedlings on the normal MS medium, but strong GUS activity was also detected in the fresh leaves of the seedlings under 150 mM and 200 mM sucrose treatment (Figure 9a). Meanwhile, no GUS signal was detected in the leaves of seedlings under 0 mM and 50 mM sucrose treatment (Figure 9a). qRT-PCR analysis also showed that the expression level of*NtMYB12a* was highest in the seedlings under 150 mM sucrose treatment, while the transcription level of this gene in the seedlings under 200 mM sucrose treatment was also significantly higher than that in the control seedlings (Figure 9b).

RNA-seq analysis showed that the expression levels of 1367 genes were up-regulated under 150 mM sucrose treatment, while those of 2266 genes were down-regulated (Supplemental Figure S5a & 5b). Gene annotation showed that these DEGs were mainly involved in plant hormone signal transduction, phenylpropanoid biosynthesis, response to tress, and so on (Supplemental Figure S5c).Venn analysis showed that the transcription levels of 29 genes were up-regulated by both the *NtMYB12a* OE and sucrose treatment (Figure 9c). Among the 29 genes, there were three genes encoding NtGDSL2, NtGDSL3, and NtLOX6 (Table 2), respectively. When germinated on MS medium containing 150 mM sucrose, the*NtMYB12a-OE* seedlings were obviously smaller than the WT, RNAi, and mutant plants (Figure 10a). The expression levels of *NtLOX6*and *NtGDSL2* genes in the WT seedlings increased by about 4 folds under 150 mM sucrose treatment (Figure 10b). However, the expression levels of these two genes in the *NtMYB12a-RNAi* and mutant seedlings under 150 mM sucrose treatment showed no significant difference with those in the *RNAi* and mutant seedlings under normal condition (Figure 10b). By contrast, the transcription of*NtLOX5* and *NtSFAR4* genes were not affected in the WT seedlings treated with sucrose, but sucrose significantly decreased the expression levels of these two genes in the *NtMYB12a-RNAi* and mutant seedlings (Figure 10b).

When grown on the normal MS medium, the FA content in the*NtMYB12a-OE* seedlings was about 33% less than that in the WT seedlings (Figure 10c). Sucrose treatment reduced FA content in the WT seedlings by about 44%, and the FA content in the *OE* lines under sucrose treatment reduced by 60% compared to that in the normal WT seedlings, suggesting that sucrose treatment enhanced the effect of NtMYB12a on FA degradation in tobacco seedlings. The*NtMYB12a-RNAi* and mutant seedlings under normal condition contained 64% and 56% more FA than the normal WT seedlings, respectively. The FA content of the *ntmyb12a* mutant seedlings under sucrose treatment was 21% less than that in the normal WT seedlings,

but was significantly higher than that in the WT seedlings under sucrose treatment (Figure 10c), indicating that loss function of NtMYB12a partially suppressed the decrease of FA content in tobacco seedlings caused by sucrose treatment.

4 DISCUSSION

Flavonoids are important secondary metabolites in plants, and the biosynthesis of flavonoids is precisely controlled by many transcription factors. MYB11, MYB12, and MYB111 are close R2R3 homologous proteins, which acts partially redundantly to determine the accumulation and distribution of flavonol in Arabidopsis (Stracke et al., 2007). In the present study, six R2R3 *NtMYB* genes were cloned from the *N. tabacum* genome, and grouped into three pairs, which were subsequently named as*NtMYB11* , *NtMYB12* , and *NtMYB111* , respectively. The NtMYB12b is a flavonol-specific regulator, while the NtMYB12a promotes the biosynthesis of both flavonol and anthocyanin in tobacco. Moreover, we find that NtMYB12a can directly induce the transcription of*NtSFAR4* , *NtLOX6* , *NtLOX5* , and *NtGDSL2* genes, and indirectly promotes the transcription of *NtGDSL1* ,*NtGDSL3* , *NtGDSL4* , and *NtFAH1* genes, which leads to the reduction of FA content in tobacco leaves and seeds. We further show that high concentration of sucrose enhances the activity of NtMYB12a in promoting the dehydration of FA in tobacco.

4.1 The neofunctionalization of NtMYB12a in tobacco

The AtMYB12 has been well characterized as a flavonol-specific regulator in Arabidopsis (Mehrtens et al., 2005). AtMYB12, AtMYB11 and AtMYB111 share high sequence identities in the MYB motifs, and all of these three MYBs retain the function of regulating flavonol biosynthesis in Arabidopsis, but their regulatory activities are tissue-specific (Stracke et al., 2007). The AtMYB12 mainly promotes the biosynthesis of flavonol in the roots, while the AtMYB111 works mainly in the cotyledons (Stracke et al., 2007). Tobacco is an allotetraploid specie with many duplicated genes presented in its genome (Edwards et al., 2017), and the functional evolution of homologous genes in tobacco is more alternative. NtMYB11, NtMYB12, and NtMYB111 were all encoded by two close homologous genes (Figure 1a), which shared high sequence identities in the R2R3 MYB motifs (Figure 1b). The tissue-specific expression patterns of*NtMYB11* , *NtMYB12* , and *NtMYB111* genes (Figure 2a) suggested that their regulatory functions in flavonol biosynthesis might also be differentiated to tissue-specific, which is similar to that occurred to the AtMYB11, AtMYB12, and AtMYB111 in Arabidopsis (Stracke et al., 2007). It's noteworthy that the transcription of *NtMYB11b* and *NtMYB111a* genes could hardly be detected in all the tissues examined, but both the*NtMYB12a* and *NtMYB12b* showed high expression levels in many tissues (Figure 2a), indicating that NtMYB12a and NtMYB12b might have further functional differentiation.

The hypothesis about the functional differentiation of NtMYB12a and NtMYB12b was verified by generating RNAi plants of these two genes. The NtMYB12b was shown to be a flavonol-specific regulator, while the NtMYB12a was involved in the biosynthesis of both flavonol and anthocyanin (Figure 3c & 3d). RNA-seq was then performed to fully characterize the function of NtMYB12a, and found that the transcription levels of several lipase and *LOX* genes were significantly up-regulated in the *NtMYB12a* overexpression lines (Table 1, Figure 5a & 5b). The FA contents in the leaves and seeds of*NtMYB12a* overexpression plants also significantly decreased (Figure 5c, 6d). The function of MYB12 in regulating FA metabolism has not been described in any other plant species so far, but it's interestingly to see that several transcription factors involved in regulating flavonoids biosynthesis are also proved to affect the accumulation of FA or the embryo development in plants, including TT2 (Z. Wang et al., 2014), TT8 (Chen et al., 2014), *NAC03*(Dalman et al., 2017). The NtMYB12a might be a new transcription factor involved in the metabolism of both flavonoids and FA in tobacco.

The neofunctionalization of NtMYB12a in tobacco might be caused by two reasons. One is the sequence variation occurred to the C terminal of NtMYB12a protein. The amino acid sequence of NtMYB12a shares 87.91% identity with that of NtMYB12b (Supplementary Table S2). In detail, the amino acids of the R2R3 motifs within the two proteins were almost the same (only one substitution of Alanine to Glycine at 94^{th} position), while the identity between the rest amino acid sequences of the two proteins was 83.97% (Figure

1b). Considering that both the *NtMYB12a* and *NtMYB12b* genes show strong transcriptional activities, and the variation of amino acids in the C terminals of the two homologous proteins, it is quite possible for NtMYB12a to have new functional differentiation. The other reason is that the function of MYB12 varies with different plant species. The AtMYB12 is a flavonol specific regulator in Arabidopsis (Mehrtens et al., 2005), but AtMYB12 could induce the expression of genes in both CQA (caffeoyl quinic acids) and flavonol biosynthetic pathways in tomato, and affect the contents of both flavonol and anthocyanin in tobacco (Luo et al., 2008). When overexpressed the citrus *CsMYBF1* gene belonging to the flavonol-specific MYB subgroup in tomato, the contents of 5 sugars and 2-ketoglutaric acid significantly reduced in the transgenic fruits, while the contents of isocitric acid and glucuronic acid significantly increased (C. Liu et al., 2016). Therefore, the*NtMYB12a* might be a valuable candidate gene, which can increase flux to flavonoids metabolites and significantly affect the FA metabolism in tobacco.

4.2 NtMYB12a acts downstream of sucrose signaling to stimulate FA degradation in tobacco

The major FA of tobacco leaves are linolenic acid (18:3) and linoleic acid (18:2), which comprise about 60% and 15% of total FA, respectively. The contents of FA increase rapidly during tobacco seed development, and linoleic acid comprises 75% of the tobacco seed oil (Chu & Tso, 1968). The linolenic acid and linoleic acid are the major PUFAs, which are preferred substrates of LOX enzymes for generating a diverse class of phyto-oxylipins, including jasmonates, divinylethers and green leaf volatiles (GLVs) (Nalam et al., 2013; ul Hassan, Zainal, & Ismail, 2015). In the present study, we show that NtMYB12a directly bind to the regulatory regions of *NtLOX5* , *NtLOX6* , *NtSFAR4* , and*NtGDSL2* genes (Figure 7), and thus promote the transcription levels of these four genes (Figure 5a & 5b). The percentage of PUFAs significantly decreased in the leaves of *NtMYB12a-OE* lines, but significantly increased in the leaves of *NtMYB12a-RNAi* and mutant lines (Figure 5d). We further showed that over expression of*NtLOX5* and *NtLOX6* genes lead to the reduction of FA content and percentage of PUFAs in tobacco seeds. The NtSFAR4 is a close homologous protein of the Arabidopsis AtSFAR4, which plays an important role in FA degradation during post-germination and seedling development in Arabidopsis, thus reducing the FA content (Huang et al., 2015). Several GDSL esterase/lipase have been proved to be involved in FA degradation in Arabidopsis seeds (Chen, Du, et al., 2012), and the *BnGLIP* gene of *Brassica napus* expressed in*N. benthamiana* also shows lipase activity (Tan et al., 2014). Therefore, the inhibition of FA accumulation by NtMYB12a in tobacco is achieved by directly promoting the transcription of *NtLOX5* , *NtLOX6* ,*NtSFAR4* , and *NtGDSL2* genes.

The transcripts of *CHS* , *CHI* , *F3H* , *F3'H* ,*FLS* , *DFR* , and *LDOX* genes were evidently induced by sucrose in Arabidopsis seedlings (Solfanelli, Poggi, Loreti, Alpi, & Perata, 2006). The expression level of *NtMYB12a* gene was also significantly induced by sucrose treatment (Figure 9), suggesting the regulation of sucrose on the EBGs might be mediated by MYB12 in tobacco. We further showed that sucrose treatment significantly reduced the FA content in the WT seedlings (Figure 10c). The content of FA in*ntmyb12a* seedlings under sucrose treatment was higher than that in the WT seedlings under sucrose treatment, but lower than that in the WT seedlings under normal condition (Figure 10c), suggesting that loss function of NtMYB12a partially suppressed the decrease of FA content in tobacco seedlings caused by sucrose treatment.

The expression levels of *NtMYB12a* , *NtLOX6* , and*NtGDSL2* genes were all significantly elevated by sucrose treatment, and knock-out of *NtMYB12a* repressed the induction of sucrose on the expression of *NtLOX6* and *NtGDSL2* (Figure 10b), indicating that NtMYB12a mediates the promotion of sucrose on the transcription of *NtLOX6* and *NtGDSL2* . It's noteworthy that the transcription of *NtLOX5* and *NtSFAR4* genes was not affected in the WT seedlings under sucrose treatment, but sucrose could significantly reduce the transcription of these two genes in the absence of NtMYB12a function (Figure 10b), suggesting that sucrose and NtMYB12a might play opposite roles in regulating the expression of *NtLOX5*and *NtSFAR4* genes. It has been proved that sucrose can block GA (gibberellin acid) mediated degradation of DELLA proteins, which further induces the biosynthesis of anthocyanin (Y. Li, Van den Ende, & Rolland, 2014), and prevents the degradation of FA by repressing the *SFAR* genes in Arabidopsis (Chen, Du, et al., 2012). But meanwhile sucrose can also promote the accumulation of auxin in plant by degrading the auxin conjugates (Meir, Philosoph-Hadas, Epstein, & Aharoni, 1985; Meir, Riov, Philosoph-Hadas, & Aharoni, 1989) and

inducing the transcription of auxin biosynthesis gene *YUCCA* (Le, Schmelz, & Chourey, 2010; Lilley, Gee, Sairanen, Ljung, & Nemhauser, 2012). High concentration of auxin could inhibit the accumulation of FA in *microalgae Scenedesmus*(Dao et al., 2018). Therefore, sucrose can enhance the activity of NtMYB12a, which further suppresses the accumulation of FA in tobacco by targeting important lipoxygenase and lipase genes. Meanwhile, sucrose might also participate in regulating FA metabolism by balancing GA and auxin signals, which remains to be further studied.

In conclusion, we summarize the functions of NtMYB12a via a schematic that illustrates a regulatory network in which NtMYB12a promotes the biosynthesis of flavonoids and the degradation of FA in tobacco (Figure 11). We found that NtMYB12a can directly bind to the regulatory regions of *NtLOX5* , *NtLOX6* , *NtSFAR4* , and *NtGDSL2*genes, and promote the transcription of these four genes. These genes encode the lipoxygenase or lipase enzymes, which have been proved to reduce the FA content in plants by catalyzing the degradation of FA. Sucrose significantly induces the transcription of *NtMYB12a* gene, as well as the *NtLOX6* and *NtGDSL2* genes, and loss function of NtMYB12a interrupts the induction of sucrose on the expression of*NtLOX6* and *NtGDSL2* , indicating NtMYB12a mediates the promotion of sucrose on the transcription of *NtLOX6* and*NtGDSL2* . The regulation of NtMYB12a on the transcription of*NtLOX5* and *NtSFAR4* genes is independent of sucrose.

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AUTHORS' CONTRIBUTIONS

JY and ZW conceived the research and designed experiments; ZW, SSW, YSX, XDX, MZW, JLY performed the experiments; ZFL and LRC analyzed transcriptomic datasets; ZW wrote the manuscript with contributions from all authors; AGY and JY supervised the research.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Table S1. Sequence information of *NtMYB11* ,*NtMYB12* , and *NtMYB111* genes.

Table S2. Identities of amino acid and CDS sequences among*NtMYB11* , *NtMYB12* , and *NtMYB111* genes.

Table S3. All the primers used in this study.

Table S4. All the DEGs in the leaves of *NtMYB12a-OE* and WT plants.

Table S5. All the DEGs between the seedlings of CK and sucrose treatment.

Supplemental Figure S1. Relative expression levels of*NtCHS* , *NtCHI* , *NtF3H* , and *NtFLS* genes in the*NtMYB12a-RNAi* and *NtMYB12b-RNAi* plants.

Supplemental Figure S2. Characterization of the RNA-seq analysis between the WT and *NtMYB12a-OE* plants.

Supplemental Figure S3. Relative expression levels of*NtCHS* , *NtCHI* , *NtF3H* and *NtFLS* genes in the*35S:NtMYB12a-GR* seedlings under DEX, CYC, and DEX+CYC treatment.

Supplemental Figure S4. NtMYB12a indirectly promotes the transcription of *NtFAH1* , *NtGDSL1* , *NtGDSL3* , and*NtGDSL4* genes.

Supplemental Figure S5. Characterization of the RNA-seq analysis between the CK and sucrose treatment.

Supplemental Figure S6. Generation and identification of the*NtMYB12a* transgenic and mutant plants.

REFERENCES

Ballester, A. R., Molthoff, J., de Vos, R., Hekkert, B., Orzaez, D., Fernandez-Moreno, J. P., . . . Bovy, A. (2010). Biochemical and molecular analysis of pink tomatoes: deregulated expression of the gene encoding transcription factor SlMYB12 leads to pink tomato fruit color. *Plant Physiol, 152* (1), 71-84. doi:10.1104/pp.109.147322

Bannenberg, G., Martinez, M., Hamberg, M., & Castresana, C. (2009). Diversity of the enzymatic activity in the lipoxygenase gene family of Arabidopsis thaliana. *Lipids, 44* (2), 85-95. doi:10.1007/s11745-008-3245-7

Baud, S., & Lepiniec, L. (2009). Regulation of de novo fatty acid synthesis in maturing oilseeds of Arabidopsis. *Plant Physiol Biochem, 47* (6), 448-455. doi:10.1016/j.plaphy.2008.12.006

Baudry, A., Heim, M. A., Dubreucq, B., Caboche, M., Weisshaar, B., & Lepiniec, L. (2004). TT2, TT8, and TTG1 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in Arabidopsis thaliana. *Plant J, 39* (3), 366-380. doi:10.1111/j.1365-313X.2004.02138.x

Bolger, A. M., Lohse, M., & Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data.*Bioinformatics, 30* (15), 2114-2120. doi:10.1093/bioinformatics/btu170

Buer, C. S., Imin, N., & Djordjevic, M. A. (2010). Flavonoids: new roles for old molecules. *J Integr Plant Biol, 52* (1), 98-111. doi:10.1111/j.1744-7909.2010.00905.x

Chauvin, A., Caldelari, D., Wolfender, J. L., & Farmer, E. E. (2013). Four 13-lipoxygenases contribute to rapid jasmonate synthesis in wounded Arabidopsis thaliana leaves: a role for lipoxygenase 6 in responses to long-distance wound signals.*New Phytol, 197* (2), 566-575. doi:10.1111/nph.12029

Chen, M., Du, X., Zhu, Y., Wang, Z., Hua, S., Li, Z., . . . Jiang, L. (2012). Seed Fatty Acid Reducer acts downstream of gibberellin signalling pathway to lower seed fatty acid storage in Arabidopsis. *Plant Cell Environ, 35* (12), 2155-2169. doi:10.1111/j.1365-3040.2012.02546.x

Chen, M., Wang, Z., Zhu, Y., Li, Z., Hussain, N., Xuan, L., . . . Jiang, L. (2012). The effect of transparent TESTA2 on seed fatty acid biosynthesis and tolerance to environmental stresses during young seedling establishment in Arabidopsis. *Plant Physiol, 160* (2), 1023-1036. doi:10.1104/pp.112.202945

Chen, M., Xuan, L., Wang, Z., Zhou, L., Li, Z., Du, X., . . . Jiang, L. (2014). TRANSPARENT TESTA8 Inhibits Seed Fatty Acid Accumulation by Targeting Several Seed Development Regulators in Arabidopsis. *Plant Physiol, 165* (2), 905-916. doi:10.1104/pp.114.235507

Chu, H., & Tso, T. C. (1968). Fatty Acid composition in tobacco I. Green tobacco plants. *Plant Physiol, 43* (3), 428-433. doi:10.1104/pp.43.3.428

Czemmel, S., Stracke, R., Weisshaar, B., Cordon, N., Harris, N. N., Walker, A. R., . . . Bogs, J. (2009). The grapevine R2R3-MYB transcription factor VvMYBF1 regulates flavonol synthesis in developing grape berries. *Plant Physiol, 151* (3), 1513-1530. doi:10.1104/pp.109.142059

Dalman, K., Wind, J. J., Nemesio-Gorriz, M., Hammerbacher, A., Lunden, K., Ezcurra, I., & Elfstrand, M. (2017). Overexpression of PaNAC03, a stress induced NAC gene family transcription factor in Norway spruce leads to reduced flavonol biosynthesis and aberrant embryo development. *BMC Plant Biol, 17* (1), 6. doi:10.1186/s12870-016-0952-8

Dao, G. H., Wu, G. X., Wang, X. X., Zhuang, L. L., Zhang, T. Y., & Hu, H. Y. (2018). Enhanced growth and fatty acid accumulation of microalgae Scenedesmus sp. LX1 by two types of auxin. *Bioresour Technol, 247* , 561-567. doi:10.1016/j.biortech.2017.09.079

Edwards, K. D., Fernandez-Pozo, N., Drake-Stowe, K., Humphry, M., Evans, A. D., Bombarely, A., . . . Mueller, L. A. (2017). A reference genome for Nicotiana tabacum enables map-based cloning of homeologous

loci implicated in nitrogen utilization efficiency. *BMC Genomics, 18* (1), 448. doi:10.1186/s12864-017-3791-6

Gonzalez, A., Zhao, M., Leavitt, J. M., & Lloyd, A. M. (2008). Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings. *Plant J, 53* (5), 814-827. doi:10.1111/j.1365-313X.2007.03373.x

Huang, L. M., Lai, C. P., Chen, L. O., Chan, M. T., & Shaw, J. F. (2015). Arabidopsis SFAR4 is a novel GDSL-type esterase involved in fatty acid degradation and glucose tolerance. *Bot Stud, 56* (1), 33. doi:10.1186/s40529-015-0114-6

Jeanmougin, F., Thompson, J. D., Gouy, M., Higgins, D. G., & Gibson, T. J. (1998). Multiple sequence alignment with Clustal X. *Trends Biochem Sci, 23* (10), 403-405.

Kim, D., Langmead, B., & Salzberg, S. L. (2015). HISAT: a fast spliced aligner with low memory requirements. *Nat Methods, 12* (4), 357-360. doi:10.1038/nmeth.3317

Kumar, G. S., & Krishna, A. G. (2015). Studies on the nutraceuticals composition of wheat derived oils wheat bran oil and wheat germ oil. *J Food Sci Technol, 52* (2), 1145-1151. doi:10.1007/s13197-013-1119-3

Le, C. S., Schmelz, E. A., & Chourey, P. S. (2010). Sugar levels regulate tryptophan-dependent auxin biosynthesis in developing maize kernels. *Plant Physiol, 153* (1), 306-318. doi:10.1104/pp.110.155226

Li, B., Zhao, L., Chen, H., Sun, D., Deng, B., Li, J., . . . Wang, F. (2016). Inactivation of Lipase and Lipoxygenase of Wheat Germ with Temperature-Controlled Short Wave Infrared Radiation and Its Effect on Storage Stability and Quality of Wheat Germ Oil. *PLoS One, 11* (12), e0167330. doi:10.1371/journal.pone.0167330

Li, Y., Van den Ende, W., & Rolland, F. (2014). Sucrose induction of anthocyanin biosynthesis is mediated by DELLA. *Mol Plant, 7* (3), 570-572. doi:10.1093/mp/sst161

Lilley, J. L., Gee, C. W., Sairanen, I., Ljung, K., & Nemhauser, J. L. (2012). An endogenous carbon-sensing pathway triggers increased auxin flux and hypocotyl elongation.*Plant Physiol, 160* (4), 2261-2270. doi:10.1104/pp.112.205575

Liu, C., Long, J., Zhu, K., Liu, L., Yang, W., Zhang, H., . . . Deng, X. (2016). Characterization of a Citrus R2R3-MYB Transcription Factor that Regulates the Flavonol and Hydroxycinnamic Acid Biosynthesis. *Sci Rep, 6* , 25352. doi:10.1038/srep25352

Liu, K., Qi, S., Li, D., Jin, C., Gao, C., Duan, S., . . . Chen, M. (2017). TRANSPARENT TESTA GLABRA 1 ubiquitously regulates plant growth and development from Arabidopsis to foxtail millet (Setaria italica). *Plant Sci, 254* , 60-69. doi:10.1016/j.plantsci.2016.10.010

Liu, Q., Wang, C., Jiao, X., Zhang, H., Song, L., Li, Y., . . . Wang, K. (2019). Hi-TOM: a platform for high-throughput tracking of mutations induced by CRISPR/Cas systems.*Sci China Life Sci, 62* (1), 1-7. doi:10.1007/s11427-018-9402-9

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods, 25* (4), 402-408. doi:10.1006/meth.2001.1262

Luo, J., Butelli, E., Hill, L., Parr, A., Niggeweg, R., Bailey, P., . . . Martin, C. (2008). AtMYB12 regulates caffeoyl quinic acid and flavonol synthesis in tomato: expression in fruit results in very high levels of both types of polyphenol.*Plant J, 56* (2), 316-326. doi:10.1111/j.1365-313X.2008.03597.x

Matsui, K., Oshima, Y., Mitsuda, N., Sakamoto, S., Nishiba, Y., Walker, A. R., . . . Takami, H. (2018). Buckwheat R2R3 MYB transcription factor FeMYBF1 regulates flavonol biosynthesis. *Plant Sci, 274* , 466-475. doi:10.1016/j.plantsci.2018.06.025

Mehrtens, F., Kranz, H., Bednarek, P., & Weisshaar, B. (2005). The Arabidopsis transcription factor MYB12 is a flavonol-specific regulator of phenylpropanoid biosynthesis.*Plant Physiol, 138* (2), 1083-1096. doi:10.1104/pp.104.058032

Meir, S., Philosoph-Hadas, S., Epstein, E., & Aharoni, N. (1985). Carbohydrates stimulate ethylene production in tobacco leaf discs : I. Interaction with auxin and the relation to auxin metabolism. *Plant Physiol, 78* (1), 131-138. doi:10.1104/pp.78.1.131

Meir, S., Riov, J., Philosoph-Hadas, S., & Aharoni, N. (1989). Carbohydrates Stimulate Ethylene Production in Tobacco Leaf Discs : III. Stimulation of Enzymic Hydrolysis of Indole-3-Acetyl-l-Alanine. *Plant Physiol, 90* (4), 1246-1248. doi:10.1104/pp.90.4.1246

Misra, P., Pandey, A., Tiwari, M., Chandrashekar, K., Sidhu, O. P., Asif, M. H., . . . Tuli, R. (2010). Modulation of transcriptome and metabolome of tobacco by Arabidopsis transcription factor, AtMYB12, leads to insect resistance. *Plant Physiol, 152* (4), 2258-2268. doi:10.1104/pp.109.150979

Nagano, M., Takahara, K., Fujimoto, M., Tsutsumi, N., Uchimiya, H., & Kawai-Yamada, M. (2012). Arabidopsis sphingolipid fatty acid 2-hydroxylases (AtFAH1 and AtFAH2) are functionally differentiated in fatty acid 2-hydroxylation and stress responses. *Plant Physiol, 159* (3), 1138-1148. doi:10.1104/pp.112.199547

Nalam, V. J., Keereetaweep, J., & Shah, J. (2013). The green peach aphid, Myzus persicae, acquires a LIPOXYGENASE5-derived oxylipin from Arabidopsis thaliana, which promotes colonization of the host plant. *Plant Signal Behav, 8* (1), e22735. doi:10.4161/psb.22735

Palmgren, G., Mattson, O., & Okkels, F. T. (1993). Treatment of Agrobacterium or leaf disks with 5-azacytidine increases transgene expression in tobacco. *Plant Mol Biol, 21* (3), 429-435.

Schmidt, G. W., & Delaney, S. K. (2010). Stable internal reference genes for normalization of real-time RT-PCR in tobacco (Nicotiana tabacum) during development and abiotic stress. *Mol Genet Genomics, 283* (3), 233-241. doi:10.1007/s00438-010-0511-1

Shcherbo, D., Merzlyak, E. M., Chepurnykh, T. V., Fradkov, A. F., Ermakova, G. V., Solovieva, E. A., . . . Chudakov, D. M. (2007). Bright far-red fluorescent protein for whole-body imaging. *Nat Methods, 4* (9), 741-746. doi:10.1038/nmeth1083

Siloto, R. M., Findlay, K., Lopez-Villalobos, A., Yeung, E. C., Nykiforuk, C. L., & Moloney, M. M. (2006). The accumulation of oleosins determines the size of seed oilbodies in Arabidopsis. *Plant Cell, 18* (8), 1961-1974. doi:10.1105/tpc.106.041269

Sjovall, O., Virtalaine, T., Lapvetelainen, A., & Kallio, H. (2000). Development of rancidity in wheat germ analyzed by headspace gas chromatography and sensory analysis. *J Agric Food Chem, 48* (8), 3522-3527. doi:10.1021/jf981309t

Solfanelli, C., Poggi, A., Loreti, E., Alpi, A., & Perata, P. (2006). Sucrose-specific induction of the anthocyanin biosynthetic pathway in Arabidopsis. *Plant Physiol, 140* (2), 637-646. doi:10.1104/pp.105.072579

Song, Z., Luo, Y., Wang, W., Fan, N., Wang, D., Yang, C., & Jia, H. (2019). NtMYB12 Positively Regulates Flavonol Biosynthesis and Enhances Tolerance to Low Pi Stress in Nicotiana tabacum. *Front Plant Sci, 10* , 1683. doi:10.3389/fpls.2019.01683

Stracke, R., Ishihara, H., Huep, G., Barsch, A., Mehrtens, F., Niehaus, K., & Weisshaar, B. (2007). Differential regulation of closely related R2R3-MYB transcription factors controls flavonol accumulation in different parts of the Arabidopsis thaliana seedling. *Plant J, 50* (4), 660-677. doi:10.1111/j.1365-313X.2007.03078.x

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., & Kumar, S. (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol, 28* (10), 2731-2739. doi:10.1093/molbev/msr121

Tan, X., Yan, S., Tan, R., Zhang, Z., Wang, Z., & Chen, J. (2014). Characterization and expression of a GDSL-like lipase gene from Brassica napus in Nicotiana benthamiana.*Protein J, 33* (1), 18-23. doi:10.1007/s10930-013-9532-z

Trapnell, C., Hendrickson, D. G., Sauvageau, M., Goff, L., Rinn, J. L., & Pachter, L. (2013). Differential analysis of gene regulation at transcript resolution with RNA-seq. *Nat Biotechnol, 31* (1), 46-53. doi:10.1038/nbt.2450

Trapnell, C., Williams, B. A., Pertea, G., Mortazavi, A., Kwan, G., van Baren, M. J., . . . Pachter, L. (2010). Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. *Nat Biotechnol, 28* (5), 511-515. doi:10.1038/nbt.1621

ul Hassan, M. N., Zainal, Z., & Ismail, I. (2015). Green leaf volatiles: biosynthesis, biological functions and their applications in biotechnology. *Plant Biotechnol J, 13* (6), 727-739. doi:10.1111/pbi.12368

Wang, F., Kong, W., Wong, G., Fu, L., Peng, R., Li, Z., & Yao, Q. (2016). AtMYB12 regulates flavonoids accumulation and abiotic stress tolerance in transgenic Arabidopsis thaliana. *Mol Genet Genomics, 291* (4), 1545-1559. doi:10.1007/s00438-016-1203-2

Wang, N., Xu, H., Jiang, S., Zhang, Z., Lu, N., Qiu, H., . . . Chen, X. (2017). MYB12 and MYB22 play essential roles in proanthocyanidin and flavonol synthesis in red-fleshed apple (Malus sieversii f. niedzwetzkyana). *Plant J, 90* (2), 276-292. doi:10.1111/tpj.13487

Wang, Z., Chen, M., Chen, T., Xuan, L., Li, Z., Du, X., . . . Jiang, L. (2014). TRANSPARENT TESTA2 regulates embryonic fatty acid biosynthesis by targeting FUSCA3 during the early developmental stage of Arabidopsis seeds. *Plant J, 77* (5), 757-769. doi:10.1111/tpj.12426

Wang, Z., Wang, S., Wu, M., Li, Z., Liu, P., Li, F., . . . Yang, J. (2018). Evolutionary and functional analyses of the 2-oxoglutarate-dependent dioxygenase genes involved in the flavonoid biosynthesis pathway in tobacco. *Planta* . doi:10.1007/s00425-018-3019-2

Wang, Z., Wang, S., Xiao, Y., Li, Z., Wu, M., Xie, X., . . . Yang, J. (2020). Functional characterization of a HD-ZIP IV transcription factor NtHDG2 in regulating flavonols biosynthesis in Nicotiana tabacum. *Plant Physiol Biochem, 146* , 259-268. doi:10.1016/j.plaphy.2019.11.033

Xie, X., Qin, G., Si, P., Luo, Z., Gao, J., Chen, X., . . . Yang, J. (2017). Analysis of Nicotiana tabacum PIN genes identifies NtPIN4 as a key regulator of axillary bud growth.*Physiol Plant, 160* (2), 222-239. doi:10.1111/ppl.12547

Xu, W., Dubos, C., & Lepiniec, L. (2015). Transcriptional control of flavonoid biosynthesis by MYB-bHLH-WDR complexes. *Trends Plant Sci, 20* (3), 176-185. doi:10.1016/j.tplants.2014.12.001

Xu, W., Grain, D., Bobet, S., Le Gourrierec, J., Thevenin, J., Kelemen, Z., . . . Dubos, C. (2014). Complexity and robustness of the flavonoid transcriptional regulatory network revealed by comprehensive analyses of MYB-bHLH-WDR complexes and their targets in Arabidopsis seed. *New Phytol, 202* (1), 132-144. doi:10.1111/nph.12620

Yoo, S. D., Cho, Y. H., & Sheen, J. (2007). Arabidopsis mesophyll protoplasts: a versatile cell system for transient gene expression analysis. *Nat Protoc, 2* (7), 1565-1572. doi:10.1038/nprot.2007.199

Zhai, R., Zhao, Y., Wu, M., Yang, J., Li, X., Liu, H., . . . Xu, L. (2019). The MYB transcription factor PbMYB12b positively regulates flavonol biosynthesis in pear fruit.*BMC Plant Biol, 19* (1), 85. doi:10.1186/s12870-019-1687-0

Zou, J., Wei, Y., Jako, C., Kumar, A., Selvaraj, G., & Taylor, D. C. (1999). The Arabidopsis thaliana TAG1 mutant has a mutation in a diacylglycerol acyltransferase gene.*Plant J, 19* (6), 645-653. doi:10.1046/j.1365-313x.1999.00555.x

TABLE CAPTIONS and FIGURE LEGENDS

Table 1 Differentially expressed genes (DEGs) related to FA motabolism in the WT and *NtMYB12a-OE* tobacco leaves. DEGs with FDR < 0.05 and |log2 ratios|[?]1.00 are listed here.

Table 2 The DEGs induced by sucrose treatment and*NtMYB12a* over expression.

FIGURE 1 The phylogenetic tree and sequence analysis of six NtMYBs. (a) The selected R2R3-MYB transcription factors was classified into SG5, SG6, and SG7 subgroups, which was associated with the biosynthesis of PA, anthocyanin, and flavonol, respectively. (b) Alignment of the six NtMYB protein sequences. The amino acids identical in six sequences, five sequences, and four sequences are highlighted in black, dark gray, and light gray, respectively. The R2R3-type MYB domains are indicated above the alignment, while the SG7 and SG7-2 motifs are highlighted by red boxes. (c) Exon/intron structure of the six *NtMYB* genes. Black boxes represent exons, and lines represent introns. Numbers indicate length of the exons and introns. The triangle indicates the PAM positon for Crispr/cas9.

FIGURE 2 Expression pattern of the tobacco *NtMYB* genes. (a) Relative expression levels of six *NtMYB* genes in various tobacco tissues. (b) Relative expression levels of *NtMYB12a* and*NtMYB12b* genes in tobacco leaves from different development stages. Values are means of three independent replicates. Error bars denote standard deviations. (c) Subcellular localization of NtMYB12a-GFP and NtMYB12b-GFP fusion proteins. NLS is a marker protein for nuclear localization; Merge, merging of NLS, GFP, Chloroplast autofluorescence and Brightfield images.

FIGURE 3 Functional differentiation of NtMYB12a and NtMYB12b in regulating the biosynthesis of flavonoids in tobacco. (a) Gene-specific fragment used for the construction of RNAi vectors. (b) Relative expression levels of the *NtMYB12a* and *NtMYB12b* genes in different positive RNAi lines. Asterisks represent statistically significant differences between WT and RNAi plants determined by two-tailed paired Student's t-test (**, $P < 0.01$; *, $P < 0.05$). (c) Flavonol content in the tobacco leaves of different RNAi lines. (d) Anthocyanin content in the tobacco leaves of different RNAi lines. Values are means of three independent replicates. Error bars denote standard deviations. The significant differences among different lines are detected by Tucky's test.

FIGURE 4 Quantitative assay of the flavonoids content in the tobacco leaves and flowers. (a) Inflorescence phenotypes of the WT,*NtMYB12a-OE* and *ntmyb12a* mutant plants. Bar=1cm. Anthocyanin content in the flowers (b) and leaves (c) of WT,*NtMYB12a-OE* and *ntmyb12a* mutant plants. (d) Flavonol content in the flowers and leaves of WT, *NtMYB12a-OE* and*ntmyb12a* mutant plants. Asterisks represent statistically significant differences between WT and transgenic plants determined by two-tailed paired Student's t-test (**, $P < 0.01$; *, $P < 0.05$).

FIGURE 5 NtMYB12a repressed the accumulation of FA in tobacco leaves. (a &b) Relative expression levels of the FA-related DEGs in the leaves of WT, *NtMYB12a-OE* , *NtMYB12a-RNAi* , and*ntmyb12a* mutant plants. Total FA content (c) and percentage of PUFAs (d) in the leaves of WT, *NtMYB12a-OE* , *NtMYB12a-RNAi* , and *ntmyb12a* mutant plants. Asterisks represent statistically significant differences between WT and transgenic plants determined by two-tailed paired Student's t-test (**, $P < 0.01$; *, $P < 0.05$).

FIGURE 6 Comparison of the gene expression levels and FA content in tobacco seeds. (a) Relative expression levels of the eight FA-related DEGs in the developing tobacco seeds. DAF, days after flowering. Morphological appearance (b), dry weight (c), and FA content (d) of the mature seeds from the WT, *NtMYB12a-OE* ,*NtMYB12a-RNAi* , and *ntmyb12a* mutant plants. Asterisks represent statistically significant differences between WT and transgenic plants determined by two-tailed paired Student's t-test (**, $P < 0.01$; *, $P < 0.05$).

FIGURE 7 NtMYB12a activates the transcription of *NtLOX5* ,*NtLOX6* , *NtSFAR4* , and *NtGDSL2* genes by binding to their promoter regions. (a) Relative expression levels of *NtLOX5* ,*NtLOX6* , *NtSFAR4* , and *NtGDSL2* genes in the*35S:NtMYB12a-GR* seedlings under DEX, CYC, and DEX+CYC treatment. (b) Western blotting showing the NtMYB12a-GFP band. E, eluate; I, Input. (c) The promoter structures of *NtSFAR4* , *NtLOX5* ,*NtLOX6* , and *NtGDSL2* genes. The black line represents the 3 kb sequence upstream the ATG of each target gene. The black triangles indicate the position of putative MYB recognition element (MRE) required for activation by MYB12. The while boxes represent the DNA fragments amplified in

the ChIP assay. (d) ChIP enrichment test of the promoter regions bound by NtMYB12a-GFP. Asterisks represent statistically significant differences between WT and transgenic plants determined by two-tailed paired Student's t-test (**, $P < 0.01$; *, $P < 0.05$).

FIGURE 8 NtLOX6 and NtLOX5 prevent the accumulation of FA in tobacco seeds. (a) Phylogenetic analysis of the two NtLOXs with the 6 Arabidopsis AtLOX proteins. MEGA5.0 was used to generate the neighbor-joining tree (Jones-Taylor-Thornton model). The statistical reliability of the tree topology was assessed by performing a bootstrap analysis with 1000 replicates. (b) Relative expression levels of*NtLOX5* gene in the *NtLOX5-OE* and *NtLOX5-RNAi* lines. (c) Relative expression levels of *NtLOX6* gene in the*NtLOX6-OE* and *NtLOX6-RNAi* lines. (d) The FA content in the mature seeds of WT and *NtLOX5* transgenic plants. (e) Percentage of PUFAs in the leaves of WT and *NtLOX5* transgenic plants. (f) The FA content in the mature seeds of WT and *NtLOX6* transgenic plants. (g) Percentage of PUFAs in the leaves of WT and *NtLOX6*transgenic plants. Values are means of three independent replicates. Error bars denote standard deviations. Asterisks represent statistically significant differences between WT and transgenic plants determined by two-tailed paired Student's t-test (**, $P < 0.01$; *, $P < 0.05$).

FIGURE 9 Expression pattern of *NtMYB12a* under sucrose treatment. (a) Gus staining of the$Pro_{NtMYB12a}$*:GUS* seedlings treated with 0 mM, 50 mM, 88 mM (CK), 150 mM, and 200 mM sucrose. Bar=5mm. (b) Relative expression level of NtMYB12a gene under different concentration of sucrose. Values are means of three independent replicates. Error bars denote standard deviations. The significant differences among different lines are detected by Tucky's test. (c) Venn diagram of the DEGs from two RNA-seq anslyses. URG-OE, up-regulated genes in the*NtMYB12a-OE* plants; DRG-OE, down-regulated genes in the*NtMYB12a-OE* plants; URG-Suc, up-regulated genes by 150 mM sucrose; DRG-Suc, down-regulated genes by 150 mM sucrose.

FIGURE 10 Effect of sucrose on FA metabolism in tobacco seedlings. (a) Phenotype of WT, *NtMYB12a-OE* ,*NtMYB12a-RNAi* , and *ntmyb12a* mutant seedlings under sucrose treatment. Bar=1cm. (b) Relatives expression levels of *NtLOX5* ,*NtLOX6* , *NtSFAR4* , and *NtGDSL2* genes. Asterisks represent statistically significant differences between WT and transgenic plants determined by two-tailed paired Student's t-test (**, $P < 0.01$; *, $P < 0.05$). (c) FA content of the WT,*NtMYB12a-OE* , *NtMYB12a-RNAi* , and *ntmyb12a* mutant seedlings. Values are means of three independent replicates. Error bars denote standard deviations. The significant differences among different lines are detected by Tucky's test.

FIGURE 11 Schematic illustrating the regulatory network for NtMYB12a regarding promotion of flavonoids synthesis and FA degradation in tobacco.

Table 1

Gene ID	OE RPKM	WT RPKM	Log2 (OE/WT)	p_value	Regulation	Annotation	Function & Reference
Nitab4.5_0002531g0050	2.146	0.17	3.62	0.0123	Up	GDSL esterase/lipase/SFAR4	fatty acid degradation (Huang *et al*., 2015)
Nitab4.5_0003282g0010	2.63	0.26	3.36	0.0098	Up	GDSL esterase/lipase/SFAR4	
Nitab4.5_0005085g0010	17.56	2.01	3.12	5.00E-05	Up	GDSL esterase/lipase/NtGDSL2	lipid degradation (Chen et al., 2012)
Nitab4.5_0003489g0010	5.60	0.71	2.98	0.0001	Up	GDSL esterase/lipase/NtGDSL3	
Nitab4.5_0000318g0060	5.30	0.97	2.46	0.00145	Up	Oleosin	lipid accumulation (Siloto et al., 2006)
Nitab4.5_0002518g0010	1.03	0.33	1.65	0.01475	Up	Lipase/lipooxygenase/LOX5	lipid oxidation and oxylipin biosynthesis (Nalam et al., 2012)
Nitab4.5_0001375g0040	13.54	4.97	1.45	0.0001	Up	GDSL esterase/lipase/*NtGDSL1*	lipid degradation (Tan et al., 2014)
Nitab4.5_0000874g0030	13.24	5.02	1.40	5.00E-05	Up	3-ketoacyl-CoA synthase 6/CUT1	cuticular wax biosynthesis (Lee et al., 2013)
Nitab4.5_0000084g0490	7.63	3.08	1.31	0.0062	Up	Fatty acid hydroxylase	Fatty acid hydroxylation (Nagano et al., 2012)
Nitab4.5_0001473g0020	6.68	2.88	1.21	0.00125	Up	3-ketoacyl-CoA synthase 11/KCS11	cuticular wax biosynthesis (Lee et al., 2013)
Nitab4.5_0000240g0150	2.09	0.97	1.11	0.0058	Up	Lipase/lipooxygenase/LOX6	lipid oxidation and oxylipin biosynthesis (Chauvin et al., 2012)
Nitab4.5_0003094g0030	6.15	2.90	1.08	0.001	Up	3-ketoacyl-CoA synthase 1/KCS1	cuticular wax biosynthesis (Lee et al., 2013)
Nitab4.5_0000376g0070	4.32	2.11	1.03	0.0317	Up	GDSL esterase/lipase/NtGDSL4	lipid degradation (Tan et al., 2017)

Table 2

Gene ID	Suc RPKM	CK RPKM	Log2 (Suc/CK)	p_value	Regulation	Annotation
Nitab4.5_0000697g0070	145	4.07	5.15515	5.00E-05	Up	DnaJ domain, DnaJ domain, conserved site
Nitab4.5_0000246g0260	196	7.67	4.6756	5.00E-05	Up	Bulb-type lectin domain
Nitab4.5_0006365g0040	2.13	0.14	3.95556	0.001	Up	FAD/NAD(P)-binding domain
Nitab4.5_0008969g0020	0.8	0.06	3.65005	0.02375	Up	Pyridoxal phosphate-dependent transferase
Nitab4.5_0004048g0010	3.92	0.36	3.46163	5.00E-05	Up	Proton-dependent oligopeptide transporter family
Nitab4.5_0007160g0050	1.96	0.18	3.45835	0.0059	Up	Transcription factor, TCP, Transcription factor TCP subgroup
Nitab4.5_0000227g0020	61.8	7.36	3.07057	5.00E-05	Up	Protein kinase domain
Nitab4.5_0005720g0020	26.9	3.56	2.91709	5.00E-05	Up	Glycoside hydrolase family 3
Nitab4.5_0007554g0010	2.71	0.36	2.90891	0.00015	Up	LPS-induced tumor necrosis factor alpha factor
Nitab4.5_0003639g0010	66.8	9.23	2.85618	5.00E-05	Up	Pectinesterase inhibitor domain
Nitab4.5_0005393g0020	44.1	6.35	2.79603	5.00E-05	Up	Domain of unknown function DUF4005
Nitab4.5_0001301g0090	39.2	5.83	2.74884	5.00E-05	Up	Glycoside hydrolase family 3
Nitab4.5_0000839g0060	11.9	1.81	2.70898	5.00E-05	Up	Rossmann-like alpha/beta/alpha sandwich fold
Nitab4.5_0000677g0100	1.59	0.25	2.66564	0.0028	Up	Glycoside hydrolase
Nitab4.5_0002485g0020	0.69	0.11	2.59953	0.0066	Up	Terpene synthase
Nitab4.5_0000008g0110	4.1	0.73	2.49815	0.0001	Up	Unknown
Nitab4.5_0000015g0380	32.5	6.09	2.41724	5.00E-05	Up	Pectinesterase inhibitor domain
Nitab4.5_0003309g0010	23.8	4.47	2.41446	5.00E-05	Up	ZF-HD homeobox protein
Nitab4.5_0000163g0340	6.88	1.33	2.37287	5.00E-05	Up	PTR2 family proton/oligopeptide symporter
Nitab4.5_0003489g0010	**164**	**33.7**	**2.28048**	**5.00E-05**	**Up**	**GDSL esterase/lipase/NtGDSL3**
Nitab4.5_0005085g0010	**368**	**77.5**	**2.24909**	**5.00E-05**	**Up**	**GDSL esterase/lipase/NtGDSL2**
Nitab4.5_0000240g0150	**18.9**	**4.04**	**2.22694**	**5.00E-05**	**Up**	**Lipase/Lipoxygenase 6/LOX6**
Nitab4.5_0006246g0010	10.7	2.34	2.19729	5.00E-05	Up	Leucine-rich repeat
Nitab4.5_0002236g0010	279	62.7	2.15469	5.00E-05	Up	DNA-binding domain, AP2/ERF domain
Nitab4.5_0000956g0150	29.7	7.02	2.08028	5.00E-05	Up	Major intrinsic protein, Aquaporin-like
Nitab4.5_0006696g0030	1.37	0.33	2.04248	5.00E-05	Up	Leucine-rich repeat4
Nitab4.5_0011489g0010	7.17	1.75	2.03826	5.00E-05	Up	Protein of unknown function DUF620
Nitab4.5_0001759g0020	22.4	5.5	2.0277	5.00E-05	Up	ZF-HD homeobox protein
Nitab4.5_0000874g0030	6.45	1.59	2.02034	5.00E-05	Up	FAE1/Type III polyketide synthase-like protein

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