ABA homeostasis is mediated by a feedback regulation of MdMYB88 and MdMYB124

Yinpeng Xie[1,2,4], Chana Bao[1,4], Pengxiang Chen[1,4], Fuguo Cao[1], Xiaofang Liu[1], Dali Geng[1], Zhongxing Li[1], Xuewei Li[1], Nan Hou[1], Fang Zhi[1], Chundong Niu[1], Shuangxi Zhou[3], Xiangqiang Zhan[1], Fengwang Ma[1], Qingmei Guan[1*]

[1]State Key Laboratory of Crop Stress Biology for Arid Areas/Shaanxi Key Laboratory of Apple, College of Horticulture, Northwest A&F University, Yangling, Shaanxi 71200, P.R. China.

[2] Institute for Advanced Studies, Wuhan University, Wuhan, 430072, P.R. China.

[3] The New Zealand Institute for Plant and Food Research Ltd., Hawke's Bay 4130, New Zealand.

[4] These authors contribute equally.

[*]To whom correspondence should be addressed:

Qingmei Guan（email: qguan@nwafu.edu.cn; Phone: +86-29-87082864）

Highlight

We demonstrate how MdMYB88 and MdMYB124 mediate ABA homeostasis by regulating expression of ABA metabolism-related genes in a negative feedback response.

Abstract

Plant phytohormone abscisic acid (ABA) is involved in various plant biological processes. In response to drought stress, plants quickly accumulate ABA to cope with environmental changes, but the regulatory mechanism of ABA accumulation is largely unknown, especially in woody plants. In this study, we report that MdMYB88 and MdMYB124 are critical transcription factors for ABA accumulation under control and drought conditions in apple trees (*Malus* x *domestica*), and this regulation is negatively controlled by ABA. We first show that MdMYB88 and MdMYB124 positively regulate leaf water transpiration, photosynthetic capacity, and stress endurance in apple trees under drought conditions. We further show that they regulate the expression of biosynthetic and catabolic genes of ABA, as well as drought- and ABA- responsive genes. In addition, we illustrate that they associate with promoter regions of the ABA biosynthetic gene *MdNCED3*. We finally display that MdMYB88 and MdMYB124 are repressed by ABA. Our results identified a feedback regulation of MdMYB88 and MdMYB124 in modulating ABA homeostasis in apple trees.

Key words: MdMYB88/MdMYB124, drought resistance, ABA homeostasis, apple

Introduction

Abscisic acid (ABA) is a phytohormone that regulates various plant growth and development processes, including seed germination, seed maturation, dormancy, stomatal movement, senescence, and root growth. ABA also regulates a plant's response to both biotic and abiotic stimuli. Under drought stress, the concentration of ABA in leaves increases rapidly to induce stomatal closure to avoid water loss (Shinozaki and Yamaguchi-Shinozaki, 2000; Wilkinson and Davies, 2002; Shinozaki et al., 2003; Chinnusamy et al., 2004; Zhu, 2016). Besides ABA, plant hydraulic function also regulates stomatal control (Hernandez-Santana et al., 2016). It is reported that the increase of root ABA concentration under drought is correlated with increased root hydraulic conductivity (Thompson et al. 2007). Exogenous ABA application to roots or ABA-overproducing transgenic plants increases root hydraulic conductance (Hose et al. 2000; Thompson et al. 2007). In barley (*Hordeum vulgare*), the roots of wild type plants accumulate more ABA than ABA deficient barley mutant *Az34*, resulting in the increased root hydraulic conductivity of wild type plants and higher water flow from the roots. Therefore, wild type plants are capable of maintaining higher leaf water potential and higher transpiration rates to respond to air temperature increasing (Veselov et al., 2018).

The regulation of ABA levels is mediated by a balance of ABA biosynthesis and catabolism. ABA is primarily produced by the *de novo* biosynthetic pathway from carotenoids, in which zeaxanthin is converted to all-trans-violaxanthin catalyzed by zeaxanthin epoxidase (ZEP/ABA1) (Marin et al., 1996). Then subsequently catalyzed by an unknown enzyme, 9-cis-epoxycarotenoid dioxygenase (NCED), dehydrogenase/reductase (SDR/ABA2) and aldehyde oxidase (AAO/AO) step by step to ABA (Tan et al., 1997; Burbidge et al., 1999; Chernys and Zeevaart, 2000; Iuchi et al., 2001; Qin and Zeevaart, 2002; Cheng et al., 2002; Gonzalez-Guzman et al., 2002; Seo et al., 2004). A second biosynthetic pathway occurs *via* two glucosidases in Arabidiopsis, BG1 and BG2, which catalyze the hydrolysis of Glc-conjugated ABA (abscisic acid-glucose ester [ABA-GE]) to ABA (Lee et al., 2006; Xu et al., 2012). In Arabidopsis, ABA catabolism is regulated by hydroxylation and conjugation. Hydroxylation is mediated by CYP707A1 to CYP707A4. Conjugation is regulated by uridinediphosphate glucosyltransferases (UGT71B6, UGT71C5, UGT71B7, and UGT71B8) (Xu et al., 2002; Kushiro et al., 2004; Saito et al., 2004; Okamoto et al., 2006; Priest et al., 2006; Dong et al., 2014; Liu et al., 2015).

A few transcription factors have been identified to transcriptionally regulate genes involved in ABA metabolism. The bZIP transcription factor VIP1 directly binds to DNA fragments of the *CYP707A1* and *CYP707A3* promoters and enhances their expression (Tsugama et al., 2012). Another transcription factor, bHLH122, directly represses *CYP707A3* (Liu et al., 2014). SVP, a MADS-box transcription factor, negatively regulates the expression of *CYP707A1* and *CYP707A3*, but positively controls the *AtBG1* gene by associating with their promoter regions (Wang et al., 2018).

Drought stress is one of the adverse environmental conditions restricting fruit crop production and quality. To breed drought-tolerant fruit crops, traditional and biotechnological approaches (e.g., marker-assisted selection and genetic transformation) have been applied (Marguerit et al., 2012; Wang et al., 2012; Cao et al., 2013; Wang et al., 2014; Li et al., 2015; Virlet et al., 2015; Liu et al., 2018; Sun et al., 2018). However, traditional breeding is time- and labor-consuming due to the long juvenile period of fruit trees. Biotechnology has been proven feasible for improving drought resistance in woody perennial plants (Cao et al., 2013; Wu et al., 2016; Liao et al., 2017; Sun et al., 2018; Ma et al., 2019), which needs a thorough understanding of drought response at the molecular level.

MYB transcription factors are reportedly involved in various plant processes, including primary and secondary metabolism, cell fate and identity, developmental processes, and responses to biotic and abiotic stresses (Dubos et al., 2010). Numerous MYB genes have been characterized for their roles in response to drought stress (Baldoni et al., 2015). The apple tree (*Malus* x *domestica*) genome contains 229 MYB genes, many of which are responsive to various abiotic stresses, indicating the potential participation of these genes in apple stress resistance. Overexpression of one of these *MYB* genes, *MdoMYB121*, remarkably enhances apple tree resistance to high salinity, drought, and cold stress (Cao et al., 2013). In addition, overexpression of *MdSIMYB1* increases apple tree resistance to polyethylene glycol (PEG) (Wang et al., 2014). Previously, we characterized the positive roles of MdMYB88 and its paralogous MdMYB124 in improving freezing tolerance of apple trees (Xie et al., 2018). We further revealed that both MdMYB88 and MdMYB124 mediate the drought resistance of apple roots by regulating root xylem development and secondary cell wall formation (Geng et al., 2018). However, it is less certain whether both genes are involved in ABA homeostasis. Here, we provide evidence that MdMYB88 and MdMYB124 enhance ABA accumulation under control and drought conditions, and this accumulation is negatively mediated by ABA

level. Our results highlight the roles of MdMYB88 and MdMYB124 in ABA homeostasis in perennial apple trees, thereby providing genetic determinism for apple breeding in the future.

Materials and methods

Plant materials, growth conditions, and stress treatment

For gene cloning, 'Golden Delicious' (*M.* x *domestica*) apple trees grown in a greenhouse were used for RNA extraction. *MdMYB88/124* RNAi plants, *MdMYB88* or *MdMYB124* overexpression plants were previously prepared (Xie et al., 2018). GL-3, a seedling selected from Royal Gala (*M.* x *domestica*), was used as the genetic background to generate transgenic apple plants (Dai et al., 2013). Because of the sequence similarity of MdMYB88 and MdMYB124, transcripts of both *MdMYB88* and *MdMYB124* were reduced in *MdMYB88/124* RNAi plants (Xie et al., 2018).

Transgenic apple and GL-3 plants were rooted in 1/2 MS medium (2.215 g/L MS salts, 20 g/L sucrose, and 7 g/L agar, pH 5.8) supplemented with 0.5 mg/L indole-3-butytric acid (IBA) and 0.5 mg/L indoleacetic acid (IAA) under dark conditions for 3 d and then held under long-day conditions (14 h light/ 10 h dark) for an additional 45 d. Then they were transplanted into soil and grown in a light growth chamber with 60% humidity under long-day conditions. After 2 months, transgenic apple and GL-3 plants were transplanted to garden pots (43.5 cm x 20 cm x 11 cm, length × width × depth) with 4.5 kg soil (peat to vermiculite in a ratio of 3:1) for additional one month. Overexpression or RNAi plants were grown together with GL-3 in the same pots, and the position of each plant in each pot was random. Alternatively, GL-3, overexpression, and RNAi plants were grown individually in each pot. Drought treatment was carried out by withholding water for 30 d. The soil relative water content (SRWC) was measured every two d at 18:00 gravimetrically (Supplemental Figure 1).

SRWC (%) = 100 * [(Maximum water content -Pot of heavy) / (Maximum water content - Drying weight of soil)]

The photosynthetic parameters were measured with a LiCor-6400 portable photosynthesis system (LI-COR, Nebraska USA) when SRWC was 75-85% (day 0), 45-55% (day 12), and 25%-35% (day 18), respectively. The environmental condition: light intensity was 1000 μmol m^{-2} s^{-1}, CO_2 concentration was 450 ± 10 cm^3 m^{-3}, the leaf temperature was 25± 2 ℃, and the relative humidity of sample cell was 22±2 %. Fifteen plants (a pair of transgenic/non-

transgenic plants planted in the same pot) or twenty-one plants (plants of each treatment planted in single pots) of each genotype were collected. After treatment, plants were re-watered and recovered for 11 d to calculate survival rate. Thirty-six plants (a pair of transgenic/non-transgenic plants planted in the same pot) or twenty-one plants (plants of each treatment planted in single pots) of each genotype were collected and each 12 or 7 plants were used as a biological replication, respectively.

PEG treatment was carried out using hydroponically cultured plants (Geng et al., 2019). Briefly, rooted plants were transferred to soil for two months and then to 1/2 Hoagland solution. After one month, PEG6000 (Sigma) was added to the solution to a final concentration of 20% (w/v) for 6 h. Leaves and roots were collected for RNA (twenty-seven plants were collected and each 9 plants were used as a biological replication) or ABA (five plants of each genotype were collected) extraction.

To examine the mRNA level of *MdMYB88* and *MdMYB124* under ABA treatment, 'Golden Delicious' plants were sprayed with 100 μM ABA for 0, 1, or 3 h, and the leaves were collected for RNA extraction. Twenty-seven plants were collected and each 9 plants were used as a biological replication. For expression of *MdMYB88* and *MdMYB124* under drought treatment, 'Golden Delicious' plants were treated with drought for 6 d. The mature leaves (the 4^{th}, 5^{th} and 6^{th} leaves) were collected on the 0, 2^{th}, 4^{th}, and 6^{th} day after drought and used for RNA extraction. For water loss experiment, leaves of three-month-old plants were detached and air dried. Water loss was calculated based on the weight after dehydration for 30, 60, 120, 240, and 360 min. Fifteen plants of each genotype were collected.

Measurement of leaf relative water content

The leaf relative water content (LRWC) was determined by Li et al. (2019). Plant leaf water status was measured when SRWC was 75-85% (Control), 45-55% (Moderate), 25%-35% (Severe), or after dehydration for 0 h and 2 h. Nine leaves from three plants were collected randomly and weighed quickly and then transferred to deionized water overnight to measure turgid weight. Leaves were then dried and weighed to measure dry weight.

LRWC (%) =100*[(fresh weight−dry weight)/ (turgid weight−dry weight)]

Measurement of leaf water potential

Leaf water potential (Ψ_{leaf}) was measured with young leaves (one leaf per plant was collected, and nine plants of each genotype were used) using a Model 600 Pressure Chamber (PMS Instrument Company, USA) as described by the manufacturer's manual.

Measurement of leaf hydraulic conductivity

Leaf hydraulic conductivity (K_{leaf}) of both transgenic and non-transgenic plants was performed with an HPFM (Dynamax, Houston) as described by Geng et al. (2018). In brief, after drought treatment, leaves were soaked in de-gassed water and connected to HPFM. Leaf hydraulic conductivity was measured using a quasi steady-state method in accordance with the HPFM manual. Nine plants of each genotype were collected.

RNA extraction and qRT-PCR analysis

Total RNA of the apple leaves was extracted by CTAB methods (Chang et al., 1993) and then treated with RNase-free DNase I (Fermentas) at 37°C for 30 min to eliminate residual DNA. 2 ug RNA was then used for reverse transcription with a RevertAid First Strand cDNA Synthesis Kit (K1622; Thermo Fisher Scientific, Waltham, MA, USA). Quantitative reverse transcription PCR (qRT-PCR) was performed on an Applied Biosystem Step One Plus™ instrument (Life Science), using a ChamQ™ SYBR® qPCR Master Mix (Vazyme) according to the manual. The malate dehydrogenase (*MdMDH*) gene in apple trees was used as the reference gene. Primers used for qRT-PCR analysis are listed in Supplemental Table 1. Three replicates were used for each sample and relative quantitation was calculated by the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).

RNA-seq analysis

Plant leaves were collected from two-month-old GL-3 and transgenic apple plants and dehydrated for 0 and 2 h. Three biological replicates were applied for each of the control and the treatment and five leaves were used as a biological replication. Total RNAs were used for qRT-PCR and library construction according to manufacturer's instruction. Libraries were sequenced in an illumina NovaSeq 6000 platform and data of 44.5 million per sample were generated (Annoroad, Beijing, China). After removing the adaptors and low-quality reads, the remaining reads were mapped to the reference genome of *M.* × *domestica* 'Golden Delicious' (GDDH13 v1.1, https://iris.angers.inra.fr/gddh13/) using HISAT2 version 2.1.0 (Kim et al.,

2015) with default parameters. The HTSeq version 0.11.0 (Anders et al., 2014) was then used to quantify the read counts per gene. DEGs between the drought-treated and the control samples were identified using DEseq2 R packages (Love et al., 2014) based on the read counts with the adjusted Q value < 0.05 and 1.5-fold change in gene expression. Gene ontology (GO) annotation and enrichment analyses were conducted using the online tool agriGO (http://bioinfo.cau.edu.cn/agriGO/; Tian et al., 2017) and KOBAS (http://kobas.cbi.pku.edu.cn/index.php) (Ai and Kong, 2018).

Measurement of stomatal aperture

For stomatal aperture measurements, we used leaves of transgenic apple and GL-3 plants grown in soil for 2 months. Leaves were cut off and plunged into stomatal opening solution (30 mM KCl, 0.1 mM $CaCl_2$, and 10 mM MES-KOH, pH 6.15) under light (120 μmol m^{-2} s^{-1}) for 2 h, as described to induce stomatal opening (Kwak et al., 2001). Then, ABA was added to the stomatal opening solution to a final concentration of 5 μM. Leaf strips were observed for stomata with an EX30 microscope (SDPTOP) after ABA treatment for 1 h. Stomatal aperture was measured by ImageJ software.

ChIP-qPCR

ChIP-qPCR assay was performed as previously described (Xie et al.,2018). Leaves from GL-3 plants with or without drought treatment for 0 and 6 d were used for cross link and the ChIP assay was performed with an anti-MdMYB88/MdMYB124 antibody (Genscript, USA). The antibody specificity was provided in Supplemental Figure 2 and a specific 53KD protein band was detected using anti-MdMYB88/MdMYB124 antibody in immunoblots of plant extracts (An et al., 2017). Primers used for ChIP-qPCR are listed in Supplemental Table 1.

Electrophoretic mobility shift assay (EMSA)

MdMYB88 protein was previously generated (Xie et al., 2018). An EMSA assay was performed according to the manual of LightShift Chemiluminescent EMSA Kit (#89880; Thermo Scientific). The oligonucleotide probes labeled with biotin are listed in Supplemental Table 1.

Measurement of ABA content

ABA was extracted as described (Müller and Munné-Bosch, 2011). About 100 mg of the fresh sample was ground into powder in liquid nitrogen and then extracted with 500 μL of cold extraction buffer (methanol: isopropanol: acetic acid =20: 79: 1, v: v: v), followed by vortex for 5 min. After centrifugation at 4°C and 12,000 rpm for 10 min, the supernatant was collected and the pellet was re-extracted with 500 μL of cold extraction buffer. The extraction process was repeated three times and a constant amount of internal standard was added. Finally, the combined supernatant was filtered through a 0.22 μm PTFE filter (Shimadzu, Kyoto, Japan). Eight standard ABA (Sigma-Aldrich, Steinheim, Germany) solutions were prepared ranging from 0.5 to 100 ng/ml. Samples were then analyzed by QTRAP® 5500 LC-MS/MS (AB SCIEX, Redwood City, USA). Gradient elution was performed with solvent A (water with 0.1% formic acid) and solvent B (methanol with 0.1% formic acid) at the following flow rate: 0-2 min, B=20%, 2-6 min, B increment to 90%, 6.1-11 min, B=90%, 11-12 min, B decrement to 20%, 12-15 min, B=20%. Experiments were conducted in negative ionization mode. The capillary voltage was -4.5 kV and temperature was 600°C. The parameter of de-clustering potential was -60V and collision energy was -14 V (153.3) and -27 V (204.2). Five independent replicates were used for each treatment. Data were analyzed and processed using Multiquant software.

Dual-luciferase assay

The assay was carried out as described previously (Xie et al., 2018). The CDS of *MdMYB88* was cloned into pGreen Ⅱ62-SK, then the vectors was transformed into *Agrobacterium tumefaciens* GV3101 as effectors. The promoter of *MdNCED3* was cloned into pGreen Ⅱ0800 vector, to drive the firefly luciferase reporter gene. Then the vectors were each co-transformed with the helper plasmid pSoup19 into GV3101 as reporters. The reporter and effector then were mixed together in a 2: 3 volume ratios to transform *Nicotiana benthamiana* leaves. The empty pGreen Ⅱ62-SK vector was used as a negative control. The constitutive 35S promoter-drive Renilla luciferase was used as an internal reference. Ten biological repeats were measured for each sample. A Dual-luciferase assay was performed according to the manual of Dual-Luciferase® Reporter（DLR™）Assay System Kit (Promega, USA). Primers used for Dual-luciferase assay are listed in Supplemental Table 1.

Quantification and statistical analysis

For all experiments, results are shown as means ± SD and statistical significance was determined by one-way ANOVA (Tukey's test) analysis using SPSS (version 21.0, USA). Variations were considered significant if P <0.05, 0.01, or 0.001.

Accession numbers

Sequence data can be found in NCBI under the following numbers: MdMYB88 (KY569647), MdMYB124 (KY569648), MdNCED3 (XM_008380174.2), MdCYP707A1 (XM_008383813.2), MdCYP707A2 (XM_008358695.2), MdCYP707A4 (XM_008374924.2). Sequence data can be found in GDR under the following numbers: UGT71B6 (MD09G1141100), MdSPL3 (MD10G1291800), MdNF-YA7 (MD07G1011300), MdHB6 (MD15G1319800), MdHB7 (MD01G1226600), MdPUB9 (MD10G1154600), MdAHK3 (MD16G1068400), MdOEP16 (MD11G1108300).

Results

MdMYB88 and MdMYB124 act as positive regulators for drought resistance of the aboveground of apple trees

Previously, we characterized the molecular function of MdMYB88 and MdMYB124 in response to drought stress in apple roots (Geng et al., 2018). We observed that *MdMYB88* and *MdMYB124* were also drought inducible in apple leaves (Figure 1A). This led us to examine the involvement of MdMYB88 and MdMYB124 in drought resistance by examining phenotypes of the aboveground portions of apple trees. We first measured the relative water content of GL-3 and *MdMYB88/124* RNAi plants that we generated previously (Xie et al., 2018). We found that *MdMYB88/124* RNAi plants evaporated more water under dehydration conditions, especially after 6 h of dehydration (Figure 1B). We then investigated their survival ability under drought stress. After 30 d of drought treatment followed by 11 d of recovery, only 6-8% of *MdMYB88/124* RNAi plants survived while 24% of non-transgenic GL-3 plants were still alive (Figure 1C and D). Additionally, compared to GL-3 plants, *MdMYB88/124* RNAi plants had lower photosynthetic rate (An), stomatal conductance (Gs), rate of transpiration (E) and instantaneous water-use efficiency (WUEi) when treated with drought for 12 and 18 d (Figure 1E-H). Furthermore, the ion leakage level of

MdMYB88/124 RNAi plants was significantly higher than that of GL-3 plants after 7 d of drought exposure (Supplemental Figure 3A).

We also tested the drought resistance of *MdMYB88* overexpression plants (OE) and *MdMYB124* OE plants. Compared to GL-3 plants, *MdMYB88* OE and *MdMYB124* OE plants were more tolerant to drought when withheld from irrigation for 30 d (Figure 2A). After an 11d re-watering period, 50-60% *MdMYB88* OE and *MdMYB124* OE plants recovered while only 28% GL-3 plants survived (Figure 2B). Dehydration experiments showed that detached leaves of *MdMYB88* OE and *MdMYB124* OE plants lost less water than GL-3 plants (Figure 2C; Supplemental Figure 3B). Moreover, *MdMYB88* OE and *MdMYB124* OE plants performed better than GL-3 in regards to An, Gs, E and WUEi after being deprived of water for 12 and 18 d (Figure 2D-G). In addition, ion leakage assays determined that the cell membranes of *MdMYB88* OE and *MdMYB124* OE plants were less damaged than those of GL-3 plants under drought stress (Supplemental Figure 3A). To further confirm our results, we planted the individual non-transgenic and transgenic plants in different pots and examined their drought responses under the same soil water content. Similar results were obtained (Supplemental Figures 4 and 5).

To understand the leaf water status of the transgenic and non-transgenic plants, we measured the leaf relative water content (LRWC) in GL-3 and transgenic plants when SRWC was 75-85% (Control), 45-55% (Moderate), and 25%-35% (Severe). Results showed that LRWC of the overexpression plants was higher than that of GL-3 while LRWC of RNAi plants was lower under drought (Supplemental Figure 6). We also measured the leaf water potential (Ψ_{leaf}) and leaf hydraulic conductance (K_{leaf}) of non-transgenic and transgenic plants under drought (SRWC was 45-55%) (Supplemental Figure 7). The K_{leaf} and Ψ_{leaf} of the *MdMYB88* OE lines were higher than those of GL-3 while *MdMYB88/124* RNAi lines had lower K_{leaf} and Ψ_{leaf} than GL-3 under control and drought conditions (Supplemental Figure 7).

Together, our results suggest that MdMYB88 and MdMYB124 play positive roles in apple trees under drought stress.

MdMYB88 and MdMYB124 regulate the expression of drought-responsive genes

To further understand the molecular function of MdMYB88 and MdMYB124 in the drought resistance of the aboveground portion of apple trees, we performed RNA-seq analysis using dehydrated GL-3 and *MdMYB88/124* RNAi leaves. RNA-seq data showed that 4554 genes were induced while 3101 genes were repressed in GL-3 leaves after 2 h of dehydration (using both Q value of <0.05 and 1.5-fold as a cutoff) (Supplemental Table 2). Compared with GL-3 leaves, the expression of 273 genes increased, while the expression of 276 genes decreased in *MdMYB88/124* RNAi plants under dehydration conditions (Supplemental Table 3). Under control conditions, only 52 genes were up-regulated by MdMYB88 and MdMYB124, while 69 genes were down-regulated (Supplemental Table 4). Additionally, 55 out of 276 genes were dehydration inducible, whereas 15 out of 273 genes were repressed by dehydration. These data indicate that MdMYB88 and MdMYB124 regulate the expression of drought-responsive genes. A gene ontology (GO) enrichment analysis suggested that the differentially expressed genes (DEGs) in *MdMYB88/124* RNAi were significantly enriched in response to stimuli and ABA, and that the phenylpropanoid biosynthetic process was enriched under control and dehydration conditions, indicating the potential roles of MdMYB88 and MdMYB124 in ABA response and secondary metabolite accumulation (Figure 3, Supplemental Figure 8).

To verify the gene regulation by MdMYB88 and MdMYB124, we selected nine genes and performed qRT-PCR analysis using GL-3, *MdMYB88/124* RNAi, *MdMYB88* OE, and *MdMYB124* OE plants under dehydration conditions. The expression patterns for seven out of nine genes were confirmed (Figure 4, Supplemental Table 3), suggesting the reliability of the RNA-seq data. Our RNA-seq and qRT-PCR data suggest that MdMYB88 and MdMYB124 positively regulate the expression of *MdSPL3*, *MdNF-YA7*, *MdHB6*, *MdHB7*, *MdOEP16*, and *MdPUB9*, but negatively modulate the expression of *MdAHK3* under dehydration conditions (Figure 4, Supplemental Table 3). Among these seven genes, SPL3, HB7, NF-YA7, and OEP16 were positive regulators for drought stress resistance in *Arabidopsis* or wheat while AHK3 was a negative regulator (Wang et al., 2015; Valdés et al., 2012; Lee et al., 2015; Pudelski et al., 2012; Zang et al., 2017; Tran et al., 2007).

MdMYB88 and MdMYB124 mediate ABA accumulation by modulating the expression of ABA biosynthetic and catabolic genes under drought

ABA content is regulated by ABA biosynthetic genes and catabolic genes. *NCED3* catalyzes the rate-limiting step in ABA *de novo* biosynthesis (Tan et al., 1997; Burbidge et al., 1999; Chernys and Zeevaart, 2000; Iuchi et al., 2001; Qin and Zeevaart, 2002), whereas *UGT71B6* and *CYP707A1-A4* catalyze ABA to ABA-GE (Xu et al., 2002; Kushiro et al., 2004; Okamoto et al., 2006; Priest et al., 2006; Dong et al., 2014; Liu et al., 2015). From the RNA-seq data, we found that the gene homologous to *UGT71B6* in *Arabidopsis* was negatively regulated by *MdMYB88* and *MdMYB124* (Figure 5A, Supplemental Table 3). We also investigated the expression levels of *MdNCED3* and *CYP707A1*, *A2*, *A4* in GL-3, as well as *MdMYB88* and *MdMYB124* transgenic plants. We found that under control and dehydration conditions, *MdNCED3* transcript levels were much lower in *MdMYB88/124* RNAi plants but higher in *MdMYB88* OE and *MdMYB124* OE plants (Figure 5B). *MdCYP707A4* expression was increased in *MdMYB88/124* RNAi plants, but was reduced in *MdMYB88* OE and *MdMYB124* OE plants under dehydration conditions (Figure 5C). However, no transcript changes of *MdCYP707A1* and *MdCYP707A2* were detected in GL-3 and transgenic plants under control or dehydration conditions (Supplemental Figure 9).

We next sought to determine if MdMYB88 and MdMYB124 were involved in ABA accumulation under drought stress. ABA content was measured with the LC-MS/MS approach in GL-3 and transgenic plants under control and drought conditions. As shown in Figure 5D and 6, ABA accumulated rapidly and substantially after dehydration for 2 h (Figure 5D) and drought stress (Figure 6). ABA levels in *MdMYB88/124* RNAi plant leaves were much lower than those of GL-3 plants under drought stress conditions, whereas *MdMYB88* OE and *MdMYB124* OE plant leaves contained more ABA than GL-3 plants under drought (Figure 5D and 6).

The above data suggest that MdMYB88 and MdMYB124 positively regulate ABA accumulation under control and drought stress, and this regulation should be achieved by the integrative regulation of ABA biosynthetic and catabolic genes.

MdMYB88 directly regulates *MdNCED3* expression

MdMYB88 and MdMYB124 can bind to *cis*-elements in the promoter regions of their direct target genes. To determine whether MdMYB88 and MdMYB124 directly regulate promoters of these ABA biosynthetic and catabolic genes, we analyzed promoter sequences of *MdNCED3*, *MdUGT71B6* and *MdCYP707A4*. We found MdMYB88 and MdMYB124 binding sites in three regions of the *MdNCED3* promoter (Supplemental Figure 10): -1830 bp to -1826 bp, -1368 bp to -1364 bp and -880 bp to -876 bp. A ChIP-qPCR analysis using anti-MdMYB88/124 antibody suggested that MdMYB88 and MdMYB124 can bind to the promoter region of *MdNCED3* from -880 bp to -876 bp and after drought the enrichment of MdMYB88/MdMYB124 on *MdNCED3* promoter was enhanced (Fragment c in Figure 7A and B). An EMSA assay confirmed this direct binding by MdMYB88 on Fragment c (Figure 7C). Dual-luciferase assay further verified the positive regulation of *MdNCED3* by MdMYB88 (Figure 7D). However, we did not identify MdMYB88 and MdMYB124 binding sites in the promoter regions of *MdUGT71B6* and *MdCYP707A4*. These results suggest that MdMYB88 and MdMYB124 regulate ABA accumulation under control and drought conditions through directly activating *MdNCED3* transcription, while indirectly repressing the expression of *MdUGT71B6* and *MdCYP707A4*.

Sensitivity to ABA-induced stomatal closure of *MdMYB88* and *MdMYB124* transgenic plants

The involvement of MdMYB88 and MdMYB124 in ABA response and accumulation suggested the possibility of their involvement in stomatal movement. To test this hypothesis, we examined the sensitivity to ABA-induced stomatal closure of GL-3, *MdMYB88/124* RNAi plants, *MdMYB88* OE, and *MdMYB124* OE plants. When treated with 5 μM ABA for 1 h, *MdMYB88/124* RNAi plants were less sensitive to ABA-induced stomatal closure, while *MdMYB88* OE and *MdMYB124* OE plants displayed a strong sensitivity to ABA inhibition of stomatal opening, compared with GL-3 plants (Supplemental Figure 11).

Drought-stimulated ABA accumulation predominantly occurs in apple leaves instead of roots

In response to drought stress, ABA quickly accumulated in plants (Zhu, 2016). To investigate the ABA accumulation in apple trees in response to drought, we measured ABA content from leaves and roots in GL-3, *MdMYB88*, and *MdMYB124* transgenic plants under control and

PEG treatment. LC-MS/MS measurement revealed that, after simulated drought conditions for 6 h, ABA quickly accumulated, in apple tree leaves and roots. However, the accumulation was significantly lower in the roots of the apple tree after PEG treatment, though a slight accumulation was seen (Figure 8A). Although MdMYB88 and MdMYB124 positively regulate *MdNCED3* and *MdCYP707A4* expression in apple tree roots, the fold change of *MdNCED3* in response to simulated drought in GL-3 roots was significantly less than that in leaves (Figure 8B and C, Supplemental Figure 12), which is also consistent with the lower ABA content in apple tree roots in response to simulated drought (Figure 8A). These results demonstrate that both leaves and roots can biosynthesize ABA, although this capacity is higher in leaves than in roots.

Discussion

In this study, we illustrated the feedback regulation of ABA by MdMYB88 and MdMYB124 under control and drought conditions in apple plants. MdMdMYB88 and MdMYB124 positively regulate ABA accumulation which in turn represses expression of both genes (Figure 9).

Stomatal conductance regulation is one of the early responses to drought. Stomatal control is regulated by plant hydraulic function, as well as ABA. Increase of root ABA concentration under drought is observed to be correlated with increased root hydraulic conductivity (Thompson et al. 2007). Exogenous ABA application to roots results in increased root hydraulic conductance (Hose et al. 2000; Thompson et al. 2007). It is reasonable that the higher root hydraulic conductivity of *MdMYB88* or *MdMYB124* OE results in higher water flow from the roots; therefore, higher LRWC and higher stomatal conductance. In contrary, lower root hydraulic conductivity of the *MdMYB88/124* RNAi plants leads to lower LRWC and stomatal conductance under control and drought conditions.

ABA not only plays a positive role in the root hydraulic conductivity, but also leaf hydraulic conductivity (Morillon and Chrispeels 2001). Exogenous ABA application or genetically manipulated to increase endogenous ABA level results in increased leaf hydration (Thompson et al. 2007; Parent et al. 2009), and can increase leaf expansive growth under water deficit via improvement of plant water relations (Sansberro et al., 2004). Moreover, ABA can also induce the mRNA and protein levels of leaf aquaporin PIPs which play a key role in regulating water transport in roots and leaves (Tardieu et al. 2010; Morillon and Chrispeels 2001; Aroca et al. 2006; Parent et al. 2009), thereby contributing to the leaf hydraulic conductivity (Morillon and Chrispeels 2001). We previously found that root hydraulic conductivity (K_{root}) is lower in *MdMYB88/124* RNAi plants but higher in *MdMYB88* or *MdMYB124* OE plants under drought (Geng et al., 2018). In this study, we found that the leaf relative water content (LRWC) of overexpression lines of *MdMYB88* and *MdMYB124* was higher than that of GL-3 while LRWC of RNAi lines was lower under control and drought conditions (Supplemental Figure 6). To understand the coordination of leaf water status, we measured the leaf hydraulic conductance (K_{leaf}) of transgenic plants and GL-3 (Supplemental Figure 7). The results showed that K_{leaf} of *MdMYB88* overexpression lines was higher than that of GL-3 under control and drought conditions (SRWC was 45-55%) while *MdMYB88/124* RNAi lines has lower K_{leaf}. Given that MdMYB88 and MdMYB124 positively regulated ABA biosynthesis, our results support the notion that ABA positively correlated with K_{leaf} and K_{root}, as well as LRWC, which is consistent with previous observations (Morillon and Chrispeels 2001; Thompson et al. 2007).

The detached leaves of *MdMYB88/124* RNAi plants lost more water than GL-3 plants under dehydration conditions, while both *MdMYB88* and *MdMYB124* OE plants maintained more water after dehydration (Figures 1B and 2C). However, stomatal conductance in the *MdMYB88/124* RNAi plants under drought was lower but higher in the *MdMYB88* or *MdMYB124* OE plants (Figures 1 and 2). In detached leaves, the leaf hydraulic conductance is still existing (Coupel-Ledru et al., 2017). In these leaves, almost all water evaporates through stomata; therefore, the stomatal aperture regulated by ABA is the important factor during this process. Thus, the detached leaves of *MdMYB88* and *MdMYB124* OE and *MdMYB88/124* RNAi plants regulated their stomata closure by ABA accumulation.

We also observed that *MdMYB88/124* RNAi plants have lower stomatal conductance compared to GL-3 and OE plants have higher stomatal conductance at the beginning of

drought treatment (day 0). Previously, we found that MdMYB88 and MdMYB124 regulate stomatal development (Xie et al., 2018) as stomatal clusters are observed in the *MdMYB88/124* RNAi plants. The abnormal stomata might contribute to the basal lower stomatal conductance in the *MdMYB88/124* RNAi plants. In addition, the root hydraulic conductivity of *MdMYB88* or *MdMYB124* OE is higher compared to GL-3 under control (Geng et al., 2018), which should result in higher water flow from the roots; therefore, higher LRWC and higher stomatal conductance.

Endogenous ABA levels are determined by ABA biosynthesis and catabolism. In response to drought stress, ABA accumulated quickly to induce stomatal closure and avoid water loss through transpiration (Zhu, 2016). The primary pathway to biosynthesize ABA in plants is *de novo* biosynthesis from carotenoids. Many genes are identified in this pathway, such as *ABA*, *NCED*, and *AAO3* genes (Zhu, 2016). In *Arabidopsis*, NCED3 has a major role in ABA biosynthesis. Overexpression of *NCED3* in transgenic *Arabidopsis* leads to an increase in the endogenous ABA level and improves drought resistance (Luchi et al., 2001). In addition, NCED5 also contributes to ABA production in response to dehydration (Frey at al., 2012). In our study, we found MdMYB88 and MdMYB124 positively regulate the expression of *MdNCED3* by associating with its promoter regions (Figures 7). In contrast to the dramatic change of *MdNCED3* expression, no *MdAAO3* transcript changes were observed in *MdMYB88* and *MdMYB124* transgenic plants under drought or control conditions (Supplemental Figure 13). The catabolic pathway involves ABA hydroxylation mediated by cytochrome P450 members (*CYP707A1* to *CYP707A4*) and ABA conjugation mediated by UGTs- (*UGT71B6*, *UGT71C5*, *UGT71B7*, *UGT71B8*) (Kushiro et al., 2004; Okamoto et al., 2006; Priest et al., 2006; Dong et al., 2014; Liu et al., 2015). *Arabidopsis* UGT71B6, together with its two homologs (UGT71B7 and UGT71B8), contributes to endogenous ABA content (Priest et al., 2006; Dong et al., 2014). UGT71C5 itself regulates ABA homeostasis, implicating a major role of UGT71C5 in ABA accumulation (Liu et al., 2015). Suppression of tomato *SIUGT71C5* also leads to elevated ABA levels (Sun et al., 2017). Our results show that both MdMYB88 and MdMYB124 negatively regulate the expression of *MdUGT71B6* and *MdCYP707A4* under drought stress (Figure 5A and C; Supplemental Table 3). Interestingly, there was no altered expression level of *MdCYP707A4* among non-transgenic and transgenic plants under control conditions (Figure 5C). Besides the *de novo* biosynthetic pathway of ABA, BG1 and BG2 genes are also responsible for ABA accumulation by catalyzing ABA-GE to ABA (Xu et al., 2012). However, we could not identify the exact *BG*

genes, *UGT71B7*, *UGT71B8*, and *UGT71C5* in the apple genome (Velasco et al, 2010; Li et al., 2016; Daccord et al., 2017), which might be due to the incompleteness of the apple genome assemblies and annotation. Hence, our current results suggest that MdMYB88 and MdMYB124 promote ABA accumulation in response to drought in apple trees, possibly by activating the expression of ABA biosynthetic gene (*MdNCED3*) and repressing ABA catabolic genes (*MdCYP707A4* and *MdUGT71B6*). These three proteins are closely related with their close homologs in *Arabidopsis* (Supplemental Figure 14). This further indicates they play the same role in apple tree ABA signaling pathways as in *Arabidopsis*.

Altered ABA homeostasis results in altered expression levels of drought-and ABA-responsive genes and drought stress response (Nambara and Marion-Poll, 2005; Tuteja, 2007). Among the DEGs from RNA-seq data under control and dehydration conditions, we identified 55 drought-inducible genes which were down-regulated in *MdMYB88/124* RNAi plants in response to air drying (Supplemental Table 2 and 3), including *MdHB7*, *MdMYB102*, *MdPUB9*, *and MdOEP16*. This number is close to one-fifth of the 276 down-regulated genes in the *MdMYB88/124* RNAi plants. These 55 genes might be drought positive regulators and positively regulated by MdMYB88/MdMYB124, therefor they should play important roles for the contribution of drought tolerance by MdMYB88 and MdMYB124. Some drought-repressing genes were up-regulated in *MdMYB88/124* RNAi plants after dehydration treatment. Among the 273 up-regulated genes in *MdMYB88/124* RNAi plants, we identified 15 drought-repressed genes such as *MdAHK3* (Supplemental Tables 2 and 3). These genes should be drought negative factors and negatively regulated by MdMYB88 and MdMYB124. These 15 genes should also contribute to the drought tolerance by MdMYB88 and MdMYB124, though it is possible that their roles might be not as critical as those 55 genes. Homologs of *HB7* and *OEP16* in *Arabidopsis* and wheat are positive regulators for drought improvement, whereas AHK3 is a negative regulator (Söderman et al., 1996; Tran et al., 2007; Zang et al., 2017).

The compounds of phenylpropanoids are a class of plant secondary metabolites, which are activated under abiotic stress to cope with environmental factors, such as oxidative stress and drought stress (Sharma et al., 2019). Plant phenylpropanoids have a prominent role in ROS scavenging (Agati et al., 2012). Go enrichment analysis of DEGs in *MdMYB88/124* RNAi plants revealed the involvement of MdMYB88 and MdMYB124 in phenylpropanoid metabolism (Figure 3). Our recent study also revealed the accumulation of metabolites in the phenylpropanoid biosynthesis pathway in *MdMYB88* or *MdMYB124* OE plants under drought

stress (Geng et al., 2020). Therefore, the stronger ROS scavenge ability of *MdMYB88* or *MdMYB124* OE plants by higher phenylpropanoids contributed to their stronger ability of drought endurance.

Collectively, we demonstrated that, in response to drought stress, MdMYB88 and MdMYB124 modulate the ABA accumulation by activating ABA biosynthetic genes and repressing ABA catabolic genes in apple leaves, thus regulate the drought response. However, MdMYB88 and MdMYB124 were repressed by ABA (Figure 9). Overaccumulation of ABA homeostasis is not always good as ABA can induce senescence (Becker and Apel, 1993), pollen sterility and reduces growth directly and indirectly (Blum 2015). The benefit or damage of ABA depends on the dynamics of the seasonal regimen of ABA and the crop drought stress profile (Blum 2015). In our study, the *MdMYB88* or *MdMYB124* OE plants accumulated more ABA than GL-3 and higher LRWC to maintain the balance between drought resistance and plant biomass. It will be worthy to test the reality of field performance of our transgenic plants to evaluate the positive and negative effects of ABA on drought resistance and plant production in the future.

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Conflicts of Interest

The authors have no conflict of interest to declare.

Author contributions

Q.G. designed experiments. Y.X., C.B., P.C., X.W.L., D.G., X.F.L., Y.Y., N.H., F.Z. performed experiments. Q.G., and C.B. wrote the manuscript. Z.L. analyzed RNA-seq data. S.Z., F.M., X.Z., and C.N. analyzed data.

Data availability

The RNA-seq data is deposited to NCBI under BioProject accession PRJNA529852.

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Figure legends

Figure 1. *MdMYB88/124* RNAi plants were sensitive to drought. (A) Transcription level of *MdMYB88* and *MdMYB124* in 'Golden Delicious' (*Malus* x *domestica*) under drought stress for 0, 2, 4, and 6 d. Data are means ± SD (n = 3). (B) Water loss of detached leaves at room temperature. Data are means ± SD (n = 15). Leaves were detached from fifteen plants. (C) Drought resistance of GL-3 and transgenic *MdMYB88/124* RNAi plants. Bars = 5 cm. Five-month-old plants were treated with drought for 30 d, and then reirrigated for 11 d. (D) Survival rate of GL-3 and transgenic *MdMYB88/124* RNAi plants under drought stress. Thirty-six plants were collected and each 12 plants were used as a biological replication. (E-H) The rate of photosynthesis (E), stomatal conductance (F), rate of transpiration (G), and instantaneous water-use efficiency (H) of GL-3 and transgenic *MdMYB88/124* RNAi plants under drought stress. Data are means ± SD (n = 15). One-way ANOVA (Tukey's test) was performed and statistically significant differences were indicated by * (P<0.05), ** (P<0.01) or *** (P<0.001).

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Figure 5. MdMYB88 and MdMYB124 modulate ABA accumulation after dehydration by regulating expression of ABA biosynthetic and catabolic genes. (A-C) Relative mRNA level of *MdUGT71B6*, *MdNCED3*, and *MdCYP707A4* in GL-3, *MdMYB88/124* RNAi, and overexpression (OE) plants under control or dehydration conditions. (D) ABA content in GL-3 and transgenic plants under control or dehydration conditions. One-way ANOVA (Tukey's test) was performed and statistically significant differences were indicated by ** ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$). Error bars indicate standard deviation (n = 3 in A-C; 5 in D).

Figure 6. ABA content in leaves of GL-3, *MdMYB88*, and *MdMYB124* transgenic plants under drought stress. Error bars indicate standard deviation (n = 5). One-way ANOVA (Tukey's test) was performed and statistically significant differences are indicated by ** ($P<0.05$), ** ($P<0.01$) or *** ($P<0.001$). Control: SRWC was 75-85%; Moderate: SRWC was 45-55%; Severe: SRWC was 25%-35%.

Figure 7. MdMYB88 directly binds the promoter region of *MdNECD3*. (A) Diagram of *MdNCED3* promoter regions. Fragments a and b contain *cis*-element of AGCCG from -1830 bp to -1826 bp, -1368 bp to -1364 bp. fragments c contains a *cis*-element of CGCGG from -880 bp to -876 bp, fragment d serves as a negative control. TSS, transcription start site. (B) ChIP-qPCR analysis of *MdNCED3* using GL-3 plants drought treated for 0 and 12d. *MdMDH* serves as the reference gene. Data are means ± SD (n = 3). (C) MdMYB88-His is able to bind the promoter region of *MdNECD3* determined by EMSA analysis. Arrowheads indicate protein-DNA complex or free probe. *MdNCED3* probe contains CGCGG element. (D) The regulation of *MdNCED3* by MdMYB88 by Dual-luciferase assay. Effects of MdMYB88 on *MdNCED3*

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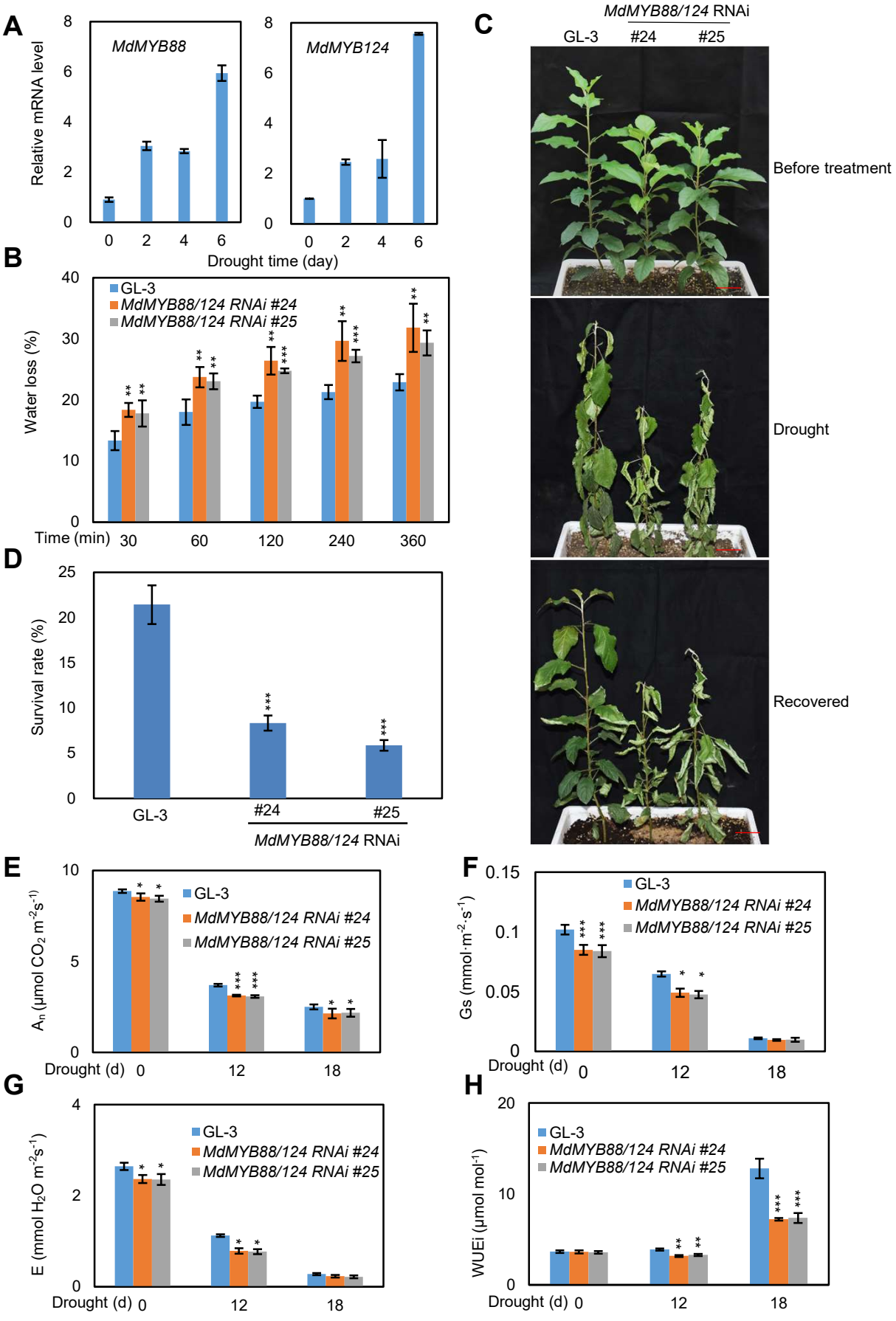

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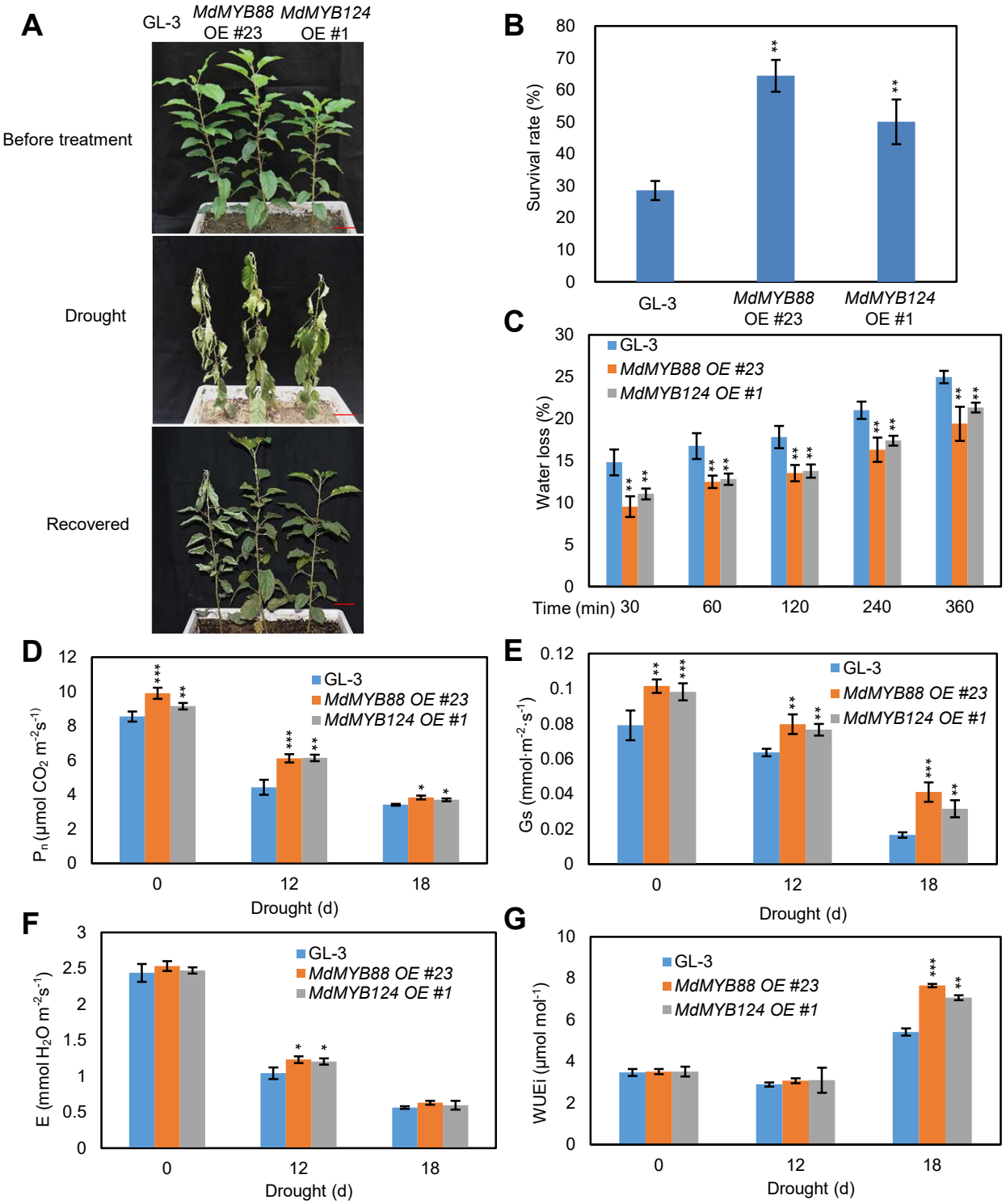

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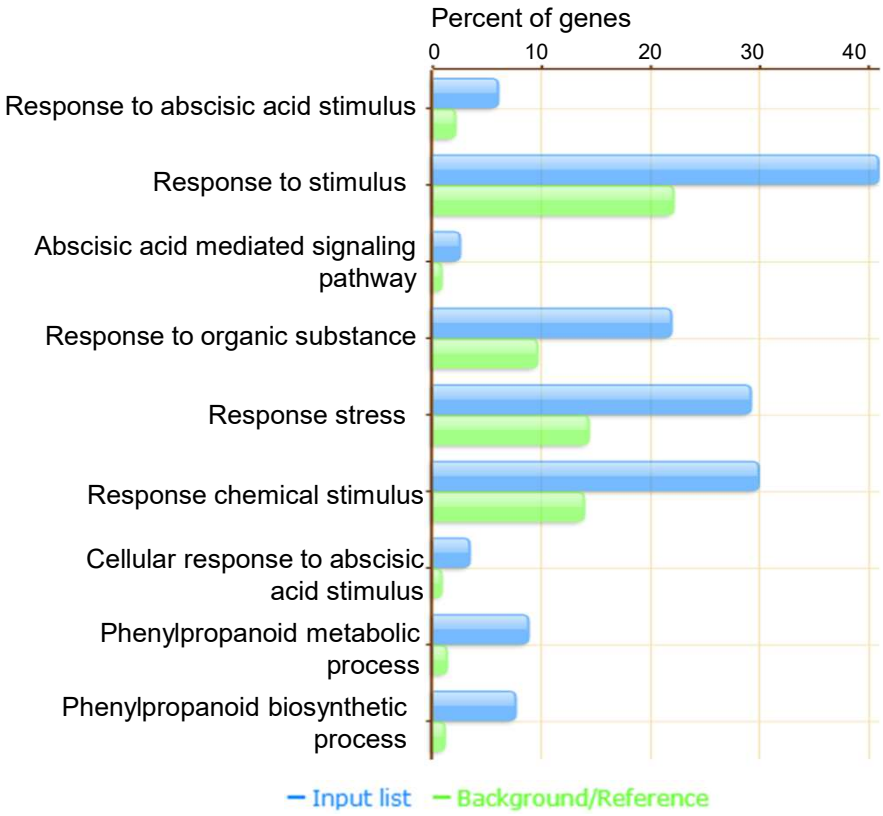

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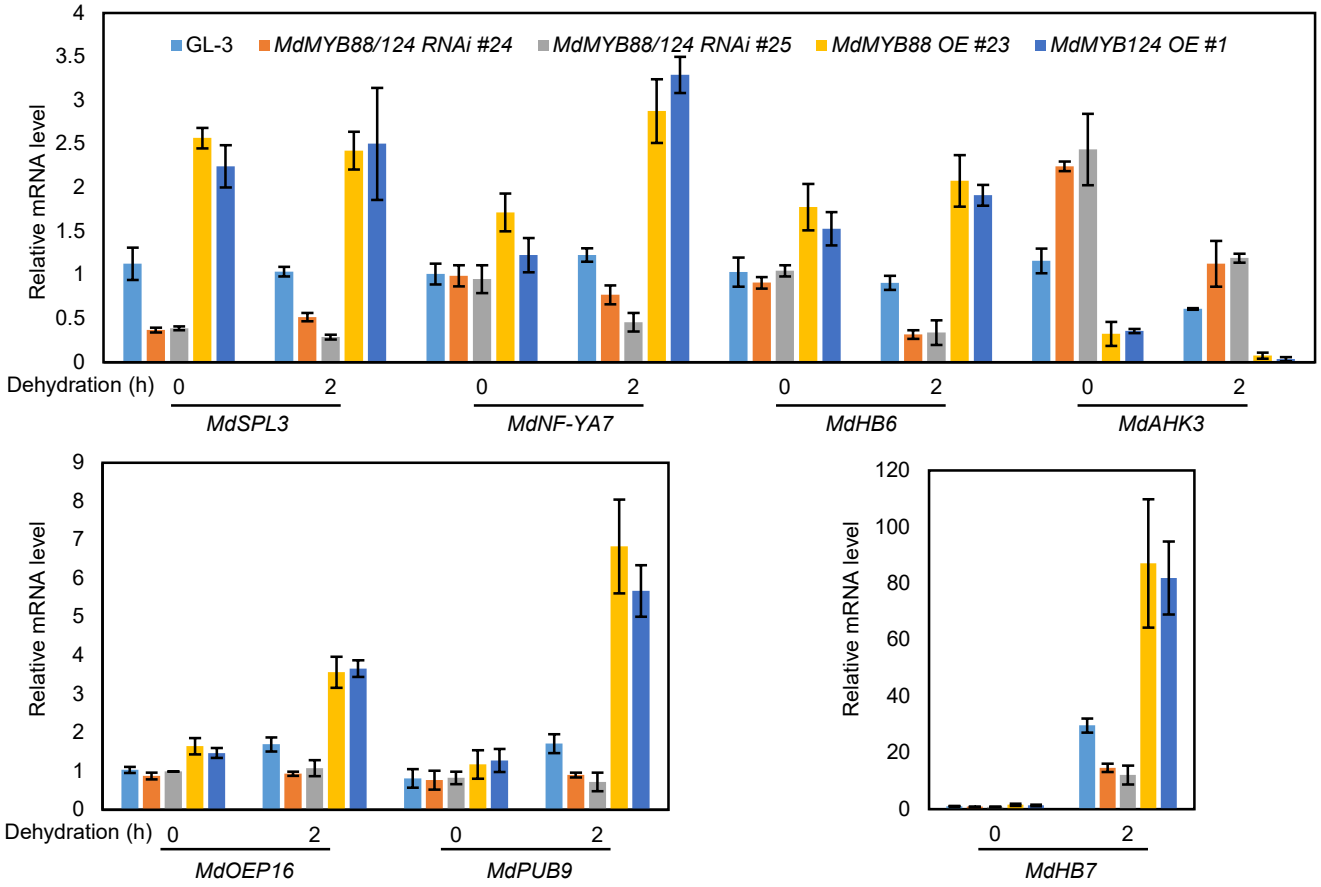

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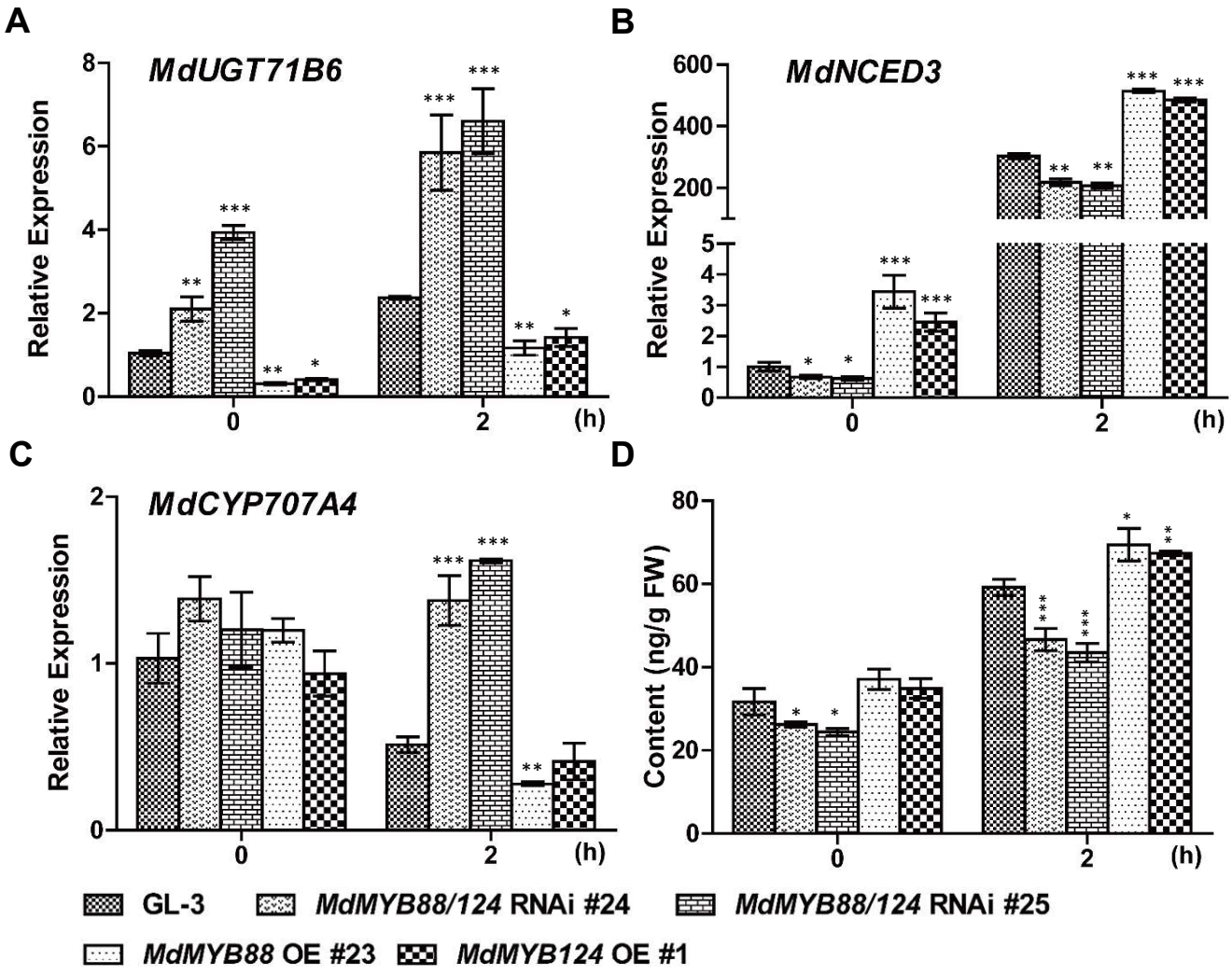

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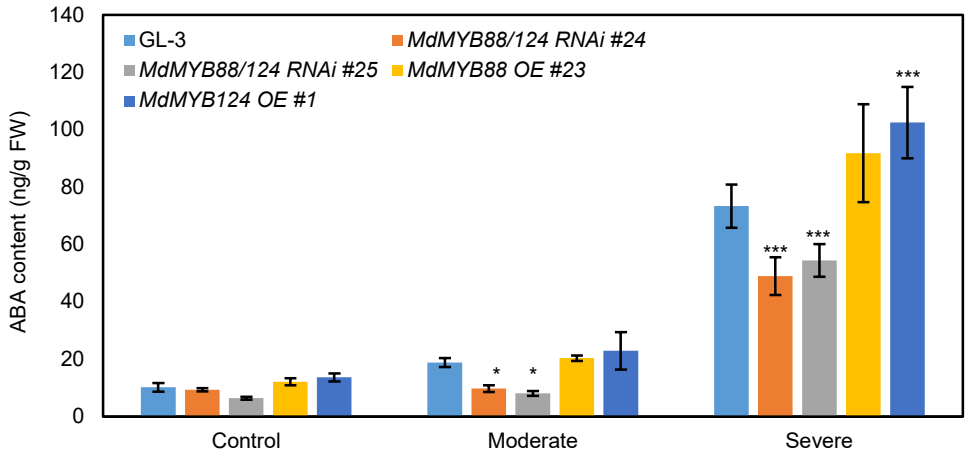

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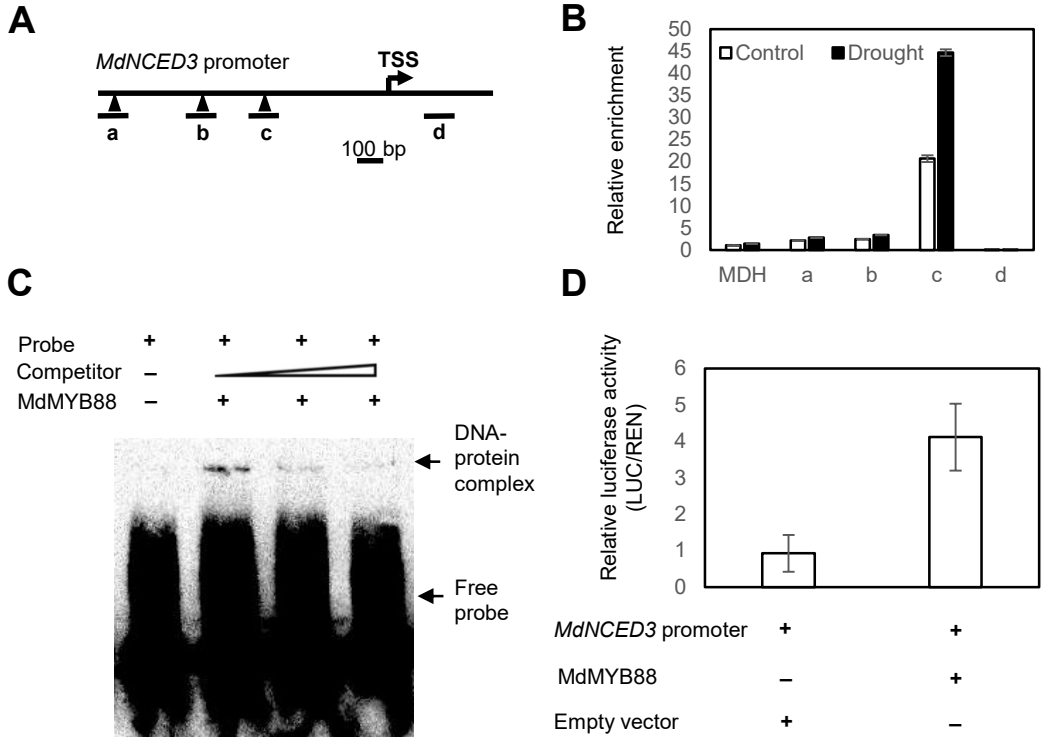

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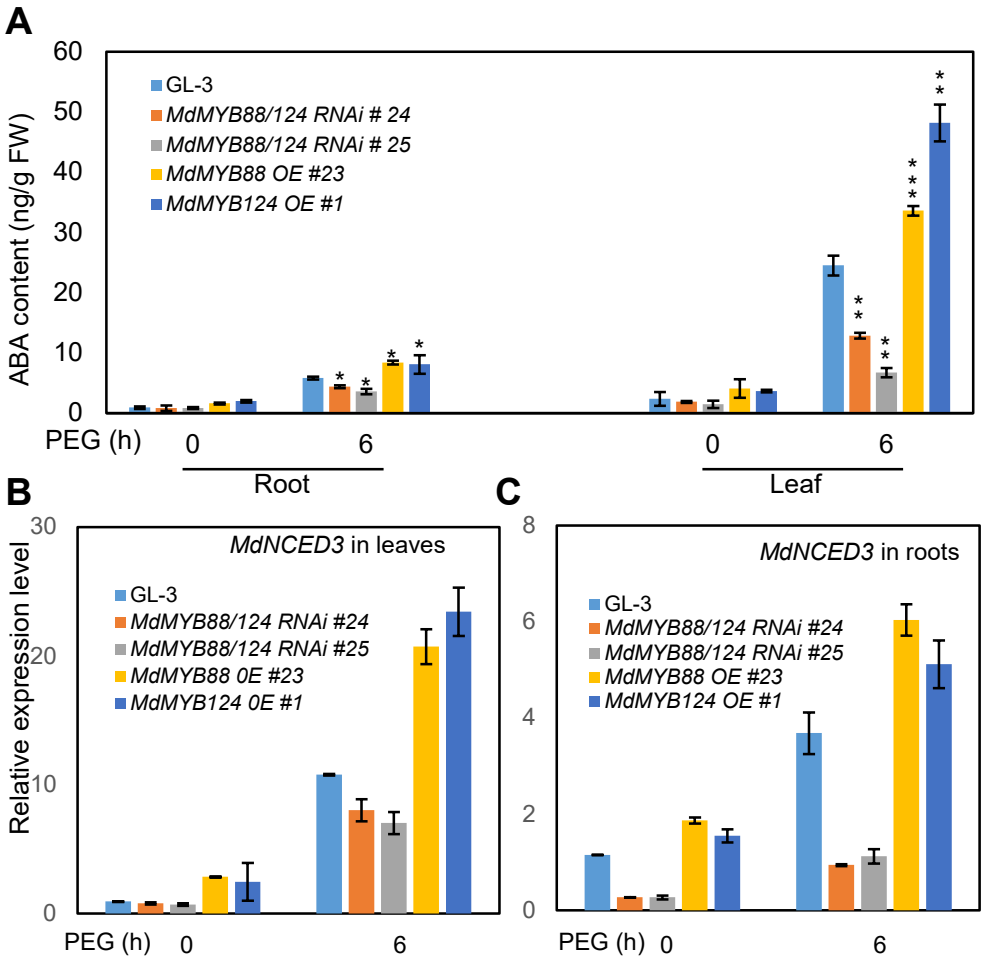

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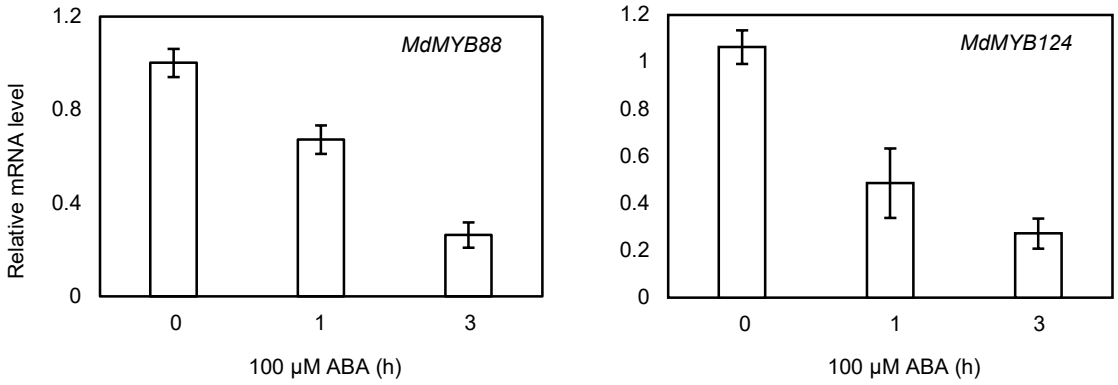

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