Abscisic Acid Hormone Induces Anthocyanin Synthesis in Arabidopsis thaliana Seedlings: Postprint

Authors: Chen Junjie, Mei Song, Hu Yanru

Date: 2019-10-17T00:00:00+00:00

Abstract

Abscisic acid (ABA) is an important plant hormone that regulates various physiological processes in plants. Anthocyanins are flavonoid compounds produced through plant secondary metabolism, playing important roles in plant growth, development, and responses to abiotic stress. In recent years, numerous studies have investigated the transcription factors and biosynthetic enzyme genes involved in anthocyanin biosynthesis; however, the signaling molecules controlling its synthesis and their mechanisms of action remain to be thoroughly explored. This study used Arabidopsis thaliana as a model to investigate the regulatory function and mechanism of ABA signaling in anthocyanin biosynthesis. Experimental results demonstrated that exogenous application of ABA significantly enhanced anthocyanin accumulation in the shoot apices of wild-type seedlings. Consistently, ABA could induce the expression of certain transcription factors and biosynthetic enzyme genes related to anthocyanin synthesis. Furthermore, genetic analysis revealed that ABA-induced anthocyanin synthesis partially depends on core transcription factors in the MBW complex, such as TTG1, TT8, and MYB75. Preliminary mechanistic studies revealed that the bZIP transcription factor ABI5 in the ABA signaling pathway can interact with TTG1, TT8, and MYB75 to form protein complexes. In summary, this study suggests that ABA signaling induces anthocyanin accumulation in Arabidopsis seedlings and may regulate anthocyanin synthesis through the synergistic action of ABI5 with the MBW complex.

Full Text

Abscisic Acid Induces Anthocyanin Synthesis in Arabidopsis Seedlings

Junjie Chen[1],[2], Song Mei[1], Yanru Hu[1],* [1]Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming 650223, China [2]University of Chinese Academy of Sciences, Beijing 100049, China

Abstract: Abscisic acid (ABA) is an important phytohormone that participates in regulating various physiological processes in plants. Anthocyanins are flavonoid compounds produced through plant secondary metabolism and play important roles in plant growth, development, and stress responses. In recent years, numerous studies have investigated the transcription factors and synthetase genes involved in anthocyanin biosynthesis; however, the signaling molecules controlling their synthesis and the underlying mechanisms remain to be further explored. This study used *Arabidopsis thaliana* as a model to investigate the regulatory function and mechanism of ABA signaling in anthocyanin biosynthesis. Experimental results demonstrated that exogenous application of ABA significantly enhanced anthocyanin accumulation in the shoot tips of wild-type seedlings. Consistently, ABA induced the expression of certain transcription factors and synthetase genes associated with anthocyanin synthesis. Furthermore, genetic analysis revealed that ABA-induced anthocyanin synthesis is partially dependent on core transcription factors in the MBW complex, such as TTG1, TT8, and MYB75. Preliminary mechanistic studies revealed that the bZIP-type transcription factor ABI5 in the ABA signaling pathway can interact with TTG1, TT8, and MYB75 to form protein complexes. In summary, this study suggests that ABA signaling induces anthocyanin accumulation in Arabidopsis seedlings and may regulate anthocyanin synthesis through the synergistic action of ABI5 with the MBW complex.

Keywords: Arabidopsis, ABA, Anthocyanin, ABI5 transcription factor, MBW complex

---

CHEN Junjie[1],[2], MEI Song[1], HU Yanru[1],*
[1]CAS Key Laboratory of Tropical Plant Resources and Sustainable Use, Xishuangbanna Tropical Botanical Garden, Chinese Academy of Sciences, Kunming, China
[2]University of Chinese Academy of Sciences, Beijing 100049, China

Abstract: Abscisic acid (ABA) is a critical phytohormone and widely modulates various biological processes in plants. Anthocyanins are flavonoids produced by plant secondary metabolism and play crucial roles in plant growth and stress responses. Recently, several transcription factors and synthetase genes involved in anthocyanins biosynthesis have been well studied; however, the upstream regulatory signals mediating their synthesis remain to be further

explored. In this study, we investigated the function and mechanism of ABA in the control of anthocyanin biosynthesis. Phenotypic analysis showed that exogenous application of ABA significantly increased the accumulation of anthocyanins in the stem ends of wild-type Arabidopsis seedlings. Consistently, ABA induced the expression of certain transcription factors and synthetase genes associated with anthocyanin synthesis. In addition, genetic analysis revealed that ABA-stimulated anthocyanin synthesis is partially dependent on core transcription factors in the MBW complex that positively regulates anthocyanin synthesis, such as TTG1, TT8, and MYB75. Preliminary mechanism studies revealed that the bZIP-type transcription factor ABI5 in the ABA signaling pathway physically interacts with TTG1, TT8 and MYB75 to form a protein complex. Taken together, this study shows that ABA signaling induces anthocyanin accumulation in Arabidopsis seedlings and may regulate the synthesis of anthocyanins by synergizing the ABI5 with the MBW complex.

Keywords: Arabidopsis, ABA, Anthocyanin, ABI5 transcription factor, MBW complex

Introduction

Anthocyanins are water-soluble natural pigments produced through plant secondary metabolism, belonging to the flavonoid compounds that hold significant value in food nutrition and medicinal health care (Peiffer et al, 2016; Wei et al, 2018). They are widely present in angiosperms and constitute important components formed during plant growth. Comprehensive studies have demonstrated that anthocyanins also play crucial roles in enhancing plant tolerance to adverse environmental stresses, which is significant for plant growth, reproduction, and environmental adaptation (Rowan et al, 2009; Fan et al, 2016; Liang and He, 2018). Genes involved in the anthocyanin synthesis pathway can be divided into two categories: structural genes and regulatory genes. Structural genes include early biosynthetic genes (such as CHS, CHI, and F3H) and late biosynthetic genes (such as DFR, ANS, and UF3GT) (Tanaka et al, 2008; Zhang & Schrader, 2017). Currently, numerous studies have found that regulatory genes involved in anthocyanin synthesis primarily encode MYB, bHLH, and WD40 family proteins (Deng & Lu, 2017; Ma & Constabel, 2019). PAP1 is a member of the R2R3 MYB family, MYB75, which coordinately upregulates the expression of anthocyanin synthesis-related genes, such as PAL, CHS, and DFR, with its homologous protein PAP2/MYB90 (Maier et al, 2013; Shin et al, 2015). Additionally, Gonzalez et al demonstrated that overexpression of MYB113 or MYB114 also leads to significant increases in Arabidopsis anthocyanins (Gonzalez et al, 2008). TT8, GL3, and EGL3 proteins belong to the bHLH family of transcription factors, all homologous to the maize R transcription factor, and positively regulate anthocyanin biosynthesis in Arabidopsis (Baudry et al, 2004; Escaray et al, 2017). TTG1 belongs to the PAC1 clade of the WD40 protein family and was reported as early as 1981 to control seed coat color, anthocyanin accumulation, seed mucilage, and root hair development (Koornneef,

1981). MYB, bHLH, and WD40 regulatory factors typically form a ternary MBW complex to exert regulatory functions, directly controlling the expression of anthocyanin synthesis genes such as DFR, BAN, LDOX, TT12, TT19, and AHA10 (Xu et al, 2014). In-depth investigation of anthocyanin biosynthetic pathways and regulatory signals will help researchers understand related plant physiological mechanisms and holds potential application value for improving plant growth status and enhancing crop economic benefits.

In recent years, plant hormone regulation of anthocyanin biosynthesis has received widespread attention. For example, exogenous hormone application can activate or inhibit the expression of anthocyanin synthesis-related genes to control anthocyanin accumulation in fruits (Shen et al, 2014; Chen et al, 2016). ABA is one of the important growth-regulating substances in plants and widely participates in regulating various physiological processes, including embryonic development, seed dormancy and germination, seedling growth, root development, fruit ripening, leaf senescence, and responses to abiotic stresses such as drought, high salinity, high osmotic pressure, and low temperature (Nakashima & Yamaguchi-Shinozaki, 2013; Dejonghe et al, 2018; Brunetti et al, 2019). ABI5, a key transcription factor in the ABA signaling pathway, belongs to the bZIP family and can be phosphorylated by SnRK2 kinases, primarily participating in the regulation of seed germination and post-germination growth in plants (Yu et al, 2015; Pan et al, 2018). Recent studies have found that ABA can promote anthocyanin synthesis and accumulation in certain plant fruits (Hiratsuka et al, 2001; Jiang & Joyce 2003; Shen et al, 2014; An et al, 2018). Despite these findings, the biological function and molecular mechanism of ABA in regulating anthocyanin synthesis in Arabidopsis remain unclear. This study used Arabidopsis as experimental material to further investigate the molecular mechanism of ABA in plant anthocyanin synthesis. We found that anthocyanin accumulation in the shoot tips of wild-type Arabidopsis seedlings increased significantly under different concentrations of ABA treatment, showing a positive correlation with ABA concentration changes. Real-time quantitative PCR (qRT-PCR) experiments further verified that ABA treatment could elevate the expression levels of anthocyanin synthesis structural genes such as LODX, DFR, and UF3GT, with an increasing trend corresponding to higher ABA concentrations. Further investigation revealed that ABA-promoted anthocyanin synthesis may be partially dependent on core transcription factors in the MBW complex, such as TTG1, TT8, and MYB75. Protein interaction experiments demonstrated that proteins including MYB75, MYB113, TT8, and TTG1 can interact with the ABI5 transcription factor, thereby establishing the molecular link between the ABA signaling pathway and anthocyanin synthesis. In summary, this study preliminarily reveals the molecular mechanism by which ABA hormone signaling regulates anthocyanin synthesis in Arabidopsis seedlings.

Materials and Methods

1.1 Materials and Plant Growth Conditions

All mutants were in the wild-type *Arabidopsis thaliana* Columbia-0 genetic background. Mutant seeds tt8-1, myb-RNAi, and pap1-D were kindly provided by Professor Yang Hongquan. For experimental studies, wild-type Arabidopsis seeds and mutant seeds tt8-1, myb-RNAi, and pap1-D were surface-sterilized with 20% 84 disinfectant for 8 minutes, then sown on 1/2 MS medium containing 0.6% agar and 1% sucrose (pH 5.8). After stratification at 4°C for 24 hours, they were grown under long-day conditions at 22°C (light/dark: 16 h/8 h). ABA used in experiments was purchased from Sigma-Aldrich, Taq DNA polymerase from Takara Biotechnology, and other common reagents from Sangon Biotech (Shanghai) Co., Ltd.

1.2 ABA Treatment

To observe changes in plant anthocyanin accumulation under ABA treatment, Arabidopsis seeds were sown on 1/2 MS agar medium supplemented with different concentrations of ABA (0, 0.25, 0.5, and 0.75 mol·L ^{1}) following the experimental method of An et al (An et al, 2018). After stratification at 4°C for 24 hours, they were placed under long-day conditions and grown at 22°C for 6-12 days. Anthocyanin accumulation in seedlings was observed and samples were photographed using an electron microscope. Each sample had at least three biological replicates for ABA treatment analysis, and each experiment was repeated at least three times.

1.3 Anthocyanin Content Measurement

Anthocyanin content was measured according to the method of Laby et al. Seven-day-old seedlings grown on 1/2 MS agar medium containing 0.25 mol·L ^{1} ABA, including wild-type Col, mutant pap1-D, myb-RNAi, and tt8, were sampled and weighed (W, in grams) on an electronic balance. One milliliter of hydrochloric acid methanol extract (methanol:HCl volume ratio of 99:1) was added, and the samples were oscillated in the dark at 4°C for 24 hours. After centrifugation at 13,000 rpm for 10 minutes, the supernatant was measured for absorbance at wavelengths of 530 nm and 657 nm (OD values). The relative anthocyanin content was calculated using the formula (A - 0.25 × A) g ^{1} FW (Xie et al, 2016). Experiments were repeated at least three times.

1.4 RNA Extraction and qRT-PCR

As described by Hu et al, total RNA was extracted from Arabidopsis seedlings using Trizol reagent (Invitrogen) and reverse-transcribed into cDNA for quantitative real-time PCR (qRT-PCR). Briefly, first-strand cDNA was synthesized from 1.5 g DNase-treated RNA using M-MuLV reverse transcriptase (Fermentas, EU) with oligo(dT) primers in a 20 L reaction volume. qRT-PCR was performed on a Roche Light Cycler 480 real-time PCR instrument using 2×SYBR

Green I master mix according to the manufacturer' s instructions (Hu et al, 2014). In this experiment, the Arabidopsis ACTIN2 gene was used as an internal reference for gene expression. Each sample had at least three biological replicates for qRT-PCR analysis, and at least two technical replicates were analyzed for each biological replicate. Gene-specific primers used for transcript detection are listed in Table 1 .

Table 1. Primers of qRT-PCR

Gene Name	Forward Primer	Reverse Primer
UF3GT	CTTCTTATACGAACAAGCAGCC	CGAGACCATTTTCCGTACAATC
MYB75	TGAAGGTACGTTATATTCGGGG	CTAGAGGCGTCTTAGCTAACTC
MYB90	CTGATTCGATTGTGATGCACAT	ACAATCTTATCCTTTGGGGGTT
MYB113	GAAAAAGAGAGACATTACGCCC	ATTAACGTCAACTTTTGGTGGG
MYB114	TTCCTTCGATTGGAACGATGTA	GCAAGTCTTAACAAAGGCGTAT
TT8	ACTAAAGATAAGAGGCTACCGC	ATGATTTACGTACGCAATGGTG
TTG1	ACTCAAGAAAAATAATGTTTGTGAAAA	AGGAACAATCGCATCAGCTTCT
DFR	ATAAAAATAGTTGCAACGATGTCAA	TGCTGTTTCCGTAGCTTCTGG
LDOX	ACTCAAGAAAAATAATGTTTGTGAAAA	CACGGTTCATGCTTCTTACTCAGA
UF3GT	GTCAAAGGAAGCGAGGGAGGAC	TGAGCTGAAACTCTGTTCCTCAA
HY5	AGAAGGTGTCGGTTAACAATGTTG	CCGATCCTTAAATTATCGGTTAAAC
ACTIN2	ATGTGAATGTAGGAGAAGATGAACCA	TGACAGTTAAGCAGAGTAAACCGTC

1.5 Yeast Two-Hybrid Assay (Y2H)

The full-length CDS of ABI5 is 1326 bp, encoding 442 amino acids. To verify the interaction between ABI5 protein and anthocyanin synthesis-related proteins, the full-length ABI5 sequence was cloned into pGBKT7 to construct plasmid BD-ABI5. Following Chen et al (Chen et al, 2012), truncated plasmids were constructed: BD-ABI5 , BD-ABI5 , BD-ABI5 , BD-ABI5 , and BD-ABI5 . The coding sequences of anthocyanin synthesis-related activators were cloned into the pGADT7 vector to generate plasmids AD-MYB75, AD-MYB90, AD-MYB113, AD-MYB114, AD-TT8, and AD-TTG1. Following Xie et al (Xie et al, 2016), these were divided into N-terminal and C-terminal fragments: amino acids 1-122 of MYB75 as the N-terminus and amino acids 123-249 as the C-terminus to construct truncated plasmids AD-MYB75-N and AD-MYB75-C; amino acids 1-123 of MYB113 as the N-terminus and amino acids 124-247 as the C-terminus to construct AD-MYB113-N and AD-MYB113-C; amino acids 1-358 of TT8 as the N-terminus and amino acids 359-519 as the C-terminus to construct AD-TT8-N and AD-TT8-C; amino acids 1-174 of TTG1 as the N-terminus and amino acids 175-342 as the C-terminus to construct AD-TTG1-N and AD-TTG1-C (Xie et al, 2016). These fusions in different vectors were used for yeast two-hybrid experiments. Primers used for constructing various clones in this study are listed in Table 2 .

Table 2. Primers used for generating various clones

Plasmid Name	Forward Primer	Reverse Primer
AD-MYB90	GATTACGCTCATATGATG	AGAGGTCTCGAACTACTG
AD-MYB114	GATTACGCTCATATGATG	AGAGGTCTCGAACTACTG
AD-MYB75	GATTACGCTCATATGATG	AGAGGTCTCGAACTACTG
AD-MYB75-N	GATTACGCTCATATGATG	AGAGGTCTCGAACTACTG
AD-MYB75-C	GATTACGCTCATATGAAA	ACATCGACCTCGTACTTCTAACAATCAAAG
MYB75-nYFP	ATAGGATCCATGGAGGGA	TTACTCTACAAATG
AD-MYB113	GATTACGCTCATATGATG	AGGCGAGTCACACTACTG
AD-MYB113-N	GATTACGCTCATATGATG	AGGCGAGTCACACTACTG
AD-MYB113-C	GATTACGCTCATATGAAG	ACACGAGTCGACAATGCTCTA
MYB113-nYFP	ATAGGATCCATGGGCGAA	ATCTACTCAAATG
GAD-TT8	GAGGCCAGTGAATTCATG	GAACTCAATCAAGTCTTATAGGT
GAD-TT8-N	GAGGCCAGTGAATTCATG	GAACTCAATCAAGTCTTGTCACGC
GAD-TT8-C	GAGGCCAGTGAATTCGTG	AACATCGAGTCCCACTCC
TT8-nYFP	ATACTCGAGATGGATGAA	ATCATCTACTTACTTCTG
GAD-TTG1	GATTACGCTCATATGATG	AGATAGTTCAGATCTCCAATAC
GAD-TTG1-N	GATTACGCTCATATGATG	AGATAGTTCAGATCTCAGATGC
GAD-TTG1-C	GATTACGCTCATATGGAT	ACACGAGGGTTCAATGACTATAGCTT
TTG1-nYFP	ATATCTAGAATGGATAAT	TTCATCTAGAAACTTCTG
GUS-nYFP	CGCGGATCC ATGGTCCGTCCTGTAGAAAC	GCTCTAGA TCATTGTTTGCCTCCCTGCT
GUS-cYFP	GCTCTAGA ATGGTCCGTCCTGTAGAAAC	CGCGGATCCTCATTGTTTGCCTCCCTGCT
BD-ABI5	ATAGAATTCTCAGAGCGA	TAGAGATAGTAGTAGGTCT
BD-ABI5	ATAGAATTCTCAGAGCGA	TAGAGATAGTAGTATCT
BD-ABI5	ATAGAATTCCATAGAGGT	ATGTGATACTCTAGATT
BD-ABI5	ATAGAATTCCATAGAGGT	ATGTGATACTCTAGATT
BD-ABI5	ATAGAATTCCCCACTAAT	TCTAGAACCCTAATCCAATA
BD-ABI5	ATAGAATTCCCAGTGGAA	GTAAGATACTTCAACGTCA
ABI5-cYFP	ATAGAGCTCATGGTAACT	TAGGATACGACTCTGAAG

1.6 Bimolecular Fluorescence Complementation Assay (BiFC)

The cDNA sequences of N-terminal enhanced YFP (nYFP) comprising 173 amino acids and the C-terminal fragment (cYFP) comprising 64 amino acids were PCR-amplified and cloned into pFGC5941 to generate pFGC-nYFP and pFGC-cYFP, respectively (Kim et al., 2008). The coding sequence of ABI5 was fused with pFGC-cYFP to construct plasmid ABI5-cYFP, while the coding sequences of MYB75, MYB90, MYB113, MYB114, TT8, and TTG1 were introduced into pFGC-nYFP to create in-frame N-terminal fusions with nYFP. The resulting plasmids were introduced into *Agrobacterium tumefaciens* (strain GV3101) and tobacco infiltration was performed as described by Hu et al (Hu et al, 2014). Different plasmid combinations were co-infiltrated into tobacco leaves, which were placed in the dark at room temperature for 48 hours, then stained

with fluorescent dye. Infiltrated leaf mesophyll tissues were excised, mounted, and observed for YFP and DAPI fluorescence under a confocal laser scanning microscope (Olympus, Tokyo, Japan). Primers used for constructing various clones in this experiment are listed in Table 2.

Results

2.1 ABA Promotes Anthocyanin Accumulation in Arabidopsis Seedlings

To investigate the biological function of ABA hormone in regulating anthocyanin accumulation in Arabidopsis seedlings, this study sowed wild-type (WT) seeds of Columbia ecotype background on 1/2 MS medium containing different concentrations of ABA (0, 0.25, 0.50, and 0.75 $\text{mol} \cdot \text{L}^{1}$) to verify whether exogenous ABA could induce anthocyanin synthesis. We observed that increasing ABA concentrations could induce anthocyanin accumulation in plants, manifested as darkened coloration in seedling shoot tips, presenting a purple-red phenotype. The study further observed the phenotypes of ABA-treated seedlings under microscopy and photographed seedlings at different days. Images showed that with increasing ABA concentration gradients, wild-type seedlings grew more slowly while anthocyanin accumulation in seedling shoot tips became more pronounced (Figure 1 [Figure 1: see original paper]A). To further clarify the function of ABA in inducing anthocyanin synthesis, this study measured anthocyanin content in seedlings treated with different ABA concentrations (grown under light for 6 days). Results showed that wild-type seedlings had the lowest anthocyanin content on 1/2 MS plates without ABA and the highest anthocyanin content on plates containing 0.75 $\text{mol} \cdot \text{L}^{1}$ ABA, with anthocyanin content increasing as ABA concentration increased (Figure 1B). The measurement results were consistent with the observed trend in anthocyanin accumulation phenotypes. These results indicate that exogenous ABA treatment can promote anthocyanin biosynthesis in Arabidopsis seedlings, thereby promoting its accumulation in plants, with an increasing trend corresponding to higher ABA concentrations.

Figure 1. ABA induces anthocyanin synthesis in wild-type Arabidopsis seedlings. Significant differences were based on Student' s t-test: *, $P < 0.05$; **, $P < 0.01$.

2.2 ABA Induces Expression of Anthocyanin Synthesis-Related Genes

Since ABA can induce anthocyanin synthesis in Arabidopsis seedlings (Figure 1), does ABA regulate the transcriptional expression of anthocyanin synthesis-related genes? PAL, C4H, 4CL, CHS, CHI, F3H, DFR, LODX, and UF3GT are key structural genes for anthocyanin synthesis, while MYB75, MYB90, MYB113, MYB114, TT8, GL3, EGL3, TTG1, HY5, and TT2 are important regulatory genes controlling structural gene expression. All these genes participate in essential enzymatic reactions of the anthocyanin synthesis pathway, and their increased expression can activate the anthocyanin synthesis pathway in

Arabidopsis, thereby elevating anthocyanin content in plants (Dubos et al, 2008; Gonzalez et al, 2008; Petroni & Tonelli, 2011). To analyze whether ABA affects the expression of the aforementioned anthocyanin synthesis-related genes, we extracted total RNA from wild-type seedlings treated with different ABA concentrations, reverse-transcribed it into cDNA, and performed qRT-PCR experiments to detect the relative expression levels of all these anthocyanin synthesis structural and regulatory genes. Results showed that in ABA-treated plants, the expression of anthocyanin structural genes such as C4H, DFR, LODX, and UF3GT increased significantly, showing a positive correlation with increasing ABA concentration (Figure 2 [Figure 2: see original paper]). Additionally, the expression levels of regulatory genes including MYB75, MYB90, TT8, GL3, EGL3, TTG1, TT2, and HY5 were also induced by ABA (Figure 2). These experimental results demonstrate that ABA hormone can induce anthocyanin synthesis in Arabidopsis seedlings by upregulating the expression levels of anthocyanin synthesis-related genes.

Figure 2. ABA induces expression of anthocyanin synthesis related genes in wild-type Arabidopsis seedlings.

2.3 ABA-Induced Anthocyanin Synthesis Is Partially Dependent on MYB75 and TT8 Regulatory Proteins

Gene expression analysis results showed that ABA hormone can induce the expression of anthocyanin synthesis-related regulatory genes such as MYB75 and TT8 (Figure 2). Therefore, this study further analyzed through genetic experiments whether ABA-promoted anthocyanin synthesis depends on the normal function of these regulatory proteins. We collected wild-type seeds of Columbia ecotype background, anthocyanin-deficient mutant seeds myb-RNAi and tt8, and anthocyanin synthesis-enhanced plant seeds pap1-D from the same batch, and sowed them on 1/2 MS medium with 0.25 $\mathrm{mol \cdot L^{1}}$ ABA concentration. As shown in Figure 3 [Figure 3: see original paper]A, wild-type plant seedlings grown under light for 6 days displayed light purple coloration in shoot tips, whereas mutant myb-RNAi and tt8 seedlings showed no obvious color change, with entire shoot tips and leaves remaining green. Conversely, pap1-D plant shoot tips were markedly darker than those of wild-type plants. To further verify these results, we measured anthocyanin content in the relevant plants. Results indicated that anthocyanin content in mutant myb-RNAi and tt8 plants on 1/2 MS medium with 0.25 and 0.5 $\mathrm{mol \cdot L^{1}}$ ABA concentrations was significantly lower than in wild-type plants, while anthocyanin content in pap1-D plants was significantly higher than in wild-type plants (Figure 3B). Further analysis revealed that the ratio of anthocyanin content in wild-type plants on 1/2 MS medium with 0.5 $\mathrm{mol \cdot L^{1}}$ ABA to that on medium with 0 $\mathrm{mol \cdot L^{1}}$ ABA was significantly higher than the corresponding ratios in mutant myb-RNAi and tt8 plants (Figure 3C). Additionally, we examined the expression levels of anthocyanin synthesis-related structural genes (such as DFR, LDOX, and UF3GT). Total RNA was extracted from wild-type plants and relevant

mutant plants treated with 0 and 0.5 $mol \cdot L^{1}$ ABA, reverse-transcribed into cDNA, and subjected to qRT-PCR analysis to detect their relative expression levels. Analysis results showed that under ABA treatment, the relative expression levels of DFR, LODX, and UF3GT in mutant myb-RNAi and tt8 plants were significantly lower than in wild-type plants, while their expression levels in pap1-D plants were significantly higher than in wild-type plants (Figure 3D). In summary, this study suggests that ABA-promoted anthocyanin synthesis in Arabidopsis seedlings is partially dependent on the normal function of regulatory proteins such as MYB75 and TT8.

Figure 3. The ABA-induced anthocyanin synthesisinArabidopsis seedlings is dependent on TT8 and MYB75 transcription factors. Significant differences were based on Student' s t-test: *, $P < 0.05$; **, $P < 0.01$.

2.4 MYB75, TT8, and TTG1 Proteins Interact with ABI5 Transcription Factor

ABI5 transcription factor is one of the core transcription factors in the ABA signal transduction pathway, participating in the regulation of plant seed germination and seedling growth and development. To explore the molecular mechanism by which ABA hormone regulates anthocyanin synthesis in Arabidopsis seedlings, this study used the yeast two-hybrid system to detect interactions between relevant proteins. We constructed plasmid BD-ABI5 as bait and plasmids AD-MYB75, AD-MYB90, AD-MYB113, AD-MYB114, AD-TT8, and AD-TTG1 as potential prey. In the experiments, relevant bait and prey plasmids were co-transformed into yeast cells. Results showed that ABI5 strongly interacted with MYB75, MYB90, MYB113, MYB114, TT8, and TTG1 in yeast cells (Figure 4 [Figure 4: see original paper]). To further verify these interactions, this study additionally employed bimolecular fluorescence complementation assays for detection and analysis in planta. ABI5 was fused with the C-terminus of YFP protein to form ABI5-cYFP, while MYB75, MYB113, TT8, and TTG1 were each fused with the N-terminus of YFP protein to form MYB75-nYFP, MYB113-nYFP, TT8-nYFP, and TTG1-nYFP. When ABI5-cYFP was co-transformed with MYB75-nYFP, MYB113-nYFP, TT8-nYFP, or TTG1-nYFP into tobacco leaves, strong YFP fluorescence signals were observed (Figure 5 [Figure 5: see original paper]). Correspondingly, no fluorescence signals were observed in relevant control experiments (Figure 5). These experiments demonstrate that ABI5 transcription factor can interact with anthocyanin synthesis-related regulatory proteins such as MYB75, TT8, and TTG1 to form complexes.

Figure 4. ABA-related ABI5 transcription factor physically interacts with the anthocyanin synthesis-associated TT8, MYB75, and TTG1 proteins in yeast.

Figure 5. ABA-related ABI5 transcription factor physically interacts with the anthocyanin synthesis-associated TT8, MYB75, and TTG1 proteins in plant cells.

2.5 MYB75, TT8, and TTG1 Proteins Interact with the C-Terminal Domain of ABI5 Transcription Factor

Through the previous experimental analysis, we have established that ABI5 transcription factor can interact with anthocyanin synthesis-related regulatory proteins including MYB75, TT8, and TTG1 to form complexes. To further define the amino acid domains through which ABI5 transcription factor interacts with MYB75, TT8, and TTG1 regulatory proteins, this study generated several ABI5 truncations and constructed corresponding bait plasmids (Figure 6 [Figure 6: see original paper]). Yeast two-hybrid experiments revealed that the C-terminal 278 amino acids of ABI5 transcription factor (containing the conserved bZIP domain) are essential for its interaction with MYB75, TT8, and TTG1 regulatory proteins. As shown in Figure 6, when ABI5 transcription factor lacked the C-terminal 278 amino acids, it could not interact with MYB75, TT8, and TTG1 regulatory proteins. Conversely, when ABI5 transcription factor lacked the N-terminal 164 amino acids, it could still interact with MYB75, TT8, and TTG1 regulatory proteins. These results indicate that the C-terminal amino acid domain of ABI5 mediates its interaction with regulatory proteins such as MYB75, TT8, and TTG1.

Figure 6. C-terminal region of ABI5 transcription factor physically interacts with the anthocyanin synthesis-associated TT8, MYB75, and TTG1 proteins in yeast.

2.6 ABI5 Transcription Factor Interacts with the N-Terminus of MYB75, MYB113, and TTG1, and the C-Terminus of TT8

Similarly, this study further employed yeast two-hybrid experiments to analyze the amino acid domains of MYB75, TT8, and TTG1 regulatory proteins that interact with ABI5 transcription factor. We truncated MYB75, MYB113, TT8, and TTG1 proteins into N-terminal and C-terminal fragments and constructed corresponding prey plasmids (Figure 7 [Figure 7: see original paper]). Yeast two-hybrid experiments revealed that the N-terminus of MYB75, MYB113, and TTG1 is crucial for their interaction with ABI5 transcription factor. When the N-terminus of MYB75, MYB113, and TTG1 was deleted, they could not interact with ABI5 (Figure 7). Conversely, the C-terminus of TT8 protein is essential for its interaction with ABI5 transcription factor; when the C-terminus of TT8 was deleted, it could not interact with ABI5 (Figure 7).

Figure 7. ABI5 transcription factor physically interacts with the N-terminal regions of MYB75 and TTG1 in yeast.

Conclusion and Discussion

In the core ABA signaling pathway, ABA binds to receptor proteins such as PYR/PYL/RCAR, thereby inducing conformational changes that stabilize the interaction between ABA receptors and PP2C phosphatases, leading to PP2C

inactivation and derepression of SNF1-related protein kinases (SnRK2). Subsequently, SnRK2 phosphorylates and activates many downstream signaling components, such as ABI5 transcription factor, ion channels, and NADPH oxidases, to achieve abiotic stress tolerance (Fujii & Zhu, 2009; Park et al, 2009; Bauer et al, 2013; Yu & Xie, 2017). Additionally, recent studies have shown that ABA hormone can promote anthocyanin synthesis and accumulation in certain plant fruits, thereby affecting fruit quality (Koyama et al, 2010; Jia et al, 2011; Shen et al, 2014; An et al, 2018). For example, ABA functions as a signaling molecule regulating anthocyanin synthesis to promote sweet cherry fruit ripening (Shen et al, 2014). Similarly, in grape berry skins and cell suspension cultures, exogenous ABA application can induce the expression of multiple anthocyanin synthesis structural and regulatory genes, promoting anthocyanin accumulation (Jeong et al, 2004; Gagné et al, 2011). In the study by An et al, they found that apple MYB transcription factor MdMYB1 activates anthocyanin biosynthetic gene expression in response to ABA (An et al, 2018). Loreti et al discovered that ABA can enhance sucrose-induced anthocyanin synthesis and accumulation in Arabidopsis, indicating that ABA and sucrose have a synergistic activating effect on anthocyanin synthesis (Loreti et al, 2008). The few existing studies mentioned above suggest an intrinsic link between ABA and plant pigments or fruit color, but the biological function and molecular mechanism of ABA in regulating anthocyanin synthesis in Arabidopsis remain to be further investigated.

This study used Arabidopsis as experimental material to investigate the inductive effect of ABA on anthocyanins in Arabidopsis seedlings through genetic and molecular biology-related experimental methods. The study found that ABA treatment can promote anthocyanin accumulation in wild-type Arabidopsis seedlings (Figure 1A), and anthocyanin content measurement results verified the same conclusion (Figure 1B). By analyzing the expression of related genes, experimental results showed that in ABA-treated wild-type plants, the expression of anthocyanin structural genes such as C4H, DFR, LODX, and UF3GT increased significantly, showing a positive correlation with increasing ABA concentration (Figure 2). In addition, the expression levels of regulatory genes including MYB75, MYB90, TT8, GL3, EGL3, TTG1, TT2, and HY5 were also induced by ABA (Figure 2). These experimental results demonstrate that ABA hormone can induce anthocyanin synthesis in Arabidopsis seedlings by upregulating the expression levels of anthocyanin synthesis-related genes. This study further treated anthocyanin synthesis-related mutant plants such as myb-RNAi, tt8, and pap1-D with exogenous ABA hormone. Phenotypic analysis revealed that anthocyanin content in mutant myb-RNAi and tt8 plants was significantly lower than in wild-type plants, while anthocyanin content in pap1-D plants was significantly higher than in wild-type plants (Figure 3). Consistently, under ABA treatment, the relative expression levels of structural genes such as DFR, LODX, and UF3GT in mutant myb-RNAi and tt8 plants were significantly lower than in wild-type plants, while their expression levels in pap1-D plants were significantly higher than in wild-type plants (Figure 3D). In summary, this study suggests that ABA-induced anthocyanin synthesis in Arabidopsis

seedlings likely requires regulatory proteins such as MYB75 and TT8.

ABI5 transcription factor is one of the key transcription factors in the plant ABA signal transduction pathway, participating in the regulation of physiological processes such as seed germination and seedling growth (Finkelstein, 2006; Pan et al, 2018). It can also mediate crosstalk between different signaling pathways through interactions with other proteins (Yu et al, 2015). So does ABA affect anthocyanin biosynthesis by mediating interactions between ABI5 and anthocyanin synthesis regulatory factors (such as MYB75, TT8, and TTG1)? Experimental studies revealed that ABI5 transcription factor can indeed interact with important regulatory proteins related to anthocyanin synthesis, such as MYB-type MYB75, MYB90, MYB13 and MYB114, bHLH-type TT8, and WD40-type TTG1 (Figures 4 and 5). We also performed truncation experiments to verify the specific amino acid domains through which ABI5 interacts with anthocyanin synthesis-related regulatory proteins. Experimental results indicated that the C-terminal 278 amino acids of ABI5 transcription factor (containing the conserved bZIP domain) are crucial for its interaction with regulatory proteins such as MYB75, TT8, and TTG1. As shown in Figure 6, when ABI5 transcription factor lacked the C-terminal 278 amino acids, it could not interact with MYB75, TT8, and TTG1 regulatory proteins. Similarly, this study further clarified that ABI5 transcription factor interacts with the N-terminus of MYB75, MYB113, and TTG1, and the C-terminus of TT8. Based on these experimental results, this study proposes that the bZIP-type transcription factor ABI5 in the ABA signaling pathway can interact with anthocyanin synthesis-related proteins such as MYB75, TT8, and TTG1 to form complexes. Future detailed analysis of ABI5 transcription factor-related mutants or overexpressing transgenic plants is expected to further clarify the function and possible molecular mechanism of ABI5 transcription factor in regulating anthocyanin synthesis. This study preliminarily reveals the intrinsic link between the ABA signaling pathway and anthocyanin synthesis, and further research will uncover the regulatory mechanism of ABA-induced anthocyanin synthesis in Arabidopsis seedlings.

References

AN JP, YAO J F, XU RR, et al, 2018. Apple bZIP transcription factor MdbZIP44 regulates abscisic acid-promoted anthocyanin accumulation[J]. Plant Cell Environ, 41(11):2678-2692.

BAUDRY A, HEIM M A, DUBREUCQ B, et al, 2004. TT2, TT8, and TTG1 synergistically specify the expression of BANYULS and proanthocyanidin biosynthesis in Arabidopsis thaliana[J]. Plant J, 39(3):366-380.

BAUER H, ACHE P, LAUTNER S, et al, 2013. The stomatal response to reduced relative humidity requires guard cell-autonomous ABA synthesis [J]. Curr Biol, 23(1):53-57.

CHEN JX, MAO L C, MI HB, et al, 2016. Involvement of abscisic acid

in postharvest water-deficit stress associated with the accumulation of anthocyanins in strawberry fruit[J]. Postharvest Biol Technol, 111:99-105.

CHEN R, JIANG H L, LI L, et al, 2012. The Arabidopsis mediator subunit MED25 differentially regulates jasmonate and abscisic acid signaling through interacting with the MYC2 and ABI5 transcription factors[J]. Plant Cell, 24(7):2898-2916.

DEJONGHE W, OKAMOTO M, CUTLER S R, 2018. Small molecule probes of ABA biosynthesis and signaling[J]. Plant Cell Physiol, 59(8):1490-1499.

DENG Y X, LU S F, 2017. Biosynthesis and regulation of phenylpropanoids in plants[J]. Crit Rev Plant Sci, 36(4):257-290.

DUBOS C, LE GOURRIEREC J, BAUDRY A, et al, 2008. MYBL2 is a new regulator of flavonoid biosynthesis in Arabidopsis thaliana[J]. Plant J, 55(6):940-953.

ESCARAY F J, PASSERI V, PEREA-GARCIA A, et al, 2017. The R2R3-MYB TT2b and the bHLH TT8 genes are the major regulators of proanthocyanidin biosynthesis in the leaves of Lotus species[J]. Planta, 246(2):243-261.

FAN XP, FAN BH, WANG YX, et al, 2016. Anthocyanin accumulation enhanced in Lc-transgenic cotton under light and increased resistance to bollworm[J]. Plant Biotechnol Rep, 10: 1-11.

FANG H, DONG Y, YUE X, et al, 2019. The B-box zinc finger protein MdBBX20 integrates anthocyanin accumulation in response to ultraviolet radiation and low temperature[J]. Plant Cell Environ, (1365-3040 (Electronic)):T - aheadofprint.

FINKELSTEIN RR, 2006. Studies of abscisic acid perception finally flower[J]. Plant Cell, 18(4):786-791.

FUJII H, ZHU JK, 2009. Arabidopsis mutant deficient in 3 abscisic acid-activated protein kinases reveals critical roles in growth, reproduction, and stress[J]. Proc Natl Acad Sci USA, 106(20):8380-8385.

GAGNE S, CLUZET S, MERILLON J-M, et al, 2011. ABA initiates anthocyanin production in grape cell cultures[J]. J Plant Growth Regul, 30 (1): 1-10.

GONZALEZ A, ZHAO MZ, LEAVITT JM, et al, 2008. Regulation of the anthocyanin biosynthetic pathway by the TTG1/bHLH/Myb transcriptional complex in Arabidopsis seedlings[J]. Plant J, 53(5):814-827.

HIRATSUKA S, ONODERA H, KAWAI Y, et al, 2001. ABA and sugar effects on anthocyanin formation in grape berry cultured in vitro[J]. Sci Hortic, 90(1-2):121-130.

HU YR, YU DQ, 2014. BRASSINOSTEROID INSENSITIVE2 interacts with ABSCISIC ACID INSENSITIVE5 to mediate the antagonism of brassinos-

teroids to abscisic acid during seed germination in Arabidopsis[J]. Plant Cell, 26(11):4394-4408.

JEONG ST, GOTO-YAMAMOTO N, KOBAYASHI S, et al, 2004. Effects of plant hormones and shading on the accumulation of anthocyanins and the expression of anthocyanin biosynthetic genes in grape berry skins[J]. Plant Sci, 167 (2): 247-252.

JIA H F, CHAI YM, LI CL, et al, 2011. Abscisic acid plays an important role in the regulation of strawberry fruit ripening[J]. Plant Physiol, 157(1):188-199.

JIANG Y M, JOYCE D C, 2003. ABA effects on ethylene production, PAL activity, anthocyanin and phenolic contents of strawberry fruit[J]. Plant Growth Regulation, 39(2):171-174.

KOORNNEEF M, 1981. The complex syndrome of ttg mutanis[J]. Arabidopsis Inf Serv, 18(1981):

KOYAMA K, SADAMATSU K, GOTO-YAMAMOTO N, 2010. Abscisic acid stimulated ripening and gene expression in berry skins of the Cabernet Sauvignon grape[J]. Funct Integr Genomics, 10(3): 367-381.

LIANG J, HE JX, 2018. Protective role of anthocyanins in plants under low nitrogen stress[J]. Biochem Biophys Res Commun, 498(4):946-953.

LORETI E, POVERO G, NOVI G, et al, 2008. Gibberellins, jasmonate and abscisic acid modulate the sucrose-induced expression of anthocyanin biosynthetic genes in Arabidopsis[J]. New Phytol, 179(4):1004-1016.

MA D, Constabel CP, 2019. MYB repressors as regulators of phenylpropanoid metabolism in plants [J]. Trends Plant Sci. 24(3):275-289.

MAIER A, SCHRADER A, KOKKELINK L, et al, 2013. Light and the E3 ubiquitin ligase COP1/SPA control the protein stability of the MYB transcription factors PAP1 and PAP2 involved in anthocyanin accumulation in Arabidopsis[J]. Plant J, 74(4):638-651.

NAKASHIMA K, YAMAGUCHI-SHINOZAKI K, 2013. ABA signaling in stress-response and seed development[J]. Plant Cell Rep, 32(7):959-970.

PAN J, WANG H, HU Y, et al, 2018. Arabidopsis VQ18 and VQ26 proteins interact with ABI5 transcription factor to negatively modulate ABA response during seed germination[J]. Plant J, 95(3): 529-544.

PARK S-Y, FUNG P, NISHIMURA N, et al, 2009. Abscisic acid inhibits type 2C protein phosphatases via the PYR/PYL family of START proteins[J]. Science, 324(5930):1068-1071.

PEIFFER DS, WANG LS, ZIMMERMAN NP, et al, 2016. Dietary consumption of black raspberries or their anthocyanin constituents alters innate immune cell trafficking in esophageal cancer[J]. Cancer Immunol Res, 4(1):72-82.

PETRONI K, TONELLI C, 2011. Recent advances on the regulation of anthocyanin synthesis in reproductive organs[J]. Plant Sci, 181(3):219-229.

ROWAN DD, CAO MS, LIN-WANG K, et al, 2009. Environmental regulation of leaf colour in red 35S:PAP1 Arabidopsis thaliana[J]. New Phytol, 182(1):102-115.

SHEN XJ, ZHAO K, LIU LL, et al, 2014. A role for PacMYBA in ABA-regulated anthocyanin biosynthesis in red-colored sweet cherry cv. Hong Deng (Prunus avium L.). [J]. Plant Cell Physiol, 55(5):862-880.

SHIN DH, CHO M, CHOI MG, et al, 2015. Identification of genes that may regulate the expression of the transcription factor production of anthocyanin pigment 1 PAP1/MYB75 involved in Arabidopsis anthocyanin biosynthesis[J]. Plant Cell Rep, 34(5):805-815.

TANAKA Y, SASAKI N, OHMIYA A, 2008. Biosynthesis of plant pigments: anthocyanins, betalains and carotenoids[J]. Plant J, 54(4):733-749.

WEI J Y, WU H J, ZHANG HQ, et al, 2018. Anthocyanins inhibit high glucose-induced renal tubular cell apoptosis caused by oxidative stress in db/db mice[J]. J Mol Med, 41(3):1608-1618.

XIE Y, TAN HJ, MA ZX, et al, 2016. DELLA proteins promote anthocyanin biosynthesis via sequestering MYBL2 and JAZ suppressors of the MYB/bHLH/WD40 complex in Arabidopsis thaliana[J]. Mol Plant, 9(5):711-721.

XU W J, GRAIN D, BOBET S, et al, 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[J]. New Phytol, 202(1):132-144.

YU FF, WU YR, XIE Q, 2015. Precise protein post-translational modifications modulate ABI5 activity[J]. Trends Plant Sci, 20(9):569-575.

YU FF, XIE Q, 2017. Non-26S proteasome endomembrane trafficking pathways in ABA signaling[J]. Trends Plant Sci, 22(11):976-985.

ZHANG BP, SCHRADER A, 2017. TRANSPARENT TESTA GLABRA 1-Dependent Regulation of flavonoid biosynthesis[J]. Plants (Basel), 6(4), pii: E65.

ZHAO D, TAO J, 2015. Recent advances on the development and regulation of flower color in ornamental plants[J]. Front Plant Sci, 6:261.

Note: Figure translations are in progress. See original paper for figures.