VIGS approach reveals the modulation of anthocyanin biosynthetic genes by CaMYB in chili pepper leaves

Zhen Zhang1†, Da-Wei Li1,2†, Jing-Hao Jin1, Yan-Xu Yin1, Huai-Xia Zhang1, Wei-Guo Chai3 and Zhen-Hui Gong1,2*

1 College of Horticulture, Northwest A&F University, Yangling, China, 2 State Key Laboratory for Stress Biology of Arid Region Crop, Northwest A&F University, Yangling, China, 3 Institute of Vegetables, Hangzhou Academy of Agricultural Sciences, Hangzhou, China

*Correspondence: Zhen-Hui Gong, College of Horticulture, Northwest A&F University, Yangling, China, zhongg@nwsuaf.edu.cn
†These authors have contributed equally to this work.

The purple coloration of pepper leaves arises from the accumulation of anthocyanin. Three regulatory and 12 structural genes have been characterized for their involvement in the anthocyanin biosynthesis. Examination of the abundance of these genes in leaves showed that the majority of them differed between anthocyanin pigmented line Z1 and non-pigmented line A3. Silencing of the R2R3-MYB transcription factor CaMYB in pepper leaves of Z1 resulted in the loss of anthocyanin accumulation. Moreover, the expression of multiple genes was altered in the silenced leaves. The expression of MYC was significantly lower in CaMYB-silenced leaves, whereas WD40 showed the opposite pattern. Most structural genes including CHS, CHI, F3H, F3’5’H, DFR, ANS, UFGT, and GST were repressed in CaMYB-silenced foliage with the exception of PAL, C4H, and 4CL. These results indicated that MYB plays an important role in the regulation of anthocyanin biosynthetic related genes. Besides CaMYB silenced leaves rendered more sporulation of Phytophthora capsici Leonian indicating that CaMYB might be involved in the defense response to pathogens.

Keywords: Capsicum annuum L., purple leaves, CaMYB, virus-induced gene silencing, anthocyanin biosynthesis, P. capsici resistance

Introduction

Flavonoids are secondary plant metabolites that fulfill numerous physiological functions such as pigmentation, health-promoting components, and protection against damage by ultraviolet light and phytopathogens (Lepiniec et al., 2006; Davies et al., 2012). Anthocyanins are soluble flavonoids pigments and its biosynthetic pathway has been elucidated. The enzymes involved in the pathway include phenylalanine ammonia-layse (PAL), cinnamate 4-hydroxylase (C4H), 4-coumarate: CoA ligase (4CL), chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F3H), flavonoid 3’,5’-hydroxylase (F3’5’H), dihydroflavonol 4-reductase (DFR), anthocyanin synthase (ANS), and UDP-glucose: flavonoid 3-glucosyltransferase (UFGT) (Aza-González et al., 2013; Figure 1). Also, an anthocyanin permease (ANP) and a glutathione S-transferase (GST) have been suggested to participate in sequestration of anthocyanin in the vacuole (Mathews et al., 2003).

A protein complex consisting of a MYB transcription factor, a basic helix-loop-helix (bHLH) protein and WD40 protein (MBW complex) has been proposed to regulate the expression of
The appearance of purple color is due to the abundance of anthocyanin. The anthocyanin biosynthetic pathway has been previously investigated in Petunia (Quattrocchio et al., 1999). Stommel et al. (2009) evaluated the expression of anthocyanin biosynthetic pathway in pepper leaves. In addition, the regulation of structural genes in Capsicum and also provided incomplete data on the expression of rest genes in anthocyanin biosynthetic pathway in chili pepper, especially in leaves.

Virus-induced gene silencing (VIGS) is a well-established technology in many species to characterize the function of genes, but it has not been applied in studying the anthocyanin biosynthetic pathway in pepper foliage. In this study, we explored the VIGS application in studying the regulation of anthocyanin biosynthetic pathway in pepper leaves. CaMYB was knocked down in purple pepper line Z1 using the tobacco rattle virus (TRV) based gene silencing technique (Wang et al., 2013). We examined the anthocyanin content and expression of anthocyanin regulatory gene (CaMYB, MYC, and WD40) and 12 structural genes in silenced leaves. Based on these results, we propose that CaMYB is a key factor in anthocyanin biosynthesis pathway in pepper leaves. In addition, CaMYB may be involved in the defense response to pathogens in the purple pepper line.

Materials and Methods

Plant Materials and Growth Conditions

The purple pepper line Z1 and green pepper line A3 were provided by College of Horticulture, Northwest A&F University. Z1 is an anthocyanin pigmented cultivar having purple stem and immature fruit under regular growth conditions. Its leaf is purple under the sixth position, and interspersed with green portions above the sixth position. Pepper seeds were germinated on damp sponge at 28°C in the dark for 2 days. Sprouted seedlings were sown into plastic trays containing steam-sterilized growing media in the illumination incubator at day/night temperatures of 23/18°C with relative humidity of 60% and a 16-h light and 8-h dark photoperiod.

VIGS of CaMYB in Pepper

The pTRV vector and Agrobacterium tumefaciens strain GV3101 were prepared for VIGS (Wang et al., 2013; Ji et al., 2014). We cloned CaMYB gene in purple pepper Z1 and found that the sequence in the coding region of CaMYB is the same as that deposited in the GenBank (AJ608992). Multiple sequence alignment was performed using MEGA6 to reveal the homology among genes involved in anthocyanin biosynthesis from different species. Online software siRNA-scan1 was used to avoid off-target silencing (Xu et al., 2006). A 332-bp fragment harboring a conserved and a non-conserved region of CaMYB was cloned into the pTRV2 vector by One Step Cloning Kit to yield the pTRV2: CaMYB construct. Empty vector (pTRV: 00) was served as a negative control (NC). The pTRV2: PDS, which contains the phytoene desaturase sequence to induce a photo-bleaching phenotype, was used as a positive control (Figure 2). The primers were shown in Table 1. A 10-mL culture of each strain was incubated for 24–36 h at 28°C in LB broth containing 50 mL⁻¹ kanamycin, 50 mL⁻¹ Gentamicin, and 50 mL⁻¹ rifampicin. The primary culture was resuspended into Induction Medium containing 50 mL⁻¹ kanamycin, 20 mg

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1http://bioinfo2.noble.org/RNAiScan/RNAiScan.htm
ml−1 rifampicin, 50 ml−1 Gentamicin, 200 μM acetylsyringone, and was shacked at 28°C for 20–24 h according to Velasquez et al. (2009). The cells were precipitated by centrifugation for 10 min at 3,000 × g and resuspended in the same volume of Agrobacterium infiltration buffer containing 10 mM MgCl2 and 10 mM MES at pH 5.7 till the OD600 reaching 0.5. A. tumefaciens GV3101 containing pTRV1 was mixed with GV3101 containing either pTRV2:00 or pTRV2:CaMYB in a 1:1 ratio. After mixing them, 400 μM acetylsyringone was added.

The plants with the fourth leaf fully expanded were used for VIGS. We punched holes with a needle on both sides of main veins. Then the leaves were infiltrated with a 1 ml syringe without needle. The inoculated plants were grown at 18°C for 48 h in 60% relative humidity under dark and then placed in a growth room at 25°C with a 16-h light/8-h dark photoperiod.

**Pathogen Preparation**

Preparation and identification of Phytophthora capsici inocula was conducted according to Zhang et al. (2013b). The strain PC of P. capsici was grown on potato dextrose agar (PDA) medium in darkness at 28°C. Twenty-one days after the induction of TRV-mediated gene silencing, detaching leaves from non-infiltrated, pTRV2:00 and pTRV2:CaMYB plants were exposed to an 8 mm diameter mycelium plug from P. capsici. Then the leaves were placed in a petri dish containing moist filter paper followed by incubation in the dark at 28°C for 3 days.

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**TABLE 1 | List of primers used in this study.**

| Abbreviation | Forward Primer (5’ to 3’) | Reverse Primer (5’ to 3’) |
|--------------|---------------------------|--------------------------|
| VIGS-CaMYB   | GTAAGGTTACCGAATTGCAGTTGAGGATGCGTGC | AGCTCTGGATCCGATCGTTCTATGCTGAGGATGCGTGC |
| RT-CaMYB     | AGATTGCCGGGAAAGAACAGCAAGC | TTTCACCTAGAGCGCCGAGG |
| PAL          | ATGGATTCTTTCGCAAGATCAGCT | GCTCCTGGTCTGATGAGG |
| C4H          | GTAATTCATTCTGCTGAG | CCTTCTGATGAGG |
| 4CL          | CTGGACCAATGGTGCACGCGAT | GTTACGGGGGGGCAAGCAG |
| MVD          | GAAAGGAGAAAGAACACAC | GGAAGGACCAAGTCAGG |
| WDA          | GTGACACGGATGCGCTG | GCAACACACAGGCAAGG |
| CBS          | GTGACCGGTAGCTTGGTTG | GCCACCGGGGGCAGCC |
| PGL          | GTGACGGACAGGTGTATG | GCAAGGAGGAGG |
| DFS          | GATGACGGATGTGCTG | GCCACCGGGGGCAGCC |
| DFR          | AATGGTCCAGGCTCTCAGCAATG | GTGACGGGAGGAGG |
| ANS          | CAAATGCCCTTACAAAGAAG | GACAGGACAGGCTCTG |
| UFI          | GCGTACATGCCTCTGAG | GACAGGACAGGCTCTG |

The restriction enzyme sites added at 5’ end of primers are underlined.
RNA Analysis

The total RNA was extracted from the leaves of the inoculated plants of Z1 at the same position as the bleaching leaves of pTRV2: PDS plants using the RNA simple kits (Tiangen Biotech, Beijing, China). RNA samples (1 µg) were reverse transcript into complementary DNA (cDNA) using the High Fidelity PrimeScript RT-PCR kit (TaKaRa) according to the protocol. For extraction of anthocyanin, 5 g leaves of Z1 line were homogenized in a mixture of HCl : water : MeOH, 1:3:16 (v/v/v; Gould et al., 2000). The extracts were centrifuged. High-performance liquid chromatography (HPLC) analysis of anthocyanin was carried out with a HP1200 Liquid Chromatograph equipped with a diode array detector (Agilent Technology, Palo Alto, CA, USA) using the method as described by Stomme et al. (2009). The anthocyanin was identified by comparing both the retention time and the absorption spectrum with those of a commercial standard (delphinidin-3-p-coumaroyl-rutinoside-5-glucoside).

Results

Phylogenetic Analyses

CaMYB is clustered with the R2R3 MYB transcription factors that are involved in the regulation of anthocyanin biosynthesis in other plant species, such as *Arabidopsis*, maize, petunia, tobacco, and eggplant (Figure 3). CaMYB is closely related to the subgroup 6 MYBs in *Arabidopsis* (Dubos et al., 2010), and the nearest MYB is SmnMYB2 in eggplant which belongs to the Solanaceae. In pepper genome, there are six genes showing high similarity to CaMYB, and the similarity is heterogeneous ranging from 49 to 67% at the protein level.

Expression of Biosynthetic Genes in both Anthocyanin-Pigmented and Non-Pigmented Pepper

The expression of genes involved in the anthocyanin biosynthetic pathway was monitored in leaves of pepper line A3 (non-anthocyanin pigmented) and Z1 (anthocyanin pigmented; Figure 4). These genes included phenylpropanoid pathway genes (*PAL*, *C4H*, *4CL*, *CHS*, *CHI*, *F3H*, *F3′H*, *DFR*, *ANS*, *UFGT*, *ANP*, and *GST*) and regulatory genes (*CaMYB*, *MYC*, and *WD40*). The transcription level of *CaMYB* was undetectable in A3, but maintained a high level in Z1. The expression of *MYC* was extremely low in A3 as opposed to Z1. On the contrary, the expression of *WD40* was lower in Z1 than in A3. Structural genes could be classified into three groups on the basis of expression pattern. Nine genes including *PAL*, *C4H*, *F3H*, *F3′H*, *DFR*, *ANS*, *UFGT*, *ANP*, and *GST* showed higher expression in Z1 than in A3, whereas *CHI* showed higher expression in A3 than in Z1. The transcript level of *4CL* and *CHS* was similar between A3 and Z1.

Silencing of CaMYB Reduced the Accumulation of Anthocyanins

As an indication of successful silencing, *CaPDS*-silenced plants showed photo bleaching (Figure 5A). Z1 leaves infiltrated with pTRV2: 00 (NC) appeared to be less purple colored compared with non-infiltrated Z1 leaves (CK). Z1 leaves infiltrated with pTRV2: CaMYB exhibited green coloration, and *CaMYB* abundance was significantly decreased in *CaMYB*-silenced plants compared to the NC (Figure 5B).

Anthocyanin from leaves of CK, NC plants, *CaMYB*-silenced plants and *CaPDS*-silenced plant were extracted and measured by HPLC (Figure 6). The color intensity of the extracted solutions was different among four samplings (Figure 6B). *CaMYB*-silenced foliage with a green phenotype accumulated far less anthocyanin; a 31- and 37-fold increase of anthocyanin.
accumulation was detected in NC and in CK, respectively, compared to that in CaMYB-silenced leaves (Figure 6C).

**Expression of Anthocyanin Biosynthetic Genes in CaMYB-Silenced Plants**

Real-time quantitative PCR was carried out to examine the transcription of anthocyanin biosynthetic genes in leaves of CK, NC and CaMYB-silenced plants (Figure 7). The expression of MYC was significantly lower in CaMYB-silenced leaves than in CK and NC. In contrast, WD40 showed higher expression in CaMYB-silenced leaves than in CK and NC. The transcription of nine structural genes including CHS, CHI, F3H, F3′5′H, DFR, ANS, UFGT, ANP, and GST were repressed in CaMYB-silenced foliage, while PAL, C4H, and 4CL maintained stable and even higher expression in silenced foliage.

**Detached Leaves Inoculation Assays**

To determine the role of CaMYB in the basal defense, detached leaves from CK, NC, and pTRV2: CaMYB plants were exposed to an 8 mm diameter mycelium plug from *P. capsici* 21 days after...
FIGURE 5 | Silencing efficiency of CaMYB in pepper plants using a tobacco rattle virus (TRV)-based Virus-induced gene silencing (VIGS) system. (A) Phenotypes of pepper plants 21 days after infiltration of different vectors. a: non-infiltrated control (CK); b: negative control (NC) plants (PTRV2:00); c: CaMYB-silenced plant (PTRV2:CaMYB); d: PDS-silenced plant (PTRV2:PDS). (B) Quantitative real-time PCR analysis of CaMYB expression levels in leaves of CK, NC plants and CaMYB-silenced plants. Ten silencing plants were analyzed (numbered 1–10). Error bars represent mean ± SD for three technical replicates for each plant. Bars with different lower case letters in each group indicate significant differences using Duncan’s multiple range test at \( p < 0.05 \).

FIGURE 6 | Variation of anthocyanin accumulation in leaves infiltrated with different vectors. (A) Leaves were detached from: a: non-infiltrated control (CK); b: NC plants (PTRV2:00); c: CaMYB-silenced plant (PTRV2:CaMYB); d: PDS-silenced plant (PTRV2:PDS). (B) Extracted solutions from the corresponding four samplings. (C) Anthocyanin content of the four samplings by High-performance liquid chromatography (HPLC). Means ± SD (\( n = 3 \)).

| Tissue | CK | pTRV2:00 | pTRV2:CaMYB | pTRV2:PDS |
|--------|----|----------|-------------|-----------|
| Foliage | 374.9 ± 29.3 | 313.3 ± 26.2 | 10.1 ± 2.4 | 130.7 ± 20.9 |
FIGURE 7 | Expression of anthocyanin biosynthetic genes in foliage infiltrated with different vectors. CK, non-infiltrated control leaf; NC, negative control leaf (PTRV2:00); SP, CaMYB-silenced plants. Error bars represent the mean ± SD of three independent biological replicates. Bars with different lower case letters in each group indicate significant differences using Duncan’s multiple range test at \( p < 0.05 \).

the performance of the VIGS. Water-soaked lesions occurred 40 h after inoculation on the silenced Z1 leaves, and 8 h later the whole leaves became water-soaked (Figure 8). In contrast, water-soaked lesions were visible on CK and the NC plants at 72 h after inoculation and the symptoms developed slowly.

Discussion

It has been reported that purple pepper leaves can enhance the photosynthesis and alleviate the oxidative stress. The anthocyanin epidermal layer may limit photo-inhibitory effects on PSII, the formation of reactive oxygen species and oxidative cellular damages of Tradescantia pallida leaves, supporting the role of anthocyanin pigments in the regulation of photosynthesis for excess absorbed light irradiance (Dewez and Perreault, 2013). Also, pepper seedlings can be consumed fresh and the purple pigments adds nutritive value. Thus it is worthwhile to expand the understanding of anthocyanin biosynthetic pathway in leaves.

Phylogenetic analysis indicates that CaMYB may execute similar functions as that in other plant species (Figure 2). In Arabidopsis, plants harboring a silencing construct targeting MYBs showed decreased MYB gene expression and visible depletion of pigmentation (Gonzalez et al., 2008). In this study, silencing of CaMYB resulted in considerable loss of anthocyanin accumulation in pepper leaves (Figures 5 and 6). Likewise, disabling of CaMYB in pepper fruits reduced the anthocyanin content with lower efficiency (Aguilar-Barragán and Ochoa-Alejo, 2014) in comparison with the results from leaves in this study. These evidence supports that MYBs are essential
for anthocyanin production in various organs, including pepper leaves.

The transcription levels of CaMYB and MYC were undetectable in non-pigmented foliage (A3), but an increase in expression of WD40 was observed in A3 compared with Z1 (Figure 4), which is inconsistent with the results from Stommel et al. (2009) probably owing to different lines used in the two studies. The expression of MYC was significantly lower in CaMYB-silenced leaves, whereas WD40 showed the opposite pattern (Figure 7). It has been noted that R2R3-MYB gene alone is capable of regulating the expression of the bHLH (MYC) partner (Baudry et al., 2006; Albert et al., 2014). MYB may induce the expression of MYC, co-regulating the accumulation of anthocyanin. WD40 transcription factor serves as a scaffold that allows the interactions between different MYC and MYB proteins, such as Transparent Testa Glabra1 (TTG1) in Arabidopsis thaliana (Gonzalez et al., 2008). In Petunia sp., AN11 (WD40) protein activated AN2 and AN4 (MYB domain proteins; Quattrocchio et al., 1993; Spelt et al., 2000). More direct evidence is needed to clarify the mechanisms of MBW complex in the anthocyanin biosynthesis in pepper foliage.

Based on the different expression mode of A (CaMYB) and four anthocyanin biosynthetic related genes in both anthocyanin-pigmented and non-pigmented pepper lines, Borovsky et al. (2004) concluded that the early genes (CHS, CHI) in the flavonoid pathway are regulated independently of A, while expression of the late genes (DFR, ANS) is A-dependent. In this study, the expression modes of the anthocyanin biosynthesis related genes in Z1 and A3 indicated that the transcription of LBGs and EBGs except CHS and CHI may be dependent on CaMYB (Figures 1 and 4). This observation was further confirmed by VIGS showing that the expression of flavonoid pathway genes were altered in CaMYB-silenced plants (Figure 7). The expressions of CHS, CHI, F3′5′H, DFR, and 3GT (UFGT) genes were decreased in pepper fruits transformed with the TRV2-MYB construct (Aquilar-Barragán and Ochoa-Alejo, 2014), which also indicated that CHS, CHI were dependent on the expression of MYB (CaMYB). The transcription of F3H did not differ between fruits treated with either the empty TRV2 or TRV2-MYB and the normal fruits, indicating that F3H might be modulated in a different manner in pepper leaf from that in pepper fruits.

It was noted that the flavonoid pathway genes were effectively suppressed, while phenylproanoid pathway genes showed a stable (PAL) or an even higher (C4H, 4CL) expression level in CaMYB-silenced leaf (Figure 7). Presumably CaMYB directly activates transcription of CHS, which is the first gene with lower expression in the anthocyanin biosynthetic pathway in CaMYB-silenced plants. Espley et al. (2007) identified the interaction signature...
motif in MdMYB10, showing that the protein interacts with bHLH proteins to activate transcription of DFR in apple (Espley et al., 2007). In A. thaliana, ternary complex (MYB–MYC–WD40) is important in stimulating anthocyanin formation by activating transcription of DFR and ANS along with trichome formation (Nemie-Feyissa et al., 2014). Here, VIGS provides a useful tool to preliminary screen the target sites of transcription factors, however, sophisticated experiments are needed to identify the authentic targets of transcription factors.

CaMYB-silencing plants rendered susceptibility to P. capsici (Figure 8). It has been observed that anthocyanins often served as the first line of defense against pathogen attacks (Wang et al., 2011). Disruption of Del/ROS1 partially reduced the accumulation of anthocyanins on purple tomato fruits, and areas with less anthocyanin were more susceptible to Botrytis cinerea than purple areas (Zhang et al., 2013a). In potato, common scab resistance was correlated with the expression of MYB and three bHLH genes, indicating that they might be involved in the regulation of the defense response against the common scab pathogen (Tai et al., 2013). Presumably CaMYB gene plays a role, at least partially, in resistance against P. capsici. Therefore effective deployment of CaMYB may aid to develop pepper cultivars with favorable anthocyanin production and resistance level.

Conclusion

This study provides a more comprehensive study on the regulation of anthocyanin biosynthetic genes in pepper leaves. Differential gene expression between leaf of A3 and Z1 and in pepper leaves by VIGS suggests that CaMYB in C. annuum. L regulates the expression of all the flavonoid pathway genes. The gene expression analysis in CaMYB-silenced leaves demonstrates that the transcription factor of CaMYB plays a vital role in the MBW activation complex for anthocyanin accumulation, and MYC might be positively regulated by CaMYB, while WD40 might be negatively regulated by CaMYB in pepper leaves. Furthermore, detached leaves inoculation assay from VIGS system revealed that CaMYB may play an important role in resistance to Phytophthora capsici Leonian in purple pepper. The evidence presented in this study in conjunction with results acquired from further research will help clarify the mechanisms of MBW complex in the anthocyanin biosynthesis and defense response in pepper and other plants.

Acknowledgments

The following organizations are acknowledged for their financial support: National Natural Science Foundation of China (No.31401879, 31272163), Northwest A&F University Basic Fund for Scientific Research (QN2013016), and the Hangzhou Municipal Science and Technology Commission Project (20140932H01).

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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