Calmodulin-binding protein CBP60g functions as a negative regulator in Arabidopsis anthocyanin accumulation

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Abstract

Anthocyanins, a kind of flavonoid, normally accumulate in the flowers and fruits and make them colorful. Anthocyanin accumulation is regulated via the different temporal and spatial expression of anthocyanin regulatory and biosynthetic genes. CBP60g, a calmodulin-binding protein, has previously been shown to have a role in pathogen resistance, drought tolerance and ABA sensitivity. In this study, we found that CBP60g repressed anthocyanin accumulation induced by drought, sucrose and kinetin. The expression pattern of CBP60g was in accordance with the anthocyanin accumulation tissues. Real-time qPCR analysis revealed that the anthocyanin biosynthetic genes CHS, CHI and DFR, as well as two members of MBW complex, PAP1, a MYB transcription factor, and TT8, a bHLH transcription factor, were down regulated by CBP60g.

Introduction

Anthocyanins, a kind of natural hydrosoluble pigment produced by the flavonoid biosynthetic pathway, extensively exists in all kinds of plants and color the petals and fruits. Plant benefits from these pretty colors to attract the insects to pollinate and transmit the seeds [1]. In vegetative tissues, anthocyanins also have photoprotective roles by providing a light-absorbing screen for photosynthetic cells [2, 3], and serve as scavenger to remove reactive oxygen species under light stress conditions [4]. To fulfill these functions, anthocyanin biosynthesis deserves an accurate regulation in plant.

Anthocyanin biosynthetic and regulatory genes show temporal and spatial expression pattern differently and this enable the fine control of anthocyanin accumulation [1, 5, 6]. The understanding in flavonoid biosynthetic pathway and its regulatory components is increasing [1]. The early biosynthetic genes (EBGs) include CHS (chalcone synthase), CHI (chalcone isomerase), F3H (favone 3-hydroxylase), and FLSM (favonol synthase). The late biosynthesis genes (LBGs) include DFR (dihydrofavonol 4-reductase), LDOX (leuco anthocyanidin dioxygenase), and UF3GT (UDP-glucose:favonoid 3-Oglucosyl transferase) and so on [7]. Many
anthocyanin biosynthetic genes were regulated by MBW (MYB-bHLH-WD40) protein complex consisting of R2R3-MYB, bHLH and a WD-repeat containing protein [8, 9]. R2R3-MYBs, owing to their multiple expression pattern compared with bHLH and WD40, are the essential regulatory components in the complex. [2, 10, 11].

PAP1 (PRODUCTION OF ANTHOCYANIN PIGMENT 1), a R2R3-MYB transcription factor, can interact with a bHLH transcription factor TT8 (transparent test 8), EGL3 (enhancer of glabra3) or GL3 (glabra3), and a WD-repeat transcription factor TTG1 (transparent testa 1), and these ternary complexes regulate anthocyanin synthesis [12]. The pap1 knockout mutant showed a less anthocyanin accumulation phenotype than wild-type, while overexpression of PAPI increased anthocyanin accumulation [11, 13].

The accumulation of PAPI transcripts is regulated by temperature, concentration of sucrose, hormone treatment, strength and wavelength of light [14]. Cytokinin positively regulated anthocyanin accumulation caused by sucrose, and this process is PAPI mediated [15, 16]. Ethylene has been proved to play a negative role in anthocyanin accumulation [14, 17]. However, the exact regulatory components upstream PAPI are not clear.

CBP60s are plant specific calmodulin-binding proteins first identified in maize [18–20]. In Arabidopsis thaliana, there are eight CBP60s, named CBP60a-g, and SARD1 [21]. Five members of CBP60s can interact with CaM (calmodulin) in a Ca\(^{2+}\) dependent manner following treatment with elicitors [21]. CBP60g lacks the CaM interaction C-terminal, with a CaM interaction N-terminal instead [22]. Previous studies have shown that most of the members of CBP60 family play roles in pathogen and drought resistance [18, 23, 24]. CBP60g acts synergistically with SARD1 in the pathogen resistance, while antagonistically with CBP60a [18, 23–25].

Previous studies focused on the role of CBP60g in the pathogen resistance. Wang et al. revealed that CBP60g participated in SA (salicylic acid) synthesis and contributed to pathogen resistance [22]. The cbp60g mutant was found to support more bacterium growth than the wild-type in a bacterium growth assay [24]. Zhang et al. further confirmed that SARD1 (Systemic Acquired Resistance Deficient 1) and CBP60g regulated the SA biosynthetic gene, ICS1 (Isochorismate synthase 1), while the induction of ICS1 was blocked in the cbp60g mutant. CBP60g fulfilled this role by binding to the promoter of ICS1 and functioning as a transcription activator [26]. Wan et al. found that CBP60g overexpression plants accumulated more ICS1 transcripts and SA, and were more resistant to pathogen [23].

In addition to its role in pathogen resistance, Wan et al. indicated that CBP60g was also involved in drought tolerance and ABA sensitivity. In this paper, we found that CBP60g could regulate the expression of two members of MBW complex, PAP1, a MYB transcription factor, and TT8, a bHLH transcription factor, thus control the anthocyanin synthesis, and for the first time linked calcium signaling to the anthocyanin accumulation.

**Materials and methods**

**Plant materials and growth conditions**

*Arabidopsis thaliana* wild-type was Columbia-0, the cbp60g mutant and CBP60g overexpression lines were also in the Columbia background. The T-DNA insertion allele of CBP60g (cbp60g-1; At5g26920; SALK-023199) was obtained from the Arabidopsis Biological Resource Center (ABRC).

Seeds were surface sterilized by sequentially immersed in 75% ethanol or 100% ethanol with 0.05% Tween-20 for 10 min each. After 3 days stratification at 4°C on half strength MS medium, plants were set into 22°C growth chamber with a 16h/8h of light/dark cycle. After 10 days, the seedlings were transferred to a 1:1 mixture of peat soil and vermiculite in the same
growth chamber. Swimming plants were grown in GC (gas chromatography) vial and performed as previously reported [23].

Drought treatment

We use two or three-weeks-old plants to observe the anthocyanin accumulation under drought treatment. Either 4 three-weeks-old plants or 200 two-weeks-old seedlings grown in a pot with 80 g mixture soil were undergoing a water limitation, that 30 mL water each pot was supplied every 3 days. Six biological replicates were performed.

Measurement of anthocyanin content

About 0.1 g samples were grounded in 1.5 mL Eppendorf tube and 1 mL methanol contain 1% HCl was added. After centrifugation at 13000 rpm for 20 min, the absorbance of supernatants were measured at 528 nm and 657 nm using Beckman DU800 (USA). The content of anthocyanin was quantified using the formula A530-1/4(A657) to compensate for the contribution of chlorophylls. Three biological replicates were performed.

Anthocyanin Induced Condition (AIC)

About 100 seeds were sown in half strength liquid MS medium with 3% sucrose. After 12 days, anthocyanin accumulation can be observed. To observe anthocyanin among different lines in the same plate, we use half strength solid MS medium contain 3% sucrose, 40 μM kinetin or 7% sucrose as AIC. Each plate contains 30 seedlings for one line, and observed 14 days after treatment. Three biological replicates were performed.

Histochemical GUS assay

ProCBP60g::GUS transgenic plants generated by previously study [23] were grown in half strength liquid MS medium with 3% sucrose for 14 days. GUS staining was performed as previously described [23]. Samples were immersed in staining buffer (50 mM Na₂HPO₄–NaH₂PO₄, pH 7.3, 2 mM K₄Fe(CN)₆, 2 mM K₃Fe(CN)₆, 0.1% Triton X-100) with 0.5 mg mL⁻¹ X-Gluc and incubated in 37˚C for 6–12 h. After decolorized by ethanol, the GUS staining patterns were photographed under a dissecting microscope (Nikon SMZ800).

Kinetin and sucrose treatment

Kinetin (KT) and sucrose were used to induce the expression of anthocyanin synthesis related genes. Twelve -day-old seedlings growing in 20 mL GC vials were treated with a final concentration of 100μM KT or 150 mM (5.13%) of sucrose. Samples were harvested at various time points.

Quantitative real-time PCR analysis

Total RNA was extracted from 30 mg samples. After DNase I (Ambion Cat# AM2224) treatment, 500 ng total RNA was used for reverse transcription using M-MLV reverse transcriptase kit (TaKaRa). After 40-fold dilution, 5 μL cDNA was used as the template of real-time PCR using SYBR Premix Ex Taq (TaKaRa), with a Roche LightCycler 480. The PCR was performed with the following cycling profile: 95˚C for 1 min; 40 cycles at 95˚C for 5 s, 60˚C for 30 s, and 72˚C for 10 s. AtEF1α was used as reference gene. Three technical repetitions were performed for each experiment, and at least three independent biological replicates were performed. The primers used for qRT-PCR were listed in S1 Table.
EGTA treatment
Ethyneglycol-bis(beta-aminoethylether)-N,N'-tetraacetic acid (EGTA) was added into half strength MS medium contain 40μM KT and 3% sucrose to chelate the Ca^{2+}. The 18-day-old seedlings were treated and observed.

Results
CBP60g affected the anthocyanin accumulation during drought stress
Previously, it was reported that CBP60g play an important role in Arabidopsis disease resistance [18, 23, 24]. And CBP60g overexpression lines exhibited a drought-tolerance phenotype [22]. To further investigate the mechanism of CBP60g in plants drought tolerance, different genotypes of plants were exposed to drought stress by limiting the water supply for two weeks, anthocyanin did not accumulate as much in the CBP60g overexpression line (OE16-8) as in wild-type (Columbia-0) plants, especially in the abaxial side of the rosette leaves and the stems (Fig 1A and 1B). Consistent with this, measurement of anthocyanin content of the rosette leaves indicated that it was three times less in CBP60g overexpression line than that in wild-type (Fig 1C). This suggests that CBP60g suppresses the anthocyanin accumulation under drought stress.

CBP60g as a negative regulator in sucrose and kinetin induced anthocyanin accumulation
As the mechanism of anthocyanin accumulation under drought stress is not clear, in this study, we used sucrose and kinetin containing medium as anthocyanin inductive conditions (AIC) as previously described [27]. Accumulating data revealed that sucrose containing medium induced anthocyanin synthesis either independently or synergistically with cytokinin. The presence of sucrose appears to promote anthocyanin accumulation via controlling the expression of PAP1, which is a component of the MBW (MYB-bHLH-WD40) and regulate the expression of anthocyanin synthase [16, 28–30].

Seedling of the cbp60g mutant, CBP60g overexpression line and WT was growing in solid half strength MS medium containing either 3% sucrose or 7% sucrose for 14 days, OE16-8 plants accumulated less anthocyanin than wild-type, while the cbp60g mutant accumulated more anthocyanin than wild-type in 7% sucrose containing medium (Fig 2A and 2B). Furthermore, in 3% sucrose and 40 μM kinetin containing solid half strength MS medium (AIC), the accumulation of anthocyanin is higher in the cbp60g mutant and lower in the CBP60g overexpression lines compared with WT (Fig 2C and 2D). In addition, the CBP60g overexpression lines showed greater growth inhibition than the wild-type and the cbp60g mutant when the concentration of kinetin was increased to 100 μM (Fig 2C and S1 Fig).

Taken together, these results indicated that CBP60g was a negative regulator in anthocyanin accumulation.

The expression pattern of CBP60g was coincident with anthocyanin accumulation pattern
Our previous study has shown that CBP60g is expressed in various tissues and organs [23]. In this study, we used the Pro_{CBP60g::GUS} transgenic plants [23] grown in the liquid half strength MS medium with 3% sucrose to observe the histochemical localization of GUS activity, along with anthocyanin accumulation. Anthocyanin accumulated in the abaxial side of leaves, especially in true leaves (Fig 3A and 3B). While in the adaxial side, anthocyanin accumulated in cotyledons and the edge of the true leaves (Fig 3C and 3D). In the flank side, we also observed that anthocyanin accumulated in cotyledons and true leaves. GUS activity was also detected in
We also found that CBP60g itself was responsive to kinetin (S2A Fig). The anthocyanin biosynthetic and regulatory genes were negatively regulated by CBP60g. It has been pointed out that the anthocyanin biosynthetic genes are induced under sucrose and cytokinin treatment [31, 32]. We detected the anthocyanin regulatory genes and their expression under different conditions. The anthocyanin accumulation was affected by CBP60g, as shown in Fig 1B. (C) The content of anthocyanin in (A) was quantified. Three biological replicates were performed for each experiment. Each data point represents the average of three technical replicates ±SD.

Fig 1. Anthocyanin accumulation under drought treatment was affected by CBP60g. Anthocyanin accumulation of three-week-old (A) or two-week-old (B) plants was observed among wild-type, the \textit{cbp60g} mutant and CBP60g overexpression lines under water limitation condition. Water was supplied 30 mL per pot containing 80g mixture soil every three days. (C) The content of anthocyanin in (A) was quantified. Three biological replicates were performed for each experiment. Each data point represents the average of three technical replicates ±SD.

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the same tissues Fig 3E and 3F). We also found that \textit{CBP60g} itself was responsive to kinetin (S2A Fig).
biosynthetic genes among the wild-type, the cbp60g mutant and the CBP60g overexpression line under sucrose and cytokinin treatment using qRT-PCR. ARR5 was used as a marker gene to validate the treatment (S3 Fig). The induction of biosynthetic genes CHS, CHI and DFR in the cbp60g mutant was more intensive than that in wild-type, however, it was reduced in the CBP60g overexpression line (Fig 4A, 4B and 4C). The induction of anthocyanin regulatory genes such as PAP1 and TT8 (belonging to the MBW) was similar to the synthetic genes (Fig 4D and 4E). The induction of PAP2, TT2, EGL2, MYBL2 and GL3, which also belongs to the MBW complex, were not affected among different genotypes (S3 Fig). The result indicated that CBP60g negatively regulated the anthocyanin synthesis through PAP1 and TT8. We also checked the expression of CHS, CHI, DFR, PAP1 and TT8 under 150 mM sucrose treatment, and confirmed that CBP60g suppressed the expression of these genes. (S4 Fig).

**Anthocyanin accumulation regulated by CBP60g was calcium-independent**

CBP60g, as a plant specific calmodulin binding protein, has the innate relationship with Ca$^{2+}$ [23]. We had known that the expression of CBP60g is induced by Ca$^{2+}$ (S5 Fig). Then we try to investigate whether Ca$^{2+}$ can influence the repression of anthocyanin synthesis caused by...
CBP60g. Ethyleneglycol-bis(beta-aminoethylether)-N,N'-tetraacetic acid (EGTA) was used to chelate Ca\(^{2+}\) in the AIC medium. Wild-type, the cbp60g mutant and the CBP60g overexpression lines grown in the solid half strength MS medium supplemented with 3% sucrose, 40 \(\mu\)M kinetin and various concentrations of EGTA were observed (Fig 5A). We found that anthocyanin accumulation was weakened along with the decrease of Ca\(^{2+}\) (Fig 5B). The extent of reduction in anthocyanin accumulation was similar among wild-type, the cbp60g mutant and CBP60g overexpression lines. This result showed that CBP60g regulated anthocyanin accumulation is calcium independent.

Interestingly, the growth retardation of wild-type and the cbp60g mutant caused by kinetin was greatly reduced by the reduction of Ca\(^{2+}\), however, CBP60g overexpression lines did not appear to recover in any concentration of EGTA (Fig 5C).

Fig 3. The comparison of the anthocyanin accumulation tissues and the CBP60g expression pattern. (A), (C) and (E) The anthocyanin accumulation tissues. (B), (D) and (F) CBP60g expression tissues. (A) and (B) The abaxial side of 14 days seedlings. (C) and (D) The adaxial side of 14 days seedlings. (E) and (F) The flank side of 14 days seedlings. doi:10.1371/journal.pone.0173129.g003
Fig 4. CBP60g repressed the expression of anthocyanin biosynthetic and regulatory genes under AIC. Twelve-day-old wild-type, the cbp60g mutant and CBP60g overexpression lines grown in the liquid 1/2 MS medium were treated with 100 μM kinetin. The expression of anthocyanin biosynthetic genes CHS, CHI and DFR, along with the anthocyanin regulatory genes PAP1 and TT8 were detected. Three biological replicates were performed for every experiment. Each data point represents the average of three technical replicates ±SD.

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Fig 5. CBP60g repressed the anthocyanin accumulation in a calcium independent manner. Wild-type, the cebp60g mutant and CBP60g overexpression lines grown in the solid 1/2 MS medium with 3% sucrose, 40 μM kinetin and various concentrations of EGTA were observed (A). The fresh weight and anthocyanin content were quantified (B) and (C). Three biological replicates were performed for every experiment. Each data point represents the average of three technical replicates ±SD.

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Discussion

1 CBP60g function as feedback repressor in Ca\(^{2+}\) promoted anthocyanin accumulation

The role of calcium signaling in anthocyanin accumulation has been reported recently. Shin et al. discovered that Ca\(^{2+}\) induced Arabidopsis anthocyanin accumulation, it was depressed while Ca\(^{2+}\) was inactivated by adding EGTA into medium [17]. Peng et al. clarified that *Fragaria vesca* UDP-glucosyltransferase (FvUGT1) activity was inhibited by anthocyanidin, and was alleviated via calmodulin binding to FvUGT1 [33]. CDPKs, as a part of calcium signaling, have an important role in plant growth and stress response. CDPKs regulate the activity of transcription factors and metabolic enzymes via the molecular interaction [34]. Schulz et al. revealed that PARP suppressed anthocyanin accumulation via controlling the anthocyanin related transcription factors expression, and this is similar to the role of CBP60g, one of the transcription factors in anthocyanin accumulation [35]. The anthocyanin synthesis in *Medicago truncatula* was negatively controlled by MYB2, which itself was regulated by CBP60g[23, 36]. In our study, anthocyanin content decreased in Arabidopsis grown in medium containing EGTA (Fig 5), which was in accordance with the previous studies that calcium induced anthocyanin accumulation. However, it increased when grown in medium with extra Ca\(^{2+}\) (S5 Fig). In brief, calcium induces anthocyanin accumulation and the expression of CBP60g. However, CBP60g represses this anthocyanin accumulation conversely. An explanation is that CBP60g mediates a negative feedback, which suppresses anthocyanin accumulation (illustrated in S6 Fig). After stress treatment and signaling activation, intercellular calcium concentration increases which stimulates anthocyanin accumulation in turn and the expression of CBP60g. Then, CBP60g induces the expression of PAP1 and TT8 which are components of the MBW complex. However, this interaction is not quite clear so far. Finally, the anthocyanin synthetic genes are repressed and anthocyanin accumulation is blocked. It is without doubt that CBP60g, as a transcription factor, regulates anthocyanin accumulation in a currently unknown manner, perhaps by regulating anthocyanin synthesis related genes and competitively inhibiting the calmodulin function. The negative feedback appears to be calcium independent (Fig 5).

2 How do CBP60g regulate the anthocyanin biosynthetic or regulatory genes

Plant anthocyanin synthesis is regulated by the MBW complex. MYB transcription factor was a key component in the MBW complex [11, 16]. PAP1, a MYB2 transcription factor, plays an important role in sucrose induced anthocyanin accumulation [11, 37]. MBW complex regulates the expression of many anthocyanin synthesis genes. Our results show that CBP60g also regulates the expression of many anthocyanin synthesis related genes, such as PAP1, TT8, CHS, CHI and DFR (Fig 4 and S4 Fig). Zhang et al. revealed that CBP60g regulates the expression of ICS1 via directly binding to the GAAATTT motif of its promoter[26]. We searched the promoters from the 2000bp upstream to 200bp downstream of the start codon of PAP1, TT8, CHS, CHI and DFR for the CBP60g binding motif and found it is only present in the promoter of CHS and PAP1. This suggests that CBP60g may regulate other genes in an indirect way, such as via MYB2.

CBP60g blocked calcium regulated growth inhibition caused by kinetin

The crosstalk between cytokinin and calcium signaling pathway has been discovered. Peng et al (1996) has revealed that Ca\(^{2+}\) inhibits senescence-retarding effect of cytokinins in
detached rice leaves [38]. Calcium antagonists and calmodulin inhibitors repress cytokinin-induced bud formation in Funaria [39]. In this study, we also found that EGTA, as a calcium chelator, could block the growth retardation caused by kinetin (Fig 5).

The reduced growth phenotype caused by kinetin was not removed by decreasing Ca\(^{2+}\) in the CBP60g overexpression lines. This indicated that CBP60g participated in cytokinin signaling pathway, and played a role in the crosstalk between cytokinin and Ca\(^{2+}\).

Supporting information

S1 Fig. The CBP60g overexpression plants were lethal under 100μM kinetin treatment.
Plants were vertically grown on the 1/2 MS medium with 100μM kinetin and photographed 14 days after treatment.
(TIF)

S2 Fig. CBP60g and ARR5 were induced by kinetin treatment.
Twelve-day-old wild-type, the cbp60g mutant and CBP60g overexpression lines grown in the liquid 1/2 MS medium were treated with 100 μM kinetin kinetin. Three biological replicates were performed for every experiment. Each data point represents the average of three technical replicates ±SD.
(TIF)

S3 Fig. The expression of PAP2, TT2, EGL2, MYBL2 and GL3.
Twelve-day-old wild-type, the cbp60g mutant and the CBP60g overexpression lines grown in the liquid 1/2 MS medium were treated with 100 μM kinetin kinetin. Three biological replicates were performed for every experiment. Each data point represents the average of three technical replicates ±SD.
(TIF)

S4 Fig. The expression of PAP1, TT8, DFR, CHI and CHS under sucrose treatment.
Twelve-day-old wild-type, the cbp60g mutant and CBP60g overexpression lines grown in the liquid 1/2 MS medium were treated with 150 Mm sucrose. Three biological replicates were performed for every experiment. Each data point represents the average of three technical replicates ±SD.
(TIF)

S5 Fig. Calcium induced anthocyanin accumulation.
(A) Twelve-day-old wild-type, the cbp60g mutant and CBP60g overexpression lines grown in the solid 1/2 MS medium with 3% sucrose and 100 mM CaCl\(_2\) were observed. (B) The expression of CBP60g was induced under 50 mM CaCl\(_2\).
(TIF)

S6 Fig. CBP60g suppresses Arabidopsis anthocyanin accumulation.
Light and sucrose induce anthocyanin accumulation, cytokinin and calcium signaling participate in this process. Calcium signaling increases the anthocyanin accumulation, while on the other hand, induces CBP60g expression thus represses the anthocyanin accumulation. These form a negative feedback. We suggest that CBP60g regulates anthocyanin accumulation through PAP1 and TT8, which are the components of MBW complex.
(TIF)

S1 Table. Primers used for qRT-PCR analysis in this work.
(DOC)

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