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Cytokinins Enhance Sugar-Induced Anthocyanin Biosynthesis in Arabidopsis

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In higher plants, the regulation of anthocyanin synthesis by various factors including light, sugars and hormones is mediated by numerous regulatory factors acting at the transcriptional level. Here, the association between sucrose and the plant hormone, cytokinin, in the presence of light was investigated to elucidate cytokinin signaling cascades leading to the transcriptional activation of anthocyanin biosynthesis genes in *Arabidopsis* seedlings. We showed that cytokinin enhances anthocyanin content and transcript levels of sugar inducible structural gene *UDP-glucose:flavonoid 3-O-glucosyl transferase* (*UF3GT*) and regulatory gene *PRODUCTION OF ANTHOCYANIN PIGMENT 1* (*PAP1*). Genetic analysis showed that cytokinin signaling modulates sugar-induced anthocyanin biosynthesis through a two-component signaling cascade involving the type-B response regulators ARR1, ARR10 and ARR12 in a redundant manner. Genetic, physiological and molecular biological approaches demonstrated that cytokinin enhancement is partially dependent on phytochrome and cryptochrome downstream component HY5, but mainly on photosynthetic electron transport. Taken together, we suggest that cytokinin acts down-stream of the photosynthetic electron transport chain in which the plastoquinone redox poise is modulated by sugars in a photoreceptor independent manner.

INTRODUCTION

Numerous regulatory transcription factors control the expression of different anthocyanin biosynthetic genes in Arabidopsis (Gonzalez et al., 2008; Nesi et al., 2000; 2001; Wade et al., 2003; Winkel-Shirley, 2001). The Arabidopsis MYBs/bHLH/WD-repeat (MBW) complex, which includes the transcription factors PRODUCTION OF ANTHOCYANIN PIGMENT 1 (PAP1) and PAP2, ENHANCER OF GLABRA3 (EGL3), GLABRA3 (GL3), and TRANSPARENT TESTA 1 (TTG1), predominantly regulates the expression of the 'late' anthocyanin biosynthetic genes including *dihydroflavonol 4-reductase* (*DFR*), *leucoanthocyanidin dioxygenase* (*LDOX*), and *UDP-glucose:flavonoid 3-O-glucosyl transferase* (*UF3GT*) over the expression of the 'early' anthocyanin biosynthetic genes such as *phenylalanine ammonium lyase* (*PAL*), *chalcone synthase* (*CHS*), *chalcone isomerase* (*CHI*), and *flavanone 3-hydroxylase* (*F3H*) (Gonzalez et al., 2008). Some WD-repeat independent MYBs, such as MYB11, MYB12, and MYB111 (Mehrtens et al., 2005; Stracke et al., 2007) regulate the 'early' gene expression. *Flavonoid 3'-hydroxylase* (*F3'H*) may be dually regulated by WD-repeat-dependent and -independent mechanisms, consistent with its requirement for the production of both quercetin-based flavonols and cyanidin-based anthocyanin (Gonzalez et al., 2008).

In *Arabidopsis*, transcription factors such as PHYTOCHROME-INTERACTING FACTOR 3 (PIF3) and LONG HYPOCOTYL 5 (HY5), which are downstream components of light signaling perceived and transduced by photoreceptors including UV-B photoreceptor, cryptochrome 1 (CRY1) and phytochrome (PHY) A and B, positively regulate anthocyanin biosynthesis through direct binding to the promoters of anthocyanin structural genes, including *CHS*, *CHI*, *F3H, F3'H*, *DFR* and *LDOX* (Shin et al., 2007).

Plant hormones and sugars have adopted different mechanisms to interact with the components of the MBW complex, which are regulated at either the transcriptional or post transcriptional level. The gaseous hormone ethylene inhibits sugar- and photosynthesis- induced anthocyanin accumulation by suppressing the expression of positive transcription factors including GL3, TT8, PAP1, while stimulating the concomitant expression of the negative R3-MYB regulator MYBL2 (Jeong et al., 2010). Jasmonate (JA) and brassinosteroids (BRs) also regulate MBW complex activity at the posttranslational level. JA ZIM-domain proteins (JAZs), which are substrates of the SCF^{COI1} complex and act as negative regulators of JA-responsive genes (Chini et al., 2007; Thines et al., 2007), interact with bHLHs (TT8, GL3, and EGL3) and R2R3 MYB transcription factors (MYB75 and GL1), repressing JA-regulated antho-

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cyanin accumulation (Qi et al., 2011). BR affects anthocyanin biosynthesis by regulating the expression of JA-induced MYB/bHLH transcription factors such as *PAP1*, *PAP2*, and *GL3* (Peng et al., 2011).

Cytokinis (CKs) are also implicated in the modulation of anthocyanin accumulation presumably *via* the regulation of the development of the photosynthetic apparatus, through reactive oxygen species-mediated oxidative stress (Argyros et al., 2008), or through the stimulation of Sucrose (Suc)-induced anthocyanin biosynthesis in the presence of light (Guo et al., 2005). In relation to anthocyanin biosynthesis, the CK receptors AHK2, 3, and 4 and the type-B response regulators ARR1, 10, and 12 were implicated in light-induced pigmentation in a redundant manner (Argyros et al., 2008). However, the regulatory genes involved in this process are largely unknown.

Light is a prerequisite factor for anthocyanin biosynthesis for sugar and hormone signalings to anthocyanin biosynthesis. Without light supplementation, other signals such as sucrose (Jeong et al., 2010), CK (Guo et al., 2005) and ethylene (Jeong et al., 2010) failed to induce anthocyanin pigmentation. Thus, it is most likely that signalings pathways leading to anthocyanin biosynthesis are merged at a certain point of the synthesis pathway.

Here, we investigated the interplay of sucrose Suc, CK and light signaling pathways resulting in the anthocyanin pigmentation of Arabidopsis seedlings using their signaling component mutants. We showed that CK enhances Suc-induced anthocyanin biosynthesis via transcriptional activation of the MBW complex, while suppressing *MYBL2* expression, which involves positive regulation by a subset of type-B response regulators. Further, we show that CK-mediated enhancement of anthocyanin synthesis is dependent on the redox state of the photosynthetic electron transport mediated by Suc signaling but independent of HY5/PIF3-mediated light signaling pathways.

MATERIALS AND METHODS

Plants and growth conditions

Seeds of wild-type Columbia (Col-0), Wassilenskijja (Ws) and Landsberg (Ler) ecotypes were used as controls depending on the genetic background of the mutants. The mutant lines used in this investigation were *cwinv1* (Salk-091455C), *stp1, suc1-2,* and *gin2-1* for studying the role of sugar metabolism and sensing. The CK signaling pathway components involved in anthocyanin accumulation were studied using the CK receptor mutants *ahk2*, *ahk3,* and *ahk4*, and the double mutants *ahk2*/*3* and *ahk3*/*4*(*ahk4* in Ws background). The genetic CK response regulator mutants were the type-B response regulator mutants *arr1-3* (CS6971), *arr10-5* (Salk_098640), *arr12-1* (CS6978) single, *arr1-3*/*12-1* (CS6981), *arr1-3/10-5*, *arr10-5*/*12-1* (CS39991) double, and *arr1-3*/*10-5*/*12-1* (SALK_054752) triple mutants, as well as the type-A ARR hexaple mutant *arr3/4/5/6/8/9* (CS25279). Genetic mutant lines used for light signaling included *hy1-1* (CS67), the Cry signaling double mutant *cry1*/*cry2* and the transcription factor mutant *hy5-221*. Besides these, transgenic lines over-expressing the CKX2 gene (*CKX2ox*) were used. The redox state signaling *stn7* (SALK_072531C) and *stn8* (SALK_ 064913C) mutant germplasm stocks were obtained from TAIR. Unless otherwise mentioned, all mutants were under Col-0 eoctype background.

Sterilized seeds were sown on agar plates containing 1/2-strength MS medium supplemented with various concentrations of Suc (0, 15, 30, 60, and 90 mM Suc), and various metabolic sugars such as maltose (Mal, 90 mM), glucose (Glc, 90 mM) and fructose (Fru, 90 mM). A 1:1 mixture of Glc:Fru (45 mM:45 mM) was used. In addition, a sugar alcohol, mannitol (Man, 90 mM), and a non-metabolic sugar, palatinose (Pal, 90 mM) were added to the medium. All media were used in the presence or absence of various concentrations of synthetic (0, 0.1, 0.5, 1, 2, and 5 μM benzyl adenine) and natural [0.5 μM *trans*-zeatin and N6-(2-isopentenyl) adenosine] CKs. Seedlings were grown under an 18 h light/6 h dark photoperiod (22/20°C) for 7 days depending on the experimental condition. For the photosynthesis electron transport inhibition study, seeds were surface sterilized and sown in 1/2-strength MS media agar plates and grown for 4 days before being transferred to 90 mM Suc-containing growth media. When required, the photosynthetic electron transport inhibitors DCMU (5 μM) or DBMIB (10 μM) were included in the Suc-containing growth media. The plates were then incubated for 24 h under 140 μmol m^{-2} s^{-1} of continuous white light.

Measurement of anthocyanin and soluble sugars

Anthocyanin contents of whole seedlings of Arabidopsis plants were determined using the method described by Rabino and Mancinelli (1986). Absorbance (*A*) of the supernatant extract was measured at 530 and 657 nm, and the concentration of anthocyanin was calculated by A_{530}-0.25A_{657}. To extract soluble sugars, 20 root-excised seedlings were ground to powder in liquid N_2, and then extracted in 80% (v/v) ethanol at 80°C for 30 min. After centrifugation at 12,000 rpm for 2 min, the supernatant was decanted and stored on ice. This extraction procedure was performed on the pellet three more times and the collected supernatants were combined. After depigmentation with chloroform (1:3, v/v, extract:chloroform), ketose sugars were degraded with 1.0 N NaOH (1:1, v/v) for 5 min (Jeong et al., 2010). The sugar content was determined spectrophotometrically at 520 nm using the Resorcinol method, with Suc as the standard (Roe, 1934). Mean values were obtained from three or four independent replicates.

RNA isolation and quantitative real-time RT-PCR analysis

Total RNA was extracted with the TRI reagent (Molecular Research Center) and complementary DNA was synthesized from 1 μg of total RNA with the iScript cDNA Synthesis Kit (Bio-Rad) (Jeong et al., 2010). Quantitative PCR was performed using the CFX96™ Real Time System (Bio-Rad, USA) and all reactions were performed with the Dynamo HS SYBR Green qPCR Kit (FINNZYMES) and specific primers according to the procedure described previously (Jeong et al., 2010). Reactions were performed in triplicate using 5 μl of 2X Dynamo HS master mix, 0.5 μM of each primer, 2 μl of 20-fold diluted cDNA and nuclease-free water (Roche Diagnostics) to a final volume of 10 μl. A negative water control was included in each run. After incubation for 15 min at 95°C, 40 cycles of amplification were run for 10 s at 94°C and 30 s at 62°C, followed by a final extension step for 30 s at 72°C. The raw data were analyzed with the CFX Manager™ software (version 1.1), and expression was normalized to *actin* 2 (AT3G18780) to minimize variation in cDNA template levels. The relative expression level was calculated by using the comparative CT (threshold cycle value) method. Fold changes ($2^{-\Delta\Delta CT}$) were expressed relative to wild-type seedlings grown in the Suc-containing medium in the light. Mean values of three to five biological replicates, each with triple values, are given.

Statistical analysis

The significance of differences between data sets was evalu-

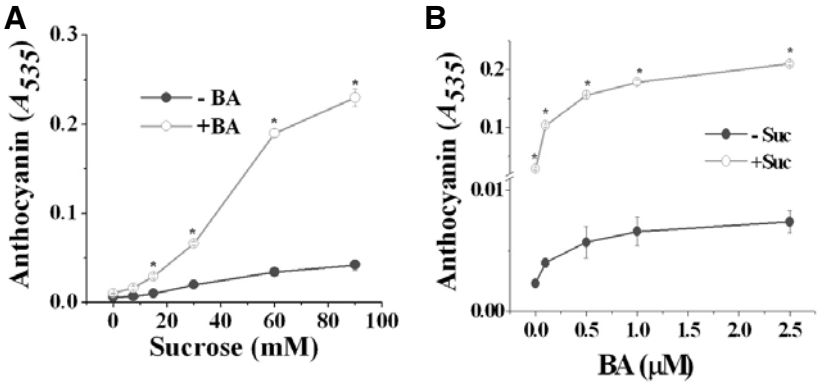

Fig. 1. CK enhances Suc-mediated anthocyanin pigmentation. Col-0 seedlings were grown in 1/2-strength MS media supplemented with various concentrations of Suc (0, 7.5, 15, 30, 60, and 90 mM) with (+BA) or without (-BA) 0.5 μM BA (A) or with various BA concentrations (0, 0.1, 0.5, 1, 2, and 5 μM) with (+Suc) or without (-Suc) 90 mM Suc (B) under growth light conditions for 7 days. Mean values were calculated from results of at least five biological replicates. Symbols over bars indicate significant differences ($P < 0.05$; *t*-test) between control and treatment.

ated using paired student's *t*-test using OriginPro8 (OriginLab).

RESULTS

Cytokinins enhance sugar induction of anthocyanin accumulation

Anthocyanin induction by sugars is differentially regulated by plant hormones such as GA, ABA, ethylene, CK, and JA (Jeong et al., 2010; Jung et al., 2010; Loreti et al., 2008). To investigate how CK modulates sugar-induced anthocyanin accumulation, Arabidopsis wild-type Col-0 seedlings were grown on 1/2-strength MS medium supplemented with different Suc concentrations ranging from 0-90 mM with and without 0.5 μM benzyl adenine (BA) under 140 μmol m^{-2} s^{-1} light for 7 days. As shown in Fig. 1A, Suc induced anthocyanin accumulation in a dose-dependent manner, being consistent with a previous report (Jeong et al., 2010). Co-treatment of BA with Suc significantly enhanced the Suc-induced increase in anthocyanin levels (1.8-5.8-fold increase compared to Suc-fed seedlings). To validate whether the effect of CK and Suc is additive, Col-0 seedlings were grown in various concentrations of BA ranging from 0-2.5 μM with and without 90 mM Suc (3.24%). Anthocyanin accumulation in seedlings treated with BA alone or in combination with 90 mM Suc showed saturation curves beyond 0.5 μM BA, but Suc supplementation enhanced pigmentation levels *ca.* 27-fold compared to treatment with BA (Fig. 1B), which is significantly higher than the effect of each agent alone.

The total soluble sugar content was determined in Col-0 seedlings treated with 90 mM Suc and 0.5 μM BA alone or in combination. Consistent with a previous report (Jeong et al., 2010), the total soluble sugar increased four-fold in seedlings grown on Suc-containing media compared to Suc-free media. However, BA treatment did not change the level of total soluble sugar (Fig. 2A). Thus, the stimulatory effect of CK on sugar induction is attributable to the modulation of sugar signaling amplitude rather than changes in endogenous sugars levels.

Next, we investigated whether the interaction between CK and sugar is a naturally occurring phenomenon, in which case a transgenic line (*CKX2ox*) over-producing CK oxidase 2 should show less sensitivity to Suc treatment due to 30-45% lowered bioactive CK contents (Werner et al., 2003) as well as differential responsiveness to synthetic and natural CKs; natural CKs would show less enhancement effect than the synthetic form, as natural CKs would be cleaved by CKX2, while synthetic CKs would be kept intact. Treatment with 90 mM Suc resulted in a slightly lower (17%) anthocyanin accumulation in *CKX2ox* compared to Col-0 plants and minor changes in the sensitivity to exogenously applied synthetic CK, BA, with respect to anthocyanin accumulation (Fig. 2B). On the other hand, *CKX2ox* supplemented with natural CKs such as *trans*-zeatin (*tZ*) and N6-(2-isopentenyl) adenosine (iP) that are degradable by CKX2 showed 50-60% less induction of anthocyanin compared to BA-fed *CKX2ox* control plants, presumably because synthetic CKs are resistant to CKX2 action due to structural differences.

Suc induction of CK-mediated anthocyanin accumulation (Figs. 1 and 2) strongly suggests that CK modulates sugar signaling via transcriptional activation of the MBW regulatory complex and the corresponding structural genes (Jeong et al., 2010). Therefore, quantitative qRT-PCR analysis of the representative regulatory genes *PAP1* and *MYBL2* and a structural gene *UF3GT*, which are light- and sugar-responsive genes (Jeong et al., 2010), was performed on samples grown on Suc and natural CK tZ and iP supplements. Suc induction of anthocyanin biosynthesis in Col-0 was correlated with an increase in the transcript levels of the positive transcription factor *PAP1* (Fig. 3A) but a decrease in the negative transcription factor *MYBL2* (Fig. 3B), and hence transcriptional activation of structural genes such as a *UF3GT* (Fig. 3C). Stimulation of Suc-induced anthocyanin pigmentation by both synthetic (BA) (Fig. 1) and natural (*tZ* and iP) (Fig. 2) CKs appeared to be mediated by further amplification of *PAP1* expression (*ca* seven-fold) (Fig. 3A) with concomitant suppression of *MYBL2* expression (*ca* 0.16-0.28 fold) (Fig. 3B). Consistent with the decrease in anthocyanin levels (Fig. 2), transcript levels of *PAP1* in natural CK-treated *CKX2ox* plants were about 30-40% of those in Col-0 (Fig. 3A), while expression levels of the negative regulator *MYBL2* were comparable between Col-0 and *CKX2ox* lines. The fact that CK treatment decreased *MYBL2* expression to similar levels in *CKX2ox* and Col-0 plants indicated that CKs are responsible for the decrease in *MYBL2* expression levels (Fig. 3B). Our results suggest that CK amplifies the effect of Suc signaling on anthocyanin pigmentation by increasing MBW complex activity and decreasing MYBL2 expression.

CK enhances metabolizable sugar-mediated accumulation of anthocyanin

To investigate whether CK treatment enhances Suc- and maltose (Mal)-dependent, but monosaccharide and osmoticum-independent signaling to anthocyanin biosynthesis (Jeong et al., 2010; Solfanelli et al., 2006), Col-0 seedlings were grown in different types of sugars at a concentration of 90 mM singly or in combination, and supplemented with or without 0.5 μM BA for 7 days in 140 μmol $m^{-2}s^{-1}$ light intensity. As previously reported (Jeong et al., 2010; Solfanelli et al., 2006), metabolizable sugars induced anthocyanin accumulation to different extents,

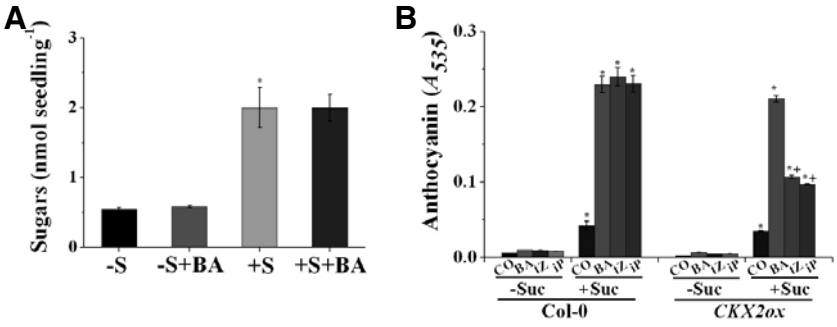

Fig. 2. Cytokinin modulates Suc regulation of anthocyanin accumulation. Total soluble sugar (A) and anthocyanin (B) contents in Col-0 wild-type (A, B) and *CKX2ox* transgenic (B) plants were extracted from 7-day-old seedlings grown either in 1/2 strength MS media (-S) or in 1/2-strength MS media supplemented with 90 mM Suc (+S) singly with (+BA) or without 0.5 μM BA (A) or in 1/2 strength MS media (-Suc) or in media supplemented with 90 mM Suc (+Suc) with [BA, *trans*-zeatin (tZ) and N6-(2-isopentenyl) adenosine (iP)] or without (CO) 0.5 μM CKs under growth light conditions. Values represent averages from results of at least four biological replicates. Symbols over bars indicate significant differences ($P < 0.05$; *t*-test) between control and treatment (asterisk) or wild-type and transgenic plants (cross).

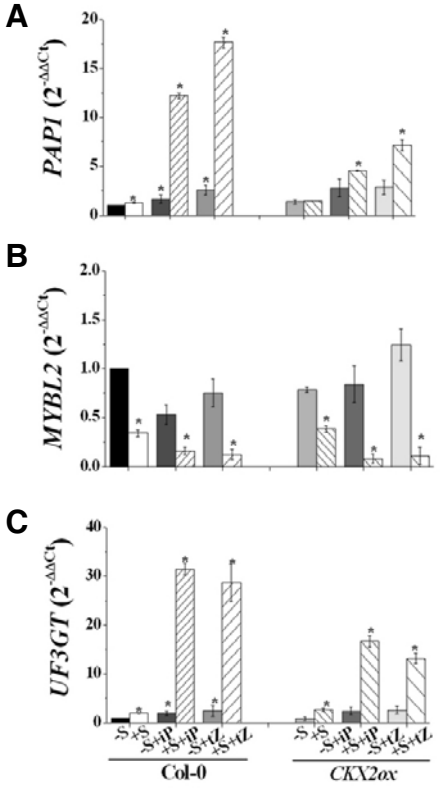

Fig. 3. Cytokinin modulates transcript levels of anthocyanin biosynthesis related genes. Transcript levels of *PAP1* (A), *MYBL2* (B) and *UF3GT* (C) were determined in Col-0 wild-type and *CKX2ox* transgenic plants grown either in 1/2 strength MS media (-S) or 1/2-strength MS media supplemented with 90 mM Suc (+S) singly or with 0.5 μM of natural CKs (+tZ and +iP) under growth light conditions for 7 days. Values represent averages from results of at least three biological replicates. Symbols over bars indicate significant differences ($P < 0.05$; *t*-test) between control and treatment (asterisk) or wild-type and transgenic plants (cross).

while the disaccharide sugar analogue palatinose (Pal) and an osmoticum, mannitol (Man), caused only a slight accumulation of anthocyanin (Fig. 4A). Despite the variable effects of metabolizable sugars on anthocyanin pigmentation, sugar type did not affect the sensitivity to exogenous CK treatment; BA treatment amplified sugar induction by 5-9-fold compared to negligible induction in Pal- or Man-fed plants (Fig. 4A). Thus, CK enhances sugar signaling-mediated induction of anthocyanin pigmentation irrespective of the type of metabolizable sugar.

This effect was further tested with several Arabidopsis mutants defective in sugar metabolism and Glc sensing. Mutants in cell wall invertase (*cwinv1)*, hexose transporter (*stp1)* and Suc transporter 1 (*Suc1*), which is partially responsible for Suc-induced anthocyanin pigmentation (Jeong et al., 2010; Sivitz et al., 2008), were grown in 90 mM Suc with and without 0.5 μM BA for 7 days. As shown in Fig. 4B, CK-modulated anthocyanin accumulation in the *stp1* mutant was comparable to that of Col-0 plants, while the *cwinv1* mutant showed 66% of the anthocyanin level of Col-0 plants, indicating that cell wall invertase is involved in CK-mediated anthocyanin induction. As expected, anthocyanin accumulation in the *suc1-2* mutant was almost half of that in the WT in Suc media without decreased responsiveness to exogenous CK (Col-0, 5.24-fold increase, versus *suc1-2*, 4.91-fold increase), which could be attributed to lower amounts of Suc transported into the signal generating sites of plants (Jeong et al., 2010). To rule out the involvement of AtHXK1-generated Glc signaling in the sugar induction of anthocyanin, the *gin2-1* mutant line exhibiting only catalytic activity but no hexose sensing activity (Moore et al., 2003) was grown along with wild-type Ler in MS media containing 90 mM Suc with or without 0.5 μM BA. As shown in Fig. 4B, no differences in the sensitivity to CK were noted between *gin2-1* and WT Ler plants. Thus, CK modulation of anthocyanin in the presence of Suc seems to follow a pathway distinct from the conventional hexokinase sensing and regulatory mechanism, but one that responds predominantly to Glc, a cleavage product of Suc.

CK signaling is partially mediated by type-B ARRs in a redundant manner

To elucidate the role of the membrane-bound CK receptors AHK2, AHK3, and AHK4 (Riefler et al., 2006) in the CK amplification of Suc induction observed above, single or double CK receptor mutants were studied. Anthocyanin levels in the *ahk2*, *ahk3* and *ahk4* single mutants and the *ahk2*/*3* and *ahk3*/*4* double mutants grown on 90 mM Suc-only media were lowered by 25-40% compared to WTs Col-0 and Ws (parent background for *ahk3*/*4*) plants (Fig. 5A). Supplementation of the Suc medium with 0.5 μM BA caused variable levels of anthocyanin accumulation in the single receptor mutants, with the *ahk4* mutant showing the greatest decrease. Furthermore, CK insensitivity was more severe in the *ahk2*/*3* and *ahk3*/*4* double mutants than in the respective single mutants, indicating the re-

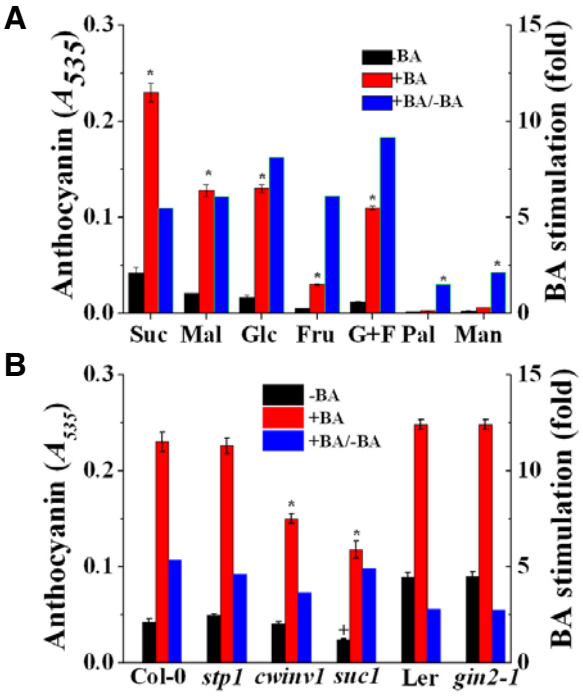

Fig. 4. Cytokinin stimulates metabolizable sugar-mediated anthocyanin accumulation. Wild-type Col-0 (A, B) or Ler (B) and sugar metabolism (*stp1*, *cwinv1* and *suc1-2*)- and Glc sensing (*gin2-1*)-defective mutant (B) seedlings were grown in 1/2 strength MS media supplemented with sugars singly (-BA) or with 0.5 μM BA (+BA) under growth light conditions for 7 days. The concentrations of Suc, Mal, Glc, Fru, Pal, and an osmoticum, Man, were 90 mM and the concentration of G+F (Glc + Fru) was 45 mM of each. Values represent averages from results of three biological replicates. Symbols over bars indicate significant differences ($P < 0.05$; *t*-test) between control and treatment (asterisk) or wild-type and mutant plants (cross).

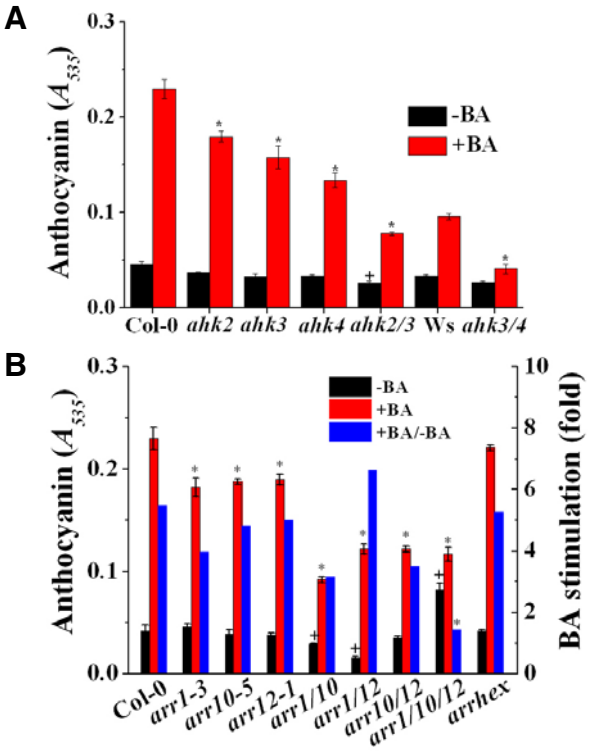

Fig. 5. Cytokinin signaling is mediated by AHK4 and type B ARRs. Anthocyanin contents were measured in single and double CK receptor (A) and type-A and -B response regulator (B) mutants. Wild-type Col-0 or Ws or receptor (*ahk2*, *ahk3*, *ahk4*, *ahk2/3*, and *ahk3/4*)- or ARR [*arr1-3*, *arr10-5*, *arr12-1*, *arr1/10*, *arr1/12*, *arr10/12*, *arr1/10/12*, and *arrhex* (*arr3/4/5/6/8/9*)]-defective mutant seedlings were grown in 1/2 strength MS media supplemented with 90 mM Suc singly (-BA) or with 0.5 μM BA (+BA) under growth light conditions for 7 days. Values represent averages from results of three biological replicates. Symbols over bars indicate significant differences ($P < 0.05$; *t*-test) between control and treatment (asterisk) or wild-type and mutant plants (cross).

dundancy of AHK2, AHK3, and AHK4 in CK modulation of Suc-mediated anthocyanin accumulation.

Among 11 type-B ARRs in the Arabidopsis genome, ARR1, ARR2, ARR10, ARR11 and ARR12 reportedly function as positive regulators of CK signaling in a redundant manner (Mason et al., 2005). To ascertain the response regulators that work in concert with AHKs in amplifying sugar signals to the downstream anthocyanin biosynthesis components, single, double and triple mutants of the type-B family of transcriptional regulators ARR1, ARR10 and ARR12 were investigated (Fig. 5B). The single mutants of *arr1-3*, *arr10-5* and *arr12-1* showed statistically insignificant differences in pigmentation compared to Col-0 seedlings when grown in 90 mM Suc, while they showed *ca.* 20% lower pigmentation in response to exogenous CK treatment. Analysis of double mutants revealed that *arr1-3/10-5* (*arr1/10*), *arr10-5/12-1* (*arr10/12*) and *arr1-3/12-1* (*arr1/12*) showed a significantly reduced response to Suc as well as exogenous CK in terms of anthocyanin pigmentation (38% in *arr1/10* compared to Col-0) (Fig. 5B). The *arr1/10/12* triple mutant showed three-fold higher anthocyanin accumulation than Col-0, consistent with previous findings reported by Argyros et al. (2008). Despite this higher anthocyanin accumulation, *arr1/10/12* triple mutants grown at a light intensity of 140 $\mu mol^{-2} s^{-1}$ were almost insensitive to exogenous CK treatment in the presence of Suc (only 0.4-fold increase compared to 5.4 fold in Col-0). Type-A response regulators are negative regulators of CK signaling and are the primary response factors for most of the CK-induced genes (To et al., 2004). Type-A ARRs in turn are subjected to transcriptional regulation by type-B ARRs. Interestingly, the hexaple type-A mutant of *arr3/4/5/6/8/9* retained sensitivity to exogenous CK application (Fig. 5B), indicating that type-B ARRs are the primary response factors in anthocyanin biosynthesis.

CK stimulates photoreceptor-independent Suc signaling regulating anthocyanin pigmentation

Irrespective of the presence of exogenous Suc, CK failed to induce anthocyanin accumulation in the dark (Argyros et al., 2008; Guo et al., 2005; Jeong et al., 2010), indicating that light is indispensable for CK activation of Suc signaling. As sugar signaling-dependent anthocyanin biosynthesis is under the control of light *via* photosynthesis-related factors (Das et al., 2011; Jeong et al., 2010), we assessed the effect of CK and sugar on known light signaling components to determine the point of convergence of the two signaling pathways.

Light signaling mutants such as *hy1*, which is deficient in the

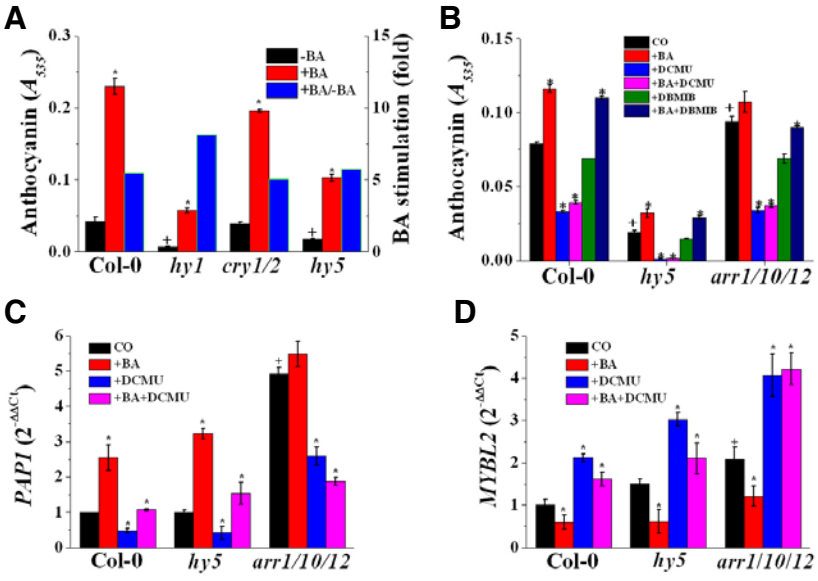

Fig. 6. CK stimulation is not dependent on PHY, CRY and HY5 signaling, but on photosynthesis. Anthocyanin (A, B) and transcript (C, D) levels in photoreceptors and type-B response regulator mutants. (A) Col-0, *hy1*, *cry1/2*, and *hy5* mutant seedlings were grown in 1/2-strength MS media supplemented with 90 mM Suc singly (-BA) or with 0.5 μM BA (+BA) under growth light conditions for 7 days. (B-D) Col-0, *hy5* single and *arr1/10/12* triple mutants were grown in 1/2-strength MS media for 4 days under growth light conditions and then transferred to 1/2 strength MS media containing 90 mM Suc (CO), supplemented with 0.5 μM BA (+BA), 5 μM DCMU (+DCMU), 10 μM DBMIB (+DBMIB) singly or in combination (+BA+DCMU, +BA +DBMIB), and further incubated under white light (140 μmol m^{-2} s^{-1}) for 1 day. Values represent averages from results of three biological replicates. Symbols over bars indicate significant differences ($P < 0.05$; *t*-test) between control and treatment (asterisk) or wild-type and mutant plants (cross).

PHY binding chromophore, the *cry1/2* double mutant, and *hy5*, were grown in 90 mM Suc-containing media supplemented with and without CK for 7 days under illumination. Accumulation of anthocyanin in *hy1* and *hy5* single mutants was significantly lower than in Col-0 plants, while the *cry1/2* double mutant showed comparable pigment level (Fig. 6A), consistent with previously published results (Jeong et al., 2010). Supplementation of Suc-containing growth media with 0.5 μM BA increased anthocyanin accumulation in all light signaling mutants, albeit to varying extents. Despite lowered anthocyanin contents in photoreceptor and HY5 mutants, the CK-induced fold change (*ca.* 5-8) in the light signaling mutants was comparable to that of Col-0 (Fig. 6A). Retention of CK sensitivity with respect to anthocyanin accumulation despite the absence of functional photoreceptors and a bHLH transcription factor, HY5, indicates that either the photoreceptors compensate for each other with varying efficiency, or CK induction of pigmentation is independent of the known photoreceptors involved in anthocyanin biosynthesis. A plausible candidate for such a signal could be the redox state of the photosynthesis electron transport that regulates sugar induction of anthocyanin biosynthesis in Arabidopsis (Das et al., 2011; Jeong et al., 2010).

CK stimulates photosynthesis-derived Suc signaling regulating anthocyanin pigmentation

To test whether a photosynthesis-derived signal, but not a photoreceptor-derived HY5-mediated signal, is modulated by CK in the regulation of anthocyanin accumulation, 4-day-old Col-0, *hy5* and *arr1/10/12* triple mutants grown in the light (140 μmol m^{-2} s^{-1}) in 1/2-strength MS media were transferred to 90 mM Suc liquid media supplemented with and without 5 μM DCMU (an electron transport inhibitor at photosystem II) or 10 μM DBMIB (an electron transport inhibitor that acts by binding to the cytochrome B_6f complex) (Trebst, 1980), and then were incubated for 24 h under white light (140 μmol m^{-2} s^{-1}). We chose transfer experiments here rather than co-treatments of DCMU and DBMIB with or without BA in Suc-containing growth media for 7 days from the beginning of germination under illumination to avoid inhibitor-induced seedling death under such long-term treatment conditions. In addition, high light intensity (140 μmol m^{-2} s^{-1}) rather than lower light (for instance 30 μmol m^{-2} s^{-1}) was chosen because the stimulatory effect of Suc on anthocyanin pigmentation in low light was not as obvious as in high light (Jeong et al., 2010). DCMU treatment for 1 day caused a severe inhibition of Suc induction of anthocyanin in Col-0 as well as in *hy5* and *arr1/10/12* triple mutants. However, DBMIB treatment showed a minimal effect on Suc induction (Fig. 6B), confirming previous findings implicating the photosynthesis-dependent light signaling pathway in anthocyanin induction (Das et al., 2011; Jeong et al., 2010). BA supplementation of the Suc-containing media increased pigmentation levels 1.5- and 1.7-fold in Col-0 and *hy5* plants, respectively, while the pigment level remained almost unchanged in *arr1/10/12* triple mutants (Fig. 6B). The BA-induced stimulation of Suc-induced pigmentation was significantly inhibited by DCMU treatment, but not by DBMIB treatment (Fig. 6B). Thus, CK modulation appears to act downstream of the redox poise of the photosynthetic electron transport chain that generates light and sugar signals.

The effect of short term BA treatment for 1 day on the stimulation of positive and negative regulatory genes involved in sugar-induced anthocyanin biosynthesis was investigated (Jeong et al., 2010). Although transcript levels were several-fold lower than those from 7-day-treated Col-0 seedlings (Fig. 3), *PAP1* and *bHLH*s (*TT8*, *GL3*, and *EGL3*) transcript levels were enhanced and the *MYBL2* transcript level was lowered by BA treatment, while expression of the WD40 repeat protein TTG1 remained unchanged (Supplementary Figs. 1A and 1B), confirming earlier findings (Fig. 3) that BA enhancement is mediated by selective activation of the MBW complex and inhibition of MYBL2 expression.

Next, we investigated the transcript levels of regulatory genes coding for the MBW complex and the negative regulator MYBL2, and the structural gene *UF3GT* in WT Col-0, *hy5* and *arr1/10/12* triple mutants treated with or without BA and DCMU for 1 day. Consistent with anthocyanin contents, CK stimulation correlated with increased transcript levels of the positive tran-

scription factors *PAP1* (Fig. 6C), *EGL3* (Supplementary Fig. 1C), *GL3* (Supplementary Fig. 1D) and *TT8* (Supplementary Fig. 1E) and a modest decrease in the transcript level of the negative transcription factor *MYBL2* (Fig. 6D), and hence the transcriptional activation of the structural gene *UF3GT* (Supplementary Fig. 1F). This stimulatory effect of CK on Suc-induced transcript levels was not significantly affected by the *hy5* mutation, but was significantly reduced by the inhibition of photosynthetic electron transport (DCMU treatment). As expected, *arr1*/*10*/*12* defective mutants were almost insensitive to CK treatment in terms of the accumulation of regulatory gene transcripts, but retained sensitivity to DCMU treatment, suggesting that CK acts downstream of the photosynthetic electron transport chain. In contrast to other regulatory factors in which transcript levels were almost unchanged by CK treatment, *TT8* transcript levels were raised 50% by exogenous CK treatment (Supplementary Fig. 1E) in the presence of mutations of *ARR1*, *10*, and *12*, indicating that additional type-B ARRs could maintain a certain level of redundancy.

DISCUSSION

AHKs and a subset of type-B ARRs that act as part of the CK two-component pathway positively regulate anthocyanin biosynthesis in the presence of sugars

Arabidopsis has five receptor histidine kinases (AHKs), of which three transmembrane hybrid kinases containing an extracellular cytokinin-binding CHASE domain sense the CK stimulus in response to changes in external cues (Anantharaman and Aravind, 2001). CK regulation of anthocyanin biosynthesis was previously shown to be mediated by AHK2, AHK3 and AHK4 in Arabidopsis (Argyros et al., 2008). The current results confirmed this finding and further pinpointed the variable degree of redundancy of these three receptors during amplification of sugar signals regulating anthocyanin biosynthesis. AHK4 acts as a major CK sensor for amplification of the sugar signal, as shown by a lower level of redundancy of its mutant compared to AHK2 and AHK3 with respect to the accumulation of anthocyanin in response to CK treatment (Fig. 5A). Such variable degrees of redundancy among AHK2, AHK3 and AHK4 are highly expected, as they share a similar CHASE domain required for CK binding (Schmülling, 2004).

Analysis of the single, double and triple mutants of type-B ARRs, *arr1-3*, *arr10-5*, and *arr12-1* and the hexaple mutant of type-A ARRs indicated that CK signaling leading to anthocyanin accumulation is channeled primarily through ARR1, ARR10 and ARR12, as double mutations in *arr1/10* and *arr1/12* and triple mutations in *arr1*/*10*/*12* resulted in an almost complete loss of sensitivity to exogenous CK in a redundant manner (Fig. 5B). Interestingly, high anthocyanin pigmentation was observed in the *arr1*/*10*/*12* triple mutant in response to Suc, even under lower growth light (30 μmol $m^{-2}s^{-1}$) conditions (Supplementary Fig. 2). This hypersensitivity of the *arr1*/*10*/*12* triple mutant to growth light intensity was also reported by Argyros et al. (2008). The hypersensitivity to Suc and light could be attributed to changes in root-mediated sugar responses, as the root-localized Suc transporter AtSUC1 is partially responsible for anthocyanin pigmentation (Jeong et al., 2010; Sivitz et al., 2008). However, detached leaves of the *arr1*/*10*/*12* triple mutant without root and hypocotyls floated on 90 mM Suc under illumination (140 μmol $m^{-2}s^{-1}$) for 24 h were fully capable of anthocyanin production in response to Suc treatment in comparison to Col-0 leaves (Supplementary Fig. 2). Thus, genetic changes in shoots as a consequence of the triple mutation including chloroplast development (Argyros et al., 2008) and overall increases in the transcript levels of both positive and negative transcription factors for anthocyanin biosynthesis (Fig. 6 and Supplementary Fig. 1) seem to be responsible for the acquired hypersensitivity to Suc and light although the mechanism underlying this phenomenon needs further study.

CK amplifies sugar-induced anthocyanin accumulation in Arabidopsis

In Arabidopsis, the effect of sugars on increasing the anthocyanin content in seedlings (Solfanelli et al., 2006; Teng et al., 2005) is independent from photoreceptors, PHY and CRY1/2, but is dependent on photosynthesis (Das et al., 2011; Jeong et al., 2010). CKs are also implicated in the regulation of anthocyanin accumulation presumably by affecting photosynthetic apparatus development or reactive oxygen species-mediated oxidative stress (Argyros et al., 2008) or by modulating sugar signaling (Guo et al., 2005). Thus, it is most likely that positive signaling pathways related to anthocyanin biosynthesis, such as those mediated by sugars, CK and light, are merged at a certain point of the synthesis pathway. In the present study, we found that CKs stimulate sugar-induced anthocyanin biosynthesis *via* transcriptional activation of the positive regulators PAP1, (E)GL3 and TT8 (Figs. 3 and 6; Supplementary Fig. 1). Comparable endogenous soluble sugar contents between plants grown with or without BA (Fig. 2A) suggest that CK functions downstream of sugar signaling, rather than modulating sugar signaling through changes in endogenous sugar levels as suggested previously (Guo et al., 2005).

Sugars such as Suc and Mal stimulate anthocyanin accumulation in a concentration-dependent manner when supplied exogenously (Jeong et al., 2010). Consistently, Suc was the most effective sugar at enhancing anthocyanin pigmentation. However, CK enhanced the effect of Mal, Glc, and Fru on anthocyanin induction almost to the same degree as that of Suc, suggesting that CK enhancement of the effect of sugars is metabolism-dependent. Furthermore, CK did not significantly induce anthocyanin pigmentation in the presence of the Suc analogue Pal and the osmoticum Man (Fig. 4A), ruling out the involvement of disaccharide sugar signaling or internal Glc sensor AtHXK1-mediated signaling. This view is further strengthened by the fact that the effects of CK on anthocyanin accumulation were partially but significantly reduced by mutations in either cell wall invertase (*cwINV1*) or Suc transporter 1 (*suc1-2*) (Fig. 4B). The amplification of sugar signals by CK seems independent of known AtHXK1-mediated signaling (Moore et al., 2003). However, the existence of such a pathway is not beyond contemplation, as HXK1 independent pathways are involved in the regulation of stress signaling genes such as *CHS*, *PAL1,* and *PAL3*, and starch metabolism-related *AGPase* and *CIN1* genes (Xiao et al., 2000). The role of plant hormones in sugar regulation with respect to anthocyanin biosynthesis is paramount, as demonstrated by the failure of methyl JA to induce anthocyanin synthesis in sugar-free media (Shan et al., 2009), and the involvement of sugars in the ethylene and GA repression of anthocyanin pigmentation (Jeong et al., 2010; Loreti et al., 2008).

CK stimulation of anthocyanin pigmentation involves photosynthesis-dependent signaling components

Light is an essential factor governing sugar- and CK-induced accumulation of anthocyanin pigmentation in Arabidopsis seedlings, as dark grown seedlings failed to produce any anthocyanin even in the presence of sugar and CK (Argyros et al., 2008;

Guo et al., 2005). In Arabidopsis, the PAP1 transcription factor, a principal target of Suc and light signaling pathways, was shown to be subject to regulation by CK and light, as revealed by the increase in transcript levels in response to treatment with both synthetic and natural CKs (Fig. 3A). Further, in the *hy5* mutant, PAP1 transcript levels and sensitivity to exogenous CK treatment were comparable to those of WT Col-0, while the photosynthesis inhibitor DCMU resulted in almost full repression of transcripts (Fig. 6C) along with anthocyanin content (Figs. 6A and 6B), suggesting that CK stimulation of anthocyanin biosynthesis via PAP1 regulation is independent of HY5 but dependent on photosynthesis signaling. This is in contrast to the study by Vandenbussche et al. (2007), who concluded that HY5 is the point of convergence between light and CK signalings, and the contribution of HY5-independent pathways is very minor in the light. In fact, recent reports showed that a HY5-independent pathway of anthocyanin biosynthesis operates in Arabidopsis and is repressed by ethylene (Das et al., 2011; Jeong et al., 2010).

CK activates sugar signals that regulate photosynthesis pathways *via* the redox state of the PQ pool

Inhibition of photosynthetic electron transport at the acceptor side of PS II by DCMU drastically inhibited anthocyanin pigment accumulation in the presence of sugar alone and sugar and CK together, while another photosynthetic electron transport inhibitor, DBMIB, had negligible effects on pigmentation (Fig. 6B). DCMU treatment causes the oxidation of the PQ pool while DBMIB prevents the reoxidation of the PQ pool by the cytochrome B_6f complex, resulting in the reduction of the PQ pool. Thus, changes in the redox state of the PQ pool in response to light may regulate sugar and CK signaling, leading to anthocyanin accumulation. This is consistent with earlier reports that anthocyanin biosynthesis is decreased in the presence of photosynthesis inhibitors in Arabidopsis (Das et al., 2011; Jeong et al., 2010) as well as other plants such as turnip seedlings (Schneider and Stimson, 1971) and corn leaves (Kim et al., 2006). Research into redox signaling identified several candidates that might mediate the retrograde signaling directly or indirectly. In Arabidopsis, two-thylakoid-associated kinases, STN7 and STN8, sense redox signals generated by the PQ pool in chloroplasts and play distinct roles in the short- and long-term acclimation of the photosynthetic apparatus to varying a light environment (Bellafiore et al., 2005; Vainonen et al., 2005). In the present study, *stn7* as well as *stn8* mutants failed to show any insensitivity to sugar and CK (Supplementary Fig. 3), ruling out these kinases as putative redox sensors responsible for anthocyanin pigmentation unless they function redundantly during the CK amplification of light- and sugar- signaling cascade for anthocyanin biosynthesis. A recent study suggested that anthocyanin biosynthesis in Arabidopsis, is regulated in a redox-dependent manner, based on correlative changes in the ascorbic acid content and flavonoid pigment levels under high light conditions (Page et al., 2011). This is further supported by results that ascorbic acid is involved in ABA- and JA-mediated anthocyanin accumulation (Shan et al., 2009). If this is the case, then the redox state of the PQ pool might be a signal regulating ascorbic acid biosynthesis, which needs to be examined further.

Based on the current findings, we propose a regulatory interaction among light, sugar and CK in anthocyanin biosynthesis in Arabidopsis. Light and sugar signals generated by photosynthetic electron transport (PET) regulate anthocyanin biosynthesis by activating the MBW complex and inhibiting MYBL2 expression (Jeong et al., 2010). Here, we have shown that CK signaling components could act as intermediaries downstream of PET signaling by activating the MBW complex. Our results that the disruption of PET signaling significantly hindered the CK-mediated induction of anthocyanin accumulation via transcriptional regulation of the MBW complex support this model, as increase exogenous cytokinin under DCMU treatment failed to retain the level of anthocyanin accumulation observed without DCMU treatment when treated in sugar medium.

In summary, we demonstrated that CK modulates sugar-induced anthocyanin accumulation *via.* a subset of type-B ARRs, ARR1, ARR10 and ARR12, which eventually activates the MBW transcriptional complex. In addition, we showed that CK activity is independent of HY5 and other photoreceptors, but highly dependent on the redox state of the photosynthetic electron transport chain whereby the redox poise of the PQ pool regulates the pigment biosynthesis.

Note: Supplementary information is available on the Molecules and Cells website (www.molcells.org).

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