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Responses to Environmental and Chemical Signals for Anthocyanin Biosynthesis in Non-Chlorophyllous Corn (*Zea mays* L.) Leaf

Jin-Seog Kim*, Byung-Hoi Lee, So-Hee Kim, Kwang-Hoon Oh, and Kwang Yun Cho
Biological Function Research Team, Korea Research Institute of Chemical Technology, Daejeon 305-600, Korea

The effects of environmental and chemical signals on anthocyanin biosynthesis in non-chlorophyllous (white) corn leaves were investigated. Carbohydrates that caused the greatest stimulation of anthocyanin formation included fructose, glucose, and sucrose, followed by maltose, raffinose, trehalose, cellobiose, melibiose, galactose, and lactose. Sucrose enhanced the expression of anthocyanin biosynthesis genes on a transcriptional level. Carbohydrate concentration, duration of light exposure, and incubation temperature also had quantitative effects. Low temperatures stimulated anthocyanin biosynthesis whereas water stress had no effect. Abscisic acid, jasmonic acid, and ethephon also enhanced anthocyanin accumulation, although the degree of its accumulation depended on co-supplied sucrose concentrations, and was relatively lower in white tissue than in green tissue. Gibberellic acids and 6-benzylaminopurine were significantly inhibitory at the nanomolar level. Indole 3-acetic acid and salicylic acid did not influence anthocyanin synthesis in the white tissue system. Diuron inhibited its formation only in green tissue. These results indicate that the white leaf segments of corn would be good systems for research on the signal networks related to chloroplast functioning in anthocyanin biosynthesis. Additionally, this experimental system could be practical for identifying hormone-like substances, especially gibberellic acids and benzylaminopurine.

Keywords: anthocyanin biosynthesis, carbohydrates, environmental factors, non-chlorophyllous tissue, plant hormones, signal network

Anthocyanins are the red, purple, and blue flavonoid pigments produced mainly in plant epidermal cells, where they accumulate in vacuoles, causing the tissue to become colored. The biochemical and genetic basis for anthocyanin production is relevant to both the development of neutroceuticals, because of its antioxidant activity and resistance to environmental stress, and to its modification of flower colors (Mol et al., 1996; Chalker-Scott, 1999; Winkel-Shirley, 2001; Kong et al., 2003). The anthocyanin biosynthetic pathway has been studied intensively. Expression of the genes involved is regulated by a complex array of environmental and developmental signals, such as light quality, temperature, plant hormones, and sugars (Mol et al., 1996; Chalker-Scott, 1999; Winkel-Shirley, 2001). Some of these signals promote the activity of master transcription regulators that control various secondary metabolic pathways (Endt et al., 2002). Negative regulators of anthocyanin biosynthesis also have been reported (Aharoni et al., 2001; Wade et al., 2003). Nevertheless, although the physiology and basic genetics of the anthocyanin biosynthetic pathway are well characterized, an integrated understanding of the regulatory network is still elementary.

Park et al. (1998) have reported that anthocyanin formation is accelerated by sucrose supplementation to a greater extent in non-chlorophyllous (white) leaf segments of corn, as induced by fluridone treatment, than in green tissue. This suggests that a more intense investigation of the role of chloroplast and details of the signal network related to anthocyanin biosynthesis might be possible using white leaf tissue. To determine this possibility, one must first evaluate the effect of various environmental and chemical signals on the induction of anthocyanin biosynthesis in those white tissues. Therefore, objective of the present study was to examine maintenance of a normal anthocyanin formation system in non-chlorophyllous leaf segments with several unique characteristics. We also assessed their suitability for signal network research on anthocyanin biosynthesis, especially as it relates to chloroplast functioning.

*Corresponding author; fax +82-42-861-4913
e-mail jskim@krict.re.kr

MATERIALS AND METHODS

Plant Materials

Corn seeds (*Zea mays* L. cv. Suweon 19) were soaked in a 0.15 mM fluridone solution at room tem-

perature for 1 d. They were then sown in pots (350 cm^2) and grown in a greenhouse (30 to 35°C/20 to 25°C, day/night; 14-h photoperiod) for 3 d. Afterward, the pots were transferred to a growth chamber (29 ± 1°C; light, 70 ± 5 μmol m^{-2} s^{-1}; 12-h photoperiod) and incubated for 4 d (Kim et al., 2003, 2004). The second leaves of the resultant white seedlings were cut into 5-mm segments after their mid-ribs were removed.

Carbohydrate Treatments

We investigated the effects of 16 different carbohydrates, including sucrose, on anthocyanin biosynthesis in non-chlorophyllous tissues. Leaf segments were floated on solutions of 1% carbohydrate in 1.0 mM MES buffer, then incubated for 2 d in a growth chamber (constant temperature of 28°C, continuous light, 110 ± 5 μmol m^{-2} s^{-1}). The effect of sucrose concentration on anthocyanin formation relative to the timing of illumination was also investigated, using two methods. With the first technique, leaf segments were floated on various sucrose solutions (0.3, 1.0, 2.0, or 3.0%) and immediately incubated for 2 d with light (80 ± 5 μmol m^{-2} s^{-1}). The second method involved supplying sucrose prior to illumination; here, leaf segments were floated on various sucrose solutions (1, 2, 3, or 4%) in the dark for 1 d, then transferred to a sucrose-free buffer and incubated for 1 d in the light (80 ± 5 μmol m^{-2} s^{-1}).

Temperature and Dehydration Treatments

The effect of incubation temperature on anthocyanin biosynthesis was tested. Leaf segments were floated on a buffer containing 1.0% sucrose, then exposed for 2 d under continuous light (75 μmol m^{-2} s^{-1}), between 15 to 30°C. The influence of transient low temperatures was also examined before the non-chlorophyllous tissues, both seedlings and leaf segments, were placed on the sucrose solution. White seedlings in the early third-leaf stage were treated at various temperatures (15, 20, 25, or 30°C) in the light (40 μmol m^{-2} s^{-1}) for 1 d. Leaf segments were placed on wet paper and treated with illumination (40 μmol m^{-2} s^{-1}) for 1 d at 20°C or 30°C. To examine the effects of dehydration, fresh leaf segments were air-dried for 160 min, weighed, and incubated for their anthocyanin accumulation. Induction of anthocyanin biosynthesis in the samples above was carried out essentially as described by Kim et al. (2003). Leaf segments (0.15 g fresh weight) were placed on 6 mL 1.0 mM MES buffer (pH 6.0 to 6.5) that contained 1% sucrose. They were then incubated for 2 or 4 d at a constant temperature of 25 to 28°C and under continuous light of 90 to 100 μmol m^{-2} s^{-1}.

Treatment with Hormones and Defense Signal Messengers

Various compounds were evaluated for their effect on anthocyanin accumulation. These included the plant hormones indole 3-acetic acid (IAA), gibberellic acids (GAs), 6-benzylaminopurine (BA), and abscisic acid (ABA), as well as 2-chloroethylphosphonic acid (ethephon), methyl-jasmonic acid (MeJA), and salicylic acid (SA). Diuron, a photosystem II inhibitor, was also tested. Both non-chlorophyllous and chlorophyllous leaf segments were placed on 6 mL 1.0 mM MES buffer (pH 6.0 to 6.5) that contained 0.3% or 1.0% sucrose, then incubated for 2 d at a constant temperature (26°C) and under continuous light (85 μmol m^{-2} s^{-1}). The results were expressed as the percent of anthocyanin accumulation relative to the untreated control.

Extraction and Assay of Anthocyanin

All the above experiments were carried out with three replicates. Leaf segments were macerated, in 10 mL methanol and 1% concentrated HCl, at 28000 rpm for 30 s in a Polytron PT3000 Homogenizer (Kinematica, USA). They were then centrifuged at 27200g for 20 min. The absorbances of the supernatants were measured at 528 nm and 657 nm in a 2401PC UV-VIS Spectrophotometer (Shimazu, Japan). Anthocyanin was quantified as described by Mancinelli (1990), using the formula A_{528}-0.25(A_{657}) to compensate for the contribution of chlorophylls. Data were expressed as the mean ± standard deviation (SD) of three replicates.

Expression Analysis of Anthocyanin Biosynthesis Genes

Non-chlorophyllous leaf segments were floated on a 0.3% sucrose solution and immediately incubated for 2 d with light (85 μmol m^{-2} s^{-1}) at 26°C. Quantitative reverse-transcription polymerase chain-reaction (QRT-PCR) was performed to analyze the expression of anthocyanin biosynthesis genes from those sucrose-treated tissues. Total RNA isolation was carried out with the Easy-spin™ [DNA free] Total RNA Extraction Kit (Intron Biotech, Korea), following the manufacturer's instructions. Two micrograms of total

Table 1. Primers and PCR conditions for analyzing the expression of anthocyanin biosynthesis genes from sucrose-treated non-chlorophyllous leaf segments of corn.

Genes	Primers	PCR conditions
CHS	Forward 5′-GATCACGCACCTAGTGTTCT-3′ Reverse 5′-TCGATGTTCTTGGAGATGAGC-3′	35 cycles at 94°C for 30 s, 52°C for 40 s and 72°C for 90 s
CHI	Forward 5′-GTCGAGATCGGAGGCAAC-3′ Reverse 5′-CTCCCCGATGATGGACTC-3′	35 cycles at 94°C for 30 s, 55°C for 40 s and 72°C for 90 s
F3H	Forward 5′-CGTGAGATCGTGACCTACTT-3′ Reverse 5′-GAACTCCTTGTTGGCACTCT-3′	35 cycles at 94°C for 30 s, 52°C for 40 s and 72°C for 90 s
DFR	Forward 5′-AAGACCCTGAGAATGAGGTAAT-3′ Reverse 5′-GTCGTACTCGGGGTACCTAT-3′	35 cycles at 94°C for 30 s, 52°C for 40 s and 72°C for 90 s
ANS	Forward 5′-AGGACTACCTCTTCCACCTT-3′ Reverse 5′-GCTGCTGTTTCTTCTTGAAAAGC-3′	35 cycles at 94°C for 30 s, 52°C for 40 s and 72°C for 90 s
orp-2	Forward 5′-AAGGACGTGCACACCGC-3′ Reverse 5′-AACAGGGGGGCACTACC-3′	35 cycles at 94°C for 40 s, 56°C for 40 s and 72°C for 40 s

RNA from each treatment was reverse-transcribed with a Maxime RT PreMix Kit (Intron Biotech) at 45°C for 60 min. With the reverse-transcribed treatment (cDNA), chalcone synthase (*CHS*), chalcone isomerase (*CHI*), flavanone 3-hydroxylase (*F3H*), dihydroflavonol 4-reductase (*DFR*), and anthocyanidin synthase (*ANS*) were PCR-amplified using the Maxime PCR PreMix (i-Taq) and a Geneamp™2700 Thermal Cycler (Applied Biosystems, USA). The *orange pericarp-2* (*opr-2*) gene, which encodes the β-subunit of tryptophan synthase (Wright et al., 1992), was used to standardize the concentrations of different samples (Piazza et al., 2002). Primer sequences and PCR conditions are listed in Table 1. Afterward, the PCR products were analyzed by gel electrophoresis on 2% agarose gels.

RESULTS

Effect of Carbohydrates on Anthocyanin Biosynthesis

In the non-chlorophyllous corn, anthocyanin could not be biosynthesized without supplemental carbohydrates. Here, 16 different carbohydrates were exogenously supplied to fluridone-treated tissues, and their effects on anthocyanin formation were compared (Table 2). Those that proved most stimulating, i.e., producing anthocyanin values >1.0, were fructose, glucose, and sucrose. These were followed, in order of their effectiveness, by maltose, raffinose, trehalose, cellobiose, melibiose, galactose, and lactose, with anthocyanin values ranging from 0.02 to 1.0. In contrast, mannose, ribose, xylose, isomaltose, isopropyl β-D-1-thiogalactopyranoside (IPTG), and mannitol had no effect.

Table 2. Effect of exogenously applied carbohydrates on anthocyanin formation[1)] in non-chlorophyllous corn leaf segments. Mean ± SD.

Carbohydrate	Anthocyanin content (A_{528}/0.15 g FW/10 mL MeOH)
Cellobiose	0.1275 ± 0.006
Fructose	1.8083 ± 0.223
Glucose	1.6570 ± 0.109
Galactose	0.0487 ± 0.026
IPTG	ND
Isomaltose	ND
Lactose	0.0472 ± 0.005
Maltose	0.8841 ± 0.077
Mannitol	ND
Mannose	ND
Melibiose	0.0541 ± 0.017
Raffinose	0.3993 ± 0.107
Ribose	ND
Sucrose	1.7853 ± 0.175
Trehalose	0.1423 ± 0.025
Xylose	ND

[1)]For anthocyanin formation, leaf segments inoculated on a buffer solution containing 1% sucrose were incubated under continuous light (110 μmol m^{-2} s^{-1}) at 28°C for 2 d. ND, not detected.

The influences of sucrose concentration and supplementation method were also investigated. When sucrose was incorporated into the tissue prior to illumination, the anthocyanin content was increased in a linear manner ($y = 0.434x - 0.063$, $r^2 = 0.993$) (Fig. 1B). However, when sucrose was supplied simultaneously with illumination, the increase in anthocyanin

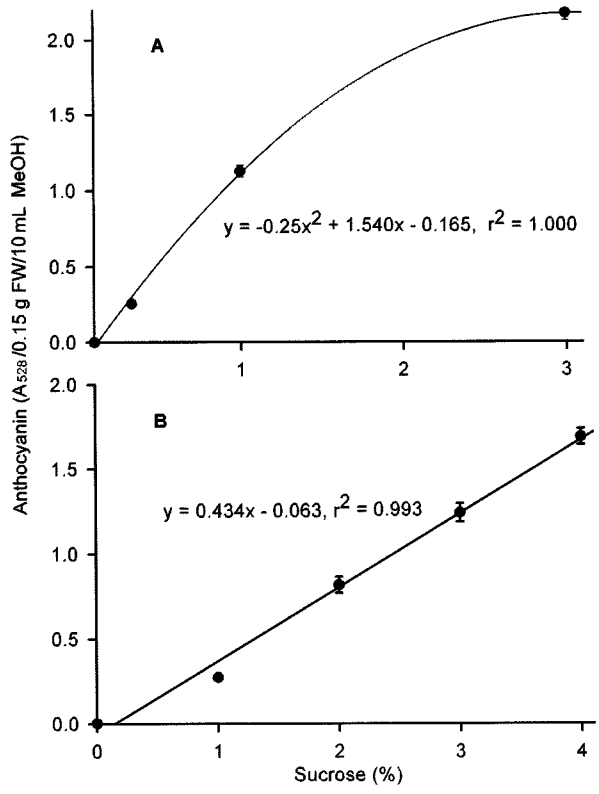

Figure 1. Effect of exogenous sucrose supplements on anthocyanin accumulation in non-chlorophyllous corn. **A,** Leaf segments were floated on buffers containing different concentrations of sucrose, then immediately incubated in the light for 2 d. **B,** Leaf segments were floated on various concentrations of sucrose in the dark for 1 d, transferred into sucrose-free buffer, then incubated in the light for 1 d.

content followed a second-order curve ($y = -0.25x^2 + 1.540x - 0.165$, $r^2 = 1.00$) (Fig. 1A). This indicates that the carbohydrate species and concentration were highly related to the magnitude of anthocyanin accumulation in white tissue.

The effect of sucrose supplement on the expression of structural genes associated with anthocyanin biosynthesis was also evaluated. When white leaf segments were floated on 0.3% sucrose and incubated in the light for 48 h, expression of *CHS*, *CHI* and *ANS* was already enhanced at 12 h after sucrose supplement. Expression of *F3H* and *DFR* was remarkably increased at 24 h after the addition of sucrose (Fig. 2A).

Effect of Environmental Signals on Anthocyanin Biosynthesis

Effect of incubation temperature on sucrose- and light-dependent anthocyanin production was determined in white leaf segments. Biosynthesis in tissues floated on a 1% sucrose solution in the light was linearly increased ($y = 0.093x - 1.389$, $r^2 = 0.996$) when the incubation temperature rose from 15 to 30°C (Fig. 3).

Moderate to low temperatures (10 to 15°C) can stimulate gene expression for anthocyanin biosynthesis in some plant species (Christie et al., 1994; Mol et al., 1996). Here, we examined whether this was also true for white corn seedlings or their leaf segments. Seedlings grown at 30°C were incubated in a temper-

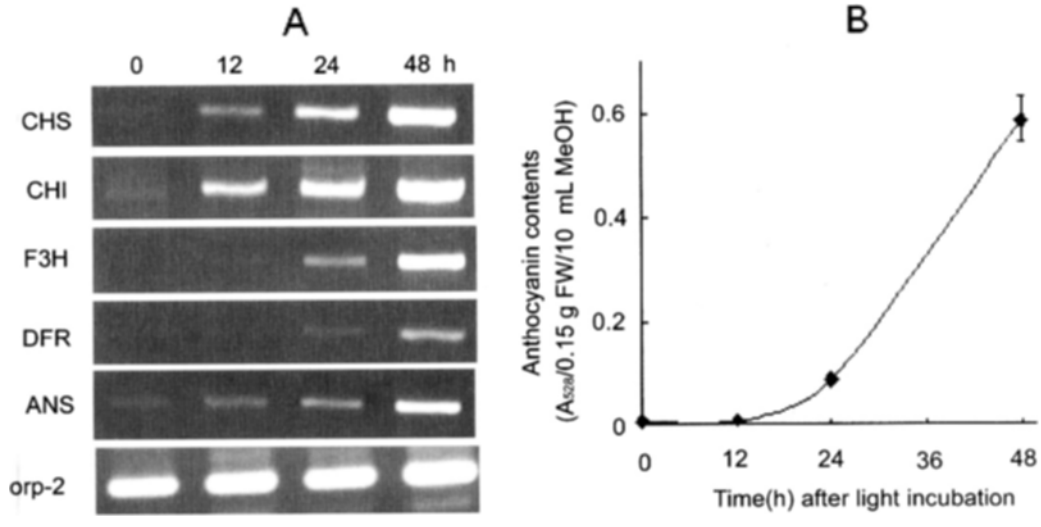

Figure 2. Effect of 0.3% sucrose on expression of anthocyanin biosynthesis genes (**A**) and accumulation of anthocyanin under the Light (**B**) in non-chlorophyllous corn. Leaf segments were floated on a buffer containing 0.3% sucrose, then incubated under continuous light (85 μmol m^{-2} s^{-1}) at 26°C for 48 h.

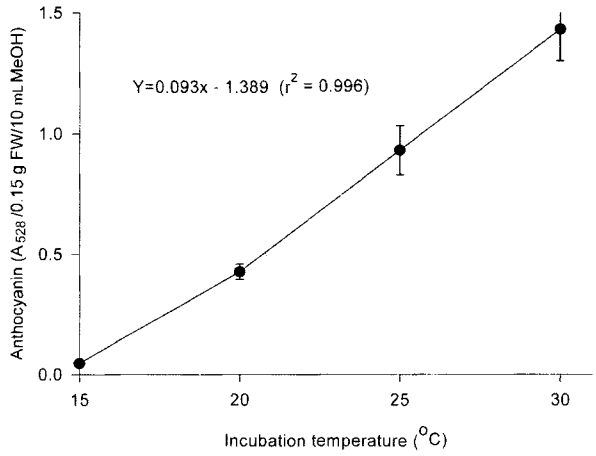

Figure 3. Effect of incubation temperature on anthocyanin formation in non-chlorophyllous corn. Leaf segments were floated on buffer solution containing 1.0% sucrose, then incubated under continuous light (75 μmol m^{-2} s^{-1}) and at indicated temperature for 2 d.

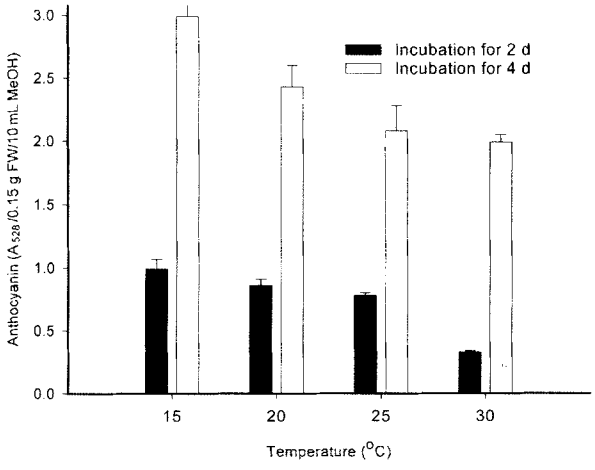

Figure 4. Effect of moderate- to low-temperature treatments on induction of anthocyanin biosynthesis in non-chlorophyllous corn. White seedlings in early third-leaf stage were placed at various temperatures under continuous light (40 μmol m^{-2} s^{-1}) for 1 d. Segments were then prepared from second leaves of treated seedlings, and incubated for 4 d as described in Materials and Methods.

ature-regulated chamber for 1 d, and then anthocyanin formation was tested with the treated leaves. Accumulation was greater when seedlings were exposed to 15°C than to 25 or 30°C (Fig. 4). In addition, anthocyanin accumulation was 231% higher when leaf segments were treated at 20°C than at 30°C (Table 3). These results indicate that the transient treatments of moderate temperature and low light imposed on either intact white seedlings or leaf segments had a stimulatory effect on anthocyanin

Table 3. Effect of moderate temperature and light treatments on anthocyanin formation in non-chlorophyllous corn leaf segments. Mean ± SD.

Treatment[1]		Anthocyanin induction [2] (A_{528}/0.15 g FW/10 mL MeOH)
Temperature (°C)	Illumination	
20	Light	1.568 ± 0.032
30	Light	0.676 ± 0.060

[1]Leaf segments were incubated at the indicated temperatures in the light (40 μmol m^{-2} s^{-1}) for 1 d. [2]For anthocyanin induction, treated leaf segments were floated on a buffer containing 1% sucrose, and incubated under continuous light (90 μmol m^{-2} s^{-1}) at 26°C for 2 d.

Table 4. Effect of water stress on anthocyanin formation[1] in non-chlorophyllous corn leaf segments. Mean ± SD.

Air-drying duration (min)	Decrease in water content (μg/0.15 g FW)	Anthocyanin content (A_{528}/0.15 g FW/ 10 mL MeOH)
0	00.0	2.2912 ± 0.11
60	42.8 ± 1.84	2.2122 ± 0.10
80	51.7 ± 1.76	2.1733 ± 0.10
100	59.9 ± 7.94	1.9413 ± 0.06
120	67.7 ± 6.56	1.9875 ± 0.02
140	71.2 ± 1.65	1.9210 ± 0.06
160	77.3 ± 1.41	1.9728 ± 0.10

[1]For anthocyanin biosynthesis, air-dried leaf segments were floated on a buffer containing 1.0% sucrose, and incubated under continuous light (110 μmol m^{-2} s^{-1}) at 28°C for 2 d.

induction. However, we believe the significant difference in accumulation between 20 and 30°C was likely not induced only by low temperature because we also observed somewhat weak deterioration (discoloration) in white tissues exposed to the higher temperature.

Although water stress can enhance the synthesis of anthocyanin (Mol et al., 1996), our dehydration treatment did not increase its formation. In fact, anthocyanin accumulation decreased by 13.9% when seedlings showed a level of 48.5% dehydration compared with their initial fresh weight (Table 4).

Effects of Several Compounds on Anthocyanin Biosynthesis

Application of ABA (at 0.01 to 1.00 μM) to non-chlorophyllous corn leaf segments had a stimulatory effect on anthocyanin accumulation. The higher ABA rate, however, decreased pigment accumulation because of the disintegration of cell membranes (Fig. 5).

GA analogues inhibited sucrose- and light-dependent anthocyanin accumulation in white tissues at nanomolar levels: their effects differed according to the particular GA species applied. Of all the compounds tested, GA_7 was the most inhibitory (Fig. 5). BA also inhibited anthocyanin accumulation in white tissues at nanomolar levels (Fig. 5). IAA and SA showed no significant effects on anthocyanin biosynthesis.

Supplements of MeJA and ethephon, in the range of 1 to 10 μM, to 1% sucrose enhanced anthocyanin formation only in green tissues, and response to ethephon treatment was relatively minor. Higher concentrations of MeJA and ethephon inhibited anthocyanin accumulation in both green and white leaf segments (Fig. 5). Supplements of either one in 0.3% sucrose enhanced anthocyanin production in green tissue and, to a lesser extent, in white tissue (Table 5).

Diuron significantly inhibited anthocyanin formation in green tissue, but showed no effect in the white tissue, suggesting that photosynthetic electron transport could be closely connected to anthocyanin formation (Table 5).

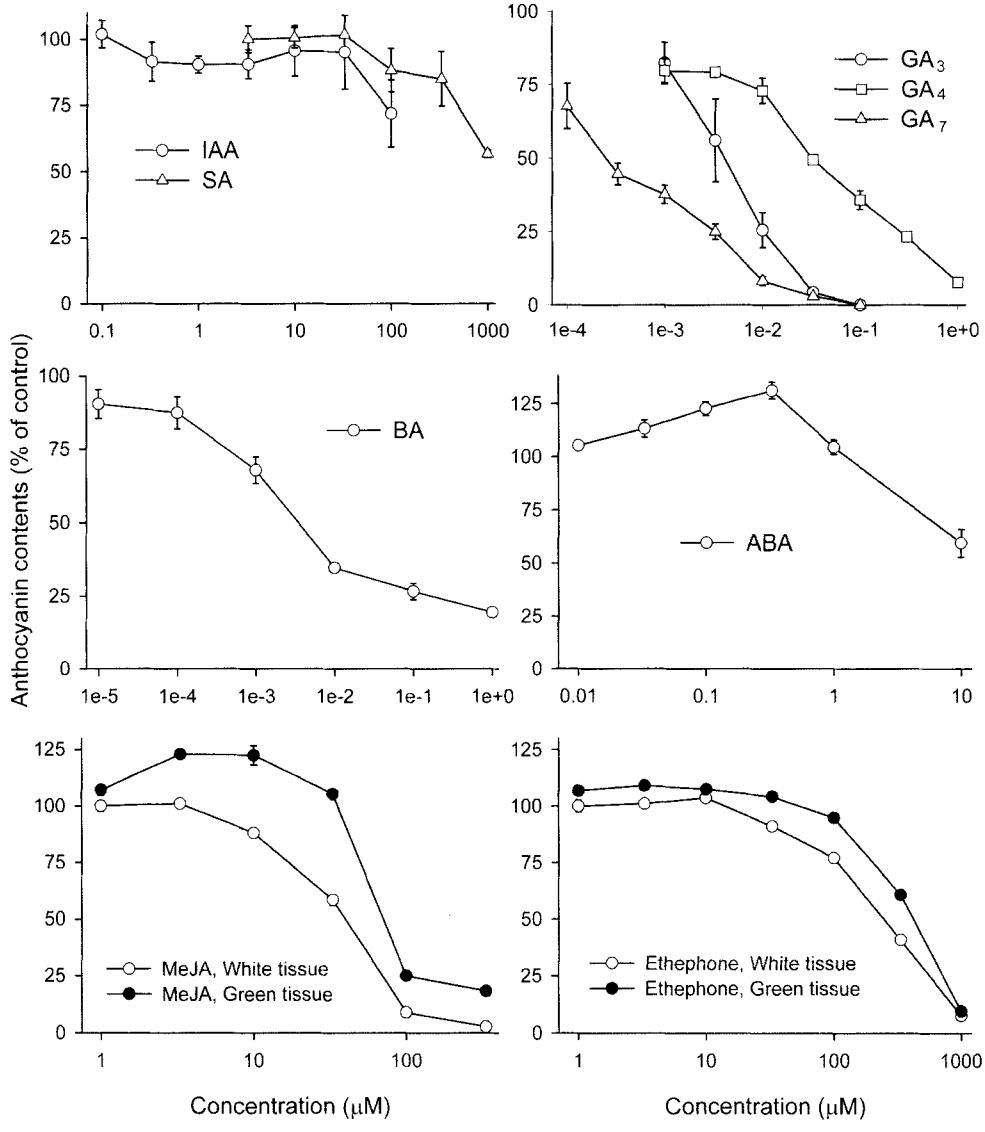

Figure 5. Effects of plant hormones and defense signal messengers on the induction of anthocyanin synthesis in non-chlorophyllous and chlorophyllous (for MeJA and ethephon) leaves of corn. Segments were obtained from second leaves of seedlings and floated on solutions of IAA, SA, GAs, BA, ABA, MeJA, or ethephon. They were then incubated under continuous light (85 μmol m^{-2} s^{-1}) at 26°C for 2 d.

Table 5. Effect of methyl-jasmonic acid, ethephon, ABA, and diuron on sucrose-induced anthocyanin formation[1)] in green and white leaf segments of corn seedlings. Mean ± SD.

Compound	Concentration (μM)	Anthocyanin content (% of control)	
		White leaf segment	Green leaf segment
Methyl jasmonic acid	3.3	113.8 ± 1.0	128.5 ± 7.4
	10	128.2 ± 2.9	151.2 ± 9.5
Ethephon	3.3	110.4 ± 4.1	108.4 ± 3.8
	10	105.2 ± 6.4	128.5 ± 8.0
Abscisic acid	0.10	119.1 ± 9.3	138.0 ± 8.8
	0.33	102.1 ± 5.6	131.5 ± 13.2
Diuron	3.3	94.5 ± 5.5	4.7 ± 0.4
	10	93.5 ± 3.3	1.0 ± 0.8

[1)]For anthocyanin formation, leaf segments were floated on a buffer containing 0.3% sucrose, and incubated under continuous light (85 μmol m^{-2} s^{-1}) at 26°C for 2 d.

DISCUSSION

Anthocyanin biosynthesis is regulated through the complex interactions of various internal and external influences, including temperature, light, carbohydrates, plant hormones, and water stress. These stimulate transcription factors (TFs) through an intricate signal transduction network, and the activated TFs then trigger the expression of various genes related to anthocyanin biosynthesis (Mol et al., 1996; Chalker-Scott, 1999; Weiss, 2000; Winkel-Shirley, 2001; Endt et al., 2002; Kong et al., 2003). The relevant TFs and their precise functions are currently being elucidated in several plant species, but their coordinated regulation in that signalling network is still unclear (Mol et al., 1996; Winkel-Shirley, 2001; Endt et al., 2002; Rolland et al., 2002; Gyula et al., 2003; Wade et al., 2003).

Sugar sustains plant growth and development by functioning in the up- and down-regulation of large sets of genes through still-unknown mechanisms (Rolland et al., 2002; León and Sheen, 2003). It also contributes to anthocyanin biosynthesis (Mol et al., 1996; Mita et al., 1997; Weiss, 2000; Hara et al., 2003). Sugar binds to responsive domains on DNA and regulates various physiological functions at the transcriptional level (Mita et al., 1997; Chalker-Scott, 1999; Weiss, 2000; Rolland et al., 2002; León and Sheen, 2003). We also observed this activity in our white leaf tissues, with sucrose beginning to increase the expression of anthocyanin biosynthesis genes, e.g., *CHS, CHI,* and *ANS,* from 12 h after treatment (Fig. 2). However, the role of sucrose in white leaf tissues is not likely to be limited only to the induction of gene expression. Sucrose supplements delay the senescence of white leaf tissues (Kim et al., 2004). In addition, it is expected that carbohydrates are used as a carbon sources of anthocyanidin biosynthesis and for a glycosylation of anthocyanidins.

The non-chlorophyllous seedlings used in the present study could not produce carbohydrates in leaf tissue (Kim et al., 2004), but survived mainly by consuming the nutrients that remained from the endosperm. In a previous investigation, total sugar contents for white and green leaves just before incubation for anthocyanin formation were 31.0 and 166.3 mg g^{-1} dry weight, respectively. Therefore, the carbohydrate-deficient tissues seemed to respond more quantitatively and sensitively to exogenous carbohydrates, making them useful tools for studying the role of carbohydrates in anthocyanin production. Using this system, we compared the effects of various carbohydrates on anthocyanin biosynthesis (Table 2). Fructose, glucose, and sucrose produced the greatest stimulation of anthocyanin formation, probably because they are not only main energy sources for plant metabolism but are also important in gene regulation (Fig. 2). Furthermore, this formation was quantitatively dependent on the concentration of sucrose supplement (Fig. 1). In contrast, maltose, raffinose, trehalose, cellobiose, melibiose, galactose, and lactose had minor effects. These various responses could have been just a function of the differential uptake and/or degradation of particular compounds, as well as their differential effect on gene regulation. Therefore, further study is needed to determine their roles in the plant cell.

Anthocyanin biosynthesis in white tissues that were floated on sucrose solution in the light was linearly increased as the incubation temperature rose from 15 to 30°C (Fig. 3). When white seedlings or leaf segments were exposed to a transient, low temperature (15-20°C) prior to their placement on the sucrose solution, anthocyanin accumulation increased during the incubation period (Fig. 4). These results suggest that the cooler temperature stimulates gene expression for anthocyanin biosynthesis (Christie et al., 1994; Mol et al., 1996) despite the existence of smaller amount of endogenous carbohydrates and the fact that low temperature is not necessarily associated with chloroplast functioning.

Water stress may enhance anthocyanin synthesis (Mol et al., 1996) as a direct result of osmotic potential regulation (Chalker-Scott, 1999). However, our

results did not support this idea. That is, the dehydration treatment of white leaf segments did not increase anthocyanin accumulation (Table 4). One alternative hypothesis is that water stress might cause a rise in cell carbohydrate concentration (Müller et al., 1995) and the increased carbohydrates act as a direct signal for anthocyanin induction. In fact, we found here that increasing levels of anthocyanin coincided with higher sucrose concentrations (Fig. 1), and that the exogenous supply of trehalose, commonly known to be increased by water stress, induced small amounts of anthocyanin accumulation in the white leaf tissue (Table 2).

Various chemicals, including plant hormones, affect anthocyanin biosynthesis either directly or indirectly (Mol et al., 1996). In the present study, relatively low concentrations of ABA, MeJA, and ethephon stimulated its accumulation in white tissues. While GA and BA inhibited this response, IAA and SA had no effect on anthocyanin biosynthesis (Fig. 5).

ABA can be practically applied to agricultural products as a color-enhancing substance, because relatively low concentrations stimulate anthocyanin formation (Han et al., 1996; Lee et al., 1996). Ethylene and jasmonate, substances generally known to induce senescence, also may increase the production of anthocyanin (Franceschi and Grimes, 1991; Tamari et al., 1995; Han et al., 1996; Ei-Kereamy et al., 2003). Our results showed that these substances were less effective stimulators than ABA in white tissue (Fig. 5). The relatively greater effect in green tissue than in white tissue (Table 5) may be partially related to the fact that these compounds cause senescence. Further study will allow us to test this hypothesis.

The effect of GA on anthocyanin synthesis differs among plant species. GA stimulates anthocyanin formation in the corolla tissue of petunia (*Petunia hybrida*) (Moalem-Beno et al., 1997), in detached *Hyacinthus orientalis* (Hosokawa et al., 1996), and in the sheaths of *Hordeum vulgare* (Martinez and Favret, 1990). In contrast, GA inhibits anthocyanin formation in suspension cultures of wild carrot (Ilan et al., 1994) and in strawberry fruits (Martinez et al., 1996). In our study of non-chlorophyllous corn leaf segments, GA strongly inhibited the sucrose-dependent biosynthesis of anthocyanin (Fig. 5). Ronchi et al. (1997) have also linked anthocyanin accumulation in *Zea mays* with gibberellin inhibition. Cytokinins may also stimulate its accumulation in tissue cultures and plant organs. For instance, Deikman and Hammer (1995) have reported that BA promotes anthocyanin biosynthesis in *Arabidopsis*. Here, however, BA applied to our non-chlorophyllous leaf segments strongly inhibited sucrose-dependent anthocyanin induction at 0.1 nM (Fig. 5). Further investigations are needed to clarify the mechanisms by which GA and BA inhibit this accumulation. It has been suggested that GA regulates the transcriptional expression of anthocyanin biosynthesis genes (Ilan et al., 1994). Furthermore, GA and BA might affect the formation of cell structures such as anthocyanoplasts (Pecket and Small, 1980; Grotewold et al., 1998).

Diuron, a photosystem II inhibitor, profoundly inhibited sucrose- and light-dependent anthocyanin induction in green tissue but not in the white tissue (Table 5). This indicates that photosynthetic electron transport is closely related to anthocyanin formation through an as yet unidentified mechanism. Therefore, this white corn system might be useful for further studies of that mechanism.

We demonstrated here that non-chlorophyllous corn leaf segments maintained a normal anthocyanin formation system, with several interesting characteristics. Accordingly, the use of such plant material to assay for sucrose-dependent anthocyanin production could become a more convenient research tool for studying signalling networks and the regulatory mechanisms of anthocyanin biosynthesis. This assay system quantitates the response to sugar concentration, incubation time, temperature, and illumination. Furthermore, the experimental method is relatively simple and efficient for evaluating the effects and interactions of various factors on anthocyanin biosynthesis, and large quantities of uniform white leaf segments can be easily obtained via fluridone treatment. In addition, the role of the chloroplast in anthocyanin biosynthesis can now be intensively investigated, because the chloroplast does not function in white tissues. Finally, this assay can be applied to the screening of plant hormone-like substances, such as GA and BA, as well as herbicides that inhibit shikimic acid biosynthesis (Kim et al., 2003). With the present assay method, GA and BA activity appeared at 0.3 nM and 0.1 nM, respectively, indicating sensitivity levels higher than those obtained with other systems.

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