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Constitutive Expression of the Maize Genes *B1* and *C1* in Transgenic Hi II Maize Results in Differential Tissue Pigmentation and Generates Resistance to *Helicoverpa zea*

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Anthocyanin biosynthesis in maize protects tissues from biotic and abiotic stresses. Constitutive expression of the maize *B1* and *C1* genes, which induces anthocyanin biosynthesis, resulted in transgenic plants with varied phenotypes. Some colored leaves were substantially resistant to thrips damage, while only leaves with the highest levels of cyanidin, the predominant anthocyanidin detected in all colored transgenic tissues, were resistant to corn earworm (CEW) larvae. Colored anthers were resistant to CEW feeding, and reductions in CEW growth were significantly correlated to levels of cyanidin in the anthers. Cyanidin chloride and cyanidin-3-glucoside chloride added to insect diet slowed the growth of CEW larvae. Attempts to produce 3′5′-hydroxylated anthocyanins in colored maize with the expression of a petunia F3′5′H hydroxylase gene were unsuccessful.

KEYWORDS: Insect; resistance; Thysanoptera; Lepidoptera; *Zea mays*

INTRODUCTION

Insect herbivores cause yield losses due to physical damage to maize and also promote the colonization of the plants by fungi, some of which produce toxins deleterious to humans and livestock. Enhancing plant resistance to insect herbivory is an important aspect of crop management, leading to lower fungal toxin levels (*1*). The production of transgenic corn with a toxin from *Bacillus thurigienesis* greatly enhanced plant resistance against some insect herbivores and also reduced fungal toxin levels (*1*). Because resistance to *B. thurigiensis* toxins is possible (*2*) and the toxins do not kill all the pests of corn, new insect resistance molecules need to be discovered and incorporated into maize via traditional breeding or genetic engineering.

In many plants, anthocyanins are synthesized in floral tissues to attract pollinators (*3*). Anthocyanins present in maize are likely produced for other functions, as this species is wind pollinated. Anthocyanins protect tissues from light stress, serve as antioxidants, or function as osmoregulators (*4*−*6*). Additionally, anthocyanins may be part of a defense strategy against potential herbivores. Experiments with two different tree species determined that leaf anthocyanins possibly play a role in herbivore protection (*7*,*8*). Cyanidin, delphinidin, and cyanidin-3-glucoside from cotton inhibited the growth of tobacco budworm (*9*, *10*), while petunia anthocyanins, such as malvidin 3-*cis*-/3-*trans*-*p*-coumaroylrutinoside-5-glucoside, contributed to growth retardation of corn earworm and cabbage looper larvae (*11*).

The anthocyanin biosynthetic pathway of maize has been studied at both the biochemical and genetic level (*3*, *12*). A number of chemical studies determined that maize tissues synthesize anthocyanins derived from the three following anthocyanidins: cyanidin, pelargonidin, and peonidin (**Figure 1**) (*13*−*15*). If flavonoid 3′-hydroxylase (F3′H), a cytochrome P450 enzyme, is active, the cyanidin or peonidin derivatives (hydroxylated at the 3′-carbon) will accumulate, while a null F3′H activity results in pelargonidin derivatives (**Figure 1**). The absence of 3-*O*-glycosides of delphinidin, petunidin, and malvidin (delphindin is hydroxylated at the 3′- and 5′-carbon, petunidin has a 3′-methoxy group, and malvidin has 3′- and 5′-methoxy groups) in maize is likely due to the lack of flavonoid 3′5′-hydroxylase (F3′5′H) enzyme, another cytochrome P450 enzyme.

Studies of anthocyanins in the *Graminaceae* have determined that very few members synthesize 3′5′-hydroxylated anthocyanins (*13*, *14*). An exception is blue wheat (cv. Purendo), where delphinidin glycosides account for ∼69% of the total anthocyanins in aleurone tissue (*14*). These findings in other cereals indicate that transformation of maize with a functional F3′5′H could result in production of 3′5′-hydroxylated anthocyanins. Pilot studies in our laboratory indicated delphinidin chloride slowed the growth of insect larvae (data not shown). This study documents the transformation of Hi II maize with the petunia F3′5′H gene with the intent of producing delphinidin glycosides that could potentially contribute to insect resistance.

MATERIALS AND METHODS

Plasmids. *B1*+*C1*. The maize *B1*-*Peru* and *C1* cDNAs (provided by Dr. V. Chandler), each regulated by a cauliflower mosaic virus 35S promoter, Adh1 intron 1, and NOS (*Agrobacterium tumefaciens* nopaline synthase) terminator, were cloned as a *Not* I and *Xba* I fragment, respectively, into an empty ligated pCR2.1 vector (Invitrogen, Carlsbad, CA) using standard molecular biology techniques (*16*).

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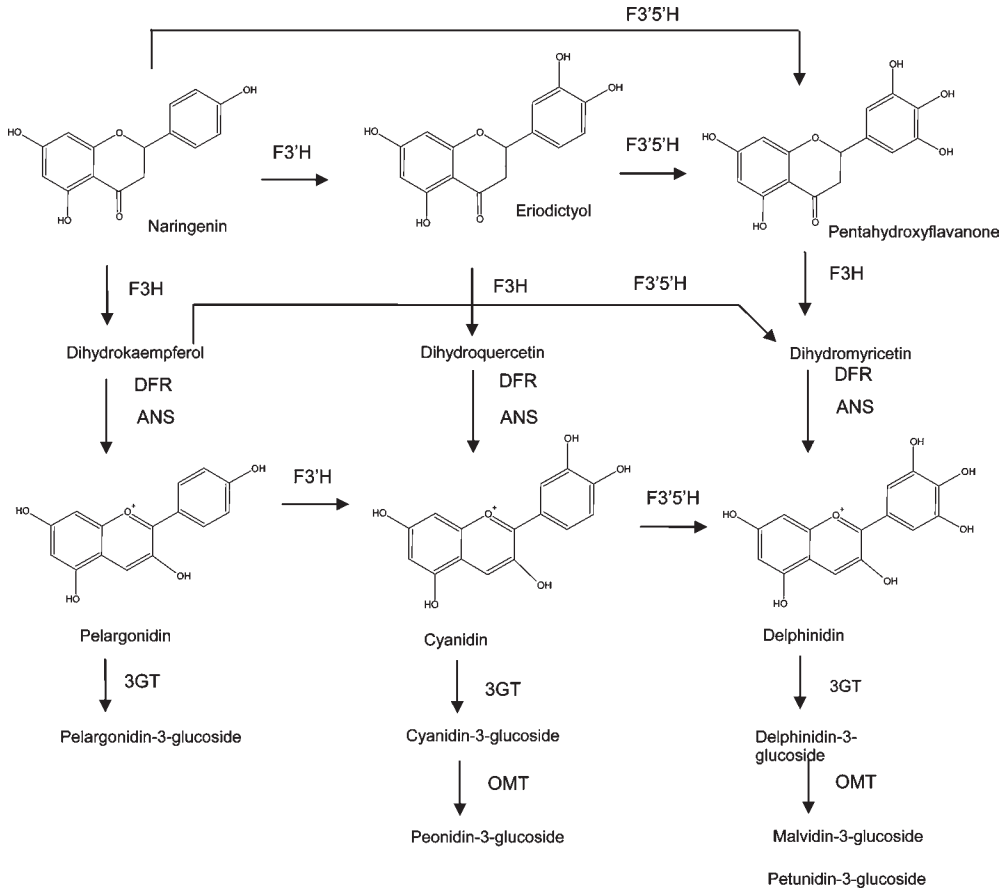

Figure 1. Anthocyanin biosynthesis pathway. Enzymes involved in the pathway are indicated as follows: F3H, flavonone 3-hydroxylase; F3′H, flavonoid 3′-hydroxylase; F3′5′H, flavonoid 3′5′-hydroxylase; DFR, dihydroflavonol-4-reductase; ANS, anthocyanidin synthase; 3GT, UDP-glucose:flavonoid 3-*O*-glucosyltransferase; OMT, *O*-methyltransferase. Maize does not contain functional F3′5′H activity and therefore only synthesizes pelargonidin-, cyanidin-, and peonidin-3-glucosides. This figure was adapted from refs *12* and *17*.

F3′5′H. The *Petunia hybrida* F3′5′H cDNA obtained from International Flower Developments, Pty. Ltd., Australia encodes a protein that matched the *Petunia hybrida* (cultivar QL01) protein sequence ABN42195 in Genbank. However, this sequence has four different amino acids than the F3′5′H sequence first cloned from petunia (*17*). The Holton et al. sequence (Z22545, 1521 bp) is conserved among seven other petunia F3′5′H sequences in Genbank and its substrate specificity verified in yeast (*17*). To ensure the insertion of a functional F3′5′H into maize, the obtained F3′5′H cDNA was mutated to the Holton et al. sequence using the Quick Change Kit (Stratagene, La Jolla, CA) as follows: phenylalanine 262 to tyrosine, glycine 263 to glutamate, threonine 322 to alanine, and arginine 325 to lysine. The altered F3′5′H sequence (confirmed by sequencing with BigDye Terminator Cycle Sequencing Kit, Applied Biosystems, Foster City, CA) was cloned using standard techniques (*16*) into an altered pAHC25 (*18*) that no longer contained β-glucuronidase. F3′5′H mRNA should be transcribed by the maize ubiquitin promoter in the modified pAHC25 vector.

Plants. Maize transformation of Hi II embryos was performed by the Iowa State Plant Transformation facility using the biolistic protocol (*19*). Two different bombardments were completed: (1) *B1*+*C1* plasmid only; and (2) equimolar amounts of *B1*+*C1* and F3′5′H plasmid. Transgenic callus was shipped to Peoria and the colored callus was picked for embryo hardening at 25 °C in the dark (*19*). Some additional callus lines exposed to light under laboratory conditions developed color and were also transferred to embryo hardening media. Hardened embryos were then placed on regeneration medium (*19*) and placed in a 25 °C incubator with 16 h of illumination by a growth light. Transgenic plantlets (the T_0 generation) were transferred to a soil mixture described previously (*20*) and kept in a greenhouse with the target temperature conditions of 25 °C and a 14:10 light:dark photoperiod maintained by supplemental lights. To help acclimation to soil, the plantlets were initially covered with plastic cups as described (*20*). Mature plants were those surviving the transformation and regeneration process and were phenotypically scored during development. T_1 (the progeny of T_0 plant crosses) seed was sown in the same soil mixture (*20*) in a walk-in plant growth facility that contained 1000 W sodium and halide lighting. The growth facility was kept at 24 ± 2 °C day and 18 ± 2 °C night temperatures with 50 ± 10% relative humidity. Plant tissue samples were placed at −80 °C until future chemical analysis (unless otherwise noted).

Analysis of Anthocyanidins and Anthocyanins. Fresh leaves were added directly to 2 M HCl or frozen at −80 °C, weighed after removal from the ultralow freezer, and then added to 2 M HCl. Leaf−2 M HCl solutions were stored at 4 °C at least overnight. Freeze-dried tissue was added to 2 M HCl just prior to heat treatment. Anthocyanidins were extracted as described (*21*) with the following modifications. Acid-extractable pigment was treated multiple times with amyl alcohol to recover as much pigment as possible. The pigment in amyl alcohol was evaporated under nitrogen and the pigment resuspended in methanol with 0.12% HCl. All samples were filtered through 0.45 μm nylon membranes before injection. This acid-extraction method is likely not complete, as there was still color in the boiled tissue samples, which means the actual anthocyanidin concentration of each sample is higher than reported.

Samples were run on an Applied Biosystems/MDS Sciex QStar Elite Q-TOF mass spectrometer with a Turboionspray electrospray source and an Agilent 1100 series HPLC system (G1379A degasser, G1357A binary capillary pump, G1389A autosampler, G1315B photodiode array detector, and a G1316A column oven) all running under Applied Biosystems Analyst 2.0 (build 1446) LC-MS software. The MS was calibrated at least once daily with a standard calibration mixture recommended by Applied Biosystems, and the signal detection was optimized as needed. The data was acquired in the MOF MS mode, positive. The MS parameters were as follows: accumulation time 1 s, mass range 200−1000 Da, source gas

1−50 units, source gas 2−35 units, curtain gas 25 units, ion spray voltage 4500 V, source heater 400 °C, declustering potential 80, focusing potential 265, declustering potential 2−15, ion release delay 6, ion release width 5. The column used was an Inertsil ODS-3 reverse phase C-18 column (3 μ, 150 × 3 mm, with a Metaguard column, from Varian). For LC-MS analysis, the initial conditions were 5% methanol and 0.2% acetic acid in water, at a flow rate of 0.25 mL/min. The effluent was monitored at 520 nm on the PDA. After a delay of 2 min, the column was developed to 100% methanol with a linear gradient over 60 min. Quantifying the PDA peaks of maize anthocyanidins was based on linear plots of cyanidin chloride, peonidin chloride, and pelargonidin chloride standards (Chromadex, Irvine, CA). The PDA peak used for each anthocyanidin calculation had a lagging (~0.4 s) mass ion peak that corresponded to the correct mass.

Insects and No-Choice Bioassays. Corn earworms (*H. zea*) were reared as previously described (*22*). Bioassays of plantlet and mature leaf tissue were performed as previously described (*20*). Briefly, 10 first instar *H. zea* larvae were added (by carefully grasping their silk with a dissection probe) to tight-closing plastic dishes containing plant material or diet discs with pigment (see below) sufficient for *ad libitum* feeding. Pieces of plantlet tissue were removed for bioassays as the plantlets were being transferred from tissue culture boxes to soil. For mature T_0 leaf tissue, the seventh leaf of 10 leafed plants was used for the bioassay. The second leaves of T_1 plants were used for bioassays; the distal half of the leaf (including the tip) was used in the bioassay, while the proximal half of the leaf was saved for pigment analysis (described above). Twenty anthers were used for each bioassay and scored for the number of holes after ~24 h, while after ~48 h the score reflected the number of anthers with >50% of material still intact. Cyanidin chloride (Chromadex, Irvine, CA) was dissolved in 90% acetone, while cyanidin 3-glucoside chloride (Apin Chemical, Abingdon, U.K.) was dissolved in distilled water and added to freeze-dried insect diet as previously described (*20*). Mortality of larvae in all the bioassays was low (< 10%) and therefore not reported. Surviving caterpillars were frozen at −20 °C and subsequently weighed to 0.01 mg using an analytical balance (Mettler-Toledo AE163 or AX105DR, Columbus, OH). Some T_0 plants were assayed for thrips (present in the greenhouse) damage 10−16 days after CEW bioassays with the seventh leaf. The total millimeters of linear leaf scraping was determined by counting each scraped feeding site on the leaf, which occurs in a linear fashion.

Maize Lesion Assays. The youngest leaf of four Hi II plants (each ~1 foot high) was used for assays. The leaves were ~2 cm wide and were cut into four approximately square sections. Three slits were cut into each leaf perpendicular to the veins using a sharpened jeweler's screwdriver with a 1.5 mm wide blade, avoiding the center vein. Cuts were made approximately 5 mm from the edges. Two cuts were made on the right side of the leaf piece, and one cut was made on the left side (the cut nearest the midvein). Sequential sections from each leaf were used for alternating treatments. A 2 μL portion of a 1% solution of cyanidin chloride or delphinidin chloride (Chromadex) was added to each cut. Leaf pieces were placed in 5 mm wide Petri dishes with tight-fitting lids (Falcon 351006, Becton Dickinson, Franklin Lakes, NJ) along with a 42.5 mm diameter filter paper disk (Whatman #1, Maidstone, England) that had 120 μL of sterile deionized water added. Leaf disks were examined daily for 3 days, and the width of any chlorotic zones present at the slits was determined to the nearest millimeter.

RT-PCR Analysis. Fresh or frozen tissue (90−100 mg) was ground to a powder with liquid nitrogen using a mortar and pestle. Total RNA was isolated using Trizol (Invitrogen) according to the manufacturer's instructions. A portion of RNA was treated with DNase (Qiagen, Valencia, CA) and purified using the RNeasy MinElute Cleanup Kit (Qiagen). A 500−600 ng portion of purified RNA was used to synthesize cDNA with the Accuscript High Fidelity first Strand cDNA synthesis kit (Stratagene). PCR (20 μL reactions) was performed with PCR Master mix (Roche Diagnostics, Mannheim, Germany) using 5% DMSO, 2 μL of cDNA or RNA control (purified RNA diluted in distilled H_2O), and primers (2 ng/μL each) to amplify the entire petunia F3′5′H gene: 5′-ATG ATG CTA CTT ACT GAG CTT GGT GCA-3′ and 5′-CTA TGG TAC ATA AAC ATC CAA TTG TAA CCT TG-3′. Amplification was performed with the PTC-200 thermal cycler (MJ Research, Waltham, MA) under the following conditions: 95 °C for 2 min; 40 cycles of 95 °C for 30 s, 61 °C for 30 s, and 72 °C for 1 min 30 s; 72 °C for 10 min. PCR products were separated by agarose gel electrophoresis and visualized with SYBR Safe DNA stain (Invitrogen).

Table 1. CEW Feeding Ratings on T_0 Transgenic Plantlets[a]

line	day 1 (*N*)	day 2 (*N*)	survivor wt ± SE (*N*)
B1+*C1* colored	21 ± 1.7 (8) a	12 ± 2.0 (7) a	0.093 ± 0.0080 (45) a
B1+*C1* green	25 ± 2.2 (12) a	19 ± 2.3 (11) a	0.13 ± 0.0086 (84) b
B1+*C1*+F3′5′H colored	14 ± 2.0 (9) a	11 ± 3.3 (9) a	0.092 ± 0.0093 (50) a
B1+*C1*+F3′5′H green	28 ± 2.3 (19) b	16 ± 1.7 (16) a	0.11 ± 0.0051 (120) a

[a] Mean feeding rating ± SE: day 1, total 0.25 mm^2 holes; day 2, total 1 mm^2 holes. Mean values with different letters were significantly different ($P < 0.05$).

Table 2. Survival of Plantlets and Phenotypes of T_0 Transgenic Plants

B1+C1

green plantlets: 75% survival (9 of 12)
colored plantlets: 25% survival (2 of 8)
mature survivors
- event 8: green leaves, male sterile (7 plants)
- event 16: green leaves, purple anthers (3 plants)
- event 26: green leaves, purple anthers (12 plants)

B1+C1+F3′5′H

green plantlets: 84% survival (16 of 19)
colored plantlets: 44% survival (4 of 9)
mature survivors:
- 3 events: purple leaves, purple anthers (4 plants)
- 3 events: green leaves, purple anthers (14 plants)[a]
- 5 events: green leaves, tan/yellow/pink anthers (18 plants)

[a] One event produced one plant with purple anthers and two other plants with tan anthers.

Statistical Analysis. Statistical differences in feeding and larvae survivor weights were determined by analysis of variance (ANOVA; proc GLM) using SAS version 9.1 (Cary, NC). Correlation analyses were completed using "proc reg corr" with the same SAS software.

RESULTS

Insect Resistance of T_0 Plantlets. Two types of transgenic plants were generated: *B1*+*C1* only, and *B1*+*C1*+F3′5′H. When they are expressed using constitutive promoters, the *B1-peru* and *C1* gene products coordinately induce pigment production in any tissue with a functional anthocyanin biosynthetic pathway (*3*). At the plantlet stage, colored tissue, generated by the presence of the *B1*+*C1* gene combination, was observed in both groups of plants. Corn earworm larvae (CEW) feeding ratings were lower (though not significantly) on colored plantlet tissue from both *B1*+*C1* and *B1*+*C1*+F3′5′H transformed tissues, and significantly lower ($P < 0.05$) on Day 1 of *B1*+*C1*+F3′5′H colored tissue compared to *B1*+*C1*+F3′5′H green tissue (**Table 1**). Larvae feeding on *B1*+*C1* colored tissue weighed significantly less than those feeding on *B1*+*C1* green tissue with a similar trend noted between *B1*+*C1*+F3′5′H colored and green tissues.

Studies of Mature T_0 Plants. Survival of colored plantlets was much lower compared to that of green plantlets (**Table 2**). It should be noted that not all regenerated plants were assayed for CEW resistance at the plantlet stage. Leaves of the two *B1*+*C1* survivors (event 16) were colored at the plantlet stage but became green upon maturity. Green *B1*+*C1* plants from events 16 and 26 produced green tassels with purple anthers, while event 8 plants were male sterile. There were no *B1*+*C1* mature plants with colored leaves. Of the 4 colored *B1*+*C1*+F3′5′H survivors rated for CEW resistance at the plantlet stage, only 1 plant had colored leaves at maturity, 2 grew into green plants with purple anthers,

Table 3. Feeding Ratings of CEW and Thrips on Mature T_0 Transgenic Plants[a]

line	CEW day 1	CEW day 2	thrips rating (*N*)[b]
	2-13-07		
B1+*C1* green	65	44	39 ± 13 (9) a
B1+*C1*+F3′5′H green	47	28	ND
B1+*C1*+F3′5′H-36pur1[c]	62	34	6 ± 3 (7) b
	3-4-07		
B1+*C1* green (11 plants)	31 ± 4.8 a	24 ± 4.2 a	42 ± 4.3 (56) a
B1+*C1*+F3′5′H green (3 plants)	35 ± 8.7 a	33 ± 4.7 a	60 ± 9.9 (17) a
	3-25-07		
Hi II green (2 plants)	76/83	44/52	147 ± 17 (19) a
B1+*C1*+F3′5′H-36pur1[c]	89	57	48 ± 13 (8) b
B1+*C1*+F3′5′H-44pur	41	32	31 ± 12 (8) b

[a] Mean ± SE, where noted; see **Table 1** for rating descriptions. Mean values with different letters were significantly different ($P < 0.05$). ND = not determined. [b] *N* is the total number of analyzed damage sites. [c] The same plant was assayed twice.

Table 4. CEW Feeding Ratings[a] and Survivor Weights[b] on T_0 Transgenic Anthers

line	D1 rating (*N*)	D2 rating (*N*)	survivor wt (*N*)
B1+*C1* purple[c]	27 ± 5.2 (12) x	14 ± 0.89 (12) x	0.30 ± 0.018 (104) x
B1+*C1*+F3′5′H purple[d]	21 ± 2.7 (10) x	15 ± 1.6 (10) x	0.16 ± 0.013 (67) y
B1+*C1*+F3′5′H t/ly/p[e]	43 ± 4.8 (11) y	6.2 ± 1.1 (11) y	0.45 ± 0.027 (99) z

[a] For day 1 (D1), number of holes ± SE; for day 2 (D2), number of anthers ± SE (out of 20) with >50% of material remaining. [b] Weight in mg ± SE. Mean values with different letters were significantly different ($P < 0.05$). [c] Eleven plants tested representing two events. [d] Ten plants tested representing two events. [e] Nine plants tested representing four events: t, tan; ly, light yellow; p, pink.

and the last was green with yellow anthers. In *B1*+*C1*+F3′5′H event 45, 9 of 10 regenerated mature plants were green-leafed with purple anthers. Two of the *B1*+*C1*+F3′5′H colored plants (Figure SI1, Supporting Information) displayed no consistent resistance against CEW larvae when compared to green *B1*+*C1*, *B1*+*C1*+F3′5′H, or Hi II plants (**Table 3**). However, these same two *B1*+*C1*+F3′5′H colored plants were significantly more resistant to thrips damage compared to *B1*+*C1* or Hi II green plants (**Table 3**). The higher thrips rating (**Table 3**) for *B1*+*C1*+F3′5′H-36pur1 on 3-25-07 (compared to 2-13-07) was likely due to continued thrips herbivory since the date of the first rating.

CEW larvae ate less of the purple anthers than the white-pink anthers on both days of the bioassay (**Table 4**). There was no statistical difference in comparing feeding ratings of purple *B1*+*C1* anthers and purple *B1*+*C1*+F3′5′H anthers. However, feeding ratings on white and pink *B1*+*C1*+F3′5′H anthers were significantly higher than the feeding ratings of purple *B1*+*C1* anthers and purple *B1*+*C1*+F3′5′H anthers. Larval survivors feeding on purple *B1*+*C1*+F3′5′H anthers weighed significantly less than those feeding on purple *B1*+*C1* anthers; those survivors feeding on white or pink anthers weighed significantly more than the other two test groups.

LC-MS analysis found that cyanidin was the predominant anthocyanidin in all the colored tissues (**Table 5**). The concentrations of cyanidin, pelargonidin, and peonidin in *B1*+*C1*+F3′5′H-3A anthers are means of duplicate analyses. A significant negative correlation ($R = -0.31$, $P = 0.04$) was found between the cyanidin concentration of anthers (samples noted by footnote *e* in **Table 5**) and the weights of CEW survivors feeding on anthers from the respective plant (**Table 4**). LC-MS analysis was completed on purple anther sample *B1*+*C1*-26-9, but a bioassay was not done with this material. Therefore, only two *B1*+*C1* anther sample concentrations were used in the correlation, which was statistically significant with the data utilized. No delphinidin, malvidin, or petunidin was detected in any purple *B1*+*C1*+F3′5′H T_o tissue.

Table 5. Distribution of Acid-Extractable Anthocyanidins[a] from Various Freeze-Dried T_o Tissues

tissue	cya	pel	peo
B1+*C1*+F3′5′H-36pur1[b] husk	0.69	0.20	CE[c]
B1+*C1*+F3′5′H-36pur1 leaf	1.2	0.11	ND
B1+*C1*+F3′5′H-36pur1 tassel[d]	2.9	0.34	0.29
B1+*C1*+F3′5′H-44pur leaf	0.78	ND	ND
B1+*C1*+F3′5′H-38-3 green leaf	ND	ND	ND
B1+*C1*+F3′5′H-45-5 green leaf	ND	ND	ND
B1+*C1*-26-1 pur anther	2.7[e]	0.29	0.26
B1+*C1*-26-4 pur anther	2.3[e]	0.36	0.34
B1+*C1*-26-9 pur anther	2.4	0.24	0.22
B1+*C1*+F3′5′H-45-2 pur anther	2.0[e]	0.26	0.20
B1+*C1*+F3′5′H-45-3A pur anther	6.5[e]	1.2	0.45
B1+*C1*+F3′5′H-45-4A pur anther	2.9[e]	0.61	ND
B1+*C1*+F3′5′H-45-8A pur anther	1.0[e]	0.54	ND
B1+*C1*+F3′5′H-31-3 g/t[b] anther	ND	0.14	ND
B1+*C1*+F3′5′H-44-2A g/t anther	ND	0.11	ND
B1+*C1*+F3′5′H-44-7 g/t anther	ND	0.10	ND

[a] Acid-extractable micrograms of cyanidin (cya), pelargonidin (pel), and peonidin (peo) per milligram of dry weight. ND = Not detectable. [b] Legend: pur, purple, g/t, green-tan. [c] CE = coeluted with an unidentified pelargonidin derivative. [d] Sample included some immature anthers. [e] Correlation (*R*) of these values to survivor weights (**Table 4**) is −0.31 ($P = 0.04$).

Table 6. CEW Bioassays on T_1 *B1*+*C1*+F3′5′H-36pur2 Plants

	D1[b]	D2[b]	survivor wt[c] (*N*)	Cya[d]	corr[e]
			Set 1[a]		
purple-veined-1	41	18	0.21 ± 0.045 (7) x	0.21	
green-1	43	19	0.21 ± 0.032 (10) x	0	$R = -0.33$
purple-1	22	9	0.10 ± 0.025 (7) y	0.36	$P = 0.058$
purple-2	33	15	0.19 ± 0.025 (10) x	0.21	
			Set 2		
green-11	30	35	0.25 ± 0.022 (7) x	0.0	
purple-10	19	20	0.15 ± 0.018 (8) y	1.4	$R = -0.25$
purple-veined-12	32	52	0.25 ± 0.039 (8) x	1.3	$P = 0.16$
purple-veined-14	35	44	0.19 ± 0.027 (9) xy	0.82	

[a] Bioassays of plant lines grouped in rows were run simultaneously. [b] Day 1 and 2 feeding ratings; see **Table 1** for rating descriptions. [c] Mean weight in milligrams ± SE. [d] Acid-extractable micrograms of cyanidin per milligram of fresh weight. [e] Correlation of survivor weights to cyanidin concentration. Mean values with different letters were significantly different ($P < 0.05$).

Studies of T_1 Plants. LC-MS analysis of purple-leafed T_o plant *B1*+*C1*+F3′5′H-36pur2 was not performed, but this plant was self-pollinated and the T_1 generation was analyzed for CEW resistance and anthocyanidin content (**Table 6**). Some of the young plants were green and others had dark purple leaves, while others were light purple-red with purple midveins (Figure SI2, Supporting Information). Bioassays (with the second leaf) determined that the leaves with the most cyanidin (purple-1 and purple-10) in each set resulted in the lowest feeding ratings and CEW larvae with the lowest weights. However, purple-veined-12 had cyanidin levels very similar to those of purple-10, but the CEW larvae feeding on purple-veined-12 weighed significantly more than those larvae feeding on purple-10. The weights of larvae feeding on purple-10 were not significantly different from

Table 7. Cyanidin Chloride and Cyanidin-3-glucoside Chloride Reduces CEW Larva Growth after 3 Days

soln	mean insect wt in mg ± SE (*N*)	*P*[a]
control	0.98 ± 0.15 (13)	0.138
0.35% cyanidin chloride	0.71 ± 0.10 (14)	
control	0.66 ± 0.11 (14)	0.24
0.5% cyanidin chloride	0.50 ± 0.071 (13)	
control	0.47 ± 0.058 (16)	0.060
0.3% cyanidin-3G-chloride	0.30 ± 0.024 (8)	

[a] *P* value computed by the GLM procedure, which compares the variances.

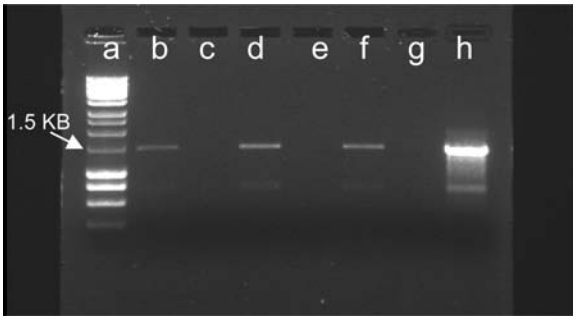

Figure 2. Representative PCR reactions with primers for petunia F3′5′H: (a) DNA ladder with arrow pointing to 1.5 KB fragment; (b) cDNA of T_1-*B1*+*C1*+F3′5′H-36pur2-6; (c) RNA control of T_1-*B1*+*C1*+F3′5′H-36pur2-6; (d) cDNA of T_1-*B1*+*C1*+F3′5′H-36pur2-3; (e) RNA control of T_1-*B1*+*C1*+F3′5′H-36pur2-3; (f) cDNA of T_1-*B1*+*C1*+F3′5′H-31 × *B1*+*C1*+F3′5′H-36pur1-6; (g) RNA control of T_1-*B1*+*C1*+F3′5′H-31 × *B1*+*C1*+F3′5′H-36pur1-6; (h) positive control using F3′5′H plasmid.

the weights of larvae feeding on purple-veined-14 leaves. Cyanidin levels were negatively correlated to CEW larvae weights for each set of bioassays, though not at statistically significant levels. Cyanidin chloride (at 0.35% and 0.5% in insect diet) and cyanidin-3-glucoside chloride (at 0.3% in insect diet) reduced the mean weight of CEW larvae compared to the control mean weight after 3 days (**Table 7**).

Lack of 3′5′-Hydroxylated Anthocyanins. RNA was extracted from colored leaves of progeny produced by the following crosses: *B1*+*C1*+F3′5′H-31 (noncolored, pollen donor) × *B1*+*C1*+F3′5′H-36pur1 and *B1*+*C1*+F3′5′H-36pur2 (selfed). Full length petunia F3′5′H mRNA was detected in all five *B1*+*C1*+F3′5′H-31×*B1*+*C1*+F3′5′H-36pur1 progeny analyzed and two of three *B1*+*C1*+F3′5′H-36pur2 progeny analyzed (see **Figure 2** for a representative gel). No delphinidin, petunidin, or malvidin was detected in the T_1 plants expressing F3′5′H mRNA (data not shown). The lack of 3′5′-hydroxylated anthocyanins in *B1*+*C1*+F3′5′H tissues in this study prompted experiments exploring the relative toxicity of delphinidin to Hi II maize leaves, the transformation host. The mean lesion length of spots treated with 1% delphinidin chloride was significantly larger than lesions resulting from 1% cyanidin chloride treatment (3.3 ± 0.42 mm vs 0.75 ± 0.24 mm, respectively, $P < 0.0001$ by GLM procedure).

DISCUSSION

The steps of maize anthocyanin biosynthesis have been extensively studied at the genetic level (*3*). The presence of a MYB protein (C1 or PL1) and helix−loop−helix protein (R or B1) together is sufficient to initiate anthocyanin biosynthesis in any maize tissue (*3*). This is achieved by the MYB and bHLH proteins coordinately binding DNA regulatory sequences that initiate transcription of the genes responsible for anthocyanin biosynthesis (*3*). Pale aleurone color 1, a WD40 regulator that is constitutively expressed in maize, is also necessary for anthocyanin biosynthesis, but its molecular function is not known (*3*). The use of constitutive promoters for both *B1* and *C1* in the *B1*+*C1* plasmid should induce anthocyanin biosynthesis in all maize tissues. The small number of colored mature plants recovered in this study (**Table 2**) suggests that the transformed *B1*+*C1* plasmid was susceptible to further genetic regulation. Experiments with *Lotus japonicus* indicated that multiple transgenes with the same gene promoter may be prone to transcriptional gene silencing (*23*). In addition, maize transformation by particle bombardment, as done in this study, appears to result in plants with high transgene copy numbers (*24*). Therefore, having *B1* and *C1* expressed by the same 35S promoter in the *B1*+*C1* plasmid may have caused some gene silencing, which resulted in a high proportion of green transgenic plants.

The anthocyanin biosynthetic pathway utilizes malonyl CoA and 4-coumaroyl CoA, which is derived from phenylalanine. 4-Coumaroyl CoA is also utilized by hydroxycinnamoyl-CoA shikimate/quinate hydroxycinnamoyl transferase (HCT) in the biosynthesis of two important lignin building blocks, the guaiacyl and syringyl units (*25*). When HCT mRNA was efficiently suppressed in *Arabidopsis thaliana*, the plants appeared stunted, had an altered lignin composition, and accumulated a number of flavonoid molecules, including anthocyanins (*25*). When chalcone synthase, the initial biosynthetic step of anthocyanins, was suppressed in HCT-deficient plants, the double transformants had green leaves and normal growth with altered lignin composition (*25*). The authors concluded that the altered growth rate was due to inhibition of auxin transport by high levels of anthocyanins and flavonol glycosides. If the regulatory mechanisms of phenylpropanoid biosyntheis between maize and *A. thaliana* are conserved, high anthocyanin biosynthesis rates in some of the putative transgenic lines of this study could compromise the lignin biosynthetic pathway and/or slow growth through inhibition of auxin transport. Interestingly, plants purple-1 and purple-10 (**Table 6**), which had high insect resistance and cyanidin concentrations, reverted to green leaves as the plant matured (at approximately the sixth leaf). While it would be difficult to determine if the observed reduction in color was due primarily to metabolic difficulties or a genetic change, it does suggest that high levels of *B1* and *C1* expression and/or anthocyanin production in maize leaves are not sustainable.

It is not clear why a large proportion of T_o survivors (both *B1*+*C1* and *B1*+*C1*+F3′5′H plants) had green leaves with purple anthers (**Table 2**). Hi II plants normally have yellow or tan anthers. The phenomenon was also apparent in the next generation, as T_1 progeny of *B1*+*C1* T_o plant 26-5 (green with purple anthers) had green plants with tan anthers or green plants with purple anthers. More experiments are needed to define the mechanism of the reversal of silencing in anthers. The colored anthers from both *B1*+*C1* and *B1*+*C1*+F3′5′H plants were more resistant to CEW than white or pink *B1*+*C1*+F3′5′H anthers (**Table 4**; there were no *B1*+*C1* plants with tan or pink anthers produced). Most (90%) of the *B1*+*C1*+F3′5′H colored anthers tested were from one transformation event (#45) and none of the four *B1*+*C1*+F3′5′H-45 anther samples tested contained 3′5′-hydroxylated anthocyanidins (**Table 5**). It is possible that the lower survivor weights of CEW feeding on colored *B1*+*C1*+F3′5′H anthers (**Table 4**) compared to *B1*+*C1* colored anthers is due to higher levels of a cyanidin glycoside; the average level of cyanidin (taken from **Table 5**) in *B1*+*C1*+F3′5′H anthers was 3.1 μg/mg dry weight ($N = 4$) while in *B1*+*C1* anthers it was 2.5 μg/mg dry weight ($N = 3$). The significant correlation between cyanidin level and insect weight found when using data of four *B1*+*C1*+F3′5′H anthers and two *B1*+*C1* anthers (**Table 5**) is additional

evidence for a significant bioactivity of a maize cyanidin glycoside inhibiting CEW larvae development.

There was significant resistance to thrips in colored T_o leaves (**Table 3**). Further experiments with controlled release of thrips will be needed to fully investigate anthocyanins as a possible source of plant resistance. The thrips infestation occurred while the T_0 plants were growing and then flowering. The plantlets (**Table 1**) and T_1 seedlings (**Table 6**) did not show any signs of thrips damage. While it is conceivable that the thrips may have triggered defense responses of T_0 plants, it is likely that both control and experimental plants were equally exposed. Thus, the reported biological effects on CEW larvae (**Tables 3−5**) of the inserted gene combinations (*B1*+*C1* and *B1*+*C1*+F3′5′H) were in addition to any endogenous response to thrips. Cultivated maize is frequently attacked by multiple insect species, and thus the results of CEW resistance (**Tables 3−5**) with a concurrent thrips attack may be closer to actual field conditions.

While there was no consistent CEW resistance in colored T_o leaves, some colored T_1 leaves had significant insect resistance (compare **Tables 3** and **6**). On the basis of correlation analysis, CEW larvae growth rates diminished when they fed on young leaves with the highest cyanidin levels (**Table 6**). Perhaps the T_o leaves tested were not pigmented enough to slow the growth of CEW larvae. The cyanidin levels in T_1 set 1 plants were lower than for set 2 (**Table 6**). The leaves for set 1 were placed directly in 2 M HCl, while the leaves for set 2 were frozen and then placed into the 2 M HCl. It is possible that the freeze−thaw method released more pigment into the 2 M HCl, resulting in higher cyanidin levels. Regardless of this difference in methodology between the two sets, the plant with the highest concentration of cyanidin had the lowest amount of feeding and smallest CEW survivors (purple-1 and purple-10). The high feeding yet high cyanidin levels of purple-veined-12 might be explained by a concentration of pigment in the midvein that was predominately avoided by the larvae.

In this study, the maize anthocyanins were hydrolyzed to anthocyanidins (cyanidin, delphinidin, pelargonidin, and peonidin) for analysis because of the availability of commercial standards and ease of comparison among tissue samples. The anthocyanidins are biosynthetic intermediates that are ultimately conjugated to a variety of sugars and functional groups. In maize the following cyanidin glycosides have been identified by three laboratories: cyanidin 3-glucose, cyanidin 3-(6″-malonylglucoside), cyanidin 3-(3″,6″-dimalonylglucoside), cyanidin-3-(6″-ethylmalonylglucoside), cyanidin-3-rutinoside, cyanidin succinyl-glucoside, cyanidin malonyl-succinyl-glucoside, and cyanidin disuccinyl glucoside (*13*, *14*, *26*). The significant correlation between cyanidin content and CEW larvae weight in colored anthers (both *B1*+*C1* and *B1*+*C1*+F3′5′H plants, **Table 5**), a similar trend among the T_1 leaves (**Table 6**), and reductions in mean CEW weight with cyanidin chloride and cyanidin-3-glucoside chloride (**Table 7**) suggest that some cyanidin glycoside(s) from maize slows CEW growth. Cyanidin from cotton leaves at ∼0.16% added to insect diet reduced the weight of first instar tobacco budworm larvae 50% compared to controls (*9*, *10*). The same studies found that ∼0.07% of cyanidin-3-glucoside added to diet reduced the larval weight to 50% of controls, which suggests that tobacco budworm larvae may be more sensitive to cyanidin than CEW larvae. Alternatively, beneficial components of the CEW diet in this study may reduce the toxicity of cyanidin and require a higher concentration to achieve a 50% weight reduction. The tobacco budworm studies (*9*, *10*) also show that cyanidin is a toxic molecule but reaches its target location more effectively as a glucoside. Additional studies with purified maize cyanidin-type anthocyanins may ultimately determine what specific compound reduces the growth of CEW larvae.

Experiments with Black Mexican Sweet maize suspension cells found that a fusion of the maize *C1* and *R* (homologous to *B1*) gene (called the *CRC* gene) effectively induced anthocyanin biosynthesis (*27*). Analysis of the mRNAs induced by the CRC protein identified 663 differentially regulated transcripts, which included the genes (∼6) coding for enzymes involved in anthocyanin biosynthesis (*27*). Only a handful of the CRC differentially regulated genes were identified (*27*). This study (*27*) indicates that either a large number of genes are required for maize biosynthesis and processing or that the maize C1/Pl1 and B1/R proteins regulate a number of genes unrelated to anthocyanin biosynthesis. Maize anthocyanins are produced in response to a variety of stresses or pathogens. Evidence suggests that maize anthocyanins protect plants from DNA damage caused by ultraviolet light (*28*). Other data indicate that anthocyanins are involved in maize cellular responses to pathogens, including *Bipolaris maydis* (*29*). However, it is not clear from these studies if other molecules coordinately synthesized with the anthocyanins contribute to the defense response. Therefore, while cyanidin glycosides may indeed function as CEW resistance molecules in anthers or young leaves, cyanidin accumulation may co-occur with other unidentified molecules that slow CEW development.

In this study, no 3′5′-hydroxylated anthocyanins were detected in any *B1*+*C1*+F3′5′H tissues, despite the presence of petunia F3′5′H mRNA in some maize T_1 colored tissues (**Figure 2**). There are a number of possible explanations. Although F3′5′H mRNA was present, there was not enough enzyme produced to make detectable levels of 3′5′-hydroxylated anthocyanins. If sufficient F3′5′H enzyme was produced, it did not find enough substrate. F3′5′H possibly competes with other enzymes for substrate (**Figure 1**). Anthocyanin biosynthesis may take place along a metabolic channel (*30*). If this is true in maize, the anthocyanin metabolic channel may not be able to accommodate an additional enzyme (F3′5′H) from a different species to produce 3′5′-hydroxylated anthocyanins. Alternatively, it may be possible that high production of 3′5′-hydroxylated anthocyanins is deleterious to the cell, as larger leaf lesions were noted with application of 1% delphinidin chloride compared to 1% cyanidin chloride. Therefore, high-level expression of the *B1*, *C1*, and F3′5′H genes in transformed cells may have led to premature cell death. More studies need to be done to determine if maize can synthesize and accumulate 3′5′-hydroxylated anthocyanins.

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Supporting Information Available: Figures showing a colored T_0 *B1*+*C1*+F3′5′H plant and colored T_1 *B1*+*C1*+F3′5′H tissues. This material is available free of charge via the Internet at http://pubs.acs.org.

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