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Effects of Gibberellic Acid and Uniconazole on the Activities of Some Enzymes of Anthocyanin Biosynthesis in Carrot Cell Cultures

A. Ilan* and D. K. Dougall†

Department of Botany, University of Tennessee, Knoxville, Tennessee 37996-1100, USA

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Abstract. Gibberellic acid (GA_3) inhibition of anthocyanin accumulation by carrot cell-suspension cultures was reversed by supplying dihydroquercitin or naringenin to the culture and not by supplying 4-coumaric acid or malonic acid. This suggested that gibberellic acid was inhibiting chalcone synthase, chalcone isomerase, or acetyl CoA carboxylase. Acetyl-CoA-carboxylase specific activity was the same in GA_3-treated and untreated cultures and was not detected in cultures treated with uniconazole, an inhibitor of gibberellic acid biosynthesis. Chalcone-isomerase specific activity was lower in GA_3-treated cultures than in untreated cultures and was lower in uniconazole-treated cultures than in the GA_3-treated cultures. The total chalcone synthase activity in extracts from GA_3- and from uniconazole-treated cells was not significantly different from that in extracts of untreated tissue. When these extracts were chromatographed on a Mono Q column, three peaks of chalcone synthase activity were found in extracts of nontreated cells, whereas only two of these peaks were detected in extracts of GA_3-treated cells. The extracts from GA_3-treated cells did not contain the peak of chalcone synthase activity that, in untreated cells, preceded the main peak. The correlation between the absence of this peak and the inhibition of anthocyanin accumulation suggests that this form of chalcone synthase is responsible for anthocyanin synthesis and that GA_3 prevents this form from appearing in the cells.

The study by Hinderer and Seitz (1986) of the inhibition of anthocyanin accumulation in a carrot cell-suspension culture, grown in the presence of gibberellic acid (GA_3), suggests the following hypothesis for the effect of GA_3. Little or no 3′-nucleotidase activity is present in carrot cell suspensions. The 3′-nucleotidase activity is markedly stimulated by GA_3 application. The 3′-nucleotidase dephosphorylates malonyl CoA, producing 3′-dephospho-malonyl CoA, which is an inhibitor of chalcone synthase. Consequently, the accumulation of anthocyanin is inhibited. This hypothesis was examined by Ilan et al. (1994). Considerable activity of 3′-nucleotidase was found in nontreated cell extracts. Only moderate effects of GA_3 and uniconazole, a growth retardant that is known to inhibit GA biosynthesis on 3′-nucleotidase activity, were found. Ilan et al. (1994) suggested that alteration of 3′-nucleotidase activity in response to exogenous GAs may not be responsible for the observed differences in anthocyanin accumulation.

To identify steps in anthocyanin biosynthesis inhibited by GA_3, intermediates of anthocyanin biosynthesis were fed to cultures growing in the presence of GA_3. Intermediates that restore anthocyanin accumulation are beyond the point of GA_3 inhibition. As a result of these feeding experiments, the activities of chalcone isomerase (CHI) and chalcone synthase (CHS) were assayed in extracts from cells grown in the presence of GA_3 or uniconazole. Uniconazole was used because it increases anthocyanin accumulation by the carrot cell cultures, is an inhibitor of GA biosynthesis (Ilan and Dougall 1992), and was expected to provide a contrast to GA_3 treatments. The activities of acetyl CoA carboxylase, an enzyme which catalyzes the conversion of acetyl CoA into malonyl CoA, a precursor of anthocyanin synthesis, were also examined under the above conditions. Only in CHS was a difference found, which correlated with GA_3 inhibition of anthocyanin. This difference was the absence of a peak of CHS activity in extracts from GA_3-treated tissue.

* Present address: Rosh Hanikra, 22825, Israel.
† Author for correspondence.

Materials and Methods

Cultures Maintenance

Carrot (*Daucus carota* spp. *carota*) cell-suspension cultures were maintained as described elsewhere (Ilan and Dougall 1992). Subclone WC 63-1-9-1-6-1-1-2 (clone 6) was used in all experiments. Subclones WC-63-1-9-1-15-3-3-2-1-2-1 (clone 15) and WC 63-1-9-1-13-1-1-1-2 (clone 13) were used in one experiment each. These clones have been described by Vogelien et al. (1990). Stock cultures of the clones were used to inoculate WCM-4 medium (Dougall and Weyraugh 1980) (1:20 v/v) and treated as shown in the Results. GA_3 (Sigma Chemical Co., St. Louis, MO) was prepared as a concentrated stock solution in water at pH 4.5, filter sterilized, and aseptically added to the cooled autoclaved media as required. Cell suspensions were grown in 250 or 500-ml Erlenmeyer flasks containing either 50 or 100 ml of medium.

Anthocyanin and Growth Measurements

At the end of the growth period, triplicate samples of 2.5 ml were taken from each flask for both anthocyanin and dry-weight measurements as described by Vogelien et al. (1990). For each treatment, three Erlenmeyer flasks were used.

Feeding Experiments

Carrot cell-suspension cultures were grown for 12 days on WCM-4 in the presence, supplied at day 0, or absence of GA_3. GA_3, at a concentration of 10^{-6} M, was used for feeding experiments with naringenin and dihydroquercetin (DHQ) and at 3×10^{-6} M in feeding experiments with coumaric acid and malonic acid. Naringenin, DHQ, malonic acid, and 4-coumaric acid at concentrations of 0.1, 0.25, 0.003, and 0.02 mM, respectively, and 0.5% v/v dimethylsulfoxide (DMSO) were added at days 0, 4, and 8. These were the highest concentrations that did not inhibit growth of the cultures. Clone 6 was used for feeding experiments with malonic acid and coumaric acid, and clone 13 was used with naringenin and DHQ.

Enzyme Extraction and Purification

Extraction of tissue and treatment of extracts for measurement of CHS, CHI, and ACCase activity was done by using the procedure described for 3′-nucleotidase (Ilan et al. 1993). The tissue extract after the first centrifugation is referred to as "crude extract," and the extract after ammonium sulfate precipitation and desalting on a Sephadex column is referred to as "desalted extract."

Synthesis of 4-Coumaroyl CoA

The synthesis of 4-coumaroyl CoA through the hydroxysuccinamide ester of 4-coumaric acid was performed as described by Stockigt and Zenk (1975) with some modification. 4-Coumaroyl CoA was separated from other components of the reaction mixture by thin-layer chromatography (TLC) on cellulose plates (250 μm, Whatman International Ltd., Maidstone, UK) by using *n*-butanol:glacial acetic acid:water (5:2:3). The components on the TLC plates were located by UV light (254 and 350 nm). Compounds with phenolic hydroxyl groups were detected by using Pauly's reagent. Thiol esters were detected with Nitroprusside reagent followed by methanolic NaOH (Stadman 1957).

Acetyl CoA Carboxylase Assay

The assay was based on the method of Wurtele and Nikolau (1990). The assay mixture contained 5.6 mM $MgCl_2$, 2 mM DTT, 5.6 mM ATP, 94 mM KCl, 6 mM $NaHCO_3$, and 0.48 mM $NaH^{14}CO_3$ (52 μCi/μmol) in 20 μl plus 12 μl of enzyme preparation. The assay was initiated by the addition of 8 μl of 1.5 mM acetyl CoA. Assay mixtures were incubated for 30 min at 30°C. The reactions were terminated by the addition of 4 μl of 6N HCl. Forty microliters of each mixture was transferred to a 2.4-cm glass fiber paper disk (grade 391, S/P Baxter Healthcare Corporation, McGaw Park, IL) and dried by a hot-air stream. The disks were placed in scintillation vials containing 5 ml of Scintiverse II (Fisher Scientific Company, Fair Lawn, NJ) and counted in a liquid scintillation counter (Beckman LS-3800, Irvine, CA). HPLC was used to separate components of the reaction mixture after the incubation and to show that $^{14}CO_2$ was incorporated into malonyl CoA. A flow rate of 1 ml/min^{-1} was used with a 3.9 × 300-mm μ-Bondpack C-18 column (Waters, Milford, MA). The elution buffers were 0.05 M phosphate buffer at pH 5.0 (buffer A) and 40% methanol in buffer A (buffer B). A linear gradient of 5–35% of buffer B in A over 35 min was used to elute the column. Radioactivity measured in the HPLC fractions showed that more than 90% of the radioactivity co-chromatographed with authentic malonyl CoA.

Chalcone Isomerase Assay

2′,4′,4,6′-Tetrahydroxychalcone was prepared from naringenin by the method of Moustafa and Wong (1967). The reaction mixture for the CHI assay of tissue extracts contained 980 μl of 0.05 M Tris-HCl (pH 7.6) and 10 μl of extracts. The assay mixture for Mono Q fractions contained 890 μl of 0.05 M Tris-HCl (pH 7.6) and 100 μl of a column fraction. The reaction was initiated by the addition of 10 μl of 2′,4′,4,6′-tetrahydroxychalcone solution (1 mg/ml in ethylene glycol monomethyl ether). The conversion of tetrahydroxychalcone into naringenin was measured by the reduction in absorbance at 375 nm. A mixture without enzyme preparation was used as a blank.

Chalcone Synthase Activity

The assay of CHS was a modification of the method used by Hinderer and Seitz (1985). The assay mixture, with a final volume of 210 μl, contained 5 μl (1 nmol) of 4-coumaroyl CoA, 5 μl (1.87 nmol, 35,000 dpm) of [2-^{14}C]malonyl CoA (Amersham Corporation, Arlington Heights, IL), the amount of enzyme preparation chosen for each assay and buffer (100 mM KH_2PO_4, 1.4 mM β-mercaptoethanol, 1% w/v BSA, pH 7.9) to complete the volume. The assay mixtures were incubated for 30 min (unless otherwise stated) at 30°C, the reactions were stopped by an addition of 20 μl of naringenin solution (1 mg/ml in methanol), and the products were extracted into 250 μl of ethyl acetate. Of the ethyl acetate phase, 200 μl was mixed with 5 ml of Scintiverse II

and counted in a liquid scintillation counter. More than 90% of the radioactivity of the ethyl acetate phase comigrated with naringenin when chromatographed on Whatman #1 paper, using 15% ethanol in water as a solvent.

Protein Assay

Protein was determined using bicinchoninic acid (BCA) reagent (Sigma Chemical Company). Bovine serum albumin solution (Sigma Chemical Company) was used as a standard. Crude extracts were treated as follows, prior to the protein assay. Of each crude extract, 10 µl was precipitated twice in 0.5 ml of 5% trichloroacetic acid (TCA). The TCA was then removed, and 50 µl of 0.1 N NaOH were added.

Statistical Analysis of Data

The results were subjected to nested analysis of variance (ANOVA) using PC SAS Version 6 (SAS Institute, Inc., Cary, NC). Data are given as mean plus or minus the standard deviation. Within columns, means with different letters differ significantly at 5% by multiple t test.

Table 1. Effect of intermediates in anthocyanin biosynthesis on anthocyanin production and growth of carrot cell-suspensions grown in the presence of GA_3 for 12 days.

Treatment	Anthocyanin[a] (% of control)	Dry weight[b] (% of control)
Experiment 1		
Control	$4.5 \pm 0.2_b$ (100)	$4.2 \pm 0.4_a$ (100)
GA_3	$1.2 \pm 0.1_d$ (28)	$4.0 \pm 0.4_{ab}$ (96)
GA_3 + naringenin	$2.1 \pm 0.2_c$ (47)	$4.0 \pm 0.2_b$ (95)
GA_3 + DHQ	$5.2 \pm 0.5_a$ (115)	$3.7 \pm 0.2_b$ (88)
Experiment 2		
Control	$1.6 \pm 0.1_a$ (100)	$2.9 \pm 0.1_a$ (100)
GA_3	$0.17 \pm 0.04_b$ (11)	$2.0 \pm 0.1_b$ (67)
GA_3 + 4-coumarate	$0.18 \pm 0.03_b$ (11)	$2.0 \pm 0.2_b$ (70)
Experiment 3		
Control	$1.9 \pm 0.2_a$ (100)	$3.8 \pm 0.2_a$ (100)
GA_3	$0.15 \pm 0.05_b$ (8)	$2.3 \pm 0.3_b$ (61)
GA_3 + malonate	$0.16 \pm 0.03_b$ (8)	$2.3 \pm 0.3_b$ (59)

Note. See Materials and Methods. Data given as mean ± SD. Means within a column that did not differ significantly at 5% when compared by multiple t test are followed by the same subscript.
DHQ, dihydroquercetin.
[a] Anthocyanin · (ml culture)$^{-1}$ is expressed as absorbance at 530 nm.
[b] Dry weight is expressed as mg · ml^{-1}.

Results and Discussion

Feeding Experiments

Intermediates of anthocyanin biosynthesis were fed to carrot cell-suspension cultures in the presence of GA_3 to locate the step of anthocyanin biosynthesis inhibited by GA_3. Naringenin partially restored the levels of anthocyanin, whereas DHQ gave levels of anthocyanin above those of the control (experiment 1 in Table 1). Malonic acid was fed in these experiments on the assumption that it was converted into malonyl CoA and thus supply a precursor for anthocyanin synthesis. The potential conversion of malonic acid into malonyl CoA in plants was reviewed by Stumpf (1976). Neither malonic acid (experiment 3 in Table 1) nor 4-coumaric acid (experiment 2 in Table 1) restored anthocyanin production in the presence of GA_3. These results suggest that anthocyanin accumulation is inhibited by GA_3 at steps located between either coumaric acid or malonic acid and naringenin in the anthocyanin biosynthesis pathway. Cheng et al. (1985) showed that 4-coumarate CoA ligase was present in these cultures grown in the presence of GA_3, so that the steps in anthocyanin biosynthesis that might be inhibited by GA_3 are those that are catalyzed by the enzymes chalcone synthase and chalcone isomerase. Another step that was not investigated by the feeding experiments, but which could be inhibited by GA_3, is the conversion of acetyl CoA into malonyl CoA catalyzed by acetyl CoA carboxylase.

The Effects of GA_3 and Uniconazole on the Measured ACCase Activity

The relationship between the amount of enzyme preparation and the CO_2 fixed into acid-stable material was linear up to 3.74 pmole CO_2 fixed · min^{-1}, and the CO_2 fixed was strictly proportional to incubation time up to 30 min (data not shown). The CO_2 fixed was not altered when 10^{-3}–10^{-7} M GA_3 was added to the ACCase assay mixture. More than 90% of the CO_2 fixed was in malonyl CoA.

The activities of ACCase in desalted extracts from tissue grown with GA_3, with uniconazole and from untreated control tissue, are shown in Table 2. Growth in the presence of GA_3 had no effect on the ACCase specific activity. However, no ACCase activity was found in extracts from uniconazole-treated cells. Growth and increased anthocyanin accumulation, both of which require malonyl CoA, occurred in the presence of uniconazole (Ilan and Dougall 1992). There are two possible explanations for the apparent lack of ACCase in the presence of uniconazole. First, the method used for ACCase measurement could not detect the small, but suffi-

Table 2. Acetyl CoA carboxylase activity in desalted extracts from carrot cell-suspension cultures of clone 6 grown for 5 days on WCM-4 either unsupplemented (control) or supplemented with 3×10^{-6} M GA_3 or 1.7×10^{-6} M uniconazole.

Treatment	Activity[a]	Protein[b]	Specific activity[c]
Control	$49 \pm 1_a$	2.8	$17 \pm 0.5_a$
GA_3	$37 \pm 8_b$	1.6	$23 \pm 5_a$
Uniconazole	0_c	2.1	0_b

Note. Numbers are the mean ± SD from four measurements on each of three extracts. Means within a column that did not differ significantly at 5% when compared by multiple t test are followed by the same subscript.

[a] ACCase activity in the extracts is expressed as picomoles of malonyl CoA · ml^{-1} · min^{-1}. If activity in the extracts is multipled by 4.8, it is then converted into activity · gFW^{-1} (picomoles of malonyl CoA · gFW^{-1} · min^{-1}).

[b] Protein is expressed as mg · ml^{-1}.

[c] Specific activity is expressed as picomoles malonyl CoA · mg $protein^{-1}$ · min^{-1}.

cient, ACCase activity needed. Second, malonyl CoA is supplied from other cellular sources such as oxaloacetate through malonic acid (Stumpf 1976).

The Effect of GA_3 and Uniconazole on the Measured CHI Activity

Preliminary experiments (data not shown) showed that the relationship between the amount of the CHI preparation and the CHI activity up to 4.74 μmol 2′,4′,4,6′-tetrahydroxychalcone converted · min^{-1} was linear. The disappearance of 2′,4′,4,6′-tetrahydroxychalcone was strictly proportional to time up to 80 s. No effect on CHI activity was detected when 10^{-3}–10^{-6} M GA_3 was added to the assay mixture.

CHI activity was measured in desalted extracts from control (untreated) tissue, uniconazole-treated, and GA_3-treated tissues. The results (Table 3) show that the CHI activity and specific activity in extracts from GA_3-treated tissue were reduced relative to the control. CHI activity was further reduced in extracts from uniconazole-treated tissue, an observation that appears to be inconsistent with GA_3 reducing CHI activity because uniconazole is expected to reduce endogenous GAs. Measurement of CHI activity in extracts from control and GA_3-treated cells grown for 3, 6, and 9 days (Table 4) showed that CHI-specific activity in the extracts from the untreated tissue increased with time, whereas the specific activity in the extracts from the GA_3-treated tissue decreased with increased

Table 3. Chalcone isomerase activity in desalted extracts from control GA_3-, and uniconazole-treated carrot cell-suspension cultures (clone 6) grown for 5 days on WCM-4.

Treatment	Activity[a]	Protein[b]	Specific activity[c]
Control	$216 \pm 3_a$	2.76	$78 \pm 1_a$
GA_3	$45 \pm 13_b$	1.62	$28 \pm 8_b$
Uniconazole	$7 \pm 0.3_c$	2.10	$3.5 \pm 0.1_c$

Note. The numbers are the means of three measurements on one preparation from each treatment ± SD. Means within a column that did not differ significantly at 5% when compared by multiple t test are followed by the same subscript.

Control, no supplement; GA_3, 3×10^{-6} M GA_3; uniconazole, 1.7×10^{-6} M uniconazole.

[a] CHI activity in the extracts is expressed as micromoles chalcone converted into naringenin · ml^{-1} · min^{-1}. If activity in the extracts is multiplied by 4.8 then it is converted into activity · gFW^{-1} (micromoles chalcone converted into naringenin · gFW^{-1} · min^{-1}).

[b] Protein is expressed as mg · ml^{-1}.

[c] Specific activity is expressed as micromoles chalcone converted into naringenin · mg $protein^{-1}$ · min^{-1}.

Table 4. Effect of the growth period on chalcone isomerase activity in desalted extracts from carrot cell-suspension cultures (clone 6) in the presence and absence of GA_3.

Treatment	Activity[a]	Protein[b]	Specific activity[c]
3 days			
Control	120 (15)	2.25 (0.07)	53 (7)
GA_3	79 (13)	1.76 (0.03)	45 (7)
6 days			
Control	226 (21)	2.60 (0.42)	87 (8)
GA_3	46 (1)	1.47 (0.12)	31 (1)
9 days			
Control	142 (13)	1.55 (0.21)	92 (8)
GA_3	26 (3)	1.43 (0.04)	18 (2)

Note. Clone 6 was grown for 3, 6, or 9 days on WCM-4 ± 3×10^{-6} M GA_3 (GA_3). In parentheses are the range of three measurements from one preparation of each treatment.

[a] CHI activity in the extracts is expressed as micromoles of chalcone converted into naringenin · ml^{-1} · min^{-1}. If activity in the extracts is multiplied by 4.8 then it is converted into activity · gFW^{-1} (micromoles of chalcone converted into naringenin · gFW^{-1} · min^{-1}).

[b] Protein is expressed as mg · ml^{-1}.

[c] Specific activity is expressed as micromoles chalcone converted into naringenin · mg $protein^{-1}$ · min^{-1}.

time. GA_3 treatment of the tissue decreased the CHI-specific activity relative to the control at 6 and 9 days.

Desalted extracts of untreated, GA_3-treated, and uniconazole-treated cells were chromatographed on a Mono Q column, and the CHI activity in the frac-

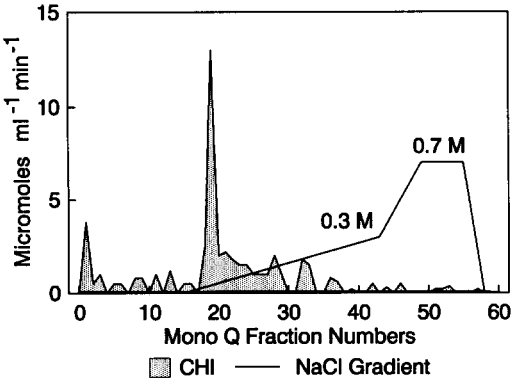

Fig 1. Chalcone isomerase activity in fractions from a desalted extract of carrot cell-suspension cultures grown for 5 days on WCM-4 (control) and chromatographed on a Mono Q column.

tions was measured. Figure 1 shows a typical profile of CHI activity in Mono Q fractions. Most of the extracts produced two peaks of CHI activity. The first peak occurred at fractions 1–2, the second at fractions 19–20. Although the peak at fractions 1–2 was missing in some extracts, no consistent difference was found between the activity profiles of the different treatments. Based on the results shown in Fig. 1, it is possible that two forms of CHI are present in carrot cells of clone 6. Two genes for CHI, which could give rise to two different forms of CHI, were found in petunia (van Tunen et al. 1988, 1990).

The results (Tables 3 and 4) showed that CHI activity was reduced in the GA_3-treated extracts and was reduced much more in the uniconazole-treated extracts. This reduced activity in the presence of uniconazole, however, is sufficient for anthocyanin production because uniconazole increases anthocyanin accumulation (Ilan and Dougall, 1992). From this, we conclude that CHI is not the location of GA_3 inhibition of anthocyanin accumulation.

The Effect of GA_3 and Uniconazole on the Measured CHS Activity

The relationship between the amount of CHS preparation and the formation of naringenin was linear up to 7 pmol naringenin · min^{-1} (data not shown). The amount of naringenin produced was proportional to the time of incubation, up to 1 h. No effect of 10^{-4}–10^{-6} M GA_3 added to the assay mixture on CHS activity could be detected. Naringenin, rather than 2′,4′,4,6′-tetrahydroxychalcone, the expected product of CHS, was found here as a result of CHI

Table 5. Chalcone synthase activity in desalted extracts from carrot cell-suspension cultures (clone 6) grown for 5 days on WCM-4 (control), WCM-4 supplemented with 3×10^{-6} M GA_3, or WCM-4 supplemented with 1.7×10^{-6} M uniconazole (uniconazole).

Treatment	Activity[a]	Protein[b]	Specific activity[c]
Control	$5.1 \pm 1.7_a$	1.8	$2.9 \pm 0.7_a$
GA_3	$5.8 \pm 2.6_a$	2.1	$3.1 \pm 1.7_a$
Uniconazole	$7.8 \pm 4.1_a$	2.3	$3.5 \pm 1.8_a$

Note. Numbers are the mean ± SD of two measurements from three preparations of each treatment. Means within a column that did not differ significantly at 5% when compared by multiple t test are followed by the same subscript.

[a] CHS activity in the extracts is expressed a picomoles naringenin · ml^{-1} · min^{-1}. If activity in the extracts is multipled by 4.8 then it is converted into activity · gFW^{-1} (picomoles of naringenin · gFW^{-1} · min^{-1}).

[b] Protein is expressed as mg · ml^{-1}.

[c] Specific activity is expressed as picomoles naringenin · mg $protein^{-1}$ · min^{-1}.

present in the extracts, and of spontaneous isomerization of 2′,4′,4,6′-tetrahydroxychalcone to naringenin.

The CHS activity of desalted extracts from control, GA_3- and uniconazole-treated tissue (Table 5) shows no significant difference between the treatments. To determine whether GA_3 altered CHS activity during growth, CHS activity was measured in extracts from carrot cell-suspension cultures grown on WCM-4 or WCM-4 supplemented with 3×10^{-6} M GA_3, and harvested at days 3, 6, and 9 (Table 6). Table 6 shows that substantial CHS activity was present in both control tissue and GA_3-treated tissue at the days checked during growth. CHS-specific activity is not greatly increased by Sephadex treatment (Table 6), which, in part, rules out the possibility that a low-molecular-weight inhibitor of CHS was present in the GA_3-treated tissue. The reduction of CHS-specific activity to 50% of the control value in extracts of GA_3-treated cells grown for 9 days parallels the observations of Hinderer et al. (1984) but is probably not important because anthocyanin accumulation was completed at this time (Dougall and Frazier, 1989).

When desalted extracts from untreated (control) cells were chromatographed on a Mono Q column, three peaks of CHS activity were usually found. These regions of CHS activity were fractions 20–23, 24–30, and 42–47 (Fig. 2). One explanation of this finding is that three forms of CHS are present in the extracts. The peak found at fractions 42–47 was not present in the control, shown in Fig. 2, but was

Table 6. Effect of growth period and GA_3 on chalcone-synthase-specific activity.[a]

	Crude extract		Desalted extract	
Day	Control	GA_3	Control	GA_3
3	3.7 (0.1)	4.9 (0.1)	5.4 (3.3)	6.0 (0.4)
6	4.0 (0.6)	3.2	4.1 (0.2)	5.7 (2.8)
9	3.2 (0.5)	1.6 (0.2)	5.7	2.4 (0.2)

[a] The units of CHS-specific activity are picomoles of naringenin · mg protein^{-1} · min^{-1}. Cells of clone 6 were grown on WCM-4 or WCM-4 supplemented with 3×10^{-6} M GA_3, harvested at days 3, 6, and 9 and extracts were prepared. In parentheses are the range of two measurements from one preparation of each treatment.

present in another control preparation and in GA_3 treatment, as shown in Fig. 2. Thus, this peak is not associated only with the GA_3 treatment. When extracts from GA_3-treated cells were chromatographed on a Mono Q column, two peaks of CHS activity were found in fractions 24–30 and 42–47. The peak of CHS activity at fractions 20–23 in the chromatography of the control extract (Fig. 2), which was present in three separate control extracts, was not present in the chromatography of any of the three independent extracts from GA_3-treated tissues. This suggests either that GA_3 inhibits the synthesis of the CHS form in fractions 20–23 or that GA_3 stimulates the synthesis of a CHS inhibitor, which cochromatographs with this CHS form. Gleitz and Seitz (1989), using UV irradiation of cultures and specific tissue of wild carrot plants, showed a correlation between the presence of a 40-kD CHS form and anthocyanin accumulation, whereas a 43-kD form was ubiquitous. Their observations support the idea of several forms of CHS specifically in carrot tissue. Hinderer et al. (1984) and Hinderer and Seitz (1985) found considerable CHS activity in extracts of the anthocyanin-accumulating cell line (DC_b) grown in the absence of GA_3, and Hinderer et al. (1984) demonstrated CHS activity in crude extracts of GA_3-treated cells of DC_b. Hinderer and Seitz (1986) did not detect any CHS activity in extracts from another, anthocyanin-free, cell line (DC_w) that was derived from DC_b by maintenance on media containing GA_3 for 10 years, or after chromatography of the DC_w extract on a Mono Q column. An explanation for the lack of CHS activity in the cell line DC_w is that this cell line became different from DC_b during the 10 years it was maintained on GA_3-containing medium, whereas DC_b was maintained in the absence of GA_3. As a result of changes that occurred, DC_w does not produce CHS, but it does produce 3′-nucleotidase, which can cause inhibition of CHS.

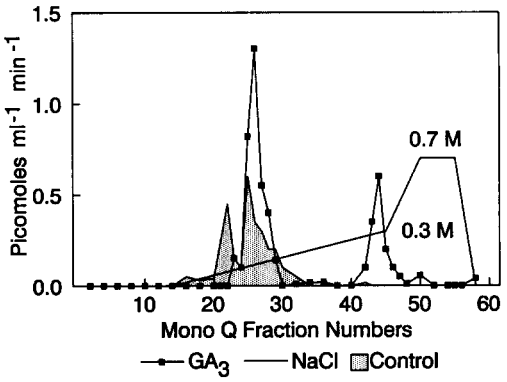

Fig 2. Chalcone synthase activity in fractions from chromatography of extracts from carrot cell-suspension cultures on a Mono Q column. Desalted extracts of cells grown for 5 days on WCM-4 and WCM-4 supplemented with 3×10^{-6} M GA_3 were chromatographed. The activities are given as picomoles of naringenin · ml^{-1} · min^{-1}.

These changes would account for the lack of anthocyanin production by DC_w.

Assays of enzymes of anthocyanin biosynthesis suggested that ACCase and CHI are not the sites of GA_3 inhibition of anthocyanin production. Furthermore, these assays suggest that uniconazole has effects on ACCase and CHI that are independent of its effects on GA biosynthesis. The lack of CHS activity in fractions 20–23 of extracts from GA_3-treated cells chromatographed on a Mono Q column suggests that this form of CHS is responsible for anthocyanin biosynthesis and is the site of GA_3 inhibition of anthocyanin synthesis. Further study is required to confirm the GA_3 inhibition of this step and to elucidate the mechanism of GA_3 inhibition. Included in possible mechanisms of GA_3 inhibition is inhibition of the synthesis of a CHS isozyme and synthesis of a high-molecular-weight inhibitor that cochromatographs with this CHS form under the conditions used here.

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