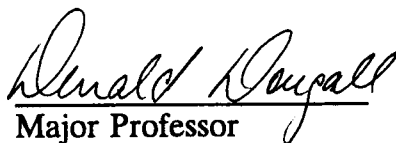
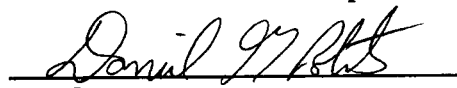


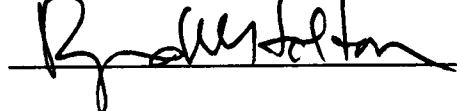
To the Graduate Council:

I am submitting herewith a dissertation written by Avihai Ilan entitled "Biological and biochemical studies of the effects of gibberellic acid and growth retardants on anthocyanin production by carrot cell suspension cultures." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Botany.


Major Professor

We have read this dissertation
and recommend its acceptance:





Accepted for the Council


Associate Vice Chancellor
and Dean of the Graduate School

**BIOLOGICAL AND BIOCHEMICAL STUDIES OF THE EFFECTS OF
GIBBERELIC ACID AND GROWTH RETARDANTS ON ANTHOCYANIN
PRODUCTION BY CARROT CELL SUSPENSION CULTURES**

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Avihai Ilan

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ABSTRACT

The inhibition of anthocyanin accumulation by gibberellic acid (GA) was investigated in carrot cell suspension cultures.

Several types of growth retardants including quaternary ammonium compounds (CCC, AMO-1618), substituted pyrimidines (ancymidol), triazoles (paclobutrazol, uniconazole) and a norbornenodiazetidine (tetcyclacis) increased anthocyanin production in carrot cell suspensions. The inhibitory effect of exogenously supplied GA₃, which reduced anthocyanin accumulation to 35% of non-treated control, was partially reversed in the presence of paclobutrazol to 77% of the control. The hypothesis that growth retardants exert their effect on anthocyanin production through the inhibition of synthesis and thus decreased levels of endogenous GAs was investigated.

An hypothesis for the action of GA₃ can be constructed from the study of Hinderer and Seitz (Archives Biochem Biophys 246:217-224, 1986) which suggested that GA stimulates 3'-nucleotidase activity in carrot cell suspensions. According to this hypothesis, the 3'-nucleotidase dephosphorylates malonyl CoA giving 3'-dephosphomalonyl CoA, which inhibits chalcone synthase, an enzyme essential for anthocyanin biosynthesis and as a result, anthocyanin accumulation is suppressed. An investigation of this hypothesis showed that 3'-nucleotidase activity in cell extracts was not greatly altered when cells were grown in the presence of GA₃ or uniconazole. This finding supports the rejection of the above

hypothesis and raises the following questions. What step in anthocyanin biosynthesis is inhibited by GA₃ and what is the mechanism of this inhibition?

In an attempt to identify the enzymatic step at which GA₃ inhibits anthocyanin synthesis, intermediates of anthocyanin biosynthesis were fed to carrot cell suspension cultures in the presence of GA₃. Naringenin and dihydroquercetin reversed GA₃ inhibition. Malonic acid, a potential precursor for malonyl CoA, and 4-coumaric acid did not restore anthocyanin production to the level of the non-treated control. These findings suggested that anthocyanin biosynthesis is inhibited by GA₃ at the enzymatic step catalyzed by chalcone isomerase or chalcone synthase. A major source for malonyl CoA, which is required for anthocyanin biosynthesis, is acetyl CoA. The conversion of acetyl CoA into malonyl CoA is catalyzed by the enzyme acetyl CoA carboxylase. For this reason, the effects of GA₃ and uniconazole on acetyl CoA carboxylase were also investigated.

The activities of chalcone isomerase, chalcone synthase and acetyl CoA carboxylase were measured in extracts from untreated, from GA₃-treated and from uniconazole-treated cells. Acetyl CoA carboxylase activity was the same in extracts from untreated cells and GA₃-treated cells but no activity was found in extracts from uniconazole-treated cells. Chalcone isomerase activity was lower in extracts from GA₃-treated cells and much lower in extracts from uniconazole-treated cells than in extracts of untreated cells. Anthocyanin accumulation was increased in the presence of uniconazole which implies that the low level of

chalcone isomerase in this treatment and the higher level in the GA₃ treatment are sufficient for the anthocyanin biosynthesis. The total chalcone synthase activity in extracts from GA₃ 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 non-treated cells while only two of these peaks were detected in extracts of GA₃-treated cells. The extracts from GA₃-treated cells did not contain the peak of chalcone synthase activity in untreated cells which preceded the main peak and was about ¼ of the total activity. 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 is the site of GA inhibition of anthocyanin biosynthesis.

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LIST OF ABBREVIATIONS

ABA	- Absciscic acid.
ACCase	- Acetyl Coenzyme A carboxylase.
ADP	- Adenosine diphosphate.
AMP	- Adenosine monophosphate.
AS	- Ammonium sulfate.
ATP	- Adenosine triphosphate.
BCA	- Bicinchoninic acid.
CHI	- Chalcone isomerase.
CHS	- Chalcone synthase.
2,4-D	- 2,4-dichlorophenoxyacetic acid.
DBP	- DNA binding protein.
DC	- <i>Daucus carota</i> .
DHFR	- Dihydroflavanol reductase.
DHQ	- Dihydroquercetin.
DNA	- Deoxyribonucleic acid.
DW	- Dry weight.
EKS	- Ent-kaurene synthase.
FPLC	- Fast protein liquid chromatography.
F₃OH	- Flavanone 3-hydroxylase.
FW	- Fresh weight.

GA	- Gibberellic acid.
GC-MS	- Gas chromatography - mass spectrometry.
GC-SIM	- Gas chromatography - selected ion monitoring.
HPLC	- High performance liquid chromatography.
KD	- Kilo Dalton.
KRI	- Kovats retention index.
MeTMSi	- Methyl trimethylsilyl.
NADPH	- Reduced nicotinamide adenine dinucleotide phosphate.
NTCE	- Non-treated crude extract.
O.D.	- Optical density (absorbance).
STCE	- Sephadex-treated crude extract.
TCA	- Trichloroacetic acid.
TLC	- Thin layer chromatography.
UV	- Ultraviolet.
WCM	- Wild carrot medium.

PART 1
INTRODUCTION

1. INTRODUCTION

Plant cell suspension cultures have been used to explore the effect of different factors on the biosynthesis and accumulation of secondary metabolites (for review see Sakuta and Komamine 1987, Yamada and Hashimoto 1984).

Anthocyanins, one group of secondary metabolites, are flavonoids synthesized through phenylalanine, a product of the shikimic acid pathway. Phenylalanine is converted to cinnamic acid, which is hydroxylated to give 4-coumaric acid. The addition of coenzyme A to coumaric acid completes the synthesis of 4-coumaroyl CoA. Another precursor of flavonoid biosynthesis is malonyl CoA which is derived from acetyl CoA, supplied by glycolysis or fatty acid metabolism. An early step in flavonoid synthesis involves the condensation of malonyl CoA and 4-coumaroyl CoA to give naringenin-chalcone, which is then converted into dihydroquercetin and anthocyanidin. The anthocyanidin thus produced is modified by the attachment of sugars such as glucose or galactose and acids such as ferulic, caffeic, sinapic, coumaric, malonic acid, etc. These modifications are typical of specific species or sub-species. The anthocyanin produced either with or without a cinnamoyl residue accumulates in the cells (Baker et al. 1993, Griesbach 1982, Hopp and Seitz 1987).

Different anthocyanins were detected in carrot cells. The major anthocyanin isolated from carrot cell suspension cultures of black carrot (*Daucus carota* ssp. *sativa*) was cyanidin-3-(sinapoylxylosylglucosylgalactoside) [Gläbgen et al. 1992, Hopp and Seitz 1987]. The predominant anthocyanin found in cell

suspension cultures of wild carrot (*Daucus carota* ssp. *carota*) was cyanidin-3-(xylosyl-galactoside) [Baker et al. 1993]. Figure 1 summarizes the present knowledge of anthocyanin biosynthesis.

Among the enzymes which catalyze anthocyanin biosynthesis are chalcone synthase (CHS), chalcone isomerase (CHI), flavanone 3-hydroxylase (F_3OH) and dihydroflavanol reductase (DHFR). The enzymes catalyzing the conversion of leucocyanidin to cyanidin (two putative steps) were not reported. The enzyme catalyzing the glucosylation of cyanidin was reported in petunia (Kho et al. 1978), parsley (Sutter and Griesbach 1975), and others. The glycosyl transferase, which transfer galactose to carrot cyanidin was not reported. One suggestion is that leucocyanidin is glycosylated first and converted later to anthocyanin (Heller and Forkmann 1988). The activities of enzymes which catalyze anthocyanin biosynthesis are subjected to developmental (i.e. internal) and environmental (i.e. external) regulation.

Chalcone synthase (CHS) catalyzes the first committed step in flavonoid biosynthesis, the condensation of 3 molecules of malonyl CoA with a molecule of 4-coumaroyl CoA to form chalcone (4',2',4,6'-tetrahydroxychalcone) [Heller and Forkmann 1988]. Hrazdina et al. (1978) investigated the CHS activity of subcellular fractions of protoplasts obtained from *Hippeastrum* and tulip petals and suggested that the cellular location of this enzyme is the cytoplasmic face of the endoplasmic reticulum membrane. CHS has been detected in many plants. Among them are pine (Fliegmann et al. 1992), walnut (Claudot and Drouet 1992),

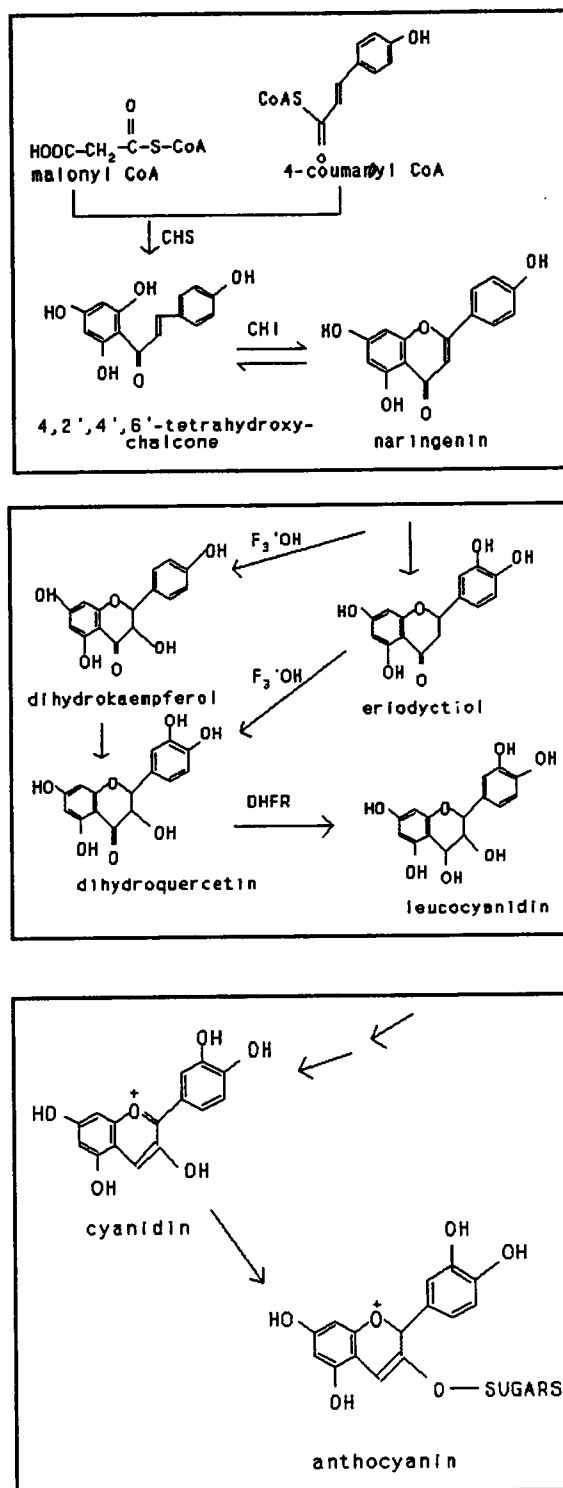


Figure 1: Biosynthesis of anthocyanin in carrot and the enzymes catalyzing it. CHS - chalcone synthase, CHI - chalcone isomerase, F₃'OH - flavanone-3-hydroxylase, DHFR - dihydroflavanol reductase.

parsley (Hrazdina et al. 1976), *Antirrhinum* (Spribille and Forkmann 1982), *Haplopappus* (Saleh et al. 1978), *Matthiola* (Rall and Hemleben 1984), oat (Knogge et al. 1986) and carrot (Ozeki et al. 1985). CHS is a homodimer made of two sub-units of approximately 41 KD each (Martin 1993). Two forms of CHS have been identified by DEAE chromatography in spinach (Beerhues and Wiermann 1985) and by isoelectric focusing and SDS-PAGE in carrot (Gleitz and Seitz 1989). CHS is regulated by different environmental factors, such as: light, injury and fungal elicitors, and by developmental factors such as growth regulators and regulatory genes. CHS is encoded by a multigene family. Eight genes for CHS were detected in petunia (Koes et al. 1987), seven in bean (Schmid et al. 1990), six in soybean (Wingender et al. 1989), three in pea (Harker et al 1990). One gene for CHS was detected in cell suspensions of parsley. This gene has two alleles differing by a transposon-like element (Hermann et al. 1988). The regulation of CHS occurs mostly at the transcriptional level of CHS gene expression (Dooner et al 1991, Lancaster 1992, Martin 1993). GA stimulated CHS transcription and anthocyanin synthesis in petunia corolla (van der Krol et al. 1990, Weiss et al 1992). Inhibition of anthocyanin accumulation by GA₃ in radish seedlings has been described by Jain and Guruprasad (1989). Hinderer et al. (1984) demonstrated a 50% decrease in anthocyanin accumulation and in CHS specific activity in GA₃-treated carrot cell suspension cultures grown for 10 days.

Chalcone isomerase (CHI) catalyzes the isomerization of 2',4',4,6'-tetrahydroxy chalcone into naringenin. Hrazdina et al. (1978) investigated CHI

activities in different sub-cellular fractions of protoplasts from *Hippeastrum* and tulip petals. They suggested that the cellular location of this enzyme is the cytoplasmic face of the endoplasmic reticulum membrane. Chalcone isomerase was detected in many plants. Among them are beans (Dixon et al. 1983), soybeans (Moustafa and Wong 1967), parsley, garbanzo bean (Hahlbrock et al. 1970) and petunia (Mol et al. 1985). Two isozymes of CHI of 62.5 Kd and 44 Kd were detected in petunia. These isozymes were expressed selectively in flower and anther tissue (van Weely et al. 1983). Other investigations in petunia and in other plants reported different molecular weights for CHI (for review see Heller and Forkmann 1988). A gene for CHI was isolated from bean and two genes for CHI were found in petunia. One of them, *chiA*, has no introns and the other, *chiB*, has 2 introns. van Tunen et al. (1990) showed that two different promoters are responsible for the differential transcription of the *chiA* gene in the corolla and anthers of petunia. CHI is known to be regulated by environmental and developmental factors including UV light (Kakegawa et al. 1991, van Tunen et al. 1988) and fungal elicitors (Dixon et al. 1983, Ebel et al. 1984, Pare et al. 1992). Weiss et al. (1990) detected increased levels of mRNA for CHI following GA₃ application in petunia corolla.

Acetyl CoA carboxylase (ACCase) catalyzes the carboxylation of acetyl CoA to give malonyl CoA. This is the first committed step in fatty acid biosynthesis. The malonyl CoA produced in this reaction serves also as a precursor for flavonoid biosynthesis. Unlike ACCase of other eukaryotes which is

located in the cytoplasm, plant ACCase is found in the stroma of plastids, the site of plant fatty acid synthesis, and it is encoded by nuclear DNA (Gornicki and Haselkorn 1993). ACCase has a biotin-containing prosthetic group which transfers CO_2 from bicarbonate to acetyl CoA. ACCase of 220-240 KD was isolated from several plant tissues (for review see Harwood 1991). ACCase was purified from soybean seeds (Charles and Cherry 1986), rape seed (Slabas and Hellyer 1985), wheat (Egin-Bühler et al. 1980, Gornicki and Haselkorn 1993), corn (Nikolau and Hawke 1984) and other plants. ACCase which was isolated from spinach chloroplasts was found to be regulated by environmental factors, such as light, and by internal factors such as Mg^{2+} and adenine nucleotide levels (Sauer and Heise 1984). This pattern of ACCase regulation has been established in other plant systems and it is thought that the increase of stromal pH, Mg^{2+} and ATP which occurs during illumination increases the activity of this enzyme (Harwood 1991). An elicitor increased ACCase activity, together with the activities of other enzymes of general phenylpropanoid metabolism, phenylalanine ammonia-lyase and chalcone synthase, in soybean cell suspensions (Ebel et al. 1984).

Anthocyanin is often found in small vesicles called anthocyanoplasts in anthocyanin producing tissues. These vesicles has been identified in hypocotyls of red cabbage (Pecket and Small, 1980) and of radish (Yasuda and Shinoda, 1985). Anthocyanoplasts can be detected only during anthocyanin synthesis. Once anthocyanin synthesis has been completed and all anthocyanin is accumulated in the vacuole, anthocyanoplasts can no longer be detected (Harborne and Grayer

1988). Activities of enzymes involved in anthocyanin biosynthesis were associated with the endoplasmic reticulum fraction in *Hippeastrum* protoplasts (Wagner and Hrazdina 1984). Therefore, Hrazdina and Wagner (1985) suggested that anthocyanins are produced in the endoplasmic reticulum by a multi-enzymatic complex, deposited in vesicles and transported to the vacuole.

Various factors affect anthocyanin biosynthesis and accumulation in plant cells. Among these factors are light (Mancinelli et al. 1991, Takeda 1990) and growth regulators including 2,4-D and gibberellic acid (GA) [Ehnmann et al. 1991, Ozeki and Komamine 1986, Peter et al. 1991], availability of cinnamic acid derivatives (Dougall 1989), sucrose concentration (Cormier et al. 1990) and aspects of mineral nutrition such as phosphorus concentration, nitrogen concentration and ammonium/nitrate ratio (Ishikura et al. 1989, Sakuta and Komamine 1987).

Gibberellins are one group of plant growth regulators. The other groups of growth regulators (plant hormones) are auxins, cytokinins and ethylene. Other phytochemicals, such as salicylic acid are often considered as growth regulators. GAs are produced from mevalonic acid through the terpenoid pathway (Figure 2). GAs have been found in many fungi and plant species. They affect many phenomena in plants such as cell growth, internode elongation, anthocyanin accumulation, production of hydrolytic enzymes in germinating graminaceous seeds, etc. (for review see Graebe, 1987).

Growth retardants are synthetic compounds which reduce internode length

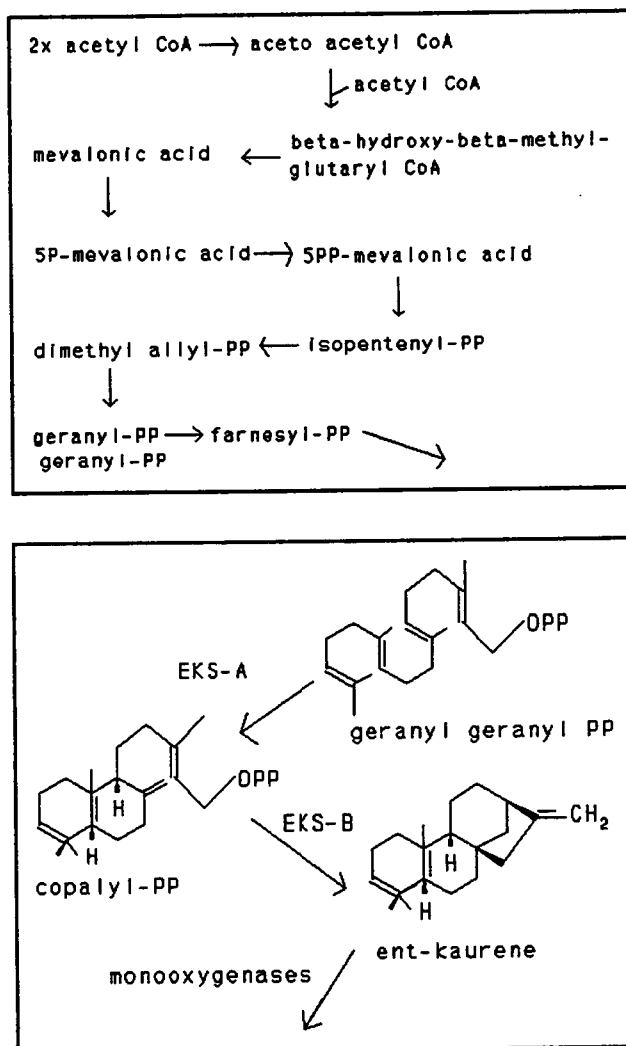


Figure 2: Biosynthesis of GA, enzymes involved in it and steps inhibited by growth retardants.

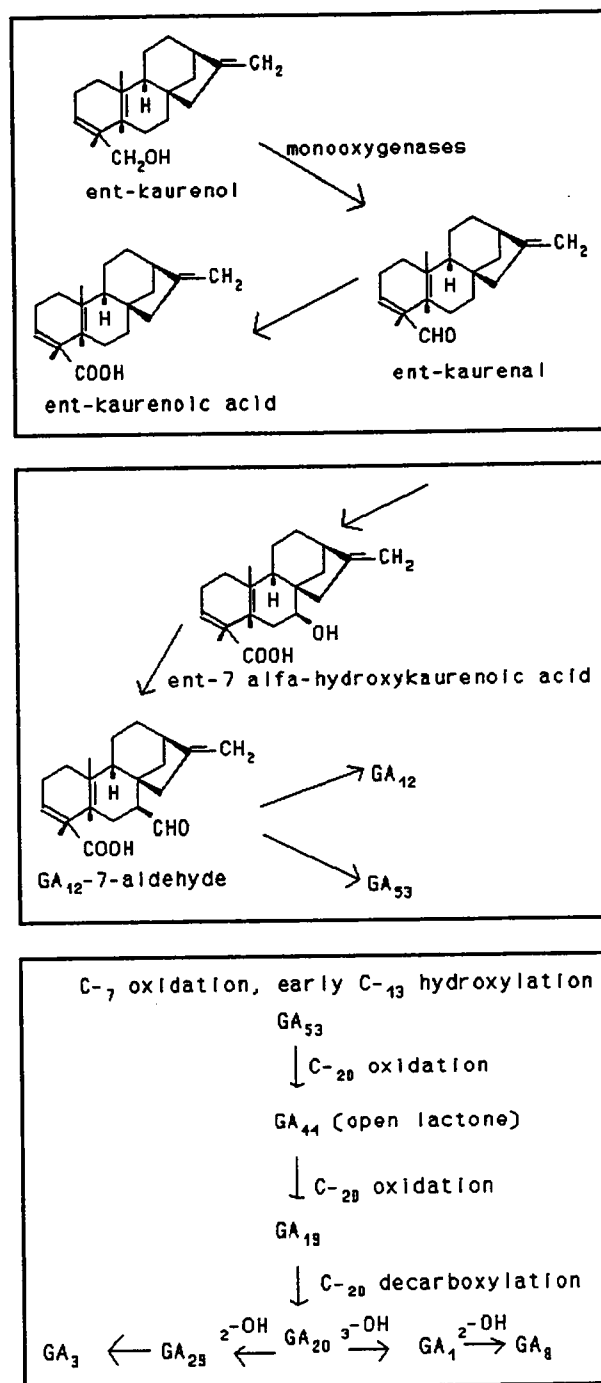


Fig. 2 (cont.): Biosynthesis of GA. 2-OH - 2-hydroxylation, 3-OH - 3-hydroxylation.

in higher plants. Their effect is the result of the inhibition of GA biosynthesis (for review see Rademacher 1991). Growth retardants are characterized by their structure and site of inhibition in the GA biosynthetic pathway. The three major divisions in this pathway that are sites of inhibition, are:

1. The conversion of geranyl-geranyl pyrophosphate to ent-kaurene which is catalyzed by the two enzymes ent-kaurene synthase A and B (EKS-A and EKS-B).

The growth retardants which inhibit this site are quaternary ammonium compounds such as 2-chloroethyl trimethylammonium chloride (CCC) and (2-isopropyl-5-methyl-4-trimethylammonium chloride)-phenyl-1-piperidinium carboxylate (AMO-1618). Their structures are shown in Figure 3.

2. The three oxidation steps involved in the conversion of ent-kaurene to ent-kaurenoic acid which are catalyzed by cytochrome P-450 dependent monooxygenases. Monooxygenases are enzymes which catalyze the addition of one atom of molecular oxygen to an organic compound. They require O_2 and NADPH for their activity. Growth retardants of several groups block these cytochrome P-450-dependent monooxygenase activities by their interaction with the protoheme iron of cytochrome P-450 which interferes with oxygen binding at this site (Sauter, 1984). The growth retardants which inhibit this site (Figure 3) are:

- a)- substituted pyrimidines such as α -cyclopropyl-4-methoxy- α -(pyrimidine-5-yl) benzyl alcohol (ancymidol).
- b)- triazoles such as (2RS,3RS)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1,2,4-triazol-1-

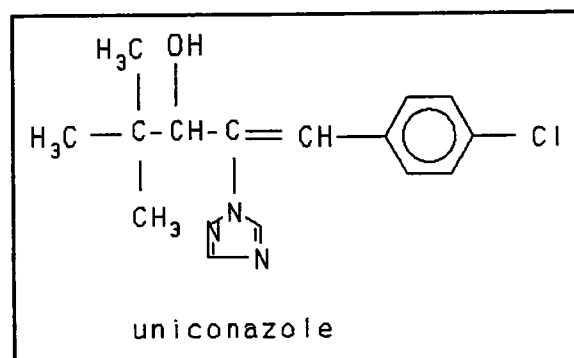
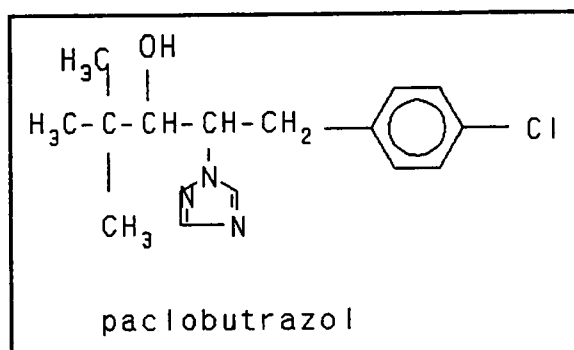
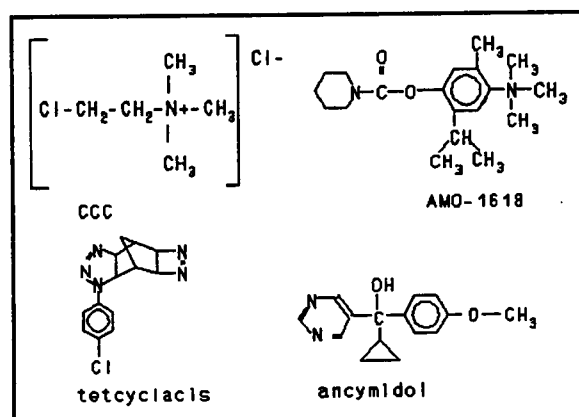


Figure 3: Structures of growth retardants used in the current research.

yl)-penten-3-ol (paclobutrazol) and (E)-1-(4-chlorophenyl)-4,4-dimethyl-2-(1H,1,2,4-triazol-1-yl)-1-penten-3-ol (uniconazole).

c)- norbornenodiazetines such as 5-(4-chlorophenyl)-3,4,5,9,10-pentaaza-tetracyclo-5,4,10^{2,6},0^{8,11}-dodeca-3,9-diene (tetcyclacis).

3. Later steps in GA biosynthesis including the conversion of GA₁₂-aldehyde to GA₁₂ are catalyzed by different microsomal and soluble enzymes. The growth retardant which inhibits some of these steps is prohexadione (3,5-dioxo-4-propionylcyclohexane carboxylic acid) which inhibits soluble dioxygenases, enzymes which catalyze the addition of two atoms of molecular oxygen to an organic compound and require α -keto glutarate as a co-substrate.

Inhibition of anthocyanin production by GA in carrot cell suspension cultures has been demonstrated in several studies. Hinderer et al. (1984) showed inhibition of anthocyanin accumulation by GA₃ in *Daucus carota* ssp. *sativa*. Cheng et al. (1985) demonstrated the inhibition of anthocyanin accumulation in cell suspension cultures of *Daucus carota* ssp. *carota*. They rejected the hypothesis that the inhibition of anthocyanin accumulation by GA₃ is due to GA₃ inhibition of the synthesis of one isozyme of 4-coumarate CoA ligase which had been earlier advanced by Heinzmann and Seitz (1977). Ozeki and Komamine (1986) showed that, in addition to GA₃, other plant hormones, 2,4-dichlorophenoxyacetic acid (2,4-D) and abscisic acid (ABA), also inhibited anthocyanin accumulation in carrot cell suspensions. Hinderer and Seitz (1986) investigated the lack of anthocyanin production in a carrot cell line (DCw) which was obtained by culturing their

anthocyanin producing cell line (DCb) in the presence of 3.0 or 0.3 μM GA_3 for 10 or more years. Extracts from the DCw line showed no chalcone synthase activity and inhibited the partially purified chalcone synthase from the cell line DCb and from other sources. The proximal inhibitor of chalcone synthase was a 3'-nucleotidase which, *in vitro*, dephosphorylated malonyl-CoA converting it from a substrate into an inhibitor of chalcone synthase. The 3'-nucleotidase removed 3'-phosphate groups from a number of compounds but did not remove 5'-phosphate groups. Hinderer and Seitz (1986) did not demonstrate the presence of 3'-nucleotidase in DCb, did not show an increase of 3'-nucleotidase activity in extracts of GA_3 -treated DCb and did not show a decrease in 3'-nucleotidase activity in DCw grown in the absence of GA_3 . In addition, Hinderer and Seitz (1986) emphasized that they have no direct evidence that the malonyl-3'-dephospho-CoA inhibits chalcone synthase *in vivo*.

Hinderer and Seitz's study (1986) suggests the following hypothesis. GA -induced production of 3'-nucleotidase, causes the dephosphorylation of malonyl CoA producing dephospho malonyl CoA which inhibits chalcone synthase (CHS), a key enzyme in anthocyanin biosynthesis.

The current investigation was aimed at extending the description of GA inhibition of anthocyanin production and examining of the hypothesis based on Hinderer and Seitz's study (1986). The research addressed the following objectives:

1. Determination of the effects of different growth retardants on anthocyanin

production by carrot cell suspension cultures (the results of this research are described in Part 2).

2. Identification of endogenous GAs, the measurement of their levels and correlation of these levels with anthocyanin accumulation in control and growth retardant-treated cultures (described in Part 2).
3. Investigation of the influence of GA₃ and growth retardants on the activity of 3'-nucleotidase in the tissue (described in Part 3).
4. Identification of steps in anthocyanin biosynthesis where GA₃ inhibits the pathway (described in Part 4).
5. The investigation of the effects of GA₃ and growth retardants in the culture medium on the activities of chalcone synthase, chalcone isomerase and acetyl CoA carboxylase in the tissue (described in Part 4).

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PART 2

THE EFFECT OF GROWTH RETARDANTS ON ANTHOCYANIN PRODUCTION IN CARROT CELL SUSPENSION CULTURES

Ilan A, Dougall DK (1992) The effect of growth retardants on anthocyanin production in carrot cell suspension cultures. *Plant Cell Reports* 11:304-309. (modified for dissertation).

1. INTRODUCTION

Growth retardants are synthetic compounds which reduce internode length in higher plants. This effect is the result of the inhibition of gibberellin biosynthesis (for review see Graebe 1987). Growth retardants differ in their sites of inhibition of gibberellin biosynthesis. Among the sites is the conversion of geranylgeranyl pyrophosphate to ent-kaurene which is catalyzed by the enzyme ent-kaurene synthetase (EKS). This enzyme is inhibited by quaternary ammonium compounds, such as AMO-1618 and CCC (Frost and West 1977). Other sites of inhibition of gibberellin biosynthesis are the oxidation steps of ent-kaurene to ent-kaurenoic acid catalyzed by cytochrome P-450 dependent monooxygenases. These steps are inhibited by substituted pyrimidines such as ancymidol, triazoles such as paclobutrazol or uniconazole and norbornenodiazetines such as tetcyclacis (Graebe 1987).

In addition to monooxygenases catalyzing the conversion of ent-kaurene to ent-kaurenoic acid, NADPH-requiring microsomal monooxygenases together with 2-oxoglutarate-requiring soluble oxygenases catalyze the conversion of GA₁₂-aldehyde to GA₁₂ and other steps in the interconversion of gibberellins. These reactions are affected by the growth retardant prohexadione (Nakayama et al. 1990).

Growth retardants, in addition to their effects on the whole plant, also

affect plant tissue cultures. Grossman (1988) showed that several growth retardants inhibited growth of different species in tissue culture. In addition, plant cell suspension cultures have been used to explore the effects of different factors on secondary metabolite biosynthesis and accumulation (for review see Sakuta and Komamine 1987) but growth retardants are not amongst these factors.

Factors affecting anthocyanin biosynthesis and accumulation in cell cultures have been studied in several plant species (Buchenauer and Rohner 1981, Cormier et al. 1990, Erikson 1967, Hall and Yeoman 1987, Hedden and Graebe 1985, Ishikura et al. 1989, Martinez and Favret 1990, Matsumoto et al. 1973, Nouse et al. 1987, Yamamoto et al. 1982).

One of the medium components which inhibits anthocyanin accumulation is GA₃ (Cheng et al. 1985, Hinderer et al. 1984, Ozeki and Komamine 1986). Noma et al. (1982) demonstrated GA production by carrot cells in suspension culture. These findings lead to the following questions: Does the alteration in endogenous GA level alter anthocyanin accumulation? Are differences in endogenous GA levels responsible for the differences in anthocyanin accumulation by different cell lines and clones? We addressed these questions by examining the effects of seven growth retardants on anthocyanin accumulation and growth in carrot cell suspension cultures.

2. MATERIALS AND METHODS

Cell cultures

Wild carrot (*Daucus carota* L.) cell suspension cultures were maintained on wild carrot medium (WCM-1, described by Wetherell, 1969) in 250 ml Erlenmeyer flasks containing 50 ml medium each. The cell suspensions were subcultured at intervals of 10 days at a 1:10 inoculation rate. The cultures were shaken on a gyrotary shaker at 120 rpm in the dark at 25°C.

The source for the different sub-clones used in this study was the parental line WC 63-1-9-1 (described by Dougall et al. 1980). The following sub-clones which have been described by Vogelien et al. (1990) were used: 6.1.1.2 and 13.1.1.1.2 which are high accumulating sub-clones; 2.5.1.2.1, 15.3.3.2.1.2.1 and 10.1.1.2 which are low accumulating sub-clones. In addition, we used three sub-lines which were obtained from the sub-clone 13.1. These sub-lines were obtained by random choice of subcultures which were then maintained independently by serial subcultures. These sub-lines were: 13.1.H - medium-high anthocyanin accumulating, 13.1.L - medium-low accumulating and 13.1.skz - high accumulating sub-line.

Experimental methods

The medium in all the experiments was WCM-4 (Dougall and Weyrauch, 1980) which gave increased anthocyanin accumulation relative to WCM-1. Cultures were grown in 50 ml of this medium in shaken 250 ml Erlenmeyer flasks. The inoculation rate was 1:10 and the growth period was 12 days unless otherwise

stated. Samples of 2.5 ml were taken from each flask for both anthocyanin and dry weight measurements as described by Vogelien et al. (1990).

In each treatment three Erlenmeyer flasks were used and three samples were taken from each flask unless otherwise stated. The results were analyzed by "nested analysis of variance" (SAS/STAT Guide for Personal Computers, Version 6). Data are given as mean $\pm$ standard deviation. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter. Anthocyanin was converted into $\mu\text{moles anthocyanin}\cdot\text{L}^{-1}$ culture by multiplying the $A_{530\text{nm}}$ by 33.3 based on a molar extinction coefficient of 30000 for cyanidin chloride (Dougall and Frazier 1989). This value was divided by the dry weight to give $\mu\text{moles}\cdot\text{gDW}^{-1}$.

3. RESULTS AND DISCUSSION

Seven growth retardants were tested for their effect on anthocyanin accumulation and dry weight in wild carrot cell suspension cultures.

The most effective of the tested concentrations of paclobutrazol in increasing anthocyanin accumulation/ml culture in both 13.1.1.1.2 (high anthocyanin accumulating sub-clone) and 15.3.3.2.1.2.1 (low accumulating sub-clone) was $1.7 \times 10^{-6} \text{M}$ (Table 1). This concentration was also the most effective in increasing anthocyanin accumulation/g.dry weight in these sub-clones. Paclobutrazol concentration of $17 \times 10^{-6} \text{M}$ yielded similar increase in anthocyanin accumulation gDW^{-1} as did $1.7 \times 10^{-6} \text{M}$ in the low accumulating sub-clone.

Paclobutrazol increased anthocyanin accumulation in all wild carrot sub-clones and cell lines tested (Table 2). These results suggest a general enhancing effect of paclobutrazol on anthocyanin accumulation in wild carrot cell suspension cultures. Paclobutrazol concentration of $0.34 \times 10^{-6} \text{M}$ was used in this experiment since no significant difference in anthocyanin accumulation was found between this concentration and $1.7 \times 10^{-6} \text{M}$, which was used in previous experiments.

Both $0.34 \times 10^{-6} \text{M}$ and $3.4 \times 10^{-6} \text{M}$ uniconazole gave statistically significant ($P=5\%$) increases in anthocyanin in sub-clone 13.1.L relative to the control and relative to the $6.8 \times 10^{-6} \text{M}$ uniconazole treatment (Table 3).

Tetcyclacis at 10^{-6}M and 10^{-7}M significantly increased anthocyanin accumulation in sub-clone 13.1.1.1.2 (Table 3). Higher concentrations of tetcyclacis (10^{-5}M , 10^{-4}M) inhibited anthocyanin accumulation (data not shown).

Table 1: The effect of paclobutrazol on anthocyanin and dry weight in carrot cell suspension cultures of sub-clone 15.3.3.2.1.2.1 and 13.1.1.1.2. The inoculation rate was 1:5. The growth period was 7 days. Each treatment contained two Erlenmeyer flasks and five samples from each flask were measured. Pac - paclobutrazol, DW - dry weight. Data are given as mean $\pm$ standard deviation. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter. Anthocyanin was converted into $\mu\text{moles anthocyanin} \cdot \text{L}^{-1}$ culture by multiplying the $A_{530\text{nm}}$ by 33.3 based on a molar extinction coefficient of 30000 for cyanidin chloride and divided by the dry weight to give $\mu\text{moles gDW}^{-1}$.

Sub-clone	Pac (μM)	Anthocyanin $\cdot \text{ml culture}^{-1}$ ($A_{530\text{nm}}$)	DW ($\text{mg} \cdot \text{ml}^{-1}$)	$\mu\text{moles} \cdot \text{gDW}^{-1}$	% of control
15.3.3.2.1.2.1	0.0	0.051 ± 0.003 b	3.13 ± 0.21 a	0.54	100
	1.7	0.064 ± 0.004 a	2.89 ± 0.17 a	0.74	136
	17.0	0.025 ± 0.002 c	1.12 ± 0.13 c	0.74	136
	170.0	0.016 ± 0.002 d	1.46 ± 0.06 b	0.36	67
13.1.1.1.2	0.0	0.43 ± 0.04 b	4.00 ± 0.15 a	3.58	100
	1.7	1.07 ± 0.17 a	3.61 ± 0.16 b	9.87	276
	17.0	0.21 ± 0.01 c	1.65 ± 0.04 d	4.24	118
	170.0	0.18 ± 0.02 c	2.11 ± 0.18 c	2.84	79

Table 2: The effect of paclobutrazol on anthocyanin accumulation and growth in several wild carrot sub-clones. Pac - paclobutrazol. 2 flasks x 5 samples were measured for sub-clones 2.5.1.1.2.1, 6.1.1.2 and 13.1.H. 3 flasks x 3 samples were measured for sub-clones 10.1.1.2, 13.1.skz and 13.1.L. Anthocyanin was converted into $\mu\text{moles anthocyanin}\cdot\text{L}^{-1}$ culture by multiplying the $A_{530\text{nm}}$ by 33.3 based on a molar extinction coefficient of 30000 for cyanidin chloride and divided by the dry weight to give $\mu\text{moles gDW}^{-1}$.

Sub-clone	Pac (μM)	Anthocyanin ml culture^{-1} ($A_{530\text{nm}}$)	DW ($\text{mg}\cdot\text{ml}^{-1}$)	$\mu\text{moles gDW}^{-1}$	% of control
2.5.1.1.2.1	0	0.95 ± 0.17	3.99 ± 0.88	7.93	100
	0.34	1.35 ± 0.28	3.82 ± 0.64	11.77	148
6.1.1.2	0	2.70 ± 0.46	4.36 ± 0.75	20.62	100
	0.34	3.73 ± 0.31	4.05 ± 0.64	30.67	149
10.1.1.2	0	0.47 ± 0.06	7.32 ± 1.63	2.14	100
	0.34	0.59 ± 0.12	5.35 ± 0.71	3.67	172
13.1.skz	0	1.45 ± 0.09	5.27 ± 0.34	9.16	100
	0.34	1.84 ± 0.19	5.88 ± 0.51	10.42	114
13.1.L	0	0.24 ± 0.03	6.23 ± 0.68	1.28	100
	0.34	0.33 ± 0.03	6.29 ± 0.24	1.75	136
13.1.h	0	0.37 ± 0.01	5.18 ± 0.21	2.38	100
	0.34	0.54 ± 0.01	5.07 ± 0.23	3.55	149

Table 3: The effect of uniconazole, tetcyclacis, and CCC concentration on growth and anthocyanin accumulation in carrot cell suspensions of sub-clones 13.1.L and 13.1.1.1.2. Conc. - concentration, DW - dry weight. Uniconaz. - uniconazole.

Data are given as mean $\pm$ standard deviation. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter. Anthocyanin was converted into μ moles anthocyanin $\cdot$ L⁻¹ culture by multiplying the A_{530nm} by 33.3 based on a molar extinction coefficient of 30000 for cyanidin chloride and divided by the dry weight to give μ moles $\cdot$ gDW⁻¹.

Sub-clone	Growth retardant	Conc (μ M)	Anthocyanin $\cdot$ ml culture ⁻¹ (A_{530nm})	DW (mg $\cdot$ ml ⁻¹)	μ mole $\cdot$ gDW ⁻¹	% of control
13.1.L	uniconaz.	0.00	0.23 $\pm$ 0.05 c	5.31 $\pm$ 0.33 a	1.44	100
		0.34	0.37 $\pm$ 0.01 a	5.40 $\pm$ 0.23 a	2.28	158
		3.40	0.38 $\pm$ 0.03 a	4.99 $\pm$ 0.24 a	2.54	176
		6.80	0.31 $\pm$ 0.01 b	4.80 $\pm$ 0.30 a	2.15	149
13.1.1.1.2	tetcyclacis	0.00	0.49 $\pm$ 0.06 c	6.01 $\pm$ 0.52 a	2.71	100
		0.01	0.47 $\pm$ 0.06 c	5.86 $\pm$ 0.68 a	2.67	98
		0.10	0.63 $\pm$ 0.17 b	5.74 $\pm$ 0.39 a	3.65	135
		1.00	0.96 $\pm$ 0.23 a	5.74 $\pm$ 0.48 a	5.57	205
13.1.1.1.2	CCC	0	0.47 $\pm$ 0.14 c	4.94 $\pm$ 0.74 b	3.17	100
		0.01	0.57 $\pm$ 0.13 b	5.45 $\pm$ 0.35 a	3.48	110
		0.10	0.69 $\pm$ 0.17 a	5.38 $\pm$ 0.31 a	4.27	135
		1.00	0.57 $\pm$ 0.07 b	5.49 $\pm$ 0.71 a	3.46	109

Ancymidol significantly increased anthocyanin accumulation by 19% in the cell line 13.1.L when applied at 10^{-6} M ($A_{530\text{nm}}$ of 0.273 compared to 0.230 in the control). Maximum increase of anthocyanin accumulation was found at this concentration.

All the cytochrome P-450 monooxygenase inhibitors tested increased anthocyanin accumulation. However, ancymidol was less effective than uniconazole, paclobutrazol and tetcyclacis. This observation parallels that of Grossman et al. (1984) who demonstrated greater inhibition of growth of carrot cell suspension cultures by tetcyclacis than by ancymidol.

CCC at the concentrations of 10^{-8} M, 10^{-7} M and 10^{-6} M significantly increased anthocyanin accumulation in sub-clone 13.1.1.1.2 (Table 3).

AMO-1618, however, increased anthocyanin accumulation only when applied at the concentrations of 10^{-5} M and 10^{-6} M to cultures which were previously grown for one passage on WCM-4 medium. The application of AMO-1618 at the second passage enhanced the anthocyanin level and restored it to near that of the first passage (Table 4). The reduction in anthocyanin accumulation as a result of several passages on WCM-4 medium was also observed by Dougall et al. (1980). The different anthocyanin levels found in clone 13.1.1.1.2 in different experiments on WCM-4 medium is presumed to result from changes in the capability of this clone to accumulate anthocyanin over time.

Prohexadione at 10^{-6} M, 10^{-7} M and 10^{-8} M significantly decreased anthocyanin accumulation in the clone 6.1.1.2 by 11%, 10% and 12.5%,

Table 4: The effect of AMO-1618 on anthocyanin accumulation and dry weight in carrot cell suspension cultures of sub-clone 13.1.1.1.2 in each of two successive passages in the medium WCM-4. Cell suspensions were grown on WCM-4 supplemented with different concentrations of AMO-1618 at each of two passages. Each treatment contained two flasks and five samples from each flask were measured after each passage for anthocyanin-(ml culture)⁻¹ (A_{530nm}) and dry weight (D.W. in mg.ml⁻¹). Data are given as mean $\pm$ standard deviation. Means within a row which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter. Anthocyanin was converted into μ moles anthocyanin.L⁻¹ culture by multiplying the A_{530nm} by 33.3 based on a molar extinction coefficient of 30000 for cyanidin chloride and divided by the dry weight to give μ moles.gDW⁻¹.

Passage on WCM-4		0 μ M AMO-1618	1 μ M AMO-1618	10 μ M AMO-1618
1 st	anthocyanin	2.91 $\pm$ 0.31 a	2.85 $\pm$ 0.26 a	2.94 $\pm$ 0.05 a
	dry weight	3.39 $\pm$ 0.53 a	3.27 $\pm$ 0.29 a	3.45 $\pm$ 0.32 a
	μ moles.gDW ⁻¹	28.58	29.02	28.32
	% of control	100	102	99
2 nd	anthocyanin	1.97 $\pm$ 0.28 c	2.55 $\pm$ 0.07 a	2.34 $\pm$ 0.21 b
	dry weight	3.35 $\pm$ 0.42 a	3.22 $\pm$ 0.17 a	3.30 $\pm$ 0.22 a
	μ moles.gDW ⁻¹	19.58	26.37	23.61
	% of control	100	135	121

respectively. Prohexadione appears to have a different effect on anthocyanin accumulation compared to the other growth retardants tested. Further research is needed to elucidate the inhibition site of this growth retardant in this plant system.

Growth retardant concentrations which were effective in increasing anthocyanin accumulation were found to be distinct from those required for growth inhibition. Furthermore, the enhancement of anthocyanin accumulation induced by growth retardants was not due to increases in culture dry weight. Paclobutrazol at a concentration of $1.7 \times 10^{-6} \text{M}$ significantly increased anthocyanin accumulation but only slightly decreased the dry weight. Higher concentrations of paclobutrazol ($1.7 \times 10^{-5} \text{M}$, $1.7 \times 10^{-4} \text{M}$) significantly decreased the dry weight (Table 1). AMO-1618 at concentrations of 10^{-6}M and 10^{-5}M applied to WCM-4-treated cultures significantly increased anthocyanin level but did not affect the dry weight (Table 4).

Grossman et al. (1984) demonstrated that several growth retardants at a concentration of 10^{-4}M inhibited the growth of cell cultures from different species. Nitsche et al. (1985) demonstrated in seedlings that tetcyclacis at concentrations below 10^{-6}M decreased cell elongation and above 10^{-6}M inhibited cell division. Grossman et al. (1985) found that sterols could fully reverse the inhibition of cell division caused by 10^{-4}M tetcyclacis while gibberellins and their precursors could not. Based on these finding and others, Graebe (1987) suggested that growth retardants inhibit gibberellin biosynthesis at lower concentration and at higher concentration they also inhibit sterol biosynthesis.

Absciscic acid (ABA) inhibited anthocyanin accumulation in carrot cell suspensions (Ozeki and Komamine 1986). However, Hauser et al. (1990) and Belefant and Fong (1991) demonstrated an increase in ABA biosynthesis in growth retardant treated plants. These observations make it unlikely that the increase of anthocyanin by growth retardants is due to inhibition of ABA biosynthesis.

We conducted the following experiment to determine whether the increased anthocyanin accumulation in the presence of growth retardants is due to their inhibition of gibberellin biosynthesis: cell suspensions were grown on WCM-4 medium or WCM-4 supplemented with 10^{-6} M GA₃, 1.7×10^{-6} M paclobutrazol, 10^{-6} M GA₃ plus 1.7×10^{-6} M paclobutrazol and 10^{-5} M GA₃ plus 1.7×10^{-6} M paclobutrazol and the anthocyanin accumulation was measured. The results of this experiment demonstrated that GA₃ at 10^{-6} M inhibited anthocyanin accumulation to 35% of control (Table 5). The addition of paclobutrazol at 1.7×10^{-6} M reversed the inhibitory effect of 10^{-6} M GA₃ on anthocyanin accumulation and increased the anthocyanin accumulated to 77% of control. Increasing GA₃ concentration to 10^{-5} M prevented the effect of paclobutrazol (Table 5).

We postulate that paclobutrazol inhibits the endogenous synthesis of GA and thus decreases the endogenous GA in the tissue. Based on this postulate we interpret the experiment as follows. 10^{-5} M GA₃ (in the presence of paclobutrazol) had the same effect on anthocyanin accumulation as 10^{-6} M GA₃

Table 5: The effects of GA₃, paclobutrazol and their combinations on anthocyanin accumulation in carrot cell suspension cultures of sub-clone 13.1.1.1.2 Cultures were grown for 15 days on the following media: WCM-4 (control), WCM-4 + 1.7x10⁻⁶M paclobutrazol (I), WCM-4 + 10⁻⁶M GA₃ (II), WCM-4 + 1.7x10⁻⁶M paclobutrazol + 10⁻⁶M GA₃ (III), WCM-4 + 1.7x10⁻⁶ M paclobutrazol + 10⁻⁵M GA₃ (IV). Data are given as mean ± standard deviation. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter. Anthocyanin was converted into μmoles anthocyanin L⁻¹ culture by multiplying the A_{530nm} by 33.3 based on a molar extinction coefficient of 30000 for cyanidin chloride and divided by the dry weight to give μmoles·gDW⁻¹.

Treatment	Anthocyanin ·ml culture ⁻¹ (A _{530nm})	Dry Weight (mg·ml ⁻¹)	μmoles gDW ⁻¹	% of control
Control	2.23±0.09 b	5.91±0.33 a	12.56	100
I	2.41±0.25 a	5.99±0.56 a	13.40	107
II	0.73±0.07 d	5.52±0.26 b	4.40	35
III	1.70±0.09 c	5.86±0.32 a	9.66	77
IV	0.73±0.18 d	5.44±0.28 b	4.47	36

provided exogenously plus endogenously synthesized GA (i.e. in the absence of paclobutrazol). The 10^{-6} M GA₃ provided exogenously alone (in the presence of paclobutrazol) had less effect than 10^{-6} M GA₃ provided exogenously plus the endogenously synthesized GA (in the absence of paclobutrazol).

Hinderer and Seitz (1986) suggested a mechanism of GA inhibition of anthocyanin accumulation. They suggested that GA increased the level of the enzyme 3'-nucleotidase, that this enzyme catalyzes the dephosphorylation of malonyl-Coenzyme A and that the dephospho product is an inhibitor of chalcone-synthase, a key enzyme in anthocyanin biosynthesis. It should be noted that in other systems such as rose petals, gibberellin enhanced anthocyanin accumulation (Zieslin et al. 1974). A possible explanation for these contradictory effects of gibberellin on anthocyanin accumulation is that the gibberellin effect is tissue and developmental stage dependant (van Tunen et al. 1988).

These results suggest that growth retardants inhibit the biosynthesis of endogenous gibberellins and, as a result, reverse the inhibition of anthocyanin accumulation by endogenous gibberellins.

To the best of our knowledge this is the first report on the enhancement of anthocyanin production in plant cell suspension cultures by growth retardants. Growth retardants may enhance the production of other secondary metabolites by plant cell cultures.

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PART 3

GIBBERELLIC ACID DECREASES ANTHOCYANIN ACCUMULATION IN WILD CARROT CELL SUSPENSION CULTURES BUT DOES NOT ALTER 3'-NUCLEOTIDASE ACTIVITY

Ilan A, Zanewich KP, Rood SB, Dougall DK (1993) Gibberellic acid decreases anthocyanin accumulation in wild carrot cell suspension cultures but does not alter 3'-nucleotidase activity (a paper submitted to *Physiol Plant*, modified for dissertation).

1. INTRODUCTION

Various factors affect anthocyanin biosynthesis and accumulation in plant cell suspension cultures. Among these factors are light, availability of intermediates for biosynthesis, the composition of the medium, e.g., minerals, ammonium/nitrate ratio, sucrose and growth regulators including gibberellins (GAs) (Cheng et al. 1985, Cormier et al. 1990, Dougall 1989, Dougall and Frazier 1989, Ehnmann et al. 1991, Hinderer et al. 1984, Hirasuna et al. 1991, Ishikura et al. 1989, Mancinelli et al. 1991, Ozeki and Komamine 1986, Peter et al. 1991, Takeda 1990).

Gibberellins (GAs) are terpenoid phytohormones which occur in some fungi as well as in plants (Graebe 1987). They are involved in many processes in plants including cell growth, internode elongation, production of hydrolytic enzymes in germinating cereal seeds, flowering and anthocyanin production (for review see Graebe 1987). The mode of action of GA in different processes has been investigated in several plant systems and mode of action is proposed to follow the general model suggested for plant hormones in which GA is secreted, it binds to specific receptors in different cells and the receptor conformation is altered. As a consequence, enzymes affected by the receptor are regulated and in turn regulate different processes in the cells such as gene activation and metabolism. The final outcome of these processes can be morphogenetic changes in the plant (Libbenga and Mennes 1987). Much research has been done on the action of GA in the aleurone layer of germinating cereal seeds. In this system,

GA secreted by the germinating embryo stimulates the transcription of mRNA for hydrolytic enzymes in the aleurone layer. Among these enzymes are amylases, proteases, acid phosphatases and nucleases (Holstein et al. 1991). These enzymes release from storage forms, the material that is required by the germinant.

Another plant system investigated for the mode of action of GA is the stimulation of pigmentation by GA in the corolla of petunia flowers. In this system, GA secreted by the stamens activates the transcription of flavonoid biosynthetic genes in the corolla and results in anthocyanin production (Weiss et al. 1992).

Inhibition of anthocyanin production by GA has been described in at least two plant systems: carrot cell suspension cultures (Cheng et al. 1985, Hinderer et al. 1984, Ozeki and Komamine 1986) and radish seedlings (Jain and Guruprasad 1989). These systems may also be useful for study of the mode of action of GAs. In one such study, Hinderer and Seitz (1986) investigated the lack of anthocyanin production in a carrot cell line (DCw) which was obtained by culturing an anthocyanin producing cell line (DCb) in the presence of 3.0 or 0.3 μM GA₃ for 10 or more years. Extracts from the DCw line showed no chalcone synthase activity and inhibited the partially purified chalcone synthase from the cell line DCb and from other sources. The proximal inhibitor of chalcone synthase was a 3'-nucleotidase which, *in vitro*, dephosphorylated malonyl-CoA converting it from a substrate into an inhibitor of chalcone synthase. The 3'-nucleotidase removed 3'-phosphate groups from a number of compounds but did not remove 5'-phosphate

groups. Hinderer and Seitz (1986) emphasized that they have no direct evidence that the malonyl-3'-dephospho-CoA inhibits chalcone synthase *in vivo*.

Hinderer and Seitz's (1986) observations *in vitro* are used here as the basis of a hypothesis for the mechanism of GA inhibition of anthocyanin accumulation by carrot cell suspension cultures during a single growth period. The hypothesis is that when anthocyanin producing carrot cells are cultured in the presence of suitable levels of GA₃ supplied in the medium, a 3'-nucleotidase is rapidly synthesized and this enzyme dephosphorylates malonyl-CoA converting it into an inhibitor of chalcone synthase thus inhibiting the accumulation of anthocyanin by the cells. In addition, the demonstration that growth retardants such as uniconazole which are inhibitors of GA biosynthesis (for review see Rademacher 1991) increase anthocyanin accumulation by the carrot cell cultures (Ilan and Dougall 1992) suggests that endogenous GAs control anthocyanin accumulation by the same mechanism. The hypothesis has several testable predictions. These include:-

- 1) that cells grown in the absence of GA₃ must have little or no 3'-nucleotidase activity.
- 2) that 3'-nucleotidase activity is greatly increased when cells are grown in the presence of GA₃.
- 3) that the level of malonyl-3'-dephospho-CoA is increased when tissue is grown in the presence of GA₃.
- 4) that growth retardants will decrease the 3'-nucleotidase activity in the cells.

- 5) that growth retardants will decrease the malonyl-3-dephospho-CoA in the cells.
- 6) that growth retardants will decrease the endogenous levels of GAs in the tissues.

We have tested four of these predictions, namely the effects of GA₃ and growth retardants on the 3'-nucleotidase and the effects of growth retardants on endogenous GAs. We could detect no substantial effect of GA₃ or growth retardants on 3'-nucleotidase activity in the tissue. Further, growth retardants did not totally inhibit GA accumulation. We conclude that alteration of the level of a 3'-nucleotidase in response to exogenous GAs during a single growth cycle of the carrot cell cultures is not principally responsible for the observed differences in anthocyanin accumulation.

2. MATERIALS AND METHODS

Plant material

Wild carrot (*Daucus carota* spp. *carota*) cell suspension cultures were maintained as previously described (Ilan and Dougall 1992). Sub-clone WC 63.1.9.1.6.1.1.2 ("clone 6") was used in all experiments. In addition subclones WC 63-1-9-1-6-3-3-3 (clone 6-3), WC 63-1-9-1-13-1-1-1-2 (clone 13) and WC-63-1-9-1-15-3-3-2-1-2-1 (clone 15) were used for measurement of endogenous GAs. These clones have been described by Vogelien et al. (1990). Stock cultures of the clones were used to inoculate WCM-4 medium (Dougall and Weyrauch 1980) (1:20 v/v) and these cultures were treated as shown in the results. GA₃ 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 50 or 100 ml medium, respectively.

Anthocyanin and growth measurements

At the end of the growth period, 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 3 Erlenmeyer flasks were used and 3 samples were taken from each flask for dry weight and for anthocyanin measurements. The results were analyzed by nested analysis of variance (SAS/STAT Guide for Personal Computers, Version 6). Data are given as mean $\pm$ standard deviation. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter.

Endogenous gibberellin content

GAs were identified from tissue grown in medium WCM-4 for 12 days from clones 15 and 6. The tissue was recovered by filtration on Miracloth, washed with deionized water, frozen in liquid nitrogen and freeze-dried. Tissue was homogenized in 80% cold MeOH and GAs were extracted and purified using sequential stepwise silicic acid chromatography (Rood et al. 1983) and C₁₈ reversed-phase HPLC (Koshioka et al. 1983). Fractions were grouped, methylated and silylated prior to analysis by gas chromatography-mass spectrometry with selected ion monitoring (Zanewich and Rood 1993). GA identification was based on Kovats Retention Indices (KRIs) (Gaskin et al. 1971) and comparison of ion abundances with authentic GAs.

Tissues for measurement of endogenous GAs were grown in medium WCM-4 for 3, 5 or 12 days. For tissue grown for 12 days, the amounts from each batch were sufficient to permit 3 analyses. For tissue grown for 3 or 5 days, several batches of tissue were combined and then divided into 2 samples for analysis. Endogenous GAs in these samples were extracted, and purified as above and analyzed by GC-MS using (²H₂) GA standards for quantitation (Zanewich and Rood 1993).

Enzyme purification

At the end of the specified growth period, cells for the extraction of nucleotidase were collected by filtration, washed with deionized water and frozen in liquid nitrogen. The frozen cells were kept at -80°C until used.

The procedure used for 3'-nucleotidase purification was based on that described by Hinderer and Seitz (1986). One gram of frozen cells were ground in a mortar and pestle with liquid nitrogen and 300 mg of insoluble polyvinylpyrrolidone (Polyclar AT). The following steps were done at 4°C. Four ml of 100 mM Bistris HCl buffer (pH 8) were added to the ground cells and after thawing, the mixture was centrifuged at 17300 x g for 15 minutes. Two ml of the supernatant was brought to 85% saturation with pulverized ammonium sulfate by stirring for 30 minutes and then centrifuged at 17300 x g for 15 minutes. The supernatant was discarded and the precipitate dissolved in 2.5 ml of 20 mM Tris-HCl buffer at pH 7.5 (buffer C). This entire solution was desalted, according to the manufacturer's directions, on a PD-10 (Pharmacia Biotech Inc., Piscataway, NJ) column containing Sephadex G-25 which was previously equilibrated with buffer C to give 3.0 ml of "Ammonium sulfate/Sephadex treated crude extract" (AS/STCE). Unless an AS/STCE was used immediately, it was frozen in liquid N₂ and stored at -80°C. Two ml of AS/STCE were loaded on a Mono Q column (HR 5/5) in an FPLC system (Pharmacia Biotech Inc., Piscataway, NJ) which was previously equilibrated with buffer C and eluted with buffer C for the first 15 fractions, then by a sodium chloride gradient of 0-0.3 M over fractions 16-45, and 0.3-0.7 M over fractions 46-50. Fractions (58-59) of 2 ml each were collected. The Mono Q fractions were frozen in liquid nitrogen and kept at -80°C until they were assayed. No loss of nucleotidase activity was observed over 3 month following this procedure (data not shown). Protein was measured in the extracts

and Mono Q fractions by the Bicinchoninic acid assay (Sigma Chemical Co., St. Louis, MO). The results were analyzed by "nested analysis of variance" (Luginbuhl and Scholtzhauer 1987). Data are given as mean $\pm$ standard deviation. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter.

Nucleotidase activity measurements

The phosphate released by enzymes was measured as described by Lanzetta et al. (1979). The standard solution of phosphate was from Sigma Chemical Company (St. Louis, MO). Preliminary experiments (data not shown) revealed that this method is more sensitive than the method of Lin and Morales (1977) which was used by Hinderer and Seitz (1986). The enzyme assay mixture consisted of: 700 μ l of 20 mM Bistris HCl (pH 6.5), 100 μ l of substrate (10 mM 3'-AMP or 5'-AMP in 20 mM Bistris HCl buffer, pH 7.0) and enzyme preparation in buffer C to give 1 ml total volume. The volume of enzyme preparations assayed was 100 μ l of Mono Q fraction or 10 μ l AS/STCE. The tubes were incubated at 30°C for 20 minutes. Activity was expressed as nanomoles PO_4 released $\text{ml}^{-1} \cdot \text{min}^{-1}$.

3. RESULTS AND DISCUSSION

Effects of time of GA₃ addition on anthocyanin accumulation.

For the measurement of the levels of 3'-nucleotidase, we attempted to select a harvest time at which GA is still important in controlling the synthesis of anthocyanins and yet significant growth had occurred. To do this we examined the effects of delaying the addition of GA₃ on the amounts of anthocyanin accumulated. Delaying the addition of GA₃ to the medium should decrease the inhibition of anthocyanin accumulation, i.e., increase the anthocyanin accumulation. In this way, the time period during which GA is increasing the 3'-nucleotidase activity in the cells can be identified.

GA₃ at a final concentration of 3×10^{-6} M was added at days 0, 2, 4, 6 and 8 to carrot cell suspension cultures grown on WCM-4. Anthocyanin accumulation and tissue dry weight were measured at these times and at the end of the growth period (day 12). Both the growth and the anthocyanin accumulation in the untreated cultures was linear when the $\log \text{ dry wt } \text{ml}^{-1}$ or $\log \text{ anthocyanin (ml culture)}^{-1}$ was plotted against time but each showed a 2-day lag period (data not shown). Similar observations were reported by Hinderer et al. (1984), Cheng et al. (1985), Dougall and Frazier (1989) and Zwayyed et al. (1991). The results (Figure. 1) showed that anthocyanin accumulation was progressively increased as GA₃ was added later in the growth period. Approximately half of the inhibition of anthocyanin accumulation occurred when GA₃ was added at day 6 compared to the addition at day 12. Day 5 was chosen as harvest time for 3'-nucleotidase

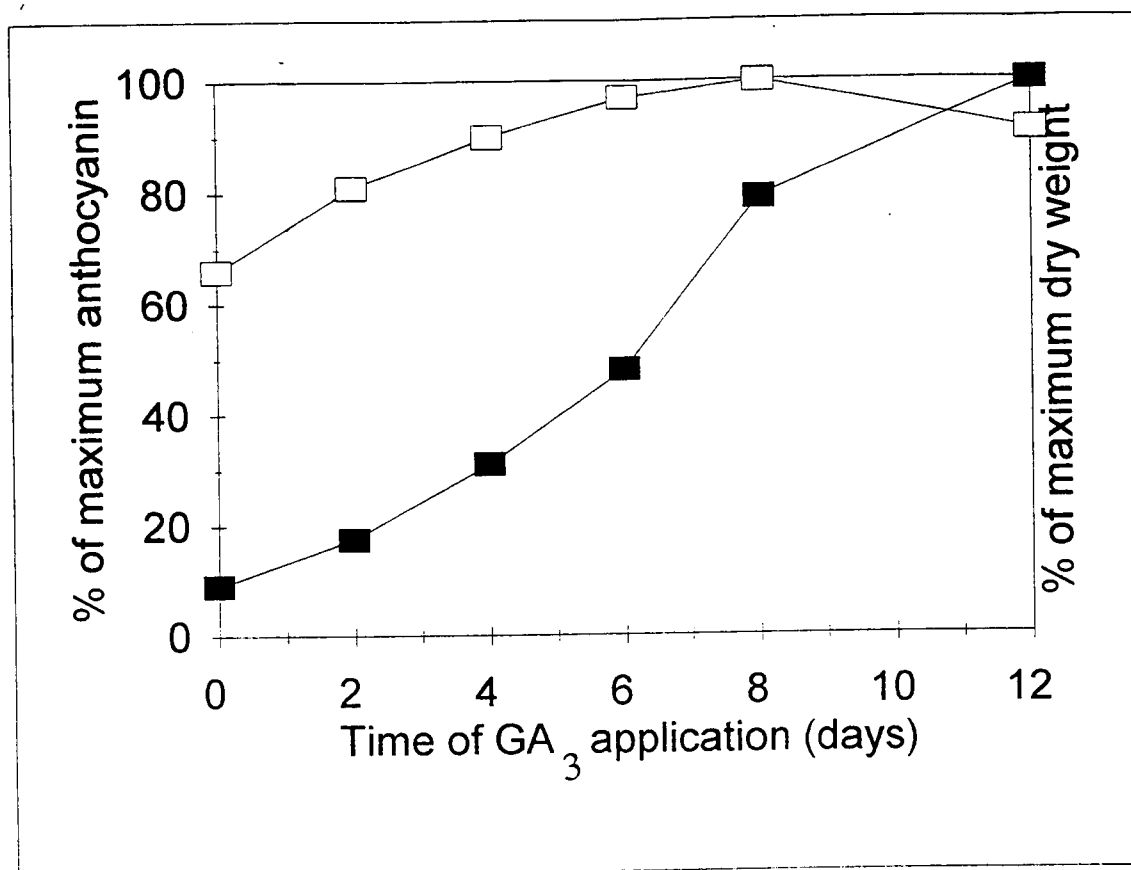


Figure 1: The effect of $3 \times 10^{-6} \text{M}$ GA₃, added to carrot cell suspension cultures of sub-clone 6 at days 0, 2, 4, 6, 8 or 12 of the growth period, on anthocyanin production and growth at 12 days. Anthocyanin is expressed as percentage of the maximum anthocyanin accumulation $(\text{ml culture})^{-1}$ ($A_{530\text{nm}} = 1.86$) recorded when GA₃ was added at day 12. Dry weight is expressed as percentage of the maximum dry weight obtained ($2.95 \text{ mg} \cdot \text{ml}^{-1}$) recorded when GA₃ was added at day 8. Closed boxes - % of maximum anthocyanin, open boxes - % of maximum dry weight.

measurement since at this point in the growth cycle the addition of GA inhibited anthocyanin accumulation by 50% or more (Figure. 1) showing that GA₃ was still exerting much of its effect. At this point, more than 30% of the final dry weight had been accumulated so that adequate biomass for enzyme extraction, etc., was produced.

Endogenous GA content

Although the occurrence of GA_{1,3,4,5,7,8,9,12,15,17,19,20,24,29,34,44,51,53} was specifically investigated, only GA₁ and GA₃ were abundant enough for identification by GC-SIM from carrot cell suspension cultures of clone 15 (Table 1). GC-SIM data also indicated the probable trace occurrence of GA_{8,19,20}. Koshioka et al. (1988) detected GA_{1,3,8,29} in carrot suspension cultures and Noma et al. (1979) identified GA_{1,4,7} in embryonic carrot cell suspension cultures. Thus, the 3,13-dihydroxylated GAs, GA₁ and GA₃ occur in relative abundance in carrot cultures. These GAs are probably physiologically active as in many other plant systems (Graebe 1987).

Application of growth retardants known to inhibit GA biosynthesis have previously been shown to increase anthocyanin accumulation (Ilan and Dougall 1992). In the present study, the application of uniconazole, another inhibitor of GA biosynthesis, to carrot cell suspension cultures was also expected to reduce endogenous GA concentrations. While GA₁ and GA₃ could be detected in the presence of uniconazole, the results of the measurements of endogenous GAs (data not shown) were very variable and could not show whether the increase in

Table 1: The expected and found Kovats retention indices (KRI) and characteristic fragments of GA₁ and GA₃ and their abundances, measured by capillary GC-SIM of methyl ester trimethylsilyl (MeTMSi) derivatives of putative GAs from carrot cell suspensions. Extracts of carrot cells from 12-day old cultures were purified by SiO₂ chromatography and HPLC and analyzed by GC-MS. Selected ion monitoring (SIM) programs were used to detect the abundance of ion fragments of the GAs in the GC-MS fractions. They were compared with the abundance of ion fragments of authentic GAs. KRI of the GAs in GC-MS fractions were compared to KRI of authentic GAs. For additional details see Materials and Methods.

	KRI	Ion fragments characteristic of GA					
		(% abundance relative to molecular ion)					
GA ₁	2661	506(100)	491(12)	448(14)	447(8)	416(11)	390(10)
GA ₃	2687	504(100)	489(10)	445(13)	431(8)	347(23)	208(45)

anthocyanin accumulation was accompanied by a corresponding reduction in the endogenous levels of GAs in the growth retardant treated tissue.

Measurements of nucleotidase activity

The phosphate released from 3'-AMP and from 5'-AMP was proportional to the amount of enzyme in the assay up to 7.5 nanomoles $\text{PO}_4 \cdot \text{min}^{-1}$ and proportional to incubation time up to 20 minutes (data not shown). The pH optimum for the release of phosphate from 3'-AMP was 6.5. At pH 6 and 7, the rate of phosphate released from 3'-AMP was more than 90% of the phosphate released at pH 6.5. Hinderer and Seitz (1986) gave the optimum pH for their 3'-nucleotidase as pH 7. The 3'-nucleotidases from other plant species showed pH optima in the range of 6-8 (for review see Schomburg and Salzmann 1990).

Measurements of nucleotidase activities with 3' and 5' nucleotides and the protein concentrations of the AS/STCE from the control, GA_3 and uniconazole treatments are shown in Table 2. No significant difference was found between the treatments in either 3'- or 5'-nucleotidase activity or in their specific activities.

The partially purified 3'-nucleotidase of Hinderer and Seitz (1986) had no activity with 5'-AMP whereas the AS/STCE from the 3 treatments measured here, all show considerable activity with 5'-AMP. To determine whether the 5'-nucleotidase or other non-specific phosphatases were obscuring a specific 3'-nucleotidase, the AS/STCE was chromatographed on a Mono Q column (FPLC system) and nucleotidase activities and protein were measured in the fractions. The elution profiles of extracts from the different treatments and their replicates

Table 2: 3'- and 5'-nucleotidase activities and protein content of ammonium sulfate/Sephadex-treated crude extracts from control, GA₃-treated and uniconazole-treated tissue. Activity in the extracts is expressed as nanomoles PO₄·ml⁻¹·min⁻¹. If activity in the extracts is multiplied by 4.8, then it is converted into activity gFW⁻¹ (nanomoles PO₄·(g FW)⁻¹·min⁻¹). Specific activity is expressed as nanomoles PO₄·(mg protein)⁻¹·min⁻¹. The data is the mean ± standard deviation of the values determined in enzyme preparations from 3 different batches of 5-day-old cell suspension cultures. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter.

	3'	5'	protein	3'activity	5'activity
Treatment	activity	activity	(mg ml ⁻¹)	mg protein ⁻¹	mg protein ⁻¹
Control	103±10a	65±16a	2.2±0.4	49±10a	30±6a
GA	111±19a	67±19a	2.0±0.5	59±16a	35±9a
Uniconazole	106±20a	81±13a	2.2±0.2	48±7a	36±2a

were similar. Figure 2 shows a typical elution profile.

The major 3'-nucleotidase peak was found between fractions 20-31. Highest 3'-nucleotidase activity was detected in fraction 25 eluted at 100 mM NaCl. The 3'-nucleotidase of Hinderer and Seitz (1986) eluted at a similar NaCl concentration. The main 3'-nucleotidase and 5'-nucleotidase peaks were not completely separated. The 3'- and 5'-nucleotidase activities did not separate when combined peak fractions were chromatographed on Sepharose 12 column (data not shown).

The total 3'-nucleotidase activity/ total protein, obtained by summing the 3' activities and protein in all the fractions from the Mono Q chromatography and calculating the ratio of these for the extracts from the control, GA₃ and uniconazole treatments, were 63 ± 19 , 68 ± 10 and 76 ± 32 nanomoles PO₄·mg protein⁻¹·min⁻¹, respectively. The total 5'-nucleotidase activity/total protein in the fractions from the Mono Q chromatography calculated the same way for the extracts from the control, GA₃ and uniconazole treatments were 24 ± 8 , 32 ± 9 and 27 ± 9 , respectively. These results when compared with those in Table 2 show recoveries for the Mono Q chromatography for the 3'-nucleotidase activity of 108, 86 and 105% respectively, of 5'-nucleotidase of 69, 66 and 50% respectively and for total protein of 81, 70 and 73% respectively, for the three treatments. While there was a loss of approximately 40% of the 5'-nucleotidase and approximately 25% of the protein after chromatography, there was no significant difference between the 3'- or 5'-nucleotidase activities for the three

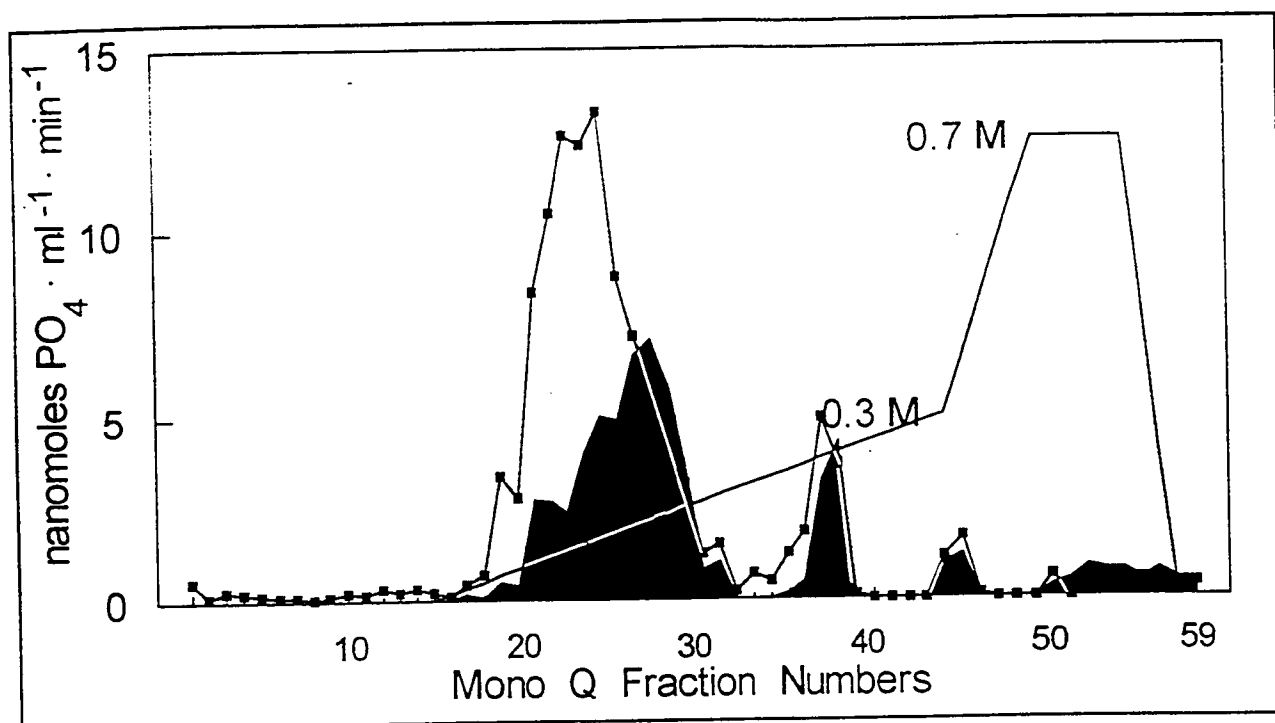


Figure 2: The 3'- and 5'-nucleotidase activities of a carrot cell culture grown in the presence of GA_3 and chromatographed on a Mono-Q column. The ammonium sulfate and Sephadex-treated extract of cells grown for 5 days on WCM4 supplemented with $3 \times 10^{-6} M$ GA_3 was chromatographed. The activities are given as nanomoles $PO_4 \cdot ml^{-1} \cdot min^{-1}$ from 3'- and 5'-AMP for each fraction (2 ml). The symbols are: ■, 3'-nucleotidase activity; shaded area, 5'-nucleotidase activity; —, NaCl gradient.

treatments. In order to adjust the total activities measured in the Mono Q fractions for the different amounts of protein in AS/STCE preparations loaded on the column, the total activities recovered were divided by the AS/STCE protein loaded. These respective values for the control, GA₃ and uniconazole treatments were 54 ± 18 , 53 ± 16 , 52 ± 17 nanomoles PO₄ (mg protein)⁻¹·min⁻¹ for 3'-nucleotidase activity and 21 ± 7 , 25 ± 11 , 33 ± 13 nanomoles PO₄ (mg protein)⁻¹·min⁻¹ for 5'-nucleotidase activity. These values are not significantly different.

In the chromatography of the nucleotidases, the peak of the nucleotidase activity obtained with 5'-AMP is delayed about 3 fractions relative to the peak of activity with 3'-AMP. Further, all fractions of the main peak of 3'-nucleotidase have 5'-nucleotidase activity but the ratio of these activities varied between fractions. This suggests that the 3' and 5'-nucleotidase activities were not fully separated on the Mono Q column. The enzyme activities and protein were summed over the main peak of nucleotidase activity for each chromatography (i.e., over fractions 20 - 31 in Figure. 2). The means and standard deviations of the total and specific activities for 3'- and 5'-nucleotidase over the peak are shown in Table 3. These specific activities are 3 and 2 times higher than those found before the chromatography (Table 2). Again, there were no significant differences in the total activities or the specific activities which associated with the treatments. In particular, these data show no evidence for the presence of a 3'-nucleotidase which is increased in the presence of GA₃.

All the tissue examined in the above experiments had been treated for 5

Table 3: The 3'- and 5'-nucleotidase activities and protein in the main peak of nucleotidase activity (fractions 20-31) from Mono Q chromatography of extracts of carrot cell cultures grown in the absence and the presence of GA or uniconazole. Activity in the extracts is expressed as nanomoles $\text{PO}_4 \cdot \text{ml}^{-1} \cdot \text{min}^{-1}$. If activity in the extracts is multiplied by 4.8, then it is converted into activity $\cdot \text{gFW}^{-1}$ (nanomoles $\text{PO}_4 \cdot (\text{gFW})^{-1} \cdot \text{min}^{-1}$). Specific activity is expressed as nanomoles $\text{PO}_4 \cdot (\text{mg protein})^{-1} \cdot \text{min}^{-1}$. The result for each treatment is the mean $\pm$ standard deviation of the sum of fractions 20 - 31 from the chromatographies of 3 different preparations. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter.

Treatment	3' activity	5' activity	protein ($\text{mg} \cdot \text{ml}^{-1}$)	3'activity $\cdot \text{mg protein}^{-1}$	5'activity $\cdot \text{mg protein}^{-1}$
Control	75 $\pm$ 33a	31 $\pm$ 22a	0.4 $\pm$ 0.1a	168 $\pm$ 35a	72 $\pm$ 39a
GA	70 $\pm$ 26a	30 $\pm$ 12a	0.4 $\pm$ 0.1a	144 $\pm$ 28a	63 $\pm$ 19a
Uniconazole	85 $\pm$ 27a	30 $\pm$ 6a	0.5 $\pm$ 0.1a	192 $\pm$ 91a	65 $\pm$ 24a

days. It was possible that the samples had been taken at a time when there was no difference in the 3'-nucleotidase present in the tissue. To test this possibility, suspension cultures of clone 6 were grown in the presence and the absence of GA₃ for 3, 6 and 9 days and the nucleotidase activities and protein in AS/STCE were measured. The data are in Table 4 and show that considerable 3'-nucleotidase activity was present in the controls at each time. The highest 3'- and 5'-nucleotidase specific activities were in the extracts from the control tissue grown for 3 days and from the GA treated tissue grown for 3 days. GA₃ increased both 3'- and 5'-nucleotidase specific activity relative to the control at each time with the maximum increase in the presence of GA₃ being 50% of the corresponding control value.

The 5'-nucleotidase activity measured in all the purification steps and growth periods was always lower than the 3'-nucleotidase activity. Hinderer and Seitz (1986) did not detect any 5'-nucleotidase activity in Mono Q fractions which demonstrated high 3'-nucleotidase activity.

We could detect considerable levels of 3'-nucleotidase in extracts of non-treated tissue and we could detect no substantial increase in the amount, specific activity or forms of 3'-nucleotidase when GA₃ was provided to the cultures. Therefore, we conclude that the amount of 3'-nucleotidase was not greatly altered by GA₃ and hence, that changes in the amount of 3'-nucleotidase caused by GA₃ may not be responsible for the decrease in anthocyanin accumulation caused by GA₃. Our findings are different from those expected from the hypothesis based

Table 4: The effects of the time of growth of carrot cell suspension cultures in the presence and absence of GA on the nucleotidase activities. Ammonium sulfate/Sephadex-treated crude extracts were prepared from carrot cell suspension cultures (clone 6) grown on WCM-4 with 0 or $3 \times 10^{-6} \text{M}$ GA₃. Activity in the extracts is expressed as nanomoles $\text{PO}_4 \cdot \text{ml}^{-1} \cdot \text{min}^{-1}$. If activity in the extracts is multiplied by 4.8, then it is converted into activity gFW^{-1} (nanomoles $\text{PO}_4(\text{g FW})^{-1} \cdot \text{min}^{-1}$). Specific activity is expressed as nanomoles $\text{PO}_4(\text{mg protein})^{-1} \cdot \text{min}^{-1}$. The values were obtained from two preparations of each treatment and each preparation was assayed in duplicate. Data are given as mean $\pm$ standard deviation. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter.

Treatment	3' activity	5' activity	protein (mg ml^{-1})	3' activity mg protein^{-1}	5' activity mg protein^{-1}
Control - 3 days	84 $\pm$ 8 a	59 $\pm$ 9 a	2.0 $\pm$ 0.3 b	42	30
GA - 3 days	84 $\pm$ 5 a	54 $\pm$ 5 ab	1.6 $\pm$ 0.2 c	53	34
Control - 6 days	65 $\pm$ 6 b	47 $\pm$ 8 b	2.5 $\pm$ 0.2 a	26	19
GA - 6 days	63 $\pm$ 11 b	50 $\pm$ 19 b	1.6 $\pm$ 0.2 c	39	31
Control - 9 days	53 $\pm$ 9 c	40 $\pm$ 14 c	1.8 $\pm$ 0.3bc	29	22
GA - 9 days*	63 $\pm$ 11 b	50 $\pm$ 15 b	1.6 $\pm$ 0.3 c	39	31

*One of the two preparations was made from 0.5 g cells. The values used were the measurements multiplied by 2 to correct for this difference.

on Hinderer and Seitz's (1986) findings, which are that little or no activity of 3'-nucleotidase is present in non-treated cells and that GA₃ greatly increases 3'-nucleotidase activity in carrot cell suspension cultures. The hypothesis may not be valid in either culture systems because it contained untested assumptions or because the changes which occurred over 10 years in the presence of GA₃ to give the line DCw, are not relevant to the effects of GA₃ during one culture passage.

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Zwayyed SK, Frazier GC, Dougall DK (1991) Growth and anthocyanin accumulation in carrot cell suspension cultures growing on fructose, glucose or their mixtures. *Biotechnol Prog* 7: 288-290.

PART 4

**EFFECT OF GIBBERELLIC ACID ON THE ACTIVITIES OF SOME
ENZYMES OF ANTHOCYANIN BIOSYNTHESIS**

Ilan A, Dougall DK (1993) Effect of gibberellic acid on the activities of some enzymes of anthocyanin biosynthesis (a paper submitted to Journal of Plant Growth Regulation, modified for dissertation).

1. INTRODUCTION

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 giving 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. (1993). Considerable activity of 3'-nucleotidase was found in non-treated cell extracts. Only moderate effects of GA_3 and uniconazole, a growth retardant which is known to inhibit GA biosynthesis, on 3'-nucleotidase activity were found. They (Ilan et al. 1993) suggested that alteration of 3'-nucleotidase activity in response to exogenous GAs may not be responsible for the observed differences in anthocyanin accumulation.

In order 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 which restore anthocyanin accumulation will be 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. The activities of acetyl CoA carboxylase, an enzyme which catalyzes the conversion of acetyl

CoA into malonyl CoA and thus is involved in the supply of an important precursor for anthocyanin synthesis, were also examined under the above conditions. Only in CHS was a difference found which correlated with GA₃ inhibition of anthocyanin. This difference was the absence of a peak of CHS activity in extracts from GA₃-treated tissue.

2. MATERIALS AND METHODS

Cultures maintenance

Carrot (*Daucus carota* spp *carota*) cell suspension cultures were maintained as previously described (Ilan and Dougall 1992). Sub-clone WC 63-1-9-1-6-1-1-2 ("clone 6") was used in all experiments. Sub-clone WC-63-1-9-1-15-3-3-2-1-2-1 (clone 15) and sub-clone 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 Weyrauch, 1980) (1:20 v/v) and treated as shown in the results. GA₃ (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 ml or 500 ml Erlenmeyer flasks containing 50 or 100 ml 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, 3 Erlenmeyer flasks were used.

Feeding experiments

Carrot cell suspension cultures were grown for 12 days on WCM-4 in the presence or absence of GA₃ supplied at day 0. GA₃ at a concentration of 10⁻⁶ M was used for feeding experiments with naringenin and dihydroquercetin (DHQ) and at 3x10⁻⁶ M in feeding experiments with coumaric acid and malonic acid.

Naringenin, DHQ, malonic acid and 4-coumaric acid at concentrations of 0.1 mM, 0.25 mM, 0.003 mM and 0.02 mM respectively and 0.5% v/v dimethylsulfoxide (DMSO) were added at days 0, 4 and 8. Clone 6 was used for feeding experiments with malonic acid and coumaric acid and clone 13 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 using the procedure described for 3'-nucleotidase (Ilan et al. 1993). The tissue extract after the first centrifugation is referred to as the non-treated crude extract (NTCE) and the extract after ammonium sulfate precipitation and desalting on a Sephadex column is referred to as the ammonium sulfate and Sephadex treated crude extract (AS/STCE).

Synthesis of 4-coumaroyl CoA

The synthesis of 4-coumaroyl CoA through the hydroxysuccinamide ester of 4-coumaric acid was preformed as described by Stockigt and Zenk (1975) with some modification. 4-Coumaroyl CoA was separated from other components of the reaction mixture by TLC on cellulose plates (250 μ m, Whatman International Ltd., Maidstone, UK) 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 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 and 6 mM NaHCO_3 and 0.48 mM $\text{NaH}^{14}\text{CO}_3$ (52 $\mu\text{Ci}/\mu\text{mole}$) 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 minutes at 30°C. The reactions were terminated by the addition of 4 μl of 6N HCl. 40 μl 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 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 $^{14}\text{CO}_2$ was incorporated into malonyl CoA. A flow rate of 1 ml. min^{-1} was used with a 3.9x300 mm μ -Bondpack C-18 column (Waters, Milford, MA) The elution buffers were 0.05M phosphate buffer at pH 5.0 (Buffer A) and 40% methanol in buffer A (Buffer B). A linear gradient of 5-35% buffer B in A over 35 minutes 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 μ l of 0.05 M Tris-HCl (pH 7.6) and 10 μ l of extracts. The assay mixture for Mono Q fractions contained 890 μ l 0.05 M Tris-HCl (pH 7.6) and 100 μ l of a column fraction. The reaction was initiated by the addition of 10 μ l 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 final volume of 210 μ l, contained 5 μ l (1 nmole) of 4-coumaroyl CoA, 5 μ l (1.87 nmole, 35000 dpm) of [2-¹⁴C]malonyl CoA (Amersham Corporation, Arlington Heights, IL), the amount of enzyme preparation chosen for each assay and buffer (100 mM KH₂PO₄, 1.4 mM β -mercaptoethanol, 1% w/v BSA, pH 7.9) to complete the volume. The assay mixtures were incubated for 30 minutes (unless otherwise stated) at 30°C, the reactions were stopped by an addition of 20 μ l of naringenin solution (1 mg/ml in methanol) and the products were extracted into 250 μ l of ethyl acetate. 200 μ l of the ethyl acetate phase 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 co-migrated with naringenin when chromatographed on Whatman #1 paper using 15% ethanol in water as solvent.

Protein assay

Protein was determined using bicinchoninic acid (BCA) reagent (Sigma Chemical Company, St. Louis, MO). Bovine Serum Albumin solution (Sigma Chemical Company, St. Louis, MO) was used as a standard. Non-treated crude extracts (NTCE) were handled prior to the protein assay in the following way. 10 μ l NTCE were precipitated twice in 0.5 ml of 5% trichloroacetic acid (TCA). The TCA was then removed and 50 μ l of 0.1 N NaOH were added.

Statistical analysis of data

The results were subjected to "nested analysis of variance" (Luginbuhl and Schlotzhauer, 1987). Data are given as mean $\pm$ standard deviation. Within columns, means with different letters differ significantly at 5% by multiple t test.

3. RESULTS AND DISCUSSION

Feeding experiments

Intermediates of anthocyanin biosynthesis were fed to carrot cell suspension cultures in the presence of GA₃ to locate the step of anthocyanin biosynthesis inhibited by GA₃. Naringenin partially restored the levels of anthocyanin while DHQ gave levels of anthocyanin above those of the control (experiment 1, Table 1). Malonic acid was fed in these experiments on the basis of 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, Table 1) nor 4-coumaric acid (experiment 2, Table 1) restored anthocyanin production in the presence of GA₃. The results suggest that anthocyanin accumulation is inhibited by GA₃ 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 GA₃ so that the steps in anthocyanin biosynthesis which may be inhibited by GA₃ are those which are catalyzed by the enzymes chalcone synthase and chalcone isomerase. Another step which was not investigated by the feeding experiments but might be inhibited by GA₃ is the conversion of acetyl CoA into malonyl CoA catalyzed by acetyl CoA carboxylase.

The effects of GA₃ and uniconazole on the measured ACCase activity

The relationship between the amount of enzyme preparation and the CO₂

Table 1: The effect of intermediates in anthocyanin biosynthesis on anthocyanin production and growth of carrot cell suspensions grown in the presence of GA₃ for 12 days. Anthocyanin-(ml culture)⁻¹ is expressed as absorbance at 530 nm. Dry weight is expressed as mg·ml⁻¹. In parenthesis: % of control. Other details in Materials and Methods. Data are given as mean ± standard deviation. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter. DHQ - dihydroquercetin.

TREATMENT	ANTHOCYANIN	DRY WEIGHT
Experiment 1		
Control	4.5±0.2 b (100%)	4.2±0.4 a (100%)
GA ₃	1.2±0.1 d (28%)	4.0±0.4 ab (96%)
GA ₃ + naringenin	2.1±0.2 c (47%)	4.0±0.2 b (95%)
GA ₃ + DHQ	5.2±0.5 a (115%)	3.7±0.2 b (88%)
Experiment 2		
Control	1.6±0.1 a (100%)	2.9±0.1 a (100%)
GA ₃	0.17±0.04 b (11%)	2.0±0.1 b (67%)
GA ₃ + 4-coumarate	0.18±0.03 b (11%)	2.0±0.2 b (70%)
Experiment 3		
Control	1.9±0.2 a (100%)	3.8±0.2 a (100%)
GA ₃	0.15±0.05 b (8%)	2.3±0.3 b (61%)
GA ₃ + malonate	0.16±0.03 b (8%)	2.3±0.3 b (59%)

fixed into acid stable material was linear up to 3.74 picomoles CO_2 fixed $\cdot \text{min}^{-1}$ and the CO_2 fixed was strictly proportional to incubation time up to 30 minutes (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 AS/STCE 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. The first explanation is that the method used for ACCase measurement could not detect the small but sufficient ACCase activity needed. The second explanation is that 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 μmoles 2',4', 4, 6' tetrahydroxychalcone converted $\cdot \text{min}^{-1}$ was linear. The disappearance of 2', 4', 4, 6' tetrahydroxychalcone was strictly proportional to time up to 80 seconds. No effect on CHI activity was detected when 10^{-3} M - 10^{-6} M

Table 2: Acetyl CoA carboxylase activity in carrot cell suspension cultures of clone 6 grown for 5 days on WCM-4 either unsupplemented (control) or supplemented with 3×10^{-6} M GA₃ or 1.7×10^{-6} M uniconazole. ACCase activity in the extracts is expressed as picomoles malonyl CoA $\cdot \text{ml}^{-1} \cdot \text{min}^{-1}$. If activity in the extracts is multiplied by 4.8 then it is converted into activity gFW^{-1} (picomoles malonyl CoA $(\text{gFW})^{-1} \cdot \text{min}^{-1}$). Protein is expressed as $\text{mg} \cdot \text{ml}^{-1}$. Specific activity is expressed as picomoles malonyl CoA $\cdot \text{mg protein}^{-1} \cdot \text{min}^{-1}$. The numbers are the mean $\pm$ standard deviation from 4 measurements on each of three extracts. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter.

TREATMENT	ACTIVITY	PROTEIN	SPECIFIC ACTIVITY
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

GA₃ was added to the assay mixture.

CHI activity was measured in extracts (AS/STCE) from control (untreated) tissue, uniconazole treated and GA₃ treated tissues. The results (Table 3) show that the CHI activity and specific activity in extracts from GA₃-treated tissue were reduced relative to the control. CHI activity was even further reduced in extracts from uniconazole treated tissue, an observation which appears to be inconsistent with GA₃ reducing CHI activity because uniconazole is expected to reduce endogenous GAs. Measurement of CHI activity in extracts from control and GA₃-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 while the specific activity in the extracts from the GA₃-treated tissue decreased with increased time. GA₃ treatment of the tissue decreased the CHI specific activity relative to the control at 6 and 9 days.

Extracts (AS/STCE) of untreated, GA₃-treated and uniconazole-treated cells were chromatographed on a Mono Q column and the CHI activity in the fractions was measured. Figure 1 is a typical profile of CHI activity in Mono Q fractions. Most of the extracts gave 2 peaks of CHI activity. The first peak was at fractions 1-2 and 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 Figure 1, it is possible that 2 forms of CHI are present in carrot cells of the clone 6. Two genes for CHI which could give rise to 2 different forms of CHI were found

Table 3: The chalcone isomerase activity in extracts from control, GA₃ and uniconazole treated carrot cell suspension cultures (clone 6) grown for 5 days on WCM-4. Control = no supplement, GA₃ = 3x10⁻⁶M GA₃, uniconazole = 1.7x10⁻⁶M uniconazole. 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). Protein is expressed as mg ml⁻¹. Specific activity is expressed as micromoles chalcone converted into naringenin mg protein⁻¹ min⁻¹. The numbers are the means of 3 measurements on one preparation from each treatment ± standard deviations. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter.

TREATMENT	ACTIVITY	PROTEIN	SPECIFIC ACTIVITY
Control	216± 3 a	2.76	78±1 a
GA ₃	45±13 b	1.62	28±8 b
Uniconazole	7±0.3 c	2.10	3.5±0.1 c

Table 4: The effect of the growth period on chalcone isomerase activity in extracts from carrot cell suspension cultures in the presence and absence of GA₃. Clone 6 was grown for 3, 6 or 9 days on WCM-4 minus and plus 3x10⁻⁶M GA₃ (GA₃). 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). Protein is expressed as mg ml⁻¹. Specific activity is expressed as micromoles chalcone converted into naringenin mg protein⁻¹·min⁻¹. Parenthesis are the range of 3 measurements from one preparation of each treatment.

TREATMENT	ACTIVITY	PROTEIN	SPECIFIC ACTIVITY
Control-3 days	120 (15)	2.25 (0.07)	53 (7)
GA ₃ - 3 days	79 (13)	1.76 (0.03)	45 (7)
Control-6 days	226 (21)	2.60 (0.42)	87 (8)
GA ₃ - 6 days	46 (1)	1.47 (0.12)	31 (1)
Control-9 days	142 (13)	1.55 (0.21)	92 (8)
GA ₃ - 9 days	26 (3)	1.43 (0.04)	18 (2)

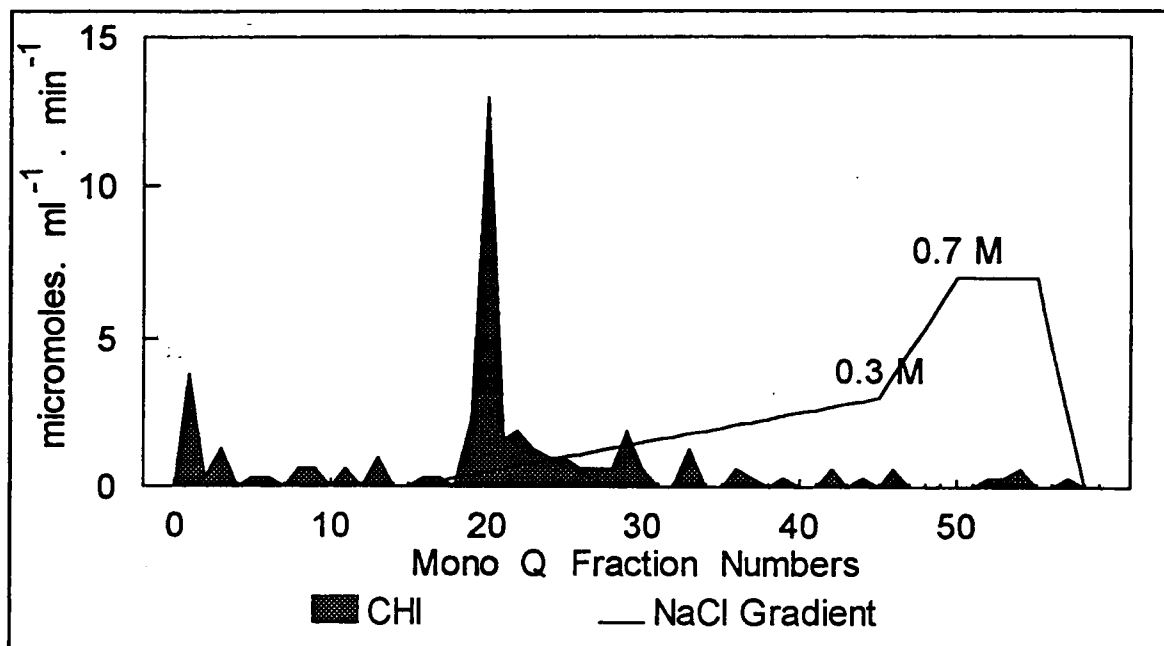


Figure 1: Chalcone isomerase activity in fractions from an ammonium sulfate and Sephadex-treated extract of carrot cell suspension cultures grown for 5 days on WCM-4 (control) and chromatographed on a Mono Q column.

in petunia (van Tunen et al. 1988, van Tunen et al. 1990).

The results (Table 3 and Table 4) showed that CHI activity was reduced in the GA₃-treated extracts and reduced much further 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 GA₃ inhibition of anthocyanin accumulation.

The effect of GA₃ 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 picomoles naringenin. min⁻¹ (data not shown). The amount of naringenin produced was proportional to the time of incubation up to 1 hour. No effect of 10⁻⁴ M - 10⁻⁶ M GA₃ 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 present in the extracts, and of spontaneous isomerization of 2',4',4,6'-tetrahydroxychalcone to naringenin.

The CHS activity of AS/STCE from control, GA₃ and uniconazole-treated tissue (Table 5) show no significant difference between the treatments. In order to determine whether GA₃ 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 3x10⁻⁶M GA₃, and harvested at days 3, 6, and 9 (Table 6). Table 6 show that substantial CHS activity was present in both control

Table 5: Chalcone synthase activity in ammonium sulfate/Sephadex treated crude 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). CHS activity in the extracts is expressed as picomoles naringenin $\cdot ml^{-1} \cdot min^{-1}$. If activity in the extracts is multiplied by 4.8 then it is converted into activity gFW^{-1} (picomoles naringenin $\cdot (gFW)^{-1} \cdot min^{-1}$). Protein is expressed as $mg \cdot ml^{-1}$. Specific activity is expressed as picomoles naringenin $\cdot mg$ protein $^{-1} \cdot min^{-1}$. The numbers are the mean and standard deviation of 2 measurements from 3 preparations of each treatment. Means within a column which did not differ significantly at 5% when compared by multiple t-test, are followed by the same letter.

TREATMENT	ACTIVITY	PROTEIN	SPECIFIC ACTIVITY
Control	5.1 ± 1.7 a	1.8	2.9 ± 0.7 a
GA_3	5.8 ± 2.6 a	2.1	3.1 ± 1.7 a
Uniconazole	7.8 ± 4.1 a	2.3	3.5 ± 1.8 a

Table 6: Effect of growth period and GA₃ on chalcone synthase specific activity. The units of CHS specific activity are picomoles naringenin·mg protein⁻¹·min⁻¹. Cells of clone 6 were grown on WCM-4 or WCM-4 supplemented with 3x10⁻⁶M GA₃, harvested at days 3, 6 and 9 and extracts were prepared. Parenthesis are the range of two measurements from one preparation of each treatment.

DAY	NTCE		AS/STCE	
	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)

NTCE = non-treated crude extract.

AS/STCE = ammonium sulfate precipitated and sephadex-treated crude extract.

tissue and GA₃-treated tissue at the 3 times during the growth. The 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 GA₃ treated tissue. The reduction of the CHS specific activity to 50% of the control value in extracts of GA₃-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 extracts from untreated (control) cells were chromatographed on a Mono Q column, 3 peaks of CHS activity were usually found. These regions of CHS activity were fractions 20-23, 24-30 and 42-47 (Figure 2). One explanation of this finding is that 3 forms of CHS are present in the extracts. The peak found at fractions 42-47 was not present in the control shown in Figure 2 but was present in another control preparation and was present in GA₃ treatment as shown in Figure 2. Thus, this peak is not associated only with the GA₃ treatment. When extracts from GA₃-treated cells were chromatographed on a Mono Q column, 2 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 (Figure 2) which was present in 3 separate control extracts, was not present in the chromatography of any of the 3 independent extracts from GA₃-treated tissues. This suggests either that GA₃ inhibits the synthesis of the CHS form in fractions 20-23, or that GA₃ stimulates the synthesis of a CHS inhibitor which co-

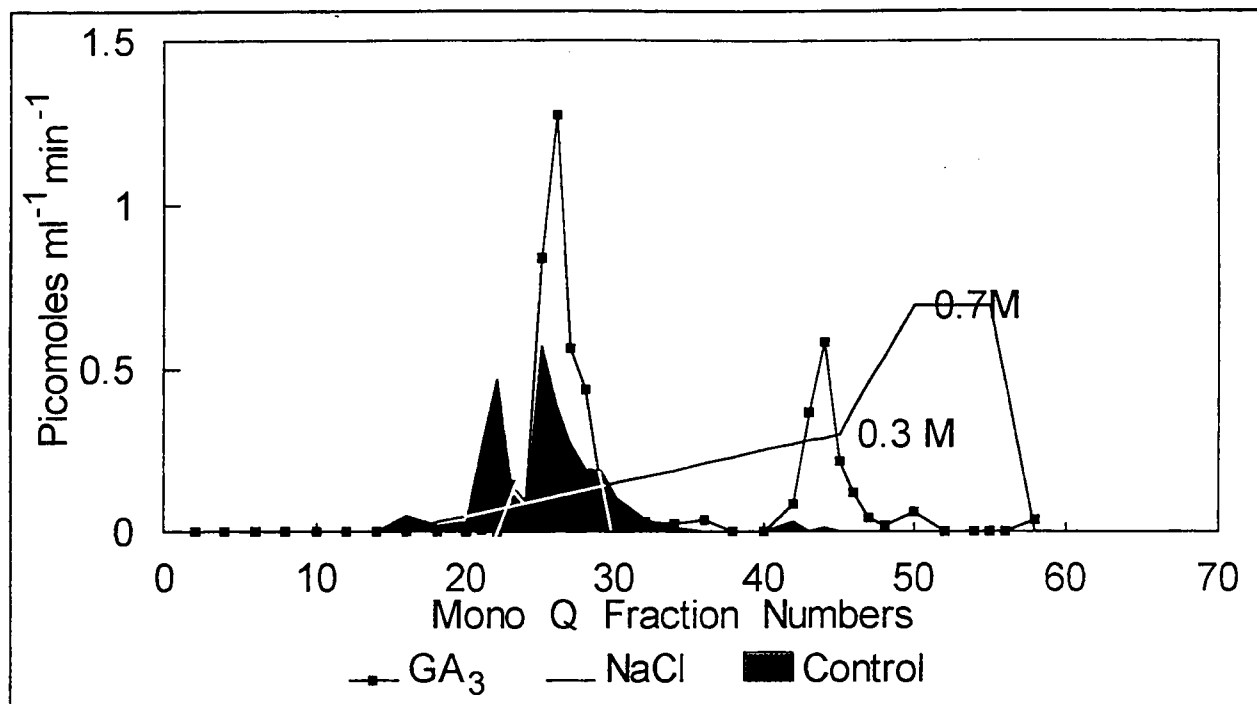


Figure 2: Chalcone synthase activity in fractions from chromatography of extracts from carrot cell suspension cultures on a Mono Q column. The ammonium sulfate and Sephadex treated extracts of cells grown for 5 days on WCM-4, and WCM-4 supplemented with 3×10^{-6} M GA₃ were chromatographed. The activities are given as picomoles naringenin $\cdot$ ml⁻¹ $\cdot$ min⁻¹.

chromatographs 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 while 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 GA₃ and Hinderer et al. (1984) demonstrated CHS activity in crude extracts of GA₃-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) which was derived from DC_b by maintenance on media containing GA₃ 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 GA₃-containing medium while DC_b was maintained in the absence of GA₃. As a result of changes which occurred, DC_w does not produce CHS but does produce 3'-nucleotidase which can cause inhibition of CHS. These changes 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 GA₃ inhibition of anthocyanin production. The lack of CHS activity in fractions 20-23 of extracts from GA₃-treated cells chromatographed on a Mono Q column suggests that this form of CHS is

responsible for anthocyanin biosynthesis and is the site of GA₃ inhibition of anthocyanin synthesis. Further study is required to confirm the GA₃ inhibition of this step and to elucidate the mechanism of GA₃ inhibition. Included in possible mechanisms of GA₃ inhibition is inhibition of the synthesis of a CHS isozyme and synthesis of a high molecular weight inhibitor which co-chromatographs with this CHS form under the conditions used here.

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PART 5

SUMMARY OF PARTS 2-4

1. SUMMARY

The phenomenon of anthocyanin inhibition by exogenously added GA₃ in carrot cell suspension cultures was investigated. This phenomenon was previously described (Cheng et al. 1985, Hinderer et al. 1984). The results of the current study (part II) showed that the addition of different growth retardants, substances which inhibit GA biosynthesis (for review see Rademacher 1991), to carrot cell suspension cultures increased anthocyanin accumulation up to 50%. Paclobutrazol supplied in the presence of 10⁻⁶ M GA₃ reversed the inhibition of anthocyanin caused by GA₃. When the concentration of GA₃ was increased to 10⁻⁵ M in the presence of paclobutrazol, anthocyanin accumulation was reduced to its level in GA₃-treated tissue. Because growth retardants are known to inhibit GA biosynthesis these results suggested that endogenous GAs also inhibit anthocyanin accumulation in the carrot cells, that the accumulation of endogenous GAs is inhibited by paclobutrazol and that exogenously supplied GA₃ can replace the endogenous GAs whose synthesis is inhibited by paclobutrazol. The assumption that endogenous GAs inhibit anthocyanin production was examined (part III of the current study) through the application of growth retardants to carrot cell suspension cultures and the measurement of the levels of endogenous GAs. The results of these measurements were very variable and did not show whether the increase of anthocyanin accumulation was accompanied by a reduction in the levels of endogenous GAs in the growth retardant-treated tissue. It is possible, however, that growth retardants affected an endogenous GA which was not

detected by the methods used but which controls anthocyanin accumulation. If the increase of anthocyanin accumulation in the presence of growth retardants was not due to inhibition of synthesis and decreased levels of endogenous GA, then the growth retardant effects are difficult to explain. For example, there are 2-3 steps in anthocyanin biosynthesis which are catalyzed by P-450-dependent monooxygenases. Inhibition of these enzymes (which is the mechanism of inhibition of GA biosynthesis by some growth retardants) would decrease anthocyanin biosynthesis and presumably accumulation rather than stimulate anthocyanin accumulation. As a general observation, inhibitors can not be regarded as specific. Indicative of the lack of specificity of growth retardants for GA biosynthesis is the reversal of the tetcyclacis inhibition of plant cell cultures growth by sterols (Grossmann et al. 1985) and the lack of ACCase activity extracts of uniconazole-treated carrot cells shown here.

The increase of anthocyanin accumulation in the presence of growth retardants may be the result of increased stability of the anthocyanin produced and not the result of enhanced synthesis. Anthocyanins are not known to accumulate in the cytoplasm and may be degraded unless transported into the vacuole. Various factors can contribute to increased stability of anthocyanins. Among these factors are vacuolar pH (Lancaster 1992), the type and pattern of sugars added to the anthocyanidin, acylation at the sugar residue (Hopp and Seitz 1987, Lancaster 1992) and the rate of transport of anthocyanin to the vacuole. Growth retardants may have increased anthocyanin stability through their effect

on these factors. The likelihood of this situation is, however, limited in view of Dougall and Vogeliën's (1987) demonstration that when anthocyanin synthesis in carrot cell suspension cultures was blocked by the addition of 2-amino-oxyphenylpropionic acid or phenylpropionic to the medium, at concentrations which did not alter the growth, the level of anthocyanin remain unchanged. These findings indicated that differences in anthocyanin levels between different cultures are more likely to be due to differences in rates of synthesis or accumulation rather than due to differences in anthocyanin stability.

A model, based on the findings of Hinderer and Seitz (1986), was proposed to explain the inhibition of anthocyanin accumulation by GA_3 . According to this model GA_3 greatly increases the amount of the enzyme 3'-nucleotidase which converted malonyl CoA into 3'-dephosphomalonyl CoA. The increased levels of 3'-dephosphomalonyl CoA inhibit the enzyme chalcone synthase, a key enzyme of anthocyanin biosynthesis. As a result, anthocyanin production is inhibited. However, Hinderer and Seitz never measured the endogenous levels of 3'-dephosphomalonyl CoA and this model remained unsubstantiated. If this model is valid, then greatly increased amounts of 3'-nucleotidase are expected in GA_3 -treated tissue. The current research (part III) showed no significant difference in 3'-nucleotidase activity measured *in vitro* in Sephadex-treated extracts and fractions prepared from GA_3 -treated cells and non-treated control tissue grown for 5 days and less than 50% increase over control levels when compared at different growth periods in a second series of cultures. The 3'-nucleotidase activity

measured *in vitro* was proportional to the amount of protein in the assay and is, therefore, likely to be proportional to the amount of enzyme present in the tissue. The above findings reduced the likelihood that this model is correct. Demonstration that in the presence of GA₃ endogenous levels of 3'-dephosphomalonyl CoA in the tissue are increased relative to the control is essential as the ultimate test of this model.

Because the 3'-nucleotidase model based on Hinderer and Seitz's (1986) work appears to be an unlikely explanation to the inhibition of anthocyanin accumulation by GA, the following questions were raised again. What step in anthocyanin biosynthesis is inhibited by GA₃ and what is the mechanism of this inhibition? In order to locate potential steps inhibited by GA₃, intermediates of anthocyanin biosynthesis were fed in the presence of GA₃ to carrot cell suspension cultures. Naringenin and dihydroquercetin reversed the inhibition of anthocyanin accumulation caused by GA₃ and so are expected to be located after the inhibited step. Malonic acid was fed in this experiment on the basis of the assumption that it would be converted into malonyl CoA. Conversion of malonic acid into malonyl CoA may occur in plants (Stumpf 1976). Malonic acid and 4-coumaric acid did not reverse the inhibition of anthocyanin accumulation by GA₃. One plausible explanation of this finding is that these compounds are located prior to the inhibited step. Cheng et al. (1985) did not detect any reduction in 4-coumarate CoA ligase, the enzyme which catalyzes the conversion of 4-coumaric acid into 4-coumaroyl CoA, in GA₃ treated carrot suspension cultures. This rules out this

enzyme as the one inhibited by GA. The activities of enzymes catalyzing the steps in anthocyanin biosynthesis, which are located between malonyl CoA, 4-coumaroyl CoA and naringenin i.e. chalcone isomerase and chalcone synthase were compared in GA₃-treated and non-treated control tissue (part IV). The activities of acetyl CoA carboxylase (ACCase) which catalyze the conversion of acetyl CoA into malonyl CoA, a major precursor for anthocyanin biosynthesis, were also measured.

ACCase specific activity was not altered in extracts from GA₃-treated cells, suggesting that the levels of ACCase in the tissue was not altered by GA₃. No ACCase activity was detected in extracts of uniconazole-treated tissue, showing that uniconazole inhibits functions of cells other than GA biosynthesis. The lack of ACCase activity in tissue grown for one passage on a medium containing uniconazole, which was accompanied by normal cell growth and enhanced anthocyanin production, indicates the existence of another source for malonyl CoA in this tissue. Residual amounts of cellular malonyl CoA from the previous passage of cultivation on a medium without uniconazole is a potential source. Another potential source for malonyl CoA in the tissue is the supply of malonyl CoA by a by-pass of the ACCase. Oxaloacetate can be decarboxylated to give malonic acid in a reaction catalyzed by peroxidase and CoA may be added to malonic acid to give malonyl CoA (Stumpf 1976).

CHI activity in extracts of GA₃-treated tissue was reduced compared to extracts of non-treated tissue (part IV). CHI activity was further reduced in

uniconazole-treated tissue. This reduced CHI 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 likely to be the site of the GA₃ inhibition of anthocyanin accumulation.

The total CHS activity was not altered significantly in extracts from GA₃-treated tissue compared with extracts of non-treated tissue except at 9 days of treatment (part IV). Nevertheless, different patterns of CHS activities were obtained when these extracts were chromatographed on a Mono Q column. Three peaks of CHS activity were detected in the chromatography of the control extracts, while only two of these peaks were found in the chromatography of extracts from GA₃-treated cells. These observations make it unlikely that the difference in patterns found is due to chromatographic artifact. The different numbers of CHS forms in the extracts of the control and extracts of GA₃-treated cells may be explained in the following ways.

- 1) GA₃ inhibited the synthesis of one CHS isozyme which is responsible for anthocyanin synthesis.
- 2) GA₃ inhibited a post-translational modification in one of the CHS forms, resulting in two CHS forms instead of the three forms found in the control.
- 3) A high molecular weight inhibitor, stimulated by GA₃, co-chromatographed with and inhibited the specific isoform of CHS which is responsible for anthocyanin biosynthesis.

- 4) The activity of a specific protease was altered (either enhanced or inhibited) in the presence of GA₃. As a result, one CHS isoform was destroyed or, if the protease activity was inhibited, the isoform was not converted into two enzymatically active forms.
- 5) Proteolysis in post extract sample yielded two or three apparent enzymatic forms.

An initial step to establish the different CHS peaks found as forms would be the demonstration that each of the peaks of CHS activity found retains its chromatographic behavior when re-chromatographed under the same conditions. A second step would be to demonstrate the presence of these forms by additional methods such as isoelectric focusing. Subsequent steps would include examining the possibility that these different forms originate from different genes.

Distinct forms of CHS in carrot cells, which are regulated differently were shown by Gleitz and Seitz (1989) who demonstrated the presence of the two forms of CHS by isoelectric focusing and SDS-PAGE in UV irradiated cells of anthocyanin accumulating cell line. One form was not detected in non-irradiated anthocyanin accumulating cultures, or in a non-accumulating cell line with or without UV irradiation. The two forms were also detected in anthocyanin-accumulating carrot purple petals but only one was detected in non-accumulating carrot leaves. Gleitz and Seitz (1989) suggested a correlation between the presence of the one form and anthocyanin accumulation. Two different forms of CHS were detected in spinach (Beerhues and Wiermann 1984). These forms had

the same subunit molecular weight but differed in their apparent native molecular weights and in their chromatography on DEAE cellulose (Beerhues and Wiermann 1988).

The suggestion that CHS is the enzyme regulated by GA₃ is consistent with CHS being a regulated enzyme in flavonoid biosynthesis. CHS is regulated by developmental (i.e. internal) and environmental (i.e. external) factors primarily at the transcriptional level (Martin 1993). It is also regulated at the translational level and by turnover. No evidence was found for post-translational modification of CHS (for review see Martin 1993).

Based on previous investigations and on the current research, the following model can explain GA₃ inhibition of anthocyanin production. GA₃, either directly or through a bioconversion into another GA, is bound to a specific cellular receptor and alters it. As a result, the transcription of a specific CHS gene is altered and a specific isoform of CHS is missing. This isoform is the one responsible for most or all of the anthocyanin production.

The presence of receptors for GA₃ on the plasma membrane was suggested by Hooley et al. (1990). They used anti-idiotypes for GA₃ and demonstrated their attachment to specific regions on the surface of oat aleurone protoplasts.

One potential mechanism for the control of transcription which is mediated by the action of GA may be the activation of DNA binding proteins (DBPs). Ou Lee et al. (1988) demonstrated the presence of GA₃-induced DBPs and identified

a specific region in the promoter for rice α -amylase gene which binds proteins induced by GA₃ in rice aleurone tissue. Rushton et al. (1992) showed that specific aleurone nuclear proteins bind to elements of the promoter region of α -amylase genes from wheat. The idea of different isozymes which are either responsive or non-responsive to GA₃ was demonstrated by Wolf (1992). He showed that one isozyme of barley β -glucanase was responsive to GA₃ while the other was not. The region of the β -glucanase gene which was required for the GA₃ effect was a specific region in the promoter.

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VITA

Avihai Ilan was born in Kibbutz Rosh Hanikra, Israel on February 17, 1952. He attended Rosh Hanikra Elementary School and graduated from Sulam Tsor Regional High School in June of 1970. The following December he was drafted to the Israeli army and served until April 1974. From 1974 until 1976 he was employed in a subtropical fruit nursery and in "Rahan Meristem" plant micropropagation laboratory at Rosh Hanikra. In November 1976 he entered the Faculty of Agriculture of the Hebrew University of Jerusalem and in 1981 received a Bachelor of Science degree in Horticulture. In 1981 he began working as a Manager of Production in the micropropagation laboratory of "Rahan Meristem." He worked in "Rahan Meristem" until he was elected for Secretary of Kibbutz Rosh Hanikra, a key leadership position in the Kibbutz society. Between 1986 and 1988 he was employed as a Scientific Director of a Research and Development project in Rosh Hanikra. In 1989 and 1990 he developed and directed the Department of Research and Development in "Rahan Meristem." During the years 1981-1982 and 1984-1990 he studied toward a Master degree in the departments of Genetics and Botany at the Faculty of Agriculture, the Hebrew University of Jerusalem, under the guidance of Professor Meira Ziv and Professor Abraham Halevy. At June 1991 he was awarded a Master of Science degree (in excellence). In August of 1990 he began his doctoral studies in the Department of Botany at the University of Tennessee with Dr. Donald K. Dougall serving as a mentor. A Doctor of Philosophy degree was awarded in May of 1994.