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# ASSESSMENT OF CELL PHENOTYPE WITH RESPECT TO ANTHOCYANIN ACCUMULATION IN CULTURES OF WILD CARROT BASED ON THE ABILITY TO USE INTERMEDIATES OF ANTHOCYANIN BIOSYNTHESIS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Dale Lynn Vogelien

December 1987

## ACKNOWLEDGMENTS

In the course of my thesis research a number of people have assisted me generously. In particular I am extremely indebted to Dr. Donald K. Dougall, who served as my Mentor. His encouragement, guidance and criticism has helped me to become the researcher that I am and hoped to be. Special thanks to my family and friends, especially to Tom Warne, Julie Dunn, and Gayle Darugh, for giving me support and encouragement throughout my graduate career at the University of Tennessee.

ABSTRACT

Information from previous studies suggests the existence of two classes of cells in wild carrot cultures, one accumulating and the other non-accumulating for anthocyanin, which are potentially reversible. The nature of the block in the low or non-anthocyanin accumulating phenotype is not known. The objective of this study was to further examine high- and low-accumulating wild carrot clones and subclones and determine the general location of the block in anthocyanin biosynthesis in the low-accumulating cultures. Preliminary experiments involved feeding coumarate, naringenin and dihydroquercetin (representing an early, middle and late intermediate, respectively, in anthocyanin biosynthesis) to the parental wild carrot line to determine the optimum concentration of each precursor that can be supplied to cultures and to confirm the ability of each precursor to result in a stimulation in anthocyanin accumulation. High- and low-yielding clones and subclones were then examined for their responsiveness to these intermediates. In general, low-accumulating clones/subclones with extremely low anthocyanin levels were nonresponsive to all three precursor feedings, while clones/subclones with the accumulating phenotype were able to respond to

all three anthocyanin precursors. The level of response in anthocyanin content due to precursor feeding appears to be related to the initial anthocyanin content of a culture, with those clones possessing the higher anthocyanin content displaying the greatest increase in anthocyanin in response to intermediates. In addition, microscopic examination of several high- and low-accumulating subclones revealed that they consist of a mixture of red (accumulating) and white (non-accumulating) cells and indicated a strong correlation between anthocyanin content and the % red or accumulating cells in the population. Together these observations suggest that the level of response to precursor feedings is, as least in part, related to the % of red cells, or accumulating cells, in the population. It was concluded that the impediment in anthocyanin biosynthesis in the non-accumulating phenotype is located at a point beyond dihydroquercetin.

TABLE OF CONTENTS

# LIST OF TABLES

# LIST OF FIGURES

# I. INTRODUCTION

Many secondary plant products are of sufficient economic, medicinal, or aesthetic value to warrant research consideration. Although naturally occurring secondary plant products are generally associated with specific plant tissues at a certain stage of plant development, it is possible to produce many natural products in dedifferentiated cultured plant cells. Consequently, there have been numerous investigations examining the ability of unorganized plant tissue cultures to produce useful chemicals. Available data indicate that plant cell cultures from the same species differ in their capacity to produce compounds. In addition, most selected high-yielding cell lines and clones tend to gradually decrease their ability to produce the compound upon prolonged maintenance by serial passage (Deus-Neumann and Zenk, 1984; Yamada and Hashimoto, 1984).

The use of plant cell cultures as a practical source of chemicals depends heavily on the yield of that chemical. Thus, its not surprising that much effort has been spent in attempts to improve yields. One approach taken to obtain this goal involves manipulating culture conditions to achieve an "optimal" culture environment.

A second approach includes selecting cultures, parts of a culture, and single cell clones that give yields which are considerably higher than the parent culture. Attempts to increase chemical production in plant cell cultures have been successful. However, researchers are still confronted with the problem of variability in yields of chemicals. It would be useful to understand the mechanism(s) involved in this phenomenon, for understanding the mechanism(s) involved might eventually lead to its control and thus result in stable high yields of desired chemicals.

The simplest explanation for the instability associated with production of chemicals in plant cell cultures is that two cell phenotypes exist in culture, one accumulating and the other non-accumulating for a chemical. Further, these two phenotypes are potentially reversible, and the reversal process occurs at a moderate frequency (Meins, 1983). At this point, two hypotheses are possible. First, the two states, accumulating and non-accumulating, are precisely interconvertible and switch from one to the other. All accumulating cells have the same capacity for producing the compound, and yield would be directly proportional to the % of producing cells in a population. An alternative hypothesis is that the interconversion

between these two states is not precise and leads to cells with different capacities to accumulate the compound. In this case, yield would be determined primarily by the capacities of cells in the population to accumulate the compound. Understanding the instability associated with the production of a secondary compound in cultured cells will involve locating possible metabolic differences between accumulating and non-accumulating phenotypes as well as determining which of these hypotheses exists in the cultured system.

## II. STATEMENT OF THE PROBLEM

Variability in anthocyanin production has been reported in cultures of wild carrot (Daucus carota) (Dougall et al., 1980). Cell populations derived from single cell clones have been isolated from a "parent" cell suspension culture of wild carrot. These clones differ greatly in their accumulation of anthocyanin when measured under standard conditions (Dougall et al., 1980). Recloning of a high-accumulating clone resulted in a wide range of anthocyanin accumulating subclones. Selection and recloning of the highest-accumulating subclone gave similar results. In each of three such selections and reclonings the highest-yielding subclone accumulated similar levels of anthocyanin, suggesting the ability to accumulate anthocyanin has an upper limit. In addition, high-yielding clones showed a decline in anthocyanin accumulation during maintenance by serial passage. Selection and recloning of wild carrot clones with the lowest anthocyanin levels also resulted in a range of accumulating subclones. Subclones were obtained that had anthocyanin levels five times that measured for the clone at the time of its selection. Yet these levels were far below the maximum level obtained by serial selection and recloning of high-accumulating subclones.

This information suggests the existence of two classes of cells in wild carrot cultures, one accumulating and the other non-accumulating for anthocyanin, which are potentially reversible. This study was undertaken to further examine high- and low-accumulating wild carrot clones and subclones and gain some insight as to the location of the obstruction in anthocyanin metabolism in the low-accumulating cultures. The aim was to characterize the different culture phenotypes by examining the ability of metabolic intermediates of anthocyanin biosynthesis to increase anthocyanin accumulation by clones and subclones. The precursors used were 4-coumaric acid, naringenin, and dihydroquercetin, representing early, middle, and late intermediates, respectively. Clones were then classified by their responsiveness to these intermediates.

Serial clonal analysis is an available methodology that can also be used in the evaluation of different phenotypes. Information from this study in conjunction with a serial clonal analysis will aid in determining if more than one accumulating phenotype exists and if the interconversion between accumulating and non-accumulating phenotypes is precise. In addition, this study begins the process of examining

the metabolic difference(s) between accumulating and non-accumulating cultures and will give insight as to the location of the impediment in the pathway of anthocyanin biosynthesis in the non-accumulating phenotype. These questions must be answered in the attempt to determine the basis of the change of phenotype in cultured wild carrot cells, be it genetic, biochemical, or physiological.

# III. REVIEW OF LITERATURE

## Production of Chemicals in Plant Cell Cultures

Higher plants are important sources of natural products. The production of secondary compounds in nature is usually associated with a particular plant tissue during a specific stage of development. However, the cells of a plant are totipotent and thus, with some manipulation, it is possible to produce some secondary metabolites in morphologically dedifferentiated cultured plant cells. There have been numerous reports citing the successful establishment of cell cultures capable of producing secondary metabolites as well as discussing selection methods and culture conditions which promote the formation of secondary products. These works have been reviewed by Tabata (1977), Dougall (1981), Deus and Zenk (1982), and Yamada and Hashimoto (1984).

There is a high level of phenotypic variability associated with the production of secondary metabolites in cultured plant cells. This variability includes both qualitative and quantitative differences in production. Variation in plant cell culture production has been reported among cultures established from individual plants of a species, among cultures obtained from a

single plant and within individual cultures (Dougall, 1985; Yamada and Hashimoto, 1984).

There have been a number of high-yielding cultures established, some of which are capable of accumulating secondary metabolites at levels comparable to or higher than those found in intact plants (Deus and Zenk, 1982; Sato and Yamada, 1984; Dougall, 1985). Some high-yielding cell lines or clones are stable in their yields of secondary products, however there are many cases where the level of production decreases upon maintenance by serial passage. These reports have been assembled and discussed by Deus-Neumann and Zenk (1984) and Yamada and Hashimoto (1984).

Dougall et al. (1980) isolated single cell clones of wild carrot (Daucus carota) which differed forty-fold in the level of anthocyanin produced when measured under standard conditions. After maintenance by serial passage both high- and low-yielding clones were recloned and found to have become heterogenous populations. Low-yielding clones produced subclones with anthocyanin levels greater than the initial value measured at the time of selection, whereas high-yielding clones produced subclones with reduced levels of anthocyanin. Although the level of accumulated anthocyanin was found to decrease during maintenance by serial passage, high-

yielding lines with anthocyanin levels comparable to those of the original clone prior to subsequent culturing were recovered through recloning. Thus, the ability to accumulate anthocyanin is both heritable and reversible.

This phenomenon is not unique to wild carrot. Deus-Neumann and Zenk (1984) report similar findings in cell suspension cultures of Catharanthus roseus accumulating alkaloids. A decrease in alkaloid production occurred during the first few months in culture, but high-yielding cell lines were recovered through clonal selection.

A major source of phenotypic diversity in plant cultures is likely to be the selection process (Meins, 1983). Cell cultures are generally initiated from a tissue explant consisting of cells at different phases of the cell cycle, differentiation stages, size, and ploidy levels. Cell lines exhibiting different spectra of secondary metabolites may reflect the diversity of the cells in the population. Variation from these sources can be eliminated by using cloned lines.

## Possible Mechanism(s) of Control

The phenotypic variation seen in plant cell cultures with respect to the production of secondary

metabolites could be the result of genetic mutations and/or epigenetic changes. A genetic mutation implies that a random permanent alteration in the genome has occurred. An epigenetic change is a heritable, directed, potentially reversible event that does not result in any permanent changes in the cells' genome (Meins, 1983). Unlike genetic mutations, epigenetic changes are not transmitted through meiosis. The mechanism(s) responsible for variation and reduced production of secondary metabolites in plant cell cultures is not known.

Gene mutation seems a doubtful explanation for unstable yields of secondary metabolites from plant cell cultures. Deus and Zenk (1982) reported that the capability for alkaloid production was negatively correlated with chromosome number in Catharanthus roseus cells. Low alkaloid producing strains showed chromosome duplication and aneuploidy, whereas high alkaloid producing strains had a higher frequency of the 2n chromsome number. However, recovery of high alkaloid yielding cell lines from these same cultures was possible by clonal selection after maintenance for eight years in culture (Deus-Neumann and Zenk, 1984). Further, changes in the ability to accumulate compounds are reversible and can to some extent, be

affected by medium composition (Dougall et al., 1980; Deus-Neumann and Zenk, 1984; Yamada and Hashimoto, 1984).

Since the quantitative changes in culture phenotype are both heritable and reversible it is possible that gene amplification is involved. Ribosomal DNA amplification is known to occur in plants (Long and Dawid, 1980). More recently, induced gene amplification has been demonstrated in alfalfa selected for resistance to L-phosphinothricin (Donn et al., 1984). Extra chromosomal DNA has been characterized in pea plants (Pisum sativum) grown without any apparent selective pressure (Krimer and van't Hof, 1982; van't Hof et al., 1983), however these fragments have not been shown to be the result of gene amplification. Anthocyanin is the product of a multienzyme pathway, and it seems very unlikely that amplification of all the genes coding for these enzymes would occur. However, increased levels of a rate-limiting enzyme in the pathway would result in increased production of a chemical if all the other enzymes in the pathway are already present or if they were co-induced.

Transposable elements were first recognized in maize but have since been found in a number of plants. Bonas et al. (1984) report that a 17 kb Tam 1 element is

responsible for unstable mutations at the nivea locus in Antirrhinum majus which codes for chalcone synthase, a key enzyme in anthocyanin biosynthesis. The element induced mutation results in a varigated phenotype in flowers and occurs in leaves as well. Excision of transposable elements is usually imprecise and results in additions, deletions or inversions at the site of integration (Bonas et al., 1984; Saedler and Nevers, 1985; Schwarz-Sommer et al., 1985). Excision of the Ac-9 element in Zea mays leaves a 6 base pair duplication at the site of integration (Pohlmann et al., 1984). Thus, insertion and subsequent excision of transposable elements may result in structurally altered genes and gene products.

Plant DNA is highly methylated but the significance of this has not been confirmed. Amasino et al. (1984) showed that phenotypic variation of an octopine-type crown gall tumor was associated with the supression of T-DNA gene expression caused by hypermethylation of T-DNA sequences. Both the unorganized tumor line and the shoot-forming variants (teratomas) contain full copies of the auxin and cytokinin T-DNA loci but differ in the T-DNA transcripts expressed. Cultured tissues from normal plants regenerated from the shoot-forming variants similarly

required phytohormones for growth in vitro, but treatment with 5-azacytidine, an inhibitor of DNA methylation, resulted in T-DNA transcription and phytohormone-independent growth. Further, the transcriptionally silent T-DNA in the shoot forming variants was highly methylated at MspI and HpaII sites, whereas the T-DNA that was re-expressed after 5-azacytidine treatment had a reduced level of methylation. A similar approach was taken to study the phenotypic variation associated with opine production in subclones established from a shooty tobacco crown gall tumor clone (Peerbolte et al., 1986). Here also inactivation of T-DNA gene expression was associated with DNA methylation. Three forms of the maize controlling element activator, Ac, have been isolated (active, inactive, active revertant) that appear to differ only in the degree of DNA methylation (Schwartz and Dennis, 1986). The inactive form is highly methylated in comparison to the active Ac element, whereas the active revertants show only partial demethylation.

## Anthocyanin Biochemistry

In the past ten years much progress has been made in the study of flavonoid biosynthesis. The sequence of reactions and many of the enzymes involved

in anthocyanin biosynthesis in plant cells have been identified. These works have been assembled and thoroughly reviewed by Hahlbrock and Grisebach (1975, 1979), Hahlbrock (1981), and Grisebach (1982). Common steps in the biosynthesis of all flavonoids are shown in Fig. 1 (Hahlbrock, 1981). The proposed sequence of reactions involved in anthocyanin biosyntheses are shown in Fig.2 (Hahlbrock, 1981; Grisebach, 1982). Cyanidin 3-(sinapoylxylosylglucosylgalactoside) has been identified as the major anthocyanin in wild carrot cultures (Harborne et al., 1983).

The method of conversion of dihydroquercetin to anthocyanin is still unknown. Putative intermediates between dihydroquercetin and cyanidin are flavan-3,4-diols, commonly refered to as leucocyanidins. The conversion of dihydroquercetin into leucocyanidin has been demonstrated in maturing barley seeds (Kristiansen, 1984). The same study proposed a biosynthetic pathway leading from dihydroquercetin to catechin and procyanidins (leucocyanidin dimers and oligomers). The enzymatic conversion of dihydroquercetin to leucocyanidin has also been demonstrated in cell cultures of Douglas fir needles (Stafford and Lester, 1982) and in flowers of Matthiola incana (Heller et al., 1985). Although the conversion of dihydroquercetin to leucocyanidin has not

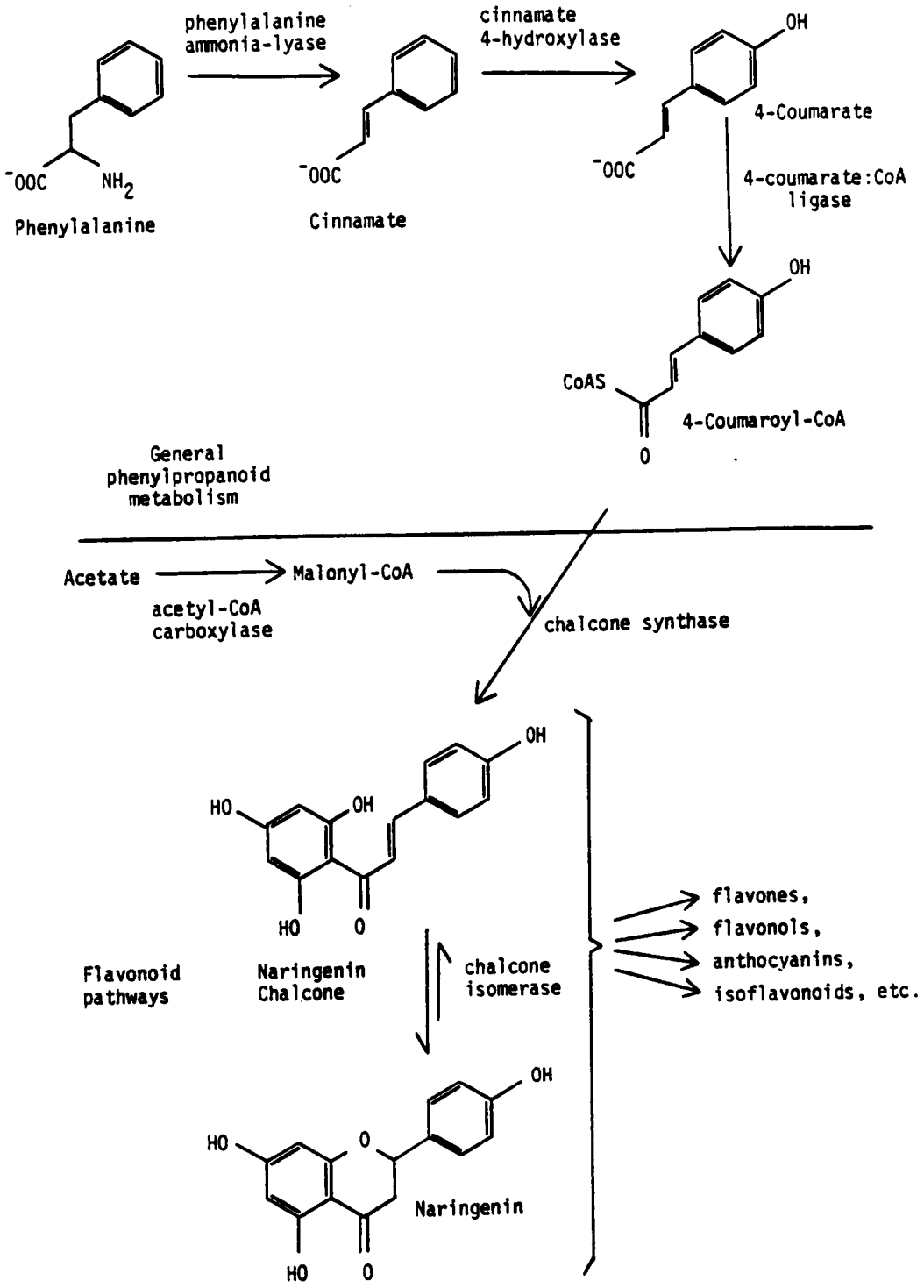



Figure 1. Common steps in the biosynthesis of all flavonoids (Hahlbrock, 1981).

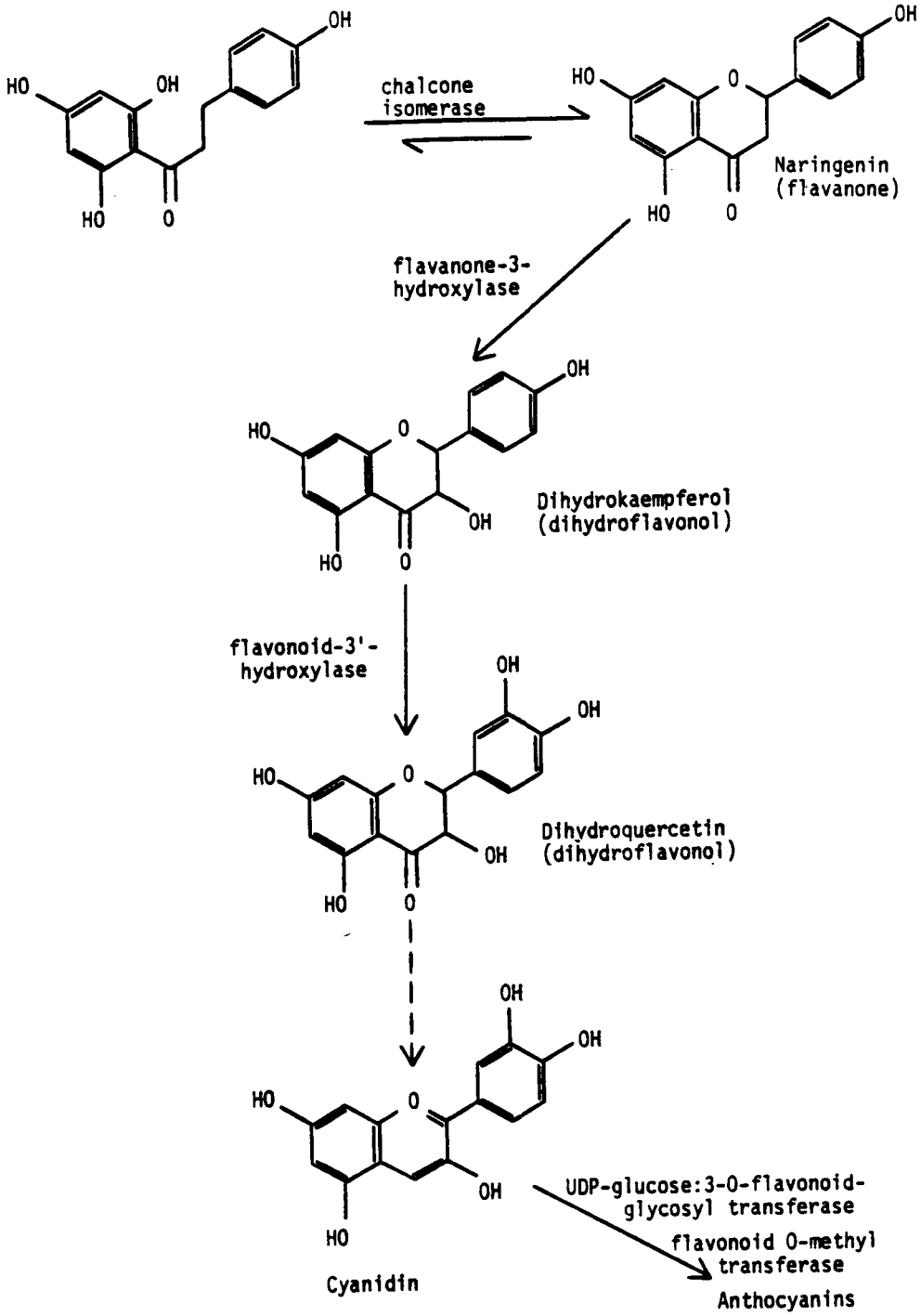



Figure 2. Proposed sequence of reactions involved in anthocyanin biosynthesis (Hahlbrock, 1981: Grisebach, 1982).

been found in wild carrot, these results are good indications for its possible role as a step in anthocyanin biosynthesis. The proposed biosynthetic pathway leading from dihydroquercetin to cyanidin, catechin, and procyanidins is shown in Fig. 3 (Kristiansen, 1984).

Heinzmann et al. (1977) examined 4-coumarate: CoA ligase in anthocyanin-accumulating cultures of wild carrot and those in which anthocyanin accumulation had been suppressed by treatment with gibberellic acid. They suggested that isozymes of the ligase may be involved in the control of anthocyanin biosynthesis. This enzyme is the last step of general phenylpropanoid metabolism and is the branch point leading to flavonoid and lignin biosynthesis (Grisebach, 1981). Cheng et al. (1985) found no differences in the 4-coumarate: CoA ligase from high and low anthocyanin accumulating subclones of wild carrot. Further, no evidence was found for the presence of isozymes of 4-coumarate: CoA ligase in either subclone.

Inhibition of anthocyanin synthesis in carrot cultures grown in the presence of gibberellic acid has been shown to be at the level of chalcone synthase (Hinderer et al., 1984). The inhibition was due to the presence of a 3'-nucleotidase that is present in cells

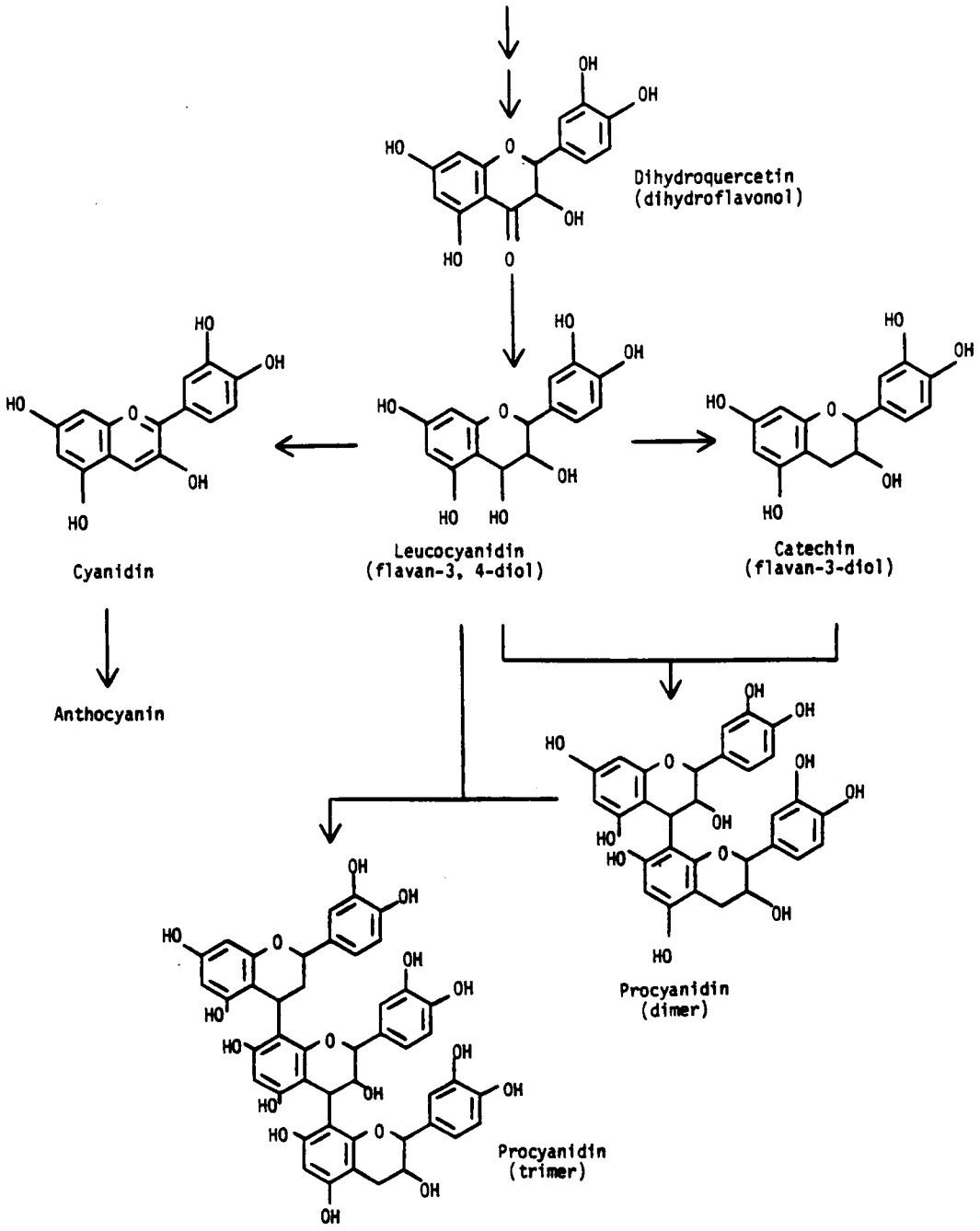



Figure 3. Proposed biosynthetic pathway leading from dihydroquercetin to catechin, procyanidins, and cyanidin (Kristiansen, 1984).

without chalcone synthase activity (Hinderer and Seitz, 1986). This enzyme functions by hydrolyzing the 3'-phosphate group of malonyl-CoA, one of the substrates of chalcone synthase, resulting in a dephosphorylated form of malonyl-CoA. Malonyl-3'-dephospho-CoA is no longer an active substrate for the synthase and acts as an inhibitor of the enzyme.

There is little available information concerning the degradation of anthocyanin. An investigation with wild carrot suspension cultures indicated that accumulated anthocyanin is not metabolized (Dougall and Vogelien, 1987). No change in the level of accumulated anthocyanin was observed over a period of 8 days in wild carrot cultures treated with phenylpropiolic acid (PPA), an inhibitor of phenylalanine ammonia lyase. Complete inhibition of anthocyanin accumulation in cultures could be achieved using PPA under appropriate conditions. Further, no differences in the rate of anthocyanin degradation were found between high- and low-accumulating subclones, indicating that differences in the rate of degradation can not account for the differences in the levels of accumulated anthocyanin in subclones.

Recent studies with buckwheat (Fagopyrum esculentum) and red cabbage (Brassica oleracea) indicate

that many of the enzymes of phenylpropanoid and flavonoid metabolism are associated with the endoplasmic reticulum (PAL, cinnamate 4-hydroxylase, 4-coumarate: CoA ligase, chalcone synthase, chalcone isomerase, and UDPG: flavonoid glucosyltransferase) (Wagner and Hrazdina, 1984; Hrazdina and Wagner, 1985). Hrazdina and Wagner (1985) demonstrated channeling between phenylalanine and p-coumarate and suggest that this complex is part of a larger multienzyme complex involving both phenylpropanoid and flavonoid metabolism. However, they were unsuccessful in demonstrating channeling of metabolites in central or late stages of flavonoid metabolism.

# IV. MATERIALS AND METHODS

## Cells, Media, and Culture Conditions

Cultures of wild carrot (*Daucus carota*), parental line WC-63 and subclones WC63-1-9-1 and WC63-2-2, described by Dougall et al. (1980), were used in this study. Cell line WC-63 was obtained through subculturing anthocyanin-containing portions of a culture established from a petiole explant of wild carrot by Dr. D.F. Wetherell (University of Connecticut, Storrs, CT, USA). Subclones WC63-1-9-1 and WC63-2-2 were obtained by the serial recloning of clones from WC63 with the highest and lowest anthocyanin accumulation, respectively.

Stock cultures of each of these were maintained in wild carrot medium (WCM) (Wetherell, 1969). The formulation of WCM is given in Table 1. Cultures were maintained by serial subculture at two week intervals using a 1/20 dilution of culture into fresh WCM. Maintenance of cultures was in the dark at 25 °C with continuous agitation on a gyratory shaker at 120 rpm.

## Pigment and Growth Measurements

The methods used to measure anthocyanin content and growth are described by Dougall and Weyrauch (1980). Samples of culture were centrifuged at 700 x g for 8

Table 1. Composition of WCM.[a]

| Original Formulation[b] | mg/l |
|---|---|
| $KNO_3$ | 4000 |
| $NH_4Cl$ | 540 |
| $MgSO_4 \cdot 7H_2O$ | 185 |
| $CaCl_2$ | 166 |
| $KH_2PO_4$ | 68 |
| $Na_2EDTA$ | 18.6 |
| $FeSO_4 \cdot 7H_2O$ | 13.6 |
| $MnSO_4 \cdot 7H_2O$ | 7.0 |
| $ZnSO_4 \cdot 7H_2O$ | 4.0 |
| $H_3BO_3$ | 2.4 |
| $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ | 0.01 |
| KI | 0.38 |
| $CuSO_4 \cdot 5H_2O$ | 0.015 |
| Thiamine·HCl | 3.0 |
| Sucrose | 20,000 |
| 2,4-D | 0.5 |

[a]Wetherell, 1969.

[b]pH of medium adjusted to 5.8 with NaOH or HCl.

minutes, and the pellet resuspended with a predetermined volume of extractant. The extractant solution consists of 95% methanol containing 1% 12 N HCl (v/v). Extraction was allowed to continue overnight at 4 °C. Samples were then centrifuged at 700 x g for 8 minutes, and the absorbance of the extracts measured at 530 nm. The optical density is directly proportional to pigment/ml culture.

After extraction, the tissue was filtered onto preweighed filter discs (2.4 cm) and washed with water. Discs were then placed in a drying oven at 70 °C overnight. Before weighing, the discs were placed in a desiccator for 15-20 minutes to allow to cool. Extracted dry weight is expressed as mg extracted tissue/ml culture.

Initial Experiments

Preliminary experiments, designed to determine the concentration of each intermediate to be provided to clones, involve feeding anthocyanin precursor(s) to cells in which anthocyanin accumulation has been inhibited at the first step in the pathway and examining changes in culture growth and in the level of anthocyanin accumulated. This approach will confirm that the cells are able to metabolize the the intermediates.

Anthocyanin accumulation in WC-63 cells was inhibited by the addition of 0.1 mM phenylpropiolic acid (PPA) to cultures at days 4 and 8 of the culture period, as described by Dougall and Vogelien (1987). Stock cultures were washed twice by centrifugation at 700 x g for 8 minutes and resuspended in an equal volume of the medium for improved anthocyanin accumulation (WCM-Imp) (Kinnersley and Dougall, 1980). The formulation for WCM-Imp is given in Table 2. Flasks were prepared using a 1/5 dilution of the washed cell suspension into fresh WCM-Imp. On days 4 and 8 of the culture period, a stock solution of 10 mM PPA was added to the cultures to obtain a final concentration of 0.1 mM PPA. The stock solution of PPA (Sigma Chemical Co.) was prepared in WCM-Imp, pH adjusted to 4.5 with NaOH or HCl, filter sterilized using a Nalgene filtration unit (0.2 micron pore size), and stored at 4 °C.

4-Coumaric acid, naringenin and dihydroquercetin were examined in separate experiments. Each of several concentrations of an intermediate were added to individual flasks described above on days 4 and 8 of the culture period. The appropriate controls and treatments were prepared in duplicate. On days 4, 8 and 12 of the culture period flasks were sampled in triplicate and anthocyanin content determined.

Table 2. Composition of WCM-IMP.[a]

| Original Formulation[b] | mg/l |
|---|---|
| $NH_4Cl$ | 320.94 |
| $MgSO_4 \cdot 7H_2O$ | 185 |
| $CaCl_2$ | 166 |
| $K_2HPO_4$ | 87.1 |
| $Na_2EDTA$ | 18.6 |
| $FeSO_4 \cdot 7H_2O$ | 13.6 |
| $MnSO_4 \cdot H_2O$ | 7.0 |
| $ZnSO_4 \cdot 7H_2O$ | 4.0 |
| $H_3BO_3$ | 2.4 |
| $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$ | 0.01 |
| KI | 0.38 |
| $CuSO_4 \cdot 5H_2O$ | 0.015 |
| Thiamine·HCl | 3.0 |
| 2,4-D | 2.5 |
| Sucrose | 20,000 |
| $K_2$ Succinate | 3885.8 |

[a]Dougall and Weyrauch, 1980.

[b]pH of medium adjusted to 4.5 with NaOH or HCl.

Extracted dry weight was also measured from days 4 and 12 samples. The intermediate concentrations examined were evaluated by their ability to cause an increase in the level of anthocyanin accumulated without causing a significant inhibition of growth. Intermediate concentrations examined were: 0.025, 0.05, 0.075, 0.1, 0.15, 0.2, 0.25, 0.35 and 0.5 mM 4-coumaric acid; 0.01, 0.025, 0.05, 0.1, 0.25, 0.5, 1 and 10 mM naringenin; 0.025, 0.075, 0.1, 0.15, 0.25 and 0.5 mM dihydroquercetin.

Stock solutions of 4-coumaric acid, naringenin and (±)dihydroquercetin (Sigma Chemical Co.) were prepared in DMSO and stored at room temperature. The final concentration of DMSO in cultures is 1%. DMSO was chosen as the solvent for the intermediates because dihydroquercetin and naringenin are insoluble in water and media. Further, DMSO is a penetrant carrier, and thus it may enhance absorption of the intermediates by the cells.

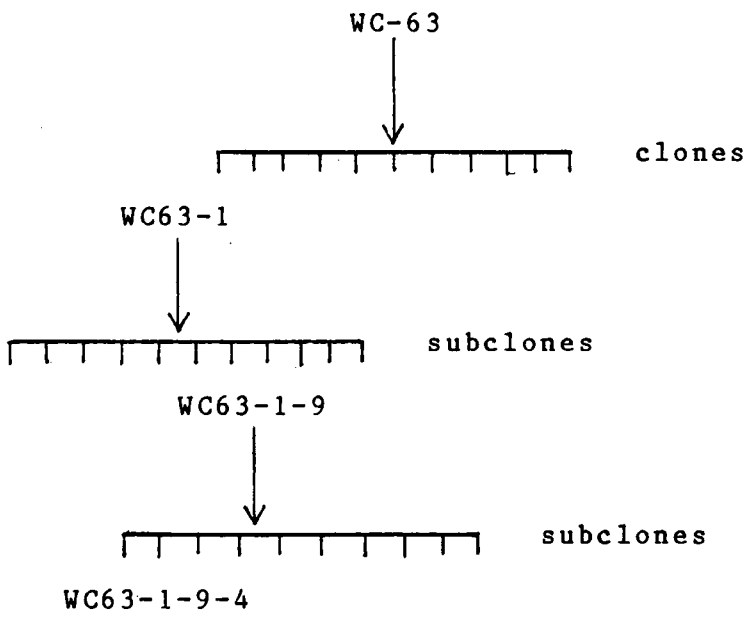
## Cloning Procedure

Clones of WC-63 cultures were obtained by the method described by Dougall et al. (1980). Cultures at or near the end of their growth cycle were screened through a 44 um stainless-steel screen. Material

retained on the screen was washed with approximately 20 ml medium. The entire filtrate was then transferred to a sterile 150 ml glass centrifuge bottle with screw cap and centrifuged at 700 x g for 8 minutes. Approximately 80% of the medium was removed, and the pellet resuspended in the remaining medium. A cell count was then made of this suspension using a Spiers-Levy Eosinophil Counter (Arthur H. Thomas and Co.). Dilution of the suspension was made in WCM-Imp. supplemented with 10 mM myo-inositol (pH 5.8) to obtain a final concentration of 100 cells/ml. One ml of this suspension was transferred to sterile 100 mm polystyrene tissue culture dishes (Corning Glass Works). Plating medium consists of WCM-Imp. supplemented with 10 mM myo-inositol (pH 5.8) and 0.75% agar purified using the method described by Binns and Meins (1979). Ten ml of melted plating medium maintained at 40 - 45 °C was added to plates and mixed carefully with inoculum. Cooled plates are sealed with Parafilm (American Can Co.) and incubated in the dark at 25 °C. When colonies were approximately 1 mm in diameter (in approximately 6 weeks) they were transferred to 16 x 150 mm glass screw cap tubes containing 1 ml of WCM-1. Tubes were maintained on a roller drum at 20 rpm in the dark at 25 °C. At the end of one week 2 ml fresh WCM-1 was added to

each tube. At the end of two more weeks contents of tubes were transferred to 25 ml of WCM-Imp. medium in 125 ml erlenmeyer flasks. After two weeks accumulated anthocyanin for each clone was measured as previously described.

Each clone that was retained for future use was designated by a number. Recloning of a clone yields subclones that were designated by a second number. In turn, any subclones obtained through the recloning of these subclones were designated by a third number. Thus, WC63-1-9-4 is subclone number 4 isolated from the cloning of number 9, which was a subclone isolated from the cloning of number 1, which was a clone from the parent WC-63 culture (see diagram below).



Assesment of Phenotype

Clones were screened following the procedure outlined in Initial Experiments but using only the "optimum" concentration determined for each anthocyanin precursor. The concentration of each intermediate used was 0.2 mM 4-coumaric acid, 0.1 mM naringenin, and 0.25 mM dihydroquercetin. Intermediates were also provided to cells in the absence of 0.1 mM PPA. On day 12 of the culture period flasks were sampled in triplicate and anthocyanin content and growth determined. The responsiveness of clones to each intermediate was assesed by comparing anthocyanin levels to the appropriate control values.

# V. RESULTS

## Initial Experiments

Initial experiments were performed to determine the concentration of 4-coumarate, naringenin, and dihydroquercetin that can be added to clones and result in an increase in anthocyanin accumulation without adversly affecting culture growth. Intermediates were supplied to cultures treated with phenylpropiolic acid (PPA), an inhibitor of phenylalanine ammonia-lyase, the first enzyme of anthocyanin biosynthesis. Anthocyanin accumulation is inhibited in WC-63 cells receiving 0.1 mM PPA at days 4 and 8 of the culture period. Addition of anthocyanin precursors following this point of the pathway should restore anthocyanin accumulation in PPA inhibited cultures. Further, any increase in anthocyanin levels of the cultures would imply that the cells are able to metabolize exogenously supplied precursors into anthocyanin. Growth and pigment production in the parental line WC-63 receiving a range of concentrations of 4-coumarate, naringenin, and dihydroquercetin are shown in Figs. 4, 5 and 6, respectively. In each case the responsiveness of WC-63 to individual intermediate concentrations was determined by comparison with treatment 4 (1% DMSO + 0.1 mM PPA).

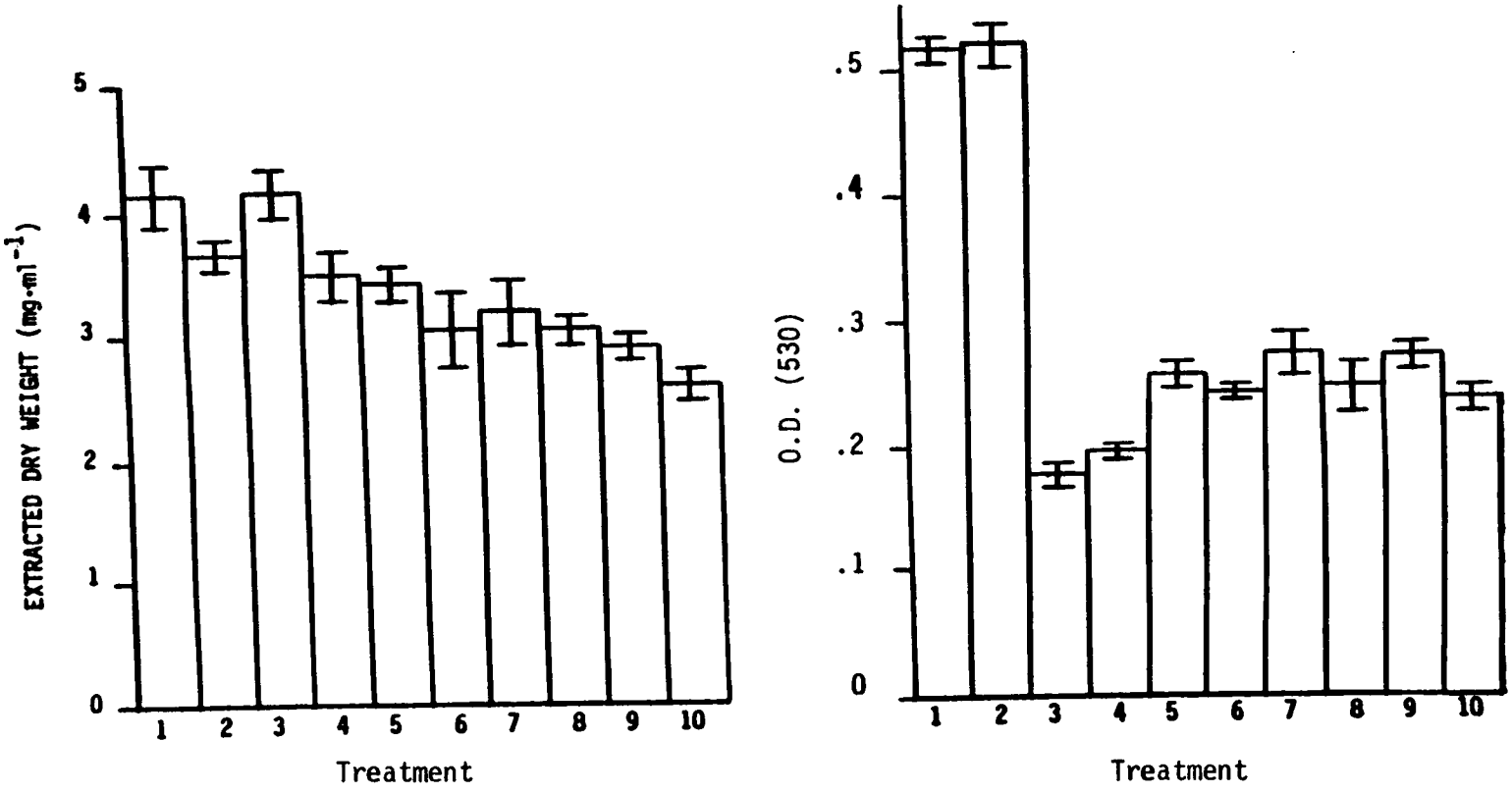



Figure 4. Growth and anthocyanin production at day 12 in WC-63 cultures treated with 0.1 mM PPA and coumarate on days 4 and 8. Treatment 1 = control, 2 = 1% DMSO, 3 = 0.1 mM PPA, 4 = 1% DMSO + PPA, 5 = 4 + 0.1 mM coumarate, 6 = 4 + 0.15 mM coumarate, 7 = 4 + 0.2 mM coumarate, 8 = 4 + 0.25 mM coumarate, 9 = 4 + 0.35 mM coumarate, 10 = 4 + 0.5 mM coumarate. Data are the mean and standard deviation of triplicate samples from each of two flasks.

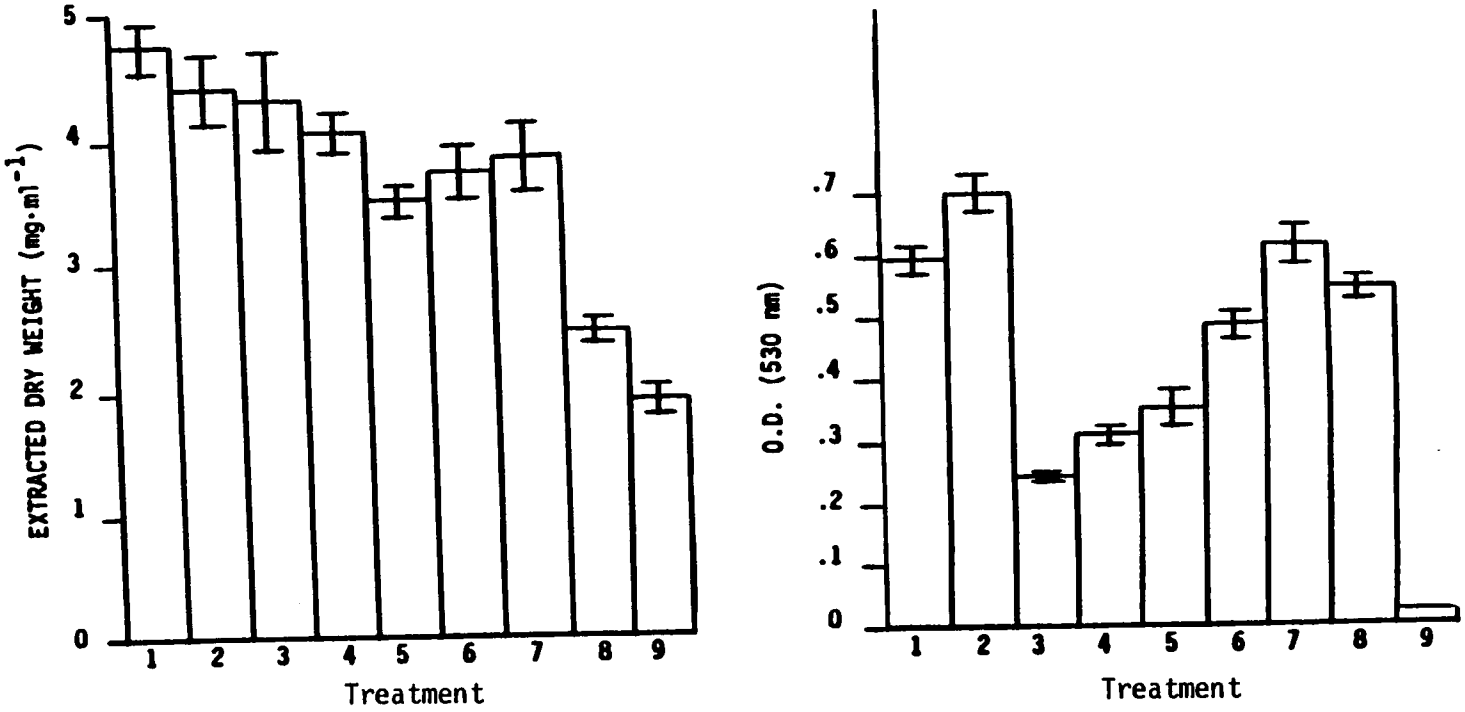



Figure 5. Growth and anthocyanin production at day 12 in WC-63 cultures treated with 0.1 mM PPA and naringenin on days 4 and 8. Treatment 1 = control, 2 = 1% DMSO, 3 = 0.1 mM PPA, 4 = 1% DMSO + PPA, 5 = 4 + 0.025 mM naringenin, 6 = 4 + 0.05 mM naringenin, 7 = 4 + 0.1 mM naringenin, 8 = 4 + 0.25 mM naringenin, 9 = 4 + 0.5 mM naringenin. Data are the mean and standard deviation of triplicate samples from each of two flasks.

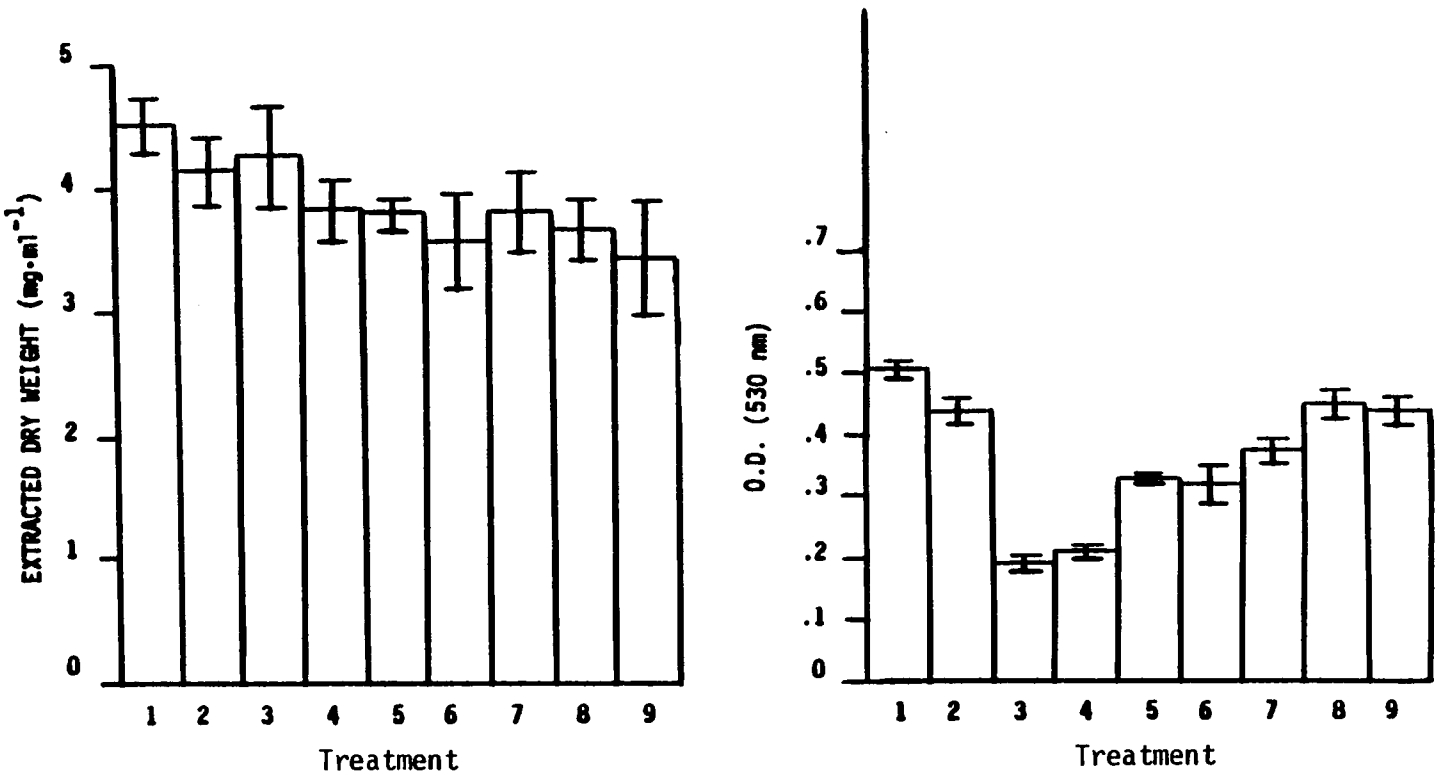



Figure 6. Growth and anthocyanin production at day 12 in WC-63 cultures treated with 0.1 mM PPA and dihydroquercetin(DHQ) on days 4 and 8. Treatment 1 = control, 2 = 1% DMSO, 3 = 0.1 mM PPA, 4 = 1% DMSO + PPA, 5 = 4 + 0.075 mM DHQ, 6 = 4 + 0.1 mM DHQ, 7 = 4 + 0.15 mM DHQ, 8 = 4 + 0.25 mM DHQ, 9 = 4 + 0.5 mM DHQ. Data are the mean and standard deviation of triplicate samples from each of two flasks.

Concentrations of coumarate below 0.1 mM (0.025, 0.05, 0.075 mM) had no effect on anthocyanin levels or growth in inhibited cultures (data not shown). Additions of 0.1 mM and above resulted in small, but measurable, stimulations in anthocyanin production (Fig. 4). There is little change in the degree of stimulation as the concentration of coumarate increases. Growth in PPA treated cultures receiving 0.1, 0.15 and 0.2 mM coumarate was comparable to control values, but the data suggests a small and gradual decline in culture growth with concentrations above 0.2 mM coumarate.

Reversal of PPA induced inhibition was also achieved with the addition of naringenin. Anthocyanin levels increased with increasing concentrations of naringenin (Fig. 5). Maximum reversal of PPA effects was achieved with the addition of 0.1 mM naringenin. Growth in cultures receiving naringenin concentrations up to 0.1 mM was comparable to that observed in control cultures, whereas higher naringenin concentrations (0.25 and 0.5 mM) severly inhibited culture growth.

Dihydroquercetin reversed PPA induced inhibition of anthocyanin production in WC-63 cultures (Fig. 6). As was the case with naringinin additions, anthocyanin levels increased in inhibited cultures with increasing concentrations of dihydroquercetin. Maximum reversal of

PPA inhibition was achieved with 0.25 mM and 0.50 mM dihydroquercetin. Further, complete reversal of PPA inhibition was observed (compare treatment 2 with treatments 8 and 9). In addition, there was no adverse affect on culture growth in response to any of the dihydroquercetin concentrations examined.

In general, it was possible to reverse PPA inhibition in WC-63 cultures with each of the precursors. The level of response to precursor additions was greatest with dihydroquercetin, followed by naringenin and coumarate. In all three cases a concentration was found that could stimulate anthocyanin production in inhibited cultures without adversely effecting culture growth. Following these criteria, concentrations of 0.2 mM coumarate, 0.1 mM naringenin and 0.25 mM dihydroquercetin were selected for use in the examination subclones.

Initially it was necessary to perform supplementation experiments on WC-63 cultures in the presence of PPA, for this approach would confirm the ability of WC-63 cultures to metabolize the exogenously supplied precursors. Once this ability is established it should no longer be necessary to treat cells with PPA. Thus, it was anticipated that future experiments analyzing WC-63 clones would be performed in the absence

of the inhibitor PPA. Further, anthocyanin accumulation is already greatly reduced in low-accumulating subclones, and treatment of these subclones with PPA would be repetitive and unnecessary. To examine and compare the responsiveness of PPA treated and untreated cultures to intermediates, 0.2 mM coumarate, 0.1 mM naringenin and 0.25 mM dihydroquercetin were supplied to the parental WC-63 culture in the presence and absence of 0.1 mM PPA. Fig. 7 shows growth and anthocyanin production at day 12 in these cultures. A small inhibition in growth was seen in cultures receiving intermediates in the absence of PPA. Since growth determinations at day 4 for all the treatments were not significantly different, differences observed at day 12 are most likely due to the addition of intermediates. Because of the small differences in growth, anthocyanin production was expressed as total O.D. and O.D./dry weight ($mg \cdot ml^{-1}$). In the absence of PPA, both naringenin and dihydroquercetin clearly cause an increase in the pigment levels of WC-63 cultures (Fig. 7a). However, the effect of 0.2 mM coumarate is less clear. O.D. values indicate no stimulation of pigment accumulation with the addition of coumarate. In fact, the data indicate a small drop in the pigment level in comparison to the control value. When expressed as O.D./$mg \cdot ml^{-1}$,

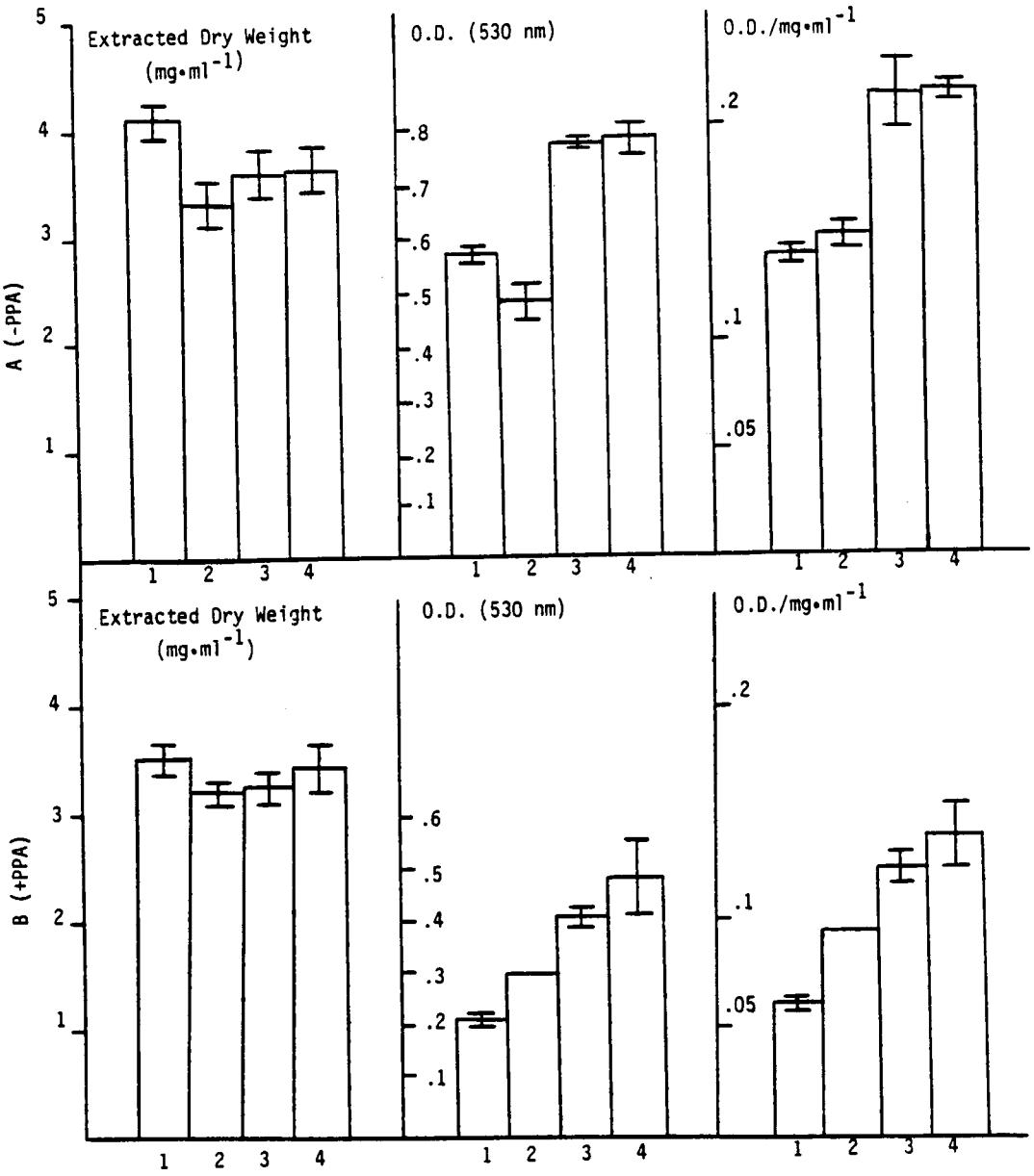



Figure 7. Growth and anthocyanin production at day 12 in WC-63 cultures receiving 0.2mM coumarate, 0.1 mM naringenin and 0.25 mM dihydroquercetin in the presence and absence of 0.1 mM PPA. A = treatments made in the absence of PPA; B = treatments made in the presence of PPA. Treatment 1 = 1% DMSO (control), 2 = 0.2 mM coumarate, 3 = 0.1 mM naringenin and 4 = 0.25 mM dihydroquercetin. Data are the mean and standard deviation of triplicate samples from each of two flasks unless the standard deviation is too small to be accurately plotted.

however, anthocyanin levels are slightly higher than that of the control. In the presence of PPA each of the three intermediates results in a clear stimulation of anthocyanin accumulation, based on both O.D. and O.D./mg. $ml^{-1}$ values. The response was similar to those observed in previous experiments, with dihydroquercetin giving the greatest response, followed by naringenin and coumarate.

WC-63 cultures receiving 1% DMSO or 0.1 mM PPA have growth patterns similar to cultures receiving no treatment. When both of these chemicals are supplied to cultures, as is the case of the control treatment for cultures receiving intermediates in the presence of PPA, a small decrease in growth at day 12 is observed (data not shown). It is quite possible that DMSO facilitates the absorption of PPA by the cells. Previous work has shown that the concentration of PPA which can be added to cultures without affecting cell growth is dependant upon culture density at the time of addition (Dougall and Vogelien, 1987). If PPA is made more available to cells by the addition of DMSO, the result could conceivably be the same as that for the addition of a higher PPA concentration.

It becomes apparent from this data that stimulations in anthocyanin content in response to

naringenin and dihydroquercetin additions are clearly detectable in both PPA treated and untreated cultures. The level of response by cultures to coumarate additions is quite small. So small that it may be necessary to evaluate anthocyanin content on an O.D./dry weight basis. Any question as to a cultures responsiveness to coumarate can also be ascertained by examining the cultures response to coumarate in the presence of PPA. The addition of the anthocyanin inhibitor appears to sharpen a cultures response to precursors.

Analysis of WC-63 Clones

A wide variety of subclones were analyzed by precursor feeding experiments. The majority of subclones examined were obtained from several clonings of a high-yielding subclone, WC63-1-9-1. Other subclones examined were those obtained through the cloning of a low-yielding subclone, WC63-2-2, and through the recloning of WC-63. When a cloning process is completed the two clones with the lowest anthocyanin yields are designated low-yielding (L), while two clones having the highest yields are designated high-yielding (H). A cell lineage is provided to show the relationship of all clones examined (Fig. 8). Also indicated is the ability

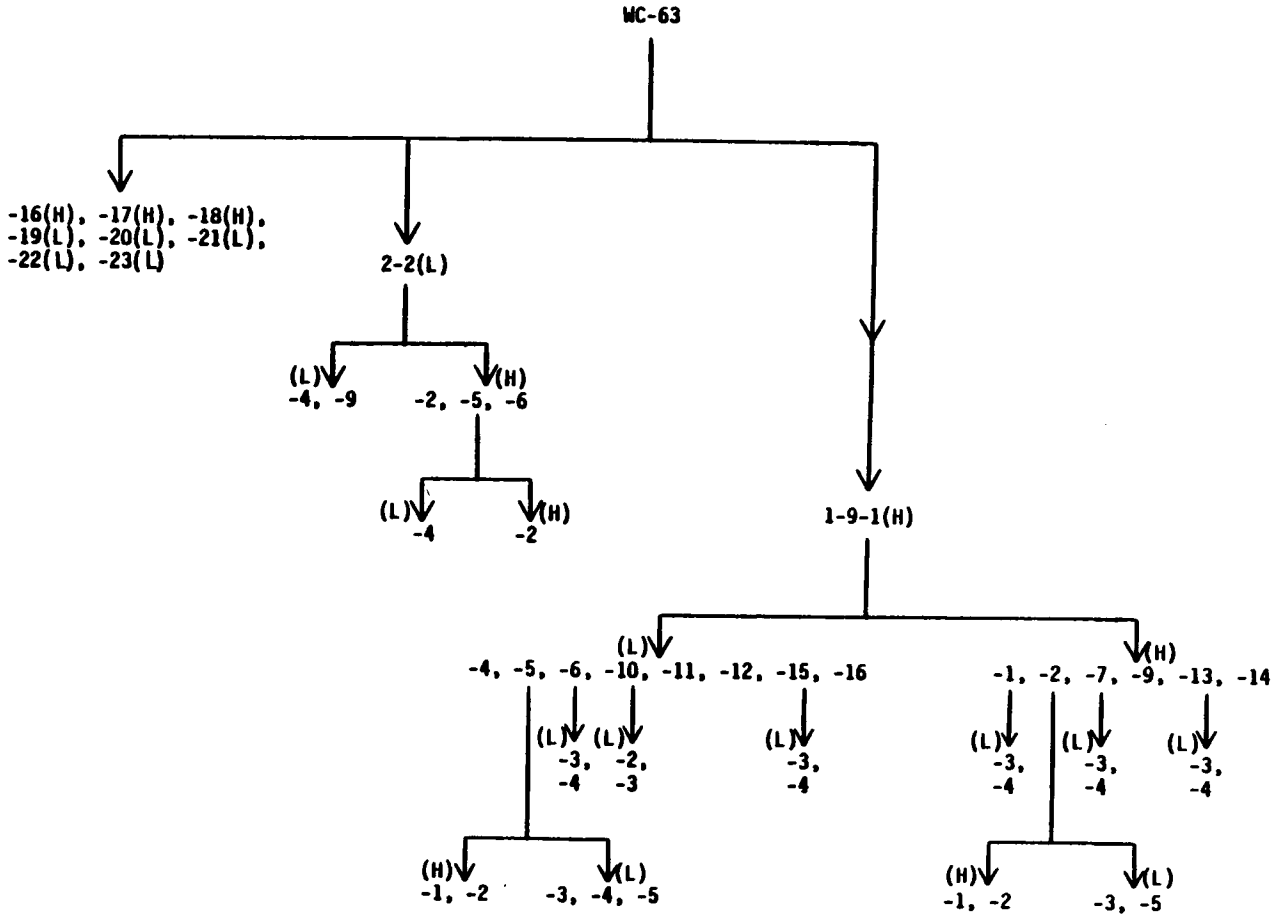



Figure 8. Diagram of clonings showing the source of all subclones and clones examined by precursor feeding experiments. Also indicated is the classification of the clone as low- or high-yielding (L or H, respectively) upon completion of the clonal analysis.

of each clone to accumulate anthocyanin immediately upon completion of a clonal analysis (indicated as L or H).

Approximately fifty individual clones were treated with coumarate, naringenin and dihydroquercetin. The effect of these intermediates on anthocyanin accumulation is shown in Table 3. Anthocyanin content for the control (receiving 1% DMSO) is given, while the response of a clone to each intermediate is reported as the increase in anthocyanin content above the control value. Further, for ease of examination subclones have been organized into the following subsets: clones generated through the recloning of WC-63, subclones generated from the cloning of 2-2, high-yielding sublcones generated from the cloning of 1-9-1, and low-yielding subclones generated from the cloning of 1-9-1. At this point it should be noted that all values in Table 3 are the average of three samples from a single flask. The experimental design makes it extremely difficult to make any strict quantitative judgements or comparisons of the data. Thus, a purely qualitative approach will be used in the discussion of this data.

It is clear from the data in Table 3 that all high-yielding subclones generated through the cloning of 1-9-1 are capable of achieving much increased levels of anthocyanin in response to each intermediate. In most

Table 3. Anthocyanin content of WC-63 clones and subclones after feeding with anthocyanin precursors.

| Clone | Anthocyanin Content as O.D./ mg/ml Control | | Increase in Anthocyanin Due to Intermediate Coum. | Nar. | DHQ |
|---|---|---|---|---|---|
| WC63-16(H) | 0.545 | | 0.109 | 0.285 | 0.321 |
| WC63-17(H) | 0.676 | | 0.009 | 0.324 | 0.459 |
| WC63-18(H) | 0.631 | | 0.131 | 0.307 | 0.298 |
| WC63-19(L) | 0.013 | | 0.000 | 0.001 | 0.014 |
| WC63-20(L) | 0.049 | | 0.016 | 0.007 | 0.019 |
| WC63-21(L) | 0.012 | | 0.000 | 0.004 | 0.008 |
| WC63-22(L) | 0.048 | | -0.007 | 0.007 | 0.020 |
| WC63-23(L) | 0.046 | | 0.000 | 0.018 | 0.017 |
| 2-2-2(H)* | 0.032 | (0.070) | 0.023 | 0.062 | 0.047 |
| 2-2-6(H)* | 0.042 | (0.095) | 0.021 | 0.048 | 0.039 |
| 2-2-5-2(H)* | 0.042 | (0.138) | 0.009 | 0.082 | 0.097 |
| 2-2-4(L)* | 0.021 | (0.037) | 0.006 | 0.018 | 0.014 |
| 2-2-9(L)* | 0.018 | (0.028) | 0.007 | 0.011 | 0.010 |
| 2-2-5-4(L)* | 0.020 | (0.036) | 0.000 | 0.009 | 0.016 |
| 1-9-1-1(H) | 2.696 | | 0.474 | 0.669 | 1.400 |
| 1-9-1-2(H) | 2.587 | | 0.819 | 0.573 | 1.132 |
| 1-9-1-9(H) | 1.865 | | 0.041 | 0.231 | 0.767 |
| 1-9-1-13(H) | 2.261 | | 0.674 | 0.485 | 0.776 |
| 1-9-1-14(H) | 1.816 | | -0.320 | 0.583 | 0.756 |
| 1-9-1-2-1(H) | 1.774 | | -0.109 | 0.696 | 0.990 |
| 1-9-1-2-2(H) | 1.709 | | 0.000 | 0.801 | 0.777 |
| 1-9-1-5-1(H) | 1.266 | | 0.134 | 0.878 | 1.617 |
| 1-9-1-5-2(H) | 0.936 | | 0.248 | 0.855 | 1.284 |
| 1-9-1-10-2 (L) | 0.013 | | 0.005 | 0.007 | 0.007 |
| 1-9-1-6-3 (L) | 0.016 | | 0.009 | 0.007 | 0.008 |
| 1-9-1-6-4 (L) | 0.021 | | 0.003 | 0.001 | -0.001 |
| 1-9-1-10-3 (L) | 0.032 | | 0.003 | 0.004 | 0.004 |
| 1-9-1-6 (L) | 0.033 | | -0.003 | 0.002 | -0.001 |
| 1-9-1-15-3 (L) | 0.056 | | 0.002 | 0.015 | 0.016 |
| 1-9-1-2-3 (L) | 0.085 | | -0.018 | 0.035 | 0.025 |
| 1-9-1-15 (L) | 0.094 | | -0.021 | 0.030 | 0.014 |
| 1-9-1-16 (L) | 0.106 | | 0.027 | 0.017 | 0.027 |
| 1-9-1-10 (L) | 0.434 | | -0.003 | 0.017 | 0.082 |
| 1-9-1-7-3 (L) | 0.179 | | -0.068 | 0.064 | 0.066 |

Table 3 (Continued)

| Clone | Anthocyanin Content as O.D./ mg/ml Control | Increase in Anthocyanin Due to Intermediate Coum. | Nar. | DHQ |
|---|---|---|---|---|
| 1-9-1-15-4 (L) | 0.152 | 0.017 | 0.083 | 0.108 |
| 1-9-1-5-3 (L) | 0.172 | -0.021 | 0.138 | 0.110 |
| 1-9-1-2-5 (L) | 0.204 | -0.042 | 0.109 | 0.134 |
| 1-9-1-12 (L) | 0.263 | -0.053 | 0.156 | 0.142 |
| 1-9-1-13-3 (L) | 0.347 | -0.019 | 0.073 | 0.174 |
| 1-9-1-5-5 (L) | 0.283 | -0.053 | 0.389 | 0.651 |
| 1-9-1-5-4 (L) | 0.441 | -0.110 | 0.322 | 0.755 |
| 1-9-1-5 (L) | 0.518 | -0.178 | 0.310 | 0.718 |
| 1-9-1-4 (L) | 0.752 | -0.029 | 0.454 | 0.243 |
| 1-9-1-11 (L) | 0.846 | 0.236 | 0.399 | 0.550 |
| 1-9-1-7-4 (L) | 1.468 | -0.017 | 0.414 | 0.678 |
| 1-9-1-1-3 (L) | 1.491 | 0.966 | 0.997 | 1.924 |
| 1-9-1-1-4 (L) | 1.667 | 0.069 | 0.539 | 1.079 |
| 1-9-1-13-4 (L) | 2.440 | 0.203 | 0.417 | 1.728 |

*Values provided were obtained from precursor feeding in the presence of 0.1 mM PPA. Anthocyanin content of the control (1% DMSO + PPA) is given, as well as values for flasks receiving 1% DMSO (in parentheses).

Precursors were dissolved in DMSO and added to cultures on days 4 and 8 of the culture period. Anthocyanin content and growth (as extracted dry weight) were determined at day 12. Data are the average of triplicate samples from a single flask. Anthocyanin content for the control (1% DMSO or 1% DMSO + PPA) is given, while precursor effect on anthocyanin content is given as the increase in anthocyanin above the control. Final concentrations: 0.2 mM coumarate, 0.1 mM naringenin, 0.25 mM dihydroquercetin, 1% (v/v) DMSO, 0.1 mM PPA.

cases the increase in anthocyanin content was greatest with dihydroquercetin, followed by naringenin and coumarate. Three of the nine 1-9-1 high-yielding subclones (1-9-1-14, -2-1 and -2-2) do not show a positive response to coumarate. Normally this result would suggest that the culture possesses a block in anthocyanin metabolism somewhere between coumarate and naringenin. This is unlikely, however, for these three subclones are obviously capable of producing large amounts of anthocyanin. It would be more reasonable to suggest that cells contain or reach a critical intracellular concentration of coumarate, such that any further uptake of coumarate becomes limited or becomes channeled to other metabolic pathways, such as lignin biosynthesis. In general, however, it appears that the subclones designated as high-yielding from clonal analyses of 1-9-1 are extremely capable of accumulating increased levels of anthocyanin when supplied with coumarate, naringenin and dihydroquercetin. This finding suggests substrate limitation exists in these subclones with respect to anthocyanin biosynthesis.

Prior to this work it was hypothesized that the low-accumulating cells possesed a block or bottleneck at some point in the pathway, be it genetic or physiological. It was anticipated that addition of

early, middle and late intermediates of the pathway to low-yielding subclones would restore anthocyanin biosynthesis and indicate the level of the block. High- and low-yielding clones generated from WC-63 were tested for their ability to metabolize exogenous supplies of coumarate, naringenin and dihydroquercetin into anthocyanin. The response of the high-yielding clones (WC63-16, -17 and -18) was, in general, similar to that given by 1-9-1 high-yielding subclones. The degree of response, however, is clearly lower than those of the 1-9-1 subclones. What is most interesting from this subset of data in Table 3 is the response, or rather the lack of response, of the low-yielding clones to all three intermediates. An increase of 0.015 or below is considered questionable, based on the sampling error observed for repeated measurements from individual flasks. All five low-yielding clones show little, if any, response to coumarate, naringenin or dihydroquercetin.

A similar pattern of response was observed in high- and low-yielding subclones obtained from the cloning of 2-2. Values of this subset of data in Table 3 are those obtained from precursor feeding experiments performed in the presence of the inhibitor PPA. In the absence of PPA, the response of the 2-2 subclones to

coumarate, and in several instances to naringenin and dihydroquercetin, was questionable. To substantiate these findings, data obtained through a concurrent precursor feeding analysis in the presence of PPA was examined and found to give a much clearer response. Those 2-2 subclones designated as low-yielding show very little, if any, capability of metabolizing coumarate, naringenin or dihydroquercetin into anthocyanin, whereas high-yielding 2-2 subclones examined are clearly able to use the intermediates, but to a limited degree. For example, the response of high-yielding 2-2 subclones to intermediates is much lower than the response of WC-63 high-yielding clones and 1-9-1 high-yielding subclones.

A wide range of responses in anthocyanin content were observed from precursor feeding experiments in the low-yielding subclones obtained from 1-9-1. Data for this subset of subclones has been listed in order of increasing response to the intermediates. The first six subclones in this subset of data show no increase in anthocyanin content in response to any of the intermediates. The following six subclones gave only small increases in pigment content, while the remaining subclones in the subset displayed strong responses and clearly possess the ability to metabolize the precursors into anthocyanin.

Negative values for a response to coumarate are seen in the data for several 1-9-1 low-yielding subclones. In many cases precursor feeding was also performed in the presence of PPA. Under this condition the non-responsive subclones 1-9-1-10-2, -10-3, -6, -6-3, -6-4, and -15-3 continued to show no response to any of the intermediates, while a positive response to coumarate, as well as naringenin and dihydroquercetin, was observed by all others. This ambiguous response to coumarate has been noted in previous experiments, but not to this extreme.

Precursor feeding experiments were repeated on selected non-responsive and low-responsive 1-9-1 subclones. Coumarate, naringenin and dihydroquercetin were added to cultures in the absence of PPA, and each treatment was performed in duplicate. The results, shown in Table 4, indicate that the two low-yielding subclones examined (1-9-1-6-4 and -10-3) are indeed non-responsive to precursor feeding, while the low-responsive subclone (1-9-1-5-3) continued to give small, but significant, increases in pigment content in response to precursor additions. Subclone 1-9-1-15-3, previously considered non-responsive, now showed a very small increase in anthocyanin content in response to dihydroquercetin. Regardless of the broad range of

Table 4. Anthocyanin content of selected 1-9-1 low-yielding subclones after feeding with anthocyanin precursors.

| | Anthocyanin Content as O.D./ mg/ml | Increase in Anthocyanin Due to Intermediate | | |
|---|---|---|---|---|
| Subclone | Control | Coum. | Nar. | DHQ |
| 1-9-1-6-4 | 0.033 | 0.006 | 0.004 | 0.003 |
| 1-9-1-10-3 | 0.054 | -0.004 | 0.005 | 0.005 |
| 1-9-1-15-3 | 0.067 | -0.004 | 0.011 | 0.018 |
| 1-9-1-5-3 | 0.055 | 0.018 | 0.046 | 0.030 |

Precursors were dissolved in DMSO and added to cultures on days 4 and 8 of the culture period. Anthocyanin content and growth (as extracted dry weight) for each treatment were determined at day 12. Data are the mean of triplicate samples from duplicate flasks. Anthocyanin content for the control (1% DMSO) is given, while precursor effect on anthocyanin content is given as the increase in anthocyanin above the control. Final concentrations: 0.2 mM coumarate, 0.1 mM naringenin, 0.25 mM dihydroquercetin, 1% DMSO.

responses by 1-9-1 low-yielding subclones, there are several subclones which give no, or extremely small, responses to coumarate, naringenin and dihydroquercetin additions.

It is interesting to note that five of the non-responsive 1-9-1 subclones were obtained from the cloning of low-yielding subclones generated from 1-9-1, or, in other words, second generation low-yielding subclones. Those subclones in which relatively small increases in anthocyanin content were observed are also second generation low-yielding subclones. Further, non- and low-responsive 1-9-1 subclones tend to have very low anthocyanin levels (< 0.100). The 1-9-1 low-yielding subclones which are very responsive to precursor feeding tend to have higher pigment levels, and a majority were obtained from the cloning of high-yielding subclones from 1-9-1. There appears to be a correlation between anthocyanin content of a subclone and the responsiveness of the subclone to precursor feeding. This observation is similar to that noted for the different degrees of response seen between high-yielding WC-63 clones and high-yielding 1-9-1 and 2-2 subclones.

The wide range in anthocyanin content found in low-yielding 1-9-1 subclones (controls) can be explained by the fact that individual subclones were obtained from

different clonal analyses (see Fig. 8, pg. 40). A subclone/clone is designated low-yielding or high-yielding purely on the basis of having the lowest or highest anthocyanin content, respectively,in comparison to all other subclones/clones from the same cloning. Table 5 lists anthocyanin content for all of the clones at the end of their individual clonal analyses. Within all clones designated as low-yielding a wide range of pigment levels are observed. For example, subclone 1-9-1-10-2 shows an anthocyanin content (based on O.D. at 530 nm) of 0.025 at the end of its clonal analysis, while subclone 1-9-1-13-4, obtained through a different clonal analysis, had a pigment level of 0.468. Although low-yielding clones differ tremendously in pigment content, each is designated a low-yielding subclone because they contained the lowest pigment level of all subclones in their respective clonal population. Also provided in Table 5 are the anthocyanin contents for clones at the time each was examined by precursor feeding experiments. Changes in anthocyanin content in clones/subclones from the time of isolation to the time of use in precursor feeding experiments contents is an example of the instability associated with anthocyanin production by wild carrot clones and subclones upon maintenance in culture. It is interesting to note that

Table 5. Anthocyanin content of WC-63 clones and subclones upon completion of their clonal analysis and during examination by precursor feedings.

| Clone | Anthocyanin Content Upon Completion of Clonal Analysis | Anthocyanin Content During Precursor Feeding Experiment |
|---|---|---|
| WC63-16(H) | 1.974 | 2.209 |
| WC63-17(H) | 1.608 | 2.267 |
| WC63-18(H) | 1.601 | 2.005 |
| WC63-19(L) | 0.018 | 0.078 |
| WC63-20(L) | 0.083 | 0.344 |
| WC63-21(L) | 0.089 | 0.141 |
| WC63-22(L) | 0.213 | 0.441 |
| WC63-23(L) | 0.308 | 0.303 |
| 2-2-2(H) | 0.824 | 0.201 |
| 2-2-6(H) | 0.403 | 0.247 |
| 2-2-5-2(H) | 0.597 | 0.369 |
| 2-2-4(L) | 0.090 | 0.111 |
| 2-2-9(L) | 0.018 | 0.061 |
| 2-2-5-4(L) | 0.064 | 0.063 |
| 1-9-1-1(H) | 3.732 | 3.823 |
| 1-9-1-2(H) | 3.244 | 2.855 |
| 1-9-1-9(H) | 2.846 | 1.669 |
| 1-9-1-13(H) | 1.980 | 3.208 |
| 1-9-1-14(H) | 1.984 | 2.555 |
| 1-9-1-2-1(H) | 2.867 | 2.644 |
| 1-9-1-2-2(H) | 2.451 | 2.289 |
| 1-9-1-5-1(H) | 2.384 | 1.969 |
| 1-9-1-5-2(H) | 2.132 | 2.276 |
| 1-9-1-10-2(L) | 0.025 | 0.023 |
| 1-9-1-6-3(L) | 0.010 | 0.037 |
| 1-9-1-6-4(L) | 0.011 | 0.057 |
| 1-9-1-10-3(L) | 0.035 | 0.049 |
| 1-9-1-6(L) | 0.064 | 0.056 |
| 1-9-1-15-3(L) | 0.022 | 0.133 |
| 1-9-1-2-3(L) | 0.084 | 0.176 |
| 1-9-1-15(L) | 0.194 | 0.213 |
| 1-9-1-16(L) | 0.194 | 0.204 |
| 1-9-1-10(L) | 0.264 | 0.545 |
| 1-9-1-7-3(L) | 0.146 | 0.223 |

Table 5 (Continued)

| Clone | Anthocyanin Content Upon Completion of Clonal Analysis | Anthocyanin Content During Precursor Feeding Experiment |
|---|---|---|
| 1-9-1-15-4(L) | 0.061 | 0.489 |
| 1-9-1-5-3(L) | 0.074 | 0.124 |
| 1-9-1-2-5(L) | 0.060 | 0.560 |
| 1-9-1-12(L) | 0.396 | 0.575 |
| 1-9-1-13-3(L) | 0.362 | 0.222 |
| 1-9-1-5-5(L) | 0.154 | 0.473 |
| 1-9-1-5-4(L) | 0.254 | 0.549 |
| 1-9-1-5(L) | 0.362 | 0.319 |
| 1-9-1-4(L) | 0.386 | 0.870 |
| 1-9-1-11(L) | 0.266 | 1.027 |
| 1-9-1-7-4(L) | 0.665 | 1.579 |
| 1-9-1-1-3(L) | 0.285 | 1.956 |
| 1-9-1-1-4(L) | 0.152 | 2.164 |
| 1-9-1-13-4(L) | 0.468 | 3.116 |

Anthocyanin content is given as total O.D. at 530 nm. Data for subclones at the end of a clonal analysis are the average of duplicate samples from a single flask. Data for subclones during examination by precursor feeding are the average of triplicate samples from a single flask.

low-accumulating subclones tend to show an increase in anthocyanin levels, while the high-accumulating subclones tend to show a drop in pigment accumulation.

It should also be noted that DMSO alone often had an effect on anthocyanin levels of the various clones and subclones, resulting in measurable increases in anthocyanin levels. This effect of DMSO on anthocyanin synthesis has been reported in previous work (Dougall and Vogelien, 1987; Hinderer et al, 1984). Regardless of the effect, stimulations in anthocyanin accumulation are still detectable from the addition of coumarate, naringenin and dihydroquercetin.

The data in Tables 3 and 4 (pgs.42 and 48, respectively) clearly indicate that there are differences between high- and low-yielding subclones/clones in the ability to metabolize coumarate, naringenin and dihydroquercetin into anthocyanin. Low-yielding clones from WC-63 and subclones from 2-2 and 1-9-1 were obtained that were non-responsive to all three precursors, while precursor feeding resulted in increased anthocyanin levels in high-yielding clones and subclones of WC-63, 2-2 and 1-9-1. In general, high-yielding 1-9-1 subclones display the greatest level of response, followed by high-yielding WC-63 clones and, lastly, high-yielding 2-2 subclones. In addition, a

wide range of responses to intermediate additions was observed in low-yielding 1-9-1 subclones, with those subclones possessing the higher anthocyanin contents displaying the greatest increase in anthocyanin. These findings indicate there is a relationship between anthocyanin content of a subclone and the ability to metabolize exogenous precursors into anthocyanin.

There are two possible explanations for this phenomenon. Since each subclone/clone arose from a single cell, it is possible that all the cells of an individual clonal population possess relatively the same metabolic ability to produce anthocyanin, and individual subclones/clones will have an anthocyanin level which is representive of that metabolic ability. Further, the level of metabolism in a clonal population determines the ability of the cells to metabolize exogenous supplies of anthocyanin precursors. Precursor additions to clonal populations with low levels of anthocyanin metabolism result in small increases in anthocyanin, while additions to clones with higher rates of metabolism result in greater increases in pigment content. An alternative explanation is that not all cells of a clonal population are producing anthocyanin. Instead, anthocyanin content of a clone is determined by the number of anthocyanin producing cells in the

population. In this case, the ability of a clone to metabolize exogenous supplies of anthocyanin precursors would be related to the number of producing cells in the population.

Cell counts were performed on four low-yielding and two high-yielding 1-9-1 subclones previously examined in precursor feeding experiments and the percentage of red cells in the population determined. In addition, cell counts were performed on several subclones from each of three different clonal analyses immediately upon completion of the cloning process. Microscopic examination of several suspensions revealed that the cultures consisted of mixtures of accumulating and non-accumulating cell types in clumps of various sizes. Suspensions were filtered through a 44 um screen and a cell count performed on the filtrate, which consisted of single cells and small cell clumps. The results given in Table 6 show a strong correlation between anthocyanin content and the % of red cells in the population (correlation coefficient: 0.749, 0.05 alpha). It should be noted that cells were counted as accumulating or non-accumulating purely on the visible detection of a pink/purple color (anthocyanin), and no attempt was made to score the intensity of the color. It is entirely possible that some cells counted as non-

Table 6. Anthocyanin content and % of red cells in WC63-1-9-1 subclones.

| Subclone | O.D. at 530 nm | % Red Cells in Population |
|---|---|---|
| 1-9-1-10-1 | 2.413 | 33 |
| 1-9-1-10-1-4 | 0.059 | 3 |
| 1-9-1-10-1-19 | 0.277 | 18 |
| 1-9-1-10-1-23 | 0.953 | 34 |
| 1-9-1-10-1-8 | 3.754 | 50 |
| 1-9-1-15-1 | 0.414 | 24 |
| 1-9-1-15-1-27 | 0.046 | 1 |
| 1-9-1-15-1-13 | 0.402 | 19 |
| 1-9-1-15-1-4 | 1.131 | 35 |
| 1-9-1-15-1-22 | 1.446 | 39 |
| 1-9-1-6-4 | 0.115 | 2 |
| 1-9-1-10-3 | 0.061 | 6 |
| 1-9-1-15-3 | 0.143 | 19 |
| 1-9-1-5-3 | 0.047 | 3 |
| 1-9-1-5-1 | 0.559 | 48 |
| 1-9-1-2-2 | 1.898 | 56 |
| 1-9-1-5-1-2 | 0.267 | 18 |
| 1-9-1-5-1-3 | 0.470 | 35 |
| 1-9-1-5-1-5 | 0.667 | 37 |
| 1-9-1-5-1-30 | 0.885 | 58 |
| 1-9-1-5-1-1 | 1.259 | 50 |
| 1-9-1-5-1-24 | 1.824 | 50 |
| 1-9-1-5-1-9 | 1.850 | 55 |

Anthocyanin content, given as O.D. at 530 nm, is the average of triplicate samples from a single flask. Cell suspensions were filtered through a 44 um screen, and a cell count performed on the filtrate. Cells were counted as accumulating or non-accumulating based on the presence or absence of a red/purple color within the cell, respectively. Two cell counts were performed on each subclone filtrate. Values for % of red cells in population were calculated on the basis of the total number of cells counted.

accumulating are in fact producing anthocyanin, but at a level below detection of the naked eye. In any event, it is clear that clonal populations are made up of a mixture of accumulating and non-accumulating cell types. It is not possible from this study to discern if only one accumulating phenotype exists in a clonal population. However, a visual difference in color intensity was noted between accumulating cells from different subclones. In general, a lower color intensity was observed in accumulating cells of subclones with lower anthocyanin contents than in the high-yielding subclones.

Several low-yielding 1-9-1 subclones were reexamined after extended maintenance by serial subculture to see if they would maintain their characteristic response to precursor feeding. The results are shown in Table 7. Three of four non-responsive subclones examined continued to show no response to precursor feeding (1-9-1-6, -6-4, and -10-3), while the fourth (1-9-1-15-3) gave a small increase in anthocyanin content in response to dihydroquercetin. Subclone 1-9-1-15 continued to give small increases in anthocyanin levels with precursor additions. Two subclones, however, gave altered responses to precursor feeding. Subclone 1-9-1-10, previously showing small

Table 7. Anthocyanin content of selected 1-9-1 low-yielding subclones in response to precursor feeding upon maintenance in culture.

| Subclone | Time in Culture (weeks) | Anthocyanin Content as O.D./ mg/ml | Increase in Anthocyanin Due to Intermediate | | |
|---|---|---|---|---|---|
| | | control | Coum. | Nar. | DHQ |
| 1-9-1-6 | 4 | 0.033 | -0.003 | 0.002 | -0.001 |
| | 21 | 0.105 | -0.007 | 0.026 | 0.020 |
| | 36 | 0.051 | -0.007 | 0.014 | 0.000 |
| 1-9-1-6-4 | 5 | 0.021 | 0.003 | 0.001 | -0.001 |
| | 22 | 0.033 | 0.006 | 0.004 | 0.003 |
| 1-9-1-10-3 | 5 | 0.032 | 0.003 | 0.004 | 0.004 |
| | 20 | 0.054 | -0.004 | 0.005 | 0.005 |
| 1-9-1-15-3 | 5 | 0.056 | 0.002 | 0.015 | 0.016 |
| | 18 | 0.067 | -0.004 | 0.011 | 0.018 |
| 1-9-1-15 | 6 | 0.094 | -0.021 | 0.030 | 0.014 |
| | 11 | 0.080 | -0.008 | 0.031 | 0.013 |
| | 28 | 0.077 | 0.007 | 0.024 | 0.063 |
| 1-9-1-10 | 8 | 0.434 | -0.003 | 0.017 | 0.082 |
| | 13 | 0.573 | 0.042 | 0.141 | 0.158 |
| | 28 | 1.081 | -0.052 | 0.169 | 0.749 |
| 1-9-1-12 | 13 | 0.263 | -0.053 | 0.156 | 0.142 |
| | 30 | 0.168 | -0.050 | 0.044 | 0.075 |

Anthocyanin precursors were dissolved in DMSO and added to cultures on days 4 and 8 of the culture period. Anthocyanin content and growth (as extracted dry weight) were determined at day 12. Data are the average of triplicate samples from single flasks. Anthocyanin content of the control (1% DMSO) is given, while precursor effect on anthocyanin content is given as the increase in anthocyanin above the control. Final concentrations: 0.2 mM coumarate, 0.1 mM naringenin, 0.25 mM dihydroquercetin, 1% DMSO.

increases in anthocyanin levels, displayed increasing responsiveness to precursor feedings upon time in culture, while 1-9-1-12 gave a lower level of response. These results suggest that low-accumulating subclones which are non-responsive are fairly stable with respect to anthocyanin production and the ability to metabolize coumarate, naringenin and dihydroquercetin into anthocyanin. If it is true that the ability to respond to precursor feeding is a function of the number of accumulating cells in the population, this would suggest that the non-responsive/non-accumulating cell phenotype is fairly stable.

# VI. DISCUSSION

There are several possible causes for a loss or drop in production of a cell metabolite. In general, the block could have a genetic basis, e.g. a mutation, or it could be caused by a physiological block. In any case the block can be identified by feeding the appropriate compound(s). Although the nature of the block in low or non-anthocyanin accumulating wild carrot cultures is not known, it should be possible to determine its location by supplementation experiments. The pathway of anthocyanin biosynthesis involves several steps. A good deal of time and expense would be required in an attempt to examine each intermediate in supplementation experiments, and some of the intermediates are not commercially available. Thus, an early, middle and late intermediate in anthocyanin metabolism (coumarate, naringenin and dihydroquercetin, respectively) were used in supplementation experiments to locate the general level of the impairment in biosynthesis in low-accumulating cultures. Supplementation experiments using naringenin, dihydroquercetin and other flavanoid compounds have been reported with cell suspension cultures of Daucus carota L. in an attempt to locate a physiological block in

anthocyanin accumulation induced by the addition of gibberellic acid (Hinderer et al., 1984).

The initial step of this study involved confirming the ability of the wild carrot parental culture to use coumarate, naringenin and dihydroquercetin and to determine the concentration that can be supplied to cultures and result in a stimulation in anthocyanin biosynthesis while showing no adverse effect on growth. Precursors were added to suspension cultures also receiving PPA. PPA is a competitive inhibitor of phenylalanine ammonia-lyase (Sato et al., 1982), and thus will result in an impairment at the first step in the anthocyanin biosynthetic pathway. This cinnamic acid derivative can be used to inhibit anthocyanin accumulation in wild carrot cultures (Dougall and Vogelien, 1987). Any increase in anthocyanin content by PPA inhibited cultures upon addition of a precursor would confirm the ability of the culture to metabolize the precursor.

It is possible to restore anthocyanin biosynthesis in PPA inhibited WC-63 cultures by adding coumarate (Fig. 4, pg. 31), naringenin (Fig. 5, pg. 32) or dihydroquercetin (Fig. 6, pg. 33) to the culture medium. Each precursor has an optimum concentration that can be used to achieve reversal of PPA induced

inhibition. In addition, supplementation is very effective with naringenin and dihydroquercetin, while only a small stimulation in anthocyanin levels are detected with coumarate. This might be due to the position of coumarate, naringenin and dihydroquercetin in the pathway. Coumarate, an early precursor in anthocyanin biosynthesis, may be channeled to other metabolic pathways, such as lignin biosynthesis or the production of flavonoid compounds other than anthocyanin. Intermediates entering the pathway at central or later stages, such as naringenin and dihydroquercetin, have less opportunity to be diverted to other pathways and are more "committed" to the production of anthocyanin. Another explanation for this observation could be the preferential use of endogenous pools of coumarate due to the functioning of enzyme complexes in phenylpropanoid metabolism. Hrazdina and Wagner (1985) have demonstrated the channeling of substrates in early phenylpropanoid biosynthesis and found that many of the enzymes involved in phenylpropanoid and flavonoid biosynthesis are associated with endoplasmic reticulum membranes. They further hypothesize that enzymes involved in phenylpropanoid and flavonoid biosynthesis exist as an endoplasmic reticulum-associated enzyme complex, such

that products are channeled through part of, and possibly the entire, pathway. The existence of multienzyme complexes in phenylpropanoid metabolism suggest preferential use of endogenous metabolites and could conceivably make it difficult for cells to incorporate exogenously supplied intermediates, such as coumarate.

The results from supplementation experiments with various high and low anthocyanin accumulating clones and subclones reveal some important information concerning anthocyanin metabolism in these two culture phenotypes. In general, low-accumulating clones and subclones with extremely low anthocyanin levels (O.D. < 0.060) are unable to metabolize exogenously supplied coumarate, naringenin or dihydroquercetin into anthocyanin (Table 3, pg. 42). Clones and subclones with the accumulating phenotype are able to metabolize all three anthocyanin precursors. However, a broad range of responses to precursor feedings are observed in the anthocyanin accumulating clones and subclones. The level of response in anthocyanin content due to precursor feeding appears to be related to the initial anthocyanin content of a culture, with those clones possessing the higher anthocyanin content displaying the

greatest increase in anthocyanin in response to intermediates.

The inability of low- or non- accumulating clones to respond to precursor feeding with coumarate, naringenin or dihydroquercetin suggests that the impediment in anthocyanin biosynthesis is located at a point beyond dihydroquercetin. The block in pigment accumulation could be due to the loss of an enzyme(s) involved in later steps of anthocyanin metabolism, such as dihydroflavonol 4-reductase, glycosyltransferases or methyltransferases. It is also possible that dihydroquercetin is being channeled to the production of other compounds, such as catechin or procyanidins (refer to Fig. 3, pg. 18). In addition, the possibility remains that the impairment in anthocyanin accumulation is beyond biosynthesis, at the level of transport of the pigment into the central vacuole. Work by Hopp and Seitz (1987) suggests that acylation of anthocyanin plays an important role in both transport and storage of the pigment in vacuoles, and that perhaps acylated anthocyanin is transported to the vacuole via a selective carrier.

Available information suggests that the enzymes of phenylpropanoid and early flavonoid biosynthesis are present in both accumulating and non-accumulating

cultures. Cheng et al. (1985) examined 4-coumarate:CoA ligase from high and low anthocyanin accumulating wild carrot subclones and concluded that no difference in the ligase existed between these subclones which could account for the difference in anthocyanin accumulation. Further, the authors found no evidence of isozymes, which could potentially be responsible for directing 4-coumarate:CoA to flavonoid biosynthesis, lignin biosynthesis or other pathways. Twelve 1-9-1 subclones, previously examined by supplementation experiments in this study, were examined for the presence and activity of chalcone synthase. Not only is the synthase present in all twelve clones, but no correlation between anthocyanin content and chalcone synthase activity is found amongst non-, low- and high- responsive subclones (G. Hrazdina and S. Reeves, Personal Communication). The preceeding information and the findings from this study strongly suggest that attention should be focused on latter stages of anthocyanin biosynthesis and the transport mechanism of the pigment into the central vacuole.

A broad range of responses in anthocyanin content were observed from precursor feeding experiments among the fifty clones and subclones examined. The level or degree of response by a clone/subclone appears

to be proportional to the initial anthocyanin content of the clonal population. Subclones possessing high anthocyanin levels (initial O.D. > 1.000) display the greatest increase in pigment levels upon precursor feeding (refer to Table 3, pg. 42). Non-responsive clones and subclones (increases in O.D < 0.015), however, have extremely low anthocyanin levels (initial O.D. of < 0.060), while those clones with moderate pigment levels display a correspondingly moderate increase in anthocyanin. Microscopic examination of several selected subclones reveal a strong correlation between anthocyanin content of the culture and the % of red cells in the population (Table 6, pg. 56). Together these observations suggest that the level of response to precursor feedings is, at least in part, related to the % of red cells, or accumulating cells, in the population.

Due to the nature of the supplementation experiments and microscopic examinations it is not possible to discern if all accumulating cells in a clonal population have or display the same capacity for accumulating anthocyanin. At this point the anthocyanin content displayed by a culture appears to be determined by the % of accumulating cells in a population, and

perhaps to a lesser extent by the capacity(s) of the accumulating cells in the population.

Since a clone/subclone is assumed to be the progeny of a single cell, one would expect all cells of a new or young clonal population to display the same phenotype, either all accumulating or non-accumulating. Simple microscopic examinations demonstrate that clones/subclones are not pure populations of accumulating or non-accumulating cells (Table 6, pg. 56). A cell population arising from two or more individual cells, one(s)accumulating and the other(s) non-accumulating, would likely consist of a mixture of accumulating and non-accumulating cells. The two states of anthocyanin accumulation are heritable and potentially reversible (Meins, 1983). Thus, clones may indeed be the progeny of a single cell, but contain cells converting from one state to the other during the time required for cloning and analysis.

No information is available describing the interconversion frequency of the two states of anthocyanin accumulation. Results from this study show that the low-accumulating subclones which are non-responsive to precursor feeding also display relatively stable levels of anthocyanin during time in culture (Table 7, pg. 58), suggesting that the non-

accumulating/non-responsive phenotype is fairly stable. Such a hypothesis would explain the gradual decrease in anthocyanin levels observed in high-accumulating clones during prolonged maintenance in culture.

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