ABSTRACT

MOLECULAR BASIS OF PIGMENTATION IN CROTON PLANTS

by

Delia K. Gutman

The striking pigmentation patterns observed in Codiaeum variegatum, commonly called croton plants, was studied at the molecular level. By hybridization experiments, we demonstrated that well known transposable genetic elements isolated from other plants systems, such as maize and snapdragon, do not control this variegation phenomenon in croton plants. The chalcone synthase gene which controls the formation of anthocyanin pigments, is present as a single copy gene in the croton genome. Northern blot experiments indicate that there is a difference in the expression of this gene in leaves located at the bottom of the plant vs. those located at the top. A genomic DNA library was constructed to perform future studies that could help to further understand variegation in crotons, as well as other plant systems.

# MOLECULAR BASIS OF PIGMENTATION IN CROTON PLANTS

by

Delia K. Gutman

A thesis submitted in partial fulfillment of

requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGY

at

FLORIDA INTERNATIONAL UNIVERSITY

Committee in charge:

Professor Case K. Okubo, Chairperson

Professor Martin Tracey

Professor Jennifer Richards

April 1989

To Professors: Case K. Okubo
Martin Tracey
Jennifer Richards

This thesis, having been approved in respect to form and mechanical execution, is referred to you for judgement upon its substantial merit.

Dean James A. Mau
College of Arts and Sciences

The thesis of Delia K. Gutman is approved.

Professor Jennifer Richards

Professor Martin Tracey

Major Professor, Case K. Okubo

Date of Examination: December 1, 1986

# MOLECULAR BASIS OF PIGMENTATION IN CROTON PLANTS

by

Delia K. Gutman

A thesis submitted in partial fulfillment of the

requirements for the degree of

MASTER OF SCIENCE

in

BIOLOGY

at

FLORIDA INTERNATIONAL UNIVERSITY

1989

ACKNOWLEDGMENTS

My deepest gratitude is extended to my good friend Dr. Case K. Okubo who as my major professor supported me with his enormous help and valuable advice during the course of this research. I am also very grateful to the other members of my committee, Dr. Martin Tracey and Dr. Jennifer Richards.

I would like to thank my husband Marcelo and my daughter Ariella for their constant encouragement and immeasurable support.

TABLE OF CONTENTS

## LIST OF TABLES

LIST OF FIGURES

# CHAPTER I

# INTRODUCTION

## The Phenomenon of Pigment Variegation in Plants.

The focal point of the research presented in this thesis is pigment variegation in plants resulting from genetic alterations implemented by transposable elements. Pigment variegation, the striped, spotted, or mosaic appearance of some individuals, has long been a source of fascination (Nevers et al., 1986). As early as 1588, multicolored and variegated kernels of maize from the New World were described by Jacob Theodor von Bergazabern, and variegation in tulips become almost a craze in the seventeenth century in the Netherlands (Nevers, et al. 1986). In plants the phenomenon of variegation is generally recognized as irregularities in pigment patterns on leaves, stems, flowers and seeds; although other characteristic such as leaf form, flower form or starch content (in maize kernels) may also be variegated. On a single variegated plant the offshoot could be all green or all white or any combination of these two. In some plants the white may or not be flushed with pink or red, or it may even be purple from the purple pigment anthocyanin (Nevers et al., 1986).

Many hypotheses have been postulated about the cause of variegation. Some of the most common cases involve no heritable changes in the different tissues of the plant and are due to an external factor which operates at random on the organism. The most common example is the variegation caused by viral infection. The cucumber mosaic virus (CMV), for example, has a broad natural host range throughout the temperate regions of the world. It

infects cereals, forages, woody and herbaceous ornamentals, vegetables, and fruit crops (Kaper and Waterworth, 1982). The most common symptom elicited by CMV is mosaic (yellow or white blotches on leaves). Severity of the disease, however, may range from no obvious symptoms in some crops to death of the host species (Kaper and Waterworth, 1982). In the latter case the external agent directly destroys tissues and the variegation persists only as long as the virus is active in the organism.

Nonhereditary variegation can also be caused by mineral deficiencies or from lack of proper nutrition at some difficult period involving food and water intake (Nevers et al., 1986). Such mutations are often seen in soils when there is improper balance between iron and manganese or lime and magnesium. Extremes of acidity or alkalinity in the soil, also contribute to this phenomenon, as well as high concentration of aluminum or deficiencies of zinc or molybdenum.

More interesting than mosaic patterns due to random effects of an environmental factor are those variegation patterns which are heritable. Because plants contain three different genomes (the chloroplast, the mitochondrion, and the nuclear), mutations that lead to a variegated phenotype can occur in any one of these organelles. Many examples of green/white variegation patterns that arise from chloroplast defects in some plant cells have been compiled by Kirk and Tilney-Bassett (1978).

Plastid mutations or plastid variegations are believed to arise in the following ways (Rhoades, 1946):

1) Two distinct types of plastid are present in the cytoplasm of the fertilized

egg and then undergo somatic segregation.

2) One type of plastid is present in the egg, but changes in plastid phenotype occur during ontogeny. These changes may be due to:
   a) plastid mutations, either induced or spontaneous
   b) varying degrees of plastid development in response to the different physiological conditions found in diverse tissues.

Plastid mutations can be identified on the basis of the following criteria (Kirk and Tilney-Bassett, 1978): 1) Segregation of the trait in crosses is non-Mendelian. 2) Inheritance is maternal, as demonstrated by nonreciprocity in crosses. 3) Cells with defective and nondefective chloroplasts can be observed. 4) The distribution of cells without color corresponds to what is expected by random sorting out of chloroplasts during cell division. These criteria were first applied in Miriabilis jalapa (Corens, 1909), Antirrihinum (Bauer, 1909, Primula (Gregory, 1915), and maize (Anderson, 1923 and Demerec, 1927), and more recently in Dianthus and Euphorbia (Stewart, 1965).

In the first decades of this century R.A. Emerson of Cornell University studied a type of variegation present in maize strains sold as Indian corn. This variegation is caused by mutations interfering with the synthesis of a red-orange pigment in the kernel pericarp. Unlike most mutations, this one was so unstable that it reverted many times in the course of each kernel's development, giving rise to alternating streaks of pigmented and unpigmented cells. This was the first published account of an unstable locus in maize (Emerson, 1914).

In the 1930's Marcus M. Rhoades provided evidence to suggest that

genetic instability in maize can be conditional. What appears to be a stable mutation can suddenly become unstable in the presence of a particular gene (Federoff, 1983). Rhoades studied the mutation that disrupts the synthesis of a purple pigment in the aleurone, rendering the aleurone colorless. He showed that when a particular gene is present in the cell, the mutations can revert, giving spots of purple pigment in the colorless aleurone (Federoff, 1983).

It was Barbara McClintock who first showed in 1947 that these instabilities were caused by transposable units that were associated with the loci in question. Her first report showing that a genetic locus could move was a troubling oddity, and for many years no one knew what to make of it. Today her work is recognized as one of the most important genetic discoveries of the century and in 1982 she was awarded the Nobel Prize in Physiology or Medicine.

McClintock proposed that discrete, transposable genetic elements, which she called "controlling elements", are capable of influencing the expression of a given locus when integrated in or next to it (McClintock, 1950a, 1956). The locus in which she first observed a transposition involved a chromosomal breakage, or dissociation. She called this the Dissociation locus or Ds. Although Ds is the site of breakage, it was not itself responsible for breakage, since it was later shown that dissociation only occurs if another locus is present.

McClintock called this second locus Activator, or Ac, for its ability to activate breakage at the Ds locus. Ac acted like a conventional locus most of the time, but in some instances it disappeared or moved to a new position on

either the same or a different chromosome. The Ds locus could transpose too, but just as it was incapable by itself of breaking the chromosome on which it resided, so it was unable to transpose in the absence of Ac. McClintock came to understand that Ac can move autonomously but Ds could only move when it is activated by Ac. (Federoff et al., 1983). Another element that has been thoroughly studied is the Spm group of transposable elements. This element was discovered in the 1950's by McClintock (McClintock, 1956), who called it the suppressor-mutator (Spm) element, and by Peter A. Peterson (Peterson, 1965) who coined the name Enhancer element. The basic behavior of Spm resembles that of Ac and Ds. These elements have provided molecular biologists with the tools necessary to study the mechanisms by which genes are expressed during different stages of differentiation and development (Federoff, 1983).

In the decades between the genetic identification of Ac and Ds and their molecular isolation, transposable elements were discovered in other organisms and the basis of their transposition began to be understood. In the mid-1960's the first indication of transposable elements in bacteria came to light when certain peculiar mutations in Escherichia coli were found to be the result, not of small changes in the sequence of nucleotides, but of the insertion in a gene of a sizable piece of foreign DNA (Cohen and Shapiro, 1980). Multiple drug resistance in bacteria is mediated by these mobile genetic elements. By the mid-1970's it was discovered that the antibiotic-resistance genes are passengers on small mobile elements, called transposons, picked up by plasmids (small circular molecules of bacterial DNA) as they traveled from cell to cell (Cohen

and Shapiro, 1980).

Bacterial transposons have been analyzed in detail. One such transposon is Tn3. It is about 5,000 base pairs (bp) and carries two genes required for transposition; while the third gene is the passenger gene encoding beta-lactamase, an enzyme which makes bacterial cells resistant to the antibiotic ampicillin (Federoff, 1983) and which is flanked at both ends by inverted repeat sequences (Calos and Miller, 1980). The arrangement is such that the sequence at one end of the transposable DNA segment is complementary, in reverse order, to a sequence on the same strand at the other end of the element; this facilitates subsequent insertion. These inverted repeats are duplicated at the target site upon transposition. These structural features are typical of transposable elements found in prokaryotes, and eukaryotes (Nevers et al., 1986).

Transposable elements have also been isolated from a number of other organisms (Lambert et al., 1988). These elements have been discovered in Drosophila melanogaster (Rubin et al., 1981), in nematodes and in yeast (Cameron et al., 1980), where they have properties similar to bacterial transposons and also resemble the Ac and Ds elements of maize (Federoff, 1983). Transposable element-like events are believed to be involved in retrovirus genomic integration into host cell genomes, causing cancerous or transformational events (Varmus, 1982). With the dawn of recombinant DNA technology in the 1970's it became possible to isolate, as physical entities, the elements which McClintock, Peterson, and others had predicted as the source of instability of mutable alleles.

The strategy of isolation of these elements was straightforward. For example, a locus which encodes a known protein product and which is thought to contain a transposable element can be cloned in the following manner. By using appropriate copy or complementary DNA (cDNA) sequences obtained by reverse transcription of its mRNA, the genomic sequence of the locus can be isolated from wild-type plant gene libraries or by using antibodies, a particular genomic sequence can also be isolated from the gene library if the gene product is expressed (Nevers et al., 1986). This sequence can then be used to isolate the corresponding sequence from the mutant, which supposedly contains the transposable element. Table 1 summarizes most of the elements that have been isolated in this manner. After a particular transposon has been cloned, it can be used to isolate similar elements which are present at different positions in the genome by virtue of sequence homology. Another technique called transposon tagging can also be used to isolate transposable elements. It consists of causing a mutation in a particular locus with a transposable element for which a molecular probe exists. This procedure of transposon tagging has been carried out in prokaryotes for some time and similar attempts have been made in plant molecular genetics (Wienand et al., 1982).

Because these elements have the ability to cause mutations quite rapidly, their role in the evolution of organisms has come under study. The rearrangements that occur in connection with transposons may be instrumental in restricting the genome during the course of evolution (Nevers et al., 1986). The evolutionary role of transposable elements has been framed into two competing hypotheses:

TABLE I

Cloned Transposable Elements and Other Insertion Elements

(Nevers et al., 1986)

| Plant | Locus | Symbol | Protein | Allele | Element |
|---|---|---|---|---|---|
| Antirrhinum majus | nivea | niv | Chalcone synthase | niv-53<br>niv-43 | Tam1<br>Tam2 |
| Glycine max<br>Zea mays | lectin 1<br>alcohol dehydrogenase-1 | le1<br>adh1 | Seed lectin<br>alcohol dehydrogenase | le1<br>adh1-Fm355<br>adh1-53034<br>adh1-2F11 | Tgm1<br>Ds1<br>Mu1<br>Ds |
| | bronze | b2 | UDP glucose-Flavonoid Glucosyl transferase | b2-m2 | Ac |
| | shrunken | sh | Sucrose synthase | sh-m5933<br>sh-m6233<br>sh-5586 | Ds5933<br>Ds<br>Tz86 |
| | waxy | wx | UDP-glucose-starch glucosyl transferase | wx-m6<br>wx-m8<br>wx-m9<br>Acwx-m9 | Ds6<br>Spm-18<br>Ds9<br>Ac9 |
| | Line C1 | lc1 | Unknown | nf1 | Cin1-001 |

1) Mobile elements are sustained by direct selection acting on genetic variability.
2) Transposable elements need not provide selective advantage to the hosts that bear them in order to account for their presence. This is the popular "selfish-gene" theory. The element is treated as a chromosomal parasite that has been selected by its own ability to maintain itself independent of selection acting on the organism (Syvanen, 1984).

Another possible role for transposons is that they may be involved in control of normal development, because the sensitivity of some elements to changes in their physical and physiological environment is manifested as altered variegation patterns (McClintock, 1965). Finally, investigators foresee the use of transposable elements in genetic engineering of plants, particularly in monocotyledons which includes the cereals, one of our most important food plants. (Nevers et al., 1986).

Hybridization Studies of Croton DNA with Known Transposable Elements.

The ornamental plant Codiaeum variegatum (Brown, 1960), commonly named croton, is found throughout many recreational sites and parks in South Florida. Figure 1 shows the croton collection in front of the Owa Ehan Building at Florida International University. The striking pigmentation patterns, which include green leaves with yellow spots and purple leaves with yellow spots overlap with purple, are the subject of this investigation.

Figure 1: Croton Collection-Owa Ehan Building at Florida International University, Miami, Florida

The possibility that transposable elements cause these pigmentation patterns in the croton "Eleanor Roosevelt" (Fig. 2) was investigated. This possibility was tested by hybridization experiments utilizing croton DNA and known transposable elements (Ac-Spm) (Table II) cloned from corn plants. Because these transposable elements cause the variegation patterns in maize kernels and leaves, a positive hybridization signal would indicate that these elements are also present in the crotons, leading to the hypothesis that they cause the leaf variegation found in "Eleanor Roosevelt".

Figure 2: Croton Plant "Eleanor Roosevelt" at Owa Ehan Building, Florida International University, Miami, Florida

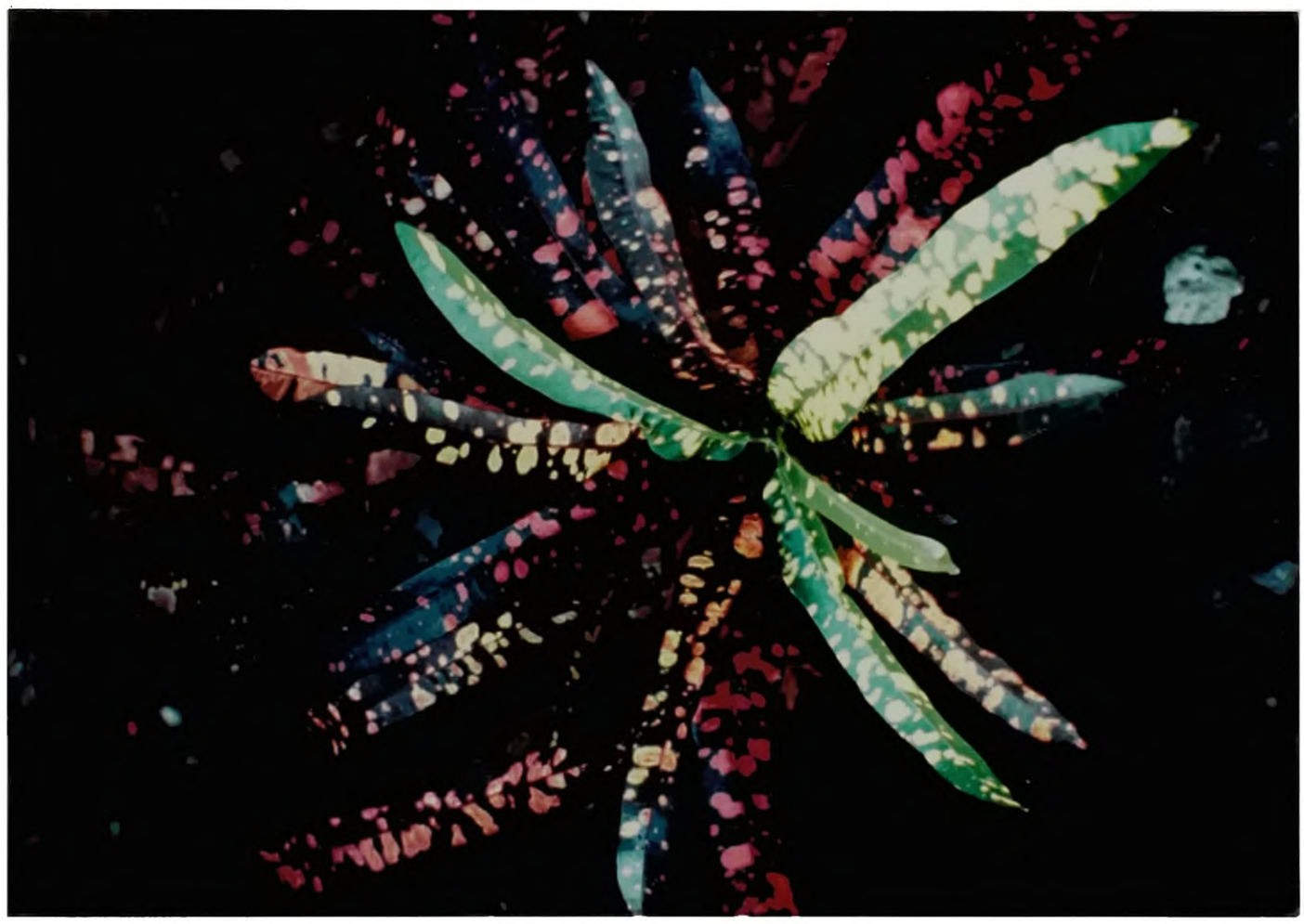


Table II

**DNA PROBES**

| Probe | pAc-9 | pUAG1 | pWx33 | pWx34 | pCA-1 |
|---|---|---|---|---|---|
| Size (bp) | 4300 | 1400 | 1100 | 1300 | 1200 |
| Cloning Vector | pKP322 | pBR322 | pUC9 | pUC9 | pBR322 |
| Cloning Site | pSTI | pSTI | pSTI | pSTI | pSTI |
| Antibiotic Resistance | Tet | Tet | Amp | Amp | Tet |
| Element/Gene | Activator | Mutator | Suppressor | Suppressor | Chalcone Synthase |

References: pAc-9, pUAG1 were kindly provided by Dr. Judy Strommer, Department of Genetics, University of Georgia, Athens, GA, U.S.A.

pWx33, pWx34, pCA-1 were gifts from Dr. Zussanne Schwarz-Sommer, Max Planck Institute, West Germany.

## MATERIALS AND METHODS

Plant DNA Extraction:

Total DNA from young (green, early stage of development) and old (purple, later stage of development) croton leaves was extracted by a modification of the method of Murray and Thompson (1981).

Ten grams of leaves were frozen in liquid nitrogen and ground to a fine powder in an electric coffee grinder (Moulinex, Regal model) pre-cooled with dry ice. The dry powder was placed in 18 ml of lysis buffer (Appendix 1) and 9 ml of distilled water. The slurry was gently mixed with a glass rod and an equal volume of chloroform-octanol (24:1) (Mallinkrodt AR) was added. The mixture was sharply inverted at least five times.

The sample was centrifuged (10,000 rpm, 10 min, 15$^{o}$C) in an SS-34 rotor in a Sorvall RC-5B refrigerated centrifuge (Sorvall Instruments, Wilmington, Del.). The viscous aqueous phase was removed with a wide bore Pasteur pipette and then heated at 65$^{o}$C for five to ten minutes to get rid of any chloroform residue. After treatment with 100 $\mu$g/ml of Proteinase K (Bethesda Research Labs) an equal volume of chloroform was added, and the sample was gently inverted 2 to 3 times before centrifugation (5,000 rpm, 5 min, 15$^{o}$C) in an SS-34 rotor.

The DNA was precipitated by adding an equal volume of precipitation buffer (Appendix I) to the aqueous phase. After twenty to thirty minutes at room temperature, the sample was respun (5000 rpm, 5 min, 15$^{o}$C). The pellet was dissolved in 2 ml of resuspension buffer (Appendix I) by gently rotating the tube or by heating at 55$^{o}$C if dissolution was difficult. The mixture was placed onto a 2.4 ml cushion of high CsCl solution (Bethesda Research Labs, Gaithersburg, Md.)

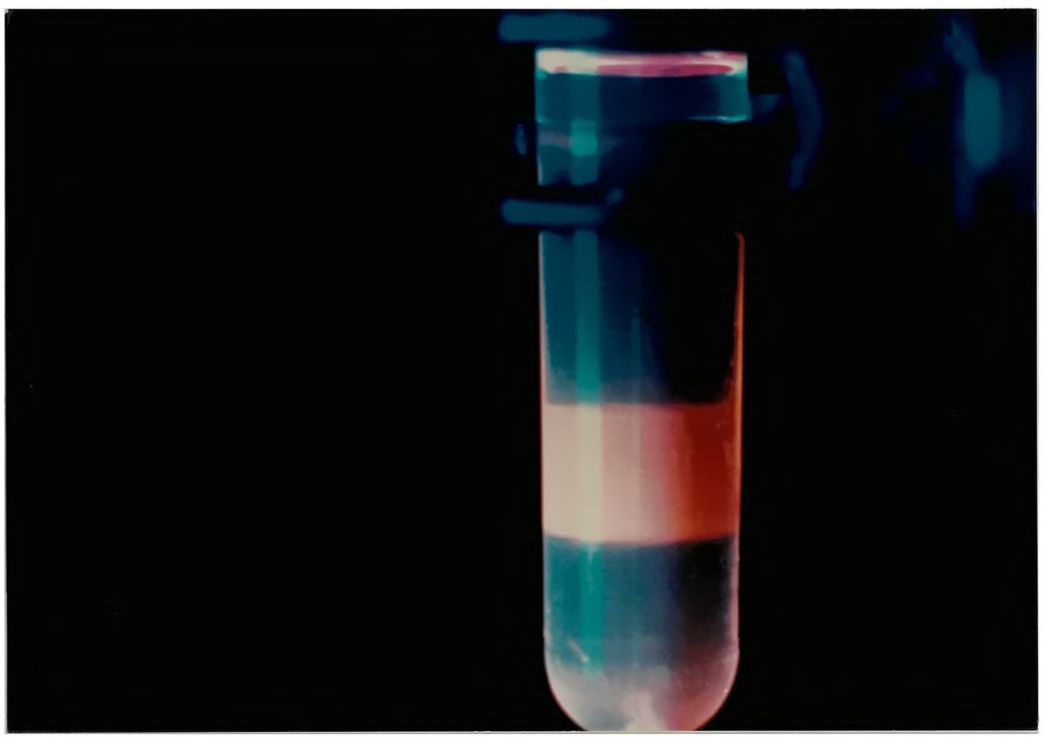
(Appendix I) and spun (45,000 rpm, 2 hr, 20°C) using the SW-65 rotor in the Beckman L8-70 ultracentrifuge (Beckman Instruments, Palo Alto, Ca.). The DNA band was observed under long wave ultraviolet light (Ultraviolet Products Inc.), and it was collected into a new centrifuge tube by side puncture of the tube using an 18 gauge needle. This tube was then filled with medium CsCl solution (Appendix I) and spun overnight (45,000 rpm, 20°C) in the SW-65 rotor. The thick fluorescent band of DNA (Fig. 3) was collected and the ethidium bromide (EtBr) was extracted four to five times with butanol saturated with NaCl (Appendix I). The plant DNA sample was dialyzed with a Spectra/Por 4 Cellulose dialysis tubing, MW cutoff 12,000-14,000 (Scientific Products) at 4°C against four changes of 1X TE buffer (Appendix I).

To determine the concentration and purity of the DNA sample, absorbance values at 260 and 280 nm of the dialyzed DNA were obtained in a Beckman Model 25 spectrophotometer (Beckman Instruments, Palo Alto, Ca.).

Dot Blot Experiments:

The technique of Kafatos et al. (1979) was followed to perform dot blot hybridization with the croton DNA and the known transposable elements from corn (Table II). Nitrocellulose filters (Schleicher & Schuell, Inc. BA85 - 0.45 $\mu$m Pore size) were first soaked in water followed by immersion in 1M $NH_4OAc$. The filters were then placed on a platform consisting, from bottom to top, of a clean glass plate covered with paper towel and moist 3MM paper (Whatman), so DNA can be applied more easily to the nitrocellulose paper.

Figure 3: The fluorescent band corresponding to "Eleanor Roosevelt" DNA stained with ethidium bromide and observed in a cesium chloride gradient.



In 10 μl of 1X TE buffer (Appendix I), 1 μg of probe (see Table 2) or plant DNA was denatured by adding one tenth volume 5M NaOH and incubating at room temperature for twenty minutes. After chilling on ice, the sample was neutralized by adding an equal volume of 2M $NH_4OAc$.

To fix the DNA onto the filter paper, samples were applied manually by holding a Rainin 100 μl micropipette vertically just above the filter paper. After each sample was spotted (two to three minutes), the individual spots were rinsed with 1 to 2 drops of 1M ammonium acetate, and the filters were washed in 4X SSC (standard saline citrate, Appendix I) and air dried for thirty minutes. The air dried filters were placed between two sheets of 3MM paper (Whatman) and baked in a vacuum oven at 80°C for two hours. The filters were stored in a vacuum dessicator for later use.

Radiolabeling of DNA by nick translation:

The nick translation reaction consisted of a 50 μl reaction mix which contained 25 μl of 2X nick translation buffer (Appendix I), 10 μl or 100 μCi of $dCTP^{32}$ (Deoxycytidine 5'-Triphosphate, tetra-Triethylammonium-salt, [$\alpha$-$^{32}$P] New England Nuclear; 3000 Ci/mmol,) and 1 μg of DNA. To nick the DNA, 1 μl of a 1/10,000 dilution of DNase (1 mg/ml stock) (Worthington Biochemicals, Freehold, N.J.) that was prepared in 1X RSB buffer, (Appendix I) and 1 μl (5 units) of DNA Polymerase I (5070 units; Cooper Biomedical) were added to the solution. After mixing, the solution was incubated in a 16°C waterbath for 90 minutes. The reaction was stopped by adding 50 μl to TE buffer (Appendix I) and 50 μl of distilled phenol (pH >7.6). After five minutes centrifugation in an Eppendorf microcentrifuge, the aqueous phase was removed with a drawn out Pasteur pipette

TE buffer (100 μl) was then added to the phenol phase and re-centrifuged. The aqueous phase was removed and pooled with the first sample making a final volume of 200 μl of nick translated DNA.

To determine the efficiency of incorporation of radioactive phoshorous a method which involves the precipitation of radiolabeled DNA with Trichloroacetic acid (TCA) was used (Maniatis et al., 1982). For total radioactivity in the sample 3 μl of a 1/100 dilution of the radioactive sample was spotted onto the center of a Whatman GF/C glass-fiber disc (2.4 cm diameter). To determine only the radioactivity incorporated into nick translated DNA 3 μl of the 1/100 dilution of the radioactive sample was added to a tube containing 50 μg of a solution of Herring sperm DNA (Sigma Chemical Co., St. Louis, Mo: 500 μg/ml in 20 mM EDTA) and then mixed with 5 ml of ice cold 10% Trichloroacetic acid solution. The tube was chilled on ice for fifteen minutes, and the precipitate was collected by filtering the solution through a GF/C glass-fiber disc. The filter was washed six times with 5 ml of ice-cold 10% TCA, followed by 5 ml of 95% ethanol.

As a control a third filter containing TCA only was employed. All the filters were dryed under a heat lamp and placed into scintillation vials and the radioactivity was counted in a liquid scintillation counter in a toluene-based scintillation fluid. The efficiency of incorporation of radioactive phosphorous was found to be 37%.

In order to separate the radioactive DNA from the unincorporated labeled dCTP column chromatography using Sephadex as support material was employed. Sephadex G-50 (30 grams) (Pharmacia) were mixed with 250 ml of 1X TE buffer and then autoclaved for fifteen minutes at 121$^{o}$C. The supernatant was decanted

and replaced with an equal volume of TE buffer pH 8.0. The resin was stored in a screw-capped bottle at 4°C. The Sephadex G-50 column was prepared in a disposable 10 ml borosilicate glass pipette plugged with sterile glass wool. This column was washed with several volumes of TE buffer. The DNA sample (200 µl) containing 10 µl of 4X stop buffer (Appendix I) was applied very slowly with a Pasteur pipette to the top of the column. A reservoir of TE buffer pH 8.0 was connected to the column so that the flow rate was about 0.5 ml/minute. The radioactive peaks were followed with a hand-held Mini-monitor g-m meter type 5.10 (Mini Instruments Ltd, Mt. Prospect, IL). The leading peak of radioactivity consisting of nick translated DNA was collected in fractions of approximately 0.5 ml each. Aliquots of 3 µl were counted with full window setting in a scintillation counter (LS 100C. Beckman Instruments, Palo Alto, Ca.). Radioactive fractions were stored at -20°C for later use.

Dot Blot and Southern Blot Hybridization:

The method used was that of Kafatos et al. (1979). The nitrocellulose filters, which contained the fixed denatured DNA were placed in a seal-a-meal bag (Sears type) with 10X Denhardt's solution, (Appendix I) and 4X SET (Appendix I). This mixture was shaken gently for at least 1 hour at room temperature. The volume of this solution was 10% of the filter area. After emptying the bag the prehybridization mixture (50% deionized formamide, 2X Denhardt's solution, 4X SET, 0.1% SDS, 100 µg/ml yeast RNA) was added and the sealed bags were incubated with gentle shaking at 42°C. After at least 1 hour, the nick translated probe which was denatured by boiling at 100°C for ten minutes and quick chilled on ice was added to the bag. The input of the $^{32}$P-labeled DNA probe was 1 X $10^6$ cpm for

DNA dot blots and 1-4 X $10^7$ cpm for Southern blots. After hybridization for 18-20 hours at 42°C, the hybridization fluid was discarded and the filters were washed twice in 2X SSC and 0.2% SDS for ten minutes each at room temperature followed by two washes in the same solution at 68°C for 20 minutes each. The filters were then rinsed in 2X SSC and air dried for at least one half hour. The dry filters were autoradiographed with XAR-5 X-OMAT X-ray film (Kodak) using an intensifying screen (Dupont-Lighting Plus) for three days at -70°C for DNA dot blots and five to seven days for Southern blots.

# RESULTS

## Dot Blot Hybridization

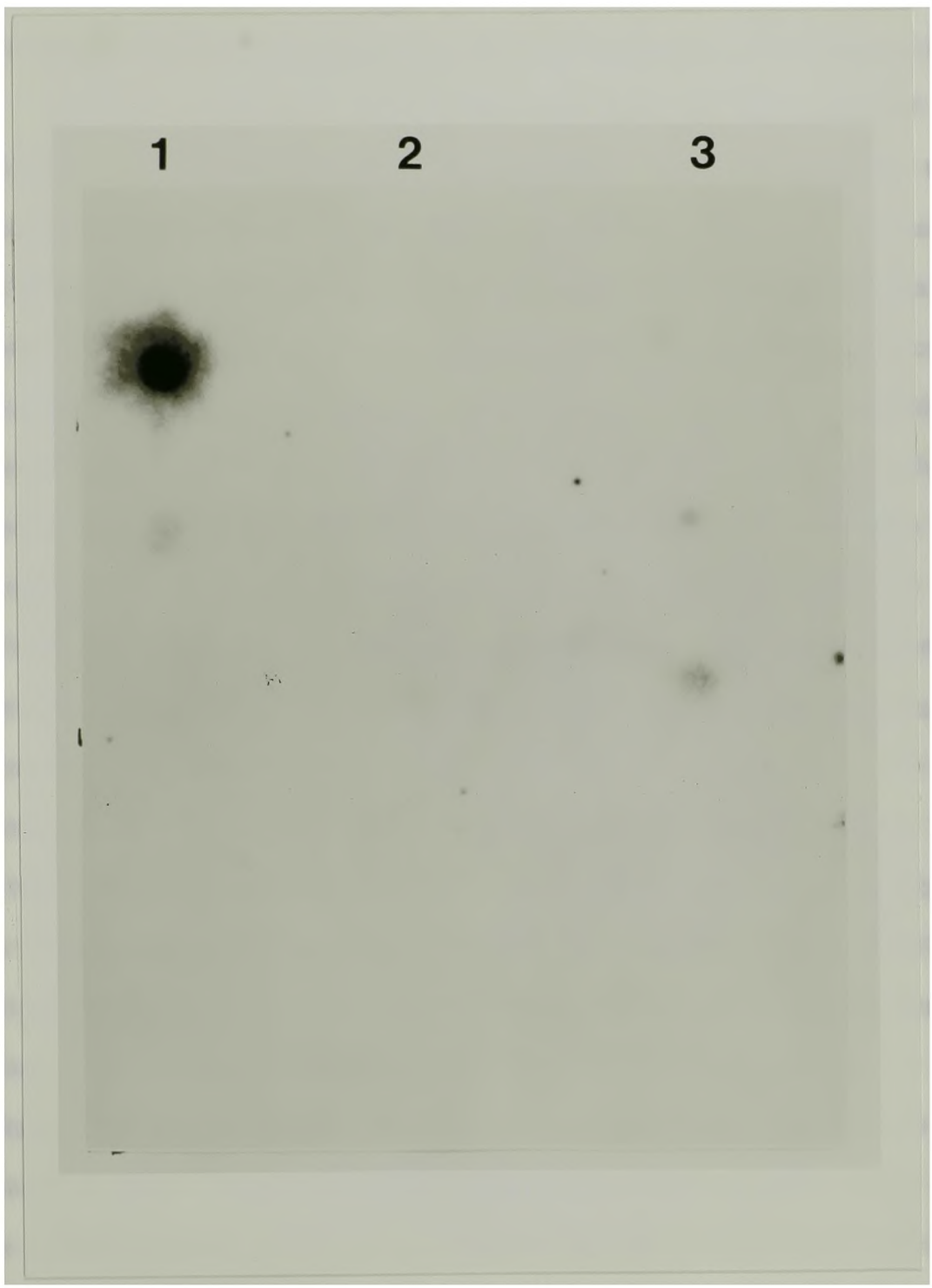
The DNA isolated from croton leaves was hybridized, using the dot blot technique to the radioactive probes which contain known transposable elements isolated from Zea mays (Table II).

At the hybridization and washing conditions described in materials and methods no homology of croton DNA to the activator transposable element (pAc-9, Table II) was observed (Fig. 4).

When pWx 34 (Table II) and pWx 33 (Table II) were employed as probes no hybridization signals were detected.

Figure 4: **Dot Blot Hybridization Experiments**

Lane 1 corresponds to the hybridization of pAc-9 DNA to 1 X $10^{-7}$ micrograms of pAc-9. Lane 2 from top to bottom corresponds to 1, 2 and 4 micrograms of croton DNA hybridized to pAc-9 probe. Lane 3 from top to bottom corresponds to 1, 2 and 4 micrograms of bacteriophage lambda DNA hybridized to pAc-9.


1
2
3

## DISCUSSION

The experiments performed in this research were undertaken to better understand the molecular mechanisms by which variegation occurs in croton leaves.

Discrete, transposable elements are responsible for some cases of variegation in maize. They influence the expression of a given locus when these elements integrate into or next to that locus (McClintock, 1950 a,b). When transposable elements from maize were hybridized to croton DNA, no hybridization occurred. If such elements existed in crotons, experiments to characterize them would have been possible. This negative outcome, however, does not preclude the possibility of some other transposable element(s) being involved in controlling the variegation mechanism in croton. Many other transposable elements have been isolated from other plants (Table I), any one of these might be homologous to croton DNA. There are a number of reasons to predict that transposable elements might be involved in these sudden color changes. For example in Drosophila melanogaster, one half of the moderately repetitive DNA in the genome (5 to 10% of the whole genome) is comprised of about 30 families of transposable elements (Rubin et al, 1981). In other words, nearly all of the middle repeat fraction of Drosophila DNA is transposable elements. It is tempting to extrapolate this information to higher plants. Their haploid DNA content ranges over two orders of magnitude among various plant species (Cavalier-Smith T., 1978). Higher plants also have a large fraction of middle repetitive DNA (Flavell et al., 1982). Maize has a DNA content about 30 times that of Drosophila melanogaster, and over 30% of the maize genome is middle repetitive (Hake et al., 1980). The Cin 1 DNA insertion element of Zea mays has structural characteristics of transposable elements (Shepherd et al., 1984),

and it also belongs to the middle repetitive class of DNA from this organism (Gupta et al., 1984).

Restriction enzyme digests of croton DNA offer good evidence for the presence of repetitive sequences in the genome of these plants. For example, repetitive sequences are visualized as discrete bands when digested DNA from crotons was run on agarose gels stained with ethidium bromide (Fig. 5). These repetitive sequences are possible middle repetitive sequences, and transposable elements belong to this middle repetitive class of DNA.

A feasible method for detecting transposable elements in the croton genome is to search for middle repetitive signals. Such experiments can be conducted by plaque hybridization of the gene library from croton DNA and probing with the radiolabeled croton DNA. The detection of intermediate strength hybridization signals, which should correspond to DNA segments of the middle repetitive class can therefore be screened to determine whether they are transposable elements or not.

Another procedure that can be tested in a future project uses the partial digestion of the DNA to 1-10 kb fragments, followed by a denaturation and then a renaturation of the DNA to detect low Cot number DNA sequences. This method produces snapback DNA, which are DNA fragments that contain inverted repeat sequences at their termini (Federoff et al., 1983). Such sequences are a typical feature of transposable elements. These fragments can thus be cloned and radiolabeled as probes for hybridization to Southern blots of genomic DNA's from related plants. They may also be used as probes in the genomic croton library (K. McElfresh and J. Strommer, personal communication).

Figure 5: "Eleanor Roosevelt" DNA digested with the following restriction endonucleases: Lane 1, Bacteriophage Lambda DNA/Hind III used as molecular weight standard. Lane 2, BamHI; Lane 3, HindIII; Lane 4, MboI; Lane 5, PstI; Lane 6, EcoRI.

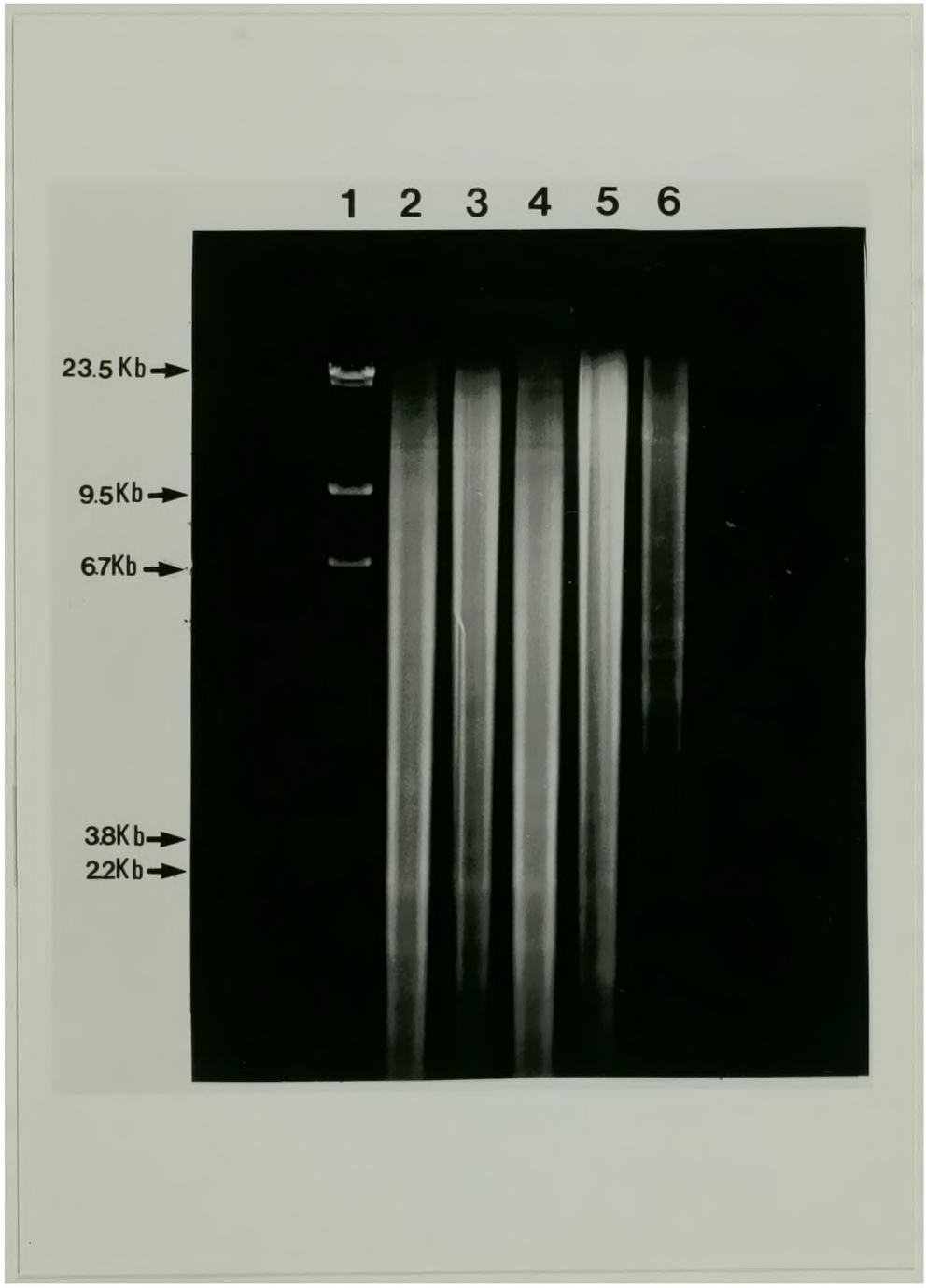
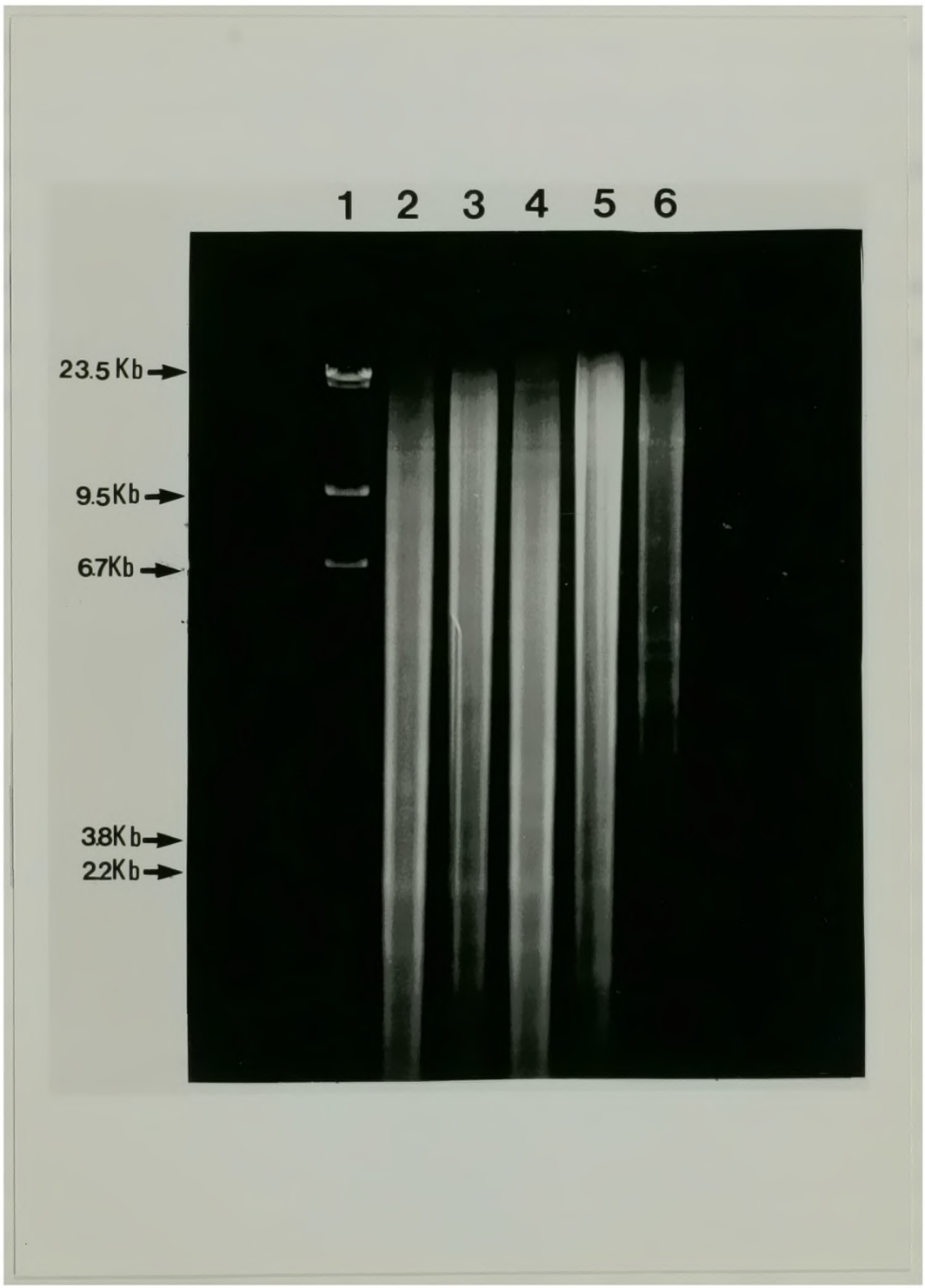

1 2 3 4 5 6
23.5 Kb
9.5Kb
6.7Kb
3.8Kb
2.2Kb

Figure 5: "Eleanor Roosevelt" DNA digested with the following restriction endonucleases: Lane 1, Bacteriophage Lambda DNA/Hind III used as molecular weight standard. Lane 2, BamHI; Lane 3, HindIII; Lane 4, MboI; Lane 5, PstI; Lane 6, EcoRI.


1 2 3 4 5 6
23.5 Kb
9.5Kb
6.7Kb
3.8Kb
2.2Kb

Genetic studies could demonstrate that the variegated phenotype is due to a mutation of the nuclear genome. To prove this, segregation of the variegated trait must be analyzed in crosses. In the most common case, integration of a transposable element at a locus causes a mutation that is recessive to wild type and exhibits monoallelic segregation (Nevers et al., 1986). Segregation ratios also indicate whether one, two, or more nuclear genes are involved in producing the variegated phenotype. A factor contributing to this variegation phenomenon factor may be a mutable allele that reverts to wild type which may be due to the presence of a transposable element (Nevers et al., 1986).

# CHAPTER II

## INTRODUCTION

### The Chalcone Synthase Gene in Croton Plants:

Light is one of the most important effectors for differentiation and development in higher plants (Mohr and Schafer, 1983). Three different sensory pigments are known to control the differentiation processes induced by light in higher plants (Kaulen et al., 1986): phytochrome (red/far red), cryptochrome (blue light), which seems to be a flavoprotein and U.V.-B photoreceptor (290 nm).

The induction of flavonoid biosynthesis is one of the most intensively studied regulatory effects of light on specific metabolic pathways (Kaulen et al., 1986). Flavonoids constitute one of the most abundant classes of phenolic compounds in higher plants and serve important functions as flower pigments, antimicrobial agents (phytoalexins) and UV-protective compounds (Kaulen et al., 1986).

The enzyme chalcone synthase catalyzes the stepwise condensation of three acetate units from malonyl-CoA with 4-coumaryl-CoA to give naringenin chalcone (Hiller and Hahlbrock, 1980). This enzyme, which is involved in the production of anthocyanin pigments, was first isolated from parsley (Petroselium hortense) (Heller and Hahlbrock, 1980), and consists of two Mr 42,000 subunits that are coded for by mRNA of approximately 1700 nucleotides (Kreuzaler et al., 1979). The gene was cloned by preparing DNA complementary to chalcone synthase mRNA's that were obtained from U.V.-irradiated cell suspension cultures of parsley (Kreuzaler et al., 1983). This cDNA was used as a probe to obtain several genomic clones containing the chalcone synthase structural gene (wild type) of Zea mays and Antirrhinum majus (snapdragon) (Wienand et al., 1982). These clones were used to clone the

Tam1 transposable element of Antirrhinum majus and Cin1 and Teo1 DNA inserts of Zea mays and Zea mexicana, respectively (Shepherd et al., 1982).

Because the light-inducible enzyme, chalcone synthase, is one of the key enzymes involved in flavonoid biosynthesis (Kaulen et al., 1986), a genetic clone containing a segment of the chalcone synthase gene will be used as a probe to determine whether this gene is responsible in some way for causing the variegation difference in "Eleanor Roosvelt" leaves and it will also be used to study the striking difference in pigmentation patterns between the younger leaves (top portion of the plant) and the older leaves (lower portion of the plant). Young leaves are green while older leaves become deep purple during development of the plant (Fig. 2). We postulate that a change at the mRNA level (transcription) of croton plants is controlling this pigmentation difference.

The cDNA from Antihirrinum majus that contains part of the structural gene chalcone synthase was used to determine a homologous sequence in the croton genome and also to determine by Northern blot hybridization if any difference exists between the mRNA from young and older croton leaves.

## MATERIALS AND METHODS

Plant DNA Extraction: Refer to Chapter I

Dot Blot Experiments:

The plasmid pCA-1 (Table II) (a gift from Dr. Zussane Schwarz-Sommer, Max Planck Institute, West Germany) containing the chalcone synthase insert was used in the dot blot hybridization experiments.

In order to estimate the copy number of the chalcone synthase sequence in the croton genome by the dot blot hybridization procedure, the following calculations were made; if the haploid genome size for croton is assumed to be $1 \times 10^9$ bp, then the chalcone synthase sequence would represent $1 \times 10^{-6}$ of the total plant genome since this sequence is 1000 bp in length (Schwarz-Sommer, personal communication). For example, if 1 $\mu$g of plant DNA were to be fixed on the filter for hybridization, then $1 \times 10^{-6}$ $\mu$g of the chalcone synthase sequence on the filter would represent one copy sequence. However, since this sequence is 1/5.3 (1000/5300) of the plasmid plus insert, the amount of plasmid plus insert to apply on the filter to represent one copy sequence in 1 $\mu$g of croton DNA must be $5.3 \times 10^{-6}$ $\mu$g of DNA. Therefore, one copy sequence of plasmid plus insert DNA is represented by $5.3 \times 10^{-6}$ $\mu$g of DNA, two copy sequence by $10.6 \times 10^6$ $\mu$g and so forth relative to 1 $\mu$g of croton DNA. The preparation of filter and the hybridization with pCA-1 probe was performed as stated in Chapter I (Materials and Methods) except for the pCA-1 probe (refer to above) which was mixed with 1 $\mu$g of yeast tRNA (concentration 3 mg/ml), that acted as carrier RNA to facilitate the ethanol precipitation of small DNA samples. The probe was applied to the nitrocellulose filter along with croton DNA (1, 2, 4 $\mu$g, respectively) and 1 $\mu$g phage lambda DNA which was used as a

negative control.

Gel Electrophoresis and Southern Blot Hybridization

Plant DNA was digested and electrophoresed in the following manner: Ten μg of dialyzed croton DNA (green leaves, concentration = 293 μg/ml; purple leaves, concentration = 263 μg/ml) were digested with 3 units of HindIII (Bethesda Research Laboratories, BRL) or EcoRI (BRL) per μg of croton DNA. The enzyme digestions were performed in a reaction volume of 100 μl under conditions specified by the manufacturer (see Appendix I). HindIII required Core buffer (BRL) and EcoRI, buffer C (BRL).

The same concentration (10 μg) of Snapdragon DNA (concentration = 102 μg/ml) was digested with 3 units/μg of EcoRI, but the reaction mix volume was changed to 200 μl. All the restriction enzyme digestions were performed at 37°C for three to four hours. The probe pCA-1 (Table II) was digested with PstI (BRL) at 37°C for 3 hours. The enzyme activity was stopped by heating the sample at 65°C for 10 min and then chilling on ice. Since the chalcone synthase fragment is spliced into the PstI site of pBR 322, this digestion was performed in order to release the insert DNA from pBR 322.

The digested DNA's were treated with an equal volume of distilled phenol and microfuged at room temperature for 2 minutes. The DNA in the aqueous phase was treated with 0.1 volume of 3 M NaOAC (pH 5.2) (Appendix I) and 2.5 volume of 95% ethanol and stored for either 1 hr at -70°C or overnight at -20°C. The DNA pellets were obtained by centrifugation for 15 minutes at 4°C in an Eppendorf microcentrifuge washed carefully with cold 70% ethanol and briefly dried in an oven at 80°C. The pellets were resuspended in 20 μl of TE buffer (Appendix I). The

digested DNA's along with pCA-1 digested DNA were electrophoresed overnight at 30V in a 0.8% agarose gel at room temperature. TEA buffer (Appendix I) was used for the electrophoretic run and was circulated between the positive and negative poles. After electrophoretic separation of fragments, the agarose gel was stained with 500 $\mu$l of Et Br (10 mg/ml) and observed under ultraviolet light in a transluminator Model TM-15 (Ultra Violet Product Inc., San Gabriel, Ca.). Prior to the Southern transfer the agarose gel containing the digested DNA's was exposed to ultraviolet light of short wavelength for a few minutes to allow for a more efficient transfer of high molecular weight DNA (Maniatis et al., 1982). The DNA was denatured by immersing the agarose gel twice for fifteen minutes in denaturing solution containing 1.5 M NaCl and 0.5 M NaOH (Appendix I), then neutralized by washing the gel twice for 15 minutes each in 3 M NaCl and 0.5 M Tris pH 7.4 (Appendix I). Both procedures were performed by gently shaking the gel at room temperature on a table top shaker.

To transfer the denatured DNA from the gel to nitrocellulose filters, a clean plastic platform was placed inside a plastic or glass tray such that the platform was about 1.5 inches from the bottom of the tray. A sheet of 3MM Whatman filter paper previously soaked in 20X SSC (Appendix I) was gently laid across this platform with its ends touching the bottom of the tray. Any air bubbles were removed by rolling a clean glass rod across the surface of the filter paper. The gel containing the denatured DNA was carefully laid on top of the filter paper and any air bubbles were again removed. A nitrocellulose filter cut to the size of the gel was first wetted with distilled water, rinsed in 2X SSC and placed on top of the gel. Three sheets of 3MM filter paper cut to the size of the gel were then laid

onto the nitrocellulose filter followed by a 3 inch thick wad of paper toweling or napkins. The transfer buffer (20X SSC) was gently poured into the tray making sure that the ends of the 3MM filter paper were immersed in the buffer to act as wicks for the transfer of the 20X SSC through the gel and into the nitrocellulose filter. To prevent any short-circuiting of the 20X SSC from the 3MM filter paper to the nitrocellulose filter, strips of parafilm surrounded the gel. A glass plate was then set on top of the stacked paper towels to act as a weight and the whole set-up was then wrapped in Saran wrap to prevent undue evaporation of the buffer liquid. The transfer was allowed to take place for 16 to 18 hours.

Plant RNA Extraction

For RNA extractions, all glassware was baked overnight at 200$^{o}$C in an oven, and all solutions were either autoclaved, or filter sterilized. Rubber gloves were worn at all times to avoid contamination by ribonucleases.

The method used was a modification of the method of Turpen and Griffith (1986). Leaf tissue (25 gram) was frozen in the presence of liquid nitrogen and ground to a fine powder in an electric coffee mill (Moulinex, Regal Model), which was pre-cooled with dry ice. The dry powder was placed into 200 ml of lysis buffer containing 8 M urea (Appendix I). The mixture was homogenized for two minutes at a medium setting of 4 in a Virtis homogenizer. The insoluble material was removed by centrifugation using the GSA rotor (8,000g, 25 min, 4$^{o}$C). To the supernatant, dry CsCl (BRL) was added to a final concentration of 0.2 gm/ml. After all the CsCl was dissolved, this solution was layered with a sterile syringe onto a 10 ml cushion of 5.7 M sterile CsCl solution containing 50 mM EDTA pH 7.3 in Polyallomer Quick-seal tubes (Beckman). A transparent pellet of plant RNA was obtained by

centrifugation in the Type 60 Beckman Ti rotor (39,000 rpm, 15-16 hours, 15$^{o}$C). The pellet was dissolved in 5 ml of sterile distilled water, by heating in a water bath at 65$^{o}$C for ten minutes.

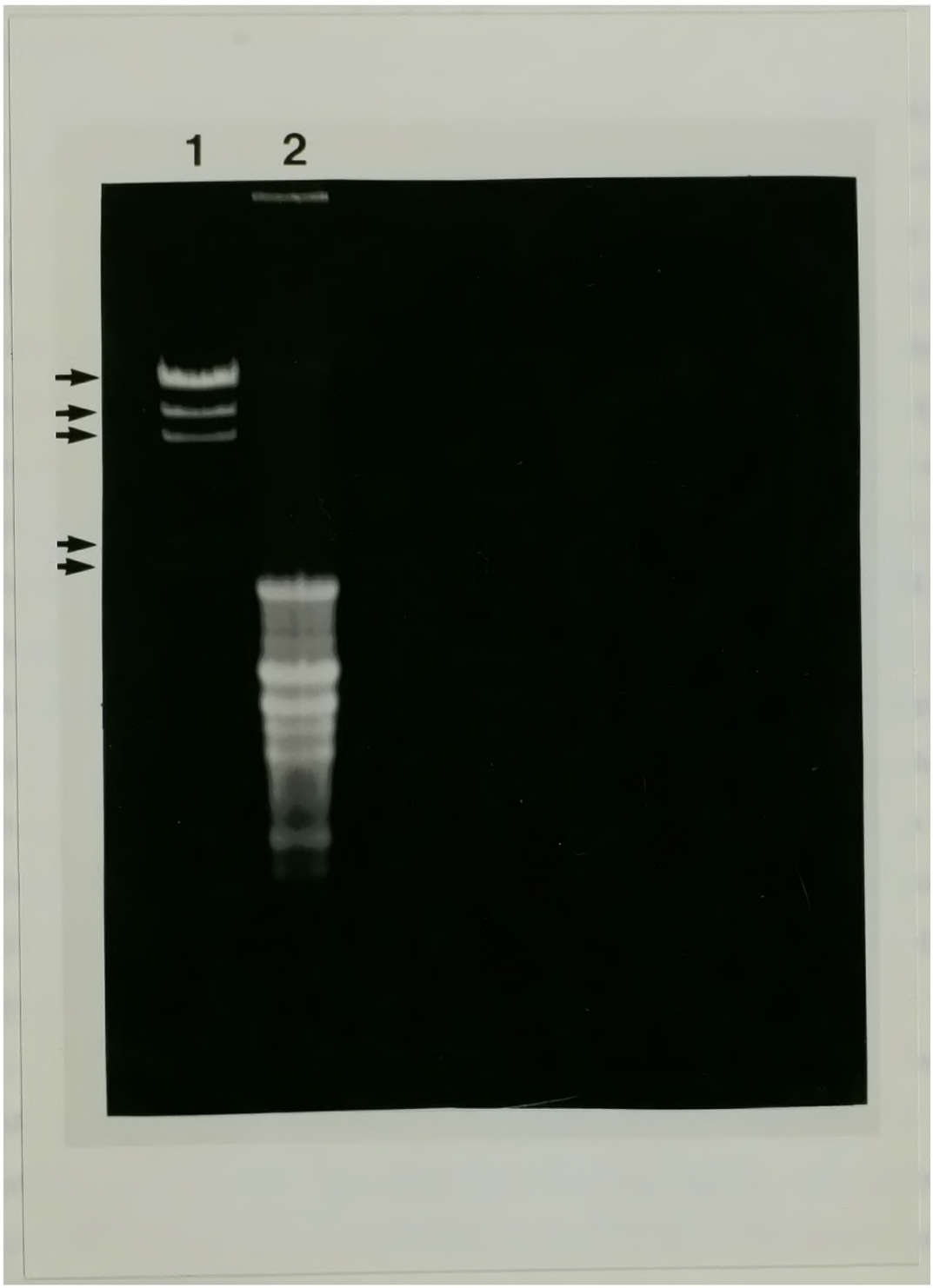
To further purify the RNA preparation, the solution was treated twice with an equal volume of a 1:1 mixture of phenol-chloroform and then with an equal volume of chloroform. As a final step the plant RNA was precipitated with four volumes of cold 95% ethanol and 1/20 volume of a 5 M NaOAC solution for one hour at -70$^{o}$C. A white RNA pellet was obtained by centrifugation in a SS-34 rotor (12,000 rpm, 15 min, 4$^{o}$C). After rinsing the pellet with 70% ethanol, it was redissolved in 1 ml of sterile distilled water.

To determine the concentration and purity of the RNA sample, absorbance values at 260 and 280 nm were obtained in a Beckman Model 25 Spectrophotometer. Since 1 O.D at 260 nm = 40 mg/ml of RNA, the concentration of croton RNA was estimated.

RNA gel electrophoresis:

Croton RNA was electrophoresed in a 1.5% agarose gel containing 1X RNA running buffer (0.2 M MOPS pH 7.0, 50 mM NaOAC, 5 mM EDTA pH 8.0). The RNA sample to be electrophoresed 5 mg) was denatured by heating at 65$^{o}$C for five minutes and quick chilled on ice. Before loading the sample onto the gel it was mixed with 1/4 volume of 4X stop buffer (Appendix I) containing 0.5% SDS. RNA was electrophoresed at 60 volts for 2 hours in a minigel electrophoretic apparatus which contained 1X RNA gel running buffer (Appendix I). Figure 6 shows the croton RNA electrophoresed under these conditions to illustrate the purity of the

Figure 6: Croton RNA electrophoresed at 40 volts on a 1.4% agarose gel with a running buffer which contains MOPS, sodium acetate and EDTA (see Appendix I). Lane 1 corresponds to bacteriophage lambda DNA digested with HindIII. Lane 2 corresponds to total croton RNA.


1
2

RNA and the various RNA fragments in the preparation.

Purification of Croton messenger RNA by chromatography on Oligothymidylic acid-cellulose:

The procedure employed was a modification of the method of Aviv and Leder (1972). In this method a clean plastic disposable column was filled with 0.5 gm of Oligo (dT)-Cellulose Type 3 (poly-A binding capacity: 112 $OD_{260\ nm}$ unit/gm. Collaborative Research, Inc.). This column was washed first with 1-2 volumes of 0.1N NaOH to remove all trace of bound material and with 1-2 volumes of sterile elution buffer (Appendix I) followed by binding buffer (Appendix I). After the column was equilibrated, the RNA sample (6 mg/ml) was diluted with an equal volume of elution buffer (Appendix I) and heated for 5 minutes at 75°C. While chilling on ice, 1/10 volume of 4 M NaCl was added to the sample and the RNA was slowly loaded onto the column. The first column volume collected after applying the sample was the flow through. This flow through volume was reapplied two more times to the column and again collected. The column was next washed with three to four volumes of mRNA binding buffer (Appendix I). The RNA in both of these washes and the flow through were precipitated with two and a half volumes of cold 95% ethanol and 1/10 volume of 3 M NaOAC at -70°C. This precipitate represents the $poly(A)^-$ fraction.

To elute the $poly(A)^+$ mRNA bound to the column three to four volumes of mRNA elution buffer (Appendix I) were used. Twenty fractions of 1 ml each were collected in autoclaved Eppendorf tubes. These samples were centrifuged for 2 minutes in an Eppendorf centrifuge to remove any resin debris, and absorbance values at 260 nm were determined for each fraction with a Beckman Model 25

Spectrophotometer. The fractions with high absorbance values (0.1 to 2.1 OD units approximately), which indicated the presence of mRNA, were pooled and precipitated with 1/10 volume at 3M NaOAC and two and a half volumes of cold 95% ethanol. After all the pooled fractions were centrifuged (12,000 rpm, 15 min., 4$^{o}$C) the pellets were resuspended in a minimum amount of sterile distilled water.

To check for the presence of the different RNA's (mRNA, tRNA, rRNA) overnight gel electrophoresis was performed at 40 volts on a 1.4% agarose gel with 1X RNA running buffer (Appendix I). The concentration of poly(A)$^{+}$ RNA for top (young green) and bottom (old purple) croton leaves were 673.6 $\mu$g/ml and 342 $\mu$g/ml respectively. Both poly (A)$^{+}$ RNA samples were ethanol precipitated and then resuspended in sterile distilled water to a concentration of 2000 $\mu$g/ml. All the samples were stored at -20$^{o}$C for later use.

Northern blot Experiments:

Before the electrophoretic run, 20 $\mu$g of each RNA sample (poly(A)$^{+}$, poly(A)$^{-}$ and total RNA) were denatured by mixing each sample with RNA gel running buffer (Appendix I), containing 50% deionized formamide (BRL) and 2.2 M formaldehyde (Baker). The mixture was heated at 65$^{o}$C for 5 minutes. The heated samples were placed on ice and 1/4 volume of 4X stop buffer (Appendix I), which contained 0.5% SDS, was added. The samples were loaded onto a 1.3% agarose gel containing 6% formaldehyde (Baker) and 1X RNA gel running buffer (Appendix I). Electrophoresis in 1X RNA gel running buffer was performed at 120 volts for three hours in a 23 cm electrophoresis tray. Similar results were obtained when electrophoresis was run at 40 volts overnight.

To visualize the electrophoretic pattern prior to Northern transfer, a duplicate

set of the RNA samples was run on the other half of the same gel. After electrophoresis, the lanes containing one set of the RNA samples was cut with a razor blade, washed for two hours with running tap water to remove the formaldehyde, and then stained with ethidium bromide (10 mg/ml stock). RNA was then visualized using a transilluminator.

If the RNA samples had migrated satisfactorily, as seen by ethidium bromide staining, the RNA in the other half of the gel was transferred without any alkali treatment directly onto Nytran membrane filters using methods (Chapter I) similar to the Southern transfer. The Nytran filters (modified nylon 66 membrane Schleicher & Schull) were presoaked in distilled water. After overnight transfer, the membrane was air dried and baked in an $80^{o}C$ vaccum oven for two hours. Prehybridization was performed for 4-24 hours at $42^{o}C$ in the prehybridization buffer (50% formamide, 5X Denhardt's, 0.1% SDS, 150 $\mu$g/ml denatured E. coli tRNA and 5X SSPE) (Appendix I). The radiolabeled pCA-1 probe ($1 \times 10^{7}$ cpm) was added to the prehybridization buffer and hybridization was performed overnight at $42^{o}C$ with gentle shaking.

The membrane was washed twice with 2X SSC, and 0.2% SDS at room temperature for ten minutes each and twice for twenty minutes at $55^{o}C$. After air drying at room temperature, the membrane was autoradiographed overnight at $-70^{o}C$ with XAR-5 film (Kodak) using an intensifying screen as previously described (Chapter 1).

# RESULTS

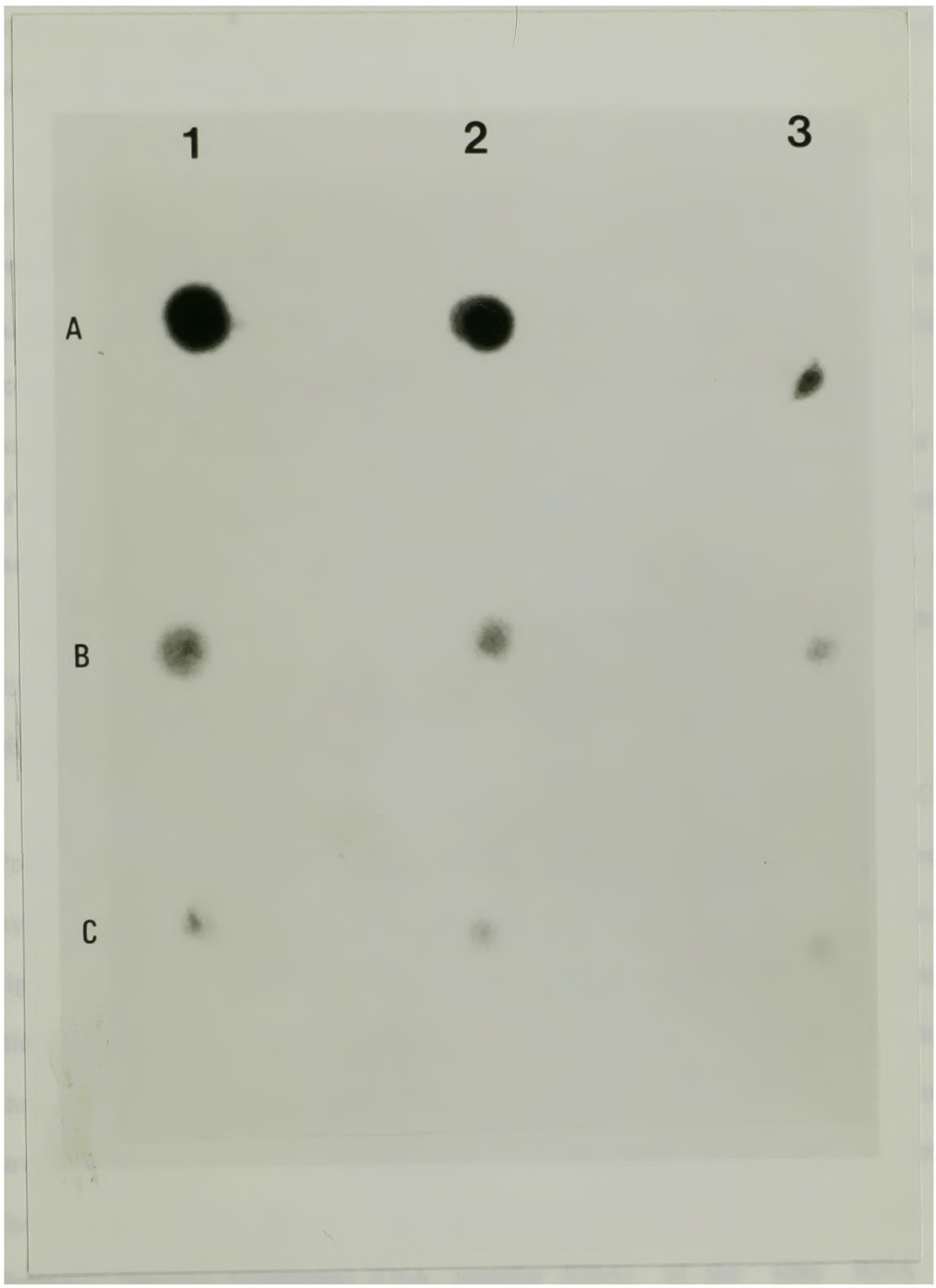
## Dot Blot Hybridization

The cDNA clone pCA-1 (Table II) which contains the chalcone synthase gene from Antirrhinum majus is a 1.2 kb fragment that contains 50 bp of the 5' leader region and most of the coding part (approximately 250 bp of the 3' end are missing). This clone was tested for possible hybridization to the croton DNA. Figure 7 shows a positive signal for 1, 2 and 4 $\mu$g of plant DNA. The intensity of hybridization decreases from the higher to the lower concentration of croton DNA as expected (Lane 1A, B, C, respectively). In lane two, A, B and C correspond to 4, 2 and 1 copies of the probe pCA-1 which represents $21.2 \times 10^{-6}$, $10.6 \times 10^{-6}$ and $5.3 \times 10^{-6}$ $\mu$g of pCA-1 DNA respectively, which were added as controls to determine the copy number of the chalcone synthase gene in the croton genome. The intensity of the hybridization signals indicates that the chalcone synthase gene is present as a single copy gene in the croton genome.

## Southern Blot Hybridization

Croton DNA from top (green, spotted, young leaves) and bottom leaves (mature leaves spotted and covered with anthocyanin pigments) of the plant was digested with either the restriction enzyme HindIII or EcoRI.

Figure 8 shows results of the hybridization of croton DNA and snapdragon DNA with the radioactive probe pCA-1. Lanes 2 and 3 correspond to the DNA from the top leaves of croton digested with HindIII and EcoRI respectively. Lanes 4 and 5 correspond to croton DNA from bottom leaves digested with the same enzymes respectively. Very weak signals are observed in the four lanes, but one band can be distinguished and its estimated size is approximately 4.7 kb.


1
2
3
A
B
C

In the hybridization pattern of pCA-1 probe to snapdragon DNA (Fig. 8, lane 6) one strong and a few weaker signals are seen. The size of the predominant DNA fragment corresponds to 4.4 kb. In the heterologous system with croton DNA few bands are seen in the Southern blot (Fig. 8).

With the Anthirrinum majus digested DNA, the results with the pCA-1 probe are different. In this case we are dealing with a homologous system in which the presence of many bands is probably due to pseudogenes that are present in the Anthirrinum majus genome. Fig. 8, lane 10 shows the hybridization pattern corresponding to a one copy sequence of the chalcone synthase gene. This result is comparable to the bands observed in rows 2-4, which corresponds to hybridization of the chalcone synthase probe to the digested croton DNA from top and bottom leaves. The chalcone synthase gene in the croton genome, therefore, appears to be a single copy sequence; this result is comparable to that obtained from the dot blot hybridization experiment.

Northern Blot Hybridization:

Fig. 9 (row 1 and 4) corresponds to the hybridization of the pCA-1 probe to Poly(A)$^+$ fractions isolated from bottom and top croton leaves, respectively. Two hybridization bands (A and B) are observed in both rows but the intensity of the signal is slightly higher for the bottom mRNA sample (Lane 1). Regarding the size difference of the Poly(A)$^+$ fractions, the migration pattern of the bands indicates that the lower hybridization band B consisting of Poly(A)$^+$ RNA from the purple lower leaves (row 1) is slightly larger in molecular weight than the lower band of the Poly(A)$^+$ RNA from the top younger leaves (row 4). Rows 2 and 3 contain the Poly(A)$^-$ fraction (ribosomal RNA, transfer RNA) of the total RNA (bottom and

**Figure 8. Southern Blot Hybridization of Croton DNA with the Chalcone Synthase Gene Probe (pAC-1).**

Lane 1: Bacteriophage lambda DNA digested with HindIII. The fragment sizes from top to bottom are represented by a large undigested multimer DNA and bands of 23.5, 9.5, 6.7, 3.8, 2.2 Kb, respectively. Lanes 2-3: 10 $\mu$g of Croton DNA from top leaves digested with HindIII and EcoRI, respectively. Lanes 4-5, 10 $\mu$g of Croton DNA from bottom leaves digested with the same enzymes as above. Lane 6: 10$\mu$g of snapdragon DNA digested with EcoRI. Lanes 7, 8, 9, 10 correspond to 1,2,4,8 micrograms of pAC-1 DNA probe digested with PstI.

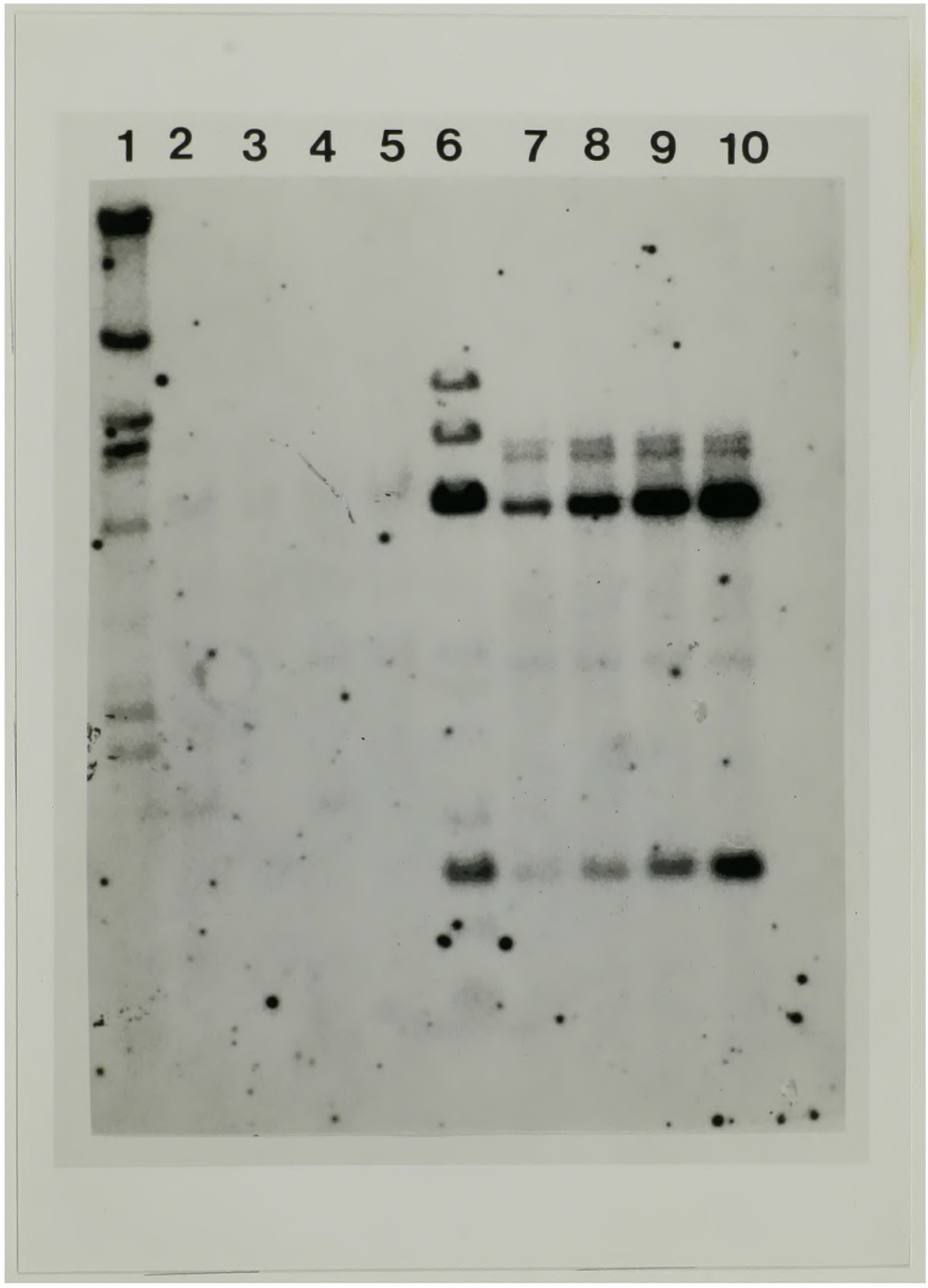
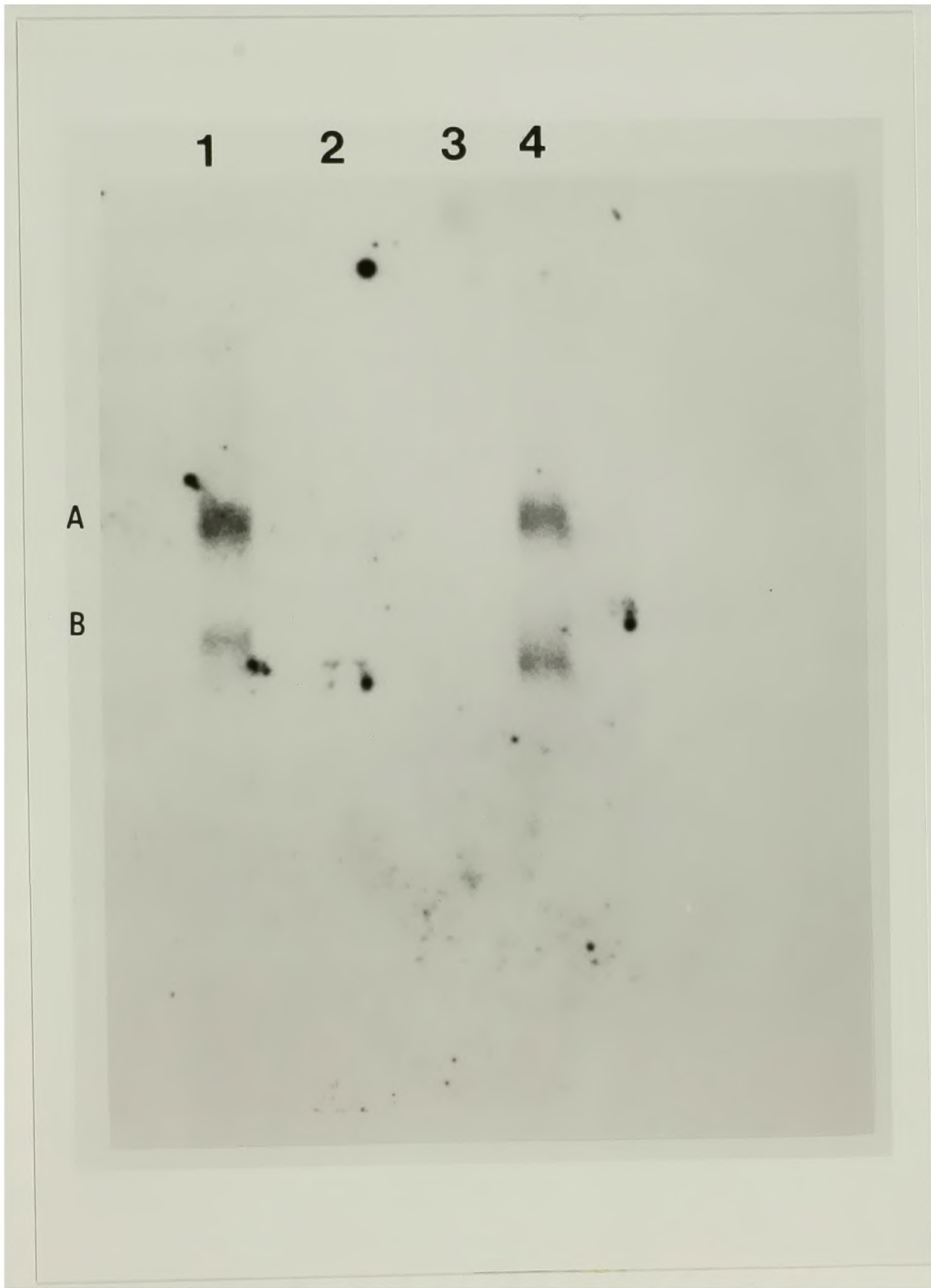

1 2 3 4 5 6 7 8 9 10

**Figure 9: Northern Blot Hybridization.**

Lanes 1 and 2 correspond to the hybridization of the pCA-1 probe to Poly(A)$^+$ fractions and Poly(A)$^-$ respectively, isolated from bottom croton leaves. Lanes 3 and 4 correspond to pCA-1 hybridization to Poly(A)$^-$ and Poly(A)$^+$ RNA respectively isolated from top croton leaves.


1
2
3
4
A
B

top leaves, respectively). No hybridization signals are detected, which indicates that the separation of Poly(A)$^+$ mRNA from Poly(A)$^-$ was successful using Oligo-dt column chromatography.

## DISCUSSION

The striking difference that exists in the pigmentation pattern between young and older leaves in the croton plant was studied at the molecular level. The problem was analyzed using the probe pCA-1 which contains most of the 5' end of the coding region of the chalcone synthase gene. The positive results from the dot blot hybridization experiment with total genomic croton DNA indicated that the chalcone synthase gene was present.

Southern hybridization experiments were used to determine if there was any difference in the hybridization pattern of DNA extracted from green top leaves and that extracted from purple bottom leaves. The results showed no difference in the hybridization pattern with the restriction enzymes tested. Only a few weak bands were observed, indicating a decrease in homology due to evolutionary divergence between the croton and snapdragon chalcone synthase gene (Fig. 8, lanes 2-5). Nonetheless, enough homology exists to give a positive signal. This homology might be due to the conservation of at least the active center of the enzyme.

Hybridization differences detect the presence of certain mutations, such as insertions or deletions, that alter the size of a gene. The alterations might be due to transposable elements. For example, we would expect a change in size of a given restriction fragment if an insertion had occurred.

Moreover, integration or deletion of a piece of DNA in or at a locus can influence the expression of the locus at a number of different levels. A number of plant transposable elements that were isolated have been found to be integrated in the 5' control region of a locus (Nevers et al., 1986). These include Tam1 and Tam3 at the nivea locus of Anthirrinum majus (Bonas et al., 1984). This

transposable element, when inserted into the 5' end of the chalcone synthase gene, causes a mutation resulting in flowers with red spots and stripes used in this study on a white background (Nevers et al., 1986). However, hybridization with the pCA-1 clone used in this study did not detect any difference between digested DNA from young and old leaves of croton. The change in pigmentation pattern between these leaves does not appear to involve mutations due to insertions or deletions which might be attributable to transposable elements. For future research, the total chalcone synthase gene should be isolated from the croton gene library using pCA-1 from snapdragon as a probe to test for hybridization differences between the DNA from young and old leaves. If we had a more homologous probe, hybridization differences between green and purple leaves might be more distinct. This could be a more effective procedure to explain the difference in pigmentation between these leaves.

Northern blot experiments were performed in order to determine if there is a difference in the expression of the chalcone synthase gene in top and bottom leaves. A slight difference in the intensity of hybridization was observed in the mRNA isolated from bottom leaves vs. that isolated from top leaves. This difference might be correlated with higher production of chalcone synthase messenger RNA as a result of a higher expression of the gene. This observed phenomenon can be used as evidence to explain the pigmentation difference that exists between the top and bottom leaves. Nevertheless, more experiments should be performed to actually determine what is causing this differential expression of the gene. A more significant observation was the change in size of the transcript as seen in the Northern blot (Fig. 9). The Poly(A)$^+$ fraction from the bottom leaves

(Fig. 9, lane 1) appears to have a higher molecular weight fragment than that of the top leaves. If the chalcone synthase gene is one of the factors which contribute to the pigmentation difference between the upper and lower leaves, a larger transcript may allow for the translation of a longer polypeptide. This peptide may be involved in the color difference found between the two types of leaves. Moreover, the larger transcript may indicate that one or more other polypeptides could be translated from the monocistronic message. Another possibility could be a post-transcriptional modification whereby a smaller or larger mRNA transcript could be synthesized.

Since the top bands migrate the same distance, this RNA fraction has the same size in the top and bottom leaves. This also indicates that the difference in size of the lower bands cannot be an artifact of the isolation procedure.

These observations indicate one difference in gene expression between the top and bottom leaves. Since the enzyme involved is known to function in the anthocyanin synthesis pathway (Kaulen et al., 1986), this difference in gene expression may be related to the pigmentation difference that exists between the croton plant leaves.

## CONCLUSION

1. No hybridization was observed between known transposable elements of maize and croton DNA. Therefore, these maize elements are not present in the croton genome.
2. The variegation phenomenon in crotons might be due to other unknown transposons or to other factors.
3. The chalcone synthase gene is present in crotons, and slight homology exists between the chalcone synthase gene from snapdragon and croton.
4. The pigmentation difference between young leaves and old leaves might be caused by a change (post-transcriptional modification) at the mRNA level.

## REFERENCES

Anderson, E. G. 1923. Maternal inheritance of chlorophyll in maize. Bot. Gaz. 76: 411-418.

Aviv, H. and P. Leder. 1972. Purification of biologically active globin messenger RNA by chromatography on oligo thymidylic acid-cellulose. Proc. Natl. Acad. Sci. 69:1408.

Bauer, E. 1909. Das Wesen und die Erblichkeitverhaltnisse der Var zlbomarginatae hort von Pelargonium zonale. Z. Indukt. Abstammungs. Vereerbungst. 1:330-351.

Bonas, U., Sommer, H., Harrison, B.J. and Saedler, H. 1984. The transposable element Taml of Antirrhinum majus is 17 Kb long. Molec. Gen. Genetics. 194:138-143.

Brown, B.F., 1960. Florida's beautiful crotons. Eau Gallie, Fla. Undersea Press. 143 pp.

Calos, M.P. and Miller, J.H. 1980. Transposable elements. Cell 20:579-595.

Cameron, J. R., Loh, E. Y. and Davis, R. W. 1979. Evidence for transposition of dispersed repetitive DNA families in yeast. Cell 16:739-751.

Cavalier-Smith, T. 1978. Nuclear volume control by nucleoskeletal DNA, selection for cell volume and cell growth rate, and the solution of the DNA c-value paradox. J. Cell Sci. 34:247-278.

Cohen, S. N. and Shapiro, J. N. 1980. Transposable Genetic Elements. Scientific American. February 1980: 40-48.

Corens, C. 1909. Vererbungsversuche mit blass (gelb) grunen and buntblaltringen Sippen bei Mirabilis, Urtica, und Lunaria, Zeitsch. Ind. Abst. Vererb. 1:291-329.

Demerec, M. 1927. A second case of maternal inheritance of chlorophyll in maize. Botanical Gazette. 84:139-155.

Emerson, R. A. 1914. The Inheritance of recurring somatic variation in variegated ears of maize. Am. Nat. 48:87-115.

Federoff, N. 1983. Controlling elements in maize. In Mobile Genetics Elements, ed. J. Shapiro, pp 1-63. New York:Academic Press.

Federoff, N., Mauvais, J. and Chaleff, D. 1983. Molecular studies on mutations at the shrunken locus in maize caused by the controlling element Ds. J. Mol. Appl. Genet. 2:11-29.

Frischauf, A. M., Lehrach, H., Poutska, A. and Murray, N. 1983. Lambda replacement vectors carrying polylinker sequences. J. Mol., Biol. 170:827-842.

Flavell, R. B. 1982. Sequence amplification, deletion and rearrangement: Major sources of variation during species divergence. In Genome Evolution, R. B. Flavell and G. A. Dover, ed. pp. 301-323. New York: Academic Press.

Gregory, R. P. 1965. On variegation in Primula sinensis. J. Genet. 4:305-321.

Gupta, M., Shepherd, N. S. Bertram, I. and Saedler, H. 1984. Repetitive sequences and their organization on genomic clones of Zea mays. EMBO J. 2:15-23.

Hake, S. and Walbot, V. 1980. The genome of Zea mays, its organization and homology to related grasses. Chromosoma 79:251-270.

Heller, W. and Hahlbrock, K. 1980. Highly purified flavanone synthase from parsley catalyses the formation of naringenin chalcone. Arch. Biochem. Biophys. 200:617.

Kafatos, F. C., Jones, W. C. and Efstratiadis, A. 1979. Determination of nucleic acid sequences homologies and relative concentrations by a dot hybridization procedure. Nucleic Acid Research 7:1541-1551.

Kaper, J. M. and Waterworth, H. E. 1982. Handbook of Plant Virus Infections Comparative Diagnosis. Edward Kurstack. Elsevier, North-Holland Biomedical Press, Amsterdam-New York-Oxford. pp 258-322.

Kirk, J.T.O. and Tilney-Bassett, R.A.E. 1978. "The plastids: their chemistry, structure, growth and inheritance," 2nd Ed. Elsevier, Amsterdam.

Kaulen, H., Schell, J. and Krevzaler, F. 1986. Light induced expression of the chimeric chalcone synthase - NPT II gene in tobacco cells. EMBO J. 5:1-8.

Kreuzaler, F., Ragg, H., Heller, R., Teich, I. and Hammer, D. 1979. Flavonone synthase from Petroselinum hortense. Eur. J. Biochem. 99:89-90.

Kreuzaler, F., Ragg, H., Fautz, E., Kuhn, D. N., Hahlbrock, K. 1983. UV-induction of chalcone synthase mRNA in cell suspension cultures from Petroselinum hortense. Proc. Natl. Acad. Sci. USA, 80:2591-2593.

Lambert, M. E., McDonalds, J. F. and Weinstein, I. B. 1988. Eukaryotic transposable elements as mutagenic agents. Cold Spring Harbor Laboratory. 345 pp.

Maniatis, T., Fritsch, E. F. and Sambrook, J. 1982. Molecular Cloning. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

McClintock, B. 1950. The origin and behavior of mutable loci in maize. Proc. Natl. Acad. Sci. 36:344-355.

McClintock, B. 1951. Chromosome organization and genetic expression. Cold Spring Harbor Symp. Quant. Biol. 19:13-47.

McClintock, B. 1952. Mutable loci in maize. Carnegie Inst. Wash. Year Book. 51:212-219.

McClintock, B. 1953. Mutation in maize. Carnegie Inst. Wash. Year Book 52:227-237.

McClintock, B. 1956. Controlling elements and the gene. Cold Spring Harbor Symp. Quant. Biology 21:197-216.

McClintock, B. 1965. The control of gene action in maize. Brookhaven Symp. Biol. 18:162-182.

Mohr, H. and Schaffer, H. 1983. Photoreception and desetiolation. Philos Trans. R. Soc. Lond. B 303:489.

Murray, K. and Murray, N. E. 1975. Phage lambda receptor chromosomes for DNA fragments made with restriction endonuclease III of Haemophilus influenza and restriction endonuclease I of Escherichia coli. J. Mol. Biol. 98:551.

Murray, M. G. and Thompson, W. F. 1980. Rapid isolation of high molecular weight plant DNA. Nucleic Acid Research 8:4321-4325.

Nevers, P., Shepherd, N. and Saedler, H. 1986. Plant Transposable Elements. Advances in Botanical Research. 12: 103-203. Academic Press, Inc.

Peterson, P. A. 1965. A relationship between Spm and Enh control systems in maize. Am. Nat. 99:931-98.

Rhoades, M. M. 1946. Plastid mutations. Cold Spring Harbor Symp. Quant. Biol. 11:202-207.

Rubin, G. M., O'Brien, W. J., Dunsmuir, P., Flavell, A. J., Levis, R., Strobel, J. J., Todle, J. J. and Young, E. 1981. "Copia-like" transposable elements in the Drosophila genome. Cold Spring Harbor Symp. Quant. Biol. 45:619-628.

Shepherd, N.S., Schwarz-Sommer, Zs., Wienand, U., Sommer, H., Deumling, B., Peterson, P.A. and Saedler, H. 1982. Cloning of a genomic frgment carrying the insertion element Cin 1 of Zea mays. Mol. Gen. Genet. 188:266-271.

Stewart, R. N. 1965. The origin and transmission of a series of plastogene mutants in Dianthus and Euphorbia. Genetics 52:925-947.

Syvanen, M. 1984. The evolutionary implications of mobile genetic elements. Ann. Rev. Genet. 18:271-293.

Turpen, T.H. and Griffith, O.M. 1986. Rapid Method for RNA Extraction (Biofeedback) Biotechniques 4:14-15.

Varmus, E. 1982. Form and function of retroviral proviruses. Science 216:812-820.

Wienand, U., Sommer, H., Schwarz, Zs., Shepherd, N., Saedler, H., Kreuzaler, F., Ragg, H., Fautz, E., Hahlbrock, K., Harrison, B. J., and Peterson P. A. 1982. A general method to identify plant structural genes among genomic DNA clones using transposable element induced mutations. Mol. Gen. Genet. 187:195-201.

# APPENDIX I
# BUFFERS AND SOLUTIONS

## Chapter I

### Plant DNA Extraction

| | |
|---|---|
| Lysis Buffer | 1.4M NaCl<br>2% Hexadecyltrimethyl ammonium bromide (CTAB)<br>100 mM Tris HCl pH 8.0<br>20 mM EDTA<br>2% Mercaptoethanol (ME)<br>50 $\mu$g/ml Ethidium Bromide |
| Precipitation Buffer | 1% CTAB<br>50 mM Tris HCl pH 8.0<br>10 mM EDTA |
| Resuspension Buffer | 1M Cesium chloride<br>50 mM Tris HCl pH 8.0<br>10 mM EDTA<br>200 $\mu$g/ml Ethidium Bromide |
| High CsCl solution | 6.6M CsCl<br>50 mM Tris HCl pH 8.0<br>10 mM EDTA<br>0.1% Sarkosyl |
| Medium CsCl solution | 4.5M CsCl<br>50 mM Tris HCl pH 8.0<br>10 mM EDTA<br>0.1% Sarkosyl<br>100 $\mu$g/ml Ethidium Bromide |
| Butanol Saturated | 1 - Butanol saturated with 5M NaCl<br>10 mM Tris HCl, pH 8.0 ml<br>5 mM EDTA |
| TE Buffer | 100 mM Tris HCl pH 8.0<br>1 mM EDTA pH 8.0 |

### Dot Blot Hybridization Experiments

| | |
|---|---|
| 20X Standard Saline Citrate (SSC) | 3M NaCl<br>0.3M $Na_3$ citrate. $2H_2O$ |
| 4X SET | 0.6M NaCl<br>0.12M Tris HCl pH 8.0<br>4 mM EDTA |

| | |
|---|---|
| 50X Denhardt's solution | 5g Ficoll (400,00 MW)<br>5g Polyvinylpyrrolidone<br>5g BSA (Pentex Fraction V)<br>to 500 ml $H_2O$ |

**Nick Translation**

| | |
|---|---|
| 2X Nick Translation Buffer | 25 $\mu$l 1M Tris pH 7.4<br>5 $\mu$l 0.5M $MgCl_2$<br>50 0.1M 2Me (50 $\mu$l of stock ME into 5 ml $H_2O$<br>25 $\mu$l BSA (1mg/ml)<br>2.5 $\mu$l dATP (4 mM)<br>2.5 $\mu$l dTTP (4 mM)<br>2.5 $\mu$l dGTP (4 mM) |
| 10X RSB Buffer | 0.1M NaCl<br>0.1M Tris Cl pH 7.8<br>15 mM $MgCl_2$ |
| 4X Stop Buffer Solution | 0.25% Bromophenol Blue<br>40% Sucrose<br>10 mM EDTA<br>0.5M NaOH |

**Gel Electrophoresis**

| | |
|---|---|
| 10X TEA Buffer | 48.44g Trizma base<br>27.22g Sodium acetate ($3H_2O$)<br>7.44g EDTA (dihydrate form)<br>10.50g NaCl<br>Adjust pH to 8.0 with glacial Acetic Acid.<br>Take up to 1L with water. |
| 3M Sodium Acetate pH 5.2 | 40.8g $NaOAc.3H_2O$<br>adjust pH to 5.2 with glacial acetic acid<br>up to 100 ml with water. Autoclaved. |

**Southern Blot Hybridization**

| | |
|---|---|
| 20X Standard Saline Citrate (SSC) | 3M NaCl<br>0.3M $Na_3$ Citrate. $3H_2O$ |
| STE buffer | 10 mM Tris HCl, pH 8.0<br>100 mM NaCl<br>1 mM EDTA |

| | |
|---|---|
| 50X Denhardt's solution | 5g Ficoll (400,000 Mw)<br>5g Polyvinylpyrrolidone<br>5g BSA (Pentex Fraction V)<br>to 500 ml $H_2O$ |
| Denaturing Solution | 1.5M NaCl<br>0.5M NaOH |
| Neutralizing Solution | 3M NaCl<br>0.5M tris pH 7.4 |

**Plant RNA Extraction**

| | |
|---|---|
| Lysis Buffer | 8M Urea<br>0.35M NaCl<br>50 mM Tris pH 7.5<br>20 mM EDTA<br>2% Sarkosyl<br>0.1M Mercaptoethanol |
| 5X RNA Running Buffer | 0.2M morpholinopropane sulfonic acid (MOPS) pH 7.0<br>50 mM NaOAc<br>5 mM EDTA pH 8.0 |

**Purification of Plant Messenger RNA**

| | |
|---|---|
| Elution Buffer | 0.01M Tris HCl pH 7.5 |
| Binding Buffer | 0.01M Tris HCl pH 7.5<br>0.5M NaCl |
| mRNA Binding Buffer | 0.01M Tris HCl pH 7.5<br>0.05% SDS<br>1 mM EDTA<br>0.5M NaCl |
| mRNA Elution Buffer | 0.01M Tris HCl pH 7.5<br>0.05% SDS<br>1 mM EDTA |

**Northern Blots**

| | |
|---|---|
| 20X SSPE | 3.0M NaCl<br>200 mM $NaH_2PO_4$ (pH 7.4)<br>0.02 EDTA<br>Adjust to pH 7.4 with 10M NaOH and adjust volume to 1 liter.<br>Sterilize by autoclaving. |

| | |
|---|---|
| Ice Cold Glucose Solution | 50 mM Glucose<br>25 mM Tris Cl pH 8.0<br>10 mM EDTA pH 8.0<br>5 mg/ml Lysozyme (Worthington Biochemicals Co.) |
| 5M Potassium Acetate | 60 ml K Acetate pH 4.8<br>pH 5.2 with glacial acetic acid.<br>Add water up to 100 ml.<br>28.5 $H_2O$ |
| STE Buffer | 10 mM Tris HCl pH 8.0<br>100 mM NaCl<br>1 mM EDTA |
| Ethidium Bromide Solutio (10 mg/ml) | 0.2g Ethidium Bromide<br>20 ml $H_2O$<br>Store 4$^{o}$C in dark |
| 10X BamHI Buffer | 0.2M Tris Cl (pH 8.0)<br>1M NaCl<br>20 mM $MgCl_2$<br>20 mM $\beta$ Mercaptoethanol |
| 10% Sucrose Solution (100 ml) | 10g Sucrose<br>1M NaCl<br>20 mM Tris HCl (pH 8.0)<br>5 mM EDTA (pH 8.0) |
| 40% Sucrose Solution (100 ml) | 40g Sucrose<br>1M NaCl<br>20 mM Tris HCl (pH 8.0)<br>5 mM Tris EDTA (pH 8.0) |
| 10X Ligation Buffer | 0.5M Tris pH 7.4<br>0.1M $MgCl_2$<br>0.2M DTT<br>10 mM ATP<br>50 $\mu$g/ml BSA |
| Packaging Buffer | 6 mM Tris HCl (pH 8.0)<br>50 mM Spermidine<br>50 mM Putrescine<br>20 mM $MgCl_2$<br>30 mM ATP<br>30 mM 2Me |
| Lambda Dilution Buffer | 0.5 ml 1M Tris HCl (pH 7.5)<br>0.5 ml 1M $MgSO_4$ |

# APPENDIX II

Culture media

Luria Broth (1 liter)

| | |
|---|---|
| Bactotrytone | 10g |
| Yeast extract | 5g |
| NaCl | 10g |

Adjust pH to 7.5 with NaOH

M-9 Medium (1 liter)

| | |
|---|---|
| $Na_2HPO_4$ | 6g |
| $KH_2PO_4$ | 3g |
| NaCl | 5g |
| $NH_4Cl$ | 1g |

Adjust to pH 7.4, autoclave, cool and add:

2 ml $MgSO_4$
10 ml 20% glucose
0.1 ml 1M $CaCl_2$

NZYM Broth (1 liter)

| | |
|---|---|
| NZ Amine | 10g |
| NaCl | 5g |
| Yeast extract | 5g |
| $MgSO_4.7H_2O$ | 2g |

Adjust pH to 7.5 with NaOH

NZYCM Broth (1 liter)

| | |
|---|---|
| NZ Amine | 10g |
| NaCl | 5g |
| Yeast extract | 5g |
| Casamino Acids | 1g |
| $MgSO_4.7H_2O$ | 2g |

Adjust pH to 7.5 with NaOH

Top Agar (250 ml)

| | |
|---|---|
| Bactotryptone | 2.5g |
| Yeast extract | 1.25g |
| NaCl | 2.5g |
| Agar | 1.3g |
| $MgSO_4$ (1M) | 2.5 ml |

Bottom Agar (1 liter)

| | |
|---|---|
| Bactotryptone | 10g |
| Bactoyeast extract | 5g |
| NaCl | 10g |
| Agar | 20g |

Adjust pH to 7.5 with NaOH

## APPENDIX III

Transformation of SK1590 ($Amp^S$ - $Tet^S$) with pWx33-pWx34 and pCA-1 (Maniatis et al. 1982)

Strain SK1590 was grown overnight in 20 ml of Luria Broth (Appendix II) in a New Brunswich incubator shaker at 37°C to an $OD_{550}$ = 0.2 units. The overnight culture (8 ml) was placed into 2-25 ml screw cap corex centrifuge tubes (sterile) and spun in a Sorvall RC-5B centrifuge using the SS-34 rotor (8000 rpm, 10 min, 4°C). The supernatant was decanted very carefully and each pellet was resuspended gently into 4 ml of MOPS pH 7.0 buffer [10 mM 3-[N-morpholino] propanesulfonic acid (MOPS) pH 7.0 and 10 mM Rubidium Chloride (RbCl)]. After resuspending the pellet, the homogenous solution was spun again in a SS-34 rotor (8000 rpm, 10 min, 4°C).

The supernatant was decanted and the pellet was resuspended as before in 4 ml of MOPS pH 6.5 buffer [100 mM MOPS pH 6.5, 10 mM RbCl, and 50 mM CaCl (calcium chloride)]. The tubes were incubated on ice for 30 minutes and then spun again as above. The cell pellets obtained were white and in a "donut" shape. The supernatant was decanted very carefully.

Each pellet was then resuspended in 0.8 ml of the MOPS pH 6.5 buffer and 200 $\mu$l of sterile cell suspension was placed into sterile 13x100 mm glass tubes. To each tube 3 $\mu$l of Dimethyl Sulfoxide (DMSO) (SIGMA) and 10 $\mu$l of transforming DNA (0.2 $\mu$g of DNA to a final volume of 24 $\mu$l with TE buffer) was added. The mixture was gently shaken and incubated on ice for 30 minutes. Each tube was then transferred to a 43.5°C waterbath and heat shocked for 30 seconds. To each tube 3 ml of Luria Broth (LB) was added, and all the samples were incubated at

37°C for at least 2 hours or until visible growth occurred.

Suspensions of 0.1 ml from each tube containing either the pWx33 (Table II), the pWx34 (Table II) or the pAC-1 transformed cells were spread onto Luria plates which contained either Ampicillin (40 μg/ml) (SIGMA) or Tetracycline (20 μg/ml) (SIGMA) and incubated overnight at 37°C.

Rapid Extraction of DNA from Transformed Cells by the Alkaline Lysis Procedure. (Maniatis, et al., 1982)

Five ml of Luria Broth (Appendix II) containing the appropriate antibiotic (20 μg/ml) was inoculated with a single bacterial colony obtained from the transformation experiment. The tubes were incubated at 37°C overnight with vigorous shaking in a water bath (Model G76-New Brunswick Scientific Co., Edison, N.J.).

One and a half ml of the overnight culture was pipetted into an Eppendorf tube and centrifuged for 1 minute. The remainder of the overnight culture was stored at 4°C.

The medium was removed by aspiration with a drawn out Pasteur pipette, leaving the bacterial pellet as dry as possible. The pellet was resuspended by vortexing in 100 μl of ice-cold glucose solution (Appendix I). The suspension was left at room temperature for five minutes with the top of the tube open). A freshly prepared, ice-cold solution (700 μl) of 0.2N NaOH, and 1% SDS was added and the tube was inverted sharply 7 to 8 times and stored on ice for 5 minutes. An ice-cold solution (150 μl) of KOAc pH 4.8 (Appendix I), was added and the tube was vortexed gently in an inverted position for 10 seconds and stored on ice for 5 minutes. The solution was centrifuged for 5 minutes in an Eppendorf centrifuge at

4°C and the supernatant was transferred to a fresh tube. An equal volume of phenol/chloroform (1:1 V/V) was added and vortexed. The sample was centrifuged for 2 minutes in an Eppendorf centrifuge and the supernatant was transferred to a fresh tube. To precipitate the DNA 2 volumes of 95% ethanol at room temperature were added. After vortexing, the sample was incubated at room temperature for 2 minutes and centrifuged for 9 minutes in an Eppendorf centrifuge at room temperature. The supernatant was removed and the tube was inverted on a paper towel to allow all the fluid to drain away. The pellet was rinsed with 70% alcohol and dried for a few minutes in an 80°C oven. Fifty μl of TE buffer, pH 8.0 (Appendix I) containing 1 μg of DNase-free pancreatic RNase (RNase stock - 10 mg/ml) was heated at 100°C for 10 minutes). The plasmid DNA was analyzed by digestion with restriction enzymes and electrophoresed in 0.8% agarose gels.

Amplification of DNA Probes pAc-9, pCA-1, pWx33 and pWx34. (Maniatis et al., 1982)

An overnight culture of a bacterial colony was prepared in 5 ml of Luria Broth (Appendix II) containing the appropriate antibiotic. For pAc-9 and pCA-1 tetracycline at 20 μg/ml was used, and for pWx33 and pWx34 ampicillin at 40 μg/ml was used.

Sterile 4000 ml Fernbach flasks containing 500 ml of M-9 medium (Appendix II) were inoculated with approximately 2.5 ml of overnight culture. The initial absorbance value at 600 nm was between 0.05 and 0.1 OD units. The flasks were vigorously shaken at 37°C on a rotary shaker (Model G-25 New Brunswick Scientific Co., Edison, N.J.). Chloramphenicol (34 mg/ml stock, SIGMA) was added to each flask to a final concentration of 170 μg/ml when the $OD_{600}$ was 0.6 OD units. The

flasks were virgorously shaken at 37°C for 12-16 hours.

Cells were harvested by centrifugation in 250 ml centrifuge bottles using the Sorvall GSA rotor (5,000 rpm, 15 min, 5°C). The cell pellet was washed with 100 ml of cold STE buffer (Appendix I).

Purification of Plasmid DNA Probes. (Maniatis et al., 1982)

Harvested cells from the plasmid amplification experiment (500 ml work-up) were resuspended in 10 ml of cold glucose buffer (Appendix I) containing 50 mg of lysozyme (5 mg/ml, Worthington Biochemicals Co., Freehold, N.J.).

The sample was transferred to two 60Ti tubes and incubated for 5 minutes at room temperature. The sample became very viscous. Cold NaOH solution (10 ml) (Appendix I) was added to each tube and inverted sharply 7-10 times. The tubes were then incubated on ice for 10 minutes. 7.5 ml of 5 M potassium acetate pH 4.8 (Appendix I) were added to each tube, and, again, the tubes were inverted sharply and incubated on ice as above.

The tubes were centrifuged in the Beckman 60 Ti rotor (27,000 rpm, 30 min, 4°C. After decanting the supernatant into a flask, 0.6 volume of isopropanol was added. After mixing, the sample was transferred immediately to 30 ml Corex tubes and incubated at room temperature for 15 minutes and then centrifuged in a SS34 rotor (12,000 rpm, 30 min, 20°C). The supernatant was discarded and the pellet was dissolved in 8 ml of TE buffer pH 8.0 (Appendix I). For each ml of solution, 1 gm of solid CsCl (Bethesda Research Lab.) was added to the tube. After mixing and dissolving, 0.7 ml of EtBr solution (Appendix I) (10 mg/ml) was added for every 10 ml of solution. After a refractive index reading of 1.3860 was attained by checking 20 $\mu$l samples in a Bausch and Lomb refractometer, the sample was centrifuged in a

Beckman type 65 rotor (45,000 rpm, overnight, 20°C).

Bands corresponding to plasmid DNAs were observed under U.V. light (Black-Ray). EtBr was extracted with butanol saturated with water (Appendix I), and the DNA samples were dialyzed against TE buffer (Appendix I).

Agarose Gel Electrophoresis of Plasmids Obtained by the Rapid Lysis and Large Scale Amplification Procedures. (Maniatis et al., 1982)

1) Plasmids from the Rapid Alkaline Lysis Method.

20 μl of plasmid DNA (approximately 1 μg DNA) obtained by the rapid alkaline lysis method was digested with PstI (Bethesda Research Labs.). The mixture was incubated at 37°C for at least 90 minutes. The DNA was then precipitated with 1/10 volume of 3M NaOAc and 2.5 volumes of cold 95% ethanol. The mixture was held at -70°C for at least 1 hour and spun for 12 minutes in the Eppendorf microfuge to pellet the DNA. The pellet was washed with cold 70% methanol, dried and resuspended in 10 ml of TE buffer (Appendix I).

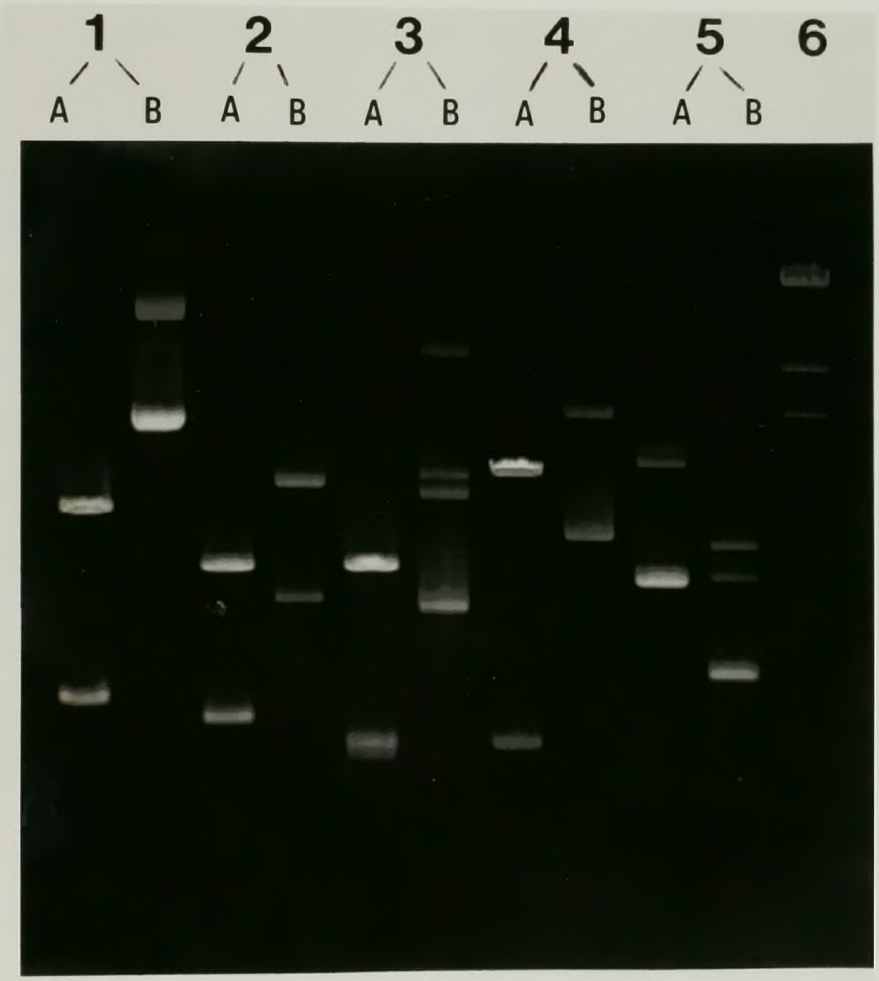
2) Plasmids from the Large Scale Amplification Method.

1 μg of the plasmid DNA's containing the maize clones were digested with PstI in a final volume of 10 μl. No ethanol precipitation was carried out in this step. Digestions were terminated by heat killing the enzyme at 65°C for 10 minutes. 4X EDTA stop buffer (Appendix I) (1/4 of the reaction volume) was added to each sample as a tracking dye. The samples were electrophoresed for 2 1/2 hours at 60 volts in a 1.5% mini-agarose gel with TEA buffer (Appendix I). The gel was then soaked for 10 minutes in approximately 200 ml of 1X TEA buffer containing 250 μl of EtBr solution (10 mg/ml in STE buffer - Appendix I). DNA fragments were visualized by using a ultraviolet transilluminator (Ultra-Violet Products Inc., San

Gabriel, Ca.). Control undigested plasmid DNA and molecular weight standards from a HindIII (BRL) digested bacteriophage lambda DNA were co-electrophoresed with the samples (Murray and Murray, 1975) (Figure 10).

**Figure 10: DNA probes digested with different restriction endonucleases.**

| | | |
|---|---|---|
| Lane 1: | A) pAc-9/PstI<br>B) pAc-9 | |
| Lane 2: | A) pWx34/PstI<br>B) pWx34 | |
| Lane 3: | A) pWx33/PstI<br>B) pWx33 | |
| Lane 4: | A) pCA-1/PstI<br>B) pCA-1 | |
| Lane 5: | A) pUAG1<br>B) pUAG1/PstI | Not used for<br>hybridization experiments |
| Lane 6: | phage lambda DNA/Hind III | |


1
2
3
4
5
6
A
B
A
B
A
B
A
B
A
B

## APPENDIX IV

Construction of a Croton Gene Library in Bacteriophage. (Maniatis et al., 1982)

The purpose of constructing a gene library is to clone a set of DNA segments which are fully representative of all the DNA sequences within a complex genome, which in this case is croton DNA. The number of clones necessary to represent all genomic sequences in such a library can be calculated from the following equation (Maniatis et al., 1982):

$$N = \frac{\ln (1-P)}{\ln (1-f)} \qquad \text{ln: natural log}$$

where N is the number of recombinants needed to have the probability P for representing the total genome where the fraction proportion of the genome f is cloned into the vector. For example, if we assume that the size of the croton genome is approximately $5 \times 10^9$ bp, it will be necessary to screen $1.2 \times 10^6$ phage in order to have a 99% probability ($p = 0.99$) of isolating a single copy sequence contained in 20 kb cloned segments ($f = 2 \times 10^4 / 5 \times 10^9$).

1) Partial digestion of high molecular weight DNA:

In order to establish conditions for the size of croton DNA necessary to construct a gene library, partial digests of genomic DNA were preexamined by electrophoresis. Croton DNA (10 $\mu$g) in BamHI buffer (BRL) in a final volume of 150 $\mu$l was mixed well. Thirty $\mu$l of the mixture was dispensed in tube 1 and 15 $\mu$l (each 15 $\mu$l contained 1 $\mu$g of croton DNA) in tubes 2 through 9. All the tubes were chilled on ice. To tube 1, 4 units of restriction enzyme BamHI (2000 U/200 $\mu$l BRL) were added and mixed well. The concentration of the enzyme was thus 2 units/$\mu$g croton DNA. 15 $\mu$l of the reaction mixture was transferred to tube 2,

making the concentration of enzyme 1 unit/$\mu$g croton DNA. The sample was mixed well and the two-fold serial dilution was continued through to tube 8. Tube 9 containing undigested croton DNA, was used as a control. All tubes were incubated at 37$^{o}$C for exactly 1 hour. The reaction was stopped by chilling the tubes in ice and by adding EDTA to a final concentration of 20 mM. 3 $\mu$l of 4X stop buffer (Appendix I) was added to each sample and the size of the fragments was checked by electrophoresis in a 0.8% agarose gel. pBR322 multimers were used as molecular weight markers. Once the conditions for the partial digestion were established a large scale partial digestion of croton DNA was performed. Two reaction tubes containing 50 $\mu$g of croton DNA (200 $\mu$g/ml concentration) in a 750 $\mu$l reaction volume were prepared. The enzyme concentration and the time and temperature of incubation were the same as those required to generate the proper size fragments. A 0.5 $\mu$g aliquot was analyzed in a 0.8% agarose gel to check if the size distribution of the digestion products was correct. The rest of the digested DNA was then extracted twice with 1/10 volume of 3 M NaAOc (pH 5.2) and 2 1/2 volumes of cold 95% ethanol. The pellet was obtained by centrifugation in a SS34 rotor (12,000 rpm, 20 min, 4$^{o}$C) and resuspended in 200 $\mu$l of TE buffer (Appendix I). A 10-40% sucrose density (Appendix I) gradient using the LKB gradient former was prepared in Beckman SW41 polyalomer tubes. DNA samples were heated for ten min at 70$^{o}$C to denature any sticky ends. After rapid cooling in ice the samples were placed onto the gradient and centrifuged (25,000 rpm, 20 hours, 20$^{o}$C) in a Beckman L8-70 centrifuge. Fractions of 0.5 ml were collected, and every third fraction was analyzed by agarose gel electrophoresis using the same conditions as stated above. The gradient fractions containing the DNA fragments in the 10-16 kb

size range were pooled and 2 ml of $H_20$ was added to each 0.5 ml fraction to dilute the sucrose. Four ml of isopropyl alcohol was then added and the DNA was precipitated overnight at -20°C. The DNA was pelleted by centrifugation (12,000 rpm, 15 min, 4°C) and resuspended in TE buffer (Appendix I). The concentration was determined by spectrophotometric readings at 260 nm.

2) Preparation of vector DNA. (Enquist and Steinberg, 1979a)

Embl-4 was used as vector DNA. Its size is 44.0 kb (left arm 19.4 kb, right arm 10.9 kb, stuffer 13.7 kb). It is a lambda replacement vector carrying polylinker sequences containing EcoRI: BamHI: SalI recognition sequences (Frischauf, 1983). This vector permits the cloning of large fragments (9-22 kb) generated by any of these enzymes. Ligated DNA was extracted twice with an equal volume of phenol: chloroform and precipitated with 1/10 volume of 3M NaAOc and 2 1/2 volumes of 95% ethanol at -70°C for 2 hours. The pellet after centrifugation in a microfuge for 10 min was resuspended in 100 $\mu$l of TE buffer. The concentration of the DNA was adjusted with TE buffer to 250 $\mu$g/ml.

Following ligation, the DNA was digested with 50 $\mu$l of restriction enzyme BamHI (BRL) in a 200 $\mu$l reaction volume that also contained 20 $\mu$l of 10X BamHI buffer (Appendix I). Incubation was for 4 hours at 37°C. A 0.5 $\mu$g aliquot was electrophoresed in a 0.8% agarose gel to check for complete digestion of Embl-4 DNA. The stuffer was then digested with SalI which cuts the stuffer fragment at both ends, thus preventing ligation to the BamHI sites of the L-R arms. The buffer concentration for the SalI digestion was changed from 100 mM to 150 mM NaCl by adding 5 M NaCl, and 50 $\mu$l of SalI (500 units/$\mu$g DNA) was added and incubated at 37°C for 5 hours.

After the SalI digestion was checked for complete digestion by agarose gel electrophoresis, the DNA was then extracted twice with an equal volume of phenol: chloroform and once with chloroform and precipitated with 95% ethanol at -70°C for 1 hour. The pellet was resuspended in 200 μl of TE buffer and heated at 70°C for 10 minutes to denature any annealed end of the BamHI complementary sequence. A 10-40% sucrose gradient was prepared as above and the DNA was loaded onto the gradient. Centrifugation was performed at 25,000 rpm for 8 hour at 20°C. Twenty fractions of 0.5 ml each were collected and precipitated overnight at -20°C with isopropanol, using the same conditions as carried out for the partially digested croton DNA. The concentration was determined by spectrophotometric readings at 260 and 280 nm and those fractions containing the ligated left-right arms were pooled, reprecipitated and used for ligation with croton DNA.

3) Ligation of arms with croton DNA.

A series of test ligations and packaging were performed to determine the ratio of arms to inserts that gave the greatest number of packaged molecules. Although this ratio is theoretically 2:1 (arms): (inserts) (Maniatis et al., 1982) for the size of the inserts (15 kb) used, some of the molecules may lack a cohesive terminus, so that the effective concentration of ends available for ligation may be less than what the calculation suggests. For the test ligations the following molar ratios of arms: inserts were used: 0.5:1, 1:1, 2:1 and 4:1. The ligation reaction was performed in a 15 μl reaction volume which contained 1.5 μl of 10X ligation buffer (BRL) and 0.1 units/μg DNA of T4 ligase (BRL). The concentration of arms was kept constant at 1 μg and the concentration of insert was varied, depending on the molar ratios required. Tubes containing only Embl-4 DNA and digested non-packaged croton

DNA were run as controls. The ligation reaction was performed overnight at room temperature, and the ligation was checked by agarose gel electrophoresis using as MW markers a bacteriophage lambda DNA HindIII digest and pBR322 ligated multimers.

4) In vitro packaging of ligated Embl-4/croton DNA:

a) Testing of bacteriophage lysogens:

To check for the presence of the mutation that renders the CI-gene product temperature sensitive, E. coli strains NS 428 (Dam II, b2, red3, CI ts 857, Sam7) (Enquist, and Steinberg, 1979) and NS433 (Lambda Eam4, b2, red3, CI ts 857) were streaked onto Luria agar plates (two plates for each strain). Plates were incubated at 32°C or at 42°C. These strains were also tested for the presence of the RecA$^-$ mutation by streaking the bacteria onto a Luria agar plate. These streaks were exposed to U.V. light in a Biogard hood for 10, 20 and 30 seconds, and then incubated at 32°C overnight (Maniatis et al., 1982). The Rec A gene product is involved in the recombinational repair mechanism and the inability to survive U.V. treatment indicates the Rec A$^-$ genotype. Positive results (no growth) for the above test indicate that the recombination mechanism is defective. This is essential because recombination between endogenous lambda DNA and recombinant DNA must be avoided.

b) Preparation of packaging extracts: (Enquist and Steinberg, 1979a)

Starter cultures of NS428 and NS433 were grown overnight at 32°C in 50 ml of NZYCM broth (Appendix II). Approximately 8 ml of overnight culture was inoculated into 500 ml of NZYCM broth in Fernbach flasks to attain an $OD_{660}$ of 0.3. The lysogens were induced by placing the flasks into a waterbath preheated to

65°C. The cultures were swirled until the temperature inside the flask reached 45°C. The flasks were then kept at this temperature an additional fifteen minutes with vigorous shaking. The cultures were transferred to a 38-39°C incubator-shaker (New Brunswick, G25) for an additional 3 hours with vigorous aeration. Cells were recovered by centrifugation (5000 rpm, 10 min 4°C), using a GSA rotor in a Sorvall RC-5B centrifuge.

b-1) Sonication of NS428:

The cell pellets were resuspended in 3.6 ml of buffer (20 mM Tris pH 8.0, 1 mM EDTA, 5 mM Beta-mercaptoethanol). The resuspended cells were placed in an 8 ml plastic tube immersed in icewater and were sonicated with 2 second bursts at a setting of four in a Sonicator (Cell Disruptor Model W185, Heat Systems-Ultrasonics) using the fine tipped probe. The sample was kept on ice water for 30 to 45 seconds between bursts to prevent overheating. The sonicated sample was transferred to a centrifuge tube and cell debris was removed by centrifugation (12,000, 10 min, 4°C) in a RC-5B Sorvall centrifuge.

To the supernatant an equal volume of buffer and 1/6 volume of freshly prepared packaging buffer (Appendix I) were added. 15 μl aliquots were dispensed into precooled (4°C) 1.5 ml Eppendorf tubes and were immediately immersed into liquid nitrogen. The tubes were kept at -70°C for long-term storage.

b-2) Freeze-thaw extracts of E. coli NS433:

The cell pellets were resuspended in 3 ml of ice-cold sucrose solution [10% sucrose in 50 mM Tris (pH 8.0]. Aliquots of 0.5 ml were distributed into batches of six precooled Epppendorf tubes and 25 μl of freshly prepared ice-cold lysozyme suspension (2 mg/ml lysozyme stock (Worthington Biochemicals) in 0.25 M

Tris (pH 8.0) was added to each tube. The tubes were then placed into liquid $N_2$. The frozen samples were thawed on ice and 2.5 $\mu$l of freshly prepared packaging buffer was added to each tube. After mixing well with a sealed Pasteur pipette, all the thawed extracts were combined and centrifuged in the Beckman L-80 using a SW 65 rotor (30,000 rpm, 1 1/2 hr, $4^{o}C$). Aliquots of 10 $\mu$l of the supernatant were dispersed into precooled $4^{o}C$ Eppendorf tubes, immediately frozen in liquid $N_2$, and stored at $-70^{o}C$.

c) Packaging reaction:

The freeze-thaw extracts of NS433 were removed from the $-70^{o}C$ freezer and thawed on ice. After complete thawing, they were transferred to the still frozen sonicated extract of NS428. Both extracts were mixed gently while the frozen NS428 extract was melting. Ligated DNA (0.8 $\mu$g) was then added to each mix. The mixture was gently stirred with a sealed Pasteur pipette and incubated for 1 hour at room temperature. After the incubation period, each sample was taken up to 1 ml with lambda dilution buffer (Appendix I) and a drop of chloroform was also added. The cell debris was removed by centrifugation in an Eppendorf centrifuge. Embl-4 DNA was also packaged and used as a control.

d) Plaque assay to measure viable bacteriophage particles:

Cultures of Q359 and Q358 were prepared in NZYCM containing 0.2% maltose (Appendix II). The cultures were grown to an $OD_{550}$ of not more than 0.2-0.3 units.

Serial dilutions ($10^{-3}$, $10^{-4}$, $10^{-5}$, $10^{-6}$) of the bacteriophage particles were made in lambda dilution buffer (Appendix I). Each dilution (0.1) was mixed with 0.2 ml of the plating bacteria (Q359 was used for Embl-4 containing recombinant DNA and

Q358 for wild type Embl-4). Samples were incubated at 37°C for 15 minutes. Melted top agar (13 ml) kept at 45°C (Appendix II) was added to each tube, and after vortexing, each sample was immediately poured out onto bottom agar plates (Appendix II). The plates were incubated overnight at 37°C and the bacteriophage titer was determined the next day.

e) Library amplification:

Plates containing a large number of plaques were placed for 4 hours in a cold room with 3 ml sterile lambda dilution buffer (Appendix I). The viral suspension was then placed in Eppendorf tubes and spun for 5 minutes at 4°C to remove debris. The plaque assay using 10-fold dilutions was performed as above to determine the new titer of the amplified library.

Plaque Hybridization. (Maniatis et al., 1982)

Dilutions of the stock library were made in order to obtain $10^4$ - $10^5$ PFU/plate. Bacteriophage particles were plated onto NZYCM bottom agar plates using Q359 as the host bacteria. After an overnight incubation at 37°C the plates were chilled for 2-3 hours in the cold room (8°C).

Nitrocellulose filters were cut the same size as the plates, and after labeling for orientation, the filters were placed over each plate for 5 minutes using Millipore tweezers. After the elapsed time, the filters were gently removed and immersed for 2 minutes in denaturating solution (0.5 M NaOH, 1.5 M NaCl) and then for 2 minutes in neutralizing solution (0.5 M Tris pH 7.5, 1.5 M NaCl). The filters were rinsed in 2X SSC (Standard Saline Citrate; Appendix I) and, after air drying, were placed in a vacuum oven to dry at 80°C.

The hybridization procedure using radioactive probes (in this case croton DNA)

and the washing of filters was similar to that used for the Southern blots. Plaque hybridization results are shown in Fig. 11.

Figure 11: Plaque hybridization of the croton DNA gene library with croton DNA as a radioactive probe.


2 R

# VITA

Title of Thesis: Molecular basis of pigmentation in croton plants.

Full Name: Delia Kaganowicz-Gutman

Place and Date of Birth: August 25, 1961
Buenos Aires, Argentina

Elementary and Secondary Education:
Escuela Normal Superior No. 4, 1966-1973, Buenos Aires, Argentina.
Escuela Normal Superior No. 4, 1973-1978, Buenos Aires, Argentina.

Colleges and Universities:
Universidad Argentina de la Empresa, Facultad de Ciencias Agrarias, 1979-1982. Buenos Aires, Argentina.
Florida International University, Department of Biological Sciences, B.S. in Biology, 1982-1983.
Florida International University, Department of Biological Sciences, M.S. in Biology, 1984-1986.

Major Department: Biology

Date: Signed: