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# STUDIES OF CHLOROPLAST TRANSMISSION AND $F_2$ HYBRID VARIEGATION IN THE COMMON BEAN *PHASEOLUS VULGARIS*

BY

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# ABSTRACT

## STUDIES OF CHLOROPLAST TRANSMISSION AND $F_2$ HYBRID VARIEGATION IN THE COMMON BEAN *PHASEOLUS VULGARIS*

By

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Many of the $F_2$ progeny in wide crosses of the common bean *Phaseolus vulgaris* display cell lineage patterns of leaf variegation. In *Oenothera* and *Pelargonium*, a similar hybrid variegation occurrs in $F_1$ plants and is called "plastome-genome incompatibility". In both of these plants, high levels of biparental non-Mendelian transmission of plastids occurs, when plastids are transmitted from both parents in a cross. The incompatibility results when one plastid is unable to develop properly in the hybrid nuclear background.

In contrast, in *Phaseolus vulgaris*, the analysis of cpDNA restriction fragment length polymorphisms described here has clearly shown that the transmission of chloroplasts in sexual crosses between cultivars is predominantly by uniparental maternal inheritance. Furthermore, the analysis of cpDNA from the $F_2$ variegated progeny of the common bean indicates that $F_2$ variegated plants contain predominantly maternal type chloroplasts.

These results have allowed the elimination of the possiblity that $F_2$ hybrid variegation in the common bean *Phaseolus vulgaris* is due to the biparental non-Mendelian transmission of plastids and subsequent plastome-genome incompatibility.

This thesis is dedicated to

my loving mother

and to the memory of my father.

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| | |
|---|---|
| bp | base pair(s) |
| Ci | Curie(s) |
| g | gram(s) |
| G | gauge |
| h | hour(s). |
| b | kilo base pair(s) |
| l | liter(s) |
| ml | milliliter(s) |
| M | Molar |
| mM | milli Molar |
| min | minute(s) |
| n | nano (1 X $10^{-9}$) |
| rpm | revolutions/minute |
| u | micro (1 X $10^{-6}$) |
| UV | ultra violet |
| w/v | weight/volume |

# CHAPTER 1

# INTRODUCTION

## VARIEGATION OF PLANT TISSUES, WITH EMPHASIS ON *PHASEOLUS VULGARIS*

Variegation of plant tissues may result from a number of causes, both environmental and genetic. Plant pathogens, particularly viral infections, may cause streaks, stripes, or mosaic patterns on leaves or petals of plants. Other types of variegation, such as that exhibited by *Coleus*, may give a uniform and predictable pattern that does not follow a cell lineage. Rather it results from the developmental regulation of pigments by the action of a single nuclear gene or multi-gene complex. Kirk and Tilney-Bassett (1978) refer to this as "non-cell lineage variegation" since the trait does not result from a clonal segregation of cells during the development of the leaf tissue; the patterns arise through physiological changes occurring at specific areas of the leaf irrespective of the cell origins.

In the common bean *Phaseolus vulgaris*, a number of different agents may be responsible for non-cell lineage leaf variegation. Some types of viral infection cause mosaicism of bean leaves. Examples include bean yellow mosaic virus (Bos 1970), bean rugose mosaic virus (Gamez 1982), bean mild mosaic virus (Waterworth 1981), bean golden mosaic virus (Goodman and Bird 1978), bean southern mosaic virus

(Shepherd 1971) and bean common mosaic virus (Bos 1971; Epko and Saettler 1974). The common mosaic virus and the southern virus can be transmitted through sexual crosses to the progeny via the pollen or by the maternal seed tissue, with transmissability ranging from 4 - 80%. In spite of the seemingly heritable nature of the mosaicism, virally-induced variegation can be distinguished from genetically based variegation by its infectivity to other plants through inoculation of the cell sap and by its non-cell lineage pattern.

Necrotic stippling (called "bronzing") may occur on older leaves in response to high levels of the air pollutant ozone (Saettler 1978; Hart and Saettler 1981). Effects are most noticeable in navy and pinto cultivars in mid-to-late August when the plants are approaching normal maturity. Unlike viral infection, "bronzing" is noninfectious and does not spread from plant to plant. Ozone levels greater than 100 parts per billion can completely defoliate plants.

A general non-cell lineage yellowing of bean plants may result in response to a deficiency of nitrogen, phosphorus, potassium, manganese, zinc, or sulfur (Vistosh *et al.* 1978), with the symptoms depending upon the particular nutrient deficiency.

Burkholder and Muller (1926) described a condition in beans which they termed "pseudo-mosaic" and which appeared to be not a virus infection but a hereditary abnormality. This hereditary disorder is governed by two recessive factors. The presence of numerous, small yellow spots on the primary and compound leaves of the common bean was found to be controlled by an incompletely dominant gene (Parker 1933). Intensely spotted plants were homozygous dominant. At first Parker had suspected that the common beans were infected with a virus, but negative results from grafting and inoculation experiments as well as Mendelian segregation in the $F_2$ and $F_3$ generations proved this hypothesis was wrong. Smith (1934) described a hereditary chlorophyll deficiency in beans. This pale green character was governed by a single recessive gene. Provvidenti and Schroeder (1969) described the inheritance of apical chlorosis in beans, which is somewhat complex and incompletely understood. $F_2$ segregations occurred, but were inconclusive and further data would be required to clarify the inheritance pattern.

In contrast to the above examples of non-cell lineage variegation, cell lineage variegations display a clonal origin. The genetic basis may be nuclear, or occasionally mitochondrial, or it may represent the sorting out of different plastid types during division of the leaf primordia.

Recently, mitochondrial DNA alterations have been shown to be involved in the maternally inherited nonchromosomal stripe of maize (Newton and Coe 1986).

In the case of nuclear mutation, if a nuclear gene mutates in the somatic tissue, and its product is necessary for chlorophyll biosynthesis or chloroplast development, the plant may be sectored. Since this kind of nuclear mutation is generally recessive, sectoring would be evident only on a plant that was originally heterozygous at the particular locus. One example is the pale green locus of maize which Peterson (1960) showed to be affected by the En-I controlling elements or the *yg* locus which can be affected by the Ac-Ds system (McClintock 1950). Other recessive alleles may be "uncovered" or null alleles may be created by chromosomal aberrations occurring during development. Examples include the loss of chromosomal fragments (e.g. Beadle 1932) and the occurrence of chromosomal deficiencies in maize (e.g. McClintock 1932, 1938), the loss of whole chromosomes in a polyploid construct of *Nicotiana* (Moav 1961) and somatic recombination (Vig and Paddock 1968; Dulieu 1974).

Mutations arising in chloroplast genes may result in a cell lineage type of variegation. In several plant species nuclear mutator genes are known to increase the frequency of

plastome mutation (e.g., Redei and Plurad 1973; Potrykus 1973; Epp 1973; Sears 1983). The resulting mutations sort out during the development of the plant and also show a non-Mendelian manner of inheritance.

An apparent chloroplast gene mutation in the common bean was reported by Parker (1934) who studied a variegated plant from a commercial field of the common bean cultivar, Pencil Pod Black Wax. The variegated trait has been interpreted to have been due to a chloroplast gene mutation (Kirk and Tilney-Bassett 1978) because the inheritance of the trait was different in reciprocal crosses between the variegated mutant and green plants. When variegated plants were used as the female parent, 72 - 95% of the offspring expressed the variegated trait; when variegated plants were used as the male parent, 0 - 30% of the offspring were variegated. This report is consistent with biparental non-Mendelian transmission of plastids in other plants. However, ultrastructural data indicate that the pollen generative cell of *Phaseolus vulgaris* may completely lack plastids (Whatley 1982), which would eliminate any possibility of biparental plastid transmission. These contradictory data are discussed further in chapter three.

Many of the $F_2$ progeny in wide crosses of the common

bean *Phaseolus vulgaris* display cell lineage patterns of leaf variegation (Zaumeyer 1938, 1942; Wade 1941; Coyne 1966, 1967; J. Kelly, personal communication; my results). When Zaumeyer (1938) crossed two normal green cultivars, all $F_1$ plants from reciprocal crosses were green. In the $F_2$ generation, a ratio of 15 normal green to 1 variegated plant was obtained from 6729 plants. The abnormality was heritable and not the result of a virus infection. However, the variegated plants found in the $F_2$ generation did not breed true. Later Zaumeyer (1942) divided the leaf variegation into two types; one type - variegation of both primary and trifolate leaves - being inherited as a double recessive. The other type of variegation was confined to the trifolate leaves, and its mode of inheritance was not discussed. The symptoms of the two are similar, but they may be inherited differently.

Wade (1941) observed variegation in the $F_2$ progeny of bean when he crossed a normal green cultivar and a variegated cultivar. Zaumeyer (1938, 1942) and Wade (1941) showed in genetic studies that the variegation was controlled by nuclear genes, but each reported a different pattern of inheritance. Zaumeyer found that the character was due to two recessive genes, while Wade reported that this was determined by any one of three recessive genes.

Coyne (1966,1967) observed variegation in the $F_2$ progeny of beans when he crossed two different normal green cultivars. Results of reciprocal crosses indicated that cytoplasmic inheritance was not involved. Coyne's data suggested that the somatic instability which produced the variegated character was due to the action of two recessive genes, one being unstable in the presence of a mutator gene. The third gene apparently had a threshold effect and was only expressed under some conditions.

Grafting experiments suggested that the variegation was not due to an infectious virus. The degree of expression and variegation appeared almost completely masked at 80°F (Coyne 1969). The inheritance of variegation in the bean crosses examined by Coyne (1969) was interpreted to indicate that as many as four loci were involved to give a Mendelian ratio of 256 green to 1 variegated. (Kirk and Tilney-Bassett 1978).

## HYBRID VARIEGATION OF *PHASEOLUS VULGARIS*: POSSIBLE CAUSES AND AN OVERVIEW OF AN EXPERIMENTAL APPROACH TO INVESTGATE THIS PHENOMENON

Hybrid variegation can arise when plastids are transmitted from both parents in a cross (non-Mendelian biparental inheritance), with one plastid being unable to develop

properly in the hybrid nuclear background. Reports on two genera, *Oenothera* (Schoetz and Reiche 1957; Stubbe 1959) and *Pelargonium* (Metzlaff *et al.* 1981, 1982) have provided details on the factors controlling biparental inheritance. Hybrids between wide crosses of *Phaseolus vulgaris* have produced a similar cell lineage type of leaf variegation but only in the $F_2$ generation. Two possible interpretations seem most plausible: one is a non-Mendelian biparental plastid inheritance and subsequent plastome - genome incompatibility, the other is a mutable gene system as described by Coyne (1966, 1967) in common bean.

In order to discriminate between these possibilities, my initial research was focused on the manner of inheritance of chloroplasts in sexual crosses of *Phaseolus vulgaris*. Specifically, these investigations were designed to determine whether biparental inheritance and subsequent plastome - genome incompatibility were responsible for the $F_2$ hybrid variegation. Traditional genetic techniques were combined with a molecular analysis for these studies. Reciprocal crosses were made between large seeded and small seeded bean cultivars to produce $F_1$ progeny. These plants were self pollinated to produce the $F_2$ progeny. Using a polyacrylamide gel system, restriction fragment length polymorphisms were identified in cpDNA from the different parental lines.

Subsequently, cpDNA was isolated from the two parental lines and from the $F_1$ progeny, and total cellular DNA was isolated from $F_2$ variegated plants. The cpDNA restriction patterns were examined to determine the inheritance of restriction fragment polymorphisms. I tried to clone variable fragments from the BamHI and HaeIII digestion of parental cpDNAs, after having extracted them from polyacrylamide gels using pBR322 as a cloning vector. However, these cloning experiments were unsuccessful. In addition, various clones from the available *Oenothera* and spinach chloroplast genomic library were used as heterologous probes for Southern analysis. These Southern analyses have increased the sensitivity of detection and thus have been a critical molecular test for non-Mendelian biparental plastid inheritance.

I have examined both $F_1$ progeny and $F_2$ variegated progeny of the common bean to determine whether those plants contain a mixture of cpDNA from the two original parents. In addition, I have performed reciprocal crosses between the variegated plants and the parental bean lines to determine whether non-Mendelian factors are involved in the hybrid variegation of the common bean.

# CHAPTER 2

# MATERIALS AND METHODS

## PLANT MATERIAL.

$F_2$ hybrid variegation in the common bean *Phaseolus vulgaris* has been reported independently by at least three groups (Zaumeyer 1938, 1942; Wade 1941; Coyne 1966, 1967). Although these research programs utilized different parental cultivars, all of the crosses involved one small-seeded parent and one large-seeded parent. In *Phaseolus vulgaris*, the small seeded cultivars are derived from the wild beans of Mesoamerica (Gentry 1968), while the large seeded cultivars are probably derived from *Phaseolus aborigineus*, the wild bean of the colder Andes (Berglund - Bruecher and Bruecher 1976). It is generally believed that there are two gene pools of the cultivated common bean. The four cultivars chosen for crosses (Table 1) are thought to represent these two centers of domestication.

Seeds of the parental lines were kindly provided by Dr. James Kelly of Michigan State University. The seeds were germinated in vermiculite and the seedlings were transferred to pots three weeks after planting for crossing. All plants were cultivated under greenhouse conditions, using air conditioning in the summer. Under these conditions, the plants begin to flower after 5 - 6 weeks.

**Table 1.** *Phaseolus vulgaris* cultivars used in this study.

| Cultivar | Commercial Class | Origin* | Seed size (gm/100 seeds) | Seed Source |
|---|---|---|---|---|
| Mecosta | lt.red kidney | Andean | 50-55 large | MSU |
| Swedish Brown | Swedish Brown | Andean | 35-38 medium | MSU |
| Tuscola | navy | Meso-american | 19-22 small | MSU |
| Swan Valley | navy | Meso-american | 17-20 small | MSU |

* Center of domestication (Gepts *et al.* 1986)

## BACTERIAL STRAINS AND PLASMIDS.

The bacteria *Escherichia coli (E. coli)* strains ED8654: *[gal K, gal T, trp R, met B, hsd R, sup E, lac Y]* (Bork *et al.* 1976) and HB101: [*hsd S* ($r_B$-, $m_B$-), *rec A, ara, pro A, lac Y, gal K, rsp L, xyl, mtl, sup E*] (Boyer and Roulland-Dussoix 1969) were used in this study. They were maintained on Luria Broth (L.B.) plates for working cultures and as frozen cultures for long term storage. L.B. medium consists of: 5 g NaCl, 10 g Bacto tryptone (Difco), 5 g yeast extract (Difco), 1 g glucose [15 g Agar (Difco or Sigma)] per liter

(Maniatis *et al.* 1982). *E. coli* storage medium consists of: 0.7 g $K_2HPO_4$. 0.3 g $KH_2PO_4$, 0.05 g Na citrate, and 0.01 g $MgSO_4$, 50% Glycerine per liter. Frozen cultures were maintained at -20°C.

Bacterial strains containing the plasmid pBR322 were maintained on L.B. plates containing either 80 ug/ml Ampicillin (Amp) or 12.5 ug/ml Tetracycline (Tet). Recombinant plasmids containing inserts cloned into the Tet resistance gene were maintained on L.B. + Amp plates, or as frozen cultures lacking antibiotics in the storage medium described above.

## CROSSING TECHNIQUE.

The common bean *Phaseolus vulgaris* is self-fertile and cleistogamous. In the flower bud, if the left-hand wing is pressed downward, the unpollinated stigma with the style-brush emerges from the keel. The stamens remain in the closed keel. This mechanical response can be used advantageously in carrying out crosses. Pollinations are conducted as follows: the flower-bud to be pollinated is held in one hand between the thumb and forefinger. A pair of forceps is held with the other hand, resting the little finger and ring-finger on the hand with the flower-bud. The standard petal of the flower bud of the female parent is opened

by scratching the suture with a sharp pointed forcep. By pressing the left-hand wing downward, the unpollinated stigma protrudes. Then the pollen-laden stigma of the male parent is hooked behind the exposed stigma and clamped between the stigma and the keel. Finally, the standard petal of the parent is closed carefully (Buishand 1956). Using this technique, it is not necessary to emasculate, thus eliminating a procedure which reduces the numbers and vigor of hybrid $F_1$ seed.

Using this pollination method, reciprocal crosses were made between small seeded and large seeded bean cultivars, with "Swan Valley" being crossed with "Swedish Brown" and "Tuscola" being crossed with "Mecosta" to produce $F_1$ progeny. These plants were self-pollinated to produce the $F_2$ progeny, some of which display cell lineage patterns of leaf variegation. In all cases, the plant used as the female parent is the first one stated when crosses are written.

## DNA PREPARATION.

### Chloroplast DNA isolation.

Chloroplasts were first purified as described below. DNA was then extracted from the isolated organelles. The chloroplast DNA (cpDNA) isolation method is based on the one described by Herrmann (1982) with modifications adapted from

Palmer (1982), as described below.

All bean cpDNAs were isolated from four-week old seedlings at approximately the five-leaf stage, since I have found that cpDNAs isolated from older bean plants are frequently subject to degradation during the isolation procedure. The seedlings were placed in the dark one to three days before harvest in order to deplete their starch reserves. Leaves from about 50 seedlings were harvested and washed in tap water, damp dried, weighed, and kept at 4°C. The leaves were homogenized for about 10 seconds in 7-10 volumes of cold homogenization medium which contains 0.35 M Sorbitol, 50 mM Tris pH 8.0, 5.0 mM EDTA, 0.1% bovine serum albumine (BSA), 5.0 mM beta-mercaptoethanol. The homogenate was filtered through one layer of 100 micron mesh gauze followed by filtration through two layers of miracloth (Calbiochem). The chloroplasts were pelleted in a Sorvall GSA rotor at 4,000 rpm at 4°C for 3 min (after top speed was reached) and were washed in the medium containing 0.35 M Sorbitol, 25.0 mM EDTA, 50 mM Tris pH 8.0. The chloroplasts were then purified over a 10% - 80% sucrose step gradient buffered with 10 mM Tris pH 8.0, 1 mM EDTA. The sucrose gradients were centrifuged in the SS-90 vertical rotor in a Sorvall superspeed centrifuge using the rate control setting at 50% for 10 min at 15,000 rpm. The band containing the chloroplasts was separated and diluted by addition of 3 - 10

volumes of wash medium and centrifuged in the HB-4 rotor at 10,000 rpm at 4°C for 10 min. The chloroplast pellet was resuspended in an equal volume of 50 mM Tris, 100 mM EDTA, 15 mM NaCl pH 8.5 for subsequent lysis. The chloroplasts were lysed and the cpDNA was liberated from the membranes with which they associate by the addition of Sarkosyl to an end concentration of 1% and Pronase to an end concentration of 1 mg/ml, followed by gentle mixing at 4°C for 4 h. Chloroplast DNA was separated from other nucleic acid by CsCl buoyant density equilibrium centrifugation in the presence of bisbenzamide (Hoechst 33258) (Mueller and Gautier 1975, and Presler 1978). 20 ug bisbenzamide and 1.1 g/ml CsCl were added per ml of lysate and the refractive index was adjusted to 1.3980. CsCl gradients were run in a Sorvall OTD-B6 ultracentrifuge with the TV865 vertical rotor for samples of small volume or a TV850 rotor for samples greater than 30 ml. Gradients were run at 40,000 rpm to 42,000 rpm at 19 to 25 °C for 12 to 16 h.

The cpDNA band was collected using a siliconized pasteur pipette under a UV-light. Bisbenzamide was removed by extracting twice with an equal volume of CsCl- or NaCl-saturated isopropanol. Two volumes of dd$H_2O$ were added to dilute the salt concentration before isopropanol or ethanol precipitation.

## Total cellular DNA isolation.

The total cellular DNA isolation method was modified from Rawson *et al.* (1982). 2 to 5 g of plant material was homogenized in a small waring blender in the chloroplast isolation buffer described above and filtered through one layer of 100 micron mesh gauze to remove large debris. The homogenate was then pelleted by centrifugation at 9,000 rpm in the Sorvall rotor at 4°C for 20 min. The pellet was resuspended in a small volume of wash buffer and spun down in a Sorvall HB-4 swinging bucket rotor at 4°C at 10,000 rpm for 15 min. The pellet was then resuspended again in 7 to 10 ml of wash buffer for lysis. Triton X-100 was added to the suspension to bring it to an end concentration of 2.5% and Sarkosyl was added to an end concentration of 2%. 1.0 g/ml solid CsCl was added and was very gently swirled until the CsCl was dissolved. The mixture was allowed to shake gently for 1 hour at room temperature. The lysate was centrifuged in the Sorvall HB4 rotor at 9,000 rpm at 4°C for 20 min yielding a pellet of starch, other debris, and a protein-lipid pellicle on the top of the sample. The aqueous material between the pellet and the pellicle was removed and further processed. CsCl was added and the refractive index

was adjusted to 1.3920. 0.1 mg/ml of Ethidium bromide (EtBr) was added. The sample was centrifuged in the TV865 rotor at 42,000 rpm at 19 to 25°C for 13 to 17 h. The DNA band was collected as described previously. EtBr was removed by extracting twice with NaCl-saturated isopropanol. Two volumes of dd$H_2O$ were added to dilute the salt concentration.

## PRECIPITATION OF DNA.

DNA was precipitated by the addition of 1/10 volume of 3 M NaOAC and two volumes of absolute ethanol or 2.5 volumes of 95% ethanol at -20°C overnight. The samples were centrifuged in the HB4 rotor at 4°C at 10,000 rpm for 50 min. A small volume of 70% ethanol was added and used to suspend the DNA pellet and transfer it to a microfuge tube. The sample was centrifuged in a microfuge at 4°C for 10 min and washed with 70% ethanol. The purified cpDNA or total cellular DNA was dried, and then dissolved in the appropriate amount of 10 mM Tris pH 8.0, 1.0 mM EDTA (TE).

## PURIFICATION OF PLASMID DNA.

### Large scale isolation of plasmid DNA.

This method has been adapted from Maniatis *et al.*

(1982) and modified in the Dodgson laboratory at Michigan State University. A 0.5 liter culture of *E. coli* containing the plasmid was grown in Luria Broth (LB medium) plus Amp (80 ug/ml) in a shaker incubator at 37°C until the cell culture reached an optical density (O.D.) $_{600}$ of 0.5. A final concentration of 170 ug/ml Chloramphenicol was added for amplification of the plasmid. Incubation of the cultures continued at 37°C for at least 12 h or overnight.

The cells were pelleted in the GSA rotor at 5000 rpm at 4°C for 10 min. The pellet was carefully resuspended in 10 ml of Solution I which contains 50 mM glucose, 25 mM Tris.HCl pH 8.0, 10 mM EDTA, transferred to two 40 ml centrifuge tubes, and kept at room temperature for 5 min. 20 ml of freshly made Solution II (0.2 N NaOH and 1% SDS) was added, and the tubes were gently inverted several times, and then incubated on ice for 10 min. 15 ml of ice cold Solution III (3 M potassium, 5 M acetate, pH 4.8; adjusted with glacial acetic acid) was added, the tubes were sharply inverted several times, and the cells were incubated on ice for another 10 min. The lysate was then centrifuged in a Sorvall HB4 rotor at 10.000 rpm at 4°C for 20 min. The top part of the supernatant containing the plasmid DNA was carefully removed and placed into centrifuge tubes. The nucleic acids were precipitated with an equal volume of isopropanol at -70°C for 10 min and were then pelleted in a HB4 rotor at

10,000 rpm at 4°C for 35 min. The pellet was air dried briefly and dissolved in 5 ml TE buffer, then followed by two extractions using equal volumes of phenol and CIA ($CHCl_3$ : isoamylalcohol in a ratio of 24 : 1). The DNA was precipitated as previously described, but with the addition of 1/10 volume of 3M NaOAC. The nucleic acids were pelleted again in an HB4 rotor at 10,000 rpm at 4°C for 20 min. The pellet was dried and redissolved in 2 ml TE buffer. CsCl was added and the refractive index was adjusted to 1.3920. 0.1 mg/ml of EtBr was added and followed by ultracentrifugation in the TV865 rotor at 42,000 rpm at 19 - 25°C for 12 to 16 h. Following ultracentrifugation, the gradient was fractionated to recover the lower band containing supercoiled plasmid DNA. The EtBr was removed by extracting it twice with an equal volume of NaCl-saturated isopropanol. Two volumes of $ddH_2O$ were added to dilute the salt concentration. The DNA was precipitated as previously described.

**Rapid small scale isolation of plasmid DNA (mini-preps).**

The mini-prep method is a modification of a procedure described by Maniatis *et al.* (1982). Mini-preps were used to isolate small quantities (1 to 5 ug) of plasmid DNA in order to screen for recombinants in cloning experiments or any time small quantities of DNA were sufficient.

Bacterial cultures were grown in centrifuge tubes containing 5 ml L.B. plus Ampicillin in a shaker incubator at 37°C for 8 h or overnight. Cells were pelleted at 3,000 rpm at 4°C for 5 min, transferred to a 1.5 ml microfuge tube, and pelleted again by a brief spin in a microfuge. The pellet was resuspended in 150 ul of ice-cold Solution I and incubated for 5 min at room temperature. 300 ul of freshly prepared Solution II was added and incubated at room temperature for 2 min. Then 225 ul of ice-cold Solution III was added. The samples were incubated at -20°C for 12 min and were followed by centrifugation in a microfuge at 4°C for 5 min. The supernatant was transferred to a fresh microfuge tube for precipitation of the DNA. Plasmid DNA was then pelleted and washed as described above and dissolved in small volume of TE buffer.

## RESTRICTION ENDONUCLEASE DIGESTION OF DNA.

Restriction endonucleases were purchased from the following companies: Bethesda Research Laboratories, New England Biolabs, Boehringer Mannheim Biochemicals, and International Biotechnologies Inc. Reactions were carried out according to manufacturer's specifications using 1 to 5 units of enzyme per ug of DNA in a reaction volume of 20 to 40 ul per 1 ug DNA for 1.5 to 3 h. If the restricted DNA

needed to be concentrated to a smaller volume, the DNA was precipitated, washed and redissolved in TE buffer.

## GEL ELECTROPHORESIS.

### Agarose gel electrophoresis.

Agarose gel electrophoresis was performed as described by Maniatis *et al.* (1982). The concentration of the agarose gels depended on the size of the DNA fragments which needed to be resolved from restriction endonuclease digestions. Electrophoresis buffer TAE (40 mM Tris, 20 mM Na acetate, 1 mM EDTA pH 8.0 with glacial acetic acid) was used for resolution of large molecular weight fragments of 10 kbp or more, and TBE (89 mM Tris, 89 mM Boric acid, 2 mM EDTA pH 8.0 with HCl) for the resolution of DNA fragments less than 10 kbp. In order to be able to monitor the progress of the electrophoresis, a running dye of 0.1% bromophenol blue (BPB) in 30% glycerol was added to the samples. EtBr was added to the running buffers at a final concentration of 0.5 ug/ml. It was possible to monitor the progress of the electrophoresis by watching the dye and by looking directly at the DNA using a hand-held UV light. Gels were run at room temperature at a current of 25 to 50 mA with constant voltage.

**Polyacrylamide gel electrophoresis.**

Polyacrylamide gel electrophoresis (PAGE) was set up according to Maniatis *et al.* (1982). The concentration of the polyacrylamide gels varied from 5% to 20% depending on the sizes of the DNA fragments. The running buffer for PAGE was TBE or TAE lacking EtBr. PAGE was run at room temperature at 100 volts for 11 to 13 h. In order to achieve the best resolution, samples were loaded in volumes not exceeding 10 ul and run with 1/10 volume of 10X loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 25% Ficol (type 400) in $H_2O$). Following electrophoresis, the gels were stained in a buffer of 1 ug/liter EtBr in TBE or TAE for 45 min, destained in TBE or TAE for 20 min, examined on a UV transilluminator, and photographed.

**RECOVERY OF DNA FRAGMENTS FROM POLYACRYLAMIDE GELS.**

**Crush and soak method.**

This method has been modified from Maniatis *et al.* (1982). The EtBr stained gel was placed on a UV transilluminator; the band of interest was located and carefully removed with a sterile scalpel. Each gel fragment was then placed into a 5 ml disposable syringe fitted with an 18 gauge needle which had been cut to 1/4 of its original length. The syringe was placed above a 1.5 ml microfuge

tube; the whole system was then placed in a 30 ml corex centrifuge tube and was spun at 5,000 rpm at room temperature in a desk top centrifuge for 10 min. Elution buffer (0.3 M LiCl, 10 mM Tris pH 7.5, 0.1 mM EDTA, 0.05% SDS) was added to the crushed acrylamide , and the slurry was incubated with shaking at 37°C for at least 8 h or for overnight. The acrylamide was separated from the DNA by centrifugation through a quick-sep (Isolab) tube. One-tenth volume of 3 M NaOAC was added and the DNA was precipitated.

**Electrophoresis onto a dialysis membrane.**

The EtBr-stained polyacrylamide gel was placed on a UV transilluminator; the band of interest was located and carefully removed with a sterile scalpel. The gel fragment was then placed in a mini gel apparatus and a low melting agarose gel (Marine Colloids) was poured around it. After that, the procedure described by Maniatis et al. (1982) and modified in the Dodgson laboratory was followed. Using a sharp scalpel, an incision was made in the gel directly in front of the gel fragment and about 2 mm wider than the band on each side. A piece of Whatman 3MM paper and a piece of dialysis membrane of the width of the slot and slightly deeper than the gel were cut and soaked in electrophoresis buffer for 5 min. Using a forceps, the 3MM paper which was backed by the dialysis membrane was inserted nearest the DNA

band, being careful that no air bubbles were trapped. Electrophoresis was continued until the band of DNA had migrated into the 3MM paper. When all the DNA had left the low melting agarose gel and was trapped on the 3MM paper, the current was turned off. A hole was pierced in the buttom of a 1.5-ml microfuge tube with a 25G needle and it was placed on the top of a disposable culture tube (VWR Scentific). The 3MM paper and dialysis tubing was withdrawn from the gel and was placed inside the 1.5-ml microfuge tube. 200 ul of elution buffer (0.2 M NaCl, 20 mM Tris pH 7.6, 0.01 M EDTA, 0.05% SDS) was added to the 1.5-ml microfuge tube containing the paper and dialysis membrane. The whole set was vortexed thoughly and was spun in a desk top centrifuge for 2 min and the eluate was recovered. This elution procedure was repeated two more times. The pooled eluates were extracted with phenol and with CIA twice each. The DNA was recovered by ethanol precipitation.

**ELECTROPHORETIC TRANSFER OF DNA FROM POLYACRYLAMIDE GEL TO NYLON MEMBRANES.**

This method was originally developed by the Bio-Rad Laboratories (Bio-Rad Bulletin 1110, 1985). I have used it with both Zeta-probe (Bio-Rad) membranes and Nytran (Schleicher and Schull) membranes. Before the transfer, the polyacrylamide DNA gel was photographed, including a ruler

in the photograph for later comparison. A Zeta-probe membrane was cut to the same size as the gel and two Whatman 3MM filter papers were cut a little bigger than the gel. The two 3MM filter papers and the Zeta-probe membrane were soaked in 1X TAE buffer (0.1 M Tris pH 7.8 adjusted with glacial acetic acid, 0.05 M Na acetate, 5 mM EDTA). The gel was soaked in the denaturation mix (0.2 N NaOH, 0.5 M NaCl) for 20 min, and followed by neutralization by soaking in 0.5 liter of 5X TAE buffer twice, 10 min each. The gel was soaked again in 1 liter of 1X TAE buffer for 10 min. The electrophoretic transfer holder was assembled with the holder, two pre-soaked Whatman 3MM filter papers, two pads with the air bubbles squeezed out, and the gel. The assembled holder was placed in the electrophoretic transfer cell which had been filled half full with 1X TAE buffer. More 1X TAE buffer was added if necessary to bring the buffer level to 1 cm below the electrode post. The buffer was circulated in the cell with a magnetic stirrer to maintain uniform temperature during the transfer in the cold room, at 20 V (0.31 A) for 12 to 17 h. After transfer, the membrane was separated from the gel, was rinsed briefly in 1X TAE, and was air-dried. Then the membrane was baked overnight at 60°C or for 2 h at 80°C.

## HYBRIDIZATION OF SOUTHERN FILTERS.

Southern hybridizations of Zeta-probe membranes were performed as described by Maniatis et al. (1982). However, the Southern hybridizations of Nytran membranes were performed according to the manufacturer's (Schleicher & Schull) instructions with a slight modification. Nytran membranes were prehybridized at 68°C for 6 h or overnight in 6X SSC (1X SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0), 1% SDS, 10X Denhardts solution (1.0 g Ficol, 1.0 g polyvinyl pyrrolidone, 1.0 g BSA), 100 ug/ml denatured salmon sperm DNA. Hybridizations with radioactive DNA probes were performed at 68°C overnight in 6X SSC, 1% SDS, 100 ug/ml denatured salmon sperm DNA. The Nytran membranes were washed twice at room temperature for at least 15 min each in 6X SSC, 0.1 - 0.5% SDS, followed by two washes at 37°C in 1X SSC, 0.5 - 1.0% SDS for 15 min each. Finally, the membranes were washed at 65°C for 1 h in 0.1X SSC, 1% SDS. For heterologous probes, the final washes were at less stringent temperatures. The membranes were allowed to air dry followed by autoradiography.

## NICK TRANSLATION.

Radioactive probes were prepared by nick translation

with $^{32}P$ labeled nucleotides (NEN-DuPont). The procedure used was a modification of that of Rigby *et al.* (1977). 0.5 to 1 ug DNA was labeled in a reaction volume of 50 ul containing 50 mM Tris pH 7.8, 7.5 mM $MgCl_2$, 10 mM mercaptoethanol, 0.05 mg/ml BSA, and one or more $^{32}P$ labeled dNTPs (20 to 40 uCi specific activity greater than 600 uCi). Cold dNTPs were added at a concentration of 12.5 nM, omitting the one(s) used to label the DNA. The DNA substrate was then digested by adding 4 ul fresh DNase I (BMB) (100 ng/ml) for 1 min at room temperature immediately followed by the addition of 1 to 5 units of DNA polymerase I (BRL, BMB, or NE Biolabs) and incubation at 14°C for 1 to 2 h. The unincorporated $^{32}P$ nucleotides were separated from the labeled DNA over a Sephacryl S-200 (Pharmacia) column. The incorporation of $^{32}P$ into the DNA was quantified with DE-81 (Whatman) ion exchange paper as described by Maniatis *et al.* (1982) and by measurement in a Beckman LS-133 scintillation counter.

The nick-translated DNA was denatured by heating at 100°C for 5 min before adding to the hybridization mix.

## CLONING.

Comparison of the cpDNA RFLPs required large amounts of material. In order to obtain sufficient quantities of these

cpDNAs, I tried to isolate and clone the cpDNA fragments of interest into plasmid pBR322 using *E. coli* HB101 or ED8654.

**Preparation of vector DNA.**

For cloning of variable cpDNA fragments from bean cultivars, such as the BamHI 1.1 kb fragment from Swan Valley and the HaeIII 3.0 kb fragment from Swedish Brown, plasmid pBR322 DNA was cut with restriction endonuclease BamHI or NruI (blunt end). In order to prevent recircularization of non-recombinant plasmids, the digested vector DNA was treated with alkaline phosphatase (Calbiochem). For this reaction, 5 units of phosphatase for each ug pBR322 DNA was added to the restriction endonuclease digestion. Following incubation at 37°C for 1 h, the phosphatase was inactivated by extraction with phenol and with CIA twice each. The phosphatased pBR322 was then precipitated.

An electrophoretic purification method for preparation of vector DNA was also utilized. The plasmid DNAs were cleaved with the appropriate restriction enzyme and treated with phosphatase. Then the resulting products were electrophorectically separated on low gelling/melting agarose (Marine Colloids, Rockland) in TAE buffer. The gel slice containing linear vector DNA was then cut out as described in the following section. The electrophoretic separation of DNA fragments should remove the enzymes used

to cleave or modify DNA (thus eliminating the need to destroy them using phenol extraction or heat inactivation) and minimizes the problems caused by incomplete digestion with restriction endonucleases (Struhl 1985).

**Ligation.**

This method was adapted from a procedure described by Struhl (1983, 1985). cpDNAs were cleaved with restriction endonucleases and were separated on polyacrylamide gels. The cpDNA fragments of interest, visualized by EtBr staining with a long-wave UV light, were cut from the gel with a clean razor blade. The polyacrylamide gel slice was put at the bottom of the gel electrophoresis apparatus, then low gelling agarose (Marine Colloids) was poured around it, and an electric current was again applied in order to transfer the DNA fragment from the polyacrylamide gel segment to low gelling agarose. The DNA band of interest in the low gelling agarose gel was cut out in as small a volume as possible. The gel slices (usually 30 to 50 ul in TAE) were melted at 70°C for 5 to 15 min and then combined with vector DNA in appropriate proportions to give a final volume of 10 ul. After equilibration of the molten gel slice to 37°C, 10 ul of ice-cold ligase reaction mixture containing T4 DNA ligase (BRL) and reaction buffer (50 mM Tris-HCl pH 7.6, 10 mM $MgCl_2$, 5% (w/v) polyethylene glycol 8000, 1 mM ATP, 1 mM

dithiothreitol) was added and mixed quickly, and incubated at 16°C for 3 to 24 h. Although the reaction mixture resolidifies into a gel, the ligation is supposed to be extremely efficient (Struhl 1983). In order to introduce the ligated products into *E. coli*, the solidified reaction mixture was remelted at 70°C and diluted by a factor of 5 to 50 into ice-cold TCM buffer (10 mM Tris pH 7.5, 10 mM $CaCl_2$, 10 mM $MgCl_2$) prior to carrying out the transformation procedure.

Unless the low melting point agarose gel technique described above was used, the ligation of DNA was carried out with a vector/insert molar ratio of 3:1 in a 20 ul reaction mixture using ligase reaction buffer and T4 DNA ligase from BRL. The amount of ligase added was 1 - 2 units/reaction. The ligation reaction was incubated at room temperature for 4 h or 16°C for 12 to 18 h. The reaction was monitored by gel electrophoresis.

**Transformation.**

The transformation competent cells of *E. coli* strains used in these experiments were HB101 and ED8654 and were prepared by Sara Kaplan using the calcium chloride method described by Maniatis et al. (1982) with modifications (Kaplan 1987). A 100 ul aliquot of competent cells of either HB101 or ED8654 was thawed on wet ice. The diluted reaction

mixture was combined with the competent cells and incubated on ice for 30 min. Then the cells were heat-pulsed at 42°C for 2 min and incubated again on ice for 10 min, followed by the addition of 1 ml of L.B. medium. The cells were incubated at 37°C with shaking for 1 h, spread on L.B. plates containing Amp, and incubated at 37°C for no more than 15 h. As a positive control, supercoiled pBR322 DNA was used. As a negative control, water was added to a small amount of competent cells. Both control experiments were performed in every transformation.

**Screening for recombinant clones.**

Screening for recombinant colonies of bacteria was done as described by Gergen et al. (1979) and Grunstein and Hogness (1975) with the following modifications. After transformation, $Amp^r$ colonies were transferred to L.B. plus Amp and L.B. plus Tet grid plates and grown overnight at 37°C. Colonies displaying an $Amp^r$ $Tet^s$ phenotype were transferred to fresh L.B. plus Amp grid plates.

The colonies were grown on these plates for no more than 15 h at 37°C. A sterile Whatman 541 circle was placed on top of the bacterial colonies. After a few minutes, the filters holding the bacterial colonies were removed from the plates. In order to lyse the cells in the colonies and denature the DNA, the filters were washed twice with agitation

for 5 min in each of the following solutions: 0.5 M NaOH, 0.5 M Tris pH 7.5, and 2X SSC (1X SSC is 0.15 M NaCl, 0.015 M Na citrate, pH 7.0). The filters were rinsed briefly in 95% ethanol and dried. The filters were prehybridized in sealed plastic bags for at least 5 h at 55°C to 68°C in 0.5% NP 40 (Sigma), 100 ug/ml denatured salmon sperm DNA, 6X SET (0.9 M NaCl, 180 mM Tris pH 8.0, 6 mM EDTA). Hybridization was done at 0°C - 3°C below the Td( temperature of denaturation) which, under the most stringent conditions in the study was 68°C. The hybridization buffer was the same as the prehybridization buffer, but included a denatured $^{32}P$ nick translated probe. The hybridization was done at 68°C overnight. Following the hybridization, the filters were removed from the plastic bags and washed three times for 5 min each in 6X SSC at just below the Td (for the most stringent condition). The filters were allowed to air dry, followed by autoradiography.

# CHAPTER 3

# CHLOROPLAST TRANSMISSION IN THE COMMON BEAN *PHASEOLUS VULGARIS*

# INTRODUCTION

Both biparental and maternal transmission of plastids have been observed in the most primitive as well as the most advanced groups of extant plants, but maternal plastid inheritance seems to predominate throughout the plant kingdom from unicellular green algae to angiosperms. Even where non-Mendelian inheritance is biparental, the maternal contribution is usually much greater than that of the paternal partner (reviewed by Kirk and Tilney-Bassett 1978; Sears 1980, 1983; Whatley 1982; Connett 1987). Transmission of plastids in the genus *Nicotiana* has been considered to be strictly maternal (reviewed by Tilney-Bassett 1978). However, using a cytoplasmic streptomycin resistance marker, 2.5% and 0.07% of the offspring were found to contain paternal plastids (Medgyesy *et al.* 1986). Also in *Epilobium*, genetic studies indicated that plastid inheritance was purely maternal (Michaelis 1935, 1958). However, a recent report shows that a low level of paternal transmission of plastids occasionally occurs in interspecific crosses of this plant (Schmitz and Kowallik 1986).

For *Phaseolus vulgaris*, the data of Parker (1934) have been interpreted to indicate biparental non-Mendelian

inheritance of bean plastids (Kirk and Tilney-Bassett 1978). Parker reported genetic results from crosses of a variegated line which was derived from a sport found in a commercial field of the Pencil Pod Black Wax bean cultivar. When variegated (V) plants were used as the female parent in crosses with the normal green (G) Pencil Pod Black Wax cultivar, all $F_1$ offspring were variegated. In the reciprocal crosses (G x V), all $F_1$ plants except one were green. Such differences in reciprocal crosses are suggestive of a non-Mendelian mutation that is transmitted predominantly from the maternal parent. However, it is surprising that the V x G crosses produced no white progeny since one would expect that sorting out of mutant and green plastids would produce at least some germ line tissue which would carry only white plastids. On the other hand, if some amount of biparental transmission of plastids occurs in all of the progeny, many of the variegated progeny could actually have received white plastids from the female parent and green plastids from the male parent. The data from Parker's reciprocal cross (G x V) at first appear to be inconsistent with a high level of biparental chloroplast inheritance, since only one of the progeny was variegated. However, two observations apply: 1) even in those angiosperms which transmit plastids from both parents, the plastids from the female parent are the predominant type (reviewed by Kirk and

Tilney-Bassett 1978; Chiu *et al.* 1988); 2) If the mutant plastids have a competitive disadvantage in multiplication, as is the case in *Pelargonium* (Hagemann and Scholze 1974; Abdel-Wahab and Tilney-Bassett 1981), the combination of these two factors could result in a very low level of biparental inheritance when the mutant plastids are contributed by the male parent.

If one proposes such a model to explain the phenotypes of the progeny of Parker's reciprocal crosses, the results of the subsequent crosses are puzzling. Of the $F_2$ progeny from the original V x G cross (N=313), 83.7% were variegated and 16.3% were green. Again, no pure white $F_2$ progeny were detected, yet they certainly would have been expected due to the somatic sorting out of mutant and green plastids within the variegated $F_1$ plants. When the $F_2$ progeny derived from the G x V cross (N=381) were examined, 7.3% showed variegation. Only one of these twenty-eight progeny came from the single variegated plant of the $F_1$ generation; the other variegated $F_2$ plants were produced by self-crosses of the totally green $F_1$ plants. Again, these results are inconsistent when compared to other examples of organelle heredity. If the mutant plastids were abundant enough to make a significant contribution to the $F_2$ generation, they should have been visible as sectors in the $F_1$ plants. In

summary, these anomalies in Parker's genetic data suggest that the inheritance of variegation in *Phaseolus vulgaris* represents a more complex genetic phenomenon than can be explained through organelle heredity. To further complicate the situation, the ultrastructural data of Whatley (1982) indicate that the pollen generative cell may completely lack plastids, which would imply completely maternal transmission of bean plastids.

The experiments described here were designed to use molecular tools to determine the manner of inheritance of chloroplasts in sexual crosses of *Phaseolus vulgaris*. First, the appropriate restriction endonucleases and gel electrophoresis regime for visualizing restriction fragment length polymorphisms (RFLPs) in chloroplast DNA (cpDNA) was determined. Then, reciprocal crosses were made between bean cultivars with variable RFLPs to produce $F_1$ progeny. Subsequently, cpDNA was isolated from the parental plant lines and from the $F_1$ progeny. Restriction digests were analyzed by gel electrophoresis and Southern hybridization to determine the manner of transmission of the cpDNA markers.

# RESULTS

## Agarose gel electrophoresis does not show restriction fragment length polymorphisms of bean cpDNA

Initially, agarose gels were used for electrophoretic separation of the bean cpDNA restriction fragments. Although resolution appeared to be quite good, no differences in restriction patterns were observed with various restriction enzymes (Fig. 1), nor with various concentrations of agarose (0.6 - 1.5%), nor with a Tris/Borate buffer system (data not shown).

## Polyacryamide gel electrophoresis shows restriction fragment length polypmorphisms of bean cpDNA

However, by running the DNA samples on polyacrylamide gels (which are usually only used for the analysis of very small fragment), polymorphisms were discerned, although the cpDNAs are strikingly similar (Figs. 2, 3, and 4). Restriction enzymes which cut the cpDNA frequently revealed RFLPs; these included HaeIII, EcoRI, MspI, and BamHI (BRL). In the experiments described in this chapter, only the results of the HaeIII restriction enzyme digestions are presented, since a heterologous probe was identified which hybridized to one of the RFLPs produced by this enzyme.

**$F_1$ plants contain only maternal cpDNA**

For both sets of reciprocal crosses, the ethidium bromide stained patterns on the polyacrylamide gels show that the cpDNA RFLPs of the $F_1$ progeny are identical to those of the maternal parents (Fig. 2A; 3A). This was confirmed by Southern hybridization analysis using a heterologous probe (a spinach chloroplast petA gene clone) which had homology to one of the RFLPs (Fig. 2B; 3B).

**Limits of reslution**

Figure 4 shows the limits of resolution of the Southern technique in this experiment. cpDNA was isolated from two cultivars and was digested with HaeIII. The two DNAs were mixed in the ratios indicated (Fig. 4). On the ethidium bromide stained gel, the variable fragment from the second cultivar is visible when that DNA composes more than 10% of the total (Fig. 4A). Through Southern hybridization DNA heterogeneity can be detected with confidence when the second DNA composes about 1% or more of the cpDNA mixture (Fig. 4B).

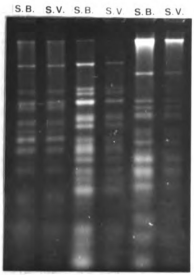


**Figure 1.** Restriction fragment patterns of chloroplast DNAs (cpDNAs) isolated from bean cultivars [Swedish Brown (lanes 1, 3, 5.) and Swan Valley (lanes 2, 4, 6.) respectively]. cpDNAs were digested with MspI (lanes 1 & 2), HaeIII (lanes 3 & 4), and EcoRI (lanes 5 & 6) respectively. They were electrophoretically separated on a 1.0% agarose gel in TAE buffer and then stained with ethidium bromide.

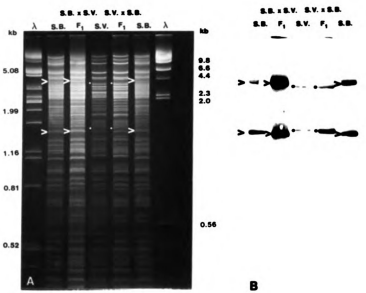


**Figure 2.** Restriction fragment patterns of cpDNAs from two bean cultivars and from $F_1$ progeny of reciprocal crosses between them. (A) Ethidium bromide stained gel. HaeIII restriction fragments of cpDNAs from Swedish Brown (SB), Swan Valley (SV), and from the $F_1$ hybrids of reciprocal crosses (SB x SV or SV x SB), were separated on a 5% polyacrylamide gel. Lambda DNAs were purchased from BRL and digested with PstI and HindIII, respectively. (B) Autoradiograph. cpDNAs were electrophoretically transferred to a Nytran membrane. Southern hybridization was performed using a $^{32}$P-labeled probe containing a spinach chloroplast *petA* (cytochrome f) gene and flanking sequences clone (pBF7) which was generously provided by W. Bottomley, Canberra, Australia.

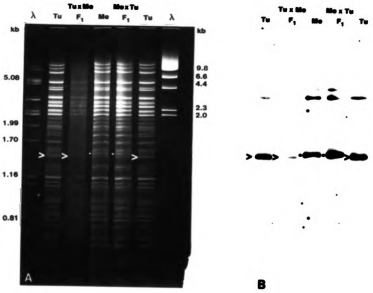


**Figure 3.** Restriction fragment patterns of cpDNAs isolated from two other bean cultivars and from $F_1$ progeny of reciprocal crosses between them. (A) Ethidium bromide stained gel. HaeIII restriction fragments of cpDNAs from Mecosta (Me), Tuscola (Tu), and from the $F_1$ hybrids of reciprocal crosses (Me x Tu) or Tu x Me), were separated on a 5% polyacrylamide gel. Lambda DNAs were digested with PstI and HindIII, respectively. (B) Autoradiograph. The cpDNAs shown in A were electrophoretically transferred to a Nytran membrane. The blot was hybridized with a $^{32}P$-labeled probe as in Figure 2.

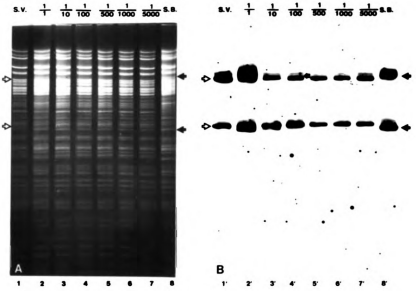


**Figure 4.** Autoradiograph showing the limits of an under-represented DNA type. cpDNAs isolated from the SV and SB cultivars were digested with HaeIII, were mixed in the ratios indicated, and then were separated on a 5% polyacrylamide gel. Lane 1 and lane 8 contain only the cpDNA of Swan Valley (SV) and Swedish Brown (SB), respectively. (A) Ethidium bromide stained gel. (B) Autoradiograph. The cpDNAs shown in A were electrophoretically transferred to a Nytran membrane. Probed with the same DNA as in Fig. 2.

# DISCUSSION

In a recent report, occasional rare transmission of paternal plastids was detected in *Nicotiana* (Medgyesy et al. 1986) and *Epilobium* (Schmitz and Kowallik 1986), which were previously thought to exhibit strict maternal plastid inheritance. The rare paternal transmission was discovered in two different ways. Using *Epilobium*, Schmitz and Kowallik (1986) pooled leaves and extracted cpDNA from a large number of progeny (N=200), while Medgyesy et al. (1986) devised a strong selection regime for *Nicotiana* by first inducing the growth of callus from the progeny and testing for transmission of a paternal antibiotic resistance marker.

Thus, Medgyesy et al. showed that 0.07% of the progeny of an interspecific cross and 2.5% of the progeny of an intraspecific cross had received the plastome marker from the male parent. From the *Epilobium* studies, it is not clear if the cpDNA from the paternal parent is present in each plant in small quantities or in only one plant or a few plants among the offspring as the predominant type of cpDNA. But, in either case, some level of biparental transmission is evident in both *Epilobium* and *Nicotiana*. Conceivably, if other "maternal" plants were tested for transmission of plastids from the male parent in one of these ways, more examples of occasional paternal contribution at a low

frequency may be discovered.

In the experiments described here, similar molecular techniques were used to reinvestigate the manner of plastid inheritance in crosses of *Phaseolus vulgaris*. Surprisingly, this plant species which has been reported as having significant levels of biparental non-Mendelian inheritance (Parker 1934; Kirk and Tilney-Bassett 1978), appears to have predominantly maternal transmission of the RFLPs analyzed. In contrast to the reports on intraspecific variation in cpDNA in other plants including *Nicotiana debneyi* (Scowcroft 1979), *Zea mays* (Timothy *et al.* 1979), *Pelargonium zonale hort* (Metzlaff *et al.* 1981), *Lycopersicon peruvianum* (Palmer and Zamir 1982), *Hordeum spontaneum* (Clegg *et al.* 1984), *Pisum sativum* and *P. humile* (Palmer *et al.* 1985), *Lupinus texensis* (Banks and Birky 1985), and *Oenothera johansen* (Johnson *et al.* 1988), much less heterogeneity of cpDNA was detected in the comparisons, and polyacrylamide gel electrophoresis was necessary in order to visualize RFLP differences.

The identification of an underrepresented cpDNA type by the visualization of fragment patterns stained with ethidium bromide has been reported to be possible if this DNA is present at 0.3% or more in an overloaded agarose gel

(Schiller *et al.* 1982). Southern hybridization expands these limits of detection to as little as 0.02% (Schmitz and Kowallik 1986), thus allowing the detection of a low level of paternal transmission of cpDNA. However, in this study, even using Southern hybridization, the DNA heterogeneity can be detected with confidence only when the second DNA composed 1% or more of the cpDNA mixture (Fig. 4B). Electrophoretic blotting of DNA fragments from polyacrylamide gels to a membrane support was less efficient than the standard method of capillary transfer developed by Southern (1975) and used for agarose gels, particularly for DNA fragments of high molecular weight. Thus, the reduced amount of DNA on the membranes may be one factor accounting for the limitation in visualization of fragments of low abundance in the mixing experiment. To increase the autoradiographic signal, nucleotides with a high specific activity label ( > 3000 Ci/m mol) were used and X-ray film was exposed for varying lengths of time. Nonetheless, resolution was limited to the ability to detect a second DNA type at the 1% level. Another contributing factor may be that the variable bands lie very close to each other in the gel. A strong signal from one band makes it difficult to see a very weak signal from the other band.

The results using this technique indicated that the cpDNA RFLPs of the $F_1$ progeny are identical to those of the maternal parent (Fig. 2A; 3A), and that the pooled $F_1$ progeny did not possess cpDNA from the male parent at the 1% level of detection (Fig. 2B; 3B). Thus, these results are consistent with Whatley's ultrastructural observations with *Phaseolus vulgaris* that plastids are absent from the pollen generative cells, which would result in purely maternal transmission of bean plastids. The results do not support the interpretation of Parker's genetic data that significant levels of biparental transmission of chloroplasts may occur in reciprocal crosses of *Phaseolus vulgaris*. It is unfortunate that the bean cultivars examined are different from the cultivar that Parker used, although all cultivated forms of the common bean are classified as belong to the same species. In order to find RFLPs, it was necessary to use two different cultivars. Actually, Parker's $F_1$ results showing predominantly maternal inheritance are consistent with those reported here. However, as discussed earlier, other aspects of Parker's genetic data including the apparent absence of sorting-out of the mutant trait and the recovery of sectored $F_2$ plants from totally green $F_1$ plants are inconsistent with the normal inheritance and segregational behavior of plastome mutations.

In many ways, Parker's data are more reminiscent of nuclear plastome mutator genes which have been characterized in several plant species (Redei and Plurad 1973; Potrykus 1973; Epp 1973). These recessive nuclear mutator genes are known to greatly increase the frequency of plastome mutations when homozygous. Conceivably, Parker's original variegated plant could have been homozygous for a *plastome mutator* allele. The $F_1$ progeny would be heterozygous; therefore the *plastome mutator* would be inactive, yet the plastome mutations induced in the original plant would be inherited in a typical non-Mendelian fashion. When the $F_1$ plants were self-pollinated, a fourth of the $F_2$ progeny would be homozygous for the *plastome mutator* allele, and new mutations would occur, giving rise to variegated plants even in those progeny derived from green $F_1$ plants.

Another explanation for Parker's results could be that the $F_2$ variegation represents a separate genetic phenomenon, different from the initial non-Mendelian variegation observed in the *Pencil Pod Black Wax* sport. At least three groups (Zaumeyer 1938, 1942; Wade 1941; Coyne 1966, 1967, 1969) have reported observing hybrid variegation following wide crosses between normal green plants of small-seeded and large-seeded cultivars of the common bean *Phaseolus vulgaris*. Their work and the results described in chapter 4

indicate that a nuclear mutator system is activated in a subset of the $F_2$ progeny (about 6-9% show variegation). Parker's results differ in that they were not derived from crosses of different bean cultivars since the variegated plant was a sport arising in a commercial field. However, it is possible that the sport arose from contamination of a seed lot by another cultivar. To determine the nuclear or non-Mendelian nature of the variegation observed in the $F_2$ progeny, reciprocal crosses would have been necessary, but were not included in Parker's study.

In the experiments described here, analysis of cpDNA RFLPs clearly indicated that the transmission of chloroplasts in sexual crosses between several cultivars of the common bean *Phaseolus vulgaris* is predominantly by uniparental maternal inheritance. Nonetheless, the possibility of occasional rare transmission of paternal plastids, as has been shown for *Nicotiana* and *Epilobium*, cannot be ruled out.

# CHAPTER 4

# $F_2$ HYBRID VARIEGATION OF THE COMMON BEAN *PHASEOLUS VULGARIS*

# INTRODUCTION

In interspecific crosses of *Oenothera* (Schoetz and Reiche 1957, Stubbe 1959) and *Pelargonium* (Metzlaff *et al.* 1982), hybrid variegation frequently arises in the $F_1$ generation when plastids are transmitted from both parents in crosses (non-Mendelian biparental inheritance), with one plastid being unable to develop properly in the hybrid nuclear background. In what was initially thought to be an analogous situation, many of the $F_2$ progeny (6-9%; Table 2) in wide crosses of the common bean *Phaseolus vulgaris* display cell lineage patterns of leaf variegation. This investigation was designed to specifically test whether biparental inheritance and subsequent plastome-genome incompatibility could be directly responsible for the $F_2$ hybrid variegation in the common bean *Phaseolus vulgaris*.

Reciprocal crosses were made between large seeded and small seeded bean cultivars to produce $F_1$ progeny. These plants were self-crossed to produce the $F_2$ progeny. Using a polyacrylamide gel system, I was able to identify RFLPs in cpDNA from the parental plant lines. Subsequently, cpDNA was isolated from the parental plant lines and total cellular DNA was isolated from the $F_2$ variegated progeny. Restriction

Table 2. Frequency of variegated $F_2$ progeny from crosses of cultivars of *Phaseolus vulgaris*. Plant designations are following: Mecosta (Me), Tuscola (Tu), Swedish Brown (SB), Swan Valley (SV). Most of these data were obtained using $F_2$ seed provided by Dr. James Kelly (JK) of the Michigan State University Bean Breeding Program. $F_2$ data from one of my crosses (PC).

| Cross | Source | % Germination of $F_2$ Progeny | Variegated Seedlings | Total # | Freq. of Variegated Seedlings |
|---|---|---|---|---|---|
| MexTu | JK | 84.7 | 10 | 111 | 9.0% |
| TuxMe | JK | 34.3 | 3 | 49 | 6.1% |
| SVxSB | JK | 74.9 | 8 | 158 | 5.1% |
| SBxSV | JK | 82.5 | 11(5)* | 137 | 8.0% |
| SBxSV | PC | 98.7 | 24 | 296 | 8.1% |

* ()indicates totally chlorotic seedlings.

digests were analyzed by gel electrophoresis to determine whether $F_2$ variegated plants contain a mixture of cpDNA from the two original parents. In addition, I have also performed reciprocal crosses between the variegated plants and the parental bean lines to determine whether non-Mendelian factors are involved in the hybrid variegation of the common bean *Phaselous vulgaris*.

## RESULTS

### cpDNA composition of $F_2$ variegated plants

Analysis of cpDNA from the $F_2$ variegated plants them-

selves should allow us to determine whether biparental inheritence is directly responsible for the $F_2$ hybrid variegation. Since the chlorotic sectors composed 25 - 50% of the plant tissue in the variegated leaves sampled, if two different plastid types from the original parents were responsible for the $F_2$ variegation, we would expect to be able to see bands from both types of cpDNA in the variegated progeny in ethidium bromide stained gel. However, Figure 5A indicates that the cpDNA restrction patterns of $F_2$ progeny were identical to those of original maternal parents. These were confirmed by Southern hybridization using a heterologous probe (pBF7) which had homology to one of the restriction fragment polymorphisms. (Fig. 5B).

**Inheritance of the variegated trait**

If the $F_2$ leaf variegation occurs because non-Mendelian mutations are induced in the bean plants, when reciprocal crosses are made using the variegated $F_2$ progeny and the green parental line, differences in the frequencies of variegated offspring will be apparent. Table 3 shows data from reciprocal back crosses of one set of $F_2$ variegated plants to the parental lines. All progeny of the back cross populations are green and there are no differences in reciprocal crosses.

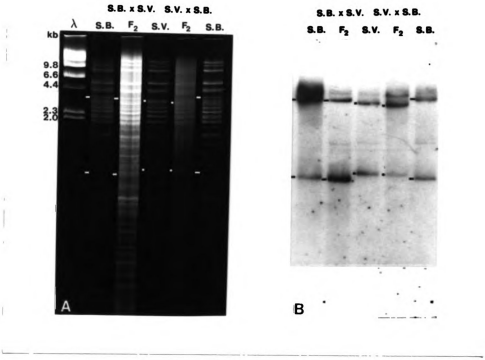


**Figure 5.** cpDNA composition of $F_2$ variegated plants. (A) Ethidium bromide stained gel. HaeIII restriction fragments of cpDNAs from Swedish Brown (SB) and Swan Valley (SV), and total cellular DNAs from the $F_2$ variegated plants of reciprocal crosses (SB x SV or SV x SB ), were separated on a 5% polyacrylamide gel. The lambda DNA was digested with HindIII. (B) Autoradiograph. The DNAs shown in A were electrophoretically transferred to a Nytran membrane. The blot was hybridized with a $^{32}P$-labeled probe as in Figure 2.

**Table 3.** Inheritance of variegation. Variegated $F_2$ progeny from the cross Swedish Brown x Swan Valley (line 5 of Table 2) were used in reciprocal crosses with either Swedish Brown (SB) or Swan Valley (SV) cultivars. Variegated plants were scored after the primary leaves were fully expanded (about 2 weeks after planting).

| Female Parent | Male Parent | % Germination | # Green Seedlings | # Variegated Seedling | Total # Seedlings |
|---|---|---|---|---|---|
| Var. $F_2$ | SB | 96.4% | 53 | 0 | 53 |
| SB | Var. $F_2$ | 100% | 44 | 0 | 44 |
| Var. $F_2$ | SV | 87.5% | 77 | 0 | 77 |
| SV | Var. $F_2$ | 100% | 14 | 0 | 14 |

## DISCUSSION

Many of the $F_2$ progeny in wide crosses of the common bean *Phaseolus vulgaris* display cell lineage patterns of leaf variegation (Zaumeyer 1938, 1942; Wade 1941; Coyne 1966, 1967, 1969). A similar hybrid variegation occurring in $F_1$ plants called "plastome-genome incompatibility" is well characterized in *Oenothera* (Stubbe 1959) and *Pelargonium* (Metzlaff *et al.* 1982), which have high levels of biparental non-Mendelian transmission of plastids. In *Phaseolus vulgaris*, the evidence regarding non-Mendelian inheritance

was conflicting: the ultrastructural data of Whatley (1982) indicate that the pollen generative cell may completely lack plastids, which would result in purely maternal transmission of bean plastids. However, an earlier report on the transmission of a variegated trait in reciprocal crosses was suggestive of significant levels of biparental non-Mendelian inheritance (Parker 1934; Kirk and Tilney-Bassett 1978). In other experiments (chapter 3 in this thesis), the analyses of cpDNA restriction fragment length polymorphisms have shown that the transmission of chloroplasts in sexual crosses between cultivars of the common bean *Phaseolus vulgaris* is predominantly by uniparental maternal inheritance. This result supports Whatley's ultrastructural observations of the generative cell in the common bean.

The analysis of cpDNA from the $F_2$ variegated plants themselves indicates that their cpDNA restriction patterns are identical to those of the original maternal parents (Fig. 5). Thus the $F_2$ variegated progeny of the common bean do not contain a mixture of cpDNA from the two original parents at significant levels.

Therefore, $F_2$ hybrid variegation of the common bean *Phaseolus vulgaris* does not appear to be due to biparental inheritance of chloroplasts and subsequent plastome-genome incompatibility.

To determine the nuclear or non-Mendelian nature of the variegation observed in the $F_2$ progeny, the manner of inheritance of the variegated trait in reciprocal crosses was determined. Since all of the progeny of reciprocal crosses were green (Table 3), the pale green sectors of the variegated plants are probably due to the expression of homozygous recessive alleles, which are returned to the heterozygous state through back crossing. The absence of differences in reciprocal crosses allows us to rule out the involvement of non-Mendelian factors in the hybrid variegation of the common bean.

These genetic data permit the conclusion that $F_2$ hybrid variegation in the common bean is not due to chloroplast mutation, but may be due to mutation(s) of nuclear gene whose product is necessary for chlorophyll biosynthesis or chloroplast development. Zaumeyer (1938, 1942), Wade (1941), and Coyne (1966, 1967, 1969) showed in genetic studies that the $F_2$ variegation was controlled by nuclear genes, but each reported a different pattern of inheritance. Based on their analyses of $F_3$ progeny, Zaumeyer (1938, 1942) and Coyne (1966, 1967) concluded that the character was due to two recessive genes, and Wade (1941) reported that this was determined by any one of three recessive genes. However, Coyne's result(1969) was interpreted to

indicate that four loci were involved (Kirk and Tilney-Bassett 1978).

By utilizing pooled $F_1$ and $F_2$ bean seed, I have been able to test several theories for $F_2$ hybrid variegation in the common bean *Phaseolus vulgaris*. However, a precise pedigree is necessary for the accurate assessment of the genetic basis of the variegation. For example, a pedigree would allow one to determine whether variegated progeny observed in the $F_2$ generation are derived from the same or different $F_1$ plants. This will give a more informative picture of the timing and nature of the mutational events than one can gain from the pooled $F_2$ data. Furthermore, complementation analysis is necessary to determine whether the $F_2$ hybrid variegation in the common bean is due to mutation of a single locus in all plants or whether it represents multiple complementation groups. To determine this, $F_2$ variegated plants derived from crosses of different $F_1$ plants should be crossed with each other. This will allow us to determine if multiple loci are affected, or if a single gene is the target of mutation or altered expression.

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# APPENDIX

# APPENDIX

I tried to clone variable fragments from cpDNAs of bean cultivars, such as the HaeIII 3.0 kb fragment from Swedish Brown and the BamHI 1.1 kb fragment from Swan Valley, after having extracted them from polyacrylamide gels using pBR322 as a cloning vector. In order to recover the cpDNA fragment from polyacrylamide gels, several methods were used including the crush and soak method, electrophoresis of fragments onto a 3MM Whatman paper, and the low melting agarose gel method (they are described in chapter 2). Ligation was followed by transformation, and then colonies were selected which displayed an $Amp^r$ $Tet^s$ phenotype from among the many transformants. Recombinant plasmids were isolated from selected colonies using the mini prep method. They were digested with a restriction enzyme and were run on the agarose gel to compare the size of recombinant plasmid. This method is specially useful for the comparison of blunt end ligation (i.e. HaeIII and NruI), because one cannot recut a vector DNA and an insert DNA in that case. The size of vector DNA was about 4.4 kb and that of insert DNA was about 3.0 or 1.1 kb. Most recombinant plasmids analyzed in these experiments were smaller than the original vector DNA. Even though transformants showed an $Amp^r$ $Tet^s$ phenotype, they did

not contain insert DNAs. They may show an $Amp^r$ $Tet^s$ phenotype, not because target DNA was inserted in $Tet^r$ region in the plasmid, but probably because they were partially deleted in the $Tet^r$ gene region in the plasmid. Similar results were encountered using the method described above. Since the variable fragments were usually very faint in the polyacrylamide gels, I could not see the DNA bands in the agarose mini gel after eluting them from the polyacrylamide gel using various methods. Therefore, the only possible explanation for these results is that the variable fragments might be degraded or lost during the eluting procedure.

Since I was unable to directly clone the variable fragments from bean cultivars, shotgun cloning was undertaken using the entire cpDNA of Swedish Brown digested with HaeIII and inserted into the NruI site of pBR322 vector. Using the colony hybridization methods, 22 signals out of 500 colonies were positive. Even though I could not directly clone variable cpDNA fragments, the same fragments could be labeled and used as probes for colony hybridization. Recombinant plasmids were isolated from 22 colonies, and were analyzed on the gel as described previously. A 7.4 kb recombinant plasmid was chosen as a candidate clone, since the size of vector DNA was 4.4 kb and one of the fragments of interest

was 3.0 kb. The 7.4 kb recombinant plasmid was amplified, labeled, and hybridized to a cpDNA filter of parental lines. However, the result of Southern analysis did not show any polymorphisms, although the recombinant plasmid contained a slightly smaller DNA fragment than the variable fragment in the parental lines.

Various clones from the available *Oenothera* and spinach chloroplast genomic library were used as heterologous probes for Southern analysis to find RFLPs in the parental lines. The variable fragment which was eluted from the HaeIII 3.0 fragment of bean cpDNA was hybridized to filters of *Oenothera* chloroplast genomic library. Seventeen positive signals from the Southern analysis were detected. Plasmids were isolated from the *Oenothera* chloroplast genomic library which showed homology with the HaeIII 3.0 kb fragment of Swedish Brown. They were labeled and used as heterologous probes to the bean cpDNAs of parental lines. All the results of Southern analysis did not show any RFLPs. Similar experiments were conducted with the BamHI 1.1 kb fragment from Swan Valley, with similar results. Only a spinach chloroplast *petA* (cytochrome f) gene clone recognized one of RFLPs of interest in the bean cultivars.

After the variable fragments were eluted from the

polyacrylamide gel using the method by electrophoresis onto the Whatman 3MM paper, they were able to be labeled and used both as a homologous probe and as a heterologous probe. However, they could not be cloned into pBR322 vector. These results may be interpreted to indicate that the variable fragments of bean cpDNA in the polyacrylamide gel may have lost their discrete restriction site ends during the elution procedures for unknown reasons.