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Title: RFLP Analyses of Interspecific Hybrids of *Phaseolus*

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Gene transfer via interspecific hybridization within *Phaseolus* has been limited by abnormal hybrid development and the rapid loss of hybridity in subsequent generations. The most advanced embryo development occurs in *P. vulgaris* x *P. coccineus* where mature seeds form. However, the reciprocal cross still results in abnormal embryos unable to reach maturity. Although hybrids can be obtained with the aid of embryo culture, abnormal embryos and plants continue to occur in subsequent generations and viable plants exhibit few hybrid or *P. coccineus* characteristics. This study was initiated to test the hypothesis of preferential transmission of *P. vulgaris* as the cause of skewed distribution of progeny phenotypes. Restriction fragment length polymorphisms (RFLPs) detected by cDNA probes were used to analyze the transmission pattern. Interspecific polymorphism was detected by 70-

86% of the 280 cDNA clones used while intraspecific polymorphism ranged between 8-37%. The segregation patterns of 63 markers complementary to 50 cDNA probes were determined in 177 F_2s of *P. vulgaris* x *P. coccineus*. Of the 28 (44%) non-Mendelian loci, 24 exhibited preferential transmission of the *P. vulgaris* alleles. Higher than expected frequency of maternal alleles was also recovered in reciprocal F_2s. Cytoplasmic-nuclear interactions and gametic selection may contribute to the preferential recovery of maternal alleles. The markers were mapped to nine linkage groups spanning 534 centi-Morgans. Eight non-Mendelian segregating loci including a histone H2A gene, were mapped to a single linkage group suggesting selective transmission of *P. vulgaris* chromosome(s).

RFLP Analyses of Interspecific Hybrids of *Phaseolus*

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CHAPTER 1

INTRODUCTION

Interspecific gene transfer through hybridization plays an important role in crop improvement. However, in many cases natural barriers to interspecific hybridization make hybrid production difficult. Even when a viable zygote is produced, incompatible genomic interactions often lead to abnormal development and unstable hybridity. These limitations also are encountered in interspecific gene transfer of *Phaseolus*. Hybrid embryos usually do not reach maturity and the extent of development is dependent on species combination and direction of the cross (Mok et al., 1978a, b, 1986; Rabakoarihanta et al., 1979). Even in the more compatible species *P. vulgaris* and *P. coccineus,* mature F_1 seeds can be obtained only in *P. vulgaris* x *P. coccineus* while the reciprocal cross produces immature embryos (Shii et al., 1982). Although the F_1 hybrids can be obtained with the aid of embryo culture (Shii et al., 1982), abnormal morphology, sterility and abnormal embryos continue to occur in subsequent generations. Rapid loss of hybridity as evidenced by a skewed distribution of progeny toward one parent, is also observed (Wall and York, 1957; Ibrahim and Coyne, 1975; Shii et al., 1982; Mok et al., 1986). The

genetic basis of these abnormalities has not been determined.

We noted that isozyme patterns of normal and abnormal F_2 embryos of *P. vulgaris* - *P. coccineus* resembled those of *P. vulgaris* and *P. coccineus* respectively (Guo et al., 1989). This observation suggests the possibility of unequal genetic transmission of parental traits. The present study was initiated to investigate this possibility using RFLP analyses. The thesis is presented in a manuscript format preceded by a literature review (chapter 1). Results of a feasibility study have been published (chapter 2) and the comprehensive analysis (chapter 3) has been submitted for publication.

CHAPTER 2.

LITERATURE REVIEW

The Success and Limitation of Interspecific Hybridization of *Phaseolus*

The production and development of interspecific hybrids of *Phaseolus*

Interspecific hybridization in *Phaseolus* is a useful method to incorporate desirable characters and to broaden the genetic diversity of cultivated species. The genus *Phaseolus* is now considered to include about 50 species (Miranda, 1966; Evans, 1978). Of these, *P. vulgaris* (common bean), *P. coccineus* (runner bean), *P. lunatus* (lima bean) and *P. acutifolius* (tepary bean) are the major cultivated species. Improvement of *P. vulgaris* has been the primary goal of interspecific crosses. Some desirable traits that are potentially useful are disease or insect resistance of *P. coccineus* (Rudorf, 1955; Hubbeling, 1957; Baggett and Frazier, 1959; Baggett et al., 1965), *P. acutifolius* (Coyne and Schuster, 1973; CIAT, 1977; Yoshii et al., 1978), and *P. lunatus* (McFarlane and Rieman, 1943; Wolfenbarger and Sleesman, 1961; CIAT, 1976); hypogeal germination and cross pollination habit of *P. coccineus* (Gates, 1951; Honma and Heeckt, 1962; Ibrahim and Coyne, 1975); high temperature

and drought tolerance of *P. acutifolius* (Rachie and Robert, 1974) and efficient nitrogen-fixing capability of *P. lunatus* (Henzell, 1977).

The most common and successful interspecific cross has been *P. vulgaris* x *P. coccineus*, species considered to be closely related (Hucl and Scoles, 1985). The first interspecific hybrid in *Phaseolus* was produced between these two species as reported by Mendel in 1865. Consistent success has been limited to crosses using *P. vulgaris* as the female parent from which mature seeds were routinely recovered (Lamprecht, 1941; Thomas, 1964; Smartt, 1970). In reciprocal crosses, only a few studies reported the recovery of mature seeds. In those cases, the success was achieved through the use of particular genotypic combinations (Lamprecht, 1948; Thomas, 1964; Al-Yasiri and Coyne, 1966; Smartt, 1970) or the use of intraspecific hybrids of *P. vulgaris* (Wall and York, 1960) and *P. coccineus* (Smartt and Haq, 1972) as parents. When *P. coccineus* was used as the female parent, pod growth was slower than in the reciprocal cross but seeds sometimes developed to near normal size. However, these seeds contained abnormal embryos. The failure to obtain mature seeds has been attributed to various causes such as the lack of fertilization (retarded growth of *P. vulgaris* pollen tubes in *P. coccineus* style) and the slow development of hybrid embryo and endosperm (Thomas, 1964). However, other studies demonstrated that pre-fertilization events (pollen germination and pollen tube growth) were

normal (Hawkins and Evans, 1973; Shii et al., 1982). It was concluded that the primary failure of *P. coccineus* (female) x *P. vulgaris* (male) crosses resided in the abnormal development of the hybrid embryos (Shii et al., 1982).

The hybrids of *P. vulgaris* x *P. coccineus* (derived from mature seeds) had lower fertility (17%-45%) than those of the reciprocal cross (75% or higher) (Thomas, 1964; Smartt, 1970; Ibrahim and Coyne, 1975; Haq et al., 1980; Shii et al., 1982). This reduced fertility could not be attributed to either lack of chromosome homology (Shii et al., 1982) or a cytoplasmic effect, since chromosome pairing in F_1s was near normal and both reciprocal F_2 populations contained plants with low fertility (Mok et al., 1986). In addition, pollen sterility and restricted embryo development appeared to be under separate genetic control as there was no correlation between the extent of embryo development and the degree of pollen fertility among plants in later generations (Shii et al., 1982). A skewed distribution toward the maternal parent was observed in F_2 and backcross generations for cotyledon position (Wall and York, 1957) and other morphological characters (Lamprecht, 1948; Ibrahim and Coyne, 1975). It was suggested that the reversion to the parental phenotype could be due in part to the elimination of seeds containing abnormal embryos which may represent specific genotypic combinations (Mok et al., 1987). Abnormal embryos were genetically more like the *P. coccineus* parent as

identified by isozyme analysis, whereas normal embryos had zymograms similar to *P. vulgaris*, a predominant phenotype in surviving progeny (Guo et al., 1989). The selective recovery of certain embryos combined with different pollen fertility may have contributed to the skewed distribution of quantitative characters observed in generations following the initial *P. vulgaris* - *P. coccineus* crosses (Wall and York, 1957; Smartt, 1970).

Abnormal plants of interspecific hybrids of *Phaseolus* have been described in several cases. F_1 populations of *P. vulgaris* x *P. coccineus* were either uniformly abnormal or segregated into normal and "crippled" plants (Lamprecht, 1948; Rudorf, 1961). The "crippled" phenotypes included dwarfism, abnormal leaf development and in extreme cases, seedling lethality. The frequencies of these plants could be explained by a two-gene system (Rudorf, 1961).

Crosses between *P. vulgaris* and *Phaseolus* species other than *P. coccineus* have been less successful. Hybridization between *P. vulgaris* and *P. acutifolius* was first investigated by Honma (1956). Crosses using *P. vulgaris* as female parent resulted in pod formation, but the pods abscised before seed maturity (Honma, 1956; Al-Yasiri and Coyne, 1966; Smartt, 1970). Fertilization and division of the embryo and endosperm occurred regularly in reciprocal crosses involving these two species but embryos aborted during early stages of development (Rabakoarihanta et al., 1979). Culturing the immature embryos on artificial medium was

required to recover hybrid plantlets (Honma, 1955; Mok et al., 1978). An exception to these observations was the formation of mature hybrid seeds using specific cultivars of *P. vulgaris* and *P. acutifolius* (Smartt, 1970).

The low fertility of *P. vulgaris* x *P. acutifolius* hybrids was related to incomplete chromosomal homologies (Rabakoarihanta et al., 1980). In other cases, genetic factors other than insufficient chromosome homology were thought to be the cause of sterility (Parker and Michaels, 1986).

Seed formation in crosses between *P. vulgaris* and *P. lunatus* was reported by Honma and Heeckt (1959), but all later attempts failed to confirm this observation (Smartt, 1970). The discrepancy may have been due to the fact that Honma and Heeckt (1959) used intraspecific hybrids as parents. In other reports, the cross between *P. vulgaris* (female) and *P. lunatus* (male) resulted in formation of pods which abscised early in development, while the reciprocal cross failed to set pods (Al-Yasiri and Coyne, 1966; Mok et al., 1978). *P. vulgaris* x *P. lunatus* embryos could be rescued using embryo culture. However, hybrids obtained by embryo culture were abnormal and did not flower (Mok et al., 1978).

Embryo development

P. vulgaris - P. coccineus:

Limited embryo development was found to be the primary barrier in

interspecific hybridization (Mok et al., 1987). The only exception was in crossing *P. vulgaris* (female) with *P. coccineus* (male) which resulted in the formation of mature seeds. The seeds produced by these crosses were as normal as selfed *P. vulgaris* seeds and germinated easily. On the other hand, seeds produced in the reciprocal cross were shrivelled, contained abnormal embryos, and had low germination (Thomas, 1964). The causes of embryo abortion and the reciprocal cross difference are not known.

In hybrids of *P. coccineus* x *P. vulgaris*, abnormalities occurred very early in development (Kroh, 1962; Thomas, 1964). During the immediate post-fertilization period, the interspecific embryos arising from *P. vulgaris* x *P. coccineus* grew faster and had a higher mean nuclei number than selfed embryos. In contrast, growth of hybrid embryos from *P. coccineus* x *P. vulgaris* during the immediate post-fertilization period was slow negligible and mean nuclei number was lower than in selfed embryos. Ten days after pollination, hybrid embryos of *P. vulgaris* x *P. coccineus* developed slower than selfed embryos. At this time embryos from *P. coccineus* x *P. vulgaris* had the lowest nuclei number compared with the F_1 and selfed embryos. It was suggested that abnormal embryos might result from the adverse interaction of embryo and endosperm (Thomas, 1964). There were two classes of abnormal embryos of *P. coccineus* x *P. vulgaris*, shrunken and underdeveloped embryos (Shii et al., 1982; Mok et

al., 1986). The shrunken embryos were characterized by wrinkled cotyledons and a deformed embryonic axis. The underdeveloped embryos were characterized by their small size at the cotyledonary stage compared to those of the reciprocal cross at the same stage. In addition, a thick layer of endosperm tissue surrounded the embryos while comparable reciprocal embryos filled the seed cavity. The occurrence of the particular type of abnormal embryos, shrunken or underdeveloped, depended on the genotype of the *P. coccineus* parent. Underdeveloped embryos were observed in all *P. coccineus* x *P. vulgaris* crosses examined, while shrunken embryos occurred only when particular genotypes of *P. coccineus* were used. Three types of F_2 embryos, normal, shrunken and underdeveloped, were observed. The degree of abnormality of the shrunken embryos varied, ranging from those with extremely wrinkled cotyledons to those with only rough edges. There were no significant reciprocal cross differences in the frequency of the different classes of F_2 embryos. It was suggested that the interaction between the embryo and maternal parent was the likely cause of abnormal development (Shii et al., 1982).

P. vulgaris - *P. acutifolius*:

Honma (1956) reported that embryos of *P. vulgaris* x *P. acutifolius* aborted within 3 to 24 days following pollination. The reciprocal cross did

not form pods or seeds. When intraspecific F_1 plants of *P. acutifolius* were used as female parents, a few pods were set but embryos failed to attain sufficient size for *in vitro* culture.

Smartt (1970) also reported that reciprocal hybrid embryos between *P. vulgaris* and *P. acutifolius* were formed but pods collapsed 17 to 21 days after pollination.

Embryo development in reciprocal crosses of *P. vulgaris* and *P. acutifolius* has been investigated by Mok et al., (1978) and Rabakoarihanta et al., (1979). Embryos of *P. vulgaris* x *P. acutifolius* usually developed to the early cotyledonary stage. A distinct characteristic of these embryos was uneven development of the two cotyledons which were apart rather than closely adjoining each other (as in selfed embryos). Hybrid embryos derived from the reciprocal cross developed until the late heart or early cotyledonary stage. The final size of the hybrid embryos ranged from 2.5 to 3.5 mm 24 days after pollination. The rate of growth and final size of these hybrid embryos seemed to be influenced by the genotype of both parents (Mok et al., 1978). It was suggested that the failure of hybrid embryos to develop normally may be caused by the relatively low levels of cytokinins (Nesling and Morris, 1979).

P. vulgaris - *P. lunatus*:

Formation of mature seeds from *P. vulgaris* x *P. lunatus* has been reported (Honma and Heeckt, 1959). However, other studies indicated that hybrid pods usually abscised early in development (Al-Yasiri and Coyne, 1966; Smartt, 1970). In a later investigation (Mok et al., 1978) all embryos obtained from *P. vulgaris* x *P. lunatus* developed only to the pre-heart stage. The maximum length of the hybrid embryo at the time of pod abscission was 0.4 mm. The reciprocal cross (*P. lunatus* x *P. vulgaris*) resulted in embryos arrested at the four-celled stage. It was suggested that the severe delay in embryo and endosperm divisions could be the major cause of early pod abscission in *P. lunatus* x *P. vulgaris* crosses. As addition of cytokinins to the female parent in a hydroponic culture advanced the four-celled embryos to the pre-heart stage (Rabakoarihanta et al., 1979), low levels of this hormone may limit embryo and pod development.

Applications of Molecular Markers in Genetic Studies and Plant Breeding

The properties of molecular markers

The development of electrophoresis and enzyme-specific stains provides biochemical markers. The advantages of using biochemical markers over conventional morphological traits in genetics studies are: (1) isozyme patterns are a direct reflection of gene activity, unlike morphological markers that are often influenced by environmental factors, (2) isozyme variations usually do not have deleterious effects and (3) codominance of alleles allows distinction between heterozygotes and homozygotes (Beckmen and Soller, 1983). Therefore, from a genetic point of view, isozymes are useful as quantitative parameters of genetic composition.

The number of useful isozyme markers is limited by the availability of enzyme-specific stains and isozymes represent expressed genes only. Tissue and/or developmental specific expression also can affect the consistency of the zymogram. Restriction Fragment Length Polymorphism (RFLP) is another approach to measure genomic composition. As RFLPs measure differences at the DNA level, they are less likely to be affected by differential gene expression, types of tissue or stages of development. In addition, the number of markers is theoretically unlimited.

RFLPs are variations in the length of DNA fragments generated with a particular restriction endonuclease digestion. Detection of RFLPs involves extraction and purification of genomic DNA and digestion of the DNA with a restriction endonuclease that recognizes and cleaves at a specific sequence of the DNA molecule. Genetic differences are reflected in the different sizes of restriction fragments. These DNA fragments can be separated by size using gel electrophoresis and then transferred and immobilized on a membrane. After hybridizing to a labelled probe (radioactive or non-radioactive), specific RFLP banding patterns for different genotypes are revealed.

RFLP linkage mapping

When parents are polymorphic at specific loci (detected as RFLP markers), progeny (i.e., F_2 or backcross populations) can be used to map each locus via linkage analysis. If the population also segregates for other traits of interest, such as disease resistance, the genetic location of these traits can be determined relative to RFLP markers.

The construction of a linkage map with RFLP markers involves three basic steps: (1) the development of probes, (2) choice of parental lines and establishment of progeny populations and (3) segregation pattern and linkage analysis (Landry et al., 1987). The source of probes for RFLP mapping usually includes clones from genomic or cDNA libraries.

Alternatively, Random Amplified Polymorphic DNA (RAPD) markers (Williams et al., 1990) are also used. cDNA clones are derived from mRNA while genomic DNA clones consists of random DNA fragments. The RAPD markers are PCR-amplified regions flanked by two random primers. However, RAPDs detect only "dominant" alleles and do not distinguish homozygotes from heterozygotes. Most maps consist of a combination of all these markers as well as markers based on morphological characters. Intra- and interspecific hybrids have been used to generate populations for RFLP mapping.

The potential impact of RFLP mapping on eukaryotic genetics was first described in 1980 by researchers in human genetics (Botstein et al.). RFLP markers have received increased attention from human geneticists due to the feasibility of saturating the human genome with polymorphic markers and thus providing a means for human gene mapping and genetic therapy. Over the past few years, plant geneticists and breeders have begun to develop RFLP maps for important species. High resolution genetic maps have been constructed for many crops such as maize (Helentjaris, 1987); tomato (Bernatzky and Tanksley, 1986); potato (Bonierbale et al., 1988; Gebhardt et al., 1989); soybean (Apuya et al., 1988); rice (McCough et al., 1988) and lettuce (Landry et al., 1987) as well as the model plant system, *Arabidopsis* (Chang et al., 1988). Among these plant species, tomato has one of the most extensive linkage maps

(markers consisting of over 600 DNA clones and isozymes). The average spacing between these markers is less than 5 cM. A high density linkage map of maize based on RFLPs consists of a few hundred loci covering all ten chromosomes. For plant species with aneuploidies, the assignment of RFLP markers to specific chromosomes facilitated the physical correlation of RFLP map with cytological and conventional genetic maps. For example, in wheat, using addition lines, RFLP loci were assigned to specific chromosomes. Furthermore, ditelosomic and nullisomic-tetrasomic stocks were used to assign these sequences to chromosome arms (Kam-Morgan et al., 1989). In maize, plants hemizygous for specific segments of each of the maize chromosomes were generated using different B-A translocations. The chromosomal region on the RFLP map was identified by each of the B-A translocations, including the short and long arms and centromere region of each chromosome (Weber and Helentjaris, 1989).

However, some inconsistences between maps generated from different populations have been reported in pea (Ellis et al., 1992), where different linkage arrangements have been observed for one region of the genome. Therefore, it is expected that maps derived from classical, cytogenetics and molecular mapping may not give exactly the same linkage relationship. Also, genetic maps with reduced recombination frequency have been reported when interspecific hybrid populations are

used to construct the linkage map. The possible causes are either less homology and reduced chromosome pairing between species (Bonierbale et al., 1988) or selective elimination of recombinant gametes or zygotes (Gebhardt et al., 1991). Therefore judicious interpretations are required in analyzing linkage relationships between different populations or in compiling a single linkage map based on results obtained from different populations (Ellis et al., 1992).

RFLP in plant breeding

The potential utility of RFLP markers in plant breeding is based on finding tight linkages between these markers and traits or genes of interest (Beckman and Soller, 1986; Bernatzky and Tanksley, 1986). Such linkage permits the inference of the presence of a desirable gene by tagging RFLP markers. Traits controlled by one or a few genes are frequently transferred from one genetic background to another by intercrossing. Major genes conferring disease resistance is an example. Simultaneous or sequential screening of plants with several different pathogens can be difficult or impractical. In other instances, breeders are unable to screen for resistance to new pathogens because of quarantine restrictions. In contrast, detecting disease resistance genes by their linkage to RFLP probes would make it possible to screen for multi-resistances simultaneously without the need to inoculate the population.

In several cases, RFLP markers have been associated with disease resistance genes. In tomato, RFLP markers have been linked to *Tm-2a* (resistance to tobacco mosaic virus)(Young et al., 1988); *Mi* (resistance to root knot nematode)(Klein-Lankhorst et al., 1991); I_1 and I_2 genes (resistance to *Fusarium oxysporum* f. sp *lycopersici* race 1 and 2) (Segal et al., 1992). Molecular markers have also been linked to genes controlling soluble solids content and fruit ripening of tomato (Osborn et al., 1987; Kinzer et al., 1990). Similarly, tight linkages between RFLP markers and other major genes are currently being sought by researchers working with a number of other crop species.

RFLP-assisted selections have been explored as a means of transferring desirable genes from wild species into wheat, rice, tomato and potato (Tanksley et al., 1989). Undesirable effects of linkage drag are pronounced when breeding with exotic germplasm. By monitoring recombinants around genes of interest with linked RFLP markers, linkage drag associated with introgression can be quickly and efficiently reduced. Using this approach, the linkage drag can be reduced as low in two generations as would require 100 backcross generations without RFLP selection (Young and Tanksley, 1989).

Most agricultural traits exhibit continuous variation resulting from the action of multiple genes modified by the environment, making selection less efficient. Complex traits resolved into individual genetic

components, might be dealt with the efficacy of single traits. High-density RFLP maps provide this opportunity by making it feasible to identify, map and measure the effects of genes underlying quantitative traits. Systematic dissection of the quantitative trait loci (QTL) using RFLP maps has been proposed by Lander and Botstein (1989), using the approach of interval mapping that is based on two linked markers flanking an interval containing a QTL. The relative effect of each component locus can be evaluated upon overall variance for a complex trait. The linked RFLP markers that contribute the major effect for the QTL can be used for selection (Helentjaris, 1987)

Paterson et al., (1988) dissected several QTLs controlling fruit mass, concentration of soluble solids and fruit pH into Mendelian factors using RFLP linkage map. Such an marker-defined sequence can then be treated in breeding program as a single complex "allele", allowing efficient selection for quantitative traits at early stages of the life cycle.

Other applications of RFLPs include distinguishing strains and varieties of agricultural importance. Markers which hybridize to repetitive DNA can generate highly specific fingerprints. RFLPs also may provide a convenient assessment of heterogeneity within and between stocks retained as germplasm resources. This kind of screening might be used to ensure that germplasm resources do indeed represent a wide range of genetic variability (Beckmen and Soller, 1986).

Map-based gene cloning

Methods are well established for isolating and cloning a gene if the product of that gene is known (Maniatis et al., 1982). Unfortunately, the products of many important plant genes are unknown, such as disease resistance genes. Until these genes are isolated and studied with the tool of molecular biology, it seems unlikely that we will fully understand their mode of action. Transposon tagging has been useful in cloning genes whose product is unknown, particularly in maize which contains well characterized transposons (Behrens et al., 1984; Federoff et al., 1984; Marrotta et al., 1989). Attempts to introduce transposons into heterologous background have been limited to a few plant species (Baker et al., 1986). Map-based cloning offers an independent method for gene isolation, often referred to as reversed genetics, which is based on physical linkage to RFLP markers (Orkin, 1986). It requires that the map position of a gene conferring a certain phenotype is known in a saturated RFLP linkage map. Begining from the closest flanking marker, one can then carry out a chromosome walk to a gene of interest, especially in organisms with very small genomes. A typical example is the fruit fly (*Drosophila melanogaster*) where several genes have been cloned by this approach (Bender et al., 1983). With the development of new techniques, such as pulse field gel electrophoresis (PFGE) and yeast artificial chromosome (YAC) vectors, it is now possible to use similar approach to

analyze organisms with large genome. Genes controlling Wilm's tumor (Gessler et al., 1990) and Cystic fibrosis (Rommens et al., 1989) have been cloned in humans.

Recently, RFLP-assisted gene cloning has been used in plants. Clones have been selected that are tightly linked to several disease resistance genes, for example, gene [*pi-2(t)*] conferring resistance to the fungal pathogen *Pycularia oryzae* in rice (Yu et al., 1991), and *ml-o*, a barley resistance gene to the fungus *Erysiphe graminis* (Hinze et al., 1991). RFLP markers tightly (<1 cM) linked to *Helminthosporium turcicum* race 1 (Ht_1) have been identified in maize (Bentolila et al., 1991). In lettuce, markers near four genes controlling resistance to downy mildew have been mapped (Landry et al., 1987). The region near the gene of interest is further defined by saturating with more markers, so that the DNA fragment flanked by these two markers can be resolved with the current PFGE technique (3-4 Mb) or is within a feasible chromosome walking distance. Once the gene of interest is bracketed by two tightly linked molecular markers and a physical map has been established, the region between these two markers can be cloned into the YAC vectors and subsequently screened for the gene of interest. In tomato, I_1 and I_2 genes have been both genetically and physically characterized. A molecular marker mapped 0.4 cM (620 kb) from I_2 has been obtained (Segal et al., 1992). A high resolution RFLP map has also been

constructed around *Mi*, the nematode resistance gene of tomato, with an interval of 0.4-2 cM (>1 Mb) to the flanking markers (Messeguer et al., 1991). One of the tightly linked markers, acid phosphatase-1, has been cloned, and can be used as a starting point for chromosome walking. With the availability of a YAC library (Aarts et al., 1991), it seems feasible to clone the *Mi* gene via chromosome walking. Functional activity of the genes cloned can be assayed by inserting DNA back into plant genomes using *Agrobacterium* transformation (Klee et al., 1988).

Segregation distortion of RFLP markers

Segregation distortion of molecular markers has been reported in tomato (Helentjaris et al., 1986); lentil (Havey and Muehlbauer, 1989); soybean (Keim et al., 1990); *Brassica napus* (Landry et al., 1991) and potato (Gebhardt et al., 1991). Such distortions were observed in both intra- and interspecific hybrids. The nature and cause of the aberrant segregation in certain regions of plant genomes remain unclear. It has been suggested that the biased segregation of RFLP markers may be due to the presence of a lethal allele (i. e., in *B. napus*, Landry et al., 1991) or a self-incompatibility locus (i.e., in potato, Gebhardt et al., 1991) within the vicinity of these markers. Selective fertilization was suggested to be involved in the segregation distortion of RFLP markers in interspecific crosses between *Lycopersicon hirsutum* and *L. esculentum* (Helentjaris et

al., 1986). The behavior of gametocidal genes is an example of selective fertilization in which abortion of certain gametes occurs after meiosis as a result of the interaction between the pollen and the anther tissues. Pollen killer genes have been identified in *Nicotiana* (Cameron and Moav, 1957), tomato (Rick, 1966) and wheat (Loegering and Sears, 1963). A gamete eliminator gene that causes preferential transmission of male and female gametes may be present on chromosome 4 of *Agropyron elongatum* (Dvorak, 1980). In tomato, a factor distorting selection ratio has been inferred to lie on chromosome 2 (Paterson et al., 1990).

Selective survival of progeny with *P. vulgaris* morphology was noted in earlier studies of interspecific hybrids of *P. vulgaris* x *P. coccineus* (Wall and York, 1957; Ibrahim and Coyne, 1975). The recovery of progeny with predominantly parental phenotypes is one of the major factors that affect the efficiency of interspecific gene transfer. However, little information is available to address the genetic mechanisms of such a selective transmission. Using RFLP to study interspecific hybrids of *Phaseolus* should provide insights to preferential transmission and the possibility of isolating genes associated with these phenomenon.

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CHAPTER 3.

ANALYSES OF *PHASEOLUS VULGARIS* L. AND *P. COCCINEUS* LAM. HYBRIDS BY RFLP: PREFERENTIAL TRANSMISSION OF *P. VULGARIS* ALLELES

Abstract

Restriction fragment length polymorphism (RFLP) was determined among *P. vulgaris* genotypes and *Phaseolus* species using 19 probes. The incidence of polymorphism was high (70 to 86%) between species but relatively low (22 to 26%) between genotypes of *P. vulgaris*. Suitable probes were identified for the analysis of *P. vulgaris* and *P. coccineus* hybrids. The segregation pattern in F_2 populations was Mendelian for two probes (LHB and VEE20) and non-Mendelian for GS-g, CHS and CHI. Statistical analyses indicated gametic selection with preferential transmission of the *P. vulgaris* alleles, which may account for the selective recovery of *P. vulgaris* progeny types observed earlier. The available hybrids of *P. vulgaris* and *P. coccineus* and the high degree of interspecific RFLP will facilitate construction of a linkage map for *Phaseolus.*

Key words: *Phaseolus* interspecific hybrid, RFLP, beans

Introduction

Interspecific hybridization of *Phaseolus* usually results in embryo abortion. We have studied the development of hybrid embryos in crosses of *P. vulgaris* to three other species, *P. lunatus*, *P. acutifolius* and *P. coccineus*. The extent of hybrid embryo development ranges from the four-celled stage to maturity and depends on the parental species combination as well as the direction of the cross (Mok et al., 1978, 1986, Rabakoarihanta et al., 1979). The most advanced development occurs in crosses with *P. coccineus*: when *P. coccineus* is used as the female parent the embryos develop to the mid to late cotyledonary stage, while in the reciprocal cross embryos normally develop to maturity (Shii et al., 1982). Hybrid plants can be obtained from the abnormal embryos of *P. coccineus* x *P. vulgaris* with the aid of embryo culture techniques.

The combination of *P. vulgaris* and *P. coccineus* is of particular interest, since in addition to the dramatic difference in hybrid embryo development between reciprocal crosses, several other abnormalities can be detected following the initial hybridization (Shii et al., 1982). For instance, reciprocal hybrids differ in pollen viability. *P. vulgaris* x *P. coccineus* F_1 plants have 25% stainable pollen, whereas the reciprocal hybrids obtained from abnormal embryos have 80% stainable pollen. Reciprocal F_2 populations segregate into normal and two classes of

abnormal embryos, the shrunken and underdeveloped types. Moreover, normal as well as non-viable plants occur in the F_2. In later progeny populations (F_4 and F_5), the plants generally resemble one or the other parent (Mok et al., 1987). This phenotypic reversion is accompanied by the recurrence of normal and abnormal embryos, varying degrees of pollen fertility, and different viability of progeny plants. Although the causes of these complex developmental problems remain unknown, cytoplasmic effects can not account for these phenomenon, since the abnormalities occur in successive generations (at least to the F_4) regardless of the direction of the initial cross.

We observed that normal and abnormal embryos exhibited specific isozyme patterns resembling *P. vulgaris* and *P. coccineus* parents respectively (Guo et al., 1989), which suggests that types of embryo development may be related to specific combinations of the parental genomes and/or with differential gene expression. We have begun to examine selected aspects of these problems, namely the possibility of preferential transmission of specific linkage groups and occurrence of genetic combinations associated with embryo or plant viability. This paper describes the initial phase of work using restriction fragments to estimate inter- and intra-specific polymorphism in *Phaseolus* and to determine if Mendelian or non-Mendelian segregation occurs in interspecific crosses.

Materials and Methods

Plant materials and sources of DNA

To detect usable DNA probes and to estimate the degree of interspecifc and intraspecific (within *P. vulgaris*) polymorphism, the following *Phaseolus* genotypes were used: *P. vulgaris* L. cvs. Great Northern (GN), Tendergreen (TG), Improved Tendergreen (IT), Sanilac (SA), Contender (CO), *P. coccineus* Lam. cv. Scarlet Runner (SR), *P. lunatus* L. cv. Kingston (K), and *P. acutifolius* A. Gray P. I. #321637 (AC2). *Vigna unguicularis* cv. Purple Hull was used for intergeneric comparison. *P. vulgaris* GN and *P. coccinues* SR were used to generate F_1 and F_2 populations as reported earlier (Guo et al., 1989). Immature F_2 embryos were classified according to the type of development, normal or abnormal. Callus cultures were established from the cotyledons of individual embryos (Mok et al., 1978b) and plantlets were derived by culturing the embryonic axes (Shii et al., 1982).

DNA isolation

Plant (stem and/or leaf) and callus tissues were collected and immediately frozen under liquid nitrogen and then stored at -80 °C until DNA preparation. DNA from 5 g of sample was extracted and purified using the methods of Dellaporta et al., (1983) with the following

modifications: 1. 2X DNA extraction buffer (DEB) was used for callus tissue. 2. Triton X 100 instead of SDS was used in DEB. 3. For plant tissue, DNA was extracted twice. The first grinding was done in mortar and pestle containing liquid nitrogen and the ground powder was suspended in 15 ml DEB. After centrifugation, the precipitated cell debris was resuspended in DEB and homogenized with a polytron homogenizer. The first extraction yielded DNA with higher molecular weight (50-100 kb). The second extraction with a polytron increased DNA recovery but gave rise to lower molecular weight (10-25 kb) fragments. Higher or lower MW DNA was used depending on the probes employed.

Southern hybridization

DNA samples (10 μg) were digested with restriction enzyme *EcoR*1 (1 μg DNA/10-20 units enzymes) at 37 °C overnight. After electrophoresis in 1% agarose gels, DNA was transferred to IMMOBILON-N membrane, according to the manufacturer's instructions (Millipore). The filters were prehybridized at 65 °C for 2-4 hr in solution containing 6X SSC (1X SSC consisted of 0.15 M NaCl and 0.15M sodium citrate), 5X Denhart solution, 0.5% SDS, 10 μg/ml salmon sperm DNA and 5% dextran sulfate, then hybridized with ^{32}P labelled probes under the same conditions for 16-24 hr. Genes of known function and random cDNA clones were used as probes. Probes were labelled using a

multiprime DNA labelling system (Amersham) and purified by centrifugal dialysis in Centricon-10 with two washings (1 ml) of 2X 0.2M EDTA. Filters were washed at 65 °C with 3 changes of 2X SSC and 0.1% SDS and then autoradiographed at -80 °C using preflashed XAR-5 film and intensifying screens.

Construction of cDNA libraries and isolation of cDNA clones

cDNA libraries were made from 5 μg poly (A^+) RNA isolated from GN or SR embryos of mixed developmental stages. The library constructions were described by Lightfoot et al., (1988) except that vector pGEM-3zf(+) (Promega) was used.

Sources of DNA of known genes

Plasmid probes were maintained as frozen cell lines at -80 °C. Plasmid DNA was prepared by alkaline or boiling lysis and $CsCl_2$ or column purification (Stratagene). After restriction digestion plasmid DNA was electrophoresed on a 1.5% ultrapure agarose gel. The appropriate bands were excised and the DNA insert recovered using Glassmilk (Bio-101). Probes were obtained from the following sources:

CHI (chalcone isomerase): Mehdy and Lamb, 1987.

CHS (chalcone synthase): Ryder et al., 1984.

CAB (chlorophyll a/b binding protein): Gallagher et al., 1982.

LHB (leghemoglobin), GS (glutamine synthetase): Bennett et al., 1989.

PAL (phenylalanine ammonia lyase): Edwards et al., 1985.

SSU (Rubisco small subunit): Bedbrook et al., 1980.

rRNA: gift, Rivin, Oregon State University.

VML series (cDNA clones): Lightfoot et al., 1988.

VEE series (cDNA clones): this work.

PIN (phaseolin): gift, Sam Sung, University of Hawaii.

ACT (actin): Lightfoot (unpublished).

Statistics

Segregation data obtained in F_2 populations were analyzed using the program Linkage 1 (Suiter et al., 1983).

Results

Embryo and plant development

F_2 embryos were recovered from pods formed on GN x SR F_1 plants. Of these embryos, 123 were classified as normal and 53 as abnormal. The ratio between normal and abnormal embryos did not differ significantly from 3:1 (Table 1). However, the abnormal embryo population was highly heterogeneous, precluding further conclusions concerning the precise mode of inheritance. Two main classes of abnormal embryos were observed, the underdeveloped and shrunken. The underdeveloped embryos were arrested at the early cotyledonary stage and most of them were surrounded by a thick layer of endosperm tissue. The shrunken embryos had either extremely or moderately wrinkled and soft cotyledons. Callus cultures were obtained from all embryos, but plants could be recovered from only a portion of the cultured embryo axes. About 48% of the plantlets obtained died before 4 weeks (Table 1). Another 21% had abnormal phenotypes, including spindly or dwarf growth habit, or wrinkled leaves and abnormal flowers, while the remaining plants (31%) appeared normal. The abnormalities at later stages did not seem to be related to abnormal embryo development since many normal embryos gave rise to spindly or excessively bushy plants.

Table 1. Distribution of F_2 embryos and plants according to developmental classes.

Embryo development			Plant development		
Normal	Abnormal		Normal	Abnormal	Lethal[a]
	Wrinkled	Under-developed			
123	28	25	43	29	66

[a] Included plantlets that perished before 4 weeks and axes that did not grow on culture medium.

Intra- and inter-specific polymorphism and identification of usable gene probes

Restriction fragment length polymorphism was determined using a total of 19 probes and several genotypes of *P. vulgaris* and other legumes (Table 2). The incidence of RFLP was high between species (from 70 to 86%) but relatively low between genotypes of *P. vulgaris* (22 to 26%). Representative patterns of LHB, CHI, GS-g and VEE20 are presented in Figure 1. Probes that showed polymorphism between GN and SR were also used to determine banding patterns in F_1s. Probes with bands that enabled unambiguous scoring of parental and F_1 types were selected to screen the F_2 populations. These included LHB, CHI, CHS, GS-g and VEE20. DNA samples obtained from callus and plant parts of selected genotypes were compared and no difference in the restriction fragment patterns were found (data not shown).

Segregation analyses

Evaluations included more than 150 F_2s and the five selected probes. Representative RFLP patterns of F_2 are presented in Figure 2. The distribution of progeny into genotypic classes (parental or F_1) is presented in Table 3. The segregation of LHB and VEE20 was Mendelian and did not deviate from the expected 1:2:1 ratio. However, the distribution of progeny for GS-g, CHS and CHI differed significantly from

Table 2. RFLPs between genotypes of *P. vulgaris* and related species.

Probes	No. of bands in GN	*P. vulgaris* genotypes					Related species			
		GN	TG	IT	SA	CO	SR	K	AC2	VU
SSU	4	1(0)	1(0)	1(0)	1(0)		2(4)	3(4)	4(4)	5(4)
CHI	1	1(0)	1(0)	1(0)	2(1)	1(0)	3(1)	4(1)	5(1)	6(1)
LHB	4	1(0)	1(0)	2(1)	2(1)	2(1)	3(4)	4(4)	5(4)	6(4)
GS-a	2	1(0)	1(0)	1(0)	2(1)	1(0)	3(2)	4(2)	5(2)	6(2)
GS-b	2	1(0)	1(0)	1(0)	1(0)	1(0)	1(0)	2(2)	3(2)	4(2)
GS-g	3	1(0)	2(1)	2(1)	1(0)	2(1)	3(3)	4(3)	5(3)	6(3)
GS-d	1	1(0)	2(1)	2(1)	1(0)	2(1)	3(1)	4(1)	5(1)	6(1)
PIN	5	1(0)	2(1)	2(1)	3(1)	4(1)	6(5)	7(5)	8(5)	9(5)
ACT	1	1(0)	1(0)	1(0)	1(0)	1(0)	1(0)	2(1)	3(1)	4(1)
CHS	5	1(0)	3(2)	3(2)	2(2)	3(2)	5(1)	6(5)	7(5)	8(5)
PAL	2	1(0)	2(1)	3(1)	4(1)	5(1)	6(2)	7(2)	8(2)	9(2)
rRNA	3	1(0)	2(1)	2(1)	2(1)		3(1)	4(1)	5(1)	6(1)
VML22	5	1(0)	2(3)	3(2)	4(2)	5(4)	6(5)	7(5)	8(5)	9(5)
VML23	2	1(0)	1(0)	1(0)	1(0)	1(0)	2(1)	2(1)	2(1)	2(1)
VEE2	2	1(0)	1(0)	1(0)	1(0)	1(0)	1(0)	1(0)	2(1)	1(0)
VEE3	2	1(0)	1(0)	1(0)	1(0)	1(0)	2(1)	3(1)	4(1)	5(1)
VEE4	2	1(0)	1(0)	1(0)	1(0)	1(0)	2(1)	3(1)	4(1)	5(1)
VEE6	2	1(0)	1(0)	1(0)	1(0)	3(1)	4(2)	5(2)	6(2)	2(2)
VEE15	1	1(0)	1(0)	1(0)	2(1)	1(0)	3(1)	4(1)	5(1)	6(1)
VEE16	3	1(0)	2(1)	2(1)	2(1)	2(1)	3(1)	4(1)	5(1)	6(1)
VEE20	2	1(0)	1(0)	1(0)	1(0)	1(0)	2(1)	3(1)	4(1)	5(1)
Polymorphic bands (to GN)		(0)	(11)	(11)	(12)	(13)	(37)	(43)	(44)	(43)
% Polymorphism		0	22	20	24	26	70	82	86	84

Number out side of the parentheses denotes banding pattern. Numerals within the parentheses indicate the number of polymorphic bands in comparison to *P. vulgaris* cv. GN.

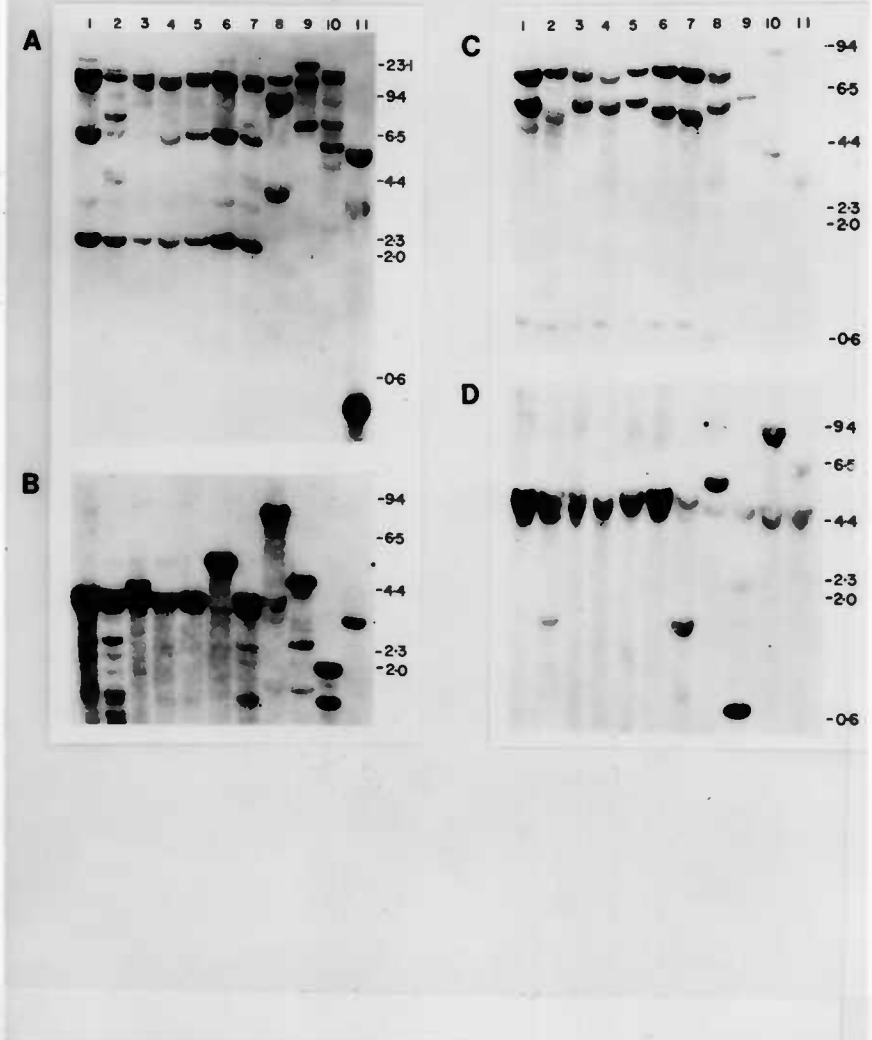

Figure 1. A-D. Polymorphism of representative probes among genotypes of *P. vulgaris* and related species. Panels A to D: leghemoglobin (LHB), chalcone isomerase (CHI), glutamine synthetase-gamma (GS-g), and clone 20 (VEE20). Lanes 1-7: *P. vulgaris* genotypes (1: P725, 2: P691, 3: CO, 4: IT, 5: TG, 6: SA, and 7: GN). Lanes 8-10: *Phaseolus* species [8: *P. coccineus* SR. 9: *P. lunatus* (K), 10: *P. acutifolius* (AC2)]. Lanes 11: *Vigna unguicularis*.

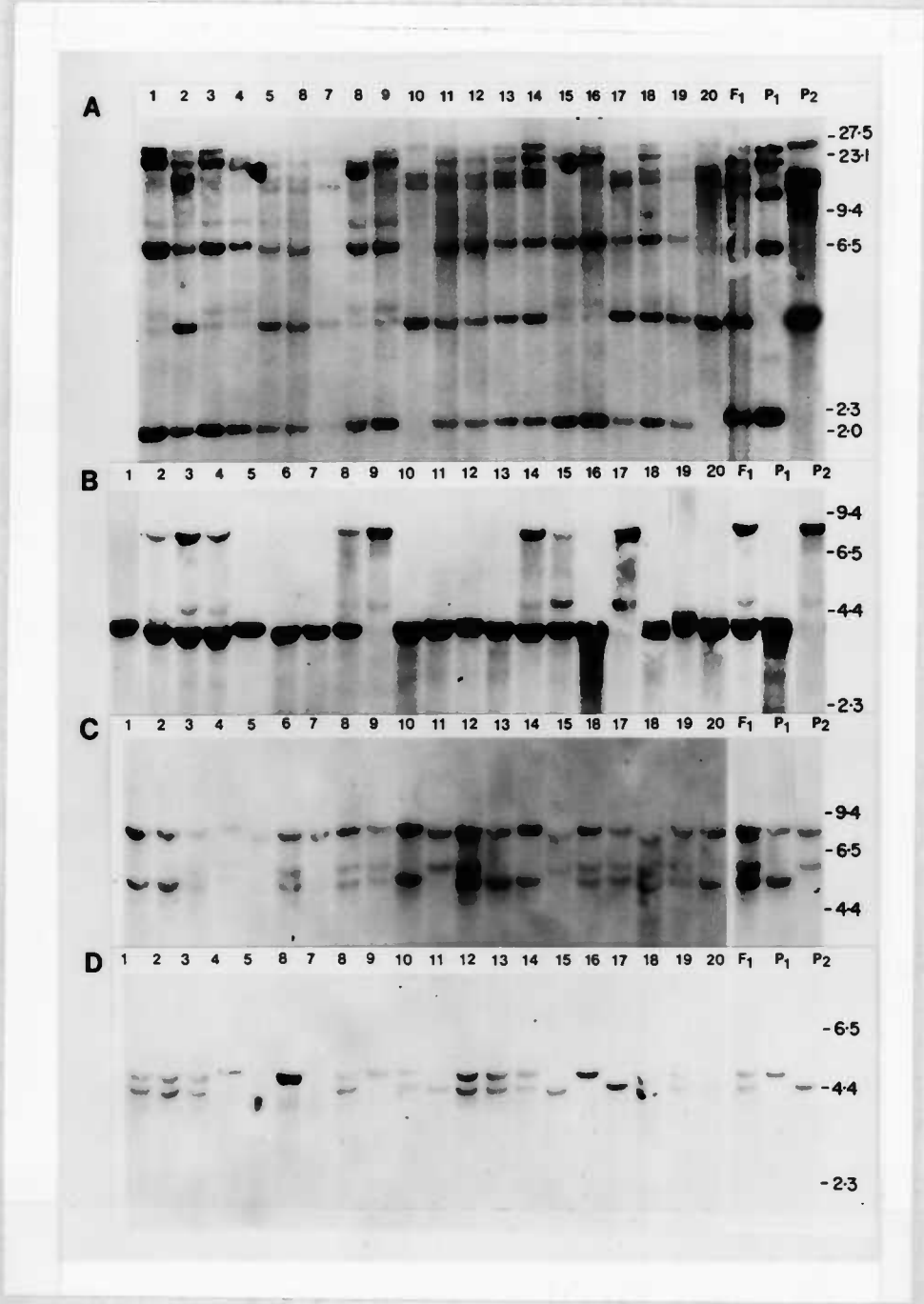

Figure 2. A-D. RFLP of *P. vulgaris* and *P. coccineus* parents, interspecific F_1, and representative F_2s. Panels A to D: Leghemoglobin (LHB), chalcone isomerase (CHI), glutamine synthetase-gamma (GS-g), and clone 20 (VEE20). Lanes 1-20: F_2s; P_1: *P. vulgaris* cv GN; P_2: *P. coccineus* cv SR; F_1: GN x SR hybrid.

Table 3. Distribution of GN x SR F_2 progeny according to genotypes of selected polymorphic probes and goodness of fit to the hypothesis of gametic selection

Probes	Progeny classes						P			Goodness of fit for gametic selection
	Expected ratio			Observed F_2s				Estimated allelic ratio		
	GN	F_1	SR	GN	F_1	SR		G	S	
LHB	1	2	1	44	79	38	0.77	0.52	0.48	NA
VEE20	1	2	1	35	73	45	0.44	0.50	0.50	NA
GS-g	1	2	1	68	76	10	10^{-7}	0.69	0.31	0.17
CHS	1	2	1	59	71	24	10^{-4}	0.61	0.39	0.94
CHI	1	2	1	81	47	19	10^{-7}	0.71	0.29	0.01

the expected ratios. For all three probes, the SR class was under-represented. In order to test the possible occurrence of gametic selection, the allelic ratio was calculated, and the expected numbers of progeny in each class was derived from these ratios (Table 3). Chi-square tests indicated that for GS-g and CHS, the expected and observed numbers of progeny were not significantly different, suggesting preferential transmission of GN alleles. The significant deviation in the case of CHI indicated that mechanisms other than gametic selection must have contributed to the skewed distribution observed in the F_2 population.

Segregation ratios of probes in sub-populations classified according to embryo development, normal and abnormal, were similar to those in the whole population, with one exception. The F_2 ratio of CHS differed between sub-populations and was Mendelian in the sub-class consisting of abnormal embryos.

A representative F_2 population obtained from the reciprocal cross (SR x GN) was also analyzed with four probes (LHB, CHI, CHS and VEE20). The segregation patterns (Table 4) were essentially identical with those obtained for GN x SR progeny.

Linkage analyses

Linkage could be detected only between two of the loci, CHI and CHS ($p = 0.00002$) in the complete population (Table 5). Analyses of the

sub-populations consisting of the normal embryo class and the abnormal embryo class also revealed linkage between CHI and CHS ($p = 0.004$ and 0.009 respectively).

Table 4. Distribution of SR x GN F_2 progeny according to genotypes of selected polymorphic probes.

Probes	Progeny classes					P
	Expected ratio			Observed F_2s		
	GN	F_1	SR	GN	F_1	SR	
LHB	1	2	1	13	24	8	0.52
VEE20	1	2	1	9	22	9	0.82
CHS	1	2	1	21	21	9	0.02
CHI	1	2	1	18	15	3	5×10^{-4}

Table 5. Linkage detected between CHI and CHS probes.

	Chi-square	df	p	r	SE
Total population	26.6	4	10^{-4}	0.29	0.034
Normal embryos	15.1	4	0.04	0.4	0.041
Abnormal embryos	13.6	4	0.009	0.27	0.058

Discussion

RFLP between species of *Phaseolus* was substantially higher than within *P. vulgaris*. This observation is in agreement with results obtained for *Lycopersicum*, *Solanum* and *Lens* (Bernatzky and Tanksley, 1986; Harvey and Muehlbauer, 1989; Helentjaris et al., 1986). In addition, the incidence of polymorphism between GN and SR was slightly lower than between GN and representative genotypes of the other two *Phaseolus* species, confirming previous studies on evolutionary relationships of *Phaseolus* species.

Based on the results presented here, it seems that RFLP analyses will be useful in identifying gene markers and chromosome sections associated with aberrations, including developmental abnormalities, resulting from interspecific crosses of *Phaseolus*. For example, the differential transmission of GN alleles (evidenced by particular probes such as GS-g, CHI and CHS) may account for the selective recovery of *P. vulgaris* progeny types observed earlier (Mok et al., 1987). Our preliminary results suggest that one of the mechanisms of selective transmission could be gametic selection. As no difference between reciprocal crosses was detected, the preferential transmission does not appear to be affected by simple cytoplasmic sources. RFLP markers deviating from the expected F_2 ratios have been observed in interspecific

crosses of *Lycopersicum* (Bernatzky and Tanksley, 1986) and *Lens* (Harvey and Muehlbauer, 1989). However, in these cases the majority of the markers examined segregated in Mendelian ratios, while in the GN x SR cross non-Mendelian segregation seems to be frequent.

Two of the probes, CHS and CHI, appeared to be linked. Although the map distance can be calculated (approximately 44 map units), it may not be accurate due to the distorted segregation ratios of the individual loci. The possible inviability or selective disadvantage of particular genetic combinations could alter the distribution of the alleles. Although less likely, it is conceivable that the two markers are located on different chromosomes but that there is an interaction between the alleles of the two loci or other loci linked to them, resulting in more and less viable genotypes.

Interspecific polymorphism has been used to construct linkage maps or to correlate particular traits with DNA markers using fertile interspecific hybrids (Keim et al., 1990; Paterson et al., 1990). Similar efforts may also be successful in *Phaseolus* since hybrids such as those between *P. vulgaris* and *P. coccineus* are sufficiently fertile to generate large numbers of F_2s and polymorphic probes are easily detected. On the other hand, much larger numbers of probes must be tested for intraspecific mapping due to limited polymorphism, but intraspecific crosses may offer the advantage of Mendelian segregation. In addition, map distances

calculated from recombination frequencies in intraspecific and interspecific progenies may differ due to the possible lower incidence of crossing over and elimination of particular recombinants in the latter. As a result, linkage may be detected over greater physical distances in interspecific combinations. Thus, although our primary objective is to study genetic abnormalities in *Phaseolus* interspecific crosses, these studies also may contribute significantly to the creation of a linkage map.

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CHAPTER 4

RFLP ANALYSES OF PREFERENTIAL TRANSMISSION AND LINKAGE MAPPING IN INTERSPECIFIC HYBRIDS OF OF *PHASEOLUS VULGARIS* AND *P. COCCINEUS*

Abstract

The inheritance of RFLP markers in interspecific hybrids of *P. vulgaris* and *P. coccineus* was analyzed. Of 280 cDNA probes used, 70-85% revealed polymorphism between species while intraspecific RFLP ranged from 8-37%. Segregation of 63 clearly scorable markers was examined in 177 *P. vulgaris* x *P. coccines* F_2s maintained as callus. Preferential transmission of the *P. vulgaris* alleles was observed in 24 of the 28 loci exhibiting non-Mendelian ratios. Nuclear-cytoplasmic interactions and gametic selection may account for the preferential transmission of *P. vulgaris* alleles. The markers were mapped in nine linkage groups. Aggregation of markers with preferential, maternal transmission, including a histone H2A gene, in a linkage group suggest that selection for individual chromosomes also may occur. These observations may explain the skewed distribution of phenotypic traits following interspecific hybridization.

Key words: *Phaseolus*, interspecific hybrid, RFLP, preferential transmission, beans

Introduction

Gene transfer via interspecific hybridization within *Phaseolus* has been limited by the abnormal development of hybrid embryos and the rapid loss of hybridity in subsequent generations. For example, crosses between *P. vulgaris* and *P. lunatus* give rise to embryos that develop to either four-cell or pre-heart shape stages depending on the direction of the cross, while hybrid embryos of *P. vulgaris* and *P. acutifolius* develop to the cotyledonary stage (Mok et al., 1978; Rabakoarihanta et al., 1979). The most advanced development occurs in crosses of *P. vulgaris* x *P. coccineus* which form mature seeds. However, the reciprocal cross still results in abnormal embryos unable to reach maturity (Kroh, 1962; Thomas, 1964; Shii et al., 1982; Mok et al., 1987). Although hybrid plants can be recovered by embryo culture, in F_2 and further generations both abnormal and normal embryos occur. Moreover, viable plants exhibit few hybrid or *P. coccineus* characteristics (Wall and York, 1957; Ibrahim and Coyne, 1975). Studies using isozyme markers revealed distinct patterns in normal and abnormal embryos, resembling *P. vulgaris* and *P. coccineus* respectively (Guo et al., 1989). We hypothesized that the preferential transmission of *P. vulgaris* types in *P. vulgaris* x *P. coccineus* hybrids may be due to selective survival of individuals which inherit a greater portion of *P. vulgaris* genome. Initial studies using a few RFLP

markers indicated that differential transmission of DNA markers occurred (Guo et al., 1991). The present paper reports results of analyzing the inheritance of 63 polymorphic cDNA markers. The degree of preferential segregation and a linkage map are presented. The possible mechanisms of selective transmission and the effects of non-Mendelian segregation on linkage relationship are discussed.

Materials and Methods

Plant materials

For the comparison of polymorphism between genera and species, the following genotypes were used: *Vigna unguiculata* cv. Purple Hull (PH), *P. lunatus* L. cv. Kinston (K), *P. acutifolius* A. Gray P.I. #321637 (AC2), *P. coccineus* Lam cv. Scarlet Runner (SR) and *P. vulgaris* L. cv. Great Northern (GN). Polymorphism between genotypes of *P. vulgaris* was determined with GN, Tendergreen (TG), Improved Tendergreen (IT), Sanilac (SA), Contender (CO), P691 (P1) and P725 (P2). Reciprocal F_2s of *P. vulgaris* cv. GN and *P. coccineus* cv. SR were generated and maintained as cotyledon-derived callus cultures as described previously (Guo et al., 1991), 177 from GN x SR and 36 from SR x GN were included.

DNA manipulations

The methods of DNA isolation and Southern hybridization have been described earlier (Guo et al., 1991). Genomic DNA of the target plant was digested with *EcoR*1. cDNA libraries were constructed from poly (A^+) mRNAs of immature GN embryos and cloned into the *SmaI* site of pGEM-3zf(+)(Promega). Inserts were released by digestion with the restriction enzymes *BamH*1 and *EcoR*1. Inserts representing expressed

genes of low copy number were used as probes.

RFLP segregation and linkage analyses

Target DNA fragments (markers) detected by a single cDNA probe were identified by the probe number and distinguished by letters. For example, E327X and E327Y were two markers detected by one probe E327. Segregation and linkage analyses were performed using the programs Linkage 1 (Suiter et al., 1983) and Mapmaker (Lander et al., 1987).

Results

Detection and selection of polymorphic probes

Over 280 cDNA probes were screened (Table 6). As expected, intergeneric polymorphism between *V. unguiculata* cv. PH and *P. vulgaris* cv. GN was detected with most (93%) of the clones. Interspecific polymorphism was detected by 70-85% of the cDNA clones while genotypes of *P. vulgaris* were polymorphic at varying degrees, between 16% to 37% in relation to GN or 8%-37% when comparisons were made among all genotypes. cDNAs such as E442 and E445 revealed polymorphism mainly between species (Figures 3A and 3B) while E454 and E90 identified intraspecies as well as interspecies RFLPs (Figures 3C and 3D).

Segregation analysis of RFLP markers

Sixty three RFLP markers involving 50 probes could be scored unambiguously and were used for analyses of F_2 populations of GN x SR. The size of the probe, the number of total genomic and polymorphic fragments detected are presented in Table 7. Most of the probes were complementary to one or two bands from each parent. Others, such as LHB, E435, E466 and E596, hybridized to several genomic fragments. Segregation patterns were analyzed using the Linkage 1 program. For GN

Table 6. Polymorphism detected between genotypes of *Phaseolus* and *Vigna*

Genotypes	No. of probes examined	No. of polymorphic probes	% of polymorphism
Between genera			
V. Unguicularis(PH) vs *P. vulgaris*(GN)	193	181	93
Between species			
P. vulgaris(GN) vs *P.coccineus*(SR)	280	195	70
P. vulgaris(GN) vs *P.acutifolius*(AC2)	215	178	83
P. vulgaris(GN) vs *P.lunatus*(K)	148	126	85
Between genotypes of *P. vulgaris*			
GN vs. CO	213	61	29
GN vs. SA	221	57	26
GN vs. TG	219	81	37
GN vs. IT	217	75	35
GN vs. P1	194	31	16
GN vs. P2	194	49	25

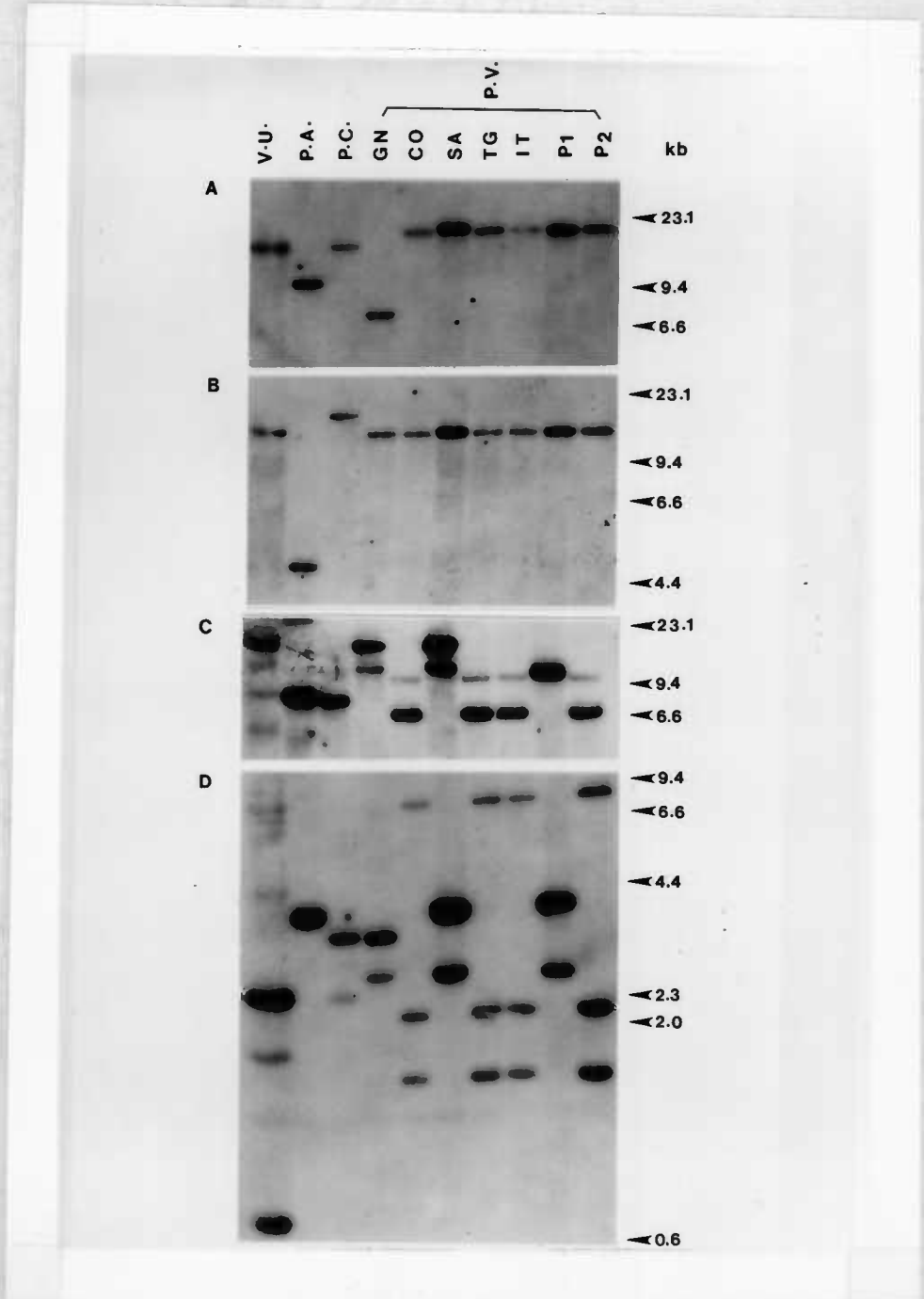

Figure 3. Representative RFLP between genotypes of *Phaseolus* and *Vigna*. Panels A to D: probed with E442, E445, E454 and E90. V.U.: *Vigna unguicularis* PH; P.A.: *P. acutifolius* AC2; P.C.: *P. coccineus* SR; P.V.: *P. vulgaris* GN.

Table 7. Description of cDNA probes used for linkage analysis

Probe	Size (kb)	No. of genomic bands		No. of polymorphic bands		No. of markers* selected for RFLP analysis (per cDNA probe)
		GN	SR	GN	SR	
LHB	0.6	5	4	5	4	1
GSg	1.4	2	2	1	1	1
CHS	1.4	5	5	3	3	1
CHI	0.85	1	1	1	1	1
E20	0.85	1	1	1	1	1
E43	0.8	4	4	2	2	2
E54	0.8	3	2	3	2	3
E83	0.3	1	1	1	1	1
E90	0.85	2	2	1	1	1
E217	0.6	2	2	1	1	1
E251	0.7	2	4	1	3	2
E267	0.6	1	1	1	1	1
E274	0.5	1	1	1	1	1
E276	0.5	1	1	1	1	1
E281	1.1	2	1	2	1	2
E284	0.5	1	2	1	2	3
E294	0.5	1	1	1	1	1
E314	0.55	3	3	1	1	1
E317	0.8	1	1	1	1	1
E326	0.55	1	1	1	1	1
E327	0.75	5	4	3	2	3
E331H	0.75	1	1	1	1	1
E333	0.6	1	1	1	1	1
E342	0.4	1	1	1	1	1
E377	0.75	1	1	1	1	1
E379	0.8	1	2	1	2	1
E384	0.8	2	2	2	2	2
E395	0.9	2	2	2	2	2
E397	0.5	1	1	1	1	1
E398	0.5	3	3	1	1	1
E403	0.5	2	2	1	1	1
E406	0.7	1	1	1	1	1
E418	0.7	1	1	1	1	1
E432	0.5	2	3	1	2	1
E435	0.7	3	3	3	3	1
E436	0.3	1	1	1	1	1
E442	0.4	1	1	1	1	1
E444	0.55	1	1	1	1	1
E450	0.5	3	4	2	3	1
E464	0.6	1	1	1	1	1
E466	0.6	5	8	4	7	1

E481	0.5	1	1	1	1	1
E483	0.55	1	1	1	1	1
E503	0.4	2	2	1	1	1
E504	0.5	1	1	1	1	1
E559	0.6	4	4	2	2	1
E573	0.85	2	2	2	2	2
E596	0.8	3	3	3	3	1
P82	0.2	2	2	2	2	2
P87s	0.5	3	3	3	3	1

* Only unambiguously scorable fragments were used .

x SR F_2 populations, approximately 56% of the polymorphic markerssegregated in Mendelian fashion as represented by E435 and E481 (Figures 4A and 4B) while the remainder deviated significantly ($P<0.05$) from the expected ratio as represented by E395 and E397 (Figures 4C and 4D). With the exception of four markers, the deviation was contributed by F_2s with higher than expected frequency of the *P. vulgaris* genotype and lower number of the *P. coccineus* class (Table 8). In more extreme situations, as in the transmission of E395, no *P. coccineus* type was recovered among 165 F_2s scored.

For non-randomly segregating RFLP loci, the possibility of gametic selection was examined (using the observed genotypic distribution) by calculating the allelic frequency. Chi-square tests indicated that 17 of the 28 loci fit the hypothesis of gametic selection ($P>0.05$)(Table 8). In 16 out of the 17 loci there was a selective transmission of gametes containing *P. vulgaris* alleles; the exception was E503, with higher recovery of the *P. coccineus* alleles.

Thirty six F_2s from the reciprocal cross were also scored (Table 8). reciprocal differences of the transmission pattern was observed for some probes: E83, E90 and E274 exhibited preferential transmission of maternal phenotypes (i.e., SR phenotypes in SR x GN and GN phenotypes in GN x SR). In other cases, such as E395 and E397, although the GN phenotypes occurred at higher than expected frequencies in both

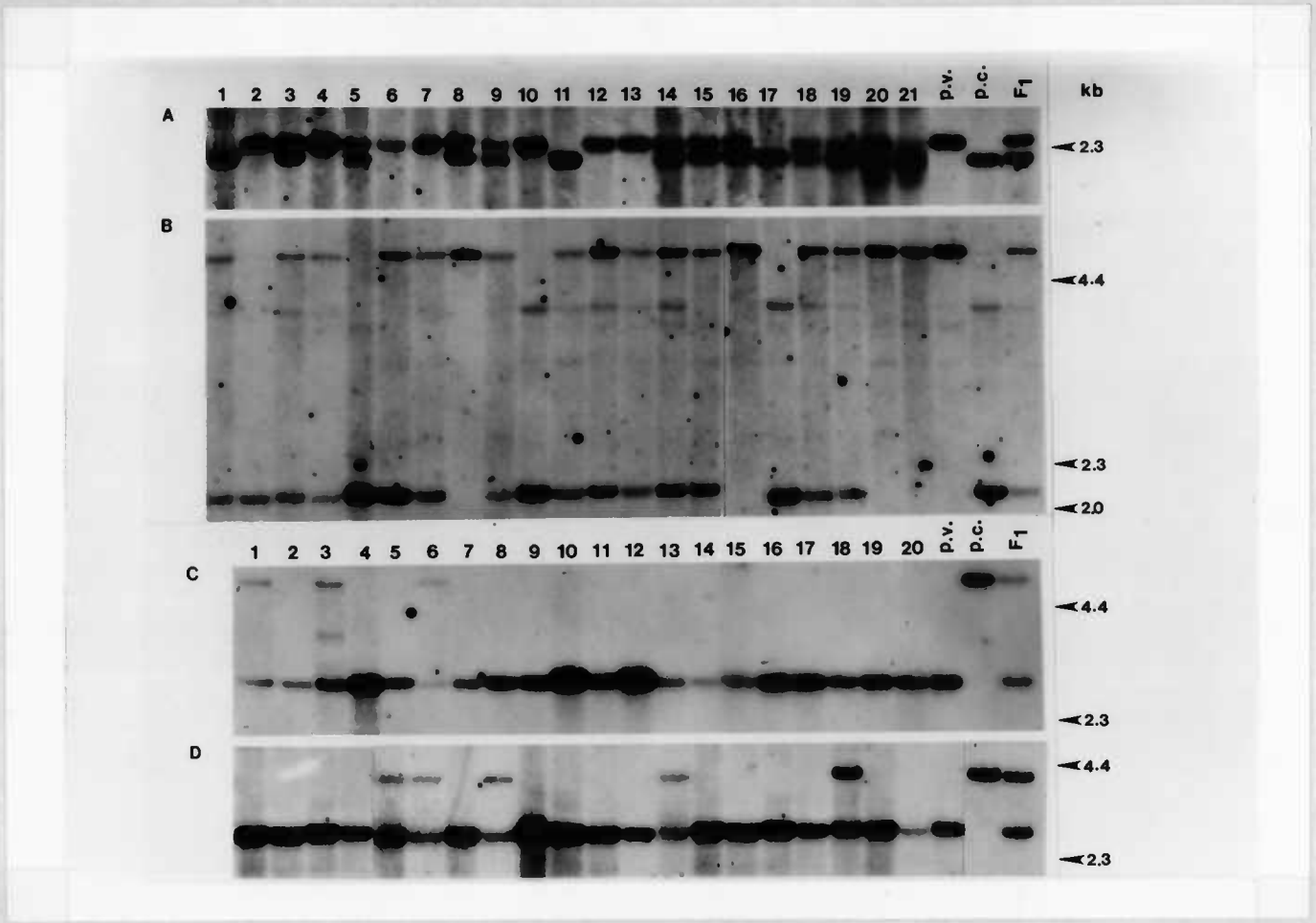

Figure 4. Representative RFLP of F_2s. Panels A and B: probed with randomly segregating clones E435 (only scored bands are shown) and E481. Panels C and D: probed with non-randomly segregating clones E397 and E395. Lanes 1-21: F_2s; P.V.: *P. vulgaris* cv. GN; P.C.: *P. coccineus* cv. SR; F_1: GN x SR hybrid.

Table 8. Analysis of non-randomly segregating markers

Probe	Observed ratio (GN X SR)			Expected ratio (GN X SR)			Estimated allelic ratio		Observed ratio (SR X GN)**		
	GN	F1	SR	GN	F1	SR	GN	SR	GN	F1	SR
GSG*	68	76	10[a]	38.5	77.0	38.5	0.69	0.31	-	-	-
CHS	59	71	24[a]	38.5	77.0	38.5	0.61	0.39	11	14	0
CHI	81	47	19	36.8	73.5	36.8	0.71	0.29	-	-	-
E43X	58	61	20[a]	34.8	69.5	34.8	0.64	0.36	-	-	-
E54Y	14	70	41[a]	31.3	62.5	31.3	0.39	0.61	-	-	-
E54Z	22	82	23	31.8	63.5	31.8	0.50	0.50	-	-	-
E83*	68	80	7	38.8	77.5	38.8	0.70	0.30	2	18	14
E90*	108	38	3[a]	37.3	74.5	37.3	0.85	0.15	1	15	13
E274	57	70	24[a]	37.8	75.5	37.8	0.61	0.39	5	13	11
E294	32	42	40	28.5	57.0	28.5	0.46	0.54	5	12	10
E314	54	76	25[a]	38.8	77.5	38.8	0.59	0.41	6	13	8
E331H*	52	28	2[a]	20.5	41.0	20.5	0.80	0.20	-	-	-
E342	62	76	24[a]	40.5	81.0	40.5	0.62	0.38	-	-	-
E384X	65	25	8[a]	24.5	49.0	24.5	0.79	0.21	-	-	-
E384Y	54	84	13	37.8	75.5	37.8	0.64	0.36	-	-	-
E395*	141	24	0[a]	41.3	82.5	41.3	0.93	0.07	14	8	5
E395Y	12	49	13	18.5	37.0	18.5	0.49	0.51	-	-	-
E397*	126	36	2[a]	41.0	82.0	41.0	0.88	0.12	13	2	3
E418*	59	26	33	29.5	59.0	29.5	0.61	0.39	4	14	8
E432	47	87	26[a]	40.0	80.0	40.0	0.57	0.43	6	15	8
E444	40	94	26[a]	40.0	80.0	40.0	0.54	0.46	9	15	5
E464*	46	67	7	30.0	60.0	30.0	0.66	0.34	6	13	3
E503	22	51	45[a]	29.5	59.0	29.5	0.40	0.60	-	4	4
E504*	38	43	9[a]	22.5	45.0	22.5	0.66	0.34	-	3	7
E573	41	54	20[a]	28.6	57.5	28.6	0.59	0.41	6	13	6
E43Y	52	◄68►		30.0	◄90.0►		NA		-	-	-
E284X	◄129►		15	◄72.0►		36.0	NA		0	22	9
E284Y	28	◄41►		17.3	◄51.8►		NA		20	12	0

* Markers on the longest linkage group.

** Statistical analyses not presented due to the small number of the progeny.

[a] Fit gametic selection.

reciprocal populations, the frequency of SR phenotypes was higher in SR x GN population. These trends were noted but the number of F_2s in SR x GN crosses were deemed too small for meaningful statistical analyses.

Linkage mapping

The segregation data of *P. vulgaris* x *P. coccineus* were used to construct a linkage map using the program Mapmaker (recombination fraction of 0.30 and LOD score at 3.0). Forty six markers were mapped to nine out of a possible 11 linkage groups (Figure 5). The remaining 17 markers were unlinked. However, when the cut-off for linkage was raised to 0.40 (as in most reported linkage mapping analysis) only 11 markers remained unlinked, and 10 instead of nine linkage groups were defined. These nine linkage groups represented 534 centimorgans (cM) of the bean genome. Of the 28 non-randomly segregating markers, nine exhibited dramatic under-representation of *P. coccineus* parental types (Table 8) and eight of these nine were clustered on the longest linkage group (Figure 5).

Sequence analysis of selected cDNA clones

In order to determine if the probes include genes important to viability, selected clones (putative full length cDNAs as determined by northern hybridization) were sequenced and compared with gene sequences deposited in GeneBank (NIH) and Swiss-Protein databanks.

E395 was of particular interest since its segregation was the most unusual, with no SR allele recovered. Sequence analyses indicated that E395 encodes a polypeptide of 146 amino acids with 56-59% homology with histone H2A of wheat (Rodrigues et al., 1985), pea (Koning et al., 1991), and sea urchin (Palla et al., 1989) (Figure 6).

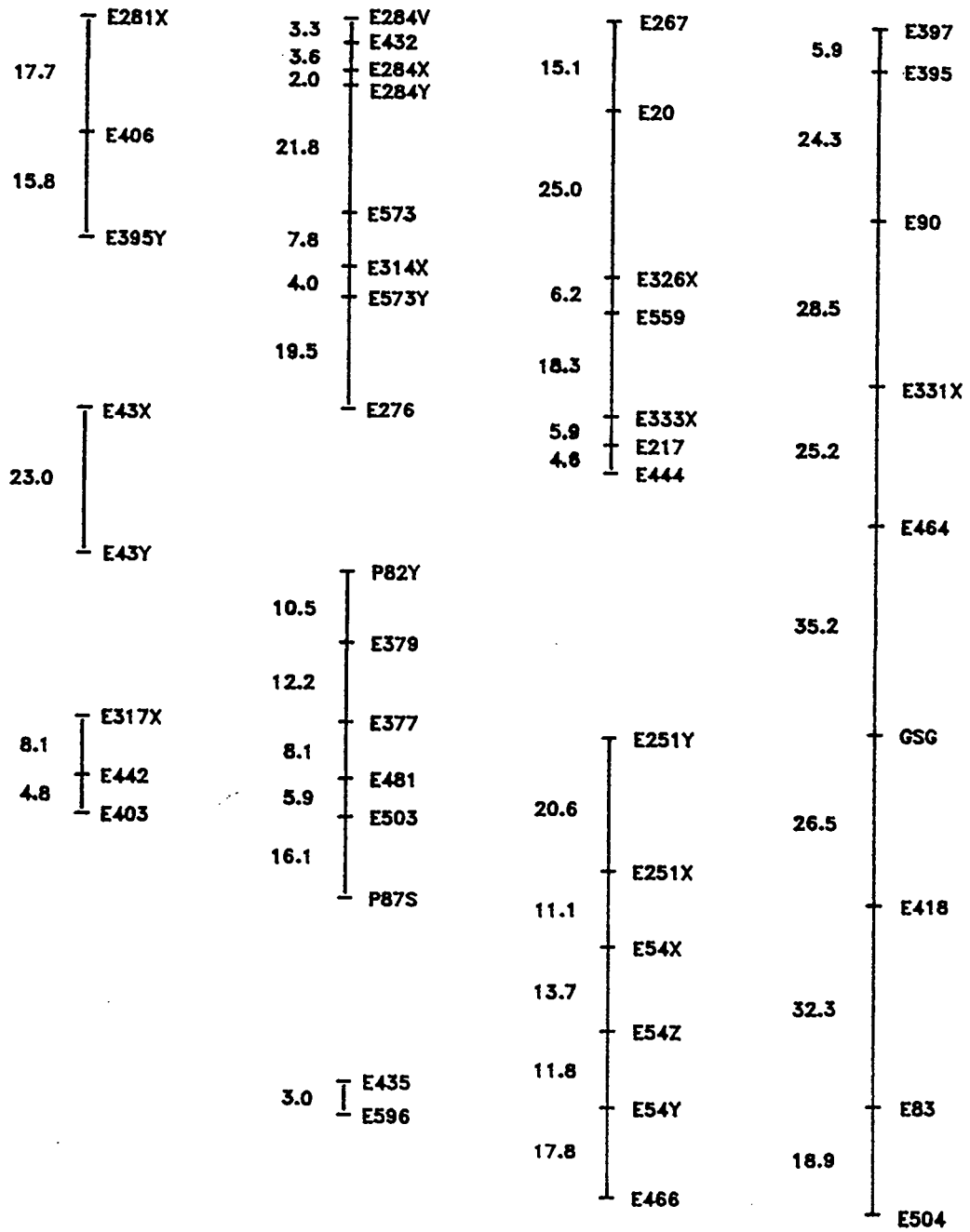

Figure 5. Linkage groups detected by cDNA clones.

```
---------- -----MSSTE VATTTKK--- GGRGK--PKT TKSVSRSSKA
---------- ---------- ---------- SGRGKSGKAR TKAKSRSSRA
---------- ---------- ---------- AGRKG--GDR KKAVTRSVKA
-LTTSENFSA RIXSSNLEMD ASTKTKKGAG GRKGG--GPR KKSVTRSVRA

GLQFPVGRIA RYLKAGRYAQ RVGSGSPVYL SAVLEYLAAE VLELAGIAAR
GLQFPVGRVH RFLRKGNYAK RVGGGAPVYM AAVLEYLTAE ILELAGNAAR
GLQFPVGRIG RYLKKGRYAQ RVGSGAPVYL AAVLEYLAAE VLELAGNAAK
GLQFPVGRVG RFLKKGRYAQ RVGTGAPVYL AAVLEYLAAE VLELAGNAAR

DNKKSRIIPR HIQLAVRNDE ELSKLMGSVT IASGGVLPKN SSKICCPRKT
DNKKSRIIPR HLQLAVRNDE ELNKLLGGVT IAQGGVLP-N IQAVLLPKKT
DNKKTRIIPR HLLLAVRNDQ ELGRLLAGVT IAHGGVIP-N INSVLLPKKS
DNKKNRISPR HLLLAVRNDE ELGKLLAGVT IAYGGVLP-N INPVLLPKRK

GKGEGEVWIY LVKGFX---- --------  E395 (1-146)
GKSS------ ---------- --------  Sea urchin H2A (1-123)
PAAAEKEAKS PKKKTSTKSP KKVAAKE-  Wheat H2A (1-145)
ENAAASTPKS PSKAKKSPKK AXTWFXVX  Pea H2A (1-166)(total 203)
```

Figure 6. Comparison of amino acid sequence of E395 with H2As of wheat, pea and sea urchin.

Discussion

Polymorphisms of intraspecific populations were sufficient for linkage mapping in maize (Helentjaris et al., 1986), *Brassica* (Figdore et al., 1988), and *Solanum* (Gebhardt et al., 1989), while in self-pollinating species, such as soybean and tomato, interspecific hybrid populations were used (Keim et al., 1989; Helentjaris et al., 1986). For *P. vulgaris*, the degree of intraspecific polymorphism (16-37%) obtained in this study with cDNA probes was comparable to that (33%) estimated using genomic clones (Chase et al., 1990). Although interspecific polymorphism in *Phaseolus* is substantially higher (70-85%), intraspecific populations may be used for RFLP analysis if unrelated genotypes and larger numbers of probes are employed.

cDNAs were more efficient than genomic clones in detecting polymorphism of lettuce (Landry et al., 1987) but gave low hybridization signals in maize (Helentjaris, 1987). Most of the genetic maps consist of a combination of markers defined by cDNA, genomic probes, and randomly amplified polymorphic DNAs (RAPDs). For our long term goal of identifying genes important to interspecific hybrid development, cDNA clones are preferred since clones of interest can be used to determine the gene product and tissue specific expression.

Segregation distortion of RFLP markers occurs in both inter- and

intra-specific populations: 34% in tomato, 26% in lentil, 13% in soybean (interspecific); 19% in *Brassica napus,* and 40% in potato (intraspecific). These segregation distortions can be either uni- or bi-directional (Helentjaris et al., 1986; Havey and Muehlbauer, 1989; Keim et al., 1990; Landry et al., 1991; Gebhardt et al., 1991). The frequency of non-random segregation (44% of the cDNA probes) observed in *P. vulgaris* x *P. coccineus* was higher, and in most cases the alleles of the *P. vulgaris* parent were favored. The extent of distortion was also more extreme than previouly reported, for one probe (E395) even resulting in the absence of *P. coccineus* alleles. Previous studies of *P. vulgaris* x *P. coccineus* hybrids have reported skewed distributions of several morphological traits, including cotyledon position, flower color and plant growth patterns, toward the *P. vulgaris* parent (Wall and York, 1957; Ibrahim and Coyne, 1975). The segregation pattern of RFLP markers in this study showed similar preferential transmission of *P. vulgaris* alleles. Selective elimination of *P. coccineus* genotypes may occur during gametic or zygotic stages or during early plant development. Both zygotic and gametic selection have been proposed as a possible mechanism to explain an excess of a specific parental homozygote in *Lycopersicon* species (Gadish and Zamir, 1987; Helentjaris et al., 1986). In our study, gametic selection could account for the distortion of 17 markers (Table 8). However, there is also the possibility of nuclear-cytoplasmic interactions,

inferring that in *P. vulgaris* x *P. coccineus* hybrids, gametes with *P. vulgaris* alleles are more compatible with *P. vulgaris* cytoplasm. Moreover, there could be interactions between embryo and endosperm resulting in favoring of maternal alleles. The results with the reciprocal cross in which *P. coccineus* genotypes were often more abundant, seem to lend support to the latter interpretations, rendering simple gametic selection less likely.

To investigate the possible functions of non-randomly segregating markers, selected cDNA clones were analyzed. The deduced amino acid sequence of E395 showed 56-59% homology with histone H2As, however the "SPKK" sequence, a motif at the C terminus of some plant histone H2A genes (Koning et al., 1991) was absent. Although there is no evidence to link the histone genes with selective transmission in *Phaseolus* at this time, it is possible that incompatible interactions of nuclear components (i.e., *P. coccineus* histone genes) and heterologous cytoplasmic factors (i.e., *P. vulgaris* binding proteins) may render *P. coccineus* homozygotes less viable. In support of this speculation is the observation that in yeast a mutation in the nuclear localization signal sequence of the histone gene resulted in the failure of the histone to be targeted to the nucleus (Lee et al., 1991). This and other possibilities are being explored by analyzing genomic clones of histone genes of *P. vulgaris* and *P. coccineus* (unpublished).

Phaseolus is a diploid with $2n = 22$. The fact that only nine of 11

linkage groups are mapped with the 46 RFLP markers may simply represent the fact that insufficient numbers of probes may have been used. As the number of marker increases, unlinked markers may be mapped to existing linkage groups or form additional linkage groups. Indeed, when the stringency of linkage was lowered to 0.40, additional markers were assigned and one more group was mapped. It is envisioned that more extensive mapping is needed to reveal more linkage groups representing all 11 chromosomes. Alternatively, the unmapped chromosomes may carry highly conserved sequences for important genes, thereby lowering the chances of detecting polymorphism with cDNA probes.

As linkage mapping programs do not take into consideration non-Mendelian segregation of markers, their effects on the segregation ratios of the other markers, the map distance, and the linkage relationships will need to be examined. For two linked loci as illustrated in Figure 7-I, non-Mendelian segregation of locus A will distort the ratio of locus B. The degree of distortion increases as the linkage becomes tighter. Unlinked loci, as illustrated in Figure 7-II, are not affected. The recombination frequency between the linked loci may be accurate if the distortion (of allele "a") affects parental (ab) and recombinant genotypes (aB) equally (Figure 7-I and 7-II), but may be inaccurate if unequal effects exist. Moreover, if unlinked loci segregate in a non-Mendelian fashion as

Figure 7-I. Loci A and B are linked with 20% recombination frequency and B is a randomly segregating marker.

P_1 P_2 F_1
AABB X aabb -----> AaBb ----> F_2:

		40 AB	10 Ab	10 aB	40 ab
40	AB	AABB 16	AABb 4	AaBB 4	Aabb 16
10	Ab	AABb 4	AAbb 1	AaBb 1	Aabb 4
10	aB	AaBB 4	AaBb 1	aaBB 1	aaBb 4
40	ab	AaBb 16	Aabb 4	aaBb 4	aabb 16

1. If A segregates randomly (no selection against any allele), segregation ratio of B locus is not affected: 25BB:50Bb:25bb = 1:2:1; recombination frequency between A and B is not affected: 10Ab/(40AB + 10Ab) = 20%

2. If A exhibits non-Mendelian segregation (e.g., allele "a" is lethal), segregation ratio of B locus is affected: 16BB:8Bb:1bb ≠ 1:2:1; recombination frequency between A and B is not affected: 10Ab/(40AB + 10Ab) = 20%.

Figure 7-II. Loci A an B are unlinked (50% recombination frequency) and B is a randomly segregating marker.

		25 AB	25 Ab	25 aB	25 ab
25	AB	AABB 6.25	AABb 6.25	AaBB 6.25	Aabb 6.25
25	Ab	AABb 6.25	AAbb 6.25	AaBb 6.25	Aabb 6.25
25	aB	AaBB 6.25	AaBb 6.25	aaBB 6.25	aaBb 6.25
25	ab	AaBb 6.25	Aabb 6.25	aaBb 6.25	aabb 6.25

1. If A segregates randomly (no selection against any allele), segregation ratio of B locus is not affected: 6.25BB:12.5Bb:6.25bb = 1:2:1; recombination frequency between A and B is not affected: 25Ab/(25AB + 25Ab) = 50%.

2. If A exhibits non-Mendelian segregation (e.g., allele "a" is lethal), segregation ratio of B locus is not affected: 6.25BB:12.5Bb:6.25bb = 1:2:1; recombination frequency between A and B is not affected: 25Ab/(25AB + 25Ab) = 50%.

Figure 7-III. Loci A and B are both non-randomly segregating markers and alleles a and b are lethal.

	AB	Ab	aB	ab
AB	AABB	AABb	AaBB	Aabb
Ab	AABb	AAbb	AaBb	Aabb
aB	AaBB	AaBb	aaBB	aaBb
ab	AaBb	Aabb	aaBb	aabb

The recovery of only "A" and "B" alleles will result in a false linkage between the two loci. The degree of distortion will depend on the severity of the selection.

Figure 7. Effects of non-Mendelian markers on segregation, map distance and linkage.

illustrated in Figure 7-III, false linkage may occur. In our study, it is interesting to note that eight out of the nine of the preferentially transmitted markers are mapped to a single linkage group. If the linkage relationship is not the result of distortion, it would suggest the chromosome is under selection pressure or contains genes important to viability. Grouping of non-Mendelian markers into regions or specific chromosomes has been reported in hybrids of *Lycopersicon esculentum* x *L. pennellii* (Kinzer et al., 1990), *B. napus* (Landry et al., 1991), *B. rapa* (Song et al., 1990) and *B. oleracea* (Slocum et al., 1990). Although the possibility of false linkage can not be excluded, most authors attributed such grouping to the presence of critical genes, such as the self-incompatibility and lethal alleles in potato and *Brassica* (Gebhardt et al., 1991; Landry et al., 1991). Physical mapping using pulse field gel electrophoresis or *in situ* hybridization may provide further information on the physical linkage relationship and possibly the mechanisms of preferential transmission.

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