Characterization of Cytoplasmic Diversity in Soybean (*Glycine max* L. Merr) Using Mitochondrial Markers

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Plant Pathology, Physiology, and Weed Science

(ABSTRACT)

Soybean, *Glycine max* L. Merr, is used worldwide as an important source of protein and oil for a wide spectrum of edible, feed and industrial purposes. Modern cultivars are derived from relatively few plant introductions (PIs) which severely limits diversity in soybean germplasm. The United States Department of Agriculture (USDA) maintains the soybean germplasm collection. Mitochondrial DNA sequences have been used as markers of diversity at the cytoplasmic level.

This project included three objectives. The first was a classification of the 208 varieties of the USDA's 'old domestic collection' of soybean varieties with two mitochondrial restriction fragment length polymorphisms (RFLP) markers. Molecular techniques were used to search for additional sources of cytoplasmic diversity available to soybean breeders. The two polymorphic markers were, a 2.3 kb *Hin*dIII fragment isolated from 'Williams 82' mitochondrial DNA, and a portion of the mitochondrial *atp*6 gene. These markers were used to distinguish cytoplasmic groups based on hybridization

analysis of *Hin*dIII-digested soybean DNA. Four major groups were observed with the 2.3 kb marker in the old domestic collection, and several minor subgroups were also detected.

The second objective included subcloning and sequencing the 0.9 kb and 1.7 kb *Hin*dIII-*Pst*I clones flanking the 2.3 kb *Hin*dIII fragment from 'Williams 82' DNA. The total 4.9 kb *Pst*I sequence from 'Williams 82' mitochondrial DNA was used to search a sequence database for any homology to known mitochondrial sequences.

The third objective compared restriction maps of the four cytoplasmic types in the regions containing homology to the 4.9 kb *Pst*I fragment. DNAs from the four cytoplasmic types were digested with five enzymes and four specific clones (0.9 kb *Pst*I-*Hin*dIII, 0.8 kb *Hin*dIII-*Xba*I, 1.5 kb *Xba*I-*Hin*dIII, 1.7 kb *Hin*dIII-*Pst*I) were used as hybridization probes in Southern analysis to examine RFLP patterns and construct comparative restriction maps of the four cytoplasmic types of DNA.

Dedicated to Elaine M. Carr and Sally S. Roraback.

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CHAPTER I

Introduction

Overview

Soybean is an important agronomic crop worldwide that provides a large supply of oil and protein products for a wide range of applications. The seed protein is processed to yield soyflour and meal. Soybean protein products include livestock feed, cereals, high-protein edibles and industrials such as adhesives. Soybean oil is processed into both edible and industrial end products. Industrial uses of soybean oil include the production of putty, linoleum, ink, and lectins for the manufacture of emulsifiers and anti-foam agents (Smith & Huyser 1987). Edible oil products are mostly margarine and vegetable oil. Plastics, lubricants, diesel fuels, sterols, and fatty acids are other products of the versatile soybean (Smith & Huyser 1987).

Classification of soybean distinguishes varieties originating in Northeast China from those originating elsewhere in Asia (Sisson *et al.* 1978). Soybean belongs to the genus *Glycine*, subgenus *Soja*. Cultivated soybean varieties are referred to as cultivars. A related member of the subgenus is *G. soja* Sieb. and Zucc., the likely progenitor of *G. max*. The USDA established the Soybean Germplasm Collection in 1949 after soybean became a major crop in the US (Sisson *et al.* 1978). This collection is a compilation of all soybean PIs originally brought to the US and Canada, as well as subsequent crosses by plant breeders. Soybeans are separated into northern (000-IV) and southern (V-IX) maturity groups based on days to seed maturity. Old domestic varieties are those that were introduced or established by 1947. Soybean varieties

established or introduced after 1947 and continuing to the present are referred to as modern cultivars.

Literature Review

Germplasm Diversity

Early soybean PIs were viney, did not produce substantial seed and were used primarily as bedding and forage. Only after the 1940s did the US grow sufficient quantities of soybean crops to warrant a breeding program (Sisson *et al.* 1978). After generations of selective breeding, plant breeders were able to incorporate favorable traits from unproductive plants into highly productive lines, which created progeny with the characteristically uniform genome of the high yield cultivar (Sisson *et al.* 1978). Species diversity is adversely affected by such breeding.

The entire genome of a cell includes the DNA of the nucleus as well as the DNA of cytoplasmic organelles. The single nucleus of a cell contains one copy of its genome which is inherited equally from each parent, in Mendelain fashion. Mitochondria and differentiated plastids (chloroplasts), are the cytoplasmic organelles which contain their own DNA. These organelles are unique in their abundance, DNA content, and mode of inheritance. Up to 200 of each of these organelles can be present in a single cell and 50 to 200 copies of their genomes are present in each organelle. The cytoplasm of a cell is inherited from only a single parent, therefore the organellar DNA is not

inherited in Mendelian fashion and this can create more uniformity in the cytoplasmic DNA relative to the nuclear DNA.

Modern soybean cultivars are derived from very few PIs, thus diversity in soybean germplasm is severely limited (Delannay *et al.* 1983). Major contributions by a limited number of cultivar introductions decreases the genetic base of soybean germplasm. Pedigree analysis can identify the location in soybean lineages where proliferation of select genotypes and loss of diversity has occurred. Delannay *et al.* (1983) traced the gene pool of 158 hybrid soybean cultivars and found it included only 50 plant introductions. Of these 50, 10 introductions reflected more than 80% of the northern gene pool, and four of the 10 contributed 50%. Seven introductions reflected more than 80% of the southern gene pool. Twelve northern introductions were shown to have contributed 88% to the nuclear gene pool, while only five of those contributed 77% to the cytoplasmic gene pool (Delannay *et al.* 1983). Different introductions from the same geographical area narrow the gene pool even further. Estimating parental contributions at 50% does not account for one parent providing more genetic information to the offspring, or plant introductions being inbred (Delannay *et al.* 1983). Delannay *et al.* (1983) investigated hybrid crosses that were released into the USA and Canada over four different time periods: prior to 1951, 1951-1960, 1961-1970, and 1971-1981. Two southern cultivars, CNS and S-100, contributed to 50% of hybrids during these time periods. From 1971-1981, S-100 contributed to 20 of 22 cultivars

and CNS contributed to all 22. Between 1951 and 1960 the northern gene pool was comprised of cultivars that arose exclusively from the crosses of 'Mandarin (Illinois)' by 'Manchu', 'Lincoln' by 'Richland', and 'Mandarin (Ottawa)' by 'A.K. (Harrow)' (Delannay *et al.* 1983). The four cultivars which contributed most heavily after 1951 were 'Mandarin (Ottawa)', 'Manchu', 'Mandarin (Illinois)' and 'Richland', respectively. The drastic uniformity that exists in cultivated soybean illustrates the need for molecular methods to be able to identify diversity. Molecular identification of diversity could provide an informative classification system not previously detected.

Diversity in the genus *Glycine* has been studied at the nuclear level by numerous laboratories. Similarity between members of the genus *Glycine* was determined by analysis of ribosomal repeat variations (Doyle & Beachy 1985). *G. max* and its progenitor, *G. soja*, varied from other *Glycine* genera but were not distinguishable from each other (Doyle & Beachy 1985). *Bam*HI digestions of total soybean DNA from *G. max* and *G. soja* were probed with a heterologous ribosomal sequence in Southern analysis (Doyle 1988). Little variation was seen between the two types of DNA. The repeat length seen in *G. max* (345 base pairs [bp]) was identical to that in *G. soja* (Doyle 1988). Lack of diversity in soybean was confirmed with isozyme markers (Griffin 1986). The old domestic collection may offer additional germplasm diversity to modern cultivars (Bernard *et al.* 1988). A system that could distinguish a diversity classification in soybean, could possibly identify additional classes of

diverslty not present in the breeding scheme of modern cultivars.

Cytoplasmic Inheritance & the Mitochondrial Genome

Mitochondria and plastids are cytoplasmic organelles that contain a DNA genome. The DNA of these organelles, together with the nuclear DNA, comprise the total DNA of a cell. Although nuclear DNA is inherited in Mendelian fashion, with the progeny receiving equal numbers of chromosomes from each parent, cytoplasmic DNAs have their own modes of inheritance. In plants, organelle DNA can be inherited maternally, paternally, or even biparentally (Birky 1983). Biparental plastid inheritance was demonstrated in *Oenothera organensis* (*Oenothera* [evening primrose]) by Stubbe (1984). Based on restriction pattern analysis of restriction digested conifer DNA, visualized by ethidium bromide staining, chloroplast DNA inheritance was shown to be paternal for both *Pinus banksiana* (jack pine) and *P. taeda* (loblolly pine) (Wagner *et al.* 1987, Neale *et al.* 1989). In contrast, chloroplast DNA is inherited paternally in loblolly pine, and mitochondrial DNA is inherited maternally (Neale *et al.* 1989). Maternal inheritance is the predominant mode of inheritance of mammalian mitochondria (Hutchison *et al.* 1974). Maternal inheritance is the predominant mode of inheritance of both mitochondria and chloroplasts of higher plants (Conde *et al.* 1979).

Hutchison *et al.* (1974) looked at restriction endonuclease digestions of mitochondrial DNA from reciprocal crosses between *Equus caballus* (horse) and

E. asinus (donkey). The mitochondrial DNA of the resulting progeny showed complete maternal inheritance based on banding patterns of restriction digests with *Hae*III. Organelle inheritance of reciprocal crosses btween the related species, *Zea mays* (maize) and *Zea perennis* (perennial teosinte) was observed by restriction fragment analysis (Conde *et al.* 1979). Mitochondrial and chloroplast DNAs were shown to be unique and distinct for each species with complete absence of paternal transmission (Conde *et al.* 1979). Intraspecific (*G. max*: PI 27890 X *G. max*: PI 290136) and interspecific (*G. max* ['Clark'] X *G. soja*: PI 65549) crosses in soybean were analyzed to determine the inheritance of soybean chloroplast DNA (Hatfield *et al.* 1985). *Cla*I digests performed on chloroplast DNA-enriched extractions showed that the hybrid pattern in each reciprocal cross was identical to the maternal banding pattern.

Beyond mode of inheritance, plant mitochondrial genomes have been investigated for their size, and structure. Mitochondrial genomes in mammals and *Saccharomyces cerevisiae* (yeast) are 15 kilobases (kb) and 75 kb, respectively. Plant mitochondrial genomes encompass a wide range of sizes and in the *Cucurbitaceae* (Cucurbit) family, include 333 kb for *Citrullus vulgaris* (watermelon), 848 kb for *Cucurbita pepo* (zucchini), 1515 kb for *Cucumis sativus* (cucumber), and 2424 kb for *C. sativus* (muskmelon) (Ward *et al.* 1981). A plant mitochondrial genome of 200 kb was identified in *Brassica campestris* (*Brassica* [Chinese cabbage]) (Palmer & Shields 1984). Size considerations of the mitochondrial genome are not related to differences in the number of

proteins encoded since the genome only codes for an estimated 20-30 proteins in all plant species (Mackenzie *et al.* 1994).

The structure of various plant mitochondrial genomes have been investigated for their organization and possible sites of rearrangement. The mitochondrial genomes of *B. campestris* (Palmer & Shields 1984), maize (Lonsdale *et al.* 1984), *Triticum aestivum* (wheat) (Quetier *et al.* 1985), *B. nigra* (black mustard) and *Raphanus sativa* (radish) (Palmer & Herbon 1986), *Spinacia oleracea* (spinach) (Stern & Palmer 1986), *B. hirta* (white mustard) (Palmer & Herbon 1987), *Beta vulgaris* (sugar beet [Owen cytoplasm]) (Breers & Lonsdale 1988), *Helianthus annuus* (sunflower) (Siculella & Palmer 1988), and *Petunia hybrida* (petunia) (Folkerts & Hanson 1989) have been mapped and investigated. These studies showed complex genomes containing a variety of repeats with distinct organization which generate subgenomic circles from the "master circle" of the mitochondrial genome. Circle sizes generated by reciprocal recombination were determined by restriction mapping and compared to predicted sizes. Analysis of soybean (Synenki *et al.* 1978) and maize (Londsdale *et al.* 1984), mitochondrial DNA by electron microscopy (EM) showed the presence of various sized circles as well as minicircles. A complete restriction map of soybean mitochondrial DNA has not been constructed.

Variation of mitochondrial genomes can be illustrated by the mapping of the maize mitochondrial genome which contains numerous repeated sequences

in its master circle as well as in several subgenomic circles (Lonsdale *et al.* 1984). These repeated sequences are present as both direct and inverted repeats. Recombination is postulated to occur at such repeated sequences to give rise to the subgenomic circles that are visualized via EM. The non-stoichiometric nature of some of the restriction fragments found in the mitochondrial genomes of angiosperms and the huge number of restriction fragments demonstrate the complexity of these genomes (Bonen & Brown 1993). Restriction map predictions of plant mitochondrial genomes include larger circular molecules than the largest (100 kb) seen with the electron microscope (Hanson & Folkerts 1992). Although isolation procedures could cause breakage of large molecules, this does not explain the presence of fewer small molecules than predicted (Hanson & Folkerts 1992). The inconsistency between electron microscope observations and genome predictions based on restriction mapping is not clear. Homologous recombination of plant mitochondrial genomes could give rise to distinct structural configurations. The complexity of variation in the mitochondrial genome is attributed to repeated and exogenous sequences as well as the large size of the genome, that could be due in part to multiple introns (Fauron *et al.* 1991). Repeated sequences are thought to be part of the recombination machinery of mitochondria (Fauron *et al.* 1991).

In contrast to the belief that plant mitochondrial DNA exists as many circular molecules, Bendich (1993) raised the possibility that the mitochondrial

genome is represented by large linear molecules and that incidental recombination between repeats on linear molecules could give rise to the small fraction of circles seen. Results from repeated mitochondrial DNA isolations showed inconsistent numbers of observed circles and it is inferred that a circular genome would give more reproducible results.

Genetically inherited traits are not entirely nuclear, plant mitochondrial genomes have been shown to carry such desirable traits as high yield, resistance to disease, pests and arid or cold conditions. Plant mitochondrial genomes have also been shown to carry susceptability to disease, for example the Texas-type cytoplasm of maize is susceptable to the toxin of the fungus *Bipolaris maydis* which is the causative agent of Southern corn leaf blight (Levings & Pring 1976) and was responsible for a 95% decrease in maize crop yields in the midwest during the late 1960s.

The best defense against the hazards of vulnerability to disease is to maintain or improve diversity within a germplasm. Defining plants with diverse cytoplasms was one of the first germplasm studies which utilized molecular techniques. Work by Levings (1976) analyzed the organelle DNA of higher plants by restriction endonuclease digests. Differences in restriction endonuclease digestion patterns can provide a measure of nucleotide sequence diversity. Gain or loss of restriction sites can result from such molecular events as nucleotide substitutions, insertions, deletions, or appropriately located rearrangements.

Searching for dissimilar soybean cytoplasms based on the mitochondrial DNA of predominant maternal ancestors, Sisson *et el.* (1978) found four different patterns using restriction analysis methods. After restriction digestions with *Sal*I, four patterns were demonstrated by the cultivars 'Arksoy', 'Tokyo', 'Lincoln', and *G. soja* (PI 81762) (Sisson *et el.* 1978).

RFLP analysis was later developed and the use of specific hybridization probes greatly enhanced the ability to distinguish between cultivars relative to the visualization of restriction fragments on ethidium bromide stained agarose gels. RFLP analysis includes the transfer and immobilization of single stranded DNA fragments from an agarose gel to a permenant filter. The filter can then be hybridized to a specific radioactively labeled DNA sequence referred to as a probe. Changes in mitochondrial genomes such as nucleotide insertions, deletions, or recombinational rearrangements, can be visualized with the labeled probe by autoradiography. Smaller restriction fragments are able to migrate faster down an agarose gel and as a result, size changes in fragments with homology to the probe appear as radioactive bands with different migration positions.

Shoemaker *et al.* (1986) continued to search for diversity in soybean germplasm by analyzing chloroplast DNA RFLP patterns. Distinction was made between five plastome types (Shoemaker et *al.* 1986). Additional work with soybean chloroplast DNA placed a single accession into a sixth unique plastome group (Close *et al.* 1989). The most prolific maternal ancestors of

modern cultivars were tested. In was concluded that modern cultivars contain more uniformity in their cytoplasm than is predicted based on nuclear diversity and Mendelian inheritance (Shoemaker *et al.* 1986). Other lines in the subgenus *Soja* showed greater cytoplasmic diversity compared to elite hybrid cultivars (Cregan *et al.* 1981, Shoemaker *et al.* 1986). Older varieties showed more diversity than modern cultivars and a few cultivars fell into different categories than expected, or previously reported (Shoemaker *et al.* 1986). Chloroplast DNA studies by Shoemaker *et al.* (1986) placed *G. soja* accessions into one of six possible groups while *G. max* fell into three of these groups.

Previous Work with the 2.3 kb Polymorphic Marker

Eight soybean cultivars of major cytoplasmic importance in modern cultivars were analyzed in a search for any obvious differences in their mitochondrial DNA patterns (Grabau *et al.* 1989). Purified soybean mitochondrial DNAs from these cultivars were examined by visualization of ethidium bromide stained *Hin*dIII-digested restriction fragments. The absence of a 10.5 kb fragment was observed exclusively in the Williams 82 cultivar (Grabau *et al.* 1989). The 10.5 kb fragment was subsequently extracted from the agarose gel and used as a probe in Southern analysis of the same eight mitochondrial DNAs. In addition to numerous repeated bands in common to all eight cultivars, the presence of a *Hin*dIII band at 2.3 kb was observed only in the Williams 82 cultivar (Grabau *et al.* 1989). This fragment of mitochondrial

DNA was isolated and cloned into the pUC19 vector and used as a marker for further diversity experiments (Grabau *et al.* 1989). When the 2.3 kb *Hin*dIII mitochondrial DNA marker was used to probe purified soybean mitochondrial DNA, the homology could be attributed exclusively to the presence of mitochondrial sequences. Use of this probe with total soybean DNA does not eliminate the possibility that the mitochondrial marker could be hybridizing to nuclear or chloroplastic DNA as well as mitochondrial DNA. However, the four polymorphic patterns originally defined by Grabau *et al.* (1992) were with purified mitochondrial DNA preparations.

The Lincoln cultivar is a maternal ancestor to 'Williams 82', which carries the atypical 2.3 kb polymorphic pattern. It was recorded that maternal 'Mandarin (Illinois)' crossed with paternal 'Manchu' gave rise to 'Lincoln' in 1943. This cross is of particular interest since both parent plants have white flowers but the resultant Lincoln cultivar has purple flowers. This surprising result could be due to a parent with purple flowers, and therefore an incorrect pedigree, or a mutation in the Lincoln cultivar. It is also one of the three crosses that contributes extensively to the northern gene pool (Delannay *et al.* 1983). 'Mandarin' and 'Manchu' exhibit the same RFLP pattern (Bedford-type) with the 2.3 kb fragment used as a probe. The Lincoln cultivar exhibits a different or atypical banding pattern (Lincoln-type) with the 2.3 kb probe. Cultivars with 'Mandarin' as a maternal parent show the Bedford-type classification at 10.5 kb with the 2.3 kb *Hin*dIII probe. Cultivars derived from

'Lincoln' as a maternal ancestor however, show the Lincoln-type classification at 2.3 kb (Grabau *et al.* 1989). Gizlice *et al.* (1994) refer to the parents of 'Lincoln' as unknown ancestors since both morphological and molecular evidence indicate an incorrect pedigree. Another possibility is that a spontaneous recombination event in the Lincoln cultivar to gave rise to the observed 2.3 kb pattern. The precise reason behind the polymorphism seen with the 2.3 kb fragment in the Lincoln cultivar is unknown.

The 2.3 kb probe was used to screen 48 modern cultivars (Grabau *et al.* 1989). In addition to the polymorphic fragments, the 2.3 kb probe was found to have a second region of homology in the mitochondrial genome of all cultivars tested. This is visualized as a common 1.8 kb *Hin*dIII fragment, indicating that a portion of the 2.3 kb fragment is repeated in the mitochondrial genome. The different restriction patterns at 10.5 kb and 2.3 kb points to a possible rearrangement in 'Lincoln' mitochondrial DNA. Continued work with the previously cloned 2.3 kb *Hin*dIII fragment (Grabau *et al.* 1992) identified cytoplasmic variation among 138 cultivars. *Hin*dIII digestions were able to differentiate four patterns. Lincoln-type, Arksoy-type, Bedford-type, and Soja-Forage-type cultivars showed bands at 2.3 kb, 3.8 kb, 10.5 kb (doublet bands), and 6.8 kb, respectively, in addition to the common band at 1.8 kb. The sequence of the 2.3 kb *Hin*dIII fragment has been determined (Hanlon, Li & Grabau manuscript in preparation).

G. soja introductions were analyzed with the 2.3 kb *Hin*dIII polymorphic

marker to see what cytoplasmic RFLP patterns would be observed. It was found that out of 304 *G. soja* introductions, 301 fell into three of the four (Grabau & Davis 1992) previously defined cytoplasmic types in *G. max* (Grabau *et al.* 1992) when probed with the 2.3 kb probe. 82% of the *G. soja* introductions were of the Soja-Forage-type classification (Grabau & Davis 1992). Three *G. soja* plant introductions did not fall into any of the four previously described patterns, suggesting the presence of additional diversity.

Several additional mitochondrial genes have been used as RFLP markers in the search for diversity in *G. max*, including *cox*I, *cox*II, *cox*III, *atp*A, *atp*6, *atp*9, and 18S rRNA (Grabau 1986, 1987, Grabau & Gengenbach 1989, Chanut *et al.* 1993, Grabau *et al.* 1988, 1990, Grabau 1985). The 2.3 kb *Hin*dIII fragment and the soybean *atp6* mitochondrial gene (Grabau *et al.* 1988) showed the greatest level of polymorphism by Southern analysis among these eight markers (Grabau *et al.* 1992).

Mitochondrial Genes & Gene Expression

Mitochondria are energy-producing organelles and must form the necessary enzyme complexes to carry out respiration and ATP synthesis. There are five inner mitochondrial membrane complexes that facilitate oxidation reduction reactions and the phosphorylation of ADP. Complexes I-IV transport electrons from NADH to ubiquinone (NADH dehydrogenase), succinate to ubiquinone (succinate dehydrogenase), ubiquinol to cytochrome *c* (cytochrome *c*

reductase), and cytochrome *c* to oxygen (cytochrome *c* oxidase), respectively. Complex V catalyzes the formation of ATP. Mitochondrial ATP formation is catalyzed by the translocation of 3 protons from the inner mitochondrial space to the matrix. The ATP synthase (ATPase) complex consists of two domains, a hydrophobic membrane spanning domain (F_0) and a hydrophilic domain (F_1) found at the matrix side (Nagley 1988). The membrane spanning F_0 portion of the ATPase acts as the proton channel while the soluble F_1 portion is the catalytic site for ATP formation (John *et al.* 1986, Nagley 1988). The F_1 catalytic domain of the ATPase complex is comprised of five subunits α, β, γ, δ, and ϵ. In plants, the α subunit of the F_1 domain (Chanut *et al.* 1993) is encoded by the mitochondrial genome. The precise number of polypeptides in the F_0 domain is unclear, but at least two subunits (ATP6, ATP9) are mitochondrially encoded (Mackenzie *et al.* 1994).

ATP 9, the proteolipid of the F_0 domain, has significant amino acid homology with the mitochondrially-encoded subunit 9 proteins of maize and yeast, as well as the nuclear-encoded proteins of bovine and *Neurospora crassa* (*Neurospera*) (Dewey *et al.* 1985). The soybean *atp*9 gene has been isolated and sequenced (Grabau *et al.* 1988) and regions of homology to *atp*9 were found elsewhere in the mitochondrial genome based on hybridization analysis. One of those locations is at the 5' end of the *atp*6 coding region (Grabau *et al.* 1988). Genes that contain regions of sequence homology to other genes are referred to as chimeric sequences and could be the result of recombination at repeated

sequences.

Many plant mitochondrial transcripts undergo post-transcriptional RNA editing. This phenomenon was first reported in trypanosome mitochondrial DNA (Benne *et al.* 1986). The mRNA of plant mitochondria differ from the genomic sequence in several plants (Gualberto *et al.* 1989, Covello & Gray 1989, Hiesel *et al.* 1989). Editing occurs most often in protein coding regions and the results frequently increase conservation between proteins across species (Hiesel *et al.* 1989, Gualberto *et al.* 1989, Covello & Gray 1989, Hernould *et al.* 1993). These editing modifications of mRNA occur before translation, thus it is speculated that unedited mRNA may be partially, if not totally nonfunctional (Hernould *et al.* 1993).

Protein coding preRNA sequences in plant mitochondria are modified by a correction mechanism that predominantly results in the conversion of specific cytidine (C) residues to uridine (U) (Bonnard *et al.* 1992). A proposed mechanism involves an editing complex that recognizes a specific sequence and deaminates an upstream C residue (Hodges & Scott 1992). This does not explain the less common reverse of U to C. An in-frame stop codon, UAA, resulting from editing CAA was reported in a mammalian apolipoprotein mRNA (Chen *et al.* 1987). CGG is often edited to UGG resulting in the amino acid Tryptophan being incorporated into the peptide chain instead of an Arginine. Stop codons are often created by RNA editing (Hanson & Folkerts 1992). Guanidine (G) insertions (1 or 2) in a stretch of Gs in the mRNA of

the Sendai virus have been reported as a *cis* viral function (Vidal *et al.* 1990). RNA editing in maize chloroplast DNA was shown to create an initiation codon (Hoch *et al.* 1991).

Editing of plant mitochondrial RNA has been demonstrated in the *atp*9 genes from *Oenothera* (Schuster & Brennicke 1990), wheat (Begu *et al.* 1990), petunia (Wintz & Hanson 1991), *Sorghum bicolor* (sorghum) lines IS1112C and Tx398 (Salazar *et al.* 1991), *Hordeum vulgare* (barley) (Rish & Breiman 1993), and soybean (Pesce & Grabau 1993). An unedited *atp*9 gene from wheat confers male-sterility in transgenic tobacco plants (Hernould *et al.* 1993).

The elucidation of ATPase subunit 9 function in yeast was investigated by mutants which were primarily characterized as nucleotide substitutions (Ooi *et al.* 1985a). Amino acid changes in these yeast mutants focused around a hairpin fold traversing the inner membrane, a putative binding site for oligomycin and venturicidin (Ooi *et al.* 1985a & b). Both antibiotics inhibit proton translocation across the inner mitochondrial membrane causing cell death in sensitive cells.

Oligomycin-resistant mutants have also been isolated the higher plant, *Nicotiana sylvestris* (tobacco) (Durand 1987, Aviv and Galun 1988, Durand *et al.* 1989). Cytoplasmic hybrids (cybrids) between oligomycin resistant tobacco suspension cells and tobacco protoplasts demonstrated a maternal pattern of inheritance (Aviv and Galun 1988). Transfer of antibiotic resistance was later accomplished with intergenically manipulated *Solanum tuberosum* (potato)

protoplasts via cybridization with tobacco (Perl *et al.* 1991). The mechanism of antibiotic resistance in higher plants has not yet been elucidated.

Objectives of Research

The focus of this project was to classify the old domestic collection of soybean cultivars based on genome diversity detected with two mitochondrial RFLP markers, a 2.3 kb *Hin*dIII fragment isolated from 'Williams 82' (a Lincoln-derived cytoplasm), and a cloned portion of the *atp6* gene (Grabau *et al.* 1988). This classification was carried out to search for cytoplasmic groups not previously detected. The 2.3 kb *Hin*dIII marker previously defined four cytoplasmic groups by RFLP patterns as described by Grabau *et al.* (1992). To characterize cytoplasmic diversity and possible origin of a polymorphic region this research project involved 3 objectives.

1) The first objective was to classify all 208 northern and southern cultivars from the collection of old domestic varieties (Bernard *et al.* 1988). Two mitochondrial markers were used to assign these cultivars to a cytoplasmic type. This classification completed a search for additional diversity not previously defined by RFLP analysis.

2) The second objective was to subclone and determine the sequence of the 0.9 kb *Hin*dIII-*Pst*I and 1.7 kb *Pst*I-*Hin*dIII flanking regions. The 2.3 kb *Hin*dIII fragment and flanking regions were first cloned as a 4.9 kb *Pst*I fragment from a 'Williams 82' genomic mitochondrial DNA cosmid library. The 0.9 and 1.7 kb

*Pst*I-*Hin*dIII flanking regions were then subcloned into the pBluescriptII vectors [see appendix] and sequenced with the use of oligonucleotide primers. The entire 4.9 kb *Pst*I fragment, including the 2.3 kb *Hin*dIII fragment was entered into a database to search for sequence homology that could possibly elucidate the function of the conserved polymorphic region.

3) The third objective was to characterize RFLP patterns of the flanking region subclones. The extent of the polymorphism observed with the 2.3 kb *Hin*dIII probe was investigated in each of the cytoplasmic groups. The flanking region subclones were used as hybridization probes with restriction digests of total DNA from each of the four cytoplasmic types in order to construct comparative restriction maps. These maps did not exhibit a clear explanation as to how the polymorphism arose in the Lincoln-type cytoplasm but did define a minimal site of divergence among 4 cytoplasmic types.

A related project involved an oligomycin resistant soybean tissue culture line. The *atp*9 was used to analyze restriction digests of normal and resistant cell lines to search for a possible genome rearrangement in the resistant line that could lead to the resistance phenotype.

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CHAPTER II

Cytoplasmic Diversity in Old Domestic Varieties of Soybean Using Two Mitochondrial Markers

This chapter is currently in press and is in the correct format for Crop Science. Consent of the coauthor (E.A. Grabau) to publish this manuscript as a chapter is appreciated.

Abstract

Modern soybean cultivars are derived from a limited number of plant introductions which restricts germplasm diversity at both the nuclear and cytoplasmic levels. This study was conducted to use molecular techniques to assess the extent of cytoplasmic diversity available to soybean breeders. We have surveyed a collection of old domestic soybean varieties with a cloned 2.3 kb *Hind*III mitochondrial DNA fragment from the cultivar Williams 82 and the mitochondrial ATP synthase subunit 6 (*atp6*) gene in order to determine sources of cytoplasmic diversity present within this collection. This strategy revealed unique mitochondrial DNA (mtDNA) polymorphisms and polymorphism combinations. In several cases the mtDNA polymorphisms provided a different classification than was predicted from recorded pedigrees. Variability was also detected among cultivars assigned the name 'Peking' that were introduced independently into the U.S. soybean collection. The results of this survey coupled with previous studies can be used to define additional cytoplasmic groups in soybean.

Introduction

Soybean cultivars have been derived from a very narrow germplasm base (Delannay *et al.* 1983) which has raised concern over the potential for vulnerability to disease due to genetic uniformity. The lack of diversity within modern cultivars is even more exaggerated at the cytoplasmic level than at the

nuclear level because the inheritance of organelles is believed to be maternal (Birky 1983, Grabau *et al.* 1989, Hatfield *et al.* 1985, Sisson *et al.* 1978). Variation at the cytoplasmic level has been studied using RFLPs from both chloroplast (Shoemaker *et al.* 1986, Close *et al.* 1989) and mitochondrial (Grabau *et al.* 1989, Grabau *et al.* 1992) genomes. Close *et al.* (1989) defined six soybean plastome groups within the subgenus *soja*. Three of the plastome groups contained *G. max* accessions while the other three groups were each characterized by single accession of either *G. gracilis* or *G. soja*. Chloroplast and mitochondrial RFLP markers have also been used to study the fate of cytoplasms in an outcrossing soybean population (Lee *et al.* 1992, Lee *et al.* 1994). Lee *et al.* (1994) utilized the three previously described *G. max* chloroplast groups (Close *et al.* 1989) and four mitochondrial groups (Grabau *et al.* 1992) to survey all parents and several subsequent generations of the outcrossing population. Of 12 possible cytoplasmic groups based on all combinations of available markers, only six groups were identified among the population, two of which were represented by only a single member.

Soybean lines in our laboratory that have previously been examined for cytoplasmic classification using mitochondrial markers were: 1) modern cultivars released in the USA and Canada, 2) a small number of old domestic varieties which represent the major germplasm contribution to modern cultivars, and 3) *G. soja* plant introductions. A 2.3 kb *Hind*III mitochondrial DNA fragment isolated from 'Williams 82' was used as a hybridization probe to identify a

mitochondrial RFLP that distinguished a total of four *G. max* cytoplasmic groups: Bedford, Lincoln, Arksoy, and Soja-Forage (Grabau *et al.* 1992). All modern *G. max* cultivars previously examined belonged to the Lincoln-type or Bedford-type cytoplasmic groups (Grabau *et al.* 1989, Grabau *et al.* 1992). All but three of 304 *G. soja* plant introductions examined were classified within three cytoplasmic groups; the majority of *G. soja* samples belonged to the Soja-Forage cytoplasmic group and none were found in the Lincoln-type cytoplasmic group (Grabau and Davis 1992). Seven additional mitochondrial probes identified mtDNA RFLPs but failed to further differentiate *G. max* cultivars examined (Grabau *et al.* 1992).

In the current study we performed a systematic analysis of a collection of northern and southern old domestic soybean varieties to search for additional sources of cytoplasmic diversity. We screened 209 entries in the collection with the 2.3 kb *Hin*dIII probe and a clone for the mitochondrial ATPase subunit 6 gene that had distinguished three mtDNA RFLP patterns in previous studies.

Materials and Methods

Soybean Cultivars

The seeds used for these studies were obtained from C. Coble and Dr. R. Nelson, USDA-ARS, University of Illinois, and represent the northern and southern collections of old domestic varieties as defined in Table 3 in the Origins and Pedigrees of Public Soybean Varieties in the United States and

Canada (Bernard *et al.* 1988).

DNA Isolation and Southern Analysis

Total DNA was purified from 7-10 day old etiolated seedlings by a modification of a previously published procedure (Dellaporta *et al.* 1983). The modification involves the addition of a phenol:chloroform extraction step prior to DNA precipitation. After digestion with restriction endonuclease *Hind*III, DNA samples were separated by agarose gel electrophoresis and transferred to nylon membranes (Magna, Micron Separations Inc.) by capillary blotting as previously described (Grabau *et al.* 1992). Southern hybridization (Southern 1975) and high stringency washes were performed according to manufacturer's specifications. The 2.3 kb *Hind*III polymorphic clone (Grabau *et al.* 1989) and a 0.9 kb *Hind*III-*Sph*I clone containing part of the *atp6* gene (Grabau *et al.* 1988) were labeled by nick-translation (Rigby *et al.* 1977). Cultivars were assigned to the cytoplasmic types Lincoln, Arksoy, Bedford, or Soja-Forage based on previously described RFLP patterns (Grabau *et al.* 1992).

Results

Classification of USDA collection of old domestic varieties

Two mitochondrial DNA probes were used to search for polymorphic DNA hybridization patterns in a survey of both northern and southern old domestic soybean varieties. A 2.3 kb *Hind*III probe was previously used to define four cytoplasmic groups (Grabau *et al.* 1992) and an *atp6* probe clone

had distinguished three of the four groups in that study. The collection of old domestic varieties (Bernard *et al.* 1988) was screened with both probes and the resulting RFLP patterns served as the basis for classifying the varieties as shown in Table 1. The varieties were placed into the Bedford, Arksoy or Soja-Forage groups differentiated by the 2.3 kb *Hin*dIII probe and any further diversity within groups was distinguished by the *atp6* results. There are only three groups listed in Table 1 because Lincoln was the only variety with an RFLP pattern characteristic of the Lincoln cytoplasmic group. Varieties were omitted from the list if they were previously surveyed (Grabau *et al.* 1992) unless the current study provided additional information. The physical evidence of mtDNA variation allowed the identification of discrepancies between cytoplasm predictions based on recorded pedigrees and direct cytoplasm determinations based on RFLP results. This study also revealed additional cytoplasmic alleles and genetic combinations that further differentiated cytoplasmic groups.

Cytoplasmic classification differing from pedigree predictions or previous reports

A number of samples that had not previously been analyzed ('A.K. [Kansas]', 'Bavender Special A', 'Hahto [Michigan]', 'Manchu', 'Montreal', and 'Manchuria') showed mtDNA RFLP patterns that differed from closely related cultivars. The expected classification for each sample was based on the cytoplasmic group(s) of recorded maternal progenitor or derivative cultivars.

Additional differences found in the survey were the classification of 'Biloxi' in the Arksoy cytoplasmic group rather than in Soja-Forage-type as previously reported (Grabau *et al.* 1992) and the assignment of 'Capital' to the Bedford group, in contrast to placement in the Soja-Forage cytoplasmic group (Lee *et al.* 1994). All samples showing unexpected patterns were retested with both probes after isolation of new DNA preparations. The seeds were obtained directly from packets provided from the USDA collection. All samples again showed the unexpected patterns which eliminated DNA sample handling error as the source of the results.

The 2.3 kb probe revealed variation among samples of the cultivar Peking. Previous classifications had placed 'Peking' in the Arksoy (Grabau *et al.* 1992) and Soja-Forage group (Lee *et al.* 1994). In this study we obtained several different cultivars that are maintained in the USDA collection as 'Peking'. In addition to the standard Peking cultivar maintained in Illinois, we received five plant introductions from the germplasm collection that were also named 'Peking' before introduction into the US. The 2.3 kb fragment was used to probe *Hin*dIII digests of 'Peking' DNA samples. The results are shown in Figure 1A and illustrate that the standard 'Peking' and four of the five 'Peking' plant introductions possess the Soja-Forage pattern similar to the studies of Lee *et al.* (1994). However, the data also indicated that one sample from the USDA Peking cultivars, P.I. 297.543, showed a Bedford cytoplasmic pattern. This differed from the results of Lee *et al.* (1994) and from our earlier results

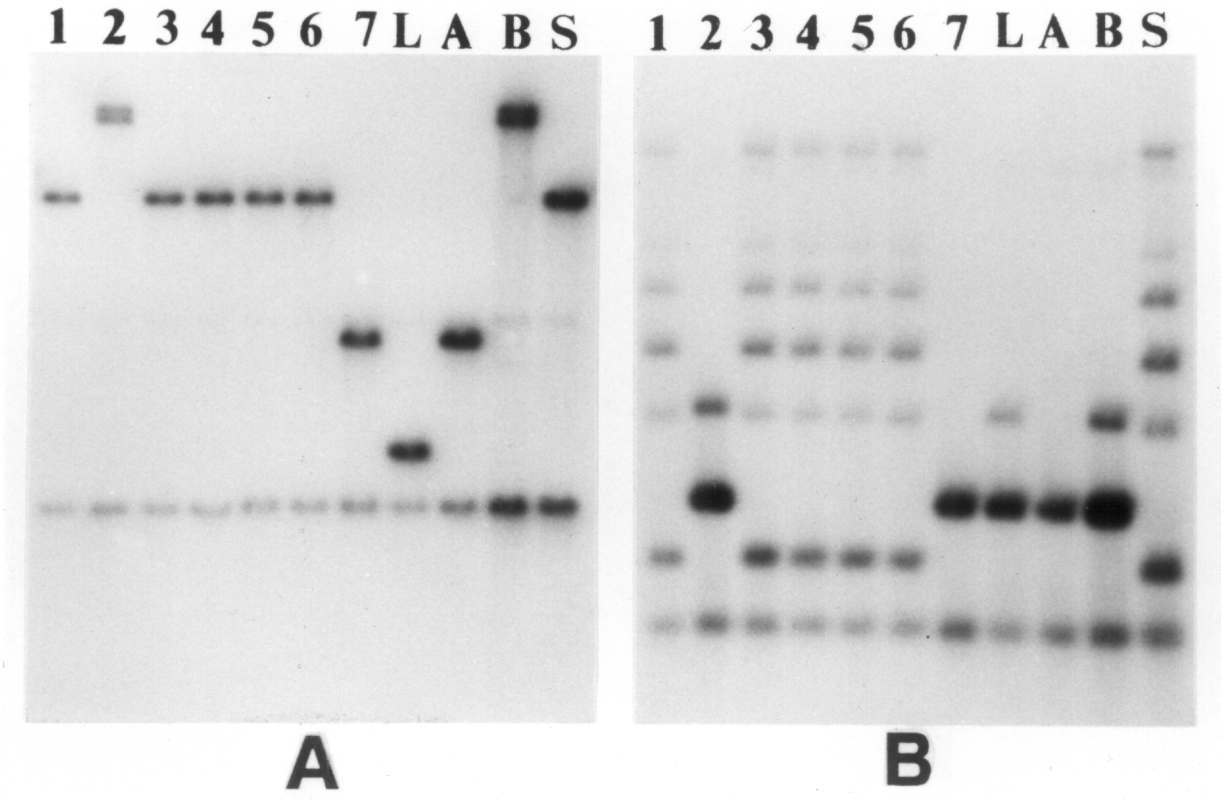

Figure 1

RFLP classification analysis of Peking cultivars with two mitochondrial markers. Southern hybridizations of *Hin*dIII digested 'Peking' DNA with A) the 2.3 kb *Hin*dIII fragment from 'Williams 82' and B) a 2 kb *Bam*HI-*Sph*I fragment of the *atp*6 gene. Duplicate blots are shown in A & B. Lane 1: standard 'Peking (Illinois)' from USDA, lane 2: P.I. 297.543, lane 3: P.I. 438.496A, lane 4: P.I. 438.496B, lane 5: P.I. 438.496C, lane 6: P.I. 438.497, lane 7: Peking from Dr. W.H. Davis of Ring Around Research, lane L: 'Lincoln', lane A: 'Ralsoy', lane B: 'Rose Non-Pop', lane S: 'Yelredo'. Lanes L, A, B, and S show the four cytoplasmic types: Lincoln-type, Arksoy-type, Bedford-type, and Soja-Forage type.

with the Arksoy-type 'Peking' sample obtained from Dr. W.H. Davis (Grabau *et al.* 1992). Figure 1B shows results using the *atp6* probe which confirms the classification with the 2.3 kb probe. For completeness, a standard Peking cultivar that had been maintained separately in the Stoneville, Mississippi collection was analyzed and found to belong to the Soja-Forage group, identical to the standard 'Peking' from the Illinois collection (data not shown). These results illustrate the need for a careful analysis of the exact origin of named cultivars when examining pedigrees.

Additional diversity within *Glycine max*

Five *G. max* cultivars belonging to the Soja-Forage cytoplasmic group according to classification with the 2.3 kb marker showed two novel RFLP patterns using the *atp6* probe. 'Midwest' and 'Gatan' shared one novel pattern while 'Rokusun', 'J.E.W.45', and 'Woods Yellow' also appeared similar to each other as shown in Figure 2. Both patterns differed from the three cytoplasmic patterns described previously for the *atp6* probe (Grabau *et al.* 1992). These new RFLP patterns potentially identify two additional mitochondrial groups. Further work is needed to resolve whether the pattern represented by Midwest and Gatan identifies a novel cytoplasmic group because Midwest was the cultivar previously used by Lee *et al.* (1994) to define one of their six cytoplasmic groups. While additional diversity at the *atp6* has been definitively demonstrated, the Midwest pattern may simply represent another characteristic

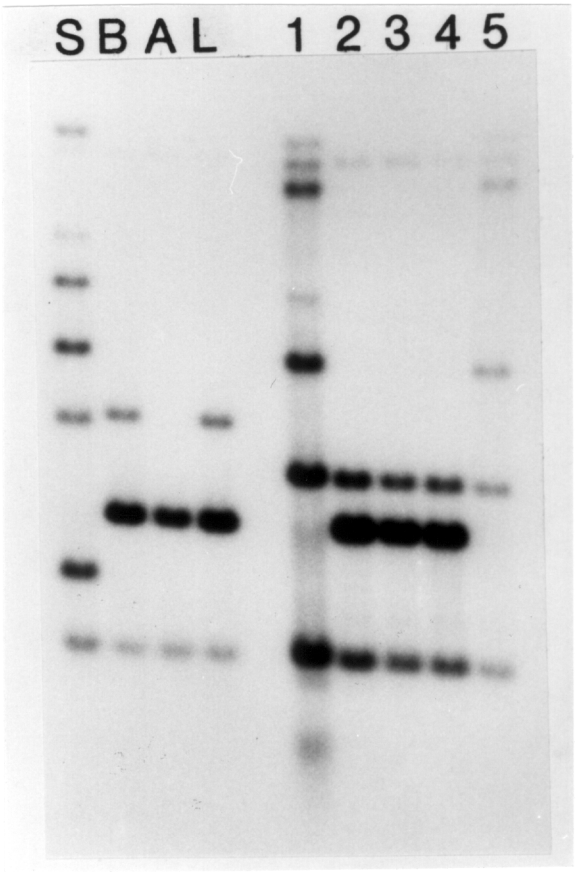

Figure 2

Additional diversity in the 'old domestic collection' detected by RFLP analysis. Total DNA from five cultivars was digested with *Hin*dIII, separated by agarose gel electrophoresis, transferred to a nylon membrane and hybridized with the 2 kb *atp*6 probe. A single northern variety and four southern varieties exhibited novel polymorphisms with the *atp*6 probe but the normal Soja-Forage-type classification with the 2.3 kb probe (data not shown). Lane S: 'Yelredo', lane B: 'Tennessee Non Pop', lane A: 'White Biloxi', lane L: 'Lincoln', lane 1: 'Midwest', lane 2: 'Woods Yellow', lane 3: 'Rokusun', lane 4: 'J.E.W. 45', lane 5: 'Gatan'. Lanes S, B, A, and L show the four cytoplasmic types, Soja-Forage type, Bedford-type, Arksoy-type, and Lincoln-type with the *atp* 6 probe. Lanes 1 and 5 represent a first novel banding pattern with the *atp*6 probe, while lanes 2-4 represent a second novel pattern. These novel banding patterns represent possible rearrangements in the Soja-Forage-type cultivars but the rearrangements are not extended to the region with homology to the 2.3 kb *Hin*dIII probe when the DNA is digested with *Hin*dIII. Such rearrangements could be to homologous recombination at repeated sequences in the genome.

of a previously identified group. The second novel *atp6* pattern appears to differentiate an entirely new cytoplasmic group.

Ten samples in the collection contain new combinations of mtDNA RFLPs found only in Bedford and Arksoy cytoplasmic groups. Two cultivars from the southern collection, 'Hahto' and 'Nanda', showed the Arksoy cytoplasmic pattern with the 2.3 kb probe and the Bedford pattern with the *atp6* probe. Eight cultivars from the northern collection, 'Agate', 'Elton', 'Kura', 'Ogemaw', 'Pagoda', 'Pando', 'Tastee', and 'Sioux', showed the Bedford cytoplasmic pattern with the 2.3 kb probe and the Arksoy pattern with the *atp6* probe. Similar to the case presented above, Hahto was previously used to define one of the six cytoplasmic groups in the study by Lee *et al.* (1994) and therefore may not identify a new group. There are eight cultivars that share the second novel combination of mitochondrial RFLP patterns which provides strong evidence for another soybean cytoplasmic group.

Discussion

The additional diversity observed in the collection of old domestic varieties fits well with the proposed route of soybean maternal ancestry (Lee *et al.* 1994). They proposed a sequence of mitochondrial mutational events which progressed as Soja-Forage→Arksoy→Bedford→Lincoln, superimposed on a sequence of chloroplast mutations through three groups, III→II→I. *G. soja* is the likely progenitor of *G. max* and would be expected to possess the largest degree of polymorphism. The identification of two additional *atp6* loci in cultivars with the Soja-Forage pattern is consistent with this hypothesis.

The ten cultivars with new combinations of RFLPs found in Arksoy and Bedford mitochondrial groups present a more complex and intriguing example. Rearrangements and recombination at repeated sequences are well documented in higher plant mitochondrial DNA (see review by Hanson and Folkerts 1992 and references therein). Soybean mtDNA contains multiple regions of homology to several identified sequences, including genes for three subunits of the ATP synthase complex (Grabau *et al.* 1988, Grabau *et al.* 1990, Chanut *et al.* 1994). Recombination at repeated sequences has been demonstrated for regions associated with the *atpA* and 5S rRNA genes in soybean mtDNA (Chanut *et al.* 1993, Morgens *et al.* 1984). The simplest explanation for cultivars containing novel combinations of the 2.3 kb *Hin*dIII and *atp6* markers would be a mutation that affected both loci (such as a rearrangement) followed by recombination between mutant and non-mutant genomes within the

mitochondrion. Subsequent cytoplasmic sorting of the four mitochondrial genome recombinational products could have fixed the cytoplasmic combinations in the population.

The existence of four combinations of the *atp6* and 2.3 kb *Hin*dIII loci within the collection of old domestic varieties is consistent with both the proposed mode and sequence of the mutations in the maternal ancestry described above. The postulated mutational event could have resulted in the shift from the Arksoy to Bedford mitochondrial pattern in the maternal ancestry scheme. The study by Lee *et al.* (1994) contains a limited population and it would not be expected that all potential recombination products would be observed. This is particularly relevant since the Arksoy cytoplasmic group postulated to have given rise to Bedford was defined by only a single member.

Speculation on the origins of different combinations of the 2.3 kb *Hin*dIII and *atp6* loci within the old domestic collection should predict that some modern cultivars in the Bedford or Lincoln cytoplasmic groups might contain a RFLP pattern at the *atp6* locus typical of an Arksoy cytoplasmic group. Alternately, it is also possible that the nuclear-cytoplasmic interaction resulting from an altered mitochondrial genome might not be favorable, as has previously been suggested for the underrepresentation of the Arksoy cytoplasm in modern domestic cultivars (Grabau *et al.* 1992). The results of soybean breeding would then have selected against the less favorable gene combinations from the modern soybean cultivars. To address possible nuclear-cytoplasmic

interactions, we previously initiated a study to follow the progeny of reciprocal crosses involving two different cytoplasms (Arksoy and Bedford). Preliminary results from those studies should be assessed after the 1994 harvest.

Table 1. Classification of old domestic soybean varieties into cytoplasmic groups. Two mtDNA clones were used as hybridization probes of soybean DNA samples digested by endonuclease *Hind*III in order to determine the RFLP patterns. Soybeans were arranged into three main groups according to the 2.3 kb probe results. Further variation using the *atp6* probe is indicated. Any discrepancies from recorded pedigrees or previous reports are noted.

† The cytoplasmic group assignment for this cultivar was Bedford based on the 2.3 kb probe and Arksoy based on the *atp6* probe.

‡ The cytoplasmic group assignment for this cultivar differed from expected based on recorded pedigrees or previously reported results.

§ The cytoplasmic group assignment for this cultivar was Arksoy based on the 2.3 kb probe and Bedford based on the *atp6* probe.

¶ The *atp6* probe results indicated a new mitochondrial RFLP pattern within the 2.3 kb Soja-Forage cytoplasmic group.

The *atp6* probe results indicated a second new mitochondrial RFLP pattern within the 2.3 kb Soja-Forage cytoplasmic group.

Bedford Group

Agate †
A.K.[Harrow]
Aksarben
Armredo
Austin
Avoyelles
Bansei[Ames]
Bavender Special B
Bavender Special C
Blackeye
Black Eyebrow
Burwell
Capital ‡
Charlee
Cherokee
Chief
Chusei
Delsoy
Earlyana
Early White Eyebrow
Elton †
Emperor
Etum
Flambeau
Fuji
Funk Delicious
Funman
Giant Green
Gibson
Goku
Goldsoy
Granger
Green and Black
Guelph
Hahto [Michigan] ‡
Hakote
Harbinsoy
Harman
Harrel
Hidatsa
Higan
Hokkaido
Hollybrook
Hoosier
Hurrelbrink
Illington
Illini
Imperial
Jefferson
Jogun
Jogun [Ames]
Kabott
Kagon
Kanum
Kura †
Linman 533
Louisiana Green
Luthy
Mamredo
Manchu [Lafayette]
Manchu [Lafayette]B
Manchu [Madison]
Manchu, Hudson
Manchu 3
Manchu 606
Manchu 2204
Manchukota
Manchuria 13177
Manchuria 20173
Mandell
Mansoy
Mendota

Miller 67
Minsoy
Missoy
Monetta
Morse
Nansemond
Nela
Norsoy
OAC 211
Ogden
Ogemaw †
Ontario
Osaya
Otootan
Pagoda †
Pando †
Patterson
Peking (P.I. 297.543) ‡
Pennsoy
Pluto
Pocahontas
Poland Yellow
Richland
Rose Non Pop
S-100
Sato
Scioto
Seneca
Shingto
Shiro
Sioux †
Sousei
Soysota
Tastee †
Tennessee Non Pop
Toku
Tortise Egg
Viking
Volstate
Waseda
Wea
Willomi
Willomi B
Wilson
Wilson B
Wolverine
Yellow Marvel

Arksoy Group

Acadian
Aoda
Arisoy
Bavender Special A ‡
Biloxi ‡
Boone
Cayuga
Chestnut
Clemson
Columbia
Creole
Delsta
Easycock
Georgian
Hahto §
Hongkong
Improved Pelican
Kingston
Magnolia
Mamloxi
Mamotan 6640
Manchuria ‡
Mingo
Nanda §
Palmetto
Peking (from W. Davis) ‡
Ralsoy
Seminole
Tanner
Tarheel Black
White Biloxi
Wing Jet

Soja-Forage Group

A.K.[Kansas] ‡
Arlington
Barchet
Dixie
Gatan ¶
Hayseed
Ilsoy
J.E.W. 45 #
Kingwa
Lexington
Manchu, Montreal ‡
Midwest ¶
Norredo
Old Dominion
Peking (Illinois)
Peking (Stoneville)
Peking (P.I. 438.496A)
Peking (P.I. 438.496B)
Peking (P.I. 438.496C
Peking (P.I. 438.497)
Pine Dell Perfection
Portugal
Rokusun #
Sanga
Sooty
Virginia
Wilson-Five
Wilson-Five B
Wilson-6
Wisconsin Black
Woods Yellow #

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CHAPTER III

Characterization of a 2.3 kb *Hin*dIII Polymorphic Marker from 'Williams 82' Mitochondrial DNA

This chapter is being prepared as a manuscript by R. Hanlon, J. Li and E.A. Grabau. The coauthors, J. Li and E.A. Grabau are responsible for sequencing the 2.3 kb *Hin*dIII fragment. PRC reactions were performed by J. Li.

Abstract

Modern domestic cultivars of soybean are derived from a very narrow germplasm base. Uniform germplasms are undesirable due to the potential for disease susceptibility. Identifying sources of diversity has been a goal of soybean crop improvement efforts. Previously, a 2.3 kb *Hin*dIII mitochondrial DNA fragment was identified that classified soybean cultivars into four cytoplasmic types. In order to further characterize this polymorphic region in the soybean mitochondrial genome, a *Pst*I restriction fragment containing the polymorphic marker and flanking regions was cloned from 'Williams 82' mitochondrial DNA. The sequence of this 4.9 kb *Pst*I fragment was determined and found to contain short regions of sequence homology to other known mitochondrial genes. Comparative restriction mapping of four different soybean cultivars using 2 subclones of the 2.3 kb *Hin*dIII fragment and 2 flanking region subclones identified potential sites of rearrangement among the four cytoplasmic types. The 4.9 kb fragment was found to contain repeated sequences which are hallmarks of higher plant mitochondrial DNA genomes and potential sites for such rearrangements. Due to the absence of any reasonable length reading frames, no obvious protein coding function can be assigned to this region, although hybridization studies indicate that it is highly conserved among soybean cultivars, suggesting an important role in mitochondrial function.

Introduction

Modern soybean cultivars are derived from a limited germplasm base (Delannay *et al.* 1983). Uniformity of soybean cultivars at the cytoplasmic level was first observed by mitochondrial DNA fragments generated by restriction endonuclease digestions and visualized by ethidium bromide staining (Sisson *et al.* 1978). Identification of four cytoplasmic types was the extent of diversity detected. RFLP analysis using chloroplast DNA probes also identified diversity at the cytoplasmic level in the genus *Glycine* (Close *et al.* 1989) which includes both modern cultivars and the likely progenitor, *G. soja.* Because uniformity in germplasm can increase vulnerability in a crop due to susceptibility to a disease or stress, identifying additional sources of diversity is important to crop breeders.

The introduction of desirable characteristics such as crop yield, pest resistance, and stress tolerance are the objectives of crop improvement efforts. Such characteristics could be associated with favorable interactions between nuclear and cytoplasmic genomes. Interactions that did not give favorable results between these two genomes would not be selected in breeding programs. Unfavorable cytoplasmic interactions can be inferred to have occurred when 'Arksoy' was the maternal parent due to the fact that Arksoy-type was a major contributor to breeding programs but pedigree and molecular analysis revealed contributions to be predominately non-cytoplasmic (Grabau *et al.* 1992).

A unique *Hin*dIII mitochondrial DNA fragment of 2.3 kb was observed in the 'Williams 82' cultivar and subsequently isolated and used as a cytoplasmic

marker (Grabau *et al.* 1989). This unique fragment was detected by hybridization with a 10.5 kb band from *Hin*dIII-digested 'Bedford' DNA that is absent in 'Williams 82' (Grabau *et al.* 1989). The 2.3 kb fragment showed the most diversity among cultivars of 8 different mitochondrial clones tested as potential markers (Grabau *et al.* 1992). The 2.3 kb *Hin*dIII marker identified four distinct patterns when used as a probe in Southern analysis of *Hin*dIII-digested soybean mitochondrial DNA. The four cytoplasmic groups are called Lincoln-type, Arksoy-type, Bedford-type, and Soja-Forage-type and the observed hybridization fragments are 2.3 kb, 3.8 kb, 10.5 kb, and 6.8 kb, respectively, in the 4 cytoplasmic groups.

A proposed route of evolutionary lineage is Soja-Forage→Arksoy→Bedford→Lincoln. This putative maternal lineage pattern was suggested following RFLP analysis of an outcrossing soybean population analyzed with a combination of chloroplast and mitochondrial probes (Lee *et al.* 1994). Loss of diversity can be seen with the 2.3 kb cytoplasmic marker by the fact that modern soybean cultivars belong to only two (Lincoln-type and Bedford-type) of the four cytoplasmic groups exhibited by the old domestic collection (Grabau *et al.* 1992). The 2.3 kb fragment identifies three of the four cytoplasmic types (Lincoln-type absent) in *G. soja* (Grabau & Davis 1992).

The 2.3 kb *Hin*dIII fragment appears to be one of very few regions that show variation within the soybean mitochondrial genome. In order to characterize the possible origin, function and organization of this region, we

subcloned a 4.9 kb *Pst*I fragment encompassing the 2.3 kb fragment from a 'Williams 82' mitochondrial DNA cosmid library and determined its sequence. Subclones of the 4.9 kb *Pst*I fragment were used to construct comparative restriction maps from the four cytoplasmic types.

Materials & Methods

Cloning of 2.3 kb flanking regions

A genomic library was constructed from 'Williams 82' mitochondrial DNA with the cosmid vector pHC79 (Grabau 1985). 24 independent cosmid clones were identified containing homology to the 2.3 kb fragment. Four cosmids (A11, C12, D5, and D7) showed identical hybridizing restriction fragments using several different restriction enzymes, indicating that these four clones encompassed the entire 2.3 kb *Hin*dIII fragment.

Cosmid A11 was digested with *Pst*I and the resulting fragments were subcloned into the *Pst*I site of the plasmid vector, pTZ19R. Colonies containing the 4.9 kb *Pst*I insert were identified by colony hybridization with the 1.5 kb *Xba*I-*Hin*dIII subclone of the 2.3 kb clone. Two clones containing 4.9 kb *Pst*I fragments (A8, A50) were digested with *Hin*dIII and *Pst*I. The resulting 0.9 kb and 1.7 kb *Pst*I-*Hin*dIII fragments were isolated from the agarose gel and subcloned into pBlueskriptII KS^- and pBluescriptII SK^- vectors [see appendix]. Digestions, ligations, and electroporations were performed as previously described (see Chapter II) and the plasmid DNA was purified using Plasmid SelectTM-100 Plasmid Purification Columns (5 Prime→3 Prime, Inc.) according to the manufacturer's specifications.

DNA isolation, Southern analysis, and sequencing

Soybean DNA was isolated as previously described (see Chapter II), digested with the restriction endonuclease *Hin*dIII, separated by electrophoresis in 0.75% agarose (1X TAE) gels, and visualized under UV light by ethidium-bromide staining. Transfer and Southern analysis procedures were previously described in Chapter II. Sequencing was performed by the Sanger dideoxy chain termination method (Sanger 1977). Sequencing reactions were performed with a Sequenase kit (U.S.Biochemical) according to manufacturer's directions.

Polymerase chain reactions

Polymerase chain reactions (PCRs) were performed using synthetic oligonucleotide primers. Total genomic DNA was isolated as described in Chapter II. The four genomic DNA samples used as template in the reactions were 'Lincoln' (Lincoln-type), 'Tennessee Non Pop' (Arksoy-type), 'White Biloxi' (Bedford-type), and 'Yelredo' (Soja-Forage-type). All PCR reactions contained 1X PCR buffer [see appendix], 2.5 mM $MgCl_2$, 0.2 mM dATP, dCTP, dGTP, and dTTP, primers at a final concentration of 0.4 μM, 500 ng of template DNA, and 0.25 μl *Taq* polymerase (5u/μl). To ensure proper reaction conditions to denature long strands of genomic DNA, the first cycle of denaturing, annealing, and synthesizing was carried out at 94° C for 5 minutes, 57° C for 5 minutes, 72° C for 2 minutes and 45 seconds. 35 cycles followed with the same conditions except that denaturing and annealing were carried out for 1 minute instead of 5

minutes. All reactions were performed in a Biosycler thermocycler.

DNA sequence analysis

The sequence of the 4.9 kb*Pst*I fragment was entered into the DNAstar analysis program. This sequence was sent to Genbank at the nucleotide level to search for homology to conserved sequences or homology to reported protein coding regions (Altschul *et al.* 1990). The "blast" search of the nucleotide sequence of the 4.9 kb *Pst*I fragment from 'Williams 82' yielded a 98% match to a 609 bp *Eco*RI-*Hin*dIII soybean mitochondrial DNA fragment (607 of 609 bp) that contains transcription initiation sites for several small RNAs of unknown function (Brown *et al.* 1991). This 609 base pair fragment is a subclone of a larger 2.8 kb *Eco*RI mitochondrial DNA fragment (designated pE2.8) from the cultivar Extra Early (Brown *et al.* 1991).

Results

In order to investigate a potential function associated with the polymorphic region identified by the 2.3 kb *Hin*dIII fragment, the nucleotide sequence was determined for a 4.9 kb *Pst*I fragment, which included the 2.3 kb region. The presence of a protein coding region could help explain the preservation of the 2.3 kb region through rearrangement events among soybean. A restriction map of this region was generated for 'Williams 82' DNA by restriction endonuclease digestions of cosmid A11 from the genomic mitochondrial DNA library. The 4.9 kb *Pst*I fragment of interest is shown in Figure 3. Location of restriction sites were deduced from Southern hybridization with the 0.8 kb and 1.5 kb subclones of the 2.3 kb *Hin*dIII fragment as probes.

The sequence of the entire 4.9 kb *Pst*I fragment is shown in Figure 4 and will be submitted to Genbank for a sequence accession number. Oligonucleotide primers used in sequencing and PRC reactions are indicated by arrows directly below the sequence. Figure 5 shows a list of restriction endonucleases with specific 6 base pair recognition sites in the 4.9 kb sequence. As shown in Figure 6 a total of nine ORFs with an ATG start were found in the 4.9 kb sequence, five extend 5'-3' from the *Pst*I site at the 1.7 kb end, and four extending 5'-3' from the *Pst*I site at the 0.9 kb end of the fragment. The longest ORF spanned 319 nucleotides.

One possible source of the 2% difference between the 4.9 kb *Pst*I sequence from 'Williams 82' and the pE2.8 sequence from the 'Extra Early' (Brown *et al.*

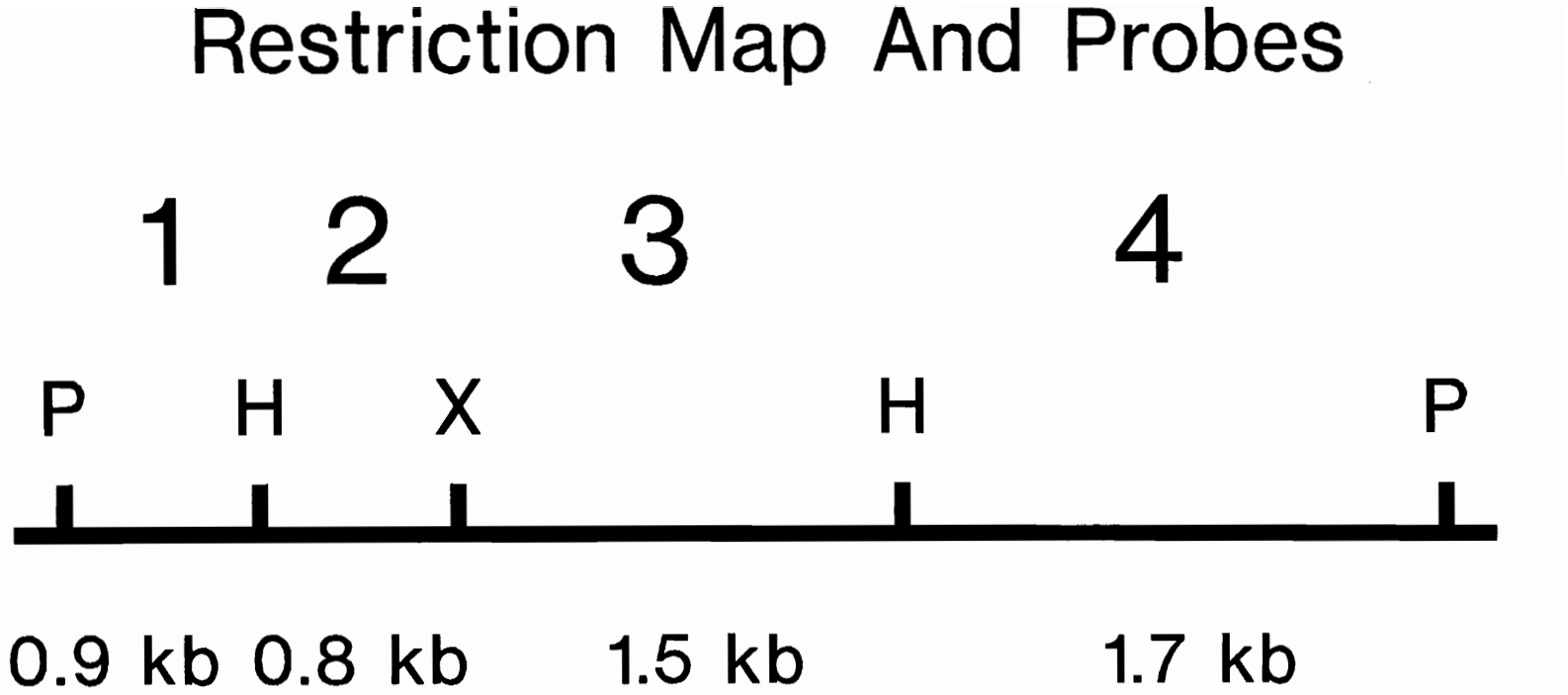
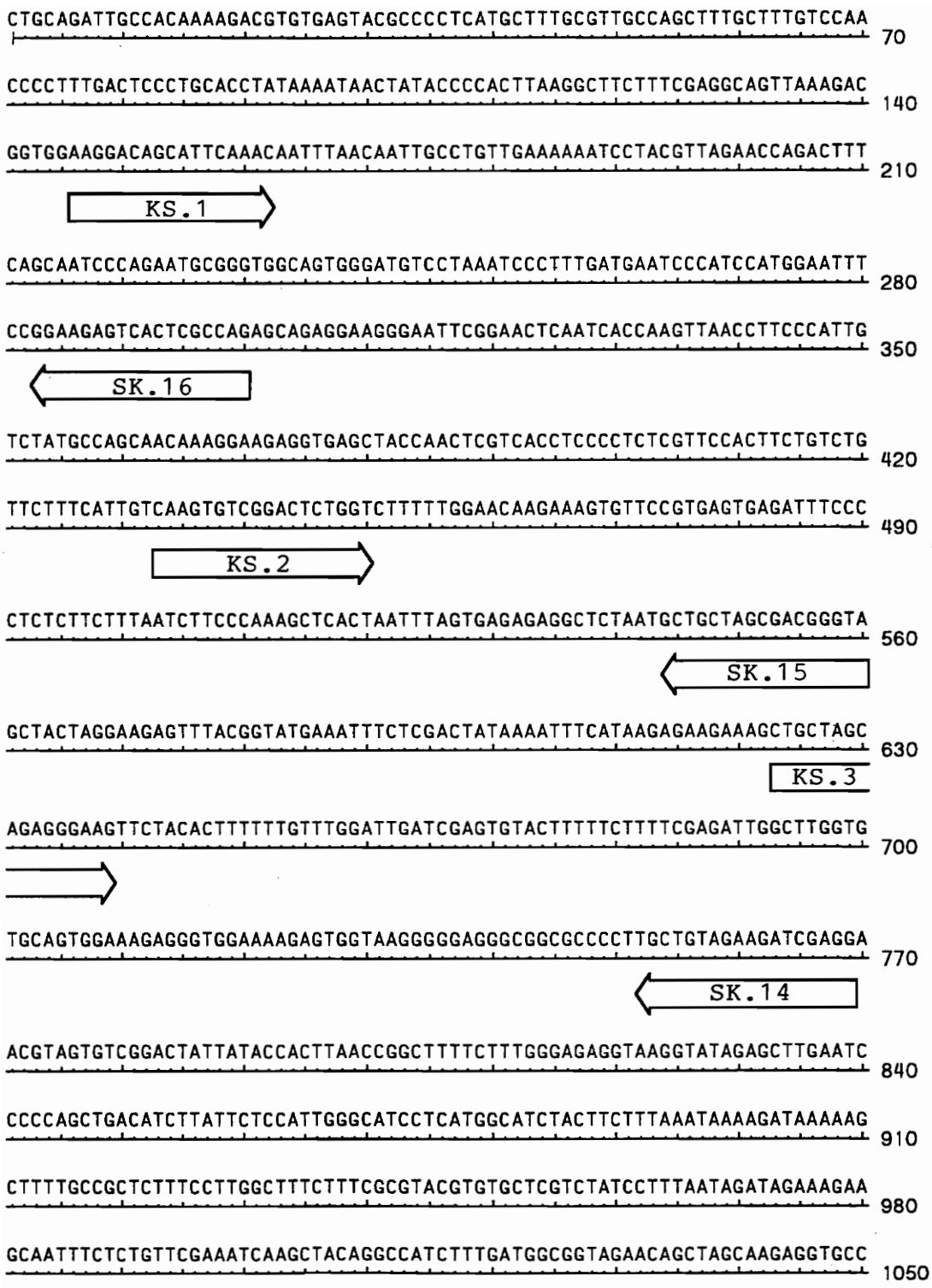


Figure 3

A restriction map of the region containing the 2.3 kb polymorphic *Hin*dIII fragment. A cosmid clone from a 'Williams 82' mitochondrial DNA library (A11) was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Pst*I alone and also with *Xba*I as double digests. Numbers 1-4 represent the four fragments within the 4.9 kb *Pst*I clone that have been subcloned and used as probes: a 0.9 kb *Pst*I-*Hin*dIII fragment, a 0.8 kb *Hin*dIII-*Xba*I fragment, a 1.5 kb *Xba*-*Hin*dIII fragment, and a 1.7 kb *Hin*dIII-*Pst*I fragment.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140
GGTGGAAGGACAGCATTCAAACAATTTAACAATTGCCTGTTGAAAAAATCCTACGTTAGAACCAGACTTT
210
KS.1
CAGCAATCCCAGAATGCGGGTGGCAGTGGGATGTCCTAAATCCCTTTGATGAATCCCATCCATGGAATTT
280
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350
SK.16
TCTATGCCAGCAACAAAGGAAGAGGTGAGCTACCAACTCGTCACCTCCCCTCTCGTTCCACTTCTGTCTG
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490
KS.2
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SK.15
GCTACTAGGAAGAGTTTACGGTATGAAATTTCTCGACTATAAAATTTCATAAGAGAAGAAAGCTGCTAGC
630
KS.3
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700
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770
SK.14
ACGTAGTGTCGGACTATTATACCACTTAACCGGCTTTTCTTTGGGAGAGGTAAGGTATAGAGCTTGAATC
840
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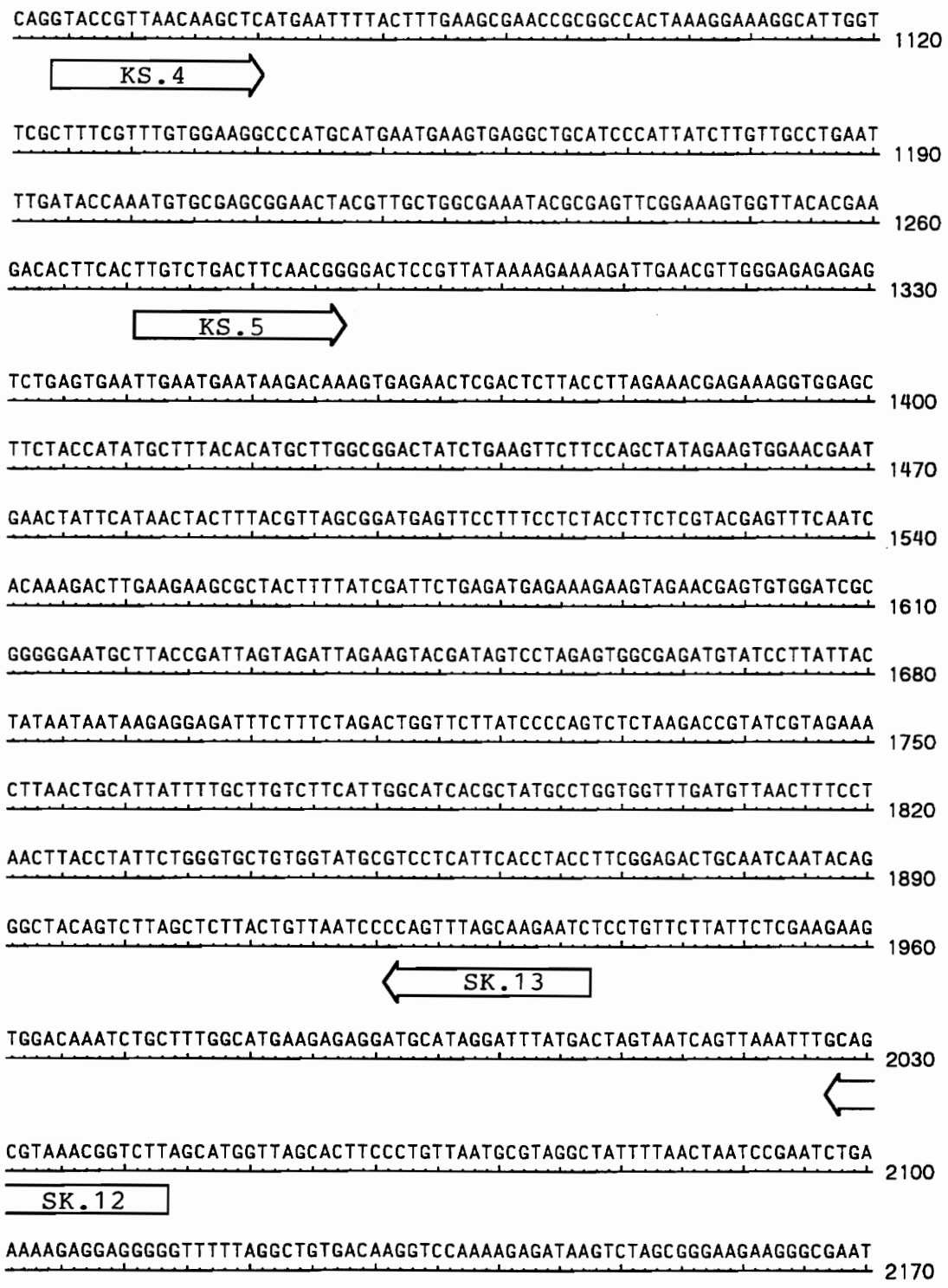
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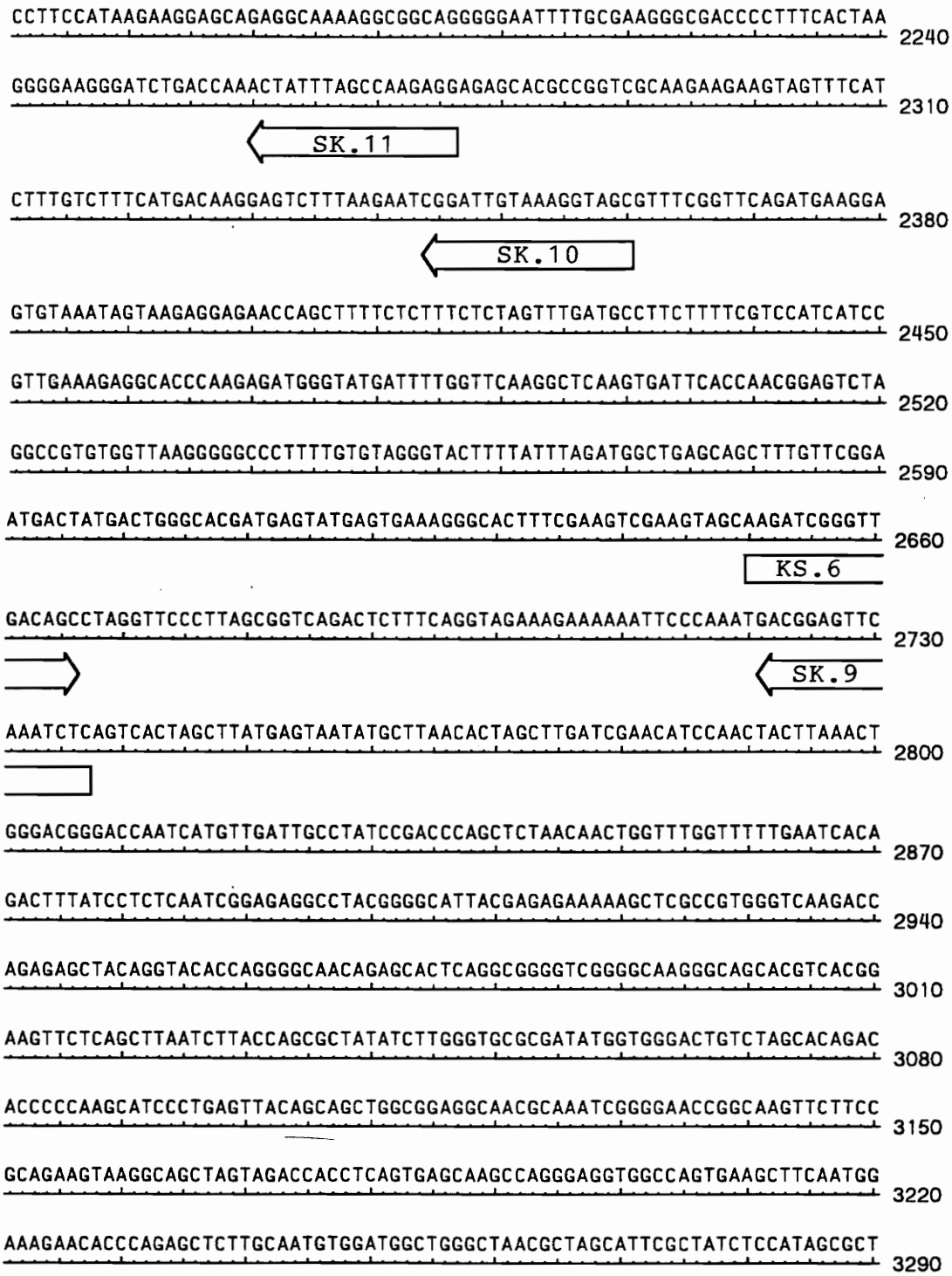
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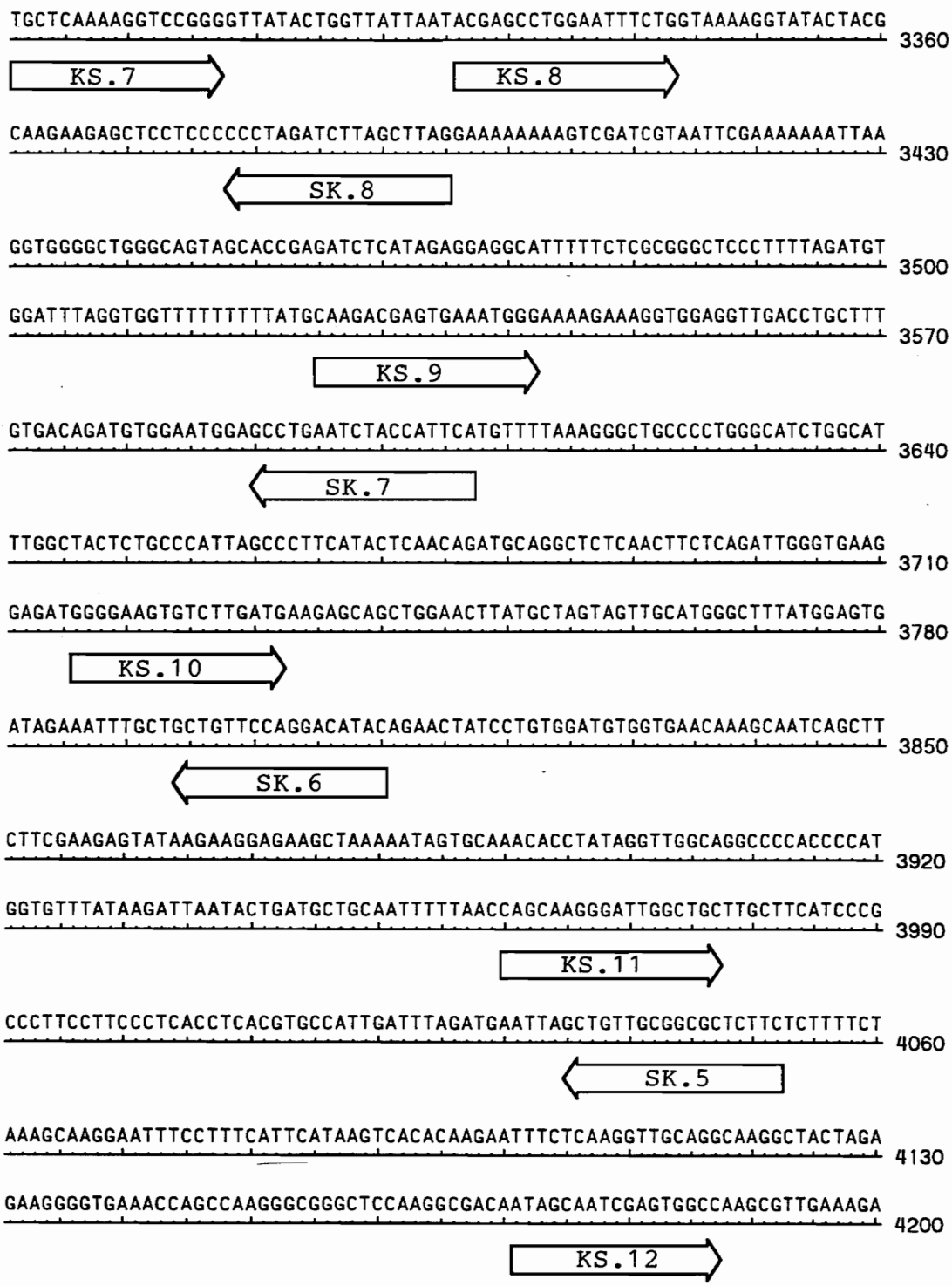
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3640
SK.7
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3780
KS.10
ATAGAAATTTGCTGCTGTTCCAGGACATACAGAACTATCCTGTGGATGTGGTGAACAAAGCAATCAGCTT
3850
SK.6
CTTCGAAGAGTATAAGAAGGAGAAGCTAAAAATAGTGCAAACACCTATAGGTTGGCAGGCCCCACCCCAT
3920
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3990
KS.11
CCCTTCCTTCCCTCACCTCACGTGCCATTGATTTAGATGAATTAGCTGTTGCGGCGCTCTTCTCTTTTCT
4060
SK.5
AAAGCAAGGAATTTCCTTTCATTCATAAGTCACACAAGAATTTCTCAAGGTTGCAGGCAAGGCTACTAGA
4130
GAAGGGGTGAAACCAGCCAAGGGCGGGCTCCAAGGCGACAATAGCAATCGAGTGGCCAAGCGTTGAAAGA
4200
KS.12

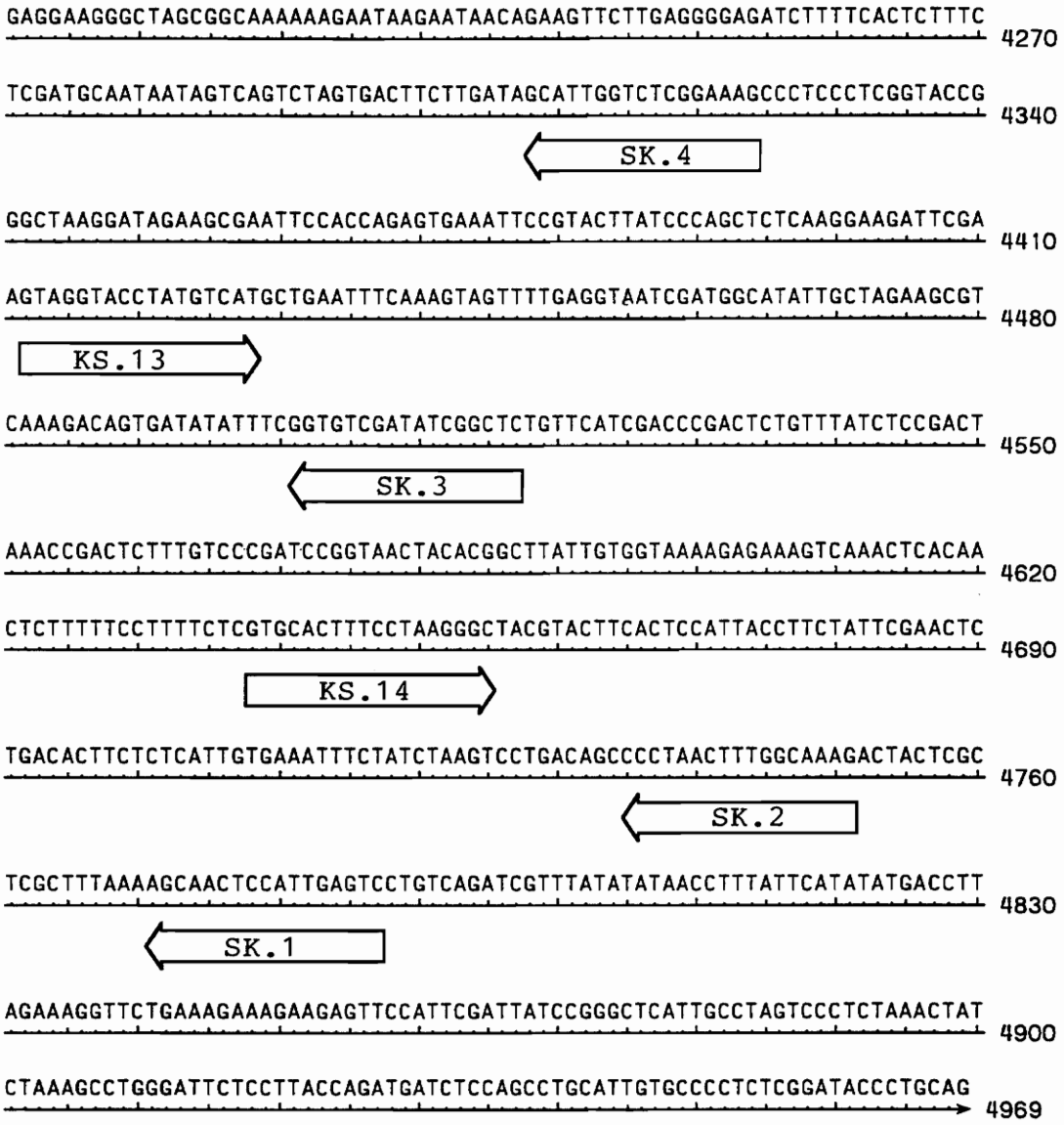


Figure 4

DNA sequence of 4.9 kb *Pst*I fragment from 'Williams 82' DNA. The 2.3 kb sequence was previously completed by deletion cloning and the 0.9 kb and 1.7 kb *Hin*dIII-*Pst*I sequences were completed with the use of oligonucleotide primers. This 4.9 kb sequence was entered into DNAStar, a DNA analysis program, and will be submitted to Genbank for an accession number. Oligonucleotide primers used in sequencing and PCR experiments are indicated by arrowed boxes below the sequence. Primers are sequentially identified as KS.1 through KS.14 in one direction and SK.1 through SK.16 in the other. A list of primer sequences is given in the appendix.

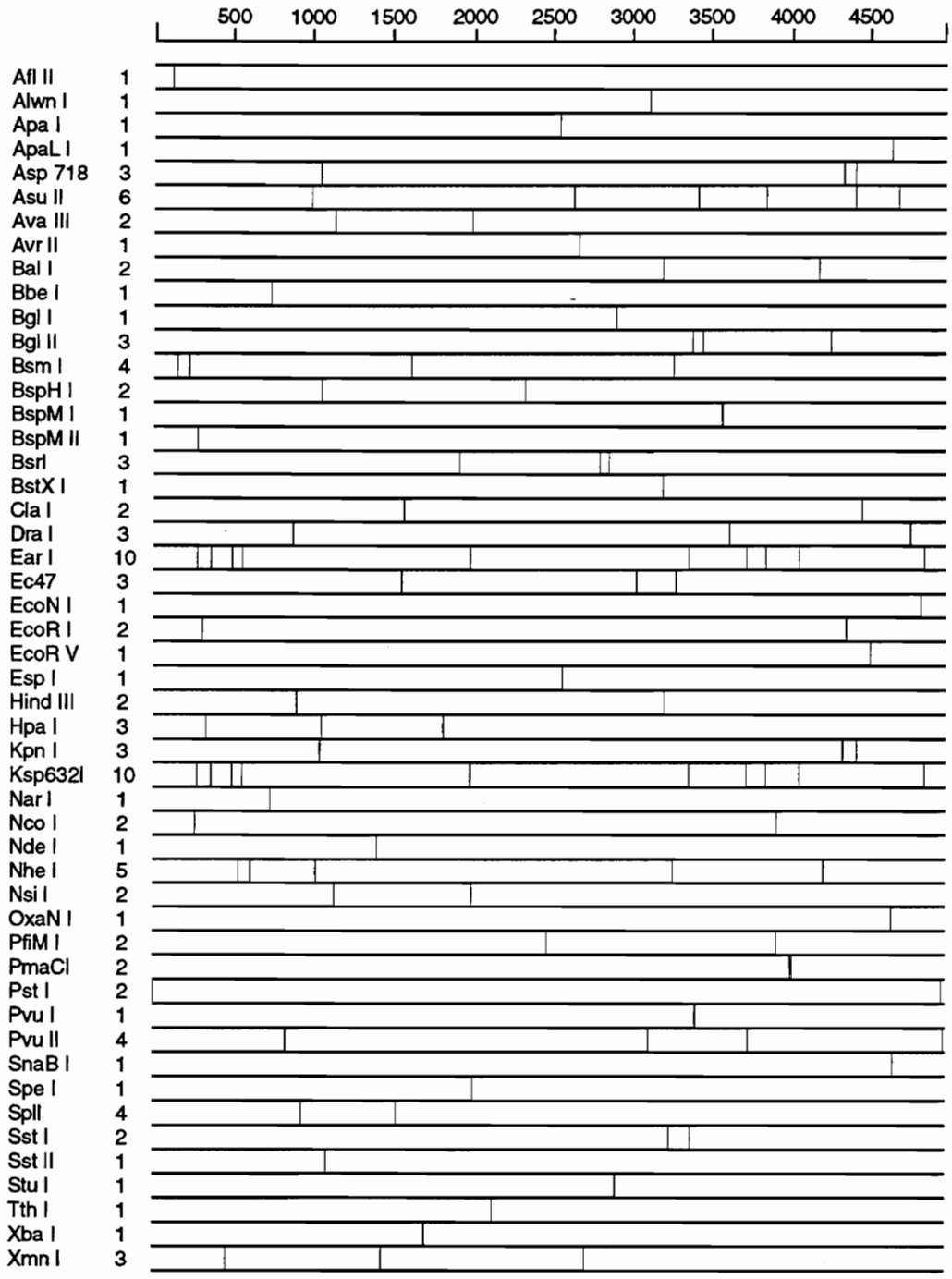


Figure 5

Location of six base pair restriction endonuclease recognition sites in the sequence of the 4.9 kb *Pst*I fragment as defined in the DNA analysis program, DNAstar.

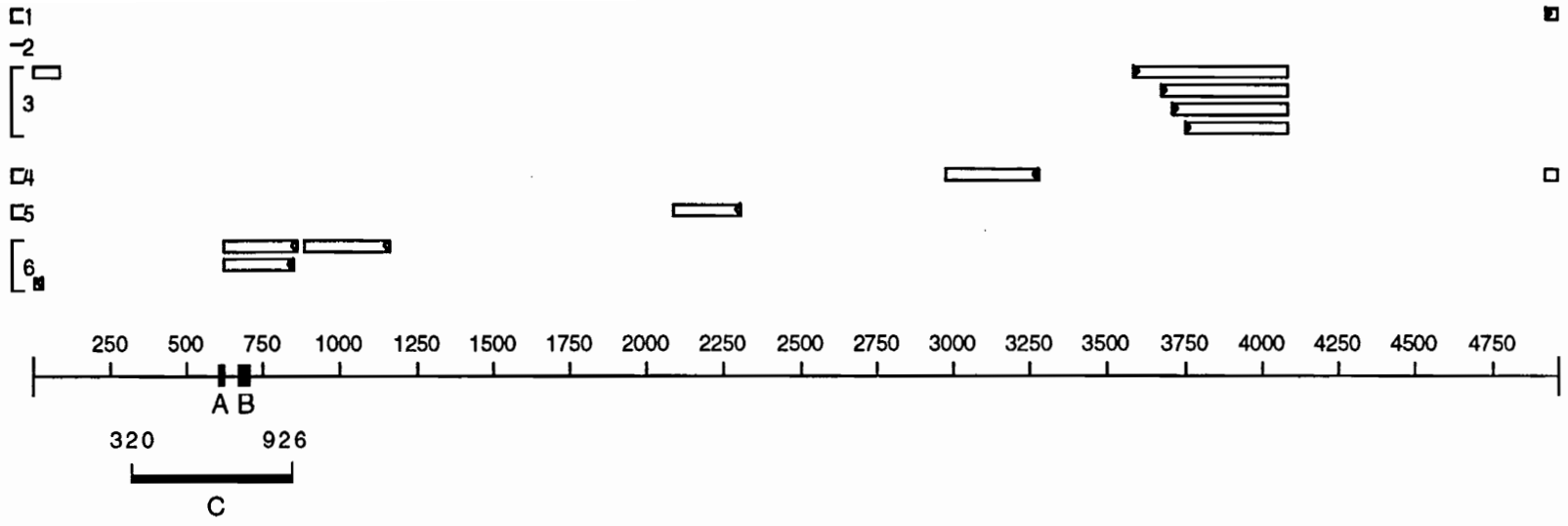


Figure 6

A map of putative open reading frames (ORFs) beginning with ATG within the 4.9 kb sequence as defined by the DNA analysis program, DNAstar. The visible number line of 250 to 4750 represents the 4.9 kb sequence. Numbers 1-6 on the left side represent the six possible protein coding reading frames (RFs) of the *Pst*I fragment. A minimum of 75 nucleotides was the criteria of the ORFs shown as boxes with an enclosed arrow indicating direction. RFs 1 and 2 do not show ORFs in the 75 nucleotide range. A total of nine of the specified ORFs were found in the sequence of the 4.9 kb *Pst*I fragment, five extending 5'-3' to the left and four extending 5'-3' to the right and ranging in size from 212-319 nucleotides. In RF 3, four ORFs are present, one ORF is present in RFs 4 and 5, and three RFs are present in RF 6. Nucleotides 602-625 are indicated by the solid box A and represent the area of homology to other plant mitochondrial transcription initiation sites (Figure 7). Nucleotides 674-709 are indicated as the solid box B and represent the area of homology to "*chi*-like" sequences in other plants (Figure 8). Nucleotides 320-929, the region of homology to the 609 bp *Eco*RI-*Hin*dIII fragment from 'Extra Early' are represented by bar C.

1991) could be a difference in the cultivar used for DNA isolation. The match extends from nucleotides 320 to 928 of the 4.9 kb fragment, which is almost entirely within the 0.913 kb *Pst*I-*Hin*dIII subclone. The 2.8 kb size of the *Eco*RI fragment suggests that 'Extra Early' would be classified in the Bedford or Soja-Forage cytoplasm based on Southern analysis and restriction mapping (see Figures 7 & 8). Another possibility is a sequencing error in the previously published sequence.

The report by Brown *et al.* (1991) demonstrated the presence of three short RNAs that are transcribed from the 609 base pair *Eco*RI-*Hin*dIII fragment. The transcripts can be capped at their 5' ends by the enzyme guanylyltransferase which indicates that the transcripts are the result of transcription initiation rather than RNA processing events. Transcripts are 133, 148, and 80 nucleotides in length and represent an overlapping set of small RNAs with two different 5' and 3' ends (see diagram below).

5'_____ 3'	RNA b	133 nt
5'_____ 3'	RNA c	148 nt
5'_____ 3'	RNA e	80 nt

The sequence directly adjacent to the soybean transcription initiation sites (nucleotides 602 to 625 of the 4.9 kb *Pst*I fragment) contains homology to several other known mitochondrial genes. An alignment of sequences is shown in Figure

Origin	Sequence	Position	% Match
4.9 kb:	AAATTTCATAAGAGAAGAAAGCTG	602-625	___
pE2.8:	AAATTTCATAAGAGAAGAAAGCTG	282-305	100%
Oenothera tRNA^{Phe}:	TAATTTCATAAGAGAGAGAAGTGC	21-44	91.6%
petunia atp9-1:	AAATTTCATAAGATAAGAGAGATG	319-342	87.5%
soybean atp9:	AAATAGCGTAAGAGAAGAAAGTAG	197-220	75%
petunia rps19:	AAATTTCATAAGAGAAGAAGAATC	991-968*	91.6%

* reverse orientation from published sequence (complementary strand)

Figure 7

Sequence alignment of nucleotides 602-625 from the 4.9 kb *Pst*I fragment from 'Williams 82' mitochondrial DNA with 5 homologous sequences found in a nucleotide level search in the Genbank data base (Altschul *et al.* 1990). Origin, sequence, nucleotide position and percentage homology are shown. Nucleotides that differ from the 4.9 kb*Pst*I fragment are shown in bold double underlines. Arrows above the nucleotides indicate transcription initiation sites.

Origin Match	Sequence	Position	%
4.9 *Pst*I fragment	CTTTTTCTTTTCGAGATTGGCTTGGTGTGCAGTGGA	674-709	___
soybean pE2.8	CTTTTTCTTTTCGAGATTGGCTTGGTGTGCAGTGGA	355-390	100%
fmet tRNA/*cox*II	CTTTTT**TC**TT-CGAGATTGGCTTGGT**C**T**T**CAGTGTG	743-777	86.1%
petunia *atp*9-1	C-TTT**AT**TTTTCGAGATTGG**G**TTGGTGT**T**CAGTG**T**A	398-432	85.5%
petunia *atp*9-2	CTTTTT**TGCG**TCGAGATTGG**G**TTGGTGT**T**CAGTG**T**A	412-447	80.6%
*Oenothera atp*9	CTTTTT**T**T**C**TTCGAGATTGGCTTGGTGT**T**CA**A**TG**TG**	178-213	85.5%
Oenothera 26S rRNA	**A**TCTTTCTT**G**TCGAGATTGGCTTGGTGT**T**CAGTG**T**A	109-144	88.9%
petunia *rps*19	CTTTTT**TGAA**TCGAGATTGGCTTGGT**C**T**T**CAGT**CT**A	880-845*	75.0%

* reverse orientation from published sequence (complementary strand)

Figure 8

Sequence alignment of nucleotides 674-709 from the 4.9 kb *Pst*I fragment from 'Williams 82' mitochondrial DNA with 7 sequences from a nucleotide level search in the Genbank data base. Origin, sequence, nucleotide position and percentage homology are shown. Nucleotides which differ from the 4.9 kb *Pst*I fragment are indicated in bold and double underlined. The *chi* sequence from *E. coli* is GCTGGTGG and is known to promote recombonation in the *E. coli* genome. "*Chi*-like" sequences in each of the plant sequences contain two additional Ts within the sequence and are indicated by a bar above the sequence.

7 with mismatches indicated in bold with double underlines. Where known, transcription initiation sites are designated with arrows. Similar alignments of transcriptional initiation sites have previously been presented by Brown *et al.* (1991) and Binder & Brennicke (1993). The alignment by Brown *et al.* (1991) includes two regions of homology to the sequences upstream of the pea *cox*II, as well as consensus transcription sites from maize and wheat. No homology was detected by the current Blast search to the pea *cox*II, soybean *atp*9 or wheat and maize initiation consensus sequences. The alignment by Binder & Brennicke (1993) included the *Oenothera* consensus promoter sequence in addition to the $tRNA^{Phe}$ transcriptional start. The homology to the petunia *atp*9 promoter and *Oenothera* $tRNA^{Phe}$ transcriptional start site were detected in our search.

A second short sequence within the 4.9 kb *Pst*I fragment (nucleotides 674 to 709) was identified that has significant homology to several other known mitochondrial sequences. The homologous regions include: 1) a sequence upstream of the soybean *f*met tRNA and adjacent non-functional copy of the *cox*II gene (Grabau 1987). The functional copy is encoded by the nuclear genome in soybean (Nugent & Palmer 1991). 2 & 3) Sequence upstream of two copies of the petunia *atp*9 genes (Rothenberg & Hanson 1987, & Young *et al.* 1986) that differs from the region in the *atp*9 sequence alignment above. This region includes a site where the flanking regions of the two petunia *atp*9 genes diverge; (the second *atp*9 copy was not found by our Genbank search,) 4) a sequence upstream of the *Oenothera atp*9 gene (Schuster & Brennicke 1989), 5) a sequence

upstream of the *Oenothera* 26S rRNA (Manna & Brennicke 1985), and 6) a petunia sequence located within a recombinational repeat, upstream of the *rps*19 gene (Sutton *et al.* 1993). An alignment of the regions is shown below with mismatches shown in bold and double underline. Brown *et al.* (1991) previously reported the homology in this region to the soybean *cox*II, *Oenothera* 26 rRNA, and petunia *atp*9 sequence. Conklin & Hanson (1993) reported homology of the petunia recombinational repeat to the petunia *atp*9 gene, the *Oenothera* 26S rRNA gene and a region from a 5.7 kb maize repeat sequence. Homology to the maize repeat sequence was not detected.

The two regions within the petunia recombinational repeat that show homology to the 4.9 kb *Pst*I soybean sequence (in reverse orientation) are located from 845-880 and 968-991 (separated by 87 nucleotides). In soybean the two regions of homology are located from 602-625 and 674-709 (separated by 48 nucleotides).

Conklin & Hanson (1993) refer to the possibility of "*chi*-like" sequences in mitochondrial DNA that may promote recombination. The *E. coli* sequence (5'GCTGGTGG3') enhances the recombination frequency (Ponticelli *et al.* 1985). The region boxed in the second short sequence alignment is similar to *chi* (5'GCTTGGTGTG3') with the exception of additional Ts shown here in bold-double underlines. The *rsp*19 sequence present within the major petunia recombination sequence is present at least 6 times in the petunia mitochondrial DNA (Conklin & Hanson 1993). Short repeated sequences in plant

mitochondrial genomes have been shown to be involved in recombination events in several plant species (review by Andre *et al.* 1992). These repeats can be as small as 6 base pairs in length and are often associated with new mutations or mitochondrial genome rearrangements that differentiate close relatives (Andre *et al.* 1992).

In an effort to determine putative rearrangement sites in soybean mitochondrial genomes, DNA from cultivars representing the four cytoplasmic types, as defined by the 2.3 kb *Hin*dIII polymorphic marker, were restriction digested and analyzed by Southern analysis. Genomic DNA of representative cultivars of these four cytoplasmic types were digested with the restriction endonucleases *Pst*I, *Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I and hybridized with four cloned mitochondrial probes to generate the results shown in Figure 9. 'Lincoln', 'Mingo', 'Willomi B', and 'Norredo' represent Lincoln-type, Arksoy-type, Bedford-type, and Soja-Forage-type, respectively are represented as repeated lanes #1-4 for each restriction enzyme. The four panels represent the same set of DNAs each probed with a different clone as shown in panels A-D (Figure 9).

Probing with the 0.9 kb flanking region shows the presence of a 4.9 kb *Pst*I fragment in Lincoln-type DNA (*Pst*I lane 1) that also hybridizes with the other 3 probes as seen in panels B-D in Figure 9. The fragment size agrees with the predicted size (4.9 kb) based on the 'Williams 82' restriction map in Figure 3. In *Pst*I lanes 2, 3, and 4, fragments of 6.4 kb, 7.6 kb, and 8.1 kb hybridize to probes 0.9 kb, 0.8 kb and 1.5 kb. With the 0.8 kb probe, only one fragment per lane

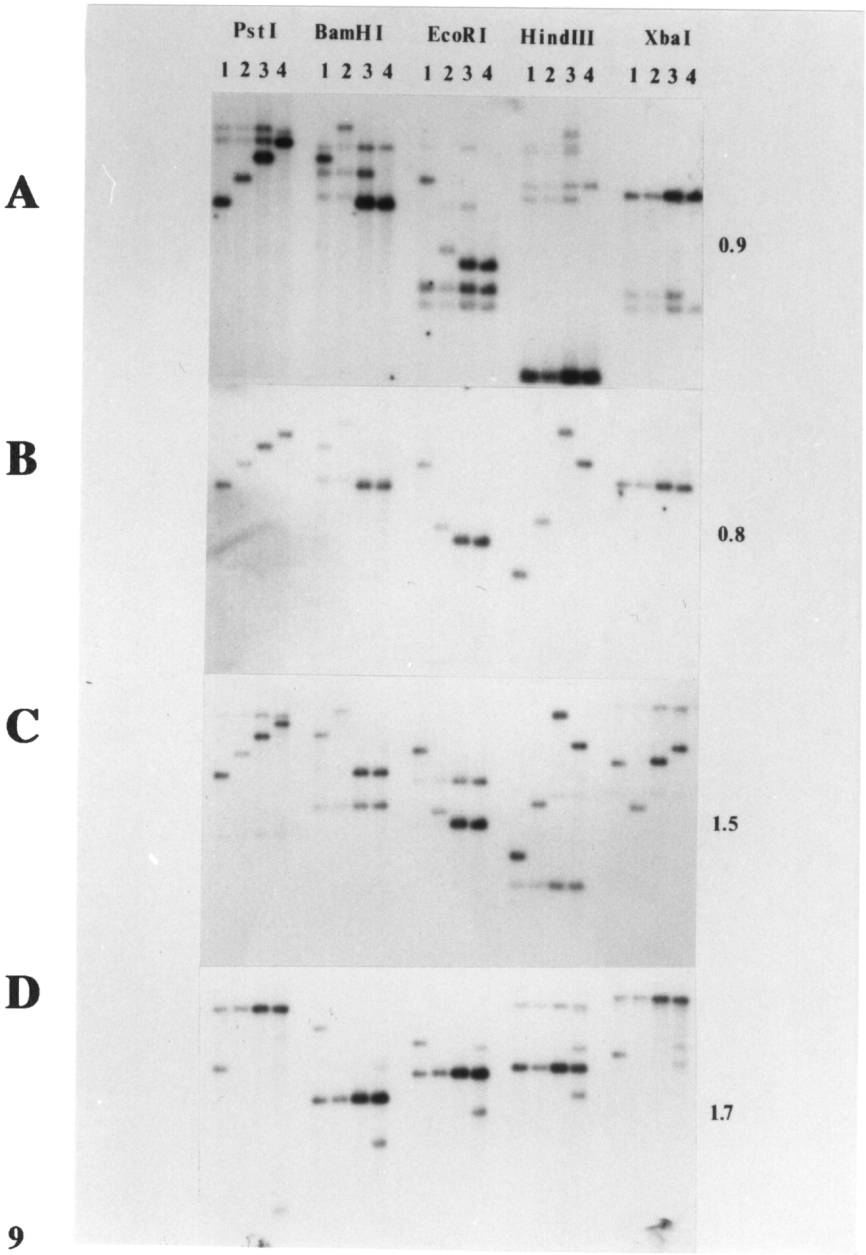


Figure 9

Southern analysis of four cytoplasmic types with cloned mitochondrial DNA probes. Total DNAs from old domestic cultivars representing the four cytoplasmic types were digested with the restriction endonucleases *Pst*I, *Bam*HI, *Eco*RI, *Hin*dIII, and *Xba*I. Lanes 1-4 in each digest represent 'Lincoln' (Lincoln-type), 'Mingo' (Arksoy-type), 'Willomi B' (Bedford-type), and 'Norredo' (Soja-Forage-type) DNA. Duplicate agarose gels were run to separate the DNA fragments. The 0.9 kb *Pst*I-*Hin*dIII fragment (panel A), the 0.8 kb *Hin*dIII-*Xba*I fragment (panel B), the 1.5 kb *Xba-Hin*dIII fragment (panel C), and the 1.7 kb *Hin*dIII-*Pst*I fragment (panel D) were used as probes in Southern analysis.

shows hybridization to the probe. Probe 0.9 clearly showed the presence of repeated sequences within the soybean mitochondrial genomes. With the 1.5 and 1.7 probes, additional repeated fragments in common to all 4 cultivar types are also observed. These common fragments indicate additional regions of homology to the probes within the mitochondrial genome. The polymorphic *Pst*I pattern is not observed using the 1.7 kb probe. Instead, a large fragment indicating a region of homology in common to all 4 cytoplasmic types is present.

In the *Hin*dIII digests, probes 0.8 kb and 1.5 kb show a polymorphic pattern similar to *Pst*I results. With the 0.8 kb probe, only one unique fragment hybridizes for each cultivar. Probe 0.8 kb hybridizes to a 2.3 kb fragment in lane 1 as expected by the 'Williams 82' map in Figure 3. In Arksoy-type, Bedford-type, and Soja-Forage-type cytoplasms, 3.8 kb, 10.5 kb (doublet), and 6.8 kb fragments, respectively, show hybridization to the 0.8 kb probe as seen previously (Grabau *et al.* 1992). Additional common bands can be seen in each lane in panels A, C, and D.

Somewhat different results were obtained for the *Xba*I digests. The 0.8 kb probe hybridized to a 5.4 kb band in all 4 cultivars. This 5.4 kb band was also observed with the 0.9 kb probe. This indicates that the region to the left of the *Xba*I site is identical in all 4 cytoplasmic types. With the 1.5 kb and 1.7 kb probes, an 11 kb band is seen in all cultivars. The Lincoln-type, Bedford-type, and Arksoy-type cytoplasms show a second band of varying size that hybridizes with the 1.5 kb probe. This is consistent with the polymorphic region beginning

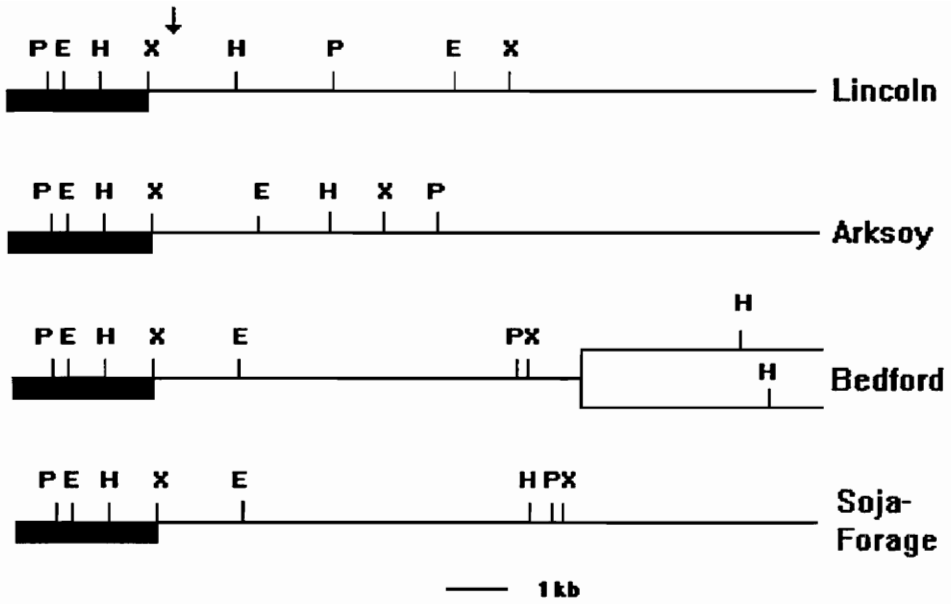


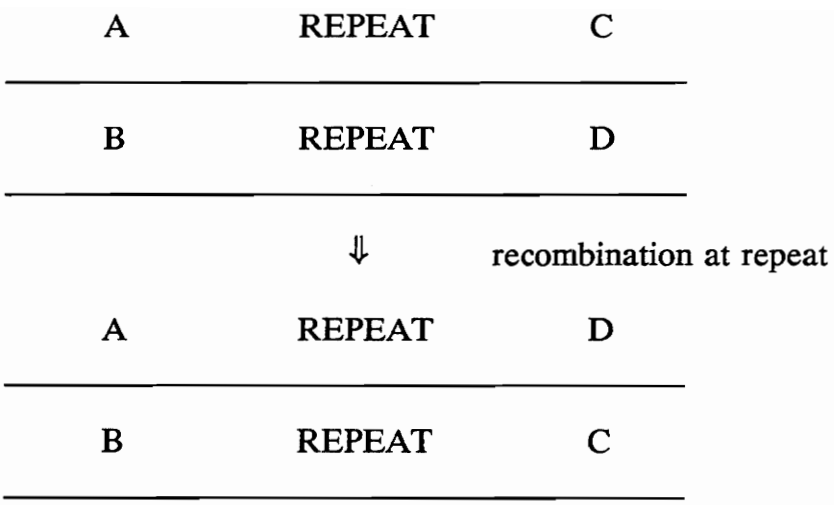
Figure 10

Restriction maps of the polymorphic region in 4 cytoplasmic types. The four DNAs used were 'Lincoln' (Lincoln-type), 'Mingo' (Arksoy-type), 'Willomi B' (Bedford-type), and 'Norredo' (Soja-Forage-type). Common regions to all cytoplasms are indicated by a solid bar. Approximate location of the site of divergence is indicated by the arrow. Two *Hin*dIII fragments in the Bedford-type cytoplasm contain repeated regions of homology. One of these fragments exists as shown, the other fragment lies elsewhere in the genome of the Bedford-type cytoplasm. Restriction enzymes: P= *Pst*I, E= *Eco*RI, H= *Hin*dIII, and X= *Xba*I.

to the right hand side of the *Xba*I site in all cultivars.

Figure 10 summarizes what is known about the organization of the polymorphic region in the four soybean mitochondrial genomes based on comparative restriction mapping. In the restriction maps, the region in common to all four cytoplasmic types is indicated by a solid bar. The arrow indicates an approximate location of divergence among the four mitochondrial DNAs. The break point must lie to the right of the *Xba*I site and before the first mapped restriction site in the polymorphic region. From the Southern analysis in Figure 7, it is clear that regions flanking the polymorphic region are repeated at additional locations in the mitochondrial genomes. These additional regions of homology are in common to all four cytoplasmic types and are revealed with the 0.9 kb *Pst*I-*Hin*dIII, 1.5 kb *Xba*I-*Hin*dIII and 1.7 kb *Hin*dIII-*Pst*I probes. From deletion subcloning of the 1.5 kb *Xba*I-*Hin*dIII probe, it is known that the repeated sequence in the Lincoln-type cytoplasm extends at least 1.2 kb from the *Hin*dIII site towards the *Xba*I site (Li and Grabau, unpublished results).

The Bedford cytoplasm, that shows a doublet in the *Hin*dIII digest with the 0.8 kb or 1.5 kb probes, has been represented by two alternative sequences. It is known that this region contains a repeat and an explanation for the doublet is probably a recombination event at the repeat resulting in two different flanking regions. The model below illustrates how such alternative flanking regions could arise.



PCR reactions were carried out with selected oligonucleotide primers in an effort to determine whether a set of specific primers could serve as a quick screen for the cytoplasmic type of a given soybean cultivar. Based on the 'Williams 82' restriction map (Figure 3) and location of primers (Figure 4), product size can be predicted for the Lincoln-type cytoplasm. Genomic DNA from 'Lincoln' (Lincoln-type), 'Tennessee Non Pop' (Arksoy-type), 'White Biloxi' (Bedford-type), and 'Yelredo' (Soja-Forage-type) was used in all PCR reactions. In the first PCR reactions all 4 cytoplasmic types showed the same sized product as predicted for the Lincoln cultivar (data not shown). Primers KS.6 & SK.5 gave a 0.666 kb product, primers KS.6 & KS.7 gave a 1.46 kb product, primers KS.1 & SK.5 gave a 1.79 kb product, and primers KS.4 & SK.5 gave a 1.31 kb product. There are three possible interpretations of these results: 1) the regions encompassed by the primers chosen are identical among the different cytoplasmic types, 2) there is a

low level of the Lincoln-type DNA arrangement in all mitochondrial genomes, or 3) all DNA samples are contaminated. Interpretations 2 & 3 were eliminated by a second set of PCR reactions utilizing three new primer combinations (data not shown). Primers KS.5 & SK.8 gave only the predicted product of 2.0 kb in the Lincoln-type DNA. Products were not detected with the other three cytoplasmic types of DNA. Primers KS.6 & SK.8 gave the predicted 0.7 kb fragment in Lincoln-type DNA, and produced a 0.8 kb fragment in the Soja-Forage-type DNA allowing these two cultivar types to be distinguished form eachother. Products were not detected with this set of primers either in Arksoy-type or Bedford-type DNA. The predicted fragment size of 1.5 kb was produced with primers KS.8 & SK.1 in all 4 types of DNA. Although no set of primers examined was able to differentiate between the 4 cytoplasmic types of DNA, additional PCR reactions might be found that could generate such diagnostic polymorphic PCR products.

Discussion

A 4.9 kb *Pst*I mitochondrial DNA fragment from 'Williams 82' that contains a previously described polymorphic marker has been cloned and sequenced. The polymorphism, that was originally detected with an internal 2.3 kb *Hin*dIII fragment, distinguishes four cytoplasmic types among the cultivars of the old domestic collection. This marker shows the highest level of cytoplasmic diversity in soybean to date. The polymorphic region from all four cytoplasmic types appears to be surrounded by repeated sequences on either side.

Previous evidence from studies on both plastid and mitochondrial genomes suggests a derivation of soybean cultivars proceeding from diverse ancestors of the Soja-Forage type to Arksoy-type to Bedford-type and finally to Lincoln-type (Lee *et al.* 1994, Hanlon & Grabau in press). Modern domestic cultivars belong to only the Bedford-type and Lincoln-type cytoplasms (Grabau *et al.* 1989). Variation among fragment sizes visualized by Southern hybridization could have resulted from rearrangements, deletions, insertions, or single nucleotide substitutions that eliminated or created new restriction endonuclease sites. Rearrangements have been shown to occur readily in plant mitochondrial genomes (reviewed by Hanson & Folkerts 1992). A comparison of the four cytoplasmic types using all four subclones of the 4.9 kb *Pst*I fragment as hybridization probes has provided a rough map of the mitochondrial DNA organization in this polymorphic region but does not provide information to suggest an exact derivation of the polymorphism. Elucidation of the exact nature

of the polymorphism will require the mapping and sequencing of cosmid clones from all four cytoplasmic types.

Retention of sequences hybridizing to the 2.3 kb *Hin*dIII probe over evolutionary time indicates that these sequences have some importance. Speculation as to the possible function of conserved mitochondrial DNA sequences includes the presence of a mitochondrially-encoded tRNA or part of a plasmid of unknown function (Hanson & Folkerts 1992). Short sequences of mitochondrial DNA have been shown to encode both functional and non-functional tRNA sequences and there is a class of reported plasmids with unknown function (Hanson & Folkerts 1992, Leon *et al.* 1989, Bedinger *et al* 1986). Approximately 16 tRNAs are present and expressed in the higher plant mitochondrial genome, allowing recognition of 14 of 23 triplet sequences that are required for proper function and maintenance of the organelle. tRNAs have also been shown to be encoded by the nuclear genome and imported into the mitochondrion (Dietrich *et al.* 1992). The only three tRNAs that have been identified in soybean are for Glu (Wintz *et al.* 1987), *f*Met (Grabau 1987), and *m*Met (Wintz *et al.* 1988).

Potential open reading frames within the 4.9 kb *Pst*I fragment are all small, ranging in size from approximately 200 to 350 base pairs, which would correspond to 66 to 116 amino acid residues. While no long stretches of homology to known mitochondrial genes have been found in the 4.9 kb sequence, the short open reading frames could function as trans-spliced elements of a complex gene.

Trans-splicing has been shown to exist in several previously identified mitochondrial genes (Wahleithner *et al.* 1990, Chapdelaine & Bonen 1991, Wissinger *et al.* 1991). The localization to this region, of a sequence that has previously been reported as a site of transcription initiation (Brown *et al.* 1991) provides further support for an important functional role for these sequences. Consensus within the 4.9 kb sequence to other plant species shows a conservation over evolutionary time that suggests the polymorphic region has been retained due to a functional need in the genome. The soybean sequence also shows consesus to a small region located within a known petunia recombination repeat.

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CHAPTER IV

Mutagenesis and Selection for Oligomycin Resistance in Soybean (*Glycine max* L. Merr) Suspension Culture Cells

This chapter has been submitted for publication and is in the correct journal format of Plant Cell, Tissue and Organ Culture. Consent of the coauthors, E. Grabau and A. Pesce to publish this manuscript as a chapter is appreciated. It is also acknowledged that the tissue culture experiments and sequencing were performed by E. Grabau and A. Pesce, respectively, and are not the work of R. Hanlon. This material is based upon work supported by the National Science Foundation, Research Training Grant DMB-9010537, and the Cooperative State Research Service, USDA, under project VA-135387.

Abstract

Soybean suspension culture cells were subjected to mutagenesis with ethyl methane sulfonate and cells resistant to the ATP synthase inhibitor oligomycin were recovered. Suspension cultures showed high levels of resistance over a period of 17 months after mutagenesis. The level of resistance to oligomycin decreased during prolonged growth in the absence of the selection agent, consistent with segregation of resistant and sensitive mitochondria in progeny cells. Cross resistance to venturicidin was also observed which suggested the possible involvement of the mitochondrial ATP synthase subunit 9 gene. The sequences of ATP synthase subunit 9 cDNA clones from resistant cells were identical to the wild type sequence. These results indicated that resistance could not be directly attributed to a mutation in the primary gene sequence or an alteration in the RNA editing pattern of transcripts. Southern blot analyses of DNA from wild type and resistant cultures did not detect rearrangements in the region containing and directly adjacent to the ATP synthase subunit 9 gene in the mitochondrial DNA from resistant cultures.

Introduction

Resistance to oligomycin in higher plants has been demonstrated in tobacco cultures (Aviv & Galun 1988; Durand 1987; Durand & Harada 1989). Studies with cytoplasmic hybrids indicated that resistance is inherited with the cytoplasmic organelles, specifically the mitochondrion (Aviv & Galun 1988; Durand and Harada 1989; Perl *et al.* 1991). The antibiotic oligomycin binds to the membrane portion of the mitochondrial ATP synthase complex (F_0-portion) to inhibit proton translocation. Oligomycin resistance in yeast resulted from mutations in the mitochondrially-encoded genes for the ATP synthase subunit 6 (*atp6*) or subunit 9 (*atp9*). Mutations in the yeast *atp6* gene were primarily nonsense or frameshift mutations, suggesting that *atp6* can accommodate amino acid substitutions at many locations without affecting the functional characteristics of the enzyme (John *et al.* 1986). Many of the oligomycin resistant mutants in the yeast *atp9* gene were nucleotide substitutions in the coding region resulting in amino acid alterations (Ooi *et al.* 1985a). A subset of oligomycin resistant *atp9* mutants in yeast also showed cross resistance to the antibiotic venturicidin, another ATP synthase inhibitor (Ooi *et al.* 1985b). Mutant studies have allowed speculation about the folding and orientation of the ATP synthase subunit 9 polypeptide (ATP9) in the mitochondrial membrane and possible contact sites of ATP9 with ATP synthase inhibitors (Ooi *et al.* 1985b). Mitochondrially-inherited oligomycin resistant mutants in mammalian cells are due to a nucleotide substitution in the

atp6 gene (Breen *et al.* 1986). In mammals and *Neurospora* the functional ATP9 polypeptide is encoded by a nuclear gene and imported into the mitochondrion (Nagley 1988).

Oligomycin resistance has been studied in higher plants as a possible selectable marker for mitochondrial transformation studies. Both the *atp6* and *atp9* genes are encoded by the mitochondrial genome in higher plants and are candidate loci for mutations conferring oligomycin resistance. Many plant mitochondrial transcripts have been shown to undergo RNA editing which may function to conserve protein sequence (Covello & Gray 1989; Gualberto *et al* 1989; Heisel *et al.* 1989). Soybean *atp9* was sequenced at both the genomic and cDNA levels and the sequence comparison indicated that the soybean *atp9* transcript undergoes post-transcriptional C-to-T RNA editing at two positions (Grabau *et al.* 1990; Pesce & Grabau 1993). From the sequence analysis of 11 soybean *atp9* cDNA clones it appears that editing is complete in soybean as has also been reported for *atp9* in two additional dicots, *Oenothera* and petunia (Schuster & Brennicke 1990; Wintz & Hanson 1991). This contrasts the partial editing found *atp9* transcripts of three monocots, wheat, sorghum, and barley (Begu *et al.* 1990; Salazar *et al.* 1991; Rish & Breiman 1993). Sequencing of the entire *atp9* coding region and most of the *atp6* genomic clone from oligomycin resistant tobacco mitochondrial DNA showed nucleotide sequences identical to the wild type genome (Perl *et al.* 1991). Based on these findings, the authors suggested that an examination of

RNA editing might help to elucidate the origin of plant mitochondrial oligomycin resistance (Perl *et al.* 1991).

This report describes the mutagenesis of soybean suspension culture cells and selection for oligomycin resistant cell lines. The levels of growth inhibition by oligomycin and venturicidin in mutagenized and wild type cultures were determined. A preliminary attempt was made to locate a possible mutation or RNA editing anomaly responsible for the resistance phenotype by sequencing *atp9* cDNA clones from the resistant cultures. In addition, Southern analyses were performed to determine whether rearrangements of mitochondrial DNA encoding the *atp9* gene had occurred in resistant cultures that could be responsible for the mutant phenotype.

Materials and Methods

Cell culture lines

Soybean suspension culture cells were obtained from Dr. K. G. Lark at the University of Utah. The culture was initiated from soybean roots of a hybrid from a 'Noir I' x 'Minsoy' cross and was maintained in media containing Gamborg's B5 basal salts (Gamborg *et al.* 1968) with minimal organics (Sigma Chemical Company) and adjusted to pH 5.5. The medium was supplemented with 20 g $^{-1}$ sucrose, 2 g l^{-1} casein hydrolysate, and 1 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and designated B5C medium. Tissue culture reagents were obtained from Sigma Chemical Company. 50 ml cultures were grown in 250 ml Erlenmeyer flasks in a shaker-incubator at 28°C in the dark and transfers were made every 3-5 days by dilution in B5C to a final packed cell volume of approximately 15%. A separate tissue culture line (from the Williams 82 cultivar) was used as a source of control DNA for Southern blot analyses. The control culture was obtained from Dr. J. Widholm at the University of Illinois and was maintained in Murashige and Skoog medium (Murashige & Skoog 1962) supplemented with 0.4 mg l^{-1} 2,4-D.

Mutagenesis and Selection

For mutagenesis, soybean suspension culture cells were utilized at 2 days after transfer, treated for 30 minutes with 1% ethyl methane sulfonate (EMS) in the

shaker-incubator used for culture maintenance, collected by centrifugation, washed twice with B5C medium, resuspended in fresh B5C, and allowed to recover for 24 hours prior to selection. For selection, oligomycin was added to a concentration of 200 μg l^{-1}, cultures were grown for three days at which time the cells were transferred to fresh medium containing 300 μg l^{-1} oligomycin. After two weeks, the oligomycin concentration was raised to 400 μg l^{-1} and at approximately four months after mutagenesis, the concentration of oligomycin was reduced to 300 μg l^{-1} for long-term maintenance. Oligomycin and venturicidin (Sigma Chemical Company) were dissolved in ethanol at concentrations of 2.5 g l^{-1} and 5.0 g l^{-1}, respectively, and stored at -20°C. Dilutions of stock solutions were made in ethanol to allow delivery of the inhibitors in a constant volume of ethanol in each experiment.

Dose response analyses

Levels of inhibition by oligomycin were determined for wild type and EMS-treated suspension cultures by transferring 20 ml aliquots of cells into 125 ml flasks. Varying concentrations of oligomycin or venturicidin (and ethanol alone as control) were added as 20 μl aliquots from stock solutions. Three days after transfer, cells were harvested by vacuum filtration and fresh weights determined as an average of duplicate or triplicate samples. Increases in fresh weight were calculated by subtracting the mean fresh weight at day zero from each mean fresh weight at day three. Increases in fresh weight were presented as a percentage of

the increase in control culture flasks maintained without the addition of oligomycin (20 μl ethanol control). The experiments were repeated at various time points after the original mutagenesis beginning at three months and continuing to 17 months. The cumulative results of the inhibition studies are presented in the dose response curves. Dose response curves were determined for the inhibitor venturicidin to test for cross-resistance. Inhibition studies were also conducted for the resistant cell line maintained in the absence of selection for up to seven months in order to test for stability of the mutants.

cDNA cloning and sequencing

RNA was isolated from resistant cultures maintained in the presence of oligomycin via an RNAzol purification kit (Cinna/Biotecx Laboratories). cDNA synthesis was carried out with 2 μg RNA and 1 ng primer, utilizing 200 units of Moloney murine leukemia virus reverse transcriptase (Gibco-BRL). The oligonucleotide primer (5'-CCAGAGCTCAGAGACTACACCTTTC-3') was complementary to the *atp9* mRNA in the 3' non-coding region which was determined previously (Pesce & Grabau 1993). Amplification was conducted via PCR and utilized a second specific upstream oligonucleotide (5'-CGAGGATCCATGTTAGAAGGTGCAA-3'). The PCR cycle included one min at 94°C, one min at 54°C, two min at 72°C and was repeated for 45 cycles. The reaction mixture included the cDNA template, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 3.5 mM $MgCl_2$, 200 μM each of the four deoxyribonucleotide triphosphates,

1 ng of each primer and 2.5 U Taq DNA polymerase (Perkin-Elmer-Cetus). Introduction of *Bam*HI or *Sst*I sites at the 5' ends of the oligonucleotide primers (underlined in the oligonucleotide sequences above) allowed directional cloning of the cDNA into *Bam*HI-*Sst*I sites of the vectors pTZ19R (U.S. Biochemical) or pAlter-1 (Promega). The pAlter-1 vector was utilized to facilitate possible site-directed mutagenesis of the soybean *atp9* sequence in the future. DNA sequencing was carried out using Sequenase sequencing kit (U.S. Biochemical).

DNA isolation and Southern hybridization

DNA was isolated from wild type and mutagenized cultures by the method of Dellaporta *et al.* (1983). DNA samples were digested with the restriction endonuclease *Hind*III, separated by agarose gel electrophoresis, and transferred to nylon membranes (Micron Separations Inc.) by capillary blotting. Southern hybridizations were carried out according to the membrane manufacturer's specifications with an *atp9* probe labeled by nick translation (Rigby *et al.* 1977). The probe was a 265 nucleotide *atp9* cDNA cloned into the *Bam*HI-*Sst*I sites of the vector pAlter-1 (Promega).

Results and Discussion

Exponentially-growing soybean suspension culture cells were mutagenized with EMS and subjected to step-wise selection for growth on media containing oligomycin. Inhibition of growth by oligomycin was tested for both the wild type and resistant soybean cell lines from three to 17 months after mutagenesis. Growth was measured as the increase in fresh weight after three days of exposure to the inhibitor. The oligomycin dose response curves for both cell lines and standard errors for repeated experiments are shown in Figure 11. Inhibition by oligomycin was determined in seven independent experiments for the wild type line and nine experiments for the resistant line. Not all concentrations were repeated for every experiment which accounts for some of the higher standard errors observed for individual data points. The oligomycin concentration resulting in 50% growth inhibition (ID_{50}) was ten-fold greater for the resistant cell line than for the wild type cell line.

In a report comparing resistance patterns of a number of oligomycin resistant tobacco cell lines, Durand (1987) found that after maintenance on basal medium some mutagenized lines showed a loss of resistance, some retained low levels of resistance (two-fold greater than wild type), and some remained much more resistant than wild type (three to eight-fold greater than wild type levels). To test our cell line for stability of the oligomycin resistance phenotype, a subline of the culture was maintained in the absence of oligomycin selection. Growth inhibition

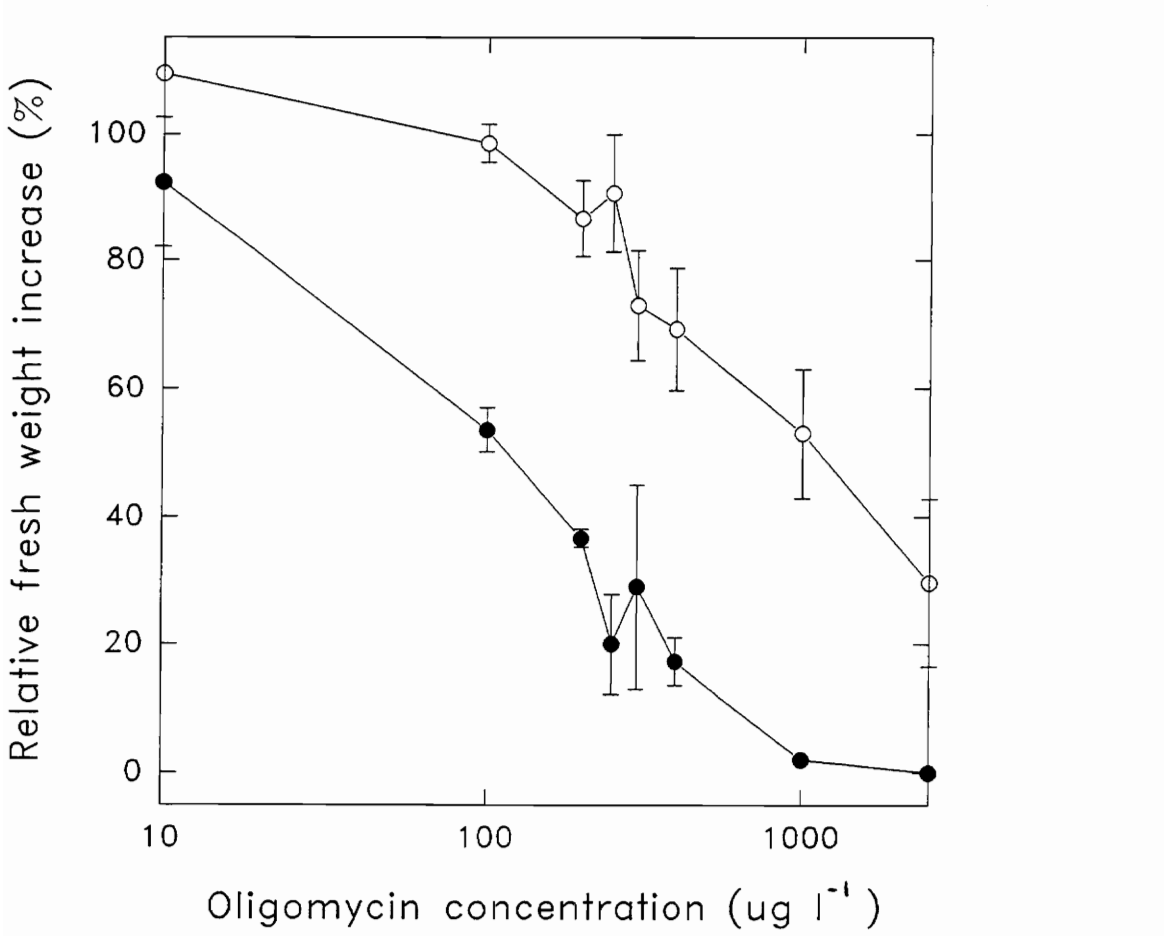

Figure 11

Cumulative results of multiple experiments of growth inhibition by the antibiotic oligomycin. Inhibition experiments carried out from three to 17 months after mutagenesis are presented in the dose response curves. For each experiment, cell suspension cultures were aliquoted into 2 to 3 flasks for each oligomycin concentration and incubated for 3 days. Increases in fresh weight were determined from the mean fresh weight value at three days for each concentration minus the mean fresh weight on day zero. The data at each oligomycin concentration were determined as a percentage of the increase in control cultures without oligomycin. Filled circles (•) indicate wild type cultures and open circles (o) indicate mutagenized cultures.

experiments were initiated after three months without selection and continued for a period of four months. The dose response curve in Figure 12 illustrates the cumulative results of four inhibition experiments with the non-selected line. The non-selected culture retained a higher level of resistance to inhibition to oligomycin than the wild type culture. However, the resistance was markedly reduced from the level displayed by the culture under selection, decreasing from a ten-fold difference in ID_{50} to an approximately two-fold difference in ID_{50} in the non-selected culture. Differences may be related to the fact that there are numerous mitochondria within a cell and each organelle contains many copies of the mitochondrial genome. It is possible that all cells that survived selection contained sufficient levels of resistant mitochondria to allow for growth in the presence of the inhibitor but that at least some cells contained non-mutant copies of the mitochondrial genome. In the absence of selection, segregation of wild type genomes could have resulted in a decrease in the proportion of resistant mitochondria in some progeny cells, thereby lowering the overall resistance of the culture when re-exposed to the inhibitor. This type of intracellular selection of mitochondria has been demonstrated in antibiotic resistant yeast (Birky 1973).

Some yeast oligomycin resistant mutants with nucleotide substitutions in the *atp9* gene display cross resistance to a second ATP synthase inhibitor, venturicidin (Ooi *et al* 1985b). Dose response curves to venturicidin were determined for both soybean cell lines as shown in Figure 13. The resulting differences in growth

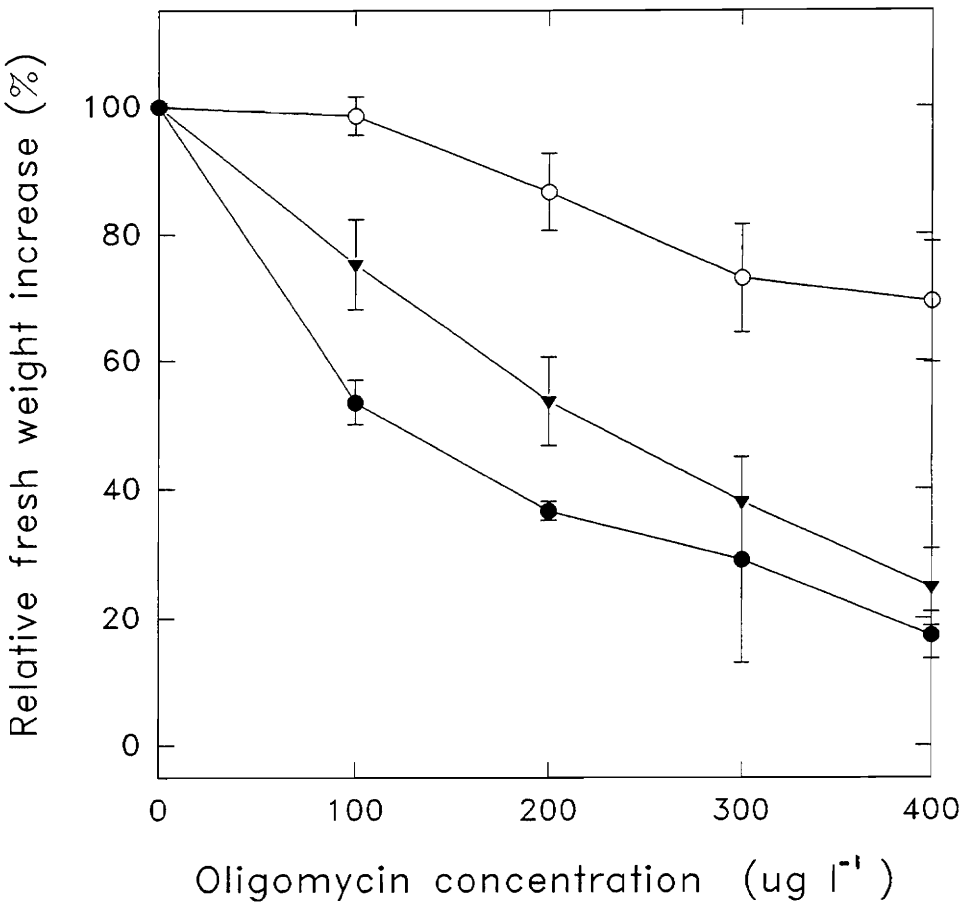

Figure 12

Stability of the oligomycin resistance phenotype. Three months after mutagenesis, a subline of the resistant culture was maintained separately in the absence of selection. After three months without selection, the subline was compared to both mutagenized cultures maintained in the presence of selection and to wild type for levels of inhibition by oligomycin. Dose response curves were determined as described for Fig. 1 above except that data were determined at oligomycin concentrations only up to 400 μg l^{-1} and results were plotted on a linear rather than semi-log scale. Data from the two previously tested cell lines (Fig. 1) were included for only those concentrations also tested in the non-selected culture line. Resistant culture maintained under oligomycin selection is indicated by the open circles (o), resistant culture maintained without selection is indicated by the filled inverse triangles (▾), and wild type culture maintained without selection is indicated by the filled circles (•).

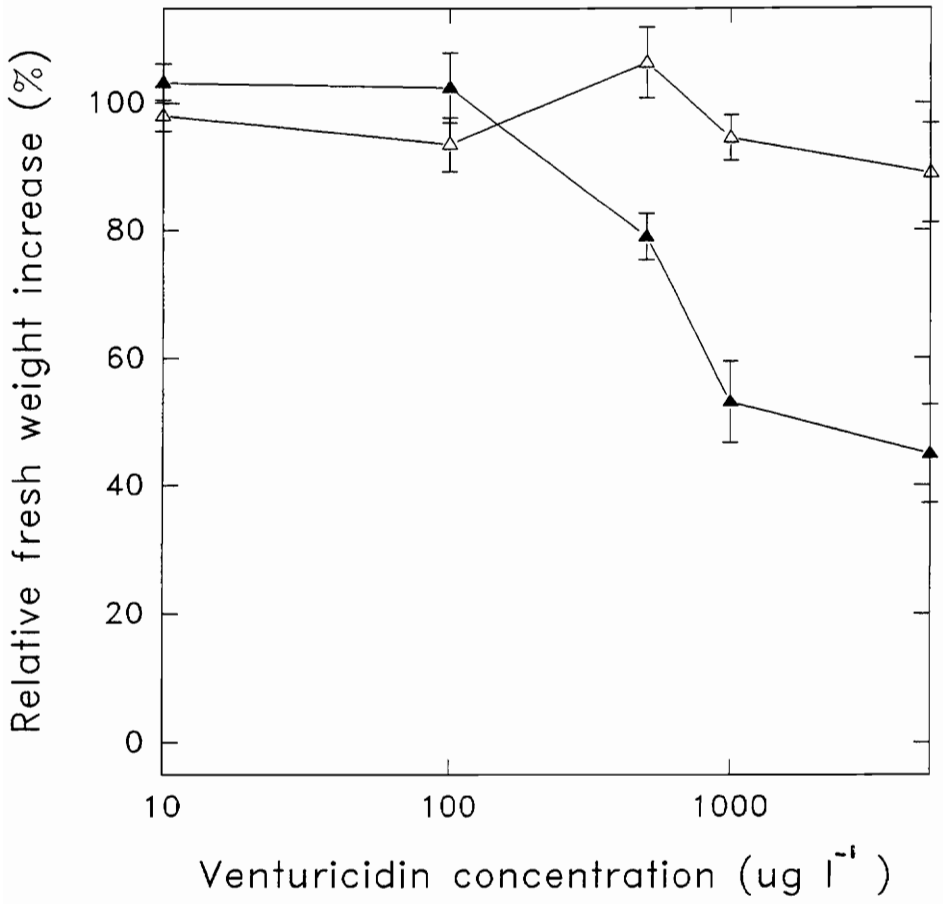

Figure 13

Growth inhibition by the antibiotic venturicidin. Dose response curves for venturicidin were determined as for oligomycin in Fig. 1 above. Inhibition to venturicidin was tested only for concentrations up to 5 mg per liter due to the problem of delivering the antibiotic in a small, consistent volume of ethanol (20 μl per 20 ml culture). Although the cultures differed in patterns of inhibition by venturicidin, ID_{50} values could not be determined. Wild-type cultures are indicated by filled triangles (▲) and mutagenized, resistant cultures are indicated by open triangles (△).

inhibition by venturicidin between wild type and oligomycin resistant cultures indicated a pattern of cross resistance in soybean similar to yeast. Because venturicidin resistance in yeast was observed only for mutations in the *atp9* gene, we focused our further investigation on the *atp9* gene in soybean. Previous oligomycin inhibition studies with tobacco failed to detect any mutations within the plant mitochondrial DNA coding for the *atp6* or *atp9* genes (Perl *et al.* 1991). Possible involvement of RNA editing in conferring resistance was suggested by Perl *et al.* (1991). To address this possibility in soybean we obtained *atp9* cDNA clones by reverse transcription of soybean RNA isolated from resistant cells maintained under selection, and subsequent DNA amplification via the polymerase chain reaction. The cDNA sequence obtained from the resistant culture was compared to the *atp9* cDNA sequence previously determined using RNA from soybean hypocotyls (Pesce & Grabau 1993). The results of sequencing six independent cDNA clones from the resistant culture showed identical sequence to the wild type, which included two editing sites relative to the known genomic sequence (Grabau *et al.* 1990; Pesce & Grabau 1993). These results were consistent with the previous lack of an identifiable *atp9* mutation leading to the oligomycin resistance phenotype in tobacco (Perl *et al.* 1991). Although sequencing six clones was not an exhaustive search, the use of RNA from cultures maintained under continuous oligomycin selection should have enhanced the proportion of mitochondria containing the oligomycin resistant determinant. Our utilization of multiple independent soybean cDNA clones extended the

observation from a single tobacco genomic clone sequence (Perl *et al.* 1991) to an examination of possible alterations in RNA editing in soybean. It is somewhat surprising that an *atp9* mutation was not identified in the soybean cultures displaying cross resistance to both oligomycin and venturicidin. Yeast *atp9* mutations showing cross resistance were attributed to nucleotide substitutions leading to amino acid alterations at several sites in the ATP9 polypeptide believed to interact directly with the inhibitors (Ooi *et al.* 1985b).

Mitochondrial DNA from higher plants contains numerous repeated sequences and undergoes extensive rearrangements which give rise to chimeric genes (for review see Hanson & Folkerts 1992). In soybean, the *atp6* gene is repeated and one copy contains sequence homologous to the 5' end of the *atp9* gene within the putative open reading frame (Grabau *et al.* 1988). Sequences homologous to the *atp9* gene are also found at multiple locations within the soybean mitochondrial genome and one region has been sequenced (Grabau *et al.* 1990). To examine whether rearrangement of the *atp9* gene could be associated with resistance to oligomycin and venturicidin, Southern analyses were carried out with DNA from wild type and resistant cultures. Figure 14 shows the results obtained from probing *Hin*dIII-digested DNA with a soybean *atp9* cDNA clone. DNA from soybean hypocotyls ('Kura') and from another soybean culture line ('Williams 82') were included as controls. Banding patterns were essentially identical among all the soybean DNA preparations indicating that no detectable rearrangements

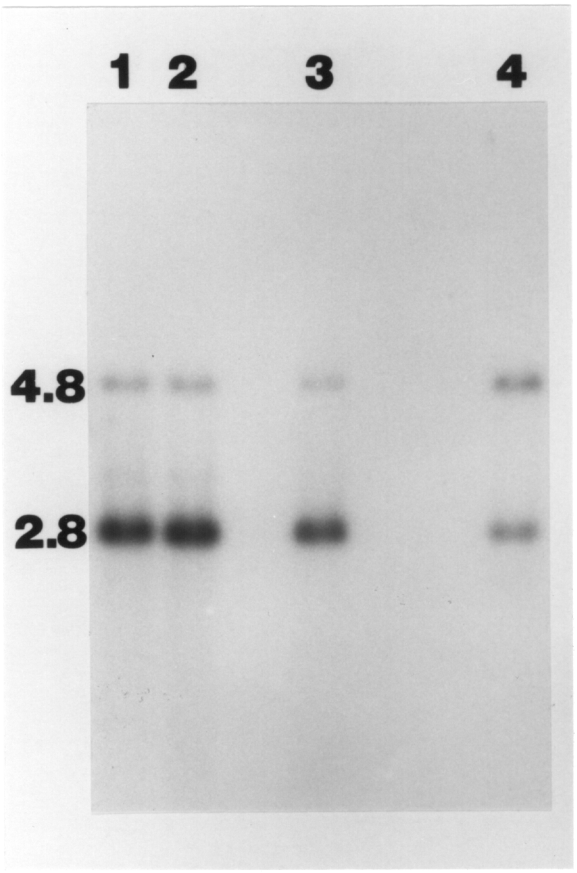

Figure 14

Southern analysis of DNA from wild type and resistant cell lines. Soybean DNA samples were probed with A) a 0.9 kb *Xba*I-*Bam*HI cDNA fragment of the *atp9* gene inserted into the pAlter vector, B) 2 kb *Bam*HI-*Sph*I fragment containing a portion of the *atp6* gene (lacking chimeric 5' end), and C) the 2.3 kb *Hin*dIII fragment from 'Williams 82' inserted into pUC19. Total DNA was purified from soybean cultures or six day old hypocotyls and digested with *Hin*dIII prior to separation by electrophoresis and transfer by capillary blotting. Lanes are identical for the three panels. Lane 1: wild type culture, lane 2: resistant culture (EMS), lane 3: second wild type soybean culture as control (MS), lane 4: soybean hypocotyls from 'Kura', lane 5: soybean hypocotyls from 'Volstate'. Fragment sizes were estimated from the mobilities of *Hin*dIII-digested lambda.

occurred to give rise to the resistant cultures. Hybridization was observed to two major bands corresponding to fragments of 2.8 and 4.8 kb. Previous sequencing of the soybean *atp9* gene was conducted by subcloning the gene from the 2.8 kb *Hin*dIII fragment (Grabau *et al.* 1990). Probing the same blot with two additional mitochondrial probes, the *atp6* gene (Grabau *et al.* 1988) and a polymorphic 2.3 kb *Hin*dIII fragment (Grabau *et al.* 1989), indicated that no rearrangements occurred at those additional locations within the mitochondrial genome (data not shown). We concluded that mutagenesis and selection for resistance to ATP synthase inhibitors are easily accomplished in soybean cultures but we cannot attribute the resistance phenotype to a specific mutation.

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Appendix

ALKALINE LYSIS: solutions

solution 1:
- 50 mM glucose
- 10 mM EDTA
- 25 mM Tris-Cl (ph 8)

solution 2:
- 0.2 N NaOH (fresh)
- 1% SDS

solution 3:
- 60 mls 5 M KOAc
- 11.5 mls gl.OAc
- 28.5 mls H20

AMPICILLIN: addition to media less than 65°C
- stock solution: 50 mg/ml
- working solution: 100 μg/ml

ß-MCE (ß-mercapto ethanol)
- stock solution: 14.3 M

DENATURING SOLUTION:
- 1 M NaCl (58.5 g/l)
- 0.5 M NaOH (20 g/l)

DENHARDTS
- stock solution: 50X Denhardts, 50 ml aliquots at -20°C
  - 5 g Ficoll (type 400, Pharmacia)[Fisher]
  - 5 g polyvinylpyrrolidone
  - 5 g bovine serum albumin (Fraction V)[Sigma]
  - DI H_2O to 500 mls

EDTA (disodium ethylenediaminetetra-acetate $2H_20$)
- stock solution: 0.5 M (pH 8)
  - 186.1 g EDTA/l
  - Adjust pH with NaOH pellets.

EXTRACTION BUFFER (EB)
- Tris 50 mM pH 8
- EDTA 10 mM

SDS 1.0%
NaCl 100 mM
ß-MCE 10 mM

ETHIDIUM BROMIDE
stock solution: 10 mg/ml
working solution: 0.5 μg/ml

HCl (HYDROCHLORIC ACID)
stock solution: 12 N
working solution: 20 mls/l, dilute HCl

IPTG (isopropylthio-ß-D-galactoside): 238.3 g/mole
stock solution: 420 mM, 1 ml aliquots, store at -20°C
10 mg IPTG to 10 ml with DI H_2O
0.22 μm filter sterilized
working solution: 4.2 mM/plate
10 μl/plate

LOADING DYE BUFFER
50mL stock solution: store at 4°C
15 ml glycerol (30% in H_2O)
0.125 g bromophenol blue (25%) [Fisher]
0.125 g xylene cyanol (25%)

LURIA-BERTONI
1 liter liquid media:
10 g Bacto-tryptone
5 g Bacto-yeast extract
5 g NaCl
1 liter media for plates:
same as liquid
15 g Bacto-agar

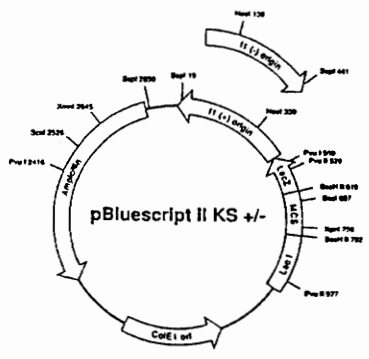
NEUTRALIZING SOLUTION
working solution: pH 7.5
0.5 M Tris (60.6 g/l)
1.5 M NaCl (87.6 g/l)

PCR BUFFER (10X)
pH 9 (25°C) & 0.2 mM dNTPs
500mM KCl
100mM Tris HCl
1% Triton-X 100

PCR PRIMERS

5'-3'	Exact strand in Figure 4	5'-3'	Opposite to strand in Figure 4
KS.1	AAGGACAGCATTCAAAC	SK.1	GACTCAATGGAGTTGCT
KS.2	CAAGTGTCGGACTCTGGT	SK.2	CTTTGCCAAAGTTAGGG
KS.3	CTGCTAGCAGAGGGAAG	SK.3	GAGCCGATATCGACACC
KS.4	GTACCGTTAACAAGCTC	SK.4	CTTTCCGAGACCAATGC
KS.5	TTGTCTGACTTCAACGG	SK.5	GAAGAGCGCCGCAACAGC
KS.6	AAGATCGGGTTGACAGC	SK.6	GTATGTCCTGGAACAGC
KS.7	TGCTCAAAAGGTCCGGG	SK.7	TGAATGGTAGATTCAGGC
KS.8	ACGAGCCTGGAATTTCTG	SK.8	CTAAGCTAAGATCTAGGG
KS.9	CAAGACGAGTGAAATGGG	SK.9	GAGATTTGAACTCCGTC
KS.10	GGGGAAGTGTCTTGATG	SK.10	GCTACCTTTACAATCCG
KS.11	GAACAAGGGATTGGCTGC	SK.11	CCTCTTGGCTAAATAGT
KS.12	ATAGCAATCGAGTGGCC	SK.12	AAGACCGTTTACGCTGC
KS.13	GTAGGTACCTATGTCAT	SK.13	GATTCTTGCTAAACTGG
KS.14	GTGCACTTTCCTAAGGGC	SK.14	CCTCGATCTTCTACAGCA
		SK.15	TACCCGTCGCTAGCAGC
		SK.16	CTGGCGAGTGACTCTTCC

pBKS-:



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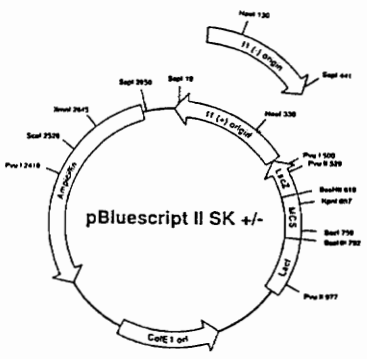
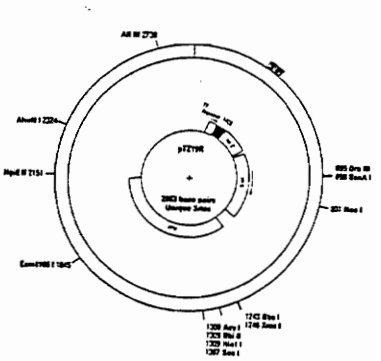


pTZ19R:



PREHYBRIDIZATION SOLUTION: 30 mls
- 6 mls 50X Denhardts (1.2 μm filtered)
- 3 mls 10% SDS
- 9 mls 20X SSPE
- 300 μl boiled salmon sperm DNA
- 11.7 mls DI H_2O

POLYACRYLAMIDE GELS: 2X 36X20 mm plates
- 42 g urea
- 17.4 mls bis-acrylamide
- 10 mls 10X TBE
- 44.4 mls H_2O
- 0.5 mls 10% APS
- 1.2 μm filter above
- 50 μl temed

SDS (sodium dodecyl or lauryl sulfate)
- stock solution: 10% SDS
  - 100 g/l SDS (68°C assists dissolution)
  - Adjust pH to 7.2 with HCl.

SSC
- stock solution: 20X SSC, pH 7.0
  - 175.3 g/l NaCl
  - 88.2 g/l sodium citrate
- working solutions: 2X SSC, 10X SSC

SSPE
- stock solution: 1 liter of 20X SSPE, pH 7.4
  - 175.3 g NaCl
  - 27.6 g NaH_2PO_4-H_2O (sodium phosphate)
  - 7.4 g EDTA

TAE BUFFER (Tris-acetate/EDTA electrophoresis)
- stock solution: 1 liter of 50X TAE
  - 242 g Tris base [Fisher]
  - 57.1 ml glacial acetic acid
  - 100 ml 0.5M EDTA (pH 8)
- working solution: 1X TAE

TE BUFFER: pH 8
- 10 mM Tris-Cl
- 1 mM EDTA

TRIS BUFFER (Tric-Cl)
stock solution: 1 M
121.1 g/l Tris base
Adjust pH with HCl; 42 mls for pH 8.

NaCl (sodium chloride)
stock solution: 5M
292.2 g/l NaCl

X-GAL (5-bromo-4-chloro-3-indolyl-ß-D-galactoside)
stock solution: 20 mg/ml, -20°C, foil wrap
dissolve in dimethylformaminde

WASH SOLUTION 1:
5X SSC
0.5% SDS

WASH SOLUTION 2:
1X SSC
0.5% SDS

WASH SOLUTION 3:
0.2X SSC
1% SDS

Vitae

I was born December 12, 1969 in Our Lady of Lords Hospital in Camden, NJ. As a child I experienced many southwestern states in the US, attended high school in Virginia Beach, and received my diploma from Kellam High School in 1988. I attended Virginia Intermont College as a Horsemanship major before transferring to Virginia Polytechnic Institute and State University where I completed a Bachelors degree in Biology with the Biotechnology option in December, 1992. I pursued a Masters in Plant Physiology at VPI&SU from January 1993 to December 1994. As of January, 1995 I will be employed as a lab specialist in plant molecular biology at VPI&SU.