APPLICATION OF MOLECULAR MARKERS TO CHARACTERIZE POTATO PLANTS DERIVED FROM ANTHER CULTURE AND PROTOPLAST FUSION

by

Vidya Ravichandran

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APPROVED:

Richard E. Veilleux, Chairman

Eric E. Beers

Glenn R. Buss

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Department of Horticulture

(ABSTRACT)

Cultivated potato is an autotetraploid (2n=4x=48), i.e., it has four complete sets of chromosomes. Tetraploids can harbor considerable heterozygosity (Jacobs, 1985) since there are potentially four possible alleles per locus. Reducing the ploidy level of potato from the tetraploid to the dihaploid level (2n=2x=24) and then the monoploid (2n=1x-12), or reducing diploid potato spp. to the monoploid level is a way of developing pure homozygous lines by doubling the monoploid genome.

Homozygous breeding lines are important since potato is a highly cross pollinated crop and inbreds are absent. The reduction of the ploidy level can be realised through anther and microspore culture. This method bears a great deal of significance in that it provides a selection against lethal recessive genes. Monoploids carry

only a single set of chromosomes and every locus carries only one allele. So the surviving monoploids are devoid of lethal alleles due to the effect of the "monoploid sieve"

Protoplasts are plant cells, the cell walls of which have been digested by a combination of pectinase and cellulase enzymes. If the protoplasts are cultured in a suitable medium, they can resynthesize the cell walls, divide and regenerate complete plantlets. But if a successful fusion of protoplasts from sexually incompatible parents is achieved, the resulting somatic hybrid carries the genome contributed by both parents.

This study aims to apply the molecular tool of RAPD mapping as an efficient agent of plant (potato) genetic characterization of anther-derived monoploids and putative hybrids of a somatic fusion of two monoploids

Dedication

This thesis is dedicated to my mom and dad, for their love, inspiration and guidance at every point in my life - no matter what, to Hari for always being there, and to Vikram for being my pillar of strength.

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CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

The cultivated potato (*Solanum tuberosum* L.) is one of the world's most valuable food crops. The potato plant is uniquely effective at converting and partioning carbon into usable forms, potentially producing more food value per unit time, land and water than any other food crop. The potato tuber also provides the best balanced dietary combination of calories and proteins, including all essential amino acids, of any major food crop (Weiser, 1985).

1.1a. APPLICATION OF CELL CULTURES TO POTATO IMPROVEMENT

The techniques of plant cell, tissue and organ culture have found newer and wider realms of application. From being a versatile tool to explore morphogenesis and demonstrate totipotentiality of plant cells as Haberlandt originally intended (Murashige, 1974), tissue culture is now commonly incorporated into several plant breeding program (Guiderdoni, 1990).

Potato breeders have been trying to develop cultivars with increased resistance to pests, diseases and environmental stress, with an emphasis on higher yield. Releasing superior cultivars is the ultimate goal of every plant breeder.

Cultivar development in potato follows three basic steps:

1. Formation of a breeding population,
2. Evaluation of individuals within the population to select a new cultivar,
3. Multiplication of seed of the new cultivar (Hoopes and Plaisted, 1987).

But this process is laborious and time consuming. For instance the development of potato cultivar "Hampton" was begun in 1972 and the cultivar was finally released in 1985. Improved methods that reduce the time for cultivar development are essential for breeding.

Cultivated potato is an autotetraploid (2n=4x=48), i.e., it has four complete sets of chromosomes. Tetraploids can harbor considerable heterozygosity (Jacobs, 1985) since there are potentially four possible alleles per locus.

Reducing the ploidy level of potato from the tetraploid to the dihaploid level (2n=2x=24) and then the monoploid (2n=1x-12), or reducing diploid potato spp. to the monoploid level is a way of developing pure homozygous lines by doubling the monoploid genome. Homozygous breeding lines are important since potato is a highly cross pollinated crop and inbreds are absent. The reduction of the ploidy level can be realised through anther and microspore culture (Guha and Maheshwari, 1966).

Another possibility in potato breeding is utilizing protoplasts i.e., somatic cells divested of their walls, for breeding. Since the first

isolation of potato protoplasts by Lorenzini (1973), and successful plantlet regeneration from potato protoplasts by Shepard (1975), protoplast culture techniques have come to be commonly incorporated into potato breeding programs as a method of developing novel genotypes.

1.1b. ANALYTIC BREEDING

The analytic breeding scheme was introduced by Chase (1962, 1963). This is a three phase process involving a) a reduction of a polyploid to its diploid components, b) intensive breeding and selection at the diploid level, and c) resynthesis and testing of the polyploid form.

A variety of germplasm is available to potato breeders, but one species of particular interest is *Solanum phureja* because of various disease resistances, specifically to *Erwinia carotovora*, Potato virus X and Potato virus Y (Qu Dongyu) and for the production of unreduced male gametes. This particular trait makes it relatively easy to obtain tetraploid offspring by crossing tetraploid with diploid parents. Selected clones of *S. phureja* can also be used to pollinate tetraploids and induce the formation of seeds containing only the maternal genome (Peloquin et al., 1966). The dihaploids arising as a result of this process are useful in breeding and genetic studies because of their simpler inheritance ratios compared to tetraploids.

Another valuable diploid species is *Solanum chacoense*. Specific selections of this species are rich in the glycoalkaloid, leptine, which

is known to convey some resistance to the Colorado potato beetle. Although the glycoalkaloid is toxic, it is restricted to the foliage and is not present in the tuber.

The diploid species of potato can be crossed to each other to obtain novel germplasm. It may be possible to use hybrids between *S. phureja* clones that exhibit good adaptation and a high frequency of 2n pollen with leptine-producing clones of *S. chacoense* to incorporate insect resistance into S. tuberosum and simultaneously minimize the negative weedy aspects of *S. chacoense* in the resulting hybrids. The plant materials used in this study are anther-derived monoploids derived from a hybrid between *S. chacoense* and*S. phureja* .

1.1c. CELL CULTURE AS A TOOL IN GERMPLASM DEVELOPMENT

In vitro culture techniques have been used more extensively in all aspects of production, improvement and germplasm handling of potato than in any other crop (Wang and Hu, 1985). Some culture techniques used routinely are meristem culture, first employed for potato virus eradication by Morel and Martin (1955), shoot tip culture (Goodwin et al., 1980), nodal segment culture (Hussey and Stacey, 1981), and callus culture (Steward and Caplin, 1951).

New germplasm can be produced by anther culture. This procedure gives rise to monoploid (2n=x=12) potato. By doubling the chromosome number of monoploids, pure homozygous lines can by

produced, which may be used as diploid true breeding lines. The potential for microspore regeneration is genetically controlled (Sopory, 1978) and is transferable by normal sexual breeding through crosses to the offspring. Hence the "tissue culture ability" (Jacobson and Sopory, 1978), or more specifically the ability to respond to the anther culture process can be integrated into any potato line.

Another useful technique is somatic hybridization of genetically diverse protoplasts to obtain a novel genotype. Theoretically, a method of somatic cell fusion, followed by elimination of most of the chromosomes from the wild parental species during subsequent mitosis, could be an ideal means of extracting valuable traits. Protoplast fusions offer the possibility of constructing highly heterozygous polyploids without the disruption of the genome that accompanies the sexual process.

Fusions can be made between autotetraploids (Gunn and Shephard, 1981), diploids, or more specifically, dihybrids, i.e., progeny of a cross between parents that differ for an allele at two loci (Binding et al., 1978), or monoploids (Roest and Bokelmann, 1983).

1.2 ANTHER CULTURE

Reduction of the ploidy level of potato can be achieved conventionally be female parthenogenesis (van Bruekelen et al., 1975). As mentioned earlier, if certain accessions of *S. phureja* are employed as pollinators, they contribute gametes to the endosperm without fertilizing the ovule, giving rise to seeds whose embryos are derived from the maternal genome and have one half the chromosome number of the mother plant. The resulting plants are haploids. But anther culture can be a more attractive option because the profuse amount of microspores naturally produced by the plant can contribute to potentially greater numbers of monoploids than the macrospore; and provide potential access to all potato species, wild or cultivated, while female parthenogenesis induces relatively fewer plants in some wild species.

Anther culture is the process of stimulating immature pollen grain to proceed along an embryogenic or androgenic pathway (Veilleux, 1994). Anthers with pollen at the uni/binucleate stage are cultured on a nutrient medium and these microspores produce embryos which can be regenerated into plants. This method bears a great deal of significance in that it provides a selection against lethal recessive genes. Monoploids carry only a single set of chromosomes and every locus carries only one allele. So the surviving monoploids are devoid of lethal alleles due to the effect of the "monoploid sieve" (Wenzel et al., 1979).

1.2a. UTILIZATION OF MONOPLOIDS

The most important use of monoploids in plant breeding is the ease of production of homozygous diploid breeding lines which can be used in sexual crosses. Not only can this be applied to production of new cultivars by employing the diploids, but breeding with these "novel" plants can be helpful in addressing some questions on the origin and evolution of a plant. For instance, Howard in 1973, was able to produce evidence that *Solanum tuberosum* was not a full autotetraploid.

Haploids find a place in mutation studies because recessive mutant alleles would be immediatly displayed phenotypically due to their hemizygous condition (Riley, 1977). Wernsman and Rufty (1987) reported that haploids also play a role as an aid in interspecific gene transfer in tobacco.

1.2b. GENETIC CHARACTERIZATION OF PLANTS USING MOLECULAR MARKERS

Research in the past few years has shown that most molecular markers in organisms are inherited in a Mendelian fashion and hence adherence to the Mendelian ratios can be expected. Genetic markers can be exploited for identification of homozygosity or heterozygosity of alleles in plants (Veilleux, 1985). Over the years, several sources of molecular markers have been utilized, the noteworthy ones being isozymes, restriction fragment length polymorphism (RFLP) (Botstein et al., 1980), random amplified polymorphic DNA (RAPD)

(Williams et al., 1989), and simple sequence repeats (SSR) (Smeets et al., 1989).

Isozymes are multiple molecular forms of an enzyme derived from a tissue of an organism. Discrete bands based on charge and molecular weight of the isozymes are formed when the gel is placed in a solution of the proper substrate for the enzyme. Enzymes are coded by genes, hence gene mapping is facilitated. In the RFLP mapping technique, random single copy DNA probes are capable of detecting DNA sequence polymorphism when hybridized to restriction digests of an organism's DNA. This procedure involves radiolabelling of the DNA.

RAPD is a DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. These polymorphisms are simply inherited as DNA segments which amplify from one organism but not the other. SSRs are short tandem repeats in DNA sequences. The repeated core sequences may be of two, three, or four nucleotides in length and are flanked by conserved DNA sequences. The polymorphism arises due to the differences in the number of repeats. The latter two techniques are PCR (polymerase chain reaction) based. All of these markers have been successfully employed to characterize plants derived from tissue culture.

Use of RFLP markers:

In an experiment by Rivard and Saba-El-Leil in 1994, RFLP markers were studied in *Solanum chacoense* plants derived from *in vitro* anther culture. RFLP analysis was conducted on two diploid parental lines, their anther culture (AC) progenies, their selfed progenies, and their reciprocal F1 hybrids. The AC plants could be unambiguously identified on the basis of their origin. A distinction could be made between the plants originating from reduced (n) or unreduced (2n) microspores. All the AC plants produced by gametic embryogenesis showed distinct RFLP patterns but similar patterns were found among some plants that regenerated via callus. An earlier experiment (Rivard et al., 1989) provided exhaustive information on the process of genetically characterizing *in vitro* anther derived plants of *Solanum chacoense*. This can be useful for constructing genetic maps, identifying lethal alleles and quantitative trait loci (QTLs).

RFLP markers have been used in similar studies by Van Eck et al., in 1993 to map the inheritance of flower color in diploid potato controlled by three unlinked loci, D, F, and P, by Thompson et al., (1991) to characterize microspore-derived plants of four spring barley crosses and their parents, and by Schweizer et al., (1992) in potato to analyze the distribution, organization, and inheritance of highly repeated genome fragments. It was found that approximately 5-7% of the Solanum genome is comprised of repeated fragments.

Using RFLPs is technically complex, costly, and involves the use of radiolabelled isotopes. However, PCR-based molecular analysis like RAPD and SSR mapping can overcome these problems.

Polymerase Chain Reaction (PCR): PCR is a method of enzymatic amplification of a DNA fragment that is flanked by two oligonucleotide primers which hybridize to opposite strands of the target sequence. Repeated cycles of heat denaturation of the template, annealing of the primer to their complementary sequences and extension of the annealed primers with a polymerase enzyme, increase the amount of the DNA fragment a million fold.

Use of RAPD markers:

The RAPD assay detects the presence of only one allele at any given locus (Rafalski et al., 1991). So the absence of an amplified band represents all other alleles at that locus that failed to amplify. Therefore RAPDs are dominant markers and cannot be used to distinguish heterozygous loci. Therefore while characterizing monoploids, the occurrence of null alleles characterized by the absence of any band at a locus, is not unusual.

There have been innumerable reports on the varied applications of RAPDs in most plant species. ranging from detecting genetic diversity to constructing genetic maps. RAPDs have been used in Solanum sp. to detect genetic variation in androgenic monoploids of diploid Solanum. (Singsit and Ozias-Akins, 1993). By

understanding the genetic relationship among monoploids, doubled monoploids and anther donor plants of diploid potato using the RAPD band pattern, they suggested that it was possible to construct a genetic linkage map, without making crosses, by utilizing monoploids and the RAPD markers.

Use of SSR markers:

Plant genomes contain polymorphic repetitive sequences that can be used as DNA markers. These may be minisatellites (16-64 bp per repeat) or simple sequence repeats (2-6 bp per repeat) (Sharon et al., 1995). The usefulness of SSRs as genetic markers has been documented in soybean by Akkaya et al. (1992). Allelic diversity of SSRs was found in an evolutionary dynamics study of barley conducted by Saghai-Maroof et al. (1994). In another study by Yu et al. (1993), SSR markers have helped in identifying a gene, Rsv, conferring resistance to soybean mosaic virus.

1.2c. GAMETIC SELECTION

It is possible that any deviation from the expected Mendelian ratios for allelic segregation is indicative of *in vitro* gametic selection. In a study on rice by Guiderdoni (1991), the segregation and recombination of heterozygous isozyme markers was monitored in anther culture (AC) derivatives. Consistent segregation distortion was obtained in the F2 as in the AC populations. These distortions were found to be specific to AC derivatives indicating the existence of *in*

vitro gametic selection. In a similar study on oilseed rape, Tanhuanpaa et al.(1993) observed 68 F2 and 40 microspore derived plants. The results showed a significant excess of "Topas" (one of the parents) alleles at five RAPD loci in the microspore derived population. This suggested that the genomic regions probably affecting microspore culture ability were segregating in a skewed manner.

In an experiment involving 62 doubled haploid lines of barley (Zivy et al., 1991), derived from an AC population, a deviation from the 1:1 ratio expected in the absence of selection was actually observed for at least one chromosome segment. This was perhaps linked to gene involved in the process of anther culture. In another study on the DH lines of spring barley (Kintzios et al., 1994), a significant level of segregation distortion occurred in favor of the DH genotypes resistant to mildew. This may have been due to linkage of a mildew resistance gene on chromosome 5 with genes for plant regeneration or unintentional screening for mildew resistance.

In a study mentioned earlier, (Rivard et al., 1994), *S. chacoense* plant materials were used to study segregation distortion. Segregation studies of RFLP markers revealed significant deviation from the expected Mendelian ratios in both AC-derived populations as well as in the selfed progenies. Such deviations were rare in the reciprocal F1 hybrids. This was probably due to the presence of genetic sieves operating during anther culture. In a study on the segregating hybrid population of *S. chacoense* X *S. phureja* (Hosaka

and Hanneman, 1994), significant departures were seen from the expected ratios.

1.3 PROTOPLAST CULTURE

Protoplasts are plant cells, the cell walls of which have been digested by a combination of pectinase and cellulase enzymes. If the protoplasts are cultured in a suitable medium, they can resynthesize the cell walls, divide and regenerate complete plantlets.

But if a successful fusion of protoplasts from sexually incompatible parents is achieved, the resulting somatic hybrid carries the genome contributed by both parents.

Fertile somatic hybrids have been successfully obtained by Jahne et al. in 1991 in barley, Serraf et al. in potato in 1991. Somatic hybrids have been used to detect introgression in Solanum spp. by Jacobsen in 1994.

Intraspecific somatic hybrids of *Solanum tuberosum* were created by Cooper-Bland et al. (1994) for studying nematode resistance. Resistance to *Verticillium dahliae* was transferred from wild to cultivated Solanum by protoplast electrofusion. (Jadari et al., 1992)

1.3a. CHARACTERIZATION OF SOMATIC HYBRIDS

Somatic hybrids have been characterized by studying morphological variation and characteristics in potato (Preiszner et al., 1991) and by the molecular methods mentioned earlier. Brown et. al.

(1993) investigated the use of PCR and RAPD techniques to analyze single protoplasts and obtained successful results. RAPD markers are a very useful tool to confirm fusion products as somatic hybrids by the presence of bands from both parents in the putative hybrid.

A study to this effect was conducted on dihaploids of potato by Takemori et al. (1994). Most of the primers they used detected polymorphism either among regenerated tetraploids or dihaploids, and polymorphism was detected even between closely related clones. The polymorphism was observed due to the amplification of the fused genome, thus characterizing the plants as true somatic hybrids. In another study by Rasmussen and Rasmussen (1995), after selecting putative somatic hybrids based on vigorous callus growth, RAPD analysis was undertaken on these plants. More than 50% of the selected plants were somatic hybrids. About 49% of the primers used confirmed hybridity. Other studies (Rokka et al., 1994) have extended the use of RAPDs to assess inheritance of disease resistance in potato.

1.4. OBJECTIVES

This study aims to apply the molecular tool of RAPD mapping as an efficient agent of plant (potato) genetic characterization. Potato monoploids are used here to study genetic segregation and also to create somatic hybrids. Segregation patterns of monoploids derived from anther culture of an F1 (CP2) between clones of *Solanum chacoense* (80-1) X *Solanum phureja* (1-3) will be studied using

RAPDs. Confirmation of somatic hybrids, a fusion product of two monoploid clones will be attempted with the same tool.
The specific objectives are:

1. To determine allelic segregation among anther-derived monoploids from a *S. chacoense* X *S. phureja* (CP) hybrid using RAPDs.
2. To use molecular markers to confirm the identity of putative somatic hybrids resulting from fusion of protoplasts of two monoploid clones using molecular markers.

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APPLICATION OF RAPD MARKERS TO STUDY SEGREGATION RATIOS IN A MONOPLOID POTATO FAMILY

by

Vidya Ravichandran
Richard E. Veilleux, Chairman
Department of Horticulture

(ABSTRACT)

A hybrid clone (CP2) between *Solanum chacoense* (80-1) and *Solanum phureja* (1-3) was used as anther donor in a series of experiments to develop an anther-derived family of 20 monoploids. DNA was extracted from *in vitro* copies of these monoploids. PCR amplification was conducted followed by RAPD (Random Amplified Polymorphic DNA) resolution on 1.4% agarose gel.

Fifty polymorphic bands produced by 13 primers were scored. Segregation patterns of RAPD markers were studied. Ratios not significantly different from the expected 1:1 (presence : absence of a band) ratio were obtained for 29 markers. Of the remaining 21 markers, 16 (76%) showed skewed segregation in favor of the *chacoense* parent, whereas five (24%) segregated in favor of the *phureja* parent. From the 29 normally segregating markers, 27 pairs of linked loci were obtained.

Highly distored segregation could have been be due to selection pressure against deleterious alleles or in favor of loci that influence the process of haploidization through anther culture.

CHAPTER II

APPLICATION OF RAPD MARKERS TO STUDY SEGREGATION RATIOS OF MONOPLOID POTATO.

INTRODUCTION

Potato (*Solanum tuberosum* L.) is a highly heterozygous autotetraploid ($2n=4x=48$). In this highly heterozygous state, potato may harbor deleterious recessive alleles at many loci without any apparent effect on growth and performance (Uijtewaal et al. 1987). But when the chromosome number of diploid breeding lines is reduced to the monoploid level through anther culture, it is safe to assume that the surviving monoploid regenerant is free of these lethal or deleterious alleles. This was the concept of the monoploid sieve proposed by Wenzel et al. in 1979. It is also possible that selection pressure in the anther culture process not only occurs indirectly by ridding the monoploids of deleterious alleles but also directly favoring alleles conducive to the anther culture process.

An anther-derived population from a heterozygous anther donor is expected to segregate according to normal Mendelian ratios. In principle, 50% of the population should reflect one parental type and 50% the other if there is no selection pressure of any form.

Some authors have reported that segregation of alleles occurred according to expected Mendelian ratios in anther-derived populations, concluding that there is absence of any selection

pressure, e.g. Winzeler et al. (1987) in wheat, Lashermes et al. (1988) in maize, Schon et al. (1990), and Chen and Beversdorf (1990) in rapeseed, but some claim otherwise.

RAPD (random amplified polymorphic DNA) (Williams et. al. 1990) analysis is a DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence. RAPD polymorphism can be used to distinguish genetically distinct individuals. The polymorphisms are usually inherited in a Mendelian fashion, so any significant deviation based on c^2 analysis can be easily determined as distorted segregation. Moreover RAPDs are dominant markers. So, while RAPD markers would not be of too much value in a heterozygous population, in a monoploid population such as the one used in this study, segregation ratios can be reliably studied. In a study conducted by Singsit and Ozias-Akins (1993), genetic relationships among monoploid potato were determined using RAPD markers.

The objectives of this study were:

1. To examine segregation patterns of anther-derived monoploid potato using RAPDs
2. To establish linkage groups among RAPD markers in this segregating population.

2.1 MATERIALS

2.1 Plant Materials

The plant material used in this study was a heterozygous diploid hybrid, CP2 obtained by crossing the *Solanum chacoense* (*chc*) clone, 80-1, as the female parent to the *Solanum phureja* (*phu*) clone, BARD 1-3, as the male. The CP2 hybrid was subjected to anther culture (Guha and Maheshwari 1966).

2.1.1 Anther culture of CP2

The procedure described by Taylor and Veilleux (1992) was adapted for the anther culture process. Buds containing anthers with microspores in the uni- or binucleate stage were surface sterilized in 70% ethanol for 30 sec and in 100% commercial bleach [Wonder Chemical Corp., 5.24% (w/v) sodium hypochlorite] containing one drop of 'Tween 20' for 5 min. The buds were then washed twice in sterile distilled water. Anthers from the buds were distributed randomly to five Delong culture flasks (Bellco Glass Co., Vineland, NJ) containing 15 ml anther culture medium, until each flask had 30 anthers. The medium was prepared according to Uhrig's (1985) procedure with some modifications. LS basal medium (Linsmaier and Skoog, 1964) supplemented with 100 mg/l myo-inositol, 6% sucrose, 0.4 mg/l thiamine and 2.5 mg/l N6-benzyladenine (BA), and 0.1 mg/l indole-3-acetic acid (IAA). The pH of the medium was adjusted to 5.8 and 2.5 g/l activated charcoal was added. The medium was sterilized

by autoclaving at 121°C and 1.1 kg/cm^2 for 20 min. The flasks were covered with Magenta 2-way caps (Magenta Plastics, Chicago, IL.), sealed with parafilm, and maintained in the dark on a shaker at 120 rpm for six weeks. The embryos were placed on regeneration medium (Taylor and Veilleux, 1992) to convert to plantlets. The plantlets were maintained *in vitro* on MS basal medium.

2.1.2 Ploidy determination

The ploidy of the regenerants was determined by flow cytometry (Owen et al. 1988) to develop the monoploid family. One gram of in vitro plant material was placed on a 6 cm glass petri plate on ice. Three ml chopping buffer containing 38.4 mM magnesium chloride, 12mM sodium citrate, 8mM MOPS, and .04% Triton X-100 was added to the petri plate and the plant sample was chopped with a sharp razor blade for 3 min. The mixture was collected in a clean glass beaker after passing through a 250 mm and a 63 mm mesh. One ml filtrate in a microcentrifuge tube was incubated with 0.5 ml RNAse (Sigma R 5503) for half an hour at room temperature and later with propidium iodide solution for 1-3 h on ice. Ploidy determination was performed at the Virginia-Maryland Regional College of Veterinary Medicine on the stained samples after filtration through a 37mm nylon mesh. The Coulter Epics laser flow cytometer (Coulter Electronics, Hialeah, FL) was used.

The regenerants identified as monoploids which were derived over a period of 12-15 months and propagated onto two types of MS

basal medium, a solid and a liquid type. The first type (MS salts, 3% sucrose, 100 mg/l myo-inositiol, 100mg/l casein hydolysate) was solidified using 0.7% Phytagar and the second type was essentially the same medium without agar. MS slants with 20 ml solidified medium containing shoot tips of the monoploids with at least two nodes were placed at 20°C under white flourescent lights at a 16 h photoperiod and 175 mmol $sec^{-1}m^{-2}$ light intensity. Nodal cuttings of the monoploids in 20 ml liquid basal medium were placed in flasks on a shaker at 120 rpm under the same settings. Copies of the anther donor (CP2) and the parents *chc* 80-1 and *phu* 1-3 were also propagated similarly. Cuttings in the liquid medium have a faster growth rate.

2.1.3 DNA Extraction

DNA was extracted from in vitro plantlets by the method of Singsit and Ozias-Akins (1993). One g of the plant material was placed in a mortar (frozen overnight) and 20 ml liquid nitrogen was added to facilitate crushing of the plant material using the pestle. Extraction buffer (3.3 ml: 0.1 M Tris-HCl, 1.4 M NaCl, .02 M EDTA, 2% CTAB, 1% 2-mercaptoethanol, ph 8.0) was added to the powdered plant material and it was further ground. A micro-centrifuge tube containing 300 ml of the mix was incubated in a water bath at 60°C for 2 h. A 300 ml mix of a chloroform:isoamyl alcohol (24:1) solution was added to the mix and the microcentrifuge tube was inverted about 10 times to mix the contents. The tube was centrifuged for 15

min at 1500 rpm. The clear, aqueous supernatant was transferred to a sterile microcentrifuge tube containing 200 ml cold isopropanol and the contents of the tube were mixed gently by shaking to precipitate DNA. The contents were stored in a -20°C freezer overnight and the thawed samples were subjected to centrifugation at 1500 rpm for 10 min. The supernatant was discarded and the pellet was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and incubated with RNAse (10 ml/ml) for 10 min. The concentration of DNA was measured using a mini-fluorometer (Hoefer Scientific Instruments) in ng/ml at the absorbance of 510 nm.

2.2 METHODS

2.2.1 RAPD Reaction

PCR was performed on the DNA samples of the monoploids, their anther donor CP2 and its parents *chc* 80-1 and *phu* 1-3. After determining DNA concentrations on the minifluorometer, a working solution of 10 ng/ml was prepared. The PCR mix (Perkin Elmer) (25ml) contained 50 ng genomic DNA, 0.6 mM primer, 200 mM dNTPs (dATP, dTTP, dGTP, dCTP), 1 unit Taq polymerase, 2.5 mM $MgCl_2$, and 1X concentration of 10X DNA buffer in double distilled water. In other words, 25ml of mix contained 11.3ml double distilled water, 2.5ml $MgCl_2$, 2.5ml 10X buffer, 2ml dNTPs 0.2ml Taq polymerase, 1.5ml primer and 5ml DNA. A drop of mineral oil was added to the reaction mix and the amplification was performed using

a Perkin Elmer Cetus Model 460 thermal cycler under the following conditions: 45 cycles denaturation at 94°C for 1 min, annealing at 37°C for 1 min, extension at 72°C for 2 min. This was followed by 4 min at 72°C and a 4°C soak until recovery.

2.2.2 Gel Electrophoresis

To the reaction tube, 5 ml tracking dye [0.25% bromphenol blue, 0.25% xylene cyanol FF, 15% Ficoll (type 400, Pharmacia) in water] was added, and the amplified products were resolved by 1.4% agarose gel. The conditions of Sambrook et al. (1989) for agarose-TBE gel electrophoresis were adopted.

2.2.3 Scoring bands

The banding patterns of the two parents, *chc* 80-1 and *phu* 1-3, the F_1, CP2, and the 20 identified anther-derived monoploids were studied. RAPD bands that were polymorphic among the monoploids and that were present in either of the two parents were scored. The presence of an allele, as indicated by the presence of a band was indicated as a "1" and the absence of the corresponding allele was scored as a "0".

2.2.4 Data Analysis

The pooled data of all bands scored was analyzed using Linkage 1 (Suiter et al. 1983) software. Chi-square analysis was performed

using 'Single Locus Goodness of Fit' test to study the segregation pattern. Linkage 1 was used to observe potentially linked loci, if any. The data were also run through the MAPMAKER software (Lander et al. 1987) version 3.0b, in order to assign the various loci to linkage groups and to perform two-point analyses to obtain distances between linked loci in centimorgans.

2.3 RESULTS AND DISCUSSION

The results of the PCR amplification varied. Some primers did not amplifiy DNA whereas others produced discrete bands. Forty primers were picked at random from OPC Kits A, C and G. Of these 13 produced scorable polymorphic bands (Table 1). Each polymorphic band was scored as a different marker (Williams et al. 1990), since single primers of arbitrary sequence can amplify genomic DNA and the target sequences amplified by the different primers are associated with different loci. The RAPD markers were named after the primer that produced the polymorphic band and the location of the band on the gel.

A total of 133 bands was scored, of which 50 were polymorphic (Table 2). The number of scorable polymorphic bands per primer varied from 1 (Primer C-7) to 7 (Primer C-1), thus averaging about 3.8 bands per primer. This was lower than the value of 7.2 RAPD bands per primer obtained by Hosaka and Hanneman (1993) for F_2 of the same parental species, *S. chacoense* and *S. phureja*.

In the present study, "0/1" banding pattern for the individuals

(Table 3) for all 50 loci was analyzed using the Linkage 1 program. The single locus goodness of fit tests provided the chi-square values for every marker. Twenty nine markers had a c^2 value between 0 and 3.9 which was a fit to a 1:1 ratio (Table 4). But 21 of the markers had significantly skewed segregation (Table 5). The skewness of the segregation was not equally distributed towards both parents but was greater towards the 80-1 parent (76%) with 16 of the 21 loci that segregated in a distorted manner favoring the *chc* allele.

In a study of segregation and linkage analysis of RAPD markers in microspore-derived population of oilseed rape, Tanhuanpaa et al. (1993) found that five RAPD markers revealed a significant segregation distortion in favor of 'Topas', the highly embryogenic cultivar. It was suggested that regions affecting 'microspore culture ability' segregated in a manner different from the normally expected 1:1 ratio.

In other studies conducted on different plant species, skewed segregation in AC progenies have always been in favor of the more 'anther culture competent' parent. Table 6 gives a list of plants and percentage of skewed markers in favor of the more responsive parent.

Of the two parents *chc* 80-1 and *phu* 1-3 used in our study, earlier work has shown that the *phu* clone is more responsive to anther culture than *chc*. But only 24% of the skewness was in favor of the 1-3 alleles. Greater segregation distortion in this experiment was associated with the less 'anther-culture competent' parent.

It is already known that *chc* 80-1 is a less anther culture competent clone. In the F_1 generation, these genes will be present in the heterozygous condition. When this F_1, i.e., CP2 is subjected to AC, Wenzel's (1979) 'monoploid sieve' comes into operation when haploids are produced. Since we are selecting for segregating alleles, no lethal alleles will be seen. The allele associated with deleteriousness is reduced in frequency and its complement surviving the sieve is inherited by the haploid. This could potentially explain the greater proportion of the 80-1 alleles. This would also be true if the *phu* clone has more deleterious genes.

The remaining 24% skewness in favor of the *phu* 1-3 parent could be due to the linkage of these markers with a gene/genes associated with anther culture selection or could also be linked to lethal or deleterious *chc* alleles. During haploidization, the population that receives the potentially deleterious allele either does not start the embryonic pathway or perishes during regeneration.

In a study on *S. chacoense* by Birhman et al. (1994) RFLP analysis of the AC plants of the F1 hybrid revealed no selection pressure in favor of the more responsive parent. Similar results were also seen in *S. chacoense* by Rivard et al. (1994) who surmised that gametic selection occurring *in vitro* was no different from that occurring *in vivo*. Rivard et al. (1994) warned that androgenic plants regenerated from single calli would not be genetically distinct and conclusions based on such a population would be incorrect.

Based on this, only regenerants of different calli were used, thereby trying to eliminate the possibility of including genetically identical genotypes. But monoploid regenerants CP2 170 and CP2171, from one of the early experiments, produced very similar RAPD profiles. (Table 3). Only 4 of 30 markers differed in the two clones. Repeating the experiment would very possibly prove the genetic similarlity of these clones.

Three point analysis to arrange the markers to a chromosomal segment failed because of two reasons. Firstly, the basic assumption of the Mapmaker program is that the segregation pattern is the Mendelian 1:1 pattern (Foisset and Delorme, 1996). Our data however, have very significant distortion ratios. Secondly for a reliable genetic map to be constructed, Mapmaker requires a large number of markers and/or large population sizes. An extended study to generate more markers will help in map construction.

Since a considerable proportion of markers used in the suudy exhibited skewness, analyzing the entire data to find linked loci would provide erroneous results. So only the normally segregating markers were again subjected to Linkage analysis. Twenty seven pairs of linked loci (at 98% probability) were obtained with linkage distances ranging from 11 to 25 map units. (Fig. 7).

In conclusion, molecular markers can be used as a valuable tool to study segregation ratios in haploid potato populations. Distorted segregation, though not expected is still seen due to selection pressure during haploidization. With a suitably large sample

population, and sufficiently large number of markers showing normal segregation, RAPD maps can be constructed using haploid populations.

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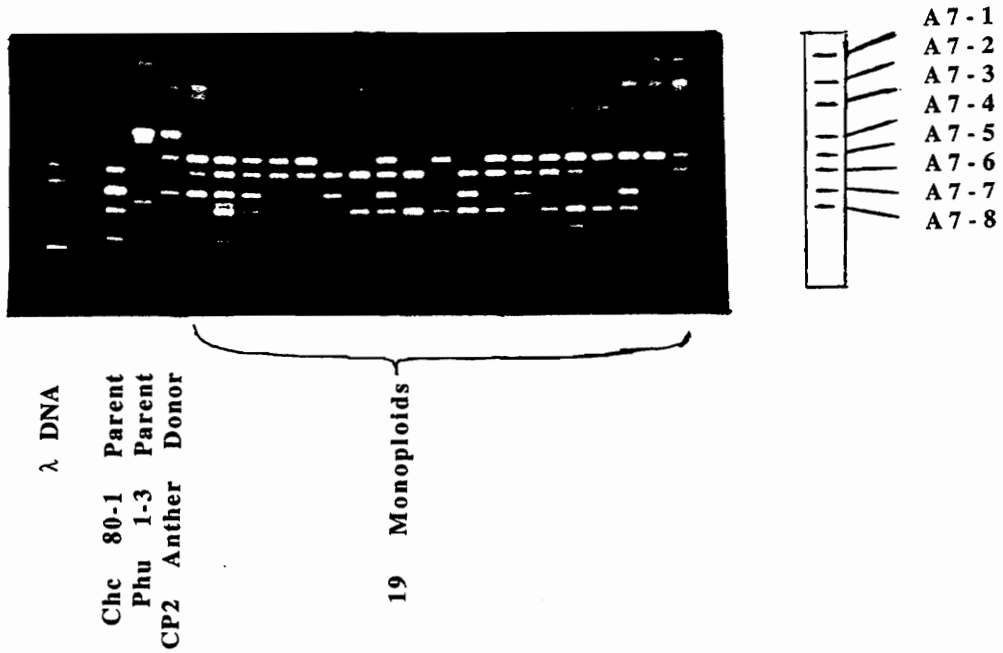

Fig. 1. Ethidium Bromide stained gel of PCR amplified RAPD fragments generated by OPC primer A7 for the parents of the anther donor (*chc* 80-1 and *phu* 1-3), the anther donor (CP2) and 19 anther-derived monoploids. A key to specific bands is on the right. Locus A7-5 exhibits skewed segregation as it appears in 15 of the 19 monoploids whereas locus A7-7 exhibits normal segregation, appearing in 8 of 19 monoploids.

Table 1. Nucleotide sequences (5'-3') of oligonucleotide primers yielding polymorphism in RAPD analysis of CP2 monoploids.

Primer	Nucleotide Sequence
C1	TTCGAGCCAG
C5	GATGACCGCC
C7	GTCCCGACGA
C8	TGGACCGGTG
C10	TGTCTGGGTG
C13	AAGCCTCGTC
C16	CACACTCCAG
C17	TTCCCCCCAG
C18	TGAGTGGGTG
C19	GTTGCCAGCC
C20	ACTTCGCCAC
A7	GAAACGGGTG
A14	TCTGTGCTGG

Table 2. Polymorphic bands of total RAPD bands scored in the anther-derived monoploids of CP2.

Primer	Total number of bands scored	Number of polymorphic bands scored
C1	14	7
C5	10	3
C7	2	1
C8	11	4
C10	15	5
C13	10	3
C16	9	3
C17	11	3
C18	12	4
C19	6	3
C20	8	4
A7	12	5
A14	13	5
Total=	133	50

Table 3. Polymorphic RAPD bands from a population of 20 CP2 monoploids, their anther donor, CP2, and parents Chc 80-1 and Phu 1- 3. "1" = band present; "0" = band absent.

					CP2 MONOPLOIDS																			
Locus	**80-1**	**1-3**	**CP2**	**7**	**103**	**155**	**165**	**170**	**171**	**200**	**256**	**303**	**308**	**314**	**318**	**319**	**331**	**335**	**337**	**341**	**354**	**373**	**374**
A7-1	0	1	1	0	.	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	0	0
A7-2	1	0	1	0	.	1	1	0	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0
A7-3	1	0	1	1	.	1	1	0	0	1	0	1	0	1	1	0	1	0	0	0	1	0	0
A7-4	1	0	1	1	.	1	1	1	1	0	0	1	0	1	0	1	1	1	1	1	1	1	1
A7-5	0	1	1	0	.	0	1	1	0	1	0	0	0	0	0	1	0	0	0	0	1	1	1
C13-1	0	1	1	1	.	1	0	0	0	1	.	0	0	1	0	1	1	1	0	1	1	0	0
C13-2	1	0	1	1	.	1	1	0	0	0	.	1	0	1	1	1	1	1	0	1	1	1	1
C13-3	0	1	1	1	.	1	0	0	0	1	.	1	0	1	1	1	1	1	0	1	1	0	0
C17-1	0	1	1	0	.	0	1	0	0	.	0	1	0	1	0	1	0	0	1	1	1	1	0
C17-2	1	0	1	1	.	1	1	1	1	.	1	1	0	1	1	1	0	1	1	1	1	1	1
C17-3	1	0	1	0	.	1	1	1	0	.	1	1	1	0	0	1	0	0	1	1	0	1	1
C5-1	1	0	1	1	.	1	1	.	.	.	.	1	.	1	1	0	0	0	.	1	1	1	1
C5-2	1	0	1	1	.	1	1	.	.	.	.	1	.	1	0	0	0	0	.	0	0	1	1
C5-3	0	1	1	0	.	1	0	.	.	.	1	0	.	0	0	1	1	1	.	1	1	0	0
C10-1	.	1	1	1	.	0	0	0	0	.	0	0	0	0	0	1	1	0	0	0	1	0	0
C10-2	.	1	1	1	.	0	0	0	0	.	0	1	0	1	0	1	1	0	1	1	1	0	0
C10-3	.	1	1	1	.	0	1	1	1	.	1	1	1	0	0	1	1	0	1	1	1	1	0
C10-4	.	0	1	0	.	0	1	1	1	.	1	0	1	0	0	1	0	0	1	0	1	1	1
C10-5	.	0	1	0	.	0	1	0	0	.	1	0	1	0	0	1	0	1	0	0	1	1	1
C20-1	0	1	1	0	.	0	0	1	1	0	0	0	1	0	0	1	1	0	1	0	1	1	0
C20-2	0	1	1	1	.	1	1	0	0	0	0	1	1	1	1	1	1	1	1	1	1	1	1
C20-3	0	1	1	1	.	1	1	1	1	1	0	1	0	1	0	1	0	0	1	1	1	1	1
C20-4	1	0	1	0	.	1	1	1	1	1	0	1	1	1	1	0	0	0	1	1	0	1	1

C18-1	.	0	1	0	.	.	1	1	1	0	0	1	1	1	0	0	0	1	0	0	1	1	0
C18-2	.	1	1	0	.	.	1	1	1	0	1	1	1	0	0	1	1	0	1	1	0	0	0
C18-3	.	1	1	1	.	.	1	1	1	1	1	1	0	1	0	1	0	0	1	1	1	1	1
C18-4	.	1	1	1		.	1	1	1	1	0	1	1	1	0	0	1	1	1	1	1	1	1
C19-1	0	1	1	0	0	0	0	1	0	1	0	0	1	0	1	1	1	0	1	0	1	0	1
C19-2	0	1	1	0	0	0	1	1	0	1	0	1	0	0	1	1	1	1	0	1	0	0	0
C19-3	1	0	1	1	1	1	1	0	1	1	0	0	0	1	0	1	0	1	1	0	0	1	1
C7-1	.	.	1	0	.	0	0	0	.	0	1	1	0	1	1	1	1	0	0	1	1	1	0
C8-1	1	0	1	1	1	1	1	1	.	.	.	1	0	1	1	1	0	1	1	0	1	0	1
C8-2	1	0	1	1	1	1	1	1	.	.	.	1	0	1	0	1	0	1	1	0	1	1	1
C8-3	0	1	1	0	1	1	0	1	.	.	.	1	1	1	1	1	1	0	1	1	1	1	0
C8-4	0	1	1	1	1	1	1	1	.	.	.	1	1	1	1	0	1	1	1	1	1	1	1
C16-1	1	0	1	1	0	0	0	0	.	.	.	0	0	1	1	0	0	1	0	0	0	0	0
C16-2	1	0	1	1	0	1	1	1	.	.	.	1	.	1	1	1	1	1	0	1	0	0	0
C16-3	1	0	1	0	0	0	0	0	.	.	.	1	.	0	0	1	1	0	0	0	1	0	0
C1-1	1	0	1	0	1	0	0	0	.	.	.	1	0	1	1	0	1	0	0	1	1	0	1
C1-2	1	0	1	0	1	0	0	0	.	.	.	1	0	1	0	1	0	1	0	1	1	0	0
C1-3	0	1	1	1	0	1	1	1	.	.	.	1	1	1	1	1	1	1	0	1	1	1	1
C1-4	1	0	1	1	0	0	1	0	.	.	.	1	0	1	1	1	1	0	0	1	1	1	1
C1-5	0	1	1	1	1	1	0	1	.	.	.	1	1	1	1	1	1	1	0	1	1	0	1
C1-6	1	0	1	1	1	1	1	1	.	.	.	1	1	1	1	1	1	0	1	1	1	1	1
C1-7	0	1	1	1	1	1	1	1	.	.	.	1	1	0	0	1	1	1	1	0	0	1	1
A14-1	1	0	1	0	0	1	1	0	.	.	.	1	0	0	0	0	0	1	0	0	0	0	1
A14-2	0	1	1	1	1	1	1	1	.	.	.	0	1	.	1	0	1	1	1	1	0	0	0
A14-3	0	1	1	0	1	1	1	1	.	.	.	1	0	.	0	1	1	0	1	1	1	1	1
A14-4	0	1	1	1	0	0	0	0	.	.	.	0	0	.	0	0	0	1	0	1	0	0	0
A14-5	0	1	1	0	1	1	1	0	.	.	.	1	0	.	0	0	0	1	1	0	0	1	1

Table 4. RAPD markers with non-significant χ^2 value for segregation (χ^2 value <=3.9)

Locus	chc:phu ratio
A7-1	12:7
A7-3	8:11
A7-5	8:11
C13-1	9:9
C13-2	13:5
C13-3	11:7
C17-1	8:10
C17-2	12:6
C5-1	10:4
C5-2	7:7
C5-3	7:7
C10-2	8:10
C10-3	13:5
C10-4	8:10
C10-5	8:10
C20-1	8:11
C20-4	13:6
C18-1	9:9
C18-2	10:8
C19-1	9:11

C19-2	9:11
C7-1	9:9
C16-2	11:5
C1-1	8:9
C1-2	7:10
C1-4	11:6
A14-1	5:11
A14-2	11:5
A14-5	8:8

Table 5. Markers showing significant segregation distortion (χ^2 values >=4.0) in favor of both chc 80-1 and phu 1-3 and linkage groups bearing the markers.

Skewed segregation (χ^2>=4.0) in favor of			
Chc 80-1		Phu 1-3	
Locus	chc:phu ratio	Locus	chc:phu ratio
A7-2	15:4	C10-1	4:14
A7-4	15:4	C18-1	4:14
C17-3	15:3	C16-3	4:12
C20-2	15:4	A14-4	3:13
C20-3	14:5	C16-1	4:13
C18-3	14:4		
C18-4	15:3		
C8-1	13:4		
C8-2	13:4		
C8-3	13:4		
C8-4	16:1		
C1-3	15:2		
C1-5	16:1		
C1-6	14:3		
C1-7	13:4		
A14-3	12:4		

Table 6. Evidence of segregation distortion in favor of the more anther culture competant parent in some plant spp.

Plant sp.	% distortion in favor of the responsive parent	Reference
Barley	100%	Thompson et.al. 1991
"	68%	Graner et.al. 1991
"	86%	Zivy et.al. 1992
Rice	50%	Guiderdoni, 1990
Maize	96%	Bentotilla et.al. 1992
"	83%	Murigneux et.al 1993

Table 7. List of linked loci as obtained by Linkage 1 analysis of normally segregating markers. Probability of recombination and distance in centimorgans is given.

Pairs of linked loci	Probability (p)	Recombination distance (cM)
A7-1/C1-2	0.016	25.0
A7-5/A14-2	0.025	20.0
C13-1/C13-3	0.001	11.1
C13-1/C5-3	0.008	15.4
C13-1/C20-4	0.009	22.2
C13-2/C13-3	0.026	22.2
C13-2/C1-4	0.004	12.5
C13-3/C10-2	0.024	23.5
C13-3/C10-4	0.001	11.8
C13-3/C1-2	0.016	25.0
C17-1/C10-2	0.02	22.2
C17-2/A14-5	0.029	26.7
C5-1/C5-2	0.018	21.4
C5-1/C5-3	0.018	21.4
C5-1/C20-4	0.006	14.3
C5-2/C5-3	0.008	14.3
C5-2/A14-5	0.021	16.7
C10-3/C20-1	0.019	27.8

C10-3/C18-2	0.006	17.7
C10-4/C10-5	0.015	22.2
C10-4/C20-1	0.015	22.2
C10-4/C16-2	0.013	20.0
C20-1/A14-1	0.01	20.0
C19-2/C16-2	0.007	18.8
C7-1/C1-1	0.012	18.8
C7-1/C1-4	0.007	18.8
A14-1/A14-5	0.007	18.8

APPLICATION OF RAPD MARKERS TO CHARACTERIZE PUTATIVE SOMATIC HYBRIDS

by

Vidya Ravichandran

Richard E. Veilleux, Chairman

Horticulture

(ABSTRACT)

Protoplasts were isolated from two independently derived monoploid ($2n=1x=12$) potato clones 13-14 203 and CP2 103. Fusions were conducted in PEG 22.5 medium using Ca^{2+} washing medium. Regenerants from p-calli of fusion plates and of unfused protoplasts, used as the control, were transferred to basal medium and cultured *in vitro*.

DNA was extracted from *in vitro* copies of the monoploids, putative somatic hybrids following protoplast fusion and somaclones derived by regeneration of the CP2 103 monoploids. Protoplasts of 13-14 203 did not regenerate. PCR amplification was conducted, followed by RAPD (Random Amplified Polymorphic DNA) resolution on 1.4% agarose gel. Banding patterns were studied to try to determine the true nature of the putative hybrids.

All five putative hybrids in this study carried markers unique to one parent (CP2 103). One of the five somatic hybrids exhibited markers unique to the other monoploid (13-14 203). Quantitative estimation of DNA by flow-cytometry of propidium-iodide stained nuclei of in vitro plantlets revealed that two of the somatic hybrids were hexaploid ($2n=6x=72$) and another was tetraploid ($2n=4x=48$).

It was thus ascertatined that one of the five putative hybrids had been derived from genomes of both monoploid parents.

CHAPTER III

CHARACTERIZATION OF PUTATIVE SOMATIC HYBRIDS OF MONOPLOID POTATO USING MOLECULAR MARKERS

INTRODUCTION

Potato is a vegetatively propagated crop, and it also responds well to tissue culture. So somatic hybridization in potato is an interesting subject of study. Successful interspecific protoplast fusion can overcome the tediousness and reduce the time consumed in classical breeding to produce the desired interspecific hybrid (Mollers and Wenzel, 1991).

There are several reports of the successful production of interspecific somatic hybrids (Pelletier et al., 1988; Glimelius et al., 1991) and intraspecific somatic hybrids (Austin et al., 1985; Deimling et al., 1988) at the tetraploid and the dihaploid levels. Very little has been reported about protoplast culture of monoploid potatoes. But Sree Ramulu et al. (1987) reported successful plant regeneration from monoploid potato and Uijtewaal et al. (1987) successfully fused protoplasts of monoploids to create somatic hybrids for the first time.

Monoploids, with their single set of chromosomes, represent a population free of lethal and deleterious alleles. Combining monoploids to produce somatic hybrids can enhance heterosis without the reassortment of dominant characters occurring at meiosis (Fish et al., 1987). More importantly, the sterility problems of the monoploids can be bypassed by using the somatic hybrids for

breeding purposes (Karp et al., 1987).

Characterization of putative somatic hybrids constitutes a very important step in somatic hybridization experiments. Several different methods have been used by researchers. Selection based on morphological characters (Austin et al., 1985; Waara et al., 1989) isozyme analysis (Chaput et al., 1990), RFLP analysis (Pehu et al., 1990; Matteij et al., 1992), and RAPDs (Baird et al., 1992). A comparison of morphological, cytological, isozyme and RAPD analysis revealed that RAPD analysis is a fast, non-laborious and reproducible method for identification of potato somatic hybrids (Rasmusssen and Rasmussen, 1995).

The objective of this study is to characterize putative somatic hybrids of monoploid potato clones using RAPD analysis

3.1 MATERIALS AND METHODS

3.1.1 Plant materials

The plant materials used in this experiment were two independently obtained monoploid clones 13-14 203 (*S. phureja*) and CP2 103 (*S. chacoense X S. phureja*) derived by anther culture as part of other experiments.

3.1.2 Protoplast isolation

In vitro copies of the two monoploids propogated on MS basal medium were placed in the refrigerator 48 h prior to protoplast

isolation. In vitro shoots and leaves were cut into fine pieces in a 10 cm petri plate and preplasmolysis medium (See appendix for composition of all media in the experiment) was added to it. After 1/2 h incubation, the preplasmolysis medium was replaced with enzyme medium and the cultures were incubated in the dark overnight. Protoplasts were gently loosened from the debris by swirling the petri plates. The mix was passed through a sterile 63 mm filter, the plate washed with 3 ml rinse medium that was added to the protoplast solution through the filter. The solution was centrifuged at 50 x g for 5 min in a sterile centrifuge tube.

The supernatant was discarded and the pellet resuspended in 10 ml flotation medium. About 1 ml rinse medium was dripped gently along the sides of the tube on top of the protoplasts in the flotation medium so that the two layers of media remained distinct. The tube was centrifuged again at 50 x g for 5 min. The protoplasts formed a band at the interface of the rinse and high sucrose medium. They were collected with a sterile pipette and transferred to sterile 15 ml centrifuge tubes and rinse medium was added to make a total of 10 ml. The tube was recentrifuged at 50 x g for 5 min and the supernatant discarded. The protoplast pellet was resuspended in CPW 13 M medium to a density of $1x10^6$.

3.1.3 Protoplast fusion

A sterile cover slip was placed on a drop sterile mineral oil in the middle of a sterile petri plate. Three drops of 13-14 203 and CP2

103 protoplasts in CPW 13M medium were placed on the cover slip and left undisturbed for 10 min to allow protoplasts to settle and stick to the cover slip. After adding six drops of PEG 22.5 around the protoplasts and a drop in the center, the plates were left undisturbed at room temperature for 20-25 min. Then, a single drop of Ca^{2+} washing medium was added every 5 min to the culture and a drop removed from the opposite side. This was repeated five times. Next, three drops of culture medium were added from one side and calcium medium was removed from the other. This step was repeated three times. Finally, the cover slip was flooded with culture medium and a few drops were placed on the plate to maintain humidity. The plates were sealed and incubated in dim light at 25°C. Control plates of unfused protoplasts were prepared to contain a protoplast density of 2.5×10^5 per ml. Fresh medium was periodically added to the plates.

3.1.4 Transfer of p-calli

Continuous division of cells led to the development of visible cell colonies (p-calli). P-calli were transferred to differentiation medium and placed in light. As regenerants appeared, the shoots were excised and transferred to basal medium. This was done for regenerants from both the fusion and control plates.

3.2 Methods

3.2.1 Ploidy determination

The ploidy of three of the regenerants was determined by flow cytometry (Owen et al. 1988). One gram of in vitro plant material was placed on a 6 cm glass petri plate on ice. Three ml chopping buffer containing 38.4 mM magnesium chloride, 12mM sodium citrate, 8mM MOPS, and .04% Triton X-100 was added to the petri plate and the plant sample was chopped with a razor sharp blade for 3 min. The mixture was collected in a clean glass beaker after passing through a 250 mm and a 63 mm mesh. One ml filtrate in a microcentrifuge tube was incubated with 0.5 ml RNAse (Sigma R 5503) for half an hour at room temperature and later with propidium iodide solution for 1-3 h on ice. Ploidy determination was performed at the Virginia-Maryland Regional College of Veterinary Medicine on the stained samples after filtration through a 37mm nylon mesh. The Coulter Epics laser flow cytometer (Coulter Electronics, Hialeah, FL) was used.

3.2.2 DNA Extraction

DNA was extracted from in vitro plantlets of 13-14 203, CP2 103, and the five putative somatic hybrids by the method of Singsit and Ozias-Akins (1993). One g of the plant material was placed in a mortar (frozen overnight) and 20 ml liquid nitrogen was added to facilitate crushing of the plant material using the pestle. Extraction

buffer (3.3 ml: 0.1 M Tris-HCl, 1.4 M NaCl, .02 M EDTA, 2% CTAB, 1% 2-mercaptoethanol, ph 8.0) was added to the powdered plant material and it was further ground. A micro-centrifuge tube containing 300 ml of the mix was incubated in a water bath at 60°C for 2 h. A 300 ml mix of a chloroform:isoamyl alcohol (24:1) solution was added to the mix and the microcentrifuge tube was inverted about 10 times to mix the contents. The tube was centrifuged for 15 min at 1500 rpm. The clear, aqueous supernatant was transferred to a sterile microcentrifuge tube containing 200 ml cold isopropanol and the contents of the tube were mixed gently by shaking to precipitate DNA. The contents were stored in a -20°C freezer overnight and the thawed samples were subjected to centrifugation at 1500 rpm for 10 min. The supernatant was discarded and the pellet was dissolved in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and incubated with RNAse (10 ml/ml) for 10 min. The concentration of DNA was measured using a mini-fluorometer (Hoefer Scientific Instruments) in ng/ml at the absorbance of 510 nm.

3.2.3 RAPD Reaction

PCR was performed on the DNA samples of 13-14 203, CP2 103, and the five putative hybrids. After determining DNA concentrations on the mini fluorometer, a working solution of 10 ng/ml was prepared. The PCR mix (Perkin Elmer) (25ml) contained 50 ng of genomic DNA, 0.6 mM primer, 200 mM dNTPs (dATP, dTTP, dGTP,

dCTP), 1 unit Taq polymerase, 2.5 mM $MgCl_2$, and 1X concentration of 10X DNA buffer in double distilled water. In other words, 25ml of mix contained 11.3ml double distilled water, 2.5ml $MgCl_2$, 2.5ml 10X buffer, 2ml dNTPs, 0.2ml Taq polymerase, 1.5ml primer and 5ml DNA. A drop of mineral oil was added to the reaction mix and the amplification was performed using a Perkin Elmer Cetus thermal cycler under the following conditions: forty five cycles denaturation at 94°C for 1 min, annealing at 37°C for 1 min, extension at 72°C for 2 min. This was followed by 4 min at 72°C and a 4°C soak until recovery.

3.2.4 Gel Electrophoresis

To the reaction tube, 5ml tracking dye [0.25% bromphenol blue, 0.25% xylene cyanol FF, 15% Ficoll (type 400, Pharmacia) in water] was added, and the amplified products were resolved by 1.4% agarose gel. The conditions of Sambrook et al. (1989) for agarose-TBE gel electrophoresis were adopted.

3.2.5 Scoring bands

The banding patterns of the two fusion parents, 13-14 203, CP2 103, and the five putative somatic hybrids were studied. RAPD bands that were polymorphic among the monoploids and that were present in either of the two parents were scored. The presence of an allele, in other words, the presence of a band was indicated as a "1" and the absence of the corresponding allele was scored as a "0".

3.3 Results and Discussion

Thirty primers were used to screen the two parents and the five putative somatic hybrids. Of the primers that produced scorable bands, six primers (OPC7, OPC8, OPC9, OPC12, OPC16, OPA7), produced inconclusive banding patterns if a primer amplified a band on the first parent and on the putative somatic hybrids, but failed to amplify a different band on the second parent and the putative hybrids.

Primer OPC2 revealed a band (C2-3) present on parent CP2 103 but not 13-14 203 and also present in four of the five putative hybrids (SH2, SH3, SH5, SH7) and a second band (C2-1) present in the other parent (13-14 203) but not CP2 103 and in SH2.

Primer OPA2 produced markers A2-1 on all five putative hybrids and CP2 103. Marker A2-13 was present exclusively on monoploid 13-14 203 and SH2.

Primer OPG3 produced a band G3-4 present uniquely on CP2 103 and SH2 and SH3 (Fig. 1). These results confirm that CP2 103 contributed to all of the putative somatic hybrids. However, bands unique to 13-14 203 were found only in SH2.

The ploidy level of three of the putative somatic hybrids (SH1, SH2, SH3) was determined. SH1 and SH2 were hexaploid and SH3 was a tetraploid. Chromosomal counting was not performed on the samples. It is possible that the somatic hybrids were aneuploids, as were the somatic hybrids in the monoploid potato fusion experiment

performed by Uijtewaal et al. (1987). The output of the flow cytometer results is shown in Figure 2.

RAPD analysis was used by Rokka et al. (1994) to characterize somatic hybrids of dihaploid potato. Most of the somatic hybrids obtained in their experiment were aneuploids. Genetic instability during tissue culture may be a cause for aneuploid production. Tetraploids were generated by Barsby et al. (1984). Tetraploids may be the result of fusion of the diploid numbers of the parents. Triploids could be the result of fusion of two cells of one parent and one cell of the other. Spontaneous doubling of triploids may result in hexaploid production.

In using RAPDs to confirm somatic hybrids, Takemori et al. (1994) suggested using a mixture of primers to generate complementary polymorphic bands for both parents. But three different primers conclusively proved that the putative hybrids were indeed true hybrids.

In conclusion, this study proves that arbitrarily chosen commercial decamer primers can be used to generate amplified sequences of genomic DNA that can be used to identify the genetic nature of potato plants. Dominantly inherited RAPD markers can be successfully used to characterize putative somatic hybrids derived from protoplast fusion of monoploid potato clones.

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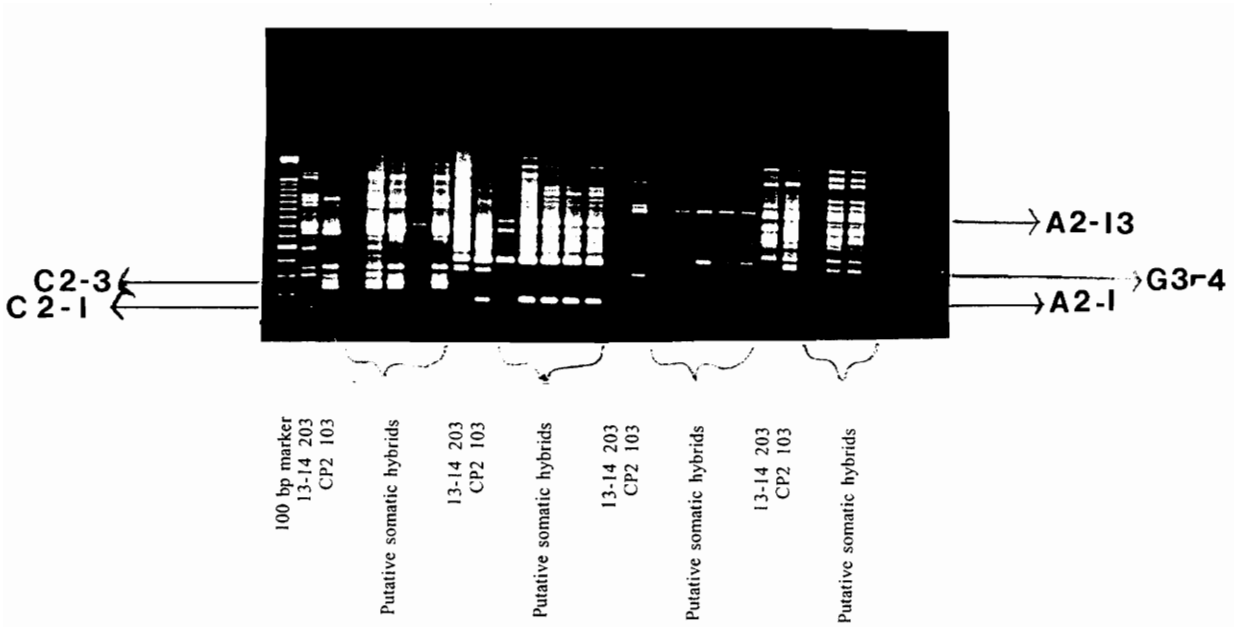
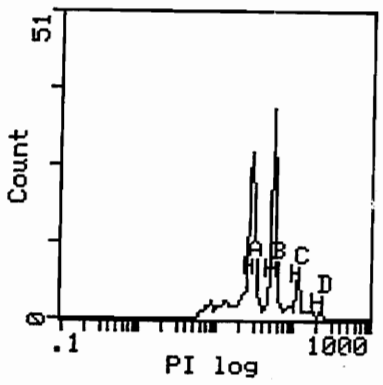
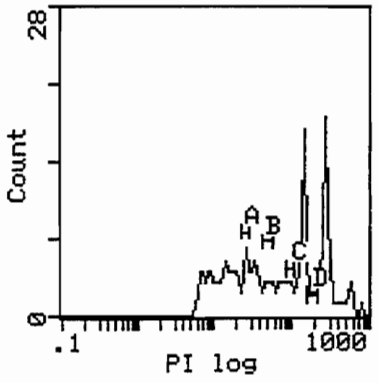


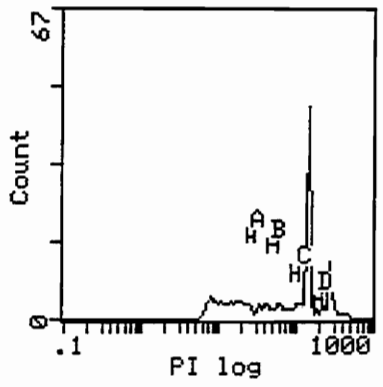
Fig. 1. Ethidium Bromide stained gel of PCR amplified RAPD fragments generated by primers C2, A2, C17 and G3 for the fusion parents, 13-14 203 and CP2 103, and five putative somatic hybrids (SH1, SH2, SH3, SH5, and SH7). Bands C2-3, A2-1, and G3-4 are unique to CP2 103 and C2-1, and A2-13 unique to 13-14 203.



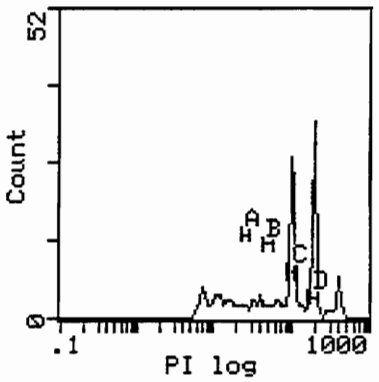
Monoploid control



Somatic Hybrid 1



Somatic Hybrid 2



Somatic Hybrid 3

Fig. 2. Comparison of flow cytometer results of somatic hybrids SH1, SH2, and SH3 with a monoploid control. SH1 and SH2 appear to be hexaploid and SH3, tetraploid.

APPENDIX

COMPOSITION OF MEDIA FOR PROTOPLAST EXTRACTION AND FUSION

ENZYME MEDIUM

Component	%
Onozuka R10 cellulase	0.5
Onozuka R10 macerozyme	0.1
Mannitol	13.0
pH 5.8	

PROTOPLAST ISOLATION (PI) MEDIUM

Component	mg l^{-1}
$CaCl_2 \cdot H_2O$	1480.0
KH_2PO_4	27.2
KNO_3	101.0
$MgSO_4 \cdot 7H_2O$	246.0
$CuSO_4 \cdot 5H_2O$	0.025
KI	0.16
pH 5.8	

PROTOPLAST CULTURE (PC) MEDIUM

Component	mg l^{-1}
$Ca(H_2PO_4)•H_2O$	100.0
$CaCl_2•2H2O$	450.0
KNO_3	2500.0
$MgSO_4•7H2O$	250.0
$NaH_2PO_4•2H2O$	170.0
$(NH_3)_2SO_4$	134.0
$CoCl_2•6H_2O$	0.025
$CuSO_4•5H_2O$	0.025
H_3BO_3	3.0
KI	0.75
$MnSO_4•4H_2O$	13.2
$Na_2MoO_4•2H_2O$	0.25
$ZnSO_4•7H_2O$	2.0
Sequestrene 330	28.0
Sucrose	10,000
Glucose	18,000
Mannitol	100,000
Inositol	100.0
Nicotinic acid	1.0
Pyridoxine-HCl	1.0
Thiamine-HCl	10.0
2,4-D	0.1
NAA	1.0
BA	1.0
pH 5.8	

PROPAGATION MEDIUM

Component	Amount (per liter)
MS1 and MS2	20 ml
MS3, MS4, MS5	10 ml
Sucrose	20 g
Myo-inositol	100 mg
KH_2PO_4	170 mg
Casein hydrolysate	500 mg
Cefotaxime[1]	250 mg
Agar 7 g	
pH 5.7	
utoclave[2]	

[1]This antibiotic is optional but is especially recommended for propagation of stock plantlets one or more clonal generations before propagation for protoplast extraction to reduce bacterial contamination. It is not autoclavable so must be filter-sterilized and added to the autoclaved medium as it cools.

[2]Filter-sterilize if using cefotaxime.

PREPLASMOLYSIS MEDIUM

Component	per 100 ml
MS2	6.7 ml
MS5	1 ml
KH_2PO_4	3.3 mg
KNO_3	10.1 mg
$MgSO_4$	600 mg
Mannitol	9 g
MES	58.6 mg
pH 5.8	
Filter-sterilize	

ENZYME MEDIUM

Component	per 100 ml
Mannitol	7.3 g
Glucose	1.8 g
Modified MS1[1]	1 ml
MS2	3 ml
MS3, MS4, MS5	0.5 ml each
Cellulase Onozuka R-10	1 g
Macerozyme R-10	0.1 g

pH 5.8
Filter-sterilize, make fresh on the day of isolation

[1](MMS1 has 9.5 g KNO3, 0.85 g KH2PO4 and 0.903 g MgSO4 per 100 ml)

RINSE MEDIUM

Component	per 200 ml
KCl	4.4 g
MS2	6 ml

pH 5.8
Autoclave

FLOTATION MEDIUM

Component	per 100 ml
Sucrose	17.1 g
MS2	3 ml

pH 5.8
Autoclave

CULTURE MEDIUM

Component	per 100 ml
Modified MS1	1 ml
MS3, MS4, MS5	0.5 ml each
MS2	3 ml
Glucose	3 g
Sucrose	1 g
Sorbitol	5 g
Casein hydrolysate	50 mg
Glutamine	10 mg
Serine	1 mg
Myo-inositol	10 mg
Thiamine	1 mg
NAA	0.125 mg
2,4-D	0.025 mg
Zeatin	0.1 mg
pH 5.8	
Filter-sterilize	

CPW 13M MEDIUM

Component	per liter
KH_2PO_4	27.2 mg
KNO_3	0.101 g
$CaCl_2 \cdot 2H_2O$	1.48 g
$MgSO_4 \cdot 7H_2O$	246 mg
KI	0.16 mg[1]
$CuSO_4 \cdot 5H_2O$	0.025 mg[2]
Mannitol	130 g
pH 5.8	
Autoclave	

[1] 18 mg/50 ml stock - use 1 ml
[2] 22.5 mg/50 ml stock - use 0.5 ml

FUSOGEN PEG 22.5 MEDIUM

Component	per 100 ml	%
PEG 8000	22.5 g	22.5
Sucrose	1.8 g	1.8
$CaCl_2 \cdot 2H_2O$	0.150 g	0.15
KH_2PO_4	0.01 g	0.01

pH 5.8 *use 1 N KOH buffer*
Autoclave

Ca^{2+} WASHING MEDIUM

Component	per 100 ml	%
$CaCl_2 \cdot 2H_2O$	0.74 g	0.74
Glycine	0.38 g	0.38
Sucrose	11.0 g	11.0

pH 5.8
Filter-sterilize

CURRICULUM VITAE

Vidya Ravichandran

Education:

Master of Science. February 1996.
Department of Horticulture, Virginia Polytechnic Institute and State University, Blacksburg, VA.

Bachelor Of Science. January 1994.
Agriculture : Majors - **Microbiology and Genetics**
University of Agricultural Sciences, Bangalore, India.

Employment:

Current position title: Sr. User Services Consultant.
Pioneer Hi-bred International, Johnston, Iowa.

Research Assistant: August 1994 - February 1996. VPI & SU, Blacksburg, VA.

- Studied molecular mapping of plants using Random Amplified Polymorphic DNA (RAPD), and Simple Sequence Repeats (SSR).
- Carried out *in vivo* and *in vitro* gametic segregation/selection studies using molecular markers through tissue culture.

Summer Intern: May 1995 - September 1995. VPI & SU, Blacksburg, VA.
Designed a Webpage on the World Wide Web for the Department of Horticulture and maintained internet resources.

Teaching Assistant: January 1995 - May 1995. VPI & SU, Blacksburg, VA.
Assisted laboratory session for a graduate level Plant Tissue Culture course.

Publication: 'Biology Digest' (Text Book of Questions and Solutions) for Pre-University Class. *Marvel Digest Publishers*, 1991, Mangalore, India.

R. Vidya

Vidya Ravichandran