UTILIZATION OF TISSUE CULTURE METHODS AND MOLECULAR MARKERS FOR IMPROVEMENT OF *SOLANUM PHUREJA* JUZ. & BUK.

by

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(ABSTRACT)

Anther-derived monoploids of *Solanum phureja* were utilized to investigate factors essential to an efficient method of regenerating doubled monoploids (DMs). The presence of silver thiosulfate (STS) in the MS basal medium did not affect the frequency of cells with $2x$ nuclei but increased the proportion of cells with $1x$ nuclei and decreased the proportion with $4x$ nuclei. Results indicated that STS lower the occurrence of endopolyploid nuclei. The production of DMs was not affected by the presence of STS in MS basal medium on which the source of leaf explants was maintained. The incubation of leaf cultures in the dark or light during the callus induction phase did not influence the subsequent regenerative ability of the monoploids. However, there was a significant genotype by incubation condition interaction. Overnight incubation on MS medium with benzyladenine (BA) pulse prior to transfer to regeneration medium did not affect regeneration.

Field evaluation showed various responses of DMs in terms of growth and yield compared to their anther donors or corresponding F_1 progeny. Female fertility was observed in a majority of the DMs verifying their applicability as parental genotypes in practical breeding.

Efforts to generate potato hybrids based on selection of genetically diverse parents using RAPD markers and to develop high yielding diploid potato germplasm that may be instrumental in new cultivar development were addressed. Genetic diversity among *in vitro* plantlets of *S. phureja* monoploids (which represent DM maternal genotypes) and diploid heterozygous pollinators (ID lines) was estimated using RAPD markers. Field evaluation revealed significant differences among fourteen F_1 hybrids of *S. phureja* DM × ID with respect to total tuber number, total tuber yield, average tuber weight and vigor. Using simple matching or Jaccard coefficients, the largest parental genetic distance was associated with the highest total tuber yield among the progenies of DM parents. Based on our results, RAPDs have the potential to facilitate the identification of diverse parents to maximize the expression of heterosis in *S. phureja* hybrids.

SSR markers were used to analyze the genetic composition of anther-derived potato plants. Anther-derived monoploids exhibited a single allele as expected. Both homozygous and heterozygous diploids were identified. SSRs were also utilized to study allelic segregation in an F_1 population. Results of this experiment revealed Mendelian inheritance of SSR alleles.

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Chapter 1: Introduction

Genetics and breeding of the potato

Potato belongs to the *Solanaceae* family. The genus *Solanum* contains about 2000 species of which about 150 are tuber-bearing (Howard 1970). The tuber-bearing potatoes occur in five cytological groups; diploids, triploids, tetraploids, pentaploids or hexaploids with chromosome numbers of 2n= 24, 36, 48, 60 or 72, respectively (Howard 1970, Hawkes 1979). For the species whose chromosome numbers have been resolved, 74% are diploids, 4.5% are triploids, 11.5% are tetraploids, 2.5% are pentaploids and 5% are hexaploids (Hawkes 1978). Some 2.5% contain a mixture of cytotypes. Most of the diploid species are gametophytically self-incompatible and are able to produce seeds only when they are pollinated with pollen bearing a different *S* allele. The triploids and pentaploids which are sterile, are able to perpetuate through vegetative reproduction.

The potato that is known as an important world crop is the tetraploid species, *Solanum tuberosum* L. ($2n=4x=48$). The cultivated potato is an outbreeder which has accrued deleterious recessive genes as well as genes for male sterility (Jacobsen and Ramanna 1994). Conventional breeding of potato has been limited by severe inbreeding depression after self-pollinations and complex segregation patterns due to its tetraploid and highly heterozygous

nature. Crosses between cultivars produce a large amount of variation only some of which is useful. Dihaploid breeding offers an alternative approach to facilitate potato breeding. Chase (1963) has outlined a procedure of employing dihaploids to produce inbred lines which may be used to generate vigorous heterozygous tetraploids. Other workers have also proposed the use of dihaploid breeding methods to facilitate potato improvement (Wenzel et al. 1979, Ross 1986, Deimling et al. 1988). In this approach, dihaploids are generated, screened for desirable traits, and then selected dihaploids are combined by somatic hybridization to produce tetraploid clones. The resynthesis of tetraploid genotypes results in the fixation of heterozygous gene combinations in a single step. Furthermore, Mendelian genetic analysis will be less ambiguous at the diploid level.

The analytic breeding scheme of Chase (1963) could be enhanced by selection at the monoploid level where there are no allelic interactions. Genotypes bearing lethal alleles would be eliminated through the monoploid seive (Wenzel et al. 1979). The generation of monoploids and subsequent production of homozygous diploids (doubled monoploids) provide the opportunity to gain more information about potential parental materials of breeding populations. Moreover, monoploids are the only means of producing homozygous potato at different ploidy levels. The homozygotes present the chance of propagating potato through true seeds.

Improvement of *S. tuberosum* is significantly contingent on its wild and cultivated tuber-bearing relatives which represent a large source of valuable germplasm. Diploid potatoes and their wild species are generally characterized by self-incompatibility, outbreeding, and high levels of heterozygosity. Interspecific crosses produce highly fertile offspring (Hawkes 1979). One primitive species which has been used extensively by potato breeders is *S. phureja* Juz. & Buk. (Ross 1986, Hawkes and Hjerting 1989). *Solanum phureja* is a cultivated diploid species ($2n=2x=24$) in the Andes region of South America (Hawkes 1963). It is early maturing, forms tubers in three to four months and it lacks tuber dormancy which makes it possible to plant two or three crops per year in frost-free regions (Hawkes 1990, 1994). The importance of *S. phureja* to the improvement of *S. tuberosum* includes such salient traits as disease resistance (e.g., bacterial wilt, potato virus Y, root-knot nematode), heat tolerance and promising high dry matter content and protein content of the tubers (Hawkes and Hjerting 1989). However, the utilization of *S. phureja* in potato improvement requires enhancing its yield components such as tuber size and tuber set because number of tubers per plant tends to be too high and average tuber size is too small in unselected populations (Veilleux 1990).

Use of molecular markers in plant breeding and genetics

Evaluation of genetic divergence among available germplasm in a crop species is essential to its improvement. This would allow the breeder to control the materials used in a breeding

program and predict potential genetic gain without doing unnecessary crosses. Characterization of potato genotypes has been carried out using morphological and physiological traits, isozymes, and tuber proteins (Desborough and Peloquin 1968, Desborough 1983, Oliver and Martinez-Zapater 1985, Douches and Ludlam, 1991). However, this approach is limited by environmental influences, and the number of enzyme loci, respectively. DNA polymorphisms such as restriction fragment length polymorphisms (RFLPs), random amplified polymorphic DNA (RAPDs) or simple sequence repeats (SSRs), on the other hand, are not subject to environmental effects and are present in unlimited numbers. Molecular markers could enhance selection efficiency in plant breeding.

RFLPs. RFLP analysis involves hybridization of labeled DNA clones to homologous sequences in plant DNA which has been cut with restriction enzymes. Alleles are determined by variations in the size of the restriction fragment to which the probes hybridize. Polymorphisms result from loss or gain of a restriction site due to a base pair substitution or an insertion or deletion (Burr et al. 1988). RFLP markers are inherited as simple Mendelian codominants (Botstein et al. 1980) and have potential uses in genotype identification, estimation of genetic diversity, or mapping quantitative characters (Tanksley et al. 1989, Smith et al. 1990, Clegg 1989, Zehr et al. 1992, Vierling et al. 1994).

Extensive linkage maps have been constructed using RFLPs in maize (Helentjaris et al.1986, Helentjaris 1987, Burr et al. 1988), tomato (Bernatzky and Tanksley 1986), rice (McCouch

et al. 1988) and potato (Bonierbale et al. 1988, Gebhardt et al. 1989b, 1991, Tanksley et al. 1992) among other crops. These maps equip breeders and geneticists with a ready tool of tagging a gene of interest as well as tagging genes controlling quantitative traits (Osborn et al. 1987, Young et al. 1988, Sarfatti et al. 1989). RFLPs have been used to evaluate population differentiation and estimate genetic relationships. RFLPs were used to identify *S. tuberosum* subsp. *tuberosum* cultivars (Gebhardt et al. 1989a, Gorg et al. 1992). RFLPs were employed to characterize the genetic composition of plants produced by anther culture in *S. chacoense* (Rivard et al. 1989, Birhman et al. 1994, Rivard et al. 1994). RFLP data of haploid plants showed only one or the other allele of each RFLP marker as expected, and were agreeable with cytological observations. Also, identification of possible lethal alleles could be achieved with RFLP data, i.e., the presence of only one allele and the absence of the alternate form would indicate possible lethality. RFLPs provide a means for transforming QTL into Mendelian entities that can be managed more easily in classical breeding programs (Beckmann and Soller 1986). In potato, quantitative traits such as tuber size, plant height, fertility, and response to anther culture could be scored in each homozygous diploid in replicated experiments while constructing the RFLP map, and mapping of such characters could be attained using computer programs that analyze QTLs (Rivard et al. 1989). Progress in the area of somatic hybridization has been facilitated by confirming somatic hybrids using RFLPs (Williams et al. 1990a). In this study, the markers were used to investigate segregation of chromosomes in the sexual progeny from a cross between a somatic hybrid and a potato cultivar.

RAPDs. RAPD analysis has been widely employed to examine genetic diversity in several crops such as rice (Fukuoka et al. 1992, Yu and Nguyen 1994), *Brassica* species (Hu and Quiros 1991, Demeke et al. 1992, Hallden et al. 1994, Jain et al. 1994, dos Santos et al. 1994), millet (Hilu 1994, M'Ribu and Hilu 1994), coffee (Orozco-Castillo et al. 1994) and cocoa (Wilde et al. 1992, N'Goran et al. 1994). In potato, RAPDs have also been extensively used for characterization of genotypes (Mori et al. 1993, Demeke et al. 1993, Hosaka and Hanneman 1994, Hosaka et al. 1994, Veilleux et al. 1995). RAPD markers and cluster analysis based on Rogers' distances (Rogers 1972) were used to study some anther donors and androgenic monoploids of diploid *Solanum* species and the cluster formed resembled classification based on parental origins and hybrid combinations (Singsit and Ozias-Akins, 1993). Also, RAPD markers have been used to characterize somatic hybrids (Baird et al. 1992, Rokka et al. 1994, Takemori et al. 1994, Rasmussen and Rasmussen 1995). Somatic hybrids have been verified based on species-specific amplification products so as to confirm the presence of fusion genomes in the putative hybrids. In sexual hybrids, RAPDs were used to study gene introgression in potato (Waugh et al. 1992) and under conditions where preferential chromosome elimination may ensue (Clulow et al. 1991), RAPD markers would help to distinguish eliminated chromosomes. Another significant application of RAPDs is the construction of genetic maps and mapping of genes (Rafalski et al. 1991, Rowland and Levi 1994). RAPD markers could fill in the gaps and saturate existing potato RFLP maps (Bonierbale et al. 1988, Gebhardt et al. 1989b, 1991, Tanksley et al. 1992). The RAPD technique has been used to identify DNA markers linked to disease resistance including

Pseudomonas resistance (Martin et al. 1991) and nematode resistance (Klein-Lankhorst et al. 1991) in tomato, and downy mildew resistance in lettuce (Michelmore et al. 1991, Paran et al. 1991). In these studies, near-isogenic lines or bulked segregants were utilized to expedite verification of linkage. RAPD-based genetic diversity has been exploited in the allocation of genotypes to heterotic groups for breeding purposes (Jain et al. 1994). The use of RAPD assay to identify cultivars and assign genotypes to heterotic groups is easier and the technique is faster when compared to the labor-intensive and time consuming RFLP analysis (Caetano-Anolles et al. 1991, Weising et al. 1991). A comparison of the genetic diversity obtained by RAPD and RFLP analyses showed that RAPDs are as reliable as RFLPs to estimate genetic relationships (dos Santos et al. 1994, Hallden et al. 1994, N'Goran et al. 1994).

The RAPD assay (Williams et al. 1990b, Rafalski et al. 1991) is based on the amplification of random DNA segments by the polymerase chain reaction (PCR) using short, random sequence oligo-nucleotides as primers. A primer usually produces several amplification products which originate from different genomic locations (Rafalski et al. 1991). RAPD markers could cover the entire genome by amplifying coding or non-coding regions, repeated or single copy sequences (N'Goran et al. 1994). The procedure is simple, requiring only small amounts of DNA and results could be obtained in one day (Rafalski et al. 1991, Horn and Rafalski 1992, Demeke et al. 1993). The amplified products are separated by agarose gel electrophoresis and variable number and size of bands which reflect sequence variation are

displayed. When the size of amplified products is less than 500 bp, a better resolution could be obtained using polyacrylamide gels (Yu and Nguyen 1994). Polymorphism based on RAPDs may result from single base changes, deletions, insertions or inversions that alter primer binding sites (Williams et al. 1990b). The primary advantage of RAPDs over RFLPs is the simplicity and ease of the technique. The main disadvantage of RAPDs compared to RFLPs is their prevailing dominant mode of inheritance which precludes the discrimination of homozygous dominant individuals from heterozygous ones.

SSRs. The genomes of higher eukaryotes comprise a large proportion of tandemly repeated DNA sequences which exhibit high levels of variability. Based on the length of the core sequence, variable-number-tandem-repeats (VNTRs) (Nakamura et al. 1987) may be categorized as: (1) macrosatellites with more than 100 basepairs (bp) (Jabs et al. 1989); (2) minisatellites with a repeat unit of 10 to 45 bp (Jeffreys et al. 1985); or (3) microsatellites with a repeat unit of 2 to 4 bp (Litt and Luty 1989). Microsatellites are also referred to as simple sequence repeats (SSRs) or short tandem repeats (STRs). SSRs are tandemly repeated short oligonucleotide sequences which have been demonstrated to be highly polymorphic and abundant in human and other mammalian genomes as well as in plant genomes (Tautz and Renz 1984, Tautz 1989, Weber and May 1989, Ostrander et al. 1992, Wang et al. 1994). SSRs have attracted considerable attention recently because of their abundance and relatively simple experimental procedure (Saghai Maroof et al. 1994; Wang et al. 1994). DNA sequences bordering SSRs are conserved which allows the synthesis of primers that will

amplify the SSR block by PCR. In earlier studies, PCR reactions included one ^{32}P-labeled nucleotide or one or two ^{32}P end-labeled primers to visualize amplification products on a standard sequencing gel (Litt and Luty 1989, Weber and May 1989). The procedure has been simplified by using MetaPhor agarose which does not require autoradiography (Senior and Heun 1993) although for smaller size differences of allelic fragments resolution may require denaturing sequencing gels.

SSRs appear to be randomly distributed throughout the genome and occur both intragenically and intergenically (Weber 1990). There are 50,000-100,000 $(CA)_n$ repeats with n ranging from 15 to 30 dispersed in the human genome (Tautz and Renz 1984). The majority of $(CA)_n$ motifs in the human genome is distributed within introns or between genes (Weber and May 1989). In plants, the majority of DNA repeats comprise the sequence AA/TT followed in abundance by AT/TA and CT/GA (Lagercrantz et al. 1993) while the GT/CA motif constitutes only a small fraction. Several microsatellite probes incorporating poly(GT), poly(GTG) and poly(GAC/TA) units recognize microsatellites which are found in the genomes of human, animals and plants (Haberfeld et al. 1991). Tzuri et al. (1991) reported that human, cattle and phage M13-derived mini- and microsatellite probes hybridized to polymorphic loci in several flower genera. In a more recent study, SSR-based primers were utilized in single primer amplification reactions (SPARs) of evolutionarily diverse eukaryotic genomes (Gupta et al. 1994). This demonstrates the potential use of SSRs in comparative mapping including species with few published gene sequences.

The presence of SSRs in plants has been widely substantiated (Cregan 1992, Poulsen et al. 1993, Rus-Kortekaas et al. 1994, Wang et al. 1994). SSRs are highly polymorphic as a function of the number of repeats and as such represent a large source of potential genetic markers for gene mapping (Akkaya et al. 1992, Morgante et al. 1994) and genetic studies (Lagercrantz et al. 1993) in plants. The extent of polymorphism detected using the tetrameric $(GATA)_4$ probe was adequate to discriminate among 15 tomato cultivars (Vosman et al. 1992). In another study, a GACA-containing SSR probe produced polymorphic and unique bands which were used to distinguish six *Lycopersicon* species and 15 *L. esculentum* cultivars (Rus-Kortekaas et al. 1994). SSR polymorphism has also been reported in maize (Senior and Heun 1993), rice (Zhang et al. 1994), soybean (Morgante et al. 1994, Yanagisawa et al. 1994, Yu et al. 1994, Maughan et al. 1995, Rongwen et al. 1995), barley (Saghai Maroof et al. 1994, Becker and Heun 1995), yam (Terauchi and Konuma 1994), sunflower (Brunel 1994, Mosges and Friedt 1994), grape (Cipriani et al. 1994, Thomas et al. 1994), rapeseed (Kresovich et al. 1995) and chickpea (Sharma et al. 1995). For the purpose of discriminating homozygous from heterozygous anther-derived potato, SSRs offer the same potential as RFLPs, i.e., both alleles of a heterozygote can be detected.

Objectives

The overall objectives of this project were to generate potato hybrids based on molecular

marker-assisted evaluation of parental genotypes and to develop high yielding diploid germplasm of *S. phureja* that may be instrumental in the development of new cultivars of the cultivated potato, *S. tuberosum*. To achieve this goal the following research objectives were addressed:

(1) to develop a diverse set of doubled monoploids of *S. phureja* from available monoploids using *in vitro* shoot regeneration and generate hybrid seeds on doubled monoploids using diploid heterozygous pollinators (ID lines);

(2) to investigate the relationship between parental genetic distance using RAPD markers and yield or vigor in F_1 hybrids; and

(3) to determine the genetic composition of anther-derived diploids using SSRs and examine the inheritance pattern of SSRs.

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Chapter 2: Influence of culture medium and *in vitro* conditions on shoot regeneration in *Solanum phureja* monoploids, and evaluation of regenerated doubled monoploids

Summary

The utilization of monoploids in potato breeding provides advantages such as the availability of lines which are free of lethal and severely deleterious alleles as well as the rapid production of homozygous lines which may allow incorporation of new genes into breeding lines and increase selection efficiency. The incorporation of *Solanum phureja* monoploids in practical breeding requires an efficient method of producing homozygous diploids either by chromosome doubling in reduced microspores or doubling the complement of haploid tissue or plants. This study investigated factors essential to an efficient method of regenerating doubled monoploids and the performance of the regenerated diploids. Anther-derived monoploids of *S. phureja* were utilized in this study. The effect of silver thiosulfate (STS) on the ploidy distribution of cells within monoploid potato plants was determined. An enhanced proportion of diploid cells which would give rise to meristematic centers preceding shoot regeneration was desired for production of doubled monoploids (DMs). However, the presence of STS in the MS medium did not affect the frequency of cells with diploid ($2x$) nuclei but increased the proportion of cells with monoploid ($1x$) nuclei and decreased the

proportion with 4*x* nuclei. A higher regeneration frequency was obtained when leaf explants were used compared to stem explants. Induction of shoots was neither affected by light or dark incubation during the callus process nor by overnight treatment with benzyladenine (BA) prior to transfer of leaf explants to regeneration medium. Based on ploidy determination of regenerants by flow cytometric analysis, leaf disk regeneration offers a dependable technique to generate DMs. A field evaluation of DMs compared to their anther donors or corresponding F_1 progeny showed variable responses in terms of growth and overall performance. Female fertility was observed in a majority of the DMs and seed set was obtained which signify their applicability as parental genotypes in practical breeding.

Introduction

Potato is a highly heterozygous crop and, when subjected to the so-called 'monoploid seive' (Wenzel et al. 1979) by anther culture, plants free of lethal alleles and severely deleterious genes are produced. Hemizygosity in monoploids exposes any lethal or recessive deleterious alleles which would cause embryos bearing them to perish. The utilization of monoploids provides the plant breeder with potentially desirable lines and a valuable tool to hasten crop improvement by allowing the rapid production of homozygous lines; thus, selection efficiency can be increased.

Monoploids ($2n=x=12$) of *Solanum phureja* Juz. & Buk. have been produced by anther culture (Irikura 1975, Veilleux et al. 1985, Pehu et al. 1987, Veilleux 1990, Snider and Veilleux 1994). *S. phureja* is a cultivated diploid species ($2n=2x=24$) in the Andes region of South America (Hawkes 1963) which has been used extensively by potato breeders (Ross 1986, Hawkes and Hjerting 1989). It is early maturing, forms tubers in three to four months and it lacks tuber dormancy which makes it possible to plant two or three crops per year in frost free regions (Hawkes 1990, 1994). The importance of *S. phureja* to the improvement of *S. tuberosum* includes such salient traits as pest resistance (e.g., bacterial wilt, potato virus Y, root-knot nematode), heat tolerance and promising high dry matter content and protein content of the tubers (Hawkes and Hjerting 1989). The most likely route to the incorporation of *S. phureja* monoploids in potato breeding requires an efficient method of producing diploid homozygotes, either by chromosome doubling in reduced microspores or doubling the complement of haploid tissue or plants. Fertile homozygous lines can be used to generate highly heterozygous but genetically uniform F_1 hybrids. If such hybrids have functional $2n$ gametes, they can be used in various breeding schemes involving sexual polyploidization to generate improved potato cultivars for traditional vegetative propagation or for true potato seed (TPS). Evaluation of fertility of *S. phureja* doubled monoploids showed reduced female fertility compared to the anther donor (M'Ribu and Veilleux 1992). They reported that male sterility was generally observed among the doubled monoploids. In a previous study, *S. tuberosum* doubled monohaploids showed poor flowering and produced few seeds (Uijtewaal et al. 1987).

The traditional method to induce chromosome doubling involves the use of colchicine which often produces ploidy chimaeras (Jacobsen 1981, De,Maine and Fantes 1983). In *S. phureja,* generation of doubled monoploids (DMs) has been attained by an alternative method that exploits naturally occurring endopolyploidization in somatic tissues through *in vitro* shoot regeneration from monoploids (M'Ribu and Veilleux 1990). Ploidy doubling has also been observed in adventitious shoots formed from leaf tissue of dihaploid *S. tuberosum* (Fleming et al. 1992) and from diploid *S. brevidens* (Valkonen 1994). Shoot regeneration from different types of explants such as leaf, stem, pith or petiole has been reported in several potato cultivars (Hermsen et al. 1981, Wheeler et al. 1985, Wareh et al. 1989). The response to shoot regeneration was shown to be genotype-dependent and some genotypes remained recalcitrant (Webb et al. 1983, Fleming et al. 1992, Hulme et al. 1992, M'Ribu and Veilleux 1990). It is important then, to determine an efficient method of producing DMs by *in vitro* shoot regeneration which is applicable to most , if not all, genotypes.

Flow cytometric methods have been used to study plant nuclear DNA (Sharma et al. 1983, Owen et al. 1988, Ulrich et al. 1988, Fuchs and Pauls 1992, Ulrich and Ulrich 1991, Nicoloso et al. 1994) and to study endopolyploidization of monoploid potato plants (Owen et al. 1988, Snider 1992). The technique involves estimation of total DNA in the cell nuclei using DNA stains preceded by treatment of cells with RNAse to eliminate staining of double stranded RNA, thus preventing artifactual increase of DNA content. Flow cytometry involves measurements made while the cells pass in single file through the apparatus in a fluid stream

(Shapiro 1995). A linear relationship occurs between fluorescence intensity and DNA content of the nuclei (Galbraith 1989).

The objectives of this study were: (1) to investigate factors essential to an efficient method of regenerating DMs and; (2) to investigate the performance of the resulting homozygous diploids (DMs).

Materials and Methods

Plant Materials. The monoploids and corresponding DMs were derived by anther culture of field selections from an adapted population of *S. phureja/S. stenotomum* (Haynes 1972). The different anther donors had diverse origins. The BARD clones were independently selected for anther culture competence from a heat tolerant subpopulation of the original "Haynes' *phureja*". AD2-4 3s.2, 3s.3 and 3s.8 were regenerated by anther culture of a DM (AD2-4) and would be expected to differ from each other only by possible gametoclonal variation. A3P2-6 6n.1 and A3P2-6 16 were derived by anther culture of a hybrid between DM AD3-4 (full sib to AD2-4) and a heterozygous pollinator. PP5 3s.21 was obtained by anther culture of PP5, a heterozygous clone which had been selected for anther culture competence and 2*n* pollen production. The PC monoploids were derived by anther culture of a hybrid between *S. phureja* and *S. chacoense*. The various plant materials used in this study are presented in

Table 1.

Experiment 1. Effect of silver thiosulfate on the cellular ploidy of anther-derived monoploids and on the subsequent production of DMs

Maintenance of monoploids. Six anther-derived monoploids of *S. phureja* (BARD 1-3 3n.29, AD2-4 3s.2, PP5 3s.21, PC 45-4, PC 21-78, PC 37-9) were utilized in this study. The ploidy level had been previously determined by Snider (1992) using flow cytometry. Nodal cuttings were transferred to Murashige and Skoog (MS) medium (Murashige and Skoog 1962) with or without 4 μM silver thiosulfate (STS). Two copies were maintained per genotype per replication. The tubes were laid out in a randomized complete block with three replications. Flow cytometric analysis to determine the frequency of monoploid ($1x$), diploid ($2x$) and tetraploid ($4x$) cells was done three weeks after subculture.

Shoot regeneration from monoploids. Explants were obtained from *in vitro* plantlets of BARD 1-3 3n.29, AD2-4 3s.2, PC 45-4, PC 21-78 and PC 37-9 which had been previously maintained for 4 weeks on MS medium with or without 4 μM STS. Shoot regeneration was induced based on the method of M'Ribu and Veilleux (1990). Leaf disks (2-4 mm diam) of the third and the fourth leaf from the top and stem sections (5 mm length) were cultured on callus induction medium (MS + 1.0 μM NAA + 10 μM BA) for 14 days, then transferred to

shoot regeneration medium (MS + 10 μM BA + 14.4 μM GA_3). The frequency of regeneration and number of shoot regenerants were observed six weeks after leaf disk culture.

Frequency of doubled monoploids. Flow cytometry was done to determine the ploidy level among regenerants of PC 45-4 and PC 37-9. Fifty shoot regenerants obtained from leaf explants were transferred to MS basal medium for each genotype. There were ten regenerants per replication, with five replications in a randomized complete block design.

Experiment 2. Effect of genotype, light and a BA pulse on shoot regeneration from leaf explants of *S. phureja* monoploids

Materials. Leaf disk regeneration was done in six *S. phureja* monoploid clones: AD2-4 3s.2, BARD 1-3 516, BARD 13-14 202, BARD 13-14 203, BARD 9-9 204 and A3P2-6 16 following the method of Hulme et al. (1992). Monoploid plantlets were maintained on MS medium with 4 μM STS for five weeks at 25°C and 16 h photoperiod.

Shoot regeneration. Leaf explants (0.5 to 1 cm^2) were derived from five week-old *in vitro* plantlets, then floated overnight in liquid pulse medium containing MS salts and vitamins, 10 g/l sucrose, 80 mg/l NH_4NO_3, 147 mg/l $CaCl_2$, 54 μM NAA and 44 μM BA. Subsequently, the explants were transferred to callus induction medium which included MS salts and

vitamins, 1 g/l sucrose, 4 g/l mannitol, 0.1 μM IAA and 10 μM BA. The leaf explants were maintained on callus induction medium for seven days in the dark or under light. In the next step prior to transfer to regeneration medium, the leaf explants were either treated or not with a second pulse to try to stimulate regeneration by overnight incubation on MS medium with 44 μM BA, 147 mg/l $CaCl_2$, 80 mg/l NH_4NO_3 and 10 g/l sucrose.

The regeneration medium was composed of MS salts and vitamins, 10 μM BA, 14 μM GA_3 and 8 μM STS. Leaf explants were transferred to fresh regeneration medium every two weeks. Cultures were maintained at 20°C under 16 h photoperiod in petri plates, using four leaf explants per plate, arranged in a randomized complete block with three replications. The frequency of regeneration and number of shoots per explant were obtained after 6 weeks on regeneration medium.

Preparation of chopped nuclei and flow cytometry

Leaf and stem tissue weighing up to 0.5 g was placed on a 6 cm diam glass petri plate into which 1.5 ml chopping buffer containing 45 mM $MgCl_2$, 30 mM Na-citrate, 20 mM MOPS and Triton X-100 (Sharma et al. 1983) was added. Chopping was done on ice for 3 min. The homogenate was filtered through a 250 μm filter and subsequently through a 63 μm filter. The filtrate (0.5 ml) was deposited in a 1.5 ml microcentrifuge tube and incubated on ice. The

RNAase solution was prepared (80 mg Sigma ribonuclease-A in 100 ml chopping buffer) and 250 µl was added to each microtube. The samples were kept at room temperature for 30 min. The chopped nuclei were stained by adding 125 µl propidium iodide (PI) solution (40 mg PI in 100 ml chopping buffer) to each sample, then incubated on ice for at least 30 min. Flow cytometric analysis was done within 3 h according to Owen et al. (1988). A sample of a previously identified monoploid was used as control. Flow cytometry was carried out using an Epics V model 752 laser flow cytometer (Coulter Electronics, Hialeah, FL). The percentage of nuclei per fluorescence peak was measured based on the total number of nuclei of a sample.

Morphological evaluation, fruit set and yield of DMs

Microtubers derived by *in vitro* tuber induction of twelve *S. phureja* DMs (DM BARD 9-9 203 and 204; DM BARD 1-3 511 and 516; DM BARD 13-14 200, 201, 202 and 203; DM AD2-4 3s.2, 3s.3 and 3s.8; DM A3P2-6 6n.1), diploid anther donors (BARD 1-3, BARD 13-14, AD2-4) and heterozygous pollinators (ID 4, ID 5, ID 8) were planted in the greenhouse in spring and fall 1994 and maintained at 16 h photoperiod. The ID lines had been selected for field adaptation in Idaho by Dr. J. Pavek, USDA/ARS, Aberdeen, Idaho with the designation AD x 463-5, AD x 497-1 and AD x 881-4, corresponding to ID 4, 5, and 8, respectively. They represent complex interspecific hybrids such that ID 4 and ID 5 comprise

1/2 *S. stenotomum* (*stn*), 3/16 *S. phureja* (*phu*), 3/16 *S. tuberosum* (*tbr*), and 1/8 *S. chacoense* (*chc*) whereas ID 8 comprises 7/16 *stn*, 11/32 *tbr*, and 7/32 *phu*. These clones represent field selections for horticultural traits and male fertility. Plants were maintained in the greenhouse and crosses were made between DMs or anther donor as maternal genotypes and the ID lines as pollinators. Data on fruit set and seeds per fruit were obtained. Minitubers were harvested at maturity.

Minitubers of eight DMs and two anther donors were stored at 4^0C for at least 3 months before planting in the field. In addition, F_1 progeny of DM AD2-4 3s.3, DM AD2-4 3s.8 and DM A3P2-6 6n.1 crossed with ID pollinators were also grown. Seven-plant-plots were laid out in a completely randomized design. However, only the mean performance of the different DMs and two anther donors (BARD 1-3 and AD2-4) was determined and statistical analysis was not carried out due to poor emergence of some genotypes, in addition to the already limited number of minitubers as planting materials. Morphological characters and yield were noted. The following data were obtained: number of inflorescences, number of flowers per inflorescence and anther length (mm) of five anthers at 10 weeks after planting (WAP); plant height (cm) at 8 WAP; terminal leaflet length (cm) and terminal leaflet width (cm) of five fully expanded leaves at 8 WAP; vigor (scale of 1=high to 4=low) at 8 WAP; maturity (scale of 1=dried up to 5=still flowering) at 18 WAP; tuber number and average tuber yield per plant.

Statistical analysis

All statistical analyses were performed using SAS General Linear Models procedure (SAS 1985). Data on the frequency of $1x$, $2x$ and $4x$ cells were analyzed using a multivariate analysis of variance (MANOVA) with Wilk's statistics.

Results

Experiment 1. Effect of silver thiosulfate on the cellular ploidy of anther-derived monoploids and on the subsequent production of DMs

DNA measurements of monoploids revealed 1C, 2C and/or 4C peaks corresponding to monoploid ($1x$), diploid ($2x$) and tetraploid ($4x$) cells, respectively (Fig. 1). Cells in the 1C peak would be at the postmitotic gap (G1) of the cell cycle; those in the 2C peak comprise cells in the premitotic gap (G2) as well as cells that have undergone a single cycle of endopolyploidization. Cells in the 4C peak would have undergone two cycles of endopolyploidization. Based on a multivariate analysis of variance using Wilk's lambda on all three peaks, significant differences among genotypes (F=15.2, p<.01) and between STS levels (F=8.45, p<.01) were obtained. A canonical discriminant analysis revealed that a substantial portion of the difference was attributed to the 1C peak. The percentage of $1x$ cells

differed significantly among the different genotypes and ranged from 12.3% to 49.8% (Table 2). Two of the genotypes (PC 21-78 and PC 37-9) exhibited more endopolyploidization than the other genotypes as evidenced by the significantly higher frequencies of 4C cells. The presence of STS in the MS basal medium did not affect the frequency of cells with $2x$ nuclei but increased the proportion of cells with $1x$ nuclei and decreased the proportion with $4x$ nuclei (Table 3). This observation supported the results of a related study using two monoploid clones wherein STS significantly increased the frequency of $1x$ cells but reduced the amount of $2x$ and $4x$ cells (Appendix 1). In both experiments the significant differences indicated that STS had the effect of lowering the occurrence of endopolyploid nuclei.

Shoot formation on explants was observed as early as two weeks after culture initiation. A higher regeneration frequency was obtained when leaf explants were used compared to stem explants (Table 4). The frequency of regeneration ranged from 4.2% to 54.2% and was significantly different among the monoploid genotypes. Likewise, the mean number of shoots per leaf disk varied significantly among the monoploids. There was a linear correlation between regeneration frequency and mean number of shoots per leaf disk ($r=0.98$, $p<.05$). The addition of STS to MS medium where sources of explants were maintained did not affect regeneration.

The production of DM regenerants was not affected by the presence of STS in MS basal medium on which the source of leaf explants were maintained (Table 5). Monoploid PC 37-9

exhibited fewer diploid and more tetraploid regenerants compared to monoploid PC 45-4 (Table 5). Significantly, PC 37-9 showed a higher proportion of 4*x* cells than PC 45-4 (Table 2) and this condition may have generated the higher frequency of tetraploid regenerants in PC 37-9.

Experiment 2. Effect of genotype, light and a BA pulse on shoot regeneration from leaf explants of *S. phureja* monoploids

The frequency of leaf disk regeneration ranged from 2.1% in BARD 13-14 203 to 100.0% in AD2-4 3s.2 (Table 6). Both monoploids derived from BARD 13-14 had significantly fewer leaf disk regenerants and significantly fewer shoots per leaf disk relative to the other genotypes. The mean number of shoots per leaf disk was significantly fewer for AD2-4 3s.2 (6.1 shoots) than for BARD 1-3 516 (13.8 shoots), BARD 9-9 204 (12.6 shoots) or A3P2-6 16 (10.3 shoots).

The incubation of leaf cultures in the dark or light during the callus induction phase did not influence the subsequent regenerative ability of the monoploids used in this study (Table 7). However, there was a significant genotype by incubation condition interaction. For example, BARD 9-9 204 had 70.8% regeneration in dark and 100% in light whereas BARD 13-14 202 had 20.8% in dark and 2% in light. Overnight incubation on MS medium with a BA pulse

prior to transfer to regeneration medium did not affect regeneration.

Morphological evaluation, fruit set and yield of DMs

There were only two anther donors evaluated as determined by the availability of minitubers for field planting. DM AD2-4 3s.2 looked to be similar to AD2-4 anther donor as expected because of homozygosity of AD2-4 anther donor (Table 8). However, the performance of DM AD2-4 3s.2 was relatively lower compared to the F_1 progeny of DM AD2-4 3s.3 or DM AD2-4 3s.8. The yield of DM BARD 1-3 511 was relatively inferior to BARD 1-3 anther donor. DM BARD 1-3 511 exhibited a decrease in mean number of tubers and average yield per plant which was consistent with the results of a related greenhouse experiment in fall 1994 (Appendix 2).

In crosses using DMs as female parents and the ID pollinators, female fertility was observed in ten of the twelve DMs (Table 9). Crosses with DM BARD 9-9 204 as the maternal parent did not set fruit. Most flower buds of DM BARD 1-3 511 aborted before pollinations could be done on them. When ID 5 was used as the pollen source, a higher success of fruit formation was attained. The number of seeds per fruit is shown in Table 10. Though seed set was low, it was adequate for using the DMs as parental genotypes in practical breeding.

Discussion

Several factors influence the induction of *in vitro* shoot regeneration: genotype, physiological state of the donor plant and the explant, cellular state of the explant and growth regulators in the medium (Bhojwani and Razdan 1983, Webb et al. 1983, Wheeler et al. 1985). Monoploid plants are polysomatic because some cells may go through one or more cycles of endopolyploidization (Uijtewaal 1987, Snider 1992). Monohaploid shoots had been shown to double spontaneously during growth (Karp et al. 1984). The level of ploidy predominant among the dividing cells can be influenced by the culture medium. In the present study the effect of STS on the cellular state of anther-derived monoploids was investigated. STS has an ethylene antagonistic effect (Perl et al. 1988) and its addition to culture medium has concomitantly improved growth and cell division capabilities of tissue cultures. The addition of STS to MS basal medium used for the maintenance of monoploids appeared to inhibit endopolyploidization as exhibited by the reduced frequencies of 4*x* cells in plantlets grown on STS. This may have been due to an alteration in morphology (higher proportion of leaf tissue compared to stem tissue) of *in vitro* plantlets on STS that was reflected in altered ploidy distribution. An increase in the proportion of diploid cells which would give rise to meristematic centers preceding shoot regeneration was desired for increased production of DMs. The frequency of 2*x* nuclei was unaffected by growth on STS, however, and the frequency of diploid regenerants was likewise unaffected.

Induction of shoot regeneration was attained in both leaf and stem explants although it was higher when leaf explants were cultured. Since growth of different monoploids varies according to the relative proportion of stem and leaves, the more prevalent tissue can be cultured (M'Ribu and Veilleux 1990). Although STS did not produce the desired effect on the source plantlets to enhance its regeneration and DM production, it could be used to produce monoploid plants with expanded leaves necessary for leaf disk regeneration especially for genotypes with very few small leaves. In related studies, successful generation of DMs was highly attributed to the monoploid source according to the degree of endopolyploidization and chromosomal changes during callus formation (Karp et al. 1984, Owen et al. 1988, M'Ribu and Veilleux 1990). This observation was in agreement with endopolyploidization of callus cultures in tetraploid potato (Hanisch ten Cate and Sree Ramulu 1987). Because PC 45-4 did not differ from PC 37-9 for frequency of $2x$ nuclei, a higher proportion of DMs in PC 45-4 (Table 5) can be attributed to endopolyploidization during callus formation resulting in the production of doubled cells and consequently, DMs. Thus, DMs can be obtained either from derivatives of diploid cells of the explant or doubled cells resulting from endopolyploidization during callus induction. The latter supports the claim that shoot regeneration originates from the proliferating cells rather than the original cells of the explant (Hermsen et al. 1981, Webb et al. 1983). Based on our results, leaf disk regeneration provides a dependable technique to generate doubled monoploids.

Significant genotypic differences concerning shoot formation among sibling monoploids or

among unrelated monoploids were observed. Genetic variation for shoot regeneration within a clone has been observed (Fish and Jones 1988, M'Ribu and Veilleux 1990) and these results suggest the quantitative character of shoot formation. Significant interaction between genotype and light or dark incubation was obtained. Gavinlertvatana and Li (1980) reported that incubation in the dark promoted callus formation compared to incubation under light. In contrast Dhingra et al. (1991) observed that growth of leaf callus was enhanced under light or intermittent light and dark incubation than under continuous dark. These results and the significant genotype × incubation condition in our study indicate a strong genotype-dependent response which is commonly observed in tissue culture experiments. BA usually promotes shoot differentiation. However, in the present study, an overnight pulse with BA of callused leaf explants prior to transfer to regeneration medium did not affect shoot induction rate (Table 7). This can be due to two reasons: (1) this treatment was not necessary; or (2) the level of BA used was not sufficient to trigger an effect. Shoots regenerated from leaf explants did not show any abnormality such as albinism or chlorosis as opposed to results obtained by Austin and Cassells (1983) in *S. tuberosum*.

Female fertility was observed in a majority of the DMs. This observation signifies the applicability of DMs as potential parental materials to speed up efforts in potato breeding and genetics. Field evaluation of the DMs (e.g., BARD 1-3 511) showed inferior performance relative to the anther donor (Table 8). This condition may be explained by the homozygous state of undesirable alleles due to chromosome doubling which corresponds to inbreeding

depression in practical breeding whereas the anther donors and progenies are highly heterozygous. In a related study, poor survival and low vigor in doubled haploids of *Coffea canephora* was associated with a strong negative effect of homozygosity (Lashermes et al. 1994). Inbreeding depression can be attributed to the fixation of unfavorable or deleterious recessives or to the loss of favorable allelic interactions at a particular locus (Allard 1960, Krebs and Hancock 1990, Golmirzaie et al. 1994). DM A3P2-6 6n.1 had a higher yield compared to the corresponding F_1 progeny. This may be attributed to the presence of unadapted germplasm in ID pollinators. Although genetic variability was increased, mean performance was diminished by genes for lack of adaptation (Bonierbale et al. 1993).

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Table 1. Source of monoploids and corresponding DMs used throughout the study.

Anther donor	Pedigree	Monoploids	Doubled monoploids
PP5	PI 225669	PP5 3s.21	
BARD 9-9	PI 225681 × PI 195192	BARD 9-9 203 BARD 9-9 204	DM BARD 9-9 203 DM BARD 9-9 204
BARD 13-14	PI 225695 × PI 195192	BARD 13-14 200 BARD 13-14 201 BARD 13-14 202 BARD 13-14 203	DM BARD 13-14 200 DM BARD 13-14 201 DM BARD 13-14 202 DM BARD 13-14 203
BARD 1-3	PI 195191 × PI 195192	BARD 1-3 3n.29 BARD 1-3 511 BARD 1-3 516	 DM BARD 1-3 511 DM BARD 1-3 516
PC 45, 21, 37	*S. phureja* × *S. chacoense*	PC 45-4, PC 21-78, PC 37-9	
A3P2-6	AD3-4 (from PP5) × heterozygous pollinator	A3P2-6 6n.1 A3P2-6 16	DM A3P2-6 6n.1
AD2-4	PP5	AD2-4 3s.2 AD2-4 3s.3 AD2-4 3s.8	DM AD2-4 3s.2 DM AD2-4 3s.3 DM AD2-4 3s.8

Table 2. Genotypic differences for the mean frequency of cells with 1*x*, 2*x* or 4*x* nuclei in six anther-derived monoploids of *S. phureja*. Data are pooled over culture medium treatments with and without silver thiosulfate.

Genotype	n	%1*x* cells	%2*x* cells	%4*x* cells
BARD 1-3 3n.29	6	47.8 a	34.4 b	17.9 b
AD2-4 3s.2	6	47.7 a	34.2 b	18.0 b
PP5 3s.21	3	37.7 b	42.5 ab	19.8 b
PC 45-4	6	49.8 a	35.4 b	14.7 b
PC 21-78	5	22.1 c	39.0 ab	38.9 a
PC 37-9	6	12.3 d	45.6 a	42.1 a

Mean separation within columns by SNK, $\alpha = .05$

Table 3. Effect of STS in the propagation medium on the frequency of cells with 1*x*, 2*x* or 4*x* nuclei in monoploid potato grown *in vitro*. Means represent six genotypes.

Silver thiosulfate	n	%1*x* cells	%2*x* cells	% 4*x* cells
absent	14	33.9 b	37.6 a	28.5 a
present	18	38.6 a	38.5 a	22.9 b

n =number of samples.
Mean separation using SNK, $\alpha = .05$

Table 4. Effect of genotype, silver thiosulfate contained in MS medium used for the maintenance of source plantlets, and type of explant on *in vitro* regeneration in five anther-derived monoploids of *S. phureja*.

Variable	n	Frequency of regeneration (%)	Mean number of shoots per leaf disk
Genotype			
PC 37-9	12	54.2 a	17.7 a
PC 45-4	12	47.9 a	17.7 a
AD2-4 3s.2	12	14.6 b	4.1 b
BARD 1-3 3n.29	12	12.5 b	3.2 b
PC 21-78	12	4.2 b	0.3 b
Silver thiosulfate			
absent	30	26.7 a	7.6 a
present	30	26.7 a	9.6 a
Explant			
leaf	30	40.8 a	14.2 a
stem	30	12.5 b	2.9 b

Mean separation within columns using SNK, $\alpha = .05$
n = number of samples.
Frequency of regeneration = number of leaf disks with shoot(s) per total number of leaf disks cultured × 100.

Table 5. Effect of genotype and culturing source plantlets in medium with or without silver thiosulfate on the ploidy of regenerants derived from leaf culture of monoploid plants.

Factor	% monoploids	% doubled monoploids	% tetraploids
STS			
absent	17.2 a	74.6 a	8.2 a
present	11.0 a	61.9 a	27.1 a
Genotype			
PC 45-4	13.0 a	85.0 a	2.0 b
PC 37-9	15.2 a	51.4 b	33.3 a

Mean separation within columns using SNK, $\alpha = .05$.

Table 6. *In vitro* regeneration of six different monoploids using pulse medium prior to placement of explants on callus induction medium (Hulme et al.1988).

Genotype	n	Frequency of regeneration (%)	Mean number of shoots per leaf disk
AD2-4 3S.2	12	100.0 a	6.1 b
BARD 1-3 516	12	93.8 a	13.8 a
BARD 13-14 202	12	20.8 b	0.3 c
BARD 13-14 203	12	2.1 c	0.0 d
BARD 9-9 204	12	85.4 a	12.6 a
A3P2-6 16	12	95.8 a	10.3 a

Mean separation within columns using SNK, $\alpha = .05$.
N = number of samples.
Frequency of regeneration=number of leaf disks with shoot(s) per total number of leaf disks cultured × 100.

Table 7. ANOVA for the frequency of regeneration and number of shoots as related to genotype, incubation and benzyladenine (BA).

Source	df	Mean Squares	
		Frequency of regeneration	Number of shoots
Rep	2	.01 ns	18.96 ns
Genotype	5	2.74 **	543.96 **
Incubation (light vs. dark)	1	.06 ns	37.71 ns
BA	1	.02 ns	10.56 ns
Genotype x Incubation	5	.13 *	65.32 ns
Genotype x BA	5	.04 ns	5.66 ns

α = 0.5
Note: ns - not significant
** - significant at α=.01
* - significant at α=.05

Table 8. Performance of anther-derived doubled monoploids and anther donors of *Solanum phureja*.

Genotype		N	NI	NFI	AL	HT
Anther donors						
BARD 1-3		3	6.2	10.8	6.8	25.0
AD 2-4		1	6.0	8.0	4.0	10.6
F_1 progenies (DM × ID)						
DM AD2-4 3s.3, 3s.8		12	3.8	11.3	6.3	9.5
DM A3P2-6 6n.1		12	6.2	9.3	6.0	8.9
Doubled monoploids						
DM BARD 1-3	511	1	1.6	11.6	7.1	26.4
DM BARD 13-14	200	10	0.9	3.3	4.9	14.6
DM BARD 13-14	201	5	0.8	5.7	4.1	10.9
DM BARD 13-14	202	3	1.6	5.1	4.4	14.6
DM BARD 9-9	203	1	4.6	5.8	4.7	24.8
DM BARD 9-9	204	1	2.6	4.4	3.2	16.4
DM AD2-4	3s.2	1	1.4	5.6	3.4	10.0
DM A3P2-6	6n.1	1	8.0	6.0	5.8	21.4

Note: N = number of sample plots; NI = number of inflorescences; NFI = number of flowers per inflorescence; AL = anther length (mm); HT = plant height at eight weeks after planting (cm).

Table 8. Continued...

Genotype	N	LL	LW	VI	M	TN	ATY
Anther donors							
BARD 1-3	3	5.9	2.9	2.3	4.3	30.0	513.8
AD2-4	1	1.6	1.0	4.2	1.0	1.5	10.3
F_1 progenies (DM × ID)							
AD2-4 3s.3, 3s.8	12	4.5	2.1	2.4	2.9	9.0	27.6
A3P2-6 6n.1	12	4.2	2.0	2.6	3.1	10.9	30.5
Doubled monoploids							
DM BARD 1-3 511	1	4.8	2.4	3.0	1.0	2.5	35.2
DM BARD 13-14 200	10	5.3	2.7	3.3	2.4	11.7	44.0
DM BARD 13-14 201	5	3.6	1.6	3.8	2.2	2.7	14.2
DM BARD 13-14 202	3	5.0	2.4	3.4	1.3	11.6	34.0
DM BARD 9-9 203	1	3.2	1.6	3.2	1.0	4.0	23.4
DM BARD 9-9 204	1	2.9	1.7	3.0	1.0	4.2	15.2
DM AD2-4 3s.2	1	1.8	1.1	4.0	1.0	2.5	13.3
DM A3P2-6 6n.1	1	2.7	1.5	4.0	1.0	2.8	85.4

Note: N = number of sample plots; LL = terminal leaf length (cm); LW = terminal leaf width (cm); VIG = vigor (scale: 1= high to 4 = low); MAT = maturity (scale:1 = dried up to 5 = still flowering); TN = mean tuber number; ATY= average tuber yield per plant.

Table 9. Frequency of fruit set (number of fruits per number of pollinations) in crosses between DM or anther donor and ID pollinator.

Female parent	Pollinator			Total
	ID 4	ID 5	ID 8	
DM BARD 9-9 203	2/36 (5.6)	26/51 (51.0)	8/31 (25.8)	36/118 (30.5)
DM BARD 9-9 204	0/3 (0.0)	0/69 (0.0)	0/24 (0.0)	0/96 (0.0)
DM BARD 1-3 511	-	0/3 (0.0)	-	0/3 (0.0)
DM BARD 1-3 516	0/7 (0.0)	9/19 (47.4)	2/11 (18.2)	11/37 (29.7)
BARD 1-3 a.d.	0/37 (0.0)	44/67 (65.7)	23/72 (31.9)	67/176 (38.1)
DM BARD 13-14 200	1/33 (3.0)	14/84 (16.7)	1/39 (2.6)	16/156 (10.2)
DM BARD 13-14 201	0/25 (0.0)	1/29 (3.4)	0/21 (0.0)	1/75 (1.3)
DM BARD 13-14 202	2/35 (5.7)	23/61 (37.7)	9/65 (13.8)	34/161 (21.1)
DM BARD 13-14 203	0/1 (0.0)	2/24 (8.3)	1/12 (8.3)	3/37 (8.1)
BARD 13-14 a.d.	0/2 (0.0)	0/16 (0.0)	0/3 (0.0)	0/21 (0.0)
DM AD2-4 3s.2	2/26 (7.7)	4/30 (13.3)	5/23 (21.7)	11/79 (13.9)
DM AD2-4 3s.3	1/53 (1.9)	14/54 (25.9)	0/17 (0.0)	15/124 (12.1)
DM AD2-4 3s.8	1/13 (7.7)	2/11 (18.2)	1/15 (6.7)	4/39 (10.2)
AD2-4 a.d.	0/4 (0.0)	0/18 (0.0)	2/18 (11.1)	3/40 (7.5)
DM A3P2-6 6n.1	9/47 (19.1)	16/34 (47.1)	13/32 (40.6)	38/113 (33.6)
Total	18/322 (5.6)	155/570 (27.2)	65/383 (17.0)	

Numbers in parenthesis are percentages; a.d. = anther donor.

Table 10. Number of seeds per fruit obtained from crosses between DM or anther donor and ID pollinator.

Female parent	Pollinator		
	ID 4	ID 5	ID 8
Doubled monoploids			
DM BARD 9-9 203	21	206	77
DM BARD 1-3 516	-	59	150
DM BARD 13-14 200	32	27	31
DM BARD 13-14 201	-	59	-
DM BARD 13-14 202	52	45	64
DM BARD 13-14 203	-	28	4
DM AD2-4 3S.2	39	77	58
DM AD2-4 3S.3	-	28	-
DM AD2-4 3S.8	87	34	78
DM A3P2-6 6n.1	12	122	230
Anther donor			
BARD 1-3	-	116	108
AD2-4	-	-	96

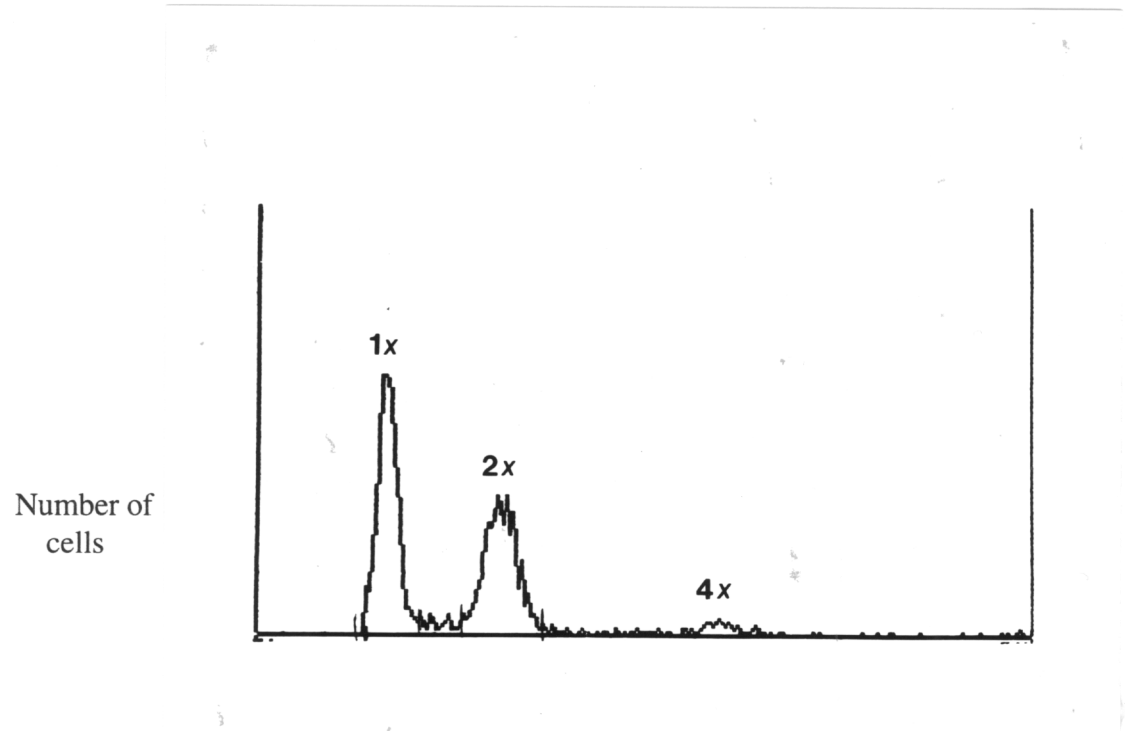

Figure 1. Ploidy distribution of cells obtained from PC 45-4 monoploid.

Appendix 1. Effect of genotype, silver thiosulfate and plant tissue on the weight and frequency of cells with 1*x*, 2*x* or 4*x* nuclei in potato monoploids grown *in vitro*.

Variable	n	Weight	%1*x* cells	%2*x* cells	%4*x* cells
Genotype					
AD 2-4 3s.2	18	.34 a	72 a	22 b	7 b
PC 21-78	18	.23 b	50 b	38 a	11 a
Silver thiosulfate					
absent	18	.31 a	57 b	32 a	10 a
present	18	.25 b	65 a	28 b	8 b
Plant tissue					
leaves	12	.34 a	66 a	28 b	6 b
stem	12	.23 b	55 b	32 a	12 a
whole shoot	12	.28 b	62 a	29 b	8 b

Mean separation within columns and variables using SNK, $\alpha = .05$
n = number of samples

Appendix 2. Mean performance of DMs and anther donor of *Solanum phureja* in the greenhouse.

Genotype		n	DTF	NI	NFI	TN	ATY
BARD 1-3 a.d.		4	56.2	6.2	7.3	23.2	90.8
DM BARD 1-3	511	2	66.5	1.5	2.5	3.0	40.6
DM BARD 1-3	516	1	66.0	7.0	8.1	1.0	7.9
BARD 13-14 a.d.		5	68.0	1.4	5.6	1.0	11.3
DM BARD 13-14	200	15	64.8	2.3	7.6	16.2	39.0
DM BARD 13-14	201	7	67.3	1.8	8.7	7.5	22.0
DM BARD 13-14	202	11	64.0	2.7	5.7	8.3	27.8
DM BARD 13-14	203	7	66.0	1.3	5.5	6.5	18.8
BARD 9-9	203	6	57.0	3.3	6.9	0.8	7.7
AD2-4 a.d.		3	66.7	5.5	6.4	2.0	2.9
DM AD2-4	3s.2	1	70.0	1.0	7.0	5.0	3.2
DM AD2-4	3s.3	1	61.0	4.0	6.2	0	0

Note: n = number of sample plants; DTF = days to flower; NI = number of inflorescence; NIF = number of flowers per inflorescence; TN = mean tuber number; ATY = average tuber yield; a.d. = anther donor.

Chapter 3: Genetic diversity based on RAPD polymorphism and its relationship with the performance of *Solanum phureja* hybrids

Summary

The overall objectives of this study were to generate potato hybrids based on selection of genetically distant parents as measured by RAPD markers, and to develop high yielding diploid potato germplasm that may be instrumental in new cultivar development. RAPD analysis was done on *in vitro* plantlets of *S. phureja* monoploids and diploid heterozygous pollinators. From among sixty decamer primers screened, eleven did not show polymorphism while some primers produced complex banding patterns or faint bands that were difficult to score. Genetic distance estimates were based on 151 polymorphic RAPD markers of 208 bands scored using 33 primers. Both simple matching and Jaccard coefficients were obtained to estimate genetic similarity (GS). Genetic distance (GD=1-GS) among genotypes ranged from 0.0 to 0.664. Cluster analysis exhibited groupings of genotypes that were consistent with known genomic compositions or genetic relationships inferred from their pedigree.

Field evaluation of fourteen F_1 families resulting from *S. phureja* doubled monoploid (DM) × IDs revealed significant differences with respect to total tuber number, total tuber yield, average tuber weight and vigor. Total tuber yield per family ranged from 174 to 404 g per

plant and was significantly lower than the cultivar check Kennebec. The average combining ability of DM BARD 13-14 202 was superior to the other DM parents. Specific combining ability was noted in AD2-4 3s.8 × ID 4 progeny. Among the male parents, ID 8 performed better than ID4 or ID 5.

Using simple matching, the largest parental genetic distance was always associated with the highest total tuber yield among the progenies of DM parents. A similar trend was obtained using Jaccard coefficients. Based on our results, RAPDs have the potential to facilitate the identification of diverse parents to maximize the expression of heterosis in *S. phureja* hybrids.

Introduction

The importance of the primitive species *Solanum phureja* Juz. & Buk. ($2n=2x=24$) to the improvement of the tetraploid potato *S. tuberosum* L. subsp. *tuberosum* includes such salient traits as resistance to bacterial wilt, virus resistance and lack of tuber dormancy (Hawkes and Hjerting 1989). Lack of tuber dormancy has been significant in the domestication of *S. phureja* since it offered the chance of having more than one crop per year in frost-free valleys at high altitude (Correll 1962). This trait would be undesirable, however, in temperate potato production as it is generally associated with poor storability of tubers. The utilization of *S. phureja* in potato improvement requires enhancing its yield components such as tuber size and

tuber set because the number of tubers per plant tends to be too high in unselected populations (Veilleux 1990).

Development of highly heterozygous hybrids by crossing clones of diverse parentage may be an essential step to improving yield and quality in *S. phureja.* Broadening the genetic base of breeding populations may be achieved by introducing germplasm of its wild and cultivated relatives. Carroll (1987) reported that 2*x* hybrids between *S. phureja* and dihaploid *S. tuberosum* showed slightly higher yield and had larger tubers than *S. phureja.* In tetraploid potato, best breeding results may be obtained by maximizing allelic diversity at as many loci as possible (Mendiburu et al. 1974). Chase (1963) has outlined a procedure of employing dihaploids to produce inbred lines which may be used to generate vigorous heterozygous tetraploids. Other workers have also proposed the use of dihaploid breeding methods to facilitate potato improvement (Wenzel et al. 1979, Ross 1986, Deimling et al. 1988). The analytic breeding scheme of Chase (1963) could be enhanced by selection at the monoploid level where there are no allelic interactions. Genotypes bearing lethal alleles and severely deleterious genes would be eliminated through the monoploid seive (Wenzel et al. 1979). Hemizygosity in monoploids exposes any lethal or recessive deleterious alleles which would cause embryos bearing them to perish. The generation of monoploids and subsequent production of homozygous diploids [doubled monoploids (DM)] provide the plant breeder with potentially desirable lines and a valuable tool to hasten crop improvement by allowing the rapid production of homozygous lines; thus, selection efficiency can be increased. Fertile

homozygous lines can be used to generate highly heterozygous but genetically uniform F_1 hybrids. If such hybrids have functional $2n$ gametes, they can be used in various breeding schemes involving sexual polyploidization to generate improved potato cultivars for traditional vegetative propagation or for true potato seed (TPS).

Heterosis or hybrid vigor has been closely related with heterozygosity at a large number of loci (Moll et al. 1965, Mendiburu et al. 1974, Mendoza and Haynes 1974, Stuber 1994) thus, heterosis may be estimated by divergence for molecular markers between parental genotypes (Hallauer et al. 1988). That is, DNA marker analysis may facilitate the evaluation of genotypes and permit selection of parental materials polymorphic for many loci. DNA polymorphism is not expected to be greatly influenced by environmental conditions and may be present in unlimited numbers. Melchinger et al. (1991) obtained a grouping of maize lines which was generally consistent with expected results based on known breeding behavior and pedigree using data from restriction fragment length polymorphism (RFLP). RFLP-based estimation of genetic distance could be used for assigning inbreds to heterotic groups (Melchinger et al. 1992). RFLP data were highly correlated with coefficients of parentage among parents and genetic distances had a higher correlation with grain yield heterosis than any other measure of similarity in maize (Smith et al. 1990). Meanwhile, Melchinger et al. (1990) reported that genetic distances between inbred lines of maize which were calculated from RFLP data as Rogers' distance (Rogers 1972) were not adequately associated with heterosis for yield. In tetraploid potato, the significance of maximum heterozygosity in

heterosis was reported to be dependent on the genetic background of the genotype being evaluated (Bonierbale et al. 1993). Another type of DNA marker which has been utilized to evaluate population differentiation and estimate genetic relationships is random amplified polymorphic DNA (RAPD). RAPD markers and cluster analysis based on Rogers' distances (Rogers 1972) were used to study some anther donors and androgenic monoploids of diploid *Solanum* species and the clusters formed resembled classification based on parental origins and hybrid combinations (Singsit and Ozias-Akins, 1993). The use of RAPD assay to identify cultivars and assign genotypes to heterotic groups is easier and the technique is faster compared to the labor-intensive and time consuming RFLP analysis (Caetano-Anolles et al. 1991, Weising et al. 1991). RAPD-based genetic distance was used to predict heterosis in *Eucalyptus globulus* although it accounted for less than 5% of the variation in SCA (Vaillancourt et al. 1995). In contrast, a lack of direct correlation between genetic distance and heterosis was observed in *Brassica juncea* by Jain et al. (1994) . Results of their study did not show the applicability of RAPD-based genetic distance alone for predicting hybrid performance. Comparisons of genetic diversity obtained by RAPD and RFLP analyses have shown that RAPDs are as reliable as RFLPs to estimate genetic relationships (dos Santos et al. 1994, Hallden et al. 1994, N'Goran et al. 1994).

The RAPD assay is based on the amplification of random DNA segments by the polymerase chain reaction (PCR) using short, random sequence oligonucleotides as primers (Williams et al. 1990, Rafalski et al. 1991). A primer usually produces several amplification products

which originate from different genomic locations (Rafalski et al. 1991). RAPD markers could cover the entire genome by amplifying coding or non-coding regions, repeated or single copy sequences (N'Goran et al. 1994). The procedure is simple and results can be obtained in one day (Rafalski et al. 1991, Horn and Rafalski 1992, Demeke et al. 1993). The amplified products are separated by agarose gel electrophoresis and variable number and size of bands which reflect sequence variation are displayed. Polymorphism based on RAPDs may result from single base changes, deletions, insertions or inversions that alter primer binding sites (Williams et al 1990).

The overall objectives of this study were to investigate the feasibility of generating potato hybrids based on selection of genetically distant parents based on RAPD markers and to develop high yielding diploid potato germplasm that may be instrumental in new cultivar development. The specific objectives were: (1) to estimate genetic distance using RAPD markers among *S. phureja* monoploids and diploid heterozygous pollinators (ID lines); (2) to evaluate progeny (DM × ID) performance under field conditions; and (3) to investigate the relationship between parental genetic distance and yield or vigor in F_1 progenies.

Materials and Methods

Experiment 1. Estimation of genetic distance among *S. phureja* monoploids and

heterozygous pollinators based on RAPD polymorphism

Plant Materials. *In vitro* plantlets of six anther-derived monoploids of *S. phureja* (BARD 1-3 516, BARD 13-14 202, BARD 9-9 203, AD2-4 3s.3, AD2-4 3s.8, A3P2-6 6n.1) and four heterozygous pollinators referred to as ID lines (ID 4, ID 5, ID 8, ID 9) were analyzed. The ID lines were kindly provided by Dr. Joe Pavek, USDA/ARS, Aberdeen, Idaho under the designation AD x 463-5, AD x 497-1, AD x 881-4 and AD x 916-12, corresponding to ID 4, 5, 8, and 9, respectively. They represent complex interspecific hybrids such that ID 4 and 5 comprise 1/2 *S. stenotomum* (*stn*), 3/16 *S. phureja* (*phu*), 3/16 *S. tuberosum* (*tbr*), and 1/8 *S. chacoense* (*chc*) whereas ID 8 and 9 comprise 7/16 *stn*, 11/32 *tbr*, and 7/32 *phu*. These clones represent field selections for horticultural traits and male fertility. The monoploids and corresponding DMs were derived by anther culture of field selections from an adapted population of *S. phureja/S. stenotomum* (Haynes 1972). The BARD clones were independently selected for anther culture competence from a heat tolerant subpopulation of the original "Haynes' *phureja*". AD2-4 3s.3 and 3s.8 were regenerated by anther culture of a homozygous DM (AD2-4) and would be expected to differ from each other only by possible gametoclonal variation. A3P2-6 6n.1 was derived by anther culture of a hybrid between DM AD3-4 (full sib to AD2-4) and a heterozygous pollinator. *In vitro* plantlets of these genotypes were maintained on MS basal medium (Murashige and Skoog 1962) for 4 to 5 weeks before DNA extraction.

DNA isolation. Genomic DNA was extracted following the method of Doyle and Doyle

(1987) with some modifications. Fresh leaves and stems (0.3 - 0.5 g) obtained from *in vitro* plantlets were ground in liquid nitrogen with a mortar and pestle until a fine powder was obtained. The DNA was isolated using 1 ml CTAB extraction buffer [0.1 M Tris-HCl (pH 8), 1.4 M NaCl, 0.02 M EDTA (pH 8), 2% hexadecyltrimethylammonium bromide (CTAB), 1% fresh 2-mercaptoethanol]. The grindate was transferred to a 1.5 ml microcentrifuge tube and incubated in a water bath at 60°C for 1-2 h. An equal amount of chloroform:isoamyl solution (24:1) was added to the tube, the contents were mixed and centrifuged for 15 min. The upper aqueous portion was deposited into a new microcentrifuge tube containing 200 µl cold isopropanol. The solution was discarded and the DNA pellet was collected and washed twice with cold 75% ethanol. The DNA was air-dried, redissolved in TE (10 mM Tris-HCl, 1mM EDTA) and RNAse (10 µg/ml). DNA concentration was determined using a TKO 100 mini-fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

RAPD analysis. Amplification reactions were done in volumes of 25 µl containing 1× *Taq* polymerase buffer II (10 mM Tris-HCl, pH 8.3; 50 mM KCl), 2.5 mM $MgCl_2$, 200 µM each of dATP, dCTP, dGTP and dTTP, and 1.0 unit *Taq* polymerase. In each of the reaction mixtures 20 ng genomic DNA was used as template for amplification by PCR using 0.6 µM 10-mer oligonucleotide. Three priming sets, each consisting of twenty random primers were tested - OPA, OPC and OPG (Operon Technologies, Alameda, CA). The reaction mix was overlaid with a drop of mineral oil. Thermal cycling conditions consisted of 45 cycles of 1 min at 94°C (denaturation), 1 min at 36 C (annealing) and 2 min at 72 C (primer extension)

followed by one cycle of 3 min at 72°C for final extension and a 4°C soak until the samples were unloaded. All PCR amplifications were carried out in a Perkin Elmer Cetus 480 thermal cycler. The amplified DNA products were analyzed on 1.4% agarose gel run with Tris-borate-EDTA buffer. The gel was stained with ethidium bromide (1 mg/100 ml) for 10 min and photographed on a UV transilluminator. Reproducibility of results was verified by doing RAPD analysis twice on separate dates, or once but always in conjunction with a positive check previously characterized. A negative control which contained all the necessary PCR components except for the template DNA was included in all PCR runs.

Data analysis. The primers revealing polymorphism were used to determine genetic distance. If a RAPD marker was present in a genotype it was designated 1, if absent it was given a score of 0. Pairwise comparisons of genotypes were done to obtain genetic similarity (GS) using simple matching (Sokal and Michener 1958) and Jaccard [Jaccard 1908 (cited by Yu and Nguyen 1994)] similarity coefficients. Genetic distance (GD) among *S. phureja* monoploids and ID lines was calculated as: GD=1-GS (Jain et al. 1994). An unweighted pair-group method with arithmetical averages (UPGMA) cluster was constructed using the NTSYS (1988) computer program.

Experiment 2. Field evaluation of F_1 hybrids of *S. phureja* DM × ID and the relationship between parental genetic distance and hybrid performance

Plant Materials. DMs were generated by leaf disc regeneration in *S. phureja* monoploid clones based on the methods of M'Ribu and Veilleux (1990) and Hulme et al. (1992). The monoploids were determined by flow cytometry from among anther-derived plants, then doubled by leaf disc regeneration (see chapter 2) and determined to have doubled by another flow cytometric analysis. Subsequently, *in vitro* microtuberization of DMs was done following the method of Hussey and Stacey (1984) with some modifications. Single nodal cuttings with one leaf were grown on MS basal medium with 5 mg/l BA and 100 mg/l chlorocholine chloride. Cultures were maintained in the dark at room temperature until microtubers were produced. Microtubers of DMs were established in the greenhouse and plants generated were maintained under 16 h photoperiod. The same procedure was done with the anther donors and diploid ID pollinators. Hybridization was carried out for two seasons (spring and fall 1994). In spring, a set of five *S. phureja* DMs (DM BARD 1-3 516, DM BARD 13-14 202, DM BARD 9-9 203, DM AD2-4 3s.8, DM A3P2-6 6n.1) was crossed in all possible combinations with a set of ID lines (ID 4, ID 5, ID 8, ID 9) to generate F_1 hybrids, and seeds obtained from these crosses were stored at 5°C except for ID 9 which did not produce seeds. In August, 1994 seeds (100 per cross) were treated overnight with 1000 µg/l GA_3, then sown in the greenhouse. The seedlings were transplanted to 25.8 cm^2 plastic pots and grown in the greenhouse until maturity. Minitubers were harvested in December 1994 and stored at 4°C for a minimum of three months prior to planting in the field.

Success of producing F_1 hybrids in spring was low; hence, more crosses were made in fall.

However, there was not enough time to generate minitubers between seed collection from the fall crosses and field planting. Instead, transplanted seedlings were used for field planting. Seeds (100 per cross) were germinated in the greenhouse. The seedlings were transferred individually to cell packs and grown in the greenhouse for one month before transplanting in the field.

Field set-up. Field evaluation was carried out from April to September, 1995. The first set-up (experiment 2A) consisted of three replications of five families (DM A3P2-6 6n.1 × ID 4, DM A3P2-6 6n.1 × ID 5, DM A3P2-6 6n.1 × ID 8, DM AD2-4 3s.8 × ID 5, DM BARD 9-9 203 × ID 5) using both tubers and seedlings in separate plots as planting materials. Sixteen-plant-plots were laid out using a strip plot design with family as the horizontal factor and planting material as the vertical factor (Appendix 1). The aim of this experiment was to determine the relative performance of tuber-generated progenies and progenies grown from seedlings. The absence of significant differences for the interaction of family and planting material would be used to support the results of experiment 2B wherein, due to the unavailability of tubers, plants were propagated exclusively from seedlings. Experiment 2B, consisting of 14 F_1 families, was laid out as a randomized complete block with three replications using seedlings as planting material. Kennebec was used as the check cultivar. Sixteen-plant-plots were used; hence, each family was represented by 48 different genotypes. Established major production, management and cultural practices concerning weed control, spraying, fertilization and irrigation for field testing of potato were carried out. The following

were obtained; tuber number per plant, tuber yield per plant, mean tuber weight, vigor (scale of 1=high to 4=low) at 8 weeks after planting (WAP), and maturity (scale of 1=dried up shoots to 5=upright, still flowering) at 18 WAP. Vigor was scored on an individual plant basis whereas maturity was scored on a plot basis.

Relationship between genetic diversity and yield or vigor. The association between genetic distance of parental genotypes and yield or vigor (based on means of all three replications) in experiment 2B was investigated. Correlation analysis was done.

Statistical analysis

All statistical analyses were performed using SAS General Linear Models procedure (SAS 1985).

Results

Experiment 1. Estimation of genetic distance among *S. phureja* monoploids and heterozygous pollinators based on RAPD polymorphism

The RAPD products were classified into two types - monomorphic (constant) or polymorphic

(variable). From among 60 primers screened, eleven (18.3%) did not show polymorphism while some primers produced complex banding patterns or very faint bands that were difficult to score. A total of 208 bands was scored using 33 primers, 57 of which were not polymorphic; thus, genetic distance estimates were based on 151 RAPD markers (Table 1). Figure 1 shows an example of gel electrophoresis of DNA amplified with OPA primers and visualized by staining with ethidium bromide. Band "a" produced by OPA-18 was monomorphic and was not included in calculating genetic distance whereas bands "b" and "c" amplified by OPA-4 were polymorphic and were used to estimate genetic distance.

Both simple matching and Jaccard coefficients were obtained to estimate genetic similarity (GS). Genetic distance (GD=1-GS) among genotypes ranged from 0.0 between AD2-4 3s.3 and AD2-4 3s.8 to 0.664 between BARD 1-3 516 and ID 9 using Jaccard coefficients (Table 2). A similar trend was achieved based on simple matching (SM) coefficients: AD2-4 3s.3 vs. AD2-4 3s.8, GD=0.0; BARD 1-3 516 vs ID 9, GD=0.537; (Table 3). Monoploids of the independently selected *S. phureja* (BARD 9-9, 1-3 and 13-14) showed as much diversity among themselves as they did compared to the ID pollinators. This underscored the diversity in the adapted population.

Cluster analysis exhibited groupings which were consistent with known genomic compositions or genetic relationships. The complex diploid pollinators of similar genomic composition (ID 4 and ID 5) were sorted from those known to have a different genomic composition (ID 8 and

ID 9; Figs. 2 and 3). AD2-4 3s.3 and AD2-4 3s.8 which are sibling monoploids derived from a homozygous DM were not separable. A3P2-6 6n.1 shares a common parent with the AD2-4 monoploids and exhibited correspondingly lower genetic distance from them. The fit of the phenogram to the similarity matrix was very high with a matrix correlation of r=0.966 (Jaccard) and r=0.945 (SM).

Experiment 2. Field evaluation of *S. phureja* DM × ID F_1 hybrids and the relationship between parental genetic distance and hybrid performance

There were no significant differences for the average number of tubers (ATN), average tuber yield (ATY) and mean vigor between similar progenies grown from tubers (tuber families) and seedlings (seedling families) (Table 4). Average tuber weight was significantly greater in tuber families and maturity was delayed in seedling families. Interaction effect was not significant for all traits (Table 5). These results show that the performance of the progenies was not affected by the type of planting material used. This observation was critical and essential in interpreting the results of the second experiment which was propagated exclusively by seedlings instead of the conventional method of using tubers.

In the seedling population, there were significant differences among the 14 DM × ID F_1 families with respect to total tuber number, total tuber yield, average tuber weight and vigor

(Table 6). Mean total tuber yield per plant ranged from 174 to 404 g for families DM AD2-4 3s.8 × ID 5 and DM BARD 1-3 516 × ID 8, respectively (Table 7). These yields were significantly lower than the cultivar check Kennebec (1320 g per plant). However, a bias could have been introduced because Kennebec was tuber-propagated. When the yield of each of the 650 genotypes used in the field trial was examined, some plants approached the yield of Kennebec (Fig. 4). The average combining ability of DM BARD 13-14 202 for total tuber yield was superior to that of the other DM parents (Table 7). Specific combining ability was noted in DM AD2-4 3s.8 × ID 4 progeny. The mean yield of the progenies of DM BARD 13-14 202 was significantly higher (368 grams) than the other DM progenies. However, the number of tubers was also high; hence, the average tuber weight was only 9 g (Table 8). Among the F_1 hybrids a wide range of the total number of tubers (13-49) was observed while Kennebec had an average of nine tubers. The mean tuber weight of these families varied from 8 to 17 g per plant compared to Kennebec at 147 g per tuber.

Among the male parents, ID 8 performed better than ID 4 or ID 5. The average tuber yield of the progenies of ID 8 was significantly higher (338 g) than ID 4 (285 g) or ID 5 (244 g) (Table 9). The progenies of ID 8 were also the most vigorous. A wide range of total tuber yield and total tuber number was noted for the progenies of each ID pollinator.

Simple correlation of genetic distance with yield among the 14 families was not significant. However, perusal of the data revealed that based on SM coefficients the highest total tuber

yield was associated with the largest genetic distance, among the progenies of each DM parent considered separately (Fig. 5). Using Jaccard coefficients the same trend was observed in families of DM BARD 1-3 516, DM BARD 13-14 202 and DM AD2-4 3s.8. Vigor was not significantly correlated with genetic distance although a relatively higher plant vigor was coupled with a large genetic distance using SM coefficients (Fig. 6). Based on Jaccard coefficients association between large parental genetic distance and high vigor was attained among the progenies of DM BARD 1-3 516, DM BARD 13-14 202 and DM AD2-4 3s.8.

Discussion

The breeding strategy undertaken for the improvement of *S. phureja* involved combination of the genome of *S. phureja* DMs with other *Solanum* genomes and exploiting the heterotic effects in hybrids. To carry out this endeavor, the relationship between genetic diversity of parental genotypes based on RAPD polymorphism and the performance of F_1 hybrids was investigated.

Six monoploids analyzed for RAPDs were chosen from a population of anther-derived monoploids of *S. phureja* on the basis of their ability for leaf regeneration to generate DMs. The ID lines which served as paternal genotypes were selected based on their diverse genetic

composition. Genetic similarity (GS) among S. *phureja* monoploids and ID lines was obtained using both simple matching (SM) and Jaccard coefficients. Comparison between genotypes based on the presence (1) or absence (0) of a RAPD marker produced four possible outcomes (a=1,1; b=0,1; c=1,0; d=0,0). To obtain the ratio of similarities to total comparisons, SM coefficients consider the four outcomes (a+d/a+b+c+d) while Jaccard coefficients do not include absence by absence comparison (a/a+b+c). The exception of (0,0) is deemed more suitable for interspecific comparisons (Skroch et al. 1992). Also, it has been asserted that Jaccard coefficients are more suitable in the study of *Sorghum* inbred lines instead of basing similarity on the mutual absence of a character (Vierling et al. 1994). However, if the probability of sequence homology is high as determined by the frequency of monomorphic bands, then mutual absence may be used (Skroch et al. 1992, Hallden et al. 1994). In *Phaseolus vulgaris*, a high level of band monomorphism (80% of detected marker loci were monomorphic for a RAPD band) was observed thus, absence by absence comparisons manifest high probability of sequence identity (Skroch et al. 1992). The majority of the primers utilized in the present study gave good amplification patterns. A bias may have been introduced in the assessment of GD because the monoploids have only one set of chromosomes while the ID lines were highly heterozygous. Since RAPD markers are dominant, homozygous or heterozygous loci in the ID lines could not be differentiated. This may result in underestimating genetic diversity between genotypes. However, our results showed that overall banding patterns reflected the natural relationships of the genotypes studied, dispelling the presence of bias in our estimation of GD. The absence of diversity

between AD2-4 3s.3 and AD2-4 3s.8 may be attributed to their being sibling monoploids derived from a single DM (homozygous diploid). This also verifies the homozygous nature of the DM parent. Overall, more closely related genotypes as deduced from their genomic compositions or diploid source were grouped together which demonstrated that the presence or absence of RAPDs reflected genetic relatedness. The approach to study DNA polymorphism by the RAPD assay using *in vitro* plantlets included the following advantages: (1) RAPD analysis requires only about 0.1 to 25.0 ng DNA (Demeke et al. 1993, Horn and Rafalski 1992) which makes it desirable for the analysis of *in vitro* plants; (2) RAPDs behave as dominant genetic markers (Williams et al. 1990, Welsh and McClelland 1990) hence, are convenient to use with monoploids which have only one set of chromosomes; (3) RAPD assay of monoploid plants would permit genotype-based selection prior to the production of DMs thus saving time and resources for the breeder.

One dynamic application of the RAPD assay is its ability to estimate genetic relationships that allows the breeder to sample the available germplasm more systematically and design matings which will keep diversity high. However, one should exercise vigilance in using DNA polymorphism to assign materials to heterotic groups and predict yield. With RAPDs, an essential factor to consider would be the number of primers to be screened for the proper assessment of genotypes. For closely related materials fifteen to twenty primers were necessary to generate a large number of amplification products and obtain heterotic groups in *Brassica juncea* (Jain et al. 1994). In addition, Bernardo (1992) reported that the

association between molecular marker heterozygosity and hybrid performance is highly dependent on the genotype being examined and listed some important conditions in order to predict hybrid performance efficiently using molecular markers. These include among others: (1) strong dominance effects; (2) high heritability for the character; and (3) at least 30-50% of the QTL must be linked to molecular markers.

Heterosis breeding is an essential means towards improving agricultural yield. For the improvement of *S. phureja,* ID lines which are complex diploid hybrids were used as sources of germplasm to maximize heterozygosity in the progenies. A trend was observed wherein higher yield among the progenies of DM parents was related with increase in parental genetic distance. However, when the ID lines were considered, no relationship or trend could be ascertained between the yield of the progenies and genetic distance. This may be attributed to the condition that the ID lines were complex hybrids involving unadapted germplasm that may have contributed allelic variance but poor agronomic performance. Hybrid performance between adapted and unadapted clones is usually diminished by genes for lack of adaptation (Bonierbale et al. 1993). Although genetic variability is increased, mean performance is curtailed and this hinders selection of good recombinants. Attempts to maximize heterosis in $4x \times 2x$ hybrids through introgression of unadapted germplasm had also mixed results (Bani-Aameur et al. 1991, Yerk and Peloquin 1990). The advantages of maximum heterozygosity may be canceled if the proportion of unadapted germplasm is too high. For an unadapted material, long term selection efforts to exclude its undesirable traits could be

done to develop a valuable breeding population (Eberhardt 1971). Selection of maize inbreds for hybridization by examination of genetic distance is a far different exercise due to the general adaptation of germplasm (Smith et al. 1990). If male fertile DMs of potato were available, a more powerful assignment of parents to generate heterotic combination would be possible. Given the germplasm available, the apparent trends in Fig. 5 are quite commendable.

To our knowledge this is the first report on the use of DMs to generate hybrids in *S. phureja*. In a related study, doubled dihaploids × cultivar crosses were used to enhance the progeny mean for the dihaploids' major trait (De,Maine 1992). Doubled dihaploids which are more homozygous than their tetraploid parents (De,Maine and Jervis 1989) but more heterozygous than inbred lines were also considered potential parents for the generation of hybrid true potato seed (TPS) (Clulow et al. 1995). Based on our results, RAPDs have the potential to facilitate the identification of diverse parents to maximize the expression of heterosis in *S. phureja* hybrids.

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Table 1. Primers resulting in the amplification of polymorphic RAPD markers used to calculate genetic distance.

No.	Primer	Primer sequence 5' to 3'	Number of RAPDs scored
1	OPA-02	TGCCGAGCTG	1
2	OPA-04	AATCGGGCTG	5
3	OPA-07	GAAACGGGTG	4
4	OPA-08	GTGACGTAGG	3
5	OPA-09	GGGTAACGCC	3
6	OPA-10	GTGATCGCAG	3
7	OPA-11	CAATCGCCGT	4
8	OPA-16	AGCCAGCGAA	5
9	OPA-18	AGGTGACCGT	5
10	OPA-19	CAAACGTCGG	6
11	OPC-02	GTGAGGCGTC	1
12	OPC-03	GGGGGTCTTT	3
13	OPC-05	GATGACCGCC	4
14	OPC-06	GAACGGACTC	6
15	OPC-08	TGGACCGGTG	2
16	OPC-09	CTCACCGTCC	7
17	OPC-10	TGTCTGGGTG	3
18	OPC-13	AAGCCTCGTC	2
19	OPC-14	TGCGTGCTTG	7
20	OPC-15	GACGGATCAG	6
21	OPC-18	TGAGTGGGTG	4
22	OPC-19	GTTGCCAGCC	6
23	OPC-20	ACTTCGCCAC	7
24	OPG-01	CTACGGAGGA	4
25	OPG-04	AGCGTGTCTG	4
26	OPG-05	CTGAGACGGA	12
27	OPG-08	TCAGGTCCAC	3
28	OPG-09	CTGACGTCAC	5
29	OPG-12	CAGCTCACGA	12
30	OPG-13	CTCTCCGCCA	2
31	OPG-14	GGATGAGACC	3
32	OPG-15	ACTGGGACTC	2
33	OPG-16	AGCGTCCTCC	7

Table 2. Genetic distance (GD) based on RAPD polymorphism among monoploids of *S. phureja* and diploid heterozygous pollinators (ID lines) using Jaccard similarity coefficients.

	Genotype	1	2	3	4	5	6	7	8	9	10
1	BARD 9-9 203										
2	AD2-4 3s.3	.509									
3	AD2-4 3s.8	.509	.000								
4	A3P2-6 6n.1	.420	.321	.321							
5	BARD 1-3 516	.598	.626	.626	.597						
6	BARD 13-14 202	.619	.610	.610	.597	.596					
7	ID 4	.586	.659	.659	.566	.655	.571				
8	ID 5	.500	.592	.592	.508	.617	.536	.340			
9	ID 8	.569	.617	.617	.550	.656	.646	.525	.439		
10	ID 9	.577	.625	.625	.547	.664	.643	.521	.434	.011	

Table 3. Genetic distance (GD) based on RAPD polymorphism among monoploids of *S. phureja* and diploid heterozygous pollinators (ID lines) using simple matching coefficients.

	Genotype	1	2	3	4	5	6	7	8	9	10
1	BARD 9-9 203										
2	AD2-4 3s.3	.377									
3	AD2-4 3s.8	.377	.000								
4	A3P2-6 6n.1	.311	.225	.225							
5	BARD 1-3 516	.429	.463	.463	.456						
6	BARD 13-14 202	.477	.470	.470	.483	.407					
7	ID 4	.450	.536	.536	.457	.490	.416				
8	ID 5	.371	.470	.470	.404	.456	.389	.225			
9	ID 8	.464	.523	.523	.470	.531	.537	.401	.331		
10	ID 9	.470	.530	.530	.464	.537	.530	.404	.325	.007	

Table 4. Average performance of five F_1 families (DM × ID) grown from tubers or seedlings.

Planting material	ATN	ATY (g)	ATW (g)	Vigor	MAT
tuber	12 a	317 a	27 a	2.5 a	2.6 b
seedling	19 a	249 a	14 b	2.7 a	3.5 a

Means within a column followed by the same letter are not significantly different using SNK at 5% level.

Note: ATN = average tuber number
ATY = average tuber yield
ATW= average tuber weight
Vigor rating: scale of 1=high to 4=low
Maturity rating: scale of 1=dried up shoots to 5=upright, still flowering
F_1 families: DM A3P2-6 6n.1 × ID 4, DM A3P2-6 6n.1 × ID 5, DM A3P2-6 6n.1 × ID 8, DM AD2-4 3s.8 × ID 5, DM BARD 9-9 203 × ID 5

Table 5. Mean squares for vigor, maturity and tuber characters among five DM × ID F_1 families grown from tubers or seedlings.

Source	df	Mean square				
		ATN	ATY	ATW	Vigor	Mat
Hybrid (A)	4	1703 *	968052 **	193 ns	11.9 *	1.2 ns
Planting material (B)	1	4883 ns	294964 ns	16688 **	6.5 ns	5.0 *
A X B	4	56 ns	328028 ns	532 ns	2.5 ns	0.5 ns

Note: *=significant at 5% level; **=significant at 1% level; ns=not significant
ATN=average tuber number; ATY=average tuber yield; ATW=average tuber weight; Mat=maturity.

Table 6. Mean squares for vigor, maturity and tuber characters in fourteen DM × ID F_1 families grown from seedlings.

Trait	Mean square
total number of tubers	5968.7 **
total tuber yield	2405135.4 **
average tuber weight	37280.9 **
vigor	12.8 **
maturity	1.8 ^{ns}

**=significant at 1% level
ns=not significant

Table 7. Mean total tuber yield (g per plant) of DM × ID F_1 families. Each mean represents 48 hybrids.

Female parent		Male parent ID 4	ID 5	ID 8	Mean
DM BARD 1-3	516		194	404	299 b
DM BARD 13-14	202	357	371	377	368 a
DM BARD 9-9	203	197	193	302	231 c
DM AD2-4	3s.8	321	174	283	259 bc
DM A3P2-6	6n.1	265	288	326	293 b
Mean		285 b	244 c	338 a	
check cultivar					
Kennebec	1320				

Mean separation by SNK at 5% level.

Table 8. Performance of progenies of female parents (DMs). Numbers in parentheses are family ranges.

Female parent		Number of families	Mean tuber yield (g)	Mean tuber number	Mean tuber weight (g)	Vigor
DM BARD 1-3	516	2	299 b (194-404)	28 b (18-37)	12 b (12-13)	2.8 a
DM BARD 13-14	202	3	368 a (357-377)	43 a (34-49)	9 c (8-11)	2.3 c
DM BARD 9-9	203	3	236 c (193-302)	27 b (16-36)	10 c (8-12)	2.2 c
DM AD2-4	3s.8	3	260 bc (174-321)	20 c (13-24)	14 ab (13-15)	2.7 ab
DM A3P2-6	6n.1	3	293 b (265-326)	22 c (19-25)	15 a (14-17)	2.6 a

Mean separation within columns by SNK at 5% level.
Vigor rating: scale of 1=high to 4=low.

Table 9. Performance of progenies of male parents (ID lines).

Male parent	Number of families	Mean tuber yield (g)	Mean tuber number	Mean tuber weight (g)	Vigor
ID 4	4	285 b (197-357)	30 b (22-46)	13 a (8-15)	2.5 b
ID 5	5	244 c (174-371)	20 c (13-34)	12 a (11-17)	2.7 a
ID 8	5	338 a (283-404)	34 a (23-49)	12 a (8-15)	2.2 c

Mean separation within columns by SNK at 5% level.
Numbers in parentheses are family ranges.
Vigor rating: scale of 1=high to 4=low.

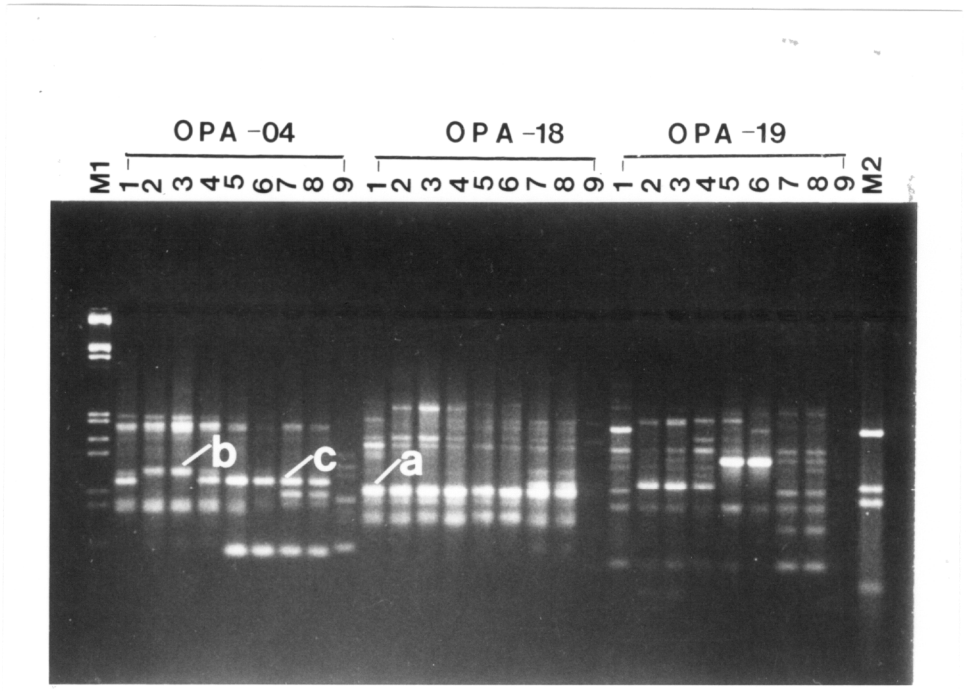

Figure 1. Agarose-gel electrophoresis of RAPD markers obtained by PCR amplification of genomic DNA obtained from *S. phureja* monoploids and ID lines using decamer primers (from left to right) OPA-04, OPA-18 and OPA-19. There are nine lanes for each primer: (1) BARD 9-9 203; (2) AD2-4 3s.3; (3) AD2-4 3s.8; (4) A3P2-6 6n.1; (5) ID 4; (6) ID 5; (7) ID 8; (8) ID 9; (9) control with no DNA. M1 contains *Eco*R I-*Hind* III digested lambda DNA and M2 has pBR322 *Bst*N1 digest. (Examples of scored bands: band "a" was monomorphic and was not included in calculating genetic distance whereas bands "b" and "c" were polymorphic and were used to estimate genetic distance).

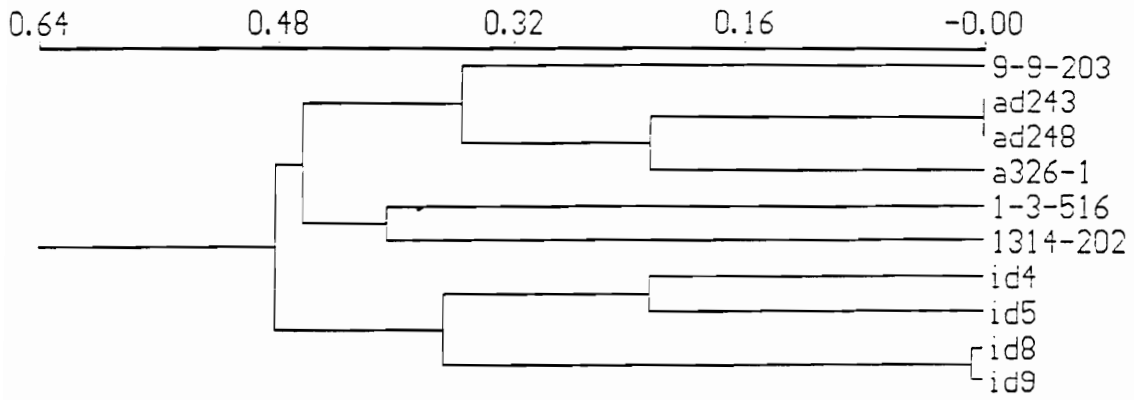

Figure 2. Relationships among *Solanum phureja* monoploids and diploid heterozygous pollinators (ID lines) disclosed by UPGMA cluster analysis of simple matching coefficients based on 151 RAPD markers and 33 primers.

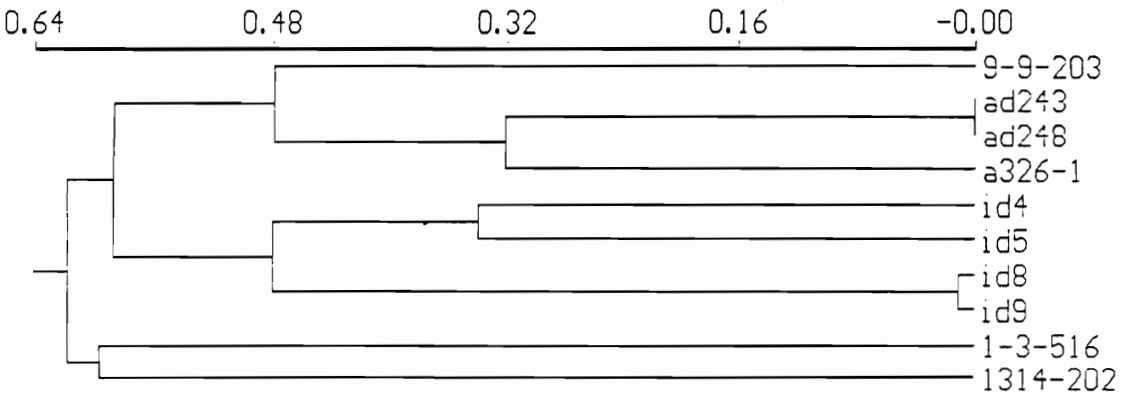

Figure 3. Relationships among *Solanum phureja* monoploids and diploid heterozygous pollinators (ID lines) disclosed by UPGMA cluster analysis of Jaccard genetic similarity coefficients based on 151 RAPD markers and 33 primers.

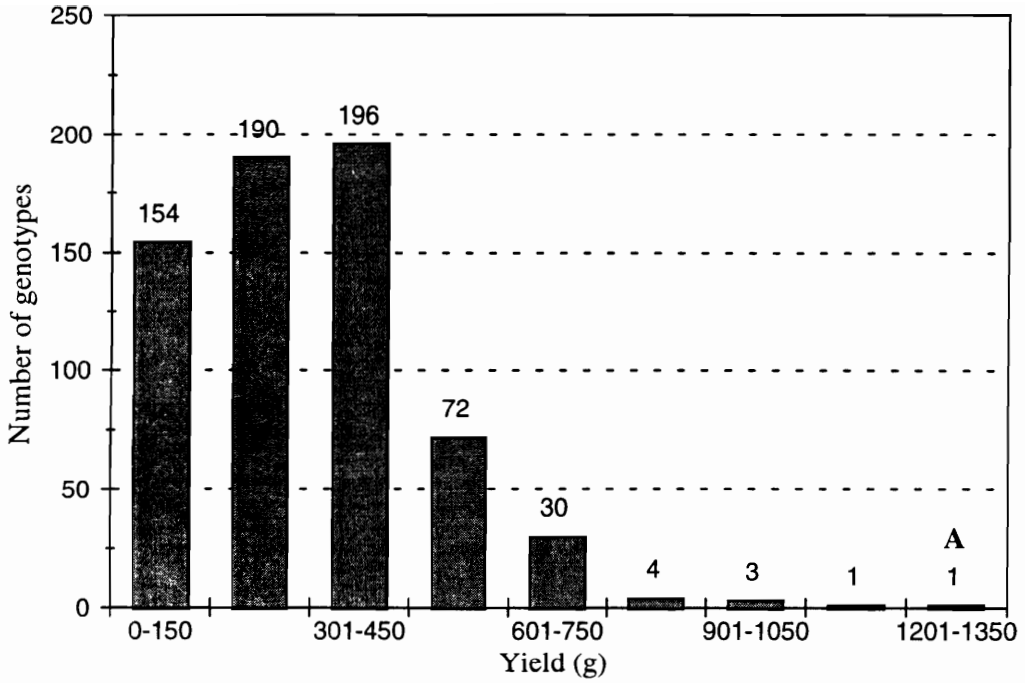

Figure 4. Distribution of yield among 650 genotypes derived from fourteen DM × ID F_1 crosses. The figures above the bars represent the number of genotypes included in each bar. (A=cultivar check Kennebec).

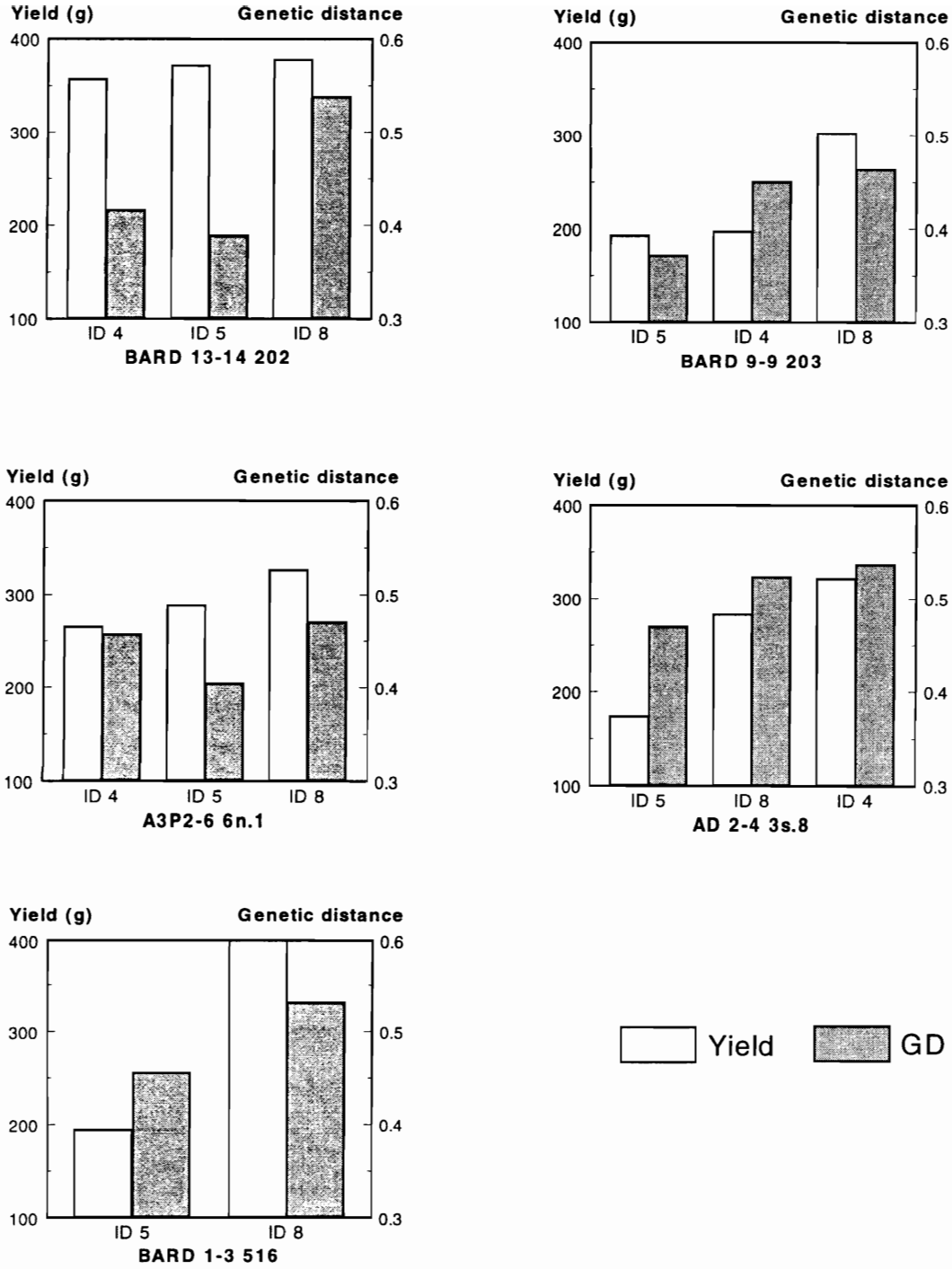

Figure 5. Relationship between parental genetic distance based on simple matching coefficients and yield among fourteen doubled monoploid × ID F_1 crosses with respect to the maternal parent.

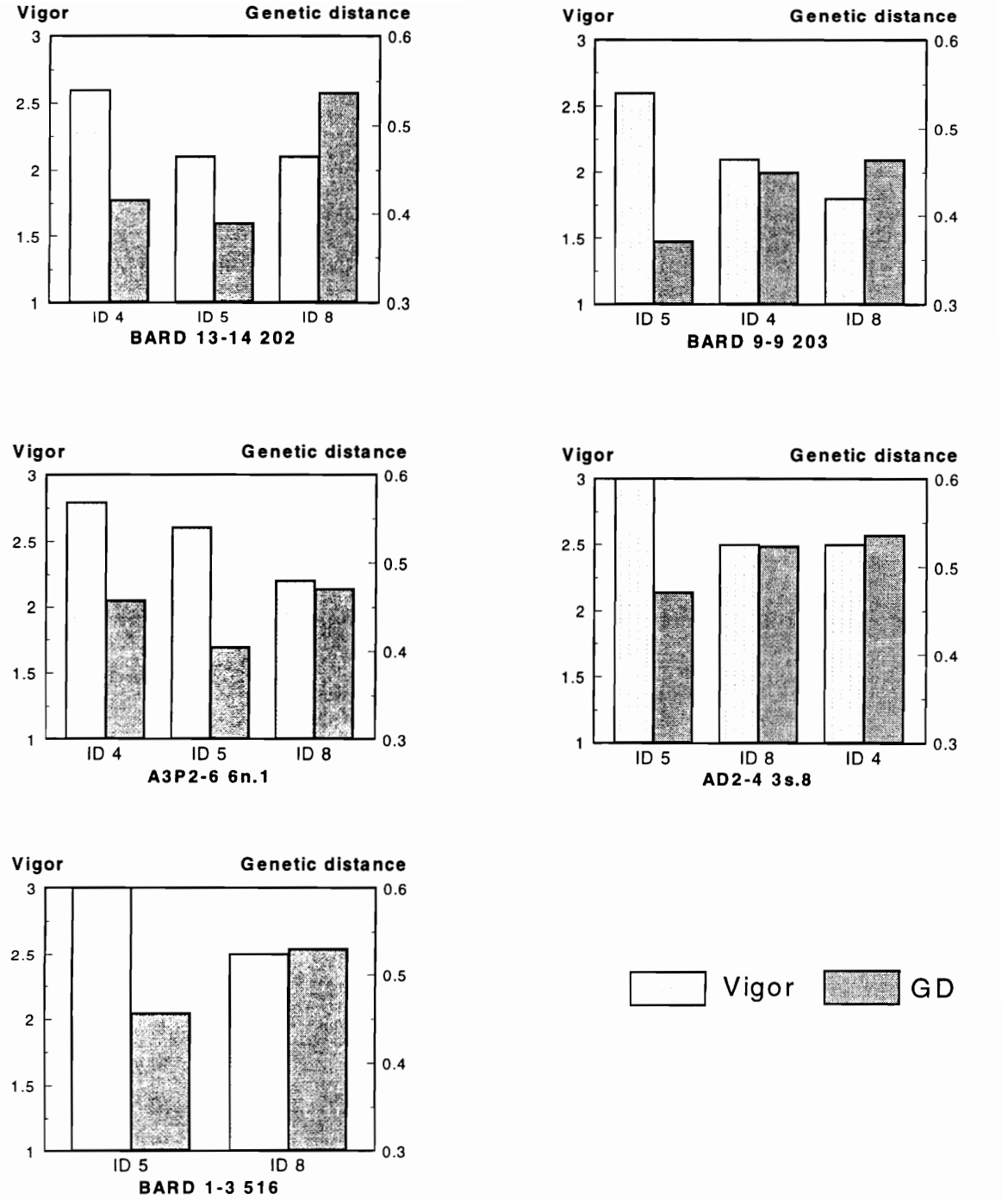

Figure 6. Relationship between parental genetic distance based on simple matching coefficients and vigor (scale of 1=high to 4=low) among fourteen doubled monoploid × ID F_1 crosses with respect to the maternal parent.

Appendix 1. Analysis of variance for a 2 x 5 factorial expriment using strip-plot design.

Source of variation	Degree of freedom	Sum of squares	Mean square	F value
Replication	r-1=2	SS1	MS1	
Horizontal factor (A)	a-1=4	SS2	MS2	MS2/MS3
Error(a)	(r-1)(a-1)=8	SS3	MS3	
Vertical factor (B)	b-1=1	SS4	MS4	MS4/MS5
Error(b)	(r-1)(b-1)=2	SS5	MS5	
A x B	(a-1)(b-1)=4	SS6	MS6	MS6/MS7
Error(c)	(r-1)(a-1)(b-1)=8	SS7	MS7	
Total	rab-1=29	SS8		

Note: Horizontal factor=progeny
Vertical factor=planting material

Chapter 4: Genetic characterization of potato using SSR markers

Summary

Anther-derived diploids may be heterozygous (arising from a somatic cell or unreduced microspore) or homozygous (arising from reduced microspores that undergo chromosome doubling). Homozygous diploids comparable to inbred lines are generally the desired product of anther culture in breeding programs. Thus, a simple procedure to determine the genetic composition of androgenic diploids would facilitate their utilization in potato improvement. In the present study, SSR markers were used to analyze the genetic composition of anther-derived potato plants. The banding pattern of the anther-derived monoploids based on SSR analysis was consistent with expected results according to the genetic composition of monoploids wherein only a single allele per locus should be present. Both homozygous and heterozygous diploids were identified. Five diploids exhibited only a single band at all five SSR loci verifying their homozygosity. The other nine diploid plants were heterozygous at one to five SSRs where the presence of more than one allele was observed. SSRs were also utilized to study allelic segregation in an F_1 population. Results of this experiment revealed Mendelian inheritance of SSR alleles. Based on our results, SSR analysis provides a powerful method for the genetic characterization of plant populations.

Introduction

Improved yield in the cultivated tetraploid potato *Solanum tuberosum* L. may be attained by maximizing levels of heterozygosity. This could be achieved by introducing the germplasm of its wild and cultivated relatives to broaden the genetic base of breeding populations. Chase (1963) has outlined a procedure of employing dihaploids to produce inbred lines which may be used to generate vigorous heterozygous tetraploids. The analytic breeding scheme of Chase (1963) could be enhanced by selection at the monoploid level where there are no allelic interactions, i.e., genotypes bearing lethal or deleterious alleles could be eliminated through the monoploid sieve (Wenzel et al. 1979). The utilization of monoploids provides the opportunity to gain more information about potential parental materials of hybrids. Moreover, monoploids are the only means of producing homozygous potato at different ploidy levels. The homozygotes present the opportunity of propagating potato through true seed, thus producing virus free plants.

Anther culture allows rapid production of homozygous lines that may be equivalent to those produced after many generations of inbreeding. Anther culture has been utilized in the development of both monoploids ($2n=1x=12$) and dihaploids ($2n=2x=24$) in several *Solanum* species. However, regenerants from diploid plants often vary in ploidy level (Bajaj and Sopory 1986). Veilleux et al. (1985) observed that a diploid clone of *S. phureja* Juz. & Buk. ($2n=2x=24$) produced more than 60% diploids ($2n=2x=24$), about 30% monoploids

(2*n*=1*x*=12) and 10% tetraploids (2*n*=4*x*=48). In *S. chacoense* Bitt., *in vitro* anther culture often produced diploid plants (Cappadocia et al. 1984). Anther-derived diploid plants may arise from: 1) reduced microspores that undergo chromosome doubling either during culture or subsequently; 2) embryogenesis of a somatic cell; or 3) unreduced microspores that undergo androgenesis (Chowdhury 1984). Homozygous diploids will be produced via the first process. Homozygous anther-derived diploids are expected to be equivalent to doubled monoploids (DMs) and can be evaluated directly without the time and bother of diploidization processes that monoploids require. Diploids which originate from somatic tissues of the anther will be genetically identical to the anther donor except for possible gametoclonal variation whereas those formed from unreduced microspores would be partially heterozygous, especially at loci distal to the centromeres (Veilleux 1985). The genetic composition of such androgenic diploids must be determined in order to distinguish homozygous from heterozygous anther-derived diploids and their utilization in potato improvement will be fully realized.

Molecular markers have been useful for assessing genetic variation and determining the genetic composition of anther-derived plants. Restriction fragment length polymorphism (RFLP) markers were used to characterize the genetic composition of plants produced by anther culture of *S. chacoense* (Birhman et al. 1994, Rivard et al. 1989). Because RFLP markers are codominant, the allelic segregation of loci that were found to be heterozygous in the anther donor could be used to distinguish homozygous anther-derived diploids expected

to have only a single allele per locus, from heterozygous diploids, expected to have two different alleles at many loci. Later, Meyer et al. (1993) screened anther-derived diploid potato by routinely using five RFLP loci found to be heterozygous in the anther donors. RFLP analysis, however, requires considerable technical skill and laboratory equipment. Also, appropriate DNA probes, Southern blotting, and radioisotopic labeling of probes are required.

Another potential marker system that could be used for characterizing anther-derived populations is simple sequence repeats (SSRs), otherwise known as short tandem repeats, or microsatellites. SSRs are tandemly repeated short oligonucleotide sequences which have been demonstrated to be highly polymorphic and abundant in human and other mammalian genomes as well as in plant genomes (Tautz and Renz 1984; Tautz 1989; Weber and May 1989; Wang et al. 1994). They appear to be evenly distributed throughout the genome and occur both intragenically and intergenically (Weber 1990). SSRs have attracted considerable attention recently because of their abundance and relatively simple experimental procedure (Saghai Maroof et al. 1994; Wang et al. 1994). The extent of polymorphism detected using the tetrameric $(GATA)_4$ probe was adequate to discriminate among 15 tomato cultivars (Vosman et al. 1992). In another study, a GACA-containing SSR probe produced polymorphic and unique bands which were used to distinguish six *Lycopersicon* species and 15 *L. esculentum* cultivars (Rus-Kortekaas et al. 1994). SSR polymorphism has also been reported in maize (Senior and Heun 1993), rice (Zhang et al. 1994), soybean (Yu et al. 1994),

barley (Saghai Maroof et al. 1994), and sunflower (Brunel 1994). For the purpose of discriminating homozygous from heterozygous anther-derived potato, SSRs offer the same potential as RFLPs, i.e., both alleles of a heterozygote can be detected.

The second section of this chapter investigates the inheritance pattern of SSRs. Several studies have established that reiterated sequences such as minisatellites which contain 10 to 35 base pairs (bp) for each repeat unit or the shorter SSRs are inherited in a Mendelian fashion (Jeffreys et al. 1985, Dallas 1988, Rogstad 1994, Smith and Devey 1994, Yanagisawa et al. 1994). Generally, the offsprings reveal alleles that are present in one or the other parent although new fragment lengths not exhibited by either parental lines have been observed (Jeffreys et al. 1985, Jeffreys et al. 1988, Traut et al. 1992, Rogstad 1994). Mutant alleles may be the result of unequal exchange and the mutation rate has been estimated at 0.004 (Jeffreys et al. 1985).

The objectives of this study were: (1) to distinguish homozygous from heterozygous diploids produced by anther culture of diploid potato by using SSR markers, and (2) to determine allelic segregation for SSRs in the F_1 population of a cross between a homozygous and heterozygous diploid potato.

Materials and Methods

Experiment 1. Use of SSR markers to determine the genetic composition of anther-derived plants

Plant materials. Plant materials included *S. chacoense (chc)* clone 80-1, *S. phureja (phu)* clone 1-3, their F_1 hybrid CP2, and 19 plants (5 monoploids -- CP2 145, 103, 7, 154, 153 and 14 diploids -- CP2 162, 160, 157, 150, 147, 146, 117, 107, 106, 111, 119, 155, 158, 132) derived by anther culture of CP2. Anther culture and plant regeneration followed the procedure described by Taylor and Veilleux (1992). The ploidy of anther-derived plants was determined by flow cytometric examination of *in vitro* leaf and stem tissue (Owen et al. 1988).

DNA isolation. Genomic DNA was extracted following the method of Doyle and Doyle (1987) with some modifications. Fresh leaves and stems (0.3 - 0.5 g) obtained from *in vitro* plantlets were ground in liquid nitrogen with a mortar and pestle until a fine powder was obtained. The DNA was isolated using 1 ml of CTAB extraction buffer [0.1 M Tris-HCl (pH 8), 1.4 M NaCl, 0.02 M EDTA (pH 8), 2% hexadecyltrimethylammonium bromide (CTAB), 1% fresh 2-mercaptoethanol]. The grindate was transferred to a 1.5 ml microcentrifuge tube and incubated in a water bath at 60°C for 1-2 h. An equal amount of chloroform:isoamyl solution (24:1) was added to the tube, the contents were mixed and centrifuged for 15 min.

The upper aqueous portion was deposited into a new microcentrifuge tube containing 200 µl cold isopropanol. The solution was discarded and the DNA pellet was collected and washed twice with cold 75% ethanol. The DNA was air-dried, redissolved in TE (10 mM Tris-HCl, 1mM EDTA) and RNAse (10 µg/ml). DNA concentration was determined using a TKO 100 mini-fluorometer (Hoefer Scientific Instruments, San Francisco, CA).

SSR analysis. Using the *DNAStar* program, the EMBL + GenBank database was searched for potato sequences containing SSRs by entering "potato" and specific SSR repeats of 20 nucleotide lengths. SSR analyses of gene sequences incorporating each of seven selected SSRs representing the dinucleotide repeats $(TA)_n$ and $(TC)_n$; trinucleotide repeats $(CTT)_n$, $(AAG)_n$ and $(AGA)_n$; and tetrameric repeats $(ACTC)_n$ and $(AATT)_n$ were done. The Primer program version 0.5 was utilized to design suitable primers (20-24 nucleotide length) flanking each SSR (Table 1). The primers were synthesized by DNAgency, Ashton, PA.

Amplification conditions were based on the procedure of Yu et al. (1994). Amplification reactions were done in volumes of 20 µl containing 1× assay buffer (50 mM KCl, 10 mM Tris-HCl at pH 9, 1% Triton X-100); 3 mM $MgCl_2$, 160 µM each of the dNTPs, 1.5 units *Taq* DNA polymerase (Promega, Madison, WI) and 0.1 µM of each primer. In each reaction mixture, 50 ng genomic DNA was used as template for amplification by PCR. The reaction mix was overlaid with a drop of mineral oil. Thermal cycling conditions consisted of 40 cycles of 1 min at 94°C (denaturation), 2 min at 55°C (annealing) and 1.5 min at 72°C

(primer extension) followed by 1 cycle of 5 min at 72°C for final extension. After thermal cycling, 5 µl loading dye was added to the reaction mix. The amplified DNA products were analyzed following the method of Senior and Heun (1993) with some modifications. Fifteen µl of the mix was analyzed on 3% MetaPhor agarose gel run with 1× TBE (Tris-borate-EDTA) buffer at 100 V for 3.5 h. The gel was stained with ethidium bromide (10 mg/ml) for 20 min and photographed under UV light.

Experiment 2. Inheritance pattern of SSR markers in *S. phureja* DM × ID 4 F_1 population

Plant materials. Microtuber-propagated plants of DM AD2-4 3s.3, a homozygous doubled monoploid derived from *S. phureja* clone PP5, and ID 4 (heterozygous pollinator), a complex interspecific hybrid, were grown under 16 h photoperiod in the greenhouse in fall 1994. Cross pollinations were done to generate the F_1 progeny. Seeds generated from the crosses were sown in the greenhouse on March 31, 1995. After two weeks, seedlings were transferred individually to cell packs and grown in the greenhouse for one month before transplanting to the Whitethorne-Kentland Farm. The parental genotypes (DM AD2-4 3s.3, ID 4) and 100 F_1 plants were assayed for SSR markers.

DNA isolation. Fresh leaves were collected two weeks after transplanting. Samples were stored at -80°C until freeze drying was done. Genomic DNA was extracted based on the

methods of Doyle and Doyle (1987) and Saghai Maroof et al. (1984) with some modifications. Freeze dried leaf samples (0.2 - 0.4 g) were ground with a mortar and pestle until a fine powder was obtained. DNA extraction and subsequent steps followed the procedure described in the first experiment.

SSR analysis. The procedure for SSR assay was similar to that described in the first experiment. Confirmation of the SSR alleles of parental genotypes was done following the method of Yu et al. (1994) using polyacrylamide gels.

Results

Experiment 1. Use of SSR markers to determine the genetic composition of anther-derived plants

Five of the seven SSR sequences screened were found to be polymorphic in the CP2 anther donor. CP2 was heterozygous with two alleles observed at four SSR loci (TC/TA, AAG, AGA, CTT) and three alleles at the ACTC locus. Except for CTT repeats, the other four SSR loci in CP2 exhibited alleles contributed by both *chc* 80-1 and *phu* 1-3. The CTT locus exhibited two alleles in CP2 and the anther-derived plants, one from *chc* 80-1 and one new allele that was not observed in either parent (Fig. 1). This could be attributed to

recombination events resulting in the creation of the new allele. Hypervariability of minisatellites (Jeffreys et al. 1985) and small size differences of SSRs (Litt and Luty 1989) have been previously reported.

The primer pairs flanking each of the five polymorphic SSRs were used to study five anther-derived monoploids and fourteen anther-derived diploids. All five monoploids showed only the allele contributed by *chc* 80-1 at all five loci. Both homozygous and heterozygous diploids were identified. Five diploids (CP2-160, 162, 157, 155, and 158) exhibited only a single band at all five SSR loci (Fig. 2 and Table 2) corroborating homozygosity. The other nine diploid plants were heterozygous at one to five SSRs where the presence of more than one allele was observed.

SSR analysis identified distinct grouping of genotypes. Among the five homozygous diploids, CP2-155 was genetically identical to the monoploids whereas the other four (CP2-157, 158, 160, and 162) formed a genetically identical group that differed from the monoploids at one of the five SSR loci. A unique group of three genetically identical heterozygous anther-derived diploids consisting of CP2-107, 117, and 150 was obtained that was heterozygous at two of the five loci. Also, CP2-106, 111, 119, and 147 formed a group that was heterozygous at all five SSR loci and therefore indistinguishable from the anther donor whereas another group (CP2-132 and 146) was heterozygous at only one of the five SSR loci.

Experiment 2. Inheritance pattern of SSR markers in *S. phureja* DM × ID 4 F_1 population

Before analyses were performed on F_1 plants, the parental genotypes (DM AD2-4 3s.3 and ID 4) were screened for polymorphic markers using seven SSR sequences (Table 1). Four SSRs [$(ACTC)_n$, $(TC)_n$ and $(TA)_n$, $(CTT)_n$ and $(AAG)_n$] were found to differ between the parental lines. These repeats were utilized to examine allelic segregation in the F_1 except for $(TC)_n$ and $(TA)_n$ motif because PCR amplification was poor and the bands were very faint and difficult to score. Polyacrylamide gel electrophoresis was carried out in an effort to obtain better visualization of PCR products but the bands remained indistinct (Fig. 3). Based on three SSRs, genotypic frequencies in the F_1 population fit the expected 1:1 Mendelian segregation ratio. The maternal parent showed allele 1 whereas the paternal genotype had alleles 1 and 2; thus two genotypic classes were observed in F_1 plants (Table 3). The allele derived from the homozygous parent, DM AD2-4 3s.3, was always present in the F_1 plants whereas alleles from heterozygous pollinator ID 4 segregated in a 1:1 fashion (Fig. 4). The presence of the DM band and a similar band from ID 4 was evidenced by a relatively intense band in the F_1. The chi-square values for observed versus expected genotypes at each of the three SSR loci are shown in Table 4. Observed frequencies of genotypes did not differ significantly from expected. No new alleles were observed in the F_1 hybrids.

Discussion

The banding pattern of the anther-derived monoploids based on SSR analysis was consistent with expected results according to the genetic composition of monoploids wherein only a single allele per locus should be present. Surprisingly, the monoploid plants displayed only the alleles contributed by the *chc* 80-1 parent. A similar study using RAPD analysis to examine the genetic composition of the same plant materials (Veilleux et al. 1995) disclosed that four monoploids were genetically identical and the fifth (CP2-7) differed from the others at 12 of 17 loci. It is unlikely that CP2-7 that was so obviously different from the group of four by RAPD analysis would have received the same allele at all five SSR loci [probability $= (0.5)^5 = 0.03$] without the presence of some selection imposed by the anther culture process. Analysis of a sufficient number of anther-derived plants and SSRs could identify putative lethal or non-functional alleles if only one allelic form in different SSR loci is observed in monoploids and homozygous diploids. Rivard et al. (1994) compared RFLP markers of anther-derived plants of *S. chacoense* and observed skewed segregation ratios at many loci. In one case, only one allele could be found among homozygous forms. The absence of the alternate homozygous genotype indicated a possible lethal allele or close linkage to one. However, lethal alleles would not have been expected among our SSR loci *per se* as they were all deduced from intragenic sequences. These alleles were apparently viable in the parents of the anther donor. Linkage of the SSR loci to lethal alleles at neighboring loci cannot be ruled out.

In the present study, the anther donor was an interspecific hybrid and therefore could be expected to be highly heterozygous for intragenic SSRs because of the reproductive isolation of the two parental species. Preliminary results using some of the same primer pairs on an intraspecific anther-derived population of *S. phureja* (data not presented), exhibited polymorphic alleles. Polymorphism at two SSR loci in *S. phureja* clone 1-3 can be observed in both Figs. 1 and 2. This clone was selected for its agronomic performance and its responsiveness to anther culture from an adapted *S. phureja* population (Haynes 1972). As such, its polymorphism should not be associated with any unusual heterozygosity that would not be expected in an outbreeding crop. As the database in the NIH genebank increases, the discriminating ability of SSRs deduced from such sequences will concomitantly increase.

The same five monoploids and fourteen diploids obtained by anther culture of CP2 were analyzed for RAPDs (Veilleux et al. 1995). Homozygosity in five diploids (CP2- 160, 162, 157, 155, and 158) as revealed by RAPD analysis was confirmed by SSR analysis, i.e., a single band was observed at all five SSR loci in these diploids. Moreover, the grouping of the 19 anther-derived plants by RAPD analysis was generally corroborated by SSR analysis. Common origin of several anther-derived plants through secondary embryogenesis in anther culture was revealed by RAPD analysis and confirmed by SSR analysis.

The results of the second experiment revealed Mendelian inheritance of SSR alleles. SSR markers observed in the progeny could be traced to both parents. The allele derived from the

homozygous parent, DM AD2-4 3s.3, was always present in the F_1 plants as expected. Alleles from ID 4 which was heterozygous at the three SSR loci segregated in a 1:1 fashion. Investigation of the occurrence of new length variants due to mutation would require more SSR loci. Jeffreys et al. (1985) observed a mutant allele out of 240 offspring bands (u = 0.004) based on minisatellites in a study of 27 human DNA samples. The rate of occurrence of new DNA fingerprinting bands is 1 in 300 per generation (Jeffreys et al. 1988). Rogstad (1994) observed non-parental bands which represented approximately 0.5% of the total bands observed. In microsatellites, high mutation rate could be attributed to slippage (Tautz et al. 1986, Schlotterer and Tautz 1992).

SSRs provide a powerful tool for molecular analysis of the genetic architecture of plant populations. In the present study, the SSRs were deduced from published gene sequences. If sufficient polymorphism can be found, as in the present study, this represents the simplest technique for such a genetic analysis. Oligonucleotide primers flanking SSRs can be constructed such that the only DNA synthesis that occurs in the PCR reaction is that of the target sequence. This approach eliminates the tedious method of probing genomic DNA with SSR sequences to identify SSR loci, and the trial and error of finding RAPD primers that give repeatable banding patterns. It must be borne in mind that the genetic analysis using SSRs is based on intragenic variation and results of such analysis may differ from that obtained from random genomic targeting such as in RAPD analysis.

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Table 1. Simple sequence repeats (SSRs) found in potato sequences derived from the NIH GenBank and their primers.

Sequence	SSRs	Product size (bp)	Pair of primers (5' to 3')
actin gene	$(TA)_{13}$	228	TTTCTATTGAAAACCTTGAGAGGG CATCTCTACATTCACGAGCATTG
proteinase inhibitor gene	$(TC)_{12}$ & $(TA)_{18}$	180	CTTGCAACTTGTTAGTACCCCC AAATCCTTTGTGACCTCCCC
waxy gene	$(CTT)_4$	246	TGATTCTCTTGCCTACTGTAATCG AGTCAGAGTATGGTTCCTGAGTCC
granule bound starch synthase gene	$(AAG)_8$	236	TTCTGATTTCATGCATGTTTCC ATGTGTGGTCTACAAAAAGGGG
mRNA for leaf type *α-starch phosphorylase*	$(AGA)_5$	220	TTCAGAGACATCATGGCAACTT ATCCTTCATCAGAGGAAGAATCC
waxy gene	$(ACTC)_5$	223	CCCATAATACTGTCGATGAGCA GAATGTAGGGAAACATGCATGA
patatin pseudogene	$(AATT)_5$	386	CAACCAACAAGGTAAATGGTACC TGGTCTGGTGCATTAGAAAAAA

Table 2. SSR marker polymorphisms in the CP2 anther donor, its parents (*chc* clone 80-1 × *phu* clone 1-3), and anther-derived plants.

Genotype			Simple sequence repeats				
			ACTC	TC&TA	CTT	AAG	AGA
chc 80-1 parent			A*	D	F	J	N
phu 1-3 parent			ABC	E	FG	JM	NP
CP2 anther donor			ABC	DE	FI	JM	NP
Monoploids							
CP2 -	7		A	D	F	J	N
	103		A	D	F	J	N
	145		A	D	F	J	N
	153		A	D	F	J	N
	154		A	D	F	J	N
Diploids							
CP2 -	155	(HOM)	A	D	F	J	N
	157	(HOM)	B	D	F	J	N
	158	(HOM)	B	D	F	J	N
	160	(HOM)	B	D	F	J	N
	162	(HOM)	B	D	F	J	N
	107	(HET)	B	DE	F	J	NP
	117	(HET)	B	DE	F	J	NP
	150	(HET)	B	DE	F	J	NP
	106	(HET)	ABC	DE	FI	JM	NP
	111	(HET)	ABC	DE	FI	JM	NP
	119	(HET)	ABC	DE	FI	JM	NP
	147	(HET)	ABC	DE	FI	JM	NP
	132	(HET)	A	D	F	J	NP
	146	(HET)	A	D	F	J	NP

* Different letters represent different allelic forms

Table 3. SSR marker polymorphism in a diploid potato F_1 progeny and its parents (DM AD2-4 3s.3 × ID 4).

Genotype	Simple sequence repeats		
	ACTC	CTT	AAG
phu DM AD2-4 3s.3	A[a]	C	E
ID 4	AB	CD	EF
F_1	A, AB	C, CD	E, EF

[a]Different letters represent different allelic forms.

Table 4. Frequencies of the observed and expected number of individuals in the F_1 population of DM AD 2-4 3s.3 × ID 4 at three SSR loci.

SSR	F_1 genotype	Observed	Expected	χ^2	P[a]
$(ACTC)_n$	A	51	50	.04	.75 - .90
	AB	49	50		
$(CTT)_n$	C	58	50	2.56	.25 - .10
	CD	42	50		
$(AAG)_n$	E	55	49	1.46	.25 - .10
	EF	43	49		

[a]Probability of a larger value of χ^2 with a 1:1 ratio.

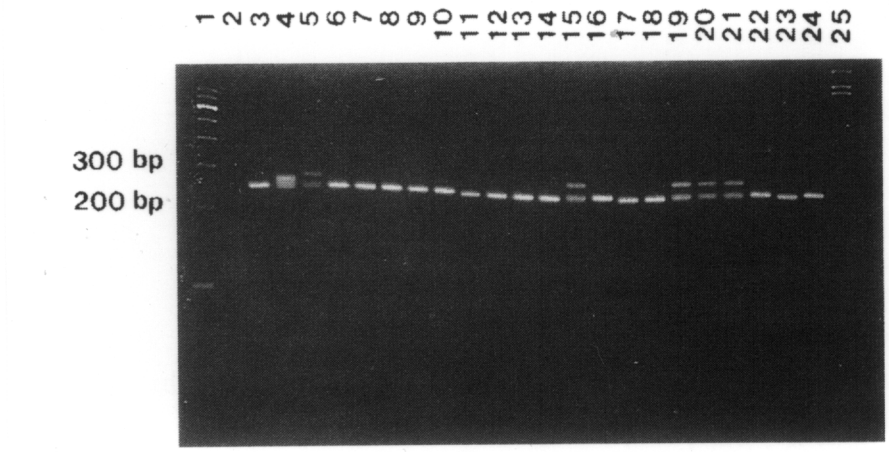

Figure 1. SSR marker polymorphism amplified by PCR using primers flanking the CTT repeats within the *waxy* gene locus of potato in; *chc* 80-1 parent (lane 3), *phu* 1-3 parent (lane 4), CP2 anther donor (lane 5), five anther-derived monoploids (lanes 6-10), 14 anther-derived diploids (lanes 11-24). Lanes 1 and 25 are the 100 bp ladder and pBR322 *Bst*N1 digest, respectively; lane 2 is the check with no DNA. Of the 14 anther-derived diploids ten are homozygous (exhibiting only the light allele of CP2) and four are heterozygous (exhibiting both alleles of CP2).

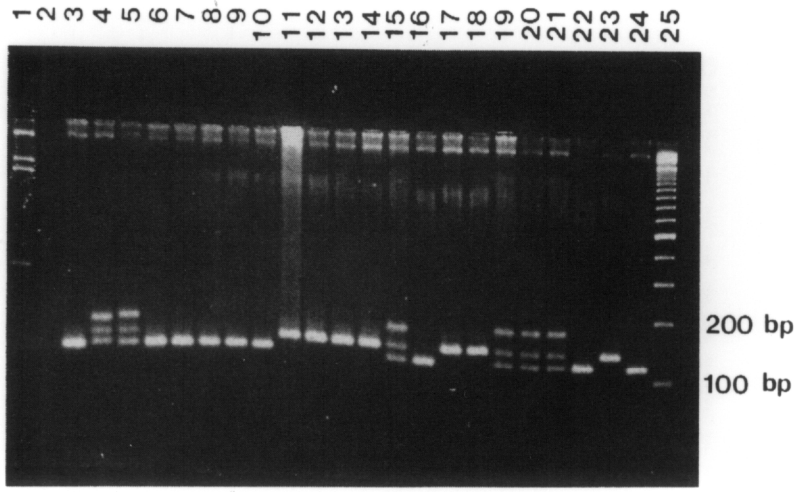

Figure 2. SSR marker polymorphism amplified by PCR using primers flanking the ACTC repeats within the *waxy* gene locus of potato in *chc* 80-1 parent (lane 3), *phu* 1-3 parent (lane 4), CP2 anther donor (lane 5), five anther-derived monoploids (lanes 6-10), and 14 anther-derived diploids (lanes 11-24). Lanes 1 and 25 are the pBR322 *Bst*N1 digest and 100 bp ladder, respectively; lane 2 is the check with no DNA. Of the 14 anther-derived diploids ten are homozygous (seven exhibiting only the middle allele of CP2 and three exhibiting the light allele) and four are heterozygous (exhibiting all three alleles of the anther donor).

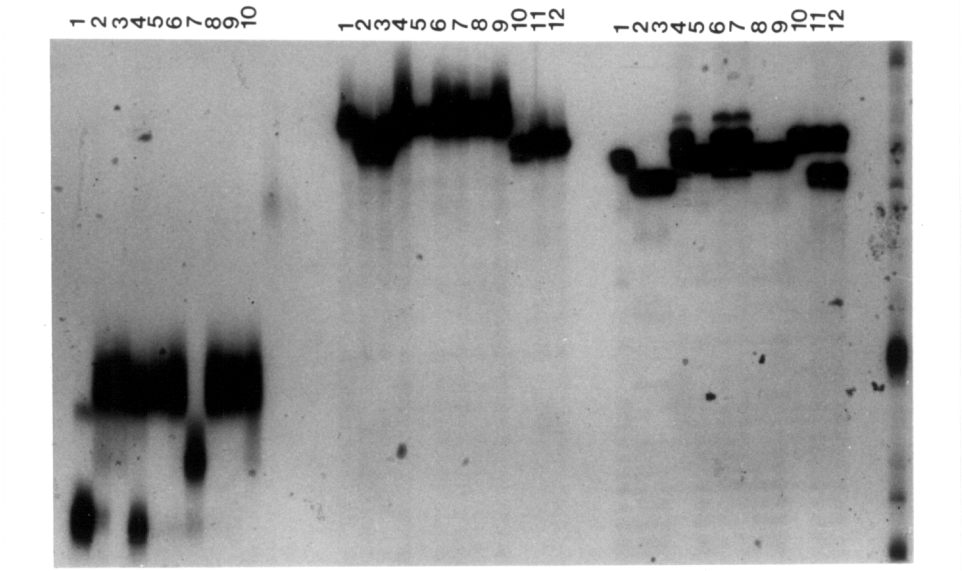

Figure 3. SSR patterns using primers flanking (left to right) TC & TA repeats within the *proteinase inhibitor* gene, CTT repeats within the *waxy* gene and AAG repeats within the *granule bound starch synthase* gene of potato; in DM AD2-4 3s.3 (lane 1), ID 4 (lane 10), other ID lines (lanes 2, 3, 11, 12) and six F_1 plants of DM AD2-4 3s.3 × ID 4 (lanes 4-9) visualized by polyacrylamide gel electrophoresis. (Analyzed by Zhaowei Liu, Dept. of Crop and Soil Environmental Sciences, VPI&SU, Blacksburg, VA).

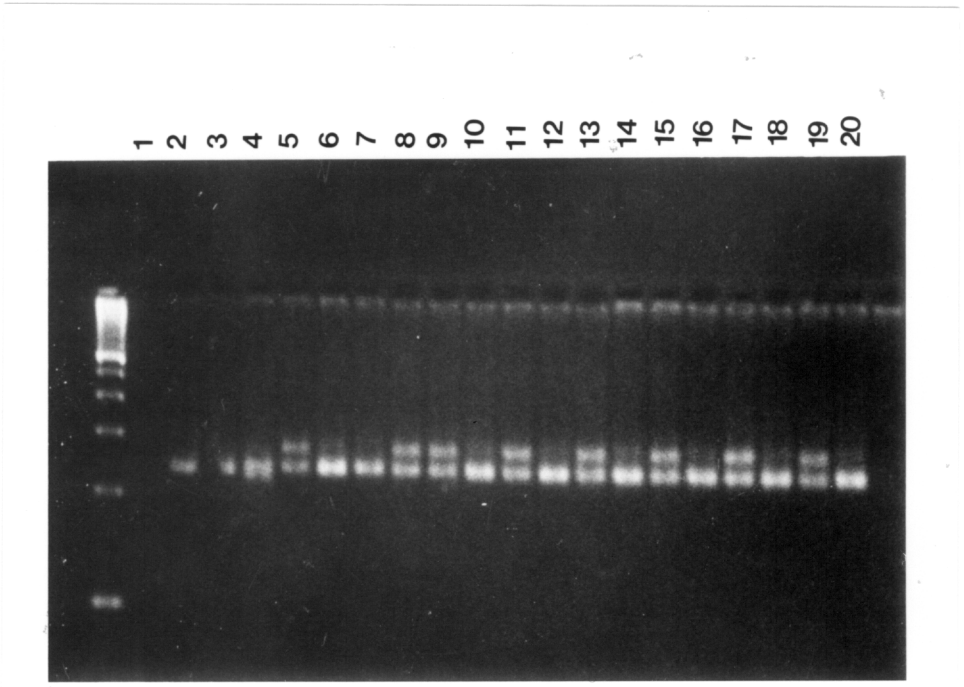

Figure 4. Segregation of alleles of CTT repeats within the *waxy* gene locus of potato in 15 F_1 plants (lanes 6-20). Lane 2 is AD2-4 anther donor; lanes 3-4 contain DM AD2-4 3s.3; lane 5 is the heterozygous pollinator ID 4; lane 1 is the check with no DNA. The left lane contains 100 bp ladder. Eight of the F_1 are homozygous and seven are heterozygous.

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