'ANALYSIS OF ANTHER-DERIVED PLANTS OF SOLANUM PHUREJA: VARIATION IN PLOIDY, PHOTOSYNTHETIC EFFICIENCY AND STRUCTURE OF THE NUCLEAR GENOME'

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(ABSTRACT)

The ultimate goal of the breeding scheme, of which the present study is a part, is to introduce exotic germplasm into existing cultivars of Solanum tuberosum through 4x X 2x crosses using the South American diploid potato species Solanum phureja as the pollen parent. The first phase of this program includes the 'reconstruction' of a highly heterozygous diploid pollen parent by first reducing the chromosome number of the S. phureja clones to the monoploid level and subsequently fusing genomes of two unrelated monoploid plants either by somatic hybridization or by cross-pollination between fertile doubled monoploids.

Within this framework, the objectives of this research were to analyze variation among anther-derived plants of Solanum phureja regarding their: 1) ploidy level and morphology, 2) net photosynthesis and its biochemical compo-

nents, and 3) nuclear genomic structure, particularly with regard to the amplification of rRNA genes as influenced by the anther-culture process.

Based on the analysis of several morphological characters of the anther-derived plants by canonical discriminant function, four characters (anther length, number of chloroplasts/pair of guard cells, leaf width, corolla width) were selected for most effective assignment of plants to their ploidy groups by clustering procedures. Clustering of the anther-derived plants proved to be an efficient means of separating monoploids from higher ploidy levels.

To assess the impact of the process of anther-culture on the physiology of the resulting plants and to evaluate the possibility of selecting anther-derived genotypes for further breeding efforts, monoploid, diploid and tetraploid anther-derived plants were studied regarding their net photosynthesis and its component characteristics. Leaf area, net photosynthesis and chlorophyll content increased with increasing ploidy. Among the monoploids, Rubisco activity and concentration displayed a significant genotypic effect, whereas in the diploid group variation among genotypes was significant for total protein content and maximum specific activity of ribulose bisphosphate carboxylase. Among the tetraploid genotypes, significant differences were found with

respect to net photosynthesis and specific leaf weight. Two exceptional genotypes were identified: a monoploid with an increase of 28% for maximum activity of ribulose bisphosphate carboxylase and a tetraploid with an increase of 30% for net photosynthesis over the anther-donor plant.

To evaluate DNA variation among the anther-derived plants, the nuclear genomes of anther-derived monoploid and diploid plant were studied by DNA reassociation kinetics. It was found that the nuclear genome of the monoploid has undergone differential replication resulting in an increase of sequences consisting of highly repetitive DNA. Free solution RNA-DNA hybridization showed that the monoploid DNA contained 30% more rDNA sequences than the diploid. Southern blot analysis using rRNA as the probe revealed variation for copy number of certain restriction fragments and for restriction enzyme cleavage sites.

DEDICATION

To my friend,

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CHAPTER 1

INTRODUCTION

THE VALUE OF SOLANUM PHUREJA TO POTATO IMPROVEMENT

In total volume of production (estimated at 290 million tons/yr for 1981), the potato ranks fourth in the world after wheat, maize and rice. Of all the food crops, the rate of potato production is currently increasing most rapidly in many developing countries; the percent increase in production from 1961 to 1981 was 147% for potatoes compared with 127% for wheat (FAO, 1982).

The ploidy levels of cultivated potato species range from diploid to pentaploid (2*n* = 24, 36, 48,60). However, the most important cultivated potatoes are tetraploid. There are two views regarding the taxonomic classification of the tetraploid potato: the Russian botanists Juzepczuk and Bukasov have divided the tetraploid potatoes into two species, *S. tuberosum* L. and *S. andigena* Juz & Buk, whereas other taxonomists recognize only one species *S. tuberosum*,

often further divided into two subspecies, namely tuberosum and andigena (Howard, 1970).

There is a similar controversy over the introduction of the tetraploid potato to Europe: the Russian botanists hold the view that tetraploid species originated in Chile; however, there seems to be more evidence suggesting that the first introduction came from the Andes and was of the Andigena type, which was subsequently selected for adaptation to long day conditions in Europe. The potato was brought to the United States by European settlers in the early eighteen century. Except for one later introduction of potato to the U.S. directly from South-America in 1851, currently grown potato cultivars in the U.S. have descended from the original introductions (Howard, 1970). Thus, the cultivars in the northern climates have been developed from a restricted gene pool. This narrow genetic base of the tetraploid cultivars has lead to increased interest in incorporation of germplasm from non-tuberosum species into cultivars of S. tuberosum.

Solanum phureja is a diploid potato species of regional economic importance in South America. Through recurrent selection, it has been adapted to long-day conditions (Haynes 1972) and is currently being used in several breeding programs in the US. Selections of S. phureja have been shown to be resistant to bacterial wilt caused by Pseudomonas

solanacearum (French and De Lindo 1982; Bowmann and Sequeira, 1982). The resistance has been characterized by reduced acropetal spread of the bacteria and by tolerance to a high titre (Bowmann and Sequeira 1982). Ethanolic extractions of tissues of wilt-resistant clones of S. phureja have been shown to inhibit the growth of Pseudomonas solanacearum (Zalewski and Sequeira 1973). Bacterial wilt resistance is of particular importance in tropical climates, where the warm temperatures and high humidity favor its spread. Two S. tuberosum clones containing wilt-resistant S. phureja in their parentage have been selected for further testing largely because of their good yield and superior bacterial wilt resistance in a CIP (International Potato Center) program in Mindanao (CIP report, 1984).

TISSUE CULTURE AND MOLECULAR TECHNIQUES FOR POTATO GERMPLASM IMPROVEMENT

Somaclonal variation among plants derived from tissue culture

Phenotypic variation among plants derived through tissue culture has been viewed as a mechanism for crop improvement. This is especially the case for potato because of the relative ease with which various explants (Cassells et al. 1983),

suspension cells (Austin and Cassells, 1983), microspores (Wenzel, 1981; Veilleux et al. 1985) and protoplasts (Shepard, 1977) can be manipulated in vitro to undergo morphogenesis.

The first successful callus culture of potato was accomplished by Steward and Chaplin as early as 1951. However, regeneration of embryoids and shoots from tuber callus was not reported until 1975 (Lam). Subsequently, plantlet regeneration has been reported from callus derived from tubers (Lam 1977; Jarret et al. 1981), cut rachises of leaves (Roest and Bokelmann 1976), leaf discs (Jacobsen 1977; Karp et al. 1984), stem pith (Cassells et al. 1983), and shoot tips (Ahloowalia 1982; Wang and Huang 1975; Roca et al. 1978).

Potato plantlets have also been regenerated from suspension cultures grown on media supplemented with an auxin and little or no cytokinin (Lam 1977; Schumann 1980). Plant regeneration from protoplast-derived callus (p-calli) has been achieved for several tetraploid cultivars, mainly by modifications to culture conditions, as described by Shepard and colleagues (Shepard and Totten 1977; Shepard 1980, 1981, 1982; Shepard et al. 1980; Gunn and Shepard 1981; Thomas 1981; Karp et al. 1982; Bokelmann and Roest 1983). Recently, Haberlach et al. (1985) published a protocol for isolation and culture of potato protoplasts that was successfully ap-

plied to 15 S. tuberosum lines. Regeneration of calli of dihaploid breeding lines of S. tuberosum (Binding et al. 1978) as well as S. phureja (Schumann et al. 1980), and the hybrid S. phureja x S. chacoense gibberulosum (Grun and Chu 1978) have been reported.

Plants derived from all of the above in vitro techniques have been demonstrated to express somaclonal variation (Van Harten et al. 1981; Karp 1984; Hu and Wang 1983; Ahloowalia 1982); however, most attention has been focused on evaluation of variation among protoplast derived plants. Useful variation for the breeder with respect to disease resistance (Alternaria solanii, Matern et al. 1978; Phytophtora infestans, Shepard et al. 1980), growth habit, maturity, tuber characteristics and photoperiodic requirements for flowering (Shepard et al. 1980; Ayers and Shepard 1981; Thomas et al. 1982) has been observed.

A comparison among the types of somaclonal variants among plants regenerated from protoplast cultures and leaf derived callus and those derived from lateral buds revealed grossly abnormal plants in both of the first two culture types, however, these were selected against during the establishment stage (Cassells et al. 1983). Thus, the variability assessed after establishment in the greenhouse was similar in the callus, single cell and lateral bud derived plants, except

that leaf variegation and anthocyanin production appeared only in the callus and single cell derived plants. The authors concluded that it was encouraging that the surviving regenerants displayed the variation within the range of character variability of the lateral bud derived plants.

For somaclonal variation to be useful in crop improvement, it must be heritable, and there must be a means of generating the desired variation while otherwise preserving the original genome. This leads to a consideration of the factors causing somaclonal variation. Only by understanding the mechanisms responsible for the variation can it be manipulated in a desired fashion. The approaches to solve the problem have included conventional Mendelian genetics and cytogenetics (Shepard, 1985), evaluation of the immediate gene products by electrophoretic analysis of enzymes (Sanford et al. 1984), and studies on the nuclear (Dhillon et al. 1983; dePaepe et al. 1983; Sree Ramulu et al. 1984; 1985) and organellar DNA (Shepard et al. 1985) by cytophotometric quantification of nuclear DNA and by molecular genetic studies of the changes in the DNA sequences which occur during culture.

Several studies have documented the association between somaclonal variation and chromosomal changes, such as polyploidization, aneuploidy and chromosome aberrations (reviews in Larkin and Scowcroft 1981; Evans 1984; Karp 1984;

Sree Ramulu et al. 1983, 1984, 1985). Accumulated mutations in the somatic cells of the explant may result in variation among the regenerated plants (Shepard, 1980); however, even among plants derived from the same callus, different phenotypes and/or ploidy levels have been reported (Sree Ramulu et al. 1983). Moreover, it has been observed that nuclear fragmentation followed by mitosis at an early stage of culture can result in variation in calli and regenerated plants (D'Amato et al. 1980).

In addition to chromosomal aberrations, mutations affecting only one or few loci have been observed (Sanford 1984; Gill et al. 1985). However, due to the high heterozygosity and sterility of tetraploid potato cultivars, detection of point mutations by conventional methods is nearly impossible.

The application of Feulgen cytophotometry for quantification of nuclear DNA of protoplasts, callus cells and regenerated plants has produced valuable information on DNA replication at the initial stages of culture, and has shown that regenerated plants contain cell populations of different ploidy levels. Sree Ramulu et al. (1984, 1985) observed nuclear DNA contents of 4C to 16C including intermediate DNA values within 8 days of culture of tetraploid potato protoplasts and of suspension culture cells derived from monohaploid, dihaploid and tetraploid potato plants. The explant (shoot

tip) in all of the cases contained only cells with a normal DNA content for the particular ploidy level.

It was also observed in the latter study that monohaploid cells had a more rapid rate of polyploidization than cells of the higher ploidy levels. This is in agreement with the findings of Veilleux et al. (unpublished results).

It has been shown by restriction endonuclease patterns that, in addition to changes in the nuclear genome, the mitochondrial genome also undergoes changes during protoplast culture (Kemble and Shepard 1985). Using Southern transfer analysis Cullis (1984) showed flax plants regenerated from callus to contain a higher copy number of 5s rDNA sequences than the source plants (see chap. 4).

From these studies, it can be concluded that DNA replication and cell division are under independent control, and thus not synchronized in in vitro cultures. A key factor to achieve synchronization is a proper balance of the exogenous growth regulators, which are known to affect both DNA polymerase activity and cytokinesis (D'Amato 1972; Mennes et al. 1978). Commonly, high auxin/cytokinin ratios are used for the establishment of protoplast, suspension and callus cultures to promote dedifferentiation and cell proliferation. This might lead to chromosomal instability, especially if 2,4-D is used

as the auxin source. Sree Ramulu et al. (1985) found that, to maintain cytogenetic stability, high cytokinin levels and culture of small richly cytoplasmic, meristematic cells with short subculture intervals gave the best results. This is of special importance for the culture of haploid protoplasts.

Other mechanisms that have been proposed as sources of somaclonal variation include DNA sequence transposition, differential DNA replication and cryptic virus elimination (Larkin and Scowcroft 1981, see chap. 4)

Anther-culture, somatic hybridization and genetic engineering in potato breeding

A combination of slow progress in potato breeding by conventional means, which is largely due to the narrow genetic base of the existing tetraploid cultivars, and, on the other hand, the success with plant regeneration from *in vitro* cultures have lead to various proposals to incorporate *in vitro* techniques into potato improvement programs (Wenzel et al. 1982; Veilleux et al. 1985, see p. 12). Wenzel and co-workers at the Max Planck Institute have developed an 'analytical-synthetic' breeding scheme, which includes the use of anther-culture and protoplast fusion in combination with hybrid production through sexual crosses (Wenzel et al. 1982).

The approach is based on stepwise reduction of the tetraploid potato to the monoploid level (the analytical part), followed by controlled reconstruction of a new heterozygous tetraploid clone (the synthetic part). The breeding scheme has been applied mainly to utilize genes responsible for virus and nematode resistance as well as in vitro culturability (Wenzel and Uhrig, 1981). So far the greatest barrier to success has been encountered with somatic fusion: of 2000 plants regenerated from protoplast fusion, none were somatic hybrids (Wenzel 1980; 1982).

Successful fusions have been accomplished between S. tuberosum and S. chacoense (Butenko and Kuchko 1979, 1980), and between S. tuberosum and S. nigrum (Binding et al. 1982). In the latter study, the objective was to transfer the maternally inherited atrazine resistance of S. nigrum biotype to S. tuberosum. Eleven putative hybrids were recovered, of which six were resistant to atrazine. Protoplasts of S. tuberosum have also been successfully fused intergenerically with those of Lycopersicon esculentum (Melchers 1978) and Nicotiana tabacum (Gleba and Evans, 1983).

The ease with which potato protoplasts can be cultured and regenerated has made it an attractive candidate for transformation studies. There are two recent reports on transformation of potato by Ti T-DNA (Ooms et al. 1983) and Ri T-DNA

(Ooms et al. 1985). In both studies, incorporation and expression of T-DNA was observed; the high endogenous cytokinin content of the Ti T-DNA transformed plants lead to strong tuberization, whereas the plants containing Ri T-DNA had abundant roots and showed reduced geotropism.

A proposed next step is to engineer the vector plasmid to contain a DNA sequence coding for a desired characteristic. So far, only the gene coding for the tuber storage protein, patatin, has been characterized (Mignery et al. 1984). However, the recombinant DNA techniques in conjunction with the cellular level breeding methods have great potential and applicability for potato improvement.

RATIONALE AND SIGNIFICANCE OF THE PRESENT WORK

There has been very little progress in potato breeding programs based on conventional breeding methods. Evidence of this is that over 60% of potato acreage in the U.S. and Europe is planted to three cultivars, all released more than thirty years ago (Howard, 1970). Barriers to potato improvement result from the narrow genetic base of existing S. tuberosum cultivars; hybrid progenies are seldom superior to their parents. The 'revolution' in potato breeding has come about by the finding that exotic germplasm can be incorporated into tetraploid cultivars via 4x X 2x crosses utilizing unreduced

gametes in diploid germplasm (Mok and Peloquin 1975; de Jong and Tai 1977; McHale and Lauer 1981; Veilleux and Lauer 1981). Genotypes of Solanum phureja have frequently been found to express 2n gametes (Veilleux and Lauer 1981). Genetic explanation for the increased vigor of 4x X 2x hybrids has been provided by Mendiburu et al. (1974). If the numerical nonreduction of chromosomes in the pollen is due to fused (Ramanna 1979; Veilleux et al. 1982;) or parallel (Mok and Peloquin 1975) spindles during the second meiotic division, then most of the heterozygosity present in the diploid parent is preserved in the 2n pollen (Fig 1). This mechanism allows maximum heterozygosity in the resulting hybrids. Moreover, as can be observed from Fig. 1, this mechanism results in a relatively homogenous population of 2n gametes, which in turn will translate into homogeneous 4x X 2x progeny. This fact then leads to the possibility of converting potato from an asexually to a sexually propagated crop.

Exploitation of hybrid vigor present in the progeny of S. tuberosum x S. phureja is the foundation for the potato breeding program designed by Dr. Veilleux. An additional element in the scheme is to improve the heterozygosity of the diploid pollen parent by in vitro techniques (Fig 2). The first step involves production of monoploid plants of two unrelated S. phureja clones. Protoplasts isolated from these plants will then be fused to produce highly heterozygous

presumably fertile, diploid, diplandroid plants, which will be used as the pollen parent in subsequent 4x X 2x crosses. We have presently identified one clone of S. phureja which produces unreduced gametes and responds to anther-culture yielding monoploid plantlets (Veilleux et al. 1985). The screening for the other clone as well as the protoplast isolation and regeneration are underway.

Broadly, the three studies included in this dissertation deal with the evaluation of gametoclonal and somaclonal variation of anther-derived plants of S. phureja. The first study examines the use of cluster analysis and canonical discriminant analysis for classifying anther-derived plants according to ploidy based on easily measurable morphological characteristics. An interpretation of the statistical information to determine the genetic mechanism through which the plants of different ploidy level arose is also presented. The objectives of the second study were to determine the ploidy and genotypic effect on net photosynthesis and its component characteristics among anther-derived plants. The evaluation of the physiological performance of the anther-derived plants, especially monoploids, enables selection at the early stages of the breeding program.

Finally, the third study was undertaken to investigate genomic changes that occur during the process of anther-culture. The involvement of rDNA sequences in these changes has been addressed.

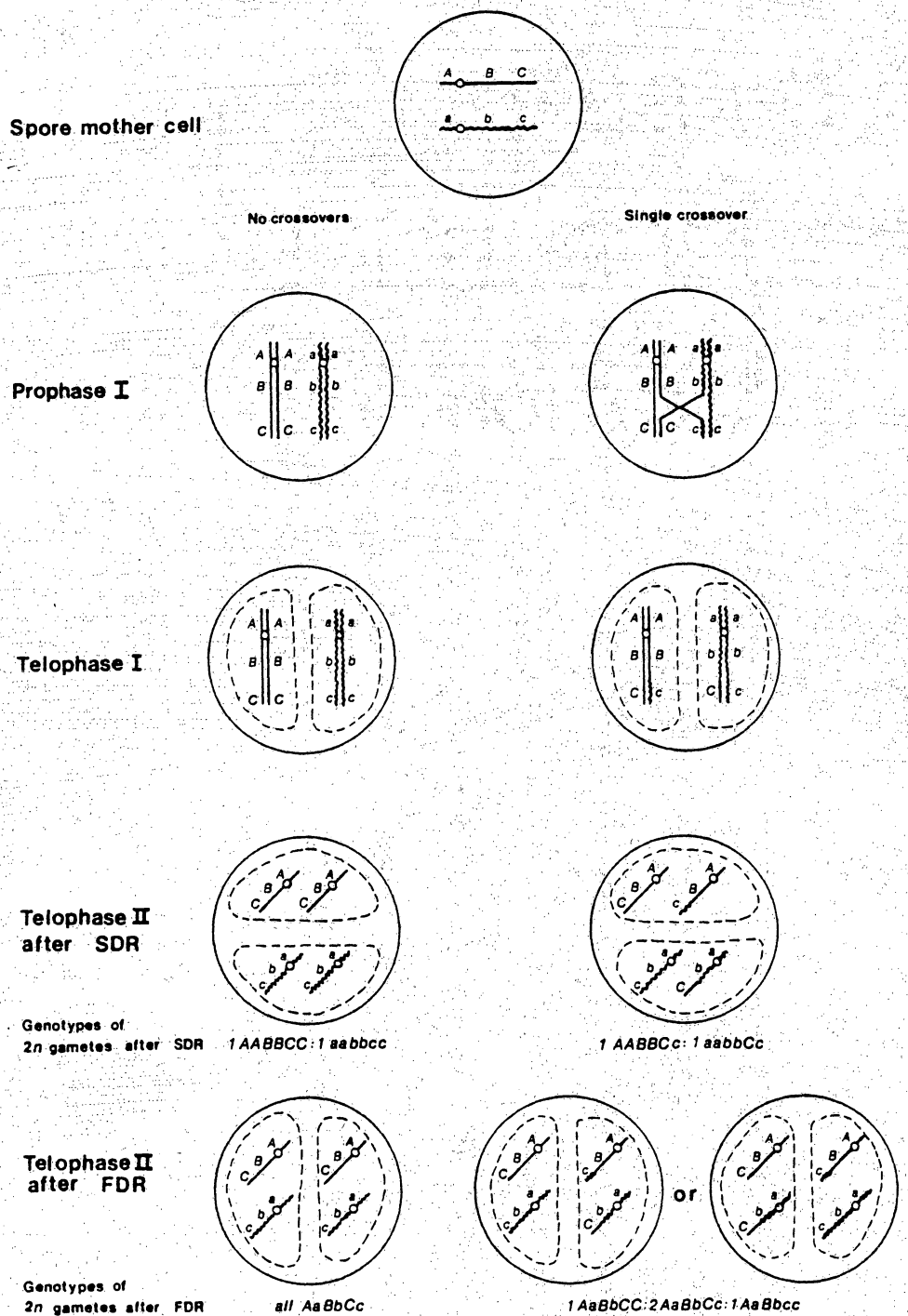

Fig.1. Segregation of three heterozygous loci on a pair of homologous chromosomes during 2n gamete formation by both first division restitution (FDR) and second division restitution (SDR).

Source: Veilleux, 1985

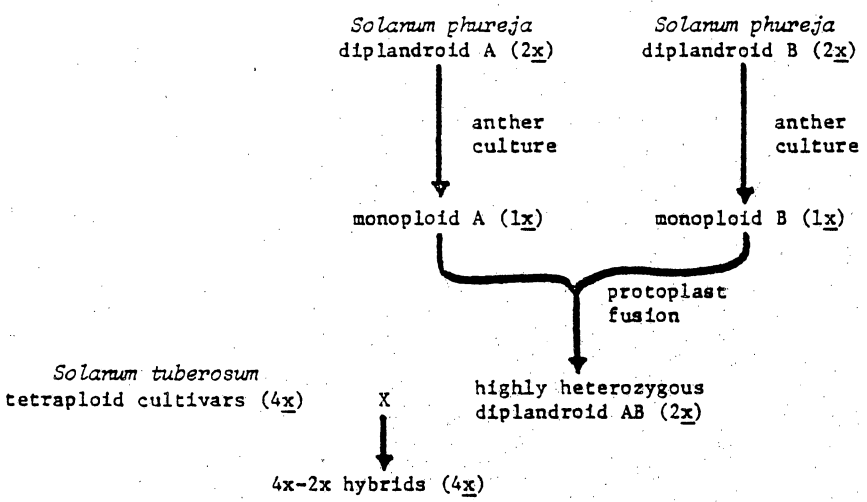

Fig. 2. The overall breeding scheme

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CHAPTER 2

CLUSTER ANALYSIS OF ANTHER DERIVED PLANTS OF SOLANUM PHUREJA BASED ON MORPHOLOGICAL CHARACTERISTICS

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ABSTRACT

The feasibility of grouping anther-derived plants of Solanum phureja according to ploidy based on their morphological characters was studied. Canonical discriminant analysis identified four characters (anther length, number of chloroplasts/pair of guard cells, leaf width, corolla width) of nine measured as the most effective combination for diagnosing ploidy. Data for these characters from two sets of plants were subjected to two clustering techniques, one of which uses the average linkage clustering (UPGMA of the NT-SYS programs) and the other centroid sorting (SAS-Fastclus). Screening of anther-derived plants by cluster analysis proved to be an efficient means of separating monoploids from the other ploidy levels.

INTRODUCTION

The main application of anther culture to plant breeding is the development of haploid and subsequently homozygous diploid plants; therefore, it is essential that the ploidy level of regenerated plants be known. Anther-derived plants have been frequently observed to vary with respect to chromosome number (Wenzel and Uhrig, 1981; Bhaskaran et al., 1983). The most common method for determining ploidy level has been Feulgen-staining of root-tip chromosomes. However, if a large number of plants is to be analyzed, this is rather a laborious method.

The overall goal of our potato breeding program is to introduce exotic germplasm into existing Solanum tuberosum cultivars ($2n = 4x = 48$) by using a South American diploid potato species, Solanum phureja Juz. & Buk. ($2n = 2x = 24$) as the pollen parent in $4x \times 2x$ crosses. The first step of the breeding program is to increase the heterozygosity of the $2x$ parent by fusion of monoploid genomes ($2n = 1x = 12$) derived through anther culture of unrelated diplandrous clones. Hence, early recognition of monoploids hastens the progress of the program.

Correlation between ploidy level and various morphological characteristics of plants regenerated from

tissue culture has been observed in several species (Tsikov et al., 1974; Hell, 1979, Fassuolis, 1977; Sree Ramulu, 1976; Santos and Handro, 1983). The objectives of this study were: 1) to determine if, with multivariate statistics, i.e., as canonical discriminant analysis and cluster analysis, a set of easily measurable morphological characteristics could be identified to diagnose the ploidy level of anther-derived plants of Solanum phureja; and 2) to quantify the variation within each ploidy group for identification of exceptional genotypes for use in further breeding.

MATERIALS AND METHODS

Analysis of greenhouse grown plants. Two separate sets of plants derived through anther-culture of a single clone of Solanum phureja P.I. 225669 (Veilleux et al., 1985) were used. One set (designated original data set) contained 58 plants, including 33 diploids, 19 monoploids and six tetraploids as determined by Feulgen staining of the root tip chromosomes (Karp, 1982). The other set (designated testing data set) consisted of 30 plants, including 22 diploids, 7 monoploids and 1 tetraploid. This set of plants was derived by anther culture of 4 diploid plants which had been regenerated by anther culture of the same genotype of S. phureja. The plants were established from in vitro shoots, cultured on Murashige and Skoog (1962) basal medium with 3% sucrose,

0.8% agar, pH 5.8 supplemented with 2 mg/l 2,4-D and 0.4 mg/l BAP, by removing the culture medium from the root mass and placing the plantlets in vermiculite under plastic for a week, followed by 3-4 days under mist. The plants were then transferred to 4 liter pots and placed in the greenhouse under ambient temperatures and natural photoperiod; the original set of plants during spring 1984, and the testing set a year later. The plants were fertilized weekly with 200ppm N from 20N-8.8P-16.6K.

At flowering, approximately six weeks after the plants had been placed in the greenhouse, the following morphological characteristics were measured: 1) leaflet length (3 leaflets/plant), 2) leaflet width (3 leaflets/plant), 3) mean number of chloroplasts/10 pairs of guard cells (Frandsen, 1968), 4) internode length (second youngest internode), 5) stem width (second internode), 6) corolla length (3 fully expanded corollas/plant), 7) corolla width (3/plant), 8) anther length at dehiscence, and 9) number of flowers in an inflorescence (3 inflorescences/plant).

The original data set was first analyzed by a canonical discriminant analysis (SAS Institute, 1982) to find the best combination of characters for determination of ploidy level. Using the resulting character combination, the data of the original data set, and, for verification, that of the testing

data set, were subjected to cluster analysis by means of nearest centroid sorting (Anderberg, 1973; SAS Institute, 1982; Veilleux et al., 1984). To examine the relationships of the different genotypes within ploidy groups, clustering of the original data set was repeated using the UPGMA (unweighted pair group method using arithmetic averages) of the numerical taxonomy package of computer programs, NT-SYS (Rohlf et al., 1978). The clustering was based on a Q-correlation matrix. Cophenetic correlation coefficients were calculated for each similarity matrix.

Analysis of in vitro plantlets. To determine if clustering according to ploidy level could be achieved at an earlier developmental stage, the following data were measured on the plants of the testing set at transfer from in vitro to the greenhouse: 1) guard cell length (5 guard cells/plant), 2) leaflet length (3 leaflets/plant), 3) leaflet width (3 leaflets/plant), 4) internode length, and 5) stem width. Peels of leaf epidermis for guard cell measurements were prepared according to the method of Hilu and Randall (1984).

RESULTS

The canonical discriminant analysis using the known ploidy as the classification variable on the original data set revealed that a combination of four characters (anther length,

chloroplast counts/pair of guard cells, leaflet width, and corolla width) was most effective for grouping the plants according to ploidy level. (Table 1). Leaflet length instead of corolla length was selected in spite of the higher character weight value of the latter to achieve a balance between floral and vegetative characters. Using this character combination the same data set was subjected to cluster analysis by SAS-fastclus procedure. Optimum clustering occurred with two clusters and yielded a cubic clustering criterion (CCC) of 13.9. All 19 monoploids were assigned to the same cluster, which included only two plants of higher ploidy groups (Table 2). The remaining diploid and tetraploid plants formed the other cluster. An attempt was made to separate the diploids from the diploid-tetraploid group by assigning the data set to three clusters. However, due to the large overlap of the character values between the diploids and tetraploids (Table 3), the tetraploids were always embedded in the predominantly diploid cluster. However, a three dimensional plot of the plants in the original data set using the characteristics most diagnostic of ploidy as revealed by canonical discriminant analysis demonstrated that the tetraploids occurred as a subgroup at the upper limits for all three characteristics (Fig. 1).

The canonical discriminant function was repeated on both of the data sets with the optimum character combination to

study the relative importance of the selected characters (Table 1). In the testing data set, the number of chloroplasts/pair of guard cells was the only variable that was not significant. There were also some differences in the relative weights of the different characters within the total canonical structure; however, in general, the selected character combination proved applicable to the testing data set. With the testing data set, two clusters were again found to be optimum (CCC = 7.5), with all of the monoploids grouped in one cluster and the majority (18 of 23) of the plants of the other ploidy levels assigned to the other.

Canonical discriminant analysis of the in vitro morphological characters revealed that all but leaflet length of the measured characteristics had a significant contribution to the between group variation (Table 1). In the cluster analysis, two clusters again were optimum (CCC = 6.9) and again, six of the seven monoploids were assigned to one cluster. However, the monoploid cluster was not distinct, because plants of the higher ploidy levels were approximately equally split between the two clusters with 44% in the monoploid cluster.

The centroid sorting method of the SAS fastclus procedure does not yield information on the relationships between the different members in each cluster. Thus, for a closer

look at the within group variation, clustering of the original data set was repeated with the UPGMA method (Fig 2). The UPGMA resulted in a better separation of the different ploidy levels. The majority of the monoploids (16 of 19) appeared in one cluster that included four diploids. The diploids were separated into two subclusters, linked to each other at a correlation coefficient level of 0.934 and to the predominantly monoploid cluster at a 0.966 level. The tetraploids separated into two very distinct subclusters that were linked to each other at the lowest correlation level (0.893) of all the other groups. The tetraploid cluster also included a heterogeneous but distinct subcluster comprised of diploids and monoploids.

DISCUSSION

Both mean chloroplast number/pair of guard cells and the guard cell length were valuable for separating the different ploidy levels of this potato species in all but the testing data set. This is in agreement with the findings of Hermsen et al., (1971). Even more diagnostic of ploidy level were the floral characters - corolla length, corolla width and anther length-, which supports the association of flower dimensions with polyploidization (Eigsti and Dustin, 1955; Narayanaswami et al., 1971; Santos and Handro, 1983). The discriminatory power of leaflet dimensions was less than that of the floral

characters, as for Petunia (Santos and Handro, 1983). Very high within-group variability for number of flowers in an inflorescence and internode length made these characters poor choices for diagnosing ploidy of mature plants. The canonical discriminant analysis not only reduced the number of characters one needs to measure, but also gave valuable information on which characters disturb clustering by having larger within than between group standard deviation; e.g., number of flowers per inflorescence and internode length.

The clustering according to ploidy level using the diagnostic character combination was good in both of the data sets from greenhouse grown plants. Thus, it is concluded that cluster analysis based on morphological characters is useful for initial screening of anther-derived plants. However, as is particularly clear in the testing data set, the ploidy level should ultimately be confirmed by a direct method. (Feulgen staining of the chromosomes, cytophotometry or flow cytometry).

Because the monoploids have developed through embryogenesis of pollen grains, the genetic complements of which result from free recombination in a cross-breeding heterozygous species, one would expect the group to be heterogeneous. Our results, however, indicate the opposite. One could speculate that with respect to the measured

morphological characteristics, the nuclear ploidy is a major determining factor leading to the uniformity of this group.

The UPGMA of the NT-SYS program was very efficient in separating the different ploidy levels. The value of the correlation coefficient at which the different ploidy groups clustered decreased with the increase in ploidy level. This would indicate higher heterogeneity at the higher ploidy levels.

Genotypes within the higher ploidy groups may have arisen either from reduced pollen followed by endopolyploidization or from unreduced or doubly unreduced pollen. The frequencies of the various mechanisms are currently unknown; however, that both non-reduction and endopolyploidization have contributed to the population of diploids in our population has been supported by genetic evidence (Veilleux et al., 1985). Those diploids arising from 2n pollen due to spindle co-orientation during the second meiotic division would be relatively homogeneous compared to those which have been derived by endopolyploidization of monoploid embryos. As a group, therefore, the diploids would be expected to exhibit less variability. This was true only for characters measured on individual flowers (anther length, corolla length and corolla width - Table 3). However, these are characters which are more stable over environments, and

because the data were collected over a period of two weeks of lengthening days, we are inclined to take this as evidence of greater genotypic homogeneity in the diploid group. However, this genotypic homogeneity may have obscured general effects of nuclear ploidy by buffering the data with a large subgroup of vigorous heterozygous diploids from 2n pollen embedded among the comparatively inbred doubled monoploids.

The clustering was not as clear on the data collected at the in vitro stage. In addition to problems in discriminatory power at this early stage, a possibility of endopolyploidization after removal of the plants from in vitro condition could have confounded the results. Because the chromosome counts were determined at the seedling stage in the greenhouse, some of the in vitro monoploids could have gone through one or more cycles of endomitosis before greenhouse establishment. Genomic lability and the tendency of monoploids to change to higher ploidy levels in potato has been reported by Wenzel and Uhrig (1981).

TABLE 1. *Canonical discriminant analysis of the 'original', 'testing' and* 'in vitro' *data sets.*

Character	Original data set		Testing data set		*In vitro* data set	
	F-value	Char. weight[a]	F-value	Char. weight	F-value	Char. weight
Chl/10 gc[b]	38.3***[c]	0.81	4.2	0.59		
Stem width	3.7	0.33				
Leaf length	12.0**	0.55			2.9	0.50
Leaf width	18.9***	0.65	6.0*	0.68	5.5*	0.64
Intern length	2.5	0.28			4.0*	0.56
Anther length	73.2***	0.94	9.8**	0.80		
Flowers/inflo	2.3	0.26				
Corol length	38.3***	0.81				
Corol width	55.2***	0.89	20.5***	0.99		
Guard cell length					8.7***	0.77

[a]Character weight in the total canonical structure.

[b]guard cell pairs

[c]*, **, and *** denote significance at the 0.05, 0.01, and 0.001 levels, respectively.

TABLE 2. *Cluster assignments of the anther-derived plants.*

	Original data set		Testing data set		*In vitro* data set	
Cluster	M[a]	O	M	O	M	O
1	19	0	7	5	6	10
2	2	37	0	18	1	13

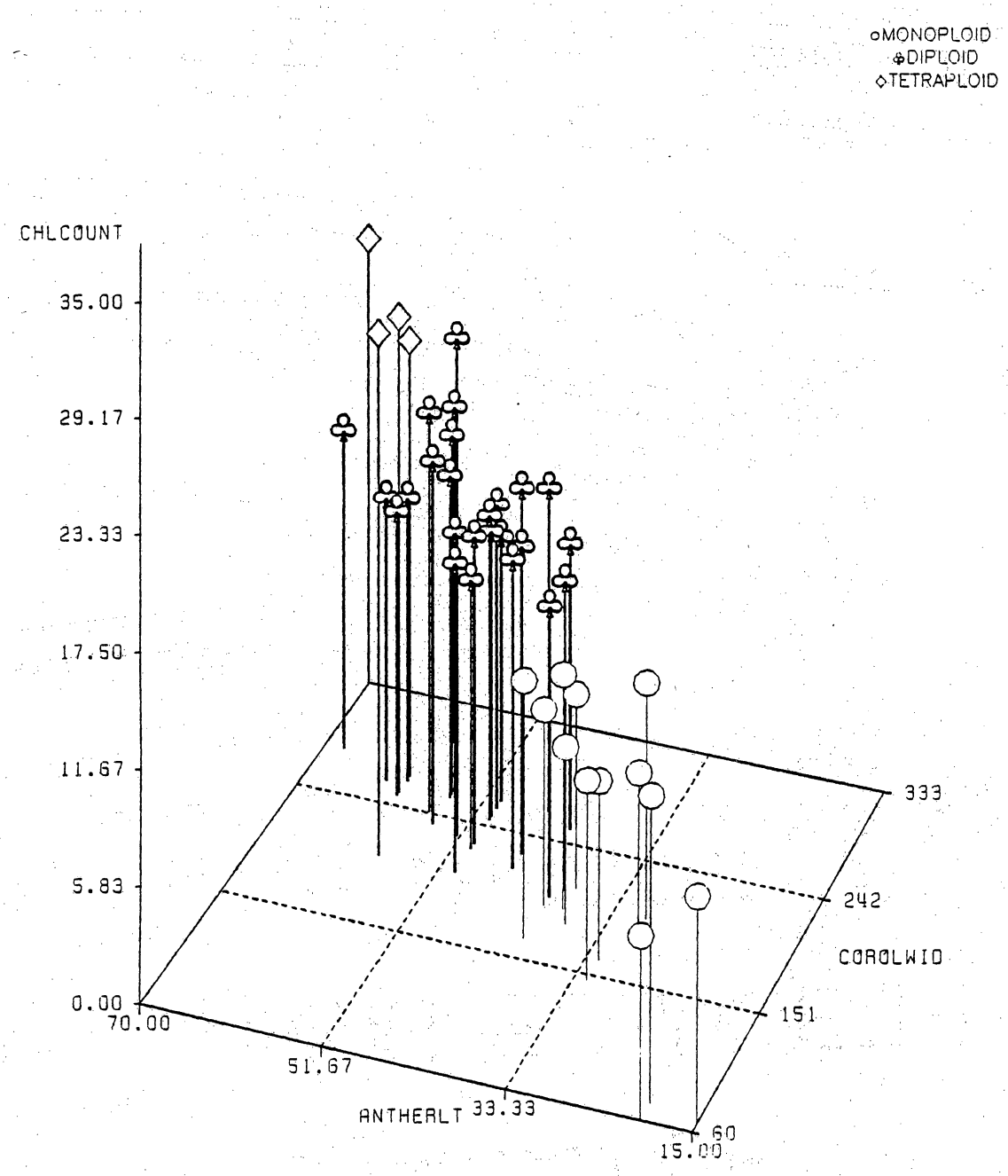
[a]M=monoploid; O=other (including both diploids and tetraploids)

TABLE 3. *Means, standard deviations (Std), and coefficients of variation (CV) of morphological characters among the anther-derived plants differing in ploidy.*

	Mean			Std			CV		
Variable	M[a]	D	T	M	D	T	M	D	T
Chl/10 g.c.	10.1	17.2	25.3	1.7	3.3	1.5	17.6	19.3	6.0
Stem width (mm)	3.3	3.7	3.9	0.7	0.7	0.6	21.9	19.3	16.8
Leaf length (cm)	2.7	3.4	3.5	0.5	0.7	1.2	20.5	21.0	35.7
Leaf width (cm)	1.4	2.2	2.9	0.3	0.5	1.2	19.1	23.5	42.7
Inter lngth (mm)	1.6	2.0	2.6	0.9	1.1	1.2	53.4	54.7	45.6
Anth lngth (mm)	3.1	5.1	5.9	0.9	0.7	0.7	27.5	13.0	11.9
Flowers/inflo	5.5	6.5	6.2	1.6	2.7	2.4	30.0	41.2	37.9
Corol lngth (cm)	0.9	1.5	1.6	0.4	0.2	0.3	42.4	14.7	18.0
Corol width (cm)	1.5	2.5	2.6	0.6	0.3	0.5	35.9	10.1	18.0

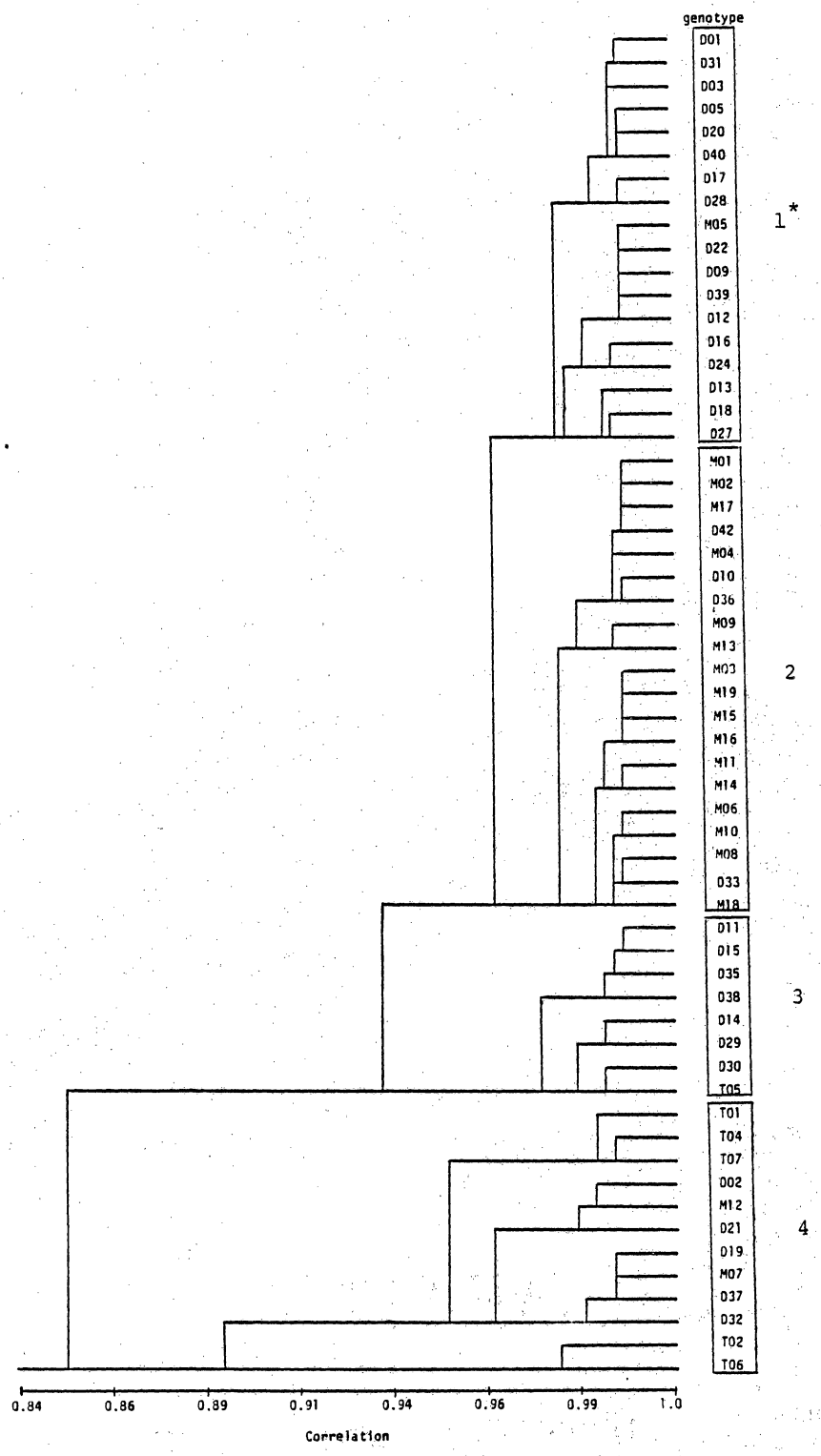
[a]M=monoploid, D=diploid, T=tetraploid

Fig. 1. Scattergram of monoploid, diploid and tetraploid plants based on three morphological characteristics. All plants were regenerated by anther-culture of one genotype of Solanum phureja



CHLCOUNT = NUMBER OF CHLOROPLASTS/PAIR OF GUARD CELLS
COROLWID = COROLLA WIDTH IN MM
ANTHERLT = ANTHER LENGTH IN MM

Fig. 2. Phenogram of anther-derived plants of Solanum phureja. Cluster 1* (17 diploids, 1 monoploid); Cluster 2 (16 monoploids, 4 diploids); Cluster 3 (7 diploids, 1 tetraploid); Cluster 4 (5 diploids, 5 tetraploids, 2 monoploids).


genotype
D01
D31
D03
D05
D20
D40
D17
D28
M05
D22
D09
D39
D12
D16
D24
D13
D18
D27
M01
M02
M17
D42
M04
D10
D36
M09
M13
M03
M19
M15
M16
M11
M14
M06
M10
M08
D33
M18
D11
D15
D35
D38
D14
D29
D30
T05
T01
T04
T07
D02
M12
D21
D19
M07
D37
D32
T02
T06
1*
2
3
4
0.84
0.86
0.89
0.91
0.94
0.96
0.99
1.0
Correlation

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CHAPTER 3

PLOIDY AND GENOTYPIC EFFECT ON NET PHOTOSYNTHESIS AND RUBP CARBOXYLASE ACTIVITY AMONG ANTHER-DERIVED PLANTS OF SOLANUM PHUREJA

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Abstract. The effect of ploidy and genotype on net photosynthesis and its component characteristics, RuBP carboxylase (E.C. 4.1.1.39) activity, chlorophyll content, leaf area and chloroplast ultrastructure among monoploid, diploid and tetraploid anther-derived plants of Solanum phureja was studied. Leaf area, net photosynthesis and chlorophyll content increased significantly with increasing ploidy. Within the monoploid group, Rubisco activity and concentration displayed a significant genotypic effect. For the diploids, variation among genotypes was significant for total protein content and maximum specific activity of Rubisco, and among the tetraploids for net photosynthesis and specific leaf weight. The anther-derived plants surpassed the anther donor plant for all characteristics except net photosynthesis, for which only the tetraploids were superior to the anther source plant. A monoploid genotype with an

increase of 28% for maximum activity of Rubisco and a tetraploid with an increase of 30% for net photosynthesis over the anther donor plant were identified.

Key words: Potato - photosynthesis - anther culture - ploidy - monoploid.

Introduction

Although originally viewed as a potentially efficient means for producing homozygous breeding lines after chromosome doubling, androgenesis in anther cultures has been demonstrated to yield transgressive segregants for traits of economic significance, or even novel phenotypic variation (Evans et al. 1984). This has been well documented for various characteristics of plant morphology, leaf color, alkaloid content and disease and insect resistance (Arcia et al. 1978; Burk and Matzinger 1976; Burk and Chaplin 1980; Thurston et al. 1977). However, variability for physiological traits among anther-derived plants has been less frequently reported (Bhaskaran et al. 1983; Medrano and Primo-Millo 1985).

A common phenomenon encountered among plants derived through anther culture is variation in ploidy resulting from cycles of endopolyploidization, nuclear fusion during the androgenetic process (Chen et al. 1984), or embryogenesis of 2n microspores (Veilleux et al. 1985). Ploidy variation

among such closely related genotypes provides the opportunity for studying gene dosage effects on various physiological characteristics. In previous studies, an increase in ploidy has been found to be positively correlated with photosynthethic rate and biochemical activities associated with CO_2 fixation rates for Medicago (Molin et al. 1982), Festuca (Byrne et al. 1981; Joseph et al. 1981) and Triticum (Dean and Leech 1982). In a polyploid series of fern (Todea barbara) DeMaggio et al. (1971) found that the ultrastructure of chloroplasts changed with increasing ploidy.

In addition to variation for ploidy among regenerated plants, the androgenetic process can also result in considerable genotypic variation within each ploidy group. This is largely due to genetic recombination and segregation during microsporogenesis of cross-pollinating, heterozygous species or to genomic instability during the callus phase for plants regenerating from indirect embryogenesis. Inbreeding depression, often present in colchicine induced polyploids, can be partly circumvented by the rigorous selection of embryogenic microspores surviving to form plantlets during androgenesis. This has been termed the 'monoploid sieve' (Wenzel et al. 1979) and may be particularly effective for species normally intolerant of inbreeding.

Selection for photosynthetic efficiency as a factor in yield improvement has met with only limited success (Zelitch 1982; Gifford et al. 1984). Crosbie et al. (1981) reported

an advance of 4.7% in carbon exchange rate per selection cycle among inbred lines of maize; however, no concomitant increase in yield was observed. Wiebold et al. (1981) observed no response to selection for net photosynthesis among soybean cultivars.

The overall goal of our breeding program is to introduce exotic germplasm to existing Solanum tuberosum L. cultivars by 4x X 2x crosses using Solanum phureja Juz. & Buk. as the diploid pollen parent. Evaluation of the genotypes derived through anther culture of the pollen parent with respect to physiological performance is important for selection of the best genotypes and methods for potato germplasm improvement. Thus, the objectives of this study were: 1) to assess the effect of ploidy on net photosynthesis and RuBP carboxylase activity among anther-derived genotypes of the diploid potato species, S. phureja; and 2) to evaluate the potential of anther culture for generating variability for photosynthetic traits among regenerated plants.

Materials and methods

Plant material. Four genotypes each of monoploid, diploid and tetraploid plants derived by direct embryogenesis in anther cultures of a single genotype of S. phureja. P.I. 225669 (Veilleux et al. 1985), which had been selected for adaptation to long photoperiods (Haynes 1972), were studied. Three individual plants of each genotype were grown from

tubers in 4 L pots in the greenhouse under ambient temperatures and natural photoperiod (March through May, 1985). The plants were watered weekly with a balanced nutrient solution.

Photosynthesis measurements. Nine weeks after planting, before the plants had flowered, net photosynthesis was determined on fully expanded leaves. One compound leaf per plant was enclosed in a plexiglas chamber (18 X 13.5 X 4 cm). Air circulation through the chamber was controlled by a small fan located directly beneath the leaf. Photosynthesis was measured using the open system described by Schaffer et al. (1985). Photosynthetic photon flux density at the leaf surface was 0.9 mM s^{-1} m^{-2}. Air temperature in the chamber was maintained at 28 ± 1°C and relative humidity at 50 ± 1%. Leaf area was measured using a Li-Cor LI-3050A leaf area meter. Following the photosynthesis and leaf area measurements, twenty 0.2 cm^2 discs were removed at random from the leaf. These discs were collected and stored in a Dewar of liquid nitrogen.

RuBP carboxylase, chlorophyll, and protein determinations. The frozen leaf material was extracted at 25°C in a glass homogenizer containing 1 ml of an extraction medium (50 mM Tris-HCl (pH 8.2), 20mM $MgCl_2$, 20 mM $NaHCO_3$ 50 mM 2-mercaptoethanol, and 1 mM Na_2EDTA). Aliquots were then

removed for measuring chlorophyll (Wintermans and DeMots 1965) and total protein concentrations. The remaining extract was clarified by centrifugation (12,000 g, 1 min). RuBP carboxylase (E.C. 4.1.1.39) activity was measured in triplicate at 25°C (Servaites, 1985). RuBP carboxylase protein concentration was determined using the 2-^{14}C-carboxy-D-arabinitol bisphosphate technique of Hall et al. (1981) as modified by Servaites (1985). Both total protein and soluble protein (that present in the extract following centrifugation) was determined in triplicate by a dye-binding assay (Esen 1978).

Electron microscopy. Leaf samples of monoploid (A205), diploid (A95) and tetraploid (A86) plants (one genotype/ploidy) were fixed in 2% gluteraldehyde in 50 mM sodium cacadylate buffer (pH 6.8) and post fixed in 1% osmium tetroxide. The samples were embedded in Spurr's resin and stained in 1% uranyl acetate in 50% ethanol and lead acetate. Grids were examined using a JEOL 100C transmission electron microscope. Number of chloroplasts/cross section of a mesophyll cell and number of starch granules/chloroplast were determined from micrographs photographed at magnification of 3300X. Chloroplast size was measured from enlarged micrographs at magnification of 1600X using a Zidas image analyzer (Carl Zeiss, Inc.).

Statistical analysis. The effect of ploidy was analyzed by SAS ANOVA procedure and the means separated by LSD test. For genotypic effect, ANOVA was repeated on data from each ploidy group separately. The relationship of net photosynthesis to the component characteristics was analyzed by correlation analysis.

Results

Area per leaf, net photosynthesis and chlorophyll content on a leaf area basis were observed to increase significantly with increasing ploidy (Table 1). Means for the three different ploidy groups were significantly different for leaf area; however, diploid and tetraploid groups did not differ significantly for net photosynthesis or chlorophyll content, although they were significantly greater than monoploid means for both traits. Specific leaf weight was not significantly different among ploidy groups.

Due to high within group variation, ploidy effect was not significant for biochemical data. However, for RuBP carboxylase activity, maximum specific activity (SAmax), and measured specific activity (SAmeas), a trend towards improved performance from monoploids to tetraploids could be observed. Means of RuBP carboxylase activity, RuBP carboxylase protein, and soluble and total protein concentration were greater for tetraploids than for diploids and monoploids. The anther-

donor plant, PP5, was inferior or similar to the mean performance of the anther-derived plants for all characteristics except net photosynthesis, for which it was only surpassed by the tetraploids.

The separate ANOVA tests for each of the three different ploidy levels revealed significant variation within each ploidy group for several of the measured traits (Table 2). For the monoploid group, RuBP carboxylase activity and concentration, and maximum as well as measured specific activity displayed a significant genotypic effect. For the diploid group, genotypic effect was significant for total protein content and maximum specific activity. The tetraploid genotypes varied significantly for net photosynthesis and specific leaf weight. The monoploid group included one genotype (A205), which had an increase of 28% in maximum Rubisco activity as compared to the anther donor plant. Among the tetraploids a genotype (A87) was identified which surpassed the anther donor plant and the mean of the tetraploids in net photosynthesis by 30 and 28%, respectively. It also had exceptionally high values for most of the measured component characteristics (Table 2).

The increase in net photosynthesis, regardless of ploidy was associated with larger leaf area, higher activity and amount of Rubisco, and higher chlorophyll concentration.

Electron microscopy. The size of the chloroplasts in mesophyll cells of the monoploid plant was significantly smaller than in the higher ploidy levels (Table 3, Fig. 1). The chloroplast size of the diploid surpassed that of the tetraploid plant; however, the number of chloroplasts in the cells of the tetraploid plant was twice that of the diploid, which resulted in similar percentages of cell face area covered by chloroplasts for both ploidy levels (Table 3). Both the number and size of the starch granules appeared to increase with increase in ploidy (Fig. 1).

Discussion

The positive association between increasing ploidy level and net photosynthetic rate reported for other crops (Joseph et al. 1981; Byrne et al. 1981; Molin et al. 1982) has been demonstrated for the diploid potato species, S. phureja. However, the increase was significant only from monoploid to diploid level, which is in agreement with a recent report by De,Maine (1984), who found similar rates of photosynthesis for a dihaploid of S. tuberosum cv. Pentland Crown and its chromosome doubled derivatives. De,Maine (1984) concluded that photosynthetic rate was not affected by ploidy manipulation.

Other parameters, which contribute to photosynthetic activity revealed similar trends in relation to ploidy level.

The larger leaf area of the tetraploid plants was not associated with reduced levels of soluble protein per unit leaf area, as has been reported for soybean (Hesketh et al. 1981) and rice (Oritani et al. 1979). The differences in protein and chlorophyll content are not the result of differences in leaf thickness because the specific leaf weight was similar for all three ploidy levels. Thus it seems that factors involving protein synthesis are not limited by the lower gene copy number at the monoploid level.

The specific activity of Rubisco did not significantly differ among the ploidy groups, which is in agreement with the findings of Joseph et al. (1981). In contrast with that study, our data showed that the amount of Rubisco as a fraction of total protein was remarkably similar (32%) in all ploidy groups. This seems to indicate that the smaller subunit of Rubisco, which is coded for by the nuclear genome, might not be a limiting factor in the biosynthesis of the enzyme at lower ploidy levels as suggested by Ellis (1975).

From the separate ANOVAs within each ploidy level, it can be concluded that useful genotypic variation was present with respect to several characteristics. Of particular interest was the variation within the monoploids and diploids for measured and theoretical maximum specific activity of Rubisco. The SAmax activities of all the diploid anther-derived plants surpassed the anther-donor plant by 14-28%. Genotype A95, which has been shown to be heterozygous

(Veilleux et al. 1985), thus likely a result of androgenetic development of a 2n microspore, had the lowest SAmax value, whereas A97, previously shown to be homozygous, thus a result of a cycle of endopolyploidy, had a high SAmax activity. It is speculated that a combination of completely homozygous nuclear loci with chloroplast DNA has resulted in higher enzyme activity than when the combination involves heterozygous loci. Similar dichotomy between high and low enzyme activity was observed among the monoploid plants, both in SAmax and SAmeas. Heterogeneity among the monoploid group was expected because of segregation during microsporogenesis. From this, one could further speculate that only a unique combination of one type of nuclear gene together with the chloroplast DNA will result in production and assembly of an enzyme of higher catalytic activity or increased expression.

An alternative explanation to the variation in Rubisco activity of the anther-derived lines could be a mutation in the chloroplast genome during anther culture. This is supported by recent work of Day and Ellis (1984), who identified large deletions in the ctDNA of anther-derived wheat plants indicating genomic lability in the organellar DNA due to anther-culture.

In spite of the improvement for Rubisco activity among the anther-derived monoploids, the source plant, PP5, still surpassed them for net photosynthesis. Thus, we are presently testing isogenic lines of A205 of different ploidy levels to

study the gene dosage effect in combination with the high enzymatic activity on the rate of photosynthesis.

The observed increase in the number of chloroplasts in the tetraploid plant as well as the smaller size of the organelle in the monoploids confirms the previously reported association between cell size and the area covered by chloroplasts (Tsuji et al. 1979; Scott and Possingham 1980; Olzewska et al. 1983; Ellis and Leech 1985). The obvious increase in the starch granule size of the tetraploids is in agreement with the finding of DeMaggio et al. (1971).

In conclusion, our results showed that anther-culture can produce genotypes which vary in net photosynthesis and its various biochemical components. Such variability may be useful for germplasm improvement. This is of particular interest in potato breeding, because several reports have shown a correlation between yield and net photosynthesis in this crop (Moll and Henniger 1978; Dwelle et al. 1981; Oparka 1985).

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Table 1. Means for photosynthetic and biochemical parameters among anther-derived plants of Solanum phureja differing in ploidy (Pn = net photosynthesis LA = Leaf area; SLW = specific leaf weight; CHL = chlorophyll; SAmax = units/mg Rubisco; SAmeas = units/mg soluble protein; SAchl = units/mg CHL)

Ploidy	Pn mg CO_2 dm^{-2} hr^{-1}	LA cm^2	SLW mg/cm^2	Rubisco activity units/cm^2	Rubisco protein mg/cm^2	Total protein mg/cm^2	Soluble protein mg/cm^2	CHL mg/cm^2	SAmax	SAmeas	SAchl
PP5	12.0	22	1.2	0.12	0.071	0.34	0.27	16	1.35	0.46	7.8
1x	8.1	26	1.2	0.14	0.100	0.43	0.31	18	1.56	0.50	8.8
2x	10.9	41	1.2	0.16	0.095	0.39	0.29	19	1.60	0.52	8.0
4x	12.8	56	1.2	0.19	0.120	0.45	0.38	25	1.63	0.52	7.0
LSD (5%)[a]	2.7	15						2.9			

[a]Anova among the ploidy groups; least significant differences (LSD) are provided for characteristics demonstrating significant genotypic effect among ploidy groups

Table 2. Means for photosynthetic and biochemical parameters for individual genotypes (3 plants per genotype) of anther-derived plants of Solanum phureja differing by ploidy (see Table 1 for heading abbreviations). Significant correlations (r*) between Pn and other characteristics, regardless of ploidy, are also given

Ploidy	Genotype	Pn mg CO_2 dm^{-2} hr^{-1}	LA cm^2	SLW mg/cm^2	Rubisco activity units/cm^2	Rubisco protein mg/cm^2	Total protein mg/cm^2	Soluble protein mg/cm^2	CHL mg/cm^2	SAmax	SAmeas	SAchl
2x	PP5	12.0	22	1.2	0.12	0.07	0.34	0.27	16	1.35	0.46	7.77
1x	A86	7.6	19	1.1	0.12	0.11	0.42	0.30	20	1.16	0.41	6.10
1x	A165	10.0	19	1.1	0.14	0.09	0.42	0.33	19	1.57	0.43	7.58
1x	A205	5.9	23	1.1	0.09	0.09	0.44	0.28	17	1.87	0.60	9.77
1x	A258	8.8	42	1.3	0.19	0.12	0.43	0.34	17	1.63	0.55	10.84
LSD (5%)[a]					0.06	0.05				0.31	0.13	
2x	A71	12.3	45	1.2	0.15	0.09	0.37	0.28	18	1.73	0.53	8.14
2x	A95	11.3	46	1.2	0.16	0.10	0.43	0.31	20	1.54	0.52	7.76
2x	A97	10.0	46	1.3	0.21	0.12	0.46	0.35	21	1.67	0.57	10.39
2x	A178	9.2	37	1.1	0.11	0.07	0.30	0.23	17	1.60	0.51	5.98
LSD (5%)							0.42			0.16		
4x	A87	17.5	83	1.5	0.23	0.14	0.54	0.44	28	1.64	0.51	8.23
4x	A119	10.8	34	1.2	0.19	0.11	0.43	0.34	24	1.69	0.57	7.75
4x	A301	15.3	58	1.1	0.19	0.13	0.45	0.36	25	1.51	0.53	7.45
4x	A330	9.3	58	1.2	0.13	0.08	0.37	0.27	24	1.70	0.46	5.15
LSD (5%)		4.8		0.3								
r* Pn to			0.36*		0.38*	0.40*			0.45*			

[a] Anova within the ploidy groups; least significant differences (LSD) are provided for characteristics demonstrating significant genotype effect within ploidy groups

Table 3. Means and standard deviations for number of chloroplasts/cross-section of a mesophyll cell (n=10), chloroplast size (n=30) and chloroplast face area as percentage of total cell face area (n=10) among anther-derived plants of Solanum phureja differing in ploidy

Ploidy	Number of chloroplasts/cell		Chloroplast size (μm^2)		Face area covered by chloroplasts (% of total area)	
	$\bar{x}$	std	$\bar{x}$	std	$\bar{x}$	std
1x	9.7	2.9	18.64	0.49	18.6	2.6
2x	7.6	1.6	23.30	0.64	26.1	4.0
4x	15.0	2.7	20.28	0.61	23.8	8.8

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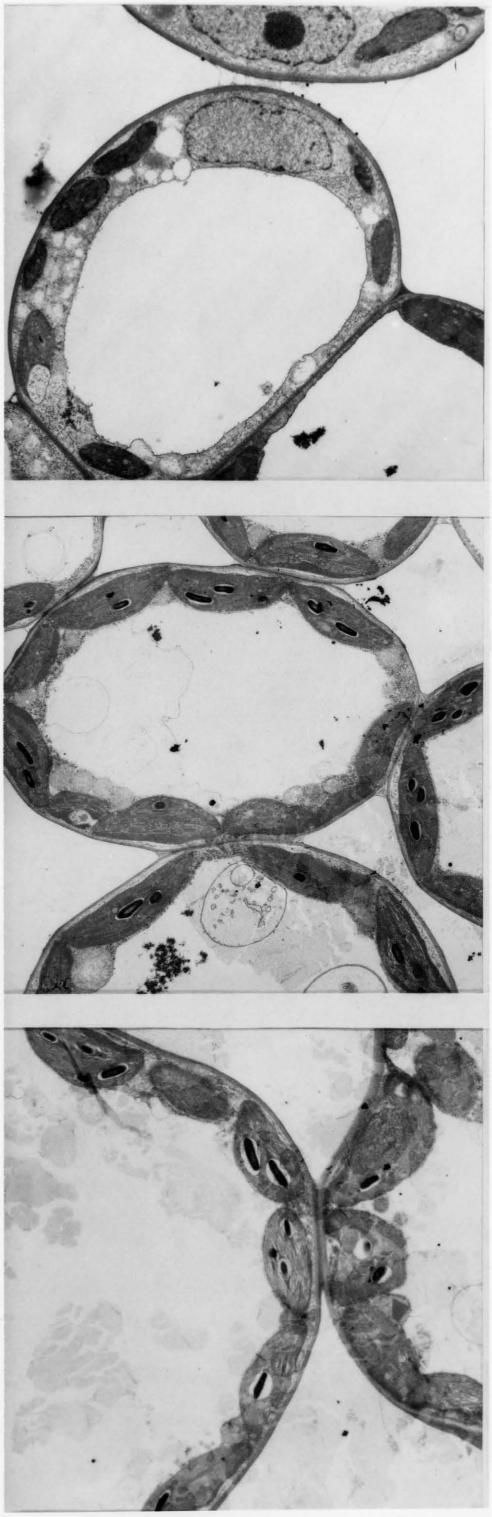
Fig. 1. Cross-section of a mesophyll cell (from top down) of a monoploid, diploid and tetraploid plant of Solanum phureja.



CHAPTER 4

CHANGES IN THE NUCLEAR GENOME OF ANTHER-DERIVED PLANTS OF SOLANUM PHUREJA: AMPLIFICATION OF RDNA SEQUENCES

(Formatted for Plant Molecular Biology)

Keywords: gene amplification, differential replication, anther-culture, monoploid

SUMMARY

The nuclear genomes of anther-derived monoploid and diploid plants of Solanum phureja were compared to that of the anther-donor plant. The Tm of nuclear DNA was determined to be 85.1°C with a corresponding mol G+C content of 38.1%. Optical reassociation kinetics revealed that DNA amplification (7%) of the anther-derived monoploid consisted of highly repetitive DNA sequences. There were no differences among the kinetic components of the nuclear genome between the anther-derived diploid and the diploid anther-source plant. RNA-DNA hybridization in free solution revealed that the monoploid

DNA contained 30% more rDNA sequences (18s and 25s components) than either diploid. Variation for copy number of certain restriction fragments and for restriction enzyme cleavage sites was revealed by Southern blot analysis using pooled 18s and 25s components of rRNA as the probe.

INTRODUCTION

Although reports of differential DNA replication during normal differentiation processes in plants (1) or during *in vitro* manipulations of plant protoplasts (2), suspension, and callus cultures (3, 4) have become more frequent, our understanding of the dynamics of these events remains limited. An amplification of repeated sequences has been demonstrated during dedifferentiation of seedling-derived callus of *Cucumis melo* (5), in callus derived from secondary phloem of carrot (6), and pith-derived callus of *Nicotiana glauca* (7) as well as suspension cultures of soybean cells (8). It seems that specific synthetic processes of DNA contribute to the developmental regulation of gene expression. Further, it has been speculated that the stress imposed on the tissue *in vitro* causes an imbalance in the regulation of DNA replication. Both of these phenomena, i.e., regulatory role of DNA amplification and the genome's response to stress, have also been shown to operate *in vivo* (9, 10, 1).

An increase in total nuclear DNA content has been demonstrated among tobacco plants regenerated by direct embryogenesis in anther cultures (11, 12). The authors speculated that the DNA increase in the vegetative nucleus, which occurs concomitantly with DNA synthesis in the generative nucleus (13), was retained though subsequent divisions of the androgenetic embryoid, resulting in higher DNA content in the anther-derived plants (11, 12). Using flow cytometric quantification of total DNA per nucleus, we have observed a similar increase of 6-7% in nuclear DNA of anther-derived monoploid plants of Solanum phureja when compared to half that of the source plant (Veilleux et al., unpublished results).

DNA overreplication has generally been associated with highly repeated sequences, specifically of genes encoding rRNA in animals (Drosophila melanogaster, 14, 15) as well as plants (16, 17). Jackson (16) found that the proportion of sequences containing rRNA genes can be altered during selective growth of soybean cells. Cullis (17) probed the genome of plants regenerated from callus as well as the callus tissue with a 5s rDNA sequence isolated from environmentally-induced genotrophs of flax known to vary for copy number of this sequence. The same variation in copy number was detected in the callus and the regenerated plants.

The ultimate goal of our breeding program is to incorporate exotic germplasm to existing tetraploid potato cultivars though 4x X 2x crosses using the diploid potato species Solanum phureja as the pollen parent. In conjuction with the traditional breeding tools, we are examining the possibilities for utilizing phenotypic variability observed among the anther-derived monoploids. Thus, a better understanding of the changes occurring in the nuclear genome during the anther-culture process is of importance.

The objectives of this study were: 1) to characterize the nuclear genome of Solanum phureja, 2) to identify the kinetic component(s) in which the observed overreplicated DNA of an anther-derived monoploid occurred, and 3) to determine if the amplified sequences included rRNA genes.

MATERIALS AND METHODS

PLANT MATERIAL:

The plant material included one monoploid (2n = 1x = 12) and one diploid (2n = 2x = 24) anther-derived genotype as well as the diploid anther donor plant of Solanum phureja Juz. & Buk., which had been selected from P.I. 225669 and 225682 (18). The plants were propagated from tubers in 4 liter pots in the greenhouse under ambient temperatures and natural photoperiod (spring, 1985). The plants were ferti-

lized weekly with a balanced nutrient solution. Leaf tissue (100g per sample) was harvested for each DNA isolation at the time of flowering.

DNA ISOLATION:

DNA from leaf tissue was extracted according to Scott and Possingham (19) with the following modifications. The tissue (100g) was ground in a Wareing blender in the presence of liquid nitrogen and extracted with 100ml of 1% SDS (sodium dodecyl sulfate) in 0.3M sodium acetate, 4% polyvinylpyrrolidone (PVP MW=10 000), 0.02M EDTA (ethylenediaminetetraacetic acid), and 720µl mercaptoethanol/l. The homogenate was centrifuged at 20 000g for 5 min and the pellet re-extracted. Pooled supernatants were incubated at 37°C with 5mg pronase, 1mg proteinase K and 100µg RNAase A for 1h. High molecular weight DNA was recovered by centrifugation at 65K for 3.5h in a 65Ti rotor. The pellet was redissolved in 0.02M EDTA, 0.05M Tris (pH 7.8), and 1% SDS. The DNA sample was then deproteinized with phenol-chloroform thee times. The DNA was precipitated by addition of 3M sodium acetate (1/10 of the total volume) and two volumes of ethanol. The DNA was pelleted, washed with 80% ethanol and dried. After drying, the pellet was resuspended in 0.02M EDTA, 0.05M Tris (pH 7.8) and purified by CsCl centrifugation. For reassociation kinetics, the DNA was fragmented by thee passages though a French pressure cell (16

000psi). Electrophoretic migration compared to size markers indicated that this procedure resulted in uniform fragment sizes of 450-600bp.

TM AND MOL % G+C DETERMINATIONS:

The melting point of each DNA sample was determined by optical melting profiles in 0.5 X SSC (standard saline citrate: 0.15M NaCl, 0.015M trisodium citrate pH 7.0) using E. coli as an internal standard. Based on the thermal melting points, the guanine plus cytosine content of the DNA preparations was determined according to Marmur and Doty (1962).

RNA ISOLATION:

RNA from leaf tissue was extracted by a variation on the method of Kirby et al., as described by Johnson (20). The tissue disruption was repeated as for DNA isolation in buffer containing 1% naphtalene disulphonate, 100µl/100ml diethyl pyrocarbonate and 75µl/100ml mercaptoethanol. The supernatant was extracted with phenol-cresol thee times and the RNA precipitated with two volumes of ethanol.

Sucrose gradient was used to isolate 18s and 25s rRNA. RNA (1mg) was loaded on a 30ml sucrose gradient (5 to 20% in 1 X SSC) and centrifuged at 25 000 rpm for 15h at 8°C. The gradient was passed though a flow cell and both the 18s and 25s

peaks were collected and pooled. The RNA preparations were stored in 1 X SSC, 0.5% SDS at -20°C.

LABELING OF THE NUCLEIC ACIDS:

DNA and RNA was labeled with ^{125}I as described by Selin et al. (21).

REASSOCIATION EXPERIMENTS:

The optical reassociation reaction was conducted in 6 X SSC at Tm-25°C and the hyperchomic shift was monitored at 260nm by a Gilford 2400 spectrophotometer with thermal denaturation apparatus. The rate constant of the reaction was calculated from initial absorbance according to Wetmur and Davidson (22). For the hydroxyapatite (HA) method, denatured DNA samples of 4 different concentrations (10 to 2000 µg/ml) were incubated at 60°C for various time periods with ^{125}I labeled DNA. After incubation, the samples were brought to 0.14 M (PB) phosphate buffer and mixed with 0.5 g equivalent of HA. The unreassociated single stranded DNA (thee washes) and the reassociated duplexes (two elutions) were eluted with 0.14 M PB and 0.40 M PB, respectively. The DNA content of each fraction was measured by a gamma counter (Beckmann, model 5500).

RNA-DNA HYBRIDIZATION IN FREE SOLUTION:

To avoid DNA reannealing, the RNA-DNA hybridization was

conducted at 55°C in 45% formamide as described by Vogelstein and Gillespie (23). The reaction mixture contained 10µl labeled RNA (0.3µg, specific activity 1.4 x 10^6 cpm/µg), 25µl of DNA (12.5µg), 25µl of 20 X SSC and 50µl formamide. The reaction mixture was incubated at 55°C for 4h. After hybridization, the reaction mixture was transferred to a test tube containing 1ml of 1 X SSC and 25 µg of RNAase A. After 1h at 50°C, the acid-precipitable radioactivity in the aliquot relative to total counts (without RNAase) was measured by a gamma counter and used to calculate the percent of hybridized RNA. Acid-precipitability at zero time was less than 2%.

DNA DIGESTION WITH RESTRICTION ENDONUCLEASES, SOUTHERN TRANSFER AND HYBRIDIZATION WITH ^{125}I LABELED rRNA PROBE:

Nuclear DNA of the anther-donor plant and an anther-derived monoploid was digested with various restriction endonucleases: EcoRI, HindIII, HincII, AluI, TaqI (BRL) under the appropriate enzyme conditions. DNA fragments were separated on a 0.7% agarose gel using lambda phage DNA digested by HindIII as the fragment size marker. The fragments were blotted on a nitrocellulose membrane as described by Southern (24). The hybridization was carried out in 5 X SSC with 55% formamide. The specific activity of the rRNA probe was 1.4 x 106 cpm/µg.

RESULTS

CHARACTERIZATION OF THE NUCLEAR GENOME OF *Solanum phureja*:

The modified DNA isolation yielded 50 µg DNA/gram fresh weight of leaf tissue. Thermal denaturation profiles indicated that the purity of the DNA samples after CsCl centrifugation was 85-90%. The thermal melting point for *Solanum phureja* was 85.1°C (std = 0.26, n = 6), which corresponds to a G-C content of 38.1% (std = 0.9, n = 6). There were no significant differences between the melting points or G-C ratios of the anther-derived plants and the source plant.

The reassociation reaction of *Solanum phureja* nuclear DNA in 6 X SSC at 60°C up to an equivalent cot of 3.5 (corrected for the salt concentration) as determined by the hydroxyapatite method is demonstrated in Fig. 1. As determined from the cot plot, there are two shifts in the rate of the reaction, one at lecot (log equivalent cot) value of 0.5 and the other at lecot value of 1.75 indicating changes in the reiteration numbers of sequences.

OPTICAL REASSOCIATION OF ANTHER-DERIVED AND THE ANTHER-DONOR PLANTS:

The cot plots of the early part of the reassociation reaction of the anther-derived monoploid and diploid plants as

well as the source plant are presented in Fig. 2. After applying the calculations described by Wetmur and Davidson (22), the reassociation reaction of the monoploid DNA was found to proceed at a faster rate (k = 1.8 liters/mole/sec) than that of the diploids (k = 1.44 liters/mole/sec) up to a cot value of 0.15, after which the reaction proceeded obeying second order kinetics, but at half reduced rate. The reduction in the rate of the reaction was proportionally greater for the monoploid than for the diploids (k = 0.46 and k = 0.52 liters/mole/sec, respectively). When HA technique was used for detection of reassociation, sensitivity was insufficient to reveal differences in the rate of the reaction among anther-derived plants and the source plant.

RNA-DNA HYBRIDIZATION IN FREE SOLUTION:

^{125}I-labeled rRNA consisting of 18s and 25s components was incubated independently with DNA from the source plant and the anther-derived monoploid and diploid in the presence of formamide to destabilize the DNA duplexes. These reaction conditions kept the RNA-DNA hybrids stable, and thus made the extent of hybridization quantifiable (23). Under these reaction conditions, approximately 8% of the labeled rRNA annealed to the DNA of the diploid source plant (Table 1). There was a significant increase in the amount of rRNA-DNA hybridization for the monoploid compared to the anther-donor plant with the monoploid having 30% more rRNA-DNA hybrids.

The anther-derived diploid showed a decrease of approximately 15% of rRNA-DNA hybridization in both experiments; however, this decrease was only significant in the second experiment.

ANALYSIS OF NUCLEAR DNA WITH rRNA PROBES:

All of the restriction endonucleases (EcoRI, HindIII, HincII, AluI, TaqI) cut the DNA into bands containing repeated sequences varying in copy number. Probing of these fragments with rRNA probe revealed both banding pattern as well as binding intensity changes in the DNA of the monoploid compared to the diploid anther-donor plant (Fig. 3). In the EcoRI digest, there was an intense light band (0.59 kb) for the source-plant which was missing for the monoploid. In addition, fragments 4.90 and 3.35 kb had different relative intensities in the two digests. In the HindIII digest, there is a large fragment showing intense binding with the probe for both of the DNAs; however, the bands corresponding to 7.71, 1.16 and 0.54 kb present for the diploid source plant were not apparent for the monoploid. The HincII digest showed clear differences in the banding patterns of the two DNAs; the monoploid DNA digest contained four bands (12.59, 7.71, 6.37, 4.92 kb) which were not present in the digest of the source plant. AluI cut the DNA into four light fragments, which hybridized with the rRNA probe; however, there were no differences between the banding patterns of the DNA digests of the two plants.

DISCUSSION

The Tm (85.1°C) and the G+C content (38.1%) of Solanum phureja determined from the optical melting profile have not been previously reported. The reassociation kinetics indicate that 60% of the DNA of Solanum phureja consists of repetitive DNA, which is consistent with the proportion of repetitive DNA found in other higher plants of similar 2C DNA content (25). The nuclear DNA content of S. phureja is 2.4 picograms (Veilleux et al., unpublished results).

Analysis of the early part of the reassociation reaction revealed a higher reassociation rate constant for the monoploid than for the diploid. Amplification of highly repetitive sequences reassociating before a cot value of 0.15 is therefore assumed for the monoploid. This is in agreement with the findings of DePaepe et al. (12) for doubled haploids of Nicotiana. The amplification in the highly repetitive component has lead to a relatively lower concentration of the other components and underlies the observed reduction in reassociation reaction of the monoploid DNA at higher cot values.

An increase of 30% rRNA-DNA hybridization for the monoploid as compared to the source plant and the anther-derived diploid demonstrated amplification of rRNA genes in

the monoploid. The Southern transfer analysis revealed both copy number variation between the monoploid and the diploid source plant as well as variation for restriction endonuclease cleavage sites. Hence, only some of the sequences of the rRNA genes had been amplified and sequence rearrangements had taken place during the process of anther-culture. To study the extent of this variation and its possible significance to phenotypic variation observed among the anther-derived lines, we are presently conducting similar experiments with a higher number of anther-derived plants.

The increase in rDNA sequences can take place during differentiation of the nucleus of the pollen grain or during the androgenetic process. Of the two anther-derived plants of Solanum phureja studied, only the monoploid displayed an increase in nuclear DNA content and in rDNA sequences. We have previously provided genetic evidence for origin of the anther-derived diploid by androgenesis of a 2n microspore (18). This leads to two speculations: if the observed amplification of rDNA sequences of the anther-derived monoploid plant resulted from replication of DNA in the vegetative nucleus, as suggested by Dhillon et al. (11) and DePaepe et al. (12), then such replication only occurs in a reduced (haploid) microspore; a diploid vegetative nucleus of an unreduced microspore would not undergo differential DNA replication. Alternatively, the monoploid DNA polymerase might

be more active during androgenesis or subsequent culture, perhaps by greater sensitivity to exogenous growth regulators, which have been shown to affect gene expression (13).

A specific gene has been reported in the genome of Drosophila melanogaster (14) which when present without its complement has lead to disproportionate replication of rDNA. Alternatively, amplification could have occurred during the embryogenic development of the anther-derived plants of both ploidy levels as a response to a temporary need for rRNA; however, only the diploids would have subsequently undergone selective underreplication to eliminate these sequences. This view is in agreement with Masuda et al. (27), who found an increase in rRNA at an early phase of carrot somatic embryogenesis; however, the extra rRNA, which was eliminated two weeks after embryoid initiation, was not attributed to increased rDNA sequences.

Besides amplification of rDNA sequences, the anther-derived monoploid demonstrated sequence rearrangements as indicated by the Southern transfer and hybridization experiment. This is rather surprising, because rDNA sequences are thought to be highly conserved within species (28). Some variation has been shown to occur among inbred lines of maize with respect to copy number and restriction endonuclease cleavage sites (29).

Amplification of rDNA and sequence rearrangements may play a role in the phenotypic variation observed among plants derived from tissue culture. The copy number of rDNA sequences can alter the amount of rRNA and thus the availability of sites for translation, or more directly, rDNA may contain enhancer sequences, the number of which can affect the expression of genes under their control. Both of these mechanisms have been reported in animal systems (15, 30).

In conclusion, this study has shown that the process of anther-culture can result in amplification and rearrangement of rDNA sequences. Such changes may have a role in the phenotypic variation observed among the anther-derived monoploids. The fact that the changes have been shown to occur at the DNA level indicates that the phenotypic variation is heritable and not only due to epigenetic differences. However, the dynamics of the plant genome are as yet poorly understood and caution should be exercised when attributing phenotypic differences to undetermined genetic events.

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TABLE 1. Binding of 125I labeled rRNA to DNA of anther-derived monoploid and diploid plant and to the diploid anther-donor plant

	Source plant		Anther-derived						
			Monoploid		Diploid				
	counts	binding*	counts	binding	counts	binding	Background counts (%)	Total counts	CV %
EXP I	11140a**	2.09	14651b	2.74	9664a	1.80	1.6	128388	11.4
LSD 5%	2151								
EXP II	9932a	1.86	12480b	2.33	8979c	1.68	1.6	128403	3.9
LSD 5%	654								

binding*= pg of RNA bound/mg of DNA
ANOVA significant in both experiments, mean separation by LSD
a**= means with the same letter are not significantly different
counts have been corrected for background
counting efficiency 70%

Fig. 1. Reassociation kinetics of Solanum phureja DNA. The fraction of DNA fragments containing duplex regions was assayed by hydroxylapatite chromatography at Tm-25°C in 6 X SSC.

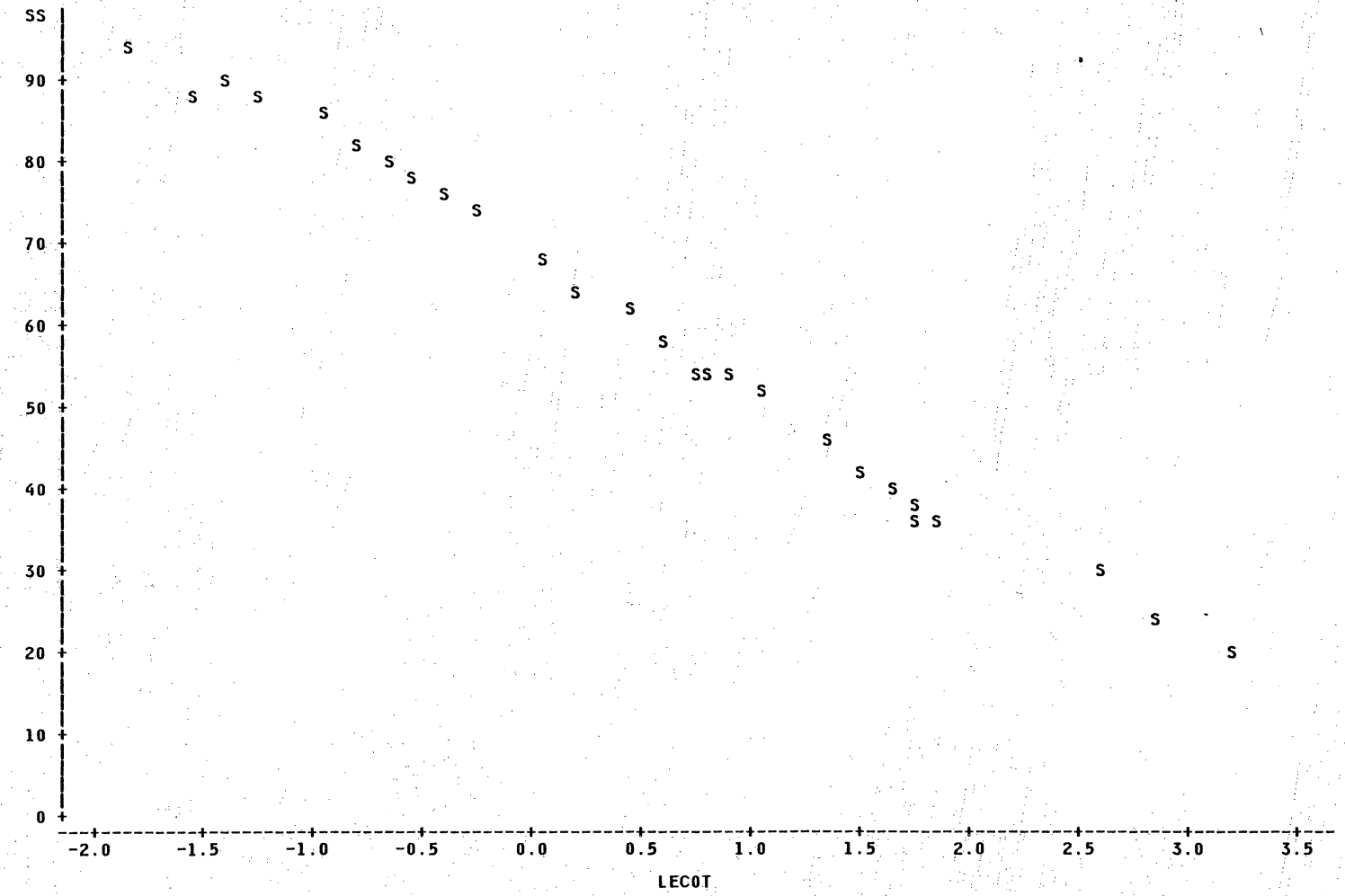

SS
90
80
70
60
50
40
30
20
10
0
-2.0
-1.5
-1.0
-0.5
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
LECOT

Fig. 2. Wetmur and Davidson plots of the early part of the reassociation reaction of the anther-derived monoploid and diploid plant. E. coli used as an internal standard.

REASSOCIATION KINETICS

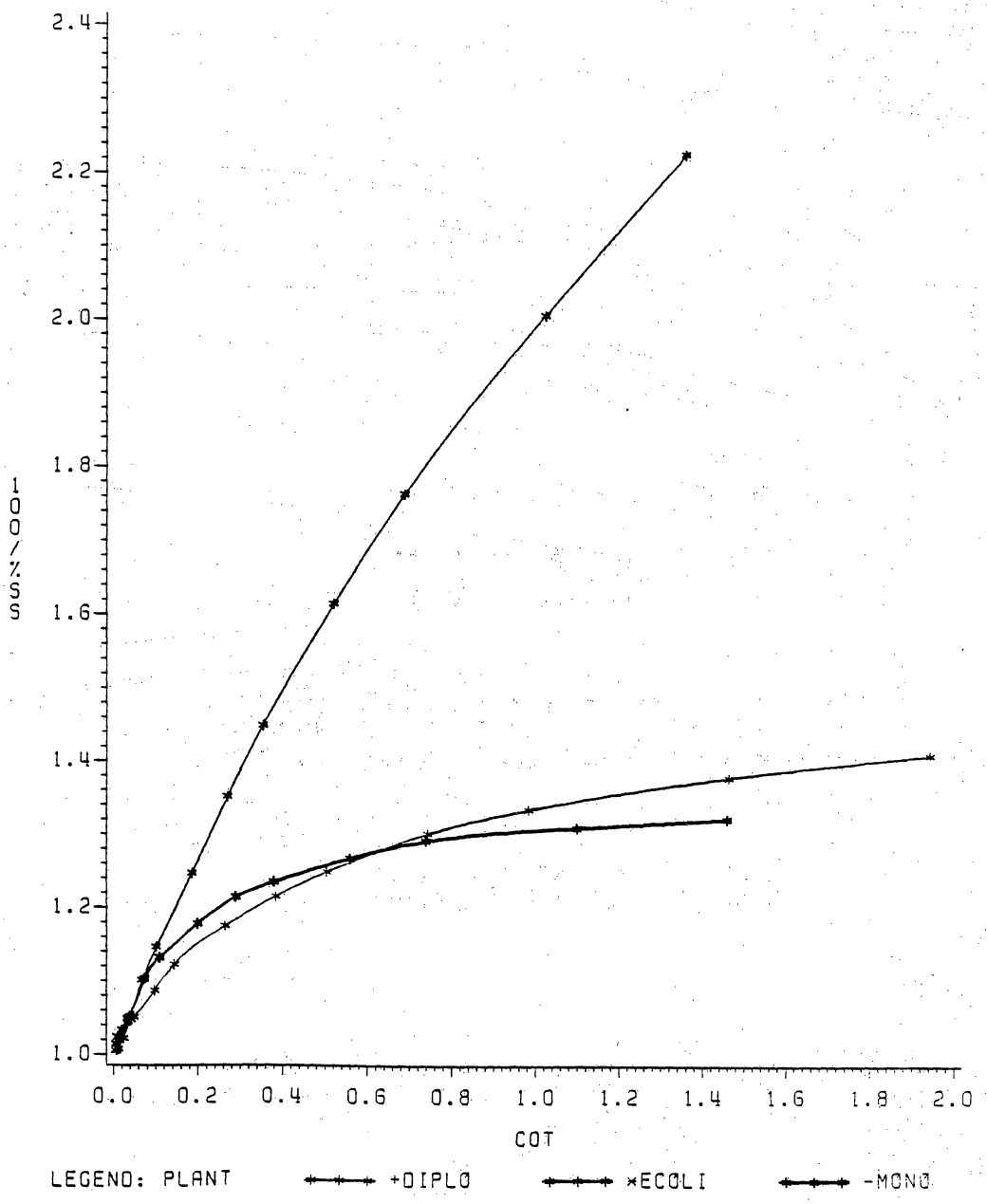
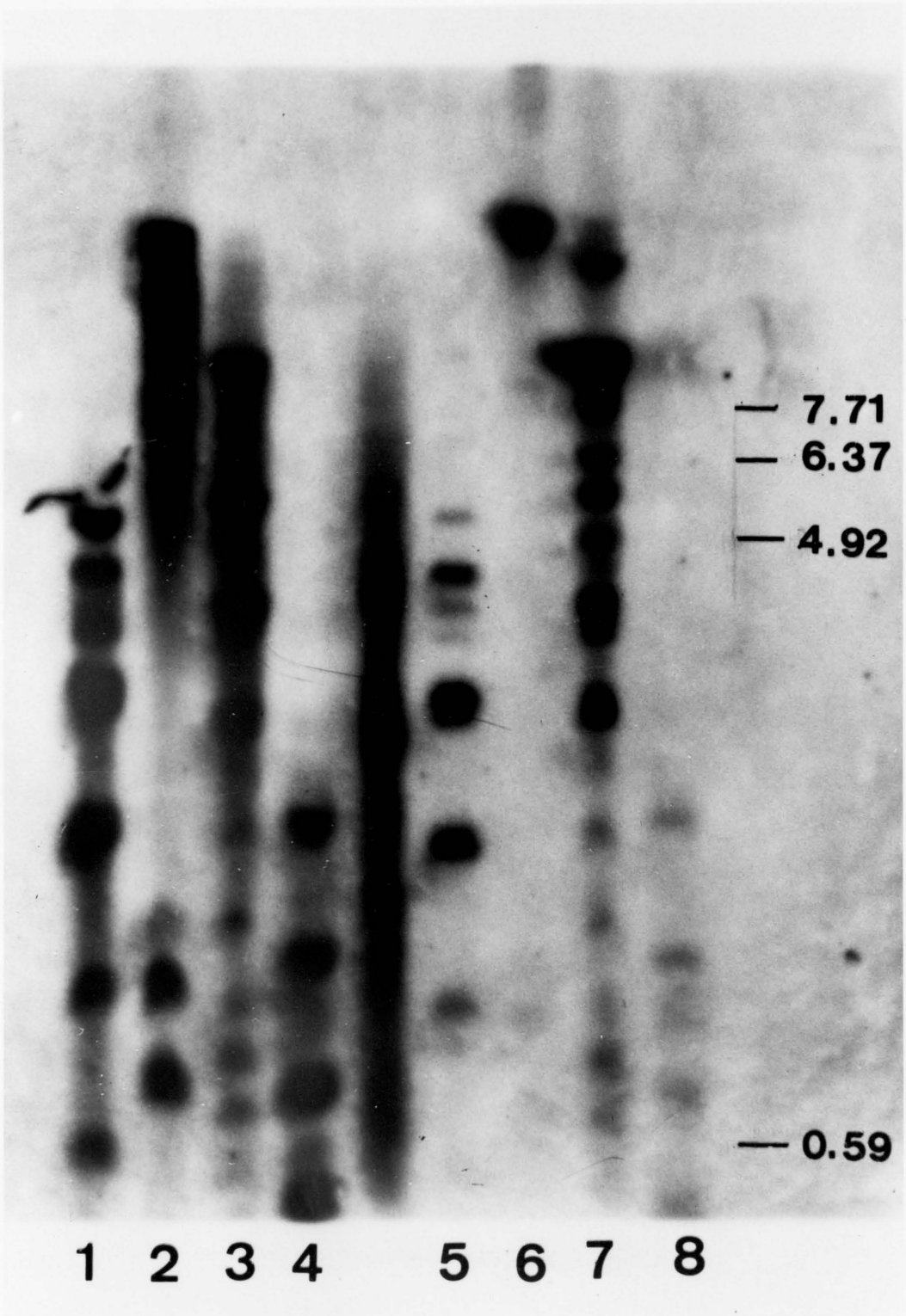


Fig. 3. Southern transfer analysis of the nuclear genome of an anther-derived monoploid and the anther-donor plant using pooled 18s-25s rRNA as the probe. Lanes 1-4, anther-donor plant DNA restricted by EcoRI, HindIII, HincII and AluI, respectively; lanes 5-8 the anther-derived monoploid DNA restricted by the same enzymes.


7.71
6.37
4.92
0.59
1 2 3 4 5 6 7 8

CHAPTER 5

SUMMARY AND CONCLUSIONS

The progress of potato breeding by conventional methods has been limited mainly by two factors; first, the narrow genetic base of the Solanum tuberosum germplasm pool in the northern climates, and second the difficulties encountered in controlling the incorporation of specific traits when crossing tetraploid cultivars of S. tuberosum. Our breeding scheme represents an attempt to tackle both of these problems by introducing wild germplasm to S. tuberosum cultivars through 4x X 2x crosses and by exploiting the variation resulting from in vitro phases included within the scheme.

The first two studies of this investigation demonstrated that the process of anther-culture has resulted in phenotypic variation regarding morphology and physiology of the anther-derived plants. Advances of 20-30% in net photosynthesis and its biochemical component characteristics among the anther-derived plants compared to the anther-donor plant were observed. This is an important contribution to the field of physiological plant breeding, where response to selection with respect to

net photosynthesis has been very limited (Gifford et al. 1984). Furthermore, there have been few reports on the impact of anther-culture on physiological traits of anther-derived plants (Bhaskaran et al. 1983; Medrano and Primo-Millo 1985). The observed improvement in net photosynthesis may be of particular importance with respect to potato breeding, because previous reports have shown an association between tuber yield and net photosynthesis in this crop (Moll and Henniger 1978; Dwelle et al. 1981).

For the variation derived by in vitro methods to be useful in subsequent breeding efforts, the characteristics must be stable, i.e., due to changes in the heritable material rather than epigenetic phenomena. The third study in this investigation showed that the process of anther-culture resulted in amplification of repetitive DNA sequences in an anther-derived monoploid. RNA-DNA hybridization revealed a 30% increase in the rDNA sequences as well as variation for restriction endonuclease cleavage sites in an anther-derived monoploid. Our results regarding the amplification of repetitive sequences confirm the findings of Dhillon et al. (1983) and DePaepe et al. (1983). However, the fact that the increase in these sequences was only observed in the anther-derived monoploid but not in an anther-

derived diploid is a novel finding which sheds some light on the process of anther-culture itself, i.e., if differentiation of the vegetative nucleus of the microspore, the source of cell division resulting in androgenesis, includes differential DNA amplification, then this process occurs only in haploid and not in diploid microspore. Furthermore, the increase in rDNA sequences as well as variation in restriction endonuclease cleavage sites as a result of anther-culture has been reported here for the first time. The occurrence of sequence rearrangements and differential replication is likely to underlie phenotypic variation observed among the anther-derived plants.

In conclusion, convincing evidence for the occurrence of gametoclonal variation for both the phenotype and genotypic composition of anther-derived plants of S. phureja has been provided in this investigation.

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