PROTOPLAST FUSION FOR THE PRODUCTION OF INTERMONOPLOID SOMATIC HYBRIDS IN CULTIVATED POTATO

by
Alexander Arthur Theodore Johnson

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Richard E. Veilleux, Chairman
Eric P. Beers
Glenn R. Buss

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Alexander A. T. Johnson

Abstract

Monoploid potato genotypes represent plant material that is free from the "genetic load" of lethal and severely deleterious alleles normally present in the highly heterozygous cultivated potato species. Field evaluations enabled the identification of agronomically superior monoploid potato genotypes from a population of more than 100 anther-derived monoploids. Chemical fusion and electrofusion between pairs selected from 31 superior monoploids resulted in the production of three different groups of intermonoploid somatic hybrids.

The hybridity of somatic hybrid plants and calluses was confirmed through PCR-based amplification of simple sequence repeat (SSR) sequences in the potato genome. Polymorphic SSR loci between the monoploid parents of a particular group of somatic hybrids were used to separate true somatic hybrids (heterozygous at the loci) from parental somaclones regenerating from unfused protoplasts (homozygous for one parental band at the loci).

One group of somatic hybrids (SH1, SH2 and SH2B) was of particular interest because it resulted from the fusion of a *S. phureja* monoploid to a high acetylleptinidine-producing monoploid derived from an F_1 hybrid between *S. chacoense* and *S. phureja*. The leptine acetylleptinidine (ALD) is produced only by some accessions of *S. chacoense* Bitt. and provides resistance to feeding by the Colorado potato beetle (*Leptinotarsa decemlineata* Say) when present in sufficient concentrations. The somatic hybrids produced moderate levels of ALD in leaves and stems (roughly 60% that of a high ALD-producing *S. chacoense* clone).

Pollinations of SH1, SH2 and SH2B by several diploid and tetraploid potato clones resulted in three fruit on SH2, one fruit on SH2B and no fruit on SH1. Two resulting progeny populations of SH2 [SH2A = SH2 × *S. andigena* 8-1 (4*x*); SH2P = SH2 × *S. phureja* 66AP11-53 (2*x*)] expressed higher fertility than the original somatic hybrids and were sexually crossed as both male and female parents to *S. tuberosum* cv. Atlantic. All of the SH2 progeny populations expressed acetylleptinidines, albeit at lower levels than the SH2 somatic hybrid, providing strong evidence that the genes controlling acetylleptinidine production are dominant. Variation for ALD expression in the SH2 progeny indicated one or a few genes with additive effect controlling the ALD trait. In addition, the choice of male parent in sexual crosses to SH2 affected subsequent ALD expression in progeny populations. The SH2 progeny represent an important first step towards transferring acetylleptinidines to cultivated potato.

SH1, SH2 and SH2B appeared to be negatively affected by an unusually high ploidy (hexaploid, 6*x*). Field-grown plants produced many tubers (mean = 35) of low weight (mean = 10.4 g) and were stunted in appearance. Anther culture of SH2 yielded triploid regenerants (3*x*). These regenerants may be more phenotypically normal than the original somatic hybrids because of lower ploidy. Segregation of SSR alleles in the triploid anther culture regenerants provided evidence that the hexaploid somatic hybrid SH2 genome is comprised of four homologous genomes of CP2-103 (the high leptine-producing monoploid) and two homologous genomes of 13-14 203 (the *S. phureja* monoploid).

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Literature Review

General Background

The potato (*Solanum tuberosum* L.) is an important staple food crop that is currently grown world-wide in most temperate, subtropical and tropical climates. The potato produces nearly twice the amount of calories per hectare as rice or wheat (Poehlman and Sleper, 1995) and the tubers possess high quantities of starch (15 to 25% of total mass), vitamin C and amino acids. Exceeded only by rice, wheat and corn in terms of total food production, potato is the most important non-cereal crop in the world. In addition, potato produces more energy and protein per unit land than any other single food crop (Bajaj 1987).

Traditional breeding efforts have focused primarily on improving the potato for disease resistance; however, commercial potato cultivars are autotetraploid ($2n = 4x = 48$) and breeding at this level is quite problematic. Tetraploidy, combined with inbreeding depression, makes it difficult and time-consuming to select for desirable characteristics after a sexual cross and to fix specific heterozygous gene combinations.

In addition to complicating breeding, the tetraploid nature of potato enables the genome to tolerate many recessive, lethal and deleterious alleles. Consequently, the majority of seedlings resulting from a cross between two superior potato cultivars often exhibit poor or undesirable growth compared to either parent due to random assortment of these unfavorable alleles among the progeny. In fact, roughly one million seedlings need to be screened by potato breeders before one is found worthy to be developed as a cultivar (Plaisted et al. 1994).

Much research involving potato has utilized clones at the diploid level, in order to simplify genetic segregation ratios. The ploidy of potato, as well as many other crops, can be reduced through anther culture (androgenesis) or through interspecific hybridization

(gynogenesis). Haploid is the name often given to a plant containing half the chromosome number (or gametic number) of the mother plant. Haploid is a suitable term when discussing diploid crops such as corn, however, haploid is too general when discussing a tetraploid crop such as potato. Dihaploid is the name given to a plant arising from androgenic or gynogenic chromosomal reduction of a tetraploid potato clone. Dihaploids are heterozygous diploids (containing the gametic chromosome number of a species, $2n = 2x = 24$) usually with limited fertility. Plants arising from androgenic or gynogenic chromosomal reduction of a dihaploid are referred to as monoploids or monohaploids. Monoploids contain only one chromosome of each chromosomal pair (the basic chromosome number of a species, $2n = 1x = 12$) and are therefore small, sterile plants. Through chromosomal doubling of monoploids, completely homozygous, somewhat fertile, diploid plants can be obtained--referred to as doubled haploids or doubled monoploids.

In contrast to tetraploids and dihaploids, monoploid potato clones are free of lethal alleles. As a genotype passes from the diploid to monoploid level, lethal genes are exposed and genotypes bearing them are unable to regenerate into functional plants—sometimes referred to as the "monoploid sieve" for lethal allele removal (Veilleux et al. 1995). By selecting for the most vigorous monoploid clones, those genotypes expressing the fewest deleterious alleles can be identified. These superior genotypes which pass through the monoploid sieve could theoretically function as inbred lines in a potato breeding program after chromosome doubling (either through colchicine treatment or leaf disk regeneration) and subsequent restoration of fertility in the doubled haploids. One could expect sexual crosses between these homozygous diploids to result in heterozygous F_1 progeny displaying a high degree of hybrid vigor. Unfortunately, the theoretical advantages of this breeding scheme have not been realized due to the presence of male sterility in currently available doubled monoploids of potato. Uijtewaal et al. (1987b) detected limited female fertility in only one of seven doubled monoploid potato clones; there was complete male sterility in all doubled monoploid clones.

Protoplast fusion, in which somatic, rather than gametic, cells are induced to fuse for the production of hybrid plants, is the only method currently available to combine two superior "monoploid sieved genomes" leading to the production of a heterozygous potato plant, and the development of such somatic hybrids has been the focus of this research.

Protoplast Fusion in Potato

Protoplast fusion has many uses in potato research (Table 1); however, this technique has been utilized most commonly to create hybrids between potato species that cannot be crossed sexually because of existing crossability barriers such as differences in the endosperm balance number (EBN) (Johnston et al. 1980). A second application has been to combine two different selected dihaploid potato clones to attempt to realize hybrid vigor in the resulting somatic hybrids, expected to be tetraploid.

Many *Solanum* species, both tuber-bearing and non-tuber-bearing, have been assigned EBNs (Hanneman 1994) based on their crossing behavior with known EBN standards (Ortiz and Ehlenfeldt, 1992). A species can be assigned a 1EBN, 2EBN, or 4EBN based on its crossing behavior, and frequently the EBN number does not correspond to the ploidy of the plant. In fact, the success of a cross between two *Solanum* species depends much more on the two involved species having the same EBN, rather than the same ploidy level. Generally if two species are of the same EBN, a successful sexual cross can be obtained.

The Endosperm Balance Number Theory (Johnston et al. 1980) states that viable endosperm (and thus a viable embryo) will result only from crosses where a 2:1 maternal to paternal EBN ratio is observed. In a hypothetical fertilization between two diploid *Solanum* species both of 2EBN, a 2*x* embryo is formed by the union of 1*x* gametes from each parent, while the 3*x* endosperm is formed by fertilization of the 2*x* maternal central cell with a 1*x* paternal gamete. The cross described above would be successful because a 2:1 maternal to paternal EBN ratio would be achieved through fusion of a 2EBN central cell with a 1EBN sperm cell. In a hypothetical cross of 2 diploid species with a 1EBN

maternal parent and a 2EBN paternal parent, the embryo would abort because a 1:1 maternal to paternal EBN ratio would result from fusion of a 1EBN maternal central cell with a 1EBN sperm cell.

Potato responds well to tissue culture and regeneration of plants from isolated protoplasts is possible with many clones--thus protoplast fusion is an ideal technique to utilize with this species. For protoplast fusion, mesophyll protoplasts are isolated from leaf tissue and purified following treatment of the leaf tissue with cellulases and pectinases to dissolve the rigid cell wall surrounding protoplasts in plant tissue. After this step, one of two methods of protoplast fusion can be used: chemical fusion or electrofusion.

In chemical fusion, the protoplasts to be fused are mixed together in a fusogenic agent (usually polyethylene glycol), which agglutinates the protoplasts and brings them into close physical contact. The protoplasts are subsequently centrifuged or washed with a high pH buffer to induce fusion of the agglutinated protoplasts. Unfortunately, chemical fusion often results in low fusion frequencies because the chemicals involved in this process can have a toxic effect on the protoplasts (Fish et al. 1988a).

Electrofusion is a non-toxic technique that minimizes damage of the target protoplasts and thereby often results in significantly higher fusion frequencies than obtained by chemical fusion. Isolated protoplasts are placed into a fusion chamber and aligned into close physical contact (often referred to as “pearl chains”) by application of a low intensity, high frequency, alternating electric current--a process known as dielectrophoresis. The alternating electric field also causes pores to form on the protoplast membrane due to a large electric potential at the two poles of the protoplast (Chang et al. 1992). Before these pores are able to “reseal,” the protoplasts are induced to fuse through application of anywhere from one to several short direct current square pulses.

Intergeneric and Interspecific Protoplast Fusion

Protoplast fusion has been used most extensively in potato for interspecific fusions where EBN differences make sexual crosses impossible (InterEBN crosses) and intergeneric fusions, generally with the aim to transfer traits of agronomic interest from distant relatives of potato to the cultivated potato gene pool. Other methods of breeding between species, such as using dihaploid *S. tuberosum* in sexual crosses to wild diploid species, or crossing wild diploids with unreduced gametes to tetraploid *S. tuberosum*, are not always possible due to EBN differences and, when possible, are inferior to protoplast fusion in that the total heterozygosity of the parents is not maintained (due to meiotic recombination and segregation).

Somatic hybrids are expected to carry many undesirable traits from the unadapted species and/or somaclonal variation induced by the tissue culture regeneration phase, and for this reason cannot be released directly as cultivars. Therefore, fertility of somatic hybrids is essential so they may be used in a potato breeding program to produce new cultivars. Potato somatic hybrids produced by fusion of two different species or genera generally have greatly reduced male-fertility or complete male-sterility; however, female-fertility in many cases remains. Incompatibility between foreign nuclei and cytoplasm often accounts for the reduction in male-fertility.

Some fusion studies have focused primarily on producing somatic hybrids between two different species or genera without focusing on the transfer of any specific trait between the two, such as the fusion of potato (*S. tuberosum*) and eggplant (*S. melongena* L.) (Li and Constabel, 1988). The idea here is to use somatic hybrids as starting material for many different potato breeding programs.

Such was also the objective in fusing potato (*S. tuberosum*) and tomato (*Lycopersicon esculentum* Mill.) (Jacobsen et al. 1992). The formation of bivalents in meiosis of resulting somatic hybrids indicated that the somatic hybrids behaved as allotetraploids; however, both male and female fertility were nearly absent and only seven fruits

containing non-viable seeds were obtained after more than 1000 pollinations of the somatic hybrids with both potato and tomato pollen. Further studies of these potato-tomato somatic hybrids (Jacobsen et al. 1994) revealed that hexaploid hybrids were more fertile than tetraploid hybrids when crossed to several genotypes of potato. The cross of a hexaploid somatic hybrid to tetraploid potato with subsequent ovule culture resulted in a female-fertile pentaploid seedling (BC_1). By analyzing progeny derived from crosses of this pentaploid seedling with tetraploid potato (BC_2), Jacobsen et al. (1995) concluded that the BC_1 plant contained only nine of the expected 12 tomato chromosomes. Therefore, elimination of tomato chromosomes occurred in the backcross generation derived from the original somatic hybrid. Such elimination could be useful for the creation of tomato addition lines (Garriga-Calderé et al. 1998). Potato has also been successfully fused with *Lycopersicon pimpinellifolium* (Jusl.) Mill. (Okamura 1988), a tomato relative displaying high resistance to bacterial wilt, late blight and an ability to thrive under high temperatures. The somatic hybrids obtained from this fusion grew vigorously, flowered in the field, and were backcrossed to *S. tuberosum*.

Intergeneric somatic hybrids have been produced through fusion of *S. tuberosum* and *Lycopersicon pennellii* (Corr.) D'Arcy protoplasts, with the objective of transferring saline tolerance from *L. pennellii* to common potato (Sherraf et al. 1994). The somatic hybrids showed increased saline tolerance; however, they were completely male-sterile. Female fertility was not investigated.

Mixoploid somatic hybrids were obtained through fusion of dihaploid *S. tuberosum* (2EBN) and *S. pinnatisectum* Dun. (1EBN) protoplasts (Ward et al. 1994). The heterokaryons showed heterosis and, unlike homokaryons and unfused protoplasts, were able to develop into calluses and regenerate shoots. The somatic hybrids grew more vigorously than the parental fusion partners when acclimated to the greenhouse.

Several wild potato species demonstrate high, if not total, resistance to many common pests that plague the cultivated potato. *S. tuberosum* (tetraploid, 4EBN) has been fused with the wild diploid species *S. bulbocastanum* Dun. (1EBN) in order to transfer

nematode resistance (race 1 of *Meloidogyne chitwoodi* Golden et. al.) to common potato (Austin et al. 1993). The somatic hybrids were female fertile, thereby showing potential in a potato nematode resistance breeding program, and also displayed levels of nematode resistance equal to that of the *S. bulbocastanum* fusion partner.

Cheng et al. (1995) used protoplast fusion to introduce rare glycolalkaloids, that are effective in resistance against insect herbivores such as the Colorado potato beetle (*Leptinotarsa decemlineata* Say), into cultivated potato through somatic hybridization between a dihaploid *S. tuberosum* clone (2EBN) and the wild relative, *S. chacoense* Bitt. (2EBN).

Resistance to both *Phytophthora infestans* (Mont.) de Bary and *Globodera pallida* (Stone) Behrens (pathotypes Pa2 and Pa3) was transferred to three of four tetraploid somatic hybrids produced between fusions of dihaploid *S. tuberosum* (2EBN) and the wild relative, *S. circaeifolium* subsp. *circaeifolium* Bitter (1EBN) (Mattheij et al. 1992). Female fertility was observed through successful crosses of the tetraploid somatic hybrids to tetraploid and diploid *S. tuberosum*. This particular interspecific combination has been done sexually (Louwes et al. 1992); however, only diploid and triploid progeny were obtained. The sexual progeny were somewhat fertile and have been backcrossed to diploid *S. tuberosum*.

Diploid breeding lines of *S. tuberosum* (2EBN) were fused to *S. pinnatisectum* Dun. (1EBN) with hopes of expressing resistance to *Phytophthora infestans* and *Erwinia* blackleg in the somatic hybrids (Menke et al. 1996). The somatic hybrids inherited complete genomes from both parents; however, fertility and disease resistance were not evaluated.

Somatic hybrids with tubers resistant to bacterial soft rot and three different species of *Erwinia* were produced through fusion of diploid *S. brevidens* Phil. (1EBN) with tetraploid *S. tuberosum* (4EBN) (Austin et al. 1988). Sexual crosses between these two species are rarely successful. The resistant traits of the somatic hybrids were transferred

to some progeny when the somatic hybrids were sexually crossed to 'Katahdin.' This case is interesting because resistance to two different pathogens affecting tubers were apparently transmitted from *S. brevidens* to *S. tuberosum*, although *S. brevidens* does not produce tubers.

Transfer of viral resistance has also served as motivation for protoplast fusion. Potato virus Y (PVY) can cause yield reductions of up to 80% in potato. In a fusion between the wild species *S. etuberosum* (1EBN) and a *S. tuberosum* × *S. berthaultii* hybrid, somatic hybrids were produced displaying increased levels of viral resistance compared to common potato cultivars; however, the resistance was not as high as that of the *S. etuberosum* parent (Novy and Helgeson 1994a). These hybrids were fertile and some of their progeny resulting from crosses to common potato were as resistant as the somatic hybrids to PVY. When the authors attempted to fuse *S. etuberosum* with a dihaploid *S. tuberosum* clone (2EBN), instead of the *S. tuberosum* × *S. berthaultii* hybrid, only weak somatic hybrids resulted (Novy and Helgeson 1994b). Apparently, in this instance the haploid × wild species hybrid was a better fusion partner with *S. etuberosum* than haploid *S. tuberosum*.

Potato leaf roll virus (PLRV) is another damaging virus affecting potato. Austin et al. (1986) produced a fertile, hexaploid somatic hybrid by fusing tetraploid *S. tuberosum* (4EBN) with diploid *S. brevidens* (1EBN). The majority of the hybrids possessed PLRV resistance from the *S. brevidens* parent and *Phytophthora infestans* (Race 0) resistance from the *S. tuberosum* parent. By fusing a dihaploid *S. tuberosum* clone with *S. brevidens*, Austin et al. (1985a) produced ten tetraploid somatic hybrids of which nine showed resistance to PLRV. Fish and Jones (1988) produced somatic hybrids between a dihaploid *S. tuberosum* clone and *S. brevidens*, of which the majority were extremely resistant to PLRV. By fusion of dihaploid *S. tuberosum* and diploid *S. brevidens* clones, tetraploid and hexaploid, female-fertile somatic hybrids were obtained displaying PLRV resistance (Fish et al. 1988b).

Williams et. al (1990) produced a fertile, hexaploid somatic hybrid between tetraploid *S. tuberosum* and diploid *S. brevidens* that has been crossed sexually to the potato cultivar 'Katahdin.' The progeny from this cross were pentaploid and fertile. RFLP analysis of the progeny revealed a slight bias toward intra-genomic pairing in the hexaploid somatic hybrid; however, inter-genomic pairing also occurred. Inter-genomic pairing facilitates transfer of desirable traits, such as PLRV resistance, from *S. brevidens* to *S. tuberosum* through chromosomal recombination.

Physiological traits have been transferred between potato and certain wild relatives using protoplast fusion technology. Protoplast fusion of dihaploid *S. tuberosum* (2EBN) with *S. commersonii* Dunal (1EBN) resulted in a female- and male-fertile, tetraploid somatic hybrid displaying increased levels of frost tolerance (Cardi et al. 1993). Similarly, Nyman and Waara (1997) produced tetraploid somatic hybrids between dihaploid *S. tuberosum* and *S. commersonii* that displayed higher capacities of non-acclimated frost tolerance and a certain degree of cold acclimation compared to the *S. tuberosum* fusion parent. Some of the somatic hybrids were both male- and female-fertile.

Herbicide resistance of certain wild potato species has been transferred to common potato using protoplast fusion. Fusion of *S. tuberosum* with an atrazine-resistant biotype of *S. nigrum* L. resulted in somatic hybrids that displayed atrazine resistance (Binding et al. 1982). Atrazine resistance could serve as an important agronomic trait as well as a selectable marker during somatic hybrid regeneration through tissue culture.

Asymmetric Fusion

Until now only symmetric somatic hybrids have been discussed, or those cases where two complete genomes from both fusion partners combine in order to form a somatic hybrid. However, symmetric fusions are not always obtained using protoplast fusion. There may be partial elimination of one or both of the fusion partner's chromosomes leading to asymmetric hybrids (aneuploids and addition lines) or even cybrid formation (entire deletion of one fusion partner's chromosomal complement, but not cytoplasm). The

retention of one fusion parent's genome preferentially over that of the other fusion partner can occur spontaneously during the development of somatic hybrids (unintentional) or it can be induced (by irradiation of one of the donor protoplasts just prior to protoplast fusion, or through fusion of microprotoplasts containing only a few chromosomes of one of the donors). It is believed that spontaneous chromosomal elimination of one of the donors occurs when there is a difference in cell cycle between the two donors. In some cases the donor with the slower cell cycle is preferentially eliminated. In other cases there is evidence for genetic control of chromosomal elimination (Oberwalder et al. 1997).

Asymmetric hybrids show potential for the transfer of multi-genic traits carried on one or several chromosomes, a feat that is extremely difficult using current genetic transformation technology. By transferring only those chromosomes or chromosomal fragments carrying the genes encoding the trait of interest, an asymmetric hybrid could theoretically be obtained expressing the desired multi-genic trait with a minimum of unwanted traits from the unadapted parent.

Elimination of nucleolar chromosomes was observed in somatic hybrids obtained from protoplast fusion of *S. phureja* Juz. & Buk. and *S. tuberosum* (Pijnacker et al. 1987). The authors believed in this case that the elimination was under genetic control, due to the fact that the *S. phureja* nucleolar chromosomes were eliminated most extensively in hexaploid somatic hybrids that contained four parts *S. phureja* and two parts *S. tuberosum* genomes, not vice versa.

Protoplast fusion between diploid potato and *Nicotiana plumbaginifolia* Viviani (Gilissen et al. 1992) resulted in three types of somatic hybrid calluses. Some calluses showed extensive loss of potato chromosomes, while others showed extensive loss of *N. plumbaginifolia* chromosomes. In addition, some calluses showed partial elimination of both fusion donors. Although no somatic hybrid plants were regenerated from these calluses, this study demonstrates spontaneous biparental elimination of chromosomes as a result of protoplast fusion.

Puite and Schaart (1993) fused *S. tuberosum* protoplasts with irradiated transgenic *S. brevidens* protoplasts in order to obtain asymmetric hybrids. Elimination of the *S. brevidens* nuclear genome ranged from 18-62%. The authors found that more of the donor DNA was retained in the asymmetric hybrids when the recipient was of a high ploidy level (e.g., tetraploid recipient retained more donor DNA than a diploid recipient). Xu et al. (1993) produced 31 asymmetric hybrids through fusion between *S. tuberosum* and irradiated *S. brevidens*. All of the asymmetric hybrids contained one or two complete genomes of *S. tuberosum* (the non-irradiated fusion partner) and anywhere from 7-22 of the *S. brevidens* chromosomes. Oberwalder et al. (1997) induced chromosomal elimination rates of up to 52% in somatic hybrids by irradiating protoplasts of the wild species *S. bulbocastanum* Dun. and *S. circaeifolium* subsp. *circaeifolium* Bitter with 210 Gy prior to fusion with *S. tuberosum*.

The problem with the irradiation experiments to create asymmetric hybrids described above is that too large a portion of the donor genome has been transferred to the hybrids. If asymmetric hybrids are to be truly advantageous over laborious backcrossing methods generally used to transfer multigenic traits, the irradiation method must be modified in order to eliminate much more of the donor fusion partner. Alternatively, other methods of producing asymmetric hybrids should be examined. Rutgers et al. (1997) created monosomic addition lines of tomato (*Lycopersicon peruvianum* Mill) possessing only 1 potato chromosome by fusing potato microprotoplasts containing only one potato chromosome to tomato protoplasts (microprotoplast-mediated chromosome transfer).

Complete elimination of one donor's chromosomal complement, leading to the formation of cybrids, is useful for production of male-sterile plants. Perl et al. (1990) fused metabolically inactivated protoplasts (the recipients) of 'Atzimba' and 'Atlantic' (through iodoacetate or rhodamine-6G treatment) with γ-irradiated protoplasts (the donors) of a male-sterile, alloplasmic *S. tuberosum* line. Many male-sterile lines were regenerated with the normal tetraploid chromosome number (48) of the recipient protoplasts 'Atzimba' and 'Atlantic,' thereby indicating that the irradiated protoplasts had not contributed to the nuclear component of the regenerates. The mtDNA restriction profiles

of the male-sterile regenerants were different from those of both the donor and recipient profiles (probably due to mtDNA recombination in the fusion product) verifying the cybrid nature of these regenerants.

Perl et al. (1991) transferred oligomycin-resistant mitochondrial DNA from *Nicotiana sylvestris* Speg. & Comes to *Solanum tuberosum* cv. Desiree by fusing irradiated *N. sylvestris* protoplasts with iodoacetate-treated *S. tuberosum* protoplasts. Because the regenerated plants resembled cv. Desiree phenotypically, it was assumed that no nuclear contribution from *N. sylvestris* was made. The chloroplast DNA restriction pattern of the regenerated plants was identical to that of *S. tuberosum*; however, the mitochondrial restriction pattern showed components of both species as well as some novel fragments, verifying that these plants were true cybrids.

Fusion Among Cultivated Forms of Potato

Protoplast fusion among sexually-compatible, commonly-cultivated forms of potato has been carried out mainly as an effort to produce highly heterozygous, tetraploid somatic hybrids. Superior genotypes can be more easily identified at the diploid, rather than tetraploid, level and therefore the majority of these cases involve the fusion of vigorous dihaploid *S. tuberosum* clones with each other or with other commonly cultivated diploid species of potato such as *S. phureja*. The fusion of two diploid potato clones results in a rapid return to the tetraploid level in the somatic hybrid, and, if the two clones are genetically different from each other, the somatic hybrid should theoretically be highly heterozygous and vigorous. This heterozygous genotype can then be preserved through clonal propagation of the tubers.

The sexual cross of two superior diploid potato clones could produce a tetraploid hybrid only if both clones had unreduced gametes. However, much of the parental heterozygosity would be lost in the hybrid due to meiotic recombination. Even more of the parental heterozygosity would be lost if the two diploid clones were doubled to the tetraploid level (through colchicine treatment) and then sexually crossed. So it is to

preserve total heterozygosity in the hybrid that fusions among sexually compatible, diploid potato clones have been carried out.

There are two types of protoplast fusion that have been performed with cultivated, sexually-compatible forms of potato: 1) dihaploid-dihaploid fusions where both fusion partners are diploid (2*x*), and to a much lesser extent 2) monoploid-monoploid fusions where both fusion partners are monoploid (1*x*).

Dihaploid Potato Fusion

Dihaploid fusion can be used to combine two superior dihaploid potato lines, perhaps displaying complementary agronomic traits, while bypassing the meiotic segregation and recombination that occurs during sexual reproduction. The fusion of two vigorous dihaploid potato lines should theoretically lead to the production of a highly heterozygous tetraploid potato clone in one step. Heterosis produced from such heterozygosity may be detectable not only at the plant level, but also at the callus level during regeneration *in vitro*. For example, faster growth of hybrid calluses, or higher rates of shoot regeneration compared to unfused parental calluses, can be viewed as an expression of heterosis in the *in vitro* environment.

Some heterosis of somatic hybrids resulting from dihaploid-dihaploid fusions may be due to the fact that the genomes of these plants have passed through a partial, or "dihaploid sieve." When dihaploid plants are extracted (from tetraploids), some of the lethal alleles originally present in the highly heterozygous tetraploid genotype are exposed and removed by degeneration of weak, unproductive plants. Many of the deleterious (yet non-lethal) alleles are subsequently selected against by the plant breeder simply by choosing the most vigorous dihaploid clones for protoplast fusion experiments. Therefore, somatic hybrids resulting from dihaploid intraspecific fusions may contain fewer lethal and deleterious alleles than progeny resulting from the sexual cross of two tetraploid potato clones, and this may account for high levels of hybrid vigor.

Alternatively, extraction of dihaploids from tetraploids and subsequent selection of the most vigorous dihaploids may lead to the loss of allelic diversity in dihaploid populations. This could be especially true if certain alleles are associated with regeneration through anther culture. If allelic diversity is lowered in dihaploid populations, then somatic hybrids resulting from the fusion of dihaploids may not be particularly heterozygous or vigorous.

Hybrid vigor among somatic hybrids resulting from dihaploid fusions gives support to the theory that fusions between monoploid clones that have undergone complete lethal allele removal should result in plants demonstrating similar or perhaps greater hybrid vigor.

Protoplast fusion of dihaploid *S. tuberosum* with *S. phureja* (both of 2EBN) resulted in hexaploid and octoploid somatic hybrids (Puite et al. 1986). Calluses resulting from similar fusions showed heterosis and grew more vigorously than parental calluses (Puite and Roest 1985). Ramulu et al. (1989) obtained hypohexaploid/hexaploid, and hypooctoploid/octoploids after fusion of dihaploid *S. tuberosum* with diploid *S. phureja*. Mattheij and Puite (1992) produced six tetraploid somatic hybrids through fusions of dihaploid *S. tuberosum* and *S. phureja* protoplasts. When tested in the field, one of the somatic hybrids outperformed cv. Bintje with respect to tuber yield. In addition, these tetraploid somatic hybrids were female and male fertile.

Austin et al. (1985b) fused two *S. tuberosum* dihaploids leading to the production of a vigorously growing callus that yielded a tetraploid somatic hybrid. Debnath and Wenzel (1987) fused a dihaploid *S. tuberosum* clone with a doubled haploid *S. tuberosum* clone. The most vigorously growing calluses were selected as putatively hybrid and placed onto shoot regeneration media 10-12 weeks after fusion. Chromosome counts revealed that 90% of the regenerated plants were tetraploid, and the hybrid nature of nearly all of these putative hybrids was verified with isoenzyme analysis. Compared to tetraploid plants resulting from fusion of the parental clones to themselves (autofused protoplasts) and to the diploid parental clones, the somatic hybrids plants showed more vigorous growth *in*

vitro and in the greenhouse with significantly increased plant height, leaflet size and leaf area. Heterosis of the somatic hybrids was not verified in the field.

Waara et al. (1989) obtained many calluses from a fusion between two potato (*S. tuberosum* L.) dihaploid clones. The fastest-growing calluses were selected for shoot-regeneration, and shoots were obtained from 15 of these calluses. However, only three calluses actually regenerated somatic hybrids; the majority was simply parental somaclones. In this case, vigorous growth at the callus level was not indicative of heterosis resulting from the fusion of two dihaploid protoplasts. In addition, only hexaploid and mixoploid plants were obtained from this fusion, rather than the expected tetraploids.

In an experiment similar to Waara et al. (1989), Karlsson and Eriksson (1988) obtained many calluses after fusing various dihaploid potato clones. After 2 months of culture, the fastest-growing calluses were placed onto shoot induction media. Of the 15 calluses that regenerated, only three plants were determined to be of somatic hybrid origin by isoenzyme testing.

Möllers et al. (1988) fused various tetraploid and dihaploid potato clones and regenerated a few somatic hybrids along with many parental somaclones. The tetraploid somatic hybrids exhibited much higher average tuber yields in a greenhouse experiment compared to the fusion partners and to parental somaclones that were of the same ploidy as the hybrids (due to spontaneous chromosome doubling *in vitro*). Chaput et al. (1990) fused various dihaploid *S. tuberosum* clones to generate intraspecific somatic hybrids, of which the majority was tetraploid. Using stem and leaf morphology and vigorous growth as selection criteria, 24 putative hybrid plants were selected from the regenerants (roughly 13% of all regenerants). Electrophoretic patterns for malate dehydrogenase verified the hybrid nature of all 24 putative somatic hybrids. The authors attributed this high frequency of somatic hybrids to heterosis brought about through fusion of two unrelated dihaploid clones. Heterosis in this case was expressed as the greater ability of hybrid

calluses to regenerate plants over unfused calluses, as well as larger leaves, thicker and taller stems and more vigorous growth compared with the fusion parents.

Using protoplast fusion to combine a low yielding, late-maturing dihaploid clone with a moderately early, high yielding dihaploid clone, Waara et al. (1992) obtained somatic hybrids with late maturity and relatively high yields. Both tetraploid and hexaploid somatic hybrids regenerated from the fusion plates. The tetraploid somatic hybrids displayed many morphological traits intermediate to both fusion parents; however, they had significantly more internodes, longer stems, and higher tuber weight and yields than either dihaploid parent. Heterosis was clearly expressed as increased plant growth of the somatic hybrids.

Möllers and Wenzel (1992) produced 308 intraspecific hybrids through 17 fusion experiments involving different dihaploid breeding clones. In all of their combinations, they did not detect a preferential ability of hybrid calluses to grow and regenerate plants. When grown in the greenhouse, somatic hybrids expressed larger leaf size and plant height, and thicker stems compared to the dihaploid fusion parents and to tetraploids resulting from chromosome doubling of the dihaploids. In some dihaploid-dihaploid combinations, the tuber yield of the somatic hybrids was much higher than that of the dihaploid parents and doubled-up tetraploid clones.

Thach et al. (1993) selected 14 dihaploid *S. tuberosum* clones containing the *Rx* and/or *Ry* genes, conferring extreme resistance to potato virus X (PVX) and potato virus Y (PVY), respectively, for protoplast fusion experiments. The recovery and ploidy of the somatic hybrids depended strongly on the genotypes of the fusion partners. Some fusion combinations resulted in all tetraploids, whereas others resulted in nearly 50% hexaploids and aneuploids. In addition, one fusion combination yielded 94% somatic hybrids (% of all obtained regenerants), while another yielded only 2% somatic hybrids. In the majority of fusions the viral resistances of both dihaploid fusion partners were expressed in the somatic hybrids.

Cooper-Bland et al. (1994) used intraspecific protoplast fusion to combine two dihaploid *S. tuberosum* lines, each with different desirable characteristics. One fusion partner, PDH 40, had high yields and good tuber shape, while the other fusion partner, PDH 417, was notable for resistance to the potato cyst nematode (*Globodera pallida*). A total of 148 *in vitro* shoots was obtained from fusion experiments, of which 86 were too weak to survive acclimation to the greenhouse. Only 62 shoots demonstrating 'fair to vigorous growth' survived. The vast majority of these (all but 2) was found to be tetraploid somatic hybrids by examining their patatin electrophoretic banding profiles. Although not tested biochemically, it is probable that the majority of the parental somaclones (weak plants *in vitro*) was discerned from the somatic hybrids using ability to survive in the greenhouse as a selective agent. More than half of the verified somatic hybrids developed tubers expressing characteristics of both parental types; however, the mean resistance of hybrids to potato cyst nematode was closer to the susceptible parent than the resistant parent.

Möllers et al. (1994) fused twelve different dihaploid clones in various combinations to produce 13 tetraploid somatic hybrids. This study is particularly useful because the analysis of somatic hybrid vigor was conducted in the field, rather than the greenhouse. The combined data from two years of field data indicated heterosis for yield in the somatic hybrids (70% to 230% compared to the mid-parent value of the dihaploid fusion clones). The heterosis for yield was expressed in increased tuber weight, but not tuber number. Starch content was significantly higher than the mid-parent value for three of the somatic hybrids. The somatic hybrids were also more vigorous than tetraploids arising from chromosomal doubling of the dihaploid parents. Yield of the tetraploid hybrids was highly correlated with yield of the dihaploid fusion partners, demonstrating the importance of selecting high-yielding dihaploids when creating somatic hybrids.

In conclusion, it appears that the expectation of heterosis, resulting from the combination of two, unrelated potato dihaploid clones, has been realized in some situations. Rapid *in vitro* growth and regeneration of calluses has not always been a reliable indication of heterosis. On the other hand, growth of hybrid plants *in vivo* is often much more vigorous than parental plants for traits such as plant height and tuber yield and suggests

that heterosis can be readily observed both in the greenhouse and in the field. Finally, the combination of physiological traits, such as viral resistances, from two different dihaploid clones in a somatic hybrid has resulted in plants showing resistance equal to the fusion parents.

Monoploid Potato Fusion

Protoplast fusion involving monoploids is more difficult than that involving dihaploids due to their relative scarcity and ploidy instability during *in vitro* culture. Regeneration of plants from such fusions has been achieved only relatively recently (Uijtewaal et al. 1987a). The rationale for fusing monoploids is similar to that for fusing dihaploids: by fusing vigorous monoploid potato clones, it is hoped that superior diploid somatic hybrids can be obtained that could be useful in a potato breeding program or for further fusion experiments. In contrast to dihaploids, these plants have been reduced from the diploid to monoploid level, thereby exposing and removing all lethal alleles in the original diploid genome, i.e., they have passed through the "monoploid sieve." Perhaps the removal of all lethal alleles, compared to only partial removal in the dihaploid sieve, would result in higher expression of heterosis.

The benefits of increasing ploidy level from monoploid to diploid has been well documented. Uijtewaal et al. (1987b) found that monoploid potatoes exhibited drastically reduced leaf area per leaf, leaf area per plant and tuber production compared to their diploid parental clones (90%, 88% and 98% reduction, respectively). Doubled *Solanum phureja* monoploids demonstrated significantly higher growth than their corresponding monoploids for 15 of 17 traits such as plant height, leaf length, and days to floral anthesis (M'Ribu and Veilleux, 1991). Because monoploids and their corresponding doubled monoploids share identical genetic material, the increase in vigor expressed in the doubled monoploids must be due to a beneficial effect of increasing gene dosage.

Somatic hybrids arising from the fusion of potato monoploids should show increased vigor relative to the monoploid parents due to both an increase in ploidy level (monoploid to diploid, or higher) and heterosis caused by the combination of two unrelated, genomes free of lethal alleles and with a minimum of deleterious alleles.

Uijtewaal et al. (1987a) fused monoploids derived from various diploid potato clones, and obtained tetraploid somatic hybrids after an 11 week regeneration period. The hybrid calluses in this study grew at equal rates compared to unfused parental calluses; however, only hybrid calluses developed shoot initials and regenerated shoots. It appears that the heterozygosity achieved through the combination of two monoploid genomes enabled plant regeneration from the hybrid calluses, regardless of ploidy of the hybrid product. In additional studies, Uijtewaal et al. (1987c) produced somatic hybrids through protoplast fusion of eight unrelated monoploid genotypes. The regeneration period *in vitro* varied from 7-20 weeks. In most cases the somatic hybrids were again tetraploid; however, in one fusion there was unconfirmed evidence of triploid heterokaryons. In some genotypic combinations there was evidence of increased growth rates of hybrid calluses, but the hybrid nature of these fast growing calluses was not verified.

Uijtewaal et al. (1987c) found the tetraploid nature of their somatic hybrids (arising from fusion of two monoploid genotypes) not surprising in light of the fact that some researchers have regenerated almost entirely diploid and tetraploid plants when monoploid protoplasts were cultured (Sree Ramulu et al. 1986). Sree Ramulu et al. (1989) regenerated only tetraploid and aneuploid plants from monoploid protoplast cultures. It appears that diploid and tetraploid protoplasts (arising from endomitosis during which chromosome sets replicate without subsequent cellular division) are better suited for regeneration through tissue culture than monoploid protoplasts. Therefore, the tetraploid somatic hybrids obtained by Uijtewaal likely arose from the fusion of two genetically different diploid, rather than monoploid, protoplasts. The triploid regenerate may have arisen from the fusion of a diploid and monoploid protoplast.

Little work has been done in the area of monoploid-monoploid fusions in potato, or interhaploid fusions in any species for that matter. Although the few studies in potato have looked for heterosis only at the callus level, evidence for heterosis in terms of increased shoot regeneration among hybrid calluses has been observed. Based on this evidence, one would expect the hybrid plants from these calluses to also show hybrid vigor.

Conclusions and Objectives

The purpose of this review was to document that protoplast fusion is a well-described and commonly utilized process for breeding in potato. Interspecific, asymmetric and intraspecific fusions have been used numerous times to achieve various breeding objectives and strategies. Somatic hybrids have been produced displaying, among other traits, improved insect and virus resistance in many instances. However, no cultivars of potato have yet been released as a direct result of these fusions.

The results of the intraspecific fusions are most relevant to my thesis. Dihaploid-dihaploid fusion in potato has shown that vigorously growing somatic hybrids can be obtained displaying, among other things, higher rates of plant growth and higher tuber yields compared to the dihaploid fusion parents and tetraploids derived from chromosome doubling of the dihaploids (Möllers and Wenzel 1992). Performance of the somatic hybrids is closely correlated with performance of the dihaploid fusion partners. Therefore, identification of superior dihaploid clones is essential for the production of agronomically useful somatic hybrids. Heterosis is usually observed in dihaploid fusions when the somatic hybrids are grown in the greenhouse and/or the field. Although not yet tested, there is reason to believe that a similar trend will be observed when somatic hybrids resulting from monoploid-monoploid fusions are grown in the field. One foreseeable problem concerns the instability of monoploid protoplasts *in vitro*. The commonly observed high ploidy of plants regenerated from *in vitro* culture of monoploid protoplasts suggests that high ploidy/aneuploidy may occur in somatic hybrids produced

by protoplast fusion of monoploids. Unusually high ploidy levels can cause severe morphological abnormalities in plants such as slow, weak, stunted growth, thick leaves and stems and short internodes (Sanford 1983).

The goal of my thesis was to produce potato somatic hybrids from protoplast fusion of monoploid clones. Superior monoploid genomes expressing the fewest deleterious alleles were identified through field evaluation of agronomic performance and selected for the protoplast culture and fusion experiments. It is expected that the fusion of two monoploid genomes completely free of lethal alleles and expressing a minimum of deleterious alleles will result in high levels of heterosis among the somatic hybrids. Heterosis will be detected by comparing the somatic hybrids to progeny derived from sexual crosses of heterozygous diploids from which the monoploid clones were derived (Figure 1).

Intermonoploid somatic hybrids could be valuable to current potato breeding efforts because the novel method utilized for their production may render them genetically superior to sexual hybrids. In addition, this technique could be used to remove recessive lethal and deleterious alleles from potato (and possibly other crop) populations.

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Table 1: Summary of protoplast fusion research conducted in potato

Type of protoplast fusion	Reason for fusion	Reference
Intraspecific fusion		
S. tuberosum dihaploid + *S. tuberosum* dihaploid	Combination of low yielding, late-maturing dihaploid and high yielding, moderately early dihaploid	Waara et al. 1992
	Combination of *Rx* and *Ry* viral resistance genes in tetraploid somatic hybrid	Thach et al. 1993
	Combination of high yields and good tuber shape with potato cyst nematode resistance	Cooper-Bland et al. 1994
	Production of heterozygous tetraploid	Austin et al. 1985b
		Waara et al. 1989
		Karlsson and Eriksson 1988
		Chaput et al. 1990
		Möllers and Wenzel 1992
		Möllers et al. 1994
S. *tuberosum* dihaploid + S. *tuberosum* doubled haploid	Production of heterozygous tetraploid	Debnath and Wenzel 1987
S. tuberosum dihaploid + *S. tuberosum* dihaploid, S. *tuberosum* tetraploid + S. *tuberosum* dihaploid	Production of heterozygous potato clones	Möllers et al. 1988
S. tuberosum monoploid + *S. tuberosum* monoploid	Production of heterozygous diploid	Uijtewaal et al. 1987a
		Uijtewaal et al. 1987c
Interspecific intraEBN fusion		
S. tuberosum dihaploid + *S. phureja* diploid	Production of heterozygous tetraploid	Puite et al. 1986

Type of protoplast fusion	Reason for fusion	Reference
S. tuberosum dihaploid + *S. phureja* diploid	Production of heterozygous tetraploid	Ramulu et al. 1989
		Mattheij and Puite 1992
S. tuberosum dihaploid + *S. chacoense* diploid	Introduce glycoalkaloids into potato gene pool	Cheng et al. 1995
Interspecific interEBN fusion		
S. tuberosum + *S. melongena*	Somatic hybrid production	Li and Constabel 1988
S. tuberosum dihaploid + *S. pinnatisectum* diploid	Somatic hybrid production	Ward et al. 1994
S. tuberosum tetraploid + *S. bulbocastanum* diploid	Transfer nematode resistance to potato gene pool	Austin et al. 1993
S. tuberosum dihaploid + *S. circaeifolium* subsp. *circaeifolium* diploid	Transfer of *Phytophthora infestans* and *Erwinia* blackleg resistance to potato gene pool	Menke et al. 1996
S. tuberosum tetraploid + *S. brevidens* diploid	Transfer of bacterial soft rot and *Erwinia* resistance to potato gene pool	Austin et al. 1988
S. tuberosum × *S. berthaultii* hybrid diploid + *S. etuberosum* diploid	Transfer of PVY resistance to potato gene pool	Novy and Helgeson 1994b
S. tuberosum tetraploid + *S. brevidens* diploid	Transfer of PLRV resistance to potato gene pool	Austin et al. 1986
S. tuberosum dihaploid + *S. brevidens* diploid	Transfer of PLRV resistance to potato gene pool	Austin et al. 1985a
S. tuberosum dihaploid + *S. brevidens* diploid	Transfer of PLRV resistance to potato gene pool	Fish and Jones 1988
S. tuberosum dihaploid + *S. brevidens* diploid	Transfer of PLRV resistance to potato gene pool	Fish et al. 1988b
S. tuberosum dihaploid + *S. brevidens* diploid	Study inter-genomic pairing in somatic hybrid	Williams et al. 1990
S. tuberosum dihaploid + *S. commersonii* diploid	Transfer of frost tolerance to potato gene pool	Cardi et al. 1993

Type of protoplast fusion	**Reason for fusion**	**Reference**
S. tuberosum dihaploid + *S. commersonii* diploid	Transfer of frost tolerance and cold acclimation to potato gene pool	Nyman and Waara 1997
S. tuberosum + *S. nigrum*	Transfer of atrazine herbicide resistance to potato gene pool	Binding et al. 1982
Intergeneric fusion		
S. tuberosum dihaploid + *Lycopersicon esculentum* diploid	Tomato-potato somatic hybrid production	Jacobsen et al. 1992
S. tuberosum + *Lycopersicon pimpinellifolium*	Tomato-potato somatic hybrid production	Okamura 1988
S. tuberosum + *Lycopersicon pennellii*	Transfer of saline tolerance to potato gene pool	Sherraf et al. 1994
Asymmetric fusion		
S. tuberosum tetraploid + *S. phureja* diploid	Study elimination of nucleolar chromosomes in somatic hybrid	Pijnacker et al. 1987
S. tuberosum dihaploid + *Nicotiana plumbaginifolia*	Study loss of chromosomes in somatic hybrid	Gilissen et al. 1992
S. tuberosum + irradiated transgenic *S. brevidens*	Partial transfer of *S. brevidens* genome to potato gene pool	Puite and Schaart 1993
S. tuberosum + irradiated *S. brevidens*	Partial transfer of *S. brevidens* genome to potato gene pool	Xu et al. 1993
S. tuberosum + irradiated *S. bulbocastanum* and *S. circaeifolium*	Partial transfer of *S. bulbocastanum* and *S. circaeifolium* genome to the potato gene pool	Oberwalder et al. 1997
S. tuberosum microprotoplasts + *Lycopersicon peruvianum*	Production of monosomic tomato lines containing one potato chromosome	Rutgers et al. 1997
Irradiated alloplasmic *S. tuberosum* + cvs. Atzimba and Atlantic	Production of male-sterile forms of 'Atzimba' and 'Atlantic'	Perl et al. 1990
S. tuberosum + irradiated *Nicotiana sylvestris*	Transfer of oligomycin-resistant mitochondrial DNA to potato	Perl et al. 1991

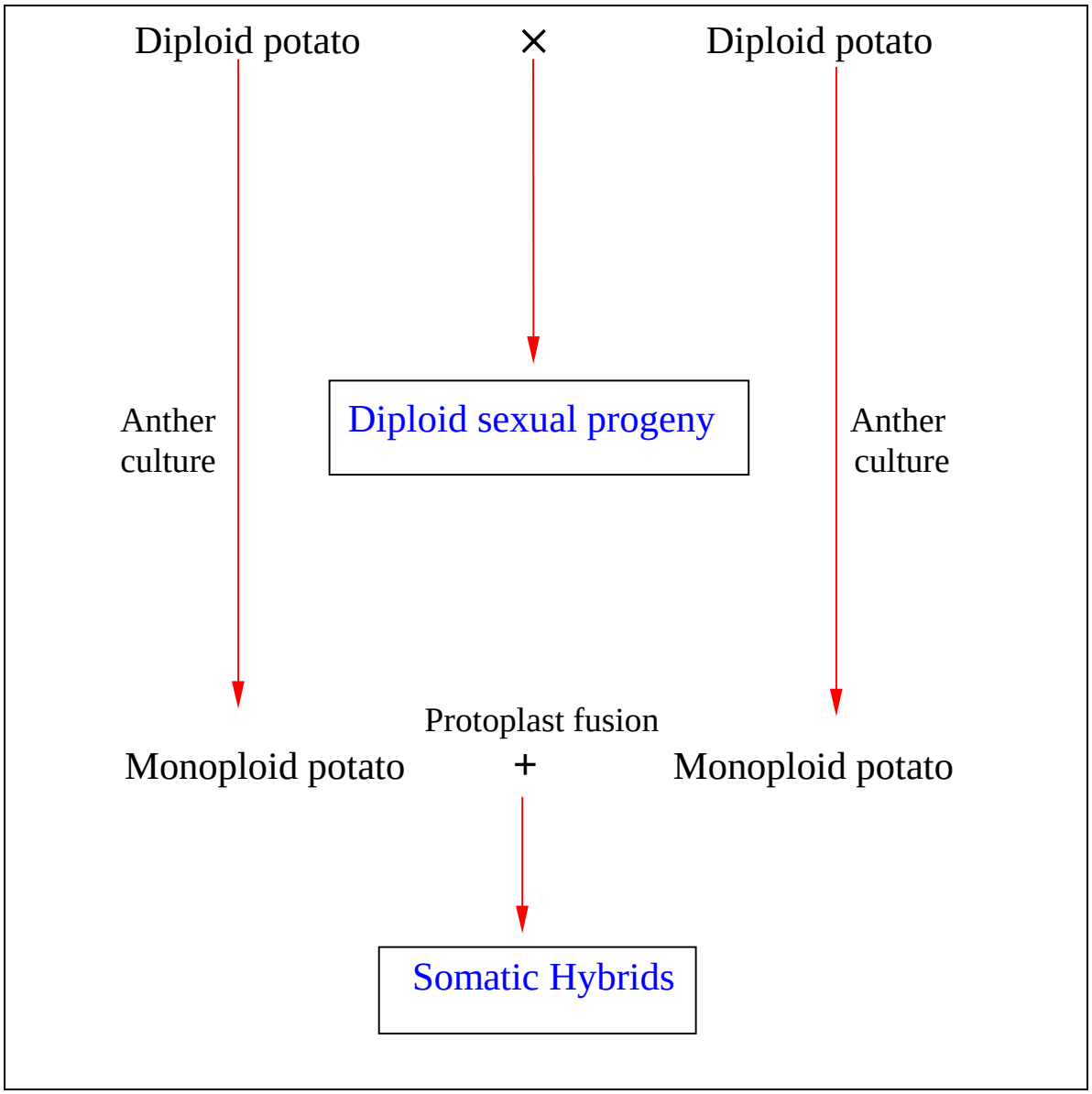

Figure 1: A depiction of the two populations (highlighted in blue) that must be generated from two unrelated diploid potato clones in order to test the effectiveness of the "monoploid sieve" for increasing the vigor of potato. The upper box depicts the sexually-derived population, which would be expected to harbor many lethal and deleterious alleles. The lower box depicts the somatic hybrid population generated by protoplast fusion of selected monoploids. The somatic hybrids, though genetically related as 'siblings' to the sexually-derived population, would be free of lethal alleles and express a minimum of deleterious alleles. Field evaluation of agronomic performance would detect any difference in vigor between the two populations.

CHAPTER 2

Selection of monoploids for protoplast fusion and generation of intermonoploid somatic hybrids

Introduction

The concept of "genetic load" refers to all of the lethal and deleterious alleles present in the individuals of a population. The majority of these alleles are recessive, and thus are tolerated by heterozygous individuals containing a corresponding, dominant, wild-type (non-lethal) allele. It is believed that all individuals of a species harbor a small number of recessive lethal alleles in their genomes (Griffiths et al. 1993). Highly heterozygous populations tend to harbor more lethal and severely deleterious alleles in their genomes than homozygous populations because the lethal alleles have less likelihood to become homozygous, and thus exert their effect. The effect of the genetic load on a species, if any, is not known.

Cultivated potato ($2n=4x=48$) is a highly heterozygous, tetraploid crop that harbors many lethal and deleterious alleles within its genome. These lethal and deleterious alleles become homozygous upon selfing, and explain the extreme inbreeding depression of potato. The large number of deleterious alleles can be observed in progeny from the cross of two superior potato cultivars. Nearly one million progeny of such a cross need to be screened before one is found worthy to be developed as a cultivar (Plaisted et al. 1994).

Monoploids of potato ($2n = 1x = 12$), and of all heterozygous species, represent gametes that have no lethal or severely deleterious alleles. When such alleles are present in gametes, regeneration of functional plants through androgenesis or gynogenesis is not possible—the basis of the "monoploid sieve" (Veilleux et al. 1995). Lethal allele-free genotypes with the fewest deleterious alleles can be obtained by selection for the most vigorous or phenotypically desirable monoploids.

Diploid potato ($2n = 2x = 24$), from which monoploids are derived, is highly heterozygous and therefore monoploids are expected to vary considerably in agronomic performance due to allelic segregation at many loci. In a study involving 118 monoploid genotypes, derived from five different diploid clones, large differences for relative vigor in a greenhouse study were detected among monoploids derived from one diploid clone, and among groups of monoploids derived from different clones (Uijtewaal et al. 1987a). These monoploid genotypes were found also to be variable for relative vigor *in vitro*; however, there was no correlation between *in vitro* and greenhouse performance. This study supports the expectation that monoploids derived from diploid potato represent a diverse genetic array that leads to differences in agronomic performance. A limitation of this study was that only relative vigor, not specific traits, such as tuber weight and number, was investigated. Also the study was not repeated to determine the stability of these differences from year to year (or season to season).

Because monoploids are expected to represent the fittest gametes due to the absence of lethal alleles, combination of distantly related monoploid genomes should result in highly heterozygous, and potentially vigorous, hybrids. Protoplast fusion represents one avenue to derive such hybrids, and electrofusion has been one of the most successful methods to induce somatic hybrids. The parameters used in electrofusion of potato have varied widely (Table 1), as have the methods used for somatic hybrid identification. Morphological features (Austin et al. 1986), restriction analysis of nuclear DNA (Mattheij et al. 1992), isoenzymes (Fish et al. 1988), and various molecular markers such as randomly amplified polymorphic DNA markers (RAPDs) (Baird et al. 1992) and simple sequence repeats (SSRs) (Provan et al. 1996) have been used for the identification of somatic hybrids in potato.

SSRs, or microsatellites, are short repeats of 1-5 nucleotides in length dispersed throughout the genome of eukaryotes (Smulders et al. 1997). Primers can be designed to flank the conserved regions surrounding a particular repeat, and PCR used to amplify the repeated region. Individuals are polymorphic if they contain different numbers of repeats at an SSR locus. SSRs are known to mutate frequently for various hypothesized reasons

(Valdes et al. 1993, Di Rienzo et al. 1994) and the mutation rate has been estimated at 10^{-2} to 10^{-3} mutations per SSR locus per gamete per generation (van Treuren et al. 1997); thus, it is not unusual for a population to contain many different alleles at a SSR locus. SSRs are codominant markers, (unlike other PCR-based molecular markers such as RAPDs) which makes them ideal for somatic hybrid identification because both SSR alleles in a somatic hybrid constructed from genetically distinct parents can be detected simultaneously using PCR. Provan et al. (1996) utilized primers to amplify two different SSR loci through PCR, one a $(TA)_{23}$ repeat in a potato proteinase inhibitor pseudogene and the other a $(T)_{12}(A)_9(TA)_7$ repeat in the IIK inhibitor gene intron, for the identification of somatic hybrids between three different dihaploid potato clones. With only these two loci, the true somatic hybrids could be distinguished unambiguously from parental somaclones.

The determination of ploidy in somatic hybrids has been accomplished through chloroplast counts of stomatal guard cells (Cardi et al. 1993), chromosome counts of root tips (Austin et al. 1993, Ehlenfeldt and Helgeson, 1987) and meristems (Binding et al. 1982), and flow cytometry (Mattheij et al. 1992, Menke et al. 1996). Flow cytometry can be used to process many samples in little time. Briefly, the cells of plant material are burst by chopping with a razor blade in a buffer with subsequent release of the nuclei. These nuclei are then stained with a dye that binds to DNA, such as propidium iodide, and the sample is run through a flow cytometer which estimates the DNA amount in each nucleus by absorption of specific wavelengths of light (from a laser beam) by the dye (Shapiro 1995).

The overall objective of this study was to generate potato somatic hybrids by protoplast fusion of selected “superior” monoploid genotypes. This process involved several specific objectives: 1) to select the most promising monoploids, based on field performance, from a large population of anther-derived monoploids regenerated from several diploid potato clones, 2) to evaluate the selected monoploid genotypes regarding their response to protoplast culture, 3) to develop appropriate techniques for electrofusion

of monoploid potato genotypes, and 4) to identify and characterize putative somatic hybrids using flow cytometry and SSRs.

Materials and Methods

Plant material: Four independently selected groups of monoploid genotypes were utilized in the 1996 field study. Three of these monoploid groups were derived from diploid *Solanum phureja* Juz. & Buk. clones, whereas one group was derived from a diploid *S. chacoense* Bitt. × *S. phureja* diploid cross. Along with the four previously discussed monoploid groups, one additional group of monoploids was utilized in the 1997 field study. This group was related to those derived from the *S. chacoense* × *S. phureja* cross, and resulted from a backcross of CP2 (a hybrid from the *S. chacoense* × *S. phureja* cross) to the *S. phureja* parent. The pedigrees of these monoploid families are given in Appendices B-F.

Field evaluation of monoploid genotypes: On May 15, 1996, 112 monoploid genotypes were planted in a randomized complete block design at Kentland Farm (Blacksburg, Va). There were three replications, or blocks, in the design, with each of the 112 monoploid genotypes represented by one plant in each replication. The monoploid plants had been started from *in vitro* plantlets which were acclimated in the greenhouse in 10.2 cm cell packs 6 wks prior to transplantation to the field. The plants were placed in rows, with 30 cm between plants in the rows and 0.91 m between rows. Straw mulch was placed around the plants to deter the growth of weeds. Four wks after transplantation to the field (June 15, 1996) the clones were evaluated on a scale of 1-5 with regard to vigor: 1) extremely vigorous, 2) vigorous, 3) fair, 4) poor, and 5) dead. On September 15, 1996, the plants were harvested and total yield and tuber number were recorded. The data was entered into SAS and the means were analyzed by general linear models (GLM) procedure with respect to tuber number, total yield and average tuber weight (SAS 1996).

On May 15, 1997, 110 monoploid genotypes were planted in a randomized complete block design with three replications at Kentland Farm (Blacksburg, Va). The

acclimatization of the plants, design of the plot, harvest (September 15, 1997) and analysis were similar to the 1996 field study except that vigor data was not collected. In addition, black landscape fabric, rather than straw mulch, was placed between the rows of plants to deter the growth of weeds. For the 34 monoploid genotypes that were included both in the 1996 and 1997 field plots, the results were analyzed statistically by SAS GLM procedure with respect to tuber number, total yield and average tuber weight.

Selection of monoploids for protoplast fusion experiments: Twenty-one of the 112 monoploid genotypes were selected for protoplast fusion experiments based on results of the 1996 field plot. Similarly, twenty of the 110 monoploid genotypes were selected for protoplast fusion experiments based on results of the 1997 field plot. Attempts were made to select the most vigorous clones; however, some vigorous clones were not used because they were not amenable to our tissue culture protocol, or they did not yield sufficient protoplasts for use in protoplast fusion experiments. In addition, some less vigorous clones were selected in order to maximize genetic variability within our selected population.

Monoploid *in vitro* growth conditions: Plantlets to be used for protoplast isolation were cleared of any systemic bacteria by culture on 30 ml cefotaxime-containing (250 mg/L, filter sterilized) MS propagation medium (Murashige and Skoog 1962) in baby food jars at 20°C, 16 h light/day, for a period of 2 wks. Tip cuttings from these plants were placed onto fresh cefotaxime-containing MS propagation medium and the 2 wk cycle was repeated two additional times. For protoplast isolation, cuttings taken from these source plantlets were cultured on 30 ml MS propagation medium in baby food jars at 20°C, 16 h light/day, for a period of 3 wks. Jars were placed in the dark at 4°C for 48 h prior to isolation.

Monoploid protoplast isolation protocol: *In vitro* leaves and shoots (roughly 1 g) from each monoploid clone was minced with a No. 10 scalpel in a 100 × 15 mm petri dish (Falcon 351029). Seven ml of enzyme solution (Cheng and Veilleux, 1991) was then added to the dish, the plate was shut and sealed with parafilm, and placed onto a gyratory

shaker (60 rpm) for 12-16 h in the dark. After enzyme digestion, large debris was removed by pouring the solution through a sterilized 63 μm filter. Rinse medium (8 ml) was poured into the petri plate and run through the filter in order to recover protoplasts stuck in the plate or filter. The 15 ml of total solution was then poured into a sterile 15 ml centrifuge tube (Corning 25317-15) and centrifuged in a Dynac II Centrifuge (Clay Adams, Division of Becton, Dickinson and Company) at 500 rpm for 5 min. The supernatant was removed and the pellet was resuspended in 10 ml of high sucrose (17.1 %) flotation medium (Cheng and Veilleux, 1991), with 1 ml of rinse medium layered on top of the flotation medium. Centrifugation at 500 rpm for 10 min resulted in the protoplasts collecting at the interface of the flotation and rinse solutions. The protoplast band was collected with a sterile Pasteur pipette (Scientific Products P5202-2) and placed into a new 15 ml centrifuge tube. Rinse medium (10 ml) was added to the tube with subsequent centrifugation at 500 rpm for 5 min. The supernatant was removed, the purified protoplast pellet was resuspended in fresh rinse medium, and the density of the protoplast solution was adjusted to 1×10^6 protoplasts/ml using a Spotlite Hemacytometer (Scientific Products B3175).

Response of selected clones to *in vitro* culture: Before each electrofusion experiment, a sample of protoplasts from each clone was suspended in 1 ml of modified Schumann and Koblitz culture medium (Cheng and Veilleux 1991) at a density of 2.5×10^5 protoplasts per ml in a 35×10 mm petri plate (Falcon 3001). The culture dish was placed at room temperature in the dark and observed on a weekly basis. The culture medium was replaced every 7 days. Each clone was rated on a scale of 1 – 5 based on response to protoplast culture. The scale was as follows: 1) protoplasts regenerated into visible calluses which eventually produced plants, 2) the protoplasts regenerated into calluses with no subsequent plant production, 3) the protoplasts showed limited divisions, but no callus formation, 4) the protoplasts expanded and regenerated a cell wall, but there was no cell division, and 5) there was no regeneration; rather, the protoplasts simply turned brown and died upon suspension in culture medium. Some monoploid clones were evaluated several times, whereas others were evaluated only once or not at all due to

scarce plant material. Each clone was placed onto the scale based on the best regeneration response observed during any evaluation.

PEG-mediated fusion protocol: A single PEG-mediated fusion was carried out by Vidya Ravichandran as part of a class laboratory exercise (Spring 1995). Protoplasts from monoploid clones were suspended in CPW 13M solution (Cheng and Veilleux, 1991). Three drops of protoplast suspension from two different monoploid clones were mixed on a sterile cover slip (in a petri plate) and left undisturbed for 10 min. Six drops of PEG 22.5 (Cheng and Veilleux, 1991) were added to induce agglutination and the protoplast suspension was incubated at room temperature for 20-25 min. To induce fusion the protoplast suspension was washed with a Ca^{2+} wash medium (Cheng and Veilleux, 1991) at a rate of 1 drop Ca^{2+} wash medium every 5 min for 20 min. The wash/PEG solution was removed with a sterile pipette and replaced with culture medium (Cheng and Veilleux, 1991). The petri plate containing the cover slide was sealed and placed at room temperature in the dark.

Electrofusion protocol and selection of parameters: A total of 15 electrofusion experiments was conducted between October 1996 and May 1997. Eight different monoploid genotypes were utilized in each fusion experiment. Using data from the protoplast isolation study, many fusions were attempted between genotypes that were capable of limited regeneration *in vitro*, but not plant regeneration, with the hope that heterosis brought about by the fusion of two unrelated monoploid genotypes would enable the production of somatic hybrids with few or no parental somaclones.

The electrofusion experiments were performed using a 2001EFS1 Electro Cell Manipulator with Enhancer 400 (Genetronics, Inc., BTX Instrument Division). Fusions were carried out in both 3.2 mm Gap Microslides (BT453) and disposable 2mm Gap Electroporation Cuvettes Plus (BT620). Just prior to fusion the protoplasts were suspended in fusion medium (1mM $CaCl_2$ in 8.5% mannitol, pH 5.6) at a density of 1×10^6 protoplasts per ml. Ten drops of protoplast suspension from each of two monoploid clones were added when using the 3.2 mm Gap Microslides, while eight drops of

protoplast suspension from each of two monoploid clones were added when using the 2 mm Gap Cuvettes. A range of electrofusion parameters reflecting those in Table 1 was applied to intermonoploid fusions. Through observation of protoplasts in fusion chambers under an inverted microscope, the electrofusion parameters were optimized to maximize alignment and fusion of protoplasts while retaining cell viability.

Five minutes after electrofusion, the protoplasts were removed from the fusion chamber using a sterile pasteur pipette and placed into a 15 ml centrifuge tube. The supernatant was removed after 5 min centrifugation (500 rpm), the protoplast pellet was resuspended in culture medium (Cheng and Veilleux, 1991) at a density of 2.5×10^5 protoplasts/ml, and the protoplast suspension was placed into a 60×15 mm petri dish (Falcon 1007). The petri plate was sealed and placed at room temperature in the dark.

Post-fusion regeneration protocol: Seven days after fusion experiments, the petri plates were examined under an inverted microscope to determine if any cell regeneration and/or division had occurred. The content of plates that contained dividing cells was centrifuged at 500 rpm for 5 min to remove the old culture media and replace with fresh media. After roughly 3 wks in liquid culture medium, the dividing cells were embedded in low gelling agarose (Agarose Type VII: Low Gelling, Sigma Cell Culture) dissolved in liquid culture medium. The protoplasts remained in agarose in the dark at room temperature for approximately 2 additional wks. At this point, visible calluses roughly 1 mm in diameter (resulting from numerous cell divisions) were removed from the agarose and placed onto solid greening medium (Tan et al. 1987) at 20°C, 16 h light/day, for a period of 2-4 wks. The calluses (now 2-3 mm in diameter and dark green in color) were then placed onto J1 callus regeneration medium (Cheng and Veilleux, 1991) for a period of 2-4 wks or until shoot initials developed. In the final step the calluses were placed on shoot proliferation medium (Austin and Cassells, 1983). When shoots regenerated from calluses they were cut off at the base of the callus and rooted in solid MS basal medium. Rooted shoots were then acclimated to the greenhouse.

Flow cytometry: All shoots arising from putatively hybrid calluses were analyzed by flow cytometry of leaf and stem tissue according to the methods of Owen et al. (1988). The cells were burst and the nuclei released by mincing 0.5 g of *in vitro* plant material in chopping buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS and 0.04% Triton X-100). The sample was treated with ribonuclease (40 mg ribonuclease in 50 ml chopping buffer) for 30 min and stained with propidium iodide (6.0 mg propidium iodide in 15 ml chopping buffer). The DNA content per nucleus was estimated by analyzing the sample with a Coulter® Epics® XL Flow cytometer (Coulter International Corp., Miami, Fla.).

DNA Extraction: Two methods of DNA extraction were utilized. Total genomic DNA was extracted from shoots and leaves of putative somatic hybrids grown in the greenhouse or *in vitro* according to the methods of Doyle and Doyle (1987) with the alterations made by Veilleux et al. (1995). Total genomic DNA was extracted from calluses according to the rapid DNA extraction procedure developed by Wang et al. (1993). Briefly, approximately 20 mg of callus tissue were ground in a 1.5 ml Eppendorf tube for 2 min using a pellet pestle grinder (Kontes Scientific Glassware/Instruments, 749515-0000) powered by a cordless motor (Kontes Scientific Glassware/Instruments, 749540-0000) with the addition of 10 µL of 0.5 M NaOH/mg of tissue. The final working solution was produced by adding 5 µL of the homogenate to 0.5 ml of 0.1 M Tris-HCl pH 8.0.

PCR amplification and SSR analysis

The amplification reactions utilized in this study were based on the protocol reported by Yu et al. (1994). A 20 µL reaction mixture was used for each amplification consisting of: 1× assay buffer (50 mM KCl, 10 mM Tris-HCl (pH 9), 1% Triton X-100), 3 mM $MgCl_2$, 160 µM each of the four dNTPs, 1.5 U *Taq* DNA polymerase (Promega, Madison, Wisconsin), and 0.1 µM of each SSR primer. The sequences of all primers utilized in this study, and the repeats that they amplify, are given in Table 2. To the reaction mixture 50 ng of genomic template DNA was added.

The conditions utilized for PCR amplification of SSRs were: 40 cycles at 94°C for 1 min, 55°C for 2 min, 72°C for 1.5 min, followed by 5 min at 72°C. The amplified fragments were separated in 3% Metaphor agarose gels (FMC Bioproducts, Rockland, Maine) in TBE buffer (Tris-borate-EDTA) for 4 h at 90-100 V (Sambrook et al. 1989).

Results

Monoploid field performance: A sample of tubers from various monoploid genotypes collected during the 1996 field study can be seen in Figure 1. The results of the 1996 and 1997 monoploid field plot evaluations are summarized in Figures 2-5. In both years genotype was a highly significant source of variation (Appendix A). According to a Ryan-Einot-Gabriel-Welsch Multiple Range Test of the 1996 field data using family as the source of variation, the 1-3 monoploid family (Appendix E) had significantly more tubers (average of 17.4) compared to the other three families (ranging from 3.5-8.1). The monoploid families did not differ significantly regarding average tuber weight, vigor or total yield. A similar analysis of the 1997 field data revealed no significant differences among the five monoploid families with respect to tuber number, average tuber weight or total yield. When data from both years were combined, the genotype by year interaction was found to be significant.

The distribution regarding vigor data from 1996 (Figure 2) demonstrates that the majority of the 112 monoploid genotypes (96) received a vigor rating of 'fair' or poor when evaluated 4 wks after transplantation. Nearly half of the monoploid genotypes (51) had five or fewer tubers (Figure 3, blue bars). Most cultivars of potato produce around 10-15 tubers/plant; however, *S. phureja* clones (the parents of most of our anther-derived monoploids) are characterized by many small tubers. To compensate for this difference, the majority of selected monoploid genotypes was taken from the 10-50 tubers/plant category, even though some clones produced well over 100 tubers/plant. Six of the monoploid genotypes produced total tuber yields of more than 100 g/plant; however, the majority (72) produced 10 g/plant or less (Figure 4, blue bars). Average tuber weights of the monoploid genotypes were quite low, with 77 of the genotypes having average tuber

weights of 2 g or less (Figure 5, blue bars). Most of the selected genotypes had average tuber weights of 2-6 g.

The 1997 field season (Figures 3-5, red bars) was more conducive to the growth of monoploids. The 1997 monoploids had significantly higher tuber numbers ($P = 0.0065$), total tuber yields ($P = 0.001$), but not significantly higher average tuber weights ($P = 0.25$). Because of the more vigorous growth, stricter selection parameters (compared to 1996) were used to select the 1997 monoploids. All but one of the 1997 selected monoploid genotypes had 10 or more tubers (Figure 3, red bars). The greatest indication of the vigorous growth of the 1997 monoploids can be seen in the distribution of total tuber yield (Figure 4, red bars). All but one of the 1997 selected monoploid genotypes had total yield of 50 g or more, whereas in 1996 eleven of the selected monoploids had less than 50g total yield. As in 1996, the majority of the selected 1997 monoploid genotypes had average tuber weights of 2-6 g (Figure 5, red bars).

Tables 3 and 4 list the field performances of all monoploid genotypes selected for protoplast fusion experiments based on the 1996 and 1997 field data, respectively. There are some genotypes listed that were not tested in the field. These genotypes were selected for other characteristics such as desirable tuber shape and form (T5-23, T5-274), high acetylleptinidine content in the foliage (CP2-103, discussed in chapter 3), or unusually high protoplast yields (PBC 9C-12, PBC-5).

Protoplast isolation study: Table 5 summarizes the results of the protoplast isolation study. Some genotypes were not evaluated with regard to protoplast response due to poor growth of source plantlets *in vitro*, persistent bacterial contamination, or poor protoplast yields. Of the 31 selected monoploids evaluated in 15 different protoplast isolations, 22 demonstrated at least some indication of growth in culture. Four genotypes were capable of regenerating into calluses that produced plants, five genotypes were capable of regenerating into calluses only, eight genotypes showed limited cell divisions, five genotypes regenerated cell walls only, and nine genotypes simply died upon protoplast isolation.

Selection of electrofusion parameters: Parameters that had been utilized previously (Table 1) for electrofusion in potato had a lethal effect on monoploid protoplasts. In particular, AC and DC pulse voltages were too high. AC voltages of 30 V or higher and DC pulse voltages of 1000 V or higher killed the protoplasts. The final electrofusion parameters which resulted in fusion frequencies of around 20% with viable fusion products were as follows:

AC Frequency – 1 MHz
AC voltage – 10 V
AC duration – 30 seconds
DC Pulse length – 60 μs
DC Pulse voltage – 450 V
Number of DC pulses – 2
Time between pulses – 0.5 s

Somatic hybrid production and ploidy analyses: Three independent fusions produced somatic hybrids (Table 6). One of these fusions was interspecific between *S. phureja* and *S. chacoense* genotypes, another involved fusion between *S. phureja* and a monoploid derived from an F_1 hybrid between *S. chacoense* and *S. phureja*, whereas the third was intraspecific between two *S. phureja* genotypes. SH1, SH2 and SH2B resulted from a PEG-mediated fusion performed by Vidya Ravichandran (Spring 1995), whereas the other somatic hybrids (SH3 – SH7) resulted from electrofusion. The regeneration phase varied from 4 to 6 months, and the ploidy of all somatic hybrids was tetraploid or greater as determined by flow cytometry (Fig. 6). SH1, SH2 and SH2B were hexaploid (Fig. 7), SH3, SH4, SH5 and SH6 appeared to be slightly higher than the tetraploid level (Fig. 8), and SH7A – SH7AD were hexaploids and octoploids (Fig. 9).

SSRs for the identification of somatic hybrids: SSRs that were polymorphic between the monoploid parents identified the somatic hybrids (Table 7). Figure 10 shows the results of PCR amplification at four different SSR loci of two monoploids, CP2-103 and

13-14 203, and two putative somatic hybrids resulting from protoplast fusion of the monoploids. STPROINI, a (tc)(ta) repeat, did not amplify well with the 13-14 203 parent, and therefore this locus was not utilized for somatic hybrid identification. STACCAS3, an (aag) repeat, STWIN12G, an (aat) repeat, and POTM 1-2, an (at) repeat, were all polymorphic between CP2-103 and 13-14 203 and were suited for somatic hybrid identification. At these three SSR loci, CP2-103 had the lower band (designated band A on Fig. 10) and 13-14 203 had the upper band (band B). Small fragments migrate faster than large fragments during electrophoretic separation in agarose gels, indicating that CP2-103 had fewer numbers of repeats at these three SSR loci compared to 13-14 203 (designated band B on Fig. 10). The somatic hybrids, SH1 and SH2, are clearly heterozygous at the STACCAS3, STWIN12G and POTM 1-2 SSR loci, indicating that they are true somatic hybrids.

The SSRs were also useful for screening out parental somaclones from true somatic hybrids. Figure 11 shows the results of PCR amplification at 2 different SSR loci (STACCAS 3 and POTM 1-2) of two monoploids, CP2-103 and T4-10, and three putative somatic hybrids resulting from protoplast fusion of the monoploids. The putative somatic hybrids contained the band of only CP2-103 (designated band A on the gel) at both loci, and were therefore unambiguously identified as parental somaclones arising from unfused CP2-103 protoplasts, not somatic hybrids.

More SSR polymorphism was observed for the monoploid parents of the interspecific somatic hybrids than the parents of the intraspecific somatic hybrids. Three of four SSR loci were polymorphic for SH1, SH2 and SH2B, four of five SSR loci were polymorphic for SH3, SH4, SH5 and SH6, while only one of five SSR loci was polymorphic for the SH7 somatic hybrids.

The rapid DNA extraction technique was used to extract DNA from 18 calluses derived from fusion of a *S. chacoense* monoploid (C80-1-4) fused to a *S. phureja* monoploid (1-3 3N-24). The DNA was then used in PCR with SSR primers flanking the STACCAS 3 locus (aag repeat) to identify the hybrid calluses (Figure 12). Eight of the calluses were

heterozygous and thus verified as somatic hybrid in nature, three calluses did not yield enough DNA to amplify with the primers, and 7 calluses were homozygous for the C80-1-4 allele and thus identified as parental clones. The rapid DNA extraction technique was also used successfully to extract DNA from 25 rapidly growing calluses derived from the fusion of two *S. phureja* monoploids (9-9 204 and O8-1) and subsequently identify a single hybrid callus regenerating along with at least 20 parental somaclones (Figure 13). In this case, only 5 of 25 callus extractions did not yield sufficient DNA to determine the nature of the calluses.

Discussion

The performance of the monoploid populations in both the 1996 and 1997 field plots indicates that there is indeed sufficient variability among monoploid genotypes to select for "superior" genotypes, or those monoploids that express the fewest deleterious alleles compared to the majority of the population. As such, our results agree with the observations made by Uijtewaal (1987a), who detected variation in relative vigor among monoploid genotypes grown in the greenhouse. However, the performance of our monoploid genotypes was much better than that observed by Uijtewaal (1987b), who recorded tuber weights per monoploid plant (total yield) ranging from only 0.01 to 0.1 g. Some of our monoploids had total yields of over 290 g per plant.

Comparison of families of monoploids, in order to see if monoploids derived from different sources varied significantly, revealed few significant differences. In fact, in 1996 the only significant difference concerned the 1-3 monoploid family (Appendix E), which had more tubers than the other monoploid families. In 1997 there were no significant differences among the monoploid families. This result is likely due to the extreme variation observed within monoploid families. For example, in the 1996 field plot RPC4-331 (a monoploid genotype eventually selected for protoplast fusion) had an average tuber yield of 143.5 g. In the same plot was a sibling monoploid RPC6-102, that had an average tuber yield of only 5.23 g. The high variation observed within monoploid families appears too great to reveal differences among families.

The fact that (for the 34 genotypes included in both the 1996 and 1997 field study) the genotype×year interaction was significant for three traits (tuber number, average tuber weight and total yield) indicates that some genotypes performed better in one year, while other genotypes performed better in the other. This finding suggests that monoploid genotypes are not consistent in agronomic performance from one year to another. However, the fact that a straw mulch was used in the 1996 field plot to deter weed growth, whereas a black landscape fabric was used in the 1997 field plot for the same purpose, could have affected growth from one year to another. The black landscape fabric may have heated the ground surrounding the monoploid clones, causing the most heat tolerant genotypes to thrive in 1997, and less heat tolerant clones to deteriorate. To determine if monoploid agronomic performance is consistent or highly variable over several years, genotypes need to be tested in the field for several years under identical conditions.

The large increases in tuber number and total tuber yield in the 1997 field plot may have been due to a more favorable season for potato monoploid growth in 1997 compared to 1996. However, the straw mulch vs. landscape fabric approach for weed control may have also been responsible for the difference in growth. Interestingly, average tuber weight was not significantly increased from 1996 to 1997. Perhaps average tuber weight is less subject to change from year to year, and therefore may be a more reliable indication of vigor among monoploid potato clones when evaluating data collected from more than one year or season.

Several months of regeneration through tissue culture are necessary in order to obtain a somatic hybrid, following the protoplast fusion event. The fact that more than 70% of our selected monoploids responded in some capacity to our *in vitro* tissue culture protocol indicated that regeneration of plants following protoplast fusion was possible. Also important from this study was the finding that only four genotypes were capable of plant regeneration following protoplast isolation and culture. The inability of the majority of selected genotypes to regenerate plants was beneficial as it should reduce the occurrence

of parental somaclones (plants arising from unfused protoplasts) developing along with true somatic hybrids. Unexpectedly, in two of the three successful fusions, parental somaclones were regenerated along with the somatic hybrids from genotypes that had not regenerated in control plates. It appears that the growth of hybrid calluses may encourage the growth of parental somaclones, due to the fact that many of the monoploid parents of the somatic hybrids (9-9 204, O8-1, CP2-103, C80-1-4) regenerated large calluses or plants from unfused protoplasts only when growing together with somatic hybrid calluses.

The parameters selected for electrofusion of our monoploid protoplasts were quite different from previously published protocols (Table 1). The most dramatic differences concerned the alternating current (AC) and direct current (DC) pulse voltages. Published protocols had utilized AC voltages ranging from 90-230 V/cm. AC voltages of this intensity caused explosion of the monoploid-derived protoplasts. An AC voltage of 10 V/cm was sufficient to cause alignment of the protoplasts. The DC voltage, which is used to induce fusion of the aligned protoplasts, ranged from 890-2000 V/cm in published protocols. DC pulse voltages of this intensity induced fusion of the monoploid protoplasts; however, the majority died following the procedure. A DC pulse voltage of 450 V/cm induced fusion of the protoplasts while maximizing viability of the resulting fusion products. These findings indicate that monoploid-derived protoplasts are more susceptible to the effects of electrofusion parameters than other types of potato protoplasts, and relatively weak AC and DC voltages are sufficient to induce fusion of the monoploid-derived protoplasts.

The high ploidy of all somatic hybrids produced from protoplast fusion of monoploid genotypes was expected, and has been observed previously in protoplast fusion experiments with monoploid potato (Uijtewaal et al. 1987b), where only tetraploid somatic hybrids were regenerated. Figure 6 shows the typical flow cytometric histogram of a monoploid potato clone. Clearly more than half of the cells in leaves and stems of monoploid plants are diploid or tetraploid. This surprising result is due to endomitosis, whereby the chromosomes in a plant cell replicate with no subsequent cellular division (Bhojwani and Razdan 1983). Endomitosis causes monoploid plants to contain

monoploid, diploid, tetraploid and even octoploid cells in the leaves and stems. Therefore, the majority of somatic hybrids resulting from the fusion of protoplasts derived from similar tissue of two monoploid clones would be expected to be tetraploid, hexaploid, or higher ploidy. In addition, the 4-6 month tissue culture regeneration phase necessary to obtain the somatic hybrid plants can raise the ploidy of somatic hybrids through spontaneous chromosome doubling, and may be responsible for the extremely high ploidy of some of the SH7 octoploid somatic hybrids.

SSRs proved to be a reliable screening method for the detection of somatic hybrids, and the elimination of parental somaclones. The presence of two bands in a plant regenerated from a fusion experiment (when examined at an SSR locus polymorphic between the monoploids involved in the fusion) quickly verified the somatic hybrid nature of the plant. Likewise, the presence of only one band indicated a parental somaclone. The finding that more polymorphic SSR loci were detected when examining the interspecific somatic hybrids compared to the intraspecific somatic hybrids was expected because high SSR polymorphism has been reported between species of *Solanum* (Provan et al. 1996). Two different species have more time to accumulate mutations and develop novel SSR alleles, causing them to be more polymorphic at SSR loci than two individuals within a species.

The rapid extraction procedure used to extract DNA from putatively hybrid calluses enabled the quick identification of somatic hybrid calluses as well as many parental somaclones. The somaclones could then be discarded before regenerating into plants while retaining only the hybrid calluses, thereby saving time and materials. The extraction method utilized very small amounts of callus, so the calluses were not adversely affected by the procedure, and DNA was extracted from all calluses in a matter of hours. The extracted DNA was of sufficient quality to amplify well when used with the SSR primers.

This study demonstrates all of the steps necessary for generation of intermonoploid somatic hybrids—from the selection of suitable plant material, to electrofusion protocol and regeneration of plants. The identification of somatic hybrids has been facilitated by

using PCR-based simple sequence repeats (SSRs) and through use of DNA extraction protocols enabling extraction of genetic material from calluses. The somatic hybrids developed during this study represent combinations of distantly related, lethal allele-free genomes which contain a minimum of deleterious alleles. Therefore, these somatic hybrids are unique in that they represent individuals that are free of the "genetic load" normally carried by all individuals of a population. Determining the effect of genetic load absence in our somatic hybrid population, and its potential for increasing vigor of potato, is the long term goal of this study.

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Table 1: Parameters previously used for electrofusion of *S. tuberosum* protoplasts.

Reference	Type of fusion	Alignment Frequency (MHz)	AC Voltage (V/cm)	AC duration (s)	DC pulse length (µs)	DC pulse voltage (V/cm)	DC pulse number	Post fusion AC voltage?	Post fusion AC duration (s)
Novy and Helgeson, 1994	*S. etuberosum* + *S. tuberosum*	0.5	90	30 - 45	100	1250	1	yes	20-30
Sherraf et al. 1994	*S. tuberosum* + *L. pennelli*	1	230	15	40	1200	1	yes	?
Fish et al. 1988	*S. tuberosum* + *S. brevidens*	1	100	?	10	1500 - 1250	1	yes	20
Möllers and Wenzel, 1991	*S. tuberosum* + *S. tuberosum*	1	100 - 120	30 - 40	20 - 40	2000	1	no	-
Cooper-Bland et al. 1994	*S. tuberosum* + *S. tuberosum*	1	120	30	20 - 50	1500	2 (0.5 s between pulses)	yes	20
Mattheij and Puite, 1992	*S. tuberosum* + *S. phureja*	1	100	?	50 or 100	1300 - 2300	1 - 3	no	-
Ward et al. 1994	*S. tuberosum* + *S. pinnatisectum*	1	180 - 450	5.5	10 or 100	890	2 (1 s between pulses)	no	-
Mattheij et al. 1992	*S. tuberosum* + *S. circaeifolium*	1	100	?	?	1300 - 1600	3	no	-
Gilissen et al. 1992	*S. tuberosum* + *N. plumbaginifolia*	1	60	?	?	1500	3	no	-
Chaput et al. 1990	*S. tuberosum* + *S. tuberosum*	1	125	?	40	1200	2	no	-

Table 2: Information regarding the amplified simple sequence repeats utilized for somatic hybrid identification.

Locus	**Type of Repeat**	**Primer Name**	**Primer Sequence**	**Accession #**	**Product Size**
G28WXST	$(actc)_5$	3 4	cccataatactgtcgatgagca gaatgtagggaaacatgcatga	X52417	223 bp
STPROINI	$(tc)_{12}(ta)_{18}$	5 6	cttgcaacttgttagtaccccc aaatcctttgtgacctcccc	Z12611	180 bp
STGBSS	$(ctt)_4$	7 8	tgattctcttgcctactgtaatcg agtcagagtatggttcctgagtcc	X83220	246 bp
STACCAS3	$(aag)_7$	15 16	aattcatgtttgcggtacgtc atgcagaaagatgtcaaaattga	Z27235	250 bp
STWIN12G	$(aat)_6$	17 18	aaatcgacacagacggaaatg cgagggactttaatttgttgga	X13497	240 bp
POTM 1-2	$(at)_{20}$	19 20	aataatactgtgatgccacaatgg gtggcatgtcttcgaaggtac	U23758	221 bp

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Table 3: Field performance of monoploid genotypes selected for protoplast fusion experiments based on the 1996 field data. Related clones are grouped together. N.T. = not tested

Clone	Tuber number	Average total yield (g)	Average tuber weight (g)
CP2-103	N.T.	N.T.	N.T.
CP2-319	12.3 ± 5.5	22.7 ± 11.1	2.0 ± 1.2
CP2-373	47.7 ± 1.2	128.4 ± 37.0	2.7 ± 0.8
1-3 202	149.3 ± 62.1	226.9 ± 30.4	1.7 ± 0.5
1-3 507	19.0 ± 8.9	38.6 ± 23.0	2.1 ± 0.8
1-3 3N-7	33.0 ± 8.9	117.7 ± 32.5	3.6 ± 0.5
1-3 3N-24	59.7 ± 23.9	59.5 ± 35.2	0.9 ± 0.4
1-3 516	8.0 ± 2.0	15.8 ± 1.9	2.0 ± 0.3
O8-1	43.7 ± 45.2	71.9 ± 57.2	2.2 ± 1.0
C80-1-4	7.7 ± 5.5	11.5 ± 12.7	1.2 ± 0.6
9-9 204	2.0 ± 0.0	5.2 ± 5.4	2.6 ± 2.7
13-14-1	51.3 ± 39.1	86.8 ± 63.0	1.8 ± 0.3
13-14-200	83.7 ± 33.5	208.6 ± 126.6	2.3 ± 0.7
T4-32	0.3 ± 0.3	0.3 ± 0.3	1.0 ± 0.0
T5-12	5.3 ± 3.1	38.7 ± 23.2	12.9 ± 16.1
T5-45	7.7 ± 5.8	5.9 ± 6.1	0.6 ± 0.5
T5-268	13.7 ± 7.4	36.2 ± 11.6	3.0 ± 1.2
T5-284	9.0 ± 10.8	8.6 ± 11.1	0.9 ± 0.1
PP53S-8	17.0 ± 14.1	69.7 ± 38.9	6.8 ± 4.9
RPC4-331	26.3 ± 16.0	143.5 ± 120.9	4.5 ± 2.6
K202	5.7 ± 4.4	26.0 ± 33.9	4.4 ± 3.8

Table 4: Field performance of monoploid genotypes selected for protoplast fusion experiments based on the 1997 field data. Related clones are grouped together. N.T. = not tested

Clone	Tuber number	Average total yield (g)	Average tuber weight (g)
PBC 8A-11	83.3 ± 33.1	182.2 ± 35.9	2.6 ± 1.4
PBC 9C-12	N.T.	N.T.	N.T.
PBC 13C-34	68.7 ± 4.0	108.3 ± 7.3	1.6 ± 0.2
PBC C5	N.T.	N.T.	N.T.
BC 1-4	14.3 ± 9.1	61.7 ± 18.1	5.2 ± 2.2
PB-4	37.7 ± 8.1	142.3 ± 76.4	3.7 ± 1.3
CP2-103	24.7 ± 25.5	66.1 ± 57.2	3.1 ± 1.7
O8-1	71.3 ± 61.0	212.0 ± 133.3	4.2 ± 2.3
1-3 3N-7	57.7 ± 10.2	291.9 ± 83.5	5.2 ± 1.9
1-3 3N-29	16.3 ± 9.3	55.8 ± 26.6	3.5 ± 0.4
1-3 521	74.3 ± 23.2	123.5 ± 11.6	1.8 ± 0.5
9-9 204	23.7 ± 26.3	58.1 ± 48.0	3.0 ± 1.9
13-14 1	96.0 ± 6.0	144.8 ± 4.2	1.5 ± 0.1
13-14 200	41.3 ± 16.0	66.1 ± 36.4	1.5 ± 0.4
T5-23	N.T.	N.T.	N.T.
T5-274	N.T.	N.T.	N.T.
T5-268	21.3 ± 11.7	40.1 ± 25.1	1.8 ± 0.9
PP5 3L-21	22.7 ± 14.4	69.2 ± 43.9	3.1 ± 0.1
AD24-3S-8	9.7 ± 2.1	86.7 ± 8.2	9.4 ± 2.7
RPC4-331	22.0 ± 23.1	115.9 ± 104.9	5.8 ± 1.9

Table 5: Protoplast response of selected monoploids (from both the 1996 and 1997 field plots) suspended in culture medium. Monoploid genotypes were ranked on a scale of 1-5: 1) protoplasts regenerated into calluses with plant regeneration, 2) callus formation only, 3) limited cell division but no callus formation, 4) regeneration of cell wall but no cellular division, and 5) protoplast death upon isolation. Related clones are grouped together. N.T. = not tested.

Clone	Number of times evaluated	Best response observed
CP2-103	5	1
CP2-319	2	3
CP2-373	3	3
1-3 202	3	3
1-3 507	1	5
1-3 3N-7	7	3
1-3 3N-24	4	3
1-3 3N-29	1	4
1-3 516	N.T.	N.T.
1-3 521	1	5
O8-1	7	1
C80-1-4	5	2
PBC 8A-11	1	2
PBC 9C-12	2	4
PBC 13C-34	N.T.	N.T.
PBC C5	1	5
BC 1-4	2	5
PB-4	1	5
9-9 204	4	1
13-14 1	8	2
13-14-200	2	2
T4-32	4	3
T5-12	6	3
T5-23	1	5
T5-45	2	4
T5-268	7	1
T5-274	2	2
T5-284	3	4
PP5-3S-8	1	4
PP5 3L-21	1	5
AD24-3S-8	2	5
RPC4-331	1	5
K202	9	3

Table 6: Intermonoploid protoplast fusions of potato that resulted in somatic hybrid plants.

Monoploid parents	**Somatic hybrid names**	**Regeneration phase duration**	**Number of shoot-producing calluses/ number of somatic hybrid calluses**	**Ploidy of hybrids**
CP2-103 (*S. chacoense* × *S. phureja* hybrid) + 13-14 203 (*S. phureja*)	SH1, SH2, SH2B	6 months	7 / 2	hexaploid
C80-1-4 (*S. chacoense*) + 1-3 3N-24 (*S. phureja*)	SH3, SH4, SH5, SH6	6 months	4 / 4	tetraploid/ pentaploid
9-9 204 (*S. phureja*) + O8-1 (*S. phureja*)	SH7A – SH7 AD (30 shoots from one callus)	4 months	27 / 1	hexaploid/ hypoaneuploids

Table 7: Six SSR-containing loci used for determining hybridity of three intermonoploid somatic hybrids

SSR Locus	**9-9 204 + O8-1**	**CP2-103 + 13-14 203**	**C80-1-4 + 1-3 3N-24**
G28WXST	+	N.T.	+
STPROINI	-	-	-
STGBSS	-	N.T.	N.T.
STACCAS3	-	+	+
STWIN12G	-	+	+
POTM 1-2	N.T.	+	+

\+ = polymorphic between parents and used to verify somatic hybrid nature
\- = monomorphic between parents and unusable for somatic hybrid testing
N.T. = not tested

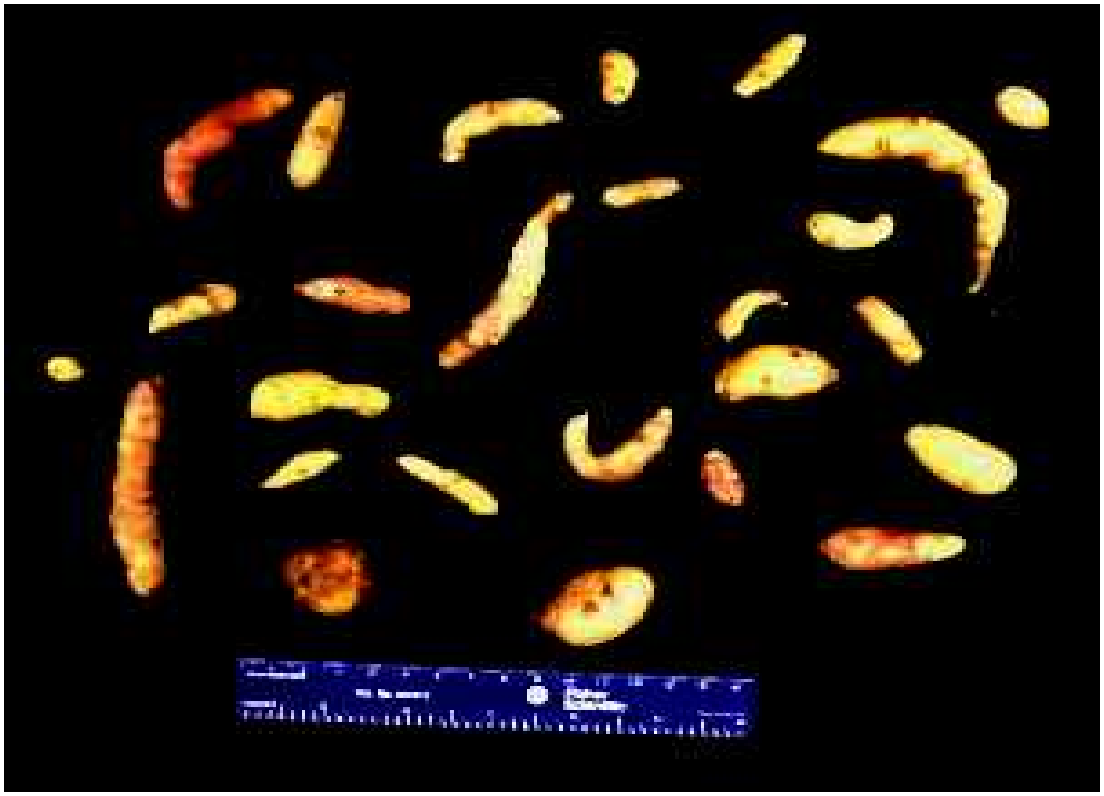


Figure 1: Sample of field tubers collected from various monoploid clones utilized in the 1996 field trial. Variability was observed among the monoploids regarding tuber weight, tuber number and total tuber yield.

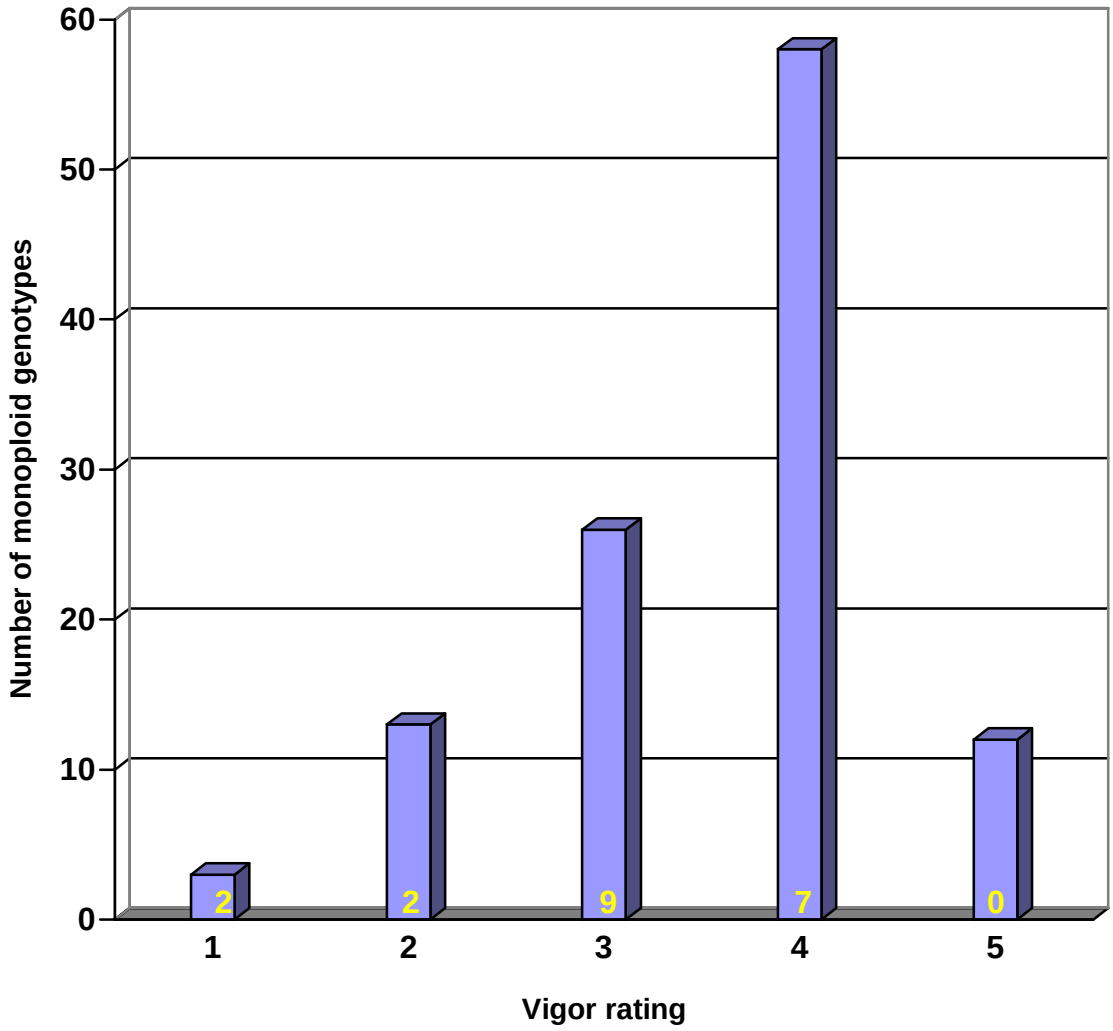


Figure 2: Distribution of 112 monoploid potato genotypes utilized in the 1996 field experiment according to the vigor rating: 1) extremely vigorous, 2) vigorous, 3) fair, 4) poor, and 5) dead. Ratings were assigned 4 wks after transplantation to the field. The number of genotypes from each category selected for protoplast fusion experiments is highlighted within bars.

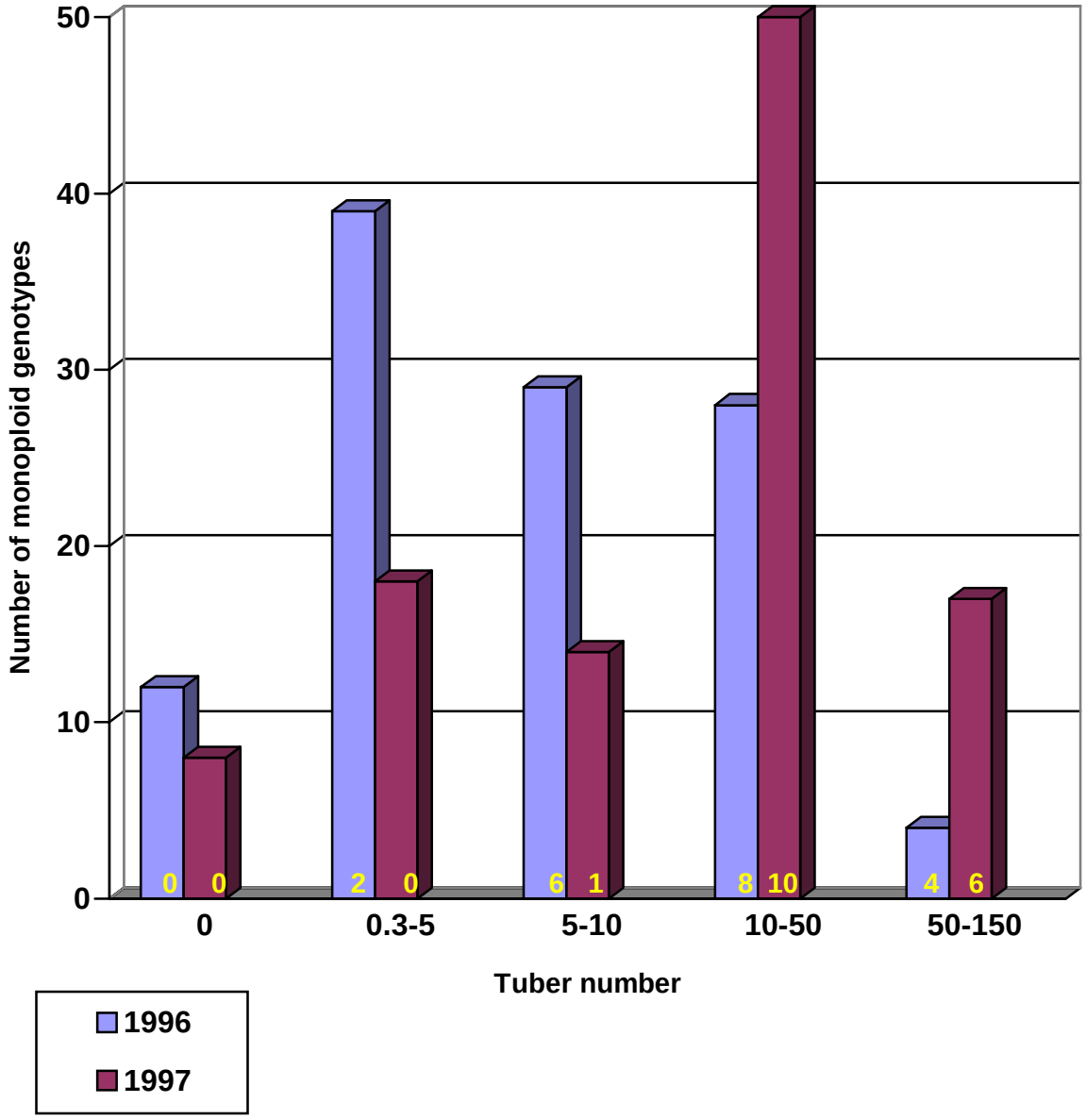


Figure 3: Distribution of 110 (1996) and 112 (1997) monoploid potato genotypes according to tuber number per plant for the 1996 and 1997 field trials. The number of genotypes from each category selected for protoplast fusion experiments is highlighted within bars.

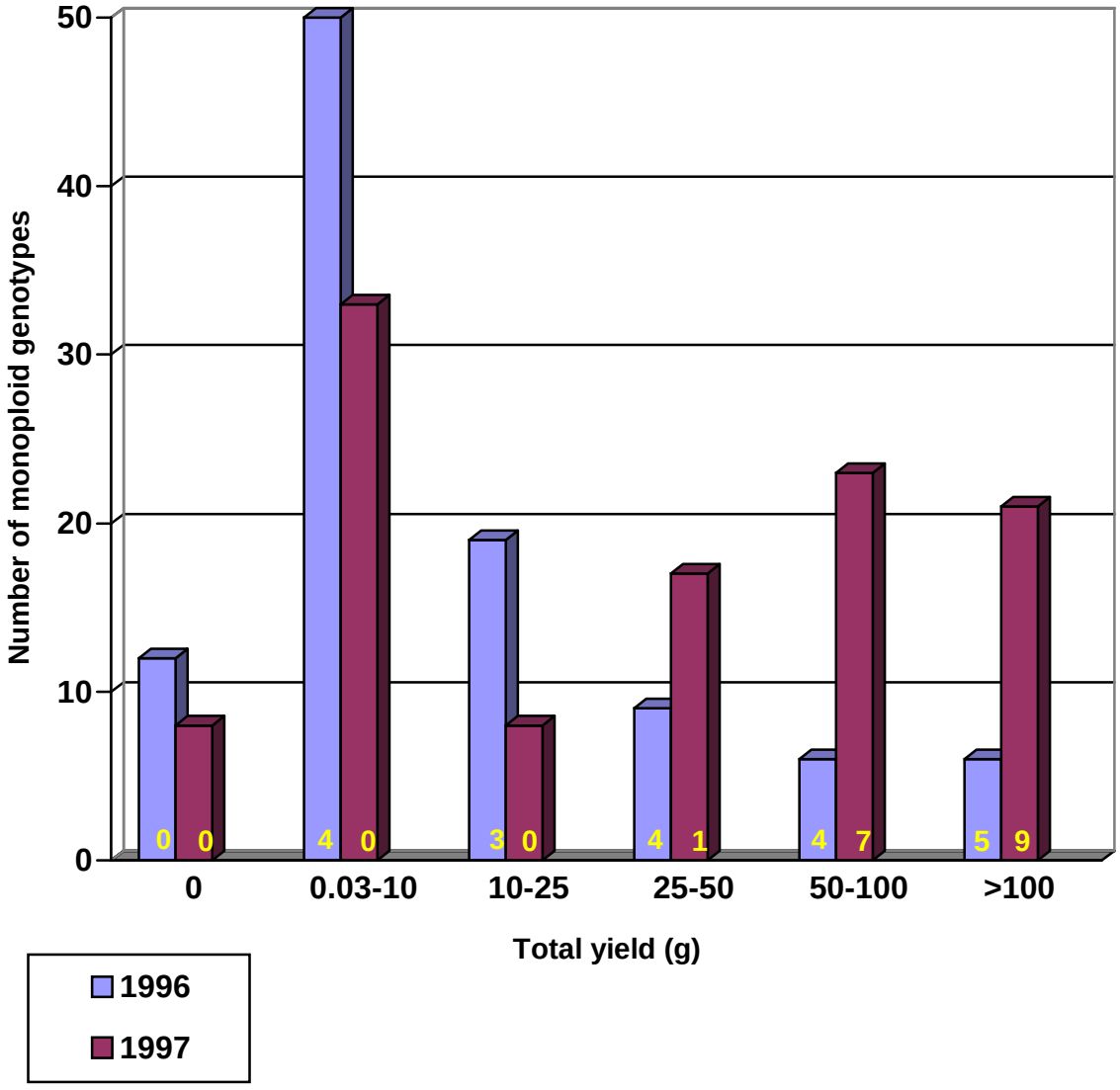


Figure 4: Distribution of 110 (1996) and 112 (1997) monoploid potato genotypes according to total tuber yield per plant for the 1996 and 1997 field trials. The number of genotypes from each category selected for protoplast fusion experiments is highlighted within bars.

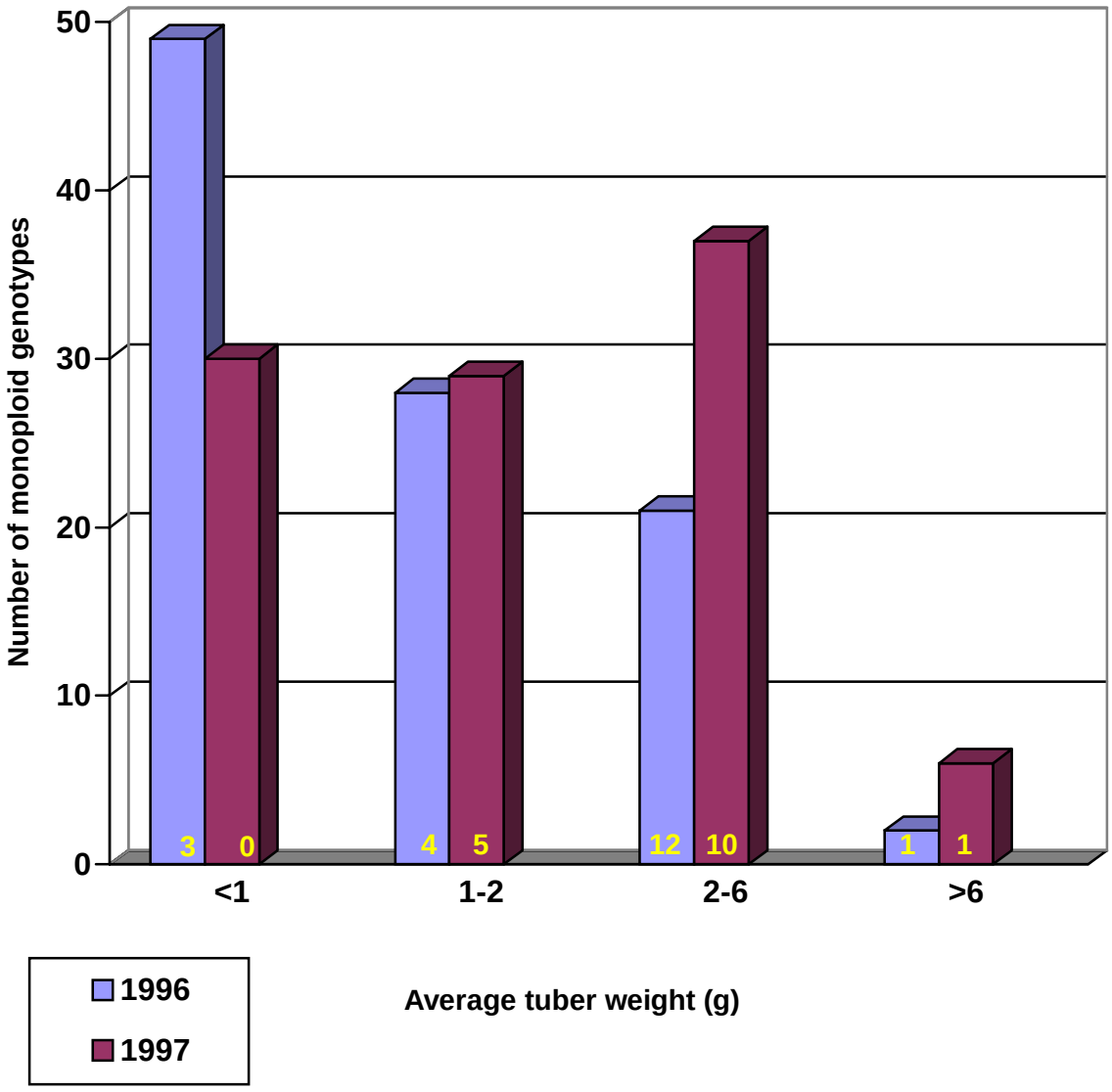


Figure 5: Distribution of 110 (1996) and 112 (1997)monoploid potato genotypes according to average tuber weight for the 1996 and 1997 field trials. The number of genotypes from each category selected for protoplast fusion experiments is highlighted within bars.

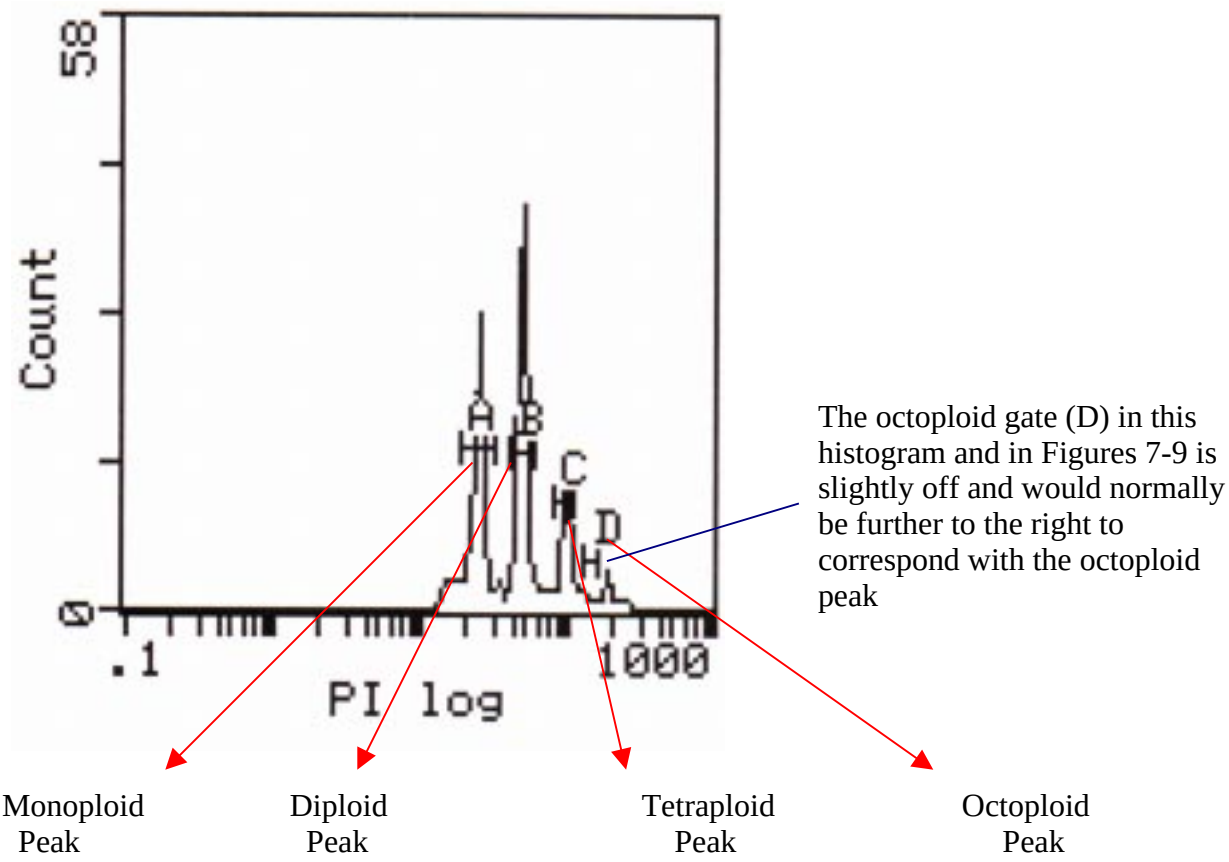
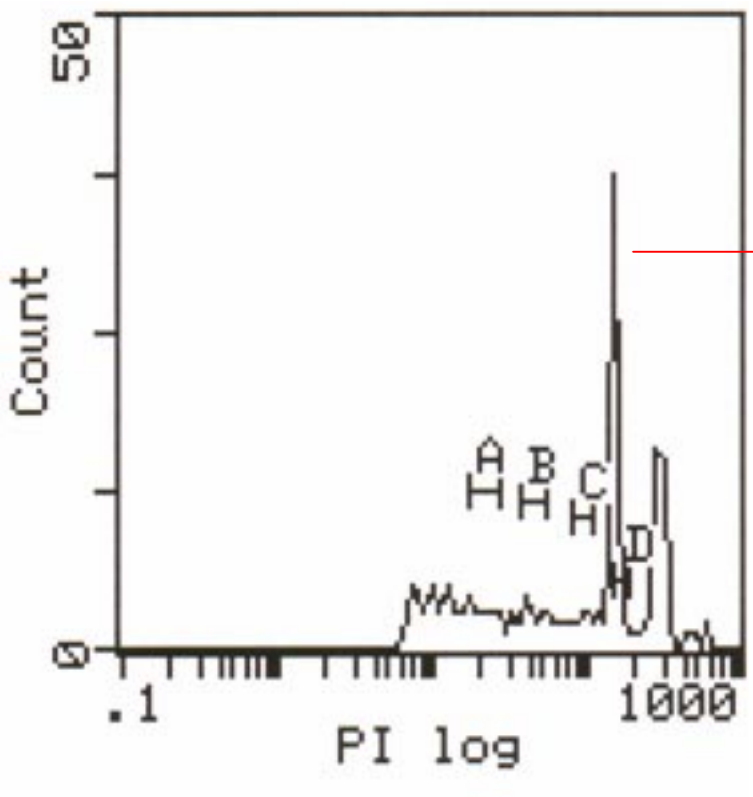


Figure 6: Flow cytometric histogram of a CP2-319 potato monoploid (1x). The four peaks represent the number of cell nuclei with a given amount of DNA (in logarithmic scale). The A, B, C, and D gates represent the monoploid, diploid, tetraploid and octoploid peaks, respectively. The first peak on a flow cytometric histogram (furthest to the left) indicates the true ploidy of the genotype. Additional peaks of higher ploidy (to the right) represent cells just prior to completion of mitosis, or mature cells that have undergone endomitosis during which the chromosomes replicate without subsequent cell division.



Hexaploid peak (halfway between tetraploid and octoploid gates)

Figure 7: Flow cytometric histogram of the SH2 somatic hybrid

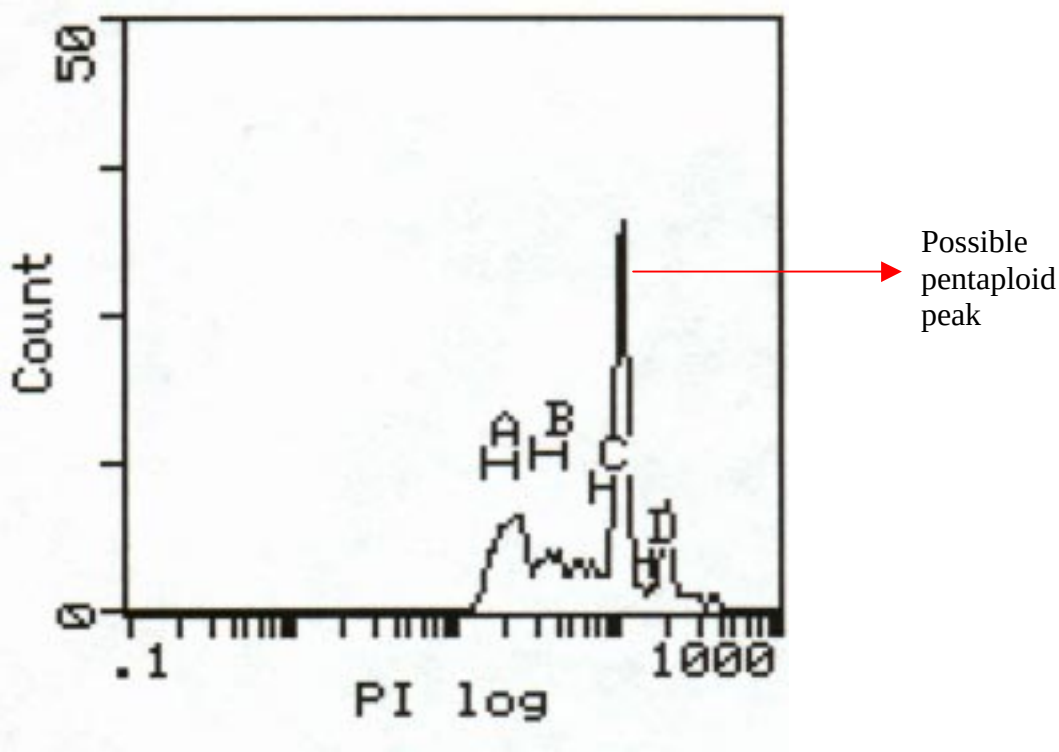


Figure 8: Flow cytometric histogram of the SH4 somatic hybrid

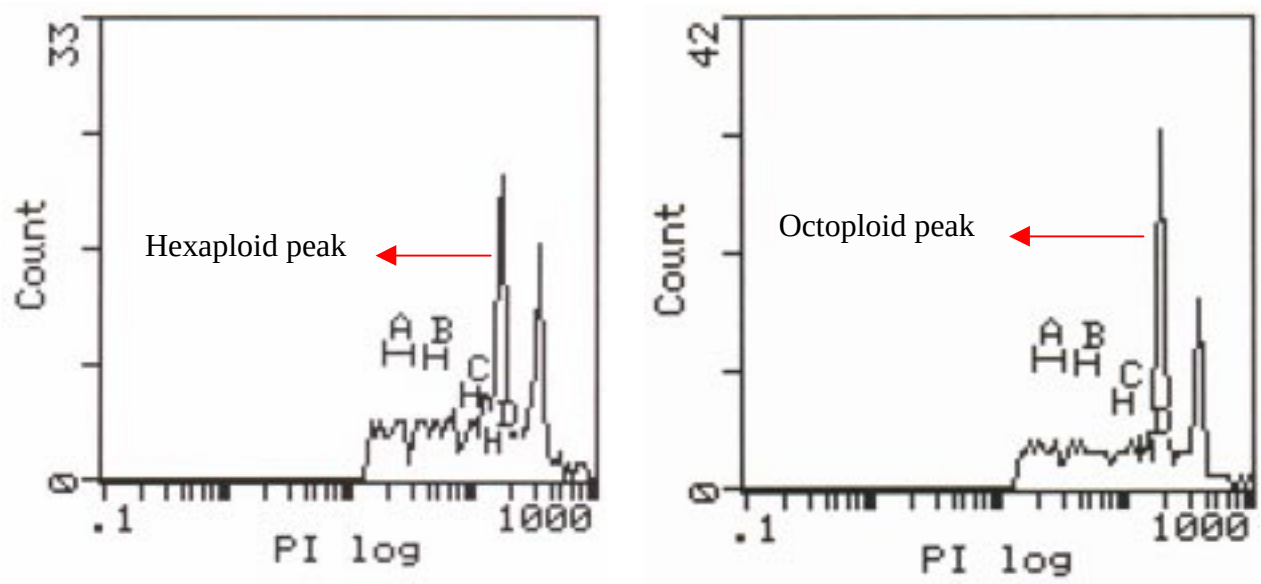


Figure 9: Flow cytometric histograms of two SH7 (SH7A, left, and SH7R, right) somatic hybrids.

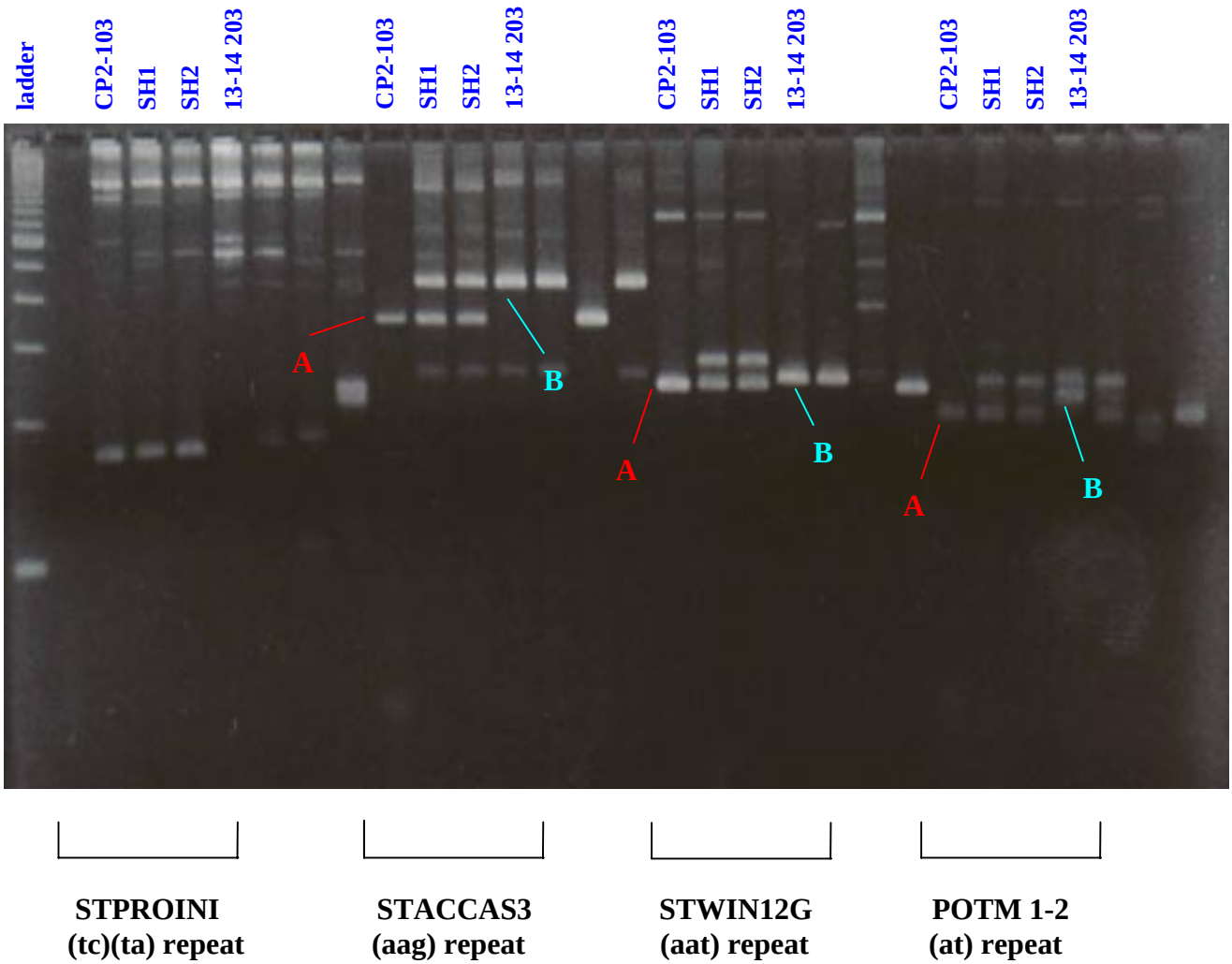


Figure 10: Use of SSRs to identify two somatic hybrids (SH1 and SH2) produced by electrofusion of the CP2-103 and 13-14 203 monoploids. Three of the four amplified SSR loci were polymorphic between the monoploids and in the somatic hybrids. The SSR loci names and their respective repeat unit is listed below the gels.

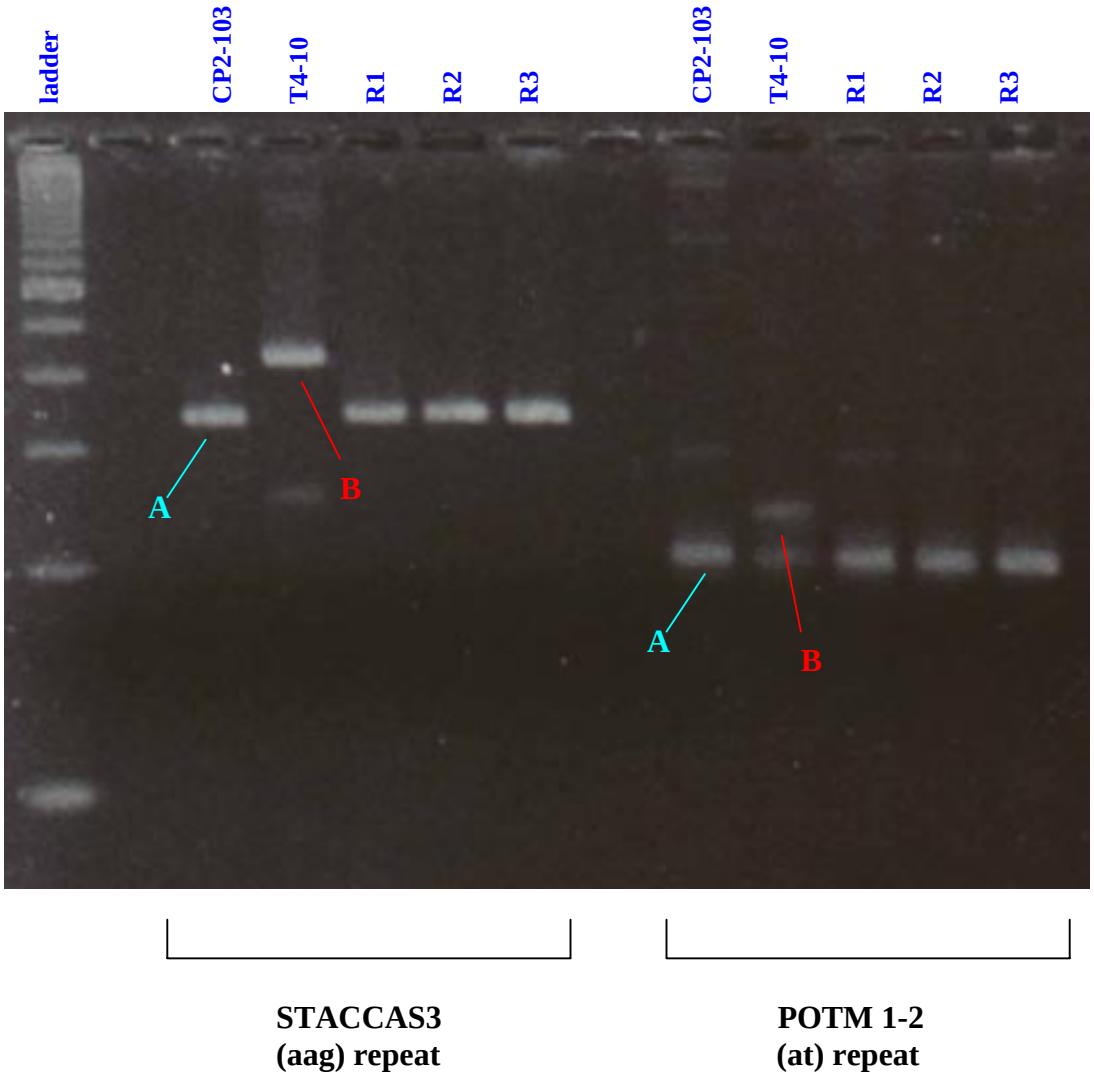


Figure 11: Use of two polymorphic SSR loci to detect three parental somaclones (R1-R3, regenerating from unfused protoplasts) which developed after a fusion of the monoploids CP2-103 and T4-10. The names of the SSR loci and their respective repeat unit are listed below the gel.

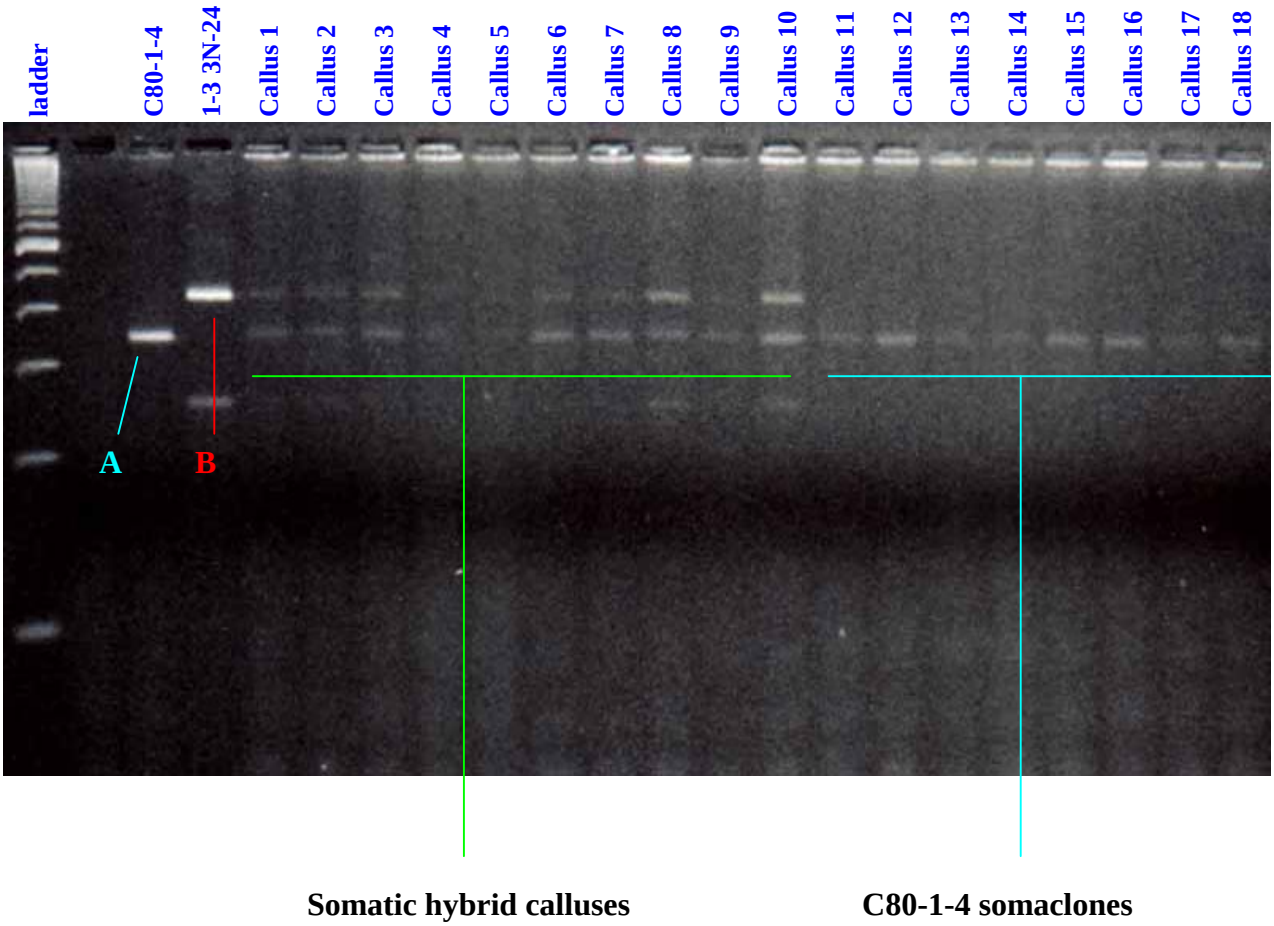


Figure 12: Rapid DNA extraction method used to analyze 18 calluses regenerated from a fusion of the monoploids C80-1-4 and 1-3 3N-24 at the STACCAS3 locus (aag repeat).

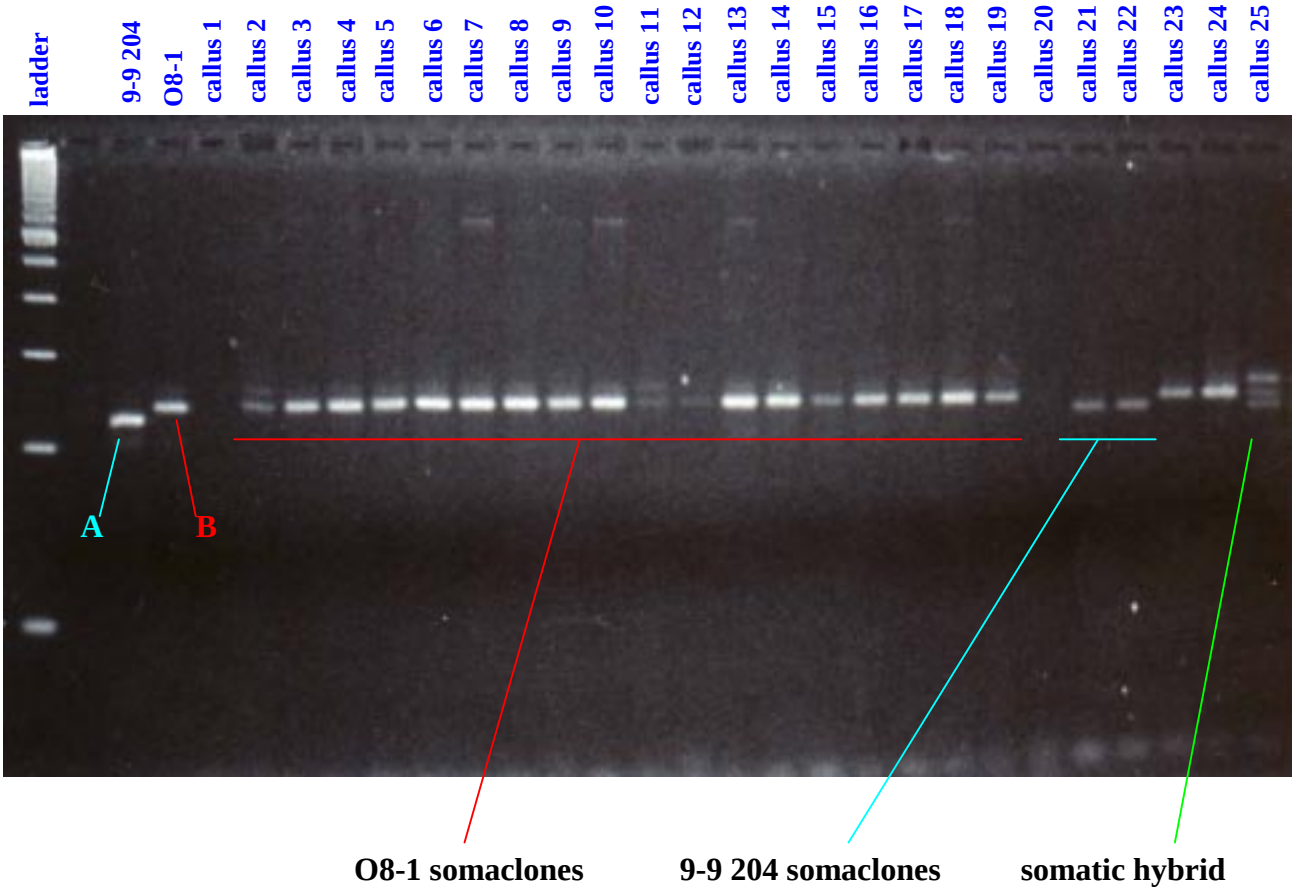


Figure 13: Rapid DNA extraction method used to analyze 25 calluses regenerated from a fusion of the monoploids 9-9 204 and O8-1 at the G28WXST locus (actc repeat in the *waxy* gene). Only one of the calluses was determined to be of hybrid origin.

CHAPTER 3

An evaluation of three hexaploid somatic hybrids (SH1, SH2 and SH2B) regarding fertility, transmission of leptines to offspring, field performance and genomic composition

Introduction

Because somatic hybrids nearly always express undesirable characteristics from an unadapted fusion partner (e.g., the wild species) or from somaclonal variation induced during the tissue culture regeneration phase, it is crucial that the hybrids be fertile for subsequent use in a breeding program. Fertile somatic hybrids produced by fusion of *S. etuberosum* and a *S. tuberosum* × *S. berthaultii* hybrid displayed increased resistance to potato virus Y (PVY); however, the resistance was less than that of the *S. etuberosum* parent (Novy and Helgeson 1994). When these hybrids were crossed to tetraploid *S. tuberosum*, some of the resulting progeny were as resistant to PVY as the somatic hybrid parents. This study demonstrates that somatic hybrids have potential for introgression of agronomically significant traits from wild species to cultivated potato.

Somatic hybrids produced from taxonomically distant fusion partners, such as potato+tomato, have been almost entirely sterile and therefore difficult to work with for breeding purposes. However, the somatic hybrids can sometimes be successfully backcrossed as female parents to one of the fusion partners with subsequent ovule culture to rescue the developing embryo (Jacobsen et al. 1994, Okamura 1988).

The Colorado potato beetle (*Leptinotarsa decemlineata* Say) is a major pest that feeds on the stem and leaf tissue of cultivated potato. Millions of dollars are spent annually in the US on its control; however, it remains a large problem to potato growers. Attempts to control this pest have employed the bacterium *Bacillus thuringiensis* var. *tenebrionis* (either applied as a spray or by insertion of the endotoxin gene of this bacterium into

cultivars giving transgenic resistance), insect traps, propane flamers, and various chemical insecticides (Stoner 1995). A natural means of resistance is highly desirable, considering the rapid life cycle of the Colorado potato beetle and thus its potential to develop resistance to prevention measures such as the *B. thuringiensis* var. *tenebrionis* endotoxin gene product.

Several wild species of potato express unusual glycoalkaloids that have been noted as having a defensive role against insects. Specific accessions of *S. chacoense* Bitt. produce glycoalkaloid leptines in leaves and stems which, in sufficient quantities, protect against feeding by the Colorado potato beetle. High levels of the leptine acetylleptinidine (ALD) in particular have been associated with resistance to the beetle. ALD levels of less than 4000 µg/g dry weight in leaf and stem tissue did not suppress feeding by the Colorado potato beetle whereas ALD levels of 7900 µg/g dry weight suppressed Colorado potato beetle feeding by 50% (Sinden et al. 1980). ALD is synthesized via the acetylation of common potato glycoalkaloids, solanine and chaconine (Sanford et al. 1996).

ALD-based resistance is highly desirable to potato breeders, as the leptine glycoalkaloids are not found in the edible product of potato—the tubers. *S. tuberosum* clones produce solanine and chaconine in leaves and stems, and it is hoped that introgression of the ALD acetylation genes from *S. chacoense* into *S. tuberosum* will result in *S. tuberosum* clones capable of producing high levels of ALD in stem and leaf tissue. Sexual crosses between *S. chacoense* and diploid forms of cultivated potato for ALD introgression have met with difficulty due to genomic incompatibility between the two species, resulting in weak hybrids not useful to a breeding program (Veilleux and Miller 1998). An alternative to sexual transfer of traits between these two species is somatic hybridization.

Somatic hybrids are often of a higher ploidy than the sum of the parent donors, due to multiple copies of one or both of the donor genomes included in the somatic hybrid. Technologies such as genomic *in situ* hybridization (GISH) and fluorescent *in situ* hybridization (FISH) can distinguish between chromosomes of even closely related

species enabling exact genomic analysis of hybrids; however, these techniques are costly, complicated procedures that involve hybridizing labeled fragments of genomic DNA or rDNA probes to the chromatin of individual cells fixed on a slide (Garriga-Calderé et al. 1998, Jacobsen et al. 1995).

In addition to their use for introgression of acetyleptinidine into cultivated potato, intermonoploid somatic hybrids can be used to test the validity of the monoploid sieve theory for improving the vigor of potato (Wenzel et al. 1979). Cultivated potato ($2n=4x=48$) is a highly heterozygous, tetraploid crop that harbors many lethal and deleterious alleles within its genome. The total genome of monoploids ($2n=1x=12$) is manifested at the plant level and therefore only those monoploid genotypes that are free of lethal and severely deleterious alleles will regenerate into functional plants through androgenesis or gynogenesis. Monoploid genotypes expressing a lethal or severely deleterious allele will not develop properly to form a viable embryo or plant—a selection referred to as the "monoploid sieve" (Veilleux et al. 1995). It is believed that intermonoploid fusions may result in highly heterozygous somatic hybrids displaying hybrid vigor due to an absence of lethal and severely deleterious alleles, also known as the "genetic load," in the somatic hybrid genome.

The following analyses of intermonoploid somatic hybrids were carried out over a 2 yr period and involved: 1) evaluation of somatic hybrid fertility through sexual crosses to several diploid and tetraploid clones of potato, 2) sexual crosses of the somatic hybrid progeny to *S. tuberosum* cv. Atlantic, 3) a field evaluation for agronomic performance of somatic hybrids, 4) use of SSR segregation in an anther culture-derived population from a somatic hybrid to determine its genetic constitution, and 5) use of gas chromatography to determine the levels of ALD, if any, expressed in the progeny of the somatic hybrids.

Materials and Methods

Somatic hybrid production: SH1, SH2 and SH2B developed from a PEG-mediated protoplast fusion of a *S. phureja* monoploid (13-14 203) with a monoploid (CP2-103) derived from a F_1 hybrid between *S. chacoense* and *S. phureja* (Figure 1). These somatic hybrids were produced in an effort to transfer the glycoalkaloid acetylleptinidine from *S. chacoense* to cultivated potato germplasm, thereby providing endogenous resistance to the Colorado potato beetle. The 13-14 203 monoploid was selected for protoplast fusion due to desirable tuber number and weight, and high total yield during a 1996 field trial. CP2-103 was selected as the fusion partner due to high ALD expression in leaves and stems (7590 μg/g dry weight, Veilleux and Miller 1998). The chemical fusion was performed by Vidya Ravichandran during Spring 1995 as part of a class exercise to demonstrate the chemical fusion process. Following the fusion, the regenerating cells were cared for by Suzanne Piovano (lab technician, Virginia Polytechnic Institute and State University). SH1 regenerated from one hybrid callus, whereas SH2 and SH2B developed as two different shoots that regenerated from another protoplast fusion-derived callus. Flow cytometry, as well as chromosome counts of root tips, indicated that these hybrids were hexaploids (see Chapter 2, Figure 7).

Plant material: Several *in vitro* shoots of SH1, SH2 and SH2B were acclimated in the greenhouse during August 1996. The plants were placed into 3.8 L nursery pots filled with ½ Sunshine All-Purpose Growing Mix (Sungro, http://www.sungro.com/profi-h.htm) and ½ sand and placed onto a ground bed under 16 h photoperiod at 25°C day/15°C night. Tubers were harvested from greenhouse grown plants after 4-5 months and utilized for further propagation of somatic hybrids in the greenhouse and for field trials.

Fertility Analyses: Unopened flower buds (2-3 days prior to anthesis) were emasculated (the yellowish-green anthers were gently removed with forceps) and pollen was applied to the stigma. In 1996 the hexaploid somatic hybrids SH1, SH2 and SH2B were crossed as female parents to several diploid and tetraploid clones of potato (Table 1). The somatic

hybrids were not used as male parents in pollinations because pollen staining revealed the pollen to be non-viable. In 1997 the progeny derived from sexual crosses of SH2 were crossed as both male and female parents to tetraploid *S. tuberosum* cv. Atlantic.

Field evaluation of SH1, SH2 and SH2B: On April 15, 1997, greenhouse-grown tubers of the three somatic hybrids (that had been stored at 2-4 °C for 12 wks) were planted in the field. Tubers of *S. tuberosum* cv. Atlantic (Dale Grant, RRI Box 635, Lee, Me.) were included, as well as tubers of ID5 (diploid, complex hybrid selected for superior field performance in Idaho by J. Pavek, USDA/ARS, Aberdeen, Id.) and 66AP11-53 (diploid, *S. phureja* selection by K. Haynes, USDA/ARS, Beltsville, Md.). The potato clones were planted in a randomized complete block design at Kentland Farm (Blacksburg, Va.). The design had three replications, with 10 hill plots of each clone per replication. The tubers were placed in rows, with 30 cm between each tuber and 0.91 m between rows. On Sept. 15, 1997, the plants were harvested and total yield and tuber number were recorded for each plot. The data were entered into SAS (SAS 1996) and analyzed statistically by SAS GLM procedure, and the genotypes were grouped according to total yield, tuber number and average tuber weight by Ryan-Einot-Gabriel-Welsch Multiple Range Test.

Anther culture of somatic hybrids: Flowers with anthers approximately 3-4 mm in length were selected for anther culture in order to obtain anthers with microspores at the late uninucleate to early binucleate stage. The flower buds (10-18 per somatic hybrid) were collected from greenhouse-grown plants during late November of 1997. The buds were wrapped in moist paper towels and stored at 4°C for 3 days. For anther culture the buds were surface-sterilized by immersion in 80% (v/v) ethanol for 1 min, then 5 min in full-strength commercial bleach (Wonder Chemical Corp., Fairless, Pa.; 5.25% (w/v) sodium hypochlorite) with one drop of 'Tween 20,' followed by two rinses in sterilized distilled water. Anthers were aseptically dissected from the flower buds under a laminar flow hood and placed into 125 ml Delong culture flasks (Bellco Glass Inc., Vineland, N.J.) containing 15 ml autoclaved liquid anther culture medium (Snider and Veilleux 1994). A total of 25-50 anthers (from 5-10 buds), depending on the availability of

suitable buds, was placed into each flask. A Magenta two-way cap (Magenta Plastics, Chicago, Ill.) was used to cover the flasks, and the flasks were incubated at 25°C in the dark on a rotary shaker at 125-150 rpm. Visible embryos were aseptically removed from the flasks after 5 wks of incubation and placed onto 20 ml solid regeneration media (Snider and Veilleux 1994) in 60 × 15 mm plastic sterile petri dishes. Shoots from regenerated embryos were transferred onto MS basal medium (Murashige and Skoog 1962) for propagation.

Flow cytometry of plants regenerated through anther culture: A sample of regenerated plants from each somatic hybrid was analyzed by flow cytometry to determine ploidy according to Owen et al. (1988). The cells were burst and the nuclei released by mincing 0.5 g *in vitro* plant material in chopping buffer (45 mM $MgCl_2$, 30 mM sodium citrate, 20 mM MOPS and 0.04% Triton X-100). The sample was treated with ribonuclease (40 mg ribonuclease in 50 ml chopping buffer) for 30 min and stained with propidium iodide (6.0 mg propidium iodide in 15 ml chopping buffer). The DNA contained in the nuclei was estimated by analyzing the sample with a Coulter® Epics® XL Flow cytometer (Coulter International Corp., Miami, Fla.).

DNA extraction from triploids and SSR analysis: Because the triploids were only recently regenerated and little plant material was available for DNA extraction, the rapid DNA extraction protocol described in Chapter 2 (Wang et al. 1993) was utilized. Approximately 20 mg leaf tissue was ground in a 1.5 ml Eppendorf tube for 2 min using a pellet pestle grinder (Kontes Scientific Glassware/Instruments, 749515-0000) powered by a cordless motor (Kontes Scientific Glassware/Instruments, 749540-0000) with the addition of 10 µL of 0.5 M NaOH/mg of tissue. The final working solution was produced by adding 5 µL of the homogenate to 0.5 ml 0.1 M Tris-HCl pH 8.0.

PCR amplification and SSR analysis: The amplification reactions utilized in this study were based on the protocol reported by Yu et al. (1994). A 20 µL reaction mixture was used for each amplification consisting of: 1× assay buffer (50 mM KCl, 10 mM Tris-HCl

(pH 9), 1% Triton X-100), 3 mM $MgCl_2$, 160 μM each of the four dNTPs, 1.5 U *Taq* DNA polymerase (Promega, Madison, Wis.), and 0.1 μM of each SSR primer. To the reaction mixture 50 ng of genomic template DNA was added.

SH2 and four triploids derived from SH2 were examined at locus G28WXST, an (actc) repeat, and locus STWIN12G, an (aat) repeat (see Table 2, Chapter 2 for primer sequence information). The PCR cycle for amplification was: 40 cycles at 94°C for 1 min, 55°C for 2 min, 72°C for 1.5 min, followed by 5 min at 72°C. The amplified fragments were separated in 3% Metaphor (FMC Bioproducts, Rockland, Me.) agarose gels in TBE buffer (Tris-borate-EDTA) for 4 h at 90-100 V (Sambrook et al. 1989).

Evaluation of leptine content in somatic hybrids and in their progeny: Roughly 5 g of greenhouse-grown leaves were collected and freeze-dried. Dried plant material was ground in a mortar and pestle and the acetylleptinidine was extracted and quantified by gas chromatography (Lawson et al. 1992) in the laboratory of Dr. A. R. Miller, Dept. of Horticulture and Crop Science, The Ohio State University, Ohio Agricultural Research and Development Center, Wooster, Ohio 44691.

Results

Fertility analysis of SH1, SH2 and SH2B: The hexaploid somatic hybrids were male-sterile, but somewhat female fertile when crossed to certain potato clones (Table 2). Unfortunately, no crosses to cultivated *S. tuberosum* cultivars were successful. SH2B set one fruit as a female parent to the tetraploid *S. andigena* clone 8-1. In this case 15 seeds were obtained, of which 8 germinated. SH2 set one fruit as the female parent to *S. andigena* clone 8-1, and in addition set two fruit as the female parent to a diploid *S. phureja* clone 66AP11-53. A total of 20 seeds was obtained from the cross of SH2 to *S. andigena* clone 8-1, of which 16 germinated. A total of 66 seeds was obtained from the crosses of SH2 to *S. phureja* clone 66AP11-53, of which 40 germinated. SH1 may be entirely sterile, as it was never crossed successfully to any potato clone.

Ploidy and morphology of the SH2 progeny: The progeny from the cross of SH2 to tetraploid *S. andigena* 8-1 (SH2A progeny) appear pentaploid by flow cytometry—the expected result of a hexaploid × tetraploid cross. The progeny from the cross of SH2 to diploid *S. phureja* 66AP11-53 (SH2P progeny) appear between the tetraploid and pentaploid level by flow cytometry. When established in the greenhouse, the SH2A progeny developed into small, weedy plants with few tubers and excessive stolon production (traits typical of the *S. andigena* parent). The SH2P progeny were much more desirable agronomically, with several large tubers and few stolons.

Fertility of the SH2A progeny: Eight plants resulting from the cross of SH2 to *S. andigena* 8-1 (SH2A progeny) were crossed to cv. Atlantic (Table 3). Four of the progeny set fruit upon pollination with Atlantic pollen. Several of the progeny; however, did not set fruit even after repeated pollinations, such as SHA#2. At least one of the SH2A plants (SH2A#15) produced functional pollen and resulted in fruit formation on 'Atlantic' when used as the male parent. The majority of the SH2A progeny, however, was used exclusively as the female parents in crosses to 'Atlantic.'

Fertility of the SH2P progeny: Seven plants resulting from the cross of SH2 to *S. phureja* clone 66AP11-53 (SH2P progeny) were crossed to cv. Atlantic (Table 4). Fruit formation was never observed when using these progeny as the female parent in sexual crosses with 'Atlantic.' However, crosses between the SH2P progeny and 'Atlantic' where the progeny were used as the male parent, or pollen donor, resulted in fruit formation. Four of the SH2P progeny appear to be both male and female fertile; however, they could not be used as the female parent in crosses to 'Atlantic.'

Field performance of the SH somatic hybrids: SH1, SH2 and SH2B did not perform well in the field trial of 1997 compared to diploid (ID5, 66AP11-53) and tetraploid ('Atlantic') check clones (Table 5). The somatic hybrids had more tubers per plant (average of 35) than the other potato clones. The somatic hybrids had significantly lower

average tuber weights (10.4 g). The combination of many tubers with low average tuber weights resulted in low total yield. SH1 and SH2B had significantly lower tuber yield compared to all of the checks. SH2 had slightly higher yield than the other somatic hybrids and was not significantly different from the diploid clones ID5 and 66AP11-53, with an average of 0.5 kg/plant. The commercially cultivated tetraploid 'Atlantic' had significantly higher yield (1.4 kg/plant) than any other entry.

Anther culture of the SH somatic hybrids: From 10 flower buds (50 anthers) of SH1 incubated in anther culture medium, 33 embryos developed and were plated on regeneration medium, and seven embryos regenerated into plants. Four of the SH1 anther culture regenerants were analyzed by flow cytometry and all were identified as hexaploid. From 18 flower buds (90 anthers) of SH2 incubated in anther culture medium, 418 embryos developed and were plated on regeneration medium, and 22 embryos regenerated into plants. Eight of the SH2 anther culture regenerants were analyzed by flow cytometry and all were identified as triploid (Fig. 2). From 10 flower buds (50 anthers) of SH2B incubated in anther culture medium, 72 embryos were obtained, and three embryos regenerated into plants. All three of the SH2B anther culture regenerants were identified as hexaploid.

SSR segregation in SH2 and four anther culture-derived triploids: An agarose gel showing amplification of DNA from the somatic hybrid SH2 and four triploids derived from SH2 at two different SSR loci is shown in Figure 3. At the G28WXST locus, two of the four triploids contained only the CP2-103 band; the 13-14 203 band was not present. At the STWIN12G locus, one of the four triploids contained only the CP2-103 band. As expected, SH2 was heterozygous at both of these loci and contained both the CP2-103 and 13-14 203 monoploid bands due to the fact that these monoploids were the fusion parents of SH2. These results were verified twice.

Leptine content of CP2-103, somatic hybrids and progeny: CP2-103 (the high leptine producing monoploid used in the fusion that created SH1, SH2 and SH2B) was derived

from a F_1 hybrid of *S. chacoense* clone C80-1 (high leptines) crossed to *S. phureja*, and produced ALD levels 62% that of C80-1 at the time of this analysis (Fig. 4). The somatic hybrids showed variation in leptine levels, with SH1 and SH2B having ALD levels roughly 24% that of C80-1 whereas SH2 had higher ALD levels almost identical to that of CP2-103 (60% of C80-1 levels). All genotypes in both groups of progeny developed from SH2 (the SH2A and the SH2P progeny) expressed acetylleptinidine. On average, the SH2A progeny expressed lower ALD than the SH2P progeny. The highest ALD-producing of the SH2A progeny (SH2A#9) had levels only 28% that of SH2. In contrast, the highest ALD-producing of the SH2P progeny (SH2P#3) had levels 65% that of SH2.

Discussion

The lack of stainable pollen indicating male-sterility of SH1, SH2 and SH2B was likely due to the hexaploid nature of these hybrids. Ehlenfeldt and Helgeson (1987) produced tetraploid and hexaploid somatic hybrids through protoplast fusion of *S. tuberosum* and *S. brevidens* clones. The tetraploid somatic hybrids had pollen stainabilities ranging from 0 to 83%. The hexaploid somatic hybrids; however, had low pollen stainabilities ranging from 0 to 23%. The greater male-fertility of the tetraploid somatic hybrids could be explained by meiotic analyses of buds. Roughly 55% of the dyads in tetraploid buds had unassociated chromosomes, whereas 86% of the dyads in hexaploid buds had unassociated chromosomes. The unassociated chromosomes had a detrimental effect on pollen tetrad formation. The tetraploid hybrids had 41% normal tetrad formation, whereas the hexaploid hybrids had only 4% normal tetrad formation. It is probable that SH1, SH2 and SH2B also have many unassociated chromosomes at meiosis, and therefore suffer from reduced fertility. The fact that less than 3% of the pollinations done on the somatic hybrids resulted in fruit set indicates the low female fertility of these plants. The high ploidy of the somatic hybrids probably had a similarly detrimental effect on megaspore production as on pollen production.

The successful cross of SH2 and SH2B to *S. andigena* 8-1 was useful for analyzing fertility of the somatic hybrids, but as expected the progeny expressed many undesirable characteristics of the *S. andigena* parent and are therefore not useful for introgression of leptines into cultivated potato germplasm. The successful cross of SH2 to *S. phureja* 66AP11-53 was more desirable, as this clone was selected for superior field performance, and the progeny displayed many desirable agronomic traits such as a small number of large tubers, and vigorous growth in the greenhouse.

The increased fertility of the somatic hybrid progeny relative to the somatic hybrids may have been due to the lower chromosome number of these plants, which approached that of tetraploid cultivated potato. The fertility of the SH2A progeny was nearly double that of the somatic hybrids, with 7% of all pollinations resulting in fruit formation. When the SH2P progeny were crossed as the male parent to cv. Atlantic, 16% of all pollinations resulted in fruit set.

Somatic hybrids produced through the fusion of monoploid clones have often not been of the expected diploid level because of a commonly occurring phenomenon in plant cells termed endomitosis (Bhojwani and Razdan 1983). Endomitotic plant cells occur when chromosomes divide without subsequent cellular division. When this process occurs in monoploid plants, it results in hemizygous 1*x* cells, and homozygous 2*x*, 4*x* and 8*x* cells present in a monoploid plant. More than half of the cells in monoploid plants have been observed to be at the diploid or higher level (Figure 6, Chapter 2), and therefore the combination of two 1*x* protoplasts from different monoploid fusion partners during protoplast fusion is quite rare. Potato intermonoploid protoplast fusions were performed previously by Uijtewaal et al. (1987), who regenerated only tetraploid somatic hybrids. The fusion of a tetraploid protoplast from one monoploid parent with a diploid protoplast from the other monoploid parent is a likely explanation for the hexaploid nature of SH1, SH2 and SH2B. Alternatively, a triploid fusion product ($2x + 1x$) may have doubled during the *in vitro* regeneration procedure to yield the hexaploid somatic hybrids.

The poor performance of the somatic hybrids in the field trial of 1997 may have been due to their high ploidy (hexaploid). The somatic hybrids appeared phenotypically abnormal in the field, with stunted stems and internodes, and short, thick, fleshy leaves. As ploidy increases, the amount of DNA that must be replicated per cell division also increases, often resulting in anatomical abnormalities and slow, weak growth. Increasing ploidy also increases cell size; hence thicker and wider leaves, and thicker branches and stems with shortened internodes (Sanford 1983).

All of the somatic hybrids regenerated plants through anther culture, even though they appeared to be male-sterile. This finding provides additional evidence that fertile pollen is not necessary for embryogenesis of microspores through anther culture (Snider and Veilleux 1994). The ploidy of plants obtained from anther culture gives an indication of microspore development of the donor plant because the plants regenerated from what would have become a male gamete (disregarding chromosome doubling of plants during tissue culture regeneration). SH2 is capable of reductional meiosis, due to the fact that triploid plants were obtained from anther culture. SH1 and SH2B, on the other hand, regenerated only hexaploid plants and therefore appeared to have mostly unreduced microspores. SH2 and SH2B developed from the same hybrid callus; however, there appeared to be large differences in their microspore development. This finding highlights the need for regeneration of many different somatic hybrids from a single protoplast fusion, particularly if the hybrids are to be used in sexual crosses. The higher female fertility of SH2 (compared to SH1 and SH2B) may be due to the formation of triploid gametes, which would be expected to be more viable than hexaploid gametes.

The segregation of SSRs in a sample of four triploids regenerated from SH2 suggest that a tetraploid CP2-103 (the high leptine-producing monoploid parent) protoplast fused with a diploid 13-14 203 (the *S. phureja* monoploid parent) protoplast to form the somatic hybrid. If SH2 consists of four CP2-103 genomes and two 13-14 203 genomes, then roughly 20% of the triploids by anther culture of this hybrid (assuming random chromosomal segregation at Anaphase I) should possess only the CP2-103 SSR allele at

any SSR locus polymorphic between CP2-103 and 13-14 203 due to segregation of the 13-14 203 allele (Figure 5). Likewise, if SH2 consists of four genomes 13-14 203 and two genomes CP2-103, then the opposite result would be true.

Two of the four triploids possessed only the CP2-103 band at the G28WXST locus, and one of the triploids possessed only the CP2-103 band at the STWIN12G locus. None of the monoploids possessed only the 13-14 203 band at either locus. The high frequency of 13-14 203 band loss provides strong evidence that SH2 contains only two copies of the 13-14 203 genome. Analysis of SSR segregation with more triploid genotypes derived from SH2 would enable testing of ratios to determine more definitively the genetic makeup of this somatic hybrid. The observation of SSR segregation at two different SSR loci with only four different triploid genotypes indicated a higher number of homologous CP2-103 genomes in SH2 compared to 13-14 203. This conclusion was made without using the costly techniques of FISH or GISH.

The ALD content of SH2 supports the conclusion made from SSR analysis that SH2 consists of four genomes CP2-103 and two genomes 13-14 203, and suggests an additive effect of the genes involved in ALD production. Veilleux and Miller (1998) found that some individuals in a potato backcross population, comprised of diploid genotypes both heterozygous and homozygous for the leptine production genes, had much higher ALD levels compared to a population comprised of only genotypes heterozygous for the leptine production genes. The homozygous individuals in the backcross population may have been capable of higher ALD production (compared to heterozygotes) due to the presence of two leptine-producing alleles in their genomes. SH2 has higher than expected ALD levels in stems and leaves (7,256 μg/g dry wt.) and expresses 60% of the ALD in the diploid, high-leptine producing *S. chacoense* clone (80-1) from which its leptine production genes were derived. Four copies of the CP2-103 leptine production genes with an additive effect in the genome of SH2 would explain why this somatic hybrid produces unusually high leptine levels. If the opposite were true, and SH2 was composed of four genomes 13-14 203 and two genomes CP2-103, then one would expect the

somatic hybrid to produce very little ALD. In addition, some sexual progeny derived from SH2 would not inherit any leptine-production genes, and thus would not express ALD.

All of the progeny in two populations produced through sexual crosses of SH2 (SH2A and SH2P progeny) had at least some ALD production (Fig. 4). Therefore, the genes controlling ALD production appear to be dominant in action. Sanford et al. (1996) concluded that leptine synthesis was controlled by a few dominant genes after analyzing leptine production in a *S. chacoense* × *S. phureja* hybrid population. The presence of some relatively high ALD-producing progeny (3500-4000 µg/g dry weight) and many low ALD-producing progeny (roughly 1000 µg/g dry weight) in the SH2P progeny, with few intermediate level progeny, also suggests one or a few genes controlling ALD production. Based on the genomic composition of SH2, one could expect 20% of the progeny to inherit three copies of the ALD gene(s) from the somatic hybrid parent (Fig. 5), and these may account for the high ALD-producing progeny.

The SH2A progeny produced lower levels of ALD compared to the SH2P progeny (average of 1,320 vs. 2,165 µg/g dry weight). The low ALD levels in the SH2A progeny may have simply been due to the choice of the male parent in the cross—*S. andigena* clone 8-1. Sanford et al. (1996) found that the choice of the male parent in sexual crosses of *S. tuberosum* to *S. chacoense* had a significant effect on the leptine production ability of progeny.

Higher expression of ALD in the SH2P progeny compared to the SH2A progeny can also be explained by the genetic contribution of the male parents used to create these two populations. The SH2P progeny resulted from crossing a diploid *S. phureja* clone to the hexaploid SH2. Assuming normal meiosis, 1*x S. phureja* gametes fused with 3*x* SH2 gametes, producing tetraploid (4*x*) progeny. These progeny would therefore inherit 75% of their genetic material from the somatic hybrid. The SH2A progeny resulted from crossing a tetraploid *S. andigena* clone to the hexaploid SH2. Again assuming normal

meiosis, 2*x* *S. andigena* gametes fused with 3*x* SH2 gametes to produce pentaploid (5*x*) progeny. These progeny would therefore inherit 60% of their genetic material from the somatic hybrid. In conclusion, because the SH2P progeny possess more of the SH2 genetic material in their genomes and less genetic material from a non-leptine producing male parent (compared to the SH2A progeny), they would be expected to have more expression of ALD.

Cheng et al. (1995) used protoplast fusion to combine a diploid leptine-producing *S. chacoense* clone with a *S. tuberosum* dihaploid clone. Several somatic hybrids were regenerated expressing the leptine aglycone, as determined by gas chromatography. These hybrids averaged a threefold reduction in feeding by the Colorado potato beetle compared to *S. tuberosum* during *in vitro* insect bioassays, and had pollen fertilities ranging from 38-57%. The *S. chacoense* clone utilized by Cheng et al. (1995) was a heterozygous diploid and thus may contain only one, dominant, leptine-producing gene. If so, some sexual progeny produced by crossing the *S. chacoense* + *S. tuberosum* somatic hybrid to other potato clones would not be expected to produce leptines, due to segregation of the leptine gene. This limits the usefulness of the somatic hybrid to a breeding program, particularly if the hybrid expresses low fertility (as many somatic hybrids do). An advantage to working with monoploid genotypes over heterozygous diploids is that the plants are hemizygous and thus express all genes present in the genome. A high-leptine producing monoploid would contribute only high-leptine producing genes to a somatic hybrid, and therefore all progeny from such a hybrid should produce leptines.

The finding that acetylleptinidine (ALD) production genes could be transferred from an intermonoploid somatic hybrid (SH2) to progeny populations demonstrates the feasibility of transferring this desirable trait to cultivated potato germplasm. Also encouraging was that certain progeny displayed ALD levels 65% that of the original somatic hybrid. Although the levels of ALD detected in the progeny populations were not sufficient to deter feeding by the Colorado potato beetle (7900 μg/g dry weight are needed to suppress

feeding by 50%), intercrosses among the highest ALD expressing progeny may lead to hybrids with ALD expression equal to or greater than the SH2 somatic hybrid.

In summary, SH1, SH2 and SH2B have contributed substantial information towards the application of somatic hybrids to potato breeding programs. Crossing to various potato clones has shown that intermonoploid somatic hybrids can be successfully crossed to cultivated forms of potato, and that traits of interest, such as leptines, can be transmitted from the somatic hybrid to progeny at sufficient levels to be useful for development of cultivars. The ploidy of plants crossed to somatic hybrids, as well as the genotype of the male parent, can affect the expression of traits in the progeny. A means of investigating the genomic composition of somatic hybrids that are of a higher than expected ploidy without the use of costly DNA hybridization techniques has been introduced and applied successfully to one of the hybrids (SH2). This method can be used with all codominant molecular markers, such as SSRs and AFLPs, and can be used with both interspecific and intraspecific somatic hybrids. Finally, the hexaploid nature of these somatic hybrids appeared to be detrimental to field performance, and thereby stressed the need for intermonoploid hybrids of a lower ploidy (such as the SH2 triploids) for use in the monoploid sieve field study.

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Table 1: Potato clones used as male parents in sexual crosses to the hexaploid somatic hybrids SH1, SH2 and SH2B during the Fall of 1996.

Name of Clone	**Ploidy**	**Genetic Background**
66AP11-53	Diploid (2*x*)	*S. phureja* field selection
CP2	Diploid (2x)	*S. chacoense* × *S. phureja* hybrid
1-3 516 × ID8	Diploid (2x)	*S. phureja* clone
ID5	Diploid (2x)	Complex hybrid (*S. phureja, S. tuberosum, S. stenotomum, S. chacoense*)
S. andigena 8-1	Tetraploid (4x)	*S. andigena* clone
'Atlantic'	Tetraploid (4x)	*S. tuberosum* cultivar
'Red Lasoda'	Tetraploid (4x)	*S. tuberosum* cultivar

Table 2: Results of crossing the SH somatic hybrids to various diploid and tetraploid clones. The female parents in bold are those which were involved in crosses that set fruit.

Female Parent	**Male Parent**	**Number of Pollinations**	**Number of Fruit**	**Number of Seeds**
SH1, **SH2**, SH2B	66AP11-53	21	2	66
SH1, SH2, SH2B	CP2	15	0	0
SH1, SH2, SH2B	1-3 516 × ID8	17	0	0
SH1, SH2, SH2B	Red Lasoda	28	0	0
SH1, SH2, SH2B	Atlantic	10	0	0
SH1, **SH2**, **SH2B**	*S. andigena* 8-1	22	2	35
SH1, SH2, SH2B	ID5	23	0	0

Table 3: Results of crossing seven SH2A progeny (resulting from SH2 × *S. andigena* 8-1) with the cv. Atlantic. The number of flowers produced by each plant determined how many crosses were performed. Each fruit indicates a successful pollination. The first plant listed is the female parent; reciprocal crosses are grouped together.

Cross	**Number of pollinations**	**Number of fruit**	**Number of seeds**
SH2A#1 × 'Atlantic'	3	0	0
SH2A#2 × 'Atlantic'	24	0	0
'Atlantic' × SH2A#2	2	0	0
SH2A#4 × 'Atlantic'	1	1	85
SH2A#5 × 'Atlantic'	12	1	45
SH2A#6 × 'Atlantic'	13	0	0
SH2A#7 × 'Atlantic'	2	0	0
SH2A#8 × 'Atlantic'	2	2	130
SH2A#15 × 'Atlantic'	19	0	0
'Atlantic' × SH2A#15	6	2	91

Table 4: Results of crossing seven SH2P progeny (resulting from SH2 × *S. phureja* 66AP11-53) with cv. Atlantic. The number of flowers produced by each plant determined how many crosses were performed. Each fruit indicates a successful pollination. The first plant is the female parent, reciprocal crosses are grouped together.

Cross	**Number of pollinations**	**Number of fruit**	**Number of seeds**
'Atlantic' × SH2P#1	1	1	48
'Atlantic' × SH2P#10	7	1	6
SH2P#14 × 'Atlantic'	16	0	0
'Atlantic' × SH2P#17	7	3	54
SH2P#17 × 'Atlantic'	12	0	0
'Atlantic' × SH2P#25	10	2	5
SH2P#25 × 'Atlantic'	6	0	0
'Atlantic' × SH2P#29	8	0	0
'Atlantic' × SH2P#37	10	0	0

Table 5: Tuber yield of entries in the 1997 somatic hybrid field plot, RCB design, 3 blocks, 10 plants of each clone per block.

Clone	Average tuber number per plant	Average tuber weight (g)	Total tuber yield (kg) per plant
SH1	36.9 ± 8.5 a[1]	7.9 ± 0.4 a	0.3 ± 0.1 a
SH2	31.4 ± 11.5 a	15.7 ± 2.1 a	0.5 ± 0.2 ab
SH2B	37.3 ± 13.6 a	7.1 ± 1.6 a	0.3 ± 0.2 a
Atlantic	20.6 ± 3.0 abc	66.4 ± 5.5 b	1.4 ± 0.3 c
ID5	13.5 ± 0.8 bc	62.7 ± 4.9 b	0.8 ± 0.1 b
66AP11-53	6.0 ± 1.4 c	118.4 ± 10.1 c	0.7 ± 0.2 b

[1]Means within columns followed by the same letter are not significantly different by Ryan-Einot-Gabriel-Welsch multiple range test, 5% level.

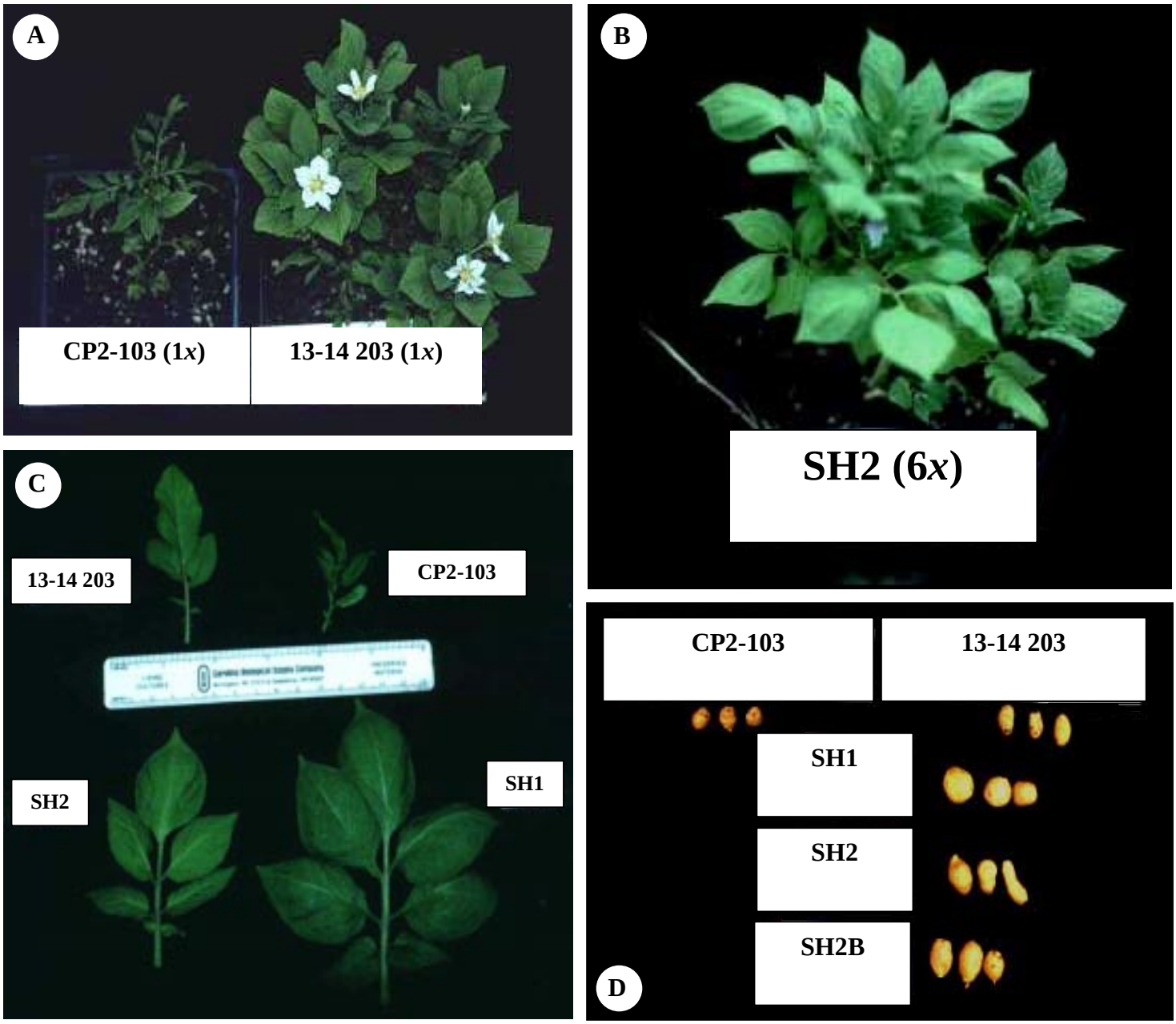


Figure 1: Morphological characterization of two potato monoploid (1*x*) genotypes, 13-14 203 and CP2-103, and two hexaploid (6*x*) somatic hybrids, SH1 and SH2, derived from protoplast fusion of leaf and stem protoplasts isolated from the monoploids. (A) Greenhouse plants of the two monoploid genotypes used for protoplast fusion. CP2-103 is a monoploid derived from an F_1 hybrid between *S. chacoense* and *S. phureja* whereas 13-14 203 is a monoploid derived from a *S. phureja* clone. (B) A greenhouse plant of the hexaploid somatic hybrid SH2 derived from fusion of 13-14 203 and CP2-103 protoplasts. (C) Leaf morphology of the two monoploids (top) and the somatic hybrids (bottom). (D) Greenhouse tubers of the monoploids (top) and the somatic hybrids (bottom).

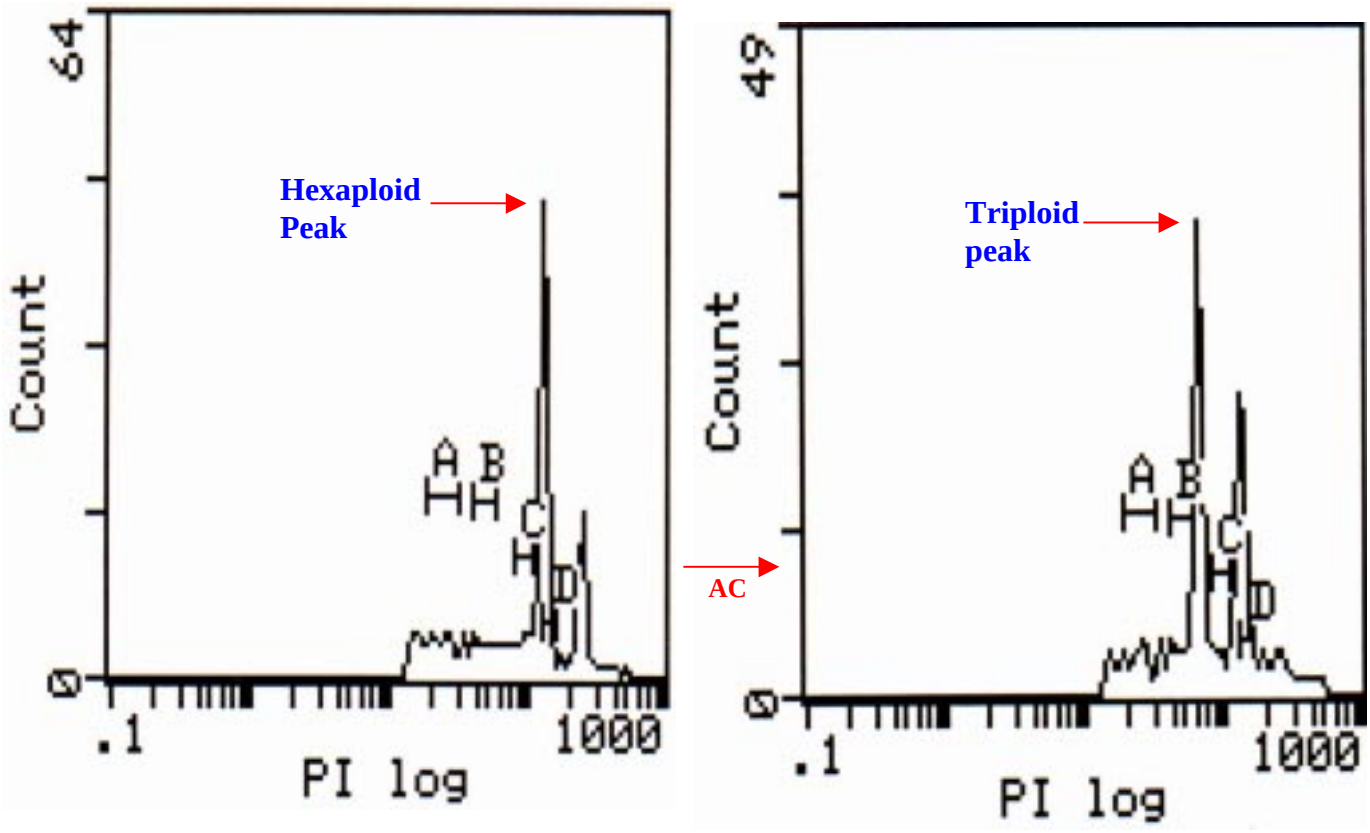


Figure 2: Flow cytometric histograms showing the hexaploid nature of SH2 (left histogram) compared to the triploid nature of an anther culture regenerant (right histogram). These histograms indicate that anther culture reduced the ploidy of SH2 by half.

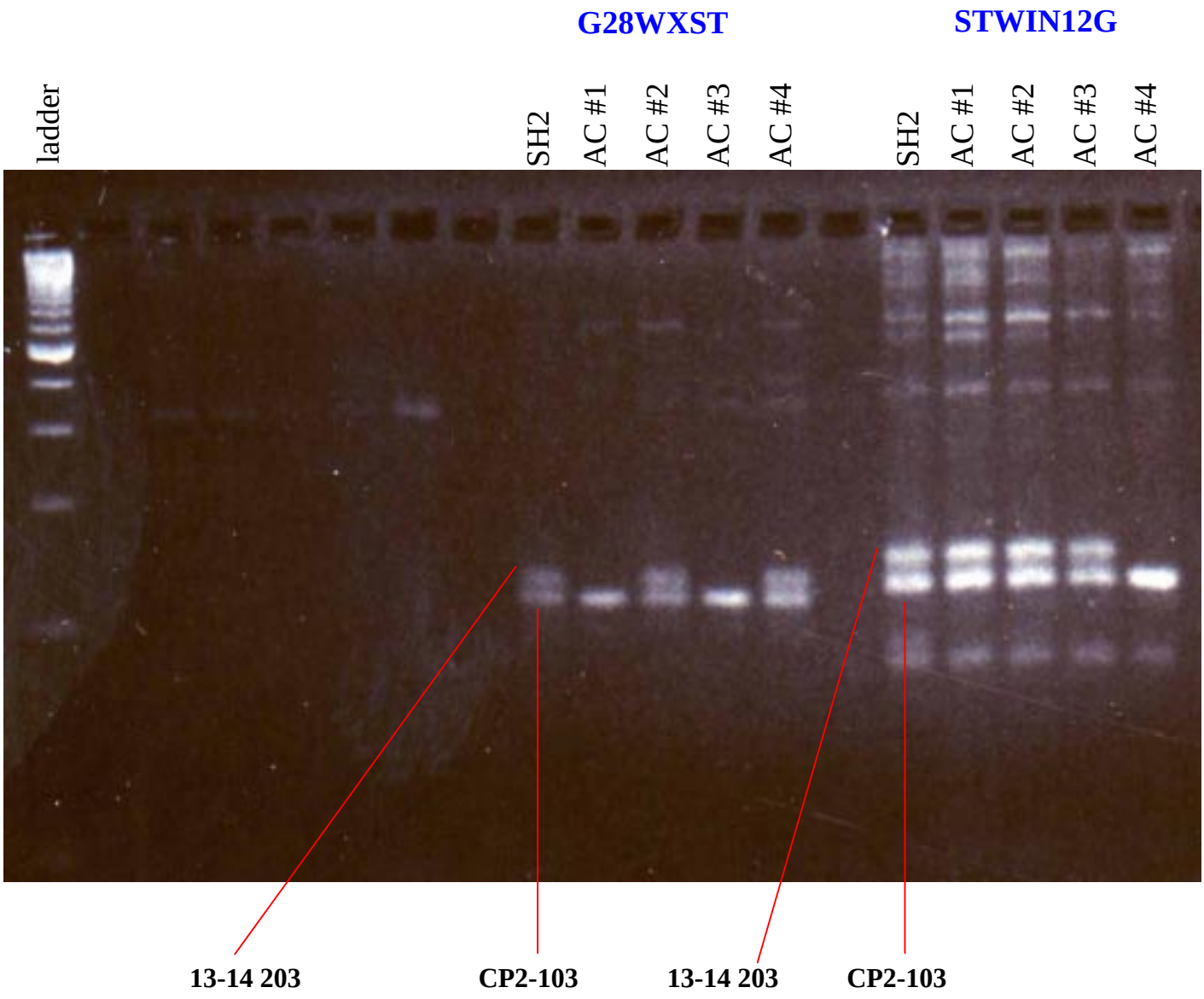


Figure 3: SSR analysis of the hexaploid somatic hybrid SH2 and four triploids developed through culture of SH2 anthers. The two parental bands of the monoploid clones that were combined to create SH2 are highlighted at both loci. At both loci, individuals can be seen lacking the 13-14 203 band. The SSR locus names are highlighted in blue above the respective lanes.

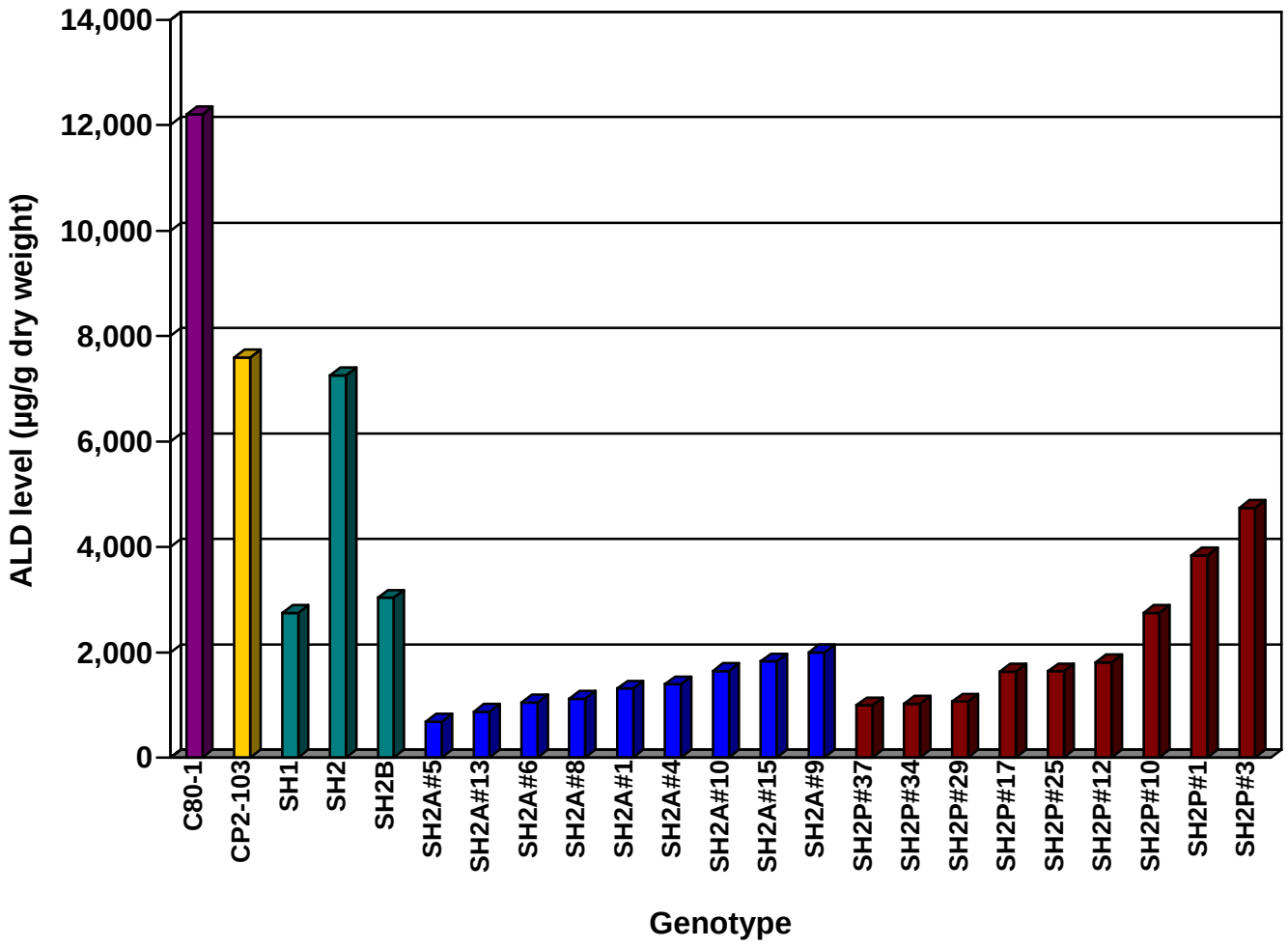


Figure 4: Gas chromotography analysis of acetylleptinidine (ALD) content in C80-1 (high leptine-producing *S. chacoense* clone, purple bar), CP2-103 (high leptine-producing monoploid derived from an F_1 hybrid of C80-1 crossed to *S. phureja*, orange bar), SH1, SH2 and SH2B (hexaploid somatic hybrids from fusion of CP2-103 to a *S. phureja* monoploid, green bars) and selected SH2 progeny (SH2A = SH2 × *S. andigena* 8-1, blue bars; SH2P = SH2 × *S. phureja* 66AP11-53, red bars). ALD was extracted from roughly 5 g of freeze-dried greenhouse-grown leaves and stems. The ALD and leptine levels are in units of µg/g dry weight.

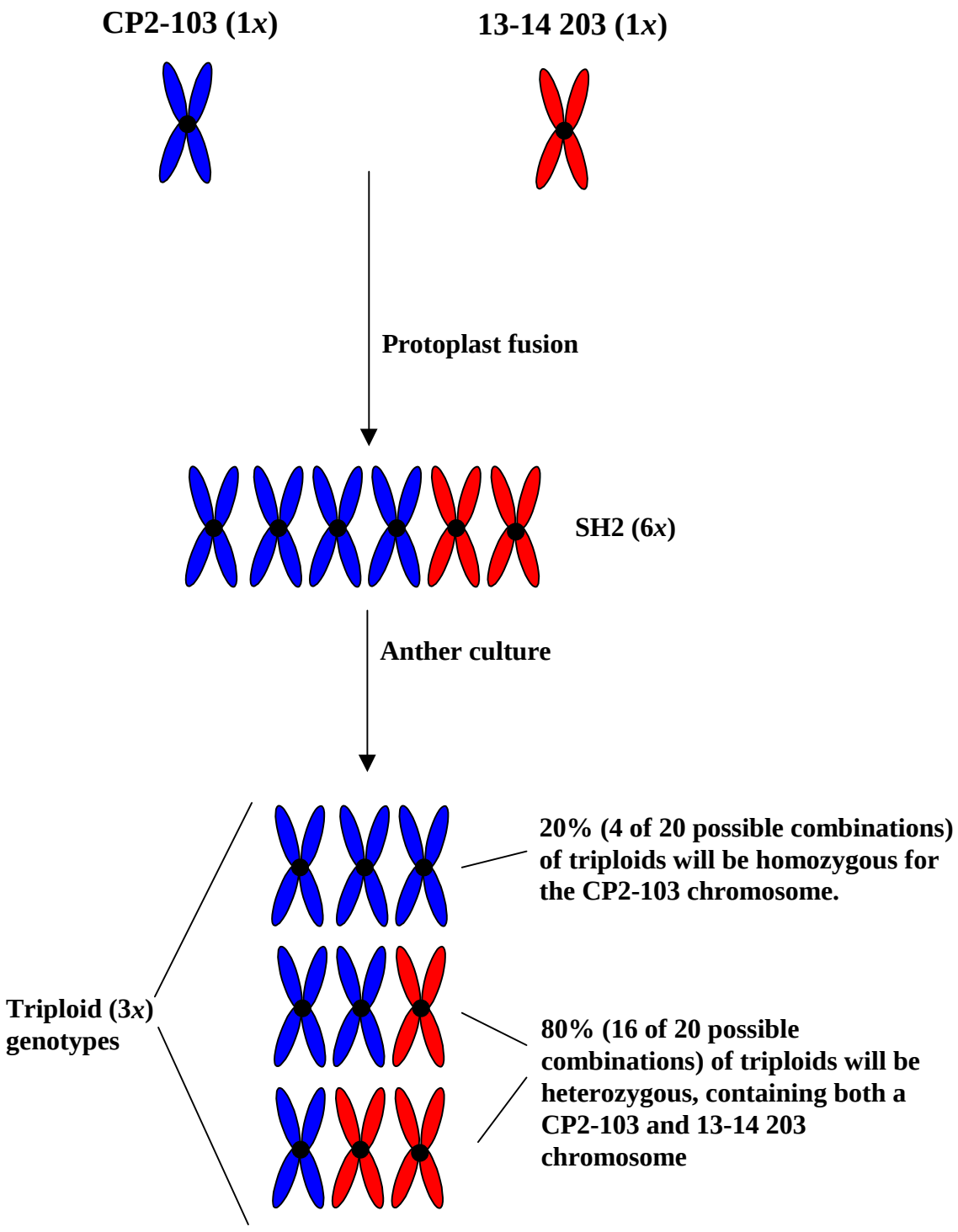


Figure 5: Expected segregation of any one of the 12 potato chromosome sets in SH2 (a hexaploid somatic hybrid produced through protoplast fusion of two monoploids) to triploid genotypes through anther culture; assuming SH2 is comprised of 4 genomes CP2-103 and 2 genomes 13-14 203. An SSR locus located on this chromosome, which was polymorphic between CP2-103 and 13-14 203, would be homozygous for the CP2-103 allele in 20% of the triploid genotypes due to loss of the 13-14 203 allele.

Appendix A: SAS General Linear Models analysis of monoploid field plot data (SAS 1996)

ANOVA of monoploid vigor rating (1996)

Source	df	SS	MS	F-value	Pr > F
Genotype	111	328.3	3.0	4.5	0.0001
Rep	2	1.5	0.8	1.1	0.3228
Exp. Error	222	146.5	0.7		
Total	335	476.3			

ANOVA of tuber number (1996)

Source	df	SS	MS	F-value	Pr > F
Genotype	111	121832.7	1097.6	8.19	0.0001
Rep	2	94.0	47.0	0.35	0.7046
Exp. Error	222	29761.3	134.1		
Total	335	151687.0			

ANOVA of total tuber yield (1996)

Source	df	SS	MS	F-value	Pr > F
Genotype	111	582113.0	5244.3	7.6	0.0001
Rep	2	2183.9	1092.0	1.6	0.2073
Exp. Error	222	152984.0	689.1		
Total	335	737280.9			

ANOVA of average tuber weight (1996)

Source	df	SS	MS	F-value	Pr > F
Genotype	99	1664.2	16.8	3.2	0.0001
Rep	2	0.3	0.2	0.03	0.9721
Exp. Error	147	779.2	5.3		
Total	248	2443.7			

ANOVA of tuber number (1997)

Source	df	SS	MS	F-value	Pr > F
Genotype	109	252557.0	2317.0	7.9	0.0001
Rep	2	772.2	306.1	1.3	0.2685
Exp. Error	218	63625.1	291.9		
Total	329	316954.3			

ANOVA of total tuber yield (1997)

Source	df	SS	MS	F-value	Pr > F
Genotype	109	4811751.3	44144.5	14.4	0.0001
Rep	2	9833.1	4916.6	1.6	0.2036
Exp. Error	218	668494.1	3066.5		
Total	329	5490078.5			

ANOVA of average tuber weight (1997)

Source	df	SS	MS	F-value	Pr > F
Genotype	101	1783.3	17.7	9.85	0.0001
Rep	2	4.6	2.3	1.28	0.2799
Exp. Error	164	294.0	1.7		
Total	267	2081.9			

ANOVA of tuber number (1996 and 1997)

Source	df	SS	MS	F-value	Pr > F
Genotype	33	126632.0	3837.3	11.31	0.0001
Rep(Year)	4	1046.2	261.5	0.77	0.5459
Year	1	4424.0	4424.0	13.04	0.0004
Year*Genotype	33	51834.3	1570.7	4.63	0.0001
Exp. Error	132	44771.2	339.2		
Total	203	228707.6			

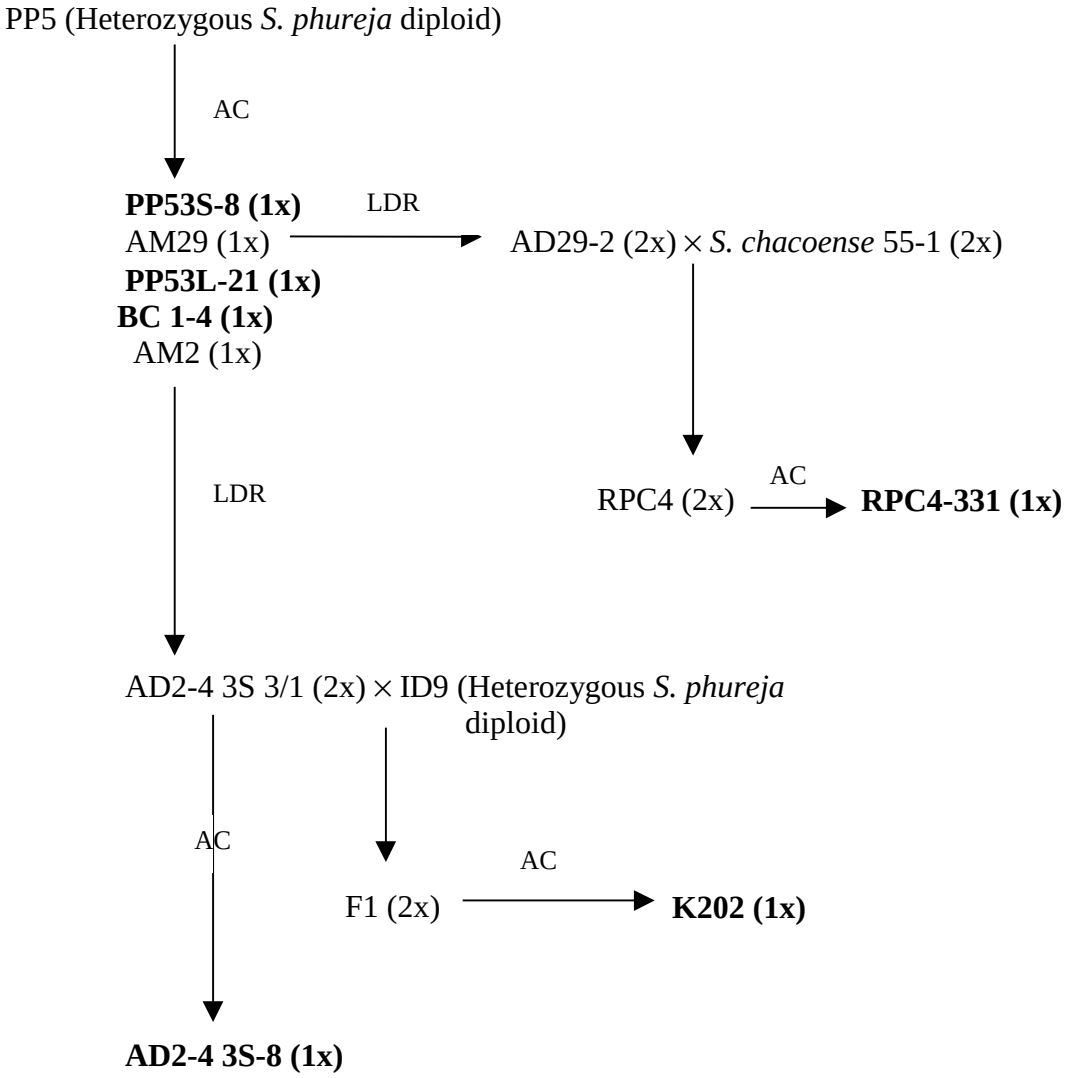
ANOVA of total tuber yield (1996 and 1997)

Source	df	SS	MS	F-value	Pr > F
Genotype	33	578568.8	17532.4	10.21	0.0001
Rep(Year)	4	8232.8	2058.2	1.20	0.3143
Year	1	45531.7	45531.7	26.53	0.0001
Year*Genotype	33	239474.0	7256.8	4.23	0.0001
Exp. Error	132	226583.3	1716.5		
Total	203	1098390.6			

ANOVA of average tuber weight (1996 and 1997)

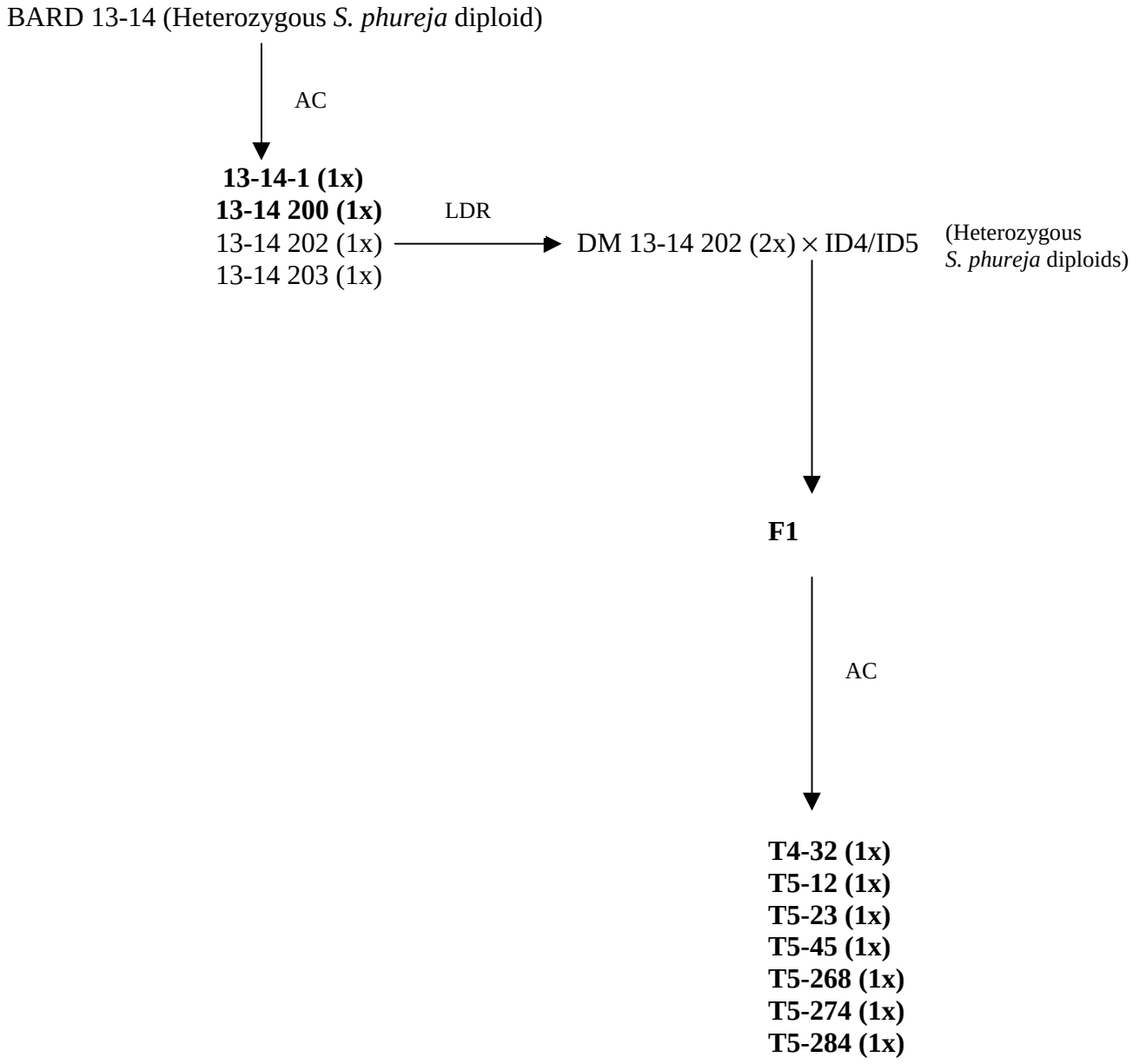
Source	df	SS	MS	F-value	Pr > F
Genotype	33	614.0	18.6	3.12	0.0001
Rep(Year)	4	9.7	2.4	0.41	0.8037
Year	1	8.6	8.6	1.44	0.2323
Year*Genotype	32	302.8	9.5	1.59	0.0406
Exp. Error	113	673.7	6.0		
Total	183	1608.7			

APPENDIX B: Genetic background of the PP5 monoploid population



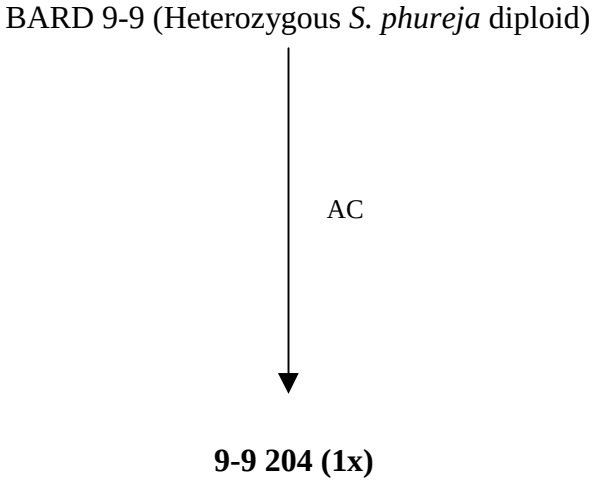
AC = anther culture
LDR = leaf disk regeneration (causing chromosome doubling)
* Selected clones are in bold

APPENDIX C: Genetic background of the 13-14 monoploid population.



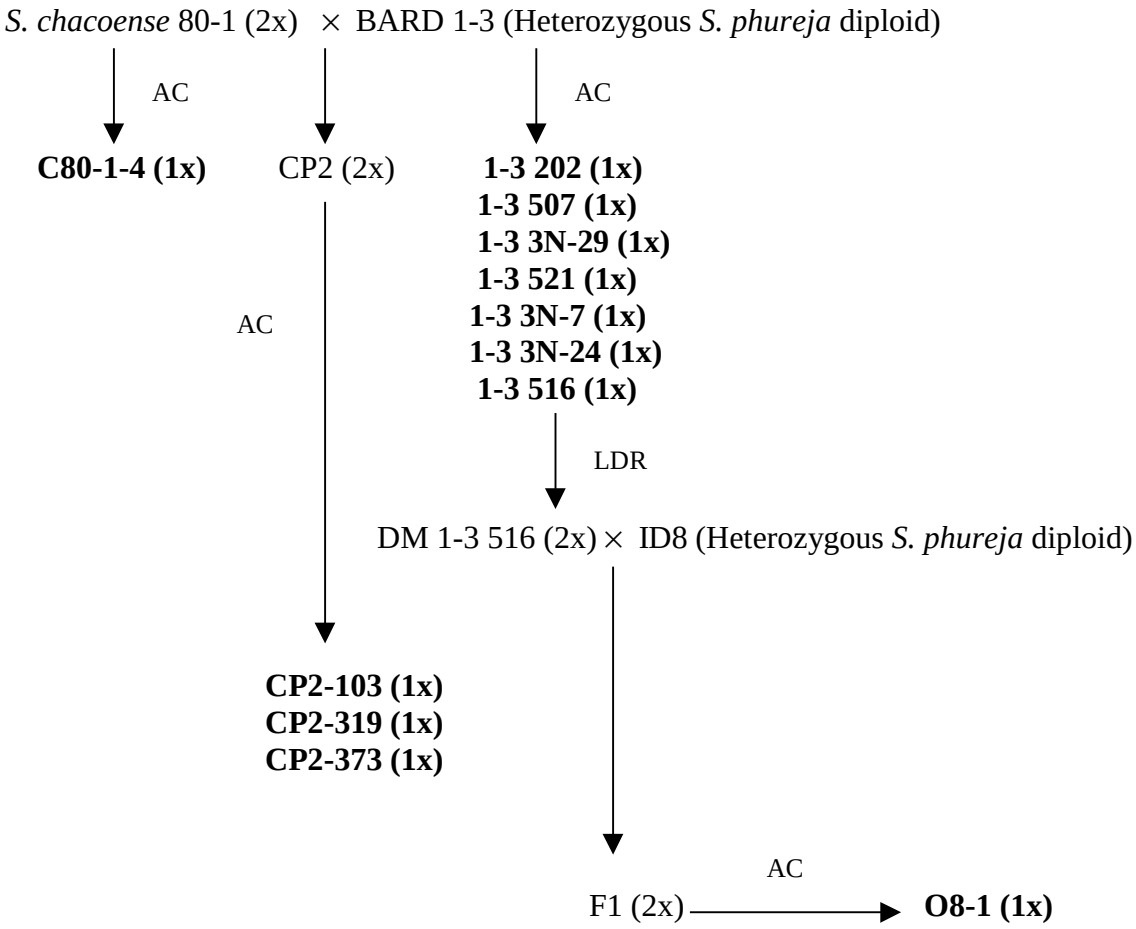
AC = anther culture
LDR = leaf disk regeneration (causing chromosome doubling)
* Selected clones are in bold

APPENDIX D: Genetic background of the 9-9 monoploid population.



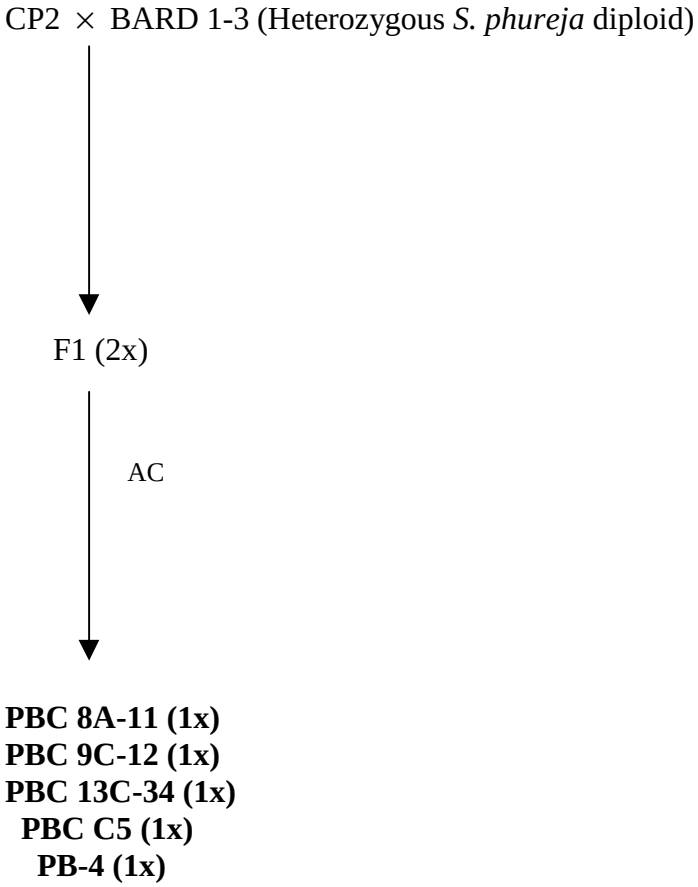
AC = anther culture
LDR = leaf disk regeneration (causing chromosome doubling)
* Selected clones are in bold

APPENDIX E: Genetic background of the 1-3 monoploid population.



AC = anther culture
LDR = leaf disk regeneration (causing chromosome doubling)
* Selected clones are in bold

APPENDIX F: Genetic background of the PBC monoploid population.



AC = anther culture
LDR = leaf disk regeneration (causing chromosome doubling)
* Selected clones are in bold

VITA

Alexander A. T. Johnson
Department of Horticulture
Virginia Polytechnic Institute and State University
Blacksburg, VA 24061-0327

Work Telephone: (540) 231-9835
Email address: aljohns6@vt.edu

Personal History

Born in Alexandria, Virginia on June 16, 1974.

Education

1. J.E.B. Stuart High School, Fairfax, Virginia, Sept. 1988 – June 1992.

2. B.A., Biology, Gustavus Adolphus College, St. Peter, Minnesota, Sept. 1992 – May 1996.

3. M.S., Horticulture, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, Aug. 1996 – Aug. 1998.

4. *Expected*: Ph.D., Horticulture, Virginia Polytechnic Institute and State University, Blacksburg, Virginia, Aug. 1998 – May 2001.

Professional Experience

1. *Employer*: Virginia Polytechnic Institute and State University
   Type of employment: Graduate Teaching Assistantship
   Inclusive dates: Aug. 1996 – May 1997, Aug. 1997 – May 1998

2. *Employer*: Virginia Polytechnic Institute and State University
   Type of employment: Graduate Research Assistantship
   Inclusive dates: May 1997 – Aug. 1997, May 1998 – Aug. 1998

Academic Honors

1. Inducted into The Honor Society of Phi Kappa Phi on November 19, 1997.

2. Awarded a 3-year Cunningham Fellowship by Virginia Polytechnic Institute and State University beginning August 1998.