DEVELOPMENT OF INTERMONOPLOID SOMATIC HYBRIDS OF POTATO AND THEIR MOLECULAR ANALYSIS BASED ON POLYMORPHISM FOR RETROELEMENT *TST1*

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DEVELOPMENT OF INTERMONOPLOID SOMATIC HYBRIDS OF POTATO AND THEIR MOLECULAR ANALYSIS BASED ON POLYMORPHISM FOR RETROELEMENT *TST1*

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Abstract

Inbred lines for hybrid crop production have been a mainstay of plant breeding. Biotechnological approaches to hasten the process are available including anther culture to halve the genome and protoplast fusion to create hybrids between incompatible partners. We applied these techniques to potato to evaluate their potential for breeding highly heterozygous, cross-pollinating species. Four families of monoploids (2n=1x=12), developed from diploid hybrids with diverse genomic constitutions but heavily favoring *Solanum phureja*, a primitive cultivated potato, were used in electrofusion experiments to create intermonoploid somatic hybrids (SH). The "monoploid sieve" results in the survival of only those gametes free of lethal and deleterious genes but generates sterile sporophytes, necessitating protoplast fusion for SH development. From six intermonoploid electrofusion combinations, 276 plants were regenerated over 6-9 months. Fusion conditions were optimized. Ploidy was determined by flow-cytometry and SH confirmed by microsatellite analysis. Field evaluations over three years revealed that intermonoploid SH were inferior to cultivars. Dihaploids derived by anther culture of a tetraploid intermonoploid SH were reduced in vigor with an increase in homozygosity, while 2x X 2x sexually derived populations had better yield than the SH, suggesting that producing SH introduced or eliminated factors required for productivity.

Molecular analysis of the SH was conducted to examine genomic stability through protoplast isolation and plant regeneration. Sequence specific amplified polymorphism (S-SAP) represents a hybrid system incorporating amplified fragment length polymorphism (AFLP) technology in conjunction with the use of a defined genomic sequence, e.g., retrotransposon display (RD) when the defined sequence is anchored into a consensus sequence of a retrotransposon such as the long terminal repeat (LTR) sequence of *Tst1*. Parental monoploids, SH and various Solanaceae were evaluated by

RD. Fluorescently-labeled retrotransposon-based primers were used in the ALFexpress automated fragment analyzer system. Eleven probes from RD were created for Southern blot analysis and used to verify taxonomic relationships between selected Solanaceae. Blots of intermonoploid somatic hybrids confirmed hybridity and occasional loss of genomic fragments. No activation or replication of retrotransposons was detected. Sequencing of inter-retrotransposon amplified polymorphism (IRAP) and S-SAP fragments revealed that all fragments had the expected *Tst1* retroelement and/or the AFLP adaptor sequence. BLAST analysis identified 4 of the 17 fragments sequenced as part of the chloroplast genome, a tobacco anther-specific gene, repetitive DNA, and the phytochrome F gene.

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1. Introduction

Potato is the most important non-cereal crop in the world and the fourth most important world crop, behind wheat, maize and rice (CIP: http://www.cipotato.org/potato/potato.htm). In 2002 the yearly production of potato was about 220 million metric tons, with the trend in global potato production showing a shift from the developed countries (169 million metric tons) to developing countries (138.5 million metric tons), both China (65 million metric tons) and India (24 million metric tons) being major producers. Other major producers include the Russian Federation (32 million metric tons), the United States of America (21 million metric tons) and Poland (15.5 million tons) (FAOSTAT database: http://apps.fao.org/page/collections?subset=agriculture). This shift has been correlated with the introduction of adapted cultivars and major seed production programs (Struik and Wiersema, 1999). Potatoes are grown in different climatic zones, including temperate regions, the subtropics and tropics, as both a lowland and highland crop. Yield of tubers ranges from 7 to 43 tons per ha, with differences depending upon climate and agronomic practices.

The common cultivated potato, *Solanum tuberosum* L. subsp. *tuberosum* (L.), is a tetraploid (2n=4x=48); whereas several Andean cultivated species exhibit ploidy levels ranging from diploid to pentaploid. In addition, there are over 225 related wild types, with a wide distribution throughout much of the Americas and extending into the USA. There is a corresponding physiological diversity as plants have adapted to the extremes of temperature and humidity. Wild potatoes also exhibit a wide range of resistance to fungal, bacterial, and viral diseases as well as insect, arachnid and nematode pests. Insect resistance has been ascribed to glandular trichomes and varying levels of alkaloid content in the leaves (Hawkes, 1990).

The use of protoplasts in potato breeding has opened new avenues for research and germplasm development. The main aim was to surmount the tedious and cumbersome hurdles of traditional breeding, such as tetrasomic inheritance, inbreeding depression and sterility in hybrids. Protoplast fusion of contrasting species separated by sexual barriers

could be used as a bridge to introduce economically important traits. Shepard *et al.* (1977), were the first to achieve large-scale success in the essential first step of plant regeneration from protoplasts. Protoplast regeneration provided opportunities for cytoplasmic substitution, *in vitro* selection for resistance to pathogen-derived toxins or environment stress, and mutation selection for improved characteristics (Grun *et al*., 1987). The initial protoplast regeneration protocols and media were quickly modified as more reliable methods were generated, e.g., Foulger and Jones (1986) found that enhancing the $CaCl_2$ level in recalcitrant genotypes could ameliorate genotypic differences observed in regeneration capacity. At the genetic level, Cheng and Veilleux (1991), working with diploid *S. phureja* Juz & Buk., proposed that the culturability of protoplasts, i.e., the ability of protoplasts to develop into calli, is controlled by two independent loci with complete dominance. This could be used as a selection scheme for somatic hybrids subsequent to protoplast fusion based on genetic complementation, where one partner is recessive for the culturability trait while the other is dominant.

1.1. Time line leading up to electrofusion

The first reported case of protoplast fusion with successful regeneration of a hybrid was by Carlson *et al.* (1972) who obtained the first parasexual interspecific hybrid plant between *Nicotiana glauca* Grah. and *N. langsdorffii* Weinm. Successful regeneration of protoplasts of *S. tuberosum*, a vital prerequisite to fusion, was achieved in the 1970's (Binding and Nehls, 1977; Binding *et al*., 1978; Shepard and Totten, 1977). Modification in regeneration medium for *Solanum* species (SKM) that allowed plating efficiencies at low density (down to 80 cell/ml) has simplified protocols (Hunt and Helgeson, 1989). The first application of electrofusion for the production of somatic hybrids in potato, *S. tuberosum* with *S. phureja*, was by Puite *et al.* (1986).

1.2. Chemical fusions

Early success with protoplast fusion involved chemical treatments consisting of 0.25 M sodium nitrate with centrifugation (Carlson *et al*., 1972; Keller and Melchers, 1973).

Polyethylene glycol and calcium chloride at high pH, to supplant the single nitrate treatment, have been the main agents for chemical fusions (Kao and Michayluk, 1974). Protoplast gel fusion, in which protoplasts have been embedded in calcium nitrate agarose gel droplets have also proved successful (Binding *et al*., 1988).

1.3. Electrofusion

Zimmermann (1981; 1982) envisioned the process of electrofusion. Electrofusion consists of the combination of (1) the process of dielectrophoresis of charged particles and (2) that of reversible membrane disassembly. The application of an initial alternating voltage field (AC current) results in the formation of pearl-like chains of aligned protoplasts displaying high membrane contact. Fusion is induced by a short high direct current pulse (0.5-10 kV/cm and pulse length 3-80 μsec), which results in temporary membrane disruption at the point of adjoining membranes.

De Vries and Templaar (1987) give a concise overview of fusion conditions. A large gap (>5 mm) electrode system can process samples of a few million protoplasts, which during the fusion process are not attracted to the electrodes, allowing for greater survival. On the other hand a small system is ideal for experimental usage where protoplasts are at a premium and are difficult to obtain and/or maintain. The fusion settings are also critical; increasing the voltage/cm of the fusion pulse and the pulse duration results in a larger numbers of protoplasts per chain, increasing the overall fusion percentage; however, there is a greater chance of multiple fusions. The source of protoplasts is also important. Suspension culture protoplasts that are the same size as leaf protoplasts tend to yield higher fusion rates; this may be due to a difference in vacuolar volume.

The start of the fusion process is quite synchronous. The more delicate protoplasts tend to fuse first. The products of fusion may sphericalarize in a few seconds to a few minutes; the presence of ions, such as Ca^{2+}, and whether the vacuoles fuse initially are factors that may affect the process. Generally the medium should have low conductivity, with a solution of mannitol normally used. The addition of spermine may further

increase contact between protoplasts, resulting in an increase in the fusion yield. Protoplast suspensions should be free of debris and the protoplasts should be swollen by means of a suitable osmolarity of the medium.

1.4. Comparison between electro-fusion and chemo-fusion

Having regenerated hybrids from dihaploid *S. tuberosum* and diploid *S. brevidens* Phil. via chemical fusion, Fish *et al.* (1988a) demonstrated, using the same fusion partners, that the new technique of electrofusion was more productive, less time consuming, with reduced levels of protoplast damage. In addition, the technique was amenable to rapid optimization of conditions. Fusion frequencies of 20-30% were achieved.

Similarly, having produced hybrids between *S. tuberosum* and frost tolerant wild type *S. commersoii* Dunal by chemical fusion and using a two step-regeneration protocol, Cardi *et al.* (1993b) compared similar hybrids obtained by electrofusion of mesophyll protoplasts of *S. commersonii* with either dihaploid or tetraploid *S. tuberosum* (Cardi *et al*., 1993c). The chemical treatment, polyethylene glycol (PEG) generally resulted in low frequencies of heterofusions and protoplast survival. In contrast, electrofusion using AC/DC conditions optimized by increasing fusion density and $CaCl_2$ concentration enhanced heterofusion frequency by 10% and 30%, respectively.

1.5. Intra-specific somatic fusions

Incompatibilities and sterilities among cultivars of *S. tuberosum* can hamper intraspecific breeding. Somatic fusion can be used to overcome such barriers but has the effect of elevating the ploidy level. This ploidy elevation may be desirable to reconstitute tetraploids from selected dihaploids. Analytic breeding of polyploids, by first reducing the tetraploid chromosome number to the dihaploid level, was first proposed by Chase (1963). After breeding has been conducted at the dihaploid level exploiting simpler segregation ratios and eliminating deleterious genes by imposing a level of inbreeding, the analytic breeder would resythesize tetraploids by sexual polyploidization. However,

breeding at the dihaploid level has been thwarted by reduced fertility and loss of vigor. Wenzel *et al.* (1979) suggested an accelerated dihaploid breeding program by protoplast fusion of selected dihaploids. Reduction of dihaploids to the monohaploid [monoploid] level to eliminate deleterious alleles completely could also be integrated into analytic breeding.

Austin *et al.* (1985a) obtained the first intra-specific fusions in *S. tuberosum* by regeneration of vigorous tetraploid hybrids, confirmed by isozyme analysis, from two diploid *S. tuberosum* "parents." Similarly Debnath and Wenzel (1987) regenerated tetraploids by protoplast fusion between dihaploids, using hybrid vigor of callus as the sole selection criterion. Because the selection system does not guarantee exclusive regeneration of hybrids, the hybrid status of regenerants was confirmed by esterase analysis.

One of the earliest cases of successful intra-specific hybridization via electrofusion was that of dihaploid potato Bf 15 with dihaploids of *S. tuberosum* cvs. Aminca and Cardinal. Somatic hybrids showed vigor (compared to parents), were tetraploid and hybridity was verified by isozymes (Chaput *et al*., 1990).

1.5.1. General and specific combining ability

In an attempt to study the effect of general and specific combining ability at the protoplast level and how this may influence regeneration and vigor of hybrids, Mollers and Wenzel (1992) fused various dihaploid *S. tuberosum* lines. Electrofusion was more efficient than PEG. Certain combinations had greater tuber yield, indicating a specific combining ability effect, while some parental types (BP32) showed good general combining ability. Dominant monogenic traits such as herbicide metribuzin tolerance and resistance gene *Rol* against the nematode *Globodera restochiensis* were fully expressed in the tetraploid somatic hybrids. Field evaluation of these hybrids and hybrids produced by Deimling *et al.* (1988) revealed heterosis in tetraploid fusions compared to dihaploid parents and doubled dihaploid parents (tetraploid protoclones). Hybrids showed a

significant increase in total tuber weight with similar numbers of tubers per plant. Other characteristics such as the tuber color and shape revealed red skin color and yellow flesh were dominant while round tuber shape was dominant to long, as previously shown by de Jong and Burns (1993).

1.5.2. Genetic stability

Somatic hybrids of varied ploidy produced by chemical fusion of dihaploid *S. tuberosum*, selected by dual fluorescent emission staining and confirmed by isozyme analysis (Waara *et al*., 1991) were cytogenetically and phenotypically analysed by Waara *et al*. (1992). Tetraploid and hexaploid somatic hybrids showed loss or gain of chromosomes and chromosomal mutations. Tetraploid somatic hybrids were taller and more vigorous than dihaploid parents. Hexaploid somatic hybrids were grouped into two classes based on the dosage of parent type and could be separated by tuber weight. The octoploid somatic hybrids were all abnormal. Ristriction fragment length polymorphism (RFLP) analysis of the chloroplast DNA revealed that either parental type could be found in the hybrid plants.

Gavrilenko *et al.* (1999) carried out an assessment of the use of intraspecific somatic hybrids for the creation of new material for potato breeding. The aim of the study was to evaluate the genetic stability of the hybrids. Ten dihaploid genotypes were electrofused pairwise in various combinations. Somatic hybrids were identified by isozyme analysis or random amplification of polymorphic DNAs (RAPDs). The majority of hybrids were tetraploid; however, aneuploids (hypotetraploids), higher polyploids and mixoploids were also produced. The tetraploids showed heterosis for tuber yield and restoration of fertility in all combinations. Cytological observations revealed numerous anomalies, including chromosome bridges, fragments and laggards at anaphase, and structural rearrangements of chromosomes. Field evaluation revealed wide phenotypic variation among hybrids. These results were consistent with Mattheij and Puite (1992) and Mollers *et al*. (1994).

1.5.3. Nematode resistance

Intraspecific hybrids have been used to incorporate nematode resistance in re-synthesized tetraploids. CooperBland *et al.* (1994) fused two agronomically good dihaploids, PDH 40 and PDH 417 derived from parthenogenic crosses with *S. phureja*. PDH 417 was resistant to white potato cyst nematode (PCN) *Globodera pallida* (Stone) Behrens pathotypes Pa2 and Pa3 (derived from parent *Solanum vernei* Bitt. et Wittm.). All somatic hybrids were tetraploid. Hybrid status was based on morphological characters and confirmed by isozyme analysis. PCN resistance varied among hybrids but in general the mean was closer to the susceptible parent, PDH 40, revealing that the PCN resistant trait is not a simple dominant gene(s) but suggesting a quantitative additive gene effect. Similar disease resistant expression patterns were obtained for dihaploid hybrid combinations, PDH 40 with PDH 727, produced by CooperBland *et al.* (1996). Newly introduced simple sequences repeats (SSR) analysis was used to confirm these somatic hybrids (Provan *et al*., 1996). The resistance evaluated was to late blight, *Phytophthora infestans* (Mont.) de Bary, which came from dihaploid PDH 727, initially derived from the wild spe*cies S. demissum* Lindley. Comparisons were made between somatic and sexual hybrids with regard to agronomic traits and late blight resistance. Selection of hybrids was based on the totipotency expressed by one fusion partner (PDH 40) where regenerants could be hybrids or somaclones of PDH 40. The tetraploid sexual and somatic hybrids showed hybrid vigor and outperformed dihaploid and doubled dihaploid parental lines and dihaploid sexual hybrids. The fusion hybrids were less variable than the sexual hybrids (2x and 4x). The resistance expressed in the sexual and somatic hybrids was intermediate between the parents, but the range expressed in the sexual hybrids was much wider than that of the somatic fusions, possibly due to meiotic segregation in the sexual progeny. In this and the previous case resistance seemed to be governed by additive genes. This result contrasts with a high degree of resistance expressed in tetraploid interspecific sexual and somatic hybrids produced from diploid *Solanum circaeifolium* Bitter and susceptible dihaploid *S. tuberosum* (Louwes *et al*., 1992; Mattheij *et al*., 1992). This result suggests that the resistance from *S. circaeifolium* is dominant and different from resistance obtained from *S. demissum*. There may also be

differences due to the expression at the interspecific somatic and sexual hybrid levels than at the intraspecific somatic and sexual hybrid levels.

1.5.4. Viral resistance

Dihaploid *S. tuberosum* clones that display dominant alleles for R*x* and R*y*, which confer extreme resistance to potato virus X (PVX) and potato virus Y (PVY), were fused to produce a multiple resistant hybrid (Thach *et al*., 1993). Deviation from the addition of resistance traits was observed in some of the hybrids.

1.5.5. Selection by mutant breeding

Amino acid analogue-resistant cell lines have been developed to aid the selection of somatic hybrids from intraspecific fusions (Devries *et al*., 1987c). Protoplasts were prepared from the cell line aec-1, which was resistant to L-aminoethylcysteine (AEC) an analogue of cysteine, and fused to cell lines 5mt-26 or 5mt-27, both resistant to 5-methyltrytophan (5MT) an analogue of tryptophan and cross-resistant to 3-fluorotyrosin (3FT) an analogue of tyrosine. Fusion products were grown on double selection medium containing AEC + 5MT or AEC + 3FT. The regenerants (aec-1 + 5mt-26) were all somatic hybrids, suggesting dominance of the traits involved. No hybrids were regenerated from aec-1 + 5mt-27, suggesting that the regeneration capacity was affected or somatic incompatibilities were present.

1.6. Inter-specific somatic fusions

Inter-specific hybrids have been conducted with the main objective of transcending sexual (breeding) barriers, with the aim of introducing traits of importance (disease resistance, heat or frost tolerance, insect resistance, novel proteins) from previously-separated sources. Such amphidiploid somatic hybrids are required to be fertile in order to accommodate the need for additional breeding cycles after protoplast fusion. Usually the parental partners consist of an unadapted species with a single desirable trait but many undesirable traits and an agronomically adapted clone lacking the specific trait of

the unadapted species. Unfortunately the resultant amphidiploids will carry the trait of interest along with many undesirable traits. Considerable backcrossing and ploidy reduction are required before agronomically acceptable germplasm into which the desirable trait has been introgressed and the undesirable traits excluded can be realized (Johnson and Veilleux, 2001).

1.6.1. Pathogen resistance

Disease resistance can come from unexpected sources; the previous fusion of *S. brevidens* with tetraploid *S. tuberosum* proved a novel source of resistance to bacterial tuber soft rot caused by *Erwinia* (Austin *et al*., 1987; 1988). The source of the novel resistance, *S. brevidens,* was a diploid non-tuber bearing wild species, and hence could not be evaluated for tuber resistance. This trait was inherited by some of the sexual progeny, which confirmed its genetic control rather than a physiological or nutritional phenomenon. These somatic hybrids and their sexual progeny with *S. tuberosum* cv. Katahdin were subsequently tested for stem soft rot (*Erwinia cartovora* subsp *atroseptica*) resistance (blackleg resistance) and ranged from highly susceptible (A206) to highly resistant (A937, T355-11), (Bains *et al*., 1999). Resistant somatic hybrids, A937 and T355-11, both confirmed sexual transmission to progeny; hence they were useful for development of cultivars with dual resistance to stem and tuber soft rot. In an attempt to study introgression of traits from *S. brevidens* into potato, McGrath *et al.* (1994) analyzed two backcross generations derived from somatic hybrid A206 crossed to Katahdin, (BC_1 and BC_2). Probes that were linked to tomato and potato were found to be syntenic along each of the 12 *S. brevidens* chromosomes. RFLP analysis detected recombination in all *S. brevidens* chromosomes – the absence of one or more RFLP specific marker was assumed to be a recombinant event for that particular chromosome. RAPD markers were also tested and correlated with RFLP data. Taken together, the loss of markers suggested recombination between *S. brevidens* chromosomes via homoeologous pairing with *S. tuberosum* chromosomes. Further studies on the stability of *Erwinia* tuber soft rot resistance, in the susceptible parent, somatic hybrids, BC_1, BC_2, BC_3, BC_4 and BC_3 F_1 (sibling crosses) revealed the highest expression in the somatic

hybrids, segregation in the backcross populations, and stabilization by BC_2 (McGrath *et al*., 2002). In addition, monosomic addition lines for *S. brevidens* revealed that synteny group 12, which corresponds to chromosome 12, appears to carry the gene for *Erwinia* resistance. In parallel study, hexaploid somatic hybrids produced from fusion of *S. tuberosum* cv. White Lady and *S. brevidens* (Polgar *et al*., 1993) were characterized by RAPD markers (Polgar *et al*., 1999). Loss of resistance in somatic hybrids was associated with the loss of three RAPD bands associated with chromosomes 5, 6, 9 and 11 of the *S. brevidens* genome.

Late blight resistance (*P. infestans)* has been reported in *S. nigrum* L. and the closely related *S. villosum* (L.) P.Mill (Colon *et al*., 1993). Earlier chemical fusions performed by Binding *et al.* (1982) of *S. nigrum* and *S. tuberosum,* to introduce atrazine resistance from black nightshade to potato, produced somatic hybrids displaying the trait of interest but successful backcross populations were not reported. A broad set of chemical fusions was carried out between *S. nigrum* and related species with three potato accessions/cultivars (Horsman *et al*., 1997). Fusion combinations consisted of four species of *S. nigrum* complex [*S. nigrum* (6x), *S. chenopodioides* Lam. (2x), *S. chenopodioides*-hygromycin resistant (2x), *S. americanum* P.Mill. (2x, 4x) and *S. villosum* (4x)] combined with three *S. tuberosum* [1029/31 (2x), 1029/31-kanamycin resistant (2x) and Desiree (4x)]. Hybridity of regenerants was confirmed by isozyme analysis and ploidy level by flow cytometry. The fusion combinations were to: (1) evaluate the use of selective markers for increasing efficiency of somatic hybridization, and (2) examine the influence of ploidy of parental types on the production of fusions. The use of kanamycin or hygromycin resistance increased selection of somatic hybrids and had no effect on somatic combining ability. The experiments with *S. americanum* and diploid potatoes were unsuccessful. All other combinations produced somatic hybrids that regenerated *in vitro*; however only the combination with *S. nigrum* survived in the greenhouse. Fusions between *S. nigrum* or *S. villosum* and tetraploid Desiree, by contrast, did not regenerate, possibly due to the high ploidy level of the expected progeny. Of the successful fusions only plants of the combination *S. americanum* (2x) + Desiree grew in the greenhouse. This implied that in addition to ploidy as a restricting

factor, the species or combining ability determined success of fusions. The first successful BC_1 and BC_2 populations were reported by Horsman *et al.* (1999) using the vigorously growing somatic hybrid 6x *S. nigrum* + 2x potato of the previous fusions. Backcrosses were made using both parental types. The first and second backcrosses with *S. nigrum* produced many progeny. Backcrosses with potato had a much lower success rate. Resistance to *P. infestans* in the somatic hybrids and BC_1 populations with potato were equal to the level displayed in *S. nigrum*. Segregation for resistance was observed in the BC_2 population. No BC_3 progeny could be produced. Successful backcross programs have shown that somatic fusions with an excess recurrent genome were more fertile (Garriga-Caldere *et al*., 1997; Horsman *et al*., 1999; Jacobsen *et al*., 1994). Horsman *et al.* (2001) in an attempt to solve the backcross infertility barrier of the *S. nigrum* + 2x potato somatic fusions made adjustments to the genomic composition of BC_1 and BC_2. Two genomes of potato were added to these backcross progeny by somatic fusions. Selection of hybrids was based on ploidy revealed by flow cytometry, with regenerants having DNA content greater than parental types considered as potential hybrids. Genomic in situ hybridization (GISH) analysis was used to confirm the hybrid status; in addition the genomic complement of parental types was confirmed in accordance with the theoretical expected values. These new somatic hybrids were pollinated with 4x potato and seeded berries were produced in a majority of the hybrids. However, extensive backcrossing and embryo rescue will be required before the potential usefulness of this somatic hybrid can be realized.

Verticillium wilt, a serious disease of potato in temperate regions, is produced by the pathogens *Verticillium albo-atrum* Reinke and Berth and *V. dahliae* Kleb. The wild eggplant relative *S. torvum* S.W. has been used successfully as a source of resistance in somatic fusions with *S. melongena* L. (Guri and Sink, 1988; Sihachakr *et al*., 1989). Jadari *et al.* (1992) in an attempt to introduce *Verticillium* wilt resistance into potato also used *S. torvum* as the source of resistance, in fusions with dihaploid *S. tuberosum.* Hybrids were selected based on vigor of calli, and confirmed by isozyme analysis. Hybrids were tetraploid (via root chromosome counts) but rooted poorly and had to be

grafted on rootstock of potato. *In vitro Verticillium* wilt tests revealed all somatic hybrids were resistant.

Resistance to frost and *Verticillium* wilt have been transferred to cultivated potato by fusion of a dihaploid clone of *S. tuberosum* subsp. *tuberosum* and the wild diploid 1 endosperm balance number (EBN) species *S. commersonii* (Bastia *et al*., 2000). Somatic hybrids were confirmed by RAPDs. Southern blot studies on the inheritance of mitochondria and chloroplast genome revealed that chloroplasts were randomly inherited but mitochondrial DNA was preferentially transferred from *S. tuberosum*, displaying a high percentage of intrageneric rearrangements. Hybrid regenerants were female fertile, more similar to the cultivated *S. tuberosum* and easily backcrossed to this parent.

Resistance to blackleg and tuber soft rot by *Erwinia carotovora* subsp. *atroseptia* (Eca) and *E. carotovora* subsp. *cartovora* (Ecc) was examined in sexual hybrids of dihaploid *S. tuberosum* and resistant wild type *Solanum* spp., and tetraploid and hexaploid somatic hybrids of *S. tuberosum* (+) *S. commersonii.* Dual resistance was displayed by the tetraploid somatic hybrid, while the hexaploid clones displayed individual resistance (Carputo *et al*., 1997b). Segregation for resistance to Eca and Ecc was observed in the BC_1 progeny, possibly due to disruption of chromosomes and re-assortment of alleles during meiosis (Carputo *et al*., 2000).

The Mexican wild species *Solanum pinnatisectum* L. is a source of resistance to *P. infestans* and to *E. carotovora* (blackleg). Protoplast fusion between *S. pinnatisectum* (2x, 1 EBN) and four different breeding lines of dihaploid *S. tuberosum* was attempted to introduce both resistances into potato (Schilde-Rentschler *et al*., 1988). Somatic hybrids were identified by RFLP analysis using an oligonucleotide $(GATA)_4$ probe and flow cytometry revealed the majority (75%) of somatic hybrids was tetraploid; however, hypotetraploid and other polyploids were also present (Menke *et al*., 1996). Two 5S rDNA repeat sequences designed from intergenic spacers unique to each species were used to identify symmetric somatic hybrids via Southern analysis (Zanke *et al*., 1995). Molecular analysis revealed preferential loss of the *S. pinnatisectum* genome in most of

the somatic hybrids that were hypotetraploid. Pijnacker *et al.* (1989) reported similar preferential loss of *S. phureja* in fusions between *S. tuberosum* and *S. phureja.*

The wild potato species *S. commersonii* has many biotic and abiotic resistances of interest, including resistance to bacterial wilt disease caused by *Ralstonia solanacearum*. With the intent of introducing this additional source of resistance into potato, Kim *et al.* (1993) developed somatic electrofusion hybrids between *S. commersonii* with both tetraploid and dihaploid *S. tuberosum*; backcross and selfed progeny were evaluated for wilt resistance (Laferriere *et al*., 1999). The fusions produced two somatic hybrid groups (1) tetraploid or hypotetraploid HA with 3 EBN and (2) hexaploid or hypohexaploid HB with 5 EBN (Kim *et al*., 1994). All the somatic hybrids were male and female-fertile (when tested with cultivars Atlantic and Superior) and self-compatible. Disease resistance was similar to that of *S. commersonii*. The fertility of the somatic fusion population facilitates introgression of resistance into potato.

Fock *et al.* (2000) introduced resistance to bacterial wilt *Ralstonia solanacearum* into *S. tuberosum* (dihaploid) via electrofusion with the cultivated diploid, *S. phureja.* In this fusion *S. phureja* showed tolerance to race 1 strain and moderate susceptibility to race 3 strain, whereas the dihaploid fusion parent *S. tuberosum* cv. BF15 was susceptible to both strains. Ten putative hybrids were selected based on morphology, callus vigor and higher ploidy (tetraploid, amphitetraploid, octoploid and mixoploid). SSR analysis between hybrids using primers specific to ctDNA suggested random distribution of parental chloroplast genomes. Resistance evaluation to bacterial wilt races 1 and 3 revealed that hybrids displayed a range of tolerance/susceptibility to race 1 and all tetraploids were susceptible to race 3 strain. Only the amphitetraploid displayed dual tolerance to both strains, with significantly less bacterial titers in stem tissue. Resistance to bacterial wilt *R. solanacearum* was also identified in *S. stenotomum* Juz. & Buk., a species that can be sexually crossed with *S. tuberosum.* However, to expedite introgression of disease resistance traits, Fock *et al.* (2001) conducted somatic electrofusions. The techniques used for selection and confirmations were as above, except genomic SSR markers were used to confirm hybrids. SSR analysis using chloroplast specific primers suggested

random distribution of parental types between the hybrids. *S. stenotomum* and its somatic hybrids showed resistance to race 1 and tolerance to race 3 of *R. solanacearum*. No significant difference was detected between disease indices of the wild parent and the hybrids. Both of the above experiments reveal that introduction of disease resistance traits from wild type into cultivars is possible (Fock *et al*., 2001), and somatic fusions can be used to complement and supplement conventional potato breeding.

Solanum bulbocastanum Dun., a wild, diploid (2n=2x=24) Mexican species, is highly resistant to *Phytophthora infestans*, the fungus that causes late blight of potato. However this 1 EBN species is virtually impossible to cross directly with potato. PEG-mediated fusion of leaf protoplasts of *S. bulbocastanum* PI 245310 and the tetraploid potato line *S. tuberosum* PI 203900 (2n=4x=48) yielded hexaploid (2n=6x=72) somatic hybrids that retained the high resistance of the *S. bulbocastanum* parent (Helgeson *et al*., 1998). RFLP and RAPD analyses confirmed the hybridity of the regenerants. Four of the somatic hybrids were crossed with potato cvs. Katahdin or Atlantic, producing viable seeds and BC_1 and BC_2 progeny that segregated for resistance to *P. infestans,* indicating that effective resistance to the late-blight fungus in a sexually incompatible *Solanum* species can be transferred into potato breeding lines by somatic hybridization and that this resistance can be further transmitted into potato breeding lines by sexual crossing.

The term partial resistance or horizontal resistance refers to several protective genes that impart a broad-spectrum resistance against a pathogen. Partial resistance can be evaluated by factors that limit the spread of the pathogen. Late blight field evaluations for partial resistance in crosses between blight resistant somatic hybrids, diploid *S. bulbocastanum* and tetraploid *S. tuberosum* (Helgeson *et al*., 1998) and resistant sexual hybrids proved that the components for partial resistance are heritable (Dorrance *et al*., 2001). Crosses with resistant X resistant parents (somatic or sexual hybrids) had lower disease levels than crosses with resistant X susceptible parents. All had higher partial resistance compared to the then current potato cultivars. Based on the synteny of RFLP markers between *S. bulbocastanum,* tomato and potato (Brown *et al*., 1996), Naess *et al.* (2001) examined the introgression of *S. bulbocastanum* DNA into *S. tuberosum* breeding

lines. RFLP analysis of BC_1 and BC_2 progeny revealed extensive loss of markers indicating that intergenomic recombination had occurred within the hybrids. Late blight resistance in *S. bulbocastanum* appears to be due to a few congregated quantitative trait loci (QTLs) on chromosome 8 (Naess *et al*., 2000). Sequence characterized amplified region (SCAR) and cleaved amplified polymorphic sequence (CAPS) markers have been developed to increase the efficiency of screening for this resistant trait.

Late blight still remains the most serious disease of potato. Two main types of resistance have been observed; race specific and general resistance, due to monogenetically and multigenetically inherited resistance, respectively. Protoplast fusion was attempted as a method of transfer of mono and polygenetic traits of sexually incompatible wild species, without segregation and possible loss of resistance, to potato. Ward *et al.* (1994) attempted to introduce late blight resistance into potato through interspecific electrofusion between *S. pinnatisectum* (2n=2x=24) and dihaploid *S. tuberosum* (2n=2x=24). No somatic hybrids had a simple addition of parental chromosomes, but were mixoploids ranging from 4x to 5x. Isozymes and RFLPs were used to identify hybrids. Although the mixoploid status did not affect the vigor of plants, their phenotypes were unstable through asexual propagation. Additional fusions were reported between the diploid *S. pinnatisectum* or the interspecific hybrid *S. pinnatisectum* X *S. bulbocastanum* with dihaploid and triploid *S. tuberosum* (Thieme *et al*., 1997). Isozyme and RAPD analysis was used to confirm hybrid status. Most somatic hybrids had the expected ploidy, tetraploid in 2x *S. tuberosum* + 2x *S. pinnatisectum* and pentaploid in 3x *S. tuberosum* + 2x *S. pinnatisectum* X *S. bulbucastanum*, with some mixoploids. Pentaploid hybrids were self-compatible and female fertile, able to produce BC_1 populations, whereas the tetraploid hybrids were sterile. On average, the resistance of the somatic hybrids was less than the mean of the parental types. The reduced resistance of the pentaploid hybrids was attributed to genome-dosage effect, with three genomes of the susceptible *S. tuberosum* and only two genomes of the resistant hybrid.

Possibly the first successful case of inter-specific fusion in potato was the production of a somatic hybrid between the cultivated, tetraploid *S. tuberosum*, and the wild potato

species, diploid *S. chacoense* Bitt., (Butenko *et al*., 1982; Butenko and Kuchko, 1979). The single fertile hybrid obtained displayed resistance to potato virus Y equal to the *S. chacoense* parent.

Both reisistance to potato leaf roll virus (PLRV) and frost tolerance were observed in fertile tetraploid somatic hybrids between diploid *Solanum brevidens* (trait bearer) and dihaploid *S. tuberosum* (Austin *et al*., 1985b). The hybrids showed increased vigor compared to parental types. Disease resistance can be combined from both fusion partners as exemplified in somatic hybrids between *S. brevidens* and tetraploid *S. tuberosum,* resulting in hexaploid plants expressing PLVR resistance of the *S. brevidens* parent as well as late blight resistance of the *S. tuberosum* parent (Austin *et al*., 1986; Helgeson *et al*., 1986). Although male fertility estimated by pollen stainability was variable, pollinations were ineffective because unilateral stylar incompatibility prevented growth of pollen tubes (Ehlenfeldt and Helgeson, 1987). The 6x somatic hybrids, however, were more fertile than the 4x somatic hybrids. Disease screening of the sexual progeny of a hexaploid (A206) somatic hybrid crossed with Katahdin revealed independent 1:1 segregation ratios for resistance to late blight and soft rot (Helgeson *et al*., 1993). In addition, agronomic traits such as tuber quality and maturity were improved in the sexual progeny. RFLP analysis of A206 and its BC_1 progeny revealed presence of all parental bands in the hybrid, so at least one copy of each parental chromosome was present (Williams *et al*., 1990; 1993). The BC_1 population revealed nulliplex individuals for one or more *S. brevidens* bands. This could be explained as chromosome loss but more probably to segregation, due to chromosome deletions and rearrangements. Williams concluded that pairing of chromosomes between *S. brevidens* and *S. tuberosum* could probably allow for recombination and introgression of desirable traits from *S. brevidens* into potato. RAPD markers were used to examine the path of introgression of *S. brevidens* chromosomes into potato (McGrath *et al*., 1996). Twelve *S. brevidens* synteny groups were identified. Intragenomic recombination between *S. brevidens* nonhomologous chromosomes was as frequent as intergenomic recombination between *S. brevidens* and *S. tubersoum* homoeologous chromosomes. It appeared that maximal recombination occurred within the early BC generations and that three *S.*

brevidens markers had been introgressed into the potato genome. Further, fluorescence in situ hybridization (FISH) technique based on ribosomal DNA (rDNA) was used to follow the introgression of chromosomes as far as the BC_3 progeny derived from a somatic hybrid. Dong *et al.* (2001) created FISH probes from chromosome-specific cytogenetic DNA markers, able to identify each of the 12 potato chromosomes (Dong *et al*., 2000) – originally derived from a bacterial artificial chromosome (BAC) library (Song *et al*., 2000). By sequential GISH and FISH analysis in a single experiment Dong *et al.* (2001) revealed the genomic origin and genetic identity of two BC_3 progeny (Williams *et al*., 1993) of A206, produced by fusion of *S. brevidens* and *S. tuberosum* (Austin *et al*., 1986). Fish *et al.* (1987), working with the same fusion combination, found that modification of fusion conditions for dihaploids, such as inclusion of growth regulators, extra $CaCl_2$ and bovine serum albumin (BSA) in the enzyme digestion mixture and reduction in centrifugation speed greatly increased the yield of viable protoplasts. Chromosome counts of the somatic hybrids revealed a range of ploidy, with euploid, tetraploid and hexaploid hybrids, as well as aneuploids at the tetra, hexa- and octoploid levels (Pehu *et al*., 1988). Molecular and cytogenetic evaluation of the somatic hybrids revealed random segregation of chloroplast types, while morphological characteristics such as tuber formation were based on nuclear dosage of *S. tuberosum* (Pehu *et al*., 1989).

The genetic basis of resistance to PLRV, PVS and PVY in the tetraploid hybrids was associated with the degree of expression of the *S. brevidens* genome, while in the hexaploid hybrids, resistance was associated with two doses of the *S. brevidens* genome and dominant phenotype (Pehu *et al*., 1990a). Increased nuclear ploidy was positively related with net photosynthesis; however, Rubisco activity was negatively affected by nuclear-organelle genome incompatibility (Pehu *et al*., 1990b). In the aneuploids, the loss of individual *tuberosum* or *brevidens* chromosomes was associated with the loss of resistance. Analysis of the same fusion combination by Barsby *et al.* (1984), revealed no chloroplast DNA alteration but a range of mtDNA alterations indicative of mtDNA recombination (Kemble *et al*., 1986) with independent segregation of organellar genomes (Xu *et al*., 1993a). Molecular characterization using newly developed species-specific

sequences clearly identified *S. tuberosum* (+) *S. brevidens* somatic hybrids (Pehu *et al*., 1990c). In field evaluations no significant differences were found between the products of chemical and electrofusion methods (Fish *et al*., 1988b); however, extensive phenotypic variation was observed among the hybrids. Significant differences were found for many morphological characters between ploidy groups, with tetraploids and near tetraploids forming a group distinct from near hexaploids. In a later study by Gibson *et al.* (1988) resistance to PLRV, PVY^{o} and PVY^{n} was demonstrated in the hybrids. Resistance or susceptibility to the three viruses was associated with loss of particular chromosomes, and hence controlled by separate genes that may be genetically linked (Pehu *et al*., 1990a).

Analysis of the steroidal glycoalkaloid aglycones (SGAA) content of the symmetric and asymmetric somatic hybrids of the previous fusion revealed novel glycoalkaloids in the somatic hybrid (Laurila *et al*., 1996). The major SGAA of *S. tuberosum* are solanidine and solanthrene, whereas that of *S. brevidens* is tomatidine. All somatic hybrids had all three SGAA in addition to demissidine. The proportion of tomatidine was correlated positively with the genomic composition of *S. brevidens* in the symmetric hybrids, suggesting a gene dosage effect. A hydrogenase enzyme from the *S. brevidens* genome was proposed to react with solanidine of the *S. tuberosum* genome, resulting in the synthesis of demissidine. The total GSAA content of the hybrids was higher than that of the *S. tuberosum* parent; however, values were close to the acceptable level of 20 mg per 100 g fresh weight.

Potato infected with PVY can show reduced yields of up to 80% (Beemster and de Bokx, 1987). *S. etuberosum* Bukasov & Kameraz possesses resistance/immunity to PVY (Hanneman and Bamberg, 1986). Novy and Helgeson (1994b) used morpholgical characters and RFLP analysis to select hybrids after electrofusion between *S. etuberosum* and either dihaploid potato or diploid hybrids between dihaploid potato and *S. berthaultii* Hawkes. RFLP analysis also revealed preferential retention of *S. etuberosum* chloroplasts. Field evaluations revealed that all but one of the somatic hybrids were significantly more resistant to PVY than the fusion parent or cultivars (Novy and

Helgeson, 1994a). However, none was as resistant as the *S. etuberosum* parent, suggesting that the PVY resistance trait was not a simple dominant but may be controlled by one or more genes.

S. etuberosum also carries resistance to PVX, PLRV and frost tolerance. Although RFLP analysis indicated one complete set of chromosomes from each fusion parent in various somatic hybrids involving *S. etuberosum* (Dong *et al*., 1999), GISH revealed that none of the hybrids had the exact complement of chromosomes expected (Novy and Helgeson, 1994b). The ultimate aim is to develop potato clones containing a single *S. etuberosum* chromosome, and with these monosomic addition lines established, locate the virus resistance genes on specific *S. etuberosum* chromosomes.

S. brevidens Phil. has been used as a source of resistance to PLRV, PVY, PVA, PVX, PVM and PVS (Valkonen *et al*., 1993). Protoplast electrofusions were performed between two anther derived dihaploid lines of *S. tuberosum* cv. Pinto and *S. brevidens* with the intent of introducing viral resistance of *S. brevidens* into potato (Rokka *et al*., 1994). RAPDs were used to verify somatic hybrids. Cytological analysis revealed that most of the somatic hybrids were aneuploid at the tetraploid or hexaploid level. All 20 somatic hybrids tested were strongly resistant to PVY^{N}. Subsequently Valkonen *et al.* (1994) reported resistance to PVY^{O}. GISH analysis was used for the identification of parental constituents of the somatic hybrids and their derivatives (Rokka *et al*., 2000). To overcome sterility of interspecific hybrids, Rokka *et al.* (1995) proposed refusion of somatohaploid with dihaploid lines of cultivated potato. GISH analysis of somatic hybrids and their derivatives revealed ploidy and genomic content as predicted in a hypothetical breeding scheme with "second generation" somatic hybrids having genomic contents of AAAAE, AAAE or AAAEE (Gavrilenko *et al*., 2002). Chromosomes of *S. brevidens* were eliminated more frequently than those of *S. tuberosum*, suggesting preferential elimination (Garriga-Caldere *et al*., 1997; Pijnacker *et al*., 1987). Contrary to other authors (Garriga-Caldere *et al*., 1997; McGrath and Helgeson, 1998; Pijnacker *et al*., 1987; Wolters *et al*., 1994), no chromosome aberrations or recombinants were observed in the first or second generations of somatic hybrids. This could be a result of

the size of the population examined and difficulties of detecting small translocation events using GISH. Laurila *et al.* (2001) examined the glycoalkaloid content in the primary somatic hybrids, their androgenic progeny and their second generation hybrid progeny in an attempt to study the effect of genome dosage on alkaloid content. The anther derived somato-haploids regenerated from the somatic hybrids had genomes consisting of AE and AEE. "A" depicts the *tuberosum* haploid genome and "E" the *etuberosum* haploid genome. Altering the genomic composition of the hybrid by adding the *S. tuberosum* (AA) genome to the somato-haploid (AEE) to give pentaploids (AAAEE) had a significant effect on the amount of aglycones. Solanidine increased significantly (A genome) while demissidine was significantly lower (E genome). Hence, changing the genomic composition of the second-generation somatic hybrids may reduce unfavorable wild species glycoaklaloid content.

Genome dosage is a factor of importance in viral resistance expression in somatic hybrids. Gavrilenko *et al.* (2003) working with the fertile somatic hybrids of *S. etuberosum* (+) dihaploid *S. tuberosum* (Thieme *et al*., 1999) and their backcross progeny concluded that many hybrids are required before enough fertile genotypes are produced. GISH analysis of the fusions could be classified into three groups, AAEE, AAEEEE and AAAAEE. Somatic hybrids were missing a few E genome chromosomes suggesting a preferential loss of the *etuberosum* genome. BC_1 plants derived from hexaploid fusion hybrids were pentaploid with the expected genomic composition of AAAEE or AAAAE. Viral resistance expression levels in the somatic hybrid population revealed a dosage effect, with hybrids AAEEEE (1A:2E) being extremely resistant to PVY, and AAAAEE (2A:1E) being susceptible. The pentaploid BC_1 plants AAAEE segregated for resistance, suggesting other genetic factors may have affected expression of resistance.

1.6.2. Insect resistance

Leptines are glycoalkaloids that have been tested on insect herbivores and shown to act as natural phytoalexins in *Solanum* species (Sinden *et al*., 1980). High levels of leptines have been observed only in *S. chacoense,* and have proved interesting to researchers

because of their restriction to green tissue, hence absence in tubers, making it selective against insect herbivores but non-toxic in the edible product. Cheng *et al.* (1995) produced somatic hybrids between high leptine-producing *S. chacoense* and dihaploid *S. tuberosum,* introducing resistance to Colorado potato beetle (CPB) into S. *tuberosum*. Hybrids were confirmed by isozyme analysis and inhibited CPB feeding compared to the *S. tuberosum* parent.

Jansky *et al.* (1999) performed electrofusion between diploid interspecific *Solanum* clones (*S. tuberosum, S. chacoense*, *S. tarijense* Hawkes and *S. berthaultii* sexual hybrid) displaying various quantities and types of glandular trichomes and *S. chacoense* with high leptine levels to combine both leptine and trichome mediated resistance. Trichome density and resistance to feeding by CPB varied among hybrids, and this was suggested to be due to somaclonal variation. In addition, type B trichomes, characteristic of *S. berthaultii*, were absent in all hybrids, indicating that this trait may be recessive or that the *S. chacoense* cytoplast inhibited the expression in the somatic hybrids.

1.6.3. Nematode resistance

Columbia root-knot nematode, *Meloidogyne chitwoodi* Golden, O'Bannon, Santo and Finley (MC) is a significant pest of potato in the Pacific Northwest of the United States of America and in the Netherlands (Evans and Trudgill, 1992; Strand and Rude, 1992). Damage consists mainly of darkening and galling on the tuber surface at the point of penetration. Two races have been separated based on host differentials of alfalfa and carrot (Mojtahedi *et al*., 1988). High levels of resistance have been confirmed in the reproductively isolated Mexican wild species *S. bulbocastanum.* Tetraploid *S. tuberosum* and nematode-resistant diploid *S. bulbocastanum* were fused to produce interspecific hexaploid somatic hybrids that displayed resistance to *M. chitwooodi* (Austin *et al*., 1993). Isozyme analyses were used to confirm somatic hybrids, which proved to be female fertile and hence potentially valuable for inclusion in potato breeding programs. Brown *et al.* (1995) examined the introgression of nematode resistance [races 1 (MC1) and 2 (MC2) and *M. hapla* (MH)] derived from *S. bulbocastanum* in F_1 and BC_1 progeny.

The F_1 hybrids showed resistance to all three nematodes; however, only MC1 resistance was consistently expressed in the BC_1 progeny, suggesting that MC1 resistance was dominant and discretely inherited. Even though F_1 and BC_1 progeny were male sterile, they could be used as the female parent in crosses, with seed yield increasing fourfold from F_1 to BC_1, allowing introgression of resistance into cultivated potato.

After two backcrosses of the above somatic hybrid (*S. bulbocastan*um + tetraploid *S. tuberosum*) to tetraploid *S. tuberosum*, Brown *et al.* (1996) associated previously mapped RFLP markers (Bonierbale *et al*., 1988) in the BC_2 population with QTLs for nematode resistance. A putative gene, R_{Mc1}, was identified on chromosome 11, and single dominant control established.

Potato cyst nematode (PCN) resistance was introduced into potato by somatic electrofusion of *S. tuberosum* cv. Brodick with *S. sanctae-rosae* Hawkes (Harding and Millam, 1999). Variable degrees of PCN resistance were found in the somatic hybrids. DNA methylation was examined in an attempt to study the regulatory factors of gene expression. Most putative hybrids had the *S. tuberosum* nuclear genome while a few showed intermediate profiles indicating hybridity or possible rearrangement of nucleolar organizer regions (NOR). Methylation status of ribosomal RNA gene of somatic hybrids varied from parental types, being reduced in some and increased in other, indicating different levels of coiling in the chromatin structure and hence possible reasons for variation in gene activity. Matthews *et al.* (1999) used a modified SSR PCR system in which 5'-anchored simple-sequence primers were used to identify hybrids. This inter-SSR PCR system has the advantage of reflecting variation in both intervening DNA sequences between SSRs and variation in the number of repeats in the microsatellites. Chromatin, nuclear DNA and organellar composition was analyzed by Harding and Millam (2000). SSR analysis targeting the nuclear genome and RFLP mtDNA analysis indicated genetic rearrangements, within a primarily S. *tuberosum* background. In contrast RFLP cpDNA analysis exhibited identical patterns between parental types and hybrids. DNA sensitivity analyzed via DNase I digest revealed resistance to degradation in the hybrids compared to parental types. This indicated alteration in the chromatin

structure, with resistance to degradation associated with a more coiled structured and inactivation of genes.

1.6.4. Herbicide resistance

The first reported case of the introduction of herbicide resistance via protoplast fusion was by Binding *et al.* (1982) working with a mixed population of haploid/dihaploid *S. tuberosum* and atrazine resistant *S. nigrum* (black nightshade). This trait is maternally inherited and thus coded by the plastid genome. Fusions were induced by PEG treatment followed by calcium nitrate. Selection of hybrids was mainly by morphological characters, particularly trichomes. Some of the hybrids were female fertile and hence this trait could possibly be introduced into breeding programs.

1.6.5. Frost-tolerance

Cardi *et al.* (1993c) introduced frost tolerance to potato by electrofusion of mesophyll protoplasts of frost tolerant *S. commersonii* (2n=2x=24) and dihaploid *S. tuberosum* (2n=2x=24). Hybrids consisted of vigorous tetraploids and somewhat less vigorous hexaploids. All the hybrids abundantly produced flowers, were female fertile but male sterile except for one fully fertile clone (SH9A). SH9A dispayed frost tolerance intermediate between the parents. Cardi *et al.* (1999) evaluated organellar DNA and male fertility by selfing SH9A and crossing the male-sterile hybrids. Maternal inheritance of fertility was confirmed. MtDNA polymorphism between the species was greater than that observed in chloroplasts, and all somatic hybrids had rearranged non-parental mitochondria. Mitochondria re-assorted independently from chloroplasts, with most hybrids having the cpDNA of *S. commersonii*. Male-sterile hybrids had the *S. tuberosum* mtDNA, while the sole male-fertile hybrid had *S. commersonii* mtDNA. Conicella *et al.* (1997) showed that early tapetal degeneration and abnormalities during meiosis II and cytokinesis were the causal agents of sterility.

The practical utilization of somatic hybrids depends heavily on intergenomic interactions between the parental types and their effect on agronomic traits. Cardi (1998) used

discriminant and cluster analyses to evaluate the usefulness of *S. commersonii* (+) *S. tuberosum* somatic hybrids for inclusion in breeding programs. Both univariate and multivariate analysis differentiated the six-ploidy groups tested. Several reproductive and vegetative traits were important for genotypic discrimination. Hybrids were generally more similar to *S. tuberosum* than the wild type parent, suggesting dominance of the cultivated phenotype, and a good chance for introgression of useful traits from *S. commersonii* into the *S. tubersosum* genome.

The evaluation of field grown F_2 progeny, derived by selfing somatic hybrid SH9A, revealed two clusters, using principal component analysis of epigeous traits (shoots, leaves, flowers) (Cardi *et al*., 2002). The major group included the *S. tuberosum* fusion parent, the somatic hybrid and the vast majority of selfed progeny, whereas the other group contained the *S. commersonii* fusion parent and the remainder of the selfed progeny. The variability among selfed progeny suggested re-assortment of the parental traits in the F_2 population, with the potential to introgress useful traits from the wild species into cultivated potato.

Before a new cultivar can be released, especially cultivars that have been produced via unconventional breeding techniques, such as transformation and interspecific hybridization by somatic fusion or sexual crosses, checks for undesirable traits and changes in chemical composition are required. In particular, the use of wild species as parental types has the potential of introducing or increasing glycoalkaloid content beyond acceptable levels for consumption. Esposito *et al.* (2002) evaluated the glycoalkaloid content of interspecific crosses between *S. commersonii* and dihaploid *S. tuberosum* via somatic hybridization (Cardi *et al*., 1993a) and sexual hybridization (Carputo *et al*., 1997a). The somatic hybrids consisted of SH9A and backcross progeny of SH9A x *S. tuberosum.* Glycoalkaloid content ranged from below that of *S. tuberosum* to as high as the wild type *S. commersoni*. Unexpectedly, the somatic hybrid had a glycoalkaloid content much less than the mean of its two parents; and some of the backcross progeny showed drastic reduction in content, suggesting that selection for low glykoalkaloid

content is possible. No significant differences were observed between somatic and sexual hybrids.

S. brevidens a diploid (2n=2x=24) cold tolerant wild species (Ross and Rowe, 1969) was fused chemically to *S. tuberosum* cv. Gracia (2n=2x=48) (Preiszner *et al*., 1991). Many aneuploids were observed among the somatic hybrids that exhibited intermediated frost tolerance compared to the parents.

Chen *et al*. (1999c) evaluated cold hardiness in the following somatic hybrids: hexaploid *S. tuberosum* (+) *S. brevidens* (Austin *et al*., 1986), tetraploid *S. tuberosum* (+) *S. brevidens* (Austin *et al*., 1985b) and tetraploid *S. tuberosum* (+) *S. commersonii* (Kim *et al*., 1993), by two independent genetic components, nonacclimated freezing tolerance (NA) and acclimation capacity (ACC) (Stone *et al*., 1993). NA is the ability to survive sudden freezing temperatures, while ACC is the ability to acquire tolerance to freezing temperatures by gradual acclimatization, while acclimated freezing tolerance (AA) is the addition of NA and ACC. The freeze tolerance expressed in tetraploid and hexaploid *S. tuberosum* (+) *S. brevidens* hybrids was mainly due to ACC. In contrast, tetraploid *S. tuberosum* (+) *S. commersonii* somatic hybrids expressed high AA. Earlier studies on interspecific potato hybrids have shown that increase in the contribution of the tolerant parent genome will affect expression of freezing tolerance (Chen *et al*., 1999a). Selfed and backcrossed progenies developed from the tetraploid somatic *S. tuberosum* (+) *S. commersonii* displayed segregation of NA and ACC, with distribution skewed towards the non-tolerant *tuberosum* parent (Chen *et al*., 1999b). Selfing progeny displayed improved freezing tolerance mainly due to an increase in ACC.

Solonum acaule Bitt. has been described as the most cold tolerant *Solanum* species (Chen and Li, 1980). It is a disomic tetraploid with 2 EBN, hence incompatible with *S. tuberosum* with 4 EBN (Brown and Adiwilaga, 1989). Leaf protoplasts from tetraploid *S. acaule* (2n=4x=48) were electrofused with two dihaploid *S. tuberosum* lines (2n=2x=24), produced by anther culture of the tetraploid cv. White Lady (Rokka *et al*., 1998a)

producing hexaploid somatic hybrids (Rokka *et al*., 1998b). Somatic hybrids were both male and female fertile.

1.6.6. Studies on combining ability

In contrast to earlier studies where single combinations of parental types were generally used and little field evaluations carried out, Mattheij and Puite (1992) tested ten different fusion combinations of *S. phureja* and *S. tuberosum* during evaluation of general and specific combining ability of parental types. Tetraploid somatic hybrids were confirmed by RFLP analysis and compared to cv. Bintje. Tuber yield in five of the six hybrids was similar to Bintje. One somatic hybrid produced significantly greater yield and was three times more productive than Bintje. They concluded that choice of parental combinations was critical and general and specific combining ability were factors requiring consideration during fusions.

1.6.7. Studies on EBN theory

The endosperm balance number (EBN) is regarded as the effective ploidy of *Solanum* species and is used to predict crossability between species (Fock *et al*., 2001). The EBN must be 2:1 maternal-paternal ratio in the hybrid endosperm for normal embryo development. The EBN acts as a strong isolating mechanism in *Solanum*. Somatic hybridization by protoplast fusion may be used as a tool to overcome EBN crossing barriers. To test this Carputo *et al.* (1998) evaluated the fertility of *S. commersonii* (+) *S. tuberosum* hybrids with different EBN and ploidy by crossing with 4 EBN *S. tuberosum* and 3 EBN somatic hybrid (SH9A). The somatic hybrids included tetraploids (2n=48, 3 EBN), hypotetraploids (2n=43–47, 3 EBN), hexaploids (2n=72, 4 or 5 EBN) and hypohexaploids (2n=63-70, 4 or 5 EBN). The EBN numbers were calculated by assuming that if there were four *S. commersonii* (2 EBN) genomes and two *S. tuberosum* (2 EBN) genomes their EBN should be 4, or if it was the reciprocal combination (two *S. commersonii* [1 EBN] and four *S. tuberosum* [4 EBN]) their EBN should be 5. Crosses between different ploidy levels but the same EBN produced more viable seeds than

crosses between genotypes with the same ploidy but different EBN, confirming that in the potato EBN is more important that actual ploidy.

1.6.8. Studies on introgression

Traits of interest from wild relatives of *Solanum* have been introduced by somatic fusion. However, for successful integration of these traits recombination between the donor and host genomes is required. This introgression is a random process that is revealed over time after several backcrosses. Evaluation of the possibility of meiotic recombination in the backcross progeny by Jacobsen *et al,* (1993) using the somatic interspecific hybrid between diploid amylose-free (*amf*) *S. tuberosum* and diploid *S. brevidens* and their BC_1 progeny revealed limited recombination. The fusion resulted in 4x and 6x female fertile somatic hybrids. Backcross tests of 4x X 4x and 6x X 4x produced seeds but only the 6x X 4x crosses resulted in progeny. Examination of meiosis was carried out on tetraploid somatic hybrids and normal bivalent formation was observed, as expected in an allotetraploid. Segregation of *amf* marker was observed in microspores of both 4x and 6x hybrids; in addition, one of the BC_1 progeny was amylose-free, indicating the occurrence of meiotic recombination in the megaspore mother cell. Similar trends were reported by Ehlenfeldt and Hedgeson (1987).

1.6.9. Marker genes as alternative methods for early selection of somatic hybrids

Marker genes present in nuclear or organellar genomes have been useful to monitor and select somatic hybrids carrying dual resistance separately derived from each parent, such as antibiotic and herbicide resistance. Successful transformation of either the cultivated potato and/or the wild type, e.g., *S. commersonii* (Cardi *et al*., 1992) is essential to realize somatic hybridization using such selection methods.

Direct gene transfer by electroporation has been used as a rapid selection method of somatic hybrids (Masson *et al*., 1989). Diploid clones of *S. tuberosum* hybrids were transformed with a plasmid conferring kanamycin, hygromycin and chlorsulfuron

resistance. Fusion combinations were selected by incorporation of the relevant combinations of antibiotics in the regeneration medium of putative tetraploid double resistant calli. This resulted in the early and accurate detection of somatic hybrids.

1.6.10. Morphological selectable markers – *rolC* (Heat and drought resistance)

Selection *in vitro* of putative somatic hybrids is facilitated when the genotypes involved carry selectable markers, such as antibiotic and herbicide resistance, or chemical markers such as β-glucuronidase (GUS) or luciferase that are identified by a color change in substrate or the emission of light, respectively. The *rolC* gene from *Agrobacterium rhizogenes,* which imparts the hairy root phenotype was introduced into diploid *S. papita* Rydb as a new selectable marker prior to electrofusion with diploid *S. tuberosum* (Kaendler *et al*., 1996) on kanamycin amended media (NPT II was part of the *rolC* gene construct). Somatic hybrids confirmed by RFLP and RAPD analysis were mainly tetraploid with one hexaploid, and all revealed a *rolC* phenotype. Tetraploid hybrids were female fertile and were backcrosses to *S. tuberosum* cvs. Datura and Desiree, yielding tetraploid BC_1 progeny where segregation was observed for the *rolC* gene.

1.6.11. Reducing sugars

Accumulation of reducing sugars in cold stored potato presents a problem to the processing industry, as this increases browning during cooking with resultant decreases in visual appeal. *S. phureja* has been identified as a cold stable genotype (Colon *et al*., 1989). In an attempt to introduce this trait into commercial cultivars, Craig *et al.* (1994) created interspecific hybrids by fusing mesophyll protoplast cells of *S. tuberosum* cv. Record with protoplasts derived from cell suspensions of diploid *S. phureja*. Morphological and RAPD evaluation for confirmation of somatic hybrids suggested that hybrids might have been asymmetric due to elimination/loss of *S. phureja* chromosomes. Glucose and fructose analysis revealed significant differences between parents Record and *S. phureja*, but no difference between *S. phureja* and the somatic hybrids, indicating that the trait for low reducing sugars, derived from *S. phureja*, was dominant. In contrast,

the sucrose levels of Record and somatic hybrids were not significantly different from each other, but significantly less than *S. phureja*. Hence, dominant genes in Record controlled this trait and *S. phureja*'s high accumulation of reducing sugars was due to recessive genes. These results suggest that the two types of sugars are under separate genetic control.

1.6.12. Glycoalkaloids

Steriodal glycoalkaloids that are reported to impart resistance to bacterial, fungal, nematode, mite and insect pests occur in many Solanaceae. Glycoalkaloids in addition to imparting bitterness have been associated with outbreaks of food poisoning when ingested at >20 mg/100 mg fresh weight of tubers (Friedman and McDonald, 1997). Many of the successful somatic fusions produced to interpose disease resistance traits to cultivated potato have the potential of introducing undesirable glycoalkaloids from the wild type parent, e.g., dihaploid *S. acuale* which carries the traits for resistance to PVX, potato spindle tuber viroid (PSTV), cyst nematode and frost tolerance. Kozukue *et al.* (1999) evaluated the potential safety of somatic hybrids in practical plant breeding programs by tabulating alkaloid content of somatic hybrids (via electrofusion) between dihaploid *S. acuale* and tetraploid *S. tuberosum* cv. Dejima. *S. tuberosum* parental type contained α-chachonine and α-solanine, while *S. acuale* parental type contained α-tomatine and demissine. All the hybrids except one contained all four glycoalkaloids. The α-tomatine and demissine levels in the somatic hybrids were less than in the *S. acuale* parent. The data suggest that the amount of α-tomatine, but not demissine, is dependent on the ploidy level of the somatic hybrids. In general the glycoalkaloid content of the somatic hybrids was intermediate between the fusion parents, but greater than cv. Dejima, making these hybrids unacceptable as immediate cultivars. However, reduced levels of glycoalkaloid should be achieved by backcrosses with the cultivar and selection of low glycoalkaloid progeny.

1.6.13. Chromosome elimination

Somatic hybrids between diploid *S. tuberosum* (clone SCP1) and diploid *S. phureja* produced by electrofusion followed by selection of heterokaryons by micromanipulator (Puite *et al*., 1986) revealed ploidy levels ranging from hypohexaploid to octoploid. Instability of the nuclear genome during regeneration and callus growth (Ramulu *et al*., 1989) resulted in preferential elimination of *S. phureja* chromosomes (Pijnacker *et al*., 1987). Tetraploid heterofusions of diploid *S. tuberosum* (2n=2x=24) and diploid *S. phureja* (2n=2x=24) were selected by flow cytometry and cytologically analysed (Puite *et al*., 1988). The somatic hybrids were hypotetraploid, missing from 1 to 7 chromosomes. Preferential elimination of chromosome 2 of the *S. phureja* genome occurred. This chromosome contains the NOR and its elimination suggests that retention of two NORs per cell (either T + T or T + PH) might be essential for survival.

1.7. Intergeneric somatic fusions

The fusion partners in intergeneric somatic hybrids belong to taxonomically distinct groups and hence differences between members are greater than at the interspecific level. This enhances the possibility of incompatibilities that either prevent regeneration or result in sterile progeny. However, a few, viable intergeneric hybrids have been produced.

Introgression of biotic traits such as disease resistance or abiotic traits such as cold tolerance found in wild species of closely related genera has been the logistic for intergeneric fusion between tomato and potato. Most of the hybrids have been sterile (Jacobsen *et al*., 1992; Melchers *et al*., 1987; Waara and Glimelius, 1995) preventing their backcross with tomato. In addition, the polyploid nature of intergeneric somatic hybrids reduces their use in tomato breeding, as tomato unlike potato is difficult to manipulate at the tetraploid level.

As an alternative to sexual backcrossing of somatic hybrids to parental types, haploidization of somatic hybrids by anther culture followed by repeated somatic fusions was hypothesized (Rokka *et al*., 1995). In an attempt to evaluate the prospects of

introgression of traits from *S. etuberosum* to tomato Gavrilenko *et al.* (2001) performed cytogenetic analysis on five intergeneric somatic hybrids (Gavrilenko *et al*., 1992; 1994) and their androgenic regenerants (Gavrilenko *et al*., 2001) by GISH. Meiotic studies of the amphidiploids revealed intragenomic bivalents pairing autosyndetically. Intergenomic bivalents, trivalents and quadrivalents were extremely rare. Androgenetic regenerants were diploid or aneuploid with one additional or one less chromosome. This suggests that the hybrids were relatively stable. The low percentage of homoeologous chromosome pairing suggested difficulty for introgression of alien genes. However, the successful production of anther-derived regenerants presents a previously unexplored avenue in potato for introgression of genes.

Due to intensive irrigation and salt-contaminated water, increased levels of soil salinity are a problem in several areas of the world. Traits for resistance to this abiotic stress reside in the wild tomato species *Lycopersicon pennellii* Corr. (Dehan and Tal, 1978). Protoplasts from a clone of *S. tuberosum* cv. Nicola and *L. pennellii* were electrofused producing intergeneric somatic hybrids (Sherraf *et al*., 1994). Two hybrid regenerants were confirmed by isozyme analysis. They were highly male sterile, rooted poorly and produced misshaped tubers (probably due to the great phylogenetic distance between the two parental types). Salinity tests revealed that the somatic hybrids had inherited the same level of salt tolerance as *L. pennellii.*

1.7.1. Potato-tomato somatic hybrids

Early attempts of intergeneric fusion of potato and tomato produced a few somatic hybrids (Melchers *et al*., 1987; Shepard, 1983); however, no progeny was reported. RFLP analysis of the plasmid DNA from Melcher's hybrids revealed that while the plastomes of potato and tomato are distinct in the hybrids they are closely related. Restriction patterns of only one parent were found in the hybrids (Schiller *et al*., 1982).

For successful introgression of traits between genomes, fertile or partially fertile progeny are required with homoeologous interchange of genetic material occurring between them.

With this in mind Jacobsen *et al.* (1992) set about creating an intergeneric somatic hybrid and backcross progeny by chemical fusion (PEG). *Lycopersicon esculentum* Mill. mutant cv. Large Red Cherry (ALRC) and kanamycin resistant transformants were fused with a diploid amylose-free (amf) mutant of *S. tuberosum*. Somatic hybrids were tetraploid and predominately formed bivalents, behaving like allotetraploids. Examination of microspores using the amf phenotype of red in the presence of iodine, revealed segregation of blue and red microspores in all hybrids, indicating some form of genomic recombination between homoeologous chromosomes or possible deletions. Successful backcrosses of the 4x and 6x somatic hybrids with various 4x *S. tuberosum* clones were obtained (Jacobsen *et al*., 1994). Both 4x and 6x somatic hybrids produced berries but only the 6x X 4x crosses resulted in a plant. This plant was pentaploid and confirmed as a true BC_1 by isozyme and DNA hybridization analysis. The BC_1 progeny was male sterile but female fertile and produced BC_2 plants when crossed with 4x potato. GISH and RFLP analyses were carried out on BC_1 and BC_2 progeny to identify alien tomato chromosomes (Jacobsen *et al*., 1995). GISH results indicated that somatic hybrids had a complete set of tomato chromosomes but the BC_1 plants only had nine chromosomes of tomato rather than the expected 12. RFLP analysis of BC_1 plants indicated six tomato chromosomes. The analysis of BC_2 progeny revealed chromosomes 1, 3 and 6 were present in duplicate while 8, 9 and 10 were haploid. The number of tomato chromosomes in the BC_2 progeny varied form 1 to 6 with the disomic chromosomes preferentially passed on to the BC_2 progeny. To conclude, the possibility of chromosome and gene introgression is highly favorable; in addition, the potential of producing a monosomic or disomic potato/tomato addition line using this family is feasible.

Intergeneric crosses between potato (*S. tuberosum*) and tomato (previously *L. esculentum,* now renamed *S. lycopersicum*) have been performed producing three fertile near hexaploid somatic hybrids, and via backcrosses to potato, several near tetraploid monosomic addition genotypes (Garriga-Caldere *et al*., 1997). The near tetraploids (2n=4x=48 + 1) had alien monosomic tomato additions that were identified using GISH and RFLP analysis (Garriga-Caldere *et al*., 1998). Converse to the previous paragraph, the aim here was the establishment of a complete set of tomato monosomic chromosome

additions in a potato genomic background. Further studies were carried out evaluating progress of introgression of alien chromosomes via verifying homoeologous pairing and crossing-over between potato and the alien tomato (Garriga-Caldere *et al*., 1999). Without homoeologous pairing and crossing-over events, introgression of desirable traits from alien chromosomes would be difficult. Still, a high rate of recombination between genomes would disrupt the integrity of the alien chromosome and prevent establishment of monosomic additions. GISH was used to investigate one of the near hexaploid somatic fusion hybrids (2n=6x –4=68) and two BC_2 near tetraploid (2n=4x=48 +1) monosomic alien addition genotypes. Contrary to previous RFLP analysis which indicated a complete haploid set of chromosomes present in the somatic hybrid (Jacobsen *et al*., 1995), GISH revealed the fusion possessed 46 potato chromosomes + 20 tomato chromosomes + 2 translocated chromosomes (Garriga-Caldere *et al*., 1997). The fusion revealed reciprocal translocation events, identified via the formation of bicolored quadrivalents in autosyndetic associations (pairing of homologs). In contrast, a low frequency of allosyndetic pairing (pairing of homoeologs) was observed in the monosomic addition lines (1.1–1.3%), suggesting possible but somewhat difficult introgression of genetic material through homoeologous recombination from tomato to potato.

1.7.2. Chromosome identification and gene location

Due to the potato's autotetraploidy and highly heterozygous genome it has been difficult to readily assign genes to chromosomes. Intergeneric somatic hybrids have been used successfully for gene mapping of the human genome (Kao, 1983); in a similar vein somatic hybrids between distant higher plants could result in unilaterial loss of chromosomes from one or the other fusion partner. With the aim of locating the gene for nitrate reductase (NR) Devries *et al.,* (1987a; 1987b) attempted fusions of monoploid *S. tuberosum* with reductase deficient (NR-) lines of *Nicotiana plumbaginifolia* Viviani. Somatic hybrids were selected by screening for nitrate reductase proficiency. Chromosome analysis of positive hybrid calli showed that hybrids were composed of tetraploid/hexaploid numbers of *N. plumbaginifolia* chromosomes with 9-12 potato

chromosomes or diplochromosomes indicating that chromosome doubling through endoreduplication had occurred. Chromosome fragments, translocations and deletions were reported but preferential loss of potato chromosomes was not observed. Although shoots were regenerated plants were not successfully hardened.

Similar studies on intergeneric electrofusion hybrids between transformed dihaploid *S. tuberosum* carrying genetic markers for kanamycin resistance, growth regulator autotrophy, opine synthesis and GUS activity (Devries-Uijtewaal *et al*., 1989; Gilissen *et al*., 1991) and diploid *N. plumbaginifolia* (Gilissen *et al*., 1992) have been carried out. Hybrid analysis after 8 and 12 month incubation revealed biparental loss of chromosomes. Elimination of chromosomes was parent specific in some lines, either *S. tuberosum* or *N. plumbaginifolia*, while in a few cases chromosome loss occurred from both parents. Interactions at the genomic level (nuclear or cytoplasm) related to the phylogenic difference between the parental types were suggested as causal agents for chromosome elimination. No shoots were regenerated.

Phylogenetic chloroplast DNA analysis has revealed that *S. etuberosa* is approximately intermediate between the genus *Lycopersicon* and the cultivated *Solanum* species (Hosaka *et al*., 1984), hence a better candidate for production of a fertile intergeneric somatic hybrid. Gavrilenko *et al.* (1992) performed chemical fusions between *L. esculentum* and the non-tuber bearing wild diploid *S. etuberosa* producing fertile hybrids (confirmed by morphology, cytology, isozyme analysis and Southern analysis). Cytological analysis of these hybrids revealed regular meiosis. However, there was a low frequency of univalent and multivalent formation suggesting the possible introgression of traits from the wild *S. etuberosum* into tomato.

1.8. Asymmetric somatic fusions

As an alternative to sexual or somatic interspecific hybridization for the introgression of a gene(s) from wild *Solanum* species that requires an extensive backcross program to eliminate undesirable wild traits, Feher *et al.* (1992) proposed the more direct route of

asymmetrical somatic hybridization. A fusion between the complete genome of the recipient line and fragments or a few relevant chromosomes from the donor line theoretically should improve fertility and shorten the breeding program. Feher *et al.* (1992) performed chemical fusions between mesophyll-derived protoplasts from *S. tuberosom* cv. Boro (recipient partner) and cell suspension derived protoplasts from transformed kanamycin resistant *S. brevidens* (donor partner). Preferential loss of *S. brevidens* chromosomes from the fusion progeny was induced by X-ray treatment. All hybrids showed potato phenotypes revealing major recipient genomic background. Cytological investigations confirmed chromosomal variation within and between regenerated clones. RFLP analysis showed omission of both *S. tuberosum* and *S. brevidens* markers and the data suggested preferential loss of *S. brevidens* markers.

Micro-manipulation and fluorescent-activated cell sorting (FACS) were successfully tested as selective tools for asymmetric hybrids produced via electrofusion between three recipient *S. tuberosum* cv. Folva, Matilda (2n=2x=48) and 161:14 (2n=2x=24) and two wild X-irradiated donor species, *S. spegazzinii* Bitter (2n=2x=24) and *S. microdontum* Bitter X *S. vernei* ssp. *balsii* (Hawkes) Hawkes & Hjert (2n=2x=24) (Rasmussen *et al*., 1997). Both selective methods are based on dual fluorescent staining of the heterofusion product. Protoplasts were fluorescently treated with either fluorescein diaceate or scopletin. The irradiation dosage negatively affected the asymmetric (hypo) tetraploid heterofusion products, with reduced growth and fewer fusion products with increasing dosage.

One of the rare cases of asymmetric intergeneric fusions was the transfer of oligomycin resistance from *Nicotiana sylvestris* Speg. & Comes to *S. tuberosum c*v. Desiree via donor-recipient protoplast fusion (Perl *et al*., 1991b). Donor protoplasts from *N. sylvestris* with oligomycin resistant mitochondria were γ-irradiated whereas recipient *S. tuberosum* protoplasts were iodoacetate treated (metabolic inhibitor) prior to fusion. The regenerated cybrids were confirmed by RFLP analysis using tobacco or maize mitochondrial probes and potato chloroplast probes.

Wolters *et al.* (1991) carried out donor-recipient asymmetric fusions between transformed (NPTII and GUS) tetraploid *S. tuberosum* and the albino mutant *Lycopersicon esculentum* cv. 'Large Red Cherry' (ALRC). Selection was based on the transfer of functional chloroplasts from potato to the albino tomato. Prior to protoplast isolation *S. tuberosum* plants were irradiated with two levels of gamma-rays. Chloroplast analysis indicated the presence of only potato cpDNA, while GUS analyses were all positive, indicating that nuclear encoded traits from *S. tuberosum* were present. No true cybrids lacking nuclear potato DNA were obtained.

In a novel attempt to produce monosomic addition lines, Ramulu *et al.* (1995) attempted fusions of microprotoplasts of donor transformed *S. tuberosum* and recipient *Lycopersicon peruvianum* (L.) Mill. *S. tuberosum* contained kanamycin resistance, GUS activity, opine synthesis, hairy root phenotype and plant growth autotrophy, whereas *L. peruvianum* contained hygromycin resistance. Hybrid callus was selected on kanamycin and hygromycin. GISH analysis revealed that many of the hybrids were monosomic additions with 24 or 48 *L. peruvianum* chromosomes and one *S. tuberosum* chromosome. Asymmetric somatic hybrids were male and female fertile and seemed to tolerate transfer of a partial genome in contrast to symmetric fusions that have been mainly sterile.

Asymmetric somatic hybrid calli were produced after fusion of gamma-irradiated protoplasts of transgenic *S. brevidens* with diploid *S. tuberosum* (Puite and Schaart, 1993). *S. brevidens* was selected as a candidate for asymmetric fusion due to its many agronomically important traits, such as PLRV and *Erwinia* soft rot resistance. Flow cytometry of calli revealed a range of DNA content per nucleus greater than the sum of the two parents, suggesting polyploidization of the recipient genome. Elimination of the *S. brevidens* DNA was confirmed by dot blot and RFLP analysis. No regenerants were produced from the calli.

Successful asymmetric hybridization between dihaploid lines of *S. tuberosum* cv. Petland Crown (PDH40, 2n=2x=24) and irradiated *S. brevidens* were performed with the aim of introducing virus resistance traits from the wild potato species (Xu *et al*., 1993b). RFLP

analysis (Xu, 1993) and chromosome counts revealed one or two complete set of *S. tuberosum* chromosomes and a partial set of *S. brevidens* chromosomes, with generally a large fraction of the *S. brevidens* genome retained. Analysis of organellar DNA content revealed rearrangements in mtDNA, whereas the cpDNA type of the hybrids was unaltered from those of the parental lines

Asymmetric fusions have also been attempted between two X-ray irradiated wild species, *S. bulbocastanum* and *S. circaeifolium,* and three dihaploid breeding line of *S. tuberosum* (Oberwalder *et al*., 1997). Flow cytometry, RFLP analysis with an oligonucleotide probe $(GATA)_4$, and single-copy probes were used for hybrids identification. Only a few asymmetric hybrids were detected with probe $(GATA)_4$, whereas flow cytometry allowed for differentiation between symmetric and asymmetric hybrids. DNA losses were higher for fusion experiments with 4x than 2x *S. tuberosum* recipients, indicating that the tetraploid recipient was better suited for production of asymmetric hybrids (Oberwalder *et al*., 1998). In addition, the genome of the asymmetric fusions was not stable, resulting in shoots regenerated from the same calli having different DNA content.

1.9. Cybrids - Cytoplasmic male sterility

Galun (1988) carried out donor-recipient protoplast fusions for the introduction of cytoplasmic male sterility (CMS) - carried on the chrondriome - into F_1 hybrid seed parents. This procedure is based on the selective transfer of the mitochondial genome from donor to recipient parent. Donor male sterile potato cultivars were X-ray treated and subsequently fused with prospective seed parent cvs. Atlantic and Atzimba. The genomic DNA of the donor was eliminated from the fusions, resulting in CMS cybrids displaying recombinant mtDNA.

The donor-recipient protoplast fusion method was further tested to study the compatibility between *S. tuberosum* nuclear genome with cytoplasm of related alien *Solanum* species of varying phylogenic distance from potato (Perl *et al*., 1990). S. *tuberosum* cv. Desiree was used as the recipient, with donor cytoplasm from *S.*

chacoense, *S. brevidens*, *S. etubersum* and *S. berthaultii* (pretreatements of iodoaceate and γ-irradiation). RFLP/Southerns using chloroplast and mitochondria specific probes characterized Organellar DNA. *S. chacoense* chloroplast and mitochondria proved compatible with the *S. tuberosum* genome. However, the more distant *S. etuberosum*, *S. brevidens* and *S. berthaultii* revealed allochondriomic incompatibility with development of cytoplast male-sterility. Abnormally pigmented cybrids were produced when *S. etuberosum* chloroplasts were transferred. *S. brevidens* fusions produced weak albino regenerants that did not survive. The establishment of de novo male-sterile plants may be useful for breeding hybrids to be propagated from true potato seed.

Donor–recipient protoplast fusions were performed between *S. tuberosum* cv. Desiree and 14 *Solanum* species (Perl *et al*., 1991a). Assessment of the diversity of these 15 species by chloroplast specific RFLP probes resulted in the construction of a plastome dendrogram of four clustal groups (Hosaka *et al*., 1984). Many donor-recipient fusions between distant clustal groups resulted in male sterility. In contrast a mtDNA dendrogram of *Solanum* species created by analysis of symmetric somatic hybrids (Lossl *et al*., 1999) revealed five distinct mitochrondrial types (α, β, γ, δ, and ε) based on RFLP patterns. Comparison between dendrograms generally revealed co-evolution of chloroplast and mitochondria genomes. Segregation of organellar genomes occurred in somatic hybrids, whereas mitochondrial recombination with novel band patterns was common.

1.10. Fusion of monoploids

Prior to attempting monoploid fusions, Uijtewaal *et al.* (1987a) studied the effect of homozygosity and polyploidization on morphology and vigor of potato. Diploid *S. tuberosum* parents (M9 and H78.01), several prickle pollination-derived monoploids and their ploidy series (x, 2x, 4x or x, 2x) were studied. The initial step of reducing the ploidy from diploid to monoploid drastically reduced plant vigor and tuber formation. However, the subsequent step of raising the ploidy level from monoploid to diploid increased performance substantially. Additional polyploidazation steps, from diploid to

tetraploid (2x to 4x), hardly produced any increase, except for tuber size. This suggested that tuber production was positively affected by gene dosage. Comparisons between the heterozygous diploid parents and the homozygous diploids and tetraploids revealed a reduction in performance of the homozygous types, suggesting that the combination of alleles in heterozygotes is more important than polyploidization, i.e. dominant effects were stronger than additive effects. Monoploids were sterile, while di- and tetraploid homozygous clones had low fertility. These results suggested a breeding strategy to combine two, three or four monoploids, possibly via protoplast fusion, to maximize heterozygosity with elimination of deleterious alleles.

Uijtewaal *et al.* (1987c) reported the first case of monoploid fusion. The monoploids used for fusion were derived from two heterozygous diploid lines (M9 and PD23-2). M9 was homozygous for maltase dehydrogenase whereas PD23-2 was heterozygous; hence monoploids derived from M9 were all homozygous for maltase dehydrogenase (banding pattern A) while monoploids derived from PD23-2 segregated for this trait (banding pattern A and B). Fusions were performed between M9 derived monoploids (banding pattern A) and PD23-2 derived monoploids (banding pattern B). All fusion regenerants were confirmed tetraploid by chromosome counts and hybridity confirmed by isozyme analysis (banding pattern AB). Further fusion combinations were carried out between monoploids derived from various dihaploids, and regenerants were evaluated. Highly significant correlations were found between callus growth and plant vigor (Uijtewaal *et al*., 1987b). The monoploid fusions resulted in mostly tetraploid regenerants except for one triploid. The tetraploid regenerants could have derived from somatic doubling of a C+C fusion product, or otherwise from 2C+2C fusions or C+C+C+C multifusions.

The use of monoploids has been extended to the production of allotriploid somatic hybrids, obtained by fusion between diploid (nitrate reductase mutant or albino mutant) *Lycopersicon esculentum* and monoploid *S. tuberosum* (Schoenmakers *et al*., 1993). The attempt here was to reduce the proportion of donor genome (*S. tuberosum*) in the hybrid by use of a haploid unirradiated genotype, instead of a diploid or higher ploidy level. Only a few allotriploids were produced and these showed high levels of cytoplasmic male

sterility. The major fusion types were allotetraploid or allohexaploid, suggesting polyploidization may be a major factor during regeneration, limiting the use of this approach.

1.11. S-SAPs

Molecular characterization of somatic hybrids has been done using various techniques. We have used sequence-specific amplification polymorphism (S-SAP) that targets the genome allowing us to examine a particular region of probable polymorphism, in our case a retroelements. S-SAP is a PCR based method that generates molecular markers capable of detecting polymorphisms in target areas between two particular types of sites within the genome. S-SAP is modification of the AFLP system (Vos *et al*., 1995; Zabeau and Vos, 1993) and as in the AFLP system consists of digesting genomic DNA with rare and frequent cutting restriction enzymes, ligating on compatible adaptors and preamplification of prepared template DNA with adaptor-homologous primers. Modification from AFLP occurs at selective amplification; here amplification is via a labeled primer (radioactive or fluorescent) designed to target a particular genomic sequence and the other site corresponds to the rare or frequent site adaptor homologous oligonucleotide. Denaturing the DNA and separation on a high-resolution gel follows this. S-SAPS is useful for genotyping and phylogenetic studies (Kumar and Hirochika, 2001). They are useful for studying high copy number sites, whereas the dispersion of the sites is dependent on the distribution of the selected elements throughout the genome.

1.11.1. Retrotransposons

There are two main types of retrotransposons, subclass I, bound by two long terminal repeats (LTRs) containing superfamilies *Ty1/copia* and *Ty3/gypsy,* and subclass II, with members that do not possess LTRs such as superfamily LINES and SINES. The internal domain of the retrotransposon encodes for analogous group specific proteins (*gag)* and polyproteins (*pol)* of retroviruses (Clare and Farabaugh, 1985; Mount and Rubin, 1985; Saigo *et al*., 1984). The *gag* polyprotein genes (*gag* and *pr*) encode the proteins that form the nucleocapsid core and the enzyme required for protein maturation. The *pol*

polyprotein gene encodes the reverse transcriptase (RT) required for creating a DNA copy from the RNA template, and an enonuclease (*endo*) gene, required for integration of the DNA copy into the host genome (Grandbastien, 1998; Mount and Rubin, 1985). Retrotransposons move/transpose via an RNA intermediate, whereas the parental retrotransposon remains fixed at the original site (Kumar and Bennetzen, 1999). With each new insertion there is an increase in the size of the genome and elevation in polymorphism detectable by retrotransposon based marker systems (Pearce *et al*., 1996b).

Many retrotransposons have been found in plants (Camirand *et al*., 1990; Grandbastien *et al*., 1989; Harberd *et al*., 1987; Hirochika and Hirochika, 1993; Jin and Bennetzen, 1989; Shepherd *et al*., 1984; Smyth *et al*., 1989; Voytas and Ausubel, 1988). The best-known LTR retrotransposons belong to the *Ty1-copia* group, originally isolated from *Saccharomyces cerevisiae* and *Drosophila melanogaster* (Boeke and Corces, 1989; Schmidt *et al*., 1996). Studies using *in situ* hybridization of metaphase chromosomes have revealed that retrotransposons preferentially but not exclusively locate in the heterochromatin (Bennetzen, 2000; Heslop-Harrison *et al*., 1997; Pearce *et al*., 1996b; Schmidt *et al*., 1996). Stress, environment challenges, protoplast manipulation and tissue culture are known to stimulate activation or expression of some mobile elements (Grandbastien, 1998; Grandbastien *et al*., 1997; Hirochika, 1993; Hirochika *et al*., 1996; Mhiri *et al*., 1997; Pearce *et al*., 1996a; Pouteau *et al*., 1991).

Recently developed retrotransposon-based marker systems are based either on the amplifation of the flanking area between adjacent retrotransposons (inter-retrotransposon amplified polymorphism - IRAP), or the area between retrotransposon and microsatellites (retrotransposon microsatellite amplified polymorphism - REMAP) (Kalendar *et al*., 1999). S-SAPs based on retroelements consists of selective amplification designed to target the LTR region of the retrotransposon and the other site corresponds to the rare or frequent site adaptor homologous oligonucleotide. The *Bare-1*-like element in barley analyzed by S-SAP revealed increased levels of polymorphsim above AFLP (Waugh *et al*., 1997). Similarly S-SAP analysis of sweetpotato revealed that S-SAP markers were more polymorphic than either AFLP or RAPD markers (Berenyi *et al*., 2002). Pearce *et*

al. (2000) used AFLP markers to study relationships between members of *Pisum* and was able to produce detailed intra- and inter-species relationships. Ellis *et al.* (1998) used the S-SAP technique on *Pisum* with the PDR1 element for both mapping and phylogenic analysis.

S-SAP polymorphisms may be due to insertions, deletions or inversions within the genome that rearrange the retrotransposons, usually resulting in loss of a band. Point mutations or changes in the methylation status at the restriction digest target site will disrupt cleavage resulting in a change in banding patterns. Finally, changes in the 5' end of the LTR region of the retrotransposon can result in ineffective binding of the retrotransposon based primer, resulting in loss of bands.

Tst1 is the first potato retrotransposon (Camirand *et al*., 1990) that has been sequenced (Camirand and Brisson, 1990). It was located in the intron of the gene coding for starch phosphorylase and belongs to *Ty1-copia* family of retrotransposons. It is 5060 bp long and is flanked by a 5 base pair target sequence repeat and possesses LTRs of 285 and 283 bp at the 5' and 3' ends, respectively. It contains four overlapping ORFs. The first two correspond to the *gag* gene and contain an RNA binding site and protease. ORF 3 and ORF 4 correspond to the *integrase* and the *reverse transcriptase* (*RT*) genes. A stop codon in the *RT* gene suggests that this element may be non-functional. For retroelement activity a termination suppression mechanism could be used to jump the stop codon sequence or a RT provided in *trans* by a functional element of *Tst1* located elsewhere.

Due to the large number of markers produced S-SAP analysis has the potential for use in identifying inter and intra-genera phylogenic relationships. Markers can also be used to evaluate changes at the genomic level, such as deletions, inversions and insertions. The activity of the retrotransposon can also be evaluated, in that new insertions should correspond to novel bands, and the frequency of these bands would be an indicator of the level of activity of the element.

1.12. Objectives

This study has used the LTR of the *Tst1* element for molecular analysis based on AFLP, S-SAP, IRAP and RFLP/Southern blot analysis to investigate the utility of retrotransposon-based markers for marker-assisted evaluation of parental monoploids, somaclones and somatic hybrids produced by electrofusion of potato monoploids. To address this broad objective we developed the following specific objectives:

1. To develop and identify a set of somatic hybrids derived from four (O, N, T and A) diverse monoploid families using electrofusion.
2. To produce a family of dihaploids derived from intermonoploid somatic hybrids, somatic hybrid by cultivar crosses, and interhybrid crosses for field evaluation of somatic hybrids and derivatives depicting different levels of heterozygosity.
3. To determine the relationship between parental monoploids, somaclones and somatic hybrids by genetic distance analysis using AFLP, IRAP and S-SAP markers.
4. To determine the relationship between members of the Solanaceae, parental monoploid and somatic hybrid families using RFLP/Southern blot marker analysis based on S-SAP derived probes.

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2. Production and evaluation of somatic hybrids derived from monoploids

2.1. Introduction

Cultivated *Solanum tuberosum* (2n=4x=48) is a highly heterozygous autotetraploid. Breeding schemes are often long and tedious with release of new cultivars taking many years. Traditional diploid crop-breeding methods of mating among sibs, backcrossing and selfing to produce homozygous lines and then crossing of appropriate lines to produce heterotic F_1 hybrids are not applicable to potato due to high inbreeding depression, self-incompatibility and frequent male sterility. To greatly reduce the time and efficiency of breeding programs, Chase (1963) suggested "to reverse the evolutionary pathway, from polypoidy back to diploidy" and conduct selected breeding at the diploid level, then resynthesize the tetraploid. With the successful development of microspore regeneration techniques in *S. tuberosum,* Wenzel (1979) extended the analytical synthetic breeding scheme to encompass reduction from the autotetraploid initially to the dihaploid and finally to the monoploid level, spontaneous production of homozygous doubled monoploids, breeding at the diploid level, and then resynthesis of highly heterozygous tetraploids.

In most crop species both highly adapted and unfavorable alleles coexist. The proportion of alleles with unfavorable properties, the genetic load, is higher in polyploids than diploids. Lethal or deleterious alleles are most likely recessive and not expressed unless exposed by homozygosity. This would be rare in crosses among heterozygous tetraploids. Hence, many such alleles will remain "hidden" and well tolerated by the population. By contrast, monoploid individuals that express lethal and deleterious alleles will be eliminated from the population. This is the basis of the "monoploid sieve" where the presence of lethal or deleterious alleles prevents regeneration of androgenic and gynogenic functional plants (Veilleux *et al*., 1995). Synthetic breeding at the monoploid level has the advantage of presenting representatives of gametes at the total plant level that have no lethal and few deleterious alleles.

Monoploids theoretically represent the fittest gametes derived from heterozygous diploids. Previous studies have shown large differences in vigor among monoploid siblings derived from the same parent clone (Uijtewaal *et al*., 1987). However, selection of monoploids based on field performance was nominal as monoploids generally performed poorly with mean weights of 2 to 6 g per tuber (Johnson, 1998).

Heterosis, the phenomenon of vigor in hybrids exceeding the midparent value, a result of increasing gene combination values and the environment, should be displayed in intermonoploid somatic hybrids, depending on the allelic diversity between monoploid fusion partners. The combination of monoploids derived from distantly related sources should result in highly heterozygous somatic hybrids, and present the greatest potential for expression of hybrid vigor. The tissue culture technique of protoplast electro-fusion surmounts sexual barriers allowing for the combination of distinct but sterile monoploid lines facilitating heterosis.

The broad objective of this study was to produce intermonoploid somatic hybrids of potato from a diverse group of monoploids in order to maximize residual allelic variation after elimination of deleterious and lethal alleles by passage through the "monoploid sieve." The specific objectives were: 1) to test the combining ability of four divergent groups of monoploids; 2) to optimize protoplast electrofusion conditions for our population; 3) to identify and verify somatic hybrids by flow cytometry and SSR analysis; and 4) to field test somatic hybrids and their progeny with comparable sexually produced populations and various cultivars.

2.2. Plant material

The monoploid families used in this study were of complex origin, derived from diverse backgrounds, with limited common ancestry. Four families of monoploids were selected and designated "A", "O", "N" and "T" according to the *Solanum phureja* PI that represents a major genetic contribution. Each family had at least three monoploid

members. The origin of the 13 monoploids used in protoplast fusions is given in Table 2.1. Most (eight of 13) of the monoploids were "second cycle," i.e., they were derived from anther culture of hybrids that had an anther-derived doubled monoploid (DM) as the maternal parent and a heterozygous pollinator as the male parent. The DMs were the result of chromosome doubling by leaf disc regeneration (Paz and Veilleux, 1999) of first cycle monoploids that were extracted by anther culture of selected clones of adapted *S. phureja*. (Haynes, 1972). The heterozygous pollinators crossed to the DMs were advanced diploid field selections kindly provided by Heilke De Jong (clone 75-21), formerly of Agriculture Canada, Fredericton, NB, and Joseph Pavek [clones ID4 (ADX463-5), ID5 (ADX497-1) and ID8 (ADX881-4), formerly of USDA/ARS, Aberdeen, ID. Although clone 75-21 is predominantly derived from adapted *S. phureja*, the ID clones are complex hybrids, with ID4 and ID5 comprised of ½ *S. stenotomum*, $^{3}/_{16}$ *S. phureja*, $^{3}/_{16}$ *S. tuberosum*, and ⅛ *S. chacoense*, and ID8 comprised of $^{7}/_{16}$ *S. stenotomum*, $^{7}/_{32}$ *S. phureja*, and $^{11}/_{32}$ *S. tuberosum*.

2.3. Culture of plants

In vitro monoploid plantlets were maintained in culture tubes (25 x 150 mm) on 30 mL of Murashige and Skoog (1962) basal medium supplemented with 100 $mg \cdot L^{-1}$ casein hydrolysate, 30 $g \cdot L^{-1}$ sucrose (MS30), 7 $g \cdot L^{-1}$ phytagar (Invitrogen Corporation) or 3.5 $g \cdot L^{-1}$ Phytagel (Sigma), at pH 5.7. Standard growing conditions of shoot cultures were 16/8 h (day/night) photoperiod at 20^{o}C with a light intensity of approximately 100-130 µmol $W \cdot m^{-2}$ supplied by white daylight fluorescent tubes. Plants were subcultured every 4 to 6 weeks.

2.4. Preparation of monoploids – pretreatments

Prior to protoplast isolation and fusion experiments, monoploid shoot cultures were treated on basal MS30 media supplemented with 250 $mg \cdot L^{-1}$ filter sterilized cefotaxime (a broad spectrum antibiotic) for two subculture cycles (each approximately 2-3 weeks duration) to curtail/eliminate endogenous bacteria. Three weeks before protoplast

isolation, three shoots per monoploid line were transferred to baby food jars containing 60 ml of basal MS30 media supplemented with 12 mM $AgNO_3$ and 96 mM $Na_2S_2O_3 \cdot 5H_2O$, to reduce ethylene production and enhance *in vitro* leaf expansion (Perl *et al*., 1988). Plantlets were conditioned at 4°C in the dark for 48 h prior to protoplast extraction to reduce starch granules in the tissue (Jones *et al*., 1989).

2.5. Protoplast isolation

Approximately 1 g of *in vitro* shoots and leaves from monoploid plants was finely minced with No. 10 scalpel blades in sterile 100 x 15 mm disposable petri plates. Ten mL of preplasmolysis medium was added to the macerated tissue and left for ½ h at RT. The preplasmolysis medium was removed with a Pasteur pipette and replaced with 7 mL modified enzyme medium (Cheng and Veilleux, 1991) containing 0.5% (w/v) cellulase "Onzuka" R-10 (Yakult Honsha Co., Ltd., Japan), 0.05% (w/v) Macerozyme R-10 (Yakult Honsha Co., Ltd., Japan) which possesses high pectinase and hemicellulase activity, 1% (w/v) BSA, ½-strength MS salts and vitamins, 0.4 M mannitol and 0.1 M glucose. The petri plates were sealed and incubated overnight, in the dark at RT, on a rotating shaker (40 rpm). Subsequent to incubation, undigested cellular debris was removed by straining the solution through a sterile 63 μm filter. The petri dish was washed with 5 mL of rinse solution to recover any remaining protoplasts and this was incorporated into the digest solution. The filtrate was centrifuged at 500 rpm for 5 min (Dynac II Centrifuge) to gently pellet the protoplasts. The pellet was resuspended in 10 mL flotation medium (Cheng and Veilleux, 1991) and 1 mL of rinse solution (approx. 20 drops) was gently layered above the flotation medium. A subsequent 10 min centrifugation at 500 rpm resulted in intact protoplasts collecting at the interface of the flotation and rinse solutions. The protoplast band was collected with a Pasteur pipette, transferred to a 15 mL centrifuge tube and washed in a 10 mL aliquot of rinse medium. This was followed by centrifugation at 500 rpm for 10 min. The protoplast pellet was resuspended in a Ca^{2+} based fusion medium (1 mM $CaCl_2$ in 8.5 % mannitol, pH 5.6) and the protoplast density adjusted to 1 x 10^6 cells per mL using a haemacytometer and visually checked for quality.

2.6. Electrofusion

Electrofusion experiments were conducted with a 2001 EFS Electro Cell Manipulator (Genetronics, Inc., BTX Instruments Division, San Diego, CA). The fusion chamber consisted of a 3.2 mm Gap Microslide (BT453). Twenty-four fusion combinations were attempted, within nine major experiments, with over 54 individual fusions performed. During each fusion experiment ten drops of fusion medium with suspended protoplasts from each of the fusion pair were mixed in a centrifuge tube prior to application to the Gap Microslide, allowing for a homogeneous mix of protoplasts. After a series of preliminary fusion trials, five fusion-settings with varied parameters were tested on protoplast combinations (Table 2.2). These settings were modifications of parameters previously found to be suitable for monoploid *Solanum* of similar parentage (Johnson, 1998).

Subsequent to electrofusion the protoplasts were allowed to sit for 10 to 20 min to facilitate stable membrane formation, before being transferred to a 15 mL centrifuge tube. The protoplasts were gently pelleted by centrifugation at 500 rpm for 5 min and the pellet resuspended in culture medium (Cheng and Veilleux, 1991). This medium was a modification of Schumann and Koblitz (1983) medium. The density of the protoplasts was adjusted to 2.5 x 10^5 per mL and transferred to 60 x 15 mm petri dishes with approximately 5 mL of culture medium per dish. The sealed petri dishes with protoplasts were incubated at RT under low indirect light.

2.7. Callus regeneration

The protoplasts were undisturbed for 3 to 7 days after which the petri dishes were examined under an inverted microscope for signs of regeneration, e.g., flow of protoplasts, vacuole enlargement, cell wall formation (irregular shape) and cell division. The old culture medium was replaced weekly by transferring the contents of each petri dish to a centrifuge tube and pelleting the cells by centrifugation at 500 rpm for 5 min.

Bacterial contamination occurred in certain fusion combinations and in these regenerants the culture medium was amended by the incorporation of 250 mg L^{-1} cefotaxime. The cultures were maintained on liquid regeneration medium for approx. 3 to 7 weeks until calli of 1 to 2 mm diam were observed. In early experiments embedding of cells in culture medium with low gelling agarose (Agarose Type VII: Low Gelling, Sigma Cell Culture) was attempted; however, this was not as productive as weekly changes of liquid medium. As protoplast derived calluses enlarged (after approx. 4 weeks) the culture medium was changed by gentle removal with a pasture pipette instead of centrifugation.

2.8. Greening

Visible calli, approx. 1-2 mm diam, were individually transferred onto greening medium [modified MS supplemented with 36.44 $g \cdot L^{-1}$ mannitol, 2.498 $g \cdot L^{-1}$ sucrose, 0.5 $mg \cdot L^{-1}$ BA, 0.05 $mg \cdot L^{-1}$ NAA and 10 $g \cdot L^{-1}$ agarose (LOW EEO); (Tan *et al*., 1987). Calli were transferred to growth chambers at 16/8 h (light/dark) photoperiod with a light intensity of approx. 100-130 $\mu mol \cdot W \cdot m^{-2}$ supplied by white daylight fluorescent tubes, 20^{o}C for 4 weeks.

2.9. Calli regeneration

Light or dark green calli (depending on the fusion combination) were transferred to J1 callus regeneration medium (Cheng and Veilleux, 1991), a modified MS basal medium amended with 100 $g \cdot L^{-1}$ casein hydrolysate, 100 $mg \cdot L^{-1}$ adenine sulphate, 2.50 $g \cdot L^{-1}$ sucrose, 10 $g \cdot L^{-1}$ mannitol, 0.2 $mg \cdot L^{-1}$ GA_3, 1 $mg \cdot L^{-1}$ trans-zeatin, 0.1 $mg \cdot L^{-1}$ IAA and 10 $g \cdot L^{-1}$ Noble agar or Bactoagar. Calli were maintained on J1 media until shoot initials developed, which was dependent on fusion combinations, with regeneration intervals ranging from 4 weeks to 6 months; some combinations did not regenerate. Calli with developing shoots were transferred to shoot proliferation medium (Austin and Cassells, 1983, Haberlach, 1985 #366); subsequently developed shoots were placed on MS basal medium with additional copies made for ploidy analysis or rooted in preparation for acclimatization in the greenhouse.

2.10. Ploidy identification

The identification of ploidy level of regenerated shoots was conducted by flow-cytometry. Analysis of samples was carried out on a Coulter Epics XL Flow cytometer (Coulter International Corp., Miami, Fla.,). Approx. 0.5 g of *in vitro* leaf and stem was chopped finely with a razor blade in 1.5 mL chopping buffer (30 mM sodium citrate, 20 mM MOPS, 45 mM $MgCl_2$ and 0.04% Triton X-100). The released nuclei were separated from cellular debris by successive filtration through a 250 µm filter above a 63 µm filter and the filtrate collected in a beaker. The filtrate (0.5 mL) was treated for 30 min with 0.25 mL ribonuclease A (40 mg per 50 mL chopping buffer) and 30 min prior to analysis stained with 0.125 mL propidium iodide (6.0 mg propidium iodide in 15 mL chopping buffer) (Owen *et al*., 1988).

2.11. Molecular marker confirmation of somatic hybrids

No selectable markers, physiological or morphological, were available for preliminary identification of somatic hybrids. All regenerants were screened and hybrid status confirmed using molecular markers based on PCR amplification and SSR analysis.

Molecular analysis was carried out on total DNA extracted by a modified CTAB (hexadecyltrimethylammonium bromide) method (Doyle and Doyle, 1987). Approx. 1.5 g of shoot and leaf tissue were treated with liquid nitrogen and ground to a fine powder with mortar and pestle. Seven mL of warmed 2X CTAB (2% CTAB, 100 mM Tris, pH 8.0, 1.4 M NaCl and 20 mM EDTA) and 0.4 g (4% w/v) PVP 40 were mixed with the ground sample and topped with 3 mL 2X CTAB used for washing the mortar, and placed in a 50 mL centrifuge tube. The grindate was incubated for 60 min at 65^{o}C and subsequently 10 mL of chloroform:isoamyl alcohol (24:1 stock solution) added and mixed to extract the non-polar cellular components. The mixture was centrifuged at 4000 rpm for 10 min and the aqueous upper layer saved in a new 50 mL centrifuge tube. Ice-cold isopropanol (5 mL) was gently mixed with the supernatant to precipitate the DNA.

The tube was stored at –20°C overnight, then centrifuged at 4000 rpm for 10 min to pellet the DNA. The supernatant was poured off and the DNA incubated at RT in 20 mL wash buffer (10 mM ammonium acetate, 75% ethanol) for 20 min. The DNA was re-pelleted by centrifugation at 4000 rpm for 10 min, the supernatant discarded and the DNA pellet allowed to air dry. The pellet was resuspended in 300 µL TE solution and RNase treated (1 µL RNase A in a 10 $mg \cdot mL^{-1}$ stock) for 30 min at RT. The DNA was subsequently quantified using a Beckman DU 7000 spectrophotometer. DNA samples were diluted in TE (1:50 or appropriate dilution according to DNA concentration) to give a total volume of 100 µL during readings.

The DNA PCR amplification reactions for SSR analysis were our current laboratory standard, a modification based on procedures reported by Yu *et al.* (1994). Amplifications were carried out in a 20 µL reaction volume containing 160 µM of each dNTP (dATP, dTTP, dGTP and dCTP), 3 mM $MgCl_2$, 1x buffer (50 mM KCl, 10 mM Tris-HCl (pH 9) and 1% Triton X-100), 1.5 U *Taq* DNA polymerase (Promega, Madison, Wis.), 0.1 µM of each primer and 50 ng genomic DNA (10 ng μL^{-1} stock). The PCR procedure for SSRs consisted of an initial denaturating step of 3 min at 4°C, followed by 40 cycles at 94°C for 1 min (denature), 55°C for 2 min (anneal), 72°C 1.5 min (extension), and a final extension cycle of 72°C for 5 min. After PCR, 15 µL of the reaction mix was separated on 3% Metaphor agarose gels (FMC Bioproducts, Rockland, Me.) in 0.5% TBE buffer (44.6 mM Tris, 34.4 mM boric acid, 100 mM EDTA) at 90 V for 4 h at room temperature (Sambrook *et al*., 1989). A 100 bp ladder or λ ladder (Promega, Madison, Wisconsin) was used as a molecular weight marker and the gel was stained with 0.1% ethidium bromide prior to visualization and photographed under UV light using Polaroid film 665.

2.12. Anther culture (Ploidy reduction)

Our first intermonoploid somatic hybrid (SH-18C) was tetraploid, presumably resulting from fusion between diploid somatic cells that had undergone a single cycle of endopolyploidization in the monoploid leaf tissue. In order to reduce the ploidy to the

diploid level, we used anther culture on SH-18C. The anther culture experiment was designed to test the effects of sugars in the culture medium on embryo yield. Five different treatments were examined in four replicates: 1) 3% sucrose, 3% maltose and 0.5% activated charcoal (AC), 2) 6% maltose and 0.5% AC, 3) 6% sucrose and 0.5% polyvinylpolypyrrolidone (PVPP), 4) a complex sugar mix (1% mannitol, 1% sorbitol, 1% sucrose, 1% fructose, 1% xylose and 1% rhamnose) and 0.5% AC, and 5) 6% sucrose and 0.5% AC (control).

Greenhouse tubers of somatic hybrid SH-18C, the result of electrofusion between monoploids O8-1 and T5-268, produced by Alex Johnson, a former graduate student, were planted in the field at Kentland Whitethorne farm on April, 2000, and buds collected in June when anthers were 3–5 mm in length, corresponding to the late uninucleate stage of microspore development (Veilleux, 1990). The method for anther culture was as described by (Taylor and Veilleux, 1992). Buds were placed on moist filter paper in plastic bags, and refrigerated at 4^{o}C for 3 days prior to culture. Surface sterilization was a two-step procedure, 80% ethanol for 30 sec followed by 100% commercial bleach (Clorox, 5.25% sodium hypochlorite) plus 2 drops of Tween-20 surfactant for 10 min. This was followed by two rinses in sterile distilled water. Anthers were excised, and placed into 125 mL Delong culture flasks containing 15 mL culture medium. The five anthers per bud were distributed across the five sugar treatments until each flask contained 30 anthers, each from a different bud. Three replicates of flasks were placed on rotary shakers, 140 rpm and incubated in the dark, at RT for 6 to 8 weeks.

Embryos were tallied and transferred to modified V1 medium (Uhrig, 1985) - B_5 medium with minimal organics (Gamborg *et al*., 1968), supplemented with 0.5 mg$\cdot$L^{-1} GA_3, 1% sucrose and solidified with 0.6% agarose, pH 5.6. Embryos were grown in an incubator (Percival) at 20^{o}C, 16/8 day/night cycle with a light intensity of approx. 100-130 µmol W$\cdot$m^{-2}. When shoots had elongated they were subcultured on MS basal medium. Subsequently, the ploidy level of the regenerants was determined by flow cytometry as described above.

2.13. Fertility of hybrids

Somatic hybrids were acclimated to the greenhouse during spring, 2001. When flowering was coordinated with cultivars the somatic hybrids were crossed as male or female parent to determine the fertility of somatic hybrids. Where possible reciprocal crosses with cvs. Atlantic and Katahdin were made. Fruit were harvested, seeds counted and resulting progeny used in field trials.

2.14. Field evaluations of somatic hybrids

Field evaluations were conduced in 2000, 2001 and 2003.

2000. A preliminary field evaluation was conducted at the Kentland Whitethorne Farm with somatic hybrid SH-18C, cv. Atlantic and seedlings resulting from crosses between Atlantic x SH-18C and SH-18C x Katahdin, in an RCB plot with 20 plants per plot and three replications. Seedlings were started in the greenhouse on 13 April and transplanted to the field on 17 May, at which time tubers of SH-18C and Atlantic were planted. The plot was harvested on 29 September, 2000. Tuber set and total yield of tubers per plant were recorded.

2001. The 2001 field plot consisted of somatic hybrid SH-18C, crosses with cultivars, SH-18C x Katahdin, Atlantic x SH-18C and, in an attempt to recreate a sexual population comparable to the somatic hybrid, we planted seeds resulting from crosses between diploid genotypes (O8-8, O8-9, T5-2 and T5-12) that were closely related to anther donors of the monoploids. Finally there was a population of dihaploids, DH-18C, derived by anther culture of the somatic hybrid, SH-18C. Seedlings and dihaploids were planted in the field from first generation greenhouse tubers produced on tuberlings that had been planted in August 2000 with tubers harvested in January 2001. The greenhouse tubers were stored at 4ºC until planting (April 15, 2001); cvs. Atlantic and Katahdin were planted but the plots had poor stand and were thus excluded from data analysis. An RBC

plot design was used with 20 plants per plot in three replicates. The plot was harvested on 14 September 2001.

In 2003 genotypes tested consisted of cvs: Atlantic and Katahdin, crosses with somatic hybrids, SH-18C and SH-N(3-2-6)4-21C and 2x X 2x crosses of ancestral "parental" types, A-1 x T-7, 1-3 x ID5, N-12 x O-3. Four fertile somatic hybrids were tested, SH-18C, SH-OT3-1B, SH-OT3-1F and SH-N(3-2-6)4-21C and the progeny of three crosses between somatic hybrids. Due to a prolonged wet spell in the spring, the plot was not planted until 11 June using greenhouse tubers grown from tuberlings as in 2002; tubers of cultivars were acquired as elite seed from Maine. An RCB design was used as in previous years. The plot was harvested on 26 September.

2.15. Results

Four monoploid families designated, A, O, N and T, were used in protoplast electrofusion experiments. A diallelic series of experiments was conducted using one member from each family (Table 2.3). Experiments were repeated according to the growth habits of the monoploids, and hence, prolific genotypes were tested more often than recalcitrant types. Recalcitrant genotypes included; A family – MH-14P, O family- 1-3 3N7, and N family – N5-6 3C; these genotypes grew slowly in culture and often did not produce enough leaves and shoots for protoplast extraction in the 3 to 4 week culture period. Other genotypes; A family – PP5-15, N family – 9-9-204 and T family- 13-14 203 produced protoplasts that degenerated during enzyme digestion or subsequent to electrofusion, suggesting that the electrofusion conditions or/and regeneration protocol were not optimized for these genotypes.

Fusion conditions tested (Table 2.2) were based on settings previously used during the creation of somatic hybrid, SH-18C, through the fusion of monoploids T5-268 and O8-1 (Johnson, 1998). Five setting conditions were tested. These setting conditions were slight variations of the standard conditions (setting 1) and consisted of modifying the AC set (V) from 10 to 8 or 12 volts, increasing the duration of AC set (V) from 30 to 45 sec,

and decreasing the set voltage (amp) from 450 to 400 and finally adjusting the number of pulses from 2 to 1 or 3.

Setting conditions three and four were the most successful, while setting conditions two and five did not produce any regenerants (Table 2.2). Surprisingly, setting condition one (the control) also did not produce any new regenerants. Successful setting condition three consisted of decreasing the AC set from 10 to 8 V while increasing the duration from 30 to 45 sec and simultaneously decreasing the amp from 450 to 400 (with two pulses), whereas setting condition four (also successful) increased the voltage applied from 10 to 12 V but reduced the number of 450 amp pulses from two to one (Table 2.2). The response to the electrofusion conditions was genotype dependent (Table 2.3), with only combinations N8-2 AR8 + O5-10 3BB regenerating under both conditions three and four (Table 2.4); however, only somaclones of O5-10 3 BB, regenerated under condition three.

A total of 276 plants was regenerated from six fusion combinations, although not all were completely characterized prior to death. However, all regenerants were evaluated for ploidy by flow cytometry (Figure 2.1). Table 2.4 gives a summary of the characterized fusion regenerants. Regeneration of fusion products was genotype dependent and occurred 4-9 months after protoplast isolation. Calli that had not regenerated after 12 months were discarded. Figure 2.2 depicts stages during the regeneration cycle and Figure 2.3 is an example of a regenerated hybrid in flower.

The ploidy of the regenerants, characterized by flow cytometry, ranged from 2x to 8x with a few displaying mixoploidy: 2x/3x, 3x/4x, 4x/5x, 4x/6x and 5x/6x. All diploid regenerants were later characterized as somaclones of one of the parental types.

SSR primer pairs RV3 and RV4 or RV15 and RV16 (Table 2.5) were used to identify somatic hybrids. Two somatic hybrids, SH-1A (6x) (Figure 2.2) and SH-18C (4x) (Figure 2.3), were identified from fusion combination O8-1 + T5-268 (population inherited from Alex Johnson), somaclones of O8-1 (2x, 4x, and a mixoploid) were also

regenerated. All 42 regenerants from fusion combination O5-10 3BB + T4-142 were identified as somatic hybrids. These regenerants were from four individual calli and had varied ploidies of 3x, 4x, 6x and 4x/6x mix. Out of a total of 89 regenerants, characterized members of the N8-2 AR8 + O5-10 3BB fusion included 19 somatic hybrids (6x) and ten O5-10 3BB (2x) somaclones; unidentified regenerants had ploidy of 2x, 6x and 8x. Seventeen of the 22 regenerants of the N8-2 AR8 + 1-3 3N-7 fusion attempt were identified as 1-3 3N-7 (2x) somaclones; unidentified members had 2x, 4x and 2x/3x ploidy. Fusion combination N8-2 AR8 + 3-2-6 6N-1 produced 85 regenerants from 37 individual calli (15* of Table 2.3); 15 were identified as 4x or 6x somatic hybrids whereas 40 3-2-6 6N-1 somaclones were 2x or 4x (Table 2.4).

Anther culture: ANOVA of mean number of embryos produced during anther culture experiments for the production of dihaploids of somatic hybrid SH-18C testing various sugars in the composition of the regeneration media (Table 2.6) revealed highly significant differences among the treatments ($P>0.002$). There was also a significant difference among reps ($P>0.01$). Of the five sugar combinations tested, 3% sucrose + 3% maltose + activated charcoal produced the greatest number of embryos (mean 4.0 ± 1.4), closely followed by treatment 6% maltose + activated charcoal (3.1 ± 3.0) (Table 2.6). The control, 6% sucrose + activated charcoal had an intermediate response (1.1 ± 0.5), while treatments substituting activated charcoal with PVPP or using a complex mix of six sugars were not beneficial and hindered embryo development.

Field trials: General trends, over the three years, revealed that the somatic hybrids produced similar tuber number but smaller tubers and hence lower tuber yield than cultivars. Crosses between somatic hybrids and cultivars increased mean tuber weight and yield compared to hybrids. The preliminary experiment conducted in 2000 demonstrated that 4x somatic hybrid SH-18C had fewer tubers and smaller total tuber weight than Atlantic or cultivar x hybrid crosses. Crosses with the hybrids differed only slightly from Atlantic for total tuber weight (Table 2.7).

For the 2001 field trial ANOVA revealed that genotype was a highly significant source of variation for mean tuber number, mean tuber weight and mean tuber yield (Table 2.8). Replication was also a significant source of variation for mean tuber number and weight (Table 2.8). Mean tuber number per plant ranged from 12.8 to 28.5, with the dihaploids of SH-18C (DH-18C) having significantly fewer tubers per plant than all of the other diploid and tetraploid hybrids (Table 2.8). The mean tuber weight per plant was greatest for the cultivar hybrids, intermediate for the 2x X 2x crosses, and least for the somatic hybrid SH-18C and its dihaploids, DH-18C (Table 2.8). A similar trend was displayed for total tuber yield, with four groups produced: cultivars X somatic hybrid crosses outperformed all other groups (2x X 2x, SH and DH respsectively). The somatic hybrid, dihaploids and three 2x X 2x crosses (T5-2 x 08-8, T5-2 x O8-9 and O8-9 x T5-2) occurred in the poorest yielding group and the remaining 2x X 2x crosses were intermediate. Samples of tubers are shown in Figure 2.6.

In the 2003 field trial, cultivars, somatic hybrids (4), crosses between somatic hybrids and cultivars, crosses between ancestral "parental" genotypes and crosses between somatic hybrids were tested. Parental crosses represented a genetically similar group to the somatic hybrid without having gone through the "monoploid sieve" while crosses between somatic hybrids represent a highly heterozygous group that would have passed through the monoploid sieve and with the potential of highest heterosis (vigor). ANOVA revealed that genotype was a significant source of variation for tuber set, mean tuber weight and mean tuber yield (Table 2.9). There were no significant differences among replications. The cultivars produced a smaller number of larger tubers per plant resulting in greater tuber yield than most of the other entries (Table 2.9). The somatic hybrids themselves were unremarkable and did not improve by intercrossing, as the SH x SH crosses representing highly heterozygous tetraploids combining alleles from as many as four different monoploids were likewise unremarkable, performing no better than the 2x x 2x control crosses that had no experience with the monoploid sieve. Because of their greater tuber number per plant, some of the cultivar x SH hybrids produced as much mean tuber yield per plant as cv. Katahdin; however, none was as productive as Atlantic (Table 2.9).

2.16. Discussion

Our study of the development and performance of intermonoploid somatic hybrids of potato serves as a model for the benefits and challenges of reducing the genome of a highly heterozygous species to complete homozygosity and then restoring heterozygosity by protoplast fusion. It bypasses sexual barriers to self- and cross-pollination and selects from millions of microspores placed into anther culture those that have the genetic composition to regenerate into viable plants. It addresses some of the same concepts employed in the development of inbred lines for breeding hybrids, only telescoping years or even decades of self-pollination and selection into two cycles: one to reach homozygosity and another to observe hybridity. Of course, there are limitations to the imposition of this synthetic hybrid breeding scheme that may well have precluded the realization of bumper crops of highly heterozygous somatic hybrid potatoes that were unrealistically but optimistically conceived at the onset of the project many years ago.

Our germplasm for testing this theory was a diploid cultivated species resembling potato but without many of the horticultural traits that have been demanded and elicited from *S. tuberosum*. This was the only potato germplasm available that could be used in the scheme – it has lots of potato-like qualities but no one would mistake it for Russet Burbank or Bintje. Furthermore the germplasm was limited to what would respond to the culture conditions. For example the production of p-calli after protoplast isolation has been shown to be genetically controlled (Cheng and Veilleux, 1991; Haberlach *et al*., 1985; Tan *et al*., 1987). Protoplast culturability is controlled by two genes and requires dominant alleles at each locus for expression of the trait (Taylor and Veilleux, 1992). Protoplast fusions between regeneration competent monoploids and non-regenerants should theoretically result in regeneration competent hybrids. There were major challenges to obtain the germplasm in the first place – we used second cycle monoploids with improved vigor, instead of Uijtewaal's sickly monoploids of *tuberosum*. Modification of protoplast fusion conditions were required to accommodate the frailty of monoploid protoplasts. We encountered some of the same limitations as using *Arabidopsis* as a model for crop plants, in that it has all the genes but not exactly in the right combination. In our case the bad traits of S. *phureja* would not be likely to be

removed just because it passed through the monoploid sieve. Comparision made with potato cultivars are therefore used as a benchmark ideal while 2x X 2x crosses are the most appropriate genome comparison to evaluate the effect of the monoploid sieve.

Tetraploid somatic hybrid regenerants between two monoploids are at best duplex at any one locus that differed allelically between the two parents. Tri- and tetra-allelism that is thought to account for some of the vigor of cultivated potato would not have been possible in our first generation somatic hybrids. The crosses between intermonoploid somatic hybrids of differing origin in our third year of field trials were our attempt to overcome this limitation by increasing allelic diversity in the germplasm while preserving only alleles that had survived the monoploid sieve. These hybrids were unexceptional, in that there was no pronounced heterosis.

Comparison with cultivar and cultivar by hybrid crosses during field evaluations revealed that the somatic hybrids were significantly less productive (mean tuber weight and mean tuber yield) than cvs. Atlantic and Katahdin, while crosses of somatic hybrids with cultivars yielded seedlings that were in the range of productivity of the cultivars. The somatic combination of monoploids did not result in significantly improved hybrids compared to cultivars. The monoploids used for the fusion experiments were derived from a complex germplasm with a large proportion of *Solanum phureja* and various wild potato species that were originally selected for heat tolerance and alkaloid content (leptene) and not for tuber size or yield. *Solanum phureja* is a prolific tuber producer but tubers are small with a short dormancy period in contrast to potato cultivars Atlantic and Katahdin. Comparision made with potato cultivars were therefore used as a benchmark ideal.

Comparison with diploid hybrids for example between the somatic hybrid and 2x X 2x sexual derived population comparable with the somatic hybrids, revealed that the sexually derived population was slightly better (but not significantly) for factors tuber weight and mean tuber yield. Diploid hybrids (2x X 2x crosses) allow for genome comparison between sexually and non-sexually derived hybrids and therefore the effect

of the monoploid sieve. This comparison is highly relevant and suggest that some step in the process of producing somatic hybrids, such as the production of parental monoploids (anther culture or IVP induced gynogenesis), protoplast electrofusion or tissue culture regeneration may be introducing factors that are negatively affecting the genome of the hybrids. Austin and Cassells (1983) has demonstrated that the calli stage of regeneration is a cause of somaclonal variation. In addition, somaclonal variation induced during the tissue culture regeneration process as represented by chromosome rearrangements or deletions has been identified during fusions (Jansky *et al*., 1999; Polgar *et al*., 1999; Waara *et al*., 1992; Williams *et al*., 1993 Wolters, 1994 #318). Comparisons between somatic hybrids and sexually derived parental cross reveal no significant differences in mean tuber weight or yield. There was great variation in mean tuber number but significantly greater numbers in the parental crosses. This again is revealing no improvement in somatic hybrids compared to sexual crosses. In addition, the anticipated increase in heterosis vigor by crossing somatic hybrids (SH-N(3-2-6)4-21C x SH-OT3-1B, SH-OT3-1B x SH-N(3-2-6)4-21C and SH-OT3-1F x SH-N(3-2-6)4-21C) was not realized when compared to the parental somatic hybrid for mean tuber weight and tuber yield.

Is there a benefit to bad alleles? With the *S. tuberosum* crosses, there would be bad alleles along with good alleles for horticultural traits. However, with the *S. phureja* crosses, there would be bad alleles and no better alleles from *phureja* than have already been distilled. So, the crosses of intermonoploid SH should have been better than the 2x *phureja* crosses if the monoploid sieve is applicable. The superiority of the cultivar crosses could be due to domesticated alleles.

Reduction of ploidy of SH18C by anther culture that is anther culture experiments conducted during the regeneration of dihaploid population, DH-18C revealed that changing the carbon source from 6% sucrose to 6% maltose or 3% sucrose + 3% maltose mix while in activated charcoal in the mix significantly improved embryo regeneration, while substituting sucrose with a six sugar sources mix (1% sucrose, 1% fructose, 1% mannitol, 1% sorbitol, 1% xylose and 1% rhamnose) hindered embryo regeneration.

These results complement earlier studies by Batty and Dunwell (1989) where substitution of maltose in the anther regeneration media improved embryo regeneration. These studies also revealed that the inclusion of activated charcoal is beneficial to embryo regeneration as substitution of AC with PVPP resulted in reduction in the number of embryos regenerated. Field evaluation of DH-18C confirmed reduction of vigor with increase in homozygosity. Parental somatic hybrid, SH-18C, is tetraploid (4x), while the DH18C, also tetraploid, but derived from a 2x gamete (reduced cell) which had spontaneously doubled during the tissue culture regeneration process to give a 4x (doubled haploid) individual. (It is also possible that the anther-derived regenerants were produced from unreduced gamete but this would have been a very rare event in the sample population examined.) Similar results have been shown in earlier studies by Uijtewaal (1987), where monohaploid potatoes derived from heterozygous diploid were vastly less vigorous, however, on restoration of the 2x ploidy level there was an increase in vigor, but, little increase from 2x to 4x. This suggests positive additive gene dosage effect only at the 2x level, and that dominant effects may have a greater role at the 4x ploidy level. The anther-derived dihaploids got even worse than the somatic hybrids, presumably through additional inbreeding. It is also possible that deleterious gametoclonal and somaclonal variation occurred during the processes of regeneration.

In conclusion, it is possible that the monoploid sieve - the removal of lethal and deleterious genes - may have eliminated epistatic and intraallelic interactions possible in heterozygotes due to lethalities of genes at the monoploid level or linkages between favorable alleles and lethals. The classic example of heterozygote superiority is the sickle cell mutation in humans, where the deleterious allele that causes anemia in homozygotes actually bestows a selective advantage to heterozygous individuals compared to homozygous wild types in resistance to malaria (Williams, 2003). It is possible that heterozygosity at loci with one or more deleterious alleles that cannot survive as a hemizygote actually improves the performance of the plants carrying them in heterozygous condition.

Maybe inbreeding without selection is too drastic. Lashermes *et al.* (1994) observed severe inbreeding depression (high mortality, sterility and loss of vigor) while working with doubled haploids of *Coffea canephora*. He assumed a high genetic load, and with strict homozygosity of DHs there could be frequent expression of lethal and sub-lethal genes. In contrast, polygenetic traits with quantitative expression (disease resistance) were favored by high heterozygosity levels.

2.17. References

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Table 2.1: Ancestry of monoploids used for electrofusion experiments. Most of the monoploids are "second cycle," derived from hybrids between "first cycle" homozygous doubled monoploids (DM) and heterozygous pollinators. The female and male parents are the maternal and paternal parents of the anther donor, respectively. Where the female parent is a DM, the anther donor of the DM is given.

Family	Monoploid	Anther donor	Female parent	Anther donor of DM	Male parent	Pedigree
A	PP5-15 3-2-6 6N-1* MH-14P	PP5 A3P2-6 Gynogenic line of PP5	PHU66P4 DM AD3-4 --	-- PP5 --	PHU66P10 75-21 --	PI 225669 x PI 225682 AD3-4 (from PP5) x 75-21
O	O5-10 3BB O8-1 1-3 3N-7	O5-10 O8 BARD 1-3	DM BARD 1-3 516 DM BARD 1-3 516	BARD 1-3 BARD 1-3 --	ID 5 ID 8 --	PI 225669 x ADX497-1 PI 225669 x ADX881-4 PI 225669
N	N8-2 AR8 N5-6 3C 9-9 204	N8-2 N5-6 BARD 9-9	DM BARD 9-9 203 DM BARD 9-9 203 --	BARD 9-9 BARD 9-9 --	ID 8 ID 5 --	PI 225709 x ADX881-4 PI 225709 x ADX497-1 PI 225709
T	T5-268/T5-284 T4-142 13-14 203*	T5 T4 BARD 13-14	DM BARD 13-14 202 DM BARD 13-14 202 --	BARD 13-14 BARD 13-14 --	ID 5 ID 4	PI 243461 x ADX497-1 PI 243461 x ADX463-5 PI 243461

*Subsequently doubled spontaneously in culture

Table 2.2. Protoplast electrofusion settings tested during generation of intermonoploid somatic hybrids.

Settings	**1**	2	3	4	5
AC set (V)	10	**12**	**8**	**12**	**8**
Duration (sec)	30	30	**45**	30	**45**
Set pulse (µsec)	60	60	60	60	60
Set voltage (amp)	450	450	**400**	450	**400**
Number of pulses	2	2	2	**1**	**1**

Setting 1:Control settings (Johnson 1998)
Figures in red indicate modifications from control

Table 2.3. Attempted protoplast electrofusions between monoploids representing four potato families.

		A			O			N			T			
		PP5-15	3-2-6 6N-1	MH-14P	O5-10 3BB	08-1	1-3 3N-7	N8-2 AR8	N5-6 3C	9-9 204	T5-268	T4-142	13-14 203	T4-284
A	PP5-15	---												
	3-2-6 6N-1	---	---											
	MH-14P	---	---	---										
O	O5-10 3BB	0	np	0	---									
	O8-1	nt	0	nt	---	---								
	1-3 3N-7	0	np	nt	---	---	---							
N	N8-2 AR8	2*	15*	0	19*	Nt	17#	---						
	N5-6 3C	nt	nt	nt	nt	nt	nt	---	---					
	9-9 204	0	0	nt	0	0	0	---	---	---				
T	T5-268	nt	nt	nt	nt	2*	nt	nt	nt	nt	---			
	T4-142	0	np	0	42*	nt	0	0	nt	0	---	---		
	13-14 203	0	0	0	0	0	0	0	nt	0	---	---	---	
	T5-284	nt	nt	nt	nt	nt	nt	nt	nt	nt	---	---	---	---

* = successful regeneration of somatic hybrids
# = regeneration of somaclones
np = no viable protoplasts after enzyme digestion of 3-2-6 6N-1
nt = not tested

Table 2.4. Evaluation of electrofusion regenerants.

Fusion settings	Parent 1	Parent 2	Somatic hybrids	Somaclones	Ploidy	Fertile
1	**O8-1**	**T5-268**	2 (1,1)	31	4x, 6x	h
3	**O5-10 3BB**	**T4-142**	42 (10,12,10,10)	0	3x,4x,6x	f, h
3,4	**N8-2 AR8**	**O5-10 3BB**	19 (9,10)	10	3x,4x,6x,8x	f
4	**N8-2 AR8**	**1-3 3N-7**	0	17	2x, 2x/3x 4x	No somatic hybrids
4	**N8-2 AR8**	**3-2-6 6N-1**	15 (4, 2, 1, 1, 1, 3,3)	40	2x,4x,6x	h
4	**N8-2 AR8**	**PP5-15**	2 (1)	0	3x,6x	died

Values within parenthesis = number of shoots regenerated from same callus
f = female fertile
h = both male and female fertile

Table 2.5. Description of simple sequence repeats (SSR) used for hybrid identification.

Primer Name	Primer Sequence	Product Size	Type of Repeat	Locus	Accession #
RV3 **RV4**	5’ ccc ata ata ctg tcg atg agc a 3’ 5’ gaa tgt agg gaa aca tgc atg a 3’	223 bp	$(actc)_5$	G28WXST	X52417
RV15 **RV16**	5’ aat tca tgt ttg cgg tac gtc 3’ 5’ atg cag aaa gat gtc aaa att ga 3’	250 bp	$(aag)_7$	STACCAS3	Z27235

Table 2.6. ANOVA and means for embryos per anther produced from cultured anthers of intermonoploid somatic hybrid SH18C on five different media differing by sugar composition.

Source	DF	SS	MS	F-value	P>F
Treatment	4	596	149	4.5	0.002
Rep	3	378	126	3.8	0.01
Trt x Rep	12	1608	134	4.1	0.0001
Error	89	2937	33		
Total	108	5519			

Treatment*	n	Mean embryos per anther ± SE
3% Sucrose + 3% Maltose + AC	22	4.0 ± 1.4
6% Maltose + AC	21	3.1 ± 3.0
6% Sucrose + PVPP	22	0.2 ± 0.1
6% Sucrose + AC	23	1.1 ± 0.5
Mix+AC	21	0.3 ± 0.2

* AC = 0.5% activated charcoal
PVPP = 0.5% polyvinylpolypropyline
Mix: 1% sucrose, 1% fructose, 1% mannitol, 1% sorbitol, 1% xylose, 1% rhamnose.

Table 2.7. Mean tuber number and weight in preliminary field trial 2000, for somatic hybrid (SH-18C), cultivar by hybrid crosses and Atlantic, RCB design, three blocks.

Genotype (Cross)	Mean ± SE Number of tubers	Mean ± SE Tuber weight (g/plant)
Atlantic	*	800
SH-18C x Katahdin	21 ± 1.5	739 ± 54
Atlantic x SH-18C	23 ± 1.7	762 ± 58
SH-18C	13 ± 2.3	453 ± 61

* = missing data

Table 2.8. Mean squares from ANOVA and means for tuber set, tuber weight and tuber yield per plant of field trial 2001 for somatic hybrid (SH-18C), SH18-C by cultivars crosses, 2x X 2x crosses and dihaploids (DH-18C) derived by anther culture of SH18C.

		Mean tuber set	Mean tuber weight	Total tuber weight
Source	**df**	**MS**	**MS**	**MS**
Genotype	9	611.7***	2358.9***	1928804.6***
Rep	2	640.3*	194.1ns	477357.8*
Error	180	160.5	146.1	1131607.3

* = significant at the 5% level; ** = significant at the 1% level;
*** = significant at the 0.1% level; ns = not significant

Type of germplasm	Genotype or family	N	Mean tuber set	Mean tuber weight (g)	Total tuber yield (g)
Cultivar X somatic hybrid	Atlantic x SH-18C	16	24.2 abc	47.9 a	1138 a
	SH-18C x Katahdin	18	25.4 abc	46.1 a	1089 a
2x X 2x	T5-12 x O8-8	21	28.5 a	29.1 b	746 b
	O8-8 x T5-12	21	25.9 ab	29.1 b	639 bc
	T5-2 x O8-8	20	16.8 abc	27.6 b	391 bcd
	O8-8 x T5-2	20	25.9 ab	19.7 bc	484 bc
	T5-2 x O8-9	19	15.3 bc	27.7 b	410 bcd
	O8-9 x T5-2	21	16.7 abc	29.9 b	447 bcd
Somatic hybrid	SH-18C	18	17.2 abc	18.8 bc	315 cd
Dihaploids of somatic hybrid	DH-18C	18	12.8 c	9.6 c	118 d

Means within columns followed by the same letter are not significantly different by Ryan-Einot-Gabriel-Wilson Multiple Range Test at the 5% level.

Table 2.9. Mean squares from ANOVA and means for tuber set, tuber weight and tuber yield per plant of field trial 2003 for somatic hybrids, inter-hybrid crosses, hybrid by cultivar crosses and 2x X 2x near parental type crosses.

		Mean tuber set	Mean tuber weight	Mean tuber yield
Source	**df**	**MS**	**MS**	**MS**
Genotype	15	1353.7***	24925.3***	1267258.5***
Rep	2	177.7 ns	285.9 ns	56018.8 ns
Error	325	189.9	473.6	43029.8

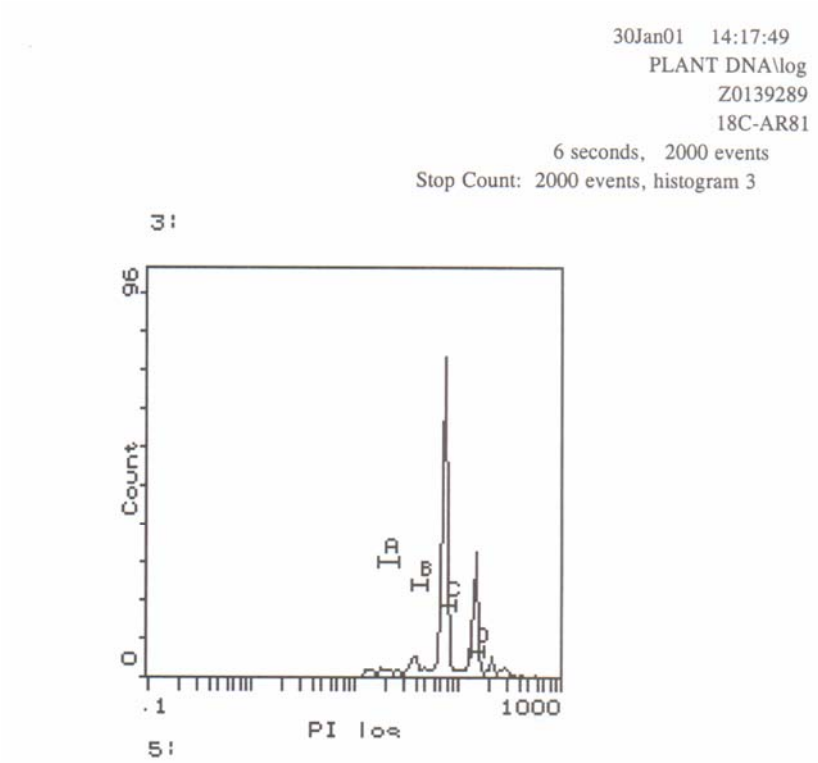
* = significant at the 5% level; ** = significant at the 1% level;
*** = significant at the 0.1% level; ns = not significant

Type of germplasm	Genotype	n	Mean tuber set per plant	Mean tuber weight (g)	Mean tuber yield (g)
Cultivar	Atlantic	22	8.5 de	139.9 a	1028.2 a
	Katahdin	7	7.7 de	105.3 b	582.0 bc
Cultivar X somatic hybrid	Katahdin x SH-N(3-2-6)4-21C	29	25.7 abc	24.2 cde	588.5 bc
	Atlantic x SH-N(3-2-6)4-21C	26	25.5 abc	27.2 cde	663.9 b
	SH-N(3-2-6)4-21C x Atlantic	23	17.3 abcde	25.7 cde	421.8 d
	Atlantic x SH-18C	28	19.9 abcde	21.6 cde	369.1 d
Diploid crosses	A-1 x T-7	26	27.8 ab	9.6 de	271.8 de
	1-3 x ID-5	26	26.3 abc	10.1 de	248.0 de
	N-12 x O-3	24	22.0 abcd	13.6 de	243.4 de
Somatic hybrids	SH-18C	13	13.0 d	33.5 cd	335.1 d
	SH-OT3-1B	11	8.6 de	26.7 cde	203. 1 de
	SH-OT3-1F	13	18.1 abcd	16.9 e	260.3 de
	SH-N(3-2-6)4-21C	11	2.5 e	41.9 c	97.6 e
SH X SH	SH-N(3-2-6)4-21C x SH-OT3-1B	27	11.1 cde	10.4 de	100.8 e
	SH-OT3-1B x SH-N(3-2-6)4-21C	28	29.3 a	8.6 e	214.1 de
	SH-OT3-1F x SH-N(3-2-6)4-21C	29	27.9 ab	10.0 de	272.6 de

Means within columns followed by the same letter are not significantly different by Ryan-Einot-Gabriel-Wilson Multiple Range Test at the 5% level.

Figure 2.1. Flow cytometry histograms of (a) tetraploid and (b) diploid. The peaks depict the counts of cell nuclei over a given interval. "Gates" A, B, C and D refer to mono-, di-, tetra- and octoploid peaks respectively.

(a)



(b)

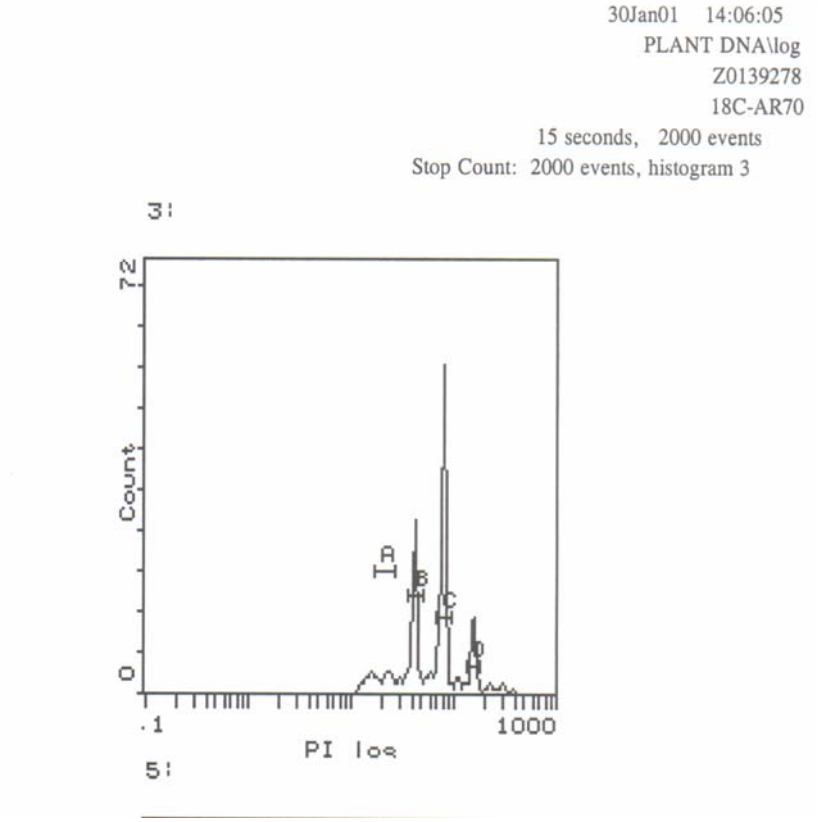
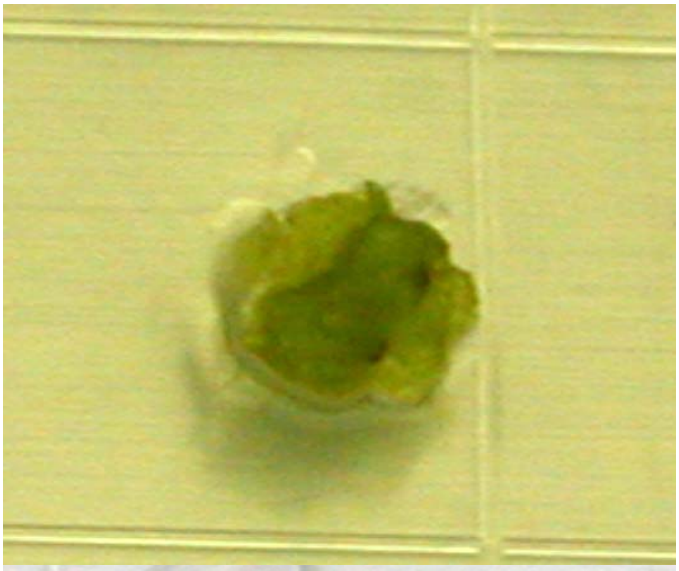
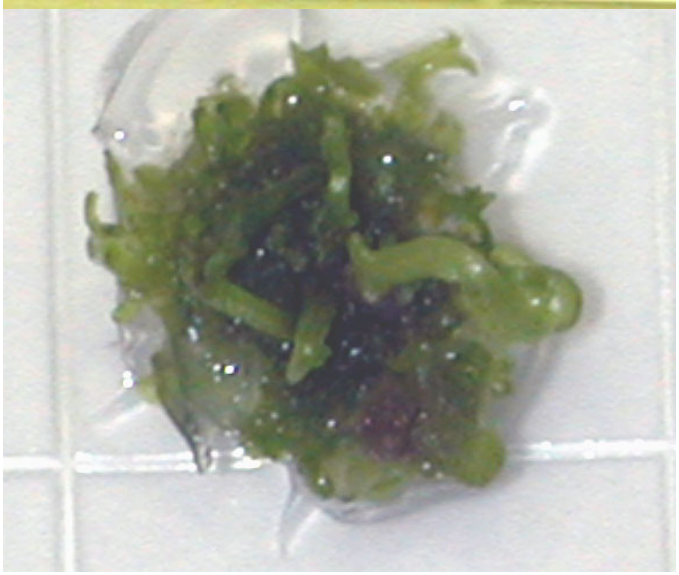


Figure 2.2 Stages in regeneration of somatic hybrids. (A) p-calli (calli regenerated from protoplasts), (B) Shoot initiation (C) Shoot regeneration.

A) p-calli



B) shoot initiation



C) Shoot regeneration

Figure 2.3. Greenhouse-grown somatic hybrid SH-NO4-1F, showing multiple flowers and vigorus growth.

Figure 2.4. Identification of somatic hybrids by SSR analysis using primers RV 3 and 4. Parental monoploids P1 and P2, hybrid (PSH-1A) depicted by heteroduplex bands, somaclones by a single band.

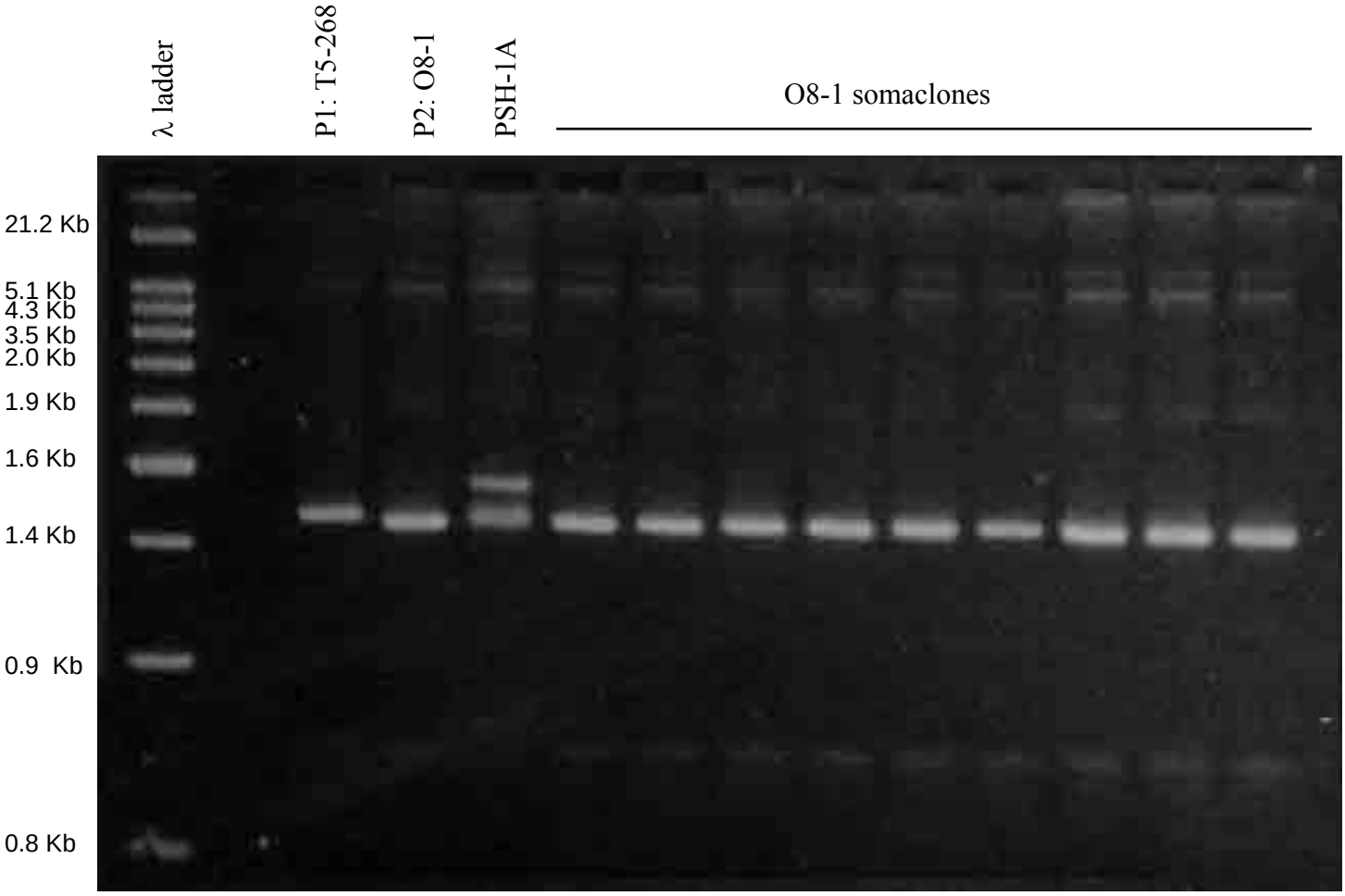


Figure 2.5. Identification of somatic hybrids by SSR analysis using primers RV 3 and 4. Parental monoploids P1 and P2, hybrids depicted by heteroduplex bands (PSH-18C), somaclones by a single band.

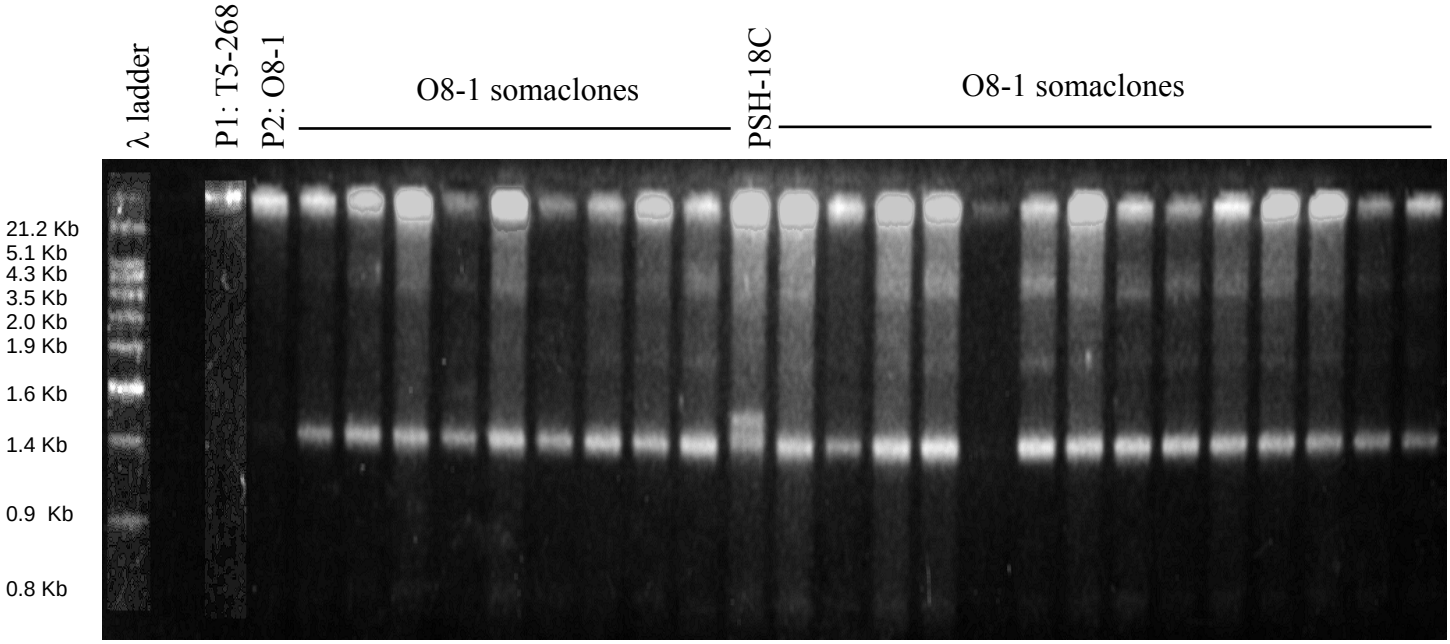


Figure 2.6 Harvested tubers of somatic hybrid SH-18C, anther culture derived DH-18C, cultivar X hybrid crosses (SH-18C x Katahdin, Atlantic x SH-18C), 2x X 2x parental types (T5-12 x O8-1, O8-8 x T5-12) and cultivar Katahdin, field trial 2001.

3. Molecular analysis of intermonoploid fusions

3.1. Introduction

Reduction of the genome of *Solanum tuberosum* cultivars in order to breed at reduced ploidy levels and eventually reconstruct synthetic tetraploids has been a goal of analytic breeding since it was first proposed by Chase (1963). The complexity of tetraploid genetic segregation and the restricted genetic base of *S. tuberosum* have hampered progress in potato breeding. Protoplast fusion can be used as part of tetraploid reconstruction by circumventing sterility barriers, allowing monoploids (2n=1x=12), the lowest ploidy level and necessarily sterile due to the absence of homologous chromosome pairs, to be incorporated into analytic breeding schemes. The advantage of monoploids is the removal of all lethal or deleterious genes, the passage of the genome through the so-called monoploid sieve (Wenzel *et al*., 1979). The introduction of new genes or genetic combinations through fusion of monoploids should result in improved hybrids by heterosis (Johnson *et al*., 2001).

We have constructed several intermonoploid somatic hybrids from selected monoploids of various genetic background to assess their breeding potential. Molecular markers must be employed in order to identify somatic hybrids from somaclones regenerants. Markers such as simple sequence repeats, SSRs (Tautz, 1989), random amplified polymorphic DNA, RAPDs (Williams *et al*., 1990), cleaved amplified polymorphisms, CAPs (Konieczny and Ausubel, 1993), random amplified microsatellite polymorphism, RAMPs (Zietkiewicz *et al*., 1994), amplified fragment length polymorphism, AFLPs (Vos *et al*., 1995) have been used in many studies to try to find a genetic basis for somaclonal variation (Johnson and Veilleux, 2001). However, the success of linking specific molecular markers with novel somaclonal phenotypes has been limited. Hirochika *et al*. (1996) found that activation of *Tos17* of a family of retrotransposons in rice was due to tissue culture manipulations and proposed that retrotransposon-based markers were more suitable than random genomic probing for associating somaclones with genomic alteration. Inter-retrotransposon amplified polymorphism (IRAP) and retrotransposon-

microsatellite amplified polymorphism (REMAP) are two PCR marker systems based on the use of retrotransposons (Kalendar *et al*., 1999), whereas sequence-specific amplification polymorphisms, S-SAPs represents a hybrid system incorporating AFLP technology in conjunction with the use of a defined genomic sequence (e.g., LTR retrotransposon sequence) in place of one of the anchor sequences of AFLP. IRAP, REMAP and S-SAP amplify sequences adjacent to retrotransposons and fall into the category of markers used for "retrotransposon display."

Retrotransposons are ancient elements with sequence similarity to retroviruses except by omission of the encapsulation gene. They are ubiquitous in higher organisms, of high copy number and widely dispersed throughout the chromosomes of higher plants (Flavell *et al*., 1992a; 1992b; Kumar, 1996; Lee *et al*., 1990; Suoniemi *et al*., 1998; Voytas *et al*., 1992). Retrotransposons transpose via an RNA intermediate, that is converted to DNA by reverse transcription prior to reinsertion, resulting in the introduction of a new element while the parental element remains fixed in the genome (Boeke and Corces, 1989; Kumar and Bennetzen, 1999). Therefore, every movement of an element results in insertion at a new site elevating the size and polymorphism of the plant genome (Pearce *et al*., 1996; 1998; SanMiguel *et al*., 1996). Eventually, the host organism shuts down the activity of a retrotransposon by some as yet unknown mechanism with the result that the genome harbors families of ancient inactive retrotransposons as well as relatively new, active ones.

Two major groups of retroelements have been recognized. The first has long terminal repeats (LTRs) and includes the *copia*-like retrotransposons (5 bp duplication at the insertion site) and the *gypsy*-like retrotansposons (4 bp duplication at the insertion site) whereas the second group does not contain the LTRs and is known as Long Interspersed Nuclear Elements, or LINEs. The most well-characterized retrotransposon belongs to the *Ty1-copia* group that was first discovered in *Saccharomyces cerevisia* and *Drosophila melanogaser.*

In plants, S-SAP, in particular, retrotransposon display, has been used as an improved genetic linkage mapping technique, due to the greater level of polymorphism compared to AFLPs (Waugh *et al*., 1997). S-SAP has been used to obtain a detailed picture of intra- and inter-species relationship within *Pisum*, using elements *Tps12* and *Tps19* (Pearce *et al*., 2000), or the polypurine track of PDR1 retroelement and *Taq* adaptor (Ellis *et al*., 1998). Yu and Wise (2000) combined markers to make a saturated map of diploid *Avena*, and found that S-SAP generated markers were evenly distributed throughout the genome, as did Waugh (1997) with barley and Berenyi *et al*. (2002) with sweetpotato. In addition, retrotransposon display can be used to study the genetic distribution of retroelements in a genome and to investigate the activity status of an element as observed by Miyao *et al*. (2003).

Tst1 is the first completely-sequenced potato transposable element and belongs to the *copia*-like transposable elements (Camirand and Brisson, 1990). It was discovered in the fifth intron of the potato starch phosphorylase gene inserted in the opposite direction of the phosphorylase (Camirand *et al*., 1990). This element is flanked by a 5 bp target sequence repeat, and possesses 5' and 3' LTRs of 285 and 283 bp, respectively. The internal domain is 4492 bp and contains four open reading frames (ORFs) that encode proteins for a reverse transcriptase, an integrase, an RNA-binding site and a protease. A stop codon located in the reverse transcriptase suggests that this element is unable to produce a functional transcriptase, but could be operative if a termination suppression mechanism is used or that reverse transcriptase is provided in *trans* by a functional allele of *Tst1*.

In an attempt to develop vigorous hybrids of potato that are free of lethal and deleterious genes, we have been working with intermonoploid somatic hybrids constructed by protoplast fusion of anther-derived monoploids of *S. phureja* Juz. & Buk. The main objective of the present study was to characterize retrotransposon-based (specifically, *Tst1*) S-SAPs in monoploids used for protoplast fusion to determine polymorphism and possible activation during the tissue culture process. The occurrence of S-SAP sequences

amplified from *S. phureja* was examined in other Solanaceae to determine their general utility as genetic markers.

3.2. Materials and Methods

3.2.1. Plant material

The potato population under investigation consisted of parental monoploids and regenerants following electrofusion of protoplasts from genetically distinct pairs of anther-derived monoploids (2*n*=1*x*=12) of the diploid primitive potato, *Solanum phureja*. Monoploid combination O8-1 + T5-268 produced somaclones of O8-1 (SC-3C, SC-5A and SC-36B) and two somatic hybrids (SH-1A and SH-18C); monoploid combination O5-10 3BB + T4-142 produced 40 somatic hybrids of which six (SH-OT3-1A, SH-OT3-1B, SH-OT3-2A, SH-OT3-2F, SH-OT3-3B and SH-OT3-4A) were selected for evaluation; monoploid combination N8-2 AR8 + O5-10 3BB resulted in ten somatic hybrids and ten somaclones whereas for combination N8-2 AR8 + 3-2-6 6N-1 five somatic hybrids were selected for evaluation (SH-N(3-2-6)4-5B, SH-N(3-2-6)4-5D, SH-N(3-2-6)4-17A, SH-N(3-2-6)4-18A, SH-N(3-2-6)4-21A and SH-N(3-2-6)4-31A). Prior to S-SAP analysis somaclones and somatic hybrids were distinguished using SSR (simple sequence repeat) primer pairs (Ashkenazi *et al*., 2001). For a broader comparison of S-SAP sequences in Southern blots we used (1) a garden blot consisting of DNA extracted from related Solanaceae (*Datura* sp., *Petunia* sp., *Lycopersicon esculentum* cv. Money Maker, *Nicotiana tabacum* cv. Samsun) as well as *Arabidopsis thaliana* ecotype Columbia 0 and (2) a family blot consisting of monoploids N8-2 AR8, 3-2-6 6n-1 and PP5-15.

3.2.2. DNA extraction and enzyme digestion

DNA was extracted from in vitro plantlets by the CTAB protocol according to Doyle and Doyle (1987). AFLP core reagent kit and pre-amp primer kit (Invitrogen Corp., Rockville, Md.) were used to perform the first two stages of the AFLP technique: (1) digestion of the DNA with restriction rare cutter *Eco*RI and frequent cutter *Mse*I, and (2)

addition of appropriate anchor sequences, a modification of the original method of Vos *et al.* (1995). Samples were stored at –20ºC.

Subsequent to AFLP preamplification, following the procedure described by Waugh *et al.* (1997) we designed primers to target the LTR region of potato *Tst1* retroelement and the *Mse*I anchor site produced by the AFLP procedure. The amplification product would be expected to contain short stretches of the LTR fragment and the intervening region of genomic DNA occurring between *Tst1* retroelements and *Mse*I sites. The 22-bp primer (Tst1-LTRFout) consisted of a consensus sequence of the LTR region (Camirand and Brisson, 1990; Camirand *et al*., 1990):

5’ CTAAATCTGCCTACTCATTCAA 3’

*Mse*I [+3] selectivity secondary amplification was carried out using the following *Mse*I homologous adaptor primer:

5’ GACGATGAGTCCTGAGTAAC 3’

3.2.3. AFLP, S-SAP and IRAP – fluorescent primers

The procedure of PCR amplification using non-fluorescent primers was optimized for [Cy5]-fluorescent primers. AFLP protocols using the AFLP® Core reagent Kit (Cat. No. 10482-016, Invitrogen) were followed. Approximately 500 ng genomic DNA were digested in a restricted reaction mix made up to 25 µL containing 1X reaction buffer (10 mM Tris-HCl, pH 7.5, 10 mM Mg acetate, 50 mM K acetate) and *EcoR I/Mse I* restriction enzyme mix (2.5 U each in 10 mM Tris-HCl, pH 7.4, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), 0.1 mg mL^{-1} BSA, 50% glycerol (v/v), 0.1% Triton® X-100). The reactions were gently mixed and collected by brief centrifugation (Galaxy mini) for 5 min, then incubated at 37^{o}C for 2 h. The mixtures were heated to 70^{o}C for 15 min to inactivate the restriction endonucleases. To the tube of digested DNA 24 µL of adaptor/ligation solution (*EcoR I/Mse I* adaptors, 0.4 mM ATP, 10 mM Tris-HCl, pH 7.5, 10 mM Mg acetate, 50 mM K acetate) plus 1 U T4 DNA ligase [1 U T4 DNA ligase μL^{-1} in 10 mM Tris-HCl, pH 7.5, 1 mM DTT, 50 mM KCl, 50% glycerol (v/v)] were added. The contents were collected by brief centrifugation, then incubated at 20^{o}C for 2 h. A

1:10 dilution was performed on the ligation mix using TE buffer (10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA) and the diluted ligation mixture was used for preamplification using AFLP® Pre-amp Primer Mix I (Cat. No. 10792-018, Invitrogen). Preamplification reaction mix was made up to a total volume of 51 µL containing 5 µL of 1X template DNA, 40 µL pre-amp primer mix, 1X PCR buffer with $MgCl_2$ and 1U of *Taq* DNA polymerase. PCR pre-amplification parameters were: denature 94^{o}C for 30 sec, anneal 56^{o}C for 1 min, extension 72^{o}C for 1 min, for X20 cycles, and a final extension of 72^{o}C for 5 min. A 1:50 dilution in TE buffer was carried out prior to final selective amplification. The AFLP PCR mixture contained 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM oJGJ108 (I.D. for [Cy5]*Eco*RI [+AA] primer) or oJGJ112 (I.D. for [Cy5]*Eco*RI [+AAC] primer), 1 µM 92H20 (I.D. for *Mse*I [+C] primer) or oJGJ122c(I.D. for *Mse*I [+CAA] primer), 1 U *Taq* DNA polymerase, 5 µL AFLP preamplification made up to a total volume of 25 µL. Primer extension selection was based on clarity of signal production. The following PCR cycling conditions were used during selective amplification: denature 94^{o}C for 2 min, for X1 cycles; step-down cycles of denature 94^{o}C for 1 min, anneal 65-54^{o}C for 2 min, 1^{o}C step-down per cycle, extension 72^{o}C for 1:30 min; regular cycles of denature 94^{o}C, for 30 sec, anneal 54^{o}C, for 2 min, extension 72^{o}C for 1 min, for X25 cycles; with a final extension of 72^{o}C for 4 min.

The S-SAP PCR mixture contained 1X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 1 µM [Cy5]*Tst1*-LTRFout primer, 1 µM oJGJ122c - *Mse*I [+CAA] primer, 1 U Taq DNA polymerase, 5 µL AFLP preamplification DNA (approx. 5-50 ng) made up to a total volume of 25 µL. PCR consisted of: denaturation step of 94^{o}C for 2 min, followed by stepdown cycles of denature 94^{o}C for 1 min, anneal 65-54 in 1^{o}C steps for 2 min, extension 72^{o}C for 1:30 min, followed by standard cycles of denature 94^{o}C for 30 sec, anneal 54^{o}C for 2 min, extension 72^{o}C for 1 min with X29 cycles, and final extension at 72^{o}C for 4 min.

The IRAP PCR reaction mix contained X1 PCR buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1.6 µM [Cy5]Tst1-LTRFout primer, 1U *Taq* DNA polymerase and 50 ng genomic DNA made up to total volume of 25 µL and was subject to the following PCR cycling

conditions: denature 94°C for 2 min, X1 cycles; denature 94°C for 45 sec, anneal 52°C for 2 min, extension 72°C for 2:03 min, for X30 cycles, with a final extension of 72°C for 10 min.

3.2.4. Gel electrophoresis and ALFexpress

PCR results were analyzed on an Automated Laser Focusing - ALFexpress® or ALFexpress® II DNA Analyzer (Amersham Pharmacia Biotech, Uppsala, Sweden) designed for automated detection of fluorescently labeled DNA molecules/fragments separated by electrophoresis. Electrophoresis was carried out in an off-vertical gel cassette with temperature control of the gel by water circulation through one of the plates forming the cassette. During electrophoresis a fixed laser beam, perpendicular to the direction of band migration, excites the fluorescently labeled DNA bands and the light emitted is detected on photodetectors positioned behind the gel. The photodetector signal is sent to the attached computer and analyzed by the ALFwin™ Fragment Analyser 1:00 software (Amersham Pharmacia Biotech). The fluorophore was incorporated in the *Eco*RI based primer of AFLP PCR selective amplification reaction while for IRAP and S-SAP fragment analysis the fluorophore was incorporated into the *Tst1* based primer. Robocycler conditions were optimized for fluorescent primers. Fluorescent PCR reaction products were separated on 5.5% denaturing polyacrylamide gels by preparing 5.5% Long Ranger® 50% gel solution (Cat No. 50611, BioWhittaker Molecular Applications, Rockland, ME), 6 M urea and 1.5X TBE buffer (133.5 mM Tris base, 133.5 mM boric acid, 3 mM $Na_2EDTA\ 2H_2O$) polymerized by 250 µL 10% APS solution and 25 µL TEMED (N,N,N',N',-tetramethylethylenediamine) (Cat No. T-9281, Sigma) per 50 mL solution or from ReproGel™ Long Read kits (Cat No. 17-6001-09, Amersham Pharmacia Biotech) with UV polymerization. For separation of amplification products on the ALFexpress we loaded 4 µL of PCR product per lane. Run conditions were 1500 V, 60 mA and 25 W for 800 min at 30ºC. Each sample was analyzed in a minimum of two and up to four separate PCR reactions. Any band that was not repeatable was excluded from the analysis.

3.2.5. Extraction, cloning and sequencing of S-SAP and IRAP fragments

The same PCR amplification procedures using modified [Cy5] fluorophore primers for analysis on the ALFexpress were followed to produce target bands with a manual adjustable height nucleic acid sequencing system (Cat. No. SG-400-20, C.B.S., Scientific Company, Inc., Del Mar, CA). An aluminum heat dispersion plate for equal heat distribution was added to the apparatus to prevent uneven fragment migration. Separations were carried out on 8% denaturing polyacrylamide gels consisting of 8% acrylamide 40% stock (37.5:1) (acrylamide:bis-acrylamide, 37.5:1) electrophoresis grade (Cat. No. BP1410-1, Fisher Scientific), 7 M urea, TBE 1X, filtered and 1 mL 10% ammonium persulfate (APS) and 60 μL TEMED added per 100 mL to initiate polymerization. The gel dimensions were 20 cm x 42 cm x 0.4 mm with 24 wells produced by a sharktooth comb with a loading capacity of 9 μL. Capillary gel casting was performed and the assembly was covered with plastic wrap during polymerization. Gels were pre-electrophoresed for 30 min at 500 V that resulted in a gel surface temperature of approximately 40-50^{o}C. Samples were heated to 95^{o}C for 5 min in a 4X formamide loading dye (100% deionized formamide, 20 mg L^{-1} Blue Dextran 2000), rapidly vortexed then chilled immediately on ice; 9 μL of sample were loaded per lane. For the ladder 9 μL of ALFexpress^{TM} Sizer^{TM} 50-500 (Cat No. 27-4539-01, Amersham Pharmacia Biotech, Sweden) were prepared as described in the protocol. Electrophoresis conditions were 500 V for 6 h. The gel was cooled and scanned with a Storm860 (Molecular Dynamics, Hercules, CA) Imaging System/Red Fluorescence/Chemi-fluorescence. The gel sandwich was opened and a 1X magnification of image (with appropriate markers for correct alignment) placed beneath the lower glass plate to locate the position of bands during excision. The bands were excised with a single edged razor blade (Cat. No. 12-640, Fisher Scientific), changing to a fresh blade for each band to minimize cross contamination. The gel was re-scanned after band excision to confirm removal of the selected bands.

Excised bands were placed in 1.5 ml centrifuge tubes and stored at 4^{o}C overnight. The DNA was eluted using QIAEX II gel extraction kit (Cat No. 20021, Qiagen Inc, Valencia, CA). Fragments were weighed and 2 volumes of diffusion buffer (0.5 M

ammonium acetate, 10 mM magnesium acetate, 1 mM EDTA, pH 8.0, and 0.1% SDS) added to 1 volume of gel. The mixture was incubated for 30 min, then centrifuged for 1 min at 14,000 rpm. The supernatant was removed and 3 volumes of buffer QX1 added. Ten microliters of QIAEXII was added to the sample and incubated at RT for 10 min with vortexing. The samples were centrifuged for 30 sec and the supernatant removed and discarded. The pellets were washed twice in 500 µL of PE buffer, air dried for 10 min and the DNA eluted by addition of 20 µL of 10 mM Tris-HCl, pH 8.5. The solution was centrifuged for 30 sec and the supernatant containing pure DNA collected in a new tube. Eluted DNA containing fluorophore [Cy5] modifications was re-amplified (same conditions as previously) with non-fluorophore primers to produce new DNA product without [Cy5] modifications. Attempts at cloning with [Cy5] modification were not successful.

DNA samples were subsequently cloned into pCR2.1 using TOPO-TA Cloning® Kit version M or the Original TA Cloning® Kit (Cat. No. K4500-01 or K4500-40, Invitrogen Corp., Rockville, MD). Six µL of TOPO® Cloning reaction (PCR product, salt solution, TOPO® vector, water) was made as directed. The reaction mix was incubated at RT for 5 min, then placed on ice. Two µL of each TOPO® cloning reaction were transferred to TOP10 One Shot Chemically Competent *E. coli* vials. The vials were incubated on ice for 10 min, then transformation of *E. coli* initiated by heat shock for 30 sec at 42^{o}C. To allow for recovery of cells 250 µL of RT SOC was added and the culture placed on a rotating incubator, 200 rpm at 37 ^{o}C for 1 h. The transformed culture was divided into 10, 50 and 100 µL aliquots and positive white or light blue transformants were selected on LB + kanamycin (50 µg mL^{-1}) + X-Gal (40 µg of a 40 mg mL^{-1} stock) plates after overnight incubation at 37^{o}C. Positive clones were tested for correct fragment size insertions by PCR using M13 forward and reverse primers that amplified the TOPO-TA cloning site plus an additional 200 bp flanking sequence. A standard PCR mix was prepared and picked colonies resuspended in the PCR cocktail. Selected colonies were simultaneously patched onto a separate plate for preservation. The reaction was incubated at 94^{o}C for 10 min to lyse cells and inactivate nucleases. DNA amplification was carried out with a ROBOcycler 46, under the following conditions: denature 94^{o}C for 3 min, X1

cycle, denature 94°C for 45 sec, anneal 48°C for 1 min, extension 72°C for 2 min, for X35 cycles, and final extension of 72°C for 10 min, X1 cycle. PCR results were separated on a 2% agarose gel in TAE, 94 V for 1:30 h. Colonies with correct sized inserts were selected and cultured overnight in 5 mL liquid LB +kanamycin (50 μg mL^{-1}).

Plasmids were purified using QIAprep Spin Miniprep Kit (Cat No. 27104 or 27106, Qiagen, Inc., Valencia, CA). Selected colonies of *E. coli* were cultured overnight in 5 mL LB (Lauria-Bertani) medium supplemented with 50 μg mL^{-1} kanamycin in a rotating incubator at 250 rpm and 37°C. Cultures were transferred to micro-centrifuge tubes and pelleted at 10,000 rpm for 1 min. The supernatant was discarded and the pellets resuspended in 250 μL buffer P1 of the QIAprep Spin Miniprep Kit and the protocol using a microcentrifuge followed. At the end of the procedure the DNA was eluted in 50 μL of EB buffer (10 mM Tris-HCl, pH 8.5), placed on a cold block at 4°C, then quantified using a spectrophotometer (DU-7000, Beckman).

3.2.6. Sequencing of fragments and BLAST analysis

Fragments excised and purified from the retrotransposon displays were sequenced by the BIG dye terminal system at the Virginia Bioinformatics Institute (VBI) using an ABI 3100 capillary automated sequencer. The PCR was performed in a total volume of 10 μL, consisting of 2 μL miniprep DNA (approx. 200 ng μL^{-1}), 1 μM M13 (forward or reverse) primer (Invitrogen Corp., Rockville, MD) and 4 μL Big Dye® Terminator v 3.1 Sequencing RR-100 (Cat No. 4336911, Applied Biosystems, Foster City, CA). PCR conditions for amplification were: denature 94°C for 1 min, one cycle; denature 94°C for 30 sec, anneal 50°C for 30 sec, extension 70°C for 1 min, X35 cycles. Sequences were analyzed and manually edited using DNAstar Editseq and Megaline. Final versions were analyzed at the National Center for Biological Information web site where BLAST N searches were performed. The identification of common nucleotides was carried out on GCG program using the Pretty sequence alignment.

3.2.7. DIG labeling of probe

Plasmids CR2.1 with inserts of non-fluorophore PCR re-amplified excised fragments of S-SAP bands were used as template for preparation of DIG (digoxigenin-3-O-methylcarbonyl-e-aminocaproic acid-N-hydroxyl-succinimide ester) non-radioactive labeled hybridization probes. The detection capacity of the labeled probe is between 0.03-0.10 pg DNA. The procedure for preparation of probe was as in the DIG Application Manual for Filter Hybridization (Roche Diagnostics Corporation, Indianapolis, IN). Primers consisted of Tst1-LTRFout (5' – CTA AAT CTG CCT ACT CAT TCA A – 3') and oJGJ122c - *Mse*I [+CAA] (5'- CGA TGA GTC CTG AGT AAC AA-3'). DIG labeled DNA probes were synthesized in reactions containing 1 ng template DNA, 0.5 μM each primer, 1U *Taq* polymerase, 200 μM dNTPs, 33 μM digoxigenin-11-dUTP alkaline labile (Cat. No. 1 573 179, Roche Diagnostics Corporation), 1.5 mM $MgCl_2$, 1X buffer in a total volume of 100 μL. PCR cycling conditions consisted of an initial denaturation step of 94^{o}C for 2 min, followed by stepdown cycles of denature 94^{o}C for 1 min, anneal 65-54 in 1^{o}C steps for 2 min, extension 72^{o}C for 1:30 min, followed by standard cycles of denature 94^{o}C for 30 sec, anneal 54^{o}C for 2 min, extension 72^{o}C for 1 min with X29 cycles, and final extension at 72^{o}C for 4 min. Control reaction without DIG-dUTP were simultaneously carried out and both amplified products separated on 2% agarose gels in TAE and stained in EtBr for visualization of retardation of DIG labeled bands as verification of successful incorporation of DIG. Electrophoresis conditions consisted of 94 V for 1:30 h.

3.2.8. Southern blots

Genomic DNA obtained by the CTAB method (see DNA extraction) was quantified (DU 7400 spectrophotometer, Beckman) by measuring A_{280}, and used directly in restriction digest or approx. 90 μg of DNA was re-precipitated with ethanol (Ausubel *et al*., 1997). The DNA pellet was re-suspended in 30 μL TE and kept overnight at 4^{o}C to ensure it was completely dissolved. The DNA was re-quantified, and 20 μg DNA used for restriction digest (40 μg of DNA was tested for increased signal level detected, however, this only slightly improved the visual signal and was not economical). To standardize the

conditions of the restriction digest, the quantified DNA was re-suspended in TE to a total volume of 30 µL. A master mix with the appropriate amount of restriction enzyme, normal concentration or high concentration (Promega or NEB) was prepared. Each restriction reaction contained 20 µg of genomic DNA, 20 U restriction endonucleases and 1X restriction buffer in a total volume of 50 µL. Initial reactions were performed in 100 µL volumes; however, this required re-precipitation of the digested DNA prior to loading of agarose gels and resulted in loss of DNA and therefore was discontinued. The restriction digest was incubated for 2 h at 20^{o}C followed by adding an equal volume of restriction enzyme, to ensure complete digestion, and allowed to incubate overnight. Following digestion of DNA the restriction enzyme was inactivated by incubation for 10 min at 65^{o}C. A 5X loading dye (12.5 µL) was added to the digest. Electrophoresis was carried out on a 0.7% agarose gel (agarose low EEO, electrophoresis grade, BP160-500, Fisher Scientific) in TAE buffer, two gel sizes were used (16 X 12.5 cm and 20.5 X 12.5 cm) according to the number of samples loaded. Five µL of DIG labeled λ ladder (DNA Molecular weight marker II, DIG-labeled, 0.12-23.1 Kbp, 1 218 590, Roche Diagnostics Corporation) was added to the end lane of the gel with preferably a separation of one lane between the ladder and samples. The samples were loaded (42.5 µL) and electrophoresis was carried out for 20 h at 20 V (approximately 1 V cm^{-1}). The gel was then stained with ethidium bromide (1X solution) for 20-30 min, destained for an equal time in distilled water and the DNA smear visualized under UV light (UV imager, BIORAD).

Southern blot transfer was carried out under neutral conditions following the procedure described in the DIG Application Manuals (Roche Diagnostics Corporation). The gel was rinsed in distilled water, then depurinated in 0.25 M HCl (250 mL) by gentle agitation on a rotating platform (50-60 rpm) until a color change of the xylene cyanole and bromophenol blue dye to green and yellow was observed (approximately 10-15 min). The HCl was removed with a water aspirator and the gel rinsed in distilled water. The gel was treated with denaturation solution (1.5 M NaCl, 0.5 M NaOH) for two 15 min intervals with continued agitation on a rotating platform, followed by neutralization (1.5 M NaCl, 0.5 M TRIS, pH 8.0) under similar conditions. The gel was then pretreated with

20X SSC (3 M NaCl, 0.3 M Na citrate·$2H_2O$, pH 7.0) for 10 min prior to assembly of the Southern blot transfer pyramid.

A wick (Whatman 3MM paper) was prepared (31.5 X 12.5 cm) and placed on a block in an appropriate size dish. The membrane was wet with 20X SSC and the gel placed on top of the filter paper. Positively charge nylon membrane (MAGNACHARGE, Nylon, transfer Membrane, 0.45 Micron, 15 cm X 3 m, roll, NB0HYB0010, Osmonics Inc., Westborough, MA) were cut to match the gel size (16 X 12.5 - small gel or 12.5 X 20.5 - large membrane) and layered over the gel for capillary transfer. Three pieces of Whatman 3 MM paper of the size of the membrane were placed on top of nylon membrane, wet with 20X SSC and air bubbles removed by gently rolling with a test tube. Paper towels the size of the membrane were stacked on top, 5 cm high, and a weight placed on the paper towels. Four strips of plastic wrap were placed between the paper towels and the gel to prevent disruption of the capillary column between the wick and paper towels. The pyramid was left overnight for transfer. The following day the pyramid was disassembled and orientation of the gel recognized by cutting the lower right side of nylon membrane. The membrane was placed on a clean sheet of Whatman 3MM paper and allowed to air dry. The membrane was subsequently dampened with 2X SSC and DNA fixed by UV cross-linking at 1200 X 100 μJ cm^{-2} UV light with a LX-1000 UV crosslinker, Spectrolinker™ (Spectronic Corporation, Westbury, NY). The membrane was rinsed briefly in distilled water, allowed to air dry, and checked with a manual UV light for the presence of DNA. The membrane was stored dry or hybridized with an appropriate probe.

Hybridization was carried out in borosilicate glass bottles in a hybridization oven (model 1000, Robbins Scientific) with rotation controls set at 4 rpm. The membrane was prehybridized with 20-40 mL (dependent on size of hybridization bottle) of pre-warmed DIG Easy Hyb solution (Cat. No. 1 603 588, Roche Diagnostics Corporation) for 30 min at 42°C in a preheated incubator. This urea-based hybridization buffer allows for higher stringency at lower temperatures. A volume of 20-40 mL of DIG-labeled *β-ATPase* probe solution was prepared (2 μL of DIG-labeled PCR product per 1 mL DIG EASY

Hyb solution). To produce single stranded denatured probe, 40 µL of new DIG labeled PCR product (probe) was placed in 50 µL of distilled water and boiled for 5 min followed by chilling on ice, then making up to 40 mL with DIG EASY (without formamide) hybridzation solution. Activation of previously used probes was achieved by reheated in boiling water for 5 min. Pre-warmed probe, in DIG EASY Hyb solution, was used to replace the DIG EASY Hyb solution. The membrane was incubated for 16 h at 42^{o}C (low stringency hybridization conditions).

The probe solution was removed and stored at –20^{o}C for later reuse. The non-specific and excess probe was washed from the membrane by submerging in 60 mL of low stringency buffer (2X SSC with 0.1% sodium dodecyl sulfate, SDS) with incubation at RT (2 X 5 min). The low stringency buffer was poured off and replaced with 60 mL of pre-warmed high stringency buffer (0.5X SSC with 0.1% SDS) and membrane incubated at 65^{o}C (2 X 15 min). The membrane was transferred to a plastic container and submerged in 100 mL washing buffer (100 mM maleic acid, 150 mM NaCl, 0.3% Tween 20 v/v, pH 7.5) and incubated for 2 min at RT with shaking. The washing buffer was discarded and replaced with 100 mL blocking solution [1% blocking reagent w/v, (Cat. No. 1 096 176, Roche Diagnostics Corporation) in 100 mM maleic acid, 150 mM NaCl, pH 7.5] and incubated for 30 min, with shaking. The blocking solution was removed and the probe target hybrid detected by the addition of 40-80 mL antibody solution [0.1 µL of anti digoxigenin-AP conjugate, Fab fragment (Cat. No. 1 093 274, Roche Diagnostics Corporation) per mL blocking solution] and incubated for 30-60 min with shaking. The antibody solution was discarded and the unbound antibody removed from the membrane by washing twice (2 X 15 min) with 100 mL washing buffer. This was followed by equilibration for 3 min in 40 mL detection buffer (100 mM Tris-HCl, 100 NaCl, pH 9.5). The membrane was placed on a clear acetate sheet and 3 mL chemiluminescent substrate [5 µL of CDP-Star^{TM} (Cat. No. 1 685 672, Roche Diagnostics Corporation) per mL detection buffer] added to assay for alkaline phosphatase. The acetate sheet protector was closed and membrane incubated for 5 min at RT. Chemiluminescent signal was visualized in 20.3 X 25.4 cm autoradiography cassettes (FBAC 810, Fisher Scientific) on BIOMAX^{TM} ML, MS or MR scientific imaging film (Eastman Kodak Company,

Rochester, NY). Exposure time varied from 5 min to 2 h depending on the intensity of probe signal and/or film type.

3.2.9. Marker analysis

Statistical analysis of banding patterns produced by AFLP, S-SAP and IRAP procedures using the ALFexpress automated system and during manual RFLP/Southern blot preparation was analysed using the computer package *Tools for Population Genetic Analyses* (TFPGA) (Miller, 1997). Genetic distances were calculated using Nei's (1972) original distance and identity measures. Phenograms, depicted as trees, were produced using an unweighted pair group method with arithmetical averages (UPGMA) cluster analysis option. Markers present in a genotype were designated 1, if absent it was scored as 2, while ambiguous bands, due to weak signal intensity were scored as 0. Both monomorphic and polymorphic bands were scored.

3.3. Results

3.3.1. ALFexpress analyses

Data sets were obtained from each of the three PCR-based marker analysis systems (AFLP, S-SAP and IRAP) produced from somatic fusion experiments.

AFLP analysis, experiment 1

Plant material consisted of DNA from parental monoploids (O8-1 and T5-268), a equal mix of the monoploids (O8+T5) representing a synthetic somatic hybrid for comparative purposes, somatic hybrids (SH-1A and SH-18C) and somaclones of monoploid O8-1 (SC-3C, SC-5A and SC-36B). The selective amplification primer pair used consisted of oJGJ112 ([Cy5]*Eco*RI [+AAC]) and oJGJ122c (*Mse*I [+CAA]). ALFexpress fragment analysis settings was as follows: algorithm type, Jaccard, with peak comparisons based on a virtual lane derived from O8+T5 with a 2-base acceptance range, peak shape 10 (scale 1-50), minimum peak height 0.2 (scale 0.1-100%), baseline type: minima

detection, with a sensitivity of 175 (scale 1-200). Subsequent to automated peak detection, manual editing were carried out. Genetic distance (GD) among genotypes was based on 90 bands, ranging in size from 24 to 805 bp, representing DNA fragments with consistent expression in duplicate samples based on separate PCR amplification (Table 3.1). The minimum genetic distance was 0.0112 between somaclones SC-5A and SC-36B, and the maximum was 0.2094 between parental monoploids O8-1 and T5-268 using Nei's distance coefficient. Somaclones showed little band diversity (GD = 0.0225 to 0.0455) compared to the parental monoploid, O8-1, and similarly the somatic hybrids showed little diversity (GD = 0.0455 to 0.0690) compared to the parental mix. UPGMA cluster analysis produced groupings consistent with expected genetic relationships (Figure 3.1a). The parental monoploids occurred in distinct groups, with monoploid O8-1 and derived somaclones forming one major group and monoploid T5-268, with the parental mix and somatic hybrids forming the second group. Somatic hybrids SH-1A and SH-18C, although closely grouped, were on separate hierarchy forks depicting a level of variation between hybrids, with SH-18C closer to the parental mix.

AFLP analysis, experiment 2

Experiment 2 was a repeat of experiment 1, with identical primers and plant material. ALFexpress fragment analysis algorithms were as previously. Due to improvements in signal detection, we based genetic distance among genotypes on more fragments (146) ranging in size from 23 to 749 bp (Table 3.2). Genetic distance among genotypes ranged from a minimum of 0.0075 between somaclone SC-5A and SC-36B to a maximum of 0.1719 between parental monoploids O8-1 and T5-268. As in the previous experiment there was great similarity between somaclones and their monoploid parent O8-1 (GD = 0.0230 to 0.0462) and between the somatic hybrids and the parental mix (GD = 0.0383 and 0.0782 respectively). UPGMA cluster analysis produced major groupings consistent with expected genetic relationships but minor groups differed slightly from previous experiment (Figure 3.1b). One difference was that monoploid T5-268 occurred closer to the parental mix (GD = 0.0305) in contrast to expected similarities between the parental mix and the somatic hybrids (GD = 0.0383 and 0.0782). In addition, increased polymorphism detected in SH-1A (possibly due to increased number of loci scored)

placed it further from its parental mix and the other somatic hybrid (SH18C) than in the previous analysis.

AFLP analysis, experiment 3

Plant material consisted of parental monoploids (O5-10 3BB and T4-142), a parental DNA mix (O5+T4) representing a synthetic somatic hybrid for comparative purposes, and four somatic hybrids (SH-OT3-1A, SH-OT3-1B, SH-OT3-2A and SH-OT3-2F). The number of bands scored was 157 and ranged in size from 23 to 526 bp. Parental types O5-10 3BB and T4-142 displayed the maximum genetic distance coefficient (0.3649; Table 3.3), whereas somatic hybrids produced two groups with almost identical minimal distances, SH-OT3-1A and SH-OT3-1B (GD = 0.0129) and SH-OT3-2A and SH-OT3-2F (GD = 0.0128). UPGMA cluster analysis produced three major groupings, with parent O5-10 3BB by itself, parent T4-142 occurring with the parental mix, and the third group consisting of the somatic hybrids, which were further divided into two subgroups that reflected the two different calluses from which they had been derived (Figure 3.1c). These results were consistent with expected genetic relationships among the genotypes.

S-SAP experiment 1

This experiment was designed to compare the effect that different levels of selective primers, *Mse*I [+1] or [+3], as developed for AFLP selection, had on the production of S-SAP markers and the relationships derived using these primers. Plant material consisted of parental monoploids (O5-10 3BB and T4-142), a parental DNA mix (O5+T4) representing a synthetic somatic hybrid for comparative purposes, and somatic hybrid SH-OT3-1A, with duplicate PCR reactions for all except the somatic hybrid. A total of 154 loci was scored with the *Mse*I [+1] selective primer, ranging in size from 23 to 454 bp, whereas a total of 138 loci was scored with the *Mse*I [+3] selective primer, ranging from 23 to 465 bp (Table 3.4). The maximum genetic distance coefficient tabulated for *Mse*I [+1] reactions was between the parental monoploid, T4-142 R1, and the parental mix, O5+T4 R1, (0.2102), surprisingly greater than between the two parental monoploids (Table 3.4). The minimum genetic distance was between reaction 1 and reaction 2 of parental monoploid O5-10 3BB, (0.0671), in contrast, repeat reactions 5 and 6 were not

closely paired (e.g., O5+T4 GD = 0.1928 between reactions) suggesting instability when using the *Mse*I [+1] primer. Similar results were obtained from reactions involving the *Mse*I [+3] selective primer with the greatest genetic distance between parental monoploid, T4-142, and the parental mix, O5+T4 R2, (0.1517). The minimum genetic distance was between parental monoploid O5-10 3BB R1 and R2. However, UPGMA cluster analysis produced unique trees for the two different reactions (Figures 3.2a,b). In the *Mse*I [+1]-based tree parental monoploid T4-142 R2, was paired with the somatic hybrid, SH-OT3-1A, and not with the expected T4-142 R1; neither were the parental mix reactions 1 and 2 paired. In the *Mse*I [+3] tree, all duplicate reactions were paired and the somatic hybrid and parental mix were adjacent to each other and sandwiched more reasonably between the parental types.

S-SAP experiment 2

In this experiment and all subsequent relevant experiments the *Mse*I [+3] primer was used due to its better match to the expected results obtained in S-SAP experiment 1. Plant material tested consisted of parental monoploids (O5-10 3BB and N8-2 AR8), a parental DNA mix (O5+N8), and nine regenerants consisting of two somaclones, SC-NO3 2A and SC-NO3–2B, and seven somatic hybrids (SH-NO3 1A, 1B, 3A, 3B, 4I, 10A and 11A). The number of bands scored was 149 and ranged in size from 23 to 435 bp (Table 3.5). The parental monoploids showed the greatest genetic distance coefficient (0.3064) while the minimum distance was between somaclones, SC-NO3-2A and SC-NO3-2B, (0.0141). UPGMA cluster analysis produced a complex tree (Figure 3.3a), with paired regenerants falling between the O5-10 3BB parent and the parental mix (O+N), somaclones SC-NO3-2A, SC-NO3-2B and SH-NO3-11A were grouped together suggesting closer relationship than with rest of the somatic hybrids. In general cluster analysis depicted the known genetic relationships.

S-SAP experiment 3

Monoploids O8-1 and T5-268, parental mix (O8+T5) and somatic hybrid SH-18C, each consisting of two PCR reactions were tested. Genetic distance was based on the scoring of 74 bands (Table 3.6), with the greatest genetic distance reported for the parental

monoploids, O8-1 and T5-268 (0.2610 by one reaction but only 0.1961 in a duplicate comparison), and the minimum genetic distance tied between repeat reactions for both the monoploid parent, O8-1, and the somatic hybrid, SH-18C, (0.0556). These results were consistent with previous results for the same family previously scored using AFLP based markers. UPGMA cluster analysis produced a tree (Figure 3.3b), reflecting the known relationships within this family. In addition, cluster analysis paired all repeat reactions.

IRAP experiment 1

The plant material tested in this experiment consisted of monoploids N8-2 AR8 and O5-10 3BB, the parental mix and ten somaclones derived from O5-10 3BB. The somaclones were generated from the same callus and should thus have been nearly identical. Genetic distance was tabulated using 114 loci ranging in size from 23 to 630 bp (Table 3.7). Genetic analysis revealed that the greatest genetic distance between the two monoploids (0.4369), whereas the minimal distance was between somaclones, SC-NO3-2A and SC-NO3-2B (0.0). Surprisingly there was considerable variation among the somaclones (GDs ranging from 0.0 to 0.1527) and between the somaclones and parental type (0.1787 to 0.3795), however, most GDs followed the expected trend of parental types being most distant and somaclones closely related to each other and the parental monoploid. UPGMA cluster analysis produced two major forks, monoploid N8-2 AR8 paired with the parental mix, and monoploid O5-10 3BB paired with the ten somaclones, which were variously related (Figure 3.4a).

IRAP experiment 2

Plant material in this experiment was as in AFLP experiments 1 and 2, and partially S-SAP experiment 3 (monoploids O8-1 and T5-268, somatic hybrids and O8-1 somaclones). Genetic distance was tabulated using 103 loci ranging in size from 36 to 598 bp (Table 3.8). Results mirrored AFLP experiment 2, with the maximal genetic distance between parental monoploids (0.2159) or somaclones of O8-1 and monoploid T5-268 and the minimal distance between somaclones SC-5A and SC-36B (0.0196).

UPGMA cluster analysis produced a tree comparable with AFLP experiment 2 with a minor rearrangement of the two somatic hybrids (Figure 3.4b).

3.3.2. Sequence analysis

To further characterize our retrotransposon-based marker systems, in particular S-SAP, and as a conclusion to IRAP marker studies Cy5 fluorophore labeled reactions were separated on 8% polyacrylamide gels using a manual sequencing system. Five IRAP fragments were excised and sequenced. All fragment analyses corresponded to the expected size and features of IRAP-based sequences, in that both the beginning and the terminal nucleotides matched the retrotransposon-based primer and were therefore authentic sequences and not an artifact of PCR. BLASTN database analysis produced three hits (Table 3.9). Fragment IRAP1 (236 bp) produced an extremely good match with (1) *Atropa belladonna* (chloroplast genome) (score 317) and (2) *Nicotiana tabacum* (chloroplast genome) (score 291) whereas partially sequenced fragment IRAP4 produced a weak hit with tobacco anther-specific gene TA-29 and stem specific gene TSJT1 (score 54, both).

A total of 11 S-SAP-based fragments was extracted, sequenced, analysed and later used for Southern blot analysis. These S-SAP sequences should correspond to the flanking regions of a retrotransposon and be delimited by the retrotransposon (*Tst1*)-based primer sequence and the *Mse*I adaptor-based primer sequence. All 11 extracted fragments had these characteristics and were therefore legitimate. The sequences ranged in size from 134 to 505 bp. BLAST N searches were conducted at the NCBI web site and two weak hits were identified. MF340-C25 produced a match (score 54) with *Solanum desimissum* chromosome 11 BAC and *Solanum lycopersicum* phytochrome F gene (score 52) (Table 3.9). MF475-C18 produced a match with *S. tuberosum* repetitive DNA (score 54) and *Lycopersicon hirsutum* RGA marker (score 50).

3.3.3. RFLP/Southern blot analysis

For Southern blot analysis genomic DNA (20 µg) was digested with *Eco*RI, separated on 0.7% agarose gels and transferred under neutral conditions to positively charged nylon membranes. Three groups of plants were examined. Group one, termed the garden blot group, consisted of six Solanaceae (*Datura* sp., *Petunia* sp., *Nicotiana tabacum* cv Samsun, *Solanum tuberosum* cvs. Atlantic and Katahdin, *Lycopersicon esculentum* cv Moneymaker) and one non-Solanaceaeous species, *Arabidopsis thaliana* 'Columbia'. Group two consisted of seven monoploid potatoes: T5-268, T4-142, O8-1, O5-10 3BB, PP5-15, 3-2-6 6N-1 and N8-2 AR8. Group three consisted of four parental monoploids families and their somatic hybrids or somaclones, consisting of combinations (1) O8-1 (+) T5-268, (2) O5-10 3BB (+) T4-142, (3) N8-2 AR8 (+) O5-10 3BB and (4) N8-2 AR8 (+) 3-2-6 6N-1. Membranes were stripped and re-probed up to a maximum of 11 times.

Probes used for analysis were derived from S-SAP fragments that had been previously extracted and sequenced. These fragments were originally derived from parental monoploids or somatic hybrids during S-SAP PCR amplification and represented sequences that exhibited strong signals and polymorphisms. The original intent was to target and extract illegitimate polymorphic bands in somatic hybrids, depicted by new non-parental bands, as identified during ALFexpress analysis; however, it was not feasible to correlate and identify polymorphisms depicted on the ALFexpress with visual signals during manual separation and excision and therefore polymorphic bands (parental or hybrid) were selected.

Experiment 1 Southern blot analysis of retrotransposon-derived RFLP markers in the Solanaceae– garden blot

The plant material analyzed in this experiment consisted of a mix of members of the Solanaceae family and one non-member, *Arabidopsis thaliana.* Eleven probes were tested and the results are presented in Table 3.10 and depicted in Figures 3.5.a.b.c. All eleven probes hybridized to *S. tuberosum* cvs. Atlantic and Kathadin; in contrast none of the probes hybridized to *A. thaliana.* Eight of the probes hybridized to *L. esculentum*, four to *Datura* but only smears with one or two probes with *Petunia* and *N. tabacum,*

respectively. Probes were separated into two groups; specific producing less than 10 bands (MF370-C01, MF310-C14, MF302-No11, H245-No01, FP146-No20, FP134-No16 and No17) or complex and not scorable producing over 13 bands (MF505-C26, MF475-C18 and MF340-C25). In general there was a loss in clarity of membranes with increased numbers of stripping and reprobing.

Experiment 2 Southern blot analysis of RFLP markers of the monoploid families

Four monoploid families were represented among the seven monoploids evaluated. The T family consisted of T5-268 and T4-142, the O family of O5-10 3BB and O8-1, the A family of PP5-15 and 3-2-6 6N-1 and the N family of N8-2 AR8. Eleven probes were tested; however, probes that produced greater than 13 bands were not scored due to the difficulty of visual separation of markers (Table 3.11 and Table 3.12). Eight probes produced 40 scorable bands. Monomorphic banding patterns were produced by probes MF505-C26 and FP134-No16, whereas complex polymorphic but unscorable bands were produced by probes MF475-C18 and MF340-C25 (Figure 3.6.a.b.c). The maximum genetic distance scored was between monoploids T5-268 and PP5-15 (0.7444), whereas the minimum distance was between members of the T family (0.1054). Members within a family displayed low genetic distances with the exception of the A family (PP5-15 and 3-2-6 6N-1) which were as distant from each other as from members of other families. Polymorphisms between families for markers ranged from 43 to 61%. UPGMA cluster analysis produced a tree, with monoploid members of the same family occurring in the same branches with the exception of the A family (Figure 3.7).

Combined experiments 3, 4, 5 and 6. Southern blot analysis of RFLP markers of the somatic hybrid O8-1 (+) T5-268 family, O5-10 3BB (+) T4-142 family, N8-2 AR8 (+) O5-10 3BB family and N8-2 AR8 (+) 3-2-6 6N-1 family.

Material tested from the first family consisted of monoploids O8-1 and T5-268, somatic hybrids SH-1A and SH-18C, and somaclones of O8-1, (SC-3C, SC-5A and SC-36B) as representatives of this family. Six probes were scored and a total of 27 markers detected. Plant material tested from the second family consisted of the monoploid O5-10 3BB and

T4-142 and four groups of pooled somatic hybrids (SH-OT3-1A to J, SH-OT3-2A to J, SH-OT3-3A to J and SH-OT3-4A to J). Data collected from somatic hybrids regenerated from the same callus were pooled as there was little polymorphism detected between members. Seven probes produced scorable bands with a total of 27 markers. The plant material tested in the third experiment consisted of monoploids N8-2 AR8 and O5-10 3BB, ten pooled somatic hybrids and ten pooled somaclones derived from O5-10 3BB. Four probes produced scorable bands with a total of 21 markers. In the fourth family material tested consisted of monoploid parental types, N8-2 AR8 and 3-2-6 6N-1 (A family) and seven somatic hybrids. Seven probes were scored and a total of 27 markers detected.

In all families statistical analysis of markers using Nei's genetic distance coefficient produced the greatest distance between the monoploid parental types (0.7419, 0.2079, 0.539 respectively) with the exception of O8-1 + T5-268 family T5-268, which produced the greatest distance between the T5-268 monoploids and O8-1 somaclones. Somaclones and their parental monoploids showed no genetic distance (0.000). Comparisons between somatic hybrids also showed no genetic distance (0.0000) with the exception of somatic hybrids SH-1A and SH-18C (0.2513). UPGMA clustal analysis produced a tree typical of the expected results, with parental monoploids and somaclones on the most diverse/distant forks and the somatic hybrids positioned between parental types (Figure 3.8.a,b). There were three cases where expected bands were missing, hybrid SH-OT3-2G (Figure 3.9) had the upper 2 bands missing (probe MF310-C14) similarly hybrid SH-OT3 3J was missing the upper 3 bands (not shown) probe MF302-No 11, whereas all hybrids were missing the upper band when examined by probe FP174-No22 (Figure 3.10).

Comparison of DNA sequences

Sequenced fragments were aligned starting from the retrotransposon based primer nucleotide sequence (*Tst1*-LTRFout). Comparison based on a Pretty alignment with a plurality of 7 of a total of 11 sequences provided evidence for preferred insertion in A/T rich regions of the genome (Table 3.13). Adjacent to the retrotransposon based primer the preferred nucleotide was A. A group of T's formed a cluster at bp 13, 14, 16 and 18

away from the primer site and may be a potential positioning site as this cannot be ascribed to random occurrence of nucleotides. Other positive pluralities were observed at bp positions 36, 37 (both T's), 61, 64 (A's) and 78 and 79 (T's). There were no G/C rich nucleotide sites or regions in the fragments analyzed suggesting that insertions may occur preferentially in non-gene areas.

3.4. Discussion

The breeding strategy used in this study consists of fusing monoploid potatoes from four different families (O, N, T and A) with the intent of improving the germplasm by exploiting the heterotic effects in hybrids derived from selected stock- plants that have gone through the "monoploid sieve" and therefore had all lethal genes removed. To investigate this, the relationship between genetic distance of the parental, hybrid and somaclone genotypes were analyzed in three systems AFLP, S-SAP and IRAP.

AFLP analysis

The number of scorable bands produced was high (90, 146, 157) and the phenograms depicting the relationships between the monoploid parents and the somatic hybrid and somaclones were as expected, with parental monoploids being most distant and hybrids located between parental types. Repeated experiments revealed inconsistent results (number of scorable bands = 90, 146) which could be a limitation of the ALFexpress system in that if plates are incorrectly aligned during the run the signal is poor and scorable bands are less. Otherwise, this may be an artifact of PCR.

S-SAP analysis

S-SAP analysis produced many scorable bands (79, 149, 154, 138) comparable to AFLP. The high band number (evidence of activation over many years) indicates that *Tst1*, the retrotransposon from which the primer was designed, is an ancient retroelement. The quantity of illegitimate polymorphisms (due to possible activation of elements) detected was few, indicating that these elements may not be active. In general, the genetic

relationships depicted were as expected with parental types being most distant, while somaclones and sibling somatic hybrids were most closely paired. Repeat PCRs within the same experiment, excluding *Mse*I [+1] primer reaction, displayed 5-7% polymorphism, thus undermining the dependability of the system. It was not possible to identify the source of the error (PCR, reader or both). Experiments varying the *Mse*I-based selective primer from [+1] to [+3] resulted in a slight reduction in the number of markers scored (154, 138). With each selective base added to the primer there should be a reducing by ¼ in the number of scorable bands. This was not observed suggesting that bands used for scoring were a complex heterogeneous mix. This may be due to the fragment size range examined; smaller fragments of similar size may actually represent more than one amplified sequence as indicated by probes FP134-No16 and FP134-No17 both extracted from the same fragment but of completely unrelated nucleotide sequence.

IRAP analysis

IRAP analysis produced many scorable bands (114, 103) comparable to both AFLP and S-SAP. As previously stated, high band number indicates that *Tst1* is an ancient retroelement. The quantity of illegitimate polymorphisms detected was high, and results of some family comparisons were excluded due to non-repeatability of fragment analysis. However, in stable reactions the genetic relationships depicted were as expected with parental types being most distant. In fusion combination N8-2 AR8 and O5-10 3BB, the regenerants were all somaclones of O5-10 3BB, however genetic distance recorded varied and were almost as high among somaclones as between parental types. Comparisons between fusion combination O8-1 and T5-268 were as expected with parental types being distant, hybrids intermediate while somaclones were closely related to parental type.

Southern blot analysis

The garden blot membrane exhibits hybridization homology that fits the relationship among members of the Solanaceae working under the assumption that the greater the percentage of matching probes the closer the taxonomic relationship (in this case to *S. tuberosum*). Current systematics by taxonomists divides the Solanaceae into (three)

subfamilies, tribes and genera (D'Arcy, 1991). *Solanum* and *Lycopersicon* are closely related and within the same tribe, Solaneae, whereas *Datura* is of the tribe Datureae, but all are of the subfamily Solanoideae. More distant *Nicotiana* and *Petunia* are both of the tribe Nicotianeae, but of the subfamily Cestroideae. These divisions are clearly reflected in the Southern blot analysis of garden blot membrane. All eleven probes hybridized to potato cultivars Atlantic and Katahdin, eight to *Lycopersicon*, four to *Datura*, and only smears with *Nicotiana* and *Petunia* (2 and 1 respectively). S-SAP based probes are therefore an additional source for revealing taxonomic relationships and can be used as molecular markers in species related to those for which they were developed.

Southern blot analysis of seven monoploid potatoes belonging to four family groups (N, O, T and A) using 11 S-SAP based probes resulted in the expected relationships, with the members of the same families grouped. The exception was the A family. T5-268 and T4-142 have the female parent of the anther donor in common (DM BARD 13-14 202) similarly O5-10 3 BB and O8-1 have the female parent of the anther donor common (DM BARD 1-3 516), however, in the A family 3-2-6 6N-1 and PP5-15 are both descendants of PP5. PP5-15 is directly from anther culture of PP5 whereas 3-2-6 6N-1 is a descendent of PP5 after an anther-derived DM had been outcrossed to an unrelated diploid clone. Therefore, the relatedness of the two clones representing the A family was less than that of the two representatives of the O and T families.

For non-disruptive integration of transposable elements there should be a selective advantage to non-random insertions. Insertions into genes or regulatory regions of the genome will potentially (high probability) be lethal or deleterious. However, insertions in the promoter regions have been observed in approximately 25% of human genes (Jordan *et al*., 2003) and have been ascribed to genome evolution especially in gene expression levels (Bennetzen, 2000). It has been reported that coding regions have high G/C content. However, targeting heterochromatin or intronic regions of the genome probably has a higher selective advantage. The proposal that low copy number transposons, e.g *Tos17*, prefer genic regions as they require active loci to maintain their copy number infers that high copy number transposons might prefer intergenic regions or

heterochromatin (Bennetzen, 2000). We have shown that *Tst1* occurs in high copy numbers and can speculate that it might prefer intergenic regions. In contrast to preferred insertion into genic regions by *Tos17* reported by Miyao *et al.* (2003) our limited analysis shows that in the 11 sequences examined there was a preference for insertion into regions of the genome with a high A/T content. With our data it is not possible to confirm that *Tst1* insertion targets non-gene areas but because there were only two HITS during database analysis, HIT3 (repetitive DNA) and HIT4 (BAC library chromosome 11, phytochrome F gene) there is the suggestion that the preferred sites are heterochromatin.

The cluster of A/T sites that are 13 to 18 bp downstream from the LTR primer sequence mirror to some extent the TATA box sequence which is used for guiding the positioning of RNA polymerase initiation during transcription (Glick and Pasternak, 1994). We can speculate that the function of the sequence may allow for easier opening of the DNA double helix structure with possible enhanced integrase activity. Target site selection by *Ty5* via interactions between integrase and regulator protein has been shown by Xie *et al.* (2001). The S-SAP markers used did not allow for comparison between the target site duplication sequence (TSD) created during insertions as only one end of the adjacent genomic sequence was examined.

Barring somaclonal variation, somatic hybrids are expected to show a combined marker total of both parents whereas somaclones should have nearly identical profiles to their parent of origin. Various molecular marker systems have been used to attempt to associate genomic regions with phenotypically obvious somaclonal variants (Johnson and Veilleux, 2001). There has been various success because both processes, i.e. somaclonal variation and marker analysis probe the genome more or less randomly. Occasionally phenotypically distinct somaclones have yielded identical marker profiles to the parent whereas phenotypically nondistinct somaclones have exhibited the appearance of new markers (Johnson and Veilleux, 2001). In order for a marker system to be more diagnostic of somaclonal variation, it should target the genome in a systematically similar way as somaclonal variation. Hirochika *et al*. (1996) made a breakthrough in associating molecular markers with somaclonal variation when they found that a specific family of

retrotransposons was activated during tissue culture of rice. This elevated retrotransposon display to the marker system of choice in analyzing somaclonal variation. Kubis *et al.* (2003) attempted to exploit this association to find retrotransposon-based markers associated with a tissue culture-induced abnormality during oil palm micropropagation. Despite their attempt to associate any of three retrotransposon-based markers to this somaclonal variant, no markers were detected. As with our study, the missing element was the knowledge of which, if any, retrotransposons are activated in tissue culture. Retrotransposon display based on ancient inactive retrotransposons is no more effective than random genomic targeting in making the desired association.

Another important criterion in identifying markers associated with somaclonal variation is the robustness and repeatability of the marker system. The lack of reproducibility in the ALFexpress system of marker genotyping leaves much to be desired in this regard. Although the system was sufficiently robust with AFLP and S-SAP analyses to confirm previously known genetic relationships, there are too many inconsistencies for precise distinctions, i.e., the same "polymorphism" may or may not reappear in duplicate runs.

This was displayed in all families tested, confirming the usefulness of this technique for somatic hybrid identification. Certain hybrids, e.g., SH-1A of the O8-1 (+) T5-268, did not possess the complete marker complement for both parents suggesting that there had been genomic rearrangement during fusion. In particular, there could have been a loss or deletion of chromosomes where markers were absent. Molecular based technique such as species-specific PCR markers based on RAPDs (Masuelli *et al*., 1995), inter-SSR PCR (Matthews *et al*., 1999), RAPDs (McGrath *et al*., 1996; Pehu, 1996) RFLP (Feher *et al*., 1992; Garriga-Caldere *et al*., 1997; Oberwalder *et al*., 1997; Xu, 1993) have been used to detect loss of chromosomes in somatic hybrids. The S-SAP markers used in this study are the flanking regions of retrotransposons. A genomic change associated with loss of band with simultaneous formation of a smaller band would be probable grounds for activation of retrotransposons via insertion into our target band. Retrotransposons do not excise from the host genome; therefore an increase in number is predicted with activation. This was not observed in our study. However, due to the limited number of

fragments identified and the inability to target illegitimate bands we cannot exclude restricted activation.

3.5. Conclusions

Electrofusion for the creation of intermonoploid somatic hybrids has the advantage of surmounting sexual barriers and combining distinct genomes in a single step. The somatic hybrids we developed were fertile and comparable with sexually derived, genetically related hybrids in field trials. The somatic hybrids had significantly lower agronomic qualities compared to cultivars or cultivar x hybrid crosses. However, this was expected as the initial monoploid germplasm was derived from a *S. phureja* background.

The ideal conditions would be to generate monoploid families with a strong *S. tuberosum* background with many good cultivar characteristics and use these for the re-synthesis of highly heterozygous somatic hybrids. Tests should be carried out against established cultivars for evaluation of heterosis and the usefulness of the monoploid sieve in a product with a potentially high commercial application. This may not be practical with *S. tuberosum* due to the high level of inbreeding depression expressed during the reduction of ploidy levels; however, it should be possible to select monoploids with particular traits such as virus resistance, cold tolerance, etc. and have these combined and evaluated in a high *S. tuberosum* background, this could also be possible with our *S. phureja* germplasm.

We have established that retrotransposon based markers, in particular *Tst1*, can be used to identify and confirm phylogenetic relationships among Solanaceae. However, due to the large number of fragments generated we were not able to identify activation of these elements. The direct targeting of mRNA using RT-PCR technology is a likely second approach to identification of active retroelements. If activation of *Tst1* can be identified we can follow this activity and see if it can be correlated with somaclonal variation. The random selection of retroelements or retroelement-based markers with the hope of

identifying a recently activated element is not efficient. Finally the use of fluorescently labeled primers with the ALFexpress, regardless of the type of marker we employed, yielded inconsistencies in duplicate reactions. The phylogenies that we observed in any given run were consistent with known relationships among genotypes. However polymorphism was unexpectedly low. We verified that the amplified products were consistent with expected products of the primers employed. There may have been too many amplified fragements in the PCR reactions, such that products of the same fragment length in different lanes obscured true genetic distances. Cloning of S-SAP fragments and using them as probes in Southern blots gave much clearer pictures of genetic relationships among individuals.

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Table 3.1. Genetic distances based on AFLP marker polymorphism among parental monoploids (O8-1 and T5-268), a parental mix, somaclones of O8-1 and electrofusion-derived somatic hybrids using Nei's (1972) original distance coefficient.

Genotype	1	2	3	4	5	6	7	8
1 O8-1	*****							
2 T5-268	0.2094	*****						
3 O8 + T5	0.1431	0.0810	*****					
4 SH-1A	0.1178	0.1304	0.0690	*****				
5 SH-18C	0.1178	0.1054	0.0455	0.0690	*****			
6 SC-3C	0.0225	0.1823	0.1178	0.0931	0.0931	*****		
7 SC-5A	0.0455	0.2094	0.1431	0.1431	0.1178	0.0455	*****	
8 SC-36B	0.0339	0.2231	0.1560	0.1304	0.1304	0.0339	0.0112	*****

Number of bands scored = 90
Size range of bands = 24 to 805 bp
Marker type: Dominant.
Hardy-Weinberg equilibrium assumed.
Allele frequencies estimated based on the square root of the frequency of the null (recessive) genotype
SH = somatic hybrid
SC = somaclone

Table 3.2. Repeat experiment - Genetic distances based on AFLP marker polymorphism among parental monoploids (O8-1 and T5-268), a parental mix, somaclones of O8-1 and electrofusion-derived somatic hybrids using Nei's (1972) original distance coefficient.

Genotype	1	2	3	4	5	6	7	8
1 O8-1	*****							
2 T5-268	0.1719	*****						
3 O8 + T5	0.1368	0.0305	*****					
4 SH-1A	0.1197	0.1112	0.0782	*****				
5 SH-18C	0.1282	0.0541	0.0383	0.0701	*****			
6 SC-3C	0.0230	0.1643	0.1292	0.1121	0.1206	*****		
7 SC-5A	0.0383	0.1630	0.1282	0.0946	0.1029	0.0153	*****	
8 SC-36B	0.0462	0.1719	0.1368	0.1029	0.1112	0.0230	0.0075	*****

Number of bands scored = 146
Size range of bands = 23 to 749 bp
Marker type: Dominant.
Hardy-Weinberg equilibrium assumed.
Allele frequencies estimated based on the square root of the frequency of the null (recessive) genotype
SH = somatic hybrid
SC = somaclone

Table 3.3. Genetic distances based on AFLP marker polymorphism among parental monoploids (O5-10 3BB and T4-142), a synthetic mix and electrofusion-derived somatic hybrids using Nei's (1972) original distance coefficient.

Genotype	1	2	3	4	5	6	7
1 O5-10 3BB	*****						
2 T4-142	0.3649	*****					
3 O5 + T4	0.2941	0.0523	*****				
4 SH-OT3-1A	0.1823	0.1446	0.1299	*****			
5 SH-OT3-1B	0.1823	0.1595	0.1299	0.0129	*****		
6 SH-OT3-2A	0.1810	0.1810	0.1510	0.0260	0.0260	*****	
7 SH-OT3-2F	0.1964	0.1964	0.1659	0.0392	0.0260	0.0128	*****

Number of bands scored = 157
Size range of bands = 23 to 526 bp
Marker type: Dominant.
Hardy-Weinberg equilibrium assumed.
Allele frequencies estimated based on the square root of the frequency of the null (recessive) genotype
SH= somatic hybrid

Table 3.4. Genetic distances based on S-SAP marker using primers with selective bases *Mse*I [+1] and *Mse*I [+3] among parental monoploids (O5-10 3BB and T4-142), a parental mix and electrofusion-derived somatic hybrid using Nei's (1972) original distance coefficient.

Genotype	**1**	**2**	**3**	**4**	**5**	**6**	**7**
*Mse*I [+1]							
1 O5-10 3BB R1	*****						
2 O5-10 3BB R2	0.0671	*****					
3 T4-142 R1	0.1552	0.1476	*****				
4 T4-142 R2	0.0953	0.1391	0.0960	*****			
5 O + T R1	0.1928	0.1771	0.2102	0.1771	*****		
6 O + T R2	0.1849	0.2007	0.1863	0.1849	0.1928	*****	
7 SH-OT3-1A R1	0.1170	0.1618	0.1629	0.0882	0.1849	0.2086	*****
*Mse*I [+3]							
1 O5-10 3BB R1	*****						
2 O5-10 3BB R2	0.0597	*****					
3 T4-142 R1	0.0850	0.0931	*****				
4 T4-142 R2	0.1013	0.1013	0.0788	*****			
5 O + T R1	0.0606	0.0924	0.0857	0.1029	*****		
6 O + T R2	0.0917	0.1242	0.1517	0.1187	0.0764	*****	
7 SH-OT3-1A R1	0.0764	0.0924	0.1029	0.1029	0.0776	0.1095	*****

Number of bands = 154, 138
Size range of bands = (23 –505) & (23 - 465) bp
Marker type: Dominant.
Hardy-Weinberg equilibrium assumed.
Allele frequencies estimated based on the square root of the frequency of the null (recessive) genotype
SH = somatic hybrid

Table 3.5. Genetic distances based on S-SAP marker polymorphism among parental monoploids (N8-2 AR8 and O5-10 3BB), a parental mix and somatic hybrids and somaclones of O5-10 3BB using Nei's (1972) original distance coefficient.

Genotype	1	2	3	4	5	6	7	8	9	10	11	12
1 O5-10 3BB	*****											
2 N8-2 AR8	0.3064	*****										
3 N8 + O5	0.1576	0.2076	*****									
4 SH-NO4-1A	0.0946	0.2603	0.1023	*****								
5 SH-NO4-1B	0.0795	0.2249	0.1023	0.0426	*****							
6 SC-NO3- 2A	0.0795	0.2970	0.1991	0.1178	0.1023	*****						
7 SC-NO3-2B	0.0800	0.2900	0.1922	0.1187	0.1030	0.0141	*****					
8 SH-NO4-3A	0.1754	0.2900	0.1837	0.1345	0.1345	0.0876	0.1038	*****				
9 SH-NO4-3B	0.1426	0.2624	0.1507	0.1030	0.1030	0.0876	0.0883	0.0286	*****			
10 SH-NO4-4I	0.1335	0.1991	0.1256	0.0946	0.0645	0.1576	0.1426	0.1588	0.1266	*****		
11 SH-NO4- 10A	0.1256	0.2603	0.1335	0.1023	0.0870	0.1178	0.1030	0.1507	0.1345	0.0795	*****	
12 SH-NO4-11A	0.1023	0.2877	0.1740	0.946	0.0795	0.0645	0.0800	0.1266	0.1266	0.1178	0.0795	*****

Number of bands scored = 149
Size range of bands = 23 to 435 bp
Marker type: Dominant.
Hardy-Weinberg equilibrium assumed.
SC = somaclone
SH = somatic hybrids
Allele frequencies estimated based on the square root of the frequency of the null (recessive) genotype

Table 3.6. Genetic distances based on S-SAP marker polymorphism among parental monoploids (O8-1 and T5-268), a parental mix and electrofusion-derived somatic hybrid (SH-18C) using Nei's (1972) original distance coefficient.

Genotype	1	2	3	4	5	6	7	8
1 O8-1 R1	*****							
2 O8-1 R2	0.0556	*****						
3 T5-268 R1	0.2610	0.1932	*****					
4 T5-268 R2	0.1961	0.1316	0.0564	*****				
5 O+T R1	0.1609	0.0994	0.1144	0.0858	*****			
6 O+T R2	0.2097	0.1769	0.1297	0.1008	0.0700	*****		
7 SH-18C R1	0.1932	0.1297	0.1144	0.0858	0.0556	0.0700	*****	
8 SH-18C R2	0.2265	0.1932	0.1452	0.1473	0.1144	0.1297	0.0556	*****

Number of bands scored = 74
Size range of bands = 23 to 492 bp
Marker type: Dominant.
Hardy-Weinberg equilibrium assumed.
Allele frequencies estimated based on the square root of the frequency of the null (recessive) genotype
SH = somatic hybrid

Table 3.7. Genetic distances based on IRAP marker polymorphism among parental monoploids (N8-2 AR8 and O5-10 3BB), a parental mix and somaclones of O5-10 3BB using Nei's (1972) original distance coefficient.

Genotype	1	2	3	4	5	6	7	8	9	10	11	12	13
1 N8-2 AR8	*****												
2 O5-10 3BB	0.4369	*****											
3 N8 + O5	0.1123	0.4187	*****										
4 SC-NO3-2A	0.2501	0.3054	0.2703	*****									
5 SC-NO3-2B	0.2501	0.3054	0.2703	0.0000	*****								
6 SC-NO3- 2C	0.2847	0.2703	0.3295	0.0634	0.0634	*****							
7 SC-NO3-2D	0.2847	0.3054	0.2935	0.0728	0.0728	0.1211	*****						
8 SC-NO3-2E	0.3219	0.1787	0.4378	0.1361	0.1361	0.0854	0.1572	*****					
9 SC-NO3-2F	0.2997	0.2165	0.4099	0.1222	0.1222	0.1024	0.1841	0.0561	*****				
10 SC-NO3-2G	0.3240	0.2501	0.3707	0.0927	0.0927	0.0546	0.1527	0.0953	0.1024	*****			
11 SC-NO3- 2H	0.3085	0.2589	0.3417	0.1112	0.1112	0.1211	0.1112	0.1155	0.1024	0.1323	*****		
12 SC-NO3-2I	0.4233	0.3795	0.4457	0.1512	0.1512	0.1411	0.2144	0.2118	0.2056	0.1527	0.1310	*****	
13 SC-NO3-2J	0.2758	0.2966	0.2966	0.0179	0.0179	0.0546	0.0927	0.1155	0.1222	0.0927	0.1123	0.1527	*****

Number of bsnds scored = 114 Size range of bands = 22 to 630 bp SC = somaclone
Marker type: Dominant. Hardy-Weinberg equilibrium assumed.
Allele frequencies estimated based on the square root of the frequency of the null (recessive) genotype

Table 3.8. Genetic distances based on IRAP marker polymorphism among parental monoploids (O8-1 and T5-268), a parental mix, somaclones of O8-1 and electrofusion-derived somatic hybrids using Nei's (1972) original distance coefficient.

Genotype	1	2	3	4	5	6	7	8
1 O8-1	*****							
2 T5-268	0.2159	*****						
3 O8 + T5	0.1021	0.1239	*****					
4 SH-1A	0.1349	0.1804	0.0498	*****				
5 SH-18C	0.1574	0.2039	0.1129	0.1239	*****			
6 SC-3C	0.0704	0.2280	0.1129	0.1239	0.1239	*****		
7 SC-5A	0.0914	0.2527	0.1574	0.1688	0.1921	0.0600	*****	
8 SC-36B	0.0704	0.2280	0.1349	0.1688	0.1921	0.0809	0.0196	*****

Number of bands scored = 103
Size range of bands = 36 to 598 bp
Marker type: Dominant.
Hardy-Weinberg equilibrium assumed.
Allele frequencies estimated based on the square root of the frequency of the null (recessive) genotype
SH = somatic hybrid
SC = somaclone

Table 3.9. Retrotransposon-based band analysis, IRAP or S-SAP, indicating origin, type, fragment size and results of data search.

Fragment type	Source	Type	Sequence size (bp)	BLAST N search	Probe made
IRAP1	Parent (T5-268)	polymorphic	236	HIT 1	NO
IRAP2	Hybrid (SH-18C)	polymorphic	269	No Match	NO
IRAP3	Somaclone (SH-3C)	monomorphic	partial	No Match	NO
IRAP4	Parent (T5-268)	polymorphic	partial	HIT 2	NO
IRAP5	Parent (O8-1)	polymorphic	588	No Match	NO
S-SAP1	Parental (T5-268)	polymorphic	505	No Match	MF505-C26
S-SAP2	Parental (N8-2 AR8)	polymorphic	475	HIT 3	MF475-C18
S-SAP3	Parental (T5-268, T4-142, N8-2, O8-1)	polymorphic	370	No Match	MF370-C01
S-SAP4	Parental (T5-268)	polymorphic	340	HIT 4	MF340-C25
S-SAP5	Hybrid (N8 + O8)		316	No Match	H316-No24
S-SAP6	Parental (T5-268, O8-1)	polymorphic	310	No Match	MF310-C14
S-SAP7	Parental (N8-2 AR8)	polymorphic	302	No Match	MF302-No11
S-SAP8	Hybrid (N8 + O8)	monomorphic	240	No Match	H240-No01
S-SAP9	Parental (N8-2 AR8)	monomorphic	174	No Match	FP175-No22
S-SAP10	Parental (N8-2 AR8)	monomorphic	146	No Match	FP146-No20
S-SAP11	Parental (N8-2 AR8)	monomorphic	134	No Match	FP134-No16
S-SAP12	Parental (N8-2 AR8)	monomorphic	134	No Match	FP134-No17

Scores over 50 accepted as HIT
HIT 1= *Atropa belladonna* (chloroplast genome), Score 317: *Nicotiana tabacum* (chloroplast genome), Score 291
HIT 2 = Tobacco anther-specific gene TA-29 and stem specific gene TSJT1, Score 54
HIT 3 = *S. tuberosum* repetitive DNA Score, 54, *Lycopersicon hirsutum* RGA, Score, 50
HIT 4 = *S. demissum* chromosome 11 BAC Score, 54; *S. lyopersicum* phytochrome F gene, Score, 52
Partial = over 1000 bp only partially sequenced

Table 3.10 Occurrence of S-SAP bands derived from *Solanum phureja* in a garden blot of other Solanaceae.

Probe	***Datura* sp.**	***Petunia* sp.**	***Nicotiana tabacum* 'Samsun'**	***Solanum tuberosum* 'Atlantic'**	***Solanum tuberosum* 'Kathadin'**	***Arabidopsis thaliana* 'Columbia'**	***Lycopersicon esculentum* 'Money Maker'**	***Total/No. of scorable Solanaceae members***
MF505-C26	0	0	0	16+	16+	0	9+	41+/3
MF475-C18	9	0	Smear	22+	22+	0	0	53+Smear/4
MF370-C01	2	0	0	5	5	0	1	13/4
MF340-C25	6+	Smear	Smear	16+	16+	0	13+	51+2Smear/6
H316-No24	11+	0	0	12+	12+	0	7	42+/4
MF310-C14	0	0	0	3	3	0	Smear	6+Smear/3
MF302-No11	0	0	0	3	3	0	Smear	6+Smear/3
H245-No01	0	0	0	9+	9+	0	3	21/3
FP146-No20	0	0	0	8	8	0	3	19/3
FP134-No17	0	0	0	2	2	0	0	4/2
FP134-No16	0	0	0	5+	5+	0	0	10+/2
Total/No. of scorable Probes	28+/4	Smear/ 11	2Smear/2	101+/11	101+/11	0/0	36+2Smear/8	266+5Smear/37

MP174-No22: No Results

Table 3.11. Occurrence of S-SAP bands derived from *S. phureja* in a panel of monoploids.

Probe	T5-268	T4-142	O5-10 3BB	O8-1	PP5-15	3-2-6 6N-1	N8-2 AR8
MF370-C01	3/8 a, c, e	3/8 a, c, e	2/8 a, d	2/8 a, d	3/8 b, e, g	4/8 b, c, d, g	5/8 b, c, d, f, h
H316-No24	1 a	1 a	1 a	1 a	1 a	1 a	1
MF310-C14	2/6 b, f	2/6 b, f	2/6 a, f	2/6 a, f	1/6 c	2/6 e, f	2/6 d, f
MF302-No11	2/6 b, f	2/6 b, f	2/6 a, f	2/6 a, f	1/6 c	2/6 e, f	2/6 d, f
H245-No01	3/14 b, e, f	3/14 e, j, k	3/14 e, j, k	5/14 a, c, f, j, k	4/14 j, k, l, m	6/14 f, g, h, i, j, k	3/16 b, c, f
FP174-No22	3/8 c, e, h	3/8 c, e, h	2/8 e, f	2/8 e, f	4/8 a, d, f, g	3/8 b, d, e	3/8 d, e, h
FP146-No20	2/4 b, c	2/4 b, c	2/4 a, c	¾ a, c, d	¼ a	2/4 a, c	0/4
FP134-No17	3/3 # a, b, c	3/3 a, b, c	3/3 a, b, c	3/3 a, b, c	3/3 a, b, c	3/3 a, b, c	3/3 a, b, c

MF505-C26: Complex: > 24 bands: 3 major monomorphic bands
MF475-C18: Complex: > 25 bands: smear: polymorphic: minor monomorphic bands
MF340-C25: Complex: > 16 bands: smear: polymorphic: minor monormorphic bands
FP134-No16: Complex: > 18 bands: smear: mainly monomorphic
2 or 3 faint bands, not scored

Table 3.12. Genetic distances between monoploid families based on Southern blot analysis of RFLP marker resulting from 8 of 11 S-SAP derived probes using Nei's (1972) original distance coefficient.

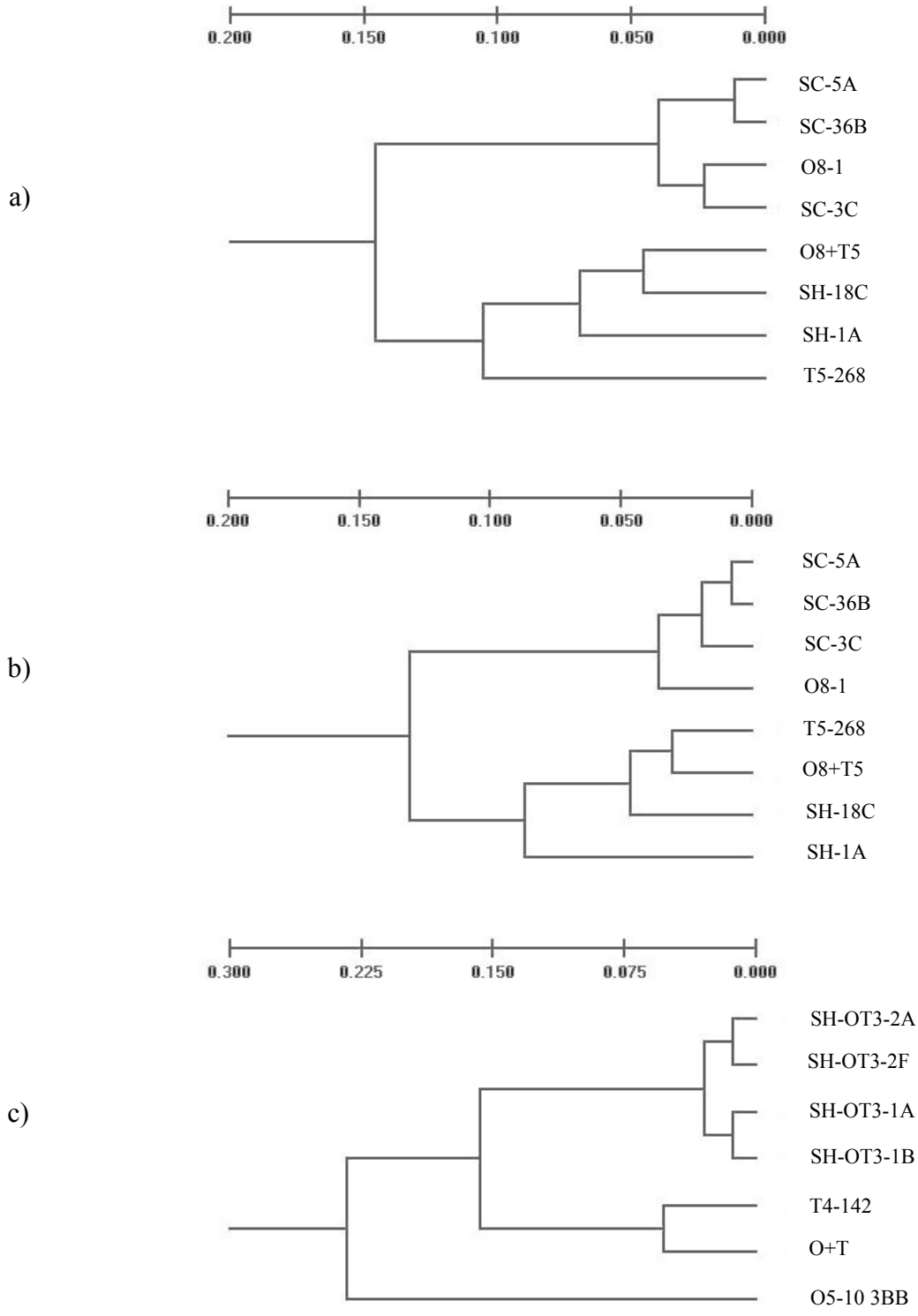
Genotype	1	2	3	4	5	6	7
1 T5-268	*****						
2 T4-142	0.1054	*****					
3 O5-10 3BB	0.4308	0.2877	*****				
4 O8-1	0.5534	0.4700	0.1335	*****			
5 PP5-15	0.7444	0.6444	0.4700	0.5978	*****		
6 3-2-6 6N-1	0.6931	0.5978	0.4308	0.4700	0.5534	*****	
7 N8-2 AR8	0.4308	0.5978	0.5978	0.5534	0.6444	0.5108	*****

Number of bands scored = 40
Marker type: Dominant.
Hardy-Weinberg equilibrium assumed.
Allele frequencies estimated based on the square root of the frequency of the null (recessive) genotype

Table 3.13. Consensus sequence depicted by a pretty pileup with a plurality of 7 comparing the DNA sequence of 11 S-SAP excised bands used to make DIG labeled probes.

```
          1                                                  50
MF475-C18  CTAAATCTGC CTACTCATTC AAACACTCTT TGGCTTTTAT ATAAAGGCTC
FP146-No20 CTAAATCTGC CTACTCATTC AAACAGCCTC GCCACTCTCT TTTCCCCCTC
FP175-No22 CTAAATCTGC CTACTCATTC AA.....GTG TACCATTTAT GAACCACCTT
MF310-C14  CTAAATCTGC CTACTCATTC AAAATATCCT CTCTTTTTTT ATCTTTTCAA
FP134-No16 CTAAATCTGC CTACTCATTC AAGCAAA.AG TCCAAAACCA AAAGGGAGTG
FP134-No17 CTAAATCTGC CTACTCATTC AAGATCA.TT GCACTTATTT GTAGAATCCA
MF340-C25  CTAAATCTGC CTACTCATTC AAAAGGG.AA CGAGTAATTT TCAACGA.AA
H240-No01  CTAAATCTGC CTACTCATTC AAACAAACCG TTACTTGTAT TCCATTACCA
MF370-C01  CTAAATCTGC CTACTCATTC AAATCCAACC ATTATCCGAA GACAATATAA
H316-No24  CTAAATCTGC CTACTCATTC AAATGTCGTC TTATTTCTTT CTTTCCAAAT
MF505-C26  CTAAATCTGC CTACTCATTC AAAACCAAAA TGCTAACCAC CCGCACATTT
Consensus  CTAAATCTGC CTACTCATTC AAA------- ----TT-T-T ----------
          51                                                 100
MF475-C18  TCATTTTTCT TTTCCTTGGC CTCTTTCCTC TCACCAAAAC ATTTTATTTT
FP146-No20 TATCTTTTTA TGTCC....C ATCTTTGCAC ACTTCAATCC CTTGTCCTTC
FP175-No22 ATATATTCAA ATTATTGGAA CGTATACACA ATAAGTATGC TTTATATAAA
MF310-C14  TTGTCACTAT CCACTTTCTT ACTCCTCTCT TTATCTTCTC TGTTATTCAT
FP134-No16 CAAGTAGCCC CCGGGTGAAT GATTTATTAT CGCGCATACT TGACAAAGTT
FP134-No17 CAAGAAATCT CTGGAAAACA CTAAGTTTTT CTGAAAGAAA CTTACAATGT
MF340-C25  AAGGTGCTCC CTGACTTGGA CATAGAAATC ATAGAAGTAA AAATGTTGGT
H240-No01  TCTTTTGTGT CCATCAGAAA AACTCCTTCA ATAGCATTGA AGCCAACACG
MF370-C01  GCATCGTCGA AAACGTTATC CTATTCAAAC TCATTCCCCC ATCTTCTAAC
H316-No24  AACCCACATG ATTGCTAAGG TGGCGGCATT ACATGCCCTT GGGCTTCTCT
MF505-C26  CCCTCCATCA CCAAAATCAA CATACCAACC CTCAGACTTT CCTTCAAAAT
Consensus  -------T-- -----T---- ---------- --A--A---- ---------T
          101                                                150
MF475-C18  TTTACTCACA CCCTCTTTTA CCTCTTTACT TAGATCGCTG CCCTCTTTCT
FP146-No20 TCCGTCATAT ACTTTACCTC GACCATTTGT TACTCAGGAC TCATCG~~~~
FP175-No22 TTTTACTTCA ACTTATGCGT ATCTTAGTTT TTTTCCTTTT TCTTGAGTAA
MF310-C14  TTCATTAGGA GTGGAATAGG CTCCAAGGGA TCCCCTTCGA GTCATTGGTA
FP134-No16 GAAGGGTCTA ATGATTTGTT ACTCAGGACT CATCG~~~~~ ~~~~~~~~~~
FP134-No17 TCATATTTAT CAAGATTGTT ACTCAGGACT CATCG~~~~~ ~~~~~~~~~~
MF340-C25  TGGAAGTAAA AATGTCTTCA GATGACTAGC CCTCTTATCG AAGGGACATG
H240-No01  GGTCACCCAA AATGGTTTTC CCCAACCAAA GTCAGCTTCA TACCAAGGCA
MF370-C01  ATCAACTTAT ACGCCTCTAA AGCATCAATA GCTAAACCTC GAGATGCATA
H316-No24  TACCCTCCTA TGAGTCCAAC TTTACAAAGC CTCCTTCACT ATGCCAGCAT
MF505-C26  TACCAAGCCA CTCAAAATGC CCAAAGTCCC TCTATTGCTC CACCACTACC
Consensus  T--------A ---------- ----A--A-- ---------- ----------
```

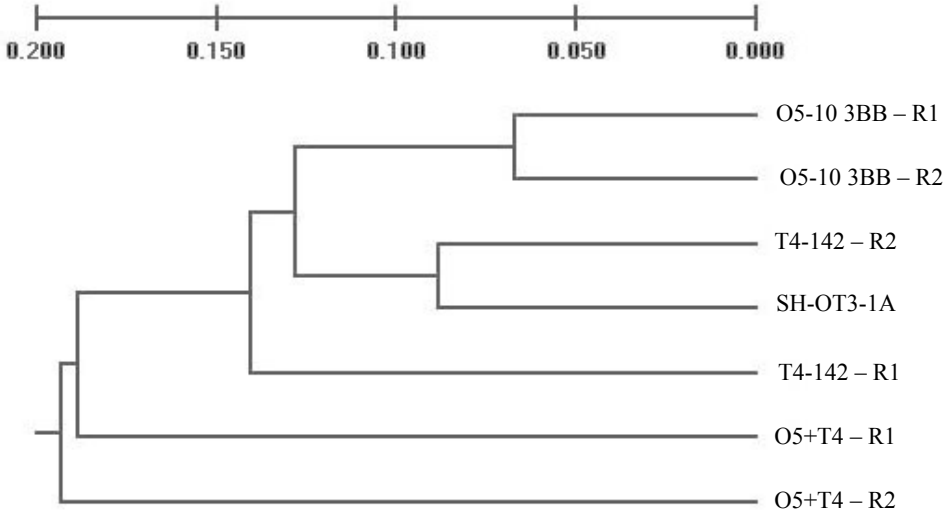
Figure 3.1 Phenograms of AFLP data generated on an ALFexpress sequencer depicting relationships between somatic hybrid families O8-1 (+) T5-268 (a), repeat comparison (b) and O5-10 3BB (+) T4-142 (c) disclosed by UPGMA cluster analysis.



SH = somatic hybrid
SC = somaclone

Figure 3.2 Phenograms of S-SAP data generated on an ALFexpress sequencer depicting relationships between somatic hybrid families O5-10 3BB (+) T4-142 using (a) *Mse*I [+1] and (b) *Mse*I [+3] selective primers, disclosed by UPGMA cluster analysis.

a) S-SAP MseI [+1]



b) S-SAP MseI [+3]

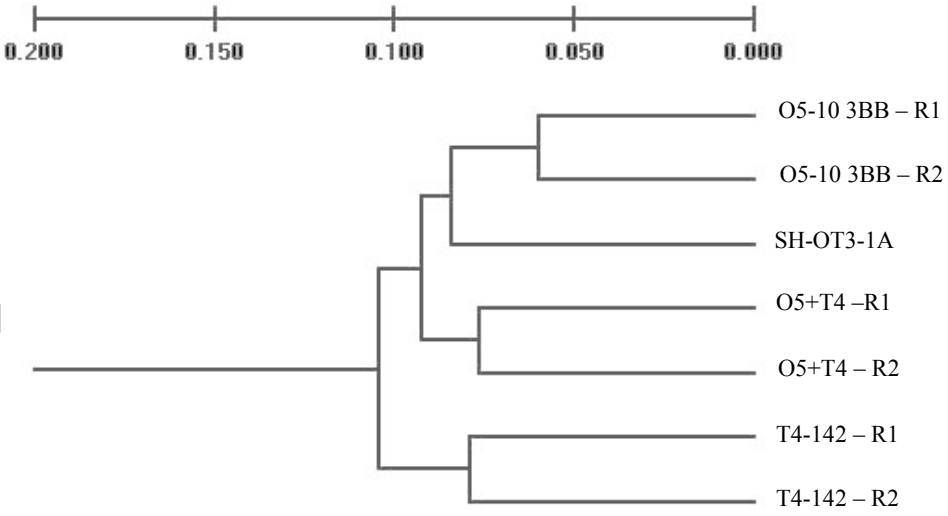
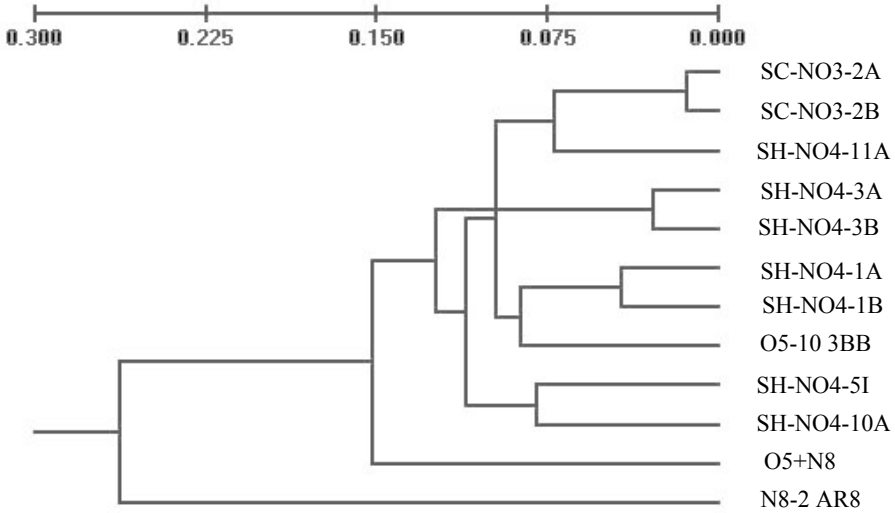


Figure 3.3 Phenograms of S-SAP data generated on an ALFexpress sequencer depicting relationships between somatic hybrid families O5-10 3BB (+) N8-2 AR8 (a) and O8-1 (+) T5-268 (b) disclosed by UPGMA cluster analysis.

a)



b)

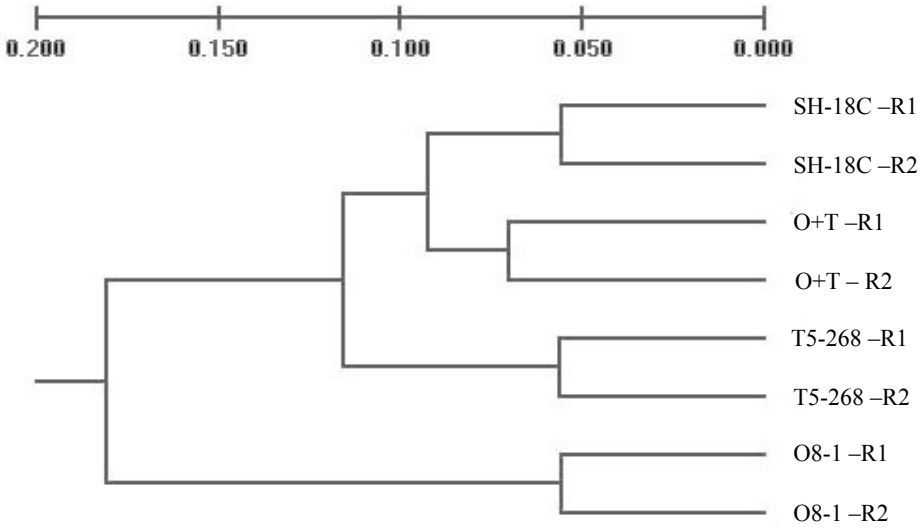
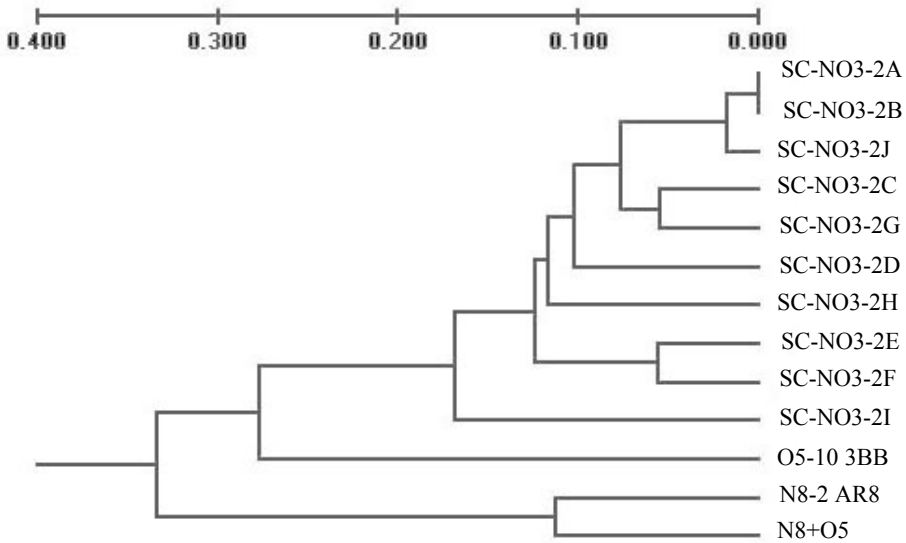
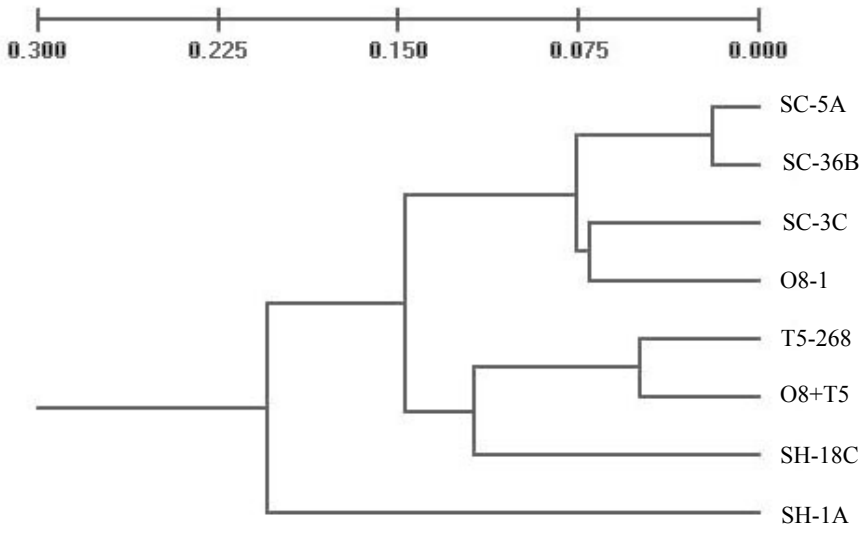


Figure 3.4 Phenograms of IRAP data generated on an ALFexpress sequencer depicting relationships between somatic hybrid families N8-2 AR8 (+) O5-10 3BB (a) and O8-1 (+) T5-268 (b) disclosed by UPGMA cluster analysis.

a)



b)



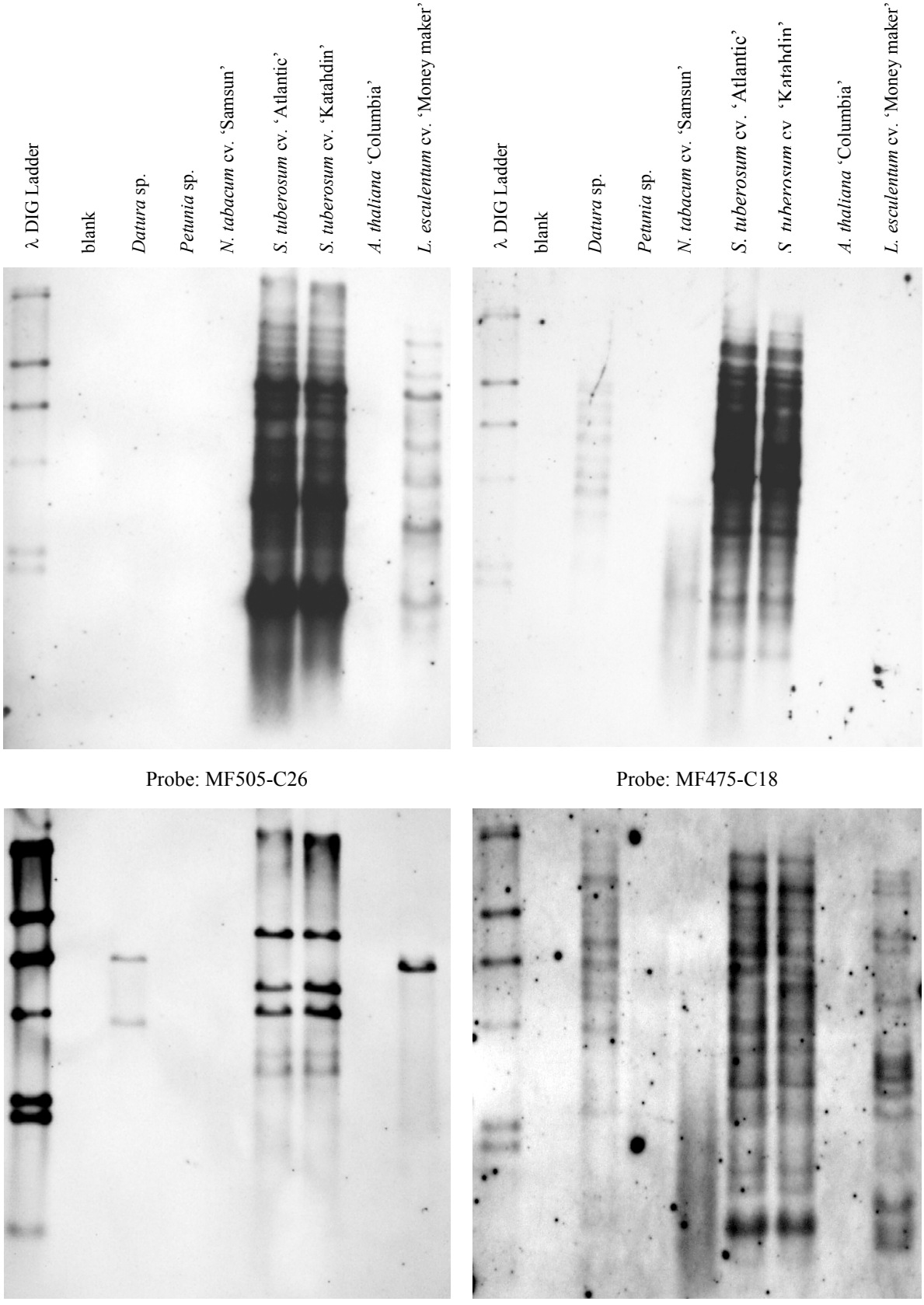
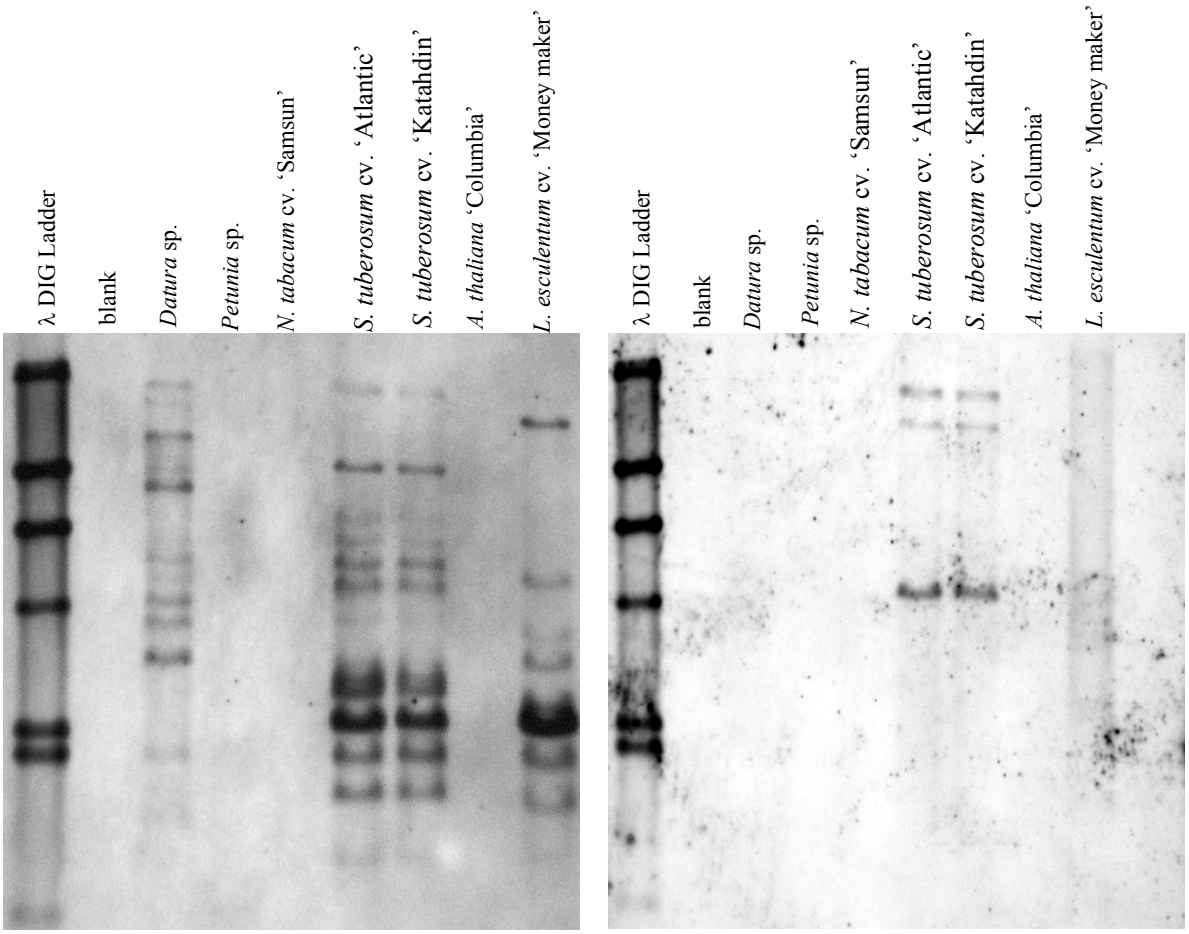


Figure 3.5.a. Solanaceae garden membrane visualized by Southern blot, 20 µg genomic DNA/lane digested with *Eco*RI, hybridized with 11 S-SAP DIG-labeled probes under low stringency DIG Easy Hyb annealing conditions.



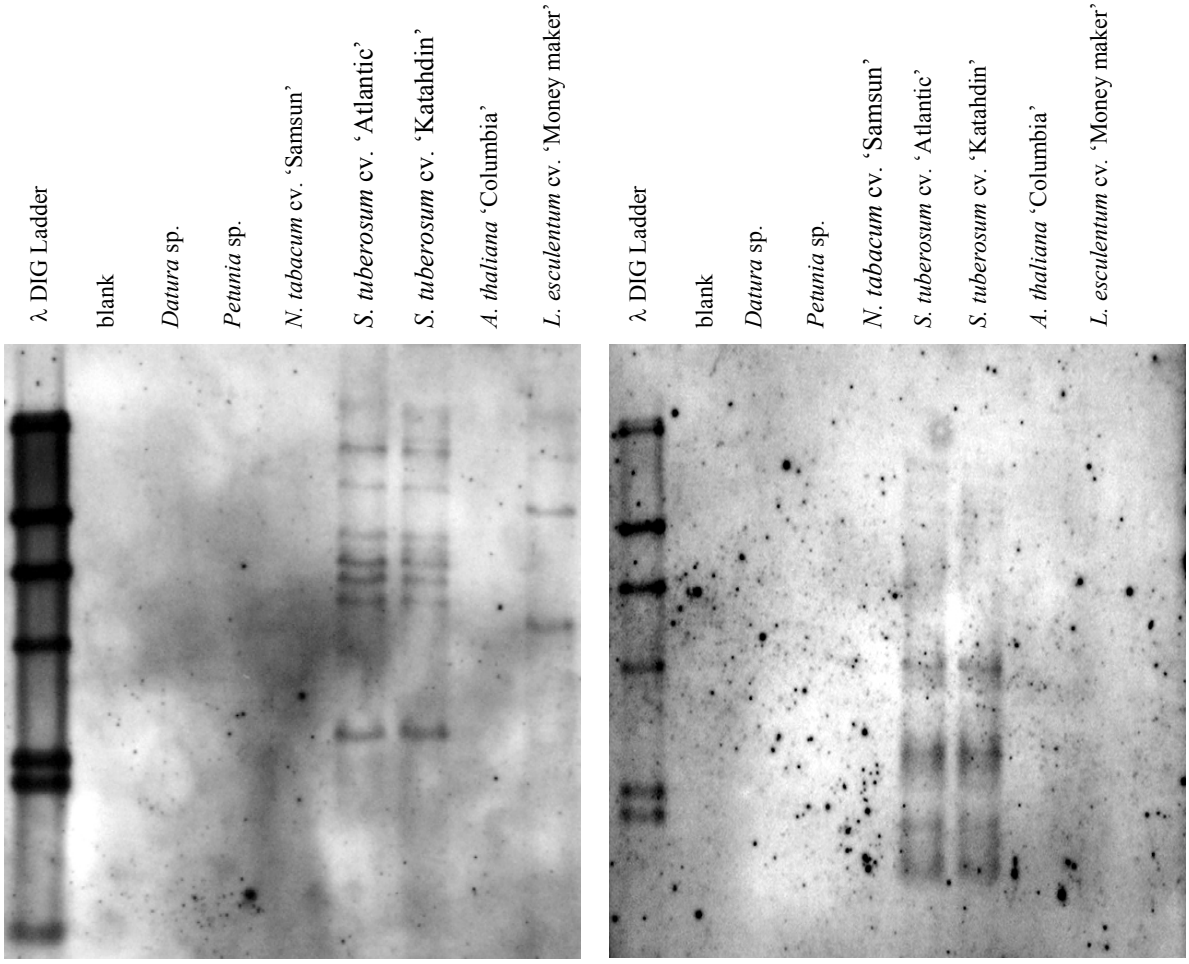
Probe: H316-No24

Probe: MF310-C14

Probe: MF302-No11

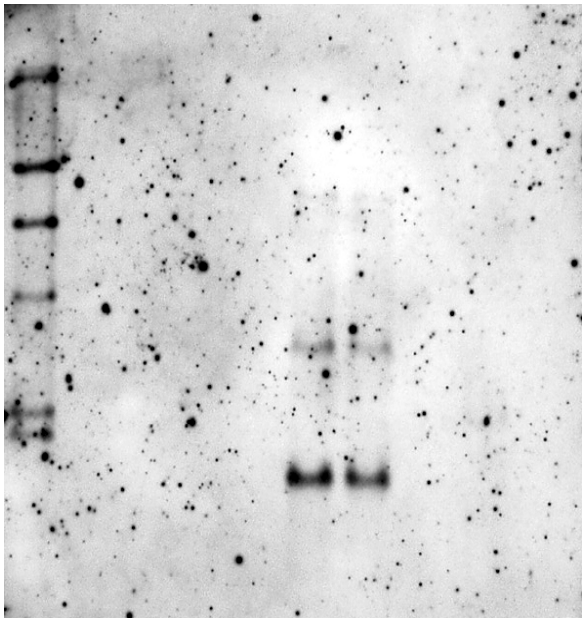
Probe: H240-No01

Figure 3.5.b. Solanaceae garden membrane visualized by Southern blot, 20 µg genomic DNA/lane digested with *Eco*RI, hybridized with 11 S-SAP DIG-labeled probes under low stringency DIG Easy Hyb annealing conditions.



Probe: FP146-No20

Probe: FP134-No16



Probe: FP134-No17

Figure 3.5.c. Solanaceae garden membrane visualized by Southern blot, 20 µg genomic DNA/lane digested with *Eco*RI, hybridized with 11 S-SAP DIG-labeled probes under low stringency DIG Easy Hyb annealing conditions.

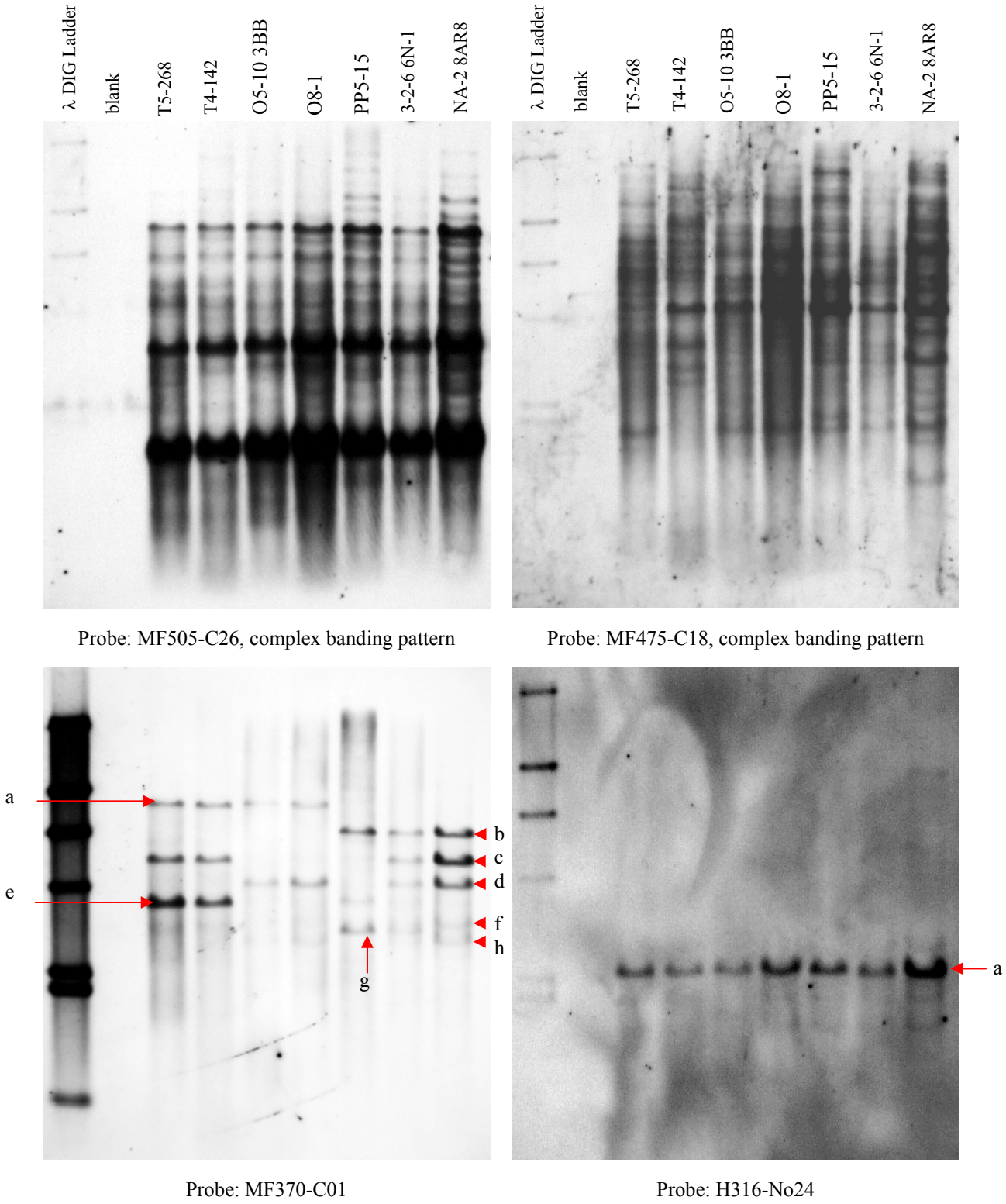


Figure 3.6.a. Monoploid family membrane visualized by Southern blot, 20 µg genomic DNA/lane digested with *Eco*RI, hybridized with 11 S-SAP DIG-labeled probes under low stringency DIG Easy Hyb annealing conditions.

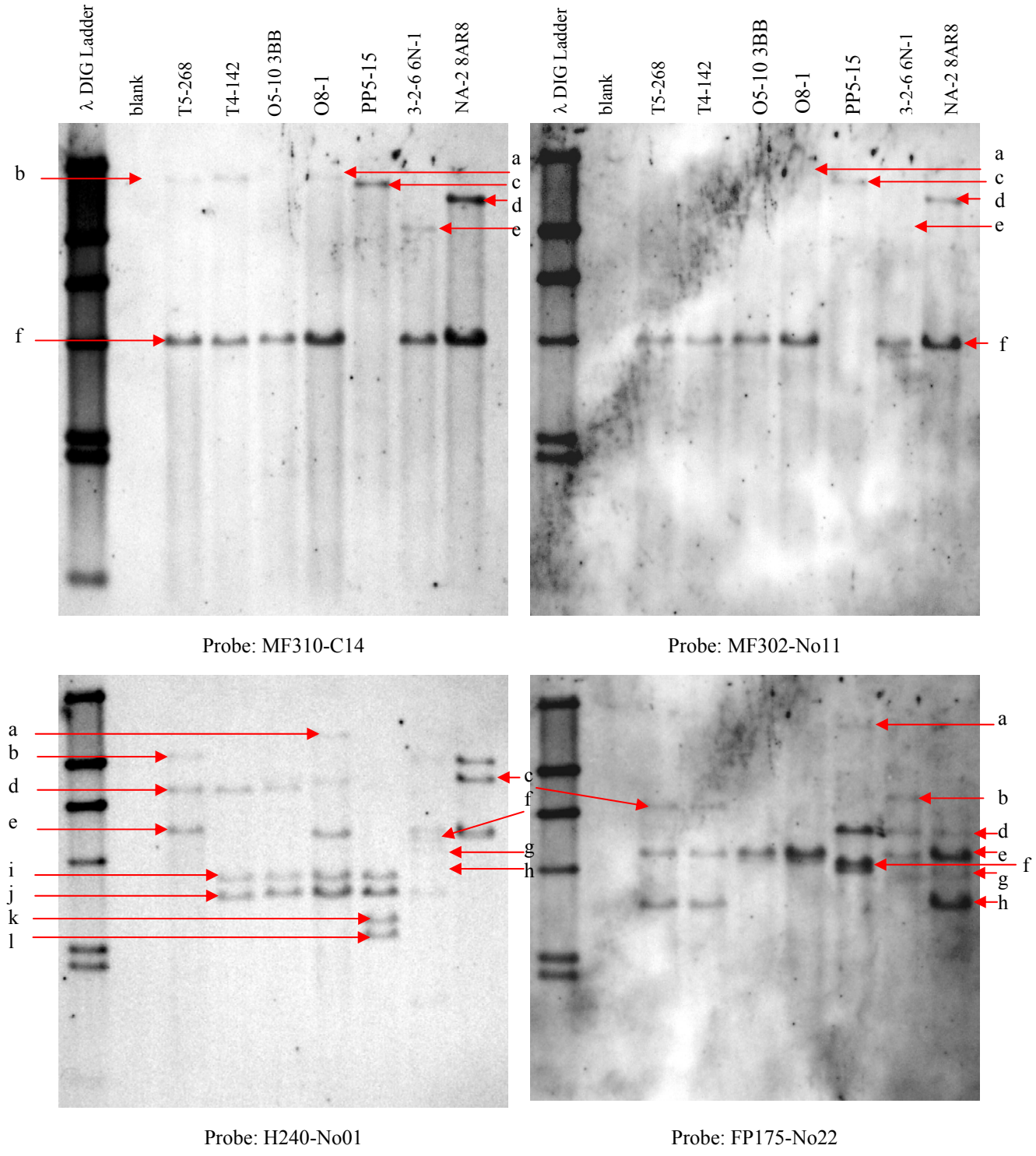
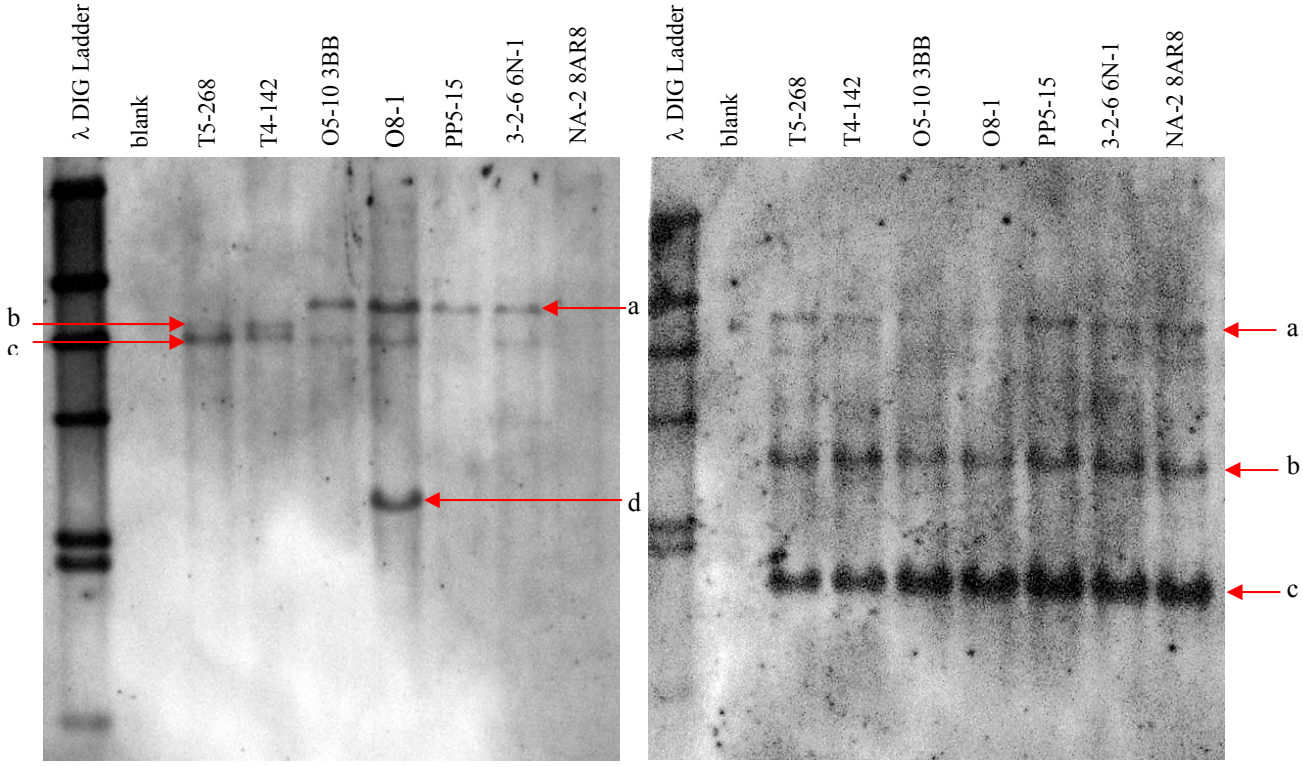
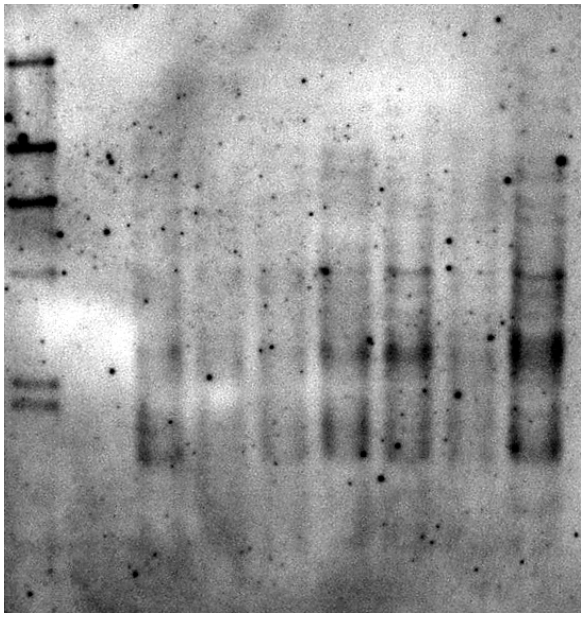


Figure 3.6.b. Monoploid family membrane visualized by Southern blot, 20 µg genomic DNA/lane digested with *Eco*RI, hybridized with 11 S-SAP DIG-labeled probes under low stringency DIG Easy Hyb annealing conditions.



Probe: FP146-No20

Probe: MF134-No17



Probe: MF134-No16, complex banding pattern

Figure 3.6.c. Monoploid family membrane visualized by Southern blot, 20 µg genomic DNA/lane digested with *Eco*RI, hybridized with 11 S-SAP DIG-labeled probes under low stringency DIG Easy Hyb annealing conditions.

Figure 3.7. Phenograms of RFLP/Southern blot data depicting relationships between monoploid families disclosed by UPGMA cluster analysis.

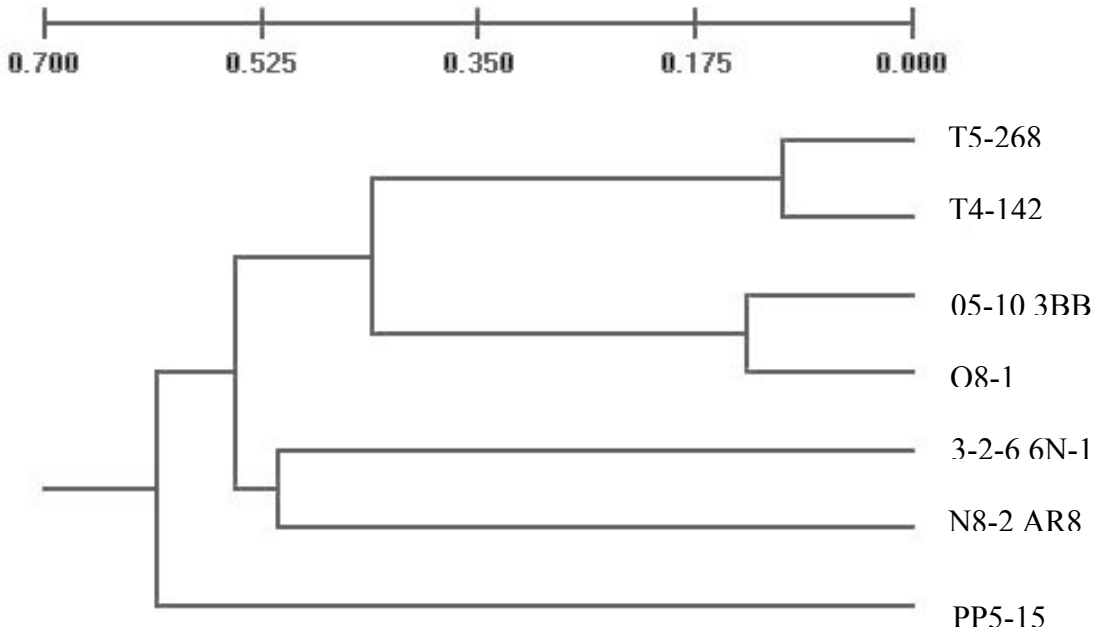


Figure 3.8.a. Phenograms of RFLP/Southern blot data depicting relationships between (a) somatic hybrid family O8-1 (+) T5-268 (b) somatic hybrid family O5-10 3BB (+) T4-142 disclosed by UPGMA cluster analysis.

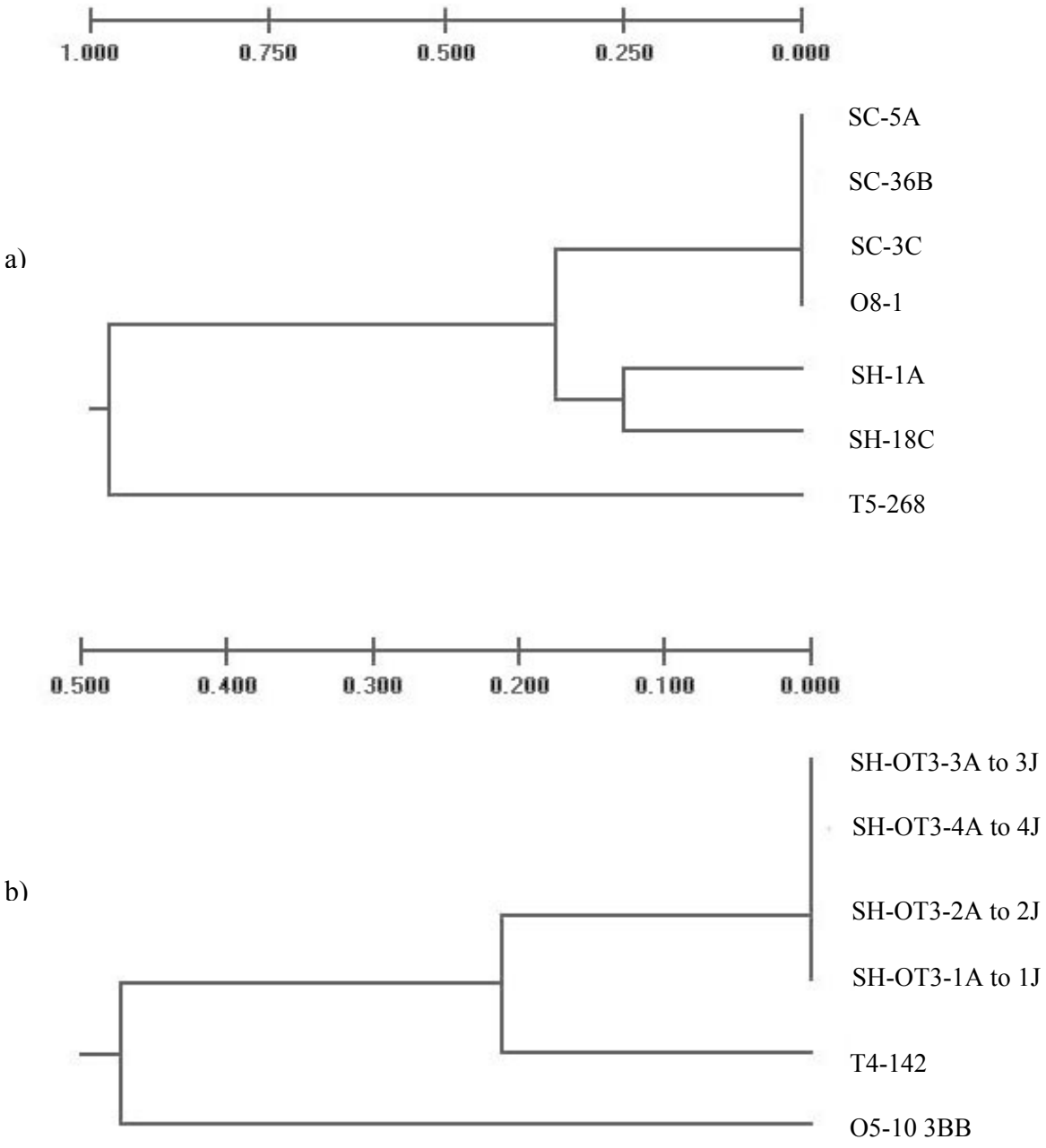
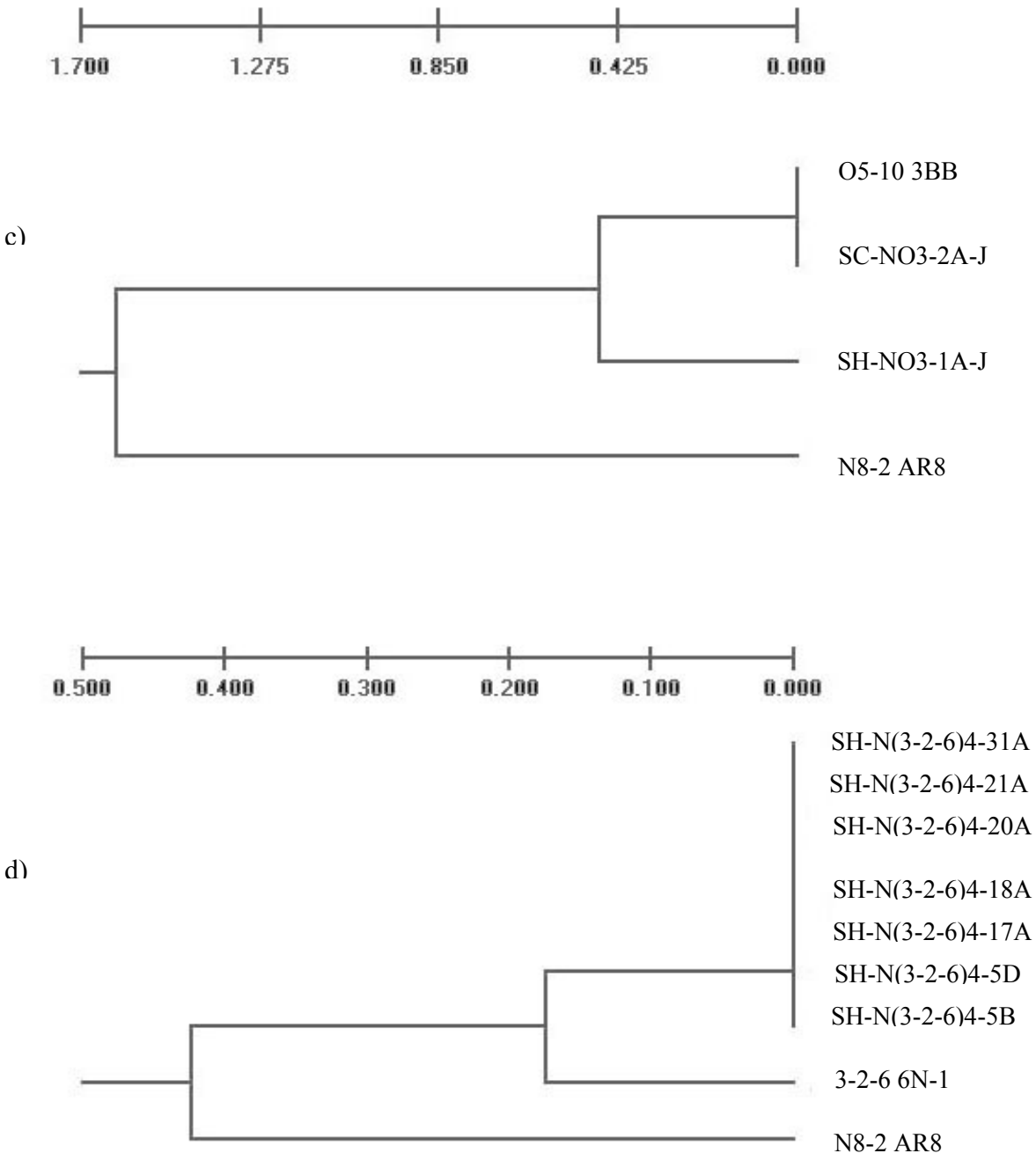
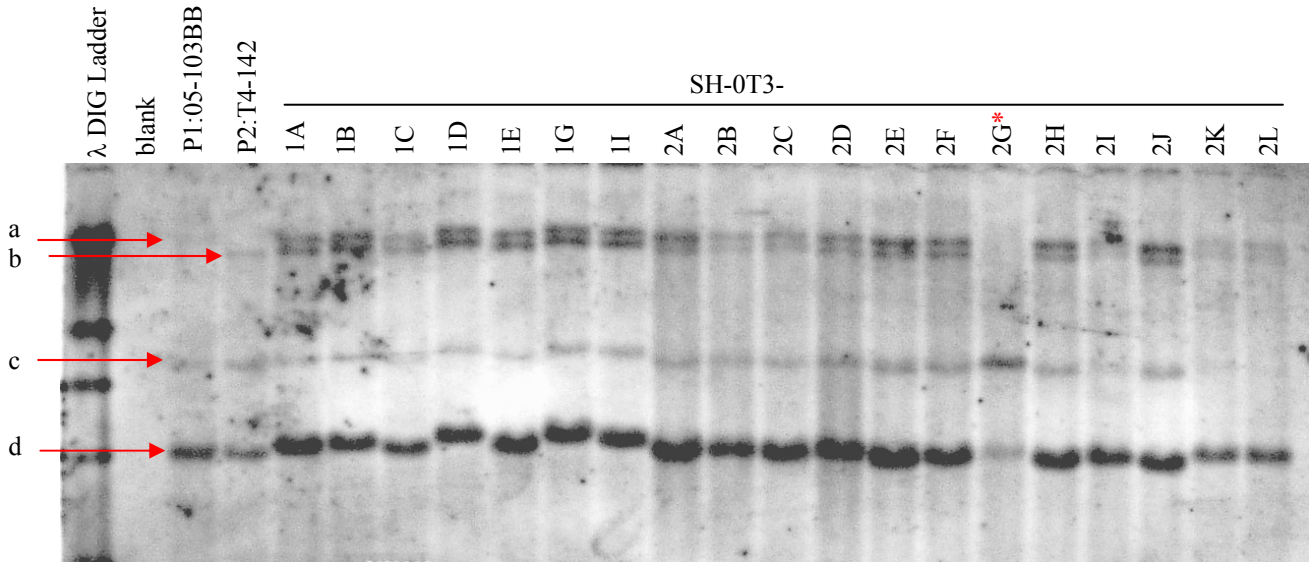


Figure 3.8.b. Phenograms of RFLP/Southern blot data depicting relationships between (c) somatic hybrid family O5-10 3BB (+) N8-2 AR8 (d) somatic hybrid family O5-10 3BB (+) T4-142 disclosed by UPGMA cluster analysis.



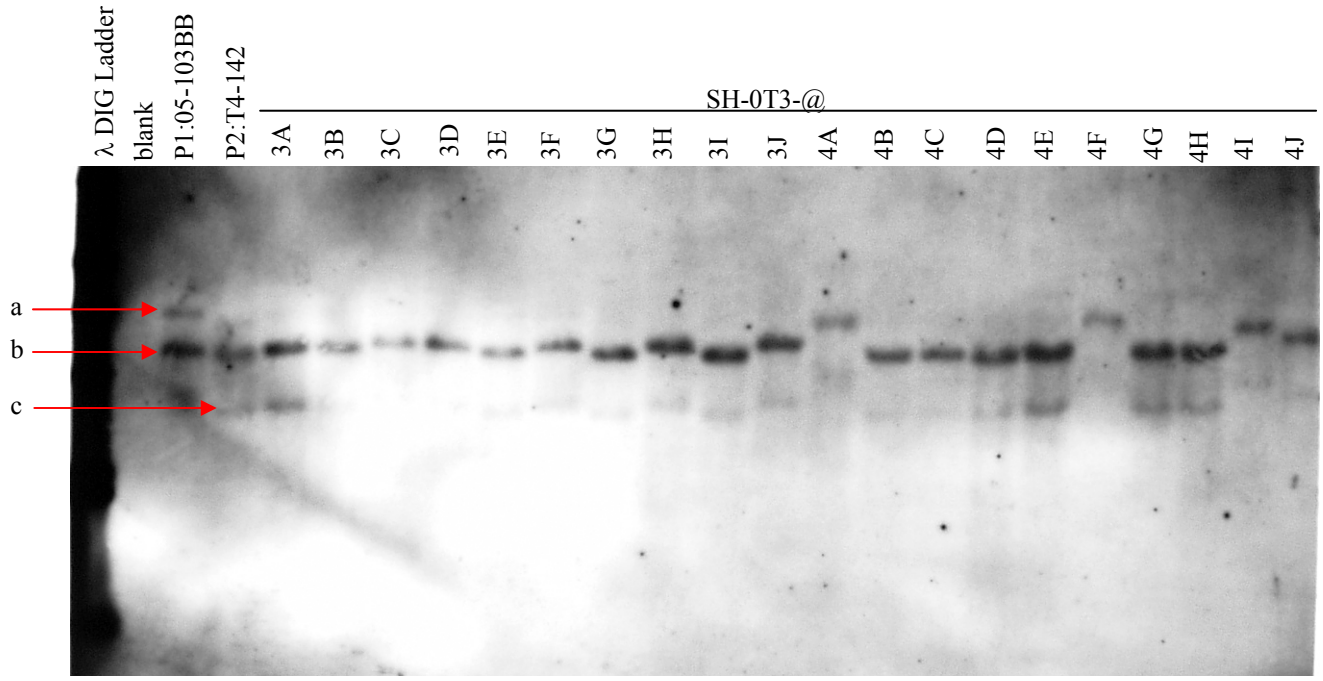
SH = somatic hybrid
SC = somaclone



Probe: MF310-C14

* SH-OT3-2G is missing bands a and b

Figure 3.9. Somatic hybrid family O5-10 3BB (+) T4-142 membrane visualized by Southern blot, 20 µg genomic DNA/lane digested with *Eco*RI, hybridized with S-SAP DIG-labeled probes MF310-C14 under low stringency DIG Easy Hyb annealing conditions, letters depict scored bands.



Probe: FP175-No22

@ All somatic hybrids are missing band a

Figure 3.10. Somatic hybrid family O5-10 3BB (+) T4-142 membrane visualized by Southern blot, 20 µg genomic DNA/lane digested with *Eco*RI, hybridized with S-SAP DIG-labeled FP175-No22 probes under low stringency DIG Easy Hyb annealing conditions, letters depict scored bands.

3.7. Vitae

Gordon James Lightbourn was born on 19 January, 1958 in Kingston, Jamaica. He is the second child from a family of three sons. He attended and graduated from Kingston College, Jamaica in 1974. He completed a Bachelor of Science in Botany and Zoology at the University of the West Indies, Mona Campus, Jamaica in 1986; a Master of Philosophy in Botany at the University of the West Indies, Mona Campus, Jamaica in 1994; and a Doctor of Philosophy in Horticulture from Virginia Polytechnic Institute & State University, Blacksburg, VA, USA in 2004.

During his Master studies in Jamaica, he gained a Jamaica Agricultural Development Foundation Scholarship (1988–1991). He later attained a "Skelton" Scholarship that was issued through Rotary International Ambassadorial Scholarship Program for studies at Virginia Polytechnic Institute & State University (1999-2003). While pursing his studies he held laboratory technician and teaching assistant positions. His working career encompassed being a Farm Manager at Jamaica Floral Exports Ltd. (1986-1988) and five years as Tissue Culture Unit Project Manager at the Scientific Research Council, Kingston, Jamaica (1994-1999). During his tenure at the Scientific Research Council a banana *in vitro* genebank was developed.