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The Comparative Research about Selection of Monoploids for Protoplast Fusion

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Abstract – Protoplasts were isolated from in vitro micro propagated sterile plantlets of the potato selection ND860-2 and the potato cultivar Russet Burbank. They are both tetraploids with chromosome number 2n=4x=48. The selection ND860-2 is derived from a long line of crosses in which the species Solanum phureja (2n=2x=24) was also included. Solanum phureja is resistant to cold-induced accumulation of reducing sugars. Some of its varieties are also resistant or tolerant to fungal and viral diseases. S. phureja is a diploid with a chromosome number 2n=24, whereas potatoes (Solanum tuberosum) are tetraploids. Protoplasts of both genotypes, 'Russet Burbank' and ND860-2, were isolated from leaves of the sterile-micro propagated plants. Protoplasts of ND860-2 were fused to protoplasts of 'Russet Burbank' using the PEG-mediated method as well as electrofusion. Calli were produced from the protoplast used in the fusion experiments and plantlets were regenerated from them. Chromosome counts of the root tip cells of the regenerated plants showed the existence of both, tetraploid (2n=4x=48) and octoploid (2n=8x=96) genetic complements. DNA was isolated from the leaves of the regenerated plantlets and was used in PCR reactions with random primers that discriminate between the two potato genotypes.

Random oligonucleotide primers were found that sufficiently discriminate between the two genotypes in PCR reactions. The basic RAPD profile of all the regenerated plantlets was that of 'Russet Burbank'. However, with two of the primers traces of the ND860-2 band pattern could also be detected in the regenerated plants. It was concluded that the regenerated plants were asymmetric hybrids in which the ND860-2 chromosomes had undergone partial or complete elimination.

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INTRODUCTION

The thought of "genetic load" points to the sum of the deadly and harmful allele's exhibit in the people of a people. The lion's share of the aforementioned alleles is latent, and subsequently is tolerated by heterozygous people holding a relating, prevailing, and wild-sort (non-deadly) allele. It is accepted that everything people of an animal group harbor a humble number of latent deadly alleles in their genomes. Exceptionally heterozygous inhabitant totals for the most part harbor progressively deadly and severely harmful alleles in their genomes than homozygous inhabitant totals for the reason that the deadly alleles have less probability to come to be homozygous, and in this way push their impact. The impact of the hereditary load on an animal type, if any, is not known.

Cultivated potato (2n=4x=48) is a profoundly heterozygous, tetraploid edit that harbors numerous deadly and injurious alleles inside its genome. The aforementioned deadly and harmful alleles come to be homozygous upon selfing, and clarify the great inbreeding sadness of potato. The huge number of harmful alleles could be watched in offspring from the cross of two unrivaled potato cultivars. Practically one million offspring of this cross need to be screened before one is recognized worthy to be advanced as a cultivar.

Monoploids of potato (2n = 1x = 12), and of all heterozygous species, act for gametes that have no deadly or severely malicious alleles. Any time such alleles are available in gametes, recovery of useful plants through androgenizes or gynogenesis is definitely not conceivable—the groundwork of the "monoploids sieve". Deadly without allele genotypes with the fewest malicious alleles might be gotten by determination for the most lively or phenotypically alluring monoploids.

Diploid potato (2n = 2x = 24), from which monoploids are inferred, is greatly heterozygous and hence monoploids are wanted to differ significantly in agronomic exhibition because of allelic isolation at numerous loci. In a study including 118 monoploid

genotypes, determined from five diverse diploid clones, huge contrasts for relative power in a nursery study were identified near monoploids determined from one diploid clone, what's more near aggregations of monoploids determined from diverse clones (Uijtewaal et al. 1987a).

The aforementioned monoploids genotypes were discovered likewise to be variable for relative life in vitro; in any case, there was no relationship between in vitro and nursery exhibition. This study underpins the need that monoploids determined from diploid potato stand for a differing hereditary exhibit that expedites distinctions in agronomic exhibition. An impediment of this study was that just relative energy, not particular characteristics, for example tuber weight and number, was explored. Likewise the study was not rehashed to verify the steadiness of the aforementioned contrasts from year to year (or season to season).

The determination of ploidy in physical cross breeds has been finished through chloroplast checks of stomatal watch units (Cardi et al. 1993), chromosome checks of root tips (Austin et al. 1993) and meristems, and stream cytometry (Mattheij et al. 1992, Menke et al. 1996). Stream cytometry can be utilized to process numerous tests in small time. Quickly, the cells of plant material are blast by hacking with extremely sharp steel in a cushion with ensuing discharge of the cores.

The aforementioned cores are then stained with a color that ties to DNA, for example propodeum iodide, what's more the specimen is run through a rush cytometer which assesses the DNA sum in each core by retention of particular wavelengths of light (from a laser bar) by the color.

Field evaluation of monoploid genotypes

Discussions at the 2003 Plant Genome Size Workshop, held at the Royal Botanic Gardens, Kew, included a review of the modern usage of several terms commonly used to describe nuclear DNA contents. The expression ‘genome size’ is often used for the DNA content of the monoploid genome or chromosome set, whereas ‘DNA C-value’ stands for the DNA content of the whole chromosome complement or karyotype irrespective of the degree of generative polyploidy of the organism. For example, Bennett et al. (1998) and Johnston et al. (2005) espoused this traditional usage. However, ‘genome size’ and ‘DNA C-value’ are often also used synonymously. Obermayer and Greilhuber (1999) and Leitch et al. (2005) are examples of this second usage. The restricted traditional use of ‘genome size’ (Bennett et al., 1998), if followed consistently, would largely eliminate from the discourse this established term, which is convenient, comprehensible and phonetically pleasing. In many cases, e.g. when the degree of generative polyploidy of a plant is unknown, a genome size in the restricted sense could not be given (Bennett et al., 1998).

Moreover, comparative genomics recently confirmed that possibly all plants, and probably most organisms, have experienced one or more polyploidization events in their ancestry (Wendel, 2000). If so, any narrow insistence now regarding the term ‘genome size’ would be altogether unfounded. Thus, a reconsideration of the terminology is clearly required. The purpose of this study is to discuss the currently unstable usage of the terms ‘genome size’ and ‘C-value’, and to propose a new unified terminology that can describe nuclear DNA contents with ease, but without ambiguity.

The term ‘genome’ was coined by Winkler. From a literal interpretation of his writing, we determined that Winkler intended that polyploids organisms have more than one genome. Winkler’s definition for ‘genome’ has been formulated more tersely by Rieger et al.

(1991): ‘in eukaryotes, the basic (monoploid) chromosome set, consisting of a species specific number of linkage groups and the genes contained therein’. So, seen from the perspective of historical priority of the term ‘genome’ and its meaning, Bennett et al. (1998) were correct in using the term ‘genome size’ (first used by Hinegardner, 1976; see below) for the DNA content of the monoploid chromosome set only. However, everyday usage now of the term ‘genome’ is not restricted to only the narrow definitions that Winkler (1920), Rieger et al. (1991) and Bennett et al. (1998) indicated. Today when we speak of the ‘wheat genome’, we may think not only of one of its monoploid genomes A, B or D, but rather of the whole complement of the 2n = 42 or n = 21 chromosomes of Triticum aestivum. Similarly, when speaking about the ‘Plant Genome Size Workshop 2003’, we would not imagine it concerned only monoploid genomes. These examples alone show that ‘genome’ and ‘genome size’ can be used in both a more inclusive or less inclusive sense. Indeed, a genome can be generatively polyploid or monoploid, reduced or nonreduced, replicated or non-replicated—but in each case the same term ‘genome’ remains appropriate. In scientific terminology, priority is not a sacred cow. Rather, convenience and consensus determine which meanings persist over time, and how the usage of terms evolves.

Ambiguity of the term ‘genome size’ is even underlined when looking at its historical roots. It was apparently used first by Hinegardner (1976) in the title of his study ‘Evolution of genome size’, where it was probably intended to denote the mass or quantity of DNA in a non-replicated haploid genome, (e.g. in fish sperm nuclei). Yet throughout the text ‘DNA content’ was used instead of ‘genome size’ and no explicit definition was given for the latter term. Thus, we note that ‘genome size’ was used by Hinegardner (1976) without an explicit connotation of monoploidy. Cavalier-Smith (1985, p. 1), who refers to Hinegardner (1976), treated genome size and C-value as synonyms, and so did Singh in his textbook (2003, p. 44). Gregory and Hebert (1999) interpreted ‘basal genome size’ and ‘C-value’ of an organism as

equivalent and defined these as 'the content of DNA (measured by weight or number of base pairs) in a single copy of the entire sequence of DNA found within a nucleus of that organism'. This definition changes the meaning of 'genome' to the chromosome complement with the number n. However, note that the expression 'basal genome size' could lead to confusion with the DNA content of the genome with the 'chromosome base number' x. Ambiguous use of the term 'genome' (relating to the meiotic ally reduced chromosome number n or monoploid chromosome base number x) is another source of potential error and misunderstanding.

Phenotypic and Transcriptomic updation in Potato Aut polyploidization events occur frequently during plant evolution. The most popular estimate of the proportion of polyploids in angiosperms is _70%. However, recent genomic investigations have revealed that many classic diploid plant species have polyploid origins, indicating the near ubiquity of polyploidy throughout the evolutionary history of the plant kingdom. This ubiquity implies that polyploidy confers selective advantages over diploidy, which are often manifested in enhanced vigor of polyploids phenotypes.

Potential selective advantages, such as increased heterozygosity, novel variation, and allelic sub functionalization, have been widely discussed.

Polyploids originate from either sexual reproduction via 2n gametes or somatic chromosome doubling. By traditional definition, there are two forms of polyploidy: allopolyploid and auto polyploidy. These terms are often used to imply the mode of polyploids formation, but more accurately describe the degree of similarity between the sub genomes in polyploids. Allopolyploids have distinct sub genomes and typically originate from interspecific hybridization between divergent progenitor species.

Autopolyploid have (nearly) identical sub genomes and typically originate from intraspecific hybridization (or self-fertilizationthrough2n gametes) or somatic chromosome doubling. Allo- and autopolyploid have traditionally been distinguished by modes of chromosome pairing and inheritance, with allopolyploids exhibiting bivalent pairing and disomic inheritance and autopolyploid exhibiting multivalent pairing and polysomic inheritance.

A number of well-known polyploid plants of agricultural interest are classical allopolyploids, which include important crops such as bread wheat (2n¼6x¼42) and cotton (2n¼4x¼56). Studies of genetic and epigenetic changes associated with polyploidization have been focused mostly on newly synthesized allopolyploid materials.

However, in allopolyploids, ploidy level per se is difficult to tease apart from many other variables, such as diverged suites of regulatory factors from different genomes. For instance, investigations in maize indicate that gene expression is altered more by genome hybridization than by genome ploidy changes. The effect of ploidy per se can only be assessed among a series of homozygous plants at different ploidy levels. There have been relatively few studies dedicated to elucidating the consequences of aut polyploidization on gene expression. Thus, the genetic impact imposed by ploidy alteration remains elusive.

We sought to identify a plant system in which the changes in gene expression are associated only with ploidy. The genus Solanum appeared to be an excellent choice due to its exceptional tolerance of ploidy manipulations.

This genus includes a wide array of wild and domesticated diploid, tetraploid, and hexaploid accessions, many of which are closely related. Meiotic mutants leading to 2n pollen and 2n eggs are prevalent in Solanum species, which could explain the repeated polyploidization events associated with several Solanum species.

Furthermore, the cultivated potato, Solanum tuberosum (2n ¼ 4x ¼ 48), has been defined as a classic autopolyploid on the basis of its tetrasomic inheritance. Genetic manipulation via meiotic mutants associated with 2n gamete formation has played a more significant role in breeding of potato than in any other crops. Thus, potato provides an excellent model system for auto polyploidy studies.

Monoploid in vitro growth conditions

Plantlets to be used for protoplast isolation were cleared of any systemic bacteria by culture on 30 ml cefotaxime-containing (250 mg/L, filter sterilized) MS propagation medium (Murashige and Skoog 1962) in baby food jars at 20^0C, 16 h light/day, for a period of 2 wks. Tip cuttings from these plants were placed onto fresh cefotaxime-containing MS propagation medium and the 2 wk cycle was repeated two additional times. For protoplast isolation, cuttings taken from these source plantlets were cultured on 30 ml MS propagation medium in baby food jars at 20^0C, 16 h light/day, for a period of 3 wks. Jars were placed in the dark at 4^0C for 48 h prior to isolation.

Monoploid protoplast isolation protocol

In vitro leaves and shoots (roughly 1 g) from each monoploid clone was minced with a No. 10 scalpel in a 15 mm petri dish (Falcon 351029). Seven ml of enzyme solution was then added to the dish; the plate was shut and sealed with parafilm, and placed onto a gyratory shaker (60 rpm) for 12-16 h in the

dark. After enzyme digestion, large debris was removed by pouring the solution through a sterilized 63 m filter. Rinse medium (8 ml) was poured into the petri plate and run through the filter in order to recover protoplasts stuck in the plate or filter. The 15 ml of total solution was then poured into a sterile 15 ml centrifuge tube (Corning 25317-15) and centrifuged in a Dynac II Centrifuge (Clay Adams, Division of Becton, Dickinson and Company) at 500 rpm for 5 min. The supernatant was removed and the pellet was resuspended in 10 ml of high sucrose (17.1 %) flotation medium, with 1 ml of rinse medium layered on top of the flotation medium. Centrifugation at 500 rpm for 10 min resulted in the protoplasts collecting at the interface of the flotation and rinse solutions. The protoplast band was collected with a sterile Pasteur pipette (Scientific Products P5202-2) and placed into a new 15 ml centrifuge tube. Rinse medium (10 ml) was added to the tube with subsequent centrifugation at 500 rpm for 5 min. The supernatant was removed, the purified protoplast pellet was resuspended in fresh rinse medium, and the density of the protoplast solution was adjusted to 106 protoplasts/ml using a Spotlite Hem cytometer (Scientific Products B3175).

PEG-mediated fusion protocol

PEG is most commonly used as a fusogen with ceils in monolayer cultures (14,17,22). The cultures are first rinsed with Hanks' balanced salt solution to remove serum proteins which are thought to interfere with fusion. The solution is then removed from the culture, and i00-150 loaded red-cell ghosts per cultured cell are added to the monolayer in a small volume of Hanks' and distributed over the plate.

PEG-1000 (40%, diluted in culture medium) at 37 ~ is added to the plate and after 1 min is diluted with #.5 ml of culture medium. The cultures are then rinsed 3 times with 10 ml of Hanks' containing 596 fetal bovine serum to remove non-fused ghosts.

The mechanism of PEG-mediated fusion appears to be similar in some ways to that induced by Sendai virus. PEG has a high capacity to bind water (28~29), which may destabilize the membrane (28,30) and promote the aggregation of intramembranous particles (28~31)~ thereby exposing lipid-rich areas of the membrane. A combination of dehydration and destabilization of the surface potential of the membrane (28) probably allows the close contact between cell membranes which is a prerequisite for fusion. Several reports suggest that PEG alone is not adequate for fusion since the fusiogenic capacity of PEG is lost or diminished (2g~32~33) upon purification by ether extraction. The crucial contaminating agents in PEG are believed to be an antioxidant and/or a polymerization catalyst (28132). However~ purification of PEG may not diminish the fusiogenic capacity of all brands of PEG (33). It is possible that levels of contaminants in PEG may be especially critical for fusion of red cells to some cell types but not to others. In any event~ the well-known variability in fusiogenic potential of commercially available PEG may be explained if the fusiogenic action requires both PEG and contaminants. In our experience% this variability applies to PEG from different manufacturers as well as to different production Jots from the same manufacturer.

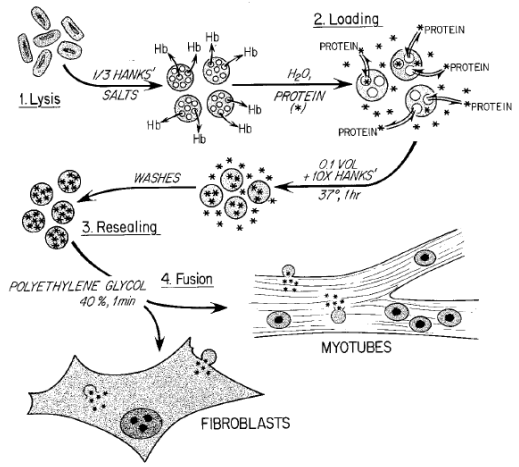

Fig. 1 Red-cell-mediated microinjection.

Polyethylene glycol (PEG) of high molecular weight is widely used to mediate ceU-ceU fusion in the production of somatic cell hybrids, including hybridoma, and more recently in the fusion injection of macromolecules from erythrocytes (1, 2) or liposomes into cultured cells . PEG offers advantages over other fusogens in that it permits fusion of a variety of cell types which may differ in species or even in kingdom and under the proper conditions produces high fusion efficiencies with minimal toxicity. Yet little is known of the mechanism by which PEG operates.

PEG causes the redistribution of intramembrane particles (IMPs) of cellular membranes, this ability being attributed to the ordering of water by high concentrations of the polymer . When aqueous solutions of PEG exceed 35%, cell aggregation and fusion are observed, although maximum fusion efficiency occurs at concentrations between 40 and 50%. Since all water is bound to PEG in solutions having concentrations of 35% by weight or greater, dehydration appears to play a role in PEG-mediated fusion. However, pure PEG does not appear to be a complete fusogen. Recently, Honda et al. have demonstrated that antioxidants and/or polymerization agents added to commercial PEG are responsible for the fusionctivity since removal of these agents through organic solvent extraction renders the PEG nonfusogenic) Earlier work had shown that membrane active chemicals such as glyceryl monooleate (GMO) are only capable of inducing cell-cell fusion when administered in conjunction with high molecular weight polymers such as dextrans (5).

Our interest in the mechanisms of PEG-induced fusion was prompted by our use of the erythrocyte-mediated microinjection method to introduce

fluorescent macromolecules into the cytoplasm of cultured cells (7) and subsequently to measure their diffusion rates by the fluorescence recovery after photo- bleaching (FRAP) technique. Experimentally, this method offers both relative biological simplicity and high fusion efficiency.

MONOPLOIDS FOR PROTOPLAST FUSION

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

Cultivated potato (2n=4x=48) is a highly heterozygous, tetraploid crop that harbors many lethal and deleterious alleles within its genome. These lethal and deleterious alleles become homozygous upon selfing, and explain the extreme inbreeding depression of potato. The large number of deleterious alleles can be observed in progeny from the cross of two superior potato cultivars. Nearly one million progeny of such a cross need to be screened before one is found worthy to be developed as a cultivar. Monoploids of potato (2n = 1x = 12), and of all heterozygous species, represent gametes that have no lethal or severely deleterious alleles. When such alleles are present in gametes, regeneration of functional plants through androgenesis or gynogenesis is not possible—the basis of the "monoploid sieve". Lethal allele-free genotypes with the fewest deleterious alleles can be obtained by selection for the most vigorous or phenotypically desirable monoploids.

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

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

SSRs, or microsatellites, are short repeats of 1-5 nucleotides in length dispersed throughout the genome of eukaryotes. Primers can be designed to flank the conserved regions surrounding a particular repeat, and PCR used to amplify the repeated region. Individuals are polymorphic if they contain different numbers of repeats at an SSR locus. SSRs are known to mutate frequently for various hypothesized reasons Chapter 2 34 and the mutation rate has been estimated at 10-2 to 10-3 mutations per SSR locus per gamete per generation; thus, it is not unusual for a population to contain many different alleles at a SSR locus. SSRs are codominant markers, (unlike other PCR-based molecular markers such as RAPDs) which makes them ideal for somatic hybrid identification because both SSR alleles in a somatic hybrid constructed from genetically distinct parents can be detected simultaneously using PCR. Provan et al. (1996) utilized primers to amplify two different SSR loci through PCR, one a (TA)23 repeat in a potato proteinase inhibitor pseudogene and the other a (T)12(A)9(TA)7 repeat in the IIK inhibitor gene intron, for the identification of somatic hybrids between three different dihaploid potato clones. With only these two loci, the true somatic hybrids could be distinguished unambiguously from parental soma clones.

The determination of ploidy in somatic hybrids has been accomplished through chloroplast counts of stomatal guard cells, chromosome counts of root tips and meristems, and flow cytometry. Flow cytometry can be used to process many samples in little time. Briefly, the cells of plant material are burst by chopping with a razor blade in a buffer with subsequent release of the nuclei. These nuclei are then stained with a dye that binds to DNA, such as

propidium iodide, and the sample is run through a flow cytometer which estimates the DNA amount in each nucleus by absorption of specific wavelengths of light (from a laser beam) by the dye (Shapiro 1995).

The overall objective of this study was to generate potato somatic hybrids by protoplast fusion of selected "superior" monoploid genotypes. This process involved several specific objectives:

1) To select the most promising monoploids, based on field performance, from a large population of anther-derived monoploids regenerated from several diploid potato clones,

2) To evaluate the selected monoploid genotypes regarding their response to protoplast culture,

3) To develop appropriate techniques for electro fusion of monoploid potato genotypes, and

4) To identify and characterize putative somatic hybrids using flow cytometry and SSRs.

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

Response of selected clones to in vitro culture: Before each electro fusion experiment, a sample of protoplasts from each clone was suspended in 1 ml of modified Schumann and Koblitz culture medium at a density of 2.5 × 105 protoplasts per ml in a 35 × 10 mm petri plate. The culture dish was placed at room temperature in the dark and observed on a weekly basis. The culture medium was replaced every 7 days. Each clone was rated on a scale of 1 – 5 based on response to protoplast culture. The scale was as follows: 1) protoplasts regenerated into visible calluses which eventually produced plants, 2) the protoplasts regenerated into calluses with no subsequent plant production, 3) the protoplasts showed limited divisions, but no callus formation, 4) the protoplasts expanded and regenerated a cell wall, but there was no cell division, and 5) there was no regeneration; rather, the protoplasts simply turned brown and died upon suspension in culture medium. Some monoploid clones were evaluated several times, whereas others were evaluated only once or not at all due to scarce plant material. Each clone was placed onto the scale based on the best regeneration response observed during any evaluation.

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

The electro fusion experiments were performed using a 2001EFS1 Electro Cell Manipulator with Enhancer 400. Fusions were carried out in both 3.2 mm Gap Microslides (BT453) and disposable 2mm Gap Electroporation Cuvettes plus (BT620). Just prior to fusion the protoplasts were suspended in fusion medium (1mM CaCl2 in 8.5% mannitol, pH 5.6) at a density of 1 × 106 protoplasts per ml. Ten drops of protoplast suspension from each of two monoploid clones were added when using the 3.2 mm Gap Micro slides, while eight drops of protoplast suspension from each of two monoploid clones were added when using the 2 mm Gap Cuvettes. A range of electro fusion parameters reflecting those was applied to intermonoploid fusions. Through observation of protoplasts in fusion chambers under an inverted microscope, the electro fusion parameters were optimized to maximize alignment and fusion of protoplasts while retaining cell viability.

Five minutes after electro fusion, the protoplasts were removed from the fusion chamber using a sterile pasteur pipette and placed into a 15 ml centrifuge tube. The supernatant was removed after 5 min centrifugation (500 rpm), the protoplast pellet was resuspended in culture medium at a density of 2.5 × 105 protoplasts/ml, and the protoplast suspension was placed into a 60 × 15 mm petri dish. The petri plate was sealed and placed at room temperature in the dark.

Post-fusion regeneration protocol: Seven days after fusion experiments, the petri plates were examined under an inverted microscope to determine if any cell regeneration and/or division had occurred. The content of plates that contained dividing cells was centrifuged at 500 rpm for 5 min to remove the old culture media and replace with fresh media. After roughly 3 wks in liquid culture medium, the dividing cells were embedded in low gelling agarose dissolved in liquid culture medium. The protoplasts remained in agarose in the dark at room temperature for approximately 2 additional wks. At this point, visible calluses roughly 1 mm in diameter (resulting from numerous cell divisions) were removed from the agarose and placed onto solid greening medium (Tan et al. 1987) at 20°C, 16 h light/day, for a period of 2-4 wks. The calluses (now 2-3 mm in diameter and dark

green in color) were then placed onto J1 callus regeneration medium for a period of 2-4 wks or until shoot initials developed. In the final step the calluses were placed on shoot proliferation medium. When shoots regenerated from calluses they were cut off at the base of the callus and rooted in solid MS basal medium. Rooted shoots were then acclimated to the greenhouse.

CONCLUSION

In conclusion, it is possible that the monoploid sieve – the removal of lethal and deleterious genes-- may have eliminated epistatic and intra-allelic interactions in heterozygotes due to lethalities at the monoploid level of otherwise beneficial alleles 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. 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.

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

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