Anther Culture of Potato and Molecular Analysis of Anther-derived Plants as Laboratory Exercises for Plant Breeding Courses

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SUMMARY. Anther culture has been one of the most successful techniques for generating haploid plants over a wide range of species. It is a reasonably simple procedure that can be accomplished successfully without sophisticated laboratory facilities; yet, the plants generated through anther culture can be used to demonstrate the application of many modern methods that have direct applicability to plant breeding. Anthers of diploid potato clones that have been selected for competence in anther culture can be cultured in a simple medium to yield androgenic embryos after 5 weeks. Plant regeneration requires an additional 3 to 4 weeks. Regenerated plants should be large enough 2 weeks after transfer to basal medium for ploidy determination by any of three methods depending on available facilities: chromosome counts in root tips; chloroplast counts in stomatal guard cells; or flow cytometry of nuclei released from in vitro plantlets. DNA can be extracted from anther-derived plantlets using a rapid extraction procedure to demonstrate segregation of PCR (polymerase chain reaction)-based markers such as RAPD (randomly amplified polymorphic DNA), RAMPs (randomly amplified microsatellite polymorphisms), or microsatellites. Microsatellite markers that were heterozygous in the anther donor can be used to verify haploidy in anther-derived plants. If an anther culture laboratory is scheduled early in a semester, such molecular analysis can be planned for late in the same semester.

Anther culture has been envisioned as a route to the rapid and efficient development of homozygous lines for hybrid breeding. Though first reported in 1964 by Guha and Maheshwari for the jimsonweed, *Datura stramonium* L., the technique was quickly adapted to commercially important crops and can now be applied to many major crops (Khush and Virmani, 1996). Anther-derived cultivars have been released for canola, *Brassica napus* L. (Stringam et al., 1998), asparagus, *Asparagus officianalis* L. (Corriols et al., 1990) and wheat, *Triticum aestivum* L. em Thell. (de Buyser et al., 1987), among others (Khush and Virmani, 1996; Veilleux, 1994). Application of haploidization techniques to self-pollinated crops has generally been more successful because of the lack of inbreeding depression in homozygous lines. A major difference of haploidization compared to self-pollination to derive homozygous lines of cross-pollinated crops is the lack of selection for fertility among doubled haploid regenerants.

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Inbred lines are per force more likely to be fertile because the infertile ones are lost during the inbreeding process. Therefore, in cross-pollinated crops, one can expect a more diverse array of homozygotes by haploidization followed by chromosome doubling compared to inbreeding, but not all regenerants may be useful. In addition, haploidization to derive homozygotes will bypass breeding barriers such as self-incompatibility or incongruency.

Haploidization of cultivated potato (*Solanum tuberosum* L.), a tetraploid ($2n = 4x = 48$), results in the recovery of largely dihaploid lines ($2n = 2x = 24$) that still embody considerable heterozygosity. The term dihaploid has been used to describe haploid plants derived from a tetraploid. It should not be confused with doubled haploid, used to describe the product of chromosome doubling after haploidization. Reduction of dihaploid potato to the monoploid level ($2n = 1x = 12$) has resulted in the recovery of weak unpromising plants. However, *Solanum phureja* Juz. & Buk. is a diploid cultivated potato that has been used extensively in potato breeding as a source of disease resistance and other valuable traits. A diverse population developed from accessions of both *S. phureja* and its close relative, *S. stenotomum*, was adapted to long photoperiod and temperate conditions (Haynes, 1972). This population comprises a diverse array of germplasm that has been selected for heat tolerance, $2n$ pollen, anther culture competence and other traits. Although *S. phureja* is considered by some to be unpromising because of irregular tuber shape, deep eyes, or poor storage capability, the population is sufficiently diverse that any of these characteristics can be subdued through selection of more agronomically acceptable traits or breeding at the diploid level with *S. tuberosum* dihaploids. Because of the dominance of anther culture competence, hybrids between clones competent to form embryos from cultured anthers and other diploid germplasm generally respond to anther culture (Singsit and Veilleux, 1988). The following protocol describes how *S. phureja* can be used as laboratory material to demonstrate the application of anther culture to a cross-pollinated crop followed by the use of microsatellite markers to examine anther-derived plants.

Handling of the plant material

Solanum phureja grows and flowers best under cool greenhouse conditions, 25 °C day / 15 °C night (77 °F / 59 °F), long photoperiod (16 h) and high light intensity provided by high pressure sodium vapor lamps. The author can supply clones that respond well to anther culture. In Virginia, there are two greenhouse seasons that induce adequate flowering to be able to conduct anther culture – planting in August for October to November experiments and planting in January for March to April experiments. Supplemental lighting should be provided starting around 15 Sept. for the August planting and as soon as the plants have emerged for the January planting. (It can be discontinued after 1 Apr. for the spring planting.) The plants will flower best given adequate space for root development, e.g., using 7.6-L (2-gal) nursery pots or growing them directly in ground beds containing 1 sand : 2 soilless mix (Sunshine, Fisons Horticulture Inc., Vancouver, BC, Canada or Pro-Mix BX, Premier Brands, Inc., Red Hill, Pa.). The plants require weekly fertilization with a water soluble fertilizer such as Peter's Fertilizer Products (W.R. Grace & Co., Fogelsville, Pa.) containing 20 N–8.4 P–14.9 K. A systemic insecticide such as Marathon (Olympic Horticultural Products, Mainland, Pa.) should be applied when planting the tubers to control thrips and whiteflies. Some type of support, such as flower support netting (product 761020; Hummert, International, Earth City, Mo.) or stakes, is necessary. Once flowering has started, ≈10 to 20 buds per plant per day can be collected three times per week.

Anther culture procedure

Flower buds with microspores at the uninucleate stage (anthers 2.5 to 3.5 mm in length) are most responsive to anther culture and can be picked and placed on paper towels moistened with distilled water in plastic sandwich bags. The buds are then refrigerated at 4 °C for 3 d in the dark. The buds (in batches of ≈30) are then surface-sterilized in 70% ethanol for 30 s and disinfected in 100% commercial bleach (5.25% sodium hypochlorite) plus Tween 20 for 5 min. Then, they are rinsed twice in sterile distilled water. Anthers (≈30 per flask) are aseptically removed and placed in 125 mL Delong culture flasks (Bellco Glass Co., Vineland, N.J.) containing 15 mL autoclaved 1 / 2 strength Linsmaier and Skoog (1965) medium with 2.5 $mg \cdot L^{-1}$ BA + 0.1 $mg \cdot L^{-1}$ IAA + 2.5 $g \cdot L^{-1}$ activated charcoal. Each flask should be closed with a Magenta two-way cap (Magenta Plastics, Chicago, Ill.) and sealed with parafilm. Flasks are then shaken on a rotary shaker, set at 125 to 150 rpm, at room temperature (≈25 °C). After 5 to 6 weeks, the embryos can be harvested by pouring the contents of the flask through an autoclaved tea strainer, rinsing with sterile water and inverting the strainer over a Petri plate. The embryos can be separated from the anthers and counted under a dissecting microscope. To demonstrate experimental design and analysis of a tissue culture experiment, treatments can be applied during anther culture. The comparison of five treatments using a randomized anther technique where each anther per bud is distributed to one of five treatments until all flasks contain 30 anthers has been found to be most convenient statistically (Snider and Veilleux, 1994). Completion of three replications per day on several days to reach 9 to 12 replications will allow rigorous statistical analysis for day and treatment effects using SAS GLM procedure (SAS, 1985) or any other statistical package that handles ANOVA. Although any number of treatments can be envisioned, those that examine the effects of different sugars or activated charcoal at various concentrations tend to give the most dramatic results.

Regeneration of anther-derived embryos

The embryos from anther culture should be transferred to regeneration medium: 3.2 $g \cdot L^{-1}$ Gamborg's B5 salts (Gamborg et al., 1968) with minimal organic compounds (Sigma G 5893, St. Louis, Mo.), 50 $mg \cdot L^{-1}$ $CaHPO_4$, 748 $mg \cdot L^{-1}$ $CaCl_2$, 250 $mg \cdot L^{-1}$ NH_4NO_3, 10 $g \cdot L^{-1}$ sucrose, 6 $g \cdot L^{-1}$ agarose (Sigma Type IIIA, No. A9793), 0.1 $mg \cdot L^{-1}$ gibberellic acid (filter-sterilized), pH 5.6, and incubated at 20 °C under high intensity light (175 $\mu mol \cdot s^{-1} \cdot m^{-2}$). At 3-week intervals, the regenerated embryos can be counted and transferred to 25 × 150 mm culture tubes containing 20 mL MS basal media (Murashige and

Skoog, 1962) supplemented with vitamins, 100 $mg \cdot L^{-1}$ myo-inositol, 100 $mg \cdot L^{-1}$ casein hydrolysate, 3% sucrose and 0.7% Gibco Phytagar (Life Technologies, Inc., Gaithersburg, Md.), pH 5.8, while the unregenerated embryos may be transferred to fresh regeneration medium. Unregenerated embryos are generally discarded 3 weeks after the last transfer. Although additional transfers may yield more regenerants, the regeneration frequency generally declines after three transfers. The regenerants are kept in a growth room at ≈20 °C under a 16-h photoperiod and 50 $\mu mol \cdot s^{-1} \cdot m^{-2}$ provided by cool white fluorescent lamps. The frequency of regenerated plants from androgenic embryos (regeneration frequency) can then be calculated.

Ploidy analysis

The ploidy of plantlets derived from the regenerated androgenic embryos may be estimated by flow cytometric examination of propidium iodide stained nuclei from *in vitro* leaf and stem tissue (Owen et al., 1988). If flow cytometry facilities are unavailable, the number of chloroplasts per pair of guard cells may be counted under a fluorescence microscope to estimate ploidy (Singsit and Veilleux, 1991). This procedure involves removing epidermal peels from in vivo or in vitro leaves with a fine forceps, painting them with 5% FDA stain prepared in acetone diluted 1:20 with water just before use. The stain can be applied with an artist's brush and the green-stained chloroplasts observed under a fluorescence microscope (250¥) equipped with a fluorescein isothiocyanate (FITC) filter combination. The number of chloroplasts should be counted for a minimum of 10 pairs of guard cells. Mean chloroplasts per guard cell pair ranges from 9 for monoploids to 15 for diploids and 20 for tetraploids. Alternatively, chromosomes can be counted in root tips from in vitro plantlets according to the methods described by Fukui and Nakayama (1996). However the small size of potato chromosomes makes chromosome counts challenging.

Simple sequence repeat (SSR) and RAPD analysis

Once sufficient anther-derived plants have been obtained, they can be analyzed with molecular markers to verify ploidy or examine segregation patterns. Total genomic DNA can be extracted from *in vitro* plant material according to Doyle and Doyle (1987) as modified by Veilleux et al. (1995). Microsatellite primers (20 to 24 base pairs; 60 °C melting temperature) flanking SSR containing potato sequences from the European Molecular Biology Laboratory (EMBL)/Genbank database have been designed with the software Primer version 0.5 (Chani, 1997). The reaction mix (20 µL) contains: 1× assay buffer (50 mM KCl, 10 mM Tris-HCl (pH 9), 1% Triton X-100), 3 mM $MgCl_2$, 160 µM of each of the dNTPs, 1.5 units *Taq* polymerase (Promega, Madison, Wis.), 0.1 µM of each primer, and 50 ng genomic DNA overlaid with a drop of mineral oil (Yu et al., 1994). A PCR protocol consisting of 40 cycles at 94 °C for 1 min, 55 °C for 2 min, 72 °C for 1.5 min, followed by 5 min at 72 °C was used. Amplified fragments are separated in 3% Metaphor agarose gels (FMC Bioproducts, Rockland, Me.) in TBE buffer for 4 to 8 h at 90 to 100 V. Chi-square tests (1 df) may be performed for each primer pair to check for skewness from the expected 1:1 ratio for each marker that is polymorphic in the anther donor. Polymorphism is determined by heterozygosity for SSR length fragments. A polymorphic anther donor has two alleles that differ by the number of repeats of the SSR motif. These different alleles have differing mobilities in the gel with shorter fragments migrating further. Monoploids have only a single allele per locus and hence are expected to express only one band. An example of the type of result expected is given in Fig. 1. Because microsatellite markers are codominant, the presence of more than a single allele at any locus in an anther-derived plant would indicate that it is not monoploid. We have identified plants as monoploid by flow cytometry but needed to discard them subsequently because microsatellite analysis revealed heterozygosity (lane 7 in Fig. 1).

We have doubled the chromosome number of anther-derived monoploids of *S. phureja* to generate homozygous lines (M'Ribu and Veilleux, 1992). These doubled monoploids have exhibited female fertility and could be crossed with heterozygous diploid potato selections. They represent the only currently available homozygous potato lines that can be used in genetic studies due to the breeding barriers that prevent derivation of homozygotes through self-pollination and the severe inbreeding depression that cultivated potato suffers.

For a laboratory exercise, the instructor should have flowering plants of *S. phureja* available at the beginning of a semester, i.e., they should be planted ≈6 weeks before the start of classes. The anther cultures can be done during the first 2 weeks. Each

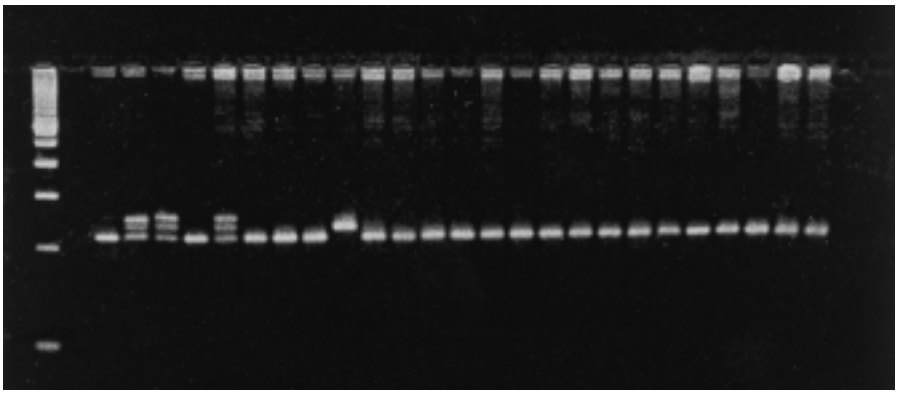

Fig. 1. Amplification of the actc simple sequence repeat (SSR)-containing region of the waxy locus of potato by primers designed from GenBank accession X52417 in an anther-derived potato family. Lane 1 is the 100 base pair ladder. Lane 2 is the negative control without DNA. Lanes 3 and 4 are the parents of the anther donor. Lane 5 is the heterozygous anther donor (*S. phureja* clone CP2). Lanes 6 through 27 are 22 anther-derived plants of CP2. All are homozygous except for Lane 7 which represents a plant that had been erroneously classified as a monoploid. The locus exhibits distortion among the monoploids with the faster migrating (shorter SSR) fragment overrepresented. Heterozygotes exhibit three bands because the uppermost band represents heteroduplex formation (adapted from Chani, 1997).

student should culture ≈100 to 200 anthers over two laboratory sessions; therefore, at least two plants per student should be grown to provide sufficient flowers. Flower buds can be collected every 2 d and refrigerated until use. Embryos can be transferred twice, once 5 to 6 weeks after anther culture and again 3 weeks later. On transfer, any regenerated embryos can be placed on MS basal medium so that regenerated plants are ready 10 weeks after the start of the semester. Ploidy analysis can then be done nondestructively using either in vitro leaflets for chloroplast counts or shoot tips for flow cytometry. By weeks 13 to 14, DNA can be extracted and marker analysis completed at the end of the semester. These exercises are more suitable for an upper division undergraduate or graduate class in plant breeding and genetics. We have done all of these exercises in a graduate plant breeding class at Virginia Tech with the assistance of a graduate teaching assistant to prepare media or reagents. The anther culture without additional analyses can be educational and does not require sophisticated equipment. A routinely equipped tissue culture lab would have most of the required equipment, perhaps with the exception of an orbital shaker to accommodate 125 mL flasks. Advantages of using potato for anther culture are the ease of dissection of flower buds and the prolific embryo production of selected clones. These clones also flower abundantly under proper environmental conditions and the regeneration of embryos has been greater than 80% for some clones.

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