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**Molecular Analysis by SSR of Genes Associated
with Alkaloid Synthesis in a Segregating Monoploid
Potato Family**

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הספריה המרכזית
 למדעי החקלאות
 בית דגן

Molecular marker analysis by SSR of genes associated with alkaloid synthesis in a segregating monoploid potato family

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Abstract

More than 15,000 anthers of an interspecific hybrid (CP2) between two diploid ($2n=2x=24$) potato species, *Solanum chacoense* (weedy) and *S. phureja* (cultivated), were cultured to generate a family of monoploid (haploid, $2n=1x=12$) plants. Of 260 regenerated plants, 34 were monoploid, 210 diploid and 16 tetraploid. SSR analysis revealed that six monoploids were genetically identical and 14 diploids were homozygous, thus limiting the population to 42 (28 monoploids and 14 homozygous diploids). New microsatellite loci were developed for potato from database sequences (15), a conventional genomic library (6), an enriched library (18) and tomato (11). Of these, 13 were polymorphic in the CP2 family and 11 were used to study genetic segregation. Four of 11 exhibited skewed segregation in the monoploid family. Seven of 18 microsatellite markers were polymorphic and informative on a set of 12 tetraploid potato cultivars. Acetylcholinesterase (ALD) is the aglycone of leptines, a natural defense against insects, especially the highly destructive Colorado potato beetle. ALD is absent in *S. phureja* but highly expressed in the *S. chacoense* parent of CP2. A backcross population between CP2 and its *S. phureja* parent was used to examine segregation for ALD. Bulks of 10 backcross individuals that expressed ALD and 10 that did not were used to identify putative RAPD markers associated with the trait. Of 80 primers tested, one putative marker amplified by OPQ02 was present in eight of ten individuals comprising the high bulk and absent in all 10 individuals comprising the low bulk. This is a putative marker for ALD expression in potato.

Objectives of the research proposal

There were three main objectives of the original research proposal:

- 1) To develop a sufficiently large population (150-200 individuals) of sibling monoploid potatoes derived from an interspecific *S. phureja* × *S. chacoense* hybrid. This family can be expected to segregate for both agronomic traits and glycoalkaloids (total as well as specific) and will serve for:
 - a. a reference potato family for the purpose of potato genome mapping. DNA from this family will be made available to the international scientific community to enhance the mapping process of the potato genome.
 - b. testing the advantages of using a haploid family for genome mapping rather than the diploid or tetraploid genotypes currently used in potato. If useful, the application of this strategy can be extended to other cultivated plant species.
- 2) To develop SSR DNA markers in potato.
- 3) To identify and map QTLs coding for alkaloid synthesis and other production traits in potato.

Data, methodology, results and discussion

Objective 1: Development of the monoploid population

Anther culture experiments were conducted for the purpose of developing a sibling monoploid family that segregates for both total glycoalkaloids and specific glycoalkaloids that convey resistance to the Colorado potato beetle. Four separate experiments were conducted during the flowering season in the greenhouse in late spring through fall of 1995. The objectives of these studies were to enhance the anther culture response of two *Solanum phureja*-*S. chacoense* hybrids. Treatments consisted of growing anther donor plants under four greenhouse environments to determine if the commonly observed day effect in anther culture experiments could be attributed to specific greenhouse conditions. A study on the effect of activated charcoal in the culture medium was also undertaken. The time of transfer of anther-derived embryos to regeneration medium was studied as this has been reported to be critical in maize anther culture. In addition, because of the recent reports of enhanced embryo yield after colchicine treatments in anther culture of *Oryza sativa* and *Brassica napus*, we investigated the influence of colchicine on the anther culture response of one of our *S. phureja*-*S. chacoense* hybrids. In each experiment the yield of androgenic embryos was scored after 5-6 weeks of culture.

Experiment 1 -- Effect of greenhouse conditions (temperature/photoperiod) to the anther donor plant on androgenic response. Plants of two PC hybrids (CP2 and RPC4) were grown under four sets of greenhouse conditions: 30° day/ 20° night, 12 h day; 30° day/ 20° night, 16 h day; 25° day/ 15° night, 12 h day; 25° day/ 15° night, 16 h day. Anthers taken from immature buds on plants growing under higher temperature and short days yielded more embryos per anther (0.36) compared to the control (0.11 embryos per anther at 25/15°C, 16 h daylength). A total of 2113 embryos was obtained from 8982 cultured anthers.

Experiment 2 -- Activated charcoal experiment. Activated charcoal (AC) has been used to stimulate androgenic response for many plants including potato and is used routinely in our lab. Five treatments were applied to try to elucidate the effect of activated charcoal: 1) routine addition of 2.5 g l⁻¹ AC, 2.5 mg l⁻¹ BA, and 0.1 mg l⁻¹ IAA to the medium before autoclaving, 2) removal of activated charcoal by filter after autoclaving the medium, 3) addition of BA to the medium after autoclaving and filtering to remove AC, 4) addition of IAA to the medium after autoclaving and filtering to remove AC, 5) addition of both BA and IAA to the medium after autoclaving and filtering to remove AC. There were no significant differences among treatments. The stimulatory effect of activated charcoal must therefore be due to removal of impurities in the medium components that may be pre-existing or form during autoclaving. Absorption of growth regulators by AC does not appear to be a problem. A total of 2113 embryos was obtained from 8982 cultured anthers.

Experiment 3 -- Time of transfer of anthers to regeneration medium. Anthers were transferred to either solid or liquid regeneration medium 2 or 3 weeks after the initiation of anther culture and the androgenic response was compared to that of control flasks from which embryos were harvested after 5 weeks on anther culture medium. No significant

differences were observed among treatments. A total of 1569 embryos was obtained from 7485 cultured anthers.

Experiment 4 -- Colchicine treatments -- Five treatments consisting of 1/2 strength Linsmaier and Skoog medium with 0, 25, 50, 100, or 200 mg l⁻¹ colchicine were used to presoak anthers of CP2 for 24 h prior to culture. The anthers were then rinsed and placed in culture under routine conditions. None of the colchicine treatments significantly increased androgenic embryo production over the control. A total of 526 embryos was obtained from 2,229 cultured anthers.

Plant regeneration from androgenic embryos of clone CP2 was better than that for clone RPC4. Hence, the CP2 family was selected for future experimentation. From all of the above and some additional experiments, 260 plants were regenerated by anther culture of CP2. Of these, 34 were monoploid, 210 were diploid and 16 tetraploid, as determined by flow cytometry. The diploid group of anther-derived plants consisted of 14 homozygotes due to chromosome doubling that occurred during the culture phase and 196 heterozygotes from androgenic development of 2n pollen. The former are desirable as they are equivalent to the monoploids except for ploidy level. Heterozygosity/homozygosity of individual genotypes in the diploid group was determined by SSR markers.

The poor yield of monoploids despite culture of more than 15,000 anthers over the course of 18 months was unexpected. Preliminary experiments had indicated that monoploids could be obtained at a much greater frequency. We can only guess as to why the anther cultures were less successful in generating monoploids than expected – growing conditions of the plants, culture conditions *in vitro*. But all of these varied over the course of the experiments. Another problem encountered was that the same microspore could regenerate many embryos through a process of secondary embryogenesis within a single flask of anthers. This resulted in genetic identity among anther-derived plants. In order for the anther-derived population to be valid in segregation analyses, genetically identical individuals would need to be eliminated because their presence would skew ratios inappropriately by presenting the same genotype repeatedly in what should be a randomly derived population. Molecular markers were required to determine if embryos regenerated from a common flask were genetically different (see attached manuscript by Teparkum and Veilleux). Our final anther-derived population consisted of 28 monoploids and 14 doubled monoploids that showed unique genotypes. (Of 34 monoploids originally identified, six were found to be duplicates by marker analysis.) Because we were dissatisfied with the size of the population for marker analysis, we augmented it with a backcross population constructed by crossing the *S. chacoense*/*S. phureja* F₁ hybrid (CP2) to its *S. phureja* parent (BARD 1-3) for the glycoalkaloid part of the study.

Objective 2: To develop SSR DNA markers in potato

Genomic libraries

Two genomic libraries were constructed from a mixture of 50 µg each of *chc* + *phu* DNA. These included a conventional library and an enriched library. In addition, the GenBank + EMBL databases were screened for SSR sequences. The conventional library included 3,500 clones of 205 bp insert size. Assuming a genome size of 1.1×10^9 and assuming no overlap between the clones, this library represents 0.065% of the potato genome. The library was screened with five microsatellite markers. Of 14 positive colonies, 13 were sequenced. In total, six microsatellites were obtained following PCR amplification.

The enriched library, developed with the repeats GT and ATT, contained 12,000 GT clones and 1,000 ATT clones with average insert size of 215. The inserts of 205 positive clones obtained with the GT probe were sequenced. For 65 clones, the sequence was not clear enough although in 15 of these 65 clones, a repeat unit was evident. The sequence of 10 positive clones did not contain a repeat unit. Twelve of the positive clones contained repeat units of less than 16 bases. Four of the positive clones were of identical sequence, presumably due to preference of these clones during the enrichment process at the PCR reaction. In two clones, a chimera was observed in which one of the flanking regions was repeated. In 100 unique sequences, repeat units were apparent. For 33 of them, the repeat unit was interrupted by different numbers of nucleotides. For 17 an additional repeat unit other than GT was observed. For 70 of the 95 clones that contained SSRs, the repeat unit was too close to the cloning site so that primers could not be obtained. In many cases proximity of the repeat unit to the cloning site was observed for only one arm. In most cases the proximity was 0 to 15 bp. In other cases the insert size was smaller than 120 bp and the number of repeats was large; therefore the insert included mainly the repeat unit without unique flanking regions. Of the 25 clones containing the SSR sequence located internally, for 16, sequencing was done in the reverse direction in order to be able to determine primer sequences. For 14 clones primers were not determined due to low annealing temperature, particularly for the nucleotides A and T or due to complementarity of the two primer sequences. In six of 18 SSR loci for which primers were designed, no product was obtained. For the remaining 14, a unique product was obtained in agreement with the expected size with a deviation of 20 bp (Table 1C). From the library enriched with ATT, 1000 clones were screened and no positive clones were observed.

Database screening

In the GenBank + EMBL databases, 380 DNA sequences of potato were searched. Altogether, 850 kb were searched for the presence of mono-, di-, and tri-nucleotide repeats. Such repeats were found in 27 different genes where the repeat units were found distributed in the regions 5'UTR, 3'UTR, introns, and exons. In total, 11 new primer pairs were synthesized for which unique PCR products were obtained (Table 1A). A group of previously published primer pairs amplifying SSRs of 12 potato and three rice sequences was also used (Table 1B). Four of the primer pairs amplified repeats (RV3-

4=RV7-8=RV9-10 and RV21-22=RV33-34) that were duplicate accessions in the database.

SSRs from the tomato genome

Tomato and potato are closely related Solanaceae and homology between the two genomes has been reported. From the tomato genome, 20 pairs of primers were sampled for possible amplification of SSRs in the potato genome: five microsatellites from a conventional library, five from an enriched library and ten from databases. The primers were examined on clones of *phu* and *chc* and their F₁ hybrid (CP2). For 8 of the 20 primer pairs, no product was obtained and for 12, a product of the expected size was obtained with a deviation of 30 bases. For one primer pair, an additional product was obtained (Table 1D).

Microsatellite analysis of the CP2 family

In vitro copies of monoploid and doubled monoploid plants obtained in Virginia were shipped to Israel where they were maintained and propagated both *in vitro* and in the greenhouse. Leaf material was harvested from greenhouse plants for DNA extraction. Of seven protocols examined for DNA extraction, that of Doyle and Doyle (1990, Cornell University) has given the most reliable yield of good quality DNA. SSR analyses were conducted both in Virginia and in Israel.

The anther-derived CP2 family obtained was used to analyze segregation patterns of SSR markers. DNA was extracted from 28 monoploids and 14 homozygous diploids. Amplification reactions were based on the procedure reported by Yu et al. (1994). The reaction mixture (20 µL) contained: 1× assay buffer (50 mM KCl, 10 mM Tris-HCl (pH 9), 1% Triton X-100), 3 mM MgCl₂, 160 µM of each of the dNTPs, 1.5 U *Taq* polymerase (Promega, Madison, Wis.), and 0.1 µM of each primer. After adding 50 ng genomic DNA template, the reaction mix was covered by a drop of mineral oil. Each primer pair was first tested with DNA from the parents (*chc* 80-1 and *phu* BARD 1-3), and the anther donor (CP2) to check for polymorphism. A primer pair was considered to be polymorphic if it amplified fragments of different size specific for each of 80-1 and BARD 1-3, and if both fragments were present in the anther-donor CP2.

Segregation ratios in the CP2 monoploid family were calculated for all primer pairs. Chi square tests (with one degree of freedom) were performed for each primer pair to check for skewness (from the expected 1:1 ratio).

Identification of SSR polymorphism in the anther-derived family

Twenty-four primer pairs were designed from potato sequences in the databases (12 of which were previously published). Two of them (RV 59+60; 61+62) did not amplify specific bands in either 80-1, BARD 1-3 or CP2. From the remaining 22, nine (RV 1+2; 13+14; 35+36; 37+38; 39+40; 63+64; 65+66; 67+68; 71+72) were monomorphic, showing either a single, or two common bands in the parents and in CP2, and these bands appeared in all the monoploids tested. Although primer pairs RV 3+4, 7+8, and 9+10,

have different sequences, they were designed using similar template sequences from the databases, or even from redundant entries in the EMBL/Genbank databases; therefore they represent the same or duplicate loci. Data obtained from these primers were identical for all the plants tested; therefore for segregation analysis only RV 3+4 was used. Similarly primer pairs RV 21+22 and 33+34 were redundant (only RV 21+22 was used for analysis). Surprisingly only one (RV 69-70) of the five primer pairs from those reported by Provan et al. (1996) was polymorphic in our population; the other four did not amplify any segment in the CP2 family (RV 63-64; 65-66; 67-68; 71-72).

Twenty-four primer pairs were developed in Israel. The melting temperatures of these primers ('T_m' in Table 1C), which in most cases determined the annealing temperature in PCR, were lower (lower G+C content) compared to those designed in Virginia. This factor predicated the use of different amplification protocols for these primers. The results obtained with these primer pairs were not satisfactory. Of the 24 primer pairs amplifying SSR loci from the genomic library, ten were monomorphic, showing one single intense band in the parents and CP2. Eight others did not amplify fragments in CP2 even after redesigning the primers. Six primer pairs amplified clear polymorphic bands in CP2 that segregated among the anther-derived monoploids.

For the 11 primer pairs obtained from tomato, only one (tom 8+9) amplified polymorphic bands in our population; two pairs did not amplify at all and the remaining eight were monomorphic. None of the chloroplast DNA primers from rice amplified in the CP2 family.

Polymorphic primer pairs exhibited specific alleles in each of the parents, and CP2 inherited one allele each from 80-1 and BARD 1-3. Only primer pair RV 19+20, showed a new allele in CP2 that was not present in either parent. Primer pair RV 17+18 did not amplify any fragment from 80-1, but in CP2, two distinct alleles could be seen, one identical to that of 1-3. It was presumed that the other allele was the one missing in 80-1.

Distorted segregation among CP2 monoploids was observed for four of 11 polymorphic loci (Table 2). The GAA repeat within the starch phosphorylase gene, amplified by primer pair RV 11+12 exhibited extreme distortion. Allele 'B' of this locus (the *phu* allele) was never detected in anther-derived monoploids. This locus was skewed towards the *chc* (A) allele. Two additional skewed loci (amplified by primer pairs RV 3+4, and 21+22) favored the *chc* allele.

Searching the public databases is the easiest and fastest way to generate SSR primers. A recent report on *Solanum* sequences in the databases (Smulders et al. 1997) shows 35 entries that contain SSRs. We limited our search to SSRs with more than one base in the core repeat, since those represent the most informative loci. Redundancy of the databases and searches done using complementary repeat sequences (such as CTT, GAA) led to false results. This was the case of primer pairs RV 3+4, 7+8, 9+10, and 41+42, that were designed from similar sequences in the databases. Accession numbers X52416, X52417, X58453, and X83220 are entries of the same gene (GBSS) sequenced from different source plants. In addition, there are two different microsatellite loci in the *waxy* gene amplified by these primer pairs; an *actc* repeat, and some 200 bp downstream, a *ctt* repeat.

Only two of the 12 new primer pairs designed from database sequences failed to amplify in the anther-derived populations. The rate of polymorphic loci among them, (9 of 24 total, or 37.5%) was similar to those obtained in other studies in potato (Schneider and Douches 1997; Provan et al. 1996) and tomato (Broun and Tanksley 1996).

The loci that exhibited monomorphism in our population may be polymorphic in a larger population. Primer pair RV 13+14, which revealed no polymorphism in our population, showed four alleles in a study with 39 North American potato cultivars (Schneider and Douches 1997). Only one of the five primer pairs taken from the study by Provan et al. (1996) showed polymorphism in our population, whereas in their study the same primer pairs revealed 2-9 different alleles among 18 potato cultivars. Hence a study of allelic diversity among 12 potato cultivars was undertaken for 18 primer pairs.

SSR polymorphism among cultivars

For fingerprinting 12 cultivars or breeding lines of *S. tuberosum* were examined. Six cultivars were first genotyped with 18 primer pairs. For three markers differences of two bases or less made differentiation difficult. For an additional eight, all cultivars were genetically similar; four were monomorphic, three had two alleles and one had three alleles. Seven markers were polymorphic and informative, for which genotyping of the 12 cultivars was performed in order to obtain a unique fingerprint of each cultivar. The minimum number of loci required to fingerprint the 12 cultivars was two, e.g., the pairs of markers synthesized by primers pot 9-19 and RV15-16 or pot 57-58 and pot 9-10.

Based on the profiles of the 12 cultivars by seven SSR markers, the number of alleles, percent of genotypes that could be discriminated by a given marker, the distribution of the number of alleles per cultivar, and the heterozygote frequency were estimated. The average number of alleles per SSR locus among the 12 cultivars was five, ranging from 3 to 6. The average heterozygosity was 66% including cultivars containing at least two alleles. Though marker pot 19-20 had only three alleles among the 12 cultivars, its level of heterozygosity was greatest (0.92). The genotype distribution reveals that, for most of the markers, most of the cultivars had two alleles.

Distorted segregation in the CP2 monoploid family was observed for four of eleven loci. For three of the four loci, over-representation of the *chc* 80-1 allele was observed. Over-representation of the *phu* BARD 1-3 allele was observed only in one case (*ag* repeat in TLS1 locus, amplified by primers RV 23+24). This allele may be linked to a gene favoring anther culture response, since the BARD 1-3 parent is known to be considerably more anther culture competent than 80-1. The SSR primer pairs developed in this study augment those of previous reports on SSR markers in potato.

Objective 3: To identify and map QTLs coding for alkaloid synthesis and other production traits in potato

Solanum steroidal glycoalkaloids are an important class of secondary metabolites found in more than 350 species (Lawson et al. 1993). Their presence in cultivated potato can be a concern regarding toxicity to humans, but on the other hand their antifeedant activity

against insect pests (especially the Colorado potato beetle) is of particular interest to breeders since they offer the possibility of introducing natural defense mechanisms into commercial cultivars. Not all the glycoalkaloids are effective deterrents of Colorado potato beetle. The cultivated potato possesses only α -solanine and α -chaconine, against which the potato beetle is resistant. Some selections of the wild tuber-bearing species *Solanum chacoense* Bitt. have demonstrated high resistance to Colorado potato beetle and this resistance has been associated with leptine glycoalkaloids (Sinden et al. 1986).

Leptine-based resistance against Colorado potato beetle is of great interest to breeders, but no cultivar incorporating this kind of resistance is yet available. This may be in part because of the polygenic inheritance of glycoalkaloid content in tetraploid potato (Sanford and Sinden 1972). In crosses between high leptine selections of *S. chacoense* (after chromosome doubling) with *S. tuberosum* breeding lines, most of the offspring produced leptines, indicating a dominant factor in *S. chacoense* (Sanford et al. 1996). However this study required chromosome doubling, which involves some inbreeding when working at the tetraploid level (only two alleles are present in the chromosome-doubled lines instead of four possible in the tetraploid clones). Selection at the diploid level seems easier due to less complicated segregation patterns encountered in diploid species. Adapted clones of *Solanum phureja* Juz. & Buk. could be used as a genetic bridge between *S. chacoense* and *S. tuberosum*, since it can be crossed easily with both species (Haynes 1972, Veilleux and Lauer 1981). Sinden et al. (1986) suggested that recessive genes control leptine synthesis, and that high levels of leptines are also under environmental control.

All these characteristics of leptine production (low heritability, polygenic trait, and environmental influence) increase the difficulties of efficient selection of high leptine clones in potato. According to the hypothetical pathway, biosynthesis of acetylleptinidine (the aglycone of leptines) occurs from solanidine, leptinidine being an intermediate step. This hypothesis is based upon the structural relationships between these alkaloids. Tagging of putative loci affecting the two steps of this pathway would be of interest to breeders, and would help the efforts to transfer leptine-based resistance to commercial cultivars of potato.

Marker-assisted selection can increase the efficiency of selection for traits of low heritability. To realize this, one should find markers tightly linked to the trait of interest. RAPD analysis (Williams et al. 1990) is based on amplification of DNA regions using random 10-mer primers. Their abundance in the plant genomes and the ease of the assay has made RAPDs a useful tool in gene mapping studies in many plant species. In cases when specific regions of the genome are investigated, bulked segregant analysis can be used to identify markers linked to genes of interest. The method, developed by Michelmore et al. (1991) involves comparing two pooled DNA samples from a population segregating for the trait of interest. Members of each pool represent extremes of expression for the trait of interest but are arbitrary for all other loci. Markers that show polymorphism between the pools are likely to be linked to the trait of interest.

Plant material populations used, and construction of bulks

Plant materials included the clones *Solanum chacoense* 80-1 (*chc*), *S. phureja* BARD 1-3 (*phu*), their F₁ hybrid CP2, the anther-derived monoploids of CP2 (42 individuals), and a backcross between CP2 and BARD 1-3 (56 individuals). Leaf samples taken from greenhouse-grown plants were sent to Dr. A.R. Miller, Ohio Agricultural Research and Development Center, Wooster, Ohio, for glycoalkaloid analysis by gas chromatography. Based on measurements of levels of total and specific glycoalkaloids, bulks were constructed with 6-8 individuals from extremes of the monoploid and backcross populations. In each bulk 100 ng of DNA solution from each individual were mixed (10 µL DNA at the concentration of 10 ng/µL from each individual).

RAPD analysis

Eighty decamer primers purchased from Operon Technologies (Alameda, Calif.) were used (kits OPA, OPC, OPG and OPQ, twenty primers each). The reaction mixture of 25 µL, contained: 20 ng genomic DNA, 0.6 µM primer, 200 µM dATP, dCTP, dGTP, dTTP, 1× PCR buffer (2.5 mM MgCl₂, 50 mM KCl, 10 mM Tris-HCl, pH 8.3), and 1 U *Taq* polymerase (Promega). For those primers that amplified intense bands, the amount of the reaction mixture was reduced to 15 µL (maintaining the concentrations of each ingredient). The reactions were overlaid with a drop of light mineral oil, and amplifications were conducted with a Perkin Elmer Cetus Model 480 thermal cycler. The amplification procedure was as follows: 45 cycles of 1 min denaturation at 94°C, 1 min primer annealing at 37°C, 2 min extension at 72°C, followed by a final extension step of 4 min at 72°C. The amplified fragments were separated in 1.4% agarose gel in TBE buffer for 4-5 hours at 90 Volts (Sambrook et al. 1989). After separation gels were stained with ethidium bromide and photographed under UV light.

The anther-donor (CP2) showed intermediate content of acetylleptinidine (2,001 µg/g dry weight). The *phu* parent (BARD 1-3) did not have any acetylleptinidine (ALD), whereas the *chc* parent (80-1) had 19,838 µg/g dry weight. The monoploids segregated for ALD content, showing values from 0-9000 µg/g dry weight (Fig. 1), approaching half of that of the *chc* parent. Eight of 20 CP2 monoploids showed no ALD content or only traces (Fig. 1). Of the 12 that exhibited ALD, the level varied by more than 100 fold. Similar segregation was seen among the backcrosses, although in this population the values were much lower than those of the monoploids or the *chc* parent (Fig. 2). This was expected, since individuals in this population were 75% *S. phureja*.

Eighty decamer RAPD primers (Operon kits A, C, G and Q) were screened using DNA from the high and low ALD backcross bulks. SSR primer pairs were not used in attempts to find correlations between markers and glycoalkaloid content because all the individuals of the monoploid family had been previously screened with all the polymorphic pairs and no linkage could be detected. Some of the RAPD primers were previously tested in studies with CP2 monoploids in our lab and were used to estimate genetic composition of the family (Veilleux et al. 1995). All the primers, except OPG 20 and OPA 8, amplified from 3 to 9 detectable bands.

Only primer OPQ 2 showed a band that was missing in the low bulk but present in the high bulk, and was consistent in all the individuals of both bulks (Fig. 3). Surprisingly, none of the primers could differentiate the high and low ALD bulks from the monoploid family.

Previous studies on the genetic basis of acetylleptinidine production proposed the presence of homozygous dominant genes in the high leptine selections of *S. chacoense* (Sanford et al. 1996; Veilleux and Miller 1998). McCollum and Sinden (1979) explained the segregation patterns of glycoalkaloids in F₂ populations of *S. chacoense* with a three locus model, involving dominance, additive effects, and epistasis. Although both populations used in our study were of limited size, the phenotypic expression of leptine content and the segregation patterns among the monoploids and the backcross progeny suggest simple inheritance and dominance.

Cooperation

Cooperation between principal investigators has been excellent. Frequent e-mail communication has been used extensively among investigators. RV and ARM visited Israel in October 1996 to discuss progress on the project. DL visited Virginia in August 1997 and JH visited Virginia in January 1998 to go over results from his laboratory. Three graduate students have been supported on the project, Eduard Chani under the direction of RV in Virginia, Varda Ashkenazi under the direction of JH at the Hebrew University in Rehovot, Israel, and Edna Fogelmann under the direction of DL at the Volcani Center in Bet Dagan, Israel. Manuscripts are currently in preparation that represent the research conducted by these students.

Evaluation of research achievements

An enhancement of embryo yield in anther culture of PC hybrids may be obtained by growing the plants under high temperatures and short photoperiod. The short photoperiod, however, has the effect of reducing the flowering period to a relatively short time. The activated charcoal in the anther culture medium can be removed after autoclaving and before anthers are placed in the medium without effecting any change in the responsiveness of the anthers, i.e., the enhancement of androgenesis due to activated charcoal is still exhibited by the charcoal treated medium, even when the charcoal does not actually come into contact with the anthers.

Despite improvements to the anther culture procedures through a series of experiment, monoploid clones from the *S. chacoense* × *S. phureja* hybrids were difficult to obtain. During the same period, other monoploid families were developed from different anther donors, emphasizing that genetic control of response to anther culture is important.

We have a population of 42 monoploids or doubled monoploids of CP2. Because of the relatively few monoploids obtained from the massive numbers of anther cultures done in the early stages of this project, we conclude that it is inefficient to rely on an anther-derived population for mapping studies in potato. Therefore, backcross populations of CP2 to both its *S. phureja* and its *S. chacoense* parents were developed. Leptine levels in

the *S. chacoense* backcross were moderate to high in all individuals whereas, in the *S. phureja* backcross, leptines were not present in most of the individuals and low in a few. Therefore, this population was used as the mapping population. It was not ideal, however, because the ideal population would segregate from low to high rather than from zero to low. Hence we are currently in the process of developing a more appropriate population through intercusses among the low individuals from the *S. phureja* backcross. We expect progeny from these will exhibit the desired segregation. The monoploid population, though insufficient in size, showed the segregation necessary (Fig. 1).

SSR markers were developed by a variety of methods including database searches, and genomic libraries. The primer pairs that amplify these new markers will be published for use by the international community. These markers were useful in characterizing the genetic composition of potato cultivars as well as our anther-derived population, allowing us to eliminate genetically identical regenerants and to discriminate heterozygous from homozygous diploid regenerants. However, the potato genome was not sufficiently saturated with SSR markers over the course of these studies for them to be used exclusively for marker assisted selection without employing other molecular markers. RAPDs were used effectively to find a marker putatively associated with leptine production in the segregating backcross population. This is a first step towards identifying glycoalkaloid genes in potato. However, it must be confirmed by additional research in a larger population.

Presentation of research results at the Potato Association of America has resulted in request for monoploid germplasm because of its recognized advantage in studies of genetic segregation. One of the difficulties in SSR analyses is a phenomenon called stuttering, i.e., the appearance of a ladder of banding patterns on a gel due to heteroduplex formation. With multiallelic organisms, determining the real from the heteroduplex "alleles" can be difficult. Due to their hemizygosity, monoploids cannot form heteroduplexes and the scoring of SSR alleles is simplified.

List of publications

- Paz, M.M and R.E. Veilleux. 1998. Influence of culture medium and in vitro conditions on shoot regeneration in *Solanum phureja* monoploids and fertility of regenerated doubled monoploids. Plant Breeding 117: (in press)
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- Boluarte, T. and R.E. Veilleux, 1998. Segregation of microsatellite alleles in reciprocal backcross populations of diploid potato. Presented at the 82nd Annual Meeting of the Potato Association of America, July 25-30, Fargo, ND
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Tables and Figures

Table 1. Primer pairs, source, and PCR conditions used to detect SSR polymorphisms in potato.

A. New primers from the public databases

RV	Sequence (5' - 3')	Locus ¹	Acc. No.	Repeat	Prod ²	T _m	Result ³
15 16	aattcatgtttgcggtacgt atgcagaaagatgtcaaa	STACCAS3	Z27235	(aag) ₇	250 bp	59°C 58°C	+
17 18	gaaatcgacacagacgg cgagggactttaattgttg	STWIN12G	X13497	(aat) ₆	240 bp	60°C 60°C	+
19 20	aataaactgtgatgccac gtggcatgtcttcaaggt	POTM 1-2	U23758	(at) ₂₀	221 bp	60°C 59°C	+
21 22	gtgattggcaatcagattg gtgtgtggactgtggagtg	ST13ST def 4	X67511	(at) ₁₁	200 bp	59°C 60°C	+
23 24	gctatgttagggtaagg atatgggatgcttggtata	STLS1	X04753	(ag) ₁₁	192 bp	59°C 60°C	+
33 34	tattcccccttctactcaa tcttcacattcctaactg	ST13ST	X67511	(at) ₁₁	202	58°C 58°C	+
35 36	caaaattaaaagaaaag accaataatcctacaaaca	STIIKA	M15186	t ₁₄ a ₁₀ (at) ₇	220	48°C 52°C	-
37 38	attgttttcttcttctgc aattctagcccttcacttg	STPROINIX	Z14027	c ₁₄ (ct) ₈ (at) ₉	200	52°C 56°C	-
39 40	tttggtggtagagtttgta ttttgtttgtttattggc	SC810LP	X72927	a ₁₉	170	54°C 51°C	-
41 42	ttatgaatcgtgttatgg caaaaaggggaatctacc	STGBSS	X58453	(tct) ₉	190	51°C 52°C	+
59 60	ttttctcctgtcactta gcttctccactaccatcc	ST20345	U20345	(gggt) ₃ (gtt) ₃	198	49°C 51°C	Np
61 62	attctccttgggttttcc atgcaggtgatgactctaa	STRBCS2	X69760	(at) ₈	224	46°C 50°C	Np

B. Primers from previously published studies obtained from the public databases

RV	Sequence (5' - 3')	Locus	Acc. No.	Repeat	Prod	T _m	Result [†]
1 2	tttctattgaaaaccttga catctctacattcagag	STPOACUTR	X55748	(ta) ₁₃	228 bp	60°C 59°C	-
3 4	cccataactgtcgatg gaatgtagggaacatg	G28WXST	X52417	(actc) ₅	223 bp	64°C 62°C	+
5 6	cttgcaacttgtagtacc aaatcctttgtgacctccc	STPROINI	Z12611	(tc) ₁₂ (ta) ₁₈	180 bp	66°C 60°C	+
7 8	tgattctctgcctactgta agtcagagtaggttctct	STGBSS	X58453	(ctt) ₄	246 bp	68°C 72°C	+
9 10	ttctgatttcatgcatgtt atgtgtggtctacaaaaa	STGBSS	X58453	(aag) ₈	236 bp	60°C 64°C	+
11 12	ttcagagacatcatggca atccttcatcagaggaag	ST STP	X73684	(aga) ₅	220 bp	62°C 66°C	+
13 14	caaccaacaaggtaaat tggtctggtgcattagaa	STPATP	X04077	(aatt) ₅	386 bp	66°C 60°C	-
63 64	agtactcagtcaatcaaa aggtaagtatgttctcca	STSNRNA10	Z11882	(a) ₁₉	173	52°C	-
65 66	ccacagtgaataagcat tagtaacggggaaagac	STRBCS3	X69763	(a) ₁₅	201	50°C	-
67 68	aaggagagaagaacat cattgaataacacaccat	STCPKIN3	Z11970	(t) ₈ (g) ₅ t(g) ₉	168	50°C	-
69 70	gacacgttcaccataaaa agaagaatagcaaagca	STGLGPB	L13771	(att) ₅ ttt	132	48°C	+
71 72	gaagttttatcagaatcc atcacctcatcagcaatc	STU6SNRN	X60506	(ttg) ₅	181	48°C	-
97 98	ggaaagggttagggttttt ccgcgatgcaataagag	Oscp80599 [†]		(t) ₃ c(t) ₁₀ a(t) ₄	95	60°C 60°C	-
99 100	gtcataaataagggttattc ggatactaaatctcgtcg	Oscp89568 [†]		(t) ₂ a(t) ₁₀ gc(t) ₃	114	62°C 64°C	-
101 102	ggaattcgtaccttctcg gaaaggaaagctagaa	Oscp134510 [†]		(a) ₄ t(a) ₁₀ g(a) ₃	140	60°C 62°C	-

C. Primers from the genomic library

RV	Sequence (5' - 3')	Repeat	Prod	T _m	Result ³
25	ggggaaacaaggctaa			58°C	Np
26	aggggacaaaagggtgg			60°C	
27	aataattcagaccaagaa	(att) ₂₀		51°C	Np
28	caatgaaaactaacgag			53°C	
29	aacacacccactcacct	40 bases rich with t and a		60°C	Np
30	ggcgcccgattttagaa			58°C	
31	ttcaaagaaatcacaaaa	20 bases rich with t and c		51°C	+
32	tcaagaccccaaaaacta			55°C	
43	actcacccactcacctc		161	54°C	Np
44	ccctcccctgcgactgt			58°C	
45	tggcaggttaggcacaaa	(ca) ₉	163	54°C	-
46	acaactcatcgtctccag			54°C	
47	aacattacaacacattag	(tg) ₁₀ (ag) ₁₀	204	47°C	-
48	aacttatctgaaactctcg			50°C	
49	gactgtgctcttgcttga	(ca) ₁₅	131	54°C	Np
50	cacactttatgctgggtc			52°C	
51	attctccgaaagtatgtct	(ca) ₁₄	129	52°C	Np
52	tgggatgagttggtgtgt			56°C	
53	gcaaaatacaggtcca	(ct) ₁₀ (ca) ₉	183	58°C	-
54	ttctcaacaactcccatc			58°C	
55	atcatcacagcctctttt	(ca) ₉	147	48°C	-
56	gcctcaatcgctacttat			52°C	
57	ttgcgtgaagcagccgt	(gt) ₁₀	111	60°C	+
58	gccagtaagtaaaaca			56°C	
73	acacacagaaaggtaaa	(ta) ₅ (tg) ₁₀	185	50°C	-
74	gtcagtacgaaaaaatgt			50°C	
75	ttgcctgatatagaat	(ca) ₁₅	224	44°C	Np
76	aaccaatccatgcctaga			56°C	
77	atcttgacaaacttactc	(tg) ₃ a(tg) ₃ a(tg) ₄	187	50°C	+
78	atttaccactagctcta			50°C	
79	ttatgaacaccttgga	(ca) ₉	135	56°C	Np
80	ttcaattaacaccataga			52°C	
81	ataaaccgcatgagaag	(ag) ₇ (gt) ₈ (ag) ₈	143	52°C	-
82	atgggatagatttgtag			48°C	
83	gggacatcacagtct	(gt) ₉	153	46°C	-
84	ggtgctcctattggtg			50°C	
85	ggcagagggaacaata	(gt) ₁₀	229	62°C	-
86	caggtaatagcaacgatg			60°C	

1C (continued)

87	aaaacaggattcggaca	(gt) ₈	250	52°C	+
88	tagtattgtgcttgctta			48°C	
89	gagcctcaatcgtcactt	(gt) ₁₀	179	54°C	-
90	ccatttgtagccagta			52°C	
91	gaccaataattcagacca	(att) ₁₉	184	58°C	+
92	gtgccagagaaaggtgt			54°C	
93	tatggaagactctgctgg	(t) ₄ ca(t) ₁₈	187	54°C	-
94	cactggtggacctaatac			54°C	
95	ctattcaaagctggatgg	(aag) ₉	236	52°C	+
96	actggcgggagcacaa			60°C	

D. Tomato primers

Tom	Sequence (5' - 3')	Locus ¹	Acc. No. ¹	Repeat	Prod ²	T _m	Result ³
M3f M3r	tcttaccttcccagatgcc cgccaaccatccgtatca	TOMCD4A	M32603	(gtt) ₅	157	68°C 64°C	-
M17f M17r	tgcaacttgtagtaccac cacccccccacacacac	POTINHW1	M17108	(at) ₉		64°C 68°C	-
8 9	gcattgattgaacttcattc attttgtccacaccaacta	Genomic library		(att) ₇	140	62°C 60°C	+
11 28	cagtaactacaaaaata attgtaatggtgatgctctt	Genomic library		(ctt) ₅ (ct) ₅	210	62°C 62°C	-
35 36	ttcttttggtggttttccttg gttatttctctgttggtgctg	LEGAST1	X63093	(ta) ₁₂	246	70°C 68°C	-
39 40	taacacattcatcaaagta ttgcgtgataatccagtaa	LEACC2G	X59139	(aatt) ₅	228	56°C 54°C	np
41* 42*		Genomic library		(tcc) ₆	157	°C °C	-
43* 44*		Genomic library		(ct) ₁₀	199	°C °C	-
45* 46*		Genomic library		t ₃₀	104	°C °C	np
47 48	caagtgattgcattacct tacaacaacatttcttcttc	LEGTOM5	X60441	(at) ₁₀	165	62°C 58°C	-
51 52	aagtgtgttgaattatc acaactattcattctcctac	Genbank		(atta) ₄	223	60°C 58°C	-
59 60	taacacatgaacattagtt cacgtaaaataaagaag	LESSRPSPG	Z34518	(c) ₁₆	130	56°C 54°C	-
61 62	ggcaaagaaggaccca ggtgcctaaaaaagttaa	LEGTPASE	Z34518	(cac) ₆	184	62°C 52°C	-
63 64	tcgtaattgttgcattgttg tcattgtagtgaggtgcta	LPTRYINH	M59427	(aatt) ₂ t(aatt) ₂	201	60°C 64°C	-

¹ Gene names and accession numbers in the EMBL/Genbank databases

² Expected product length according to the sequenced fragment used to design the primer

³ Polymorphism in CP2 family: + polymorphic; - monomorphic; np - no specific product amplified

* Primer sequences were not available at the time of submission.

Table 2. Segregation ratios for SSR markers in the CP2 monoploid family.

Locus	Segregation		
	A [§]	B [§]	χ^2 †
3+4	36	6	21.43 ^{**}
5+6	27	15	3.43 ^{ns}
11+12	41	0	41.00 ^{**}
15+16	16	23	1.26 ^{ns}
17+18	23	12	3.46 ^{ns}
19+20	14	21	1.40 ^{ns}
21+22	27	14	4.12 [*]
23+24	6	35	20.51 ^{**}
57+58	23	18	0.61 ^{ns}
69+70	19	23	0.38 ^{ns}
Tom 8+9	13	13	0.00 ^{ns}

§ At each locus 'A' represents the 80-1 allele and 'B' the 1-3 allele.

† Deviation from the expected 1:1 segregation ratio:

ns - not significant;

* - significant at 0.05% level;

** - significant at 0.01% level.

Figure 1. Acetylcholinesterase content of 20 individuals of the CP2 monoploid family.

Figure 2. Acetylptepitidine content in 55 individuals of the CP2 $\times$ BARD 1-3 backcross family.

Fig. 3. RAPD amplification using primer OPQ02. Lanes from left to right: 1=Lambda Eco RI/Hind III DNA ladder; 2=negative control; 3=chc 80-1; 4=phu 1-3 (no amplification); 5=CP2; 6-15=10 members of the high ALD bulk from the backcross family; 16-25=10 members of the low ALD bulk from the backcross family