BULK SEGREGANT ANALYSIS FOR ANTHER CULTURE RESPONSE AND LEPTINE CONTENT IN BACKCROSS FAMILIES OF DIPLOID POTATO

by

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(ABSTRACT)

Diploid potato populations between a primitive cultivated species, *Solanum phureja*, and a weedy species, *S. chacoense*, were used to examine the segregation of microsatellite markers and three traits in backcrosses. Two of the traits, anther culture competence and 2*n* pollen production, originated from *S. phureja* whereas the third, leptine production (a specific glycoalkaloid known to convey resistance to the Colorado potato beetle) originated from *S. chacoense*. Using CP2, a self-incompatible F_1 hybrid originating from a cross between *S. chacoense* clone 80-1 and *S. phureja* clone 1-3, three populations were developed: 1-3 × CP2 (PBCp), CP2 × 1-3 (PBCc), and CP2 × 80-1 (CBC).

For the microsatellite study, four simple sequence repeat (SSR) primer pairs that amplified fragments within potato sequences found in the GenBank were used to look at segregation ratios in our backcross populations and to eliminate possible spurious genotypes bearing non-parental alleles in these populations. Seventeen spurious genotypes were discarded from PBCp; none was found in PBCc or CBC. Two SSR loci showed skewed segregation in PBCp (favoring transmissnion of the allele originally found in 80-1), PBCc showed normal segregation at all loci, and CBC showed distorted segregation at one locus (revealing a deficiency of homozygotes).

In the study of anther culture, three components of ACR were investigated in a preliminary study: 1) embryos produced per anther (EPA), 2) embryo regeneration rate and 3) percentage of monoploids (2*n*=1*x*=12) among regenerants. CP2 was intermediate, 80-1 was low, and 1-3 was high for ACR. Only EPA was selected for further characterization in our populations. PBCp (78 genotypes) and CBC (57 genotypes), were characterized for anther culture response ACR/EPA in a series of studies. Nine high and ten low selections were identified in CBC, and ten high and ten low selections were

identified in PBCp. EPA selections were used for bulk segregant analysis (BSA) using 214 RAPD primers. Two bands, one amplified by OPQ-10 and another by OPZ-4 were linked in coupling and in repulsion, respectively, to ACR in PBCp. One band amplified by OPW-14 primer was linked in coupling to ACR in CBC. One-way ANOVAs for data from remaining genotypes of the populations verified linkage of the markers to ACR/EPA.

For 2n pollen production, a total of 77 PBCp genotypes was characterized; 80-1 produces low % 2*n* pollen, and 1-3 produces high % 2*n* pollen. Pollen samples were stained with propidium iodide and examined by flow cytometry. The frequency of 2n pollen varied continuously from 1.7 % to 40.6 % among the 41 genotypes that flowered sufficiently to allow three separate pollen collections. Variation due to the environment was observed where the frequency of 2n pollen appeared greater over a range of genotypes on single collection days. BSA could not be used due to limited population size and a low number of selections at the extremes of the distribution of phenotypes. The continuous variation for 2*n* pollen production suggests multigenic control of the trait.

In the study of leptine content in reciprocal backcross populations, 87 genotypes within PBCp, and 42 genotypes within PBCc were characterized using gas chromatography of leaf samples. CP2 was intermediate, 1-3 had zero, and 80-1 was high for leptine content in the foliage. Leptines were present in low levels in 43 of 87 genotypes in PBCp, indicating simple genetic control. In PBCc, only 7 of 42 genotypes expressed leptines, generally at a higher level than in PBCp, indicating cytoplasmic inheritance. Ten high and ten nil selections within PBCp, and seven high and eight nil selections within PBCc were used for BSA using 214 RAPD primers. Three primers OPQ-2, OPT-16 and OPT-20 amplified bands segregating with high bulks in both populations. These markers were linked in coupling to leptine content in PBCp. Linkage was verified by ANOVAs for leptine content in the entire population.

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Chapter I:General introduction

Potato, *Solanum tuberosum* L. ssp. *tuberosum*

The cultivated potato (*Solanum tuberosum* L. ssp. *tuberosum*) is a solanaceous autotretaploid (2*n*=4*x*=48) originating in South America. *S. tuberosum* has been the most productive and successful of the *Solanum* species, and hence became most widespread, in the course of which two subspecies have become differentiated: ssp. *andigena*, the typical Andean form of Peru and Bolivia; and ssp. *tuberosum*, developed as a result of selection under long-day conditions, as in coastal Chile and, during the last few centuries, in Europe (Burton, 1989). After its introduction to Europe during the 16th century, the potato has become one of the most important world food crops in total food production (Bajaj, 1987).

Solanum phureja Juzepczuk and Bukasov

Solanum phureja Juz. & Buk. is a tuber bearing, diploid (2*n*=2*x*=24) cultivated species originated and evolved from wild species from Peru and Bolivia. *S. phureja* lacks tuber dormancy and has been used in genetic studies and potato improvement programs. It is a source of resistance to bacterial wilt caused by *Pseudomonas solanacearum* E.F. Smith. It is also known to be resistant to *Phythophthora infestans* (Mont.) de Bary, and to potato viruses A, S, X and potato mottle virus. Some hybrids were also found to be resistant to frost. *S. phureja* is also a source for heat tolerance and adaptation to the temperate zones (Ochoa, 1990).

S. phureja is cultivated widely in northwestern Bolivia and along the entire eastern range of the Peruvian Andes. The Aymaran name “phureja” means “early” or “precocious.” It yields tubers in about 3 or 4 months and may be planted three or four times a year (Ochoa, 1990).

Solanum chacoense Bitter

Solanum chacoense Bitt. is a tuber bearing, diploid (2*n*=2*x*=24), self-incompatible species. This highly variable weedy species is found in geographically diverse regions of southern S. America, including Bolivia, Argentina, Brazil and Paraguay, where both highland and lowland accessions have been collected (Hawkes, 1962). Many characters of interest have been recorded for accessions of *S. chacoense*; the range of resistance characters is wide and includes fungal diseases such as *Phythopthora infestans*, bacterial diseases, viruses, nematodes and insect pests (Hawkes and Hjerting, 1989). A few genotypes within *S. chacoense* have been identified that produce high concentrations and proportions of leptines relative to other glycoalkaloids and are highly resistant to Colorado potato beetle (CPB), one of the most important pests of potato in the United States; therefore these accessions are of great interest to potato breeders (Sinden et al. 1986).

Potato genetics and breeding

The genetic diversity of cultivated and wild potatoes is greater than that of any other major world food crop. In addition to *S. tuberosum*, there are seven other cultivated species and over 200 wild species of potato (Horton, 1987).

Some 228 wild potato species are now known, but not all of them have been fully investigated. All of them possess the same chromosome base number (*x*=12) as the cultivated potatoes, and range from diploid (2*n*=2*x*=24) to hexaploid (2*n*=6*x*=72) (Hawkes, 1994).

Most wild and cultivated potato species can be hybridized, with some exceptions. These exceptions mainly involve the endosperm balance number (EBN) groups. The endosperm balance number (EBN) theory (Johnston et al., 1980) was developed to explain the basis for normal seed development after intra- and inter-specific crosses in potato. Many *Solanum* species, both tuber-bearing and non-tuber-bearing, have been assigned EBNs (Hanneman 1994) based on their crossing behavior with known EBN standards (Ortiz and Ehlenfeldt, 1992). According to this hypothesis, each species has a

genome-specific effective ploidy, the EBN, which must be in a 2:1 maternal to paternal ratio in the hybrid endosperm for normal development of the endosperm itself. A species can be assigned a 1 EBN, 2EBN, or 4EBN based on its crossing behavior. The EBN is more important than chromosome ploidy in determining the success or failure of interspecific crosses (Carputo et al., 1999). Crosses between diploid species with EBN of 1 and EBN of 2 are extremely difficult. However if EBN 1 species are tetraploidized, giving rise to EBN 2 progeny, the crosses can be done with comparative ease. Species that act as autotetraploid such as *S. tuberosum*, possess an EBN of 4, and can cross readily with allohexaploid species (Hawkes, 1994). The nature of the EBN mechanism is not well understood, but appears to be under genetic control (Hanneman et al., 1990).

When potato hybrids can be made, they are generally fertile at the same level of ploidy. Tetraploids with disomic inheritance can be crossed also, but not so easily with *S. tuberosum*, which possesses tetrasomic inheritance. Diploid × tetraploid crosses are easy if the species are both disomic, although the hybrids are of reduced fertility. Diploid (disomic) × tetraploid (tetrasomic) crosses are more difficult, sometimes producing tetraploid progeny through the functioning of 2*n* gametes (Hawkes, 1994).

Due to their broad genetic background, cultivated potatoes are extremely heterozygous; the offspring or progeny resulting from a cross of two parents, or even by self pollination of a cultivar, are usually highly variable. For this reason pure cultivars can only be maintained by vegetative reproduction, using tubers or other parts of stems as explants (Horton, 1987).

The cultivated potato (*S. tuberosum* ssp. *tuberosum*) is an autotetraploid and highly heterozygous. Breeding of potato is difficult for various reasons: its characters are inherited tetrasomically, and it shows strong inbreeding depression, reduction in fertility when selfed, self-incompatibility at the diploid level, and male sterility in many cultivars (Bajaj, 1987). Modern potato breeding often relies on the wild tuber-bearing species to incorporate desirable traits into cultivars (Ross, 1986). There is an extraordinarily wide range of habitats in which wild potatoes are found, and this explains the way in which they have become adapted to stress environments and have developed strong resistance to

a wide range of pests and diseases. On the other hand cultivated potatoes have been developed under a very limited range of conditions under which they are now cultivated (Hawkes, 1994). One consequence of the potato's heterozygosity is that the probability of selecting an offspring that is superior to either of its parents is extremely low. Potato breeders usually need to make several hundred crosses and evaluate hundreds of thousands of seedlings and clones over several years, often 10 or more, before releasing a single new variety (Horton, 1987).

Anther culture response

Various breeding schemes have been proposed to develop potato hybrids, utilizing haploids to combine genomes of different compositions to construct heterozygous allelic combinations (Chase, 1963; Wenzel, 1979). The potential of haploids in a plant breeding system has been studied extensively.

Haploids of potato were produced originally by pseudogamy, i.e., embryogenesis of unfertilized eggs, induced by specific pollinators (Van Breukelen, 1981). Sopory et al. (1978) proposed the use of haploids, developed by anther culture, to obtain completely homozygous lines of potato for use in breeding programs.

Monoploid plants ($2n=1x=12$), developed by reducing the ploidy level of a diploid, have only one set of homologous chromosomes; therefore they are useful for selecting against plants with recessive lethal or deleterious alleles ("monoploid sieve") and for plants with desirable characteristics (Uijtewaal, 1987).

Anther culture can be used to obtain the equivalent of inbred lines as doubled haploids. However, the use of anther culture to obtain plantlets derived from pollen embryos or calli efficiently is still limited to a few clones that are competent for androgenesis (Singsit and Veilleux, 1989).

Differences in genotype for anther culture response have been documented in *Solanum* species (Irikura, 1975; Sopory and Rogan, 1976; Jacobsen and Sopory, 1978; Wenzel and Uhrig, 1981; Dunwell, 1985; Petolino and Thomson, 1987). Working with potato, Irikura (1975) tested 118 clones of 46 species of *Solanum*, and found good anther

culture response in 11 species. He obtained monoploid plants in *S. verrucosum* Schlechtd., *S. bulbocastanum* Dun. , *S. phureja* Juz. & Buk., *S. stenotomum* Juz. & Buk., and two interspecific hybrids. These studies demonstrated that the genetic composition of the anther donor plant strongly affects the response in anther culture.

Anther culture responsiveness or androgenic competence can be separated into two phases, the first where microspores are competent to develop into embryos in anther culture and the second, where androgenic embryos are competent to regenerate into plantlets. Since there is no positive correlation between these two factors, it is possible that androgenic competence and embryo regeneration are under independent genetic control (Singsit and Veilleux, 1989). The response to anther culture was separated in various studies into different components: frequency of responsive anthers, number of embryos per anther, regeneration frequency of anther-derived embryos and frequency of regenerated plantlets per cultured anther (Foroughi-Wehr et al. 1982; Singsit and Veilleux, 1989; Murigneux et al. 1994).

Studies of anther culture responsiveness as a heritable trait have been conducted in several species like for example maize (Afele and Kannenberg, 1990), wheat (Agache et al., 1989), barley (Forughi-Wehr, et al., 1982), petunia (Raquin, 1982), rice (Miah et al., 1985) and rye (Rakoczy-Trovanowska and Malepszy, 1993). In maize, a relatively simple inheritance was determined. In wheat, anther culture response was proposed to be controlled by a few genes with major effects. A complex inheritance was found for barley. More than single gene control was proposed for petunia. A few recessive alleles are believed to control anther culture response in rice, and at least two recessive complementary loci were found to control callus regeneration after anther culture in rye. Wan et al. (1992) conducted an RFLP analysis of anther culture derived callus lines from maize hybrids. They identified six chromosomal regions in five different chromosomes associated with the formation of embryo-like structures (ELSs) or callus from ELSs from microspores. These regions are either associated with ELS formation or with callus regeneration. This work provides evidence for the genetic basis of the maize anther culture response.

The genetic basis of anther culture responsiveness has also been studied for potato. Wenzel and Uhrig (1981) as well as Sonnino et al. (1989) proposed more than one recessive allele to control anther culture response. Singsit and Veilleux (1989) suggested that one dominant allele controlled anther culture competence in a study of diploid potato species. Veronneau et al. (1992), concluded that two genes controlled induction of embryo formation and two additional genes controlled embryo regeneration in a study of anther and leaf disc culture in clones of *S. chacoense*. Taylor and Veilleux (1992), working with *S. phureja* to determine the inheritance system for leaf disc regeneration, anther culture response and protoplast culture, proposed the action of one codominant gene with additive effect for anther culture response.

The regeneration capacity of microspores is dependent on the genotype and can be transferred via sexual recombination (Wenzel and Uhrig, 1981). Cappadocia et al. (1984) concluded, after seeing segregation in crosses between *S. chacoense* x *S. tuberosum*, that one can transfer the regenerative capacity via breeding and recover highly responsive genotypes. Jacobsen and Sopory (1978) also showed the possibility for sexual transfer of the ability to form embryos in order to obtain more efficient clones in the development of embryos. Therefore, incorporating genes controlling high responsiveness to anther culture into non- or low responsive genotypes can enhance androgenesis (Singsit and Veilleux, 1989).

Leptines in potato

Tubers of potato contain small quantities of naturally occurring chemicals called steroidal glycoalkaloids, a class of potentially toxic compounds, which are found throughout the family Solanaceae (Dale and Mackay, 1994). About 95% of the total glycoalkaloids present in potatoes are accounted for by α-solanine and α-chaconine, both of which are structurally similar, being differently glycosylated forms of the aglycone, solanidine. Cultivars vary with regard to their inherent glycoalkaloid content; at low levels it is suggested that they may enhance potato flavor, but at higher concentrations (above 15 mg 100 g^{-1} fresh weight) they impart bitterness. Levels above 20 mg $100g^{-1}$

fresh weight are considered toxic for human consumption, resulting in various symptoms typically associated with food poisoning (Dale and Mackay, 1994). Glycoalkaloids present in certain tuber-bearing *Solanum* species have been implicated as resistance factors against the Colorado potato beetle (CPB) *Leptinotarsa decemlineata* (Say) (Tingey, 1984). Leptine glycoalkaloids produced by some accessions of *S. chacoense* are foliar specific (Sinden et al., 1986) and, therefore, are of special interest because the possibility of incorporating toxic products in the tubers of progeny developed by hybridization is reduced. Leaves of resistant *S. chacoense* contain solanine, chaconine, leptines, and leptinines, while most susceptible *S. tuberosum* leaves contain only solanine and chaconine. Leptine glycoalkaloids in *S. chacoense* leaves may be a resistance factor in this species. Leptine I is a potent CPB repellent, completely inhibiting feeding by the CPB (at a concentration of 1 nM). In contrast solanine and chaconine inhibit feeding only by 50% (at a concentration of 6 mM) in potato leaf discs infiltrated with the individual glycoalkaloids (Sinden et al., 1980).

2*n* pollen

As stated earlier, the tetrasomic inheritance of quantitative traits in autotetraploid potatoes is complex. The interaction pattern among four alleles in a tetraploid is potentially more complicated than that between two alleles in a diploid. The most straightforward use of 2*n* gametes in breeding would be the direct use of the dihaploids of 4*x S. tuberosum* cultivars. However such an approach is not useful in breeding since the resultant heterozygosity will be equal or below that of their parents (Maris, 1990). The use of 2*n* gametes for breeding at the diploid level, using dihaploids (2*x*), haploids derived from cultivated tetraploid (4*x*) potatoes, as source of acceptable tuber traits, and diploid species as a source of resistances to diseases, pests and environmental stresses, has been proposed by Chase (1963). Chase in his 'analytical breeding' method proposed to reduce the ploidy of tetraploid potato (2*n*=4*x*=48) to the dihaploid level (2*n*=2*x*=24), and conduct breeding at the diploid level. This method simplifies the genetic analysis, requires a smaller population and gives the possibility for introgression of genes directly

from the diploid wild *Solanum* species. Once the desirable diploid genotypes are obtained the tetraploid condition can be restored through sexual polyploidization.

Different mechanisms during the premeiotic, meiotic and postmeiotic stages of gamete formation may induce 2*n* gametes (Veilleux, 1985). Six distinct modes of 2*n* gamete formation were listed by Peloquin *et al.* (1989): (1) premeiotic doubling, (2) first division restitution (FDR), (3) chromosome replication during meiotic interphase, (4) second division restitution (SDR), (5) postmeiotic doubling and (6) apospory. In potato, the two most important modes of 2*n* gamete formation are FDR and SDR; in SDR, the abnormal event that leads to the formation of 2*n* gametes occurs as result of restitution following the second meiotic division. Abnormally fused spindles during the second meiotic division lead to FDR (Veilleux et al., 1982).

The genetic control of 2*n*gamete formation appears to be due to a few major recessive genes. Veilleux (1985) concluded that the trait is sufficiently heritable for easy manipulation in plants, regardless of the number of genes controlling it. This fact makes the 2*n* trait a good target for gene identification through methods such as bulked segregant analysis. If a gene is located it could be transferred into agronomically desirable diploids for crossing to tetraploids.

Simple sequence repeats or SSRs

SSRs are highly variable regions of animal and plant genomes that consist of tandemly repeated short DNA sequences (2-, 3- or 4-base pair elements) where frequent rearrangements result in differences in the number of repeats, even between closely related individuals. SSRs, also called microsatellites, are highly polymorphic, are distributed throughout the genomes of plants and animals (Tautz and Renz, 1984), and occur intragenically and intergenically (Weber, 1990).

The regions surrounding the repeats are highly conserved (Smulders, 1997), and can be used to design primers that will amplify across the repeat during a polymerase chain reaction (PCR). In this way, SSRs can be used as molecular markers that will

behave in a codominant manner. Differences in number of repeats between alleles will appear as different size bands after electrophoresis of the PCR products.

Veilleux et al. (1995) used seven SSR markers (among which five were found to be polymorphic in a potato diploid anther donor) to discriminate heterozygous from homozygous diploid plants regenerated from anther culture. These SSR markers were designed based on published potato gene sequences.

Eleven primer pairs derived from published sequences in the public databases, 24 primers designed from a genomic library enriched for microsatellites, 12 primers reported previously and 14 primer pairs developed in tomato were used by Chani (1998) to look for polymorphism in a monoploid potato family derived by anther culture. Eleven of those primers were found to be polymorphic (Chani, 1998).

Randomly amplified polymorphic DNA (RAPDs)

Randomly amplified polymorphic DNA (RAPDs) are molecular markers that behave as dominant alleles. With RAPDs short primers are used to target homologous sites in the genome, amplifying random sequences (Williams et al., 1991). RAPDs are not as polymorphic as SSRs. Polymorphism in RAPDs is revealed by different amplified fragments, due to differences in the frequency of target DNA sequences. RAPDs have been used in studies of *Solanum* species to select somatic hybrids (Baird et al., 1992) and to detect variation among anther-derived monoploids (Singsit and Ozias-Akins, 1993). Hosaka and Hanneman (1994) detected segregation in an F_2 potato population using RAPDs. An analysis of immature microspore-derived embryos was done in *Brassica napus* to find polymorphism between parents segregating in the embryos (Horn and Rafalski, 1992).

Many researchers have used RAPDs combined with bulk segregant analysis (BSA) to identify linkage of molecular markers to genes of interest (Penner, 1993; Wight et al., 1994; Gianfranceschi, 1994; Poulsen et al., 1995; Francis, et al., 1995; Tanhuanpää, et al., 1996; Jiang and Sink, 1996; Yang et al., 1997; Ukoskit et al., 1997; Hu et al., 1997; Chèvre et al., 1997; Ronning. et al., 1999).

Bulk segregant analysis (BSA)

Michelmore et al. (1991) developed a procedure termed bulk segregant analysis, to identify RAPD markers linked to a disease resistance gene in lettuce. BSA eliminates the need for near-isogenic lines because only a segregating population for a trait of interest is needed (when the trait can be clearly scored). BSA is based on the comparison between two DNA bulks, each comprised of DNA from individuals exhibiting the extreme phenotypes (i.e., high and low) of a particular trait in a segregating population. Due to the selection, the bulks have completely random genotype for most of the genome, except in the region around the gene confering the characteristic of interest. If one selects for resistance to a pathogen, the presence of polymorphism between the amplification pattern of the two bulks is expected only for those bands that are genetically linked to the resistance genes (Giovannoni et al., 1991). Because RAPDs are generally known to be sensitive to reaction conditions, once a RAPD band linked to the trait has been identified, the next common step is to design sequence characterized amplified region (SCARs), which are markers with specific primers that increase reproducibility.

BSA was used to identify two RAPD markers linked to *Liriomyza trifolii* Burgesss resistance in a population derived from a cross between *L. esculentum* cv. Moneymaker and *L. hirsutum* Humb. and Bonpl., known for its resistance to the pest (Moreira et al., 1999). Ronning, et al. (1999) used BSA to identify two RAPD markers linked in repulsion to leptine content in a cross between two clones of *S. chacoense*, one of them being a high leptine producer and the other that does not produce leptines (Ronning, et al., 1999). BSA was also used to identify RFLP markers in the chromosomal location of the major gene Ry_{adg} controlling extreme resistance to potato virus Y (PVY) in a segregating population of diploid *S. tuberosum* subsp. *andigena* (Hämäläinen, et al., 1997).

Objectives

The main objectives of this dissertation are:

1) To analyze the segregation patterns of SSR alleles at four loci in three backcross populations;
2) To phenotypically characterize two backcross populations for anther culture response, and use bulk segregant analysis to identify RAPD markers linked to genes that control this trait;
3) To phenotypically characterize unreduced pollen production in one backcross family segregating for the trait;
4) To phenotypically characterize two reciprocal backcross populations for leptine content to and use BSA to identify RAPD markers linked to genes that control this trait;

References

Accatino, P. and P. Malagamba. 1982. Potato production from true seed. International Potato Center, Lima, Peru.

Afele J.C. and L.W. Kannenberg. 1990. Genetic studies of corn (*Zea maiz* L.) anther culture response. Theor. Appl. Genet. 80:459-464.

Agache, S., B. Bachelie, J. de Buyser, Y. Henry, and J. Snape. 1989. Genetic analysis of anther culture response in wheat using aneuploid chromosome substitution and translocation lines. Theor. Appl. Genet. 77:7-11.

Baird, E., S. Cooper-Bland, R. Waugh, M. DeMaine, and W. Powell. 1992. Molecular characterization of inter and intraspecific somatic hybrids of potato using randomly amplified polymorphic DNA (RAPD) markers. Mol. Gen. Genet. 233:469-476.

Bajaj, Y. P. S. 1987. Biotechnology and 21st century potato. In: Y. P. S., Bajaj (ed.) Biotechnology in Agriculture and Forestry, Vol 3: Potato. Springer-Verlag, Berlin, pp.3-22.

Burton, W.G. 1989. The potato. Longman Scientific &Technical copublished in the U.S. with John Wiley & sons, Inc., New York.

Cappadocia, M., D.S.K.Cheng and R. Ludlum-Simonette. 1984. Plant regeneration from *in vitro* culture of anthers of *Solanum chacoense* Bitt. and interspecific diploid hybrids *S. tuberosum* L. x *S. chacoense* Bitt. Theor. Appl. Genet. 69:139-143.

Carputo, D, L. Monti, J.E. Werner and L. Frusciante. 1999. Uses and usefulness of endosperm balance number. Theor. Appl. Genet. 98:478-484.

Chani, Eduard. 1998. Molecular marker analysis of a segregating monoploid potato family. Ph.D. Dissertation. Virginia Polytechnic Institute and State University. Blacksburg, Virginia.

Chase, S. C. 1963. Analytic breeding in *Solanum tuberosum* L. A scheme utilizing panthenotes and other diploid stocks. Can. J. Genet. Cytol. 5: 359-363.

Chèvre, A.M., P. Barret, F. Eber, P. Dupuy, H. Brun, X. Tanguy, and M. Renard. 1997. Selection of stable *Brassica napus* B. *juncea* recombinant lines resistant to blackleg (*Leptosphaeria maculans*). 1. Identification of molecular markers, chromosomal and genomic origin of the introgression. Theor. Appl. Genet. 95:1104-1111.

Cloutier, S., M. Cappadocia, and B.S. Landry. 1995. Study of microspore-culture responsiveness in oilseed rapes (*Brassica napus* L.) by comparative mapping of a F2 population and two microspore-derived populations. Theor. Appl. Genet. 91:841-847.

Dale, M.F.B. and G.R. Mackay. 1994. Inheritance of table and processing quality. In: J.E. Bradshaw and G.R. Mackay (Eds.), Potato Genetics, Scottish Crop Research Institute, Invergowrie, Dundee pp. 285-315.

Dunwell, J. M. 1985. Anther and ovary culture. In: S. W. J. Bright and M. G. K. Jones (Eds.), Cereal Tissue and Cell Culture, Kluwer Academic Publishers. pp.1-44.

Foroughi-Wehr, B., W. Friedt, and G. Wenzel. 1982. On the genetic improvement of androgenetic haploid formation in *Hordeum vulgare* L. Theor. Appl. Genet. 62:233-239.

Francis, H.A., A.R.Leitch, and R.M.D. Koebner. 1995. Conversion of a RAPD-generated PCR product, containing a novel dispersed repetitive element, into a fast and robust assay for the presence of rye chromatin in wheat. Theor. Appl. Genet. 90:636-642.

Gianfranceschi, L., J.M. McDermott, N. Seglias, B. Koller, M. Kellerhals, and C. Gessler. 1994. Towards a marker assisted breeding for resistance against apple scab. Euphytica 77:93-96.

Giovannoni, J.J., R.A. Wing, M.W. Ganal and S.D. Tanksley. 1991. Isolation of molecular markers from specific chromosomal intervals using DNA pools from existing mapping population. Nucleic Acid Res. 19:6553-6558.

Golmirzaie, A. M., P. Malagamba, N. Pallais. 1994. Breeding potatoes based on true seed propagation. In: J.E. Bradshaw and G.R. Mackay (Eds.), Potato Genetics, Scottish Crop Research Institute, Invergowrie, Dundee. pp.499-514.

Hämäläinen, J.H., K.N. Watanabe, J.P.T. Valkonen, A. Arihara, R.L. Plaisted, E. Pehu, L. Miller, and S.A. Slack. 1997. Mapping and marker-assisted selection for a gene for extreme resistance to potato virus Y. Theor. Appl. Genet. 94:192-197.

Hanneman, R.E., Jr. 1994. Assignment of endosperm balance numbers to the tuber bearing Solanums and their close non-tuber-bearing relatives. Euphytica 74:19-25.

Hanneman, R.E., Jr., J.B. Bamberg, J.B. Dodd, M.K. Ehlenfeldt, and S.A. Johnston. 1990. The genetics of the endosperm balance number (EBN). In: MacKerron, D.K.L., Edmond, H.D., Hall, D., Kirkman, M.A., Lang, R.W., Mackay, G.R., McRae, D.C. and Oxley, S.J.P. (eds), *Abstract of the 11th Triennial Conference of the European Association for Potato Research.* Edinburgh, UK pp. 124-125.

Hawkes J.G., and J.P. Hjerting. 1989. The Potatoes of Bolivia: Their Breeding Value and Evolutionary Relationships. Clarendon Press, Oxford.

Hawkes, J.G. 1962. Introgression in certain wild potato species. Euphytica 11:26-35.

Hawkes, J.G. 1994. Origins of cultivated potatoes and species relationships In: J.E. Bradshaw and G.R. Mackay (Eds.), Potato Genetics, Scottish Crop Research Institute, Invergowrie. pp.3-42.

Horn, P. and A. Rafalski. 1992. Non-destructive RAPD genetic diagnostics of microspore-derived *Brassica* embryos. Plant Molec. Biol. Rep. 10:285-293.

Horton, D. 1987. Potatoes: production, marketing, and programs for developing countries. Westview Press. Winrock development-oriented literature series. IT Publications London.

Hosaka, K., and R.E. Hanneman. 1994. Random amplified polymorphic DNA markers detected in a segregating hybrid population of *Solanum chacoense* x *S.phureja*. Jpn. J. Genet. 69:53-56.

Hu, X.Y., H.W. Ohm and I. Dweikat. 1997. Identification of RAPD markers linked to the gene *PM*1 for resistance to powdery mildew in wheat. Theor. Appl. Genet. 94:832-840.

Irikura, Y. 1975. Induction of haploid plants by anther culture in tuber-bearing species and interspecific hybrids of *Solanum*. Potato Res. 18:133-140.

Jacobsen, E. and Sopory, S. K. 1978. The influence and possible recombination of genotypes on the production of microspore embryoids in anther cultures of *Solanum tuberosum* and dihaploid hybrids. Theor. Appl. Genet. 52:119-123.

Jiang, Chunxiao and Sink, Kenneth C. 1997. RAPD and SCAR markers linked to the sex expression locus *M* in asparagus. Euphytica 94:329-333.

Johnston, S.A., T.P.M. den Nijs, S.J. peloquin and R.E. Hanneman Jr. 1980. The significance of genetic balance to endosperm development in interspecific crosses. Theor. Appl. Genet. 57:5-9.

Maris, B. 1990. Comparison of diploid and tetraploid potato families derived from *Solanum phureja* × dihaploid *S. tuberosum* hybrids and their vegetatively doubled counterparts. Euphytica 46:15-34.

Mendiburo, A. O., Peloquin, S. J. and Mok. D. W. S. 1974. Potato breeding with haploids and 2n gametes. In: Kasha K. J. (ed.) Haploids in Higher Plants: Advances and potential. University Press, Guelph. pp. 249-258.

Meyer, R., F. Salamini, and H. Uhrig. 1993. Isolation *and* characterization of potato diploid clones generating a high frequency of monohaploid or homozygous diploid androgenetic plants. Theor. Appl. Genet. 85:905-912.

Meyer, R., F. Salamini, and H. Uhrig. 1992. Biotechnology and plant breeding: relevance of cell genetics in potato improvement. Proc. Roy. Soc. Edinburgh Sect. B 99:11-21.

Miah, M. A. A., Earle, E. D. and Kush, G. S. 1985. Inheritance of callus formation ability in anther cultures of rice, *Oriza sativa* L. Theor. Appl. Genet. 70:113-116.

Michelmore, R.W., I. Paran and R.V. Kesseli. 1991. Identification of markers linked to disease –resistance genes by bulked segregant analysis: a rapid method to detect

markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. 88:9828-9832.

Moreira, L.A., C. Mollema, and S. van Heusden. 1999. Search of molecular markers linked to *Liriomyza trifolii* resistance in tomato. Euphytica 109:149-156.

Murigneux, A., S. Bentolila, T. Hardy, S. Baud, C. Guitton, H. Jullien, S. Ben Tahar, G. Freyssinet, and M. Beckert. 1994. Genotypic variation of quantitative trait loci controlling *in vitro* androgenesis in maize. Genome 37:970-976.

Ochoa, C. 1990. The potatoes of South America: Bolivia. Cambridge University Press. Cambridge, UK. Published in collaboration with the International Potato Center.

Ortiz, R., and M.K. Ehlenfeldt. 1992. The importance of endosperm balance number in potato breeding and the evolution of tuber-bearing *Solanum* species. Euphytica 60:105-113.

Peloquin, S.J., G.L. Yerk and J.E. Werner. 1989. Ploidy manipulation in the potato. In: Adolph, K.W. (ed.) Chromosomes: Eukaryotic, Prokaryotic, and Viral. CRC Press, Boca Raton, Florida. Vol.II, pp.167-178.

Penner, G.A., CP. Wight, S.J Molnar and G. Fedak. 1993. Identification of an RAPD marker for the crown rust resistance gene *Pc68* in oats. Genome 36:818-820.

Petolino, J. F. and Thomson, S.A. 1987. Genetic analysis of anther culture response in maize. Theor. Appl. Genet. 74:284-286.

Poulsen, D.M.E., R.J. Henry, R.P. Johnston, J.A.G. Irwin and R.G. Rees. 1995. The use of bulk segregant analysis to identify a RAPD marker linked to leaf rust resistance in barley. Theor. Appl. Genet. 91:270-273.

Rakoczy-Trovanowska, M. and Malepszy, S. 1995. Genetic factors influencing the regeneration ability of rye (*Secale cereale* L.) II. Immature embryos. Euphytica 83:233-239.

Raquin, C. 1982. Genetic control of embryo production and embryo quality in anther culture of petunia. Theor. Appl. Genet. 63:151-154.

Rivard, S. R., Cappadocia, M., Vincent G., Brisson N. and Landry, B. S. 1989. Restriction fragment length polymorphism (RFLP) analysis of plants produced by *in vitro* anther culture of *Solanum chacoense* Bitt. Theor. Appl. Genet. 78:49-56.

Ronning, C.M., J.R. Stommel, S.P. Kowalski, L.L. Sanford, R.S. Kobayashi and O. Pineda. 1999. Identification of molecular markers associated with leptine

production in a population of *Solanum chacoense* Bitter. Theor. Appl. Genet. 98:39-46.

Ross, H. 1986. Potato Breeding: problems and perspectives. Verlag Paul Parey, Berlin. 132 pp.

Sinden, S.L., L.L. Sandford and K.L. Deahl. 1986. Segregation of leptine glycoalkaloids in *Solanum chacoense* Bitter. J. Agric. Food Chem. 34:372-377.

Sinden, S.L., L.L. Sanford and S. F. Osman. 1980. Glycoalkaloids and Resistance to the Colorado potato beetle in *Solanum chacoense* Bitter. Am. Potato. J. 57:331-343.

Singsit, C. and Veilleux, R. E. 1989. Intra and interspecific transmission of androgenetic competence in diploid potato species. Euphytica 43:105-112.

Singsit, C., and Ozias-Akins, P. 1993. Genetic variation in monoploids of diploid potatoes and detection of clone-specific random amplified polymorphic DNA markers. Plant Cell Rep. 12:144-148.

Smulders, M.J.M., G. Bredemeijer, W. Rus-Kortekaas, P. Arens, and B. Vosman. 1997. Use of short microsatellites from database sequences to generate polymorphisms among *Lycopersicon esculentum* cultivars and accessions of other *Lycopersicon* species. Theor. Appl. Genet. 97:264-272.

Snider, K.T. and Veilleux, R. E. 1994. Factors affecting variability in anther culture and in conversion of androgenic embryos of *Solanum phureja*. Plant Cell Tiss. Org. Cult. 36:345-354.

Sonnino, A., Tanaka, S., Iwanaga, M. and Schilde-Rentschler, L. 1989. Genetic control of embryo formation in anther culture of diploid potatoes. Plant Cell Rep. 8:105-107.

Sopory, S. K. and Rogan, R. G. 1976. Induction of pollen divisions and embryoid formation in anther cultures of some dihaploid clones of *Solanum tuberosum*. Plant Sci. Lett. 12:47-54.

Sopory, S. K., Jacobsen, E. and Wenzel, G. 1978. Production of monohaploid embryoids and plantlets in cultured anthers of *Solanum tuberosum*. Plant Sci.Lett. 12:46-54.

Tanhuanpää, P.K., J.P. Vilkki and H.J. Vilkki. 1996. Mapping of a QTL for oleic acid concentration in spring turnip rape (*Brassica rapa* ssp. *oleifera*) Theor. Appl. Genet. 92:952-956.

Tautz, D. and Renz, M. 1984. Simple sequences as a general source of polymorphic DNA markers. Nucleic Acids Res. 17:6463-6471.

Taylor, T. E. and Veilleux, R. E. 1992. Inheritance of competencies for leaf disc regeneration, anther culture, and protoplast culture in *Solanum phureja* and correlations among them. Plant Cell Tiss. Org. Cult. 31:95-103.

Tingey W.M. 1984. Glycoalkaloids as pest resistance factors. Am. Potato J. 61:157-167.

Uijtewaal, B. A., D.J. Huigen, J.G.T. Hermsen. 1987. Production of potato monohaploids (2n=x=12) through prickle pollination. Theor. Appl. Genet. 73:751-758.

Uijtewaal, B. A., Jacobsen, E. and Hermsen J. G. T. 1987. Morphology and vigour of monohaploid potato clones, their corresponding homozygous diploids and tetraploids and their heterozygous diploid parent. Euphytica 36:745-753.

Ukoskit, K., P.G. Thompson, and C.E. Watson, Jr. 1997. Identifying a randomly amplified polymorphic DNA (RAPD) marker linked to a gene for root-knot nematode resistance in sweetpotato. J. Amer. Soc. Hort. Sci. 122:818-821.

Van Breukelen, E.W., M.S. Ramanna, J.G.T. Hermsen. 1975. Monohaploids (n=x=12) from autotretaploids *Solanum tuberosum* (2x-4x=48) through two successsive cycles of female parthenogenesis. Euphytica 24:567-574.

Veilleux, R.E., N.A. McHale, F.L. Lauer. 1982. 2*n* gametes in diploid *Solanum*: frequency and types of spindle abnormalities. Canadian Journal of Genetics and Cytology 24:301-314.

Veilleux, R.E. 1985. Diploid and polyploid gametes in crop plants: mechanisms of formation and utilization in plant breeding. Plant Breeding Rev. 3:253-288.

Veilleux, R. E., L.Y. Shen and M.M. Paz. 1995. Analysis of the genetic composition of anther- derived potato by randomly amplified polymorphic DNA and simple sequence repeats. Genome 38:1153-1162.

Veronneau, H., Lavoie, G. and Cappadocia M. 1992. Genetic analysis of anther and leaf disc culture in two clones of *Solanum chacoense* Bitt. and their reciprocal hybrids. Plant Cell Tiss. Org. Cult. 30:199-209.

Wan, Y., T.R. Rocheford and J.M. Widholm. 1992. RFLP analysis to identify putative chromosomal regions involved in the anther culture response and callus formation of maize. Theor. Appl. Genet. 85:360-365.

Weber, J. L. 1990. Informativeness of human $(dC\text{-}dA)_n*(dG\text{-}dT)_n$ polymorphisms. Genomics 7:524-530.

Wenzel, G. and Uhrig, H. 1981. Breeding for nematode and virus resistance in potato via anther culture. Theor. Appl. Genet. 59:333-340.

Wenzel, G., Schieder, O., Przewozny, T., Sopory, S. K. and Melchers, G. 1979. Comparison of single cell culture derived *Solanum tuberosum* L. plants and a model for their application in breeding programs. Theor. Appl. Genet. 55:49-55.

Wight, C.P., G.A. Penner, L.S. O'Donoughue, V.D. Burrows, S.J. Molnar and G. Fedak. 1994. The identification of random amplified polymorphic DNA markers for daylength insensitivity in oat. Genome 37:910-914.

Williams, J. G. K., Kubelik, A.R., Livak, K. J., Rafalski, J. A. and Tingey S. V. 1991. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 18:6531-6535.

Yang, H.Y., S.S. Korban, J. Krüger and H. Schmidt. 1997. The use of a modified bulk segregant analysis to identify a molecular marker linked to a scab resistance gene in apple. Euphytica 94:175-182.

Chapter II: SSR analysis of backcross populations between *Solanum chacoense* and *S. phureja*

Abstract

Simple sequence repeats (SSRs) were used to analyze segregating backcross populations originating from a cross between the diploid species *Solanum phureja* (1-3) and *Solanum chacoense* (80-1). Using CP2 (a self-incompatible hybrid), three populations were developed: 1-3 × CP2 (PBCp), CP2 × 1-3 (PBCc), and CP2 × 80-1 (CBC). The analysis was done using four SSR primer pairs that amplified polymorphic fragments to look at the segregation ratios and to eliminate possible spurious genotypes from the populations. Seventeen spurious genotypes were identified and discarded from PBCp, whereas none was found in PBCc or CBC. Skewed segregation ratios were encountered for two of the four SSR loci in the PBCp populations, while its reciprocal backcross (PBCc) showed normal segregation ratios at all loci. The CBC population showed distorted segregation at one of the four loci observed. In the PBCp population the distorted segregation favored 80-1 alleles transmitted through pollen. In CBC, the distorted locus was characterized by a deficiency of homozygotes.

Introduction

Simple sequence repeats (SSRs) are regions of short tandemly repeated DNA motifs (two to five nucleotides) that are abundant in animal and plant genomes. SSRs have been widely adopted as polymerase chain reaction (PCR)-based markers to study human genetics (Hammond et al, 1994; Weber and Wong, 1993; Eduards et al., 1992). SSR polymorphism is based on differences in the number of repeated motifs within the DNA. Such differences can be observed as different size bands (alleles) during electrophoresis of PCR products amplified using primers flanking the motifs. Simple sequence repeats have demonstrated high levels of polymorphism in many plant species including soybean [*Glycine max* (L.) Merr.] (Morgante et al., 1994), barley [*Hordeum*

vulgare L.] (Saghai Maroof et al., 1994), grapevine [*Vitis vinifera* L.] (Thomas and Scott 1993), sunflower [*Helianthus annuus* L.] (Brunel, 1994), tomato, [*Lycopersicon esculentum* Mill.] (Phillips et al., 1994), rice [*Oryza sativa* L.] (Wu and Tanksley, 1993), maize [*Zea mayz* L.] (Senior and Heun, 993), *Brassica* spp. (Poulsen et al., 1993), pine [*Pinus* spp.] (Smith and Devey. 1994), wild yam [*Dioscorea tokoro* Makino](Terauchi and Konuma, 1994) and potato [*Solanum tuberosum* L.] (Milbourne et al., 1998).

The high polymorphism, abundance, and ease of assay make SSRs ideal DNA markers for genetic mapping and population studies (Liu et al., 1997). Pejic et al. (1998), in a comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, SSRs and AFLPs, showed that the number of alleles detectable in maize by SSRs was higher compared to other methods. This high level of polymorphism is expected because of the unique mechanism responsible for generating SSR allelic diversity by replication slippage (Tautz et al., 1986). PCR-based markers, like SSRs have technical advantages over RFLP analysis because the amount of DNA that PCR-based markers require is small and polymorphism is revealed without the need for probe hybridization. SSRs show co-dominant inheritance and, since they are amplified under high stringency PCR, do not have the reproducibility problem associated with RAPDs (Schierwater and Ender, 1993). Linkage maps of several crops have been enriched by the integration of SSR loci. The development of linkage maps in crop plants may increase the speed and precision of plant breeding programs (Milbourne et al., 1998).

SSRs have been used in germplasm analysis and fingerprinting of cultivars and accessions of tomato (Smulders et al., 1997), soybean (Diwan and Cregan, 1997), maize (Taramino and Tingey, 1996; Pejic et al., 1998), grape (Bowers et al., 1996), *Arabidopsis* (Van Treuren et al., 1997), barley (Liu et al., 1997), rice (Provan et al., 1997) and potato (Provan et al., 1996a; Provan et al., 1996b; Schneider and Douches, 1997). Several SSR loci have been characterized for potato (Veilleux et al., 1995; Milbourne et al., 1998) based on published sequences in databases and by screening genomic or cDNA libraries. Segregation of SSR alleles in plant populations has not been extensively studied. In

Citrus spp. two of nine SSRs showed significantly skewed segregation in segregating populations developed from intergeneric crosses with *Poncirus* (Kijas et al., 1997).

We have been working with interspecific diploid potato hybrids between the weedy species, *Solanum chacoense* Bitt. and the primitive cultivated species, *S. phureja* Juz. and Buk. Our objective in this study was to determine segregation patterns of four SSR loci in diploid backcross populations of potato.

Materials and Methods

Plant populations

CP2, a weak and self-incompatible hybrid originated from a cross between *Solanum chacoense* Bitt. (clone 80-1) and *S. phureja* Juz. & Buk. (clone 1-3), was backcrossed to both parents. Three of the resultant backcross populations, developed by S.M. Piovano, Department of Horticulture, VPI&SU, were used in this study: PBCp (1-3 × CP2), PBCc (CP2 × 1-3) and CBC (CP2 × 80-1) comprising 108, 45, and 42 genotypes, respectively. Up to 48 genotypes per family were used for segregation analysis. However, for the PBCp population, all 108 genotypes were used for analysis with two primer pairs due to the presence of spurious genotypes in this family (see Table 2). The plants were grown in a greenhouse under 16 h photoperiod at 25°C day/15°C night at different periods between 1996 and 1997. Leaflets of each genotype were picked, labeled and immediately frozen in liquid nitrogen. The samples were then stored in an -80°C freezer before DNA extraction.

SSR analysis

Total genomic DNA was extracted according to Doyle and Doyle (1987), a procedure that is a modification of the 2x CTAB method developed by Saghai Maroof et al. (1984)(procedure described in appendix). Potato DNA sequences containing SSRs were identified from the EMBL database using the DNASTAR program. Twenty-two SSRs were found and primers flanking the repeats were designed using the software PRIMER ver. 0.5. Four primer pairs proven to be polymorphic in this populations (Chani,

1998) were used: RV3-4, RV5-6; RV15-16; and RV19-20 (Table 1). Three of these loci were mapped to linkage groups by Milbourne et al. (1998). The locus amplified by primers RV 3-4 is located in linkage group VIII. The locus amplified by primers RV 15-16 was mapped to linkage group VII. The locus amplified by primers RV 19-20 was mapped to chromosome VI. The locus amplified by primers RV 5-6 was not mapped.

Amplification conditions were based on the procedure of Yu et al. (1994). PCR amplification reactions were prepared in a total volume of 25 µl containing 3 mM $MgCl_2$, 1× PCR buffer (50 mM KCl, 10 mM Tris HCl (pH 9), 1% Triton X-100), 160 µM each of the dNTPs, 0.1 µM of each reverse and forward primer, 1.5 U Taq DNA polymerase (Promega) and 50 ng genomic DNA as template for PCR. Two different thermal cyclers were used, a Perkin Elmer Cetus 480, for which the reaction mixtures were overlaid with a drop of mineral oil, and an Amersham robocycler, for which mineral oil was not required. The cycling conditions consisted of 1 min at 94°C (denaturation), 2 min at 55°C (annealing), and 1.5 min at 72°C (extension), followed by one cycle of 5 min at 72°C for final extension. After PCR, 5 µl of loading dye were added to each reaction mixture. The amplified reactions (15 µl) were run by electrophoresis in 3% Metaphor agarose (FMC Bioproducts, Rockland, Maine) gel. The gel was run with 1× TBE buffer at 100 V for 4 h and was stained with ethidium bromide (1.5 µg ml^{-1}) for 15 min, de-stained in tap water for 20 min and photographed under UV light.

DNA analysis

The primer pairs were chosen based on the relative ease to determine polymorphism between parental lines (1-3 and 80-1). Genotypes from each population were analyzed always along with the parental lines and CP2. Letter codes were assigned to the different bands based on the parental bands, and on gel pictures from monoploids developed from CP2 and 1-3 (Chani, 1998). The expected and observed segregation ratios were analyzed by Chi-square tests.

Results

Elimination of contaminating genotypes by detecting the presence of spurious bands

Based on prior RAPD analysis (Edna Fogelmann, personal communication), we suspected that the PBCp population had been contaminated with genotypes that expressed non-parental markers, perhaps through carelessness during pollination or seed extraction. However, we wanted to verify this with the more repeatable technique of SSR analysis. The gels were observed for the presence of any non-parental SSR allele at all four loci (Figures 1 and 2). Of 108 genotypes in the PBCp population, 13 were discarded due to the presence of spurious bands at one or more SSR locus (Table 2). The effective population size of PBCp was therefore reduced to 95 genotypes for segregation analysis. Spurious bands (Figures 1 and 2) were found only in the PBCp population; PBCc and CBC genotypes only expressed parental alleles.

Segregation patterns

The expected segregation patterns varied depending on whether the parents differed by one or more alleles and were determined for each primer pair as depicted in Tables 2 and 3. PBCp and PBCc are reciprocal backcrosses; therefore, similar segregation patterns were expected. PBCc exhibited normal segregation for all loci whereas PBCp showed skewed segregation for two of the four SSR loci, amplified by primers RV 15-16 ($\chi^2 = 11.48$; p=0.003), and RV 19-20 ($\chi^2 = 6.75$; p=0.009) (Figures 1 and 2, Table 3).

The CBC population showed distorted segregation ratios for one of the four SSR loci (Table 4). The skeweness was observed in the locus amplified by primers RV 19-20 ($\chi^2 = 13.8$; p= 0.003) which showed an excess of heterozygotes (AB) and a reduced number of plants homozygous for the B allele originating from 1-3 (Table 4).
(Data for all genotypes in the three populations for SSR allele assignment is shown in Tables1, 2 and 3 of the Appendix section)

Discussion

For the SSR analysis it was critical to find spurious genotypes that were contaminating one of the populations (PBCp). A group of 45 plants, from which 10 spurious genotypes were identified, was checked first with all four SSR markers. For these plants only two primer pairs (RV 3-4 and RV 15-16) were necessary to reveal spurious genotypes; therefore, the rest of the plants were screened using these two markers. The contamination of the population could be attributed to foreign pollen, from which some seeds received a different allele/alleles that were not present in CP2 or either of the parents. Plants with spurious bands always had one band from 1-3 in addition to the spurious one. This indicated that 1-3 was indeed the maternal parent but some other pollen than that of CP2 had been used to pollinate 1-3.

Few reports have examined segregation ratios of microsatellite alleles in plants. Kijas et al. (1997) found two of nine SSR loci showing distorted segregation in a segregating population from an intergeneric cross between *Citrus* and *Poncirus*. In both cases, they observed excess heterozygotes and, in one case, a deficiency of homozygous *Citrus* alleles. In a study of *Quercus robur* L., various molecular markers were used, including SSRs, to develop a linkage map (Barreneche et al., 1998). Approximately 18% of these markers showed segregation distortion; in this case as well, they observed an excess of heterozygous genotypes.

In the present study, we found differences in segregation ratios between reciprocal backcross populations (PBCp and PBCc). For microsatellites amplified by primers RV 15-16 and RV 19-20, both of which showed distorted segregation in PBCp, there was preferential transmission of a *chacoense* allele (present in CP2) through pollen, while the segregation was normal in the reciprocal population (Table 3).

Use of the interspecific hybrid as a male parent could be expected to yield gametophytes that differ greatly in their genomic composition (i.e., relative frequency of *chacoense*/*phureja* genomes). The genomic composition may actually affect gametophytic vigor, resulting in certation and subsequent skewed segregation. The same locus (amplified by primers RV 19-20) exhibited skewness on favor of the *chacoense*

allele when CP2 was used to pollinate *phureja* (Table 3), but conversely the *phureja* allele was favored when CP2 was used to pollinate *chacoense* (Table 4).

Two of the SSRs used in this study were difficult to score due to heteroduplexes present together with true alleles as bands in the gels. We were able to distinguish between heteroduplex formations and true alleles thanks to preliminary data available (Chani, 1998). These data consisted of photographed gels of monoploid genotypes originating from CP2 and 1-3; because monoploids bear only a single allele, heteroduplex formation is not possible.

Two possibilities for skewed segregation ratios in these backcross populations would be pollen selection or selective advantage of a particular genotype in the progeny. Pollen selection could occur as a result of self-incompatibility (Cipar et al., 1964), i.e., the pollen grains with an S-allele that does not match that of the stylar parent could be expected to be more successful in these crosses. Any loci linked to the S-allele would therefore appear skewed. In potato, the self-incompatibility locus (S-locus) has been mapped to linkage group I (Kreike and Stiekema, 1997). None of the mapped loci exhibiting skewness is localized in this linkage group. The location of the proteinase inhibitor gene containing our microsatellite amplified by primers RV 5-6 is unknown. However, it did not exhibit skewness in any of our three backcross populations. Therefore, self-incompatibility is unlikely to have been a cause of skewness in these populations.

Hybrid breakdown has been reported in *S. phureja* × *S. chacoense* hybrids and indeed CP2 represents a relatively vigorous plant selected from a generally weak interspecific family (Veilleux and Miller, 1998). The exact cause of hybrid breakdown is unknown, but some type of genomic incompatibility is assumed. Plants in these backcross families were generally vigorous and did not exhibit the hybrid breakdown. However, weak seedlings may easily have been lost in handling the populations prior to DNA extraction. If unfavorable genomic compositions were linked to one of our SSR loci, then skewness may have resulted.

References

Barreneche, T., C. Bodenes, C. Lexer, J.F. Trontin, S. Flush, R.Streiff, C. Plomion, G. Roussel, H. Steinkellner, K. Burg, J.m. Favre, J. Glössl, and A. Kremer. 1998. A genetic linkage map of *Quercus robur* L. (peduncle oak) based on RAPD, SCAR, microsatellite, minisatellite, isozyme and 5S rDNA markers. Theor. Appl. Genet. 97:1090-1103.

Bowers, J.E., G.S. Dangl, R. Vignani, and C.P. Meredith. 1996. Isolation and characterization of new polymorphic simple sequence repeat loci in grape (*Vitis vinifera* L.). Genome 39:628-633.

Brunel, D. 1994. A microsatellite marker in *Helianthus annuus* L. Plant Molec. Biol. 24:397-400.

Chani, Eduard. 1998. Molecular marker analysis of a segregating monoploid potato family. Ph.D. Dissertation. Virginia Polytechnic Institute and State University. Blacksburg, Virginia.

Cipar, M.S., S.J. Peloquin, and R.W. Hougas. 1964. Inheritance of incompatibility in hybrids between *Solanum tuberosum* haploids and diploid species. Euphytica 13:163-172.

Diwan, N. and P.B. Cregan. 1997. Automated sizing of fluorescent-labeled simple sequence repeat (SSR) markers to assay genetic variation in soybean. Theor. Appl. Genet. 95:723-733.

Doyle, J.J. and J.V. Doyle. 1987. A rapid DNA isolation procedure for small amounts of leaf tissue. Phytochem. Bull. 19:810-815.

Edwards, A., H.A. Hammond, L. Jin, C.T. Caskey, and R. Chakraborty. 1992. Genetic variation at five trimeric and tetrameric tandem repeat loci in four human population groups. Genomics 12:241-253.

Hammond, H.A., L. Jin, Y. Zhong, C.T. Casey, and R. Chakraborty. 1994. Evaluation of 13 short tandem repeat loci for use in personal identification applications. Am. J. Hum. Genet. 55:175-189.

Kijas J.M.H., M.R. Thomas, J.C.S. Fowler, and M.L. Roose. 1997. Integration of trinucleotide microsatellites into a linkage map of *Citrus*. Theor. Appl. Genet. 94:701-706.

Kreike, C.M. and W.J. Stiekema. 1997. Reduced recombination and distorted segregation in a *Solanum tuberosum* (2*x*) × *S. spegazzinii* (2*x*) hybrid. Genome 40:180-187.

Liu, Z.-W., R.M. Biyashev, and M.A. Saghai Maroof. 1997. Development of simple sequence repeat DNA markers and their integration into a barley linkage map. Theor. Appl. Genet. 93:869-876.

Milbourne, D., R.C. Meyer, A.J. Collins, L.D. Ramsay, C. Gebhardt, and R. Waugh. 1998. Isolation, characterization and mapping of simple sequence repeat loci in potato. Mol. Gen. Genet. 259:233-245.

Morgante, M., A. Rafalski, P. Biddle, S. Tingey, and A.M. Olivieri. 1994. Genetic mapping and variability of seven soybean simple sequence repeat loci. Genome 37:763-769.

Pejic, I., P. Ajmone-Marsan, M. Morgante, V. Kozumplick, P. Castiglioni, G. Taramino, and M. Motto. 1998. Comparative analysis of genetic similarity among maize inbred lines detected by RFLPs, RAPDs, SSRs, and AFLPs. Theor. Appl. Genet. 97:1248-1255.

Phillips, W.J., C.G.D. Chapman, and P.L. Jack. 1994. Molecular cloning and analysis of one member of a polymorphic family of GACA-hybridizing DNA repeats in tomato. Theor. Appl. Genet. 88:845-851.

Poulsen, G.B., G. Kahl, and K. Weising. 1993. Abundance and polymorphism of simple repetitive DNA sequences in *Brassica napus* L. Theor. Appl. Genet. 85:994-1000.

Provan J., G. Corbett, J.W. McNicol, and W. Powell. 1996. Chloroplast DNA variability in wild and cultivated rice (*Oriza* spp.) revealed by polymorphic chloroplast simple sequence repeats. Genome 40:104-110.

Provan, J., W. Powell, and R. Waugh. 1996a. Analysis of cultivated potato (*Solanum tuberosum*) using intermicrosatellite amplification. Genome 39:767-769.

Provan, J., A. Kumar, L. Shepherd, W. Powell, and R. Waugh. 1996b. Analysis of intra-specific somatic hybrids of potato (*Solanum tuberosum*) using simple sequence repeats. Plant Cell Rep. 16:196-199.

Saghai Maroof, M.A., K.M. Soliman, R.A. Jorgensen, and R.W. Allard. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA 81:8014-8018.

Saghai Maroof, M.A., R.M. Biyashev, G.P. Yang, Q. Zhang, and R.W. Allard. 1994. Extraordinarily polymorphic microsatellite DNA in barley: Species diversity, chromosomal locations and population dynamics. Proc. Natl. Acad. Sci. 91:5466-5470.

Schierwater, B. and A. Ender. 1993. Different thermostable DNA polymerases may amplify different RAPD products. Nucleic Acids Res. 21:4647-4648.

Schneider, K. and D.S. Douches. 1997. Assesment of PCR-based simple sequence repeats to fingerprint north american potato cultivars. Am. Pot. J. 74:149-160.

Senior, M.L. and M. Heun. 1993. Mapping maize microsatellites and polymerase chain reaction confirmation of the targeted repeats using a CT primer. Genome 36:884-889.

Smith, D.N. and M.E. Devey. 1994. Occurrence and inheritance of microsatellites in *Pinus radiata*. Genome 37:977-983.

Smulders, M.J.M., G. Bredemeijer, W. Rus-Kortekaas, P. Arens, and B. Vosman. 1997. Use of short microsatellites from database sequences to generate polymorphisms among *Lycopersicon esculentum* cultivars and accessions of other *Lycopersicon* species. Theor. Appl. Genet. 97:264-272.

Taramino G. and S. Tingey. 1996. Simple sequence repeats for germplasm analysis and mapping in maize. Genome 39:277-287.

Tautz. D., M. Trick, and G.A. Dover. 1986. Cryptic simplicity in DNA is a major source of genetic variation. Nature 322:652-656.

Terauchi, R. and A. Konuma. 1994. Microsatellite polymorphism in *Dioscorea tokoro*, a wild yam species. Genome 37:794-801.

Thomas, M.R. and N.S. Scott. 1993. Microsatellite repeats in grapevine reveal DNA polymorphisms when analysed as sequence-tagged sites (STSs). Theor. Appl. Genet. 86:985-990.

Van Treuren, R., H. Kuittinen, K. Kärkkäinen, E. Baena-Gonzalez, and O. Savolainen. 1997. Evolution of microsatellites in *Arabis petraea* and *Arabis lyrata*, outcrossing relatives of *Arabidopsis thaliana.* Mol. Biol. Evol. 14(3):220-229.

Veilleux, R.E. and A.R. Miller. 1998. Hybrid breakdown in the F_1 between *Solanum chacoense* and *S. phureja* and gene transfer for leptine biosynthesis. J. Amer. Soc. Hort. Sci. 123:854-858.

Veilleux , R.E., L. Yin Shen, and M.M. Paz. 1995. Analysis of the genetic composition of anther-derived potato by randomly amplified polymorphic DNA and simple sequence repeats. Genome 38:1153-1162.

Weber, J.L. and C. Wong. 1993. Mutation of human short tandem repeats. Hum. Mol. Genet. 2:1123-1128.

Wu, K.S. and S.D. Tanksley. 1993. Abundance, polymorphism and genetic mapping of microsatellites in rice. Mol. Gen. Genet. 241:225-235.

Yu, Y.G., M.A. Saghai Maroof, G.R. Buss, P.J. Maughan, and S.A. Tolin. 1994. RFLP and microsatellite mapping of a gene for mosaic virus resistance. Phytopathology 84:60-64.

Primer	**Sequence 5'-3'**	**Locus**	**Accession number**	Repeat
RV 3 [a] RV 4	cccataatactgtcgatgagca gaatgtagggaaacatgcatga	G28WXST (Waxy gene)	X52417	$(actc)_5$
RV 5 [a] RV 6	cttgcaacttgttagtaccccc aaatcctttgtgacctcccc	STPROINI (Proteinase inhibitor gene)	Z12611	$(tc)_{12}(ta)_1$
RV 15 [b] RV16	aattcatgtttgcggtacgtc atgcagaaagatgtcaaaattga	STACCAS3 (Gene for 1-amino cyclopropane-1-ca)	Z27235	$(aag)_7$
RV 19 [b] RV 20	aataatactgtgatgccacaatgg gtggcatgtcttcgaaggtac	POTM 1-2 *S. tuberosum* Transcription factor 1-2	U23758	$(at)_{20}$

Table 1: SSR primer sequences, loci accession numbers and nature of repeats for four SSRs used to study segregation in three backcross potato families. [a] Taken from Veilleux et al. 1995. [b] Proven to be polymorphic by Chani, 1998

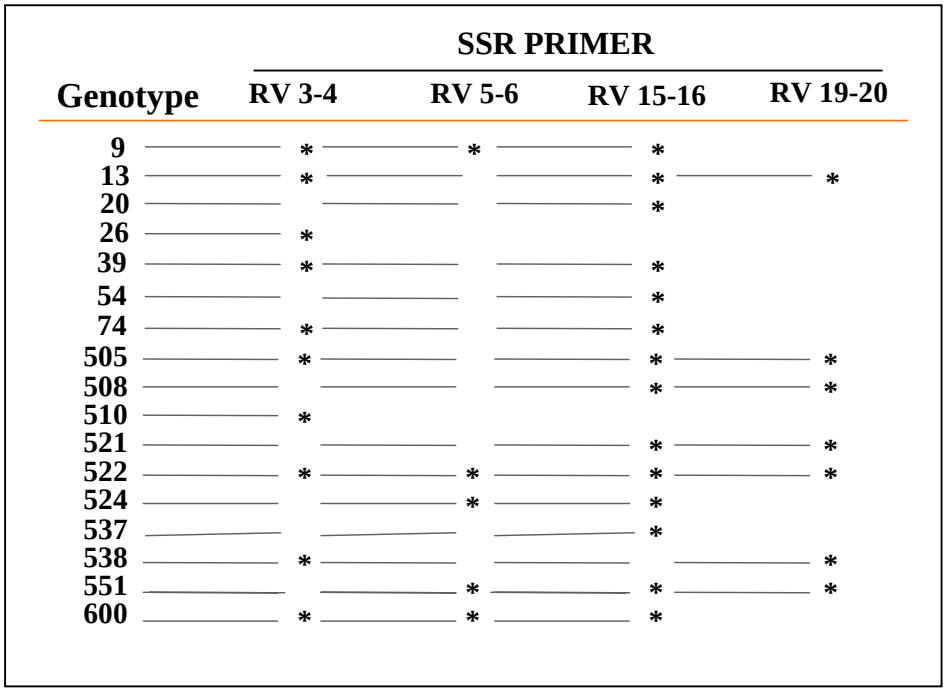

Table 2: Loci detecting spurious bands in PBCp family. Any genotype with a non-parental band detected in one or more loci was discarded from the population. All contaminating genotypes could be detected with primers RV 3-4 and 15-16.

SSR Primer	80-1 alleles	Parental Alleles 1-3	Parental Alleles CP2	Expected ratio	PBCp Observed segregation	PBCp χ^2	PBCc Observed segregation	PBCc χ^2
RV 3-4	AA	BC	AC	1AB:1AC:1BC:1CC	25:31:20:19	3.79	12:14:9:11	1.13
RV 5-6	B_[1]	C_[2]	BC	1B_:2C_[3]:1BC	10:26:12	0.50	9:23:12	0.50
RV 15-16	AA	BC	AB	1BB:2(AB+AC):1BC	15:64:16	11.48*	9:24:12	0.60
RV 19-20	AC	BB	AB	1AB:1BB	33:15	6.75*	16:26	2.40

Table 3: Allelic segregation at four SSR loci for the reciprocal populations. Skewed segregation at two loci in PBCp, show preferential transmission of 80-1 alleles through pollen

[1] Only one allele could be detected at this locus for 80-1

[2] Only one allele could be detected at this locus for 1-3

[3] Includes both C_ and CC genotypes

*Significantly skewed segregation, $P \leq 0.05$

PBCp = 1-3 × CP2; PBCc = CP2 × 1-3

SSR PRIMER	Parental alleles CP2	80-1	Expected	Observed	χ^2
RV 3-4	AC	AA	1A:1AC	23:19	0.38
RV 5-6	BC	B_[1]	1BC:2(B_+BB):1C	9:22:9	0.40
RV 15-16	AB	AA	1AA:1AB	20:21	0.12
RV 19-20	AB	AC	1AA:1AB:1AC:1BC	1:17:9:11	13.8*

Table 4: Allelic segregation at four SSR loci for CBC (CP2×80-1). Skewed segregation was found for two of the loci. For the locus amplified by primers RV 5-6, there is preferential transmission of 80-1 alleles through pollen. The skeweness in the locus amplified by primers RV 19-20 shows preference for transmission of 1-3 alleles present in CP2. [1] Only one allele could be detected at this locus for 80-1

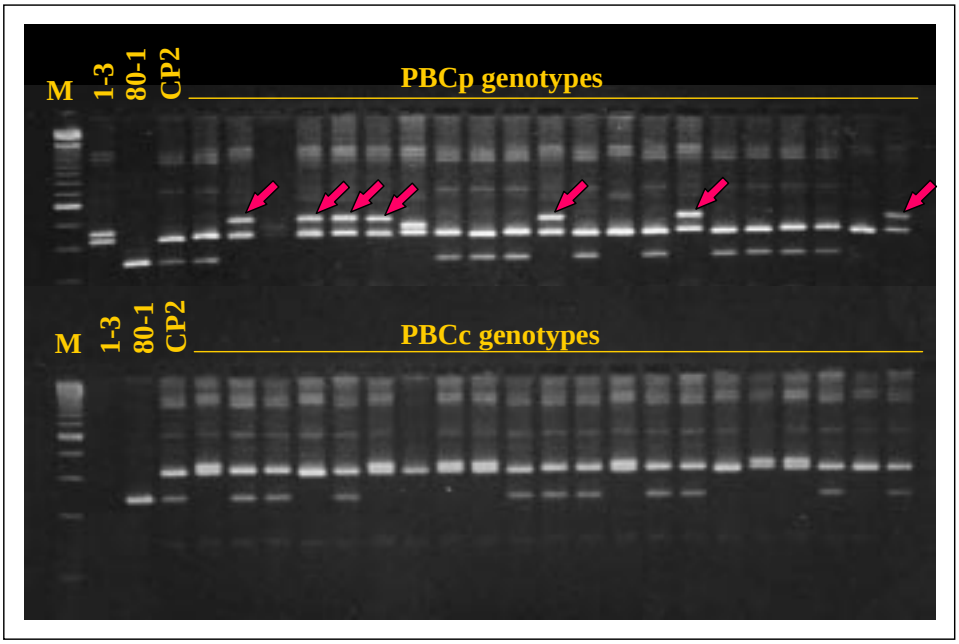

Figure 1: Amplification of a microsatellite locus by primers RV 15–16 for 1-3, 80-1, CP2 and 21 backcross genotypes of both PBCp and PBCc. CP2 and 1-3 share a common allele and each has a unique allele. Therefore the PBC backcrosses represent an AB×BC cross. It is not possible to observe the AC type, therefore a 1:2:1 segregation ratio is expected B: (AB+AC): BC. Arrows indicate non-parental bands

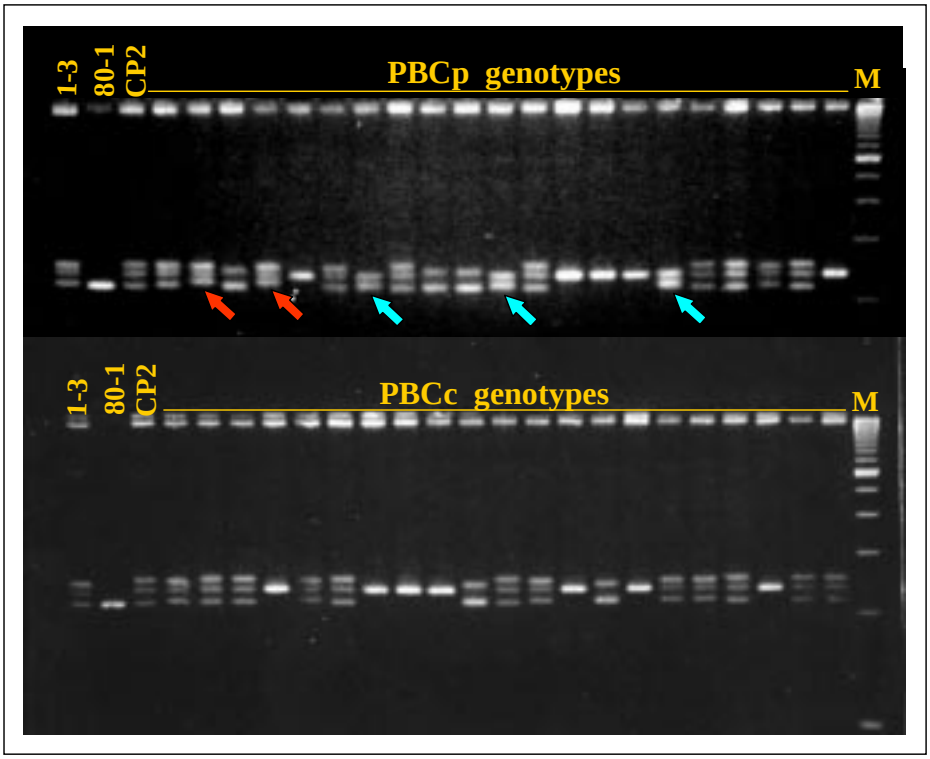

Figure 2: Amplification of a microsatellite locus by primers RV 3-4 for 1-3, 80-1, CP2 and 21 backcross genotypes of both PBCp and PBCc. CP2 and 1-3 share a common allele and each has a unique allele. The PBC backcrosses represent an AC×BC cross, with an expected 1:1:1:1 segregation ratio (AB:AC:BC:CC). Red and blue arrows indicate non-parental bands of two different patterns.

Chapter III: Phenotypic characterization and bulk segregant analysis of anther culture response (ACR) in two backcross families of diploid potato

Abstract

Two diploid (2*n*=2*x*=24) backcross potato populations (PBCp, and CBC) were characterized for anther culture response (ACR). PBCp (1-3 × CP2) and CBC (CP2×80-1) were derived from a cross between a self-incompatible hybrid, CP2 (intermediate ACR), and its parents, *Solanum chacoense* Bitt. clone 80-1(low ACR) and *S. phureja* Juz. & Buk. clone 1-3 (high ACR). Three components of ACR were investigated in a preliminary study of 24 genotypes of both PBCp and CBC: 1) embryos produced per anther (EPA), 2) embryo regeneration rate and 3) percentage of monoploids (2*n*=1*x*=12) among regenerants. Only EPA was selected for further characterization in our populations because the other two components were limited to high EPA genotypes and were too variable within genotypes for accurate phenotypic characterization. Further ACR/EPA characterization was done through a series of studies, using six to 11 genotypes per study comprising a total of 57 genotypes in CBC. In a series of five studies we identified nine high (mean EPA=3.7±2.3) and ten low (mean EPA=0.05±0.1) ACR selections within CBC. A series of four studies and a large experiment (44 genotypes) were done to identify ten high (mean EPA= 4.7±3.1) and ten low (mean EPA= 0.03±0.06) ACR selections among 78 genotypes of PBCp. High and low selections were used for bulk segregant analysis (BSA) to identify RAPD markers linked to EPA using 214 RAPD primers. A band amplified by OPQ-10 and another by OPZ-4 were linked in coupling and in repulsion, respectively, to ACR in PBCp. A band amplified by OPW-14 primer was linked in coupling to ACR in CBC. One-way ANOVAs using

presence/absence of each candidate band to classify genotypes in the entire population verified linkage of the markers to ACR/EPA.

Introduction

The first part of this study focuses on the phenotypic characterization of anther culture response (ACR), a trait of interest for potato breeding and genetics, using segregating populations developed by backcrosses of an interspecific hybrid and its parents, *Solanum phureja* Juz. & Buk. and *S. chacoense* Bitt. The phenotypic characterization was done to select extreme genotypes for the trait to be used in bulk segregant analysis.

Anther culture response is an important trait that is mostly present in a few genotypes in potato that are commercially unimportant. Anther culture is used to develop haploids; these haploid genotypes can be used in breeding programs. One major limitation in potato breeding is the unavailability of homozygous lines, because of self-incompatibility and inbreeding depression that are common in most genotypes. Doubled haploids could be developed by anther culture and used as inbred lines in breeding programs as proposed by Wenzel et al. (1979). Wenzel proposed an analytical breeding scheme based upon the development of homozygous doubled haploids by anther culture followed by intercrossing to obtain heterozygous diploids. These diploids could then be used to form tetraploid hybrids by protoplast fusion; these new tetraploid hybrids would comprise four different haploid genomes. However, to follow this or a similar approach, potato clones must be competent for androgenesis, and this competence is still limited to only a few genotypes (Singsit and Veilleux, 1989).

Another way of developing haploids is by the use of selected haploid-inducing pollinators (parthenogenesis) of *S. phureja* carrying a homozygous pleiotropic marker (Hermsen and Verdenius, 1973); nevertheless, recent research reveals that some undesirable genetic information from the haploid-inducing parent may be passed to the maternal haploids (Allainguillaume et al., 1997). Allainguillaume et al. (1997) demonstrated that haploids developed in this way are not totally of maternal origin but carry some genetic information from the pollen donor plant. Plaisted et al. (1994),

mentioned that haploids have proven to be an invaluable tool in potato genetic research, as a means of securing fertile hybrids with many wild diploid *Solanum* species. Use of haploids has been proposed as a method for cultivar development. Dihaploid extraction by anther culture of superior tetraploid clones has been limited (Rokka et al. 1996), therefore transferring this ability to commercially desirable clones or enhancing it is desirable.

Anther culture response (ACR) can be considered a two-phase process: the development of microspores into embryos and the conversion of embryos into plantlets. Uhrig and Salamini (1987) suggested that the action of a dominant gene control androgenic competence. Simon and Peloquin (1977) also suggested the action of a simply inherited dominant gene controlling callus regeneration. Research by Singsit and Veilleux (1989), working under the assumption that the responsive genotypes that they used to develop their populations were heterozygous for anther culture response, supported the action of a single dominant gene controlling anther culture response in diploid potato hybrids. All the F_1 hybrids and the backcross progeny that they studied segregated with respect to responsive and unresponsive genotypes. In contrast, Sonnino et al. (1989) suggested that several recessive genes control anther culture response as measured by embryos per anther. Meyer et al. (1993) proposed the involvement of no more than two genes for ACR. Singsit and Veilleux (1989) found no positive correlation between embryo formation and embryos regenerating into plantlets, suggesting that these two characters are inherited independently.

There have been several attempts to tag genes associated with ACR in several crops. Wan et al. (1992) identified chromosomal regions associated with the anther culture response or plant regeneration from callus using RFLP analysis of three maize anther derived callus lines from F_1 plants obtained from crosses between three inbred lines. They identified six chromosomal regions associated with the formation of embryo-like structures (ELS) or regenerable callus formation. Murigneux et al. (1994) identified three to four quantitative trait loci (QTL) involved in ACR in crosses of maize between high and non-responsive lines for ACR. Beaumont et al. (1995) mapped ACR genes in

maize conducting anther cultures and RFLP analysis in F_2 plants from different crosses. They identified six chromosomal regions associated with the ability of induce embryo-like structures from microspores. All but one of the regions that they identified were near viviparous mutant loci [known to involve the plant hormone abscisic acid (ABA)]. They suggested that ABA or its antagonist, gibberellic acid (GA-3), might somehow be related to ACR.

Devaux and Zivy (1994), used two-dimensional electrophoresis of protein markers on a population of barley anther culture derived doubled haploid (DH) lines to identify loci involved in ACR. These lines showed deviation from the expected segregation ratios at four loci. Double haploid lines having the four selected alleles were the most efficient in anther culture compared to lines with other allelic combinations. They identified markers associated with ACR in one of the distorted loci linked to genes involved in embryo production and green plant regeneration and others linked to genes involved only in green plant regeneration. Komatsuda et al. (1995) produced a backcross to characterize the function of a locus (Shd1) previously mapped to chromosome 2 of barley. The backcross involved a parent that does not promote shoot differentiation and a parent that has positive effect on shoot-differentiation rate. They located Shd1 in a chromosomal region flanking markers coming from the parent that transferred ability for shoot differentiation of immature embryo-derived callus.

He et al. (1998) used QTL analysis to identify loci that contribute to ACR in rice using a double haploid (DH) population established via anther culture on an indica/japonica hybrid. They identified five QTLs for callus induction frequency and one major QTL for albino plantlet differentiation frequency. They did not find any independent QTL for green plant differentiation. All four traits displayed continuous distributions and there was no correlation between callus induction frequency and green plantlet differentiation, but both showed strong positive correlation with green plantlet yield.

Cloutier et al. (1995) studied ACR in oilseed rape (*Brassica napus L.*) by comparative mapping of a F_2 population and two microspore-derived populations

developed from a cross between a microspore culture-responsive parent and a non-responsive parent. They identified two linkage groups as putative chromosomal regions associated with microspore-culture responsiveness, based on markers that showed segregation distortion, most of them towards the responsive parent.

The polymerase chain reaction (PCR) facilitates the use of molecular markers such as randomly amplified polymorphic DNA (RAPD; Williams et al., 1990) in genetic studies and breeding programs. Finding RAPD markers linked to genes controlling traits of interest can aid phenotypic identification during the selection process, introgression of the trait, and germplasm characterization. Molecular markers linked to a trait of interest can be identified by the use of bulk segregant analysis (BSA; Michelmore et al., 1991), a technique that consists of pooling DNA of genotypes exhibiting extreme phenotypes of a trait in a segregating population (Figure 1). Molecular markers like RAPDs are then screened using these bulks to find polymorphic DNA fragments segregating with the trait of interest.

RAPDs combined with BSA have been used successfully to identify DNA sequences linked to different traits of interest in crops, such as crown rust resistance in oat, *Avena sativa* L. (Penner et al., 1993), daylength insensitivity in oat (Whight et al., 1994), scab resistance in apple, *Malus* sp. (Yang et al., 1997), leaf rust resistance in barley, *Hordeum vulgare* L. (Poulsen et al., 1995), root knot nematode resistance in sweet potato, *Ipomoea batatas* L. Lam. (Ukoskit et al., 1997), oleic acid concentration in spring turnip rape, *Brassica rapa* ssp. *oleifera* (Tanhuanpää et al., 1996), powdery mildew resistance in wheat, *Triticum aestivum* L. em. Thell (Hu et al., 1997), locus M for sex expression in asparagus, *Asparagus officinalis* L. (Jiang and Sink, 1997) and resistance to blackleg (*Leptosphaeria maculans*) in *Brassica napus* L. (Chèvre et al., 1997).

One approach to integration of ACR into desirable potato germplasm could be to clone genes involved in the control of ACR. These genes then could be introduced by genetic transformation into any genotype in which its presence is desired. This research represents a step in this process. Our objectives were to characterize the components of

ACR in two backcross populations of diploid potato segregating for this trait in order to apply BSA to extreme phenotypes using RAPD markers.

Materials and methods

Plant material

Two backcross populations (PBCp and CBC) were established by crossing a self-incompatible interspecific hybrid (CP2) from a cross between clones of *S. chacoense* and *S. phureja* to its parents: *S. phureja* 1-3 × CP2→ PBCp and CP2 × *S. chacoense* 80-1→ CBC. *S. chacoense* 80-1 responds minimally to anther culture, *S. phureja* 1-3 was highly responsive to anther culture and CP2 was intermediate in ACR. Plants were grown in the greenhouse. The environmental conditions were 16 h photoperiod and 25-30°C day/15-20°C night. The photoperiod was controlled by a timer and was extended to 16 h when required, using halogen lamps (1000 watts). For PBCp, 78 genotypes were characterized whereas 57 genotypes were characterized for CBC.

Culture technique

Flower buds containing microspores at the late uninucleate to early binucleate stage were collected and placed at 4°C for 3 days. Buds were then sterilized by immersing them for 1 min in 80% ethanol, then 5 min in 100% household bleach with 2 drops of 'Tween 20' and finally rinsed twice in sterile distilled water. Flower buds were then dissected and 30 anthers were placed in each 125 ml culture flask containing 15 ml liquid media (Snider and Veilleux, 1994). This media contained: MS basal media (Murashige and Skoog, 1962), supplemented with 100 mg l^{-1} myo-inositol, 0.4 mg l^{-1} thiamine, 60 g l^{-1} sucrose, 2.5 g l^{-1} activated charcoal, 2.5 mg l^{-1} N_6-benzyladenine (BA), and 0.1 mg l^{-1} indole-3-acetic acid (IAA). The medium pH was adjusted to 5.8 before adding activated charcoal and then was sterilized by autoclaving at 121°C and 1.1 kg cm^{-2} for 20 min. Flasks were sealed with parafilm (American Can Co., Greenwich, Conn.)

and placed on shakers rotating at 125 rpm at 25°C in the dark. After 5 weeks, embryos were harvested, counted under a dissecting microscope and transferred to regeneration medium (Snider and Veilleux, 1994): 3.2 g l^{-1} Gamborg's B5 salts (Gamborg et al., 1968) with minimal organic compounds (Sigma G 5893), 50 mg l^{-1} $CaHPO_4$, 748 mg l^{-1} $CaCl_2$, 250 mg l^{-1} NH_4NO_3, 10 g l^{-1} sucrose, 6 g l^{-1} agarose (type III-A), 0.1 mg l^{-1} gibberellic acid (GA_3, filter sterilized), pH 5.6, poured into 100 × 15 mm Petri dishes (25 ml/dish). Embryos placed on this medium were incubated at 20°C under 16 h photoperiod. Every 3 weeks, embryos that had converted into plantlets were transferred to tubes containing 20 ml MS basal medium while the unconverted embryos were transferred to fresh embryo regeneration media for a total of three transfers. The regenerated plants were kept at 20°C under a 16 h photoperiod.

Preliminary study

A preliminary study was conducted to determine which components of anther culture response might be suitable for bulk segregant analysis. The components were: production of embryos per anther (EPA), regeneration rate of anther-derived embryos, and percentage of monoploids among regenerated plants. Figure 2 depicts the process followed during this preliminary study. A single plant of each of 24 genotypes per backcross family was grown under greenhouse conditions during the spring and summer of 1996. When possible, up to 300 anthers per genotype were cultured. These anthers were cultured when at least six flower buds (30 anthers) were available per genotype on a single day. The parents (1-3 and 80-1) were always included as controls.

After 5 weeks, the EPA were counted and transferred to regeneration medium. When the plants had grown sufficiently large *in vitro* for sampling (4 to 10 weeks after transfer) the ploidy of the regenerated plants was checked by flow cytometry, according to Owen et al. (1988) using a known monoploid ($2n=1x=12$) plants as control. Approximately 1g of leaves and stems of *in vitro* plantlets was chopped on ice for 3 min with a razor blade, in a 6 cm glass Petri dish containing 1.5 ml chopping buffer [12 mM sodium citrate, 8 mM morpholinopropanesulfonic acid (MOPS), 38.4 mM $MgCl_2$, and

0.04% Triton X-100]. The mixture was poured through a 250 μm filter and then through a 63 μm filter to remove debris; 0.5 ml filtrate was placed in a microcentrifuge tube and incubated after adding 0.25 ml RNAase solution (0.8 mg/ml chopping buffer) at room temperature for 30 min. Samples were stained by adding 0.125 ml propidium iodide (PI) solution (0.3 mg PI/ml chopping buffer) to each sample, and incubating on ice for a minumum of 30 min. The analysis was done within 3 h after sample preparation. The ploidy analysis was conducted at the Virginia-Maryland Regional College of Veterinary Medicine using an Epics X-L laser flow cytometer and cell sorter (Coulter Electronics, Hialeah, Florida). The frequencies of monoploid, diploid and tetraploid regenerants per responding genotype were calculated.

Series of studies to characterize EPA

Once we had determined that EPA was a phenotypically stable character in our populations, a series of studies were planned to obtain solid data for accurate phenotypic characterization. Each study in the series was done on at least 3 different days, to avoid confounding the day effect with the genotype effect. Three flasks per genotype per day were cultured; each flask contained 30 anthers dissected from six buds that had been estimated to contain microspores at the uninucleate stage by anther length (2.5 – 4 mm). Six to 11 genotypes were characterized in each study. For this purpose, six plants per genotype (Figure 3) were grown in a greenhouse at different times during 1996-97 in batches of ten to 12 genotypes, including the parents as controls. The plants selected in the preliminary study were also included in the series of studies to verify the results. For the PBCp and CBC families, four and five studies were conducted, respectively. Data on EPA were analyzed using SAS proc GLM procedure, after square root transformation ($\sqrt{1+\text{EPA}}$). When data consist of a wide range of whole numbers, such that mean and variance are equal, square root transformation or $\sqrt{1+n}$ is recommended (Steel and Torrie, 1980).

Final large scale study

To increase the population size for the PBCp family, the experimental design was altered to test many genotypes at the same time. In this case, 44 genotypes were tested each day, one flask of 30 anthers per genotype per day, and the anthers were cultured on 6 different days. This study was conducted during spring 1998.

BSA using RAPD primers

DNA of all the genotypes examined was extracted as described by Doyle and Doyle (1987), a modification of the 2x CTAB method described by Saghai Maroof (1984) (procedure described in appendix). Leaf samples taken from young plants grown in a greenhouse provided the starting material. Equal volumes of DNA (at concentrations of 10 ng/μl) from each selection were pooled to form the bulks (high ACR, low ACR) for the respective populations.

A total of 214 RAPD primers from sets A, C, G and Q through Z of Operon Technologies (Alameda, California) was used to screen each pair of bulks. PCR reaction mixtures of 25 μl contained: 20 ng genomic DNA, 0.6 μM primer, 200 μM dATP, dCTP, dGTP, dTTP, 1× PCR buffer (2.5 mM $MgCl_2$, 50 mM KCl, 10mM Tris-HCl, pH 8.3), and 1 U *Taq* DNA polymerase of Promega (Madison, Wis.). Amplifications were conducted in an Amersham robocycler or in a Perkin Elmer Cetus Model 480 thermal cycler. In the latter case the reactions were overlaid with a drop of sterile mineral oil. The amplification procedure consisted of 45 cycles of 1 min denaturation at 94ºC, 1 min primer annealing at 37ºC, 2 min extension at 72ºC, followed by a final extension at 72ºC for 5 min. The amplified samples were separated in 1.4% agarose gels in 1× TBE buffer [10.8 g trizma base, 5.48 g boric acid, and 4 ml EDTA (0.5 mM)/L distilled water] for 3.5 to 4.5 h at 100 V. After electrophoresis gels were stained with ethidium bromide (1.5 μg ml^{-1}) and photographed under UV light using λ DNA digested with *EcoRI* and *HindIII* as size marker and a PCR mix without template DNA as blank control. If polymorphism was verified in parents and bulks, the individual selections within the bulks were run and

then remaining genotypes in the family. Of the 214 primers used for BSA, 60 were run only on the bulks and 154 were run on both parents and bulks.

Genetic hypothesis

We are using BSA to identify RAPDs from 1-3 that occur in coupling with high EPA in backcrosses. Our parents are not necessarily homozygous because they are both selections within cross-pollinating populations. Because RAPD markers are dominant, candidate bands are either homo or heterozygous in 1-3. For the ACR bulks, assuming dominance and homozygosity for a gene in *S. phureja* 1-3 and assuming that *S. chacoense* 80-1 is homozygous recessive, the expected segregation patterns for the PBCp and the CBC populations differ. Alternatively, if we assume that *S. phureja* 1-3 is heterozygous for the trait, the expected segregation in these two backcross populations again differs (Figure 4). Thus, for the ACR bulks, any band segregating with the trait in one of the populations is not expected to segregate similarly in the other population.

Statistical analysis to determine linkage between putative bands and traits

Data were taken by assigning a "0" for the absence of a polymorphic band and a "1" for the presence of such band for each genotype within the populations. Candidate bands linked to EPA were revealed when the bulks reflected the parents, i.e., presence of band in high parent and high bulk, absence of band in low parent and low bulk. Presence/absence of a band was used to determine if such classification was a significant source of variation for the number of embryos per anther (EPA). For this analysis one-way ANOVA using SAS proc GLM procedure was used.

Isolation and sequence of linked bands

If a band was significantly linked to the trait, a PCR reaction with the same RAPD primer that differentiated the bulks was done on a single genotype that expressed the

marker and electrophoresis was run for 5-7 h depending on the size of the band of interest. The band was then excised from the gel under UV light and purified using Gene Clean (Bio 101, St. Louis, Missouri). The purified fragment was used again in a PCR reaction with the same RAPD primer to attempt to amplify a single band. When a single band was amplified, the PCR reaction was used for ligation of the fragment into a plasmid using the Pgem T-easy vector system from Promega (Madison, Wisconsin). The ligation reaction contained 5 µl of 2× Rapid ligation buffer, 1 µl pGEM-T easy vector (50 ng), 2 µl PCR product, 1 µl T4 DNA ligase (3 Weiss units/µl) and 1 µl deionized water in a total volume of 10 µl (all reagents were provided in the Pgem T-easy vector system kit). The reaction was incubated overnight at 4°C. The ligation reaction was then transformed into *E. coli* competent cells, by adding 100 µl recently thawed competent cells to a sterile 1.5 µl microcentrifuge tube containing 2 µl of each ligation reaction on ice. Colonies were grown on solid ampicillin-LB media. A number of colonies were transferred to liquid Terrific broth overnight for plasmid extraction. Plasmid DNA from each selected white colony was then digested with *NotI* restriction enzyme and run in an agarose gel together with the original fragment, purified by Gene Clean and λ DNA digested with *EcoRI* and *HindIII*. A fragment of the correct size was sent to be sequenced at the VT sequencing facility (Blacksburg, Virginia). None of the fragments resulted in a good sequence; therefore, it was not possible to design specific primers.

Results

Preliminary study for ACR

The preliminary study for anther culture response was designed to address three aspects of anther culture, (1) the number of embryos obtained per anther (EPA), (2) the frequency of embryos that converted into plants, and (3) the percentage of monoploids among the regenerated plants. In general, the PBCp backcross was much more highly responsive to anther culture than the CBC backcross by all three measures of the trait. The number of embryos per anther for PBCp (1-3 × CP2) ranged from 0 to 14.5 and for CBC (CP2 × 80-1) ranged from 0 to 3.3 (Figs. 5 and 6). Five PBCp and three CBC

genotypes, respectively, were tentatively classified as high EPA because they produced consistently more embryos ($\bar{x} = 5.25 \pm 2.34$ for PBCp and 2.23 ± 0.77 for CBC) than other genotypes in the experiment. Nine PBCp and 16 CBC genotypes, respectively, were considered variable for EPA because they exhibited different levels of embryo production at different sampling times or they consistently produced an intermediate number of embryos ($\bar{x} = 2.19 \pm 4.35$ for PBCp and 0.4 ± 0.52 for CBC). Six PBCp and four CBC genotypes, respectively, were classified as low EPA because they consistently did not produce embryos or produced very few of them ($\bar{x} = 0.04 \pm 0.04$ for PBCp and 0.02 ± 0.02 for CBC). The regeneration rate for the five high ACR genotypes in the PBCp population varied from 8 to 50% (Fig. 7) and the rate for the three high ACR genotypes in the CBC family from 3 to 17% (Fig. 8). The ploidy level of the plants regenerated from high EPA genotypes was studied to determine if the frequency of monoploids produced by anther culture was genetically controlled. For the PBCp population the percentage of monoploid plants produced by the five high EPA genotypes ranged from 0.2 to 60% compared to the 1-3 control at 20% (Fig. 9). For the CBC population the percentage of monoploid plants for the three high ACR genotypes ranged from 0 to 16%, all lower than the 1-3 control at 20% (Fig. 10).

Further EPA characterization

Because of the design of the preliminary study, i.e., culture of as many anthers as possible per day of as many genotypes as possible, statistically significant differences for EPA could not be deduced among genotypes. Therefore to further characterize EPA the populations were divided into sets of six to 11 genotypes for handling in a series of experiments that could be analyzed by ANOVA, attributing variation for EPA to day of culture and genotype as well as the interaction. When possible, both the high and low EPA parents were used in each experiment as controls. The number of genotypes was determined by how many could be effectively handled according to the processes described in Figure 3.

Variation for EPA in the PBCp population

Four such studies were conducted for PBCp. The anther culture data were analyzed separately for each study. For the four PBCp studies, genotype was always the greatest source of variation for EPA and was highly significant in each study (Table 1). Date and the clone × date interaction were also significant sources of variation in all four studies. Mean separation for clone in the four experiments revealed a total of 16 genotypes that were greater than or not significantly different from the high parent, 1-3, and 19 genotypes that did not significantly differ from the low parent, 80-1 (Fig. 11). In order to avoid any overlap between high and low EPA genotypes, only four genotypes with fewer EPA than 80-1 in these four studies were selected for the low bulk. Eight selections were obtained for the high bulk. Some high genotypes (9,13,524, 520, 538) were subsequently discarded due to the presence of spurious bands in their genetic profile (Chapter II). Therefore, a large-scale experiment was conducted to characterize more of the PBCp population for EPA. In this study 44 genotypes were characterized by culture of one flask of anthers per genotype per day for 6 days; the different dates were taken as replications for statistical analysis. Both clone and date were highly significant source of variation (Table 2). Transgressive segregation for EPA was apparent in this large-scale study. Two genotypes (516 and 641) generated significantly more EPA than 1-3 (Figure 12). Six low ACR clones were selected with EPA less than 80-1 (Fig. 12). From the four replicated studies and the large-scale experiment, a total of 78 genotypes was characterized in the PBCp backcross population revealing a range of EPA 0 to 12.3. Ten high and ten low EPA genotypes were selected for BSA. The mean EPA for the PBCp high selections and low selections was 4.7 and 0.03, respectively. By comparison, the mean EPA for the high (1-3) and low control (80-1) was 3.3 and 0.08, respectively. EPA for all the genotypes characterized in the PBCp population are represented in Figure 13 (EPA data is also presented in Table 4 of Apendix) . Even though there was variation among experiments for the controls (*S. phureja* 1-3 varied from 2 to 5.7 EPA; *S. chacoense* 80-1 varied from 0 to 0.2 EPA; Figure 14), there was always a statistically significant difference between them.

Variation for EPA in the CBC population

Five studies were conducted to characterize CBC for EPA. The anther culture data were analyzed separately for each study. For the five CBC studies, genotype was always the greatest source of variation for EPA and was highly significant in each study (Table 3). Date and the genotype × date interaction were also significant sources of variation in four of five studies. Mean separation for clone in each experiment yielded nine high EPA genotypes, and ten low EPA genotypes that did not significantly differ from the low parent, 80-1 (Figure 15). High EPA genotypes were more difficult to find than low EPA genotypes in this population. The mean EPAs for the CBC high selections and low selections were 3.7 and 0.05, respectively. By comparison, the mean EPAs for the high (1-3) and low control (80-1) were 3.8 and 0.04, respectively. All genotypes characterized for EPA in this family are represented in Figure 16. (EPA data for all genotypes characterized is presented in Table 5 of Apendix)

Bulk segregant analysis

A total of 154 primers revealed 754 RAPD bands (63%) that were polymorphic between 1-3 and 80-1. This corresponds to a genetic similarity (GS) of 0.37 [Jaccard similarity coefficient→ $n_{11}/(n-n_{00})$; (Jaccard, 1908 (cited by Yu and Nguyen, 1994] between the two parents of the interspecific hybrid populations, compared to genetic similarity values ranging from 0.596 to 0.993 for the complex interspecific hybrid potato genotypes reported by Paz and Veilleux (1997), also using RAPDs. The smaller genetic similarity (i.e., greater genetic distance) found for the parents of our hybrid may reflect greater diversication between *S. phureja* and *S. chacoense*. However the GS may be biased in comparing only two genotypes because of the absence of null loci for both genotypes, i.e., n_{00} was 0.

Within the PBCp family, two of 214 primers amplified candidate bands linked to EPA, one in repulsion and the other in coupling. RAPD primer OPQ-10 amplified a band (approx. 700 bp) (Fig. 17) that was linked in repulsion to EPA. This band was present in

80-1 and was apparently homozygous, as all genotypes in the CBC population expressed it. PCR for genotypes comprising the PBCp bulks revealed that the band was present in one of the ten high selections and in nine of the ten low selections (Fig. 17). Data from 55 genotypes (including the bulk selections) in the population in a one-way ANOVA revealed a significant difference between high EPA genotypes that did not have the band and low EPA genotypes that presented the band. The mean EPA for the genotypes with the band was 3.1 and the mean for the ones without the band was 0.5 (Table 4).

RAPD primer OPZ-4 amplified a DNA fragment (approx. 1100 bp) linked in coupling to EPA in the PBCp population (Fig. 18). This fragment was present in all ten high EPA bulk selections and in four of the ten low bulk selections. It is a fragment that was present in the 1-3 clone and appears to be associated with enhanced ACR. Analysis of data from 55 genotypes in the PBCp population (including the bulk selections) resulted in a significant difference for high EPA genotypes that expressed the band and low EPA genotypes that did not present the band. The mean EPA for the genotypes with the band was 2.2 and for the ones without the band was 0.07 (Table 4).

One RAPD primer of 214 tested amplified a fragment linked in coupling to EPA in the CBC population. RAPD primer OPW-14 generated a band (approx. 1800 bp) that was present in seven of the nine high bulk selections and in two of the ten low selections (Fig. 19). When 34 genotypes in the CBC family were amplified with OPW-14, the twelve genotypes with the fragment had a mean EPA of 1.29 whereas 22 genotypes without the fragment had a significantly lower mean EPA of 0.42 (Table 5).

The attempts to isolate and sequence the bands of interest to design SCARs were not successful, due to difficulty in amplifying a single band after a PCR amplification of DNA from an isolated band of interest. (RAPD amplification data for all genotypes and primers is presented in Tables 6 and 7 of Apendix)

Discussion

Different scientists considered different parameters or aspects of anther culture response (ACR). He et al. (1998), considered four parameters when studying ACR in rice: callus induction frequency, green plantlet differentiation, albino plantlet

differentiation and green plantlet yield. Devaux and Zivy (1994) evaluated two aspects of anther culture in barley: embryo production and green plant regeneration. Wan et al. (1992) considered embryo-like structure (ELS) formation, and the subsequent formation of regenerable callus from ELS as two components of ACR in maize. Working also in maize, Murigneux et al. (1994) looked at the percentage of responding anthers, the number of androgenetic embryos produced per 100 anthers, the number of plantlets regenerated per 100 embryos, and the number of plantlets per plated anthers. Beaumont et al. (1995) looked only at the induction of embryos in maize anther culture. Komatsuda et al. (1995) looked at formation of callus from immature embryos, and shoot differentiation from barley embryo callus. Devaux and Zivy (1994) looked at embryo production and green plant regeneration in barley. A common conclusion is that the different components of anther culture response in different plant species are under independent genetic control.

We looked at three components of ACR in potato in a preliminary study: embryos produced per anther (EPA), regeneration rate of anther-derived embryos and percentage of monoploids among regenerated plants. All the components were greater in the PBCp population (3/4 *S. phureja* 1-3) than in the CBC population (1/4 *S. phureja* 1-3); these results confirm that these components of ACR are under genetic control. Furthermore, the components of anther culture, i.e., embryo production, conversion of embryos into plants and percentage of monoploids produced by a genotype, appear to be inherited independently because high selections for EPA are variable for regeneration and percentage of monoploids. The continuous variation in both families leads us to conclude that this is a multigenic trait. This agrees with previous studies in other crops for different components of anther culturability, i.e. wheat (Lazar et al., 1984; Deaton et al, 1987), triticale (Charmet and Bernard, 1984), maize (Petolino and Thompson, 1987; Petolino et al., 1988; Afele and Kannenberg, 1990), and rice (Quimio and Zapata, 1990). The regeneration rate of anther-derived embryos was very variable in our populations, but it could only be characterized in high EPA genotypes. The percentage of monoploid regenerants was low and also very variable. The presence of diploid regenerants may

reflect 2*n* microspore frequency. This component of ACR is also under obvious genetic control, but again it is possible to characterize it only for high EPA genotypes, making it difficult to characterize sufficient genotypes.

Results of the preliminary study demonstrated that the three components of anther culture response that we studied qualify as traits with possibility of manipulation for breeding purposes. However, due to difficulties implicated in this type of phenotypic characterization, (as the fact that only a few genotypes can be handled at the same time) it was decided to pursue the characterization only for the first component (embryos per anther; EPA).

Many studies in crops like maize (Wan et al., 1992), barley (Devaux and Zivy, 1994), wheat (Agache et al., 1989), rapeseed (Uzunova et al., 1995), and diploid potato (Rivard et al, 1994) were based on the distorted segregation of markers for anther- and microspore-derived doubled haploid progenies, to find linkage to QTLs or genes controlling anther culture or microspore culture response. Murigneux et al. (1994) does not agree with Wan et al. (1992) in generalizing the hypothesis of association between QTLs controlling ACR, and segregation distortion. Unlike Wan et al. (1992), Murigneux et al. (1994) observed that some of the doubled haploid lines derived from high ACR hybrids did not show any response, in some cases the positive alleles were even underrepresented.

Murigneux et al. (1994) also brought a question that concerns us as well: whether the androgenic process is only under gametophytic control. If it is, they expected to get strong selection of positive alleles for ACR, and they did not. Therefore, they suggested that at least a part of the process, and more likely the first steps, are under sporophytic control. Physiologically active substances contained in the anther wall of barley have been shown to be involved in embryogenesis (Cowen et al., 1992). Because during anther culture the microspores are encased within the anther locule, the anther wall and tapetum may influence the embryogenic process (Cordewener et al., 1996). Beaumont et al. (1995) mapped ACR genes in maize, and pointed out that the sporophytic effect of the genotype was included in their study and that it could have been overcome by using

microspore culture rather than anther culture. We characterized ACR in backcross genotypes that should not have segregation distortion due to selection for ACR. Nevertheless, we still may have sporophytic effects by using anther culture. Experimental data on the inheritance of anther culture response are often difficult to interpret, due to the large amount of uncontrollable and environmentally induced variation. Potato is by itself a very difficult case, it cannot be selfed because it suffers from inbreeding depression and it is highly heterozygous. Barley, maize, and cabbage are the only crops that have a well-established microspore culture and also a relatively simpler genetic structure and are good candidates for gene isolation studies (Cordewener et al., 1996).

Due to the importance of anther culture as a haploid production tool for potato breeding programs, we conducted the present study, which is the first one that uses BSA and RAPD markers based in segregating populations without AC involved in its development, in a crop for which every step of the process is complex. The EPA characterization was done in a series of studies that allowed us to little by little characterize enough genotypes to get selections that could be used in BSA. The question of whether variation among studies interfered with phenotypic characterization was addressed by analyzing and comparing data from the controls (the parental clones) included in every study. EPA for the controls varied among experiments, but the difference in response between the high ACR parent and the low ACR parent was always significant (Fig.14).

The high ACR selections for the PBCp family showed a much higher EPA than those for the CBC family. It was difficult to select low ACR genotypes for PBCp and easy to select high ones. On the other hand, it was difficult to find high ACR selections in the CBC family and there were plenty of low ACR genotypes. Continuous variation was observed with respect to EPA in both families characterized for ACR, as depicted in Fig.13 and 14, depicting multigenic control of the trait. Compared with earlier studies (Singsit and Veilleux, 1989), we cannot agree that a single dominant gene controls ACR. Dominance is indicated because of the identification of high ACR genotypes in CBC.

Some additive gene action is also implicated if the high selections in PBCp are due to accumulation of ACR alleles in homozygous conditions resulting in greater EPA.

The phenotypic characterization for ACR was a difficult and time-consuming process, but it was the only way to obtain reliable selections. Due to the day effect and the genotype × date interaction, it is necessary to do experiments over several days for phenotypic characterization. The first step towards finding molecular markers by BSA is to conduct a reliable phenotypic characterization process, because the success of BSA is based on correct characterization. The future goal of the ACR study is to be able to clone genes involved in controlling ACR in order to transfer ACR genes to genotypes that could be used in potato breeding programs. For this objective the interest is to identify markers linked in coupling. Nevertheless any marker linked in coupling or in repulsion to ACR is important to identify in order to simplify the characterization/selection process. In our study, dominance or additive gene action of ACR is assumed (Fig. 4), based on the segregation of the EPA trait in both PBCp and CBC, for RAPDs (dominant markers).

The different backcross populations revealed different markers associated with ACR. Marker OPQ-10 amplified a band (approx. 700 bp) linked in repulsion in PBCp, this band was omnipresent in CBC and may have been responsible for the general suppression of ACR in this population (mean EPA for high selections = 2.49) compared to PBCp (mean EPA for high selections = 4.86). This band must be homozygous in 80-1, it segregates in a 1:1 ratio (presence:absence) (χ^2 =0.03; p<0.87) in PBCp genotypes and is highly suppressive of EPA. The mean EPA is 0.54 when the band is present. SCARs (sequence characterized amplified regions) designed to amplify this DNA fragment can be very helpful in a selection process for ACR. It would be a great advantage to be able to discard phenotypes without spending months to characterize them. However, two markers (one in each population), linked in coupling, were also identified. Both markers appear to come from the responsive parent *S. phureja* 1-3. The one amplified by OPZ-4 must be heterozygous in 1-3, this band exhibits a 28:9 (presence:absence) ratio in PBCp. It fits a 3:1 ratio (χ^2 =0.01; p<0.93), expected of a gene that would be heterozygous in 1-3. It is possible that gene dosage affects EPA, i.e., homozygotes have greater response

than heterozygotes. This band is obviously linked to a locus that affects EPA greatly because genotypes without that band have a very poor response ($\bar{x}$ EPA = 0.07). A band linked in coupling in CBC was amplified by OPW-14. The segregation ratio for this band was 12:22 (presence: absence), which is not significantly different from 1:1 (χ^2 =2.94; p<0.09). As for OPZ-4, genotypes without the OPW-14 band in CBC have a poor EPA ($\bar{x}$= 0.42). These two markers, OPZ-4 linked in PBCp and OPW-14 linked in CBC can be used not only for selection purposes but also to identify and isolate genes involved in controlling ACR by chromosome walking. This set of markers represents the first report of molecular markers linked to ACR in potato.

References

Afele J.C., and L.W. Kannenberg. 1990. Genetic studies of corn (*Zea mayz* L.) anther culture response. Theor. Appl. Genet. 80:459-464.

Agache, S., B. Bachelier, J. de Byuser, Y Henry , and J. Snape. 1989. Genetic analysis of anther culture response in wheat using aneuploid, chromosome substitution and translocation lines. Theor. Appl. Genet. 77:7-11

Allainguillaume, J., M.J. Wilkinson, S.A. Clulow, and S.N.R. Barr. 1997. Evidence that genes from the male parent may influence the morphology of potato dihaploids. Theor. Appl. Genet. 94:241-248.

Beaumont, V.H., T.R. Rocheford, and J.M. Widholm. 1995. Mapping the anther culture response genes in maize (*Zea mays* L.). Genome 38:968-975.

Charmet, G., and S. Bernard. 1984. Diallel analysis of androgenetic plant production in hexaploid triticale (×*Triticosecale* Wittmack). Theor. Appl. Genet. 69:55-61.

Chèvre, A.M., P. Barret, F. Eber, P. Dupuy, H. Brun, X. Tanguy, M. Renard. 1997. Selection of stable *Brassica napus-B. juncea* recombinant lines resistant to blackleg (*Leptosphaeria maculans*), Identification of molecular markers, chromosomal and genomic origin of the introgression. Theor. Appl. Genet. 95:1104-1111.

Cloutier, S., M. Cappadocia, and B.S. Landry. 1995. Study of microspore-culture responsiveness in oilseed rape (*Brassica napus* L.) by comparative mapping of a

F_2 population and two microspore-derived populations. Theor. Appl. Genet. 91:841-847.

Cordewener, J.H.G., J.B.M. Custers, H.J.M. Dons, and M.M. Van Lookeren Campagne. 1996. Molecular and biochemical events during the induction of microspore embryogenesis. In: In Vitro Haploid Production in Higher plants- Kluwer Academic Publishers Inc. Dordrecht, The Netherlands. Volume I:111-124.

Cowen, N.M., C.D. Johnson, K. Armstrong, M. Miller, A. Woosley, S. Pescitelli, M. Skokut, S. Belmar, and J.F. Petolino. 1992. Mapping genes conditioning in vitro androgenesis in maize using RFLP analysis. Theor. Appl. Genet. 84:720-724.

Deaton W.R., S.G. Metz, T.A. Armstrong, and P.N. Mascia. 1987. Genetic analysis of the anther-culture response of three spring wheat crosses. Theor. Appl. Genet. 74:334-338.

Devaux, P., and M. Zivy. 1994. Protein markers for anther culturability in barley. Theor. Appl. Genet. 88:701-706.

Douches, D.S., A.L. Westedt, K. Zarke, and B. Schroeter. 1998. Potato transformation to combine natural and engineered resistance for controlling tuber moth. HortScience 33:1053-1056.

Doyle. J.J. and J.V. Doyle. 1987. A rapid DNA isolation procedure for small amounts of leaf tissue. Phytochem. Bull. 19:810-815.

Gamborg, O.L., R.A. Miller, and L. Ojima. 1968. Nutrient requirements of suspension culture of soybean root cells. Exptl. Cell Res. 50:151-158.

He, P., L.H. Shen, C.F. Lu, Y. Chen, and L.H. Zhu. 1998. Analysis for quantitative trait loci which contribute to anther culturability in rice (*Oriza sativa* L.). Molecular Breeding 4: 165-172.

Hermsen, J.G.Th., and J. Verdenius. 1973. Selection from *Solanum tuberosum* group Phureja of genotypes combining high frequency haploid formation with homozygosity for embryo-spot. Euphytica 22:244-259.

Hu, X.Y., H.W. Ohm, I. Dweikat. 1997. Identification of RAPD markers linked to gene *PM*1 for resistance to powdery mildew in wheat. Theor. Appl. Genet. 94:832-840.

Jiang, C. and K.C. Sink. 1997. RAPD and SCAR linked to the sex expression locus *M* in asparagus. Euphytica 94:329-333.

Komatsuda, T., F. Taguchishiobara, S. Oka, F. Takaiwa, T. Annaka, and H.J. Jacobsen. 1995. Transfer and mapping of the shoot-differentiation locus Shd1 in barley chromosome 2. Genome 38:1009-1014.

Lazar, M.D., P.S. Baenziger, and G.W. Schaeffer. 1984. Combining abilities and heritability of callus formation and plantlet regeneration in wheat (*Triticum aestivum* L.) anther cultures. Theor. Appl. Genet. 68:131-134.

Meyer, R., F. Salamini, and Uhrig, H. 1993. Isolation and characterization of potato diploid clones generating a high frequency of monohaploids or homozygous diploid androgenic plants. Theor. Appl. Genet. 85:905:912.

Michelmore, R.W., I. Paran, R.V. Kesseli. 1991. Identification of markers linked to disease-resistance genes by bulk segregant analysis: a rapid method to detect markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA 88:9828-9832.

Murashige, T., and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. 15:473-497.

Murigneux, A., S. Bentolila, T. Hardy, S. Baud, C. Guitton, H. Jullien, S. Bentahar, G. Freyssinet, and M. Beckert. 1994. Genotypic variation of quantitative trait loci controlling in-vitro androgenesis in maize. Genome 37:970-976.

Owen, H.R., R.E, Veilleux, D. Levy, and D.L. Ochs. 1988. Environmental, genotypic, and ploidy effects on endopolyploidization within a genotype of *Solanum phureja* and its derivatives. Genome 30:506-510.

Paz, M.M. and R.E. Veilleux. 1997. Genetic diversity based on randomly amplified polymorphic DNA (RAPD) and its relationship with the performance of diploid potato hybrids.

Penner, G.A., J. Chong, C.P. Whight, S.J. Molnar, G. Fedak. 1993. Identification of a RAPD marker for the crown rust resistance gene *Pc68* in oats. Genome 36:818-820.

Petolino, J.F., and S.A. Thompson. 1987. Genetic analysis of anther culture response in maize. Theor. Appl. Genet. 74:284-286.

Petolino, J.F., A.M. Jones, and S.A. Thompson. 1988. Selection for increased anther culture response in maize. Theor. Appl. Genet. 76:157-159.

Plaisted, R.L., M.W. Bonierbale, G.G. Yencho, O. Pineda, W.M. Tingey, J.Van den Berg, E.E. Ewing and B.B. Brodie. 1994. Potato improvement by traditional

breeding and opportunities for new technologies. In: The molecular and cellular biology of the potato. Edited by W.R. Belknap, M.E. Vayda and W.D. Park. CAB International. Wallingford, UK.

Poulsen, D.M.E., R.J. Henry, R.P. Johnston, J.A.G. Irwin, R.G. Rees. 1995. The use of bulk segregant analysis to identify a RAPD marker linked to leaf rust resistance in barley. Theor. Appl. Genet. 91:270-273.

Quimio, C.A., and F.J. Zapata. 1990. Diallel analysis of callus induction and green plant regeneration in rice anther culture. Crop Sci. 30:188-192.

Rivard, S.R., M. Cappadocia, and B.S. Landry. 1996. A comparison of RFLP maps based on anther culture derived, selfed, and hybrid progenies of *Solanum chacoense*. Genome 39:611-621.

Rokka, V.M., L. Pietila, and E. Pehu. 1996. Enhanced production of dihaploid lines via anther culture of tetraploid potato (*Solanum tuberosum* L. ssp. *tuberosum*) clones. Am. Potato J. 73:1-12.

Saghai-Maroof, M.A., K.M. Soliman, R.A. Jorgensen, R.W. Allard. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA 81:8014-8018.

Simon, P.W., and S.J. Peloquin. 1977. The influence of paternal species on the origin of callus in anther culture of *Solanum* hybrids. Theor. Appl. Genet. 50:53-56.

Singsit, C., and R.E. Veilleux. 1989. Intra- and interspecific transmission of androgenetic competence in diploid potato species. Euphytica 43:105-112.

Snider, K.T., and R.E. Veilleux. 1994. Factors affecting variability in anther culture and in conversion of androgenetic embryos of *Solanum phureja*. Plant Cell Tiss. Org. Cult. 36:345-354.

Sonnino, A.S., M. Tanaka, M. Iwanaga, and L. Schilde-Rentschler. 1989. Genetic control of embryo formation in anther culture of diploid potatoes. Plant Cell Rep. 8:105-107.

Steel, R.G.D., and J.H. Torrie. 1980. Principles and procedures of statistics. A biometrical approach. Second edn. McGraw-Hill Inc., New York.

Tanhuanpää, P.K., J.P. Vilkki, H.J. Vilkki. 1996. Mapping of a QTL for oleic acid concentration in spring turnip rape (*Brassica rapa* ssp.*oleifera*). Theor. Appl. Genet. 92:952-956.

Uhrig, H., and F. Salamini. 1987. Dihaploid plant production from 4x genotypes of potato by the use of efficient anther plants producing tetraploid strains (4x EAPP clones)- proposal of a breeding methodology. Plant Breeding 98:228-235.

Ukoskit, K., P.G. Thompson, C.E. Watson Jr., G.W. Lawrence. 1997. Identifying a randomly amplified polymorphic DNA (RAPD) marker linked to a gene for root-knot nematode resistance in sweetpotato. J. Amer. Soc. Hort. Sci. 122:818-821.

Uzunova, M., W. Ecke, K. Weissleder, and G. Röbbelen. 1995. Mapping the genome of rapeseed (*Brassica napus* L.). I. Construction of an RFLP linkage map and localization of QTLs for seed glucosinolate content. Theor. Appl. Genet. 90:194-204.

Wan, Y., T.R. Rocheford, and J.M. Widholm. 1992. RFLP analysis to identify putative chromosomal regions involved in the anther culture response and callus formation of maize. Theor. Appl. Genet. 85:360-365.

Wenzel, G., O. Scheider, T. Przewozny, and S.K. Sopory. 1979. Comparison of single cell culture derived *Solanum tuberosum* L. plants and a model for their application in breeding programs. Theor. Appl. Genet. 55:49-55.

Whight, C.P., G.A. Penner, L.S. O'Donoighue, V.D. Burrows. S.J. Molnar, G. Fedak. 1994. The identification of random amplified polymorphic DNA markers for daylength insensitivity in oat. Genome 37:910-914.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 22:4673-4680.

Yang, H.Y., S.S. Korban, J. Krüger, H. Schmidt. 1997. The use of a modified bulk segregant analysis to identify a molecular marker linked to a scab resistance gene in apple. Euphytica 94:175-182.

Yu, L.X., and H.T. Nguyen. 1994. Genetic variation detected with RAPD markers among upland and lowland rice cultivars (*Oryza sativa* L.). Theor. Appl. Genet. 87:668-672.

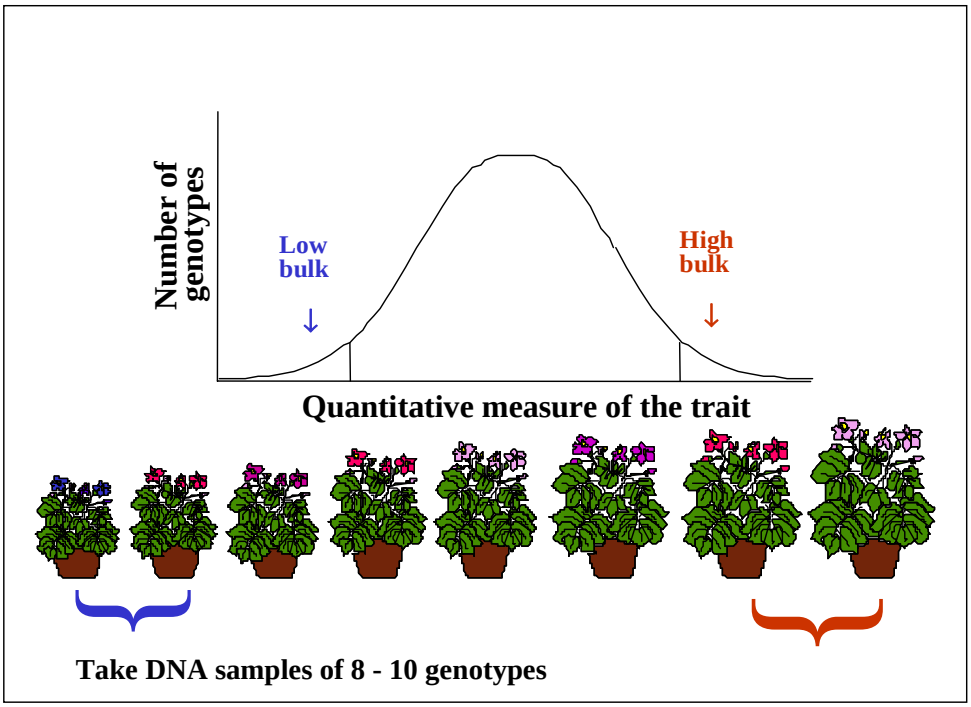

Figure 1: The "bulk segregant analysis" method consists of bulking the DNA of extreme phenotypes for a trait. These bulks can be screened with molecular markers to find a DNA fragment that segregates with the trait and is therefore linked to it.

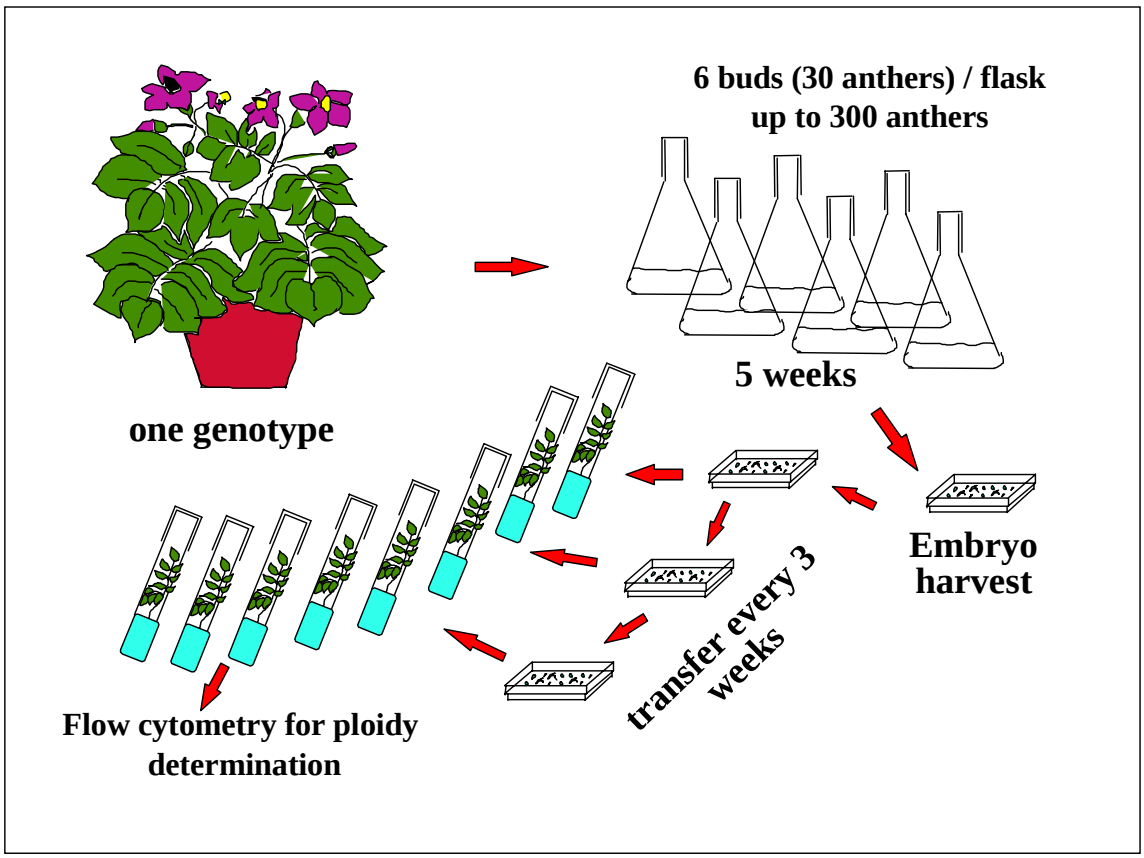

Figure 2: Process followed during a preliminary study for anther culture response (ACR). Three components of ACR were observed: embryos per anther (EPA), regeneration rate of anther-derived embryos, and percentage of monoploids among regenerated plants. The PBCp and the CBC families were part of this study, 24 genotypes of each family were used.

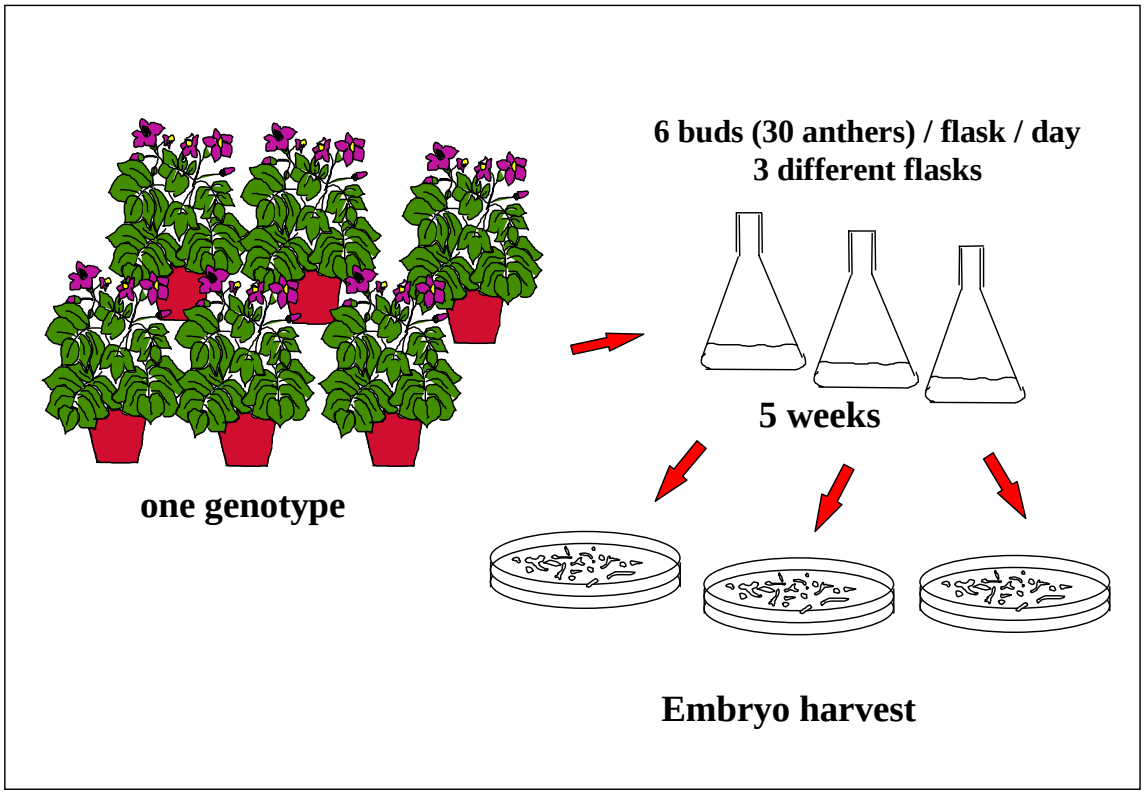

Figure 3: Process to characterize anther culture response as embryos produced per anther (EPA) in the series of studies for PBCp and CBC families. Each study comprised six to 11 genotypes, six clonally propagated plants of each genotype were grown under greenhouse conditions to have enough flower buds to culture 30 anthers per flask, three flasks per day on at least three different days. After 5 weeks of culture, the embryos produced in each flask were counted. The parental genotypes were included in each study as controls. Data were analyzed by SAS.

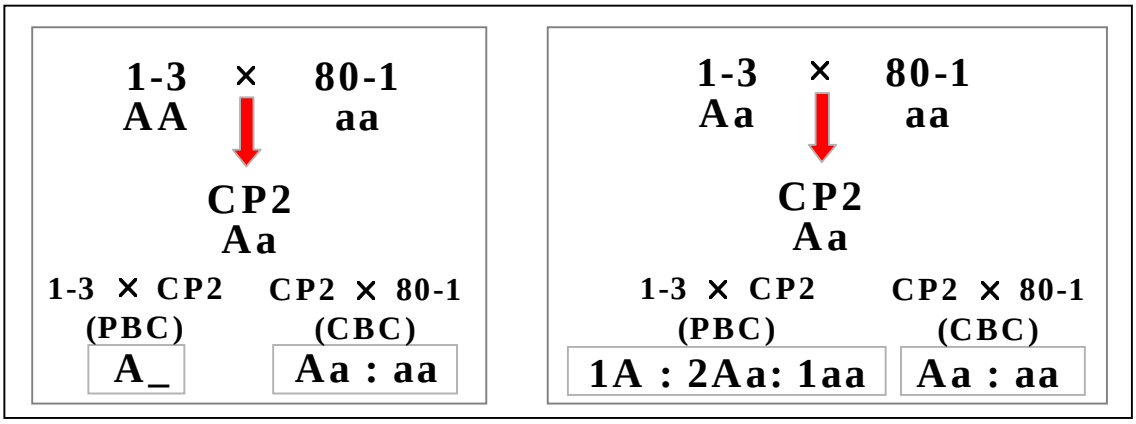

Figure 4: Possible segregation patterns for RAPD markers linked in coupling to EPA and identified by BSA. The expected segregation differs between the backcross populations. Therefore a putatively linked band identified in one population may not segregate in the other. On the left, the marker is assumed to be homozygous dominant in 1-3 whereas on the right 1-3 is assumed to be heterozygous. For a band linked in repulsion, similar segregation patterns would be expected except that dominant allele(s) would be coming from 80-1 instead of 1-3.

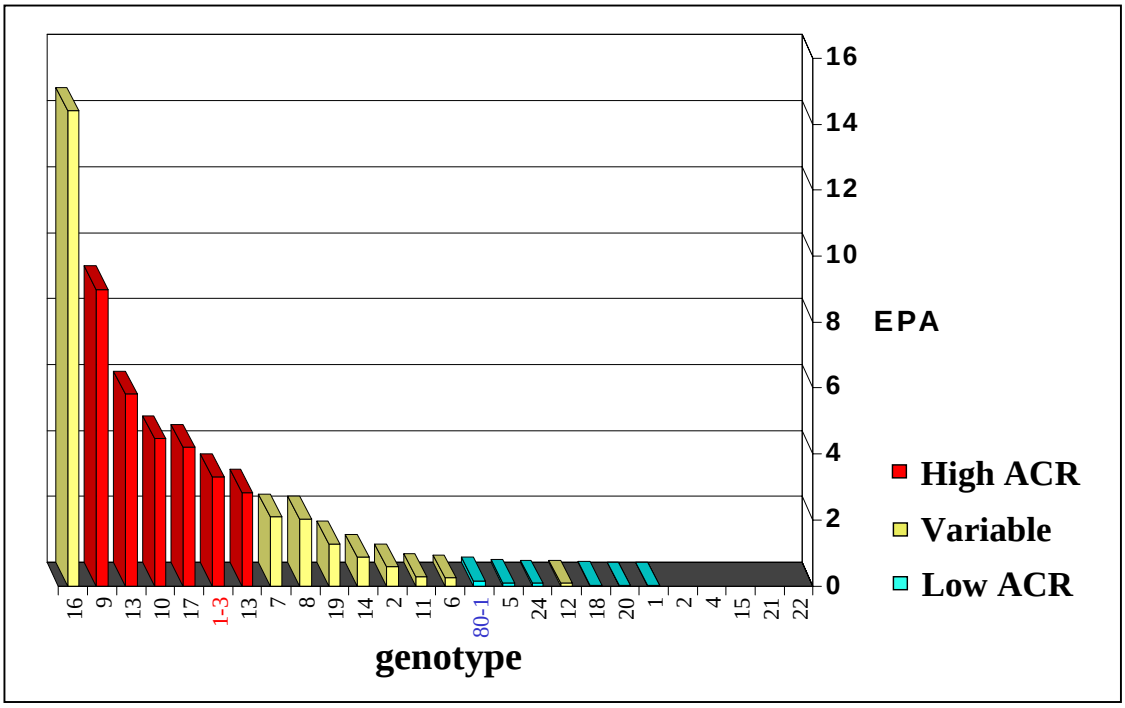

Figure 5: Embryos per anther (EPA) produced in the PBCp preliminary study for anther culture response (ACR) of 24 genotypes compared to high (1-3) and low (80-1) controls. Consistently high and low selections are indicated in red and blue, respectively, whereas yellow bars indicate variable genotypes.

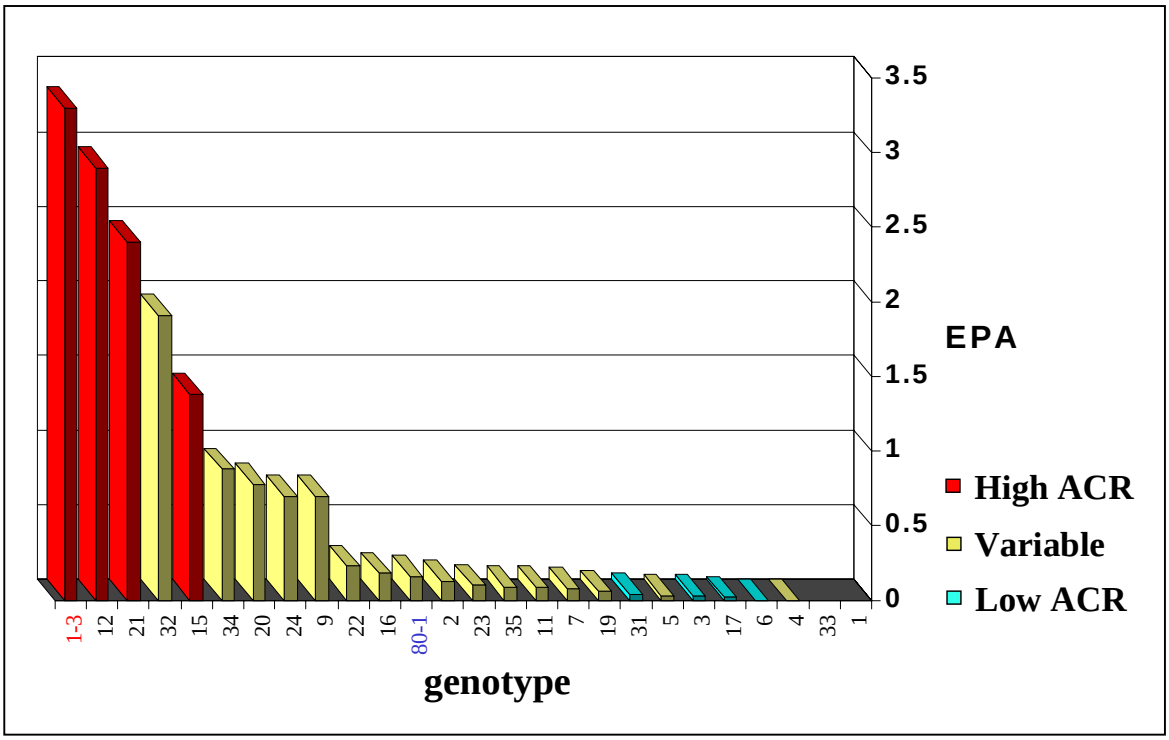

Figure 6: Embryos per anther (EPA) produced in the CBC preliminary study for anther culture response (ACR) of 24 genotypes compared to high (1-3) and low (80-1) controls. Consistently high and low selections are indicated in red and blue, respectively, whereas yellow bars indicate variable genotypes.

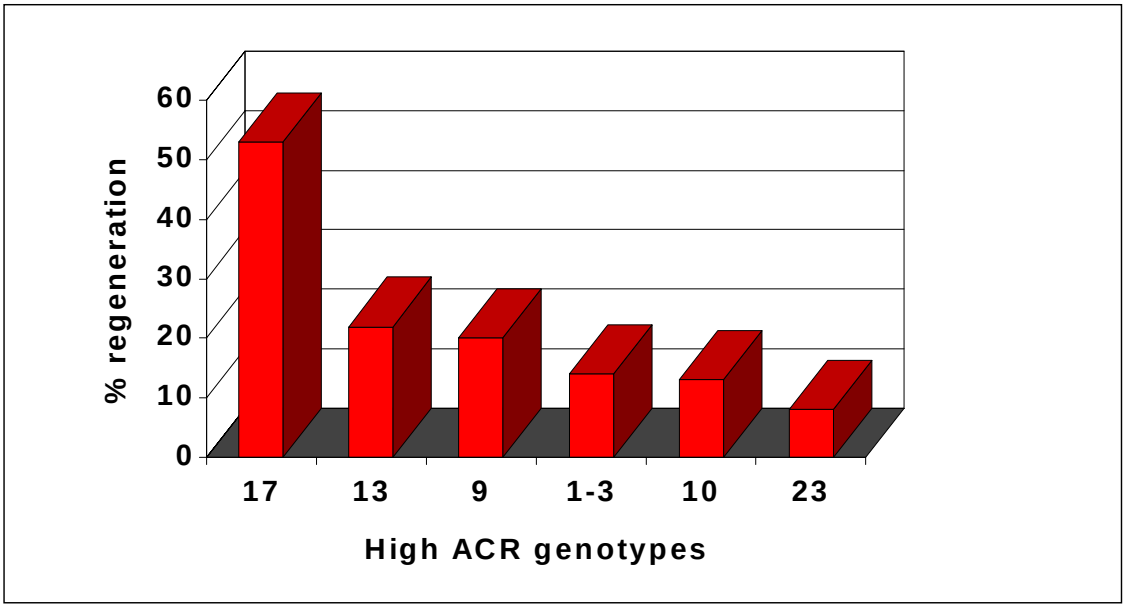

Figure 7: Regeneration frequency of anther derived embryos for five high EPA selections in PBCp and the 1-3 control.

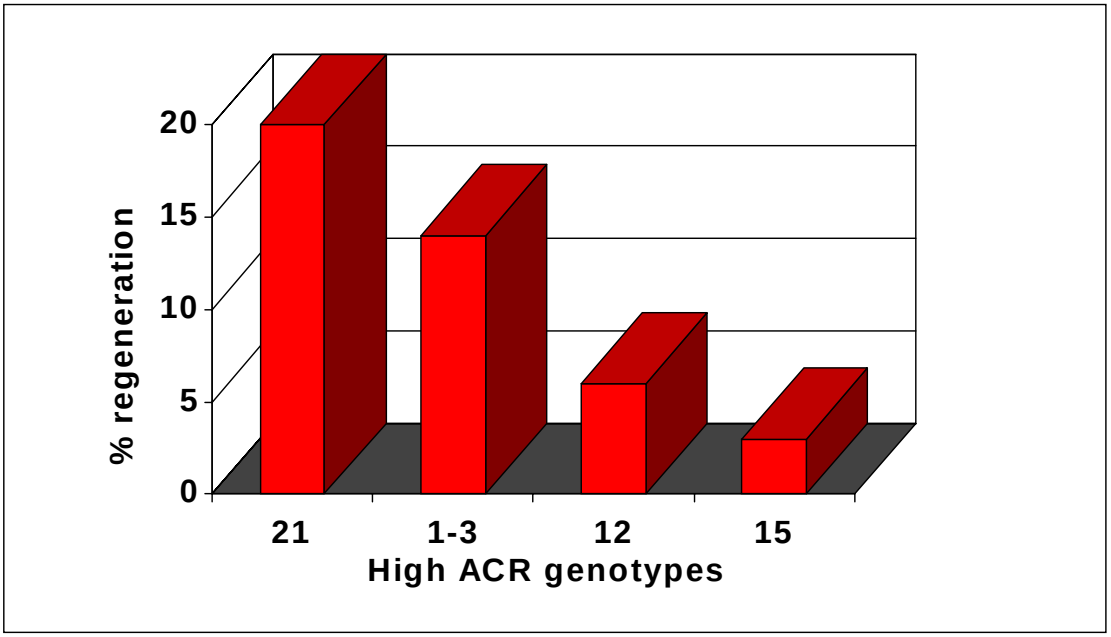

Figure 8: Regeneration frequency of anther derived embryos for three high EPA selections in CBC and the 1-3 control.

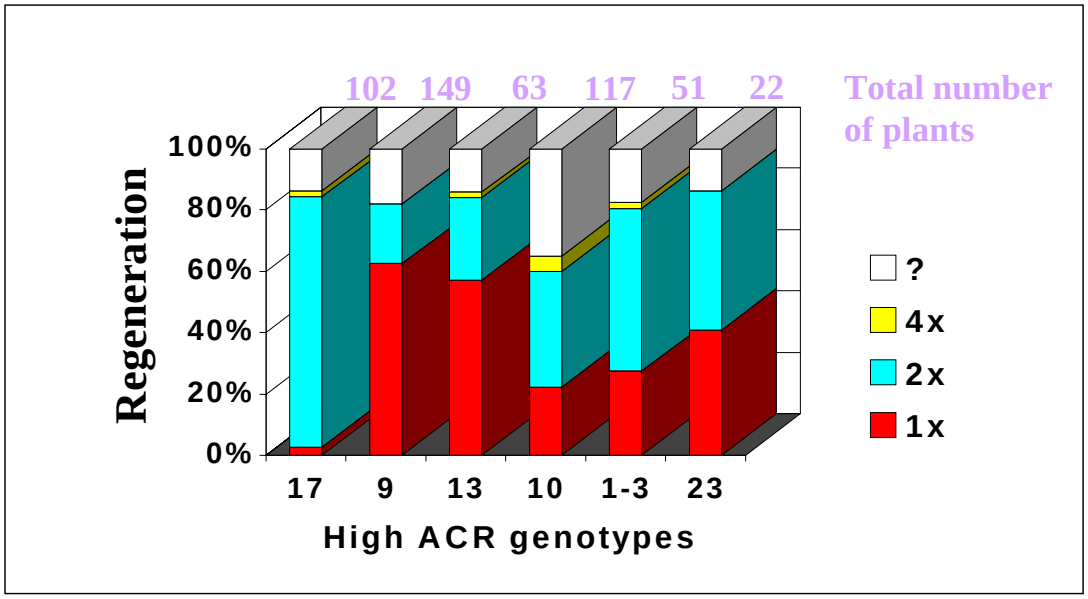

Figure 9: Ploidy of regenerants of anther-derived embryos of five high EPA PBCp genotypes and the 1-3 control. The ploidy level of the regenerated plants was checked by flow cytometry. The bars depict the percentage of monoploid, diploid, tetraploid and undetermined plants from each high EPA genotype. The total number of regenerated plants included for each genotype is given above the bars.

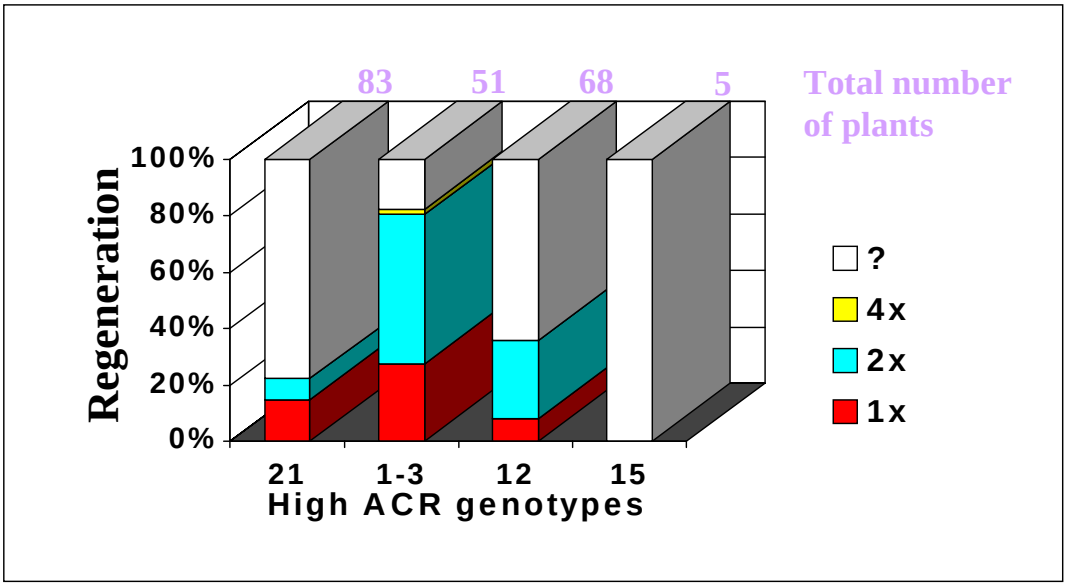

Figure 10: Ploidy of regenerants of anther-derived embryos of three high EPA CBC genotypes and the 1-3 control. The ploidy level of the regenerated plants was checked by flow cytometry. The bars depict the percentage of monoploid, diploid, tetraploid and undetermined plants from each high EPA genotype. The total number of regenerated plants included for each genotype is given above the bars.

1

Source	DF	M.S.	F value	Pr > F
Genoype	9	258.6	26.59	0.0001
Date	4	25.4	2.61	0.0442
Genot.*date	32	18.5	1.90	0.0157
Rep (date)	9	18.1	1.86	0.0758
Error	61	9.7		

2

Source	DF	M.S.	F value	Pr > F
Genotype	8	105.8	22.72	0.0001
Date	2	64.1	13.79	0.0001
Genot.*date	16	8.9	1.92	0.0045
Rep (date)	6	7.6	1.63	0.1617
Error	44	4.6		

3

Source	DF	M.S.	F value	Pr > F
Genotype	9	213.8	24.87	0.0001
Date	3	168.6	19.62	0.0001
Genot.*date	27	15.0	1.74	0.0374
Rep (date)	8	11.3	1.32	0.2523
Error	61	8.6		

4

Source	DF	M.S.	F value	Pr > F
Genotype	12	264.6	52.07	0.0001
Date	4	74.9	14.74	0.0001
Genot.*date	38	21.5	4.23	0.0001
Rep (date)	10	4.2	0.83	0.6017
Error	81	5.1		

Table 1: ANOVA tables for the series of four studies for EPA characterization of PBCp genotypes. EPA data were transformed by $\sqrt{1+\text{EPA}}$ prior to analysis.

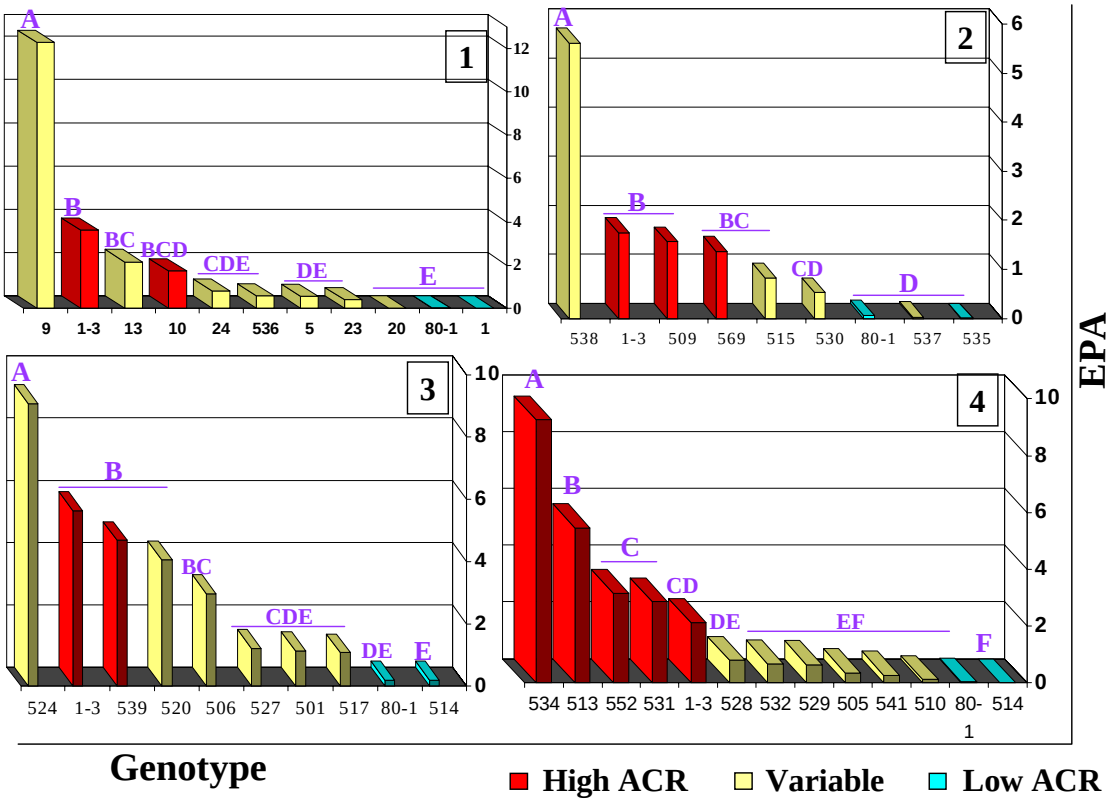

Figure 11: Results of the series of four PBCp studies for anther culture response in embryos per anther (EPA). In each study 7 to 11 genotypes were characterized, as well as the parental controls, 1-3 (high parent) and 80-1 (low parent). The studies were conducted on at least 3 different days, with three replications per day. The selections were based on the statistical grouping analysis (group letters shown in purple) given by REGWQ using SAS. Red and blue bars represent selections for high and low bulks, respectively. Some genotypes (9, 524, 538), though they generated more EPA than the high control, were not used because of dubious parentage (see chapter II).

Source	DF	Mean square	F value	Pr>F
Genotype	43	46.19	3.83	0.0001
Date	5	53.28	4.42	0.0008
Error	181	12.04		

Table 2: ANOVA for the large-scale study designed to increase the number of selected genotypes in the PBCp population. The experiment consisted of culturing 30 anthers from each of 44 genotypes in six different days following a randomized complete block design. This study allowed us to complete the characterization process for anther culture response in PBCp.

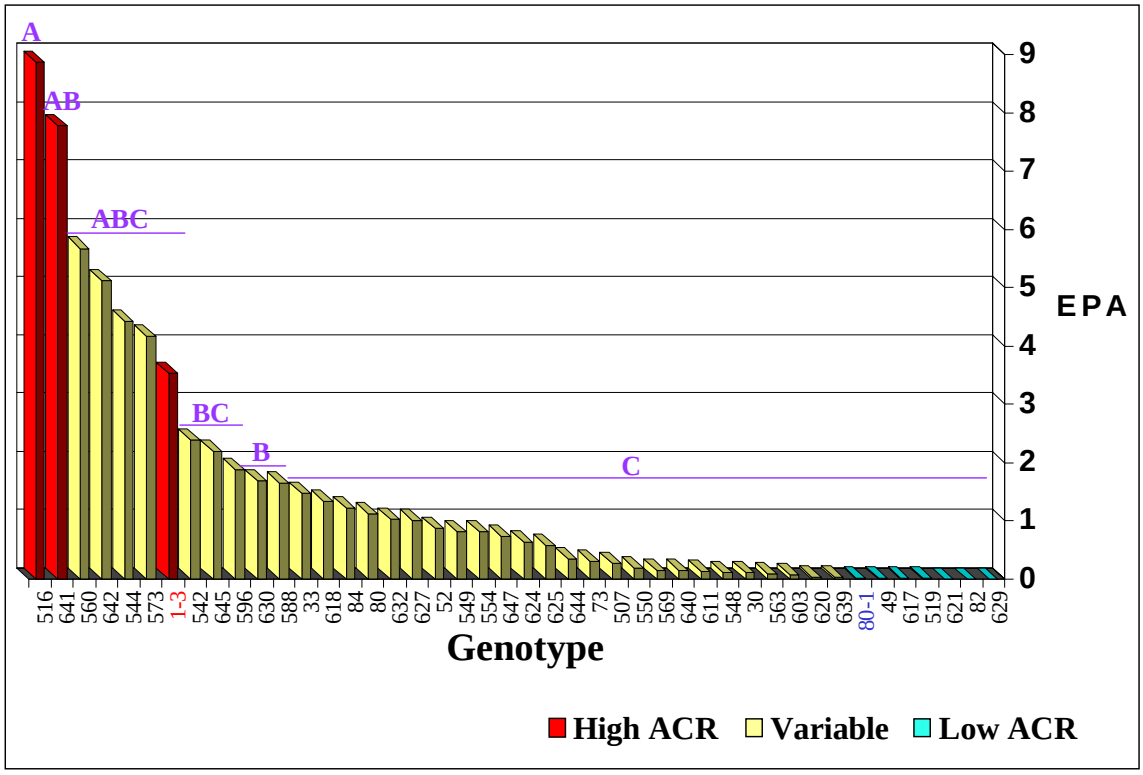

Figure 12: Results of a large-scale study for EPA in PBCp. Mean separation by REGWQ is indicated in purple letters above the bars.

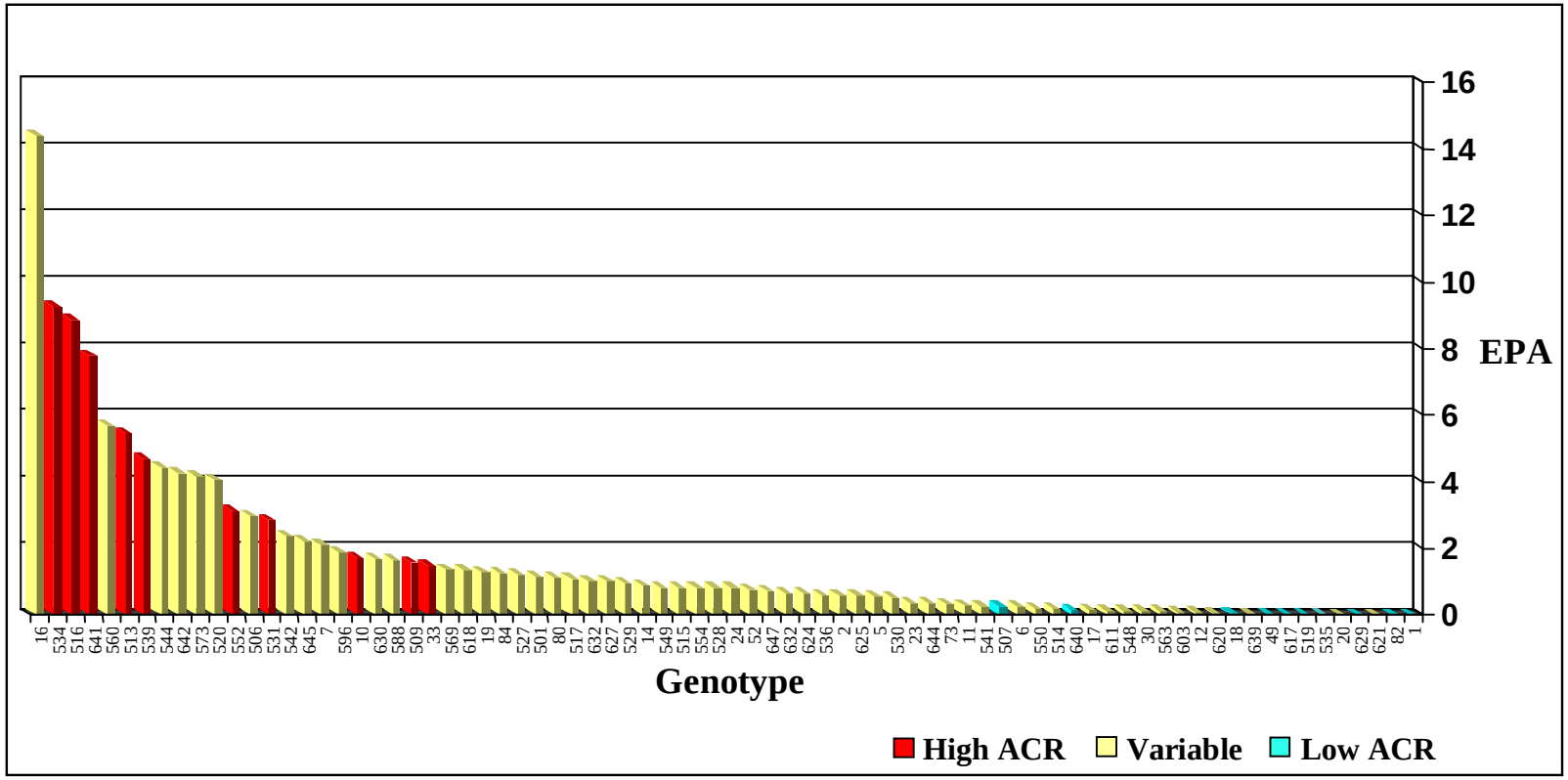

Figure13: Mean EPA for 78 sibling genotypes in the PBCp population summarized from the series of four studies and the large experiment consisiting pg 44 genotypes. Selections for the high and low EPA bulks are shown in red and blue, respectively.

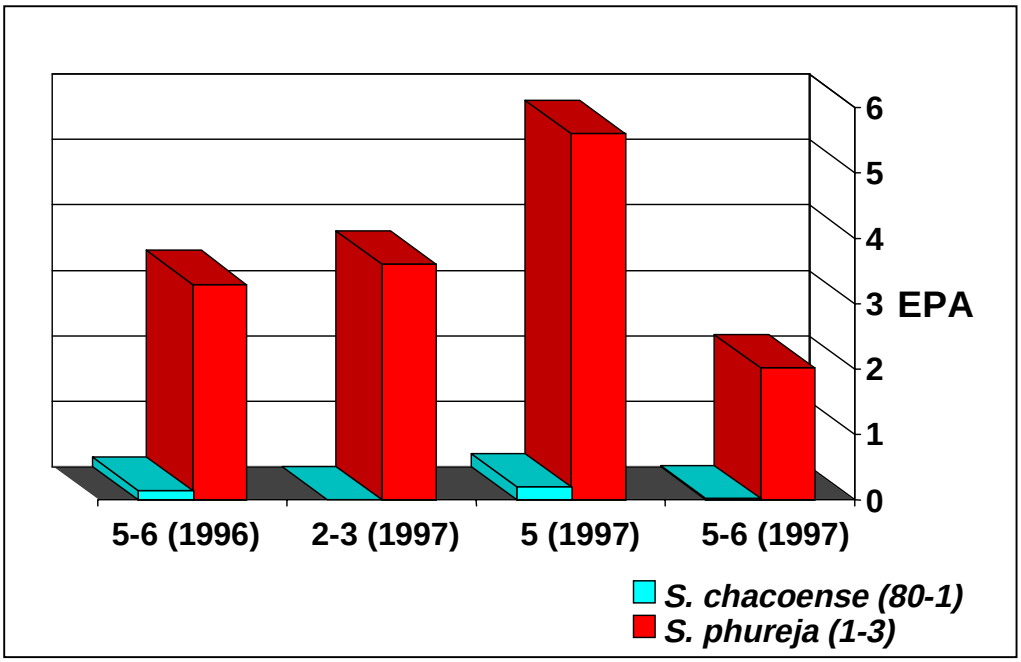

Figure 14: EPA of parental controls 80-1 and 1-3 over four studies. The numbers under the bars represent the months and year in which anthers were cultured.

1

Source	DF	M.S.	F value	Pr > F
Genotype	5	416.33	65.9	0.0001
Date	3	15.58	2.47	0.0765
Genot.*date	15	24.80	3.93	0.0003
Rep (date)	8	4.46	0.71	0.6838
Error	39	6.32		

2

Source	DF	M.S.	F value	Pr > F
Genotype	8	121.48	61.6	0.0001
Date	3	9.58	4.86	0.0046
Genot.*date	21	4.65	2.36	0.0059
Rep (date)	8	3.35	1.70	0.1197
Error	94	1.97		

3

Source	DF	M.S.	F value	Pr > F
Genotype	8	121.48	61.6	0.0001
Date	3	9.58	4.86	0.0046
Genot.*date	21	4.65	2.36	0.0059
Rep (date)	8	3.35	1.70	0.1197
Error	94	1.97		

4

Source	DF	M.S.	F value	Pr > F
Genotype	10	97.91	122.83	0.0001
Date	3	2.50	3.13	0.0302
Genot.*date	30	13.30	16.69	0.0001
Rep (date)	8	1.14	1.43	0.1986
Error	77	0.80		

5

Source	DF	M.S.	F value	Pr > F
Genotype	10	8.15	2.72	0.0073
Date	3	85.05	28.36	0.0001
Genot.*date	28	5.83	1.94	0.0140
Rep (date)	8	2.09	0.70	0.6945
Error	67	3.00		

Table 3: ANOVA tables for the series of five studies for EPA characterization of CBC genotypes. EPA data were transformed by $\sqrt{1+\text{EPA}}$ prior to analysis.

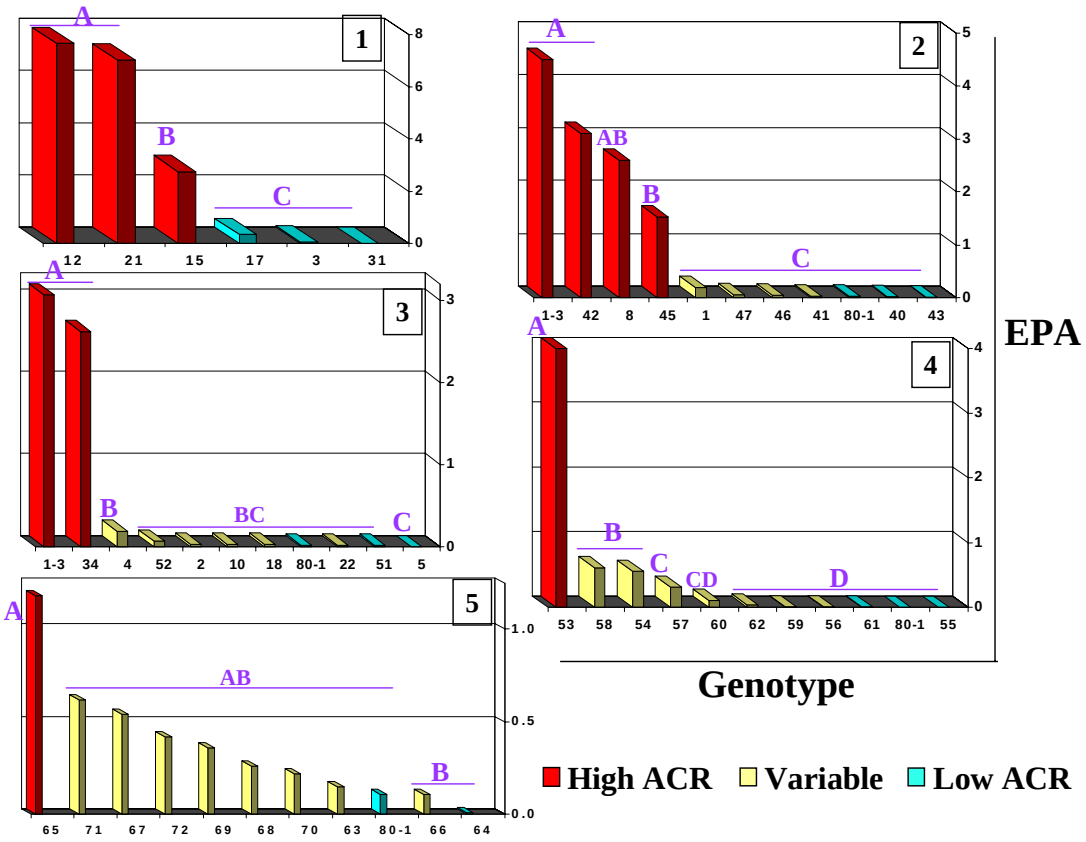

Figure 15: Results of the series of five CBC studies for anther culture response in embryos per anther (EPA). In each study 6 to 10 genotypes were characterized, and the parents 1-3 (high parent) and 80-1 (low parent) were included as controls whenever possible. The studies were conducted on at least 3 different days, with three replications per day. The selections were based on the statistical grouping analysis (group letters shown in purple) given by REGWQ using SAS. Red and blue bars represent selections for high and low bulks, respectively.

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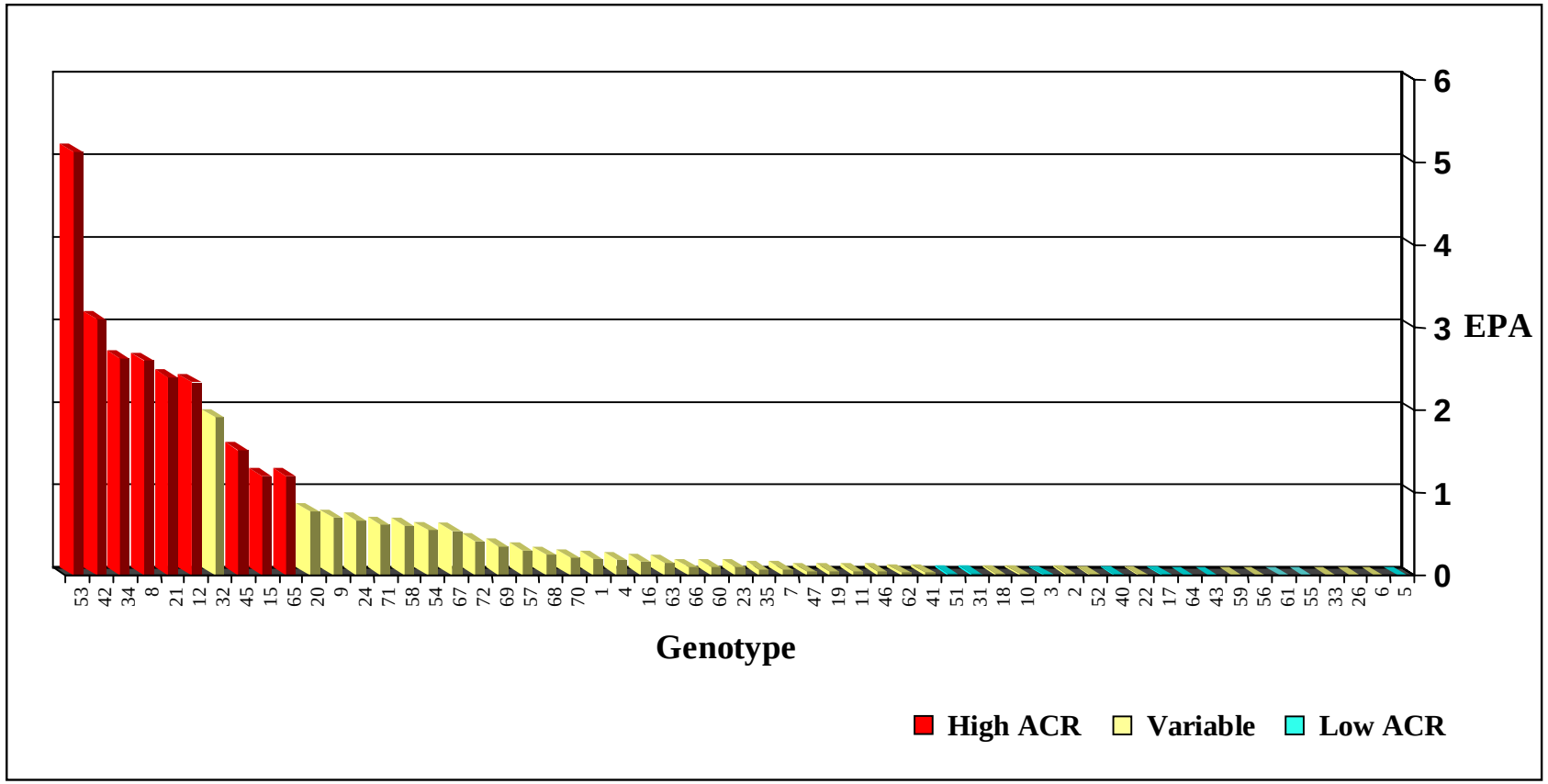

Figure 16: Mean EPA for 57 sibling genotypes in the CBC population summarized from the series of five studies. Selections for the high and low EPA bulks are shown in red and blue, respectively.

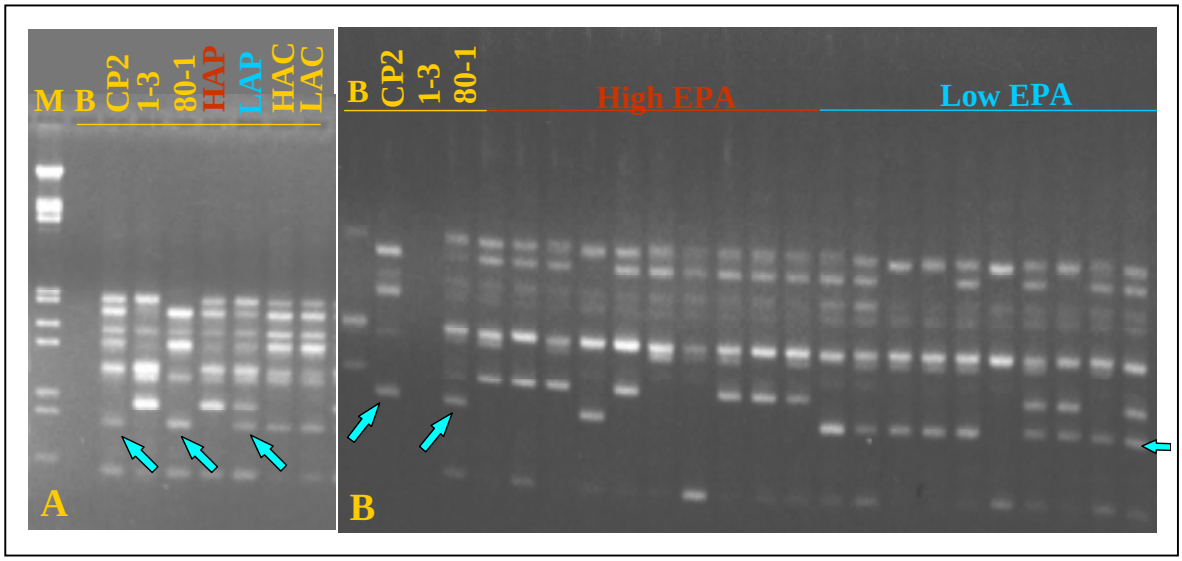

Figure 17: Bulk segregant analysis for EPA using primer OPQ-10. A band amplified by this primer was found to be linked in repulsion to ACR in the PBCp family. **A**: First primer screening where the band is present in the non-responsive parent (80-1), in the hybrid (CP2), and in the low bulk (LAP). The band is absent in the responsive parent (1-3) and in the high bulk (HAP). It is also present in both high (HAC) and low (LAC) CBC bulks. **B**: Individual selections of the PBCp bulks, the band was present in 9 of the 10 low selections and in 1 of the 10 high selections. Arrows indicate bands of interest.

A

Source	DF	M. S.	F value	Pr>F
OPQ-10	1	56.38	10.39	0.003
Error	33	5.43		
OPZ-4	1	31.54	5.32	0.02
Error	35	5.93		

B

Band in :		N	Mean EPA	REGWQ Grouping
OPQ-10	absent	17	3.07	A
	present	18	0.54	B
OPZ-4	present	28	2.22	A
	absent	9	0.07	B

Table 4: **A:** Results of one-way ANOVA analysis of EPA data from additional genotypes from the PBCp population for the markers linked to ACR. OPQ-10 is linked in repulsion and OPZ-4 is linked in coupling. **B:** Mean separation for each band is by Ryan Einot Gabriel Welsch Multiple F test (REGWQ).

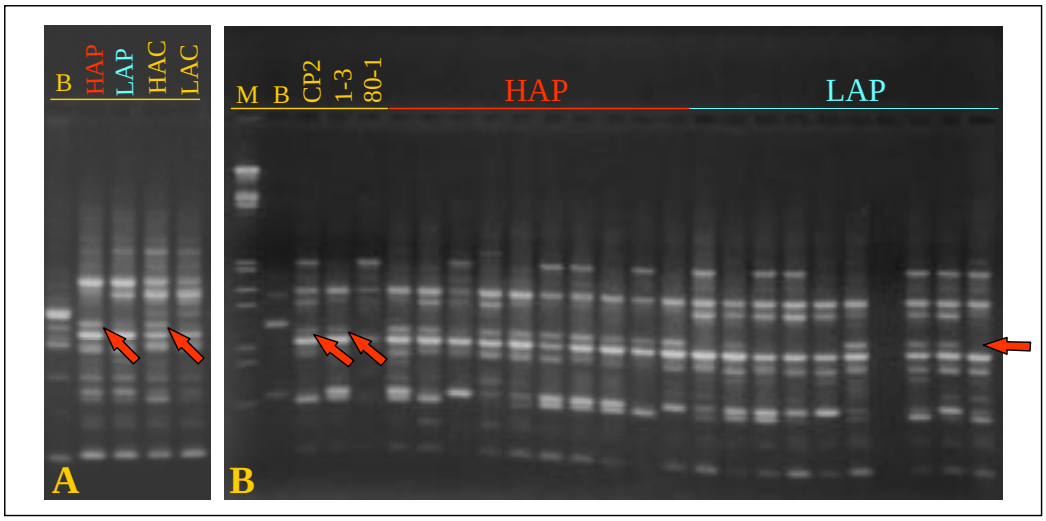

Figure 18: Bulk segregant analysis for EPA using primer OPZ-4. A band amplified by this primer was found to be linked in coupling to ACR in the PBCp family. **A**: First primer screening where the band is present in the high bulk (HAP) and not in the low bulk (LAP). It is also present in both high (HAC) and low (LAC) bulks of CBC. **B**: Individual selections of the PBCp bulks, the band was present in 10 of the 10 high selections and in 4 of the 10 low selections. Arrows indicate bands of interest.

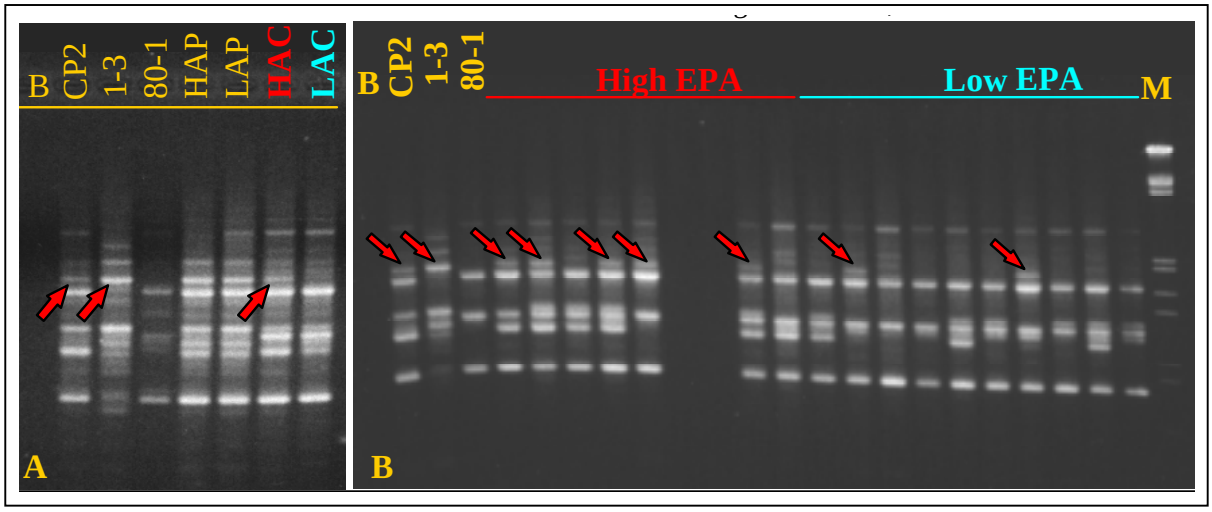

Figure 19: Bulk segregant analysis for EPA using primer OPW-14. A band amplified by this primer was found to be linked in coupling to ACR in the CBC family. **A**: First primer screening where the band is present in the responsive parent (1-3), in the hybrid (CP2) and in the high bulk (HAC), and not in the unresponsive parent (80-1), nor in the low bulk (LAC). It is also present in both high (HAP) and low (LAP) bulks of PBCp. **B**: Individual selections of the CBC bulks, the band was present in 7 of the 9 high selections and in 2 of the 10 low selections. Arrows indicate bands of interest.

A

Source	DF	M.S	F value	Pr>F
OPW-14	1	5.83	4.31	0.046
Error	32	1.35		

B

OPW-4 band	N	Mean EPA	REGWQ Grouping
present	12	1.29	A
absent	22	0.42	B

Table 5: A: Results of one-way ANOVA analysis of EPA data from additional genotypes from the CBC population for the markers linked to ACR. OPW-14 is linked in coupling. **B:** Mean separation for each band is by Ryan Einot Gabriel Welsch Multiple F test (REGWQ).

Chapter IV: Phenotypic characterization of 2n pollen production in a backcross family of diploid potato

Abstract

A backcross population, PBCp, derived from a cross between a hybrid (CP2) and its parents, *Solanum chacoense* Bitt. clone 80-1 [low % unreduced (2*n*) pollen] and *S. phureja* Juz. & Buk. clone 1-3 (high % 2*n* pollen), was characterized for 2*n* pollen production. Only 41 of the 77 genotypes bloomed for a period of time that allowed doing at least three collections. Pollen samples were examined by flow cytometry; however the trait was highly variable within genotypes and times of collection, and only six consistently high (mean=31% 2*n* pollen) and two consistently low (mean=3% 2*n* pollen) selections were obtained. The 41 genotypes showed continuous variation for 2n pollen production, suggesting multigenic control of the trait.

Introduction

This study focuses on the phenotypic characterization of a trait of interest for potato breeding and genetics using a segregating population developed by a backcross of an interspecific hybrid, CP2 (*Solanum chacoense* 80-1 × *S phureja* 1-3) and one of its parents, *S. phureja* Juz. & Buk. clone 1-3.

Production of diplandroids (male gametophytes and gametes with the somatic chromosome number) is under environmental as well as genetic control in potato (Hermsen, 1984). Mok and Peloquin (1975) described the mode of inheritance of three mechanisms of diplandroid formation. If 2*n* pollen frequencies are correlated with hybrid production, then considerable time and resources could be saved by choosing only high diplandroid pollen parents for $4x \times 2x$ crosses. This is a trait that could be introduced into

genotypes of interest for breeding programs when using genetic material of different ploidy to introgress characters such as resistance into cultivated potato.

Segregation ratios that support control of unreduced gamete formation by a single recessive gene were reported for potato (Iwanaga and Peloquin, 1982). Jacobsen (1980) concluded that major and minor genes controlled 2*n* pollen formation, and that the major genes are recessive. Veilleux (1983) concluded that the trait is heritable and could be easily manipulated.

The objective of this study was to examine variation for 2n pollen frequency in a potato family with the hope that we could identify extreme phenotypes to be able to apply bulk segregant analysis to identify molecular markers associated with the trait.

Materials and methods

Plant material

A backcross population, PBCp, was established by crossing a self-incompatible hybrid (CP2) from a cross between clones of *S. chacoense* and *S. phureja* to its *S. phureja* parent (*S. phureja* 1-3 × CP2). *S. chacoense* 80-1 produces a low percentage of unreduced (2*n*) pollen. On the other hand, *S. phureja* 1-3 produces a high percentage of 2*n* pollen.

Plants representing 77 genotypes were grown in the greenhouse. The environmental conditions were: 16 h photoperiod and 25-30°C day/15-20°C night. The photoperiod was controlled by a timer and was extended to 16 h, using halogen lamps (1000 watts).

Pollen collection and sample preparation

Pollen samples were collected over 8 weeks (once per week) from plants among the 77 genotypes that were in bloom each week during spring 1998. Pollen from each genotype was collected using an electric toothbrush as bee vibrator. We intended to do at least three collections per genotype. Collected samples were stored at -20°C in gelatin capsules inside a capped glass vial. Samples were processed as described by Owen et al.

(1988b). Pollen samples were placed in 1.5 ml microcentrifuge tubes, 0.5 ml of buffer solution [12 mM sodium citrate, 8 mM morpholinopropanesulfonic acid (MOPS), 38.4 mM $MgCl_2$, and 0.04% Triton X-100] was added. To each sample, 0.25 ml of RNAase solution (0.8 mg ml^{-1} buffer solution) was added and the samples were incubated at room temperature for 30 min, and analyzed within 3 h. The samples were stained by adding 0.125 ml of propidium iodide (PI) solution (0.3 mg PI ml^{-1} buffer solution) and incubating on ice for an additional 30 min. Stained samples were filtered through a 37 μm nylon mesh, and were ready to be analyzed within 2 h.

Flow cytometry

The analysis was conducted at the Virginia-Maryland Regional College of Veterinary Medicine using an Epics X-L laser flow cytometer and cell sorter (Coulter Electronics, Hialeah FL) to obtain graphic outputs and percentages according to pollen size and DNA distribution (measured by fluorescence of PI) as shown in Figure 1. Laser excitation was 300 mW, 488 nm from 5 W Innova 90 Argon Laser (Coherent Inc., Palo Alto, CA). The parameters recorded were: forward angle light scatter (FALS) and 90 degree light scatter (90 LS) for size measurements, and red fluorescence (RFL, 590 nm dichroic, 610 nm long pass filter) for DNA measurements. Multiparameter Data Acquisition and Display System (MDADS) and Easy 88 microcomputer analysis (Coulter Electronics) were used for data collection and analysis. FALS was collected as linear integral, 90LS log integral, and RFL linear peak red. Histograms of number of nuclei per fluorescence channel contained 256 channels and were gated by FALS and 90LS dual parameter 64×64 channel resolution histograms defining the populations of interest. The fluorescence signal from PI-stained preparations is proportional to the DNA content of pollen grains passing through the flow sheath. The calibration was done in comparison with percentages of 2*n* pollen of samples counted under a light microscope.

Results

Of the 77 genotypes from which we collected pollen, only 41 flowered for at least 3 weeks. Of those genotypes, 13 flowered during the first 3 consecutive weeks, 14 flowered during weeks 2, 3 and 4, three flowered during weeks 3, 4 and 5, three flowered during weeks 4, 5 and 6, the remaining genotypes were collected at least three times but not in consecutive weeks. A diagram of the mean 2*n* pollen at each collection for the group of 13 genotypes that flowered in weeks 1, 2, and 3 suggests that more 2*n* pollen was produced in the second week of the flowering period (Figure 2). This implies that 2n pollen production is reduced in early flowers but increases as plants mature, i.e., variation due to physiological factors. However, a similar diagram for the mean of the 14 genotypes that flowered during weeks 2, 3 and 4, showed a different trend that depicts more 2n pollen in the earliest flowers compared to those collected at the later two dates. If we consider the two groups of plants together, then 2n pollen frequency was greatest during week 2 (second collection for the first group of plants but first collection for the second group of plants that started flowering 1 week later). Thus variation for 2n pollen frequency would appear to be environmentally influenced (Figure 3).

The genotypes were highly variable for 2*n* pollen production. Figures 4 and 5 present individual 2*n* pollen percentage for the same 13 and 14 genotypes that flowered during weeks 1, 2 and 3, and weeks 2, 3 and 4, respectively. Each of the genotypes, with some exceptions (30, 33, 519, 575, 38, 588, 597, 614, 632 and 644) was highly variable between collections. We divided the 41 genotypes in four 2*n*-pollen frequency classes. Two genotypes produced less than 5% 2*n* pollen, ten produced from 5 to10 %, 14 produced from 10 to 20% and 15 produced more than 20% 2*n* pollen (Figure 6).

Six selections were found to be stable for high 2n pollen production, with an average of 31% unreduced pollen (82, 88, 568, 575, 576, 614). Only two genotypes were selected as low producers of 2n pollen, with an average of 3% unreduced pollen (33 and 626. In Figure 1, two extreme cases of unreduced pollen production are depicted. The outputs indicated percentages of peaks representing normal pollen and unreduced (large) pollen. All data collected are shown in Table 8 of the appendix section.

Discussion

Unreduced (2*n*) pollen production is an important trait in potato genetics. It is necessary to transfer the trait to germplasm of breeding interest and to speed the process of introgression of important characters from relatives of different ploidy level than commercial *S. tuberosum* cultivars.

All our genotypes produced 2*n* pollen, and many at very high percentage. Our results agree with Ramanna (1979) and Jacobsen (1980) in that 2*n* pollen production behaves as a quantitative trait. Means of three collections for 41 genotypes are represented in Figure 7, in which continuous variation, typical of quantitative genetic control can be observed.

We did not reach the same conclusion as Ramanna (1974), and Veilleux and Lauer (1981), about the increase in expression of 2*n* pollen as plants matured. By collecting pollen from plants three times over the flowering season, we were able to separate day effects from maturity effects, and conclude that environmental factors were more influential than plant maturity. Our results show that it is possible to characterize this trait by flow cytometry, but our population was not sufficiently large or did not express the required variation for BSA.

References

Hermsen, J.G. Th. 1984. Mechanisms and genetic implications of 2n gamete formation. Iowa State J. Res. 58:421-434.

Iwanaga, M., and S.J. Peloquin. 1982. Origin and evolution of cultivated tetraploid potatoes via 2n gametes. Theor. Appl. Genet. 61:161-169.

Jacobsen, E. 1980. Increase of diplandroid formation and seed set in 4x-2x crosses in potatoes by genetical manipulation of dihaploids and some theoretical consequences. Z. Pflanzenzucht. 85:110-121.

Mok, D.W.S., and S.J. Peloquin. 1975. The inheritance of three mechanisms of diplandroid (2n pollen) formation in diploid potatoes. Heredity 35:295-302.

Owen, H.R., R.E. Veilleux, F.L. Haynes, and K.G. Haynes. 1988. Photoperiod effects on 2n pollen production, response to anther culture, and net photosynthesis of a diplandrous clone of *Solanum phureja.* Am. Potato J. 65:131-138.

Ramanna, M.S. 1979. A re-examination of the mechanisms of 2*n* gamete formation in potato and its implications for breeding. Euphytica 28:537-561.

Ramanna, M.S. 1974. The origin of unreduced microspores due to aberrrant cytokinesis in the meiocytes of potato and its genetic significance. Euphytica 23:20-30.

Veilleux, R.E. 1983. Diploid and polyploid gametes in crop plants: mechanisms of formation and utilization in plant breeding. Plant Breeding Reviews 3:253-288.

Veilleux, R.E. and F.I. Lauer. 1981. Variation for 2*n* pollen production in clones of *Solanum phureja* Juz. and Buk. Theor. Appl. Genet. 59:95-100.

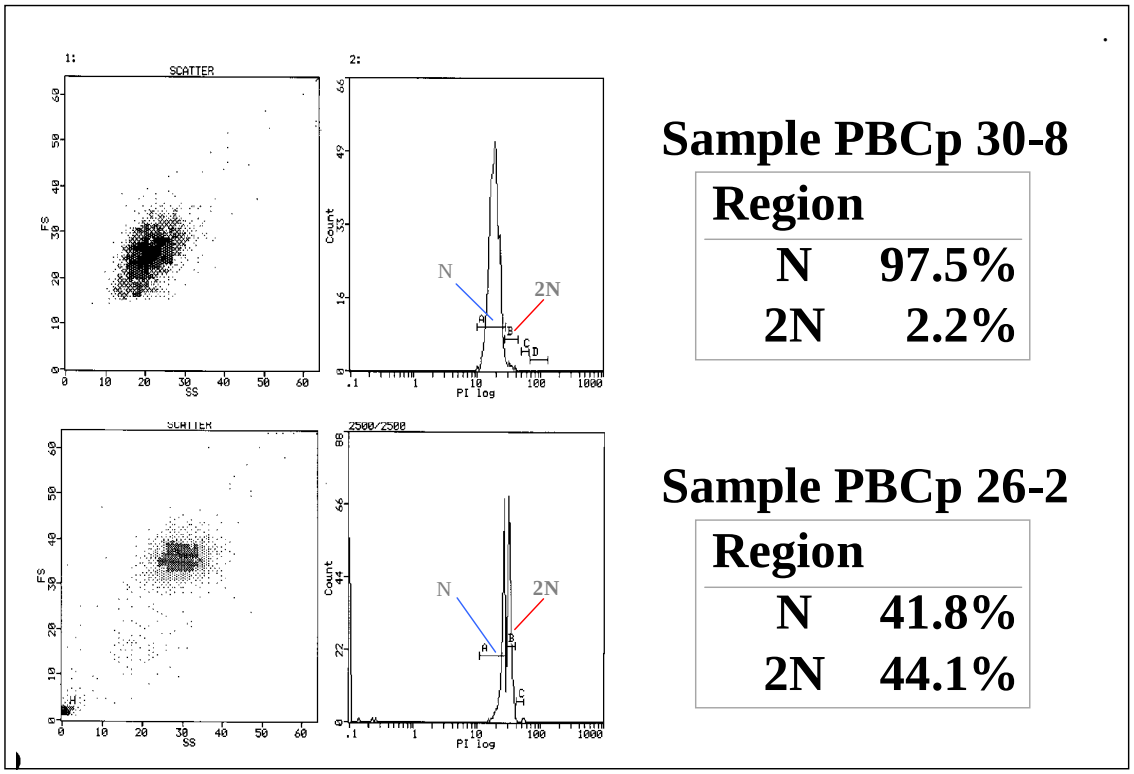

Figure 1: Flow cytometry output for unreduced pollen (2n) characterization. Pollen size was measured by forward angle (x-axis) and 90° (y-axis) light scatter and DNA distribution was measured by fluorescence of propidium iodide. X axis represents channel number, y-axis represents frequency of nuclei per channel. Graphs in the left depict the pollen population, and graphs in the right depict sub-populations peaks.

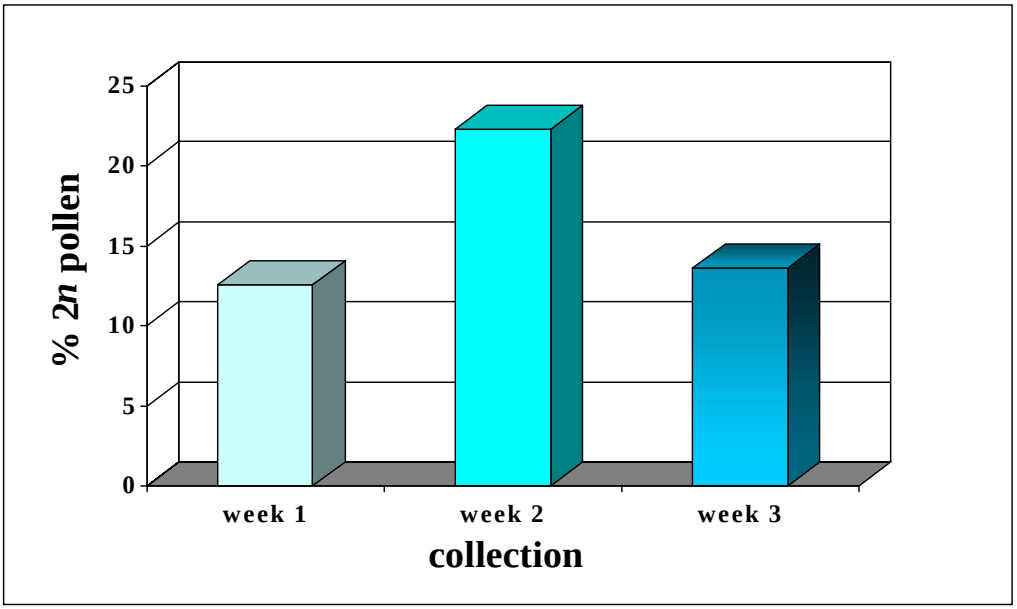

Figure 2: Mean unreduced pollen for thirteen genotypes that bloomed during the first three weeks of collection.

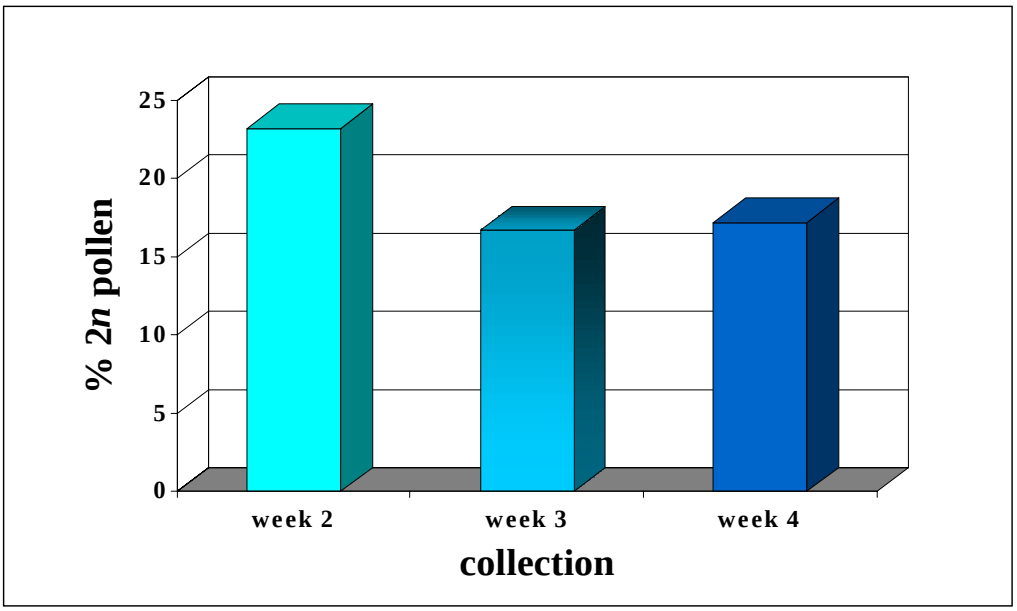

Figure 3: Percentage of unreduced pollen for 14 genotypes that bloomed during weeks 2, 3 and 4 of collection.

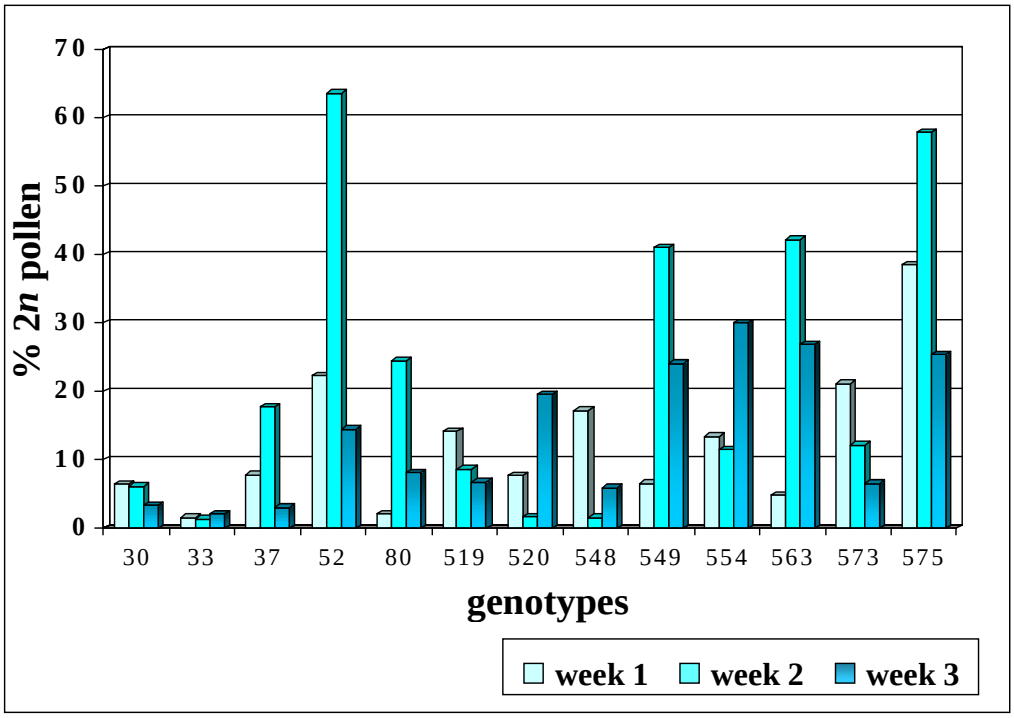

Figure 4: Percentage 2*n* pollen of each weekly collection for the 13 genotypes that bloomed during the first three weeks of collection.

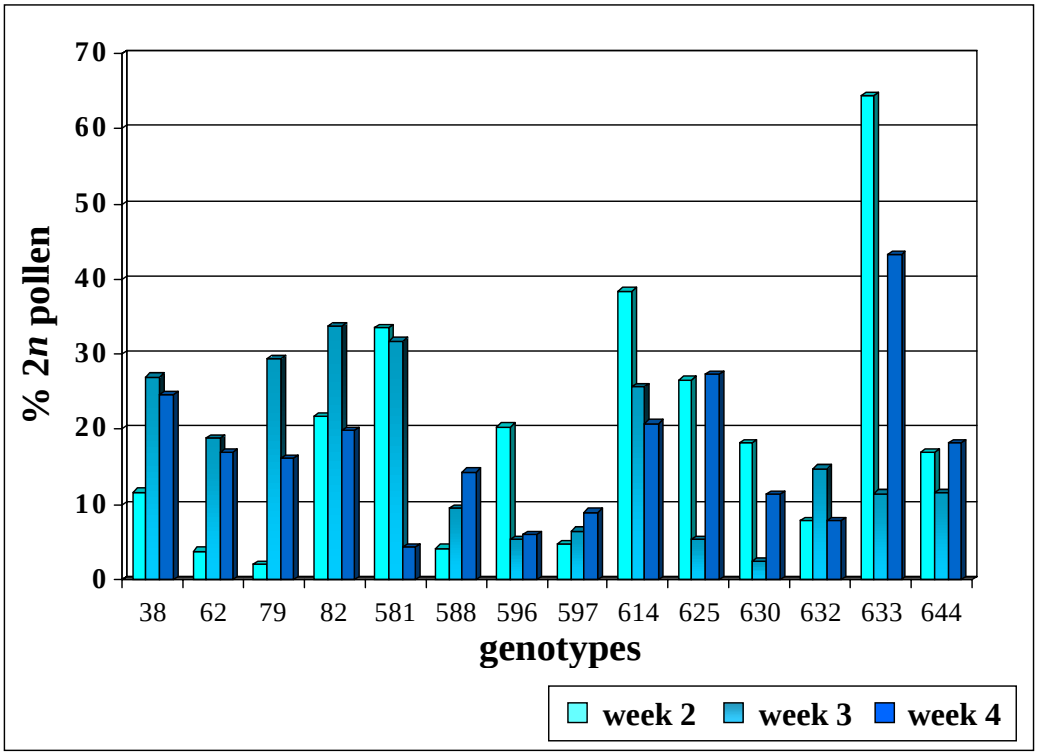

Figure 5: Mean 2*n* pollen of each weekly collection for the 14 genotypes that bloomed during weeks 2, 3 and 4.

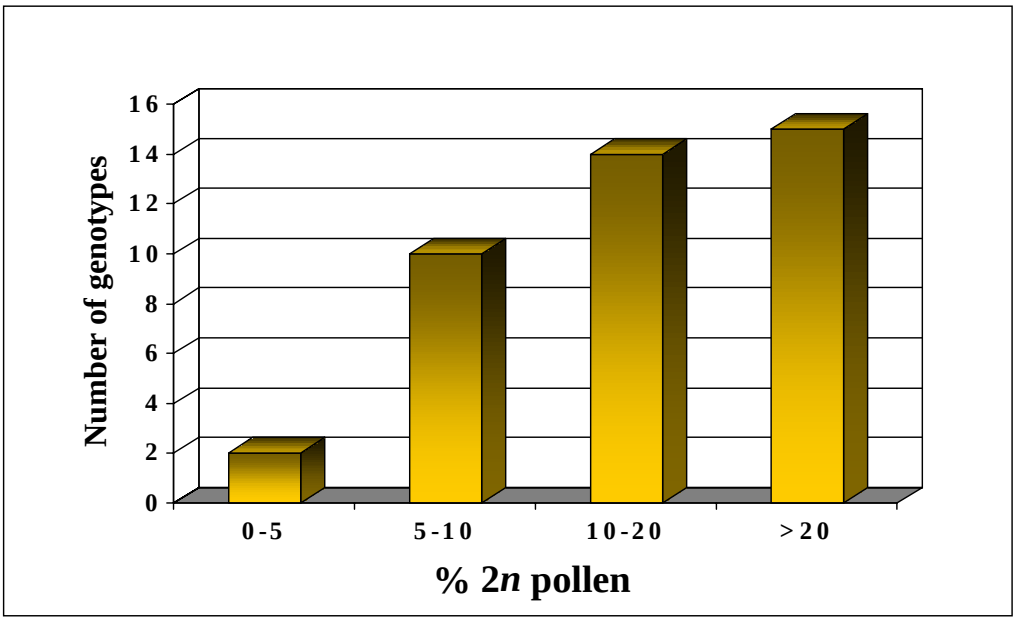

Figure 6: Unreduced pollen frequency classes for 41 genotypes that bloomed enough to allow at least three collections. Classes are based on the average of the three collections.

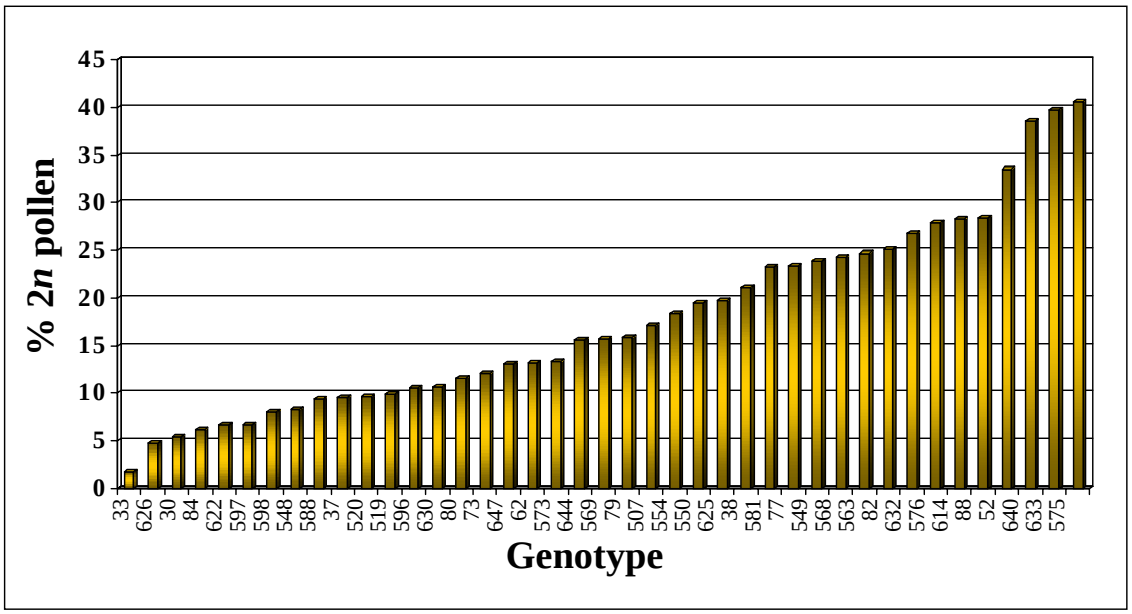

Figure 7: Mean 2*n* pollen frequency over three collections for 41 backcross potato genotypes for which three collections were possible.

Chapter V: Phenotypic characterization and bulk segregant analysis of leptine content in reciprocal backcross families of diploid potato

Abstract

Solanum phureja clone 1-3 and *S. chacoense* clone 80-1 have zero and high leptine content in their foliage, respectively. An F_1 hybrid (CP2) was intermediate for the trait, but self-incompatible. Two reciprocal backcross families, PBCp (1-3 × CP2 -- 87 genotypes) and its reciprocal PBCc (CP2 × 1-3 -- 42 genotypes) were characterized for leptine content. Leaf samples were analyzed by gas chromatography and the amounts of three specific gylcoalkaloids, solanidine, leptinidine, and acetylleptinidine (ALD, the aglycone precursor to leptines), were obtained. Leptines were present in 43 of 87 genotypes in the PBCp backcross, indicating simple genetic control by a dominant gene. However, the leptine levels were low compared to CP2. In the PBCc backcross, only 7 of 42 genotypes expressed leptines at a level generally higher than in PBCp. This is significantly different from the 1:1 segregation observed in the reciprocal backcross and indicates cytoplasmic inheritance. Ten high (mean ALD=546 μg g^{-1} dw) and ten low (mean ALD=0) selections within PBCp and seven high (mean ALD=3037 μg g^{-1} dw) and eight low (mean ALD=0) selections within PBCc were used for bulk segregant analysis (BSA) using 214 RAPD primers. Three primers OPQ-2, OPT-16 and OPT-20 amplified bands segregating with the high leptine bulks in PBCp and PBCc. These markers were linked in coupling to leptine content in PBCp. ANOVAs for leptine content using presence/absence of each band as a source of variation to classify genotypes in the entire population verified linkage of the markers to the trait.

Introduction

The Colorado potato beetle (CPB), *Leptinotarsa decemlineata* Say., is one of the most destructive pests of potato. The control of CPB has relied almost entirely on insecticides for over 125 years (Casagrande, 1987). In the 1980s its control became increasingly difficult with available chemical pesticides and potato cultivars that had little or no resistance (Sanford et al., 1984). Throughout its history the CPB has shown a remarkable ability to develop resistance to every insecticide used for its control (Forgash, 1985). Currently it shows resistance to 37 insecticides worldwide including organophosphates, carbamates, organochlorines, pyrethroids and hidrogen cyanide (Georghoiu and Lagunes-Tejeda, 1991). The CPB is now being controlled by the use of strong insecticides, like Admire and Provado (active ingredient Imidacloprid, Bayer Corp.). The introduction of new insecticides may reduce or eliminate yield losses, however, costs of resistance will continue because relatively inexpensive insecticides are no longer effective and newly developed products are up to five times more costly (Grafius, 1997). The first year Admire and Provado were introduced the amount of insecticides use on potato was reduced by three fold in Michigan (Bishop and Grafius, 1996). These insecticides have a long residual activity against CPB adults and larvae and other potato pests (Boiteau et al., 1997). However, in 1996, CPB from a field in Michigan were found to be four times less sensitive to Admire than susceptible CPB (Grafius and Bishop, 1997). Kisha et al. (1998) observed that the combination of high foliar leptines with a Bt gene increased effectiveness in controlling the CPB.

Host resistance as part of an integrated pest management program is a possible way of controlling CPB. Other than high levels of leptine glycoalkaloids (Sinden et al., 1986), glandular trichomes (Plaisted et al., 1992) are a natural form of resistance. However, the CPB is also known to adapt to factors such as glandular trichomes (Groden and Casagrande, 1986). Introduced *Bacillus thuringiensis* (Bt) toxin genes represent a different source of resistance to insect pests of potato (Douches et al., 1998), but this resistance likely will be short-lived, since artificial selection for resistance in Colorado potato beetle populations to Bt has resulted in more tolerant insect populations (Whalon

et al., 1993). Integrated pest management programs including the use of Admire and Bt "new leaf" cultivars are going on successfully at present to control the CPB. Work needs to be done to integrate plant resistance to insects with other insect control methods (Stoner, 1996).

Since the mid 1940s *Solanum* glycoalkaloids have been implicated as feeding deterrents for CPB. Stürckow and Löw (1961) compared the effect of a number of *Solanum* glycoalkaloids on CPB and determined that the leptines were the most potent. Fifty years ago resistant interspecific hybrids were developed in Europe, but this breeding research was discontinued when strong pesticides (such as DDT) became available (Schwarze, 1963; Torka, 1950). These early European hybrids between wild *Solanum* species and *S. tuberosum* L. generally had high total glycoalkaloid levels. The resistance in various wild *Solanum* species, particularly *S. chacoense* Bitt., appears to be associated with the presence of glycoalkaloids such as leptines, not found in *S. tuberosum* (Sinden et al., 1986).

Leptines first identified by Kuhn and Low (1961), are acetylated forms of the common glycoalkaloid solanidine, which is present in most *Solanum* species. Solanine and chaconine, derived from the aglycone solanidine, are the most prevalent glycoalkaloids found in cultivated potato (Deahl et al. 1993). According to Kuhn and Low (1961), leptines are not found in *S. chacoense* tubers, therefore there should be no concern about introducing the toxicity of glycoalkaloids into potato foliage by using *S. chacoense* accessions in breeding programs. Glycoalkaloids are quantitatively inherited in *S. tuberosum*, with broad sense heritability ranging from 86-89% (Sinden et al. 1984). Sanford and Sinden (1972) observed continuous variation in the level of glycoalkaloids in segregating populations (F_2 or backcross), indicating polygenic inheritance. Sinden et al. (1986) mentioned that within *S. chacoense*, only a few accessions synthesize leptines and that within some leptine-synthesizing accessions, only a few sibs are leptine-synthesizers, suggesting control of leptine synthesis by a single or few genes. Glycoalkaloids appear to have high heritability, but several environmental factors, such as temperature, light, and even mechanical stress, affect levels of glycoalkaloids (Maga 1994). Genes from *S.*

tuberosum were dominant for lower levels of glycoalkaloids in a cross between *S. chacoense* and *S. tuberosum* (Sanford et al. 1994). In crosses between *S. chacoense* parents with high and low levels of glycoalkaloids, the levels of the F_1 genotypes were closer to that of the low parent (Sanford et al. 1994, 1996). These observations suggest that suppression of glycoalkaloid expression is probably dominant and that multiple recessive alleles are required for the expression of elevated levels of glycoalkaloids. A limitation of traditional breeding for leptine biosynthesis is the probable additive nature of the trait (Sinden et al., 1984). Hybrids receiving only a single allele from *S. chacoense* express only a fraction of the leptine level found in the *S. chacoense* parent (Veilleux and Miller, 1998).

The polymerase chain reaction (PCR) facilitates the use of molecular markers such as randomly amplified polymorphic DNA (RAPD; Williams et al., 1990) in genetic studies and breeding programs. Finding RAPD markers linked to genes controlling traits of interest can aid phenotypic identification during the selection process, introgression of the trait, and germplasm characterization. Molecular markers linked to a trait of interest can be identified by the use of bulk segregant analysis (BSA; Michelmore et al., 1991), a technique that consists of bulking the DNA of genotypes exhibiting the extreme phenotypes of a trait in a segregating population (Figure 1). Molecular markers like RAPDs are then screened using these bulks, to find polymorphic DNA fragments segregating with the trait of interest.

RAPDs combined with BSA have been used successfully to identify DNA sequences linked to different traits of interest in crops, such as crown rust resistance in oat, *Avena sativa* L. (Penner et al., 1993), daylength insensitivity in oat (Whight et al., 1994), scab resistance in apple *Malus* spp. (Yang et al., 1997), leaf rust resistance in barley, *Hordeum vulgare* L. (Poulsen et al., 1995), root knot nematode resistance in sweet potato, *Ipomoea batatas* (Ukoskit et al., 1997), oleic acid concentration in spring turnip rape, *Brassica rapa* ssp. *oleifera* (Tanhuanpää et al., 1996), powdery mildew resistance in wheat, *Triticum aestivum* L. em. Thell (Hu et al., 1997), locus M for sex

expression in asparagus, *Asparagus officinalis* L. (Jiang and Sink, 1997) and resistance to blackleg (*Leptosphaeria maculans*) in *Brassica napus* L. (Chèvre et al., 1997).

Yencho et al. (1998) mapped quantitative trait loci (QTL) of foliar glycoalkaloid aglycones in *Solanum tuberosum* × *S. berthaultii* Hawkes potato progenies. Several QTLs for the accumulation of solasodine and solanidine were identified in a backcross of the hybrid (from the cross between *S. tuberosum* × *S. berthaultii*) to *S. berthaultii* and in a backcross of such hybrid to *S. tuberosum.* Two QTLs were identified for solanidine in the *S. tuberosum* backcross, one of these QTLs (the most significant one) was also identified in the *S. berthaultii* backcross. Yencho et al. (1998) suggested that these QTLs might correspond to structural and/or regulatory genes controlling the accumulation of these compounds.

Leptine production is found naturally only in genotypes that are not of commercial importance. One approach to integrate this trait into desirable germplasm could be to clone genes involved in the control of the trait. These genes then could be introduced by genetic transformation into any genotype in which its presence is desired. It is possible that multiple copies introduced through genetic transformation could result in greater expression of leptine production in hybrids. However, this would require simple genetic control and isolation of the necessary gene or genes. This research represents only a step in this process. This study focuses on the phenotypic characterization of leptine glycoalkaloid synthesis, a trait of interest for potato breeding and genetics and on the use of RAPDs and BSA to identify molecular markers linked to leptine production. The populations used in this study were developed by reciprocal backcrosses of an interspecific hybrid (cross between *S. chacoense* and *S. phureja* Juz. & Buk.) and one of its parents, *S. phureja.*

Materials and methods

Plant material

Two reciprocal backcross populations were established by crossing a self-incompatible hybrid (CP2) from a cross between clones of *S. chacoense* and *S. phureja* to

its *S. phureja* parent. *S. chacoense* 80-1 produces a high amount of leptine glycoalkaloids (range of production from 7055 to 22261 $\mu g.g^{-1}$ under greenhouse conditions) in its leaves. *S. phureja* 1-3 does not produce leptine glycoalkaloids. The hybrid, CP2, was intermediate for the trait. CP2 was backcrossed to 1-3 and these reciprocal populations 1-3 × CP2 (PBCp—87 genotypes) and CP2 × 1-3 (PBCc—42 genotypes) were used for phenotypic characterization of leptine content (the lower case letter at the end of these family acronyms represents *S. phureja* or *S. chacoense* cytoplasm.). The objective was to obtain extreme selections for bulk segregant analysis.

Backcross seedlings were grown in the greenhouse. The environmental conditions were: 16 h photoperiod and 25-30°C day/ 15-20°C night. The photoperiod was extended to 16 h using halogen lamps (1000 watts). Leaf samples (2-3 fully expanded young leaves) were taken from plants grown under greenhouse conditions, for PBCp during spring 1997 and for PBCc during spring 1998. The samples were placed in perforated paper envelopes, freeze dried in a Virtis 3L benchtop freeze drier (The Virtis company, Inc., New York) and frozen at –30°C. The dry samples were ground with liquid nitrogen, and the grindate sent in 1.5 ml ependorf tubes to be analyzed. Dr. A. Raymond Miller, at Ohio Agricultural Research and Development Center (Wooster, Ohio) did the glycoalkaloid analysis by gas chromatography, as described by Lawson et al. (1992). Data were obtained for solanidine (SL), leptinidine (LEP), acetylleptinidine (ALD), and total glycoalkaloids in $\mu g.g^{-1}$ of dry weight. The chemical structures and proposed pathway of these glycoalkaloids are depicted in Figure 2.

Bulk selections

DNA was extracted as described by Doyle and Doyle (1987), a modification of the 2x CTAB method described by Saghai Maroof (1984) (see appendix). Leaf samples taken from young plants grown in a greenhouse provided the starting material. Equal volumes of DNA (at concentrations of 10 ng/μl) from each selection were pooled to comprise high leptine and low leptine bulks for each population.

Screening of primers

A total of 214 RAPD primers from sets A, C, G and Q through Z of Operon Technologies (Alameda, California) was used to screen each pair of bulks. PCR reaction mixtures of 25 µl contained: 20 ng genomic DNA, 0.6 µM primer, 200 µM dATP, dCTP, dGTP, dTTP, 1× PCR buffer (2.5 mM $MgCl_2$, 50 mM KCl, 10mM Tris-HCl, pH 8.3), and 1 U *Taq* DNA polymerase of Promega (Madison, Wisconsin). Amplifications were conducted in an Amersham robocycler or in a Perkin Elmer Cetus Model 480 thermal cycler. In the latter case the reactions were overlaid with a drop of sterile mineral oil. The amplification procedure consisted of 45 cycles of 1 min denaturation at 94ºC, 1 min primer annealing at 37ºC, 2 min extension at 72ºC, followed by a final extension at 72ºC for 5 min. The amplified samples were separated in 1.4% agarose gels in 1× TBE buffer [10.8 g trizma base, 5.48 g boric acid, and 4 ml EDTA (0.5 mM) / L distilled water] for 3.5 to 4.5 h at 100 V. λ DNA digested with *EcoRI* and *HindIII* was used as size marker and a PCR mix without template DNA as blank control. After electrophoresis gels were stained with ethidium bromide (1.5 µg ml^{-1}) and photographed under UV light.

With 154 primers 754 bands were polymorphic between clones 1-3 and 80-1. For these 154 primers, PCR reactions were conducted with the parental clones (1-3, 80-1 and CP2). For an additional 60 primers, PCR reactions were conducted only on the bulks. In this case, when a polymorphic band was observed, the bulks were run again together with the parental clones to verify polymorphism. In all cases, if the polymorphism was verified in parents and bulks, the individual selections within the bulks were run followed by remaining genotypes in the family (70 genotypes for PBCp and 27 for PBCc).

Genetic hypothesis

Assuming that *S chacoense* 80-1 is homozygous dominant for a gene [all F_1 plants resulting from crosses with 80-1 produced some leptines (Veilleux and Miller 1998)] and *S phureja* 1-3 is homozygous recessive (it does not produce any leptines at all), the expected segregation for both PBCp and PBCc populations is the same. Therefore, a band

linked to the trait in one of the populations should also be linked in the other population (Fig. 3).

Statistical analysis to determine linkage between putative bands and traits

Data were taken by assigning a "0" for the absence of the polymorphic band and a "1" for the presence of such band from each of the genotypes within the population in which the bulks showed the same polymorphism found between the parents. These data were analyzed together with data obtained from the phenotypic characterization: the amount of solanidine, leptinidine and acetylleptinidine (leptine glycoalkaloid) were measured and expressed as in $\mu g.g^{-1}$ dry weight. One-way ANOVA tests using SAS proc GLM procedure were used to determine whether presence/absence of a candidate band was a significant source of variation for phenotypic data for glycoalkaloids.

Isolation and sequence of linked bands

If a band was significantly linked to the trait, a PCR reaction was set for a single genotype and the electrophoresis gel was run for a longer time (5 – 7 hours). The band was then excised from the gel under UV light and purified using Gene Clean (Bio 101, St. Louis, Missouri). The purified fragment was used again in a PCR reaction with RAPD primers to amplify a single band. When a single band was amplified, the PCR reaction was used for ligation of the fragment into a plasmid using the Pgem T-easy vector system from Promega (Madison, Wisconsin). The ligation reaction contained 5 µl of 2× Rapid ligation buffer, 1 µl pGEM-T easy vector (50 ng), 2 µl PCR product, 1 µl T4 DNA ligase (3 Weiss units/µl) and 1 µl de-ionized water in a total volume of 10 µl (all reactants were provided in the Pgem T-easy vector system kit). The reaction was incubated overnight at 4°C. The ligation reaction was then transformed into *E. coli* competent cells, by adding 100 µl thawed competent cells to a sterile 1.5 µl microcentrifuge tube containing 2 µl of each ligation reaction on ice. Colonies were grown on solid ampicillin-LB media. A

number of colonies were transferred to liquid Terrific broth overnight for plasmid extraction. Plasmid DNA from each selected white colony was then digested with *NotI* restriction enzyme and run in an agarose gel together with the original fragment, purified by Gene Clean and λ DNA digested with *EcoRI* and *HindIII*. A fragment of the correct size was sent to be sequenced at the VT sequencing facility (Blacksburg, Virginia).

Specific primers were designed for the sequenced fragment using Primer 0.5 computer software.

Results

Leptine content was characterized in the reciprocal populations based on gas chromatographic analysis. The analysis determined the amount of solanidine (SD), leptinidine (LD), and acetylleptinidine (ALD or leptine). From prior research (Veilleux and Miller, 1998) we know that 80-1 synthesizes an intermediate amount of SD and LD, and produces high amounts of ALD (SD = 1260; LD = 7250; ALD = 11320 $\mu g.g^{-1}$ dry wt.), 1-3 synthesizes SD, but does not synthesize any LD or ALD (SD = 3480 $\mu g.g^{-1}$ dry wt.), and CP2 is intermediate for the three compounds (SD = 940; LD = 1350; ALD = 2000 $\mu g.g^{-1}$ dry wt.) (Table 1).

From the characterized genotypes in the PBCp population, 44 produced no leptines and 43 produced low levels ranging from 50 to 790 $\mu g.g^{-1}$ dry wt. If we propose production/non production of leptines to be controlled by a single gene, disregarding absolute quantities of leptines produced per plant, then the ratio does not differ significantly from 1:1 (χ^2 = 01, p=0.92). Hence it would appear that a single gene controls leptine production in this population. However, because all genotypes expressing leptines are necessarily heterozygous and considerably lower in leptine production than the heterozygous parent, CP2, then additional genes must influence expression. The reciprocal population PBCc, differing from PBCp only by source of cytoplasm (PBCp = *S. phureja* 1-3 cytoplasm and PBCc = *S. chacoense* 80-1 cytoplasm) had a different pattern of leptine expression. In this population only 7 of 42 genotypes expressed leptines. This differs significantly from the 1:1 ratio of leptine producers found in PBCp

(χ^2 = 18.7, p<0.005). In addition, the amount of leptine produced by the seven PBCc leptine expressing genotypes ($\bar{x}$ = 3036 μg.g^{-1} dry wt.) was generally greater than for those in PBCp ($\bar{x}$ = 546 μg.g^{-1} dry wt.) and greater than that of CP2 (2000 μg.g^{-1} dry wt.). Hence there would appear to be a cytoplasmic influence on leptine production. Given the 1:1 segregation in PBCp, it would seem likely that leptine genes must be present in many nil producers in PBCc but not expressed.

Ten selections were taken for each, the high and the low bulks in the PBCp population (Fig. 4), with nil leptine for the lows and a mean of 546 ± 74 μg.g^{-1} dry wt. leptine for the highs. In the PBCc population seven and eight genotypes were selected for the high and low bulks, respectively, with nil leptine for the lows and a mean of 3007 ± 1305 μg.g^{-1} dry wt. leptine for the highs (Fig. 5).

Five RAPD bands were found that segregated with the trait. OPQ-2 amplified a fragment (aprox 1200 bp) that was present in nine of the ten high bulk selections in PBCp and in five of the seven high bulk selections in PBCc populations (Fig. 6). This fragment was present in one of ten PBCp and in three of eight PBCc low bulk selections. The one-way ANOVA test resulted in significant difference for high leptine content (ALD) genotypes presenting the band and no leptine genotypes with no such band in the PBCp population but, even though the trend was similar in the PBCc population, there was no significant difference for genotypes with and without the marker (χ^2= 0.23; P=0.63) in this population. The mean leptine content for the genotypes containing the marker amplified by this primer in PBCp was 247 μg.g^{-1} dry wt., and the mean for those without the marker was 126 μg.g^{-1} dry wt. (Table 2).

OPT-7 amplified a band (aprox. 150 bp) that was present in the high bulks and absent in the low bulks of both populations (Fig. 7). In gels ran with PCR reactions from the individual selections of the bulks with this primer, the fragment was present in seven of the ten PBCp, and four of the seven PBCc high bulk selections. The fragment was present in three of the ten PBCp and two of the eight PBCc low bulk selections. In this case the trend was similar for both populations, but the results did not show a significant difference for leptine content (ALD) in either of these populations. Interestingly, the one-

way ANOVA for leptinidine (LD) content was significant for the PBCc population. The mean LD content for the genotypes with the band was 312 $\mu g.g^{-1}$ dry wt., and for the ones without the band was 124 $\mu g.g^{-1}$ dry wt. (Table 3).

OPT-16 amplified a fragment that was linked in coupling to leptine content (approx. 1300 bp). This fragment was present in ten of the ten PBCp and in six of the seven PBCc high bulk selections (Fig. 8). It was present also in six of the ten PBCp and four of the eight PBCc low bulk selections. The results of the one-way ANOVA analysis were highly significant for the PBCp population (χ^2= 7.35; P=0.007) but not significant for the PBCc population (χ^2= 0.86; P=0.36). The mean leptine content for the genotypes in the PBCp population containing the fragment was 210 $\mu g.g^{-1}$ dry wt., and for the ones not presenting it was 68 $\mu g.g^{-1}$ dry wt. (Table 2).

OPT-20 amplified a fragment that was linked in coupling to leptine content. The fragment amplified by this primer (approx. 250 bp) was present in ten of the ten PBCp and in seven of the seven PBCc high bulk selections. It was also present in three of the ten PBCp and in two of the eight PBCc low bulk selections (Fig. 9). The one-way ANOVA results were similar to those for OPT-16 (χ^2= 2.71; P=0.1 for PBCp; χ^2= 0.12; P=0.73 for PBCc). The mean leptine content for the genotypes containing the fragment in the PBCp population was 218 in $\mu g.g^{-1}$ dry wt. and mean leptine content for the genotypes not presenting the fragment was 73 $\mu g.g^{-1}$ dry wt. (Table 2).

Five RAPD bands were found that segregated with the trait, but not all of them were significantly linked based on the one-way ANOVA results (Table 4). OPW-10 amplified a band that was not significant. This band was present in eight of the ten high selections and in five of the ten low selections of PBCp, it was also present in five of the seven high selections and three of the eight low selections of PBCc (Table 5). Data for SD, LD, and ALD for all genotypes and RAPD amplification results for the five primers are presented in Appendix Tables 9 and 10.

The data for all of these primers were also analyzed using Map-Maker Computer Program (Lander et al., 1987). Linkage was found between markers amplified by OPT-16 and OPT-20 with a genetic distance of 0.4 cM. Because the phenotypic data were similar

for the two markers it is impossible to determine which might be closer to a gene involved in leptine production.

The attempts to isolate and sequence the bands of interest to design SCARS were not successful. The only fragment for which we obtained an acceptable sequence was the one amplified by primer OPT-20, which was linked to leptine content. After designing and obtaining the primer pair, we realized that the new fragment amplified part of the vector Pgem-T, used for cloning the fragment. The remaining sequence that was not part of the vector was not suitable to design primers.

Discussion

S. chacoense 80-1, a selected clone within an accession of *Solanum chacoense,* expresses high levels of leptine glycoalkaloids in leaves. CP2, an F_1 hybrid between 80-1 and *S. phureja* 1-3 with *S. chacoense* cytoplasm, is necessarily heterozygous for genes responsible for leptine production because its *S. phureja* parent produced no leptines. Any leptine producing progeny from backcrosses of CP2 to *S. phureja* would also be heterozygous for genes for leptines. In the PBCp backcross, with *S. phureja* cytoplasm, 43 of 87 genotypes (50%) produced low levels of leptines. However, in the reciprocal backcross, PBCc, genetically similar except for *S. chacoense* cytoplasm, only 7 of 42 genotypes (17%) produced leptines, but the leptine levels in these selections were much higher than for PBCp leptine selections. Low leptine producers may obscure true genetic ratios as they challenge the limits of detection by gas chromatography. However regarding genes controlling leptines, our study implicates both dominance and cytoplasmic influence. Single gene or multigenic control of this trait is still a question. The lack of simple segregation implicates more than a single gene. However, true segregation patterns might be obscured by variable expressivity. Ronning et al. (1998) studied the segregation of leptine production in backcross families derived from leptine producer clones and non-leptine producer clones of *S. chacoense.* Their populations segregated agreeing with a single recessive gene model, but significant differences from the expected segregation in some of their populations suggested the presence of modifiers affecting the inheritance and expression of leptines. We do not disagree with that

assumption, it is possible that we are dealing with alleles for a gene controlling leptine production that is not affected by other alleles present in other *S. chacoense* accessions. If that is the case, the alleles present in our *S. chacoense* clone are acting as dominant, since the other parent in our population is a clone of *S. phureja* that may not even have an allele for leptine production. Looking at the segregation of leptines in our populations, we can not agree with Sinden et al. (1984) that proposed a multigenic control of the trait. Sanford et al. (1996) used the same *S. chacoense* 80-1 clone that we used. They doubled the chromosome number of the clone and crossed it with *S. tuberosum* (4x) clones, and proposed that the ability to synthesize leptines may be controlled by few dominant genes and the quantities synthesized may be polygenically controlled, this possibility is more acceptable for us.

The first step towards finding molecular markers by BSA is to conduct a reliable phenotypic characterization, because the success of BSA is based on correct characterization. According to our genetic hypothesis, the reciprocal populations should have revealed the same set of markers, because the populations differed only by the cytoplasm. Expression of leptines, however, differed between the two populations; PBCp showed more continuous variation suggesting multigenic control of the trait. PBCc showed more discrete variation, with only a few genotypes that contained leptines and many that did not.

The goal of this part of the study was to identify markers linked in coupling. Five primers (OPQ-2, OPT-7, OPT16, OPT-20 and OPW-10) amplified fragments that segregated with leptine content in PBCp and PBCc. Even though, looking at the entire population, the trend was similar in PBCp and PBCc (Table4), the one-way ANOVA tests resulted in significant linkage for three of them in only the PBCp population [OPQ-2 (χ^2=7.25; p=0.007), OPT-16 (χ^2=7.35; p=0.007), and OPT-20 (χ^2=2.71; p=0.1)]. For the three primers, the diagnostic band was absent in the low bulks for PBCp, but it was faint in the low bulks for PBCc. In PCR for the individual selections in both bulks, the bands that segregated with the trait were present in most or in all of the high bulk selections, but were also present in several of the low selections (Table 5). On closer examination of the

low selections, we learned that presence of one marker associated with leptines was often accompanied by presence of others for the same low/nil selection (Table 6). Some of these might be a product of genetic recombination between the marker and the gene controlling the trait. But presence of bands in so many of the low bulk genotypes is suspicious. A possible explanation could be that the leptine analysis for the phenotypic characterization was done using leaf samples taken from plants growing under greenhouse conditions, and these conditions differ from the natural conditions, especially due to stresses that plants face growing outside. Leptine production has been found to be increased by high light conditions, stress and wounding (Maga, 1994). It is possible that some of these plants have genes responsible for leptine production, but that expression is lacking due to the lack of induction, like naturally occurring insect pests, wind, etc. It may be necessary to grow these same populations under field conditions for more accurate analysis. Another factor affecting the characterization might be the use of gas chromatography. Sinden et al. (1986) mentioned that presence or absence of leptines at concentration below 2 mg % fresh weight cannot be reliably determined by gas chromatography. It is possible that our genotypes produced leptines in small amounts, due to the environmental conditions. Leptine production appears to be a polygenic trait, as could be speculated based on segregation in the PBCp population (Fig. 3). The genotypes in the PBCc population did not segregate in the same manner (as the PBCp ones) (Fig. 4), despite the similar expected segregation patterns. Nevertheless, it is important to highlight that the trend (i.e., higher leptines when each of the five candidate bands revealed by BSA was present) was similar in both populations. It is also possible that the smaller population size of PBCc compared with PBCp prevented detection of statistically significant differences in PBCc.

A recent paper (Ronning et al., 1999) reports a molecular marker linked in repulsion to leptine content. This marker was amplified by primer UBC-370 in a segregating population developed from *S. chacoense*. Our populations were checked for the presence of this marker, and it was observed in all genotypes except 80-1. Therefore as the UBC-370 marker is present in *S. tuberosum* cultivars that do not produce leptines,

it must also be in *S. phureja* 1-3. The genomic region associated with this marker suppresses leptine production, because all our genotypes have it, might be why none of the genotypes in our populations produced as high amount of leptines as *S. chacoense* 80-1.

The fragments amplified by OPQ-2, OPT-16 and OPT-20 are markers linked to the leptine trait, and we believe this is the first report of molecular markers linked in coupling to leptine production in potato.

References

Bishop, B.A. and E. Grafius. 1996. Insecticide resistance in the Colorado potato beetle. In: Jolivet and Hsaio eds. *Chrysomelidee* biology, Vol. 1, pp. 355-377. SBP Academic Publishing, amsterdan.

Boiteau, G., W.P.L. Osborn, and M.E. Drew. 1997. Residual activity of imidacloprid controlling Colorado potato beetle (Coleoptera: Chrysomelidae) and three species of potato colonizing aphids (Homoptera: Aphidae). J. Econ. Entomol. 90: 309-319.

Casagrande, R.A. 1987. The Colorado potato beetle: 125 years of mismanagement. Bull. Entomol. Soc. Amer. 33:142-150.

Chèvre, A.M., P. Barret, F. Eber, P. Dupuy, H. Brun, X. Tanguy, and M. Renard. 1997. Selection of stable *Brassica napus-B. juncea* recombinant lines resistant to blackleg (*Leptosphaeria maculans*), Identification of molecular markers, chromosomal and genomic origin of the introgression. Theor. Appl. Genet. 95:1104-1111.

Deahl, K.L., S.L. Sinden, and R.J. Young. 1993. Evaluation of wild tuber bearing *Solanum* accessions for foliar glycoalkaloid level and composition. Am. Potato J. 70:61-69.

Douches, D.S., A.L. Westedt, K. Zarke, and B. Schroeter. 1998. Potato transformation to combine natural and engineered resistance for controlling tuber moth. Hort Science 33:1053-1056.

Doyle. J.J. and J.V. Doyle. 1987. A rapid DNA isolation procedure for small amounts of leaf tissue. Phytochem. Bull. 19:810-815.

Forgash, A.J. 1985. Insecticide resistance in the Colorado potato beetle. In: Proceeding of the XVII International Congress of Entomology. D.N. Ferro, ed. Research Bulletin 704. University of Massachusetts. pp. 33-52.

Georghoiu and Lagunes-Tejeda. 1991. The occurrent of resistance to pesticides in arthropodos. FAO of the United Nations. Rome. 318 p.

Grafius, E. 1997. Economic impact of insecticide resistance in the Colorado potato beetle (Coleoptera:Chrysomelidae) on the Michigan potato industry. J. Econ. Entomol. 90:1144-1151.

Grafius, E. and B. Bishop. 1997. Resistance to imidacloprid in Michigan Colorado potato beetles. Res. Pest. Newsl. 8: 21-25. Michigan State University, East Lansing, Michigan.

Groden, E. and R.A. Casagrande. 1986. Population dynamics of the Colorado potato beetle, *Leptinotarsa decemlineata* (Coleoptera:Chrysomelidae), on *Solanum berthaultii*. J. Econ. Entomol. 79:91-97.

Hu, X.Y., H.W. Ohm, and I. Dweikat. 1997. Identification of RAPD markers linked to gene *PM*1 for resistance to powdery mildew in wheat. Theor. Appl. Genet. 94:832-840.

Jiang, C. and K.C. Sink. 1997. RAPD and SCAR linked to the sex expression locus *M* in asparagus. Euphytica 94:329-333.

Kisha, T.J., D.S. Douches, E.J. Grafius, and W.L. Pett. 1998. Effectiveness of host plant resistance traits in potato to the Colorado potato beetle. Abstract. Am. J. Potato Res. 75:282.

Kuhn, R., and I. Löw. 1961. The structure of leptines. Chem. Ber. 94:1088-1095.

Lander, E.S., P. Green, J. Abrahamson, A. Barlow, M.J. Daly, S.E. Lincoln, and L. Newburg. 1987. MAPMAKER: an interactive computer package for constructing primary genetic linkage maps of experimental and natural populations. Genomics 1:174-181.

Lawson, D.R., R.E. Veilleux, and A.R. Miller. 1993. Biochemistry and genetics of *Solanum chacoense* steroidal alkaloids: Natural resistance factors to the Colorado potato beetle. Current Topics in Bot. Res. 1:335-352.

Lawson, D.R., W.A. Erb, and A.R. Miller. 1992. Analysis of *Solanum* alkaloids using internal standardization and capillary gas chromatography. J. Agric. Food Chem. 40:2186-2191.

Maga, J.A. 1994. Glycoalkaloids in Solanaceae. Food Rev. Int. 10:385-418.

Michelmore, R.W., I. Paran, and R.V. Kesseli. 1991. Identification of markers linked to disease-resistance genes by bulk segregant analysis: a rapid method to detect markers is specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA 88:9828-9832.

Penner, G.A., J. Chong, C.P. Whight, S.J. Molnar, and G. Fedak. 1993. Identification of a RAPD marker for the crown rust resistance gene *Pc68* in oats. Genome 36:818-820.

Plaisted, R.L., M.W. Bonierbale, G.G. Yencho, O. Pineda, W.M. Tingey, J.Van den Berg, E.E. Ewing and B.B. Brodie. 1994. Potato improvement by traditional breeding and opportunities for new technologies. In: The molecular and cellular biology of the potato. Edited by W.R. Belknap, M.E. Vayda and W.D. Park. Wallingford, UK.

Plaisted, R.L., W.M. Tingey, and J.C. Steffens. 1992. The germplasm release of NYL 235-4, a clone with resistance to the Colorado potato beetle. Amer. Potato J. 60:843-847

Poulsen, D.M.E., R.J. Henry, R.P. Johnston, J.A.G. Irwin, and R.G. Rees. 1995. The use of bulk segregant analysis to identify a RAPD marker linked to leaf rust resistance in barley. Theor. Appl. Genet. 91:270-273.

Ronning, C.M., J.R. Stommel, S.P. Kowalski, L.L. Sanford, R.S. Kobayashi, and O Pineda. 1999. Identification of molecular markers associated with leptine production in a population of *Solanum chacoense* Bitter. Theor. Appl. Genet. 98:39-46.

Ronning, C.M., L.L. Sanford, R.S. Kobayashi, and S.P. Kowalski. 1998. Foliar leptine production in segregating F_1, inter F_1, and backcross families of *Solanum chacoense* Bitter. Amer. J. Potato Res. 75:137-143.

Saghai-Maroof, M.A., K.M. Soliman, R.A. Jorgensen, and R.W. Allard. 1984. Ribosomal DNA spacer-length polymorphisms in barley: Mendelian inheritance, chromosomal location and population dynamics. Proc. Natl. Acad. Sci. USA 81:8014-8018.

Sanford, L.L., R.S. Kobayashi, K.L. Deahl, and S.L. Sinden. 1996. Segregation of leptines and other glycoalkaloids in *Solanum tuberosum* (4x) × *S. chacoense* (4x) crosses. Am. Potato J. 73:21-33.

Sanford, L.L., K.L. Deahl, and S.L.Sinden. 1994. Glycoalkaloid content in foliage of hybrid and backcross populations from a *Solanum tuberosum* × *S. chacoense* cross. Am. Potato J. 71:225-235.

Sanford, .L.L., T.L. Ladd, T.L. Sinden, and W.W. Cantelo. 1984. Early generation selection of insect resistance in potato [*Empoasca fabae, Leptinotarsa decemlineata, Myzus persicae, Macrosiphum euphorbiae*]. Am. Potato J. 61:405-418.

Sanford, L.L., and S.L. Sinden. 1972. Inheritance of potato glycoalkaloids. Am. Potato J. 49:209-217.

Schwarze, P. 1963. The glycoalkaloid content and the composition of the glycoalkaloid complex in progenies of the interspecific cross *S. tuberosum* × *S. chacoense*. Zuchter 33:275-281.

Sinden, S.L., L.L. Sanford, W.W. Cantelo, and K.L. Deahl. 1988. Bioassays of segregating plants: a strategy for studying chemical defenses. J.Chem.Ecol. 14:1941-1950.

Sinden, S.L., L.L. Sanford, W.W. Cantelo, and K.L. Deahl. 1986a. Leptine glycoalkaloids and resistance to the Colorado potato beetle (Coleoptera: Chrysomelidae) in *Solanum chacoense*. Environ. Entomol. 15:1057-1062.

Sinden, S.L., L.L. Sanford, and K.L. Deahl. 1986b. Segregation of leptine glycoalkaloids in *Solanum chacoense* Bitter. J. Agric. Food Chem. 34:372-377.

Sinden, S.L., L.L. Sanford, and R.E. Webb. 1984. Genetic and environmental control of potato glycoalkaloids. Am. Potato J. 61:141-156.

Stoner, K.A. 1996. Plant resistance to insects: A resource available for sustainable agriculture. Biol. Agric. Hort. 13:7-38.

Stürckow, R., and I. Löw. 1961. Die Wirkung einiger *Solanum*-alkaloid-glykosideauf den Kartoffelkafer *Leptinotarsa decemlineata* Say. Entomol. Exp. Appl. 4:133-142.

Tanhuanpää, P.K., J.P. Vilkki, and H.J. Vilkki. 1996. Mapping of a QTL for oleic acid concentration in spring turnip rape (*Brassica rapa* ssp.*oleifera*). Theor. Appl. Genet. 92:952-956.

Torka, M. 1950. Breeding potatoes with resistance to the Colorado potato beetle. Am. Potato J. 27:263-271.

Ukoskit, K., P.G. Thompson, C.E. Watson Jr., and G.W. Lawrence. 1997. Identifying a randomly amplified polymorphic DNA (RAPD) marker linked to a gene for root-knot nematode resistance in sweetpotato. J. Amer. Soc. Hort. Sci. 122(6):818-821.

Veilleux, R.E., and A.R. Miller. 1998. Hybrid breakdown in the F_1 between *Solanum chacoense* and *S. phureja* and gene transfer for leptine biosynthesis. J. Amer. Soc. Hort. Sci. 123:854-858.

Whalon, M.E., D.L. Miller, R.M. Hollingworth, E.J. Grafius, and J.R. Miller. 1993. Selection of a Colorado potato beetle (*Coleoptera, Chrysomelidae*) strain resistant to *Bacillus thuringiensis.* J. Econ. Entomol. 86:226-233.

Whight, C.P., G.A. Penner, L.S. O'Donoighue, V.D. Burrows. S.J. Molnar, and G. Fedak. 1994. The identification of random amplified polymorphic DNA markers for daylength insensitivity in oat. Genome 37:910-914.

Williams, J.G.K., A.R. Kubelik, K.J. Livak, J.A. Rafalski, and S.V. Tingey. 1990. DNA polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res. 22:4673-4680.

Yang, H.Y., S.S. Korban, J. Krüger, and H. Schmidt. 1997. The use of a modified bulk segregatn analysis to identify a molecular marker linked to a scab resistance gene in apple. Euphytica 94:175-182.

Yencho, G.C., S.P. Kowalski, R.S. Kobayashi, S.L. Sinden, M.W. Bonierbale, and K.L. Deahl. 1998. QTL mappng of foliar glycoalkaloid aglycones in *Solanum tuberosum* × *S. berthaultii* potato progenies: quantitative variation and plant secondary metabolism. Theor. Appl. Genet. 97:563-574.

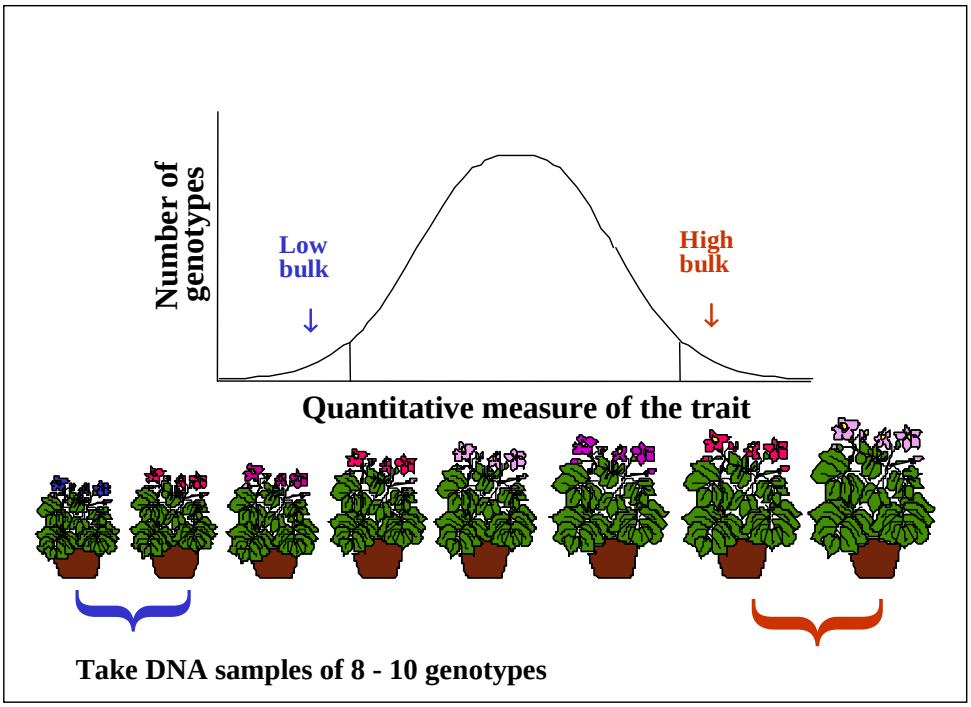

Figure 1: The "bulk segregant analysis" method consists of bulking the DNA of extreme phenotypes for a trait. These bulks can be screened with molecular markers to find a DNA fragment that segregates with the trait and is therefore linked to it.

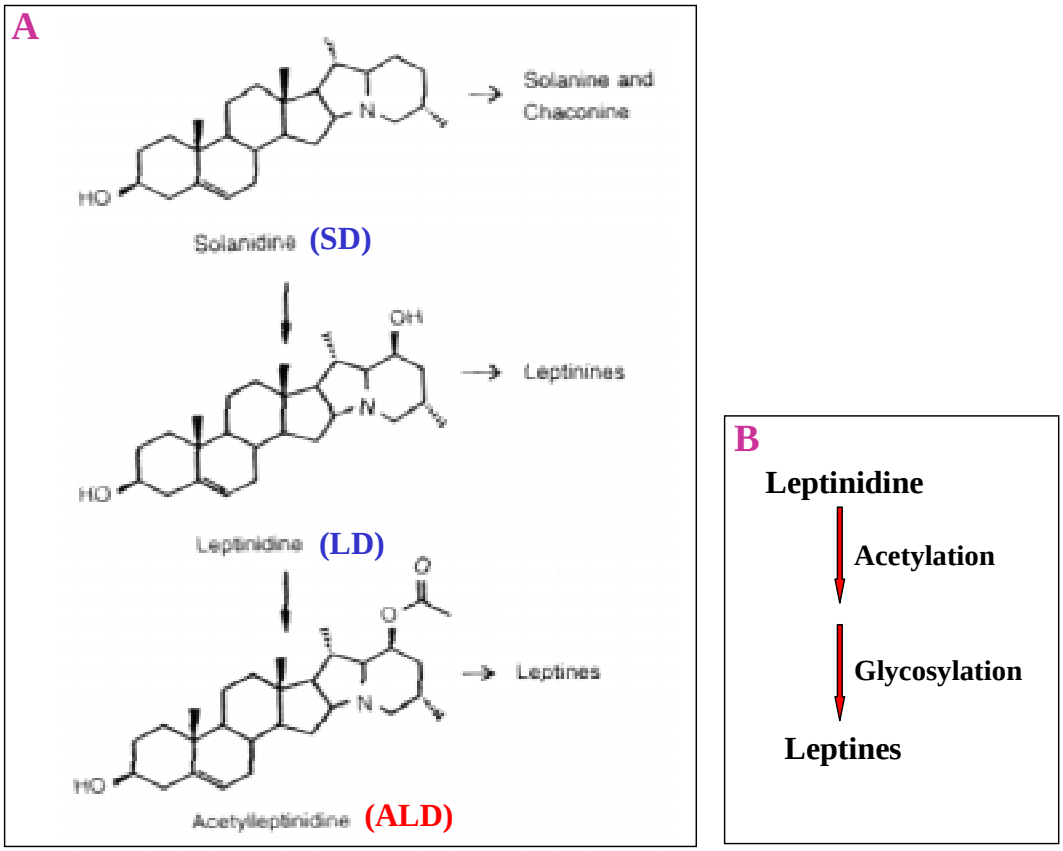

Figure 2: A: Two step pathway proposed by Lawson (1993), showing hydroxylation of carbon 23 on solanidine to form leptinidine, followed by an acetylation step to form acetylleptinidine. B: The aglycone leptinidine is acetylated and glycosylated to form leptines in leptine-producing *S. chacoense*, such as 80-1.

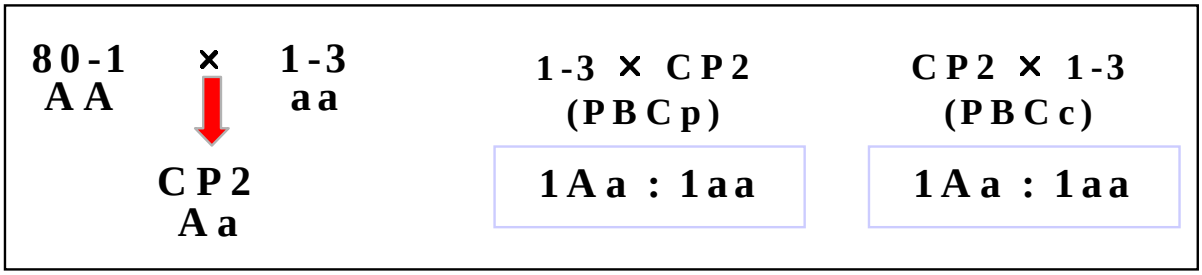

Figure 3: Expected segregation pattern for RAPD markers linked in coupling to leptine production in backcross populations of an interspecific hybrid (CP2). *S. chacoense* 80-1 produces leptines whereas *S. phureja* 1-3 does not. Because the F_1 hybrid also produces leptines, CP2 must have inherited a codominant allele present in 80-1. The expected segregation is similar in the reciprocal populations (PBCp and PBCc); therefore any band putatively linked to the trait in one of the populations should also be linked in the other.

	SD	LD	ALD
S. phureja 1-3	3480	0	0
S. chacoense 80-1	1260	7250	11320
CP2	940	1350	2000

Table 1: Solanidine (SD), leptinidine (LD), and acetylleptinidine (ALD - leptine) content of the parental lines 1-3, 80-1 and the hybrid CP2 in $\mu g.g^{-1}$ dry wt. Taken from Veilleux and Miller (1998).

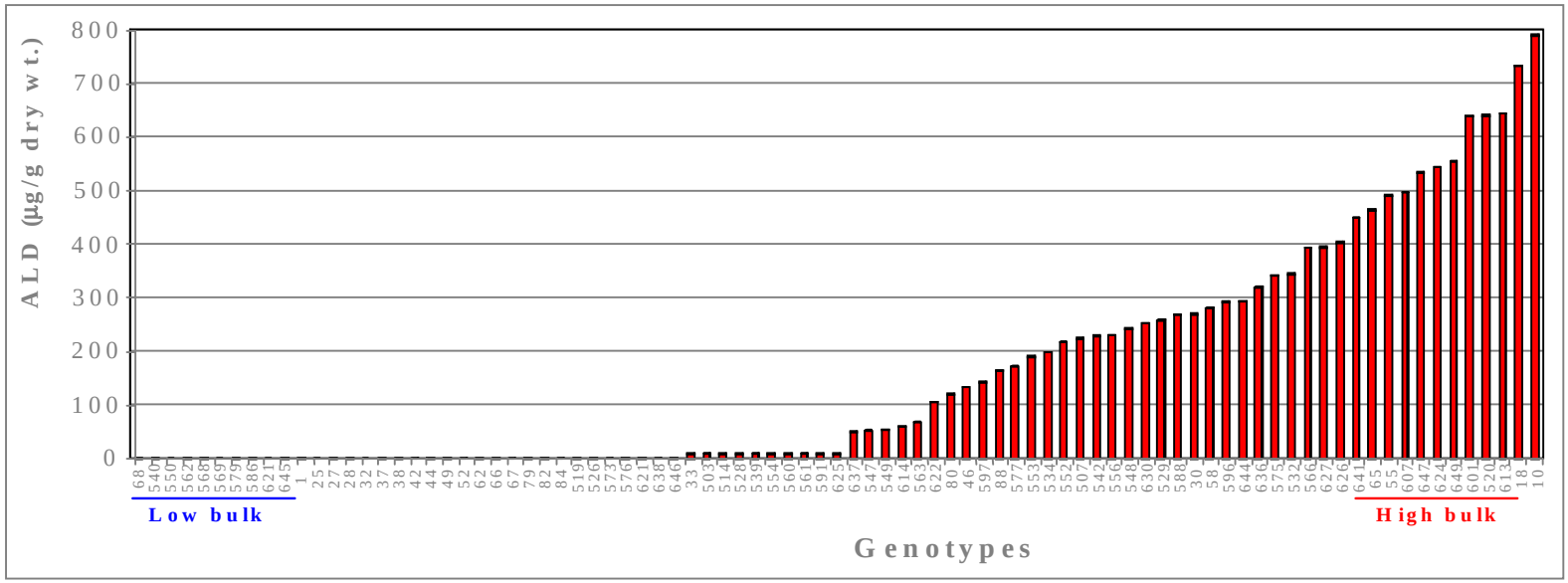

Figure 4: Ten genotypes indicated as low and high bulks from each extreme were taken as selections for BSA. ALD (leptines) content measured in leaf samples of greenhouse grown plants of 87 genotypes of a backcross population between *S. phureja* 1-3 and CP2 (PBCp).

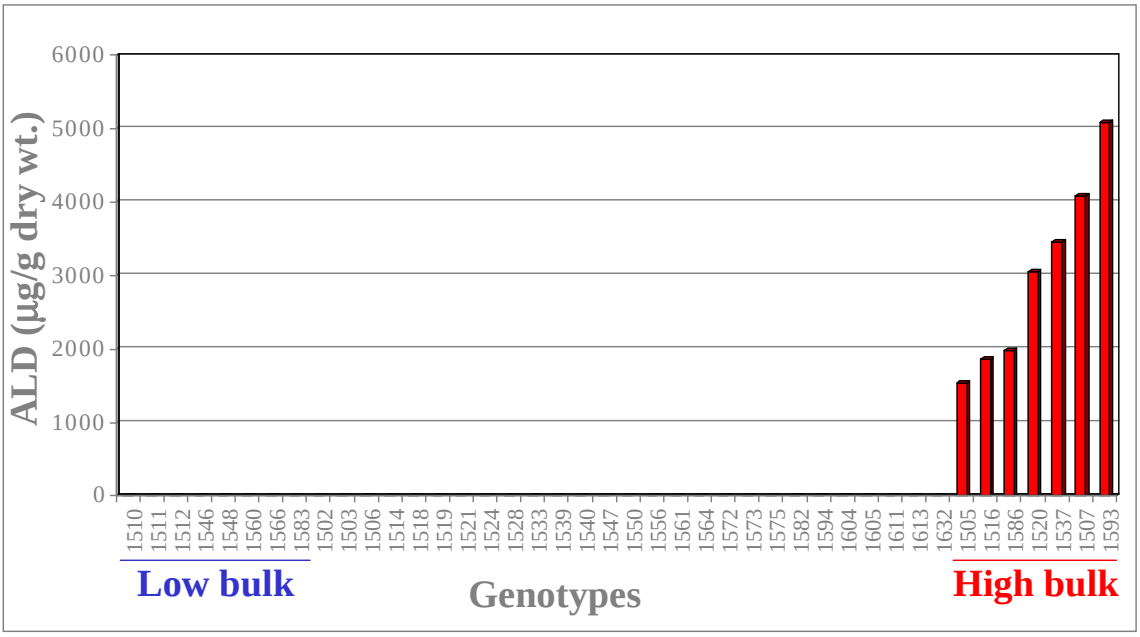

Figure 5: Eight and seven genotypes indicated as low and high bulks, respectively, from each extreme were taken as selections for BSA. ALD (leptines) content measured in leaf samples of greenhouse grown plants of 42 genotypes of a backcross population between CP2 and *S. phureja* 1-3 (PBCc).

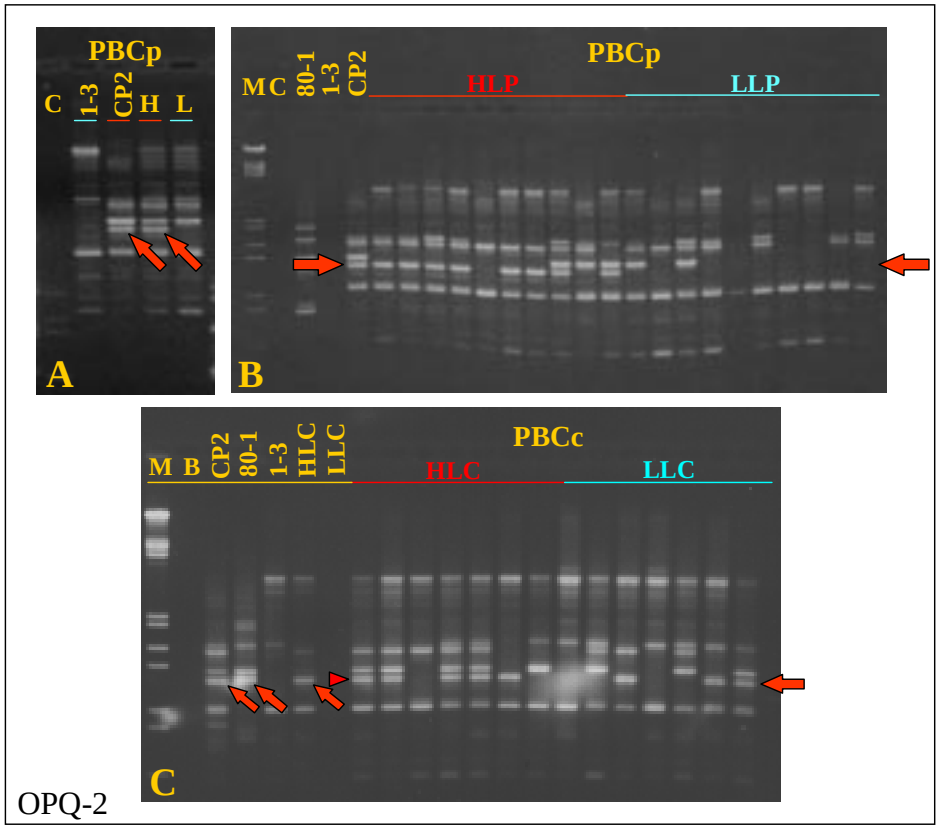

Figure 6: OPQ-2 and leptine BSA. **A**: Primer screening where a putatively linked band is identified in PBCp. **B**: 80-1, 1-3, CP2 and individual selections for the PBCp high (HLP) and low (LLP) bulks. The band was present in 8 high and 0 low selections. **C**: CP2, 80-1, 1-3, high PBCc bulk (HLC), low PBCc bulk (LLC) and individual bulk selections for PBCc, the band was present in 5 out of 7 high and 2 out of 8 low selections in PBCc. Arrows indicate linked bands.

A

Source	DF	M. S.	F value	Pr>F
OPQ-2	1	239962	5.35	0.024
Error	71	44872		
OPT-16	1	383059	9.37	0.003
Error	81	40873		
OPT-20	1	424080	10.56	0.0017
Error	81	40150		

B

Primer	band:	N	Mean ALD	REGWQ Grouping
OPQ-2	present	25	247	A
	absent	48	126	B
OPT-16	present	54	210	A
	absent	29	68	B
OPT-20	present	49	218	A
	absent	34	73	B

Table 2: **A:** Results of one-way ANOVA analysis of leptine (ALD; in $\mu g.g^{-1}$) data from all PBCp genotypes analyzed for each of the markers linked in coupling to ALD content. **B:** Mean separation for each band is by Ryan Einot Gabriel Welsch multiple range test (REGWQ).

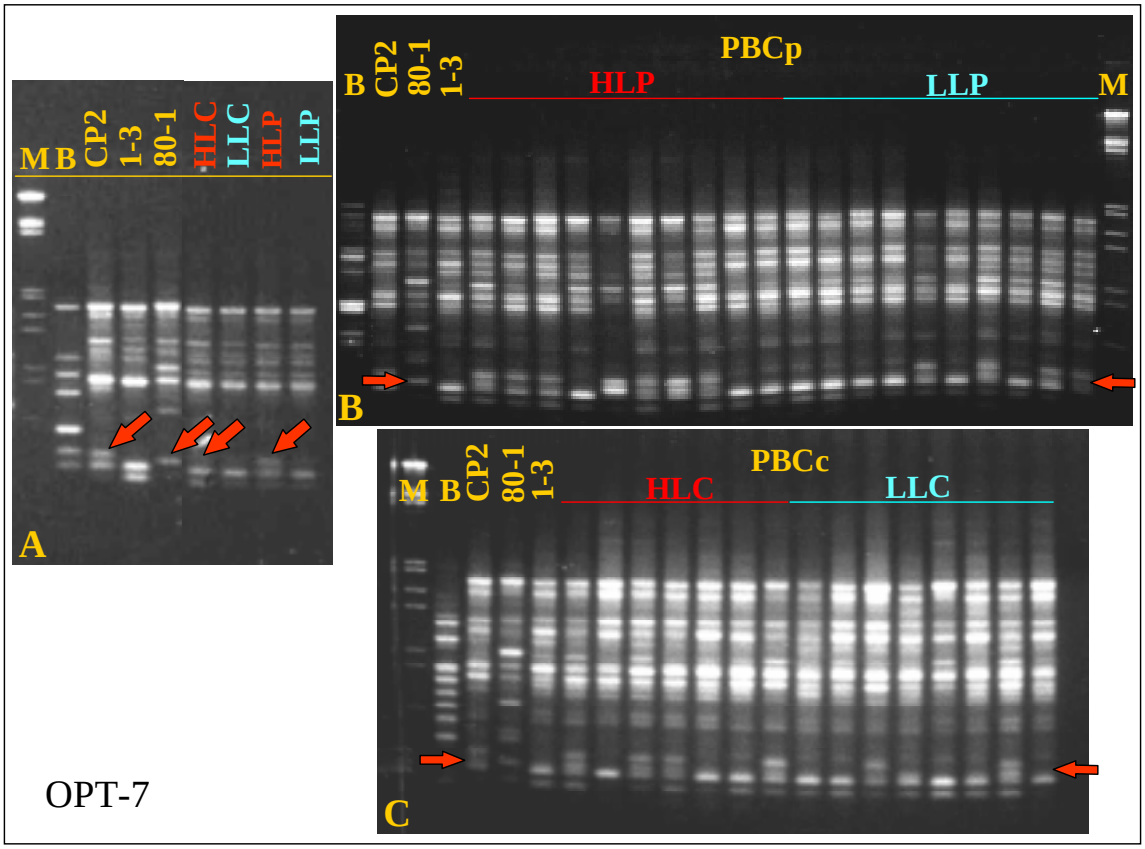

Figure 7: OPT-7 and leptine BSA. **A**: Primer screening where a putatively linked band is identified in high bulks of PBCp (HLP) and PBCc (HLC), the band is absent in the low bulks (LLP and LLC). **B**: CP2, 80-1, 1-3 and individual selections for the PBCp bulk, the band was present in 7 high and 3 low selections. **C**: CP2, 80-1, 1-3 and individual bulk selections for PBCc, the band was present in 4 out of 7 high and 2 out of 8 low selections in PBCc. Arrows indicate linked bands.

A

Source	DF	Mean square	F value	Pr>F
OPT-7	1	317450.10	4.2	0.047
Error	37	75667.41		

B

OPT-7 band	N	Mean ALD	REGWQ Grouping
present	14	312	A
absent	25	124	B

Table 3: A: Results of one way ANOVA for leptinidine (LD; in $\mu g.g^{-1}$) and presence/absence of OPT-7 band in the PBCc population revealing significance. **B:** OPT-7 segregated in coupling during BSA for leptines (ALD) in both PBCp and PBCc population. Mean separation for each band is by Ryan Einot Gabriel Welsch multiple range test.

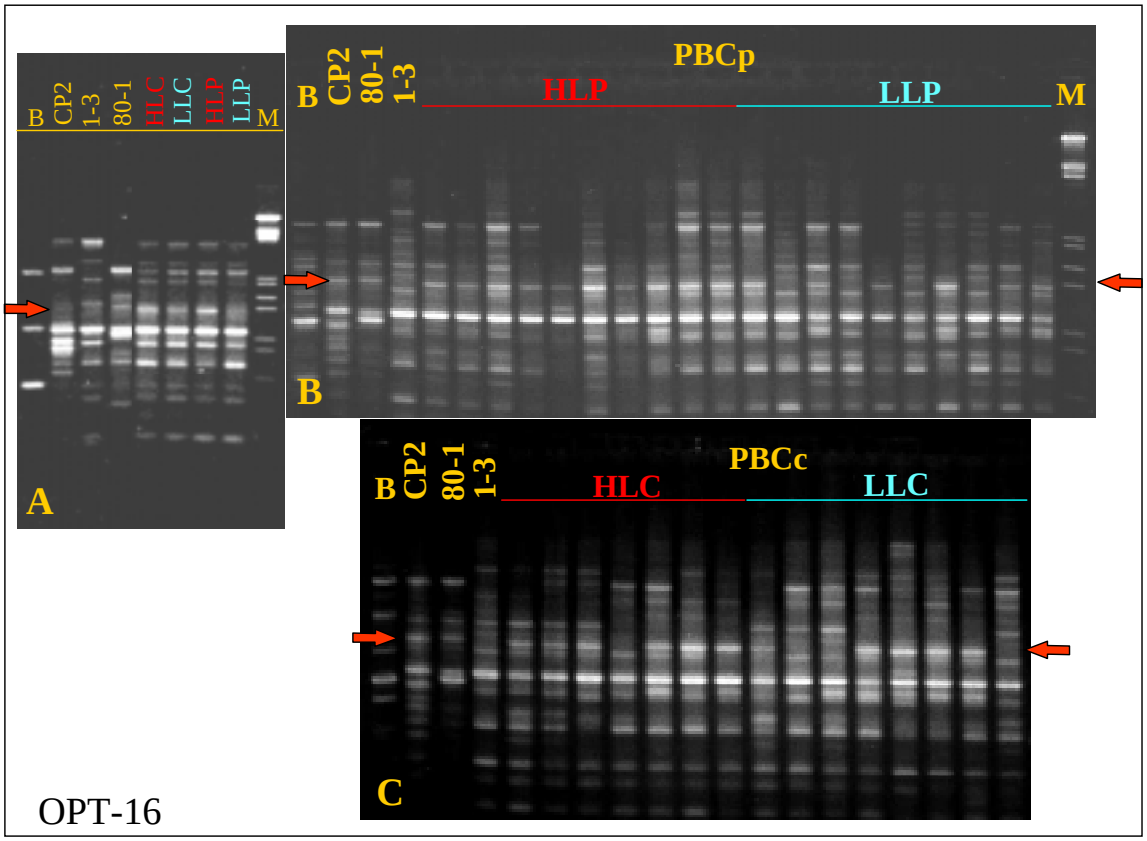

Figure 8: OPT-16 and leptine BSA. **A**: Primer screening where a putatively linked band is identified in high bulks of PBCp (HLP) and PBCc (HLC), the band is absent in the low bulks (LLP and LLC). **B**: CP2, 80-1, 1-3 and individual selections for the PBCp bulk, the band was present in all high and 6 low selections. **C**: CP2, 80-1, 1-3 and individual bulk selections for PBCc, the band was present in 6 out of 7 high and 4 out of 8 low selections in PBCc. Arrows indicate linked bands.

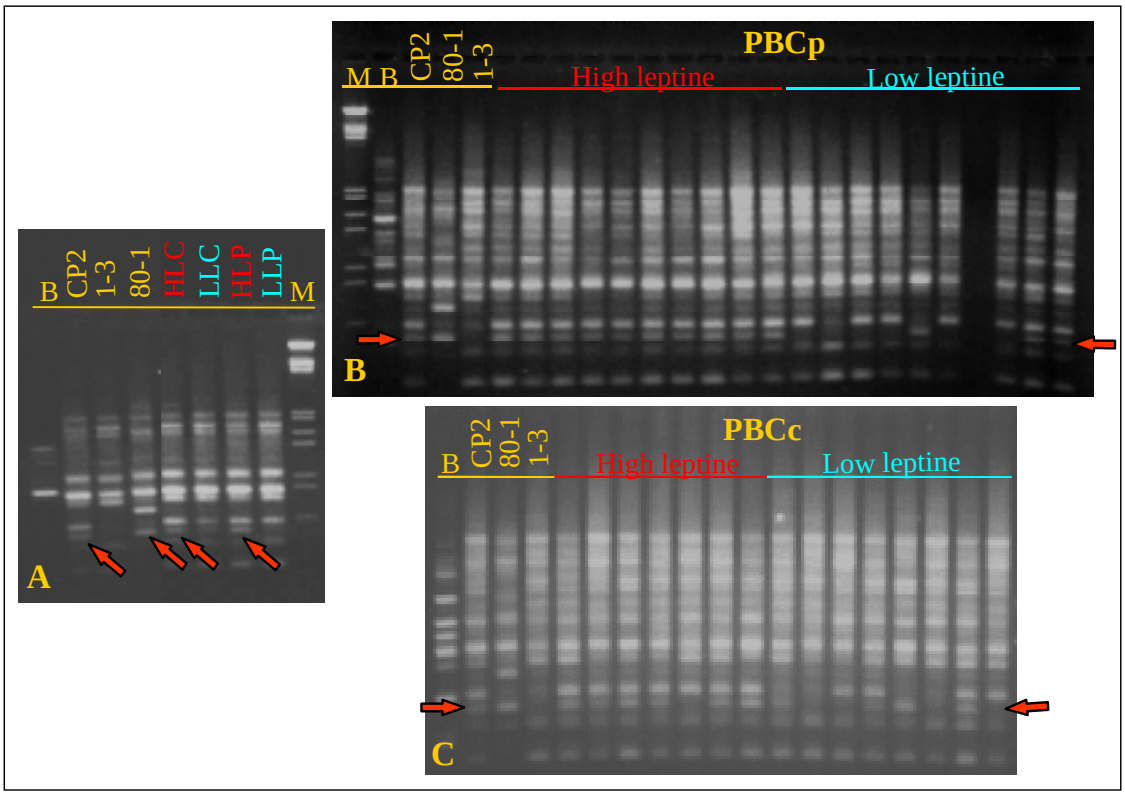

Figure 9: OPT-20 and leptine BSA. **A**: Primer screening where a putatively linked band is identified in high bulks of PBCp (HLP) and PBCc (HLC), the band is absent in the low bulks (LLP and LLC). **B**: CP2, 80-1, 1-3 and individual selections for the PBCp bulk, the band was present in all high and 4 low selections. **C**: CP2, 80-1, 1-3 and individual bulk selections for PBCc, the band was present in 6 out of 7 high and 3 out of 8 low selections in PBCc. Arrows indicate linked bands.

	PBCp		PBCc	
Primer	Mean ALD Band present	Band absent	Mean ALD Band present	Band absent
OPQ-2	247 (25)^{A}	126 (48)^{B*}	783 (18)	331 (21)
OPT-7	183 (38)	139 (47)	824 (16)	381 (26)
OPT-16	210 (55)^{A}	68 (30)^{B}	750 (24)	203 (18)
OPT-20	218 (49)^{A}	73 (34)^{B}	880 (20)	157 (22)
OPW-10	167 (55)	148 (29)	717 (25)	256 (17)

Table 4: Mean leptine content (ALD) in $\mu g.g^{-1}$ dry wt. for genotypes with or without each of five bands identified by BSA with five RAPD primers in two reciprocal backcross populations.

Numbers in parenthesis represent the number of genotypes.

* Mean separation within rows and populations by Ryan Einot Gabriel Welsch multiple F test (REGWQ).

Bands in :	PBCp		PBCc	
	High Bulk	Low Bulk	High Bulk	Low Bulk
OPQ-2*	8/10	0/10	5/7	2/8
OPW-10	8/10	5/10	5/7	3/8
OPT-7	7/10	3/10	4/7	2/8
OPT-16*	10/10	6/10	6/7	4/8
OPT-20*	10/10	3/10	6/7	3/8

Table 5: Number of genotypes within the high and low bulks of both backcross populations (PBCp and PBCc) that exhibited the bands of interest amplified by five primers that revealed a polymorphic fragment linked in coupling to leptine content. (*) significantly linked to the trait in PBCp.

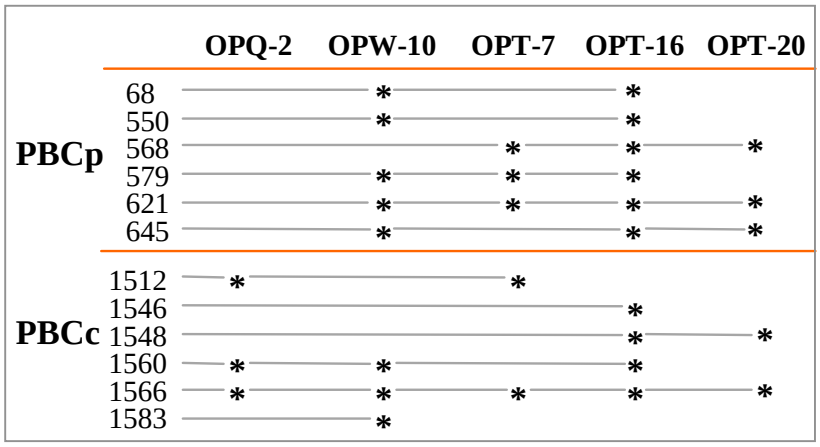

Table 6: Anomalous expression of bands linked in coupling to leptine production in 12 genotypes found in the low bulks of two reciprocal backcross populations (PBCp and PBCc). The bands were amplified by five RAPD primers shown in the top.

Appendix

Table 1: Assignment of SSR alleles to genotypes comprising the PBCp backcross family (1-3×CP2) for four different microsatellite loci.

Genotype	RV 3-4	RV 5-6	RV 15-16	RV 19-20
PBCp 1	BC	C	AB	AB
PBCp 10	AB	C	B	AB
PBCp 18	AB	C	AB	B
PBCp 25	BC		AB	
PBCp 27	BC		AB	
PBCp 28	C		BC	
PBCp 30	AB		AB	
PBCp 32	AC		AB	
PBCp 33	AC		AB	
PBCp 37	AB		AB	
PBCp 38	C		AB	
PBCp 42	BC		BC	
PBCp 44	BC		AB	
PBCp 48	BC		AB	
PBCp 49	C	BC	BC	AB
PBCp 52	AC		AB	
PBCp 55	AC	C	B	B
PBCp 58	C		BC	
PBCp 62	C		AB	
PBCp 65	AC	BC	B	B
PBCp 66	C		BC	
PBCp 67	BC		BC	
PBCp 68	BC	C	B	AB
PBCp 73	AC		AB	
PBCp 79	C		B	
PBCp 80	AB		AB	
PBCp 82	BC	C	BC	AB
PBCp 84	AC		AB	
PBCp 88	BC		AB	
PBCp 502	AC		AB	
PBCp 503	AB		AB	
PBCp 507	AC		AB	
PBCp 509	C	BC	AB	AB
PBCp 513	AC	B	AB	B

RV 3-4:	AB:AC:BC:C
n=95	25:31:20:19
	Chi sq.= 3.82
	P=0.281
RV 5-6:	B:C:BC
n=48	10:26:12
	Chi sq.= 0.5
	P= 0.779
RV 15-16:	B:AB+AC:BC
n=95	15:64:16
	Chi sq.= 11.48*
	P= 0.003
RV 19-20:	AB:B
n=48	33:15
	Chi sq.= 6.75*
	P= 0.009

PBCp 514	AB	C	AB	AB
PBCp 516	AC	C	AB	AB
PBCp 519	AB	C	AB	AB
PBCp 520	AB	C	AB	AB
PBCp 526	AB		AB	
PBCp 531	AC	B	AB	B
PBCp 534	C	C	B	AB
PBCp 535	C	C	AB	AB
PBCp 539	AC	C	AB	AB
PBCp 540	AC	C	BC	AB
PBCp 541	AC	BC	AB	B
PBCp 542	C	C	B	AB
PBCp 544	AB		AB	
PBCp 547	AB		AB	
PBCp 548	AB		AB	
PBCp 549	BC		BC	
PBCp 550	BC	B	AB	B
PBCp 552	BC	B	AB	AB
PBCp 553	AC	C	B	AB
PBCp 554	AC	BC	AB	AB
PBCp 556	AB		AB	
PBCp 560	AB		AB	
PBCp 561	BC	C	AB	AB
PBCp 562	AC	BC	B	AB
PBCp 563	AC	B	AB	AB
PBCp 566	BC		AB	
PBCp 568	BC	BC	BC	AB
PBCp 569	AC	B	B	AB
PBCp 573	AC		AB	
PBCp 575	AC		AB	
PBCp 576	AB		BC	
PBCp 577	C		AB	
PBCp 579	AB	C	AB	B
PBCp 586	BC	C	B	AB
PBCp 588	AB		AB	
PBCp 591	C	BC	AB	AB
PBCp 596	AC	B	AB	AB
PBCp 597	AC		BC	
PBCp 601	BC	BC	B	B
PBCp 607	AC	C	B	AB
PBCp 611	C		AB	
PBCp 613	AC	BC	AB	B

PBCp 614	AC	C	AB	AB
PBCp 617	C	C	BC	B
PBCp 618	AB		AB	
PBCp 620	BC		B	
PBCp 621	AB	C	AB	B
PBCp 622	C		AB	
PBCp 624	C	C	BC	AB
PBCp 625	C	B	AB	B
PBCp 626	AB		B	
PBCp 629	AB	BC	AB	AB
PBCp 630	AC		BC	
PBCp 632	AC		AB	
PBCp 638	C	B	AB	AB
PBCp 639	BC		AB	
PBCp 641	AC	C	AB	B
PBCp 644	AB		AB	
PBCp 645	AC	B	AB	AB
PBCp 647	AB	C	AB	B
PBCp 649	AB	BC	BC	AB

Table 2: Assignment of SSR alleles to genotypes comprising the PBCc backcross family (CP2×1-3) for four different microsatellite loci.

Genotype	RV 3-4	RV 5 -6	RV 15-16	RV 19-20
PBCc 1502	BC	BC	BC	AB
PBCc 1503	AC	BC	AB	AB
PBCc 1505	AC	B	AB	AB
PBCc 1506	C	B	B	AB
PBCc 1507	BC	BC	AB	AB
PBCc 1509	AC	C	BC	B
PBCc 1510	C	BC	B	B
PBCc 1511	C	BC	BC	AB
PBCc 1512	C	C	BC	AB
PBCc 1513	AB	C	AB	AB
PBCc 1514	AC	C	AB	B
PBCc 1518	BC	C	AB	B
PBCc 1519	C	C	BC	AB
PBCc 1520	AB	BC	AB	B
PBCc 1521	C	BC	B	AB
PBCc 1522	BC	C	AB	B
PBCc 1523	BC	C	BC	B
PBCc 1524	AC	C	BC	B
PBCc 1525	C	C	AB	B
PBCc 1528	AC	C	B	B
PBCc 1530	AC		AB	AB
PBCc 1533	AB	B	B	
PBCc 1534	C	C	BC	B
PBCc 1537	AB	BC	AB	B
PBCc 1539	BC	B	AB	B
PBCc 1540	BC	B	B	B
PBCc 1542	C	C	BC	B
PBCc 1545	AB	BC	AB	
PBCc 1546	AC	B	B	AB
PBCc 1547	AB	C	AB	B
PBCc 1548	BC	B	B	B
PBCc 1550	C	B	BC	AB
PBCc 1555	AC	C	AB	AB
PBCc 1556	AB	C	AB	B
PBCc 1560	BC	C	AB	AB
PBCc 1561	AC	C	BC	
PBCc 1562	AC	C	B	B
PBCc 1564	C	B	AB	B

RV 3-4:	AB:AC:BC:C
n=45	12:14:9:11
	Chi sq.= 1.13
	P= 0.77
RV 5-6:	B:C:BC
n=44	9:23:12
	Chi sq.=0.5
	P= 0.779
RV 15-16:	B:AB+AC:BC
n=45	9:24:12
	Chi sq.= 0.06
	P= 0.741
RV 19-20:	AB:B
n=42	16:26
	Chi sq.= 2.4
	P= 0.123

PBCc 1566	AC	C	AB	B
PBCc 1568	AB	BC	AB	B
PBCc 1572	AB	C	AB	B
PBCc 1573	AB	C	AB	B
PBCc 1579	AB	C	BC	AB
PBCc 1582	AC	C	AB	B
PBCc 1583	AC	BC	AB	B

Table 3: Assignment of SSR alleles to genotypes comprising the CBC backcross family (CP2×80-1) for four different microsatellite loci.

Genotype	RV 3 - 4	RV 5 - 6	RV 15-16	RV 19-20
CBC 12	A	B	AB	BC
CBC 15	A	C	A	AC
CBC 21	A	B	AB	AC
CBC 65	AC	C	AB	AC
CBC 34	AC	BC	A	AB
CBC 42	A	BC	A	BC
CBC 8	AC	B	A	AB
CBC 45	A	B	AB	BC
CBC 53	A	B	AB	AB
CBC 3	AC	B	A	BC
CBC 17	A	B	AB	BC
CBC 31	AC	B	AB	AB
CBC 64	A	B	AB	AC
CBC 51	A		A	AC
CBC 5	A	B	A	AC
CBC 40	AC	C	AB	AB
CBC 43	A		A	AB
CBC 61	A	BC	AB	AB
CBC 55	AC	B	A	BC
CBC 1	A	C	AB	AB
CBC 2	AC	BC	AB	BC
CBC 4	A	BC	A	AB
CBC 6	AC	BC	AB	A
CBC 10	AC	C	B	AB
CBC 18	A	B	A	AB
CBC 22	A	B	AB	AB
CBC 41	AC	BC	A	BC
CBC 46	AC	C	AB	AC
CBC 47	A	C	A	BC
CBC 48	AC	BC	AB	AB
CBC 52	AC	C	AB	AB
CBC 54	AC	B	A	AB
CBC 57	A	B	AB	BC
CBC 59	AC	B	AB	AB
CBC 60	A	B	A	AC
CBC 62	A	B	A	BC
CBC 63	AC	BC	A	AB
CBC 66	AC	B	A	

RV 3-4: A:AC
n=42 23:19
Chi sq.= 0.38
P= 0.537

RV 5-6: BC:B_:C
n=40 9:22:9
Chi sq.= 0.4
P= 0.819

RV 15-16: AA:AB
n=42 20:21
Chi sq.= 0.116
P= 0.733

RV 19-20: AA:AB:AC:BC
n=38 1:17:9:11
Chi sq.= 13.8*
P= 0.003

CBC 67	AC	B	AB	AC
CBC 69	A	B	AB	
CBC 71	A	C	A	
CBC 72	A	B	A	

Table 4: All genotypes characterized for embryos per anther (EPA) in the PBCp population

Genotype	EPA	ACR bulk
1	0	L
82	0	L
621	0	V
629	0	L
20	0.001	V
535	0.003	L
519	0.01	L
617	0.01	L
49	0.02	V
639	0.02	V
18	0.04	V
620	0.04	V
12	0.07	V
603	0.07	V
563	0.1	V
30	0.11	V
548	0.12	V
611	0.13	V
17	0.14	V
640	0.15	V
514	0.19	L
550	0.19	V
6	0.24	V
507	0.26	V
541	0.26	L
11	0.28	V
73	0.308	V
644	0.34	V
23	0.35	V
530	0.52	V
5	0.54	V
625	0.57	V
2	0.58	V
536	0.58	V
624	0.65	V
532	0.66	V
647	0.73	V
52	0.76	V

24	0.8	V
528	0.8	V
554	0.81	V
515	0.82	V
549	0.82	V
14	0.88	V
529	0.96	V
627	1.01	V
632	1.02	V
517	1.07	V
80	1.12	V
501	1.14	V
527	1.21	V
84	1.23	V
19	1.27	V
618	1.33	V
33	1.48	V
569	1.36	H
509	1.56	H
588	1.65	V
630	1.69	V
10	1.72	H
596	1.89	V
7	2.09	V
645	2.2	V
542	2.3	V
531	2.85	H
506	2.98	V
552	3.14	H
520	4.06	V
573	4.17	V
642	4.27	V
544	4.42	V
539	4.68	H
513	5.46	H
560	7.04	V
641	8.5	H
516	8.9	H
534	9.26	H
16	14.4	V

Table 5: All genotypes characterized for embryos per anther (EPA) in the CBC population

Genotype	EPA	ACR bulk
5	0	L
6	0	V
26	0	V
33	0	V
55	0	L
61	0.006	L
56	0.008	V
59	0.008	V
43	0.01	L
64	0.01	L
17	0.02	L
22	0.02	V
40	0.02	L
52	0.02	V
2	0.03	V
3	0.03	L
10	0.03	V
18	0.03	V
31	0.03	L
51	0.03	L
41	0.04	V
62	0.04	V
46	0.05	V
11	0.06	V
19	0.06	V
47	0.06	V
7	0.08	V
35	0.08	V
23	0.1	V
60	0.11	V
66	0.11	V
63	0.15	V
16	0.17	V
4	0.19	V
1	0.2	V
70	0.22	V
68	0.26	V
57	0.31	V

69	0.36	V
72	0.42	V
67	0.54	V
54	0.56	V
58	0.61	V
71	0.62	V
24	0.67	V
9	0.7	V
20	0.78	V
65	1.2	H
15	1.4	H
45	1.52	H
32	1.91	V
12	2.34	H
21	2.4	H
8	2.6	H
34	2.62	H
42	3.1	H
53	5.12	H

Table 6: Results for presence (1) and absence (0) of bands amplified with 3 different RAPD primers that segregated with embryos per anther (EPA). Selections for the high ACR bulk are represented in red color. Selections for the low ACR bulk are represented in blue color.

PBCp	EPA	OPQ-10	OPZ-4	OPS-2
10	1.72	0	1	1
509	1.56	0	1	1
569	1.36	0	1	1
539	4.68	1	1	1
534	9.26	0	1	1
513	5.46	0	1	1
552	3.14	0	1	1
531	2.85	0	1	1
516	8.9	0	1	1
641	8.5	0	1	1
1	0	1	0	0
18	0.04	1	1	0
535	0.003	1	0	0
514	0.19	1	0	0
541	0.26	1	0	0
629	0	0	1	1
82	0	1	0	0
519	0.01	1	1	1
617	0.01	1	1	1
49	0.02	1	0	0
30	0.11	1	0	1
52	0.76	1	0	0
80	1.12	1	1	1
84	1.23	1	1	1
507	0.26	1	1	0
520	4.06	1	0	0
528	0.8	1	1	0
529	0.96	0	1	1
532	0.66	1	1	1
542	2.3	1	0	0
544	4.42	0	1	1
548	0.12	1	1	1
549	0.82	1	1	1
550	0.19	1	0	0
554	0.81	1	1	1
560	7.04	1	1	0

563	0.1	1	1	1
573	4.17	1	0	0
588	1.65	0	1	1
596	1.89	0	1	1
603	0.07	0	1	1
611	0.13	0	1	1
618	1.33	0	1	1
620	0.04	0	1	1
621	0	1	0	0
624	0.65	0	1	1
625	0.57	0	1	1
627	1.01	0		
630	1.69	0	1	1
632	1.02	1	1	1
639	0.02	0	0	1
642	4.27	0	1	1
644	0.34	0	1	1
645	2.2	0	1	1
647	0.73	0	1	1

Table 7: Results for presence (1) and absence (0) of bands amplified with 2 different RAPD primers that segregated with the embryos per anther (EPA). Selections for the high ACR bulk are represented in red color. Selections for the low ACR bulk are represented in blue color.

CBC	EPA	OPW-14	OPU-1
12	2.34	1	0
15	1.4	1	1
21	2.4	0	0
65	1.2	1	0
34	2.62	1	0
42	3.1	1	0
8	2.6	1	0
45	1.52	1	1
53	5.12	0	1
3	0.03	0	0
17	0.02	1	1
31	0.03	0	0
64	0.01	0	0
51	0.03	0	0
5	0	0	0
40	0	1	0
43	0.02	0	0
61	0.06	0	1
55	0	0	0
1	0.2		0
2	0.03	0	1
4	0.19	0	1
6	0		1
10	0.03	1	1
18	0.03	0	0
22	0.02	0	1
41	0.04	0	1
46	0.05		1
47	0.06	0	0
52	0.02	0	0
54	0.56	1	1
57	0.31	0	1
59	0.008	0	1
60	0.11	1	1
62	0.04	0	0
63	0.15		0

66	0.11		1
67	0.54	0	1
68	0.26		
69	0.36	0	0
70	0.22		1
71	0.62		1
72	0.42		0

Table 8: Percentage of the 2*n* pollen of PBCp genotypes. Selections for the high 2*n* pollen bulk are represented in red color. Selections for the low 2*n* pollen bulk are represented in blue color. Pollen was collected during eight weeks of spring 1998.

genotype	week 1	week 2	week 3	week 4	week 5	week 6	week 8
27					25.7	7.0	
30	6.5	6.2	3.4				2.2
33	1.6	1.4	2.2				
36					25.0		
37	7.9	17.8	3.1				
38		11.7	27.0	24.6			
49	12.8			13.4			
52	22.3	63.6	14.5				
55			4.3				
56						2.0	
62		3.8	18.8	17.0			
66			2.0	1.1			
67		5.9					
73	12.6			4.6	19.0		
77				19.9	31.6	18.8	68.4
79		2.1	29.4	16.2			
80	2.1	24.5	8.2				
82		21.8	33.7	19.8			
84	2.8	13.4		2.0			
88			38.9	25.8	20.4		
503	3.6						
504	9.2						
507	6.4			3.9	8.0	22.2	
516				25.4			
519	14.2	8.7	6.8				
520	7.7	1.8	19.6				
540	26.3						
542				4.2			
544	6.1						
547			16.2	11.6			
548	17.2	1.6	6.0				
549	6.6	41.1	24.1		22.0		
550	12.2	20.5		25.8			
554	13.5	11.5	30.0				
555					13.6	12.6	
560	15.0		12.8				
563	4.9	42.2	26.9				

566			5.6				
568		28.4		19.3	25.3		
569	14.4		19.6	13.1			
570				11.5			6.0
573	21.2	12.2	6.6				
575	38.6	57.9	25.4				
576			23.8	37.5	22.3		
577		23.4		18.0			
581		33.6	31.8	4.4			
588		4.2	9.5	14.4			
596		20.4	5.4	6.0			
597		4.7	6.5	9.0			
598				15.4	5.2	3.8	
601		3.7					
602				48.7	35.0		
603		29.5		50.2			
611			4.0	8.0			
614		38.4	25.6	20.8			
617				24.2		3.4	
619		19.2	2.5				
620				18.5			
621		25.3					
622				13.1	2.7	4.2	
624			21.6	28.5			
625		26.6	5.3	27.3			
626		5.5		4.3	4.5		
627				44.8	6.9		
630		18.2	2.5	11.4			
631		12.9		74.0			
632		57.9	14.8	7.8			
633		64.4	11.5	43.3			
636		19.8					
637		20.8					
640			39.2	51.6	25.0		
641				33.6			
642				55.0			
644		16.9	11.6	18.2			
645		35.7	18.0				
647		15.5	15.6		8.2		
649		16.1		17.0			

Table 9: Results for presence (1) and absence (0) of bands amplified with 5 different RAPD primers that segregated with the leptine content trait (Ald) for genotypes of the PBCp population. Selections for the high ACR bulk are represented in red color. Selections for the low ACR bulk are represented in blue color. Sd=Solanidine; Ld=Leptinidine; Ald= Leptine glycoalkaloids measured in $\mu g.g^{-1}$dry wt.

PBCp	Sd	Ld	Ald	Total	OPQ-2	OPW-10	OPT-7	OPT-16	OPT-20
55	869	448	492	1809	1	1	1	1	1
65	589	460	464	1513	1	1	1	1	1
520	429	606	641	1676	1	1	1	1	1
601	1040	466	640	2146	1	1	0	1	1
607	460	382	497	1339	0	1	1	1	1
613	1287	505	644	2436	1	1	1	1	1
624	1180	426	545	2151	1	0	1	1	1
641	619	388	450	1457	1	1	1	1	1
647	223	506	535	1264	0	1	0	1	1
649	561	485	555	1601	1	0	0	1	1
68	137	516	0	653	0	1	0	1	0
540	360	0	0	360	0	0	0	0	0
550	121	433	0	554	0	1	0	1	0
562	741	0	0	741	0	0	0	0	0
568	1416	10	0	1426	0	0	1	1	1
569	1323	0	0	1323	0	0	0	0	0
579	753	72	0	825	0	1	1	1	
586	518	1181	0	586	0	0	0	0	0
621	800	0	0	621	0	1	1	1	1
645	233	0	0	645	0	1	0	1	1
1	201	0	0	201	1	1	0	1	1
10	48	0	790	838	0	0	0	1	1
18	648	119	733	1500	1	1	1	0	1
25	415	311	0	726	0	1	0	0	1
27	98	94	0	192	0	1	1	0	1
28	1677	0	0	1677	0	0	0	0	0
30	235	266	270	771		1	1	1	1
32	113	0	0	113	0	1	1	1	1
33	726	195	10	931		1	0	1	1
37	177	10	0	187	1	1	1	0	1
38	768	0	0	768		1	1	1	1
42	306	362	0	668	1	0	0	0	1
44	243	10	0	253	1	1	1	1	1
48	486	294	133	913	1	1	1	1	1
49	231	173	0	404		0	1	1	1

52	92	281	0	373	1	1	1	0	0
58	276	293	281	850	0	0	0	0	0
62	326	479	0	805	0	1	1	1	1
66	147	10	0	157		0	0	0	0
67	276	0	0	276	0	0	0	0	0
79	1320	0	0	1320		0	1	0	1
80	2753	459	120	3332	1	1	0	1	1
82	354	519	0	873	0	0	1	0	1
84	563	0	0	563	0	1	1	1	0
88	305	284	165	754		1	1	1	1
503	428	0	10	438	0	1	0	1	0
507	196	280	225	701	1	1	1	1	0
514	1555	0	10	1565	1	1	0	1	1
519	147	0	0	147	0	0	0	0	0
526	117	64	0	181	1	1	1	1	0
528	482	0	10	492	0	1	0	0	0
529	159	279	259	697	1	1	0	1	0
532	363	384	344	1091	1	0	0	1	1
534	285	136	199	620	1	0	0	1	1
539	1063	10	10	1083	0	1	0	0	0
542	444	296	229	969	0	0	0	0	1
547	397	431	53	881	0	1	1	0	0
548	131	277	243	651	0	0	0	1	0
549	909	0	54	963	0	0	0	1	0
552	112	219	218	549	0	1	1	1	0
553	430	172	191	793	1	0	0	1	1
554	85	431	10	526	0	1	0	0	0
556	741	230	230	1201	0	1	0	0	1
560	896	603	10	1509	0	1	0	1	1
561	1386	66	10	1462	1	1	1	0	0
563	0	83	69	152	0	1	0	1	0
566	445	319	393	1157	0	1	0	1	0
573	942	0	0	942	0	1	0	1	1
575	301	321	341	963		1	1	1	1
576	3094	0	0	3094		0	0	0	0
577	942	220	172	1334	0	1	0	1	1
588	474	232	269	975	0	1	1	1	1
591	161	494	10	665	0	1	1	0	0
596	325	262	293	880	0	1	0	1	
597	592	157	143	892	0	0	0	1	1
614	0	10	61	71		1	1	0	1
621	800	0	0	800	0	0	1	1	1

622	0	87	106	193	0	1	1	1	1
625	0	0	10	10	1	1	0	0	0
626	392	411	403	1206		0	0	1	1
627	725	347	394	1466					
630	306	292	253	851	0	0	0	1	1
636	691	297	320	1308	0	1	1	0	0
637	79	47	50	176	1	0	0	1	1
638	456	1317	0	1773			0	0	0
644	1229	317	294	1840	0	1	1	1	0
646	0	0	0	0	0	1	1	1	0

Table 10: Results for presence (1) and absence (0) of bands amplified with 5 different RAPD primers that segregated with the leptine content trait (Ald) for genotypes of the PBCc population. Selections for the high ACR bulk are represented in red color. Selections for the low ACR bulk are represented in blue color. Sd=Solanidine; Ld=Leptinidine; Ald= Leptine glycoalkaloids measured in $\mu g.g^{-1}$dry wt.

PBCc	SD	LD	ALD	Total	OPQ-2	OPW-10	OPT-7	OPT-16	OPT-20
1505	1041	233	1540	2814	1	1	1	1	1
1507	843	415	4084	5342	1	1	0	1	1
1516	626	288	1863	2777	0	0	1	1	1
1520	3186	820	3047	7053	1	1	1	0	1
1537	1238	596	3452	5286	1	1	0	1	0
1586	910	352	1977	3239	1	0	0	1	1
1593	713	492	5088	6293	0	1	1	1	1
1510	537	0	0	537	0	0	0	0	0
1511	463	0	0	463	0	0	0	0	0
1512	854	0	0	854	1	0	1	0	0
1546	292	0	0	292	0	0	0	1	0
1548	526	0	0	526	0	0	0	1	1
1560	498	0	0	498	1	1	0	1	0
1566	520	0	0	520	1	1	1	1	1
1583	280	0	0	280		1	0	0	0
1502	724	143	0	867	1	0	1	1	0
1503	265	308	0	573	0	1	1	0	0
1506	348	93	0	441	0	0	0	0	0
1514	1286	91	0	1377	1	1	1	1	1
1518	194	60	0	254	1	1	1	1	1
1519	2355	1508	0	3863	0	0	1	0	0
1521	921	196	0	1117	1	1	1	1	1
1524	0	0	0	0		0	1	0	1
1528	287	55	0	342	0	0	0	1	1
1533	570	97	0	667	1	0	0	0	0
1539	865	164	0		1	1	0	1	0
1540	2361	253	0	1029		0	0	0	1
1547	1806	0	0	2614	0	1	0	0	1
1550	429	156	0	585	0	0	0	1	0
1556	2089	75	0	2164	1	1	0	0	0
1561	342	134	0	476	0	1	0	0	0
1564	1499	0	0	1499	0	1	0	1	0
1572	513	123	0	636	0	1	0	1	1
1573	750	170	0	920	0	1	0	0	1
1575	682	270	0	952	1	1	1	0	1

1582	684	107	0	791	1	1	1	0	1
1594	1188	0	0	1188	0	1	1	1	0
1604	572	118	0	690	0	0	0	1	1
1605	408	0	0	408	0	1	0	1	0
1611	951	0	0	951	0	1	0	1	0
1613	597	361	0	958	0	1	0	1	0
1632	1559	0	0	1559	1	0	0	0	0

DNA extraction protocol:
CTAB isolation procedure of total DNA [Doyle and Doyle (1987)]

- Use 1.5 g leaf material (remove petiole and large veins).
- Crush leaves into a fine powder in a mortar & pestle using liquid Nitrogen (aprox. 50 ml).
- Add 7 ml warmed "2X CTAB isolation buffer"[1] and mix thoroughly. Add 0.4 g (4% wt/volume) PVP 40 to each sample.
- Pour the grindate into labeled 50 ml centrifuge tube. Rinse mortar with 3 ml 2X CTAB isolation buffer and add to tube.
- Incubate in 60°C water bath. Add 10 ml (or equal volume) 24:1 Chloroform: Isoamyl alcohol. Invert tube 20 times gently.
- Spin tube in clinical centrifuge (2500 rpm) for 10 minutes.
- Take off the aqueous (top) layer using a sterile pipette, and place it in a new (sterile) 50 ml centrifuge tube.
- Add 5 ml (or 2/3 volume) ice-cold isopropanol. Invert tube gently 10 times to precipitate DNA.
- Place tube in a -20°C freezer overnight.
- Take tube out of freezer, spin in clinical centrifuge 5 minutes (2500 rpm).
- Gently pour of supernatant.
- Add 20 ml "wash buffer"[2]. Gently swirl to break up the DNA pellet. Let it sit at room temperature for 20 min of in the refrigerator for up to 2 days.
- Spin in clinical centrifuge 5 min (2000 rpm).
- Pour off supernatant, invert tube on paper towel (Kimwipes) to dry excess wash buffer.
- Add 300 µl TE buffer and 6µl RNAse A (1 mg/100ml).

[1] **2X CTAB isolation buffer:**
Final concentration: 2% CTAB
100 mM Tris pH 8.0
1.4M NaCl
20 mM EDTA
Distilled water

[2] **Wash buffer** (1 L):
13.3 ml 7.5 M Ammonium acetate
800 ml 95% Ethanol
186.7 ml distilled water
(final concentration: 10 mM ammonium acetate, 75% ethanol)

Vita

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Graduate studies	Universidad Nacional Agraria La Molina, Lima – Perú Major: Plant Breeding and Genetics.
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