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ORIGINAL PAPER

Mapping of genes associated with leptine content of tetraploid potato

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Abstract High content of leptine glycoalkaloids present in *Solanum chacoense* has been associated with genetic resistance to Colorado potato beetle (*Leptinotarsa decemlineata* [Say]). From an unrecorded accession of *S. chacoense*, the North Dakota State University breeding program has developed a tetraploid genotype, ND4382-19, that contains foliar leptines. In this study, using a segregating population, ND5873 (ND4382-19 × Chipeta), and GC-MS to analyze foliar content of alkaloids, two loci, involved in the synthesis of leptines were identified. They segregated as two complementary epistatic genes that allowed the synthesis of leptinidine (Lep) and acetyl-leptinidine (AL), respectively. Partial AFLP maps for both parents were developed using 97 individuals from population ND5873. The total lengths mapped for ND4382-19 and Chipeta were 1,883 and 1,021 cM, respectively. The marker for Lep was located at the distal end of simplex-coupling linkage group R37. Expansion of the initial mapping population and analysis of Lep-containing individuals allowed us to identify the linkage group (R35) that enabled synthesis of AL. By the use of simple sequence repeat markers, linkage group R37 (Lep) and linkage group R35 (AL) have been identified as homologs of chromosomes II and VIII, respectively.

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Introduction

Colorado potato beetle (*Leptinotarsa decemlineata* [Say]) is the most important defoliating insect pest of potatoes (*Solanum tuberosum* L.) in the United States (Ferro et al. 1985; Jolivet 1991). It is widely distributed in North America and has invaded Europe and Asia. Its control requires the application of large amounts of insecticides, which are expensive and can negatively impact both the environment and human health (Friedman and McDonald 1997). This insect has demonstrated its ability to develop resistance to a wide range of pesticides (Forgash 1985; Boiteau 1988). In some cases, a new insecticide failed after 1 year or even during the first year of use (Forgash 1985). In addition, its resistance to the *Bacillus thuringiensis* subsp. *tenebrionis* delta-endotoxin has also been reported (Rahardja and Whalon 1995).

Effective control of Colorado potato beetle (CPB) may require multiple strategies (Ragsdale and Radcliffe 1999). Genetic resistance to CPB is a desirable

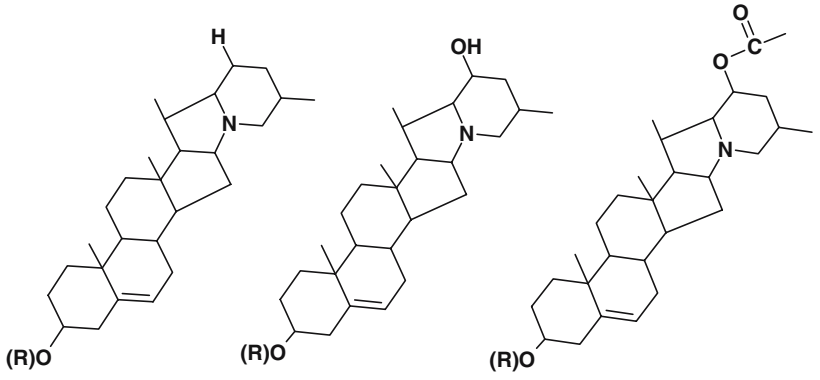
strategy with regard to cost and environmental impact. The most promising genetic resistance mechanisms to date have been glandular trichomes from *S. berthaultii* Hawkes (Tingey 1984); and leptines, unique glycoalkaloids that are found in a few accessions of *Solanum chacoense* Bitter (Kuhn and Löw 1961; Stürckow and Löw 1961; Sinden et al. 1986a). In addition to the cost and environmental benefits, host plant resistance is expected to be compatible with other biological control strategies.

Leptines are rare glycoalkaloids (GAs) that are synthesized by a few accessions of *S. chacoense*. The predominant forms of leptines found in *S. chacoense*, leptine I and leptine II (Fig. 1), both have a high CPB (Kuhn and Löw 1961; Sinden et al. 1986a) deterring capacity. They share the same steroidal aglycone and differ only by their sugar moiety. The steroidal aglycone of leptines is an acetylated (C-23) form of solanidine (Sol), the normal aglycone of *S. tuberosum* glycoalkaloids. Leptines I and II are the acetylated equivalents of α-chaconine and α-solanine, respectively (Fig. 1). Studies of leptine segregation within *S. chacoense* progenies indicate that few genes control the synthesis of leptines (Deahl and Sinden 1987). It is likely that acetyl-leptinidine (AL) is synthesized from Sol (Lawson et al. 1993; Osman et al. 1987). The Sol conversion to AL requires at least two enzymatic activities (Lawson et al. 1993; Osman et al. 1987): hydroxylation of Sol at C-23 to produce leptinidine (Lep), and acetylation of the resulting hydroxyl group to AL (Fig. 1).

Considering this postulated synthetic pathway, three phenotypic classes are possible: a class that includes plants that are able to synthesize both Lep and AL ($Sol^+Lep^+AL^+$), a second class of genotypes that only produce Lep ($Sol^+Lep^+AL^-$), and a third class with neither ($Sol^+Lep^-AL^-$). These three possible phenotypic classes were observed in *S. chacoense* progenies in which all plants contained Sol, some plants contained Lep, and a few plants contained AL (Silhavy et al. 1996). Genotypes in the class $Sol^+Lep^-AL^-$ should include two sets: individuals that do not have either enzymatic activity required to synthesize AL and individuals with acetylation activity but that lack the first hydroxylating enzyme and therefore lack substrate for acetylation (complementary epistasis). All three phenotypes are present in segregating progeny of ND4382-19, a tetraploid species backcross selection of a hybrid with *S. chacoense* that contains leptine (Lorenzen et al. 2001).

To achieve resistance against CPB in potato cultivars, high-leptine genotypes of *S. chacoense* were integrated in a breeding program (Sanford et al. 1996; Yencho et al. 2000). Successful introgression of genes involved in the synthesis of leptines could be facilitated, using marker-assisted selection (MAS). In addition, identification of markers closely associated to genes controlling leptine synthesis could facilitate

Glycoalkaloid	Aglycone	R
Chaconine Solanine	Solanidine	Chacotriose Solatriose
Leptinine I Leptinine II	Leptinidine	Chacotriose Solatriose
Leptine I Leptine II	Acetyl-leptinidine	Chacotriose Solatriose



Solanidine Leptinidine Acetyl-leptinidine

Fig. 1 Molecular structures of the aglycones solanidine, leptinidine, and acetyl-leptinidine, and the identity of the associated trisaccharides that define the respective glycoalkaloids

future isolation and cloning (Silhavy et al. 1996; Ronning et al. 1999; Hutvágner et al. 2001; Medina et al. 2002). Marker-assisted selection and map-based cloning require the generation of a molecular map from a segregating population. Mapping populations have been useful in identifying other potato resistance genes, e.g., against nematodes, viruses and late blight (reviewed in Gebhardt et al. 1993; Gebhardt and Valkonen 2001), and QTLs linked to glandular trichome expression (Bonierbale et al. 1994).

Solanum tuberosum is a highly heterozygous autotetraploid species that is vegetatively propagated. Inbreeding depression makes it difficult to obtain homozygous parents for classical mapping populations, so the F_2 pseudo-testcross approach applied to F_1 populations has been used to develop potato maps (e.g., Bonierbale et al. 1994). Although most potato maps were developed from diploid populations (e.g., Bonierbale et al. 1994; Gebhardt et al. 1989; Jacobs et al. 1995), maps from tetraploid potato populations have also been reported (Hackett et al. 1998; Meyer et al. 1998; Bradshaw et al. 1998; Luo et al. 2001).

We have developed a tetraploid population with qualitative segregation for the aglycones Lep and AL. Although differentiation of low-leptine and null-leptine individuals was difficult with HPLC, the greater sensitivity of GC/MS allowed us to differentiate these two classes (Lorenzen et al. 2001), which will be necessary to map the hydroxylation and acetylation functions. Accurate determination of chemical phenotype may allow more accurate mapping of loci that control synthesis of these two functions. We report here, the mapping of two loci, which together, enable the synthesis of leptines in potato.

Materials and methods

Plant material

Seeds of the tetraploid population ND5873 (ND4382-19 × Chipeta) were surface-sterilized and germinated in vitro. Progeny and parents have been maintained vegetatively as in vitro tissue culture plantlets (set 1: most genotypes) and/or as tubers (set 2: sub-population). The parent, ND4382-19, is a backcross from the species *S. chacoense* (Lorenzen and Balbyshev 1997). The pedigree of population ND5873 is shown in Fig. 2. After the initial alkaloid analysis indicated that there were insufficient genotypes to accurately map both functions (see below), additionally, 133 genotypes were screened for Lep and AL. Genotypes that contained Lep were kept as a subpopulation to enable genetic analysis of AL synthesis. These Lep-containing genotypes were maintained as tubers.

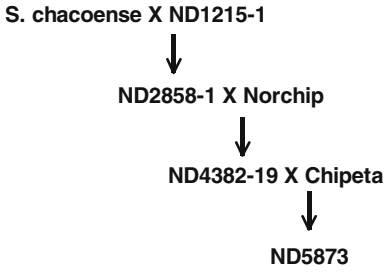


Fig. 2 Pedigree of population ND5873

Depending on the experiment, ND5873 plants and parents were grown either from tubers produced in the field, or from tissue-culture-derived plants grown in a greenhouse or growth chamber. Plant tissues for DNA extraction and glycoalkaloid analysis were obtained from plants grown in a greenhouse at North Dakota State University, Fargo, ND, in 1997 and 1998. Tissue cultured plantlets or tubers were acclimated and planted in 25-cm diameter × 19-cm deep plastic pots, filled with peat-vermiculite medium (Sunshine Mix 1, Fisons Hort., Bellevue, WA, USA) and placed in a greenhouse with 16-h photoperiod and mean daily temperature of 22.5°C. Natural lighting was supplemented by 1,000-W metal halide lamps. All plants were fertilized weekly with 15N-7P-14K (Peters, Fogelsville, PA, USA) at 1 g L^{-1} of water solution.

Chemicals and reagents

Common reagents were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). Enzymes and specialized reagents were purchased from New England Biolabs, Inc. (Beverly, MA, USA), GIBCO-BRL (Life Technologies, Inc., Rockville, MD, USA) and Promega Corp. (Madison, WI, USA). The AFLP kit was from GIBCO-BRL. Primers were ordered from IDT® (Coralville, IA, USA).

DNA extraction and AFLP reactions

DNA from each clone was extracted from fresh–frozen leaves according to Fulton et al. (1995). The recovered DNA was resuspended with 1 ml of TE (10 mM Tris–HCl and 1 mM EDTA, pH 8.0). DNA concentration and quality were estimated by absorbance at 260 nm and agarose gel electrophoresis, followed by staining with ethidium bromide, respectively, according to Sambrook et al. (1989). The AFLP reaction was performed according to Vos et al. (1995). Two pairs of restriction enzymes: *Eco*RI and *Mse*I, or *Pst*I and *Mse*I

and their respective adapters and primers were used for AFLP. Pre-amplification primer sequences of *Eco*RI, *Mse*I, and *Pst*I were 5′-GACTGCGTACCAATTC**A**-3′, 5′-GATGAGTCCTGAGTAA**C**-3′, and 5′-GACTGCG TACATGCA**G**-3′, respectively. Selective PCR reactions were performed with ^{33}P-labeled *Eco*RI (or *Pst*I) primer. The amplified DNA fragments were separated by electrophoresis in a 5% denaturing polyacrylamide sequencing gel (33 × 50 cm). The gel was transferred to filter paper and dried. AFLP amplified fragments were visualized via autoradiography. Segregating markers were numbered from small fragments to large fragments for each parent. Films were manually scored as to the presence or absence of bands (Table 1).

Simple sequence repeat assays

Simple sequence repeat (SSR) assays were performed according to Milbourne et al. (1998). The PCR reactions were done in 10 µl of final volume consisting of 20 ng genomic DNA, 1× PCR buffer [10 mM Tris–HCl (pH 8.3), 1.5 mM $MgCl_2$, and 50 mM KCl], 0.3 U of Taq DNA polymerase, 0.3 µM of forward and reverse primers, and 200 µM dNTPs. The forward primer was 5′ end labeled with [γ^{33}P]ATP and T4 polynucleotide kinase. The PCR cycling conditions in a MJ Research DNA Thermocycler (PTC-100, MJ Research, Watertown, MA, USA) were as follows: 94°C for 3 min, 25 cycles with 94°C for 30 s, Tm for 30 s, and 72°C for 30 s, followed by 72°C for 5 min. Electrophoresis was carried out on a 6% denaturing polyacrylamide sequencing gel (33 × 50 cm), and autoradiography was performed as described above.

Steroidal aglycone analysis

When plants were in the pre- to early-bloom stage, one sample of four fully expanded leaves was harvested from the upper third of each plant, placed in a plastic bag, and immediately placed in an insulated cooler over ice. Soon after harvesting, the leaves were immersed in liquid nitrogen and then lyophilized for 72 h approximately. The steroidal aglycones were extracted from ground leaf tissue and prepared according to the method described by Lawson et al. (1992). Freeze-dried leaf samples were hydrolyzed under nitrogen in 1 N HCl in methanol at 70°C for 4 h. After cooling, samples were adjusted to pH 10 with concentrated ammonium hydroxide, centrifuged at 12,000 *g*, and partitioned against toluene. Toluene fractions were dried and TMS-derivatized with N-O-*bis*(trimethylsilyl)trifluoroacetamide (BSTFA) at 65°C for 30 min. Samples were injected into an HP5890 gas chromatograph (Palo Alto, CA, USA) coupled to a Finnigan Mat Incos 50 quadrapole mass spectrometer (San Jose, CA, USA) with a 20 m × 0.18 mm × 0.20 µm Restek RTX1 (100% methylsilicone) capillary column, set either on scan mode (50–650 amu s^{-1}) or on select ion mode (125, 150, 238, 452, 454, 465, 467, 469, and 472 amu s^{-1}). The solanidine standard was purchased from Sigma, Inc. Authentic standards of leptinidine and acetyl-leptinidine standards were provided by Ray Miller, Ohio State University. Selective windows for diagnostic ions at *m/z* 150, 238, and 467 for Sol, Lep, and AL, respectively, were used to evaluate the presence or absence of the respective alkaloid, and the peak height comparison with the respective standard was used to estimate aglycone concentrations.

Linkages of genes involved in the synthesis of Lep and AL

For both markers and gene segregation, random pairing of each set of four homologous chromosomes with no double reduction was assumed. Segregation of Lep and AL was analyzed as two simplex genes (presence vs. absence) with complementary epistasis. In the cross ND4382-19 × Chipeta, the female is the donor for the two genes involved in the synthesis of Lep and AL, and the male (Chipeta) is a nulliplex for both genes. Table 2 indicates the four possible genotypes and their expected frequencies with regard to independence versus linkage of the two genes. If M1 and M2 are the genes involved in the synthesis of Lep and AL, respectively, the action of M2 depends on the product of M1 (complementary epistasis), and only three phenotypes are observable: class A [Sol^+, Lep^+, and AL^+] with both genes [$M1M2(m1m2)_3$]; class B [Sol^+, Lep^+, and AL^-] with only the gene that produces Lep

Table 1 Selective AFLP primer combination used in population ND5873

E-AAC/M-CAC	E-ACT/M-CAC	E-ACA/M-CAC	E-AGG/M-CAA	E-ACC/M-CAA
M-CAG	M-CAT	M-CAG	M-CAG	E-ACG/M-CAA
M-CCA	M-CTG	M-CGT	M-CTG	P-AA/M-CCA
M-CTG	M-CTT	M-CTG	M-CTT	P-AT/M-CAC

E, M, and P represent the sequence of universal AFLP primers designed for *Eco*RI, *Mse*I, and *Pst*I restriction sites

Table 2 Expected genotypes, ratios, and corresponding phenotypes for a two-gene model 1 given alternative genetic scenarios

All possible genotypes	Frequencies			Phenotypes	Classes
	Coupl.	Repul.	Inde.		
M1M2 $(m1m2)_3$	3/6	1/6	1/4	Sol^+, Lep^+, AL^+	A
M1m2 $(m1m2)_3$	0	2/6	1/4	Sol^+, Lep^+, AL^-	B
m1M2 $(m1m2)_3$	0	2/6	1/4	Sol^+, Lep^-, AL^-	C
$(m1m2)_4$	3/6	1/6	1/4	Sol^+, Lep^-, AL^-	

1 Two-gene model = M1-hydroxylation, M2-acetylation from Sol to Lep to AL

[M1m2$(m1m2)_3$]; and class C [Sol^+, Lep^-, and AL^-] with two genotypes, [m1M2$(m1m2)_3$] and [$(m1m2)_4$], that present identical phenotypes. Therefore, the expected frequency among the classes A:B:C is 1:1:2 if M1 and M2 segregate independently. Deviation from this ratio would indicate the possibility of linkage. Fitness to expected ratio segregations was evaluated by Chi-square and LOD score methods (Liu 1998).

Mapping of AFLP markers

Linkage analysis of AFLP segregant markers was done according to Hackett et al. (1998). Assuming random pairing and chromosome assortment during the meiosis, the smallest expected standard errors for progeny populations of size 100 over the range [0, 0.3] of recombination (r) are for simplex–simplex coupling; duplex–duplex coupling; and duplex–simplex linkages in coupling or repulsion. These are the linkages recommended for developing a tetraploid potato linkage map (Hackett et al. 1998).

Simplex-dose AFLP markers (1:1 segregation) from both parents, ND4382-19 and Chipeta, were used to define their respective simplex-coupling linkage groups using MapManagerQTX (version 0.14), with a linkage criterion of 0.0001 and Kosambi as the mapping function. Large groups with excessive length were checked with Mapmaker/Exp (version 3.0b) using a LOD = 5.0 and 30 cM as maximal distance. Binary data for chemical phenotype (presence vs. absence) for Lep and AL were included as simplex markers to identify their coupling linkage group(s). Duplex–simplex linkages in repulsion and coupling were estimated according to the maximum likelihood equation (Hackett et al. 1998), where the recombination distance is a direct function of x. A value of $0 \le x \le 0.5$ indicates coupling phase, and $r = x$; a value of $0.5 \le x \le 1$ indicates repulsion, and $r = 1 - x$. Values of x were estimated using the Newton–Raphson iteration method (Liu 1998). Consistent high LOD scores, as comparison of log-likelihood $L(r = \hat{r}) - L(r = 0.5)$, between duplex and simplex markers from coupling linkage groups were indicative of linkage. Recombination distances between simplex–simplex and duplex–simplex markers, and their respective LOD scores were used to integrate duplex markers into simplex-coupling groups, according to Stam (1993).

Linkages across homologous groups were checked as described by Bradshaw et al. (2004), using the software program TetraploidMap (Hackett and Luo 2003).

Results

Segregation and linkage analysis

The parents of family ND5873, ND4382-19 and Chipeta, contain a moderate level of leptines relative to high-leptine genotypes of *S. chacoense* (Lorenzen et al. 2001) and no leptines, respectively. In order to analyze the genetics of AL synthesis, GC-MS was used to quantify alkaloid aglycones in ND5873 tetraploid progeny. Foliar content of Sol, Lep, and AL in the parents and 93 progeny genotypes is summarized in Table 3. Solanidine was present in all genotypes, but Lep and AL segregated among progeny. Solanidine concentrations ranged from 0.23 to 2.46 mg per gram of dry weight, with an average of 1.63 mg g^{-1}

Table 3 Alkaloid content of progeny ND5873

	Aglycone content		
	Solanidine (mg g^{-1} DW)	Leptinidine (mg g^{-1} DW)	Acetyl-leptinidine (mg g^{-1} DW)
Parents			
ND4382-19	0.64	1.40	0.69
Chipeta	0.32	ND	ND
Progeny ND5873			
Number of (+) plants	93	48	15
Mean (mg g^{-1} DW)	1.63	0.11	0.16
Standard deviation	0.51	0.08	0.13

Concentrations of Sol, Lep, and AL obtained by GC-MS are indicated for parents, ND4382-19 and Chipeta, and progeny. The number of progeny genotypes that contained the respective aglycone and their average are indicated. Standard deviations are indicated for each class

ND Not detected

(SD = 0.51). Lep was present in 48 genotypes, with a range of 0.01 to 0.31 mg g^{-1} and an average of 0.11 mg g^{-1} (SD = 0.08). Content of Sol and Lep was weakly correlated among genotypes that contained Lep ($n = 48$, $r = 0.2853$, $0.05 > P > 0.01$). AL was only detected in 15 individuals of the original population, ranging from 0.05 to 0.38 mg g^{-1} with a mean of 0.16 mg DW g^{-1} (SD = 0.13). Content of AL was highly correlated with Lep content ($n = 15$, $r = 0.7182$, $P < 0.01$), but not with Sol content. Most segregating genotypes showed higher concentrations of Sol than either parent. ND4382-19 and Chipeta had Sol concentrations < 1.0 (mg DW g^{-1}), but the mean Sol content of the progeny was 1.63 (mg DW g^{-1}). Lep and AL content for progeny that contained these alkaloids was less than that of the donor parent, ND4382-19.

Two sets of plants from the same cross were tested for the segregation of alkaloids. A Chi-square test of the observed phenotypic frequencies of genes (M1 and M2) involved in the synthesis of AL from Sol indicated a significant deviation from the expected 1:1:2 ratio (0.05, 2 *df*) for set 1 (93 individuals, Table 4). However, set 2 (133 individuals) did not show deviation from the expected 1:1:2 ratio of independent segregation (Table 4). The Chi-square test (0.05, 2 *df*) of the pooled data also showed a significant deviation from the expected ratio. This deviation from the expected ratio of independent segregation suggested the possibility of linkage between the M1 and M2 genes. The total Chi-square (χ^2_{Total}) is equal to the individual contribution of each gene (χ^2_{M1} and χ^2_{M2}), and the difference between the total and individual contributions is due to the linkage contribution ($\chi^2_{Linkage} = \chi^2_{Total} - \chi^2_{M1} - \chi^2_{M2}$). Segregation of M2 can only be partially estimated because one genotypic class that carries the gene [m1M2 $(m1m2)_3$] is not detectable by alkaloid analysis, but is included in phenotypic class C. Only the observable genotypes in classes A and B were used to estimate its segregation. The segregation of class A was tested with respect to class B, with a 1:1 expected ratio. The segregation of the three classes, from progeny ND5873, indicated that if there is linkage between the M1 and M2 genes, they should be in repulsion (Table 2). Assuming repulsion, a LOD score (Ott 1992) was estimated for both sets of plants. This analysis did not detect a linkage between the two genes involved in the synthesis of AL (LOD threshold = 2.0). However, repulsion linkage has a large standard error and a very low power of detection in tetraploid species like potato (Hackett et al. 1998). A very large family size (>750) is required to detect repulsion linkage with a reasonable confidence (Wu et al. 1992).

AFLP mapping of population ND5873

Segregation of dominant AFLP markers in an autotetraploid species

To map the chromosomes of the tetraploid potato, ND4382-19, that carry the genes involved in the synthesis of AL and to identify AFLP markers closely associated to them, 20 different combinations of AFLP primers were assayed, using 98 genotypes of population ND5378. One genotype that presented very different AFLP patterns from the rest of plants was eliminated, leaving a final mapping population size of 97 individuals. A total of 592 polymorphic markers were scored, with 374 markers deriving from the leptine donor (ND4382-19) and 218 markers from Chipeta. Segregation ratios were used to identify and separate simplex, duplex, triplex, and distorted marker classes. In the case of random pairing with chromosome assortment, segregation is only expected for simplex and duplex markers, resulting in segregation

Table 4 Observed phenotypic frequency and linkage tests for genes involved in the synthesis of AL from Sol

Sets	Total	Classes				Segregation			Linkage	
		A	B	C	A + B	χ^2_{M1}	χ^2_{M2}	χ^2_{Total}	$\chi^2_{Linkage}$	LOD
Set 1	93	15	33	45	48	0.097	6.750**	7.065*	0.218	1.502
Set 2	133	24	30	79	54	4.699*	0.667	5.241	–0.125	0.145
Pooled data	226	39	63	124	102	2.142	5.647*	7.239*	–0.550	1.238
$\chi^2_{homogeneity}$						2.654	1.770	5.066	0.642	0.409

Classes A, B, and C represent plants with AL and Lep, only Lep, and neither AL nor Lep, respectively. The Chi-square test for M1 gene segregation includes all leptine-expressing plants (A + B) versus C with a 1:1 expected ratio (1 *df*). The segregation test for M2 only includes classes A and B with 1:1 expected ratio (1 *df*). Independent segregation of M1 and M2 has an expected ratio of 1:1:2 among classes A, B, and C, respectively (2 *df*). LOD scores to evaluate the possibility of linkage between M1 and M2 were based on the maximal likelihood equations

$\chi^2_{Total} = \chi^2_{Linkage} + \chi^2_{M1} + \chi^2_{M2}$

*Significant at $P \leq 0.05$; **highly significant at $P \leq 0.01$

ratios of 1:1 and 5:1, respectively. For ND4382-19, 230 markers segregated as simplex markers, of which 222 presented a 1:1 ratio and 8 markers fit a 13:15 ratio. The total number of duplex markers was 88, with 71 fitting a 5:1 ratio and 17 fitting a 22:6 ratio. There were 36 markers with a distorted ratio. For Chipeta, of the 142 simplex dose markers, only 2 did not fall in the 1:1 ratio. There were 46 duplex markers, with 38 fitting a 5:1 ratio and 8 fitting a 22:6 ratio. There were 21 markers from Chipeta with distorted segregation. There were 5 and 9 markers that fit a triplex (27:1) distribution for ND4382-19 and Chipeta, respectively. Segregation of triplex markers (27:1) has been proposed as a method to identify the fraction of chromatid assortment during the meiosis of autotetraploid species (Yu and Pauls 1993).

Mapping simplex AFLP markers linked in coupling

Simplex dose markers (1:1 segregating) were used to define the simplex-coupling linkage groups with a linkage criterion of 0.0001 and Kosambi as the map function. Lep phenotypic data (as presence or absence) were included as one of the simplex markers. Later, several AFLP markers with distorted segregation were included (slight deviation from a 1:1 segregation). Segregating SSR markers were also included to facilitate the chromosome identification. A total number of 320 simplex markers were used to map the leptine donor parent, ND4382-19, producing 46 different coupling linkage groups with 39 unlinked markers. The Chipeta parent was mapped with 168 simplex markers, producing 32 coupling linkage groups and 26 unlinked markers. The total map lengths for these parents were 1883.3 and 1021.4 cM for ND4382-19 and Chipeta, respectively. The integrated map length for diploid potato has about 1,000 cM (Jacobs et al. 1995), thus, the cumulative length of a tetraploid map, based on coupling-phase simplex markers would be expected to be four times that length (assuming similar recombination frequencies in diploid and tetraploid crosses).

Mapping Lep

GC-MS analysis allowed us to score the presence or absence of Lep in 93 progeny genotypes that were used for molecular mapping. This trait, which had a 1:1 segregation ratio, was strongly linked to the distal end of simplex-coupling group R37 (Fig. 3). The closest marker was E-AGG/M-CTTR2, separated from Lep by 3.4 cM (LOD = 15.3). This group contained a total of 20 simplex markers, including Lep as a binary marker, and it covered a total distance of 77.6 cM (Fig. 3). This simplex-coupling group included SSR markers STM0038, STM1064, STM2022, all of which reside on chromosome II (Milbourne et al. 1998).

Duplex–simplex linkages in both coupling and repulsion, have a reasonably small standard error and are useful in identifying homologous chromosomes in tetraploid potato mapping (Hackett et al. 1998). A search for duplex–simplex linkages was performed among all 89 duplex markers and the simplex markers contained in the group R37. Two duplex markers, E-AAC/M-CTGR2.1 and E-ACT/M-CACR5, were linked in repulsion to group R37. These two duplex markers were then used to screen the other simplex-coupling groups and unlinked markers. One group (R8) demonstrated strong linkage with both duplex markers, both again, in repulsion. Another small linkage group (R26) detected was in coupling with duplex E-ACT/M-CACR5. Unlinked markers P-AT/M-CACR6 and P-AT/M-CACR4 also presented coupling linkage with these duplex markers (Fig. 3). The amplification products of SSR STM1064 (chromosome II) included two fragments from the donor parent (STM1064_1 and STM1064_2), one in group R37 (Fig. 3) and the other in group R8. These two groups, R37 and R8, are linked in repulsion with the same duplex markers, E-AAC/M-CTGR2.1 and E-ACT/M-CACR5.

The simplex STM1064 alleles were converted to a duplex marker by combining the three classes, AB (11), AO (10), and OB (01), to A. Analysis of duplex marker DSTM1064 detected linkage in repulsion among the same simplex-coupling groups and unlinked AFLP markers that were detected with the AFLP duplex analysis. Later, the same procedure was done with two simplex alleles of SSR STM0038. The STM0038 loci were located on groups R37 and R26, and when combined, were linked in repulsion to group R8 and the same unlinked markers. Group R26 also contained an allele of STM3001 (chromosome II). Figure 3 shows the probable alignment among the chromosome II homologs. Therefore, it is likely that this analysis identified each of the four members of chromosome II from the donor parent, ND4382-19.

Mapping AL

Because the genes involved in the synthesis of Lep present complementary epistasis, GC-MS analysis only allows the detection of a fraction of the genotypes that carry the second gene involved in the synthesis of AL from Lep. Only those genotypes that carry the first gene are detectable, because hydroxylation must precede acetylation. Of the 48 genotypes in this set that

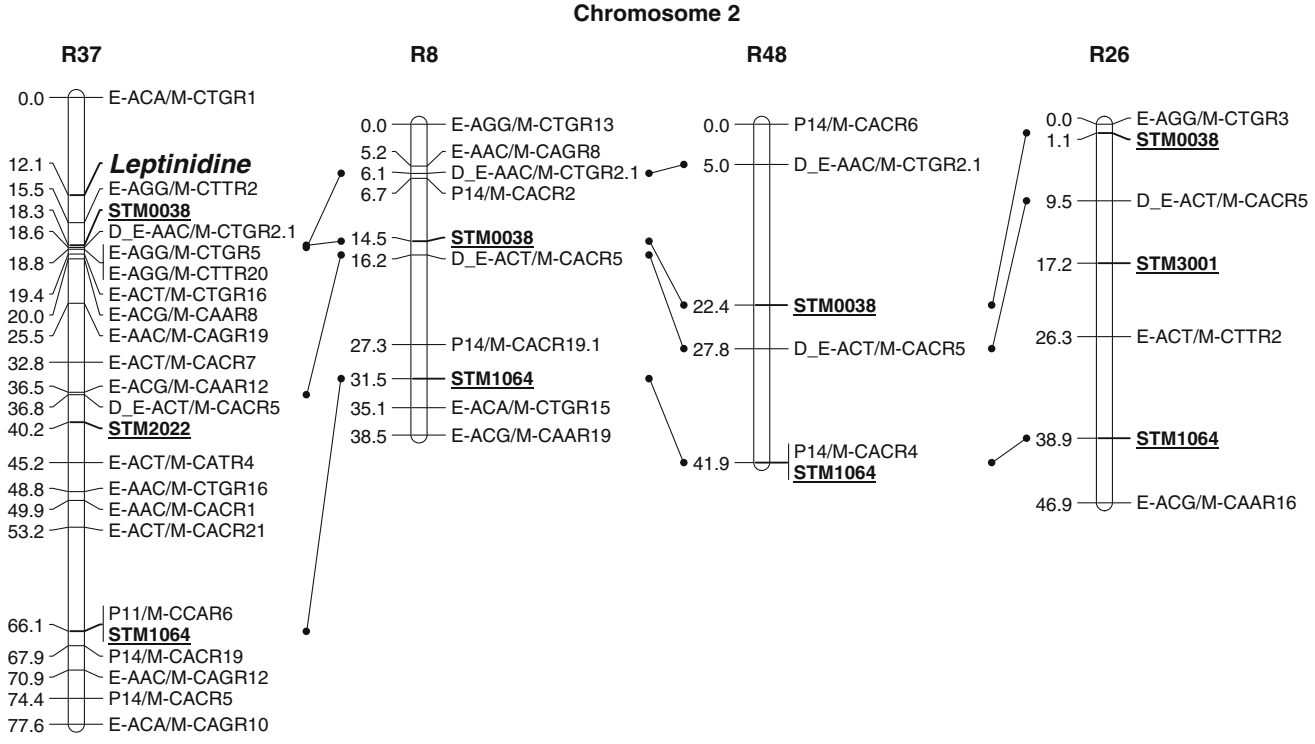


Fig. 3 Simplex-coupling group alignments for R37. The four chromosome II homologs and their probable alignment according to duplex–simplex linkage are illustrated. Lines connecting the homologous groups indicate coupling and/or repulsion with their respective duplex marker. Duplex markers are designated by "D_"

contained Lep, only 15 had detectable levels of AL. To increase the size of the sub-population that contained Lep, another 133 plants were screened for Lep and AL (Table 4). A new set of genotypes containing Lep (51 plants) was then analyzed for the same set of AFLP markers used in the previous set. This group contained 24 genotypes with AL. Single correlation analysis identified several AFLP markers that were significantly associated with AL presence that were in simplex-coupling linkage group R35. This association by single correlation strongly suggested that this group is the simplex-linkage group that contains a gene responsible for AL synthesis. Joining the two sets of Lep^+ plants, a sub-population of 99 genotypes was used to map the AL trait. AL mapped to a locus between the markers E-AAC/M-CTGR5.1 and E-ACT/M-CTTR14 of linkage group R35 (Fig. 4). The map distances of this phenotypic marker to the two closest flanking AFLP markers were 7.0 and 4.9 cM, with LOD scores of 13.6 and 12.4, respectively (Fig. 4). This simplex-coupling group included the SSR markers STM1001, STM1016 and STM1104 (chromosome VIII, Milbourne et al. 1998). A similar procedure, as described above for chromosome II, was used to identify homologous chromosomes of group R35. Analysis of duplex–simplex linkages among all 89 duplex markers and the simplex markers contained in the group R35 identified five duplex markers, E-AAC/M-CAGR2, E-AA/M-CCAR2, E-AGG/M-CTTR2, E-AGG/M-CTTR4 and E-AAC/M-CCAR20, that were linked with R35. These five duplex markers were then used to screen the other simplex-coupling groups, including unlinked markers. One group (R29) demonstrated strong linkage with two of these duplex markers. One unlinked marker E-AAC/M-CTGR17 was strongly linked to these two duplex markers. Group R29 included the SSR marker STM1024, which is also in chromosome VIII.

Discussion

Segregation and linkage analysis

Host–plant resistance from *S. chacoense* has been described as one of the most effective resistance types against CPB, and it has been associated with leptine content (Kuhn and Löw 1961; Sinden et al. 1986a). In this study, we have characterized progeny of a tetraploid potato genotype (ND4382-19) that contains leptines. This trait was inherited from an unrecorded accession of *S. chacoense* that was initially misidentified as *S. fendleri* (Tian 1998; Lorenzen et al. 2001).

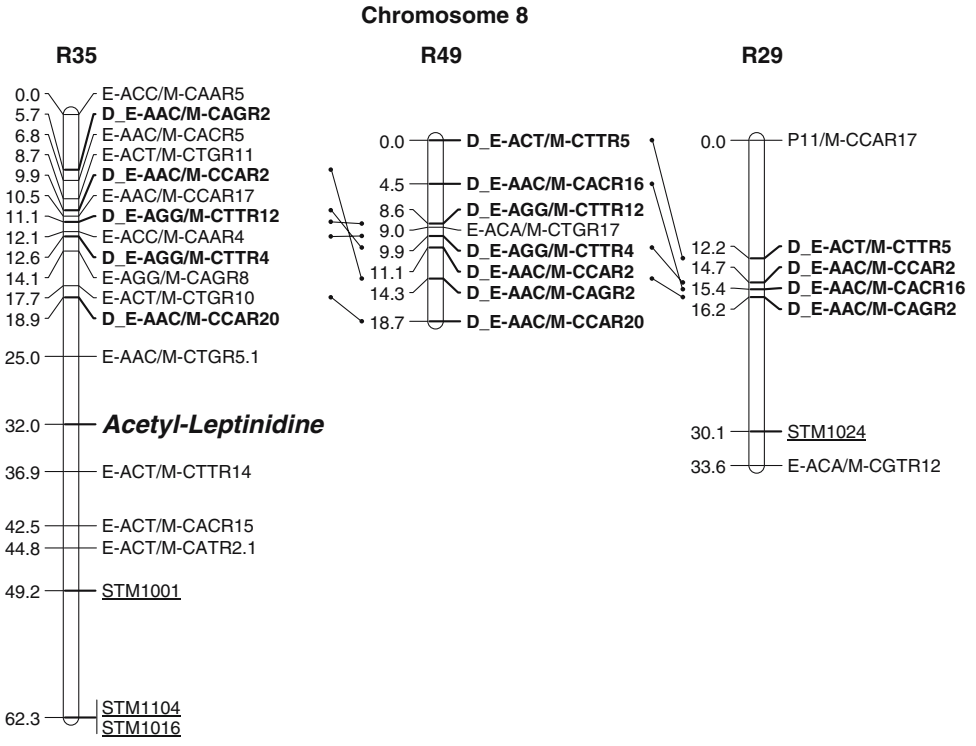


Fig. 4 Simplex-coupling group alignments for R35. The chromosome VIII homologs and their probable alignment according to duplex–simplex linkage are illustrated. See Fig. 3 for legend

Foliar GC-MS quantification of alkaloid aglycones of the segregating progeny ND5873 (ND4382-19 × Chipeta) showed quantitative segregation of Sol content, qualitative and quantitative segregation of Lep content, and qualitative and quantitative variation in AL in Lep$^+$ genotypes. The genetic analysis of the segregating phenotypes, including two sets of 93 and 133 plants, respectively, showed that genetic factors controlling AL synthesis segregated as two independent simplex genes, the first, for the synthesis of Lep and the second, enabling synthesis of AL. No linkage between these two factors was detected. This finding is consistent with previous reports, based on experiments with segregant populations of *S. chacoense*, which proposed the existence of a few genes involved in the synthesis of AL (Lawson et al. 1993, Osman et al. 1987). According to Osman et al. (1987), who recovered Lep from labeled Sol using isolated microsomal fractions of *S. chacoense* leaves in the presence of NADPH, the first enzyme involved in the hydroxylation of atom C-23 of Sol may be a P450-like enzyme. The second enzyme may be an acyltransferase, but there is no direct evidence of its identity.

The ability to develop a molecular map for species with tetrasomic inheritance, such as potato, can help to understand the genetics of important traits. Markers associated with a desirable trait could be used to facilitate the breeding process by marker-assisted selection (Meyer et al. 1998; Bradshaw et al. 1998). Constructing a map for an autotetraploid species is a stepwise process of developing simplex-coupling linkage groups and aligning them with respective homologous groups by duplex–simplex linkages (Hackett et al. 1998; Meyer et al. 1998; Bradshaw et al. 1998), chromosome anchor markers (Milbourne et al. 1998), and simplex markers common to both parents (Meyer et al. 1998). The complexity of this process is compensated by the fact that breeding populations can be used directly in the mapping process. Several agronomic traits may only be fully understood at the tetraploid level (Meyer et al. 1998). In this study, AFLP maps for two tetraploid parents were constructed with 46 and 32 simplex-coupling linkage groups. These maps covered a total length of 1883.3 and 1021.4 cM for ND4382-19 and Chipeta, respectively, representing approximately 47 and 26% of their respective genomes (Jacobs et al. 1995).

Mapping Lep

In this study, we identified a locus on chromosome II that had a qualitative effect on Lep synthesis. The location of this locus was unexpected. Several recent reports indicated the importance of chromosome I in controlling quantitative and/or qualitative variability in foliar glycoalkaloids of potato. A major QTL on chromosome I affected content of Sol-based glycoalkaloids (Yencho et al. 1998). A locus on the short arm

of chromosome I controlled whether most of the foliar glycoalkaloids were solanine+chaconine or leptine (Ronning et al. 1999). A nearby locus, termed *soh* (for Sol hydroxylation), on chromosome I enabled synthesis of leptinine (glycosylated Lep; Hutvágner et al. 2001). There are several possibilities to account for this discrepancy. In previous studies, we rejected HPLC for glycoalkaloid determination because it was not sufficiently sensitive to distinguish low content of leptine or leptinine from absence of that glycoalkaloid (Lorenzen et al. 2001). Use of GC/MS in select-ion mode gave much more sensitivity and confidence in assigning null status to an individual. The previous reports that identified chromosome I used HPLC (Yencho et al. 1998; Ronning et al. 1999) or TLC (Hutvágner et al. 2001) for glycoalkaloid determination, and may have detected loci with quantitative effects on leptine synthesis. Secondly, genetic mapping is possible only for loci that segregate in a given population. The locus discovered in the present study may not have segregated in the populations used by Ronning et al. (1999) or Hutvágner et al. (2001); it could have been homozygous in one or both parents.

Therefore, it is possible that the previous studies identified loci that are important for quantitative variation in glycoalkaloids, as mentioned by Hutvágner et al. (2001) but did not detect the qualitative Lep locus identified here. It may also be that multiple genes have a qualitative effect on synthesis of Lep. Yencho et al. (1998) mentioned that controlling loci could include either structural or regulatory genes. Tissue-specific presence of leptine in foliage but not tubers (e.g., Sinden et al. 1986b) apparently indicates a role for a regulatory gene with qualitative effect. Therefore, we do not know whether the qualitative Lep locus on chromosome II represents a structural gene that hydroxylates Sol, an enabling regulatory factor, or a member of either gene class that allows synthesis of a precursor essential for Lep synthesis. As mentioned above and by Hutvágner et al. (2001), a P450 monooxygenase would be a likely candidate gene for a structural gene. Additional studies will be required to determine the relationship between the factors on chromosomes I and II that affect synthesis of Lep.

Mapping AL

The combination of chemical phenotype, simplex, and duplex markers enabled mapping of the acetylation function to group R35, identified by SSR markers STM1001, STM1104, and STM1024 as a homolog of chromosome VIII. Therefore, chromosome VIII contains the second segregating gene that controls synthesis of AL in this population (Fig. 4). The AL phenotype showed a significant but small deviation from a 1:1 segregation pattern, mostly from the first data subset (Table 4). Segregation of the nearest AFLP marker to AL, E-ACT/M-CTTR14 was skewed in the same direction as AL, albeit not to the same degree (45:56, ns), as were the other markers on that arm of chromosome VIII. It is possible that one or a few genotypes of the subpopulation were mis-scored with regard to AL and caused the significant deviation from a 1:1 ratio. The presence of a locus on chromosome VIII that allows acetylation of Lep to AL has not previously been described. As mentioned above, it is possible that previously investigated populations did not segregate for this gene. Therefore, this study has identified two important qualitative loci that enable synthesis of Lep and AL.

Conclusions

The linked markers identified by this study could be used directly to improve the efficiency of introgression of these genes into potato cultivars in a breeding program. However, other genetic factors should be considered to improve the expression levels of leptines. ND4382-19 and its progeny population ND5873 did not synthesize high levels of leptines, suggesting that some other factor will be necessary to increase leptine production to levels effective against CPB. Ronning et al. (1998) described a recessive gene that enabled the expression of high leptines in families of *S. chacoense* that segregated for high and low levels of leptines. Although it is difficult to work with recessive genes in an autotetraploid, some segregating tetraploid genotypes had high leptine content (Sanford et al. 1996; Yencho et al. 2000). Therefore, there is much to be elucidated regarding the genetics of potato glycoalkaloids.

Since most commercial potatoes contain Sol-based glycoalkaloids, the cloning and transfer of genes for leptine synthesis in elite cultivars by transgenic methods has been proposed (Ronning et al. 1999; Silhavy et al. 1996). This study has provided important information for future map-based cloning of genes involved in the synthesis of Lep and AL. Cloning would allow us to investigate their expression and could enable genetic modification for enhanced leptine expression in transgenic potatoes.

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