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Molecular markers associated with leptinine production are located on chromosome 1 in *Solanum chacoense*

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Abstract Leptines of *Solanum chacoense* are effective natural deterrents against the Colorado potato beetle. Leptines are the acetylated forms of the glycoalkaloids solanine and chaconine and are supposed to be synthesised via hydroxylated derivatives, called leptinines. Inheritance of leptinine production was studied in crosses of closely related *S. chacoense* genotypes. The segregation data supported a single-gene model for the inheritance of leptinine production. In the segregating F_1 population of a *S. chacoense* cross, AFLP, RFLP and RAPD markers segregating with the leptinine production have been identified. The locus involved in leptinine synthesis was localised to the short arm of chromosome 1 of the potato where a major QTL for solanidine production, and markers with tight linkage to leptine production, have been mapped before. Our data further support the previous finding that the short arm of chromosome 1 is involved in steroid alkaloid synthesis in potato, and suggest that the genes involved in leptinine and leptine production are tightly linked in *S. chacoense*.

Keywords AFLP · Insect resistance · Glycoalkaloids · Leptine · Leptinine · *Solanum chacoense*

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Introduction

The Colorado beetle (CPB; *Leptinotarsa decemlineata* Say.) is a very important economical pest of the cultivated potato. In recent decades CPB control was predominantly based on excessive use of pesticides. The increasing demand for environmentally friendly and sustainable agriculture stresses the importance of developing potato varieties resistant to CPB.

One of the candidate donor species to introduce resistance against CPB into the cultivated potato is the wild species *Solanum chacoense* Bitter (Kuhn and Löw 1961). The active agents in *S. chacoense*'s defence reaction are the leptines, the acetylated derivatives of the steroid glycoalkaloids solanine and chaconine synthesised also in *Solanum tuberosum* (Stürckov and Löw 1961). Sinden et al. (1986) identified several *S. chacoense* genotypes accumulating high and low amounts of leptines. Recently, Ronning et al. (1998) reported the inheritance of foliar leptine production in genotypes derived from *S. chacoense* accessions PI 320287 and PI 458310. Most of the segregation data were consistent with a single recessive-gene model, although it was noted that a few families differed significantly from this expectation.

S. chacoense is a diploid potato species (2n = 2x = 24). Regenerants from one of the high-leptine producer genotypes of the accession PI 458310, doubled in chromosome number, were selected (Sanford et al. 1997) and crossed with tetraploid *S. tuberosum* breeding lines. All F_1 hybrids, their F_2 progeny, as well as almost all progenies from the backcross to *S. tuberosum*, synthesised foliage leptines, although the average concentration of leptines was reduced in the backcross population (Sanford et al. 1996, 1998).

The mapping of the steroid alkaloids solasodine and solanidine in *S. tuberosum* × *S. berthaultii* potato progenies revealed several QTLs located on chromosomes 1, 4, 6, 8, 11 and 12, which probably represent structural or regulatory genes controlling the accumulation of these aglycons (Yencho et al. 1998). Recently, in a segregating *S. chacoense* population, two molecular markers associated with a

high percent of solanine/ chaconine and, conversely, to a nil/ low percent of leptine were mapped to the end of the short arm of chromosome 1, in the region of a major QTL for solanidine production (Ronning et al. 1999).

Previously, we have confirmed that resistance against CPB is correlated with leptine production in the foliage. Based on the analysis of segregating populations of different accessions, including PI 320287, we have isolated *S. chacoense* lines representing the putative final enzymatic steps in leptine synthesis, such as hydroxylation resulting in leptinine formation and acetylation giving rise to leptines (Silhavy et al. 1996).

The aim of the current investigation was the identification of molecular markers linked to leptinine synthesis in a *S. chacoense* segregating population. The mapping work was mostly based on the use of AFLP markers (Vos et al. 1995), which is the most-appropriate method to map traits in the progeny of parents with a low degree of heterozygosity.

Materials and methods

Parents and crossings

Three *S. chacoense* parental lines were used in this study: A15 with a solanine/ chaconine, leptinine and leptine content; A6 synthesising solanine/ chaconine and leptinine; and H1 producing only solanine and chaconine (Silhavy et al. 1996). A15 and A6 were derived from the same accession, PI 320287, while H1 originated from the accession PI 320294. Plants were grown in greenhouses and vegetatively propagated from tubers. Crosses between the lines H1 and A15, and H1 and A6, were made under field conditions by pollinating stigmas of emasculated unopened flower buds of female parents. The pollination resulted in 16 berries containing 720 seeds and three berries with 120 seeds from the H1 × A15 and H1 × A6 crosses, respectively. On testing the glycoalkaloid composition of 91 plants derived from the H1 × A15 cross, 43 plants containing solanine and chaconine, and 48 plants producing solanine/ chaconine and leptinine, were identified. Analysis of 83 progenies of the H1 × A6 cross resulted in the detection of solanine/ chaconine in 38, and solanine/ chaconine and leptinine in 45, individual plants.

Glycoalkaloid extraction and detection

Glycoalkaloid extraction was done according to Bergenstrahle et al. (1992). A leaf sample (100 mg) was homogenised in 1.2 ml of extraction buffer containing water : acetic acid : $Na_2S_2O_5$ (100:5:0.5) with a mortar and pestle. The homogenate was transferred into a micro-centrifuge tube. After centrifugation for 15 min at 13000 rpm, 200 µl of the supernatant was transferred into another micro-centrifuge tube and vortexed with 800 µl of a mixture of chloroform : methanol : acetic acid (200:100:3). After separation by centrifugation for 3 min at 13000 rpm the upper layer was removed, evaporated to dryness and dissolved in 40 µl of methanol.

To determine the composition of total glycoalkaloids, 5–20 µl of extracts were separated on TLC silica gel plates (Merck 5554) with chloroform : methanol : 1% ammonia (70:30:5) for 30 min. The plates were dried at room temperature and heated to 105°C for 15 min. After cooling to room temperature (15 min) the glycoalkaloids were stained in the dark by immersing the plates in 0.02% Blankophor disolved in methanol for 1 min. Blankophor BA 267% was kindly provided by L. Jonsson, Swedish University of Uppsala. The plates were dried again at room temperature, heated at 105°C for 15 min and visualised by UV (Jellema et al. 1982). As standards, solanine and chaconine (Sigma) were used.

Alkaloid analysis

Glycoalkaloids disolved in methanol were hydrolysed by adding a one-tenth vol of HCl and heating for 2 h at 68°C. The liberated aglycones were extracted by adding 2 vol of concentrated ammonia and 1 vol of chloroform. After mixing and separating by centrifugation, the chloroform layer was removed and washed once with water. The aglycones were separated on TLC silica plates (Merck 5554) with ethylacetate : cyclohexane (55:54) and visualised with iodine (Gregory et al. 1981). Solanidine (Sigma) was used as a standard.

AFLP analysis

DNA isolation for AFLP was performed on fresh leaf tissue according to Shure et al. (1983). AFLP analysis was carried out by following the methods described by Vos et al. (1995). Template DNA was prepared by using the restriction enzymes *Eco*RI, and *Mse*I, and pre-amplified by the ''*Eco*RI-primer'' E+0 and the ''*Mse*I-primer'' M+0. The pre-amplification step was followed by selective amplification. Polymorphic AFLP markers resulted from the following primer combinations: E45M47 – E+ATG / M+CAA; E39M47 – E+AGA / M+CAA; E38M49 – E+ACT / M+CAG; E39M34 – E+AGA / M+AAT; E38M60 – E+ACT / M+CTC; E33M51 – E+AAG / M+CCA; E31M48 – E+AAA / M+CAC. The name of the primer combinations employed are followed by a number ''x'' on the map (see Fig. 2B) which corresponds to the x^{th} polymorphic band obtained by the same primer combination.

The degenerate primer designed for the steroid-binding motif of cytochrome P450 enzymes, and used as an AFLP primer, contained inosine (I) and had the sequence GC/GI GG/CI GTI GAI ACI ACI T/GCI.

Cloning of AFLP fragments and extended AFLP

Polymorphic AFLP fragments were cut out from the sequencing gel and boiled in 100 µl of distilled water for 10 min. From the reisolated DNA 10 µl was used in a 100-µl PCR reaction. Amplification was carried out by using the same conditions and primers as for the original AFLP reaction. The amplified DNA was run on an agarose gel and the AFLP fragment was excised, purified and cloned into a pBluescript SK (Stratagene) vector prepared for direct PCR cloning (Marchuk et al. 1990). Three independent clones from each AFLP marker were sequenced using an Applied Biosynthesis 373 DNA sequencer. To verify that the cloned DNAs and the AFLP markers were identical we performed an extended AFLP reaction where the AFLP primers contained three additional bases from the cloned and sequenced AFLP markers. The AFLP was carried out as previously described with the only exception that the pre-amplification was done with the selective AFLP primers used to identify the polymorphic AFLP fragments.

RFLP analysis

To screen for polymorphism, restriction enzyme digestions of 10 µg of genomic DNA were performed. Restriction fragments were separated on a 0.8% TAE-buffered agarose gel and transferred to a Hybond N+ membrane (Amersham). DNA probes TG14, TG17, TG23, TG25, TG42, TG44, TG46, TG47, TG65, TG128, TG367 (Tanksley et al. 1992), CP45, CP46 and CP108 (Gebhardt et al. 1991) were radiolabelled with α-[^{32}P]dATP by the random prime hexamer method. Hybridisation was done at 65°C in 10% dextran sulphate, 1% SDS, 1 M NaCl, 50 mM Tris-HCl (pH 7.5), and 40 µg ml^{-1} of denatured salmon sperm DNA. Filters were washed in 2 × SSC at 65°C for 5 min followed by a washing step in 2 × SSC, 1% SDS at 65°C for 20 min.

RAPD analysis

PCR reactions were carried out for RAPD analysis as described by Ronning et al. (1999) using the primer TCAGCCAGCG which is suitable for amplification of the RAPD marker UBC370–1500.

Mapping

The mapping populations of diploid potatoes were the progenies of the C × E (van Eck et al. 1994) and the SH × RH crosses (Rouppe van der Voort et al. 1997) characterised previously by RFLP and AFLP markers.

Linkage analysis of pairwise recombination frequencies between AFLP markers and the gene at issue was performed by the computer program JoinMap 2.0 (Stam 1993). For map-construction recombination frequencies were converted into map units (cM) by the use of the Kosambi function. Markers with LOD >3.0 were assigned to the linkage group. The order of the characterised markers was confirmed by colormapping (Kiss et al. 1998).

Results

Rapid test for glycoalkaloid and alkaloid detection

To study the inheritance of leptinine and leptine production a rapid simple chromatography assay was developed. Based on the method of Jellema et al. (1982) for the detection of solanine and chaconine in *S. tuberosum*, total glycoalkaloids of the three selected *S. chacoense* lines H1, A6 and A15 (see Materials and methods) were isolated, separated on TLC plates and stained with the fluorescent dye Blankophor (Fig. 1A). This experiment resulted in the identification of leptinines and leptines on the plate, which was confirmed by re-isolating the glycoalkaloids from the bands and analysing them by HPLC (data not shown).

The glycoalkaloid analysis of segregating populations derived from crosses was supported by analysing the corresponding alkaloids. Glycoalkaloids were hydrolysed and the resulted aglycons, solanidine from solanine and chaconine, hydroxy-solanidine from leptinine, and acetyl-solanidine from leptine, were separated on TLC plates and stained with iodine (Fig. 1B). The sensitivity of both glycoalkaloid- and alkaloid-analysis was approximately 0.5 µg of solanine, chaconine or solanidine per lane. This sensitivity is comparable to the sensitivity of HPLC analysis, although it is not so quantitative.

S. chacoense populations segregating for leptinine production

Inheritance of leptinine and leptine production was tested in the *S. chacoense* F_1 populations H1 × A15 and H1 × A6. Even though the parental lines A15 and A6 were different in respect of leptine production, i.e. A15 synthesised leptine while A6 did not, the F_1 populations of the H1 × A15 and H1 × A6 crosses segregated in the same way (see Materials and methods for details). None of the F_1 plants expressed leptine, while both F_1 families segregated 1:1 for solanine/ chaconine : leptinine

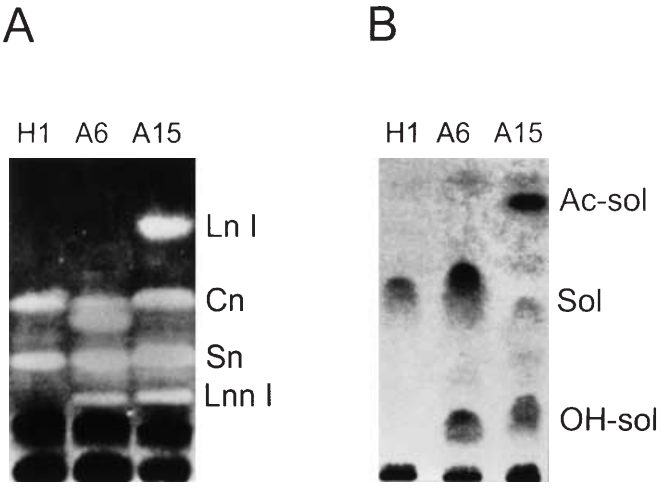

Fig. 1 Glycoalkaloids (**A**) and alkaloids (**B**) detected in the parental *S. chacoense* lines H1, A6, and A15. *Ln I* leptine I; *Cn* chaconine; *Sn* solanine; *Lnn I* leptinine I; *Ac-sol* acetyl-solanidine; *Sol* solanidine; *OH-sol* hydroxy-solanidine. *LnI* and *LnnI* have the same sugar content as *Cn*. *LnII* and *LnnII*, the derivatives of *Sn*, were not detectable in the extracts. The double band at *Cn* in the line A6 is a partial degradation product of *Cn*

(SOH^- : SOH^+ phenotype). Since the two progenies showed the same segregation ratio for leptinine production only one of them, the H1 × A15 population with the larger seed number, was used in further experiments. Based on the assumption that the aglycon of leptinine is a hydroxylated derivative of the alkaloid solanidine (Osman et al. 1987), the locus involved in leptinine production and segregating in the H1 × A15 population was designated as *soh*.

Mapping of the AFLP markers associated with the *soh* locus

To identify AFLP markers linked to the *soh* locus, we first carried out bulk segregant analysis with two pools of DNA samples, each consisting of eight individual plants with a SOH^+ and SOH^- phenotype, respectively. The primer combinations which produced DNA fragments that were polymorphic between the parental lines, as well as between the SOH^+ and SOH^- pools, were tested on a total of 70 individual plants, 37 with SOH^+ and 33 with SOH^- phenotypes. Out of 73 primer combinations seven primer pairs produced a total of 11 AFLP markers that showed co-inheritance with the *soh* locus (Fig. 2B). All the identified co-segregating markers were mapped on one side of the *soh* locus. The closest AFLP marker was located at 4.2 cM (LOD = 3.1) while the farthest AFLP marker in the linkage group was at 40.7 cM (LOD = 6.9). Two AFLP markers, E39M47–2 and E45M47–2, showed co-inheritance with the absence of leptinine in the segregating population and may be repulsion phase markers for the *soh* locus.

The hydroxy solanidine has been assumed to be synthesised by a cytochrome P450 enzyme (Osman et al.

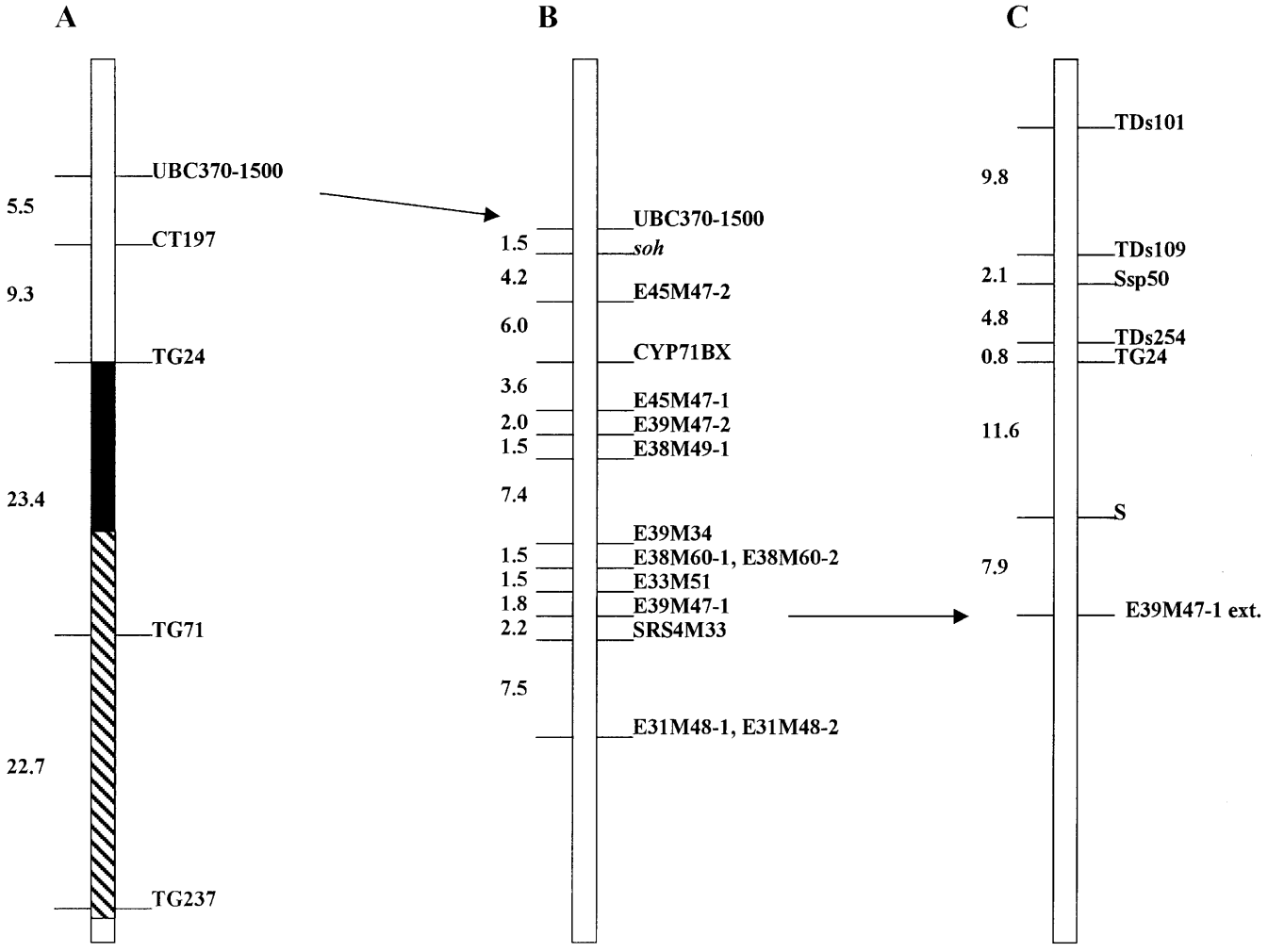

Fig. 2 **A** Map of chromosome 1 of the potato containing a RAPD marker (UBC370–1500) linked to leptine production (Ronning et al. 1999) and a major QTL involved in solanidine synthesis (Vos et al. 1995). *Solid box* $P < 0.0001$, *cross-hatch* $P < 0.0100$ significance levels. **B** Molecular markers associated with the *soh* locus in the H1 × A15 *S. chacoense* cross. **C** Map position of the E39M47–1ext., the extended AFLP marker on chromosome 1 of the C × E diploid *S. tuberosum* mapping population. Markers on the right and distances between the markers in cM on the left, are indicated. *Arrows* show the identical markers in different crosses

1987) and cytochrome P450 enzymes are known to be involved in the biosynthesis of alkaloids. Therefore, based on the sequence of known plant (Szekeres et al. 1996) and mammalian steroid-hydroxylases (Su et al. 1990), a degenerate primer was designed for the SRS4 steroid-binding motif. This primer was used as an AFLP primer in the standard AFLP reaction combined with 26 different *Mse*I primers. Out of these 26 primer combinations, one, SRS4M33, showed polymorphism in the parental lines A15 and H1 and was co-inherited with the *soh* locus in the H1 × A15 mapping population. This marker is located at 33.2 cM (LOD = 3.1) from the *soh* locus (Fig. 2B).

RFLP analysis

In a previous study we have isolated cytochrome P450 genes with unknown function belonging to the CYP71D subfamily (Hutvágner et al. 1997). To investigate whether this type of P450s cytochrome can be involved in hydroxy solanidine synthesis, the *Bgl*II/*Xho*I fragment of the clone CYP71D6 was used as a hybridisation probe to the DNAs of the H1 × A15 progenies. In this way, a co-segregating repulsion RFLP marker, designated CYP71BX in Fig. 2B, was detected which is located at a distance of 10.2 cM (LOD = 3.5) from the *soh* locus.

Position of the *soh* locus on potato chromosome maps

Chromosome maps of *S. tuberosum* are based on RFLP and RAPD markers (Tanksley et al. 1992). Identification of the linkage group carrying the *soh* locus may facilitate the mapping experiments by using described RFLP and RAPD markers in the vicinity of the *soh* locus. Therefore, 14 RFLP markers which mapped onto chromosomes 1,2,3,4,5,6,7 and 11 in *S. tuberosum* were tested in hybridisation to the DNAs of the H1 and A15 parental lines. Unfortunately, however, neither of them showed polymorphism between the two lines.

Fig. 3 Comparison of the sequence of the E39M47–1 AFLP marker derived from H × A15 *S. chacoense* with the sequence of the extended AFLP marker E39M47–1ext. that showed polymorphism in a C × E *S. tuberosum* mapping population. PCR primers used in the AFLP reaction are boxed. * = reverse complementary sequence

Another way to fit our data onto the established potato map is to map the markers co-inheriting with the *soh* locus in a potato population for which a RFLP map is available. For this reason four AFLP markers, SRS4M33, E39M47–1, E38M60–1 and E38M60–2, were isolated, cloned, sequenced, and used as CAPS, SCARs, as well as extended AFLP markers in the *S. tuberosum* mapping populations C × E (van Eck et al. 1994) and SH × RH (Rouppe van der Voort et al. 1997). One of the primers, E39M47–1, located at 29.5 cM (LOD = 3.4) from the *soh* locus in *S. chacoense* showed polymorphism in the *S. tuberosum* C × E population when it was used as an extended AFLP primer. This polymorphism co-inherited with the markers of linkage group 1 suggesting that the *soh* locus is located at the end of the short arm of chromosome 1 (Fig. 2C).

To prove that the AFLP marker which was detected in the *S. chacoense* population and the extended AFLP marker which was used to identify the linkage group are identical, we isolated and sequenced the extended AFLP marker from *S. tuberosum* and compared it to the sequence of the E39M47–1 AFLP marker isolated from *S. chacoense*. This comparison revealed a single base deletion and one nucleotide difference between the two markers (Fig. 3). This finding supports the reliability of our mapping data.

Mapping the UBC370–1500 RAPD marker

Ronning et al. (1999) published a RAPD marker which co-segregated with nil/ low leptine production in *S. chacoense* and was mapped to the end of the short arm of chromosome 1 of potato (Fig. 2A). Amplifying the UBC370–1500 RAPD marker in the progenies of the H1 × A15 *S. chacoense* cross we could detect the same, approximately 1500-bp, fragment that was mostly present in the individuals producing nil/ low leptinine. This RAPD marker was located at the other side of the *soh* locus to the AFLP and RFLP markers and at a distance of 1.5 cM (LOD = 6.8; Fig. 2B).

Discussion

In this study AFLP and RFLP markers were identified and shown to be co-segregating with the *soh* locus involved in leptinine biosynthesis in *S. chacoense*.

Leptinines are thought to be intermediates in the biosynthetic pathway between solanine/ chaconine and leptines (Sinden et al. 1988). Since commercial varieties of *S. tuberosum* produce only solanine/ chaconine, the isolation of genes responsible for both hydroxylation and acetylation will be necessary in order to obtain leptine-producing CPB-resistant transgenic potato cultivars by molecular breeding. Therefore, genetic studies on leptinine production may be as important as those on leptine synthesis.

In our experiments F_1 populations of two different *S. chacoense* crosses were analysed. The high-leptine and high-leptinine producing parents were derived from the same accession PI 320287 while the nil/ low producer was from PI 320294. Both progenies segregated in a similar way producing no plants expressing leptine, but segregating 1:1 for solanine/ chaconine : leptinine. This segregation strongly suggests that the inheritance of the ability for leptinine production in *S. chacoense* is controlled by a single gene or closely linked genes. Since the genotype of the parental lines are unknown, further crosses are required to determine whether the locus involved in leptinine production, the *soh* locus, contains a dominant or a recessive gene(s). Due to the detection limit of the method we used to analyse the phenotype of the progenies derived from the *S. chacoense* crosses, the possibility that the plants with a SOH^- phenotype produce a small amount of leptinines can not be excluded. Thus, we do not know whether we followed the segregation of a gene coding for an enzyme responsible for the hydroxylation of solanidine or that of a gene(s) with a large effect on the quantity of leptinine production.

The P450s cytochrome are a group of membrane-bound heme-containing proteins that catalyse a range of oxidative reactions in both prokaryotic and eukaryotic cells. They have an important role in steroid and alkaloid biosynthesis, and leptinines are assumed to be synthesised by a P450 cytochrome as well (Osman et al. 1987). This study revealed a marker (SRS4M33) linked to the *soh* locus when one of the original AFLP primers was replaced by an oligo designed for the steroid-binding motif of the P450s cytochrome catalysing steroid hydroxylations. Using a previously described *S. chacoense* cytochrome P450 (Hutvágner et al. 1997) fragment as a hybridisation probe, a repulsion RFLP marker was detected located at 10.2 cM from the *soh* locus. Although, P450s cytochrome can be arranged in the genome in clusters (Lin et al. 1999) the genetic distance of these two markers from the *soh* locus (Fig. 2B) excludes the possibility that they would mark a cytochrome P450 cluster containing the gene(s) responsible for the hydroxylation.

All the AFLP and RFLP markers identified belong to the same linkage group and are situated on one side of the *soh* locus. These data support the assumption that leptinine production is affected by one gene or closely linked

genes, and suggest that the *soh* locus is located at the end of one of the chromosomes. An extended AFLP marker linked to the *soh* locus showed polymorphism in the *S. tuberosum* C × E mapping population (van Eck et al. 1994) and was co-inherited with the markers of linkage group 1 (Jacobs et al. 1995), suggesting that the *soh* locus is located at the end of the short arm of chromosome 1. A major QTL for solanidine accumulation (Yencho et al. 1998) and two molecular markers, including UBC370–1500, that are closely linked to high-percent solanine/ chaconine and, conversely, to nil/ low-percent leptine (Ronning et al. 1999), have also been mapped to this region of chromosome 1 in the potato (Fig. 2A). These results, together with our finding, indicate that this part of the potato genome has a large effect on the accumulation of different steroid alkaloids. The RAPD marker UBC370–1500 had a recombination value of 3% in a *S. chacoense* F_1 progeny segregating for leptine production (Ronning et al. 1999). Since the same molecular marker had a recombination value of 11% in our mapping population it is fairly likely that the loci involved in leptine and leptinine production are tightly linked in *S. chacoense*.

The proposed biosynthetic pathway leading from cholesterol to solanidine and from solanidine to leptine assumes several intermediates; however, no genes or enzymes have yet been characterised that might be involved in these reactions. Fine-mapping and detailed analyses of this chromosome region would help to understand the synthesis of steroid alkaloids and would reveal more molecular markers assigned to the genes affecting leptine and leptinine synthesis, thereby facilitating the introgression of these traits into cultivated potato varieties.

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