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ORIGINAL PAPER

Linkage analysis of a rare alkaloid present in a tetraploid potato with *Solanum chacoense* background

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Abstract The potato genotype ND4382-19 has *Solanum chacoense* Bitt. in its genetic background. Foliar alkaloid analysis of it and its progeny ND5873 (ND4382-19 × Chipeta) by gas chromatography–mass spectrometry (GC–MS) showed that, in addition to the expected alkaloids (solanidine, leptinidine, and acetyl-leptinidine), there was an aglycone of another rare alkaloid. Its molecular mass and some of the *m/z* fragment ions were similar to leptinidine, but the major fragment ion was the *m/z* 150 peak of solanidine. This fragmentation pattern suggested that this alkaloid is a solanidine-based compound with mass equal to leptinidine. Leptinidine differs from solanidine by an extra –OH group, but the GC–MS fragmentation pattern of the rare compound indicated hydroxylation at a different position than the C-23 of leptinidine. The exact chemical structure is still unknown, and further analysis, such as NMR will be necessary to determine the structure. Segregation analysis of ND5873 (ND4382-19 × Chipeta) showed that presence of this rare compound segregated in a 1:1 ratio, indicating that a single gene controlled its synthesis and/or accumulation in foliar tissue. Analysis with AFLP and microsatellite markers indicated that the locus-controlling presence of this alkaloid resided on potato chromosome I, with the nearest flanking AFLP markers 0.6 and 9.4 cM apart. This rare alkaloid was present in the foliage and not detected in potato tubers. Its presence in leaves did not affect resistance/susceptibility to Colorado potato beetle.

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Introduction

Many Solanaceae family members synthesize steroidal glycoalkaloids; to date there are over 90 described in the literature with diverse chemical structures (Friedman 2006; Friedman and McDonald 1997; Ginzberg et al. 2009; Ronning et al. 2000; Shakya and Navarre 2008). The solanaceous glycoalkaloids identified to date are composed of a C-27-steroidal alkamine attached to a sugar moiety usually comprise a di-, tri-, or tetrasaccharide (Friedman 2006; Friedman and McDonald 1997; Maga 1994). These glycoalkaloids are considered natural toxins, and they can cause animal death at high concentration (Hall 1992). This toxicity is a very important issue for cultivated species such as *Solanum melongena* (eggplant), *S. tuberosum* (potato), and *S. lycopersicum* (tomato). Glycoalkaloid quantity can vary widely among different species and cultivars, and expression is determined by both genetic and environmental factors (Friedman 2006; Friedman and McDonald 1997; Maga 1994).

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Most commercial potatoes contain α-chaconine and α-solanine which differ only in their sugar moiety and share the same steroidal aglycone, solanidine. At low concentrations, these glycoalkaloids may enhance flavor, but high concentrations cause a bitter taste and toxicity to consumers (Friedman and McDonald 1997; Maga 1994). In general, levels in excess of 20 mg/100 g fresh weight are not allowed for any potato cultivar (Valkonen et al. 1996). Glycoalkaloids may help to protect potato plants against pests (Tingey 1984). Leptines I and II, found in a few accessions of the wild species *S. chacoense*, are a potent factor for host-plant resistance to Colorado potato beetle (*Leptinotarsa decemlineata* Say)(Kuhn and Löw 1961; Sinden et al. 1986a; Stürckow and Löw 1961). Their introgression into commercial cultivars could reduce the use of insecticides. Leptines are considered to be low risk for human toxicity, because they are only found in shoot tissue and not in tubers (Sinden et al. 1986b; Stürckow and Löw 1961). However, Shakya and Navarre (2008) using LC–MS, a very sensitive technique, recently detected trace content of leptinines and leptinidines in tubers of *S. chacoense*, S. *bulbocastanum*, *S. stenotomun* and *S. spegazzini*.

Wild potato species have been utilized in breeding programs as sources of valuable genes. Wild Solanum species, such as *S. chacoense*, *S. phureja*, *S. spegazzinii*, *S. andigena*, *S. vernei*, and *S. berthaultii*, are species that are being used in breeding programs as sources of resistance genes to insects, nematodes, late blight, viruses and other pests (Bradshaw and Mackay 1994; Plaisted et al. 1994). Traits such as high protein content or low content of reducing sugars can be improved using wild germplasm. However, these desirable traits may be associated with high glycoalkaloid content and/or may transmit new glycoalkaloids to hybrid progeny (Valkonen et al. 1996). This situation can raise concerns about toxicity for humans or livestock (Hall 1992).

This project was commenced to utilize leptines from *S. chacoense* for resistance to Colorado potato beetle (Lorenzen et al. 2001). In this report we describe the coincidental discovery of a rare alkaloid aglycone present in an insect-resistant potato, ND4382-19, and its progeny, and its genetic mapping in a segregating tetraploid population. The inheritance and map location, possible structure, and plant distribution are discussed.

Materials and methods

The tetraploid population ND5873 was generated by crossing insect-resistant ND4382-19 (Lorenzen and Balbyshev 1997) × Chipeta (Holm and Pavek unpublished). Seeds were surface-sterilized and germinated in vitro, where they were maintained vegetatively as in vitro tissue culture plantlets until planting in the greenhouse to produce seed tubers for trials with three replicates.

Plant content of alkaloids was determined in both leaves and tubers, from the different replicates. Leaf samples were a composite of four fully expanded leaves from the upper third of each plant, taken at the pre- to early bloom stage. Tuber samples were from fully matured potato tubers maintained for 2 months after harvest at 4°C. In both cases, collected samples were immediately frozen in liquid nitrogen and lyophilized. Alkaloids were extracted according to the method described by Lawson et al. (1992), TMS-derivatized, and analyzed by GC–MS as described in Sagredo et al. (2006). Briefly, freeze-dried 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 on an HP5890 gas chromatography coupled to a Finnigan Mat Incos 50 quadrapole mass spectrometer with a 20 m × 0.18 mm × 0.20 um Restek RTX1 (100% methylsilicone) capillary column, set either on scan mode (50–650 amu/s) or on select ion mode (125, 150, 238, 452, 454, 465, 467, 469, and 472 amu/s). The solanidine standard was purchased from Sigma Inc. (St. Louis, MO, USA). Authentic standards of leptinidine and acetyl-leptinidine standards were provided by Ray Miller, Ohio State University. Peak height was compared with the known standards to estimate alkaloid aglycone concentrations.

Linkage analysis of AFLP segregant markers was done according to Hackett et al. (1998), assuming random pairing and chromosome assortment during meiosis as described in Sagredo et al. (2006).

Resistance to Colorado potato beetle was determined by planting replicated trials at three sites with four replications per site. Defoliation was estimated by visual observation at 10 days intervals.

Results

GC–mass spectrum and glycoalkaloid identification

ND4382-19 was expected to contain three types of alkaloids, solanidine, leptinidine, and acetyl-leptinidine, normal steroidal aglycones found in a leptine-expressing potato (Lawson et al. 1997). GC–MS analysis of foliar content of alkaloid aglycones in ND4389-19 showed these

three expected compounds as well as tomatidenol, a spirosolane found in the "species backcross" parent, Norchip (Sagredo et al. 2006). There was an additional peak that did not correspond to known alkaloid compounds. Figure 1 shows the GC–MS and chemical structures for solanidine (a), leptinidine (b), and acetyl-leptinidine (c). In Fig. 1d the GC–MS spectrum of the rare compound and its probable chemical structures. The molecular mass of the TMS-derivatized unknown compound (*m/z* 557) and one of its larger fragment ions (*m/z* 542) was identical to that of leptinidine (Fig. 1 b, d), but smaller *m/z* fragments ions were similar to solanidine, at *m/z* 178, 150, 98, and 73. This pattern suggested that the molecular formula of the unknown is equal to leptinidine, which differs from solanidine by an –OH group. A free hydroxyl group can attach one trimethylsilyl (TMS) molecule when derivatized by BSTFA. However, the fragmentation pattern of this compound was different from leptinidine. The *m/z* 150 and 204 fragment ions are from the EF and DEF rings (Fig. 2a), respectively (Lawson et al. 1997). GC–MS of the unknown compound and possible locations of the hydroxyl group suggested by the mass increase and the known chemical structure are shown in Fig. 1d. Presence of the *m/z* 150 fragment ion precluded C-23 hydroxylation as for leptinidine (Fig. 1 b, d). However, loss of the *m/z* 204 fragment of solanidine could suggest hydroxylation at one of the D ring carbon atoms as indicated by Fig. 1d. The GC retention time for the rare alkaloid aglycone was delayed relative to solanidine. Figure 2b shows the selective window for *m/z* 150 and the respective retention time for TMS-derivatized solanidine and the rare compound. Assuming that this compound has a similar signal:mass ratio for peak *m/z* 150, this novel alkaloid is more abundant than solanidine in the foliage of parent ND4382-19 (Table 1).

Inheritance of the unknown compound

ND4382-19 was crossed with Chipeta, a genotype that contains only glycoalkaloids derived from solanidine (Table 1), producing the tetraploid progeny ND5873. Foliar glycoalkaloid content of 93 progeny genotypes was determined by GC–MS analysis. Solanidine was present in all genotypes and presented a continuous distribution for the 93 individuals (data not shown). The rare compound was present in 46 of 93 individuals but absent from the other 47 plants, consistent with a 1:1 ratio for a single copy of a dominant gene and suggestive of a single locus-controlling presence of this alkaloid (Table 2). Linkage between this locus and loci-enabling leptinidine and acetyl-leptinidine synthesis was not detected by χ^2 analysis. For the subset that contained the rare compound, concentrations of this compound were roughly equivalent to solanidine concentrations (Table 2). Solanidine concentrations ranged from 0.23 to 2.46 mg g^{-1} of dry weight, with an average of 1.63 mg g^{-1} (SD = 0.51), and concentrations of the rare compound for the subset that possessed it range from 0.09 to 3.20 mg g^{-1}, with a mean of 1.55 mg g^{-1} (SD = 0.73). There was a highly significant correlation between the concentration of solanidine and the rare compound ($r = 0.61$, $p < 0.01$) for that subset of genotypes that contained this rare alkaloid.

Map location of the rare compound

Molecular maps for ND4382-19 and Chipeta were constructed in the ND5873 population using dominant (AFLP) and codominant (simple sequence repeat, SSR) markers (Sagredo et al. 2006). The unknown alkaloid aglycone was also included as a binary phenotypic marker (±) to identify its map location. The rare alkaloid showed coupling linkage with linkage group R41 that contained 17 AFLP markers and covered a total of 64.4 cM. This linkage group included an allele of STM2030 (Chromosome I) and was linked by duplex–simplex linkage to two alleles of STM2020 (Chromosome I) located on linkage groups R15 and R46. Linkage group R15 also contained STM1029 (Chromosome I). These simplex-coupling groups also shared several common duplex AFLP markers in simplex–duplex linkage with the linkage group R7, further supporting their identification as the four homologs of chromosome I from the ND4382-19 parent. Therefore, the locus that enables synthesis and/or accumulation of this rare compound resides on chromosome I. The overall map of Chromosome I is shown in Fig. 3, where the locus for presence of the rare compound in the upper portion of Chromosome I was flanked by the AFLP markers E-ACA/M-CACR12 and E-ACA/M-CACR3 at 0.6 and 9.4 cM, respectively.

The rare compound was not detected in tubers

Tubers from a subset of population ND5873 were evaluated for presence of the rare compound using GC–MS analysis. The rare compound was not detected in any of these tubers. Of the subset of 54 genotypes analyzed for tuber alkaloids, 28 genotypes contained the rare compound in leaves but not in tubers.

The rare compound is not involved in CPB resistance

The leptine-containing genotype, ND4382-19, is highly resistant to the Colorado potato beetle. This resistance was inherited from *S. chacoense* Bitter and has been associated with the presence of leptines (Lorenzen et al. 2001; Tian 1998). Recently, Sagredo et al. (2009) found

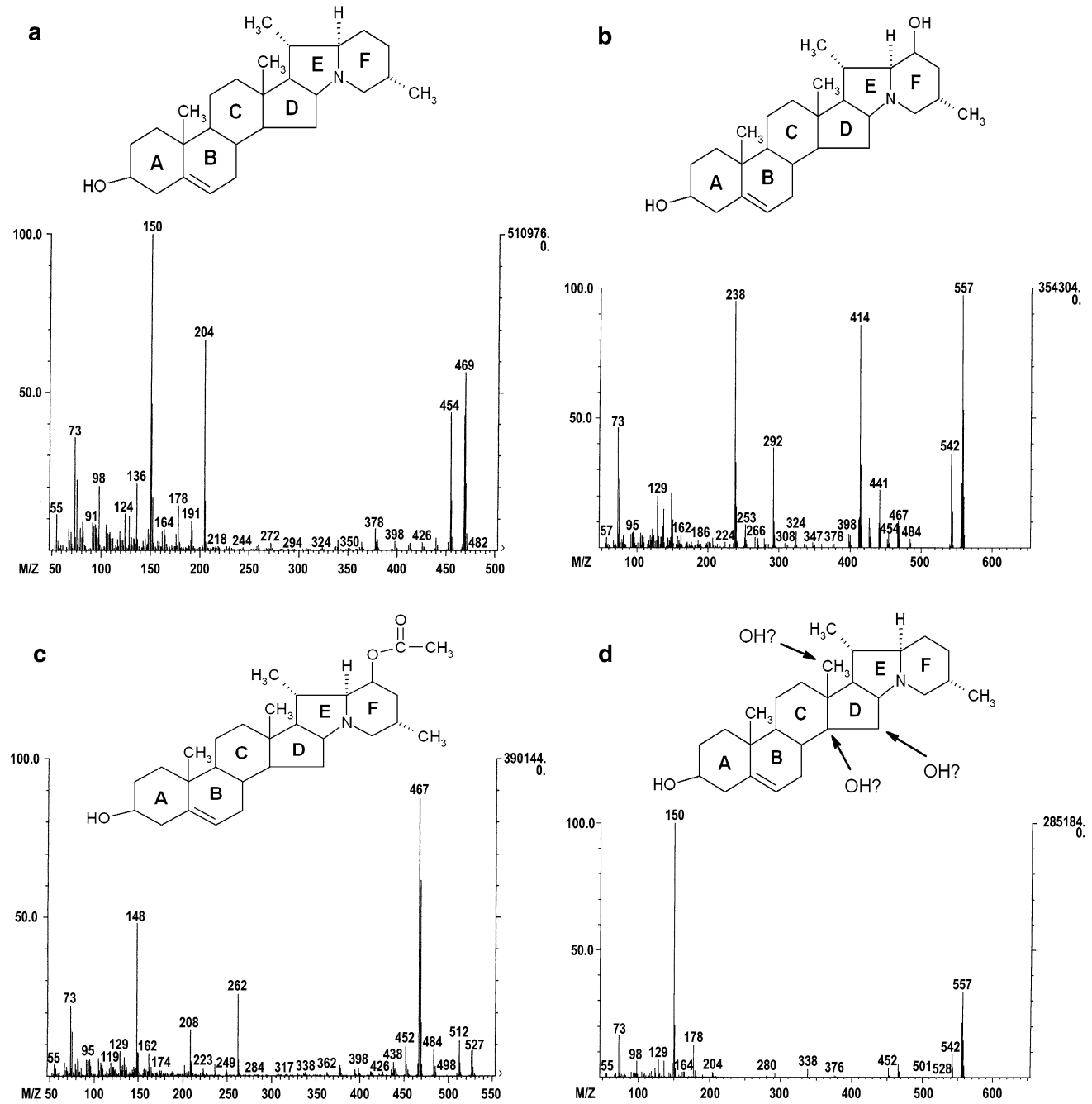

Fig. 1 GC–mass spectrum of TMS-derivatized samples of the known and rare aglycones. The figure shows the GC–mass spectrum for **a** solanidine, **b** leptinidine, **c** acetyl-leptinidine, **d** rare aglycone group (d); and their respective chemical structures for the known aglycone groups. **d** The *arrows* indicated the possible sites of hydroxylation based on a solanidine backbone for the rare aglycone group

that resistance was mainly explained by a QTL that resides on Chromosome 2. Population ND5873 (ND4382-19 × Chipeta) was evaluated for CPB resistance in three field environments during 1997. Single linear regression analysis showed that the rare compound was not associated with CPB resistance (R^2 for three environments: 0.02, 0.00, 0.01 ns).

Discussion

The GC–MS analysis of genotype ND4382-19 contained the expected alkaloid aglycones: solanidine, leptinidine, acetyl-leptinidine, and tomatidenol. However, a previously unknown alkaloid aglycone was present at a concentration similar to that of solanidine. Its molecular mass was

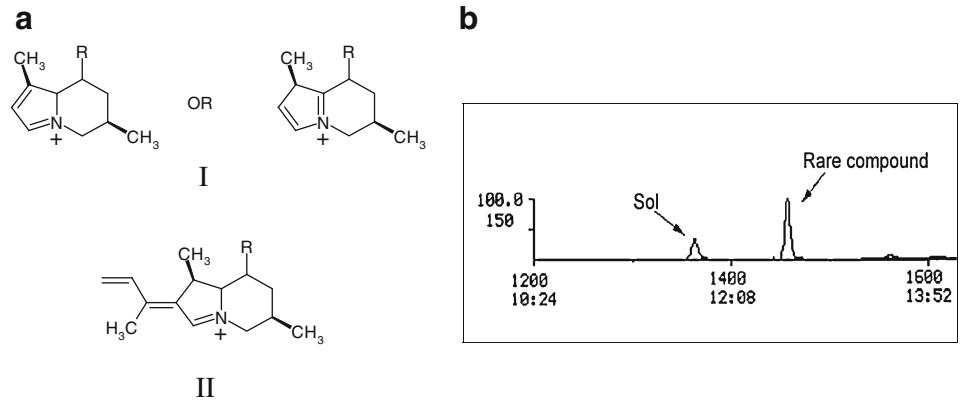

Fig. 2 Diagnostic fragment ions from GC–MS. **a** Correspond to the diagnostic fragment ions of solanidine, leptinidine, and acetyl-leptinidine (from Lawson et al. 1997). *Parentheses* indicate expected *m/z* for TMS-derivatives. **b** Retention time relative to solanidine. The chromatogram shows the GC–MS selective window for *m/z* 150

Alkaloid	Structure	Mass	Fragment Formula
Solanidine	R= H	I = 150 II= 204	$C_{10}H_{16}N$ $C_{14}H_{22}N$
Leptinidine	R= OH	I =166 (238) II = 220 (292)	$C_{10}H_{16}NO$ $C_{14}H_{22}NO$
Acetyl-leptinidine	R=OC(O)CH_3	I= 208 II= 262	$C_{12}H_{18}NO_2$ $C_{16}H_{24}NO_2$

Table 1 Foliar alkaloid content in the parents of family ND5873, as determined by GC–MS

Parent	Alkaloid (mg g DW^{-1})			
	Solanidine	Rare compound	Leptinidine	Acetyl-leptinidine
ND4382-19	0.64	1.61	1.40	0.69
Chipeta	0.32	nd	nd	nd

nd Not detected

Table 2 Segregation of alkaloids in family ND5873

Aglycone	Absent	Present			
	No. of plants	No. of plants	Mean (mg g DW^{-1})	Range (mg g DW^{-1})	SD
Solanidine	0	93	1.63	0.23–2.46	0.51
Rare compound	47	46	1.55	0.09–3.20	0.73

Foliar concentrations determined by GC–MS are expressed as mg g DW^{-1}

identical to leptinidine and it shared some larger *m/z* fragment ions with leptinidine, but the major fragment ion was the *m/z* 150 peak of solanidine, rather than the *m/z* 238 and 292 peaks of derivatized leptinidine. This suggests that it is a solanidine-based alkaloid with a mass equal to leptinidine. Leptinidine is formed by the hydroxylation of solanidine at the C-23 position. The GC–mass fragmentation pattern of this rare compound suggested hydroxylation at a different position than the C-23 of leptinidine. Analysis of the MS fragmentation pattern suggests that the site of hydroxylation could be C-14, C-15, or C-18. The exact chemical structure is still unknown, and further analysis by NMR will be necessary to determine the structure. Ronning et al. (2000) characterized 15 different accessions of *S. chacoense* from various locations and altitudes of South America and detected nine novel putative glycoalkaloids. However, the possibility of identity matching between the unknown alkaloid described here with the nine described by Ronning et al. (2000) is not yet possible because structural information for those compounds is lacking. Interestingly, Shakya and Navarre (2008), using LC–MS analysis, found several solanidine-like compounds with an additional oxygen that differed from leptinine in tubers of four different Solanum species. Whether those compounds coincide with the glycoalkaloid described here is difficult to establish due to the differences between ion fragmentations methods used.

Segregation analysis of progeny, ND5873 (ND4382-19 × Chipeta), showed that this rare compound segregated in a 1:1 ratio, indicating it is controlled by a single gene. The same type of segregation was observed in other progeny of ND4382 (ND4382-19 × N142-72; unpublished data). In the subset of family ND5873 that contained this compound, concentrations were highly correlated with solanidine concentrations, suggesting that this compound is subject to the same control mechanism(s) as solanidine. One important step toward structural identification of the unknown rare compound will be its purification, which has been difficult because of product degradation (unpublished results). If this compound is similar to other alkaloids, glycosylated forms should exist and could be analyzed by

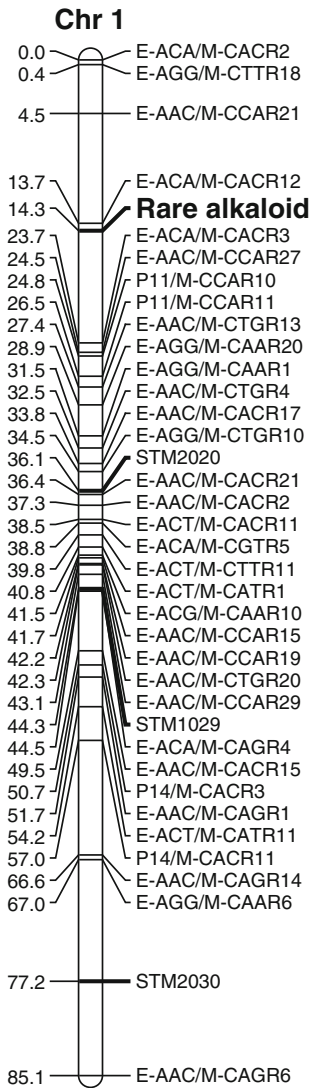

Fig. 3 Alignment of AFLP/SSR chromosome I map for parent ND4382-19 of population ND5873. SSR markers STM2030, STM2020, and STM1029 are specific for chromosome I

HPLC in reverse phase with C_{18} columns. However, we were unable to recognize an HPLC peak that corresponded with this compound in chromatograms of glycoalkaloids from ND4382-19 and its progeny (unpublished results).

The genome of genotype ND4382-19 has been partially mapped with 320 simplex AFLP markers (Sagredo et al. 2006; Sagredo et al. 2009). By considering presence or absence of the unknown alkaloid as a qualitative marker, and conducting linkage analysis with both AFLP and SSR markers (Milbourne et al. 1998), including duplex–simplex linkage analysis (Hackett et al. 1998), we were able to map it to a linkage group that represented a Chromosome I (Fig. 3). Chromosome I has been identified as having significant effects on the concentration of solanidine (Yencho et al. 1998), α-solanine, α-chaconine and TGA (Sørensen et al. 2008) and leptines (Ronning et al. 1998). The exact location of this locus relative to the loci reported to affect solanidine, α-solanine, α-chaconine, TGA and leptine synthesis in those studies cannot be determined, due to the lack of common markers across populations. However, using relative map location of the SSR markers used (Milbourne et al. 1998), this locus appears to be near the QTL for high solanidine reported by Yencho et al. (1998). The *soh* locus reported by Hutvágner et al. (2001) may be distal to this locus, since *soh* was distal to the Yencho et al. (1998) QTL and near that of the leptine locus reported by Ronning et al. (1999) and Hutvágner et al. (2001). In the case of the QTL described by Sørensen et al. (2008), which is nearby the common marker STM2030, it is clearly on the opposite end of the chromosome I. The loci involved in those studies (Yencho et al. 1998; Hutvágner et al. 2001; Ronning et al. 1999; Sorensen et al. 2008) appeared to have quantitative effects. Interestingly, Bouarte-Medina et al. (2002), using reciprocal crosses and diploid populations, identified a cytoplasmic influence on both the synthesis and level of expression of leptine glycoalkloids. The qualitative differences in this rare compound could be consistent with a locus corresponding to either a structural gene (e.g., P450 monooxygenase as a likely functional enzyme for adding the –OH group) or a regulatory gene, as mentioned by Yencho et al. (1998). P450 hydroxylases have been mapped to chromosome I (Hutvagner et al. 2001, Lorenzen et al. unpublished). The nearest flanking AFLP markers E-ACA/M-CACR12 and E-ACA/M-CACR3 were 0.6 and 9.4 cM distant from this locus, respectively. Therefore, although we were unable to purify the compound, the precision of mapping makes it unlikely that this "new rare alkaloid" represents an artifact. The recent release of the genome sequence for potato (http://www.potatogenome.net) and consequent ability to examine nearby genomic sections for candidate genes such as regulatory or structural genes involved in alkaloid biosynthesis should facilitate the identification of the genes that control the accumulation of this rare alkaloid on chromosome I. Recently, Feingold et al. (2010) reported at least 25 unique putative P-450 monoxygenase genes on chromosome I.

Analysis of tuber alkaloids from 54 genotypes of population ND5873 did not detect this compound in tubers, nor was leptinidine or acetyl-leptinidine detected in tubers. Similar organ specificity has been previously shown for leptinine and leptines (Sinden et al. 1986b). Therefore, localization of the new alkaloid in foliage but not tubers is similar to that of other novel alkaloids from *S. chacoense*, but not the solanidine-based glycoalkaloids solanine or chaconine. However, considering the results of Shakya and Navarre (2008), who found a significant diversity of traces of glycoalkaloids in potato tubers from different Solanum species, using a very sensitive technique as LC–MS, it is not possible to exclude that this compound is expressed in tubers.

In a separate experiment, leaves were harvested from a set of ND5873 plants grown in a controlled environment chamber with a photo flux density of approximately 400 μmol m^{-2} s^{-1}. The levels of the unknown compound in this experiment were undetectable by GC–MS, suggesting that high light is essential for its foliar accumulation. The level of full sun under field conditions can range up to 1,800 μmol m^{-2} s^{-1}. Light flux has been shown to be an important factor in determining content of other alkaloids (Deahl et al. 1991; Lafta and Lorenzen 2000). The tubers tested had not been exposed to light-induced greening, which may have affected our failure to detect this compound in tubers. Additionally, physiological experiments would be useful to characterize its expression. Although toxicity information for this compound is not available, its absence—at least the failure to detect by GC–MS—from non-green tubers should minimize potential concern with regard to human toxicity. It would be possible to use closely linked markers for positive or negative selection in a breeding program.

Glycoalkaloids have a wide range of bio-active effects (Maga 1994; Tingey 1984). We did not detect association of the rare compound to defoliation by Colorado potato beetle. However, it is possible that this compound affects other biological interactions and it may be useful to challenge this segregating population with other pests and diseases to determine effects on those organisms.

Glycoalkaloids can also be used for synthesis of steroidal drugs (Cham 1994). Certain types of glycoalkaloids, such as solasonine and solamargine, which are found in certain Solanum species, are used by the pharmaceutical industry to produce steroidal drugs (Balandrin et al. 1985; Cham 1994). Some glycoalkaloids present anticancer properties and are commercially produced (Cham 1994). This new steroidal compound from ND4382-19, or derivatives there of, may be of interest for pharmaceutical properties. If so, the identification and mapping of the gene in this population could be a useful first step in a map-based cloning effort. The cloning of such foliage-specific genes would also give useful information about their molecular control and expression in plant tissues. This would enhance our knowledge about glycoalkaloid synthesis and the specificity of the respective enzymes. Krits et al. (2007) studying the expression of genes involved in the steroidal glycoalkaloid pathway, correlated the level of potato glycoalkaloids with the expression of genes that participate in the isoprenoid synthesis route. Ginzberg et al. (2009) propose a genetic manipulation approach for potato glycoalkaloids, to reduce the risk of toxicity for consumers and increase their potential in plant defense. In addition, the isolation of genes coding for or allowing translation of these types of enzymes might allow us to modify alkaloids to facilitate the synthesis of some already known products or to create new ones with novel pharmaceutical properties.

In conclusion, we have presented physical and genetic information for a rare alkaloid probably derived from *S. chacoense*. The molecular weight and fragmentation pattern suggests that it is a hydroxylated solanidine distinct from leptinidine. Genetic control enabling synthesis/accumulation of this compound resides on chromosome I, which has been previously shown to have loci important in determining content of potato glycoalkaloids.

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