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Identification of a new QTL associated to reduced quinolizidine alkaloid content in white lupin (*Lupinus albus*, L.) and development of ultra-low alkaloid recombinants by stacking with the *pauper* allele

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Abstract

Background White lupin (*Lupinus albus*, L.) is a grain legume cultivated for its high agronomic and nutritional potential, despite the accumulation of bitter and potentially harmful to health quinolizidine alkaloids (QA) in the grain. Modern sweet (i.e. low alkaloid content) varieties exist, which are exploiting different recessive mutations responsible for the desired low QA chemotype. The most widely used QA-reducing determinant, *pauper*, has been recently identified enabling marker-assisted selection, but unstable QA content across growing seasons and environments remain a challenge in white lupin cultivation.

Results Through Bulked Segregant Analysis of an F_2 population, segregating for two different low QA conferring loci, we identified a novel QTL spanning a 1 Mbp region on chromosome 5, a novel source of sweetness apart from *pauper*. We present marker-trait associations for the new locus tagging low QA content in white lupin within this QTL, described in F_2 generation and validated in F_3. Together with genotyping of sweetness tagging *pauper* sweet individuals, we identified, in F_3, 50 stacked allele recombinants where the low QA chemotype is further reduced. These individuals exhibit an exceptionally low total alkaloid content (22.8 ± 10.4 ppm), even when compared to genotypes known to carry the allele associated to the most drastic reduction in QAs, *pauper* (171.7 ± 18.5 ppm).

Conclusion The discovery of this novel locus and the development of associated PACE markers, potentially applicable for marker-assisted selection together with *pauper* (especially after farther validation in a larger panel of accessions), can enhance the improvement of white lupin through the development of new varieties with very low

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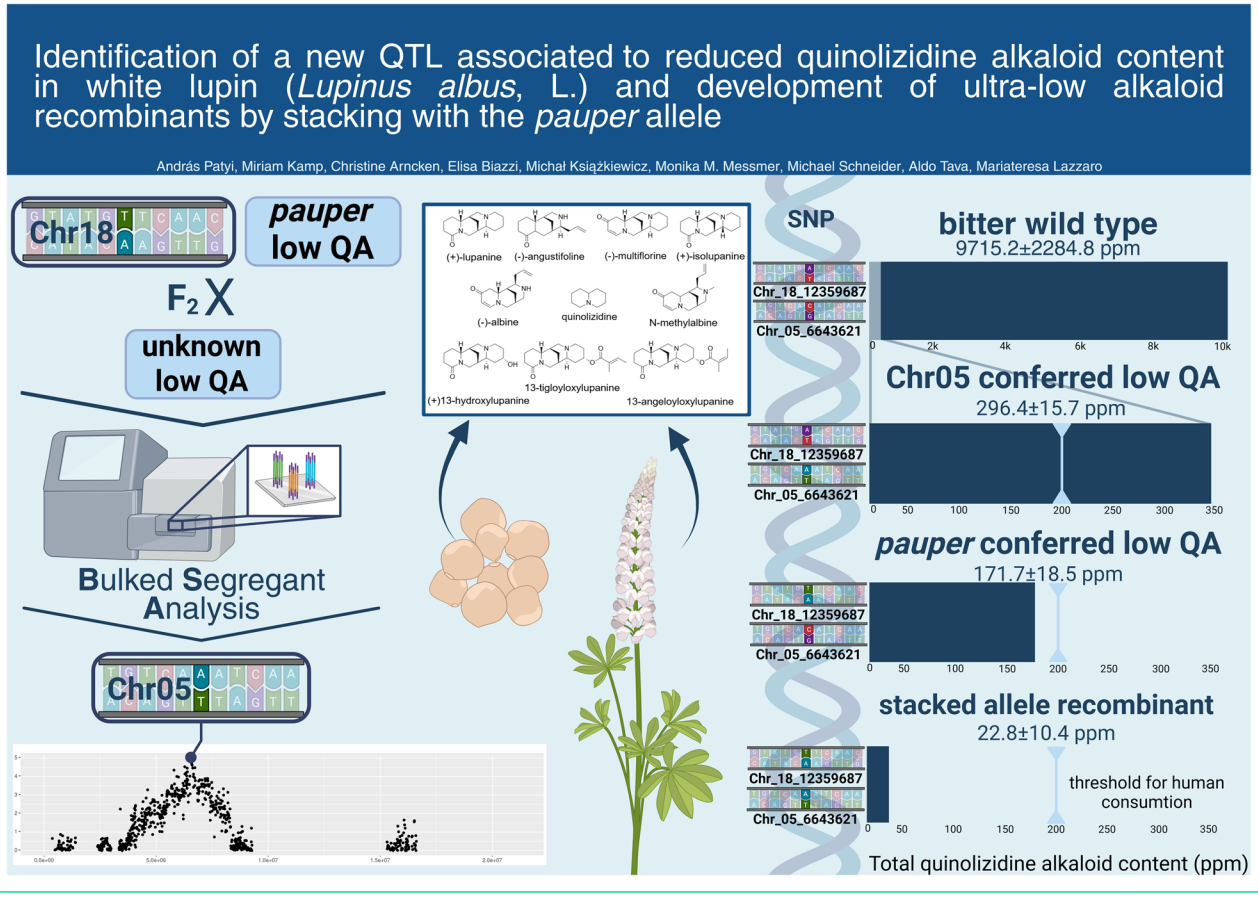
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and stable alkaloid content based on stacked allele recombinants. This can help to increase the cultivation of this useful yet underutilised crop and its use for human nutrition.

Keywords White lupin, Bulked Segregant analysis (BSA), Quantitative trait locus (QTL), Quinolizidine alkaloids (QA), PCR allelic competitive extension (PACE), Plant breeding

Graphical abstract



Background

White lupin (*Lupinus albus*, L.) is a grain legume of Mediterranean origin produced for its agronomic value and highly nutritious seeds. From the nutritional perspective, white lupin grain is appreciated for feed and food for its high protein (33–47% depending on genotype and location) and excellent fibre content [1]. Its desirable fatty acid composition ratio of omega-3 to omega-6, non-starch carbohydrate-, oligosaccharide- and antioxidant-content makes the consumption of white lupin seed a great contribution to a healthy diet [2]. Conversely, white lupin seed contains quinolizidine alkaloids (QAs), toxic (primarily due to their ability to interfere with the nervous system) and bitter compounds which set a major challenge for food producers and processors. In the closely-related blue lupin (*Lupinus angustifolius*, L.), the major site of production of QAs is the aerial epidermis, later transported to seeds during fruit development [3]. Seeds of wild forms of white lupin contain up to 127,000 ppm (mg/kg dry weight) QAs which is reduced in bitter white lupin cultivars to 2,000–4,000 ppm [4]. Historically, the grains were soaked in running water to remove the toxic alkaloids before consumption. Improved methods exist for debittering, commonly using water as a solvent, however these processes remain water-use intensive, laborious and producing toxic waste [5], constraining the use and economic viability of white lupin in the value chain, for feed and, especially, food.

Lupins contain a mixture of different alkaloids, not all of which are sufficiently known for their toxicity. Recently, Schreiber et al. [6] evaluated the toxicity of five common lupin alkaloids (sparteine, lupinine, lupanine, 13-hydroxylupanine, angustifoline) reporting that these QAs individually and as a mixture are neither cyto-, nor genotoxic in mammalian cells. Concerning their neurotoxicity, the effect of sparteine, presumably

the most toxic lupin alkaloid, has been studied best. The available risk assessment is therefore based on the knowledge of sparteine for all alkaloids present in lupins. Safety control is based on the total quinolizidine alkaloid content [7]. In Switzerland, for example, there are no legal limits for alkaloids in food or feed. The principle of self-regulation applies, which means that companies must ensure that only safe food is placed on the market [8]. The German Federal Institute for Risk Assessment (BfR) has established in 2017 the following guideline values to protect human and animal health: <200 ppm and < 500 ppm (dry weight) respectively for food and feed, relative to the final product intended for consumption [9]. Additionally, the import and export standards of the industry organisation Pulse Australia (Australia being the world's largest lupin producer) set in 2001 a maximum alkaloid content of 200 ppm [7]. The European Commission has recently recommended monitoring of the following quinolizidine alkaloids in lupins and lupin-derived food: albine, anagyrin, angustifoline, lupanine, isolupanine, multiflorine, 13α-hydroxylupanine, lupinine and sparteine [10].

Modern sweet (i.e. bitterness lacking) varieties exist with the alkaloid content ranging between 100 and 800 ppm of seed dry weight [11]. Low QA cultivars were a major achievement of the formal breeding of white lupin, started in the early 20th century with the activity of Reinhold von Sengbusch [12]. He selected against common wild type traits, amongst many others for high QA content, describing the drastic decrease (to 200–500 ppm) in QA content due to the recessive allele *pauper*, present in many modern white lupin cultivars [13]. Apart from *pauper*, other loci exist (e.g. *exiguus*, *nutricius*, *mitis*, *reductus*) representing possible recessive mutations for low QA content, described to reduce QA content to ≤1000 ppm [11]. These mutations were described based on crossings between different white lupin individuals and observing the segregation pattern in the chemotype, thus not much is known about the genes involved or transcriptomic regulation of low QA content in white lupins, except for *pauper*. Recently, the mutation causing the *pauper*-type sweetness in white lupin was identified [14]. Reportedly the presence of a single SNP (*Lalb_Chr18_12359687*) confers a greatly reduced total QA content and a distinct QA profile. This mutation is located in the coding sequence of a gene encoding an acetyltransferase, and is responsible for changes in the early biosynthetic steps of QAs, resulting in the low QA chemotype. However, phenotypically sweet variants exist, which do not carry the *pauper* mutation, confirming non-*pauper* sweetness types, earlier described in the 20th century [11]. One example was the cultivar 'Dieta', in which the genetic determinant causing low QA levels is unknown [14].

Despite the magnitude of the effect of the gene associated with *pauper*, grains harvested from reportedly sweet *pauper* accessions have been observed to exceed the advised threshold for consumption (FiBL, unpublished data). Relatively high and unstable QA content in the harvested grain remains a threat for white lupin producers and processers, creating a demand for even lower QA varieties with stable sweetness across growing seasons regardless of the environmental context. This could be achieved through gene stacking (pyramiding)– combining independent loci conferring low QA content in white lupin, which could be further aided with the availability of molecular markers tagging these loci.

The present study aimed to map an unknown source of sweetness in white lupin by Bulked Segregant Analysis (BSA) in an F_2 population resulting from the crossing of the *pauper* sweet cultivar 'Frieda' and the cultivar 'Dieta' carrying the unknown sweetness determinant. Subsequently, through PACE marker development and validation in a diversity panel, we aimed to verify the effect of the individual loci conferring the low QA level phenotype and to assess their combined effect on QA concentration and profile in the F_2 and the derived F_3 population.

Methods

Plant material

Crossing and F_2 population

Two commercial cultivars, 'Frieda' (♂, *pauper* sweetness) and 'Dieta' (♀, unknown sweetness), were crossed in 2021. Seeds were obtained from Delley Samen und Pflanzen AG (Delley-Portalban, Switzerland) for 'Frieda' and from Soya UK Ltd. (Southampton, United Kingdom) for 'Dieta'. The five harvested seeds from the crossing were propagated in 2022 under protective nets to prevent cross-pollination and seeds of the F_1 plants were harvested in a bulk.

The F_2 plants (own breeding material) were grown in 2023 in the FiBL breeding nursery located in Leibstadt, Switzerland (47°35'26.4"N 8°10'56.0"E), organized in a 10-row plot with 14 $F_{1:2}$ seeds sown per row, grown under protective nets. Young leaf tissue (ca. 100 mg) was collected and frozen from the 112 surviving individuals of the F_2 population (Supplementary Table S1).

F_3 population

Thirteen F_2 plants, all heterozygous at the *pauper* locus were selected for the F_3 population (own breeding material) and their seeds were grown in 2024 on a field in Full-Reuenthal, Switzerland (47°35'59.7"N 8°12'13.6"E), sown and processed in an identical manner as in previous years. In total, 329 individuals represented the F_3 population (Supplementary Table S3). In addition, 10 individuals each of 'Frieda' and 'Dieta' were grown in the same field and harvested as single plants.

Validation panel

A diverse panel of 34 individuals was created for the molecular marker validation, consisting of 1 to 7 representatives for 1 breeding line, 16 cultivars, 3 landraces and 1 wild type in addition to the 39 accessions of the white lupin pangenome [15] (Supplementary Table S5).

Phenotyping

The alkaloid content of F_2 and F_3 was estimated first on the field using Dragendorff paper test (Supplementary Table S1, Supplementary Table S3), a filter paper soaked in iodine/potassium iodide solution (Dragendorff solution), then dried at 40 °C [16]. Individual QA content was estimated by transferring plant sap from the petioles onto the Dragendorff paper during onset of flowering (BBCH 63–65). A visible reaction of the Dragendorff paper turning a reddish-brown colour was described to occur for QA contents of ≥ 500 ppm [17]. The colour reaction was scored from 1 to 4, 1 meaning a purely white stain, the lowest alkaloid content. A score of 2 was assigned to stains, which were mostly white with the red encircling, score 3 described red encircled pinkish stains with a light centre and lastly, a score of 4 was given to samples with an intense red stain without discolouration in the centre (Fig. 1).

Additionally, bitterness in the F_2 population was phenotyped by scoring the taste of one flower per plant in a range from 1 (absence of bitterness) to 9 (extremely bitter). For a more accurate phenotypic score in the F_2, we combined the Dragendorff phenotypic score multiplied by two to that of the bitterness tasting score. In this combined phenotypic score, the phenotypic value ranged from 3 to 15, 15 being the most bitter.

Chemotyping of F_3 individual seeds

Approximately 1 g of seeds (3–5 seeds) of a curated panel of F_3 (Supplementary Table S6) and parental individuals were milled for analytical QA quantification. QA extraction and characterization were performed according to modified versions of the protocol described in Wink [18], Resta et al. [19] and Boschin et al. [20]. Briefly, 100 mg of seed flour was suspended in 1.2 mL of 0.1 N HCl, with sparteine (CAS 90-39-1; Extrasynthese, France) added as internal standard in an appropriate concentration, and stirred at room temperature overnight. The mixture was centrifuged at 8000 g for 45 min at 4 °C, the supernatant was collected, and the solid was washed twice with 0.8 mL of 0.1 N HCl. The gathered extracts were alkalinized with 5% NH_4OH to pH 10 – 11 and then applied onto an Extrelut NT 3 column (Supelco, Merck, Darmstadt, Germany). The alkaloids were eluted with CH_2Cl_2 (3 × 5 mL), and the solvent was evaporated under vacuum. The residue was resuspended in an appropriate volume of dichloromethane and analyzed

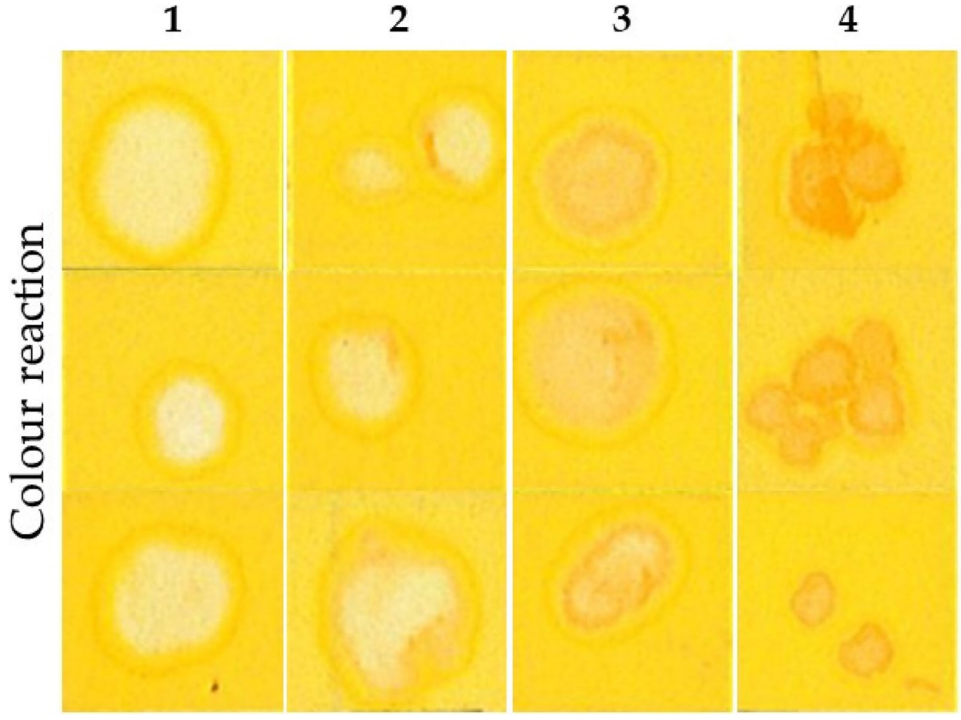


Fig. 1 Examples of the colour-reaction of Dragendorff paper according to the predefined scoring scale from 1 (very sweet) to 4 (very bitter). 1 - purely white stain; 2 - mostly white with the red encircling; 3 - red encircled pinkish stains with a light centre 4 - intense red stain without discolouration in the centre

by gas chromatography. Each sample was independently extracted, then analyzed in duplicate.

Total and individual QA content was determined quantitatively using the internal standard methods by gas chromatography/Flame Ionization Detector (Perkin-Elmer Clarus 500 GC-FID), equipped with a capillary column Elite-5 MS (DB-5, 30 m × 0.32 mm × 0.25 µm; Perkin-Elmer; Milan, Italy). The oven temperature ramp was held at 90 °C for 2 min, increased to 300 °C at 7 °C/min and held at 300 °C for 10 min. Helium was used as the carrier gas, and the flow rate was set at 2 mL/min. The temperature of the injector was set at 300 °C, and the injection volume was 1 µL; the temperatures of the FID was set at 320 °C. The absence of sparteine in the samples was demonstrated by performing GC/FID analyses without an internal standard (choosing one sample per recombinant).

The response factor of GC/FID was calculated using the ratio between the response of the internal standard (sparteine) and the response of the analyte standard lupanine. The regression coefficient between the analyte concentration and detector response was $R^2 = 0.991$.

Qualitative identification of QAs was performed by gas chromatography/mass spectrometry; GC/MS analyses were carried out using a Perkin Elmer Clarus 500 GC equipped with a Clarus 500 mass spectrometer using the same capillary column and chromatographic conditions as for the GC/FID analyses (above mentioned). Mass spectra were acquired over a range of 40–400 atomic mass units (amu) at 1 scan/sec with ionizing electron energy of 70 eV and ion source at 230 °C. The transfer line was set at 300 °C, while the carrier gas was helium at 1.0 mL/min. The QAs were identified by determination of their elution times published mass spectra [18–20], as well as a peak-matching library search [21]. The standard GC condition described above allowed a practical measurable sensitivity of 10 ng/µl per injection, i.e. a detection limit of 1 µg of lupanine g^{-1} of lupin seed flour (1 ppm).

Pooling for bulked segregant analysis

Two bulks consisting of DNA (isolated with a modified CTAB method) from eight plants of the sweet and bitter phenotypic extremes of the 'Dieta' x 'Frieda' F_2 experimental population were curated for sequencing. Homozygous individuals for the *pauper* locus were excluded from this subset to discriminate sweet individuals where the low QA content is conferred by *pauper*. Thus, the F_2 bulks consisted of individuals which were heterozygous or wild type at the *pauper* locus, suggesting that the low QA phenotype is conferred by a determinant other than *pauper*. The combined phenotypic score [3–15] of the sweet bulk was between 7 and 8.5, while that of the bitter one was 13–15. Additionally, bulks of the parental cultivars (from seedlings grown for this purpose from the same seed source used for the crossing) consisting of eight plants each were sent for sequencing.

Sequencing-data analysis

Whole genome sequencing was performed at Novogene UK (Cambridge, UK) with a NovaSeq X Plus Series, sequencing depth of 15x. Paired-end 150 bp long reads were generated. The raw sequence data was processed and analysed in a modified version of the procedure published by Schneider et al. 2022 [22]. Quality checks were performed with the *fastqc* tool [23]. Raw reads were aligned to the reference genome using *bwa mem* [24]. Subsequently, duplicated reads were removed using the markdub-function of *sambamba* [25] and sorted by their mapped position. Reads that had been mapped to multiple locations (e.g. secondary alignments) were discarded. The variant calling was conducted using *bcftools* only on SNPs that had been previously reported [15] to improve the confidence despite the low sample number and read depth. Furthermore, reads with a quality threshold below 25 for the whole read, and 30 per SNV base call were omitted, as well as SNPs with read depths below 8. The allelic depth (AD) was extracted and heterozygous SNPs in the parental bulks were omitted from further analysis. The remaining SNPs were assigned to annotated genes based on their position in the gene or in close proximity to it, as described by Schneider et al. 2022 [22]. For the resulting 23,396 gene-anchored haplotypes, the allele frequencies of 'Dieta' and 'Frieda' were calculated for the sweet and for the bitter bulk. The frequencies were aggregated with the read depth to count values, as required for *BayPass* [26], which is a Bayesian approach to identify i.e. selective sweeps between the sweet and bitter populations. *BayPass* was conducted using the effective population size of 200 to reflect the size of the 'Dieta' × 'Frieda' F_2 population. From the p-values of each haplotype and their mapped positions (Supplementary Table S8), Manhattan plots were generated, with a threshold of $-\log_{10}(\text{p-value}) > 4.5$.

Genotyping

PACE primers

Primer triplets (Table 1) for 3 selected SNPs in the newly identified QTL and for the SNP associated to *pauper* were designed by 3CR Bioscience (Essex, UK) and oligonucleotides were acquired from IDT (Coralville IA, USA). For all four loci, the primers were designed based on the sequence of the sense strand.

PACE assay

For the genotyping assays, we used the PACE master mix (2x concentration, standard ROX level) provided by 3CR Bioscience (Essex, UK). Each individual reaction

Table 1 PACE primers used in the study. Target SNP locus on the chromosome, the oligonucleotide sequence for the two forward (F1 and F2) tagging the two possible alleles at the respective locus (underlined), including the sequence of the X- and Y- Tails and the reverse (R) primers for each triplet, and melting temperatures

Locus	Primer	Sequence (5′–3′)	Tm (°C)
Lalb_Chr05_6643621	F1	GAAGGTGACCAAGTTCATGCTAGACTATGTCATGTTTTTAAACATGTCA**C**	64.2
	F2	GAAGGTCGGAGTCAACGGATTGAGACTATGTCATGTTTTTAAACATGTCA**A**	65.4
	R	GGCTTGAATAGGGTCCATGTGTTTAATAT	57.1
Lalb_Chr05_6643864	F1	GAAGGTGACCAAGTTCATGCTAATCTATTATTGTCCTATTAAAACATTACGA**G**	62.8
	F2	GAAGGTCGGAGTCAACGGATTAATCTATTATTGTCCTATTAAAACATTACGA**C**	63.3
	R	TATATTTTTTAGCTTAGGATGTTTTGGATT	52.3
Lalb_Chr05_6645052	F1	GAAGGTGACCAAGTTCATGCTCGATAAGAGAGTGGGAGGACCA**A**	67.7
	F2	GAAGGTCGGAGTCAACGGATTGATAAGAGAGTGGGAGGACCA**C**	67.8
	R	CGTGGCATGGACCCCAGTTATATAA	58.5
Lalb_Chr18_12359687 (pauper)	F1	GAAGGTGACCAAGTTCATGCTAAATGCTATCAGGATAGGGTCTATG**T**	65.3
	F2	GAAGGTCGGAGTCAACGGATTAAATGCTATCAGGATAGGGTCTATG**A**	65.7
	R	CTTCCATTGTTCTTCCTCTATCTRCACTT	57.3

consisted of 5 µl of the PACE master mix, 0.14 µl of the primer pool and 5 µl of ELGA water (VWS Ltd.; High Wycombe, UK) or DNA template, isolated from leaf samples transferred onto Qiagen FTA cards (QIAGEN GmbH; Hilden, Germany), processed according to the manufacturer`s instructions. The cycling conditions were set according to the manufacturer`s instructions of the analogously functioning Kompetitive Allele Specific PCR (KASP) assay (LGC Group; Teddington, UK) and ran on the CFX Opus 384 Real-Time PCR System (Bio-Rad Laboratories, Inc.; Hercules CA, USA).

All 112 F_2 individuals were genotyped in 2023 using a newly designed PACE marker tagging the most important SNP associated to *pauper* [14], *Lalb_Chr18_12359687* and only retrospectively genotyped in 2024 upon the availability of the PACE marker tagging the Dieta-associated sweetness. Samples from the F_3 population were genotyped with both markers in 2024.

Data analysis

All data except for the sequencing data was analyzed and visualized in R version 4.4.2 [27].

The phenotypic data (Dragendorff score) was analyzed in the F_2 and F_3 experimental populations using Linear Mixed Models (LMM). The allelic combinations at loci *Lalb_Chr18_12359687* and *Lalb_Chr05_6643621* were included as fixed effect, and the row the specific plant belonged to on the field as a random effect. The estimated marginal means from the above-mentioned linear mixed model were calculated to test the statistical difference ($p < 0.05$) using emmeans_1.10.7 [28] and were used to assess the association between the allelic states and the phenotypic value in F_2 and F_3.

Similarly, the total alkaloid content (sum of all QAs present in a sample) and the ratio between the concentrations of 13α-hydroxylupanine and lupanine per sweet variant were analysed LMM using lme4_1.4–36 [29]. For both response variables we included the allelic combinations as fixed, and the biological replicate (i.e. set of samples belonging to allelic combinations of the two loci of interest) as random factor. The estimated marginal means from the above-mentioned linear mixed model were calculated to test the statistical difference ($p < 0.05$) using emmeans_1.10.7 [28] and were used for the following downstream analysis.

Results

The phenotypic segregation in the F_2 generation confirms another determinant for low QA content

With *pauper* known to segregate in the 'Dieta' x 'Frieda' F_2 population by the genotyping at the causal mutation SNP *Lalb_Chr18_12359687*, the simplest hypothesis of 1: 3 (sweet: bitter) phenotypic segregation ratio could be rejected. Instead, the phenotypic segregation pattern was 7: 9 (sweet: bitter), which coupled with observation of the genotypic segregation 1: 2: 1 at the *pauper* locus suggests the segregation of at least one additional low QA conferring gene aside from *pauper* (Table 2).

Assuming segregation of two independent recessive genes, the phenotypic scores are expected to segregate in a ratio of 7: 9 (sweet: bitter) in QA content (following Mendelian inheritance), which hypothesis could not be rejected (Table 2), indicating two independent recessive loci conferring low QA content with no segregation distortion in the F_2 population used for the BSA. The assumption of genotypic segregation of 1: 2: 1 could not be rejected for either loci, further supporting the independence of these determinants.

Identification of a QTL associated to low QA content through BSA

After the confirmation of the presence of a determinant independent of *pauper* for low QA content in the

Table 2 Segregation ratios and χ2 statistics in the phenotype (Dragendorff score) and at the *pauper*, and Dieta-associated locus for the entire F_2 population

Segregating population		Expected ratio	Observed ratio	df	χ2	*p*-value	
Phenotypic segregation							
'Dieta'x 'Frieda'		1:3	41:69	1	8.8364	0.00295	**
		7:9	41:69	1	1.8753	0.1709	
Genotypic segregation							
'Dieta' x 'Frieda'	*pauper* segregation	1:2:1	31:47:34	2	3.054	0.2172	
	Dieta-associated segregation	1:2:1	22:63:27	2	2.1964	0.3335	

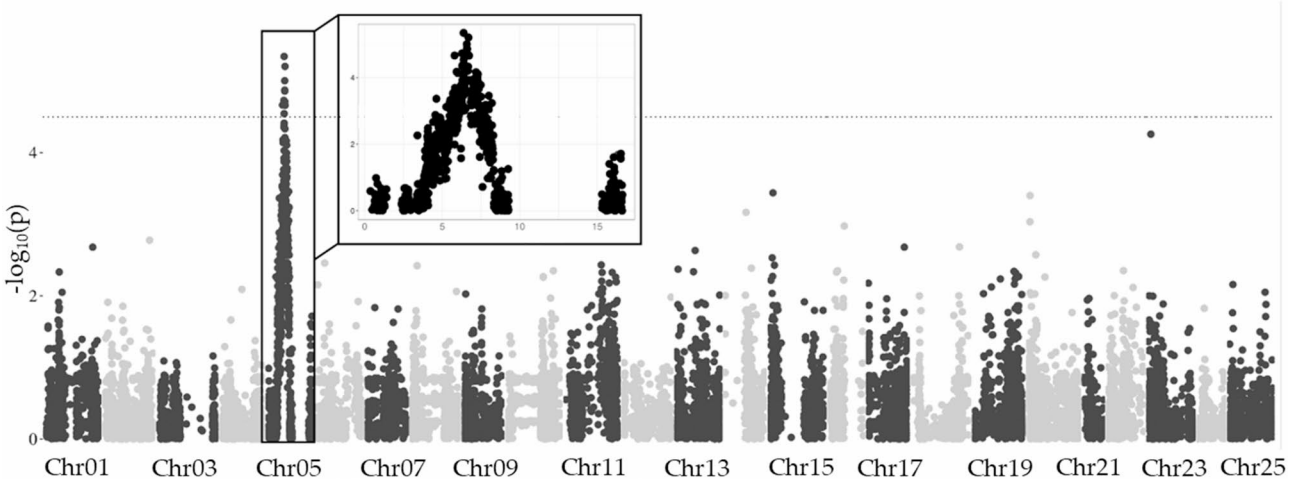


Fig. 2 Manhattan plot of -log10(p-value) (y-axis, threshold set to -log10(p-value) = 4.5), for differences in allele frequencies of haplotypes between two bulks of the F_2 population representing high and low QA levels. The x-axis indicates the position of the haplotypes in each of the 25 chromosomes. A zoomed-in plot is included for chromosome 5 for detail

segregating F_2 population, we identified the genomic region responsible for this unidentified determinant of low QA content through BSA. From the raw reads ranging between 97 and 117 million per bulk (read length of 150 bp), 47–57 million remained after quality filtering, mapping and removal of redundancies (Supplementary Table S7).

There were 1.55 million polymorphic SNPs (1.76 million before q40 filtering) from the lupin browser that were polymorphic between the parents, reported by *bcftools*, from which 514,982 remained after filtering out monomorphic, low quality and low depth variants. Following, 23,396 gene-anchored haplotypes with an average of 21 SNPs and an average read depth of 302 per haplotype were constructed. The selective sweeps analysis resulted in a clear peak on chromosome 5 (Fig. 2) that allows to differentiate the 'sweet' from the 'bitter' bulk.

Candidate gene selection and marker development

Ten genes were associated with haplotypes showing significant ($-\log_{10}$(p-value) > 4.5) differences in allele frequencies between sweet and bitter bulks within the entire QTL region spanning from 5,799,140 bp to 6,737,917 bp on chromosome 5 (Supplementary Table S9). Although none of them could be directly linked to the biosynthetic pathway, the gene *Lalb_Chr05g0222381* encoding a "Putative aminoacyltransferase" (a function similar to the gene responsible for the *pauper*-conferred low QA content) was chosen for molecular marker development. We investigated all individual SNPs within as well as 1,000 bp up- and downstream of this gene.

There are 54 polymorphic loci between 'Dieta' and 'Frieda' in this region. To determine whether these are also polymorphic within other accessions as well, we collected the allelic states for both loci of all 39 sequenced accessions from the white lupin genome browser (Supplementary Table S5). Out of these 54 SNPs, only 9 are highly polymorphic within the 39 sequenced accessions [15]. Furthermore, only 3 loci are consistent with the assumed alkaloid status of these accessions, *Lalb_Chr05_6643621*, *Lalb_Chr05_6643864*, *Lalb_Chr05_6645052*. These three were tested on the validation panel of 34 accessions to further confirm their association to alkaloid status (Supplementary Table S5), in comparison to the marker-trait association of the marker tagging *pauper*. At the locus *Lalb_Chr05_6643864*, an old cultivar, Nährquell (described to carry the sweetness mutation *nutricius*) carried the allele

associated to the Dieta-type sweetness, and the wild-type allele at the other two selected loci in the region thus this locus was not chosen for further analysis. Similarly, at the locus *Lalb_Chr05_6645052*, when comparing two different phenotypically sweet individuals of the cultivar 'Start' (described to carry the sweetness mutation *exiguus*) lead to inconsistent results, thus we selected only the SNP *Lalb_Chr05_6643621* for further genotyping of the F_3 segregating population. Interestingly, French cultivars which belong to the panel of resequenced accessions, namely 'Magnus', 'Orus', 'Luxe' and 'Clovis' carried the alleles associated to sweetness both for the *pauper* locus and the newly described one on chromosome 5.

Marker-trait associations reveal low QA stacked allele recombinants

The marker-trait association between the newly described *Lalb_Chr05_6643621* SNP, the *pauper* causal mutation SNP *Lalb_Chr18_12359687* and the phenotypic score (Dragendorff score) was confirmed for the individuals in the F_2 and F_3 populations (Supplementary Figure S1-S3), in comparison to their parental lines. In F_2, we were able to statistically differentiate between individuals carrying the sweet genotype associated to *pauper*, and discriminate for bitter individuals based on the Dragendorff score (Supplementary Table S2). However, in F_3, only *pauper* sweet individuals, and bitter individuals were clearly statistically grouped together based on the Dragendorff score only (Supplementary Table S2, Supplementary Table S4). to obtain more nuanced observations on the quantitative value of alkaloids, we performed GC/MS and GC/FID analytical QA quantification in a subset of the F3 population representing different combinations of allelic states at the pauper causal mutation SNP on chromosome 18 and the locus Lalb_Chr05_6643621 on chromosome 5, used for the following downstream analysis. This subset included the two parental lines and the most important allelic combinations at both loci (Table 3) and the corresponding estimated marginal means values from the post-hoc analysis after model fitting.

Comparing the concentrations of the total alkaloid content (Fig. 3), we observed a decrease in the estimated marginal means of the total alkaloid content in all sweet chemotypes. The F_3 plants carrying the allele *pauper* as sole contributor to the low QA phenotype (PAU) were grouped together with 'Frieda' (FRA), the parent conferring the *pauper*-type sweetness (Fig. 3). Similarly, the F_3 progeny plants in which the low QA phenotype was conferred by the parent 'Dieta' (DSW), were grouped together with the parental genotype (DIA), as well as the F_3 individuals heterozygous for *pauper* and homozygous sweet at *Lalb_Chr05_6643621* (HET), though with higher average QA content compared to the plants that are sweet because of *pauper* (PAU). The stacked allele recombinants (STA) have a very low alkaloid content (range raw data 11–62.3 ppm), lower compared to either parent or progeny, indicating a clear additive effect of stacking two low QA conferring determinants. Stacked allele recombinants (STA) represented the most drastic reduction to 12% and 8% of the total average alkaloid content of the parental cultivars, 'Dieta' and 'Frieda', respectively. The total estimated marginal mean for the alkaloid content was 22.8 ± 10.4 ppm dry weight in seeds of the stacked allele recombinants, close to 10 times lower than the suggested threshold for human consumption.

QA profiles in the F_3 population highlights the stacked allele effect

Based on the presence of specific QAs in the different allelic combinations studied, we observed distinct QA profiles associated to different low-QA determinants and their combination. In the wild-type, bitter phenotype (BIT) 14 QAs were identified (Fig. 4).

Table 3 Subset of F_3 individuals analyzed with GC/MS and GC/FID, their allelic state, number of samples (n), estimated marginal means of total Quinolizidine alkaloid (QA) content (in ppm) indicating statistical grouping, and standard error (SE). BIT– bitter wild type progeny; FRA– 'Frieda' parent, homozygous sweet at *pauper*, wild type at *Lalb_Chr05_6643621*; DIA– 'Dieta' parent, wild type at *pauper*, homozygous sweet at Lalb_Chr05_6643621; PAU– homozygous sweet at *pauper*, wild type at *Lalb_Chr05_6643621*; DSW– wild type at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*; HET– heterozygous at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*; STA– homozygous sweet at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*

Code	Allele				*n*	QA content (ppm)	SE (ppm)
	Lalb_Chr18_12359687 (pauper)		Lalb_Chr05_6643621				
BIT (bitter progeny)	AA	wild type	CC	wild type	4	9715.2	2284.8
DSW (Dieta sweet progeny)	AA	wild type	AA	homozygous sweet	10	296.4[c]	15.7
HET	TA	heterozygous	AA	homozygous sweet	10	267.1[c]	15.7
DIA ('Dieta' parent)	AA	wild type	AA	homozygous sweet	10	257.4[c]	15.7
PAU (pauper sweet progeny)	TT	homozygous sweet	CC	wild type	7	171.7[b]	18.5
FRA ('Frieda' parent)	TT	homozygous sweet	CC	wild type	10	176.2[b]	15.7
STA (stacked allele recombinants)	TT	homozygous sweet	AA	homozygous sweet	23	22.8[a]	10.4

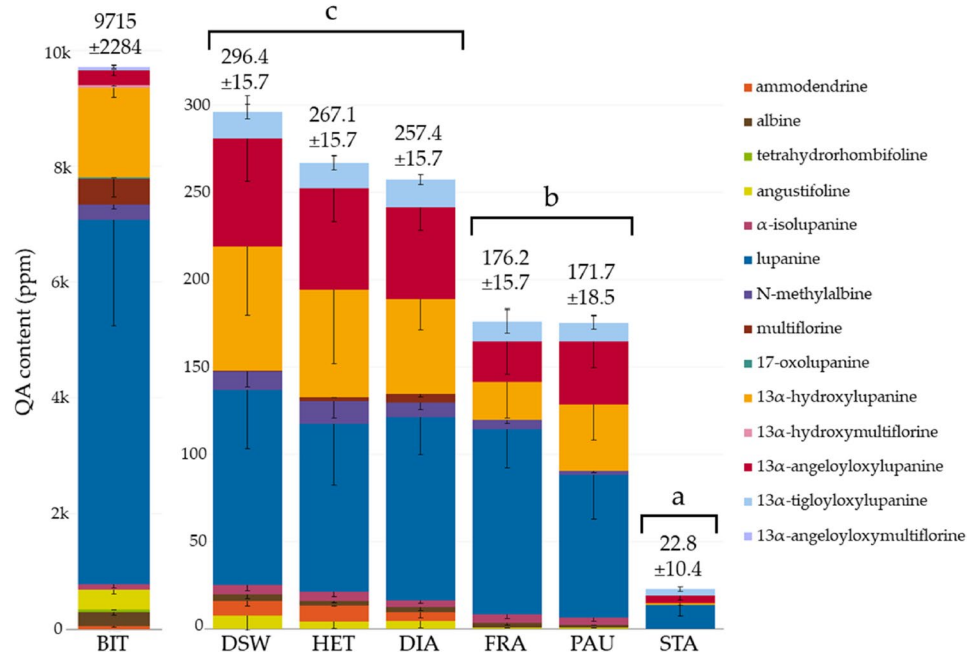


Fig. 3 Individual QA content and composition, and total QA content (estimated marginal mean value in ppm) of different recombinants in the F_3 population compared to the parental genotypes. Values with the same letter are not significantly different at 0.05 confidence level. BIT– bitter wild type progeny; FRA– 'Frieda' parent, homozygous sweet at *pauper*, wild type at *Lalb_Chr05_6643621*; DIA– 'Dieta' parent, wild type at *pauper*, homozygous sweet at Lalb_Chr05_6643621; PAU– homozygous sweet at *pauper*, wild type at *Lalb_Chr05_6643621*; DSW– wild type at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*; HET– heterozygous at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*; STA– homozygous sweet at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*

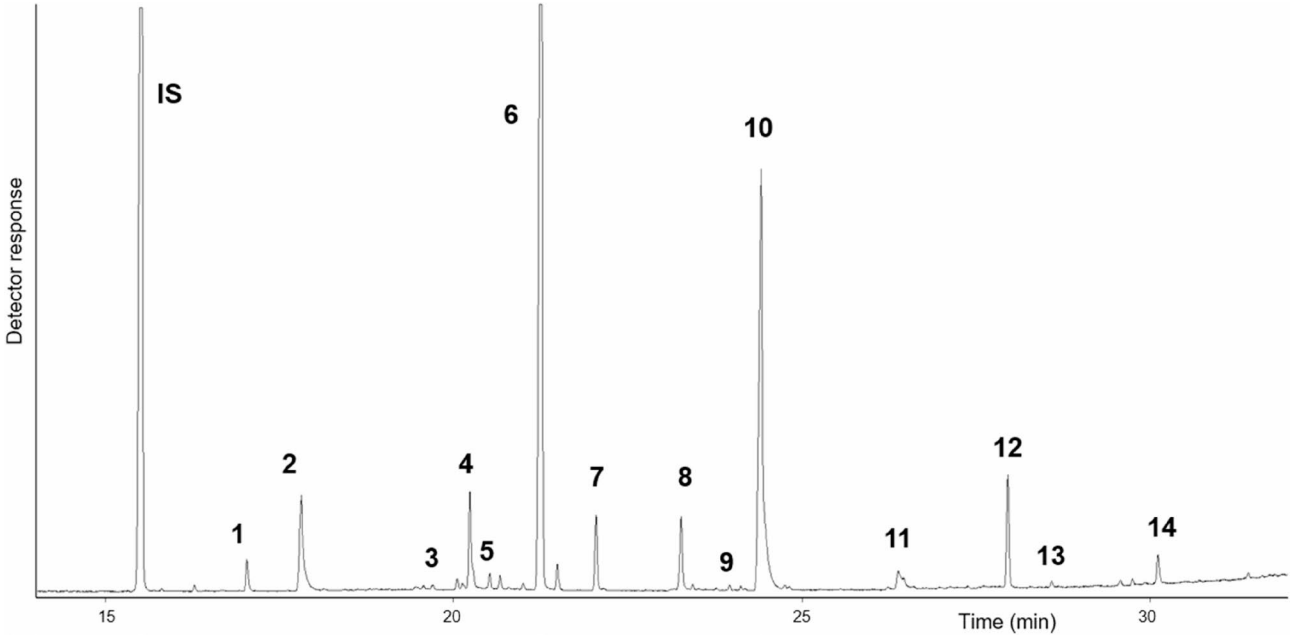


Fig. 4 Gas chromatogram of alkaloids from *L. albus* seeds. IS - internal standard (spartein); 1 -ammodendrine; 2 - albine; 3 - tetrahydrorhombifoline; 4 - angustifoline; 5 - α-isolupanine; 6 - lupanine; 7 - N-methylalbine; 8 - multiflorine; 9–17-oxolupanine; 10–13α-hydroxylupanine; 11–13α-hydroxymultiflorine; 12–13α-angeloyloxylupanine; 13–13α-tigloyloxylupanine; 14 – 13α-angeloyloxymultiflorine

Lupanine, multiflorine, 13α-hydroxylupanine, 13α-angeloyloxylupanine and 13α-tigloyloxylupanine were the most commonly occurring QAs in the bitter chemotype. Both in the individuals carrying only *pauper* (PAU) or only the 'Dieta'-associated (DIA, DSW) sweetness in a homozygous state, as well as in the line heterozygous at *pauper* and homozygous sweet at *Lalb_Chr05_6643621* (HET), there is a consistent lack of specific QAs (Fig. 5): tetrahydrorhombifoline, 17α-oxolupanine, 13α-hydroxymultiflorine and 13α-angeloyloxymultiflorine. Furthermore, the QA profile in 'Frieda' (FRA) and associated progeny (PAU) was even more reduced, lacking ammodendrine and multiflorine, while angustifoline was present only in trace amount. Stacked allele recombinants (STA) show the most drastically reduced QA profile with only lupanine and two of its esterified derivates, 13α-angeloyloxylupanine and 13α-tigloyloxylupanine present in the analysed seeds; 13α-hydroxylupanine, the second most abundant alkaloid in *L. albus*, was present only in few samples, while in others it was detected only in trace amounts (Fig. 5).

The most commonly occurring QA in all variants was lupanine making up ca. 64% of the total alkaloid content in the bitter (BIT) chemotype (Fig. 4.). A similar percentage (albeit at significantly lower ppm values as indicated on Fig. 3) was observed in the 'Frieda' parent (FRA) and the stacked allele recombinant (STA), 62.25% and 60.06%, respectively. In other variants (DIA, DSW, HET, PAU), the contribution of lupanine towards the total QA profile was reduced, ranging between 36.75 and 47.31%. The second most abundant QA in the bitter chemotype was 13α-hydroxylupanine (16.57% of the total alkaloid content), which trend was also observed in other accessions, except in the stacked allele recombinant (STA), where this QA was only present in trace amounts. The relationship between these two, most commonly occurring QAs was further investigated by calculating their ratios (Fig. 6). Figure 6 shows that in the haplotypes, where the reduced QA levels are conferred by the determinant

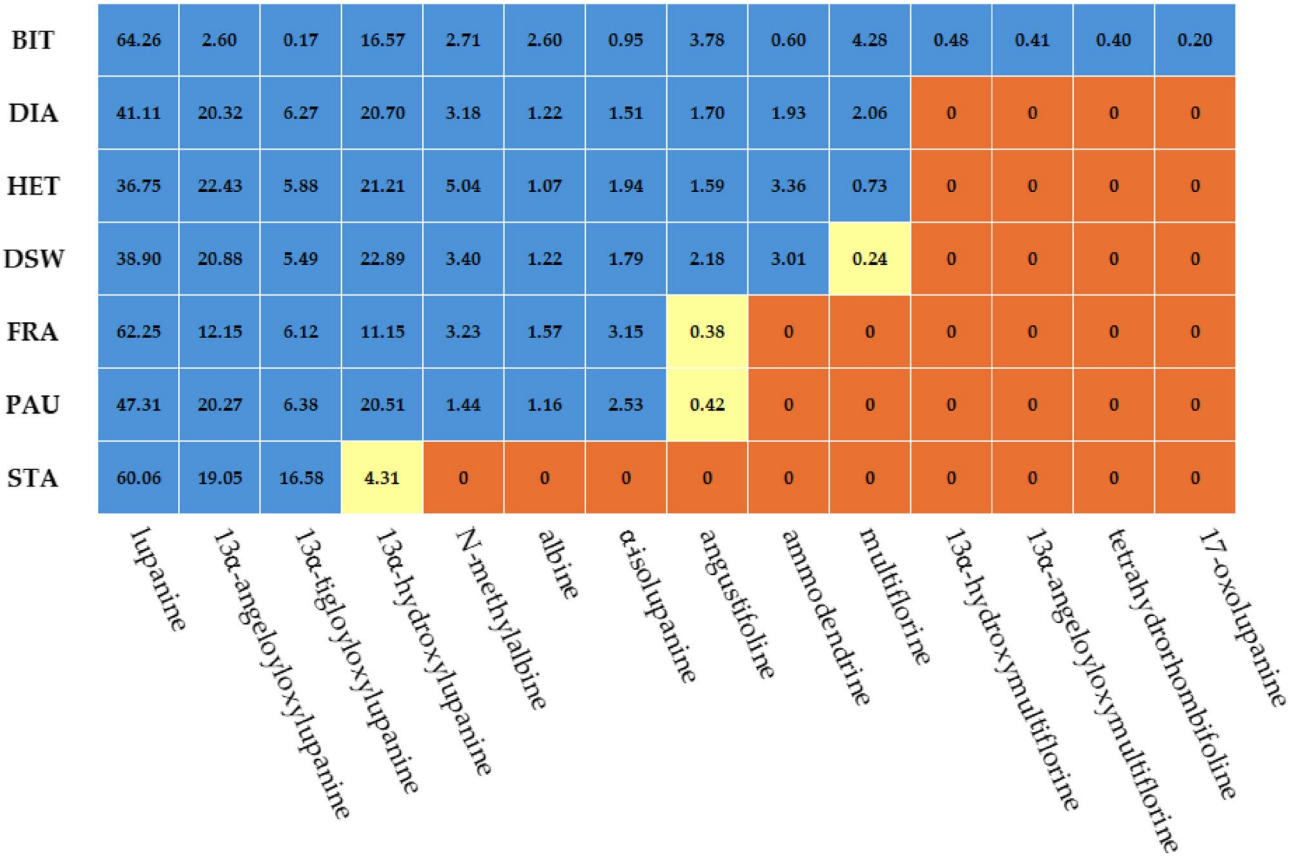


Fig. 5 Presence of QAs and their contribution in percentage in the total QA content in representatives of allelic combinations of *Lalb_Chr18_12359687* and *Lalb_Chr05_6643621*, ordered according to their abundance. A QA was assigned as present (blue cells) if its value was above 1 ppm (detection threshold of GC/FID), as present in trace amounts (yellow cell) when below 1 ppm (detection threshold of GC/FID), or as absent (red cells; GS/MS). BIT– bitter wild type progeny; FRA– 'Frieda' parent, homozygous sweet at *pauper*, wild type at *Lalb_Chr05_6643621*; DIA– 'Dieta' parent, wild type at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*; PAU– homozygous sweet at *pauper*, wild type at *Lalb_Chr05_6643621*; DSW– wild type at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*; HET– heterozygous at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*; STA– homozygous sweet at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*

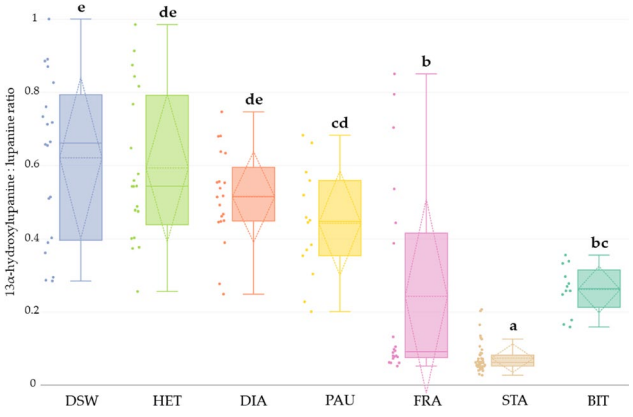


Fig. 6 Box plots of the ratio between the concentrations of 13α-hydroxylupanine and lupanine per variant. Values with the same letter are not significantly different at 0.05 confidence level. BIT– bitter wild type progeny; FRA– 'Frieda' parent, homozygous sweet at *pauper*, wild type at *Lalb_Chr05_6643621*; DIA– 'Dieta' parent, wild type at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*; PAU– homozygous sweet at *pauper*, wild type at *Lalb_Chr05_6643621*; DSW– wild type at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*; HET– heterozygous at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*; STA– homozygous sweet at *pauper*, homozygous sweet at *Lalb_Chr05_6643621*

on chromosome 5 (DIA, DSW, HET), the ratio between 13α-hydroxylupanine and lupanine was significantly higher compared to bitter (BIT) chemotype, while in stacked allele recombinant (STA), this ratio was significantly lower compared to all phenotypes.

Other, lupanine-derived QAs, 13α-angeloyloxylupanine was more abundant in the sweet chemotypes (12.15–22.43% of the total alkaloid content) compared to its presence (2.3% of the total alkaloid content) in the bitter one (Fig. 3.).

Discussion

We identified a new QTL associated to low QA content in white lupin, independent from *pauper* [14]. Furthermore, we found that the low QA associated haplotype in 'Dieta' was present (though inconsistently) in an old white lupin cultivar 'Start' which could indicate that the unidentified source of sweetness in 'Dieta' may be *exiguus* [30].

The newly identified QTL on chromosome 5 covers a large region (ca. 5.8%) of the chromosome. This could be due to the small bulk sizes, as other BSA studies include bulks of at least 20–50 individuals [31] to hundreds [32]. The limitation in our panel was created by the small sizes of the sampled subpopulations representing the phenotypic extremes, nonetheless highlighting the power of BSA even with small bulk sizes.

The QTL region consists of 10 genes with $-\log_{10}$(p-value) > 4.5, out of which the gene *Lalb_Chr05g0222381* was selected for marker development, based on its functional annotation and further investigations. The white lupin genome browser available on Phytozome, the Plant Comparative Genomics portal of the Department of Energy's Joint Genome Institute [33], describes this gene to encode a "(1 of 3) K16276 - zinc finger protein-like protein (K16276, BTS)" with further information available about domains within. Amongst others, the functional annotation suggests a RING and CTCHY-type zinc finger domain within this gene, known transcriptional repressors of alkaloids in *Catharanthus roseus* [34]. On the white lupin genome browser [15], it encodes for a "Putative aminoacyltransferase, E1 ubiquitin-activating enzyme", which function is homologous to the enzyme LaHMT/HLT, an acetyltransferase responsible for forming acetylated products of 13α-hydroxylupanine and 13α-hydroxymultiflorine [35–37]. Although the gene *Lalb_Chr05g0222381* is associated to the low QA chemotype in the F_2 and F_3 populations, its involvement in the QA biosynthetic pathway cannot be concluded by our results. In fact, in the validation panel, the varieties 'Magnus', 'Orus', 'Luxe', and 'Clovis' are genotypically stacked allele recombinants (Supplementary Table S5) but their alkaloid content and profile does not match the stacked allele recombinants` (FiBL, unpublished data), suggesting that *Lalb_Chr05g0222381* might not be the causal gene for the low QA chemotype.

Our use of the proposed PACE marker was applied on F_2 and F_3 segregating populations. Since the markers are linked to but likely not causal, further studies are required to estimate the genetic distance to the sweetness QTL on chromosome 5 and hence their use and limitations for marker assisted selection. There is very rapid LD decay (< 4 kb) in the white lupin genome [15], reported both for germplasm panels carrying only sweet-seed inbred lines or genetically diverse materials including landraces and primitive populations. Nevertheless, whether this impacts the use of the markers as diagnostic tool, depends of the genetic distance between the markers and the causal locus for the sweetness QTL on chromosome 5. Furthermore, such fast LD decay may be beneficial in attempts on further pyramiding recently reported QTLs for anthracnose resistance (gene *Lalb_Chr05g0216161*) and protein content (gene *Lalb_Chr05g0224011*), located 3,685,681 bp upstream and 1,112,291 bp downstream the *Lalb_Chr05_6643621* locus, respectively [38, 39]. Based on the LD values reported for white lupin, physical distance of 1–3 Mbp between particular QTLs should be more than enough to break the linkage in breeding programs.

The *pauper*-associated gene, *Lalb_Chr18g0051511* encodes an acetyltransferase, which is responsible to block the QA biosynthetic pathway at an earlier stage, a step immediately downstream of the production of Δ1-piperideine by the enzyme CAO [14]. This change in biosynthesis results in a distinct chemotype in accessions that are homozygous for *pauper* sweetness lacking ammodendrine (the product of

the enzyme acetyltransferase) [14]. Our study confirmed the lack of ammodendrine in the *pauper* sweet parent (FRA) and in the *pauper* sweet progeny (PAU). Comparatively, individuals that only carry the allele associated to the parent 'Dieta' and sweetness on chromosome 5 (DIA, DSW, HET) exhibited a higher QA content in these accessions due to a distinct chemotype, characterized by the additional production of ammodendrine, angustifoline and multiflorine, along with a greater accumulation of 13-OH derivatives of lupanine (13α-hydroxylupanine, 13α-angeloyloxylupanine and 13α-tigloyloxylupanine). In stacked allele recombinants (STA), only lupanine and its esterified derivates, 13α-angeloyloxylupanine and 13α-tigloyloxylupanine, were present.

Kroc et al. [4] describe that the concentrations and their ratios of white lupin QAs have specific patterns. Lupanine was the most abundant alkaloid in their panel, with a lower share in sweet accessions, which was consistent with the data presented in this study. In fact, lupanine was in all recombinants the most abundant QA and its share in the total alkaloid profile remained ca. 60% in the bitter (BIT) chemotype, in the 'Frieda' parent (FRA) and in the stacked allele recombinant (STA). While the other sweet haplotypes, such as the *pauper* associated progeny (PAU) and individuals carrying only the allele associated to the parent 'Dieta' (DIA, DSW, HET), showed a reduction of lupanine content percentage ranged from 47.31% down to 36.75%. Furthermore, Kroc et al. [4] describe the presence of lupanine being complemented only by 13α-hydroxylupanine, with a lower share in low QA accessions, i.e. higher ratio of 13α-hydroxylupanine: lupanine. This observation was confirmed in our low QA individuals carrying only the allele associated to the parent 'Dieta' (sweetness on chromosome 5; DIA, DSW, HET) which showed a significant increase in the ratio of 13α-hydroxylupanine: lupanine compared to the bitter chemotype. In the stacked allele recombinant (STA) the ratio between 13α-hydroxylupanine: lupanine was very low, due to 13α-hydroxylupanine being present only in trace amounts. However, lupanine was complemented by its esterified QAs, 13α-angeloyloxylupanine and 13α-tigloyloxylupanine indicating that 13α-hydroxylupanine has been entirely converted into its esterified QAs. These findings suggest that the determinant on chromosome 5 is involved in the biosynthetic pathway branch that begins with lupanine leading to its 13-OH derivates, in particular in the key step that catalyzes the transformation of lupanine to 13α-hydroxylupanine. This hypothesis was consistent with the putative acyltransferase function assigned to this gene in the white lupin genome browser, though it might not be the causal gene [14, 36–38].

It should be noted that recombinants carrying stacked sweetness alleles of *pauper* and *Lalb_Chr05_6643621* revealed alkaloid levels (22.8 ± 10.4 ppm) much lower than previously reported minimal values for the European white lupin gene bank [4], including domesticated materials (95414, Bialorus-1, 159 ppm; 95413, Kiev Mutant, 160 ppm and 95234, P.21525, 168 ppm). However, low QA contents in lupins have been shown to correlate to increased susceptibility to aphid infestation. In blue lupin, the highest multiplication rate of aphids was associated to the accession with the lowest QA content of 64.1 ppm [40]. Moreover, some aphids (including at least one species, *Macrosiphum albifrons*, feeding on white lupin) are tolerant for alkaloids and even use them for their own defence [41]. Although no aphid infestation occurred in the F_2 and F_3 experimental populations, the response to aphids of the stacked allele recombinants as well as general agronomic performance should be addressed. Regardless, achieved progress in white lupin seed alkaloid content reduction is similar to that reported for narrow-leafed lupin, where minimal values reached 8–55 ppm in cultivars and 4-146 ppm in breeding lines [42–44]. Such a low alkaloid content as presented in white lupin *pauper* and *Lalb_Chr05_6643621* stacked recombinants should provide a safe margin for maintaining the recommended threshold of 200 ppm according to the variability of alkaloid content between years reported in sweet narrow-leafed lupin breeding lines [42].

Conclusion

The BSA in the F_2 population between the *pauper*-sweet cultivar 'Frieda' and the sweet by unknown source cultivar 'Dieta' allowed mapping of a new QTL for QA content in white lupin located on chromosome 5. Within this QTL, candidate genes were investigated to study genotype-phenotype associations. The QTL could be exploited in the future through fine mapping, identifying other candidate genes for potential validation, and further validated in a larger and more diverse panel of accessions. Regardless, the PACE marker tagging the SNP *Lalb_Chr05_6643621* associated to the gene *Lalb_Chr05g0222381* tagging the Dieta-type sweetness, in combination with the marker tagging *pauper* are powerful tools for breeders by taking advantage of two different sources of sweetness thanks to the ability to select for the trait of low QA content at early generations discriminating for bitter and heterozygous alleles (F_2-F_3). This can support the development of white lupin cultivars with low and stable QA content regardless of the growing environment.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12870-025-06951-7.

Supplementary Material 1.

Supplementary Material 2.

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Authors' contributions
A.P.: Writing– original draft, review, editing. Conceptualization. Data collection. Analysis. Visualization. Interpretation. Data curation.; M.Ka.– Writing– original draft, review, editing. Conceptualization. Data collection. Analysis. Visualization. Interpretation.; M.S.: Writing– review, editing. Analysis. Visualization. Interpretation. Data curation.; C.A.: Writing– review, editing. Data collection. Interpretation. Field trial design. Development of segregating populations. Acquisition.; M.M.M.: Writing– review, editing. Conceptualization. Acquisition.; E. B.: Writing– review, editing. Conceptualization. Data collection. Visualization. Interpretation.; A.T.: Writing– review, editing. Conceptualization. Data collection. Visualization. Interpretation.; M.Ks.: Writing– review, editing. M. L.: Writing– original draft, review, editing. Conceptualization. Data collection. Analysis. Visualization. Interpretation. Data curation. Supervision. Acquisition.All authors have read and approve the submitted version (and any substantially modified version that involves the author's contribution to the study) and have agreed to be personally accountable for the author's own contributions and to ensure that questions related to the accuracy or integrity of any part of the work, even ones in which the author was not personally involved, are appropriately investigated, resolved, and the resolution documented in the literature.

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Data availability
Sequencing data generated and/or analysed during the current study are available in the public ENA repository, with accession number PRJEB86989. All other data generated or analyzed during this study are included in this published article and its supplementary information files.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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