A Bitter-Sweet Story: Unraveling the Genes Involved in Quinolizidine Alkaloid Synthesis in *Lupinus albus*

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Alkaloids are part of a structurally diverse group of over 21,000 cyclic nitrogen-containing secondary metabolites that are found in over 20% of plant species. *Lupinus albus* are naturally containing quinolizidine alkaloid (QA) legumes, with wild accessions containing up to 11% of QA in seeds. Notwithstanding their clear advantages as a natural protecting system, lupin-breeding programs have selected against QA content without proper understanding of quinolizidine alkaloid biosynthetic pathway. This review summarizes the current status in this field, with focus on the utilization of natural mutations such as the one contained in *pauper* locus, and more recently the development of molecular markers, which along with the advent of sequencing technology, have facilitated the identification of candidate genes located in the *pauper* region. New insights for future research are provided, including the utilization of differentially expressed genes located on the *pauper* locus, as candidates for genome editing. Identification of the main genes involved in the biosynthesis of QA will enable precision breeding of low-alkaloid, high nutrition white lupin. This is important as plant based high quality protein for food and feed is an essential for sustainable agricultural productivity.

**Keywords:** alkaloids, quinolizidine alkaloids, white lupin, *Lupinus albus*, *pauper*

**INTRODUCTION**

Lupinus, part of the Fabaceae family, is a genus of plants widely distributed around the world (Clements et al., 2008). Different cultivated species are present in the Old World (*L. albus*, *L. angustifolius*, and *L. luteus*) as well as the New World (*L. mutabilis*) (Pettersson and Mackintosh, 1994; Peterson, 2000). Despite the phenotypic differences due to environmental conditions, they are all characterized by high levels of proteins, being used as human food or animal feed (Pettersson and Mackintosh, 1994; Abraham et al., 2019; Šonta and Rekiel, 2019). The production of lupin seeds as an agricultural product occurs mainly in Australia but also it has an important role in agricultural productive system in parts of Europe, Africa, and South America (FAO, 2021). During 2019, the largest lupin producers were Australia (474,629 t), Russian Federation (103,792 t) and Poland (261,500 t) (FAO, 2021).

White lupin (*Lupinus albus*) is a legume from the Mediterranean region; its center of origin is believed to be southern Greece and Western Turkey, where its cultivation started 4,000 years ago and wild landraces still persist (Gladstones et al., 1998). White lupin (WL) is recognized as an exceptional source of protein (between 30 and 40% of the whole seed dry matter) (Bähr et al., 2014) with an adequate balance of essential amino acids, as well as an adequate protein efficiency ratio.
BIOSYNTHETIC PATHWAY

QUINOLIZIDINE ALKALOID BIOSYNTHETIC PATHWAY

Alkaloids are part of a structurally diverse group of over 21,000 cyclic nitrogen-containing secondary metabolites (Wink, 2013) that are found in over 20% of plant species (Croteau et al., 2000; De Luca and St. Pierre, 2000; Bunsupa et al., 2012b). QA are secondary metabolites that occur mostly within the Leguminosae family, but are also present in other taxa (Ohmiya et al., 1995). In lupin species, QA distinctive structure is a quinolizidine ring, which can be grouped mainly into bicyclic alkaloids, such as lupinine and its derivatives, and tetracyclic alkaloids, represented by sparteine, lupanine and hydroxilupanine (Wink, 1987). Examples exist of distinctive QA expression in different species. Isolupanine and angustifoline accumulate to high levels in L. angustifolius (narrow-leafed lupin). Albamine and multiflorine accumulate to high levels in L. albus and lupinine in L. luteus (Święcicki et al., 2019). QA vary in their toxicity and their deterrence against insect pests and mammals. Sparteine and lupanine appear to be the two most toxic QA to humans and laboratory animals (Allen, 1998; Petterson, 1998), with lupanine having the greatest impact on aphid survival, followed by sparteine, lupinine, 13α-hydroxyisolupanine and angustifoline having a moderate impact (Ridsdill-Smith et al., 2004; Philippi et al., 2015). QA toxicity against larvae from different species as well as acaricidal effect has also been reported (Hassan et al., 2019; Elma et al., 2021).

The synthesis of QA occurs through the cyclization of cadaverine, due to a L-lysine decarboxylase catalyzed by a lysine decarboxylase L/ODC (La-L/ODC) (Saito and Murakoshi, 1995; Bunsupa et al., 2012a). Cadaverine is then oxidized by a copper amine oxidase (CUAO) (Yang et al., 2017) to yield 5-aminopentanal and spontaneously cyclized to 1-piperideine Schiff base, which is a universal intermediate for the production of various Lys-derived alkaloids (Bunsupa et al., 2012b). It has been suggested that in addition to these reactions, a series of aldol-type reactions, hydrolysis, oxidative deamination and coupling gives rise to the major structural QAs [e.g., lupanine and others; (Dewick, 2002)]. The diiminium cation was proposed as an intermediate product in to yield tetracyclic alkaloids [lupanine, multiflorine, and sparteine (Fraser and Robins, 1984)]. These QA are final products, but also, can be further modified by dehydrogenation, oxygenation, hydroxylation, glycosylation, acetylation or esterification to form a wide variety of structurally related QAs (Wink and Hartmann, 1982; Saito et al., 1992, 1993; Ohmiya et al., 1995; Saito and Murakoshi, 1995; Bunsupa et al., 2012b; Boschin and Resta, 2013). Continued research will likely add more detail to the QA pathway. For example, the observation that QA can be found in high enantiomeric excess has led to the proposal that stereoselective enzyme catalysis may be involved in the QA pathway (Lichman, 2021).

GENES INVOLVED IN QUINOLIZIDINE ALKALOID BIOSYNTHESIS IN LUPINUS

Identification of genes involved in the QA biosynthesis has been partially achieved by identifying homologous genes in other species expressing QA, as in the case of Lupinus angustifolius La-L/ODC gene, which was identified as a homolog of L/ODC expressed in the distantly related species S. flavescens, E. koreensis, T. chinensis, and B. australis (Bunsupa et al., 2012a).
Genes encoding acyltransferase were described in *L. albus* and *L. angustifolius* (*LaHMT/HLT* and *LaAT*, respectively), but proof of the formation of acetylated products (13α-hydroxylycopupane and 13α-hydroxyoxymultiflorine), was only achieved for *L. albus HMT/HLT* (Saito and Murakoshi, 1995; Okada et al., 2005; Bunsupa et al., 2011).

Transcriptome experiments in different tissues of *L. angustifolius* lead to the identification of a copper amine oxidase, LaCAO (Yang et al., 2017), with cadaverine as substrate, catalyzing its transformation into 5-aminopentanal, which is then spontaneously cyclized to 1-piperideine (Yang et al., 2017). In a previous report, Okada et al. (2005) cloned and characterized an O-tigloyltransferase from WL, an enzyme involved in the final steps of QA biosynthesis. Recently, it was proven for *Lupinus angustifolius*, that RAP2-7 is a putative regulatory gene of QA biosynthesis/accumulation in aerial tissues (Kroc et al., 2019), with a S196R substitution being responsible for the bitter/sweet phenotype (Czepiel et al., 2021), but, however, additional studies are needed to determine the mechanism and effect on QA expression, and its role in different lupin species. In an effort to identify the missing enzymes of the QA pathway, the study of the existence of common enzymes between nicotine synthesis (monoterpene indole alkaloids, MIA; benzylisoquinolone alkaloids, BIA) and lupins QA has been proposed (Frick et al., 2017). Many of these enzymes (methyltransferases, decarboxylases, oxidases, acyltransferases, cytochromes-P450, oxidoreductases, demethylases, reductases, hydroxylases, and coupling enzymes) and their encoding genes have been identified in *N. tabacum*, *C. roseus*, *C. japonica*, and *P. somniferum* (Bird et al., 2003; Dewey and Xie, 2013; Hagel and Facchini, 2013; Kilgore and Kutchan, 2016; Pan et al., 2016; Thamm et al., 2016) and it is expected that they play a role in lupins QA biosynthesis (Frick et al., 2017). Sophora flavescens transcriptome analysis had also identified several genes co-expressed, such as a putative *S. flavescens* L/ODC and candidate genes clustered into the same clade as L/ODC (major latex-like protein (MLP-like), a cP450, and a ripening related protein), but their function remains unknown (Han et al., 2015). Berberine bridge and berberine bridge-like enzymes catalyze oxidative reactions for the biosynthesis of BIA (Facchini et al., 1996; Samanani et al., 2004; Kajikawa et al., 2011), possibly having similar roles in QA biosynthesis. Cytocromes-P450 have a role in hydroxylation reactions, as well as other reactions in MIA and BIA biosynthesis (Pauli and Kutchan, 1998; Thamm et al., 2016). Recently, the existence of a high number of QA biosynthesis genes controlled by a regulatory locus in NLL was reported, which supports the idea that ethylene responsive transcription factor RAP2-7 gene may control low-alkaloid phenotype in NLL, acting as a promoter of the expression of biosynthesis genes (Plewiński et al., 2019; Czepiel et al., 2021).

In addition to QAs biosynthetic genes, major loci controlling QA expression have been described in lupins. Cultivated lupins display lower alkaloid content than landraces, due to incorporation of “sweet” domestication genes, which were generated by natural mutation (Lin et al., 2009). Most of these mutations are recessive, such as *iucundus*, *esculentus*, and *depressus* in NLL, *amoenus*, *dulcis*, and *liber* in *L. luteus* (Lin et al., 2009). In *L. albus*, several loci have been reported to produce low alkaloid genotypes, with the pauper locus being the most effective and used worldwide in breeding programs (Gladstones, 1974; Harrison and Williams, 1982). In *L. mutabilis*, the low alkaloid phenotype is controlled by several alleles (Clements et al., 2008). It is worth highlighting that none of the mutations identified in lupin completely eliminate QAs (Harrison and Williams, 1982).

### Pauper LOCUS

Construction of low-density linkage maps allowed identification of genomic regions involved in alkaloid biosynthesis in white lupin (Phan et al., 2007; Croxford et al., 2008; Vipin et al., 2013). There has also been development of molecular markers to identify QTLs responsible for low alkaloid content linked to these recessive loci (Yang et al., 2010; Raman et al., 2014). The development and mass use of GBS technology as a tool for breeders (Elshire et al., 2011; Annicchiarico et al., 2017), enabled progress to identify causative genes for low QA content in *L. albus*. High-density consensus maps for comparisons between *L. angustifolius* and *L. albus*, had led to the hypothesis that the *iucundus* locus responsible for low alkaloid content in NLL differs by function from pauper in WL (Książkiewicz et al., 2017).

Worldwide, *L. albus* breeding programs have relied mainly on the effect of pauper locus to produce sweet varieties, for both food and feed. Besides its importance, little is known about the pauper locus gene(s) with respect to their effect on alkaloid content. Earlier studies identified at least two different alleles for pauper locus, controlling total alkaloid content (Harrison and Williams, 1982). The action of this recessive locus was suggested as a reduction of a common alkaloid substrate, which seems to be the ubiquitous for most lupin low alkaloid genotypes, without affecting intermediate substrates at late biosynthetic stages, when chemical differences among alkaloids are being finally specified (Harrison and Williams, 1982).

Genetic and comparative map of *L. albus*, based on a RIL population developed from Kiev (Ukrainian cv, sweet, early flowering, anthracnose susceptible) and P27174 (Ethiopian landrace, bitter, late flowering, anthracnose resistant), allowed the discovery of 220 amplified fragment length polymorphisms and 105 gene-based markers, enabling for the first time mapping of the alkaloid locus, with flanking markers located in a region within 20 cM in both directions (Phan et al., 2007). Later, with the development of Pauper M1, a molecular marker more closely linked to the pauper locus (1.4 cM), allowed discrimination of low alkaloid content genotypes with efficiencies restricted to ~95% for bitter and 91% for sweet non-pauper lines. Implementing Pauper M1 required the use of sequencing gels and radioisotope primer labeling for the correct determination of alleles, which made its application restricted to authorized facilities (Lin et al., 2009). With the aid of GBS, a high-density consensus linkage map of WL genome was constructed, integrating 453 published markers with 3,597 newly developed sequence-based markers, recovering a single linkage group per every chromosome (Książkiewicz et al., 2017). This map yielded several new markers co-segregating, or closely localized to the
pauper locus than the Pauper M1 (Książkiewicz et al., 2017). In an effort to improve Pauper M1 efficacy, CAPS markers were developed (using two identified SNPs), which were substrates for restriction enzymes, \( HhaI \) for the bitter allele, and \( Hinfl \) for the sweet allele (Rychel and Książkiewicz, 2019). One of these, LAG101_35805_F1_R1 homologous to LaAT (AB581532.1), different than pauper locus gene, showed higher efficacy than Pauper M1 (Rychel and Książkiewicz, 2019).

Recently a high quality reference \( L. \) \textit{albus} genome allowed the study of the pauper genomic region, identifying several candidate genes on Chr18. This research demonstrate the existence of a gene cluster in the pauper locus, which comprises a 958 kb region and contains 66 genes, amongst several are strong candidates genes encoding enzymatic activities, such as cinnamoyl-CoA reductase and acyltransferases (Hufnagel et al., 2020a). In addition to the reference genome, transcriptomic data from different organs, resequencing of 15 varieties and a pangenome dataset provide tools for further exploration of the genomics of alkaloid content\(^1\) (Hufnagel et al., 2020b) and the complex role of the pauper locus. Analysis of the pauper marker associated with low QA was carried out in a landrace, breeding lines and cultivars of \( L. \) \textit{albus} and QA was measured by UHPLC-HRMS. Interestingly while the marker did associate with low QA and was absent in many high QA samples, there were notable exceptions where the marker was found in high QA sample (Zafeiriou et al., 2021). Thus additional genes and regulatory elements may be important in reaching breeding objectives.

**PROSPECTIVE TECHNIQUES TO UNRAVEL BIOSYNTHESIS ALKALOID GENES IN WHITE LUPIN**

The main drawback to study candidate genes and their function in WL is the recalcitrant nature of this legume to tissue culture (Nguyen et al., 2016; Aslam et al., 2020). Several attempts have been undertaken to develop \textit{in vitro} regeneration tissue culture systems, but with limited success, which today represent a challenge in WL breeding programs (Bayliss et al., 2002; Uhde-Stone et al., 2005; Surma et al., 2013; Raza et al., 2017; Che et al., 2019; Aslam et al., 2020). It is possible, as in monocot species, that transformation is highly cultivar dependent, therefore a genetic screen for transformation aptitude in WL collections may produce a genotype amenable to transformation and thus genome editing. But, in the meantime, to overcome this situation, different reverse genetics methodologies (Till et al., 2007), such as random mutagenesis and virus induced gene silencing (VIGS) may be used to probe gene function (Gupta et al., 2013).

\(^1\)https://www.whitelupin.fr/
During the last years, the use of transient expression has facilitated gene-discovery by utilization of VIGS (Gupta et al., 2013). VIGS is an effective tool to characterize functions of candidate genes using post-transcriptional gene silencing (PTGS), which is extensively used for gene knockdowns in plants (Liu et al., 2016; Wang et al., 2016; Zhang et al., 2016). However, VIGS can also be applied as a forward genetics technique to study gene function by using cDNA libraries (Kilgore and Kutchan, 2016; Thamm et al., 2016).

In white lupin, VIGS using peanut stunt virus proved to be effective tool to silence the Phytoene desaturase gene (LaPDS) (Yamagishi et al., 2015), opening possibilities for utilization of this technique to elucidate genes participating in secondary metabolite synthesis such as QA.

Advances in CRISPR-Cas technology allow fine-tuning of gene-activity and the generation larger chromosomal variation, providing a broad toolkit for gene-function analysis (Jinek et al., 2012; Cong et al., 2013; Jung and Till, 2021). Nevertheless, optimization of CRISPR technology is needed to accommodate the tissue and transformation delivery method (Char et al., 2017). Recent developments of new transformation techniques based on the utilization of functionalized magnetic nanoparticles to deliver DNA, has been proven successful in species such as cotton, sunflower and lily (Zhao et al., 2017), opening the possibilities to bypass in vitro regeneration in legume species, such as lupin. With the aid of magnetic fields, nanoparticles can efficiently deliver CRISPR vectors through pores present in the pollen grains, producing transformed pollen which is then used to pollinate emasculated flowers, resulting in transformed seeds (Zhao et al., 2017). The utilization of functionalized magnetic particles to deliver DNA into pollen grains, and to accelerate selection of desired individuals using speed-breeding (Watson et al., 2018; Lew et al., 2020) increases transformation efficiency and bypasses tissue culture procedures to generate plants from transformed seeds within a short period of time, broadening the possibilities for WL utilization as mayor knowledge on QA synthesis is achieved. Continued advances in nanoparticle technologies may make CRISPR-Cas approaches amenable in many recalcitrant species (Ma et al., 2021).

SUMMARY

Quinolizidine alkaloid synthesis has an important number of unresolved questions, which hinders breeding efforts worldwide in a crop with high nutritional quality such as WL. Utilization of techniques to study and manipulate genes involved in alkaloid synthesis in L. albus will contribute to a better understanding of the accumulation of secondary metabolites in lupin seed (Figure 1), contributing to the development of environmentally friendly and sustainable sources of plant protein, which are expected to be a key component of conscientious population growth. Continued efforts in white lupin breeding, leveraging knowledge gained of the genetics of QA synthesis, can have an important role in human nutrition and well being.

AUTHOR CONTRIBUTIONS

CO and BT wrote, edited and reviewed the manuscript, and approved the submitted version.

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