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Title: A quinolizidine alkaloid O-tigloyltransferase gene in wild and domesticated white lupin (Lupinus albus)
Year: 2007
Journal: Annals of Applied Biology
Volume: 151
Pages: pp357-362
ISSN: 0003-4746
URL: Keywords: Alkaloids
broad-leaf lupin
HMT/HLTase gene
intron
wild white lupin.
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A quinolizidine alkaloid O-tigloyltransferase gene in wild and domesticated white lupin (*Lupinus albus*)

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Abstract

Wild white lupins have high levels of alkaloids which cause a bitter taste whereas domesticated white lupin varieties have a very low content of alkaloids in seeds. Bitter genes from wild white lupins are a contamination threat to domesticated white lupin via cross pollination. The gene(s) for alkaloid synthesis have not been clearly identified, and the associated molecular background among wild white lupin, domesticated and contaminated domesticated plant materials is unknown. So far only tigloyl-CoA:(-)-13alpha-hydroxymultiflorine/(+)-13alpha-hydroxylupanine O-tigloyltransferase (HMT/HLTase) cDNA has been cloned based on protein analysis, which was suggested as encoding a quinolizidine alkaloid transferase regulating quinolizidine alkaloid biosynthesis. This gene has not yet been well characterized in important white lupin genotypes. In this study, we found that the majority of the intron sequence of the HMT/HLTase gene differed between wild white lupin accessions P25758 and P27593, and between the commercial varieties. The expression pattern as well as the expression level of the HMT/HLTase gene showed no difference between P25758 and the low-alkaloid variety Kiev-mutant, suggesting the expression of the HMT/HLTase gene has no correlation with bitterness. However, the intron sequence is useful as a DNA marker in the identification of the contamination source of bitter seeds in commercial lupin seed lots.

Key words: Alkaloid, broad-leaf lupin, HMT/HLTase gene, intron, wild white lupin

Introduction

Alkaloids in lupins cause a bitter taste and are toxic. Wild white, or broad-leaf lupins (*Lupinus albus*) have high levels of alkaloids (>1% w/w). Fully domesticated white lupin varieties have a very low content of alkaloids in seeds (<0.02%), which makes them suitable for human food and animal feed. Such varieties are referred to as "sweet". Domesticated varieties were developed from mutants (both natural and induced) of wild white lupin, in which the mutations have disrupted the genes playing an important role in alkaloid synthesis (Gladstones 1970; Harrison & Williams 1982; Cowling *et a*l., 1998). In Australian commercial *L. albus* crops the *pauper* low-alkaloid gene is employed exclusively as it conditions a very low alkaloid level, and the phenotype is relatively insensitive to environmental conditions. Kiev-mutant is an example of a *pauper* low-alkaloid variety (originally from Ukraine), while Start is a low-alkaloid variety from Russia with a different mutation (*exiguus*). Magna was an Australian variety based on *pauper* but was withdrawn from industry following low-frequency bitter contamination in the foundation seed. Subsequently, other commercial varieties were shown to have low-frequency bitter contamination. Wild *L. albus* plants are not found in Australia, however, bitter genotypes are used in breeding as sources of valuable characters (e.g. disease resistance). Consequently, it was of interest to determine how contamination of sweet varieties had occurred.

Bitter genes from wild white lupins are a contamination threat to domesticated white lupin via cross pollination which is caused, primarily, by foraging bees. The outcrossing rate in *L. albus* has been estimated at 8% (Faluyi & Williams, 1981). The dominant bitter allele frequency increases through propagation due to within-crop outcrossing and its increased fitness level. Crosses between domesticated white lupin varieties and other lupin species (e.g. *L. angustifolius*) are incompatible, therefore, wild white lupin genotypes are the most likely contamination source. Although a method using UV light is available to detect the sweet white lupin seed lots showing bitterness contamination, the genes for alkaloid synthesis have not been clearly identified, and the associated molecular background among wild, domesticated, and contaminated domesticated plant genotypes, is unknown.

To date the only alkaloid synthesis gene cloned is tigloyl-CoA:(-)-13alpha-hydroxymultiflorine/(+)-13alpha-hydroxylupanine O-tigloyltransferase (HMT/HLTase). It was derived from cDNA and was cloned based on protein analysis (Suzuki *et al.,* 1994; Okada *et al.,* 2005). HMT/HLTase regulates the final step of the biosynthesis of ester-type quinolizidine alkaloids, converting hydroxylated alkaloids to their esters (Suzuki *et al.*, 1994). Tigloyl esters of quinolizidine alkaloids are the major forms found in *Lupinus* plants (Suzuki *et al.,* 1994). The ester-type alkaloids are assumed to be the end-products of biosynthesis and thus the forms for transport and storage (Okada *et al.,* 2005). It has been reported that the HMT/HLTase gene is expressed specifically in root and hypocotyls, and has only limited expression in cotyledon and leaf of white lupin plants (Okada *et al.,* 2005). However, this gene has not yet been well-characterized in a range of different white lupin genotypes.

In order to clarify whether this gene has any association with alkaloid content in white lupin seeds, and to detect possible molecular background alterations among white lupin genotypes, based on the published sequence data of this gene, we isolated the related sequence from the genomic DNA of two wild lupin accessions: P25758 and P27593, plus several domesticated white lupin varieties, and examined sequence polymorphism. The expression of the HMT/HLTase transcripts was also analyzed by means of reverse transcriptase polymerase chain reaction (RT PCR).

Materials and Methods

Plant materials

The following *L. albus* genotypes were studied: wild lupin accessions P25758 (originally from Crete) and P27593 (originally from the Azores islands), the low-alkaloid domesticated lupin varieties Kiev-mutant, Start, Ultra, and Magna; and contaminated seeds (designated as Pink seed) from commercial seeds lots identified by using UV light screening at 365 nm. Bitter seeds appear pink in colour and fluoresce under the UV light. P25758 and P27593 are, strictly speaking, semi-domesticated genotypes since they both have permeable (soft) seeds and non-shattering pods. However, they both retain the wild type high-alkaloid character. All the genotypes were provided by the lupin breeding program at the EH Graham Centre for Agricultural Innovation, NSW DPI, Agricultural Institute, Wagga Wagga, NSW Australia.

DNA and RNA extraction

Lupin seeds were germinated in an incubator at 22ºC, in 12 h dark/12 h light. Hypocotyls of three week-old seedlings (for each Pink seed, a single seedling was used) of all the materials were used for DNA extraction employing a DNeasy® Plant Mini Kit (Qiagen GmbH, Hilden, Germany), as well as for RNA extraction using Nucleospin® RNA Plant (Macherey Nagel, Düren, Germany). For RNA extraction, the DNase treatment was included in the procedure which followed the instructions of the kit manufacturer. In addition, for the genotypes P25758 and Kiev-mutant first true leaves and mature seeds (in addition to hypocotyls) were also used for RNA extraction.

Isolation of HMT/HLTase gene sequence

For the isolation of the HMT/HLTase gene from the genome of P25758, the middle part of the published gene sequence was amplified by PCR. PCR primers were ALF3 and ALR2, ALF2 and ALR3 (Table 1). The 25 µl reaction mixture included: 1× PCR buffer, 1.5 mM $MgCl_2$, 0.25 mM dNTPs, 0.25 µM each primer, 20 ng DNA, and 1 unit Taq DNA polymerase (Roche Diagnostics GmbH, Mannheim, Germany). The thermo cycle program was 94°C for 2 min, followed by 94 °C for 30 s, 56°C for 1 min, and 72°C for 2 min,

repeated for 40 cycles.

The 5'end and 3'end of the gene were isolated by using the Universal Vectorette™ System (Sigma-Aldrich, St Louis, MO, USA), the primers used were, ALF4 and ALFA (for 3' end walking) and ALR6 (for 5' end walking) (Table 1). ALF3 and ALR4 (Table 1) were used in PCR to isolate part of the intron area from genotype P27593. The PCR products were purified using a QIAquick Gel Extraction Kit (Qiagen GmbH, Hilden, Germany). The purified PCR products were sequenced employing a BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), and analyzed on a 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA).

cDNA synthesis and RT PCR

M-MLV reverse transcriptase (Promega, Madison, WI, USA) was used to synthesize cDNA. Anchor primers were random hexamers. 1.5 µg RNA (measured using a UV spectrophotometer at 260 nm wave length) was used in each reaction. The cDNA samples were diluted to 1:50, and 2 µl were used in each PCR reaction, the reagent of which were the same as those used in the PCR of genomic DNA. RT PCRs using 18S rRNA gene primers (18SRRNAF, 5'GAATTAGGGTTCGATTCCGGAG3', 18SRRNAR, 5'GTGCTTGATTCTGGTGATGGTG, which were designed based on the conserved sequence compared among different species) were performed to check the concentration of the samples. The reagents used were the same as those in the PCRs of genomic DNA. The thermo cycle program was: 94°C for 2 min, followed by 94 °C for 30 s, 58°C for1 min, and 72°C for 30s, repeated for 28 cycles. To measure the HMT/HLTase gene expression in different tissues of genotypes P25758 and Kiev-mutant, the primers ALF3 and ALR4 were used, and the thermo cycle program was 94°C for 2 min, followed by 94 °C 30 s, 56°C 1 min, and 72°C 1 min, repeated for 36 cycles. Other RT PCRs listed in Table 2 followed the same program.

Results

The DNA sequence of the HMT/HLTase gene isolated from genotype P25758 contained a 656 base pair intron (accession no. EF381744), and the same intron sequence was found in the domesticated varieties Kiev-mutant, Start, and Ultra (data not shown). PCR products generated by primer pairs from within the same exon showed no polymorphism (Table 2), neither did primer pairs bridging only Exon 1 and the Intron, or Exon 2 and the Intron. However, primer pairs from different exons that included the Intron in the PCR product, showed polymorphism among the genotypes (Table 2). The PCR products generated by primers ALF3 and ALR4 are shown in Figure 1A.

Primer pairs that generated RT PCR products from mRNA failed to show any polymorphism across all the genotypes tested. Some of the RT PCR results, such as the RT PCR products

produced by ALF3 and ALR4, are given in Table 2 and shown in Figure 1B.

These results indicate that the analyzed genotypes differed only in their intron sequences. Sequence data from the intron area showed that a 231 base pair region close to the 5' end was identical between P25758 (accession no. EF381744) and P27593 (accession no. EF381745). Since the primers ALF5 and ALR5 bind in this region, no polymorphism was detected when using them (Table 2). Based on those results, the genotypes analyzed can be divided into two groups, namely, group1: P25758, Kiev-mutant, Start and Ultra; and group 2: P27593, Magna and Pink seeds.

The expression of HMT/HLTase in the hypocotyls, first true leaves and mature seeds in P25758 and Kiev-mutant was examined by means of RT PCR. PCR product generated by 18S rRNA gene primers was used as an internal standard. The HMT/HLTase gene transcripts had higher levels of expression in hypocotyls and first true leaves than in seeds (Fig. 2). P25758 and Kiev-mutant shared the same expression pattern (Fig. 2). Since the primers (ALF3/ALR4) were chosen from different exons, the product shown in Fig. 2 represented the spliced fragment, which excluded any possibility of genomic DNA contamination. This result indicated that the level of HMT/HLTase gene transcripts is not associated with the alkaloid content of seeds in these genotypes.

Discussion

In this study we found that two types of intron of the HMT/HLTase gene exist among different wild and domesticated white lupin varieties. This suggests that the intron difference in this gene existed before the full domestication of the white lupin. Based on this evidence we suggest that the domesticated varieties Kiev-mutant, Start and Ultra originated from a P25758-type genetic background, and Magna from a P27593-type background. Since the domesticated varieties (all low-alkaloid) had either the P25758-type or the P27593-type intron in their HMT/HLTase gene, the intron difference is unlikely to be associated with the observed alkaloid levels in seeds.

The RT PCR expression pattern of the gene product was similar in wild and domesticated white lupin (expressed more in hypocotyls and first true leaves than in seeds), and the expression in seeds showed no significant difference between P25758 and Kiev-mutant (Fig. 2). The expression of HMT/HLTase at a high level in hypocotyls is in agreement with the results reported by Okada *et al*., (2005). It has been proposed that root and hypocotyls are the main organs for the biosynthesis of quinolizidine alkaloids (Okada *et al*., 2005). Within plant alkaloid distribution was measured in *L. albus* by Williams and Harrison (1983). They showed production in all tissues but preferential accumulation in seeds.

The sequence difference of the intron, as well as the expression of HMT/HLTase gene showed no correlation with bitterness (alkaloid content) in seeds. We assume that there are

other gene(s) blocking the transport or accumulation of alkaloids in seeds associated with the *pauper* character in domesticated varieties such as Kiev-mutant, and the *exiguus* character in Start. The *pauper* gene causes a large reduction in all of the main alkaloids found in the *L. albus* wild type and, therefore, is likely to be the mutant of a gene that operates early in the alkaloid biosynthesis pathway (Harrison & Williams 1982). This is in contrast to the HMT/HLTase gene which is thought to operate at the end of the pathway (Suzuki *et al*., 1994).

Although the result in the current study suggests that the HMT/HLTase gene is not the one mutated to produce the *pauper* character, the sequence of two different types of intron may be useful in the identification of the contamination source of bitter seeds in commercial lupin seed lots. The contaminated bitter seed samples analyzed in the current study (Pink seeds) may have originated from P27593 or Magna but not from Kiev-mutant, Start, Ultra or P25758.

Acknowledgements

This study was supported by the Grain Foods CRC (Cooperative Research Centre). We thank the Southern Cross Plant Genomics Laboratory (SCPG) for their assistance and services for DNA sequencing and analysis.

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Tables

Table 1 Primers located in different area of HMT/HLTase gene and their sequence.

Location	Primer name	Sequence (5' to 3')
Exon 1 (E1)	ALF2	TAGTGTTTAAAGTGAGGAG
	ALF6	GTTGATTGTACTGGTGAAGGAGTC
	ALR6	ACTCCTTCACCAGTACAATCAACC
	ALF3	CTCCTTTATGATGTTCCTGG
Intron (I)	ALF5	GGTTGGACAGACACTCATAGTGG
	ALR5	CCACTATGAGTGTCTGTCCAACC
Exon 2 (E2)	ALF4	CACGCCTCAAATGTGGTGG
	ALRW	CCACCACATTTGAGGCGTG
	ALR4	GTGTTCTACAACGCCATAGG
	ALR3	CCATAACGTGCATTGAGAATG
	ALR2	TTCTCAAACCTCTCCATGGC
	ALFA	CCTCATACTTCTACAATGATGTAG

Table 2 Monomorphism or polymorphism of the PCR products generated by primer pairs from the HMT/HLTase gene among different white lupin genotypes. M represents monomorphism, P1 and P2 represent two polymorphic PCR products. cDNA indicates products from RT PCR.

Primer pair	Type*	Magna	P27593	P25758	Kiev-mutant	Start	Ultra	Pink seed 1	Pink seed 2	Pink seed 3
Alkaloid level:	-	sweet	bitter	bitter	sweet	sweet	sweet	bitter	bitter	bitter
ALF2/ALR6	E1	M	M	M	M	M	M	M	M	M
ALF2/ALR5	E1-I	M	M	M	M	M	M	M	M	M
ALF4/ALR4	E2	M	M	M	M	M	M	M	M	M
ALF4/ALR3	I-E2	M	M	M	M	M	M	M	M	M
ALF4/ALR2	E2	M	M	M	M	M	M	M	M	M
ALF2/ALR3	E1-I-E2	P1	P1	P2	P2	P2	P2	P1	P1	P1
ALF6/ALR3	E1-I-E2	P1	P1	P2	P2	P2	P2	P1	P1	P1
ALF3/ALR2	E1-I-E2	P1	P1	P2	P2	P2	P2	P1	P1	P1
ALF3/ALR3	E1-I-E2	P1	P1	P2	P2	P2	P2	P1	P1	P1
ALF3/ALR4	E1-I-E2	P1	P1	P2	P2	P2	P2	P1	P1	P1
ALF2/ALR3 (cDNA)	E1-I-E2	M	M	M	M	M	M	M	M	M
ALF2/ALR2 (cDNA)	E1-I-E2	M	M	M	M	M	M	M	M	M
ALF2/ALR4 (cDNA)	E1-I-E2	M	M	M	M	M	M	M	M	M

* Type of PCR product produced: E1 = within Exon 1 only, E2 = within Exon 2 only, I = within Intron only, I-E2 = spanning Intron and Exon 2, and E1-I-E2 = spanning Exon 1, Intron and Exon 2.

Figures and figure legends

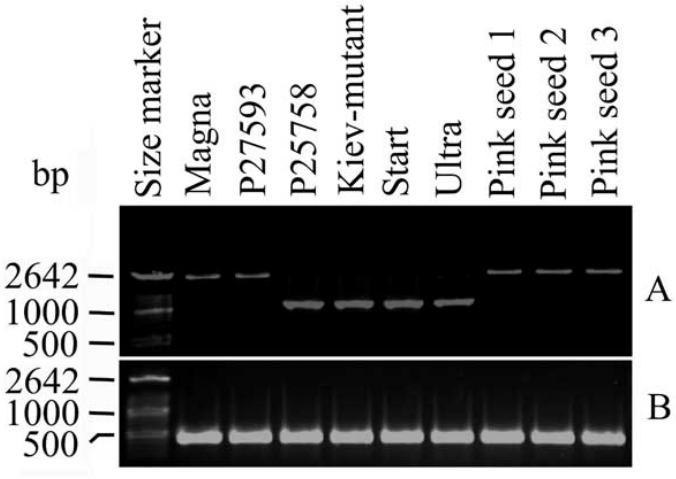

Figure 1 PCR product of the HMT/HLTase gene generated by the cross-intron primer pair ALF3/ALR4 among different white lupin genotypes. A, genomic DNAs of the genotypes were used as templates. B, cDNAs were used as templates. cDNAs were synthesized using mRNA from hypocotyls..

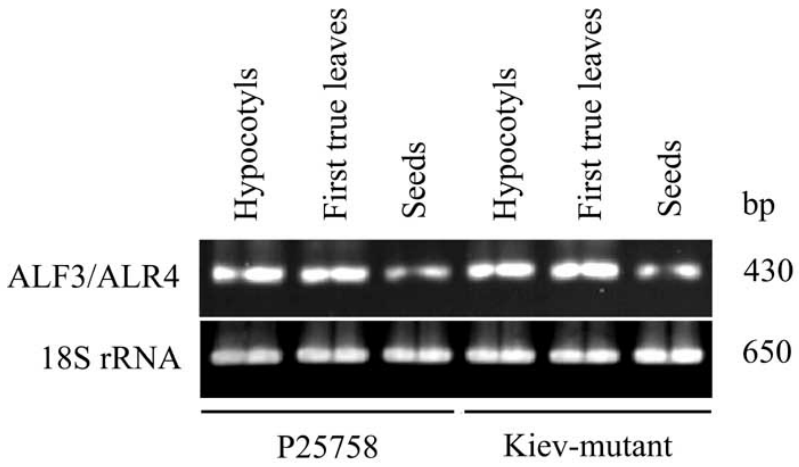

Figure 2 RT PCR product of HMT/HLTase gene generated by primer pair ALF3/ALR4 in different tissues of P25758 and Kiev-mutant. RT PCR product of 18S rRNA was used as an internal standard.

Appendix
(for reviewers)

HMT/HLTase gene sequence in genotype P25758 of *Lupinus albus*.
ATG start codon
AGGT splicing site
TGA stop codon
Italic letters indicate intron area.

AAATACCATTCATACTACTAAAAACGTATTATATATATATGTGGTAGCTAATGGAAG
GTATCTCTTCCTTAACACAAAACATGGCTCCCCAAACTCAATCTCTAGTGTTTAAA
GTGAGGAGAAACCCACAGGAGCTAGTAACACCAGCAAAGCCAACACCCAAAGA
ATTTAAGCTTCTTTCTGACATAGATGATCAAACTAGTTTGCGTTCCCTCACCCCTTT
AGTAACAATATATCGCAATAACCCATCAATGGAAGGAAAAGACCCAGTTGCAATC
ATTAGAGAAGCACTTTCTAAGACACTTGTGTTTTACTATCCATTTGCTGGAAGACT
TAGAAATGGTCCTAATGGAAAATTAATGGTTGATTGTACTGGTGAAGGAGTCATTT
TCATTGAAGCTGATGCAGATGTCACACTGGATCAGTTTGGTATTGATCTTCATCCT
CCATTCCCTTGCTTTGATCAGCTCCTTTATGATGTTCCTGGTTCAGATGGAATTCTT
GATTCTCCACTCCTTCTTATACAG*GT*TCATTCTAAACATTCTTCCTTTATGTTTTTATTT
GTTATATGTATTTTAAATTATGGTTCATGTTTTTGTTCAGTTTGGTATGATGTGATTGCGTT
GTAATGTATTTTAAATTATGGTTCATGTTTTTGTTCAGTTTGGTATGATGTGATTGCGTTGT
AGTCCGATATAGATGTACTATTATAATTCGGCATCATATTACTATTAAAGTATGATGTAGGG
ATGACAATAAGGTTGGACAGACACTCATAGTGGGCCGGGCGACATAGATAGTGTATACT
TGTACCCATAATCACTTGTCCAATTTTTGGTGTTCCAATGTATACCAATCCAGTTAAAAAC
TGACACGTAATCAATCCAGTTACATTACCTGGTATAGTGTGATTGACTTGGGACATAAAT
ACATAAATTTTTTAGAACAGATGATCCTTATTCTTATTTTTACTTTCACTGTTGAGTCATAA
TTATAATCACATTTTATAATCAACCACTCATACACTATTGGGTTTTAAACTGTACTCCTATT
GTATATACTGTAATAGCAGGAAAAGATACTGGATGATGCATATATAAATGTTATTATGACA
GTGTTGTTGCAACACTGAATGAATATATCTAATTACCATTGTGCTTATGACAGTTGAAATT
TTGAAAAACTAAATGCATATATCTAATTACCATTATATATGCTTATGAAATTGTATAAATAAT
GGT*AG*GTAACACGCCTCAAATGTGGTGGATTTATCTTTGCTGTGCGTTTAAACCAT
GCTATGTGTGATGCAATTGGAATGTCTCAGTTCATGAAGGGTTTAGCAGAAATAG
CTCGTGGTGAACCAAAACCTTTCATTCTTCCTGTGTGGSATAGAGAACTTTTATGT
GCAAGAAACCCACCAAAAGTTACATTCATCCATAATGAATACCAAAAACCACCAC
ATGATAACAACAATAACAACTTCATACTCCAACATAGTTCCTTTTTCTTTGGCCCT
AATGAGTTAGATGCTATTCGTCGTCTTCTTCCATATCACCACTCAAAATCTACTACA
AGTGACATCCTCACAGCATTCCTATGGCGTTGTAGAACACTTGCATTACAACCAG
AAAACCCTAATCACGAGTTTCGCCTATTGTACATTCTCAATGCACGTTATGGACGT
TGCAGCTTCAACCCTCCACTCCCTGAAGGTTTTTATGGCAATGCTTTTGTTTCACC
AGCAGCAATTAGCACTGGAGAAAAACTTTGCAATAACCCTCTTGAGTATGCTTTA
GAGTTAATGAAGGAAGCGAAAAGTAAAGGAACTGAGGAATATGTGCATTCTGTG

GCAGATCTTATGGTGATCAAAGGAAGACCCTCATACTTCTACAATGATGTAGGGTA
TTTAGAGGTATCAGATCTTACAAAGGCAAGGTTTAGGGATGTAGATTTTGGGTGG
GGTAAGGCTGTGTATGGTGGAGCAACTCAAGGTTATTTTAGTAGCATTCTCTATGT
TTCTTATACCAACTCCAAAGGAGTTGAAGGTATAATGGCACTAACTAGCCTTCCAA
CAAAAGCCATGGAGAGGTTTGAGAAAGAGTTGGATGATTTATTCAAGACAAAGG
ATAAGTCTCAAATATTAAGGTCACACATT**TGA**TACAAGGTTTGTTGATAGAAGGTT
TGTTAGTACATGAAAACTTGTTTGGGACAACAATAGTGGATAACATATGAGATAGT
AGATTTATGCGTATGTTGGTGAATATGTAAGTATTTTGGTTGAATATGGGCTCATAT
TCTCTAGTATTTGCTATTTGATGATCGTCAGTAAAAACAACAGATAGAGCTTTCGT
TCTTATGGAGGCTATTGGATACATTAAATTCCTTCAAGGCCAAAAATTTTAAGGGT
ATGAAATGTTAATACCCAACTGTAATATATGAATTTGGATATTGAAG

A part of the intron of the HMT/HLTase gene in genotype P27593 of *Lupinus albus*.

TCCACTCCTTCTTATAC**AG***GT*TCATTCTAAACATTCTTCCTTTATGTTTTTATTTGTTATA
TGTATTTTAAATTATGGTTCATGTTTTTGTTCAGTTTGGTATGATGTGATTGCGTTGTAGT
CCGATATAGATGTACTATTATAATTCGGCATCATATTACTATTAAAGTATGATGTAGGGATG
ACAATAAGGTTGGACAGACACTCATAGTGGGCCGGGCGACATAAATAGTGTATACTTGT
ACCCATA (above is part of the intron identical to that of P25758)

(following is part of the intron different from that of P25758)
CCCGCACTATTTGCGTGTACCGGTATGCGTAGCGGGTATCCGCGGATACATAACAAGTT
TTCAAATTGAATTATATTTTTATACATTTTACAAT--AATTAATATATAAAGATAATTATTATTTG
AATAATATGATATTTATATGATAATTTTATCATTATAGTCTCATCATTAAAAAAATTATTATGA
AATAATAATAAAATATACATGTTAAAATAATTATAATTAAGTATAAAATATATAATTTAAATATAT
ACGTTAGTACCCGTAGTTACTTTTGCAAGTACCGATTATGTGTATTTTGATGGGTACCTA
CGGGTCGAGTATTTTTGTAGTGTGCTATGATTCTGATTGTGACAACATCCACAGTATCCA
ATCAGATGTGATATGCAATGTGCA-ATTTAAAAGAATGCATCCAACTTGTTTTAACTTAGT
TGCATGATTATAAGTTATTAACGATCTTATAGCCTAAGTGATTAGTTTTTTCATA
AGCTAAACTATAATATTATTGAATACCAGTAAGGGTGTCTACAGTAGGTGTTGAATAGCT
TTTATGCACACAAAAAAATCATCTGATTTAAACACAATATTAATTTGTGT