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Comparative proteome analysis of seed storage and allergenic proteins among four narrow-leafed lupin cultivars

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Abstract

Lupin is an emerging crop worldwide due to its wide range of health benefits. In this study, a comprehensive proteome analysis was conducted using mature seed of four narrow leafed lupin cultivars, Uniharvest, Yorrel, Tanjil and Coromup, through two-dimensional gel electrophoresis followed by mass spectrometric protein sequencing. Two-dimensional gels recognized about 400 protein spots among the cultivars in the 10-100 KDa molecular weight and 5.0-8.5 PI ranges. The results revealed a considerable variation of protein expression patterns with a total of 24 proteins showed differential expression among the cultivars, among which 19 were identified as β-conglutin, and 8 were identified as allergenic proteins. Most of the α, δ and γ conglutins were showing similar expression among the cultivars. Overall, the differentially expressed proteins especially the cultivar specific proteins would be valuable markers for cultivar identification and for screening parental lines of low allergenicity in breeding process.

Keywords: Lupin, cultivars, protein, two dimensional gel electrophoresis, differential expression, allergenic

1. Introduction

Recently, functional foods are attracting more attention at the consumer level due to their potential to provide health benefits. Proteins sourced from plants are considered to be valuable ingredients by the food industry in the preparation functional foods (Sirtori, Eberini & Arnoldi, 2007). Although soybean is currently the major source of plant protein in food preparation, other grain legumes especially lupin are in rapid development as protein sources (Dijkstra, Linnemann & van Boekel, 2003). The use of lupin as a food or food ingredient is increasing due to its nutritional properties and lower levels of anti-nutritional factors (Petterson, Sipsas & Mackintosh, 1997). The nutritional properties of lupin include its high protein content (Sirtori et al., 2004) along with a higher content of fibre (Gorecka, Lampart-Szczapa, Janitz & Sokolowska, 2000), oligosaccharides (Zdunczyk, Juskiewicz, Frejnagel & Gulewicz, 1998), and phenolic compounds content (Lampart-Szczapa, Korczak, Nogala-Kalucka & Zawirska-Wojtasiak, 2003). Due to the increased concern over the GMO issues, lupin is increasingly replacing soybean in the food industries (Leduc, Moneret-Vautrin & Guerin, 2002).

Lupin flour is mainly used as an additive to wheat flour or a substitute for other protein rich flours in food preparations. Lupin enriched foods provide health benefits such as increased satiety and reduced energy intake (Lee et al., 2006), decreased blood pressure (Lee et al., 2009), decreased blood glucose level (Hall, Thomas & Johnson, 2005) and cholesterol-lowering effect (Martins et al., 2005). The lupin seed proteins have been considered to be the main contributor to these claimed health benefits. The other reported bioactivities of lupin protein include plasma cholesterol and triglyceride lowering effects (Sirtori et al., 2004), antihypertensive properties (Pilvi, Jauhiainen, Cheng, Mervaala, Vapaatalo & Korpela, 2006) and angiotensin

converting enzyme (ACE) inhibitory activity (Yoshie-Stark, Bez, Wada & Wa¨sche, 2004). Since lupin is becoming popular as a food ingredient there have been reports concerning its allergic properties. Usually seed storage proteins are considered as the cause of allergic reactions upon ingestion (Breiteneder & Radauer, 2004), suggesting that more defined information on lupin seed protein is crucial for the continued adoption of this legume grain in the food industry.

A number of studies have been carried out on the seed proteins of lupin focused on the storage protein compositions (Duranti, Consonni, Magni, Sessa & Scarafoni, 2008; Magni et al., 2007; Sironi, Sessa & Duranti, 2005), nutritional value (Brand, Brandt & Cruywagen, 2004; Lqari, Vioque, Pedroche & Millan, 2002), protein modification due to processing (Islam, Ma, Yan, Gao & Appels, 2011b; Sirtori, Resta, Brambilla, Zacherl & Arnoldi, 2010) and immunological and health properties of the protein fractions (Goggin, Mir, Smith, Stuckey & Smith, 2008; Guillamon et al., 2010; Klos, Poreba, Springer, Lampart-Szczapa & zefiak, 2010). However, the mechanisms of differential accumulation of various protein components that result in differences in seed quality (taste, allergenicity) and morphology (Kottapalli et al., 2008) remain largely unknown (Ruuska, Girke, Benning & Ohlrogge, 2002).

Two-dimensional gel electrophoresis (2-DGE)-based proteomics approaches have been used successfully to identify and profiling proteins expressed during seed development or in mature seed of model plant species including soybean (Hajduch, Ganapathy, Stein & Thelen, 2005), rapeseed (Hajduch, Casteel, Hurrelmeyer, Song, Agrawal & Thelen, 2006), *Medicago* (Gallardo, Le Signor, Vandekerckhove, Thompson & Burstin, 2003), *Arabidopsis* (Gallardo et al., 2002), wheat (Islam, Woo, Tsujimoto, Kawasaki & Hirano, 2002; Majoul, Bancel, Triboï, Ben Hamida & Branlard, 2003) and barley (Finnie, Maeda, Ostergaard, Bak-Jensen, Larsen &

Svensson, 2004). It is noteworthy that most of the previous studies on lupin proteins used the species *Lupinus albus* (Peeters et al., 2007). Very few preliminary studies using 2-DGE were reported on *Lupinus angustifolius* (Goggin et al., 2008; Islam et al., 2011b; Sirtori et al., 2010) and there is no systematic proteomics-level study on this species. Since proteomic studies on grain species (Barakat, 2004; Kottapalli et al., 2008; Liu et al., 2009; Yahata et al., 2005) reported considerable variation of storage and allergenic proteins among cultivars, it is worth to study the important cultivars of narrow leafed lupin (NLL) to understand the expression level of different seed proteins. A fingerprinting study by our research group (Islam, Ma, Ma, Buirchell, Appels & Yan, 2011a) using direct mass spectrometry on 25 cultivars of NLL grown in Australia showed considerable seed protein variation among the cultivars. Moreover, total protein extracts from three different cultivars of blue lupin showed differential effects on the plasma lipids in rats (Bettzieche, Brandsch, Schmidt, Weibe, Eder & Stangl, 2008). The latter finding in particular indicates variation of the seed proteins among the cultivars might lead to differential bioactivity that is significant for food with certain health benefits.

Although white lupin is mostly used for human consumption, the use of NLL is increasing. In recent times the inadequate knowledge regarding the functional properties of proteins of NLL grain has limited its use in food stuff (Sirtori et al., 2010). In order to characterize the proteome of NLL cultivars, we have carried out a 2-DGE based study for high resolution protein profiling of NLL cultivars. The selection of four NLL cultivars for detailed analysis was based on the phylogenetic relationship of the NLL cultivars following mass spectrometric seed protein analysis (Islam et al., 2011a). Cultivars from each of the major groups comprising pre-wild crosses, primary wild crosses and complex wild crosses were selected for this study.

The information defines the variation among the cultivars with respect to allergenicity and nutritional aspects for utilization in the breeding of lupin.

2. Materials and methods

2.1. Materials

The four cultivars were selected from 25 Australian NLL cultivars based on proteomic phylogenetic relationship by direct mass spectrometry that broadly supported the pedigree relationship (Islam et al., 2011a). The cultivars Uniwhite, Yorrel and Tanjil were selected from the group of Pre-wild, Primary wild and complex wild crosses respectively. The other cultivar Coromup had an isolated position in mass spectrometric study though its pedigree is in complex wild crossing. Seeds of the cultivars were supplied by the Department of Agriculture and Food, Western Australia (DAFWA). All the cultivars were grown in the same year at the same experimental station of DAFWA (Wongan Hills, WA). Thirty gram of seeds containing more than 100 seeds from each cultivar were taken as a working sample and ground in the "Retsch 2M 200" and sieved with 750 µm.

2.2. Protein extraction

Flour samples were defatted using hexane at 20:1 ratio (Santos, Ferreira & Teixeira, 1997). The extraction buffer (8 M urea, 4% CHAPS, 60 mM DTT and 2% (v/v) IPG buffer) was added to the defatted flour in the proportion of 20 ml/g to extract the protein at room temperature for 3 hours (Goggin et al., 2008; Islam et al., 2011b). The protein extract (supernatant) was collected by centrifugation at 12000*g* for 30 min and was precipitated by incubating with ice cold acetone at -20 ^{o}C for 16 hours followed by centrifugation. The protein pellet was then washed with 10% ethanol and then with acetone containing β-mercaptoethanol (0.07%) to remove the additional salts. Ten

milligrams of dried protein were dissolved in rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT and 2% IPG buffer for 5-6 hours at room temperature. Protein concentration was determined by using RC DC protein assay kit (Bio-Rad, Herculles, CA) and Lambda 25 UV-vis spectrometer (PerkinElmer). For each sample, 1100 μg of protein was loaded onto IPG strips (Bio-Rad, Hercules, CA) to optimize resolution and to ensure the adequate loading of minor components for MS/MS analysis (Goggin et al., 2008; Islam et al., 2011b).

2.3. Two-dimensional gel electrophoresis

Iso-electric focusing (IEF) was conducted on 17 cm IPG strips with pH 3-10. The strips were rehydrated with the buffer (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT and 2% IPG buffer) containing 1100 μg of protein for 12 hours. Strips were focussed at 60,000 Vh, with a maximum of 10,000 V, at 20 ^{o}C using Protein IEF cell (BioRad). Before running SDS-PAGE, the strips were equilibrated with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 0.002% bromophenol blue, containing 65 mM DTT for 15 min and another 10 min by substituting DTT with 135 mM iodoacetamide in the same buffer.

Protein separation was carried out on 12% acrylamide/bis (31.5:1) gels, using Protean II Xi cell (Bio-Rad). The running buffer consisted of 2.5 mM Tris-Base, 19.2 mM glycine and 0.01% SDS. The gels were stained by Coomassie Brilliant Blue (CBB). Protein standards (Bio-Rad) were used to estimate the molecular size of the proteins. To minimize experimental variability, all samples were run three times with individual extraction and IEF.

The gels were analysed by a 2-D Proteomic Imaging Systems (PerkinElmer) using ProScan 4.0 software. The digital gel maps of different samples were analysed and

compared by using Progenesis Same Spots software (Nonlinear Dynamics). Master gels were generated for each sample by matching all of the available gels. Normalisation was carried out by determining the gain factor for each sample which can be modelled as $y_{i,}/y'_i = 1/\alpha_k$ where y_i is the measured abundance of feature i on sample k, $1/\alpha_k$ is the gain factor for sample k and y'_i is the normalised abundance of feature i on sample k (Nonlinear Dynamics, n.d.).

2.4. Protein identification by MS/MS

Protein spots of interest were excised from Coomassie Brilliant Blue stained 2-D gels and analysed further by mass spectrometric peptide sequencing. To avoid the overlapping parts of closely related spots, the centre portions of each spot was sampled. The spots were analysed by Proteomics International Ltd Pty, UWA, Perth, Australia. Protein samples were trypsin digested and the resulting peptides were extracted according to standard techniques (Bringans et al., 2008). Peptides were analysed by electrospray ionisation mass spectrometry using the Ultimate 3000 nano HPLC system (Dionex) coupled to a 4,000 Q TRAP mass spectrometer (Applied Biosystems). Tryptic peptides were loaded onto a C18 PepMap100, 3 μm (LC Packings) and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v).

Spectra were analysed to identify the proteins of interest using Mascot sequence matching software (Matrix Science) with taxonomy set to Viridiplantae (Green Plants). All searches used the Ludwig NR. The software was set to allow 1 missed cleavage, a mass tolerance of ± 1.2 Da for peptides and ± 0.6 for fragment ions. The peptide charges were set at 1+, 2+ and 3+, and the significance threshold at $P<0.05$. Generally, a match was accepted where two or more peptides from the same protein were present in a protein entry in the Viridiplantae database.

3. Results

3.1. Two-dimensional gel electrophoresis

Protein extracted from seeds of each of the four cultivars (Uniharvest, Yorrel, Tanjil and Coromup) was analysed by two-dimensional gel electrophoresis and produced high resolution protein profiles as showed in the **Fig. 1**. The result indicates successful standardization of 2 DGE procedures to study the expression profile and comparative proteomic analysis of seed protein of NLL (*Lupinus angustifolious*) cultivars. The results showed considerable differences in the protein profiles among the cultivars (**Fig. 1**) although in general the protein patterns were similar. About 400 spots were revealed in the respective gels of each cultivar by 2DGE software (Progenesis Same Spots, Nonlinear Dynamics) and 97 protein spots showed some difference in their expression levels among the cultivars. However, a total of 24 protein spots were found to be either present or absent, or showing markedly differential expression among the cultivars when the difference threshold was set to 2.5 fold. Most of the proteins were located in the 10-100 KDa and 5.0-8.5 PI ranges.

The differential proteins among the cultivars were positioned largely in 3 specific areas of the gels as showed in **Fig. 1** and **Fig. 2** (A-C). Noticeably, some of the differentiating proteins were as a chain form in the gels, with similar molecular weights but different PI values. The most striking region that differentiated cultivars had proteins in the 32-35 KDa range with 5.5-8.0 PI range. In this region, 13 proteins (spot numbers 1, 2, 3, 5, 7, 9, 10, 11, 12,13, 24, 26 and 27) showed either fully present versus absent or different level of expression among the cultivars (**Fig. 2 B**, **Table 1**). Six proteins (spots number 14, 15, 16, 17, 18 and 19) from the higher molecular weight range (65-70 KDa with 5.5 to 6.5 PI; **Fig. 2 A**) showed differential

expressions. Likewise, five proteins (spot number 20, 21, 22, 23 and 25) from relatively low molecular weight range (10-20 KDa range) were found (**Fig. 2 C**) as differentiating among the cultivars.

3.2. Identifying cultivar specific proteins

3.2.1. Proteins specific to single cultivars

Eight proteins (spot number 18, 19, 20, 21, 23, 25, 26 and 27) were found present in only one of the cultivars (**Table 1**, **Fig. 2 A, B** and **C**). Cultivar Tanjil had the highest with five cultivar specific proteins (spot number 20, 21, 23, 26 and 27) and a cultivar Coromup had two cultivar specific proteins (spot number 18 and 19) at the higher molecular weight range (75KDa). Cultivar Yorrel possessed a single cultivar specific protein (spot number 25) while cultivar Uniharvest did not have any cultivar specific proteins.

3.2.1. Differentiating proteins present in two cultivars

Twelve proteins (spot number 1, 3, 5, 7, 9, 11, 14, 15, 16, 17, 22 and 24) were recognized clearly in two of the four cultivars and absent in the other two cultivars (**Table 1**, **Fig. 2**). Eleven spots out of the twelve (except 24) were expressed in cultivar Uniharvest and Tanjil and were absent in cultivars Yorrel and Coromup. In contrast, the protein corresponding to spot number 24 was clearly expressed in cultivars Yorrel and Coromup whereas absent in Uniharvest and Tanjil.

3.2.3. Proteins present in all of the four cultivars with differential expression level

Two-dimensional gel analysis by the software "progenesis same spots" recognized 77 proteins those are present in all of the four cultivars with different level of expression among the cultivars. However, only four of these proteins (spot number 2, 10, 12 and 13; **Fig. 2 B**) met our stringy threshold (2.5 fold) (**Table 1**). All of these proteins

showed relatively higher expression in the cultivars Uniharvest and Tanjil compared to Yorell and Coromup.

3.3. Protein Identification by mass spectrometry

A total of 58 different protein spots were analysed through mass spectrometry excised from the 2-dimensional gels. These included the 24 differential proteins and 34 common proteins of the cultivars, making a total of 184 proteins samples analysed. Corresponding protein spots from each of the cultivars (where available) were analysed separately and matched together and gave a very good homology of the corresponding protein spots from different cultivars. Of the 58 individual proteins analysed, 52 proteins were identified as one of the major lupin seed protein groups i.e. conglutins. Four proteins were identified with proteins from other species and two proteins could not be identified.

Out of the 24 differentially expressed proteins, 19 proteins (spots numbers 1-3, 5, 7, 9-19, 22, 23, 25; **Fig. 2**, **Table 1**) were identified as β-conglutin, the major seed storage protein of lupin. Of these 19 proteins 7 (spot numbers 1, 2, 12,14,16,17, 23; **Fig. 2**, **Table 1**) were identified as allergenic protein by matching with the accession number gil89994190, an allergenic protein of lupin (Goggin et al., 2008; Guillamon et al., 2010; Peeters et al., 2007). Protein spot 26, a cultivar specific protein of Tanjil, was also identified as allergenic protein as matched with BLAD of *Lupinus albus,* a previously reported allergenic protein of lupin (Guillamon et al., 2010). Protein spot number 20 and 24 were matched closely with an uncharacterized protein of the species *Zea mays* and *Oryza sativa* subsp. *Indica* respectively. Peptides generated from the protein spots 21 and 27 present in cultivar Tanjil only did not give any perfect match with sequences present at the database.

With the 34 common proteins among the four cultivars that were analysed through MS/MS (**Table 2** and **Fig. 3**), 33 were identified with a close match with the sequences of conglutin groups (α, β, γ and δ conglutins) in the databases. Spot number 46 was identified as glyceraldehyde-3 phospahte dehydrogenase (see the supporting document for details).

4. Discussion

In the current study, we have focused on variation in the lupin seed storage protein among different cultivars. In the past, traditional protein analysis methods failed to reveal the vast variations among germplasms especially cultivars. The current study demonstrated that 2-DGE technology coupled with mass spectrometry peptide sequencing is a powerful and high resolution approach to reveal the extent of variations among cultivars.

Allergenic variation among cultivars in other crops including peanut have been reported (Kottapalli et al., 2008). Protein isolates from different cultivars of blue lupin showing differential effects on plasma lipid regulation on rat (Bettzieche et al., 2008) indicates variation of bioactive proteins among cultivars. Moreover, dissimilar groups of conglutins have been claimed as the major allergenic protein of lupin while using different species and cultivars (Goggin et al., 2008; Guillamon et al., 2010; Klos et al., 2010) suggesting variation in expression of allergenic proteins may occur among cultivars. The study based on direct MALDI-TOF protein profiling suggested considerable variation of seed proteins among the NLL cultivars (Islam et al., 2011a). The current study applied a high resolution 2-DGE based proteomic analysis of selected four NLL cultivars to search for the differentiating seed proteins patterns with an emphasis on the allergenic and bioactive proteins.

Detection of approximately 400 spots using the 2DGE based proteomic approach indicated that in general the patterns of proteins among the cultivars were similar. Homology of many common protein spots among the cultivars allowed the identity of the differentiating proteins to be assigned on the basis of their electrophoretic mobility. A total of 97 protein spots showed some difference in their expression levels among the cultivars but we only considered the spots having more than 2.5 fold variation as qualifying for a differentiating protein. The 2.5 fold variation requirement minimized the effects of any experimental error. The analysis identified 24 differentiating proteins among the cultivars. Seed protein composition is generally genetically controlled (Bolon et al., 2010) although some environmental affects would be expected. All the samples studied in this study were grown at the same experimental conditions, suggesting that the proteomic variations revealed by the current study was due to the genetic variation. The complex crossing systems used in the breeding of cultivars (Cowling, 1999) has led to diverse genetic variation. DNA based study (Yuan, Yan, Siddique & Yang, 2005) suggested considerable genetic variation among the NLL cultivars released in Australia. Thus the information on variation at proteomic level might be useful in selecting appropriate germplasm as parental lines to breed cultivars with low or even no allergenicity.

Most of the differentiating proteins among cultivars were in the β-conglutin group, the largest seed protein family of lupin (Duranti et al., 2008). Many of the differential proteins as well as some common ones appeared as a chain in the gels at the same molecular weight with different PI values. Protein spot number 1- 6, 10-12, 14-17 and 18-19 (**Fig. 2 A** and **B**) were placed in the gels as a form of chain indicating the existence of different isoforms of the same protein. Matching of protein spots within a chain with the sequence of same protein accession also indicated their homology.

Liang, Luo, Holbrook & Guo (2006) identified differences in isoforms of basic arachin (iso-Ara h3) among peanut cultivars and suggested the cause was variation in post-translation modification. Most of the α, γ and δ conglutins including both the high and low molecular weight appeared as consistent representatives in the cultivars, indicating their consistency and stability. β-Conglutin and α-conglutin are sometimes described as vicilin-like proteins and legumin-like proteins, respectively, but we have used β-conglutin and α-conglutin consistently to avoid confusion.

The results indicate considerable variation of allergenic proteins among cultivars that provides an insight about the significance of cultivar specific lupin proteomics. All of the 8 differentiating allergenic proteins are highly expressed in the cultivar Tanjil while Cultivar Uniharvest has 6 highly expressed. However, cultivar Yorrel and Coromup have only two differentially expressed allergenic proteins at very low expression level. This predicts that cultivar Yorrel and Coromup may have low allergenic effect than the other two cultivars.

The differential expression of seed proteins among cultivars might have the potential to relate the cultivars to bioactivities of lupin proteins. Most of the differentiating proteins were identified as β-conglutin, the major seed storage protein of lupin similar to 7s glubulins of soybean which has been investigated for some biological activity (Maruyama, Maruyama, Tsuruki, Okuda, Yoshikawa & Utsumi, 2003; Prak, Maruyama, Maruyama & Utsumi, 2006). In contrast, γ-conglutins having blood glucose lowering effect is the only lupin seed protein has been reported for individual bioactivity (Duranti et al., 2008) that showed similar expression among the studied cultivars. However, the lack of information regarding the biological activity of individual proteins or individual protein groups (α, β, γ and δ) of lupin (Duranti et al.,

2008) has limited the discussion and suggested the necessity of more detailed studies of individual protein groups in terms of functionality and bioactivity.

The expression of differentiating proteins suggested two distinct groups among the cultivars. Cultivars Uniharvest and Tanjil showed similar patterns of differentiating protein expression except for the cultivar specific proteins in Tanjil whereas cultivars Yorrel and Cormup formed a separate group with similar patterns. At the 32-35 KDa molecular weight range, 11 β-conglutins were present or highly expressed in the cultivars Uniharvest and Tanjil (**Fig. 2 B**). In contrast, these proteins were absent or poorly expressed in Yorrel and Coromup. Likewise a group of high molecular weight proteins (spots 14-17) were found in cultivars Uniharvest and Tanjil and absent in Yorrel and Coromup. On the other hand one uncharacterized protein (spot number 24, **Fig. 2 B** and **Table 1**) was present in cultivar Yorrel and Tanjil but absent at cultivars Uniharvest and Tanjil. The pedigree history suggested the cultivar Coromup has one parental line from Yorrel (Buirchell, 2010). It is noted that DNA based studies suggest cultivar Tanjil is closer to the Uniharvest than the other cultivars (Yuan et al., 2005).

Cultivar specific proteins have been used for cultivar identification in some species (Kottapalli et al., 2008; Yahata et al., 2005). The eight cultivar specific proteins detected in this study will be useful for lupin cultivar identification. Cultivar Tanjil possesses the highest number (6) of cultivar specific proteins. This may be due to its complex crossing process during breeding (Cowling, 1999). Coromup, the other cultivar with complex wild crossing pedigree history had 2 cultivar specific proteins. A comparatively simpler crossing system that comprises both primary and secondary crosses (Cowling, 1999; Islam et al., 2011a) made Yorrel to have just one cultivar specific proteins. On the other hand, one of the oldest cultivar, Uniharvest, bred from

only primary crosses did not have any cultivar specific protein. These cultivar specific proteins are certainly useful for cultivar identification and for proteomic improvement of lupin through further breeding once the function of those proteins are known.

MS/MS peptide sequences of 52 different proteins out of 58 gave very good matching with the lupin protein sequences in the databases including all the conglutin groups, indicating successful identification of lupin proteins. In all analysed protein samples, the corresponding protein spots from all four cultivars were analysed separately for a better confirmation of the identification. In all cases (with few exceptions), highly similar peptide sequencing and matching with the similar accessions indicate uniformity and homogeneity of the proteins among the cultivars. Three proteins were successfully identified as proteins from other species and 3 proteins were not identified at all, suggesting lacking of sequence information in the databases.

Visible differences in the expression of important seed proteins among the four cultivars signify a valuable tool for cultivar identification for further molecular breeding. The reported differential expression of allergenic protein suggests further studies including more cultivars could lead to a targeted selection of lupin cultivars for food industries.

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Supporting information

Matching of the mass spectrometric peptide sequences to identify the common proteins among the cultivars

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Figure legends:

Fig. 1. Seed protein profile of four cultivars of *Lupinus angustifolius* as revealed by two-dimensional gel electrophoresis of total protein indicating overall variation of proteins. The rectangles indicate the regions with differentiating proteins. The elaboration of those specific areas is showed in **Fig. 2**.

Fig. 2. Comparison of specific regions on the 2-D gel demonstrating the expression differentiating proteins among the four cultivars examined. The letters A, B and C indicate the regions showed in **Fig. 1**.

Fig. 3. Identical (common) lupin seed proteins with similar expressions among the four cultivars as identified by MS/MS. The spots are shown on a 'Tanjil' background and the identification of the spots is presented in **Table 2**.

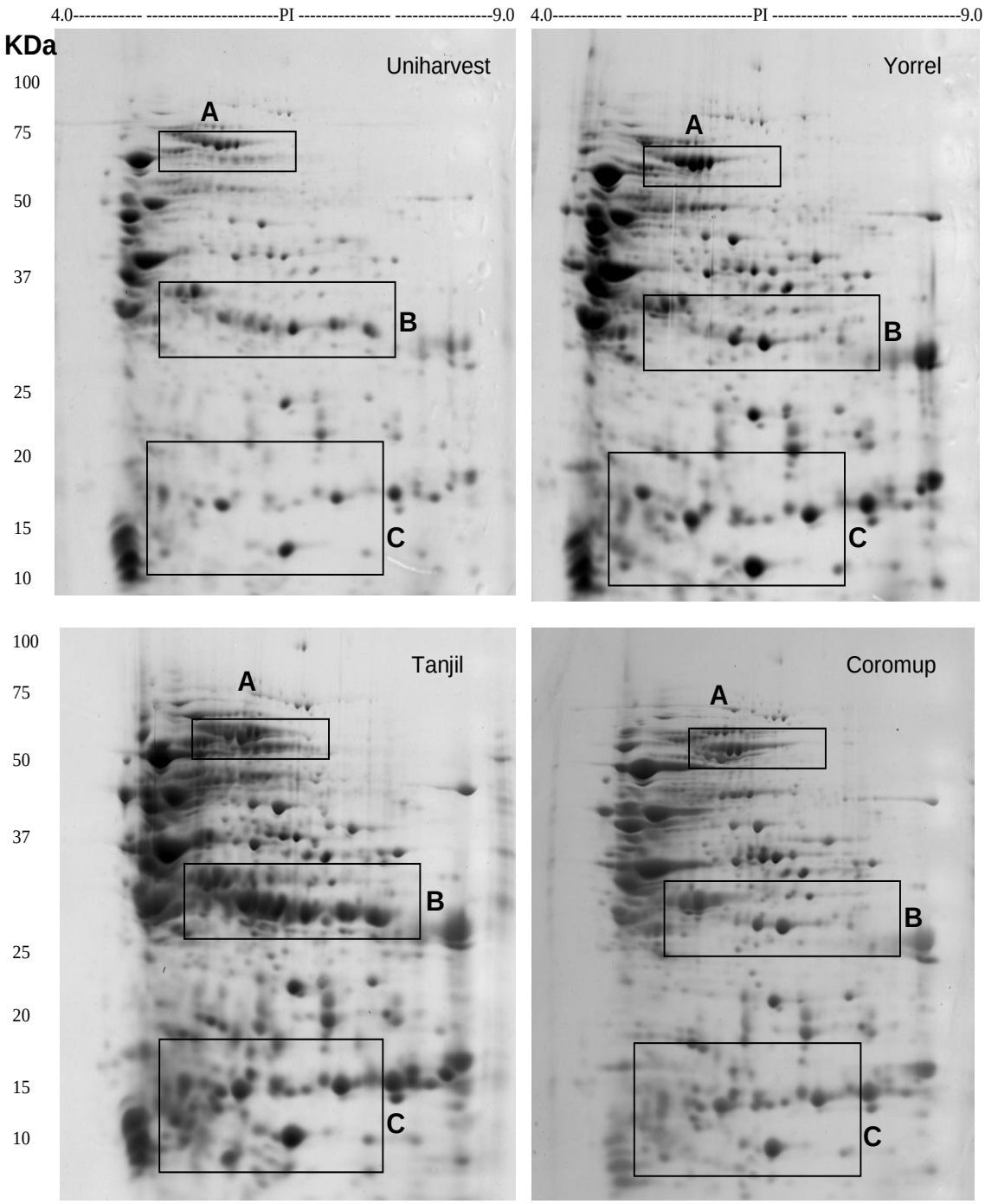
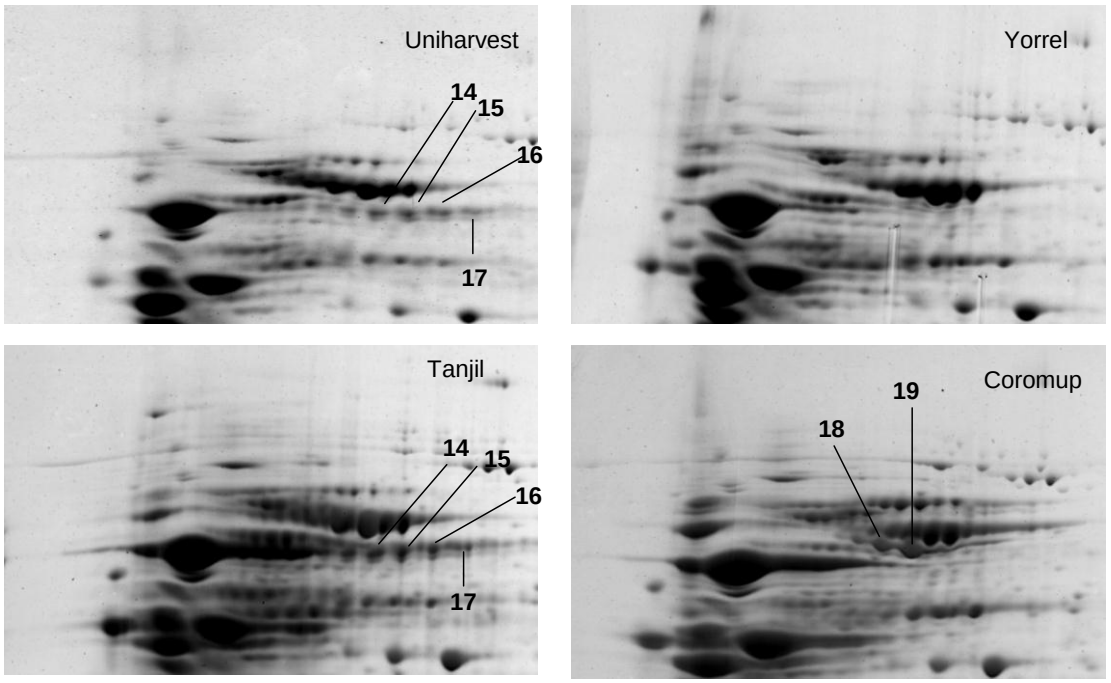


Figure 1

A



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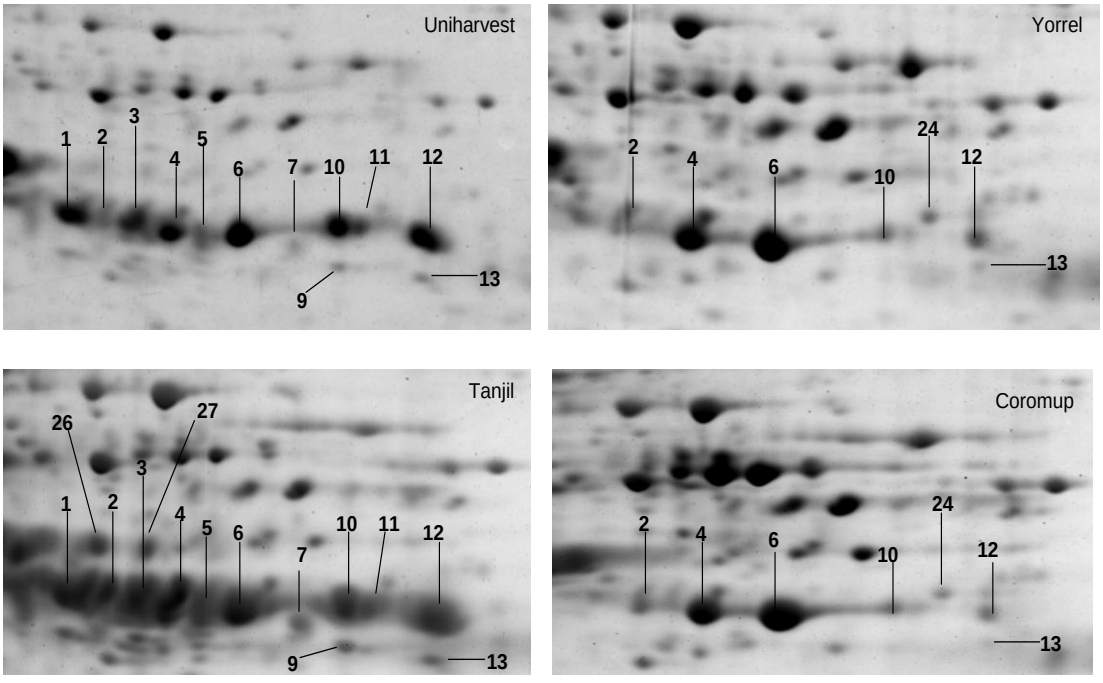

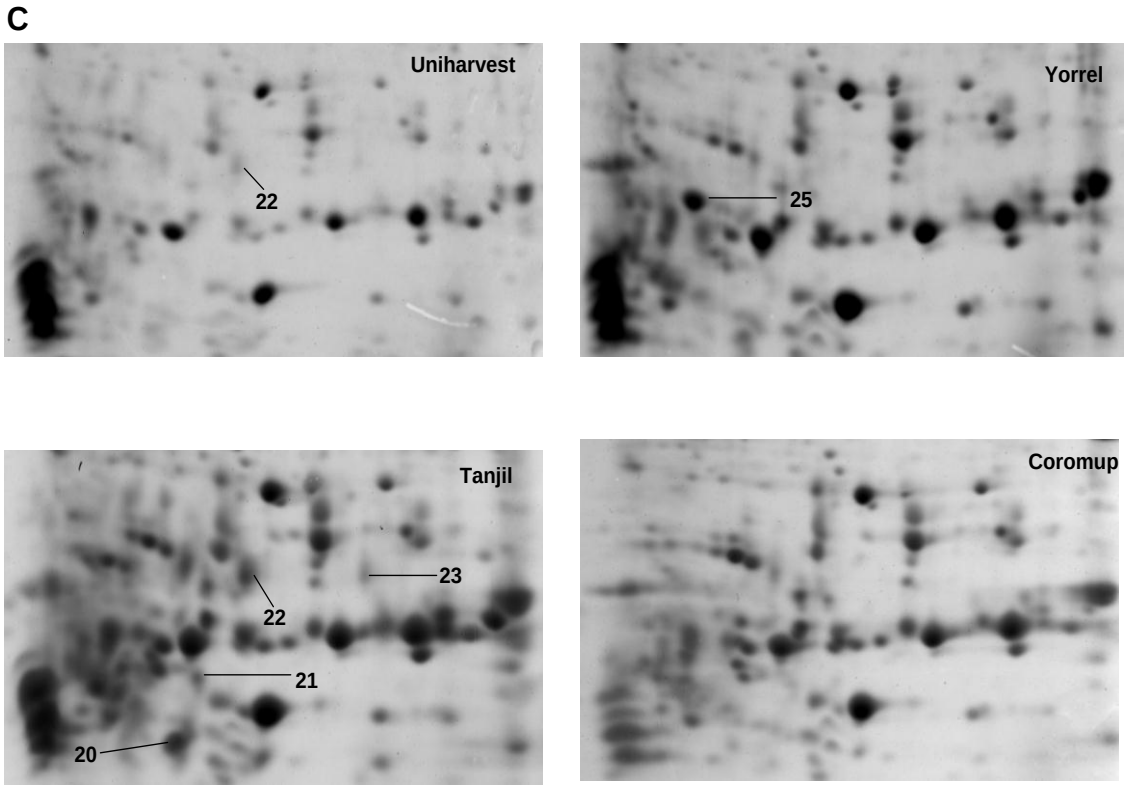

Figure 2

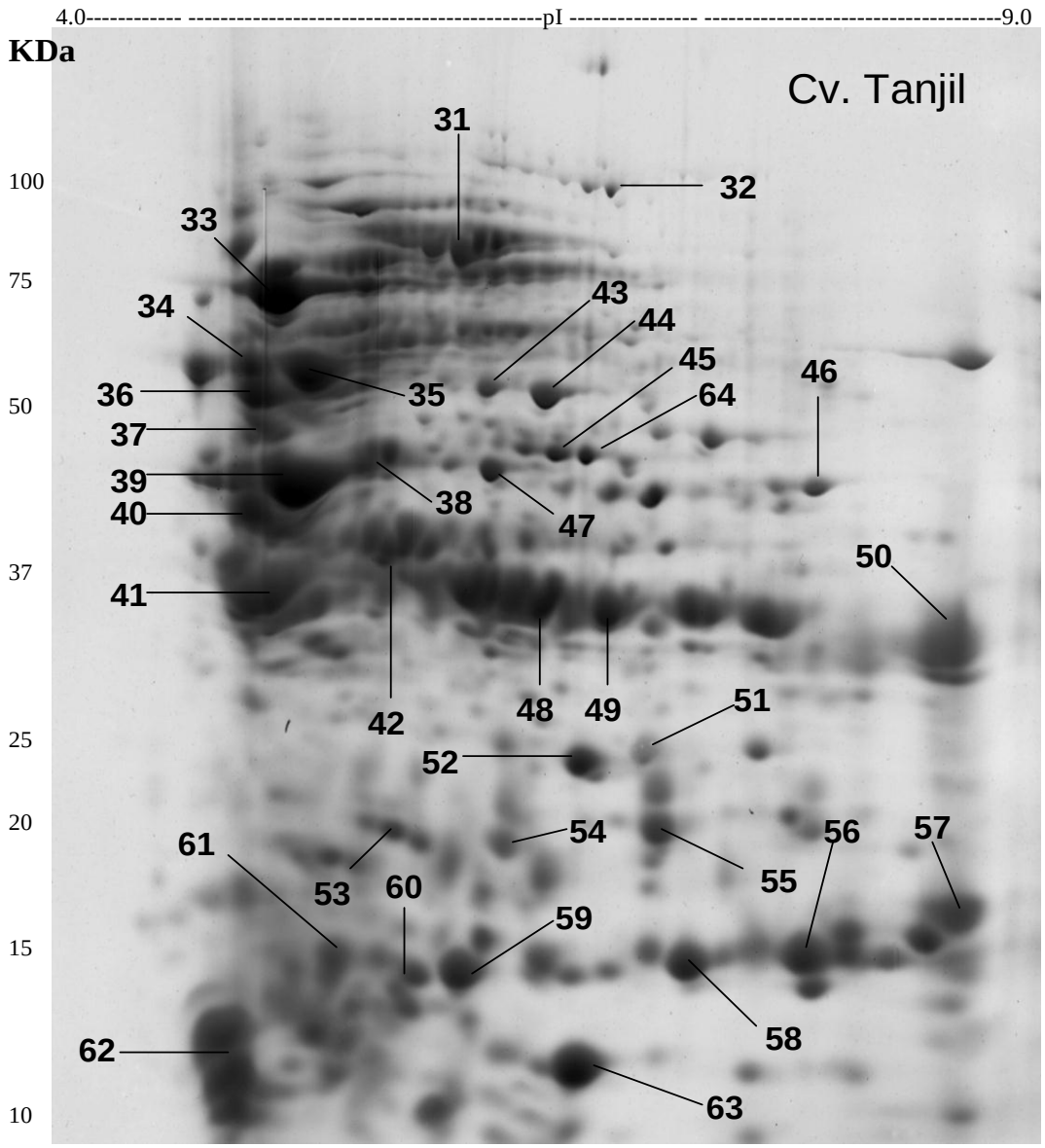

Figure 3

Table 1

MS/MS identification of differentiating proteins among the cultivars. Matching has been achieved using Mascot sequence matching software (Matrix Science) with the taxonomy set to Viridiplanate (Green Plants). β-Conglutin and α-Conglutin are sometimes referred to as vicilin-like proteins and legumin-like proteins, respectively, but we have used the term β-Conglutin and α-Conglutin for all closely matching cases to avoid confusion.

Spot no	Spot relative abundance Protein expression as fold ratio: Uniharvest/Yorell/ Coromup/Tanjil	Anova (p)	Matched protein	Accession number	Theoretical MW/PI	Observed MW/PI	Sequence coverage	MOWSE score	Matched peptides
Cultivar specific proteins									
18	--/--/2.8/--	0.008	β-conglutin [*Lupinus angustifolius]*	gil149208401	61490/5.34	60000/5.7	13%	180	GKPSESGPFNLR QAYNLEYGDALR TNRLENLQNYR HSDADYILVVLNGR AIFIVVVDEGEGNYELVGIR
19	--/--/2.6/--	0.002	β-conglutin [*Lupinus angustifolius*]	gil149208401	61490/5.34	60000/5.8	10%	167	QAYNLEYGDALR TNRLENLQNYR HSDADYILVVLNGR AIFIVVVDEGEGNYELVGIR
20	--/--/--/3.2	0.007	Putative uncharacterized protein [*Zea mays*]	gil223947879	42658/8.13	10000/5.9	3%	97	REEEEEATPAAR
21	--/--/--/3.1	2.39E-04	Not Identified	-	-	13000/6.0	-	-	-
23	--/--/--/3.8	0.006	β-conglutin [*Lupinus albus*]	gil89994190	61994/6.08	18000/7.2	8%	225	YEEIQR LENLQNYR NPYHFNSQR DQQSYFSGFSR NTLEATFNTRYEEIQR
25	--/8.6/--/--	1.08E-04	β-conglutin [*Lupinus angustifolius*]	gil149208403	54267/6.27	17500/5.0	6%	432	KQIQELR HAQSSSGEGKPSESGPFNLR KHAQSSSGEGKPSESGPFNLR
26	--/--/--/2.8	0.008	BLAD (Fragment) Tax_Id=3870 [*Lupinus albus*]	gil77994351	20393/9.66	33000/6.2	15%	134	DQQSYFSGFSR NTLEATFNTRYEEIQR
27	--/--/--/2.5	0.002	Not identified			33000/6.3			

Spot no	Spot relative abundance Protein expression as fold ratio: Uniharvest/Yorell/ Coromup/Tanjil	Anova (p)	Matched protein	Accession number	Theoretical MW/PI	Observed MW/PI	Sequence coverage	MOWSE score	Matched peptides
Proteins present in two cultivars									
1	3.6/--/--/2.1	2.45E-04	β-conglutin [*Lupinus albus*]	gi|89994190	61994/6.08	29000/6.0	9%	216	NPYHFNSQR NFLAGSEDNVIR TNRLENLQNYR NTLEATFNTRYEEIQR
3	3.6/--/--/2.2	1.99E-05	β-conglutin [*Lupinus angustifolius*]	gi|149208401	61490/5.34	28500/6.1	8%	256	TNRLENLQNYR NTLEATFNTRYEEIQR NQQQSYFANAQPQQKQQR
5	2.5/--/--/2.3	1.42E-04	β-conglutin [*Lupinus angustifolius*]	gi|149208401	61490/5.34	28000/6.5	5%	160	TNRLENLQNYR NQQQSYFANAQPQQKQQR
7	4.6/--/--/7.6	1.67E-05	β-conglutin [*Lupinus angustifolius*]	gi|149208403	54267/6.27	27000/6.7	22%	349	NPYHFSSNR DQQSYFSGFSK QQEEQEREHR TNRLENLQNYR EREQEQQPRPQR QEEEEEEEEWQPR RQEEEEEEEEWQPR NTLEATFNTRYEEIER LPAGTTSYILNPDDNQNLR
9	4.2/--/--/5.0	9.03E-05	β-conglutin [*Lupinus angustifolius*]	gi|328684565	71853/5.82	25500/7.0	4%	160	TNRLENLQNYR NQQQSYFANAQPQQQQQR
11	3.5/--/--/2.2	1.97E-05	β-conglutin [*Lupinus angustifolius*]	gi|149208401	61490/5.34	28000/7.1	3%	127	NQQQSYFANAQPQQKQQR
14	5.4/--/--/4.5	0.001	β-conglutin [*Lupinus albus*]	gi|89994190	61994/6.08	68000/5.8	9%	242	YEEIQR NPYHFNSQR DQQSYFSGFSR NFLAGSEDNVIR NTLEATFNTRYEEIQR
15	5.4/--/--/4.3	0.005	β-conglutin [*Lupinus angustifolius*]	gi|149208401	61490/5.34	68000/5.9	12%	296	EQEQQPQHGR NTLEATFNTRYEEIQR LPAGTTSYILNPDDNQNLR NQQQSYFANAQPQQKQQR

Spot no	Spot relative abundance Protein expression as fold ratio: Uniharvest/Yorell/ Coromup/Tanjil	Anova (p)	Matched protein	Accession number	Theoretical MW/PI	Observed MW/PI	Sequence coverage	MOWSE score	Matched peptides
16	4.4/--/--/4.2	0.002	β-conglutin [*Lupinus albus*]	gi|89994190	61994/6.08	68000/6.0	9%	277	NPYHFNSQR DQQSYFSGFSR NFLAGSEDNVIR NTLEATFNTRYEEIQR
17	3.9/--/--/4.5	6.89E-04	β-conglutin [*Lupinus albus*]	gi|89994190	61994/6.08	68000/6.1	9%	254	NPYHFNSQR DQQSYFSGFSR NFLAGSEDNVIR NTLEATFNTRYEEIQR
22	3.0/--/--/4.1	6.47E-06	β-conglutin [*Lupinus angustifolius*]	gi|149208401	61490/5.34	18000/6.4	8%	394	LENLQNYR TNRLENLQNYR NTLEATFNTRYEEIQR LPAGTTSYILNPDDNQNLR
24	--/4.0/4.3/--	2.51E-07	Putative uncharacterized protein [*Oryza sativa* subsp. *indica*]	gi|125539365	67761/7.12	29000/7.2	2%	56	NVSEEIICLAAALR
Proteins present in all cultivars with different expression level									
2	2.5/1.0/1.1/2.5	0.006	β-conglutin [*Lupinus angustifolius*]	gi|89994190	61490/5.34	28000/6.3	9%	250	NPYHFNSQR NFLAGSEDNVIR TNRLENLQNYR NTLEATFNTRYEEIQR
10	3.8/1.3/1.0/2.2	3.68E-05	β-conglutin [*Lupinus angustifolius*]	gi|149208401	61490/5.34	28000/7.2	3%	147	NQQQSYFANAQPQQKQQR
12	3.8/1.2/1.0/3.0	9.99E-05	β-conglutin [*Lupinus albus*]	gi|89994190	61994/6.08	28000/7.5	2%	126	NFLAGSEDNVIR
13	3.8/2.0/1.0/3.6	4.15E-05	β-conglutin [*Lupinus angustifolius*]	gi|328684565	71853/5.82	26000/7.6	6%	177	NPYHFSSNR TNRLENLQNYR NQQQSYFANAQPQQQQQR

Table 2

MS/MS protein identification results of 2-DGE gel spots. (Protein spot numbers are indicated in **Fig. 2** and **3**)

Spot number	Present in Cultivars	Identified protein
23	Tanjil	β-conglutin
20,21,26	Tanjil	Peptides did not match with any known lupin protein sequence
27	Tanjil	BLAD [*Lupinus albus*]
18,19	Coromup	β-conglutin
25	Yorrel	β-conglutin
24	Yorrel and Coromup	Peptides did not match with any known lupin protein sequence
1,3,5,7,9,11,14, 15,16,17, 22	Uniharvest and Tanjil	β-conglutin
2,10,12,13	All cultivars (with differential expression)	β-conglutin
31, 36-37, 41-45, 47-49, 52, 57,64	All cultivars (with similar expression)	β-conglutin
33-35, 38-40, 51, 53-56, 58-61	All cultivars (with similar expression)	α-conglutin
32, 50, 63	All cultivars (with similar expression)	γ-conglutin
62	All cultivars (with similar expression)	δ-conglutin
46	All cultivars (with similar expression)	Glyceraldehyde-3-phosphate-dehydrogenase [*Lupinus albus*]

- Four key lupin cultivars were analysed for their seed proteome components.
- Revealed 24 proteins with differential expression among the cultivars.
- Eight differentially expressed proteins were identified as allergenic protein.
- Differential proteins are mostly β-Conglutin; α, γ, δ-conglutins are consistent.