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In-Depth Characterization of the Phaseolin Protein Diversity of Common Bean (*Phaseolus vulgaris* L.) Based on Two-Dimensional Electrophoresis and Mass Spectrometry

María De La Fuente[1]*, *María López-Pedrouso*[2], *Jana Alonso*[3], *Marta Santalla*[1], *Antonio M. De Ron*[1], *Gonzalo Álvarez*[2] and *Carlos Zapata*[2]

[1]Department of Plant Genetic Resources, Misión Biológica de Galicia-CSIC, ES-36080 Pontevedra, Spain

[2]Department of Genetics, Santiago de Compostela University, ES-15782 Santiago de Compostela, Spain

[3]Proteomics Laboratory, University Hospital Complex of Santiago de Compostela-CHUS, ES-15706 Santiago de Compostela, Spain

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Summary

Phaseolin is the major seed storage protein of common bean. It comprises a complex set of glycoproteins heterogeneous in their polypeptide composition that is encoded by a gene family. Analyses of phaseolin banding patterns by one-dimensional electrophoresis (SDS-PAGE) have been central to the current understanding of the diversity of wild and cultivated common beans. In this work, we have carried out a detailed description and interpretation of phaseolin diversity in cultivated common beans of different geographic origins (Mesoamerican and Andean gene pools) based on the current two-dimensional electrophoresis (2-DE) technology and mass spectrometry (MS). High-quality 2-DE gel images revealed very complex phaseolin patterns across the studied cultivars. Specifically, patterns of phaseolin within cultivars were organized in a horizontal string of multiple isospot pairs varying in isoelectric point and molecular mass. The degree of similarity among phaseolin patterns was estimated from the percentage of spots shared between pairs of cultivars. Analyses of proteomic distances between phaseolin types by non-metrical multidimensional scaling revealed that 2-DE phaseolin profiles are more similar among cultivars belonging to the same gene pool. However, higher differentiation was found among cultivars of the Andean gene pool. Analysis of genetic variations of the PCR-based SCAR marker of phaseolin seed protein was in general agreement with 2-DE phaseolin patterns, but provided supplementary information regarding diversity among cultivars. Furthermore, the molecular basis responsible for the complexity of 2-DE phaseolin patterns was investigated. Thus, identification of phaseolin spots from 2-DE gels by MALDI-TOF and MALDI-TOF/TOF MS showed that each single isospot pair contained only one type (α or β) of phaseolin polypeptide, but pairs with higher and lower molecular mass corresponded to α- and β-type polypeptides, respectively. In addition, partial and total deglycosylation of seed protein extracts with the enzyme PNGase F indicated that differences between isospots of each pair are exclusively due to a different extent of glycosylation of the same

*Corresponding author; Phone: ++34 986 854 800; Fax: ++34 986 841 362; E-mail: mfuente@mbg.csic.es

Abbreviations: 1-DE: one-dimensional electrophoresis; 2-DE: two-dimensional electrophoresis; ER: endoplasmic reticulum; IEF: isoelectric focusing; IPG: immobilized pH gradient; MALDI-TOF: matrix-assisted laser desorption/ionization time-of-flight; MDS: non-metric multidimensional scaling; MS: mass spectrometry; M_r: relative molecular mass; pI: isoelectric point; PCR: polymerase chain reaction; PMF: peptide mass fingerprinting; PNGase F: protein-N-glycosidase F; SCAR: sequence-characterized amplified region; SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

type of phaseolin polypeptide. Taken together, our observations provide new insights into the study of genetic differentiation of common bean populations based on phaseolin diversity.

Key words: common bean, glycoproteins, mass spectrometry, phaseolin protein, *Phaseolus vulgaris*, seed proteome, two-dimensional electrophoresis

Introduction

Legume and oilseed proteins entail about a 15 to 50 % of the dietary proteins for humans in many countries. The common bean (*Phaseolus vulgaris* L.) is one of the most consumed grain legumes in the world, mainly in South America and Africa (*1*). Seeds of common bean have valuable nutritional properties due to the fact that they are an important source of fibre, minerals and vitamins, as well as to their low content of fat and sodium (*2*). A diet including beans provides substantial health benefits, decreasing the risk of heart and renal diseases (*3*), protecting against several cancer types (*4*) and helping in the control of overweight and obesity (*5*). In spite of these advantages, the nutritional value of common bean is affected by a number of factors, including low levels of sulphur amino acids and tryptophan, low protein digestibility and the presence of anti-nutritional compounds like proteinase inhibitors (*6*,*7*).

The major seed storage protein (40–50 %) of common bean is phaseolin, a glycoprotein that belongs to the 7S vicilin class (*8*,*9*). Phaseolin contributes, therefore, to the nutritional value of common bean seed proteins in an important way. One of the problems of phaseolin as dietary protein is its poor digestibility in native state due to partial resistance to degradation by the enzymes of the gastrointestinal tract of monogastric animals (*10*). In addition, phaseolin is deficient in essential amino acids methionine, cysteine and tryptophan, and therefore several attempts and strategies have been followed in order to improve the essential amino acid composition (*11*,*12*). Beyond its nutritional content, phaseolin has been found to have promising applications in the food industry due to its physical and biochemical properties (*13*), including applications in food formulations and beverage industry (*14*), as a component of biopolymer films (*15*) and as preservative in bread storage due to its long-term fungal inhibitory activity (*16*).

Phaseolins are a family of similar proteins but slightly heterogeneous in their polypeptide composition not only caused by divergence at the DNA sequence level, but also by co- and post-translational modifications. The genetic basis of the phaseolin protein is not completely known, but it is believed that phaseolin is encoded by a gene family constituted by approx. 6–10 co-dominant interrupted genes (five introns and six exons) per haploid genome, organized in a single close cluster on chromosome 7 (*17–22*). Nucleotide sequences of phaseolin genomic and cDNA clones showed that members of the phaseolin gene family can be subdivided into two distinct gene types (or subfamilies) termed α and β (*18–20*,*23–25*). Polysomes tightly bound to the membrane of the rough endoplasmic reticulum (ER) do translate nuclear-encoded 16S mRNAs. Translation yields phaseolin precursor polypeptides of α- and β-type containing a signal peptide of 24 amino acids in length, which initiates the transport of the growing polypeptides across the ER membrane to the ER lumen (*19*,*26–28*). Three different polypeptides have been identified by gene sequencing after cleavage of the signal sequence: two α-type polypeptides of 411 and 412 amino acids and one β-type polypeptide of 397 amino acids (*18*,*19*).

Each α- and β-phaseolin polypeptide is co-translationally glycosylated in the lumen of the ER through the transfer of either one or two N-acetylglucosamines (GlcNAc) of complex high-mannose oligosaccharides to the amide group of specific asparagine (Asn) residues giving rise to N-linked oligosaccharide side chains or glycans (*26*,*29*,*30*). Analyses of the phaseolin synthesized *in vivo* and *in vitro* demonstrated the formation of four types of glycopolypeptides in the cotyledons of common beans that corresponded to α- and β-type polypeptides either once or twice glycosylated (*29*). In the ER lumen, α- and β-type polypeptides (~50 kDa) fold and assemble post-translationally *via* their α-helical domains into trimeric protomers (~150 kDa) that associate to form a dodecameric structure (~596 kDa) (*31–33*). The phaseolin (tetramer of trimers) then travels from the ER *via* the Golgi complex, where the glycan present on the single glycosylated α- and β-type polypeptides is partially modified (*30*). Finally, phaseolin is transferred to membrane-bound organelles known as protein bodies or protein storage vacuoles, where it undergoes a further processing step that decreases the molecular mass of the phaseolin polypeptides to give the mature form (*26*). The mature phaseolin accumulates rapidly in the developing bean seed cotyledons until germination, when it is broken down by proteolytic enzymes resident in the young seedlings (*34*,*35*).

Phaseolin plays a fundamental role as molecular marker in the analysis of genetic variability of common bean populations (*25*,*36*). Diversity of phaseolin has been characterized intensively in a large number of wild and domesticated populations of common bean under denaturing conditions through one-dimensional sodium dodecyl sulphate-polyacrylamide gel electrophoresis (1-DE SDS-PAGE) (*9*,*37–44*). Thus, more than 40 different types of phaseolin have been found in wild and cultivated beans according to their polypeptide composition (*45*). Electrophoretic patterns of variation for phaseolin have revealed that common bean diversity is organized into two major ecogeographic gene pools: the Mesoamerican gene pool distributed from northern Mexico to Colombia and the Andean gene pool from southern Peru to northwestern Argentina (*9*,*39*). Phaseolin diversity data also suggest that cultivated common bean arose from two major domestication events which gave rise to Mesoamerican and Andean cultivars. Thus, bean accessions from Mesoamerica and the Andes present distinctive phaseo-

lin types: the Mesoamerican beans exhibit 'S' (Sanilac), 'M' (Middle America), or 'B' (Boyacá) types, while Andean beans present 'T' (Tendergreen), 'C' (Contender), 'H' (Huevo de Huanchaco), 'A' (Ayacucho), 'J' (Jujuy) or 'I' (Inca) patterns (*9*,*39*,*40*). Additional evidence based on morphological traits (*46*), allozyme loci (*47*), microsatellite markers (*48*), chloroplast DNA polymorphisms (*49*) and SNPs (*50*) has confirmed the organization of the common bean diversity as previously revealed from phaseolin variability. Phaseolin studies have also contributed to understanding the widespread distribution of beans from the centres of origin and to locate secondary centres of domestication (*42*). On the other hand, phaseolin variants found in wild and cultivated common beans are of practical relevance because they show important differences in digestibility and in the availability of amino acids related to differences in cleavage, susceptibility to proteolysis and to the degree of hydrolysis (*44*,*45*,*51*).

Two-dimensional electrophoresis (2-DE) is a promising proteomic tool to unravel phaseolin diversity since this technology has a higher resolving power for separation of complex mixtures of heterogeneous polypeptides than 1-DE. However, only a few studies on phaseolin diversity have used 2-DE in order to separate phaseolin polypeptides on the basis of both their charge and their molecular mass (*8*,*9*,*17*,*26*,*37*,*39*,*40*,*52*). In addition, these studies present some limitations because they were performed by the initial 2-DE technology based on carrier ampholyte-generated pH gradients for the first dimension. The current 2-DE technology for separation of proteins based on immobilized pH gradients (IPGs) offers important advantages in terms of resolution and reproducibility (*53*,*54*). In this work, we carried out a detailed characterization of phaseolin diversity through the current 2-DE technology. The types of phaseolin polypeptides in 2-DE spot patterns have been identified for the first time by mass spectrometry. The contribution of glycosylation to complexity of phaseolin patterns detected by 2-DE was also addressed. The obtained observations will be useful for a more complete description and interpretation of the polypeptide heterogeneity and diversity of phaseolin.

Materials and Methods

Plant material and seed protein extraction

Seeds of cultivated common beans displaying different phaseolin types were obtained from the germplasm collection of Mision Biológica de Galicia-CSIC (Pontevedra, Spain): Sanilac (accession PI549695) and ICA Pijao (G05773) for S-type, Boyacá 22 (PI313590) for B-type, Tendergreen (PI549633) for T-type, Contender (PI474218) and Calima (G04494) for C-type, Huevo de Huanchaco (G12588) for H-type and Ayacucho (G12053) for A-type. Total protein of bean seeds was extracted by the phenol method (*55*). This method applied to seeds of common bean has been proved to give enhanced results for the separation of proteins by two-dimensional electrophoresis in terms of resolution of protein spots, gel background and streaking, as well as for the efficient identification of proteins by mass spectrometry.

Two-dimensional gel electrophoresis

Two-dimensional electrophoresis (2-DE) was carried out from various amounts of total protein extracts (50 and 250 μg) in lysis and rehydration (7 M urea, 2 M thiourea, 4 % CHAPS, 0.002 % Bromophenol Blue) buffers. Protein extracts were loaded on 24-cm long Ready Strip IPGs (pH linear gradients 4–7 or 4.7–5.9) (Bio-Rad Laboratories, Inc, Hercules, CA, USA), together with 0.6 % dithiothreitol (DTT) and 1 % IPG buffer (Bio-Rad Laboratories). First dimensional isoelectric focusing (IEF) was carried out until 60 kVh was reached after an initial rehydration step for 12 h at 50 V. IEF was performed using PROTEAN® IEF Cell (Bio-Rad Laboratories). Focused strips were incubated in the equilibration solution (50 mM Tris, pH=8.8, 6 M urea, 30 % glycerol and 2 % SDS) with 1 % DTT for 15 min at room temperature and then with 2.5 % iodoacetamide under the same conditions. For the second dimension sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), equilibrated strips were placed on 10 or 15 % (by mass) gels and they were run in an Ettan DALTsix system (GE Healthcare, Uppsala, Sweden) using Tris-glycine-SDS (50 mM Tris, 384 mM glycine and 0.2 % SDS) as electrode buffer. Gels were stained with Sypro Ruby® stain (Lonza, Rockland, ME, USA) following the manufacturer's indications. Gel images were captured using the Gel Doc™ XR+ system (Bio-Rad Laboratories). Image analysis of digitalized gels was performed through PDQuest™ Advanced software v. 8.0.1 (Bio-Rad Laboratories). Spots were identified under the sensitivity of 1.1 and were matched among samples after being normalized using total density in gel image. Correspondence between 2-DE and 1-DE phaseolin patterns was established as follows: equilibrated IPG strips were placed on SDS polyacrylamide gels with two wells at the ends. Approximately 15 μg of total seed protein dissolved in lysis buffer were added to the solution containing 125 mM Tris-HCl, pH=6.8, 4 % (by mass) SDS, 20 % (by volume) glycerol, 0.005 % Bromophenol Blue and 5 % (by volume) 2-mercaptoethanol, until reaching a final volume of 20 μL. This mixture was loaded into one well of the gel, and standard molecular mass markers ranging from 15 to 200 kDa (Fermentas, Ontario, Canada) were loaded into the other well.

Western blot

Proteins were electro-transferred after 2-DE to polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA, USA). PVDF membranes were blocked with 10 % (by mass) non-fat dry milk in TBS (48 mM Tris, 39 mM glycine, 1.3 mM SDS and 20 % methanol) for 1 h at room temperature. Membranes were then incubated using the immune serum against *P. vulgaris* phaseolin (1:1000 dilution), as primary antibodies, overnight at 4 °C. The membranes were subsequently washed with TBS and incubated with mouse anti-rabbit IgG-FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA) (1:500 dilution), as secondary antibodies, for 1 h at room temperature. Membrane images were eventually digitalized using a LAS-3000 Imager system (FujiFilm, Tokyo, Japan).

Identification of phaseolin spots by mass spectrometry

2-DE spots of cultivars S and T, previously confirmed as phaseolin polypeptides by Western blot, were excised from the gels and digested with trypsin as described pre-

viously (*55*). The resulting peptide extracts were pooled, concentrated in a SpeedVac® (Thermo Fisher Scientific, Waltham, MA, USA) and stored at –20 °C. Identification of the type of phaseolin polypeptide (α or β) of selected 2-DE spots was performed by matrix-assisted laser-desorption-ionization time-of-flight mass spectrometry (MALDI-TOF MS) and MALDI-TOF/TOF MS according to the following procedure. Dried samples were dissolved in 4 μL of 0.5 % HCOOH. Equal volumes (0.5 μL) of peptide and matrix solution, consisting of 3 mg of CHCA dissolved in 1 mL of 50 % acetonitrile in 0.1 % TFA, were deposited using the thin layer method onto a 384 Opti-TOF MALDI plate (Applied Biosystems, Foster City, CA, USA). Mass spectrometric data were obtained in an automated analysis loop using 4800 MALDI-TOF/TOF analyzer (Applied Biosystems). MS spectra were acquired in positive-ion reflectron mode with an Nd:YAG, 355 nm wavelength laser, averaging 1000 laser shots, and at least three trypsin autolysis peaks were used as internal calibration. All MS/MS spectra were performed by selecting the precursors with a relative resolution of 300 (FWHM) and metastable suppression. Automated analysis of mass data was achieved using the 4000 Series Explorer Software™ v. 3.5 (Applied Biosystems). Peptide mass fingerprinting (PMF) and peptide fragmentation spectra data of each sample were combined through the GPS Explorer Software v. 3.6 using Mascot v. 2.1 software (Matrix Science, Boston, MA, USA) to search against a non-identical protein database (Swiss-Prot, Swiss Institute of Bioinformatics, Lausanne, Switzerland), with 30 ppm precursor tolerance, 0.35 Da MS/MS fragment tolerance, carbamydomethyl cysteine (CAM) as fixed modification, oxidized methionine as variable modification and allowing one missed cleavage. All spectra and database results were manually inspected in detail using the above software. Protein scores greater than 56 were accepted as statistically significant ($p<0.05$), considering the identification positive when protein score confidence interval (CI) was above 98 %. In the case of MS/MS spectra, total ion score CI was above 95 %. Identification of the type (α or β) of phaseolin polypeptide was manually confirmed for each spot, considering that only α-type genes contain 15- and 27-bp direct repeats in the fourth and sixth exon, respectively, as well as other differential features of the nucleotide sequences of α- and β-type gene coding regions (*18,19,23,24,56*).

Phaseolin deglycosylation

Phaseolin deglycosylation was carried out with the enzyme protein-N-glycosidase F (PNGase F, New England Biolabs, Ipswich, MA, USA) following the manufacturer's procedure. PNGase F was incubated (25 U/mL) with 50 μg of total seed protein extracted by the phenol method and diluted in reaction buffer (New England Biolabs) until a final volume of 20 μL was reached. The mixture was incubated for 12 h at 37 °C. Partial digestion with PNGase F was carried out under the conditions that resulted in cleavage of only a limited number of N-glycan chains attached to the Asn residues of phaseolin. For this purpose, the mixture was incubated for 4 h at the same temperature.

Amplification by PCR of the Phs (SCAR) marker

Two individuals from each cultivar were germinated in seedbeds under controlled conditions and young trifoliate leaves were collected, ground in liquid nitrogen and disrupted using a mill (MM-400, RETSCH, Haan, Germany). DNA extraction was performed using the procedure described by Afanador *et al.* (*57*) with modifications. Amplification by the polymerase chain reaction (PCR) of the sequence-characterized amplified region (SCAR) marker of the *Phs* (phaseolin seed protein) locus was carried out as described by Miklas (*58*) in a final volume of 25 μL. Primers designed by Kami *et al.* (*56*) from regions of complete identity between the T and S phaseolin sequence were used to amplify the *Phs* SCAR marker. The amplified fragment covers the region where the 15-bp repeat (present in α-type genes) and a 21-bp direct repeat (third intron) are located. The PCR products were electrophoresed in a 1.8 % agarose gel together with a 100- to 1000-bp ladder (Fermentas, Thermo Scientific, Ontario, Canada). The images were digitalized and analyzed by using a Gel Doc™ XR+ system (Bio-Rad Laboratories).

Results and Discussion

Optimization of 2-DE phaseolin protein profiles

A preliminary study for the optimization of 2-DE phaseolin protein profiles from bean seed samples from cultivars ICA Pijao (Mesoamerican) and Calima (Andean) was performed, which have been used as gene pool controls in a variety of studies about bean diversity (*42,48*). Protein extraction methods and electrophoretic conditions giving rise to high-quality 2-DE profiles of the proteome of bean seeds had previously been assessed in our laboratory (*55*). Fig. 1 shows a representative image of a 2-DE gel stained with Sypro Ruby obtained from 250 μg of total seed protein of the ICA Pijao cultivar, loaded on 24-cm IPG strips of 4–7 pH linear gradients and 15 % (by mass) polyacrylamide in SDS-PAGE. The presence of a very saturated gel zone can be seen because of high concentration of phaseolin protein in bean seeds, which

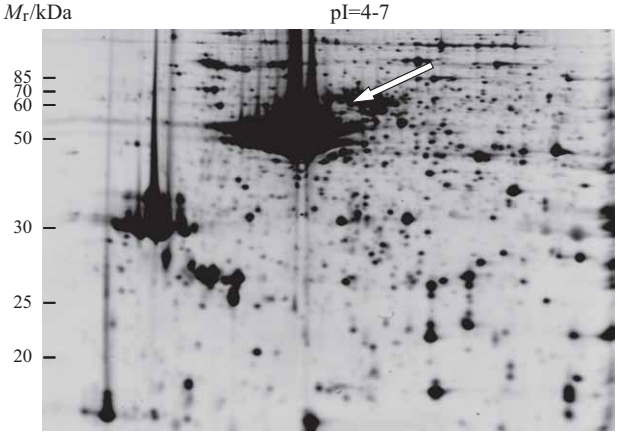

Fig. 1. 2-DE gel image of the seed proteome of *P. vulgaris* for ICA Pijao cultivar. The arrow indicates the gel zone where the phaseolin protein is located. 2-DE was performed using 250 μg of total protein loaded on 24-cm long IPG strips, pH=4–7, and 15 % (by mass) polyacrylamide gels

includes the range of pH=5.6–5.8 and M_r=45–51 kDa, previously reported for a group of phaseolin polypeptides on 2-DE gels (*37*,*38*). Based on a series of experiments, the resolution of the area of 2-DE gels where phaseolin polypeptides are located was improved using lower concentration of total seed protein (50 μg). Fig. 2 shows 2-DE gel images of seed protein samples of ICA Pijao, Calima and a homogeneous pooling of both samples, which exhibit defined patterns of phaseolin spots. The presence of phaseolin in 2-DE gels was confirmed by Western blot analyses (not shown). The relationship between 1-DE and 2-DE phaseolin patterns is also shown in Fig. 2. A direct correspondence between protein spots and 1-DE banding patterns can be established because SDS-PAGE was carried out using IPG strips after IEF together with the samples of total protein extracts loaded in adjacent gel wells. The coalescence of strings of 2-DE spots in typical 1-DE banding patterns can be seen. Note that 1-DE phaseolin profiles obtained for cultivars ICA Pijao and Calima were coincident with those previously reported for S and C cultivars, showing patterns of two and three main bands, respectively (*37*, *38*,*43*,*44*). Pooled samples were used to assess the relative positions of phaseolin spots over different cultivars regarding pI and M_r. Higher resolution of 2-DE phaseolin patterns in the first and second dimension was eventually obtained by using narrow-range (pH=4.7–5.9) IPG strips and 10 % polyacrylamide gels, respectively, reducing the occurrence of partially or totally overlapping spots (Fig. 3).

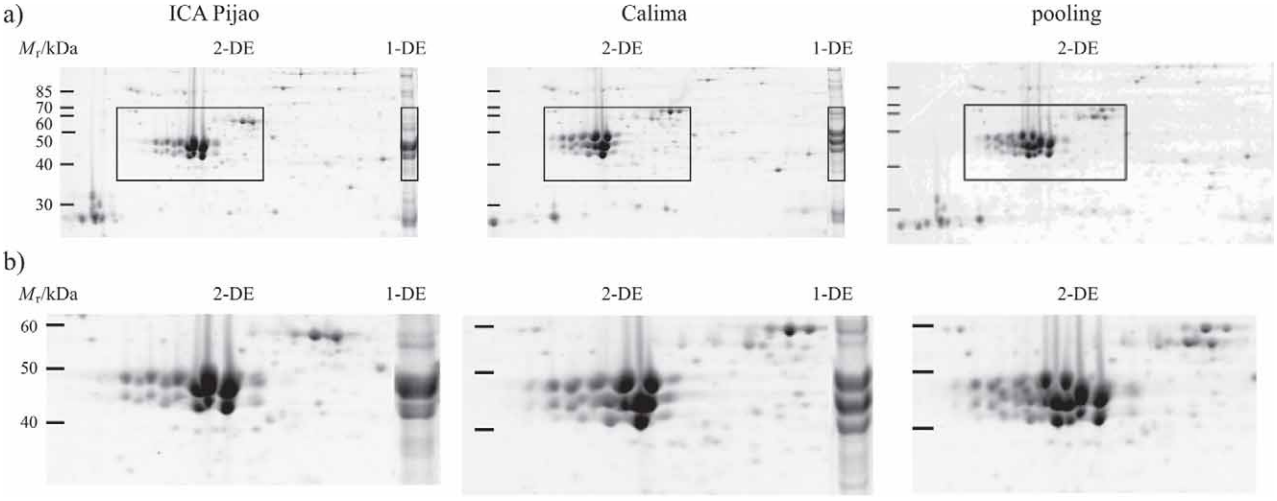

Fig. 2. 2-DE phaseolin profiles of cultivars ICA Pijao (left) and Calima (centre) and both cultivars pooled (right). 1-DE phaseolin profiles of these two cultivars are also shown. Gel regions containing phaseolin spots and protein bands are: a) enclosed in rectangles and b) enlarged. 2-DE was performed using 50 μg of total protein loaded on 24-cm long IPG strips, pH=4–7, and 15 % (by mass) polyacrylamide gels

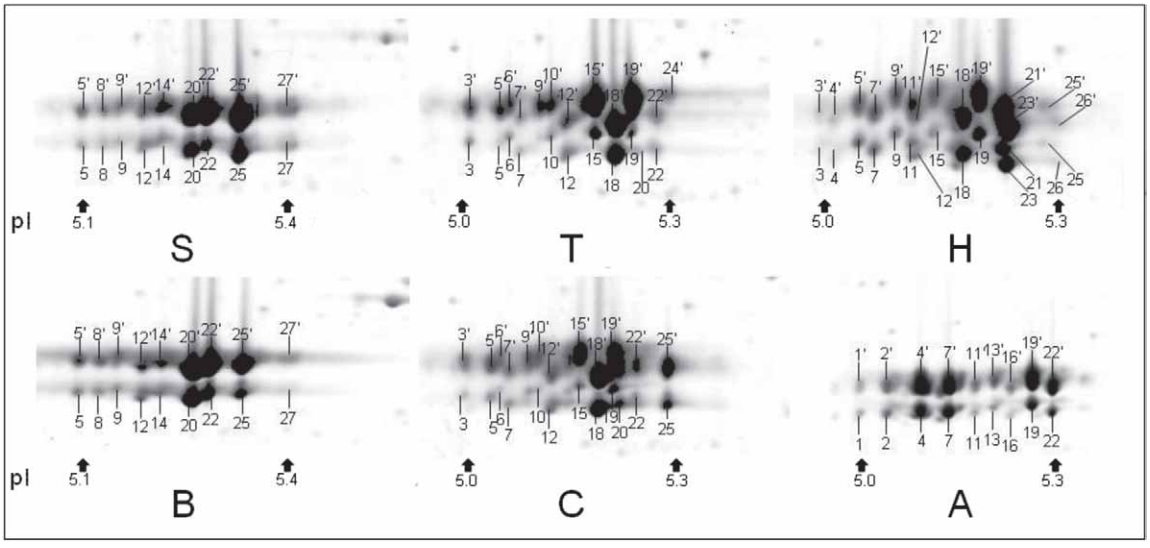

Fig. 3. 2-DE spot patterns of different types of phaseolin (S, B, T, C, H and A). Spots were numbered after matching all spots across cultivars by PDQuest. Up and down isospots of different M_r and similar pI were denoted with and without apostrophe, respectively. 2-DE was performed using 50 μg of total protein loaded on 24-cm long IPG strips, pH=4.7–5.9, and 10 % (by mass) polyacrylamide gels

Description of 2-DE phaseolin patterns

We have applied optimized 2-DE protocols to the analysis of six phaseolin types often found among domesticated varieties of common bean: S and B from the Mesoamerican gene pool, and T, C, H and A from the Andean gene pool. 2-DE gel images showing phaseolin patterns for each cultivar are presented in Fig. 3. The images are of excellent quality with well defined and highly reproducible spots, allowing the precise and in-depth description of 2-DE phaseolin patterns. 2-DE images revealed the presence of very complex phaseolin spot patterns. First of all, it can be seen that patterns of phaseolin within cultivars are organized in a horizontal string of multiple spot pairs varying in pI and M_r, each pair composed of isospots of different M_r. Pairs of isospots were then numbered in the order of their relative pI, from more to less acidic, according to their location on the master gel after matching all spots across cultivars by PDQuest software. The up/down isospots within each pair were distinguished as 1'/1, 2'/2, *etc*. Second, 2-DE protein maps of phaseolin within cultivars contained a number variable of intensive (*e.g*. 20'/20 spot pair in S cultivar) and faint (*e.g*. 5'/5 spot pair in S cultivar) spot pairs. Intensive and faint spots in most cultivars hereafter will be termed 'major' and 'minor' spots, respectively. A more detailed view of 2-DE images reveals that a 'main' spot pattern is repeated to a certain degree along the spot collection within each cultivar. For example, spot pairs 7'/7, 9'/9, 11'/11 and 12'/12, and spot pairs 18'/18, 19'/19, 21'/21 and 23'/23 in H cultivar appear to be repetitions of a similar main pattern.

Overall, our 2-DE phaseolin profiles are quite different from those reported in the literature (*8*,*9*,*17*,*26*,*37*, *39*,*59*). In particular, we have identified for the first time an important amount of minor spots located essentially in a more acidic area of the gel. This new information revealed in our 2-DE phaseolin profiles can be attributed to a variety of factors, including improvements in the protein extraction procedure, the use of IPGs, as well as the use of a wider range of pH (4.7–5.9) as compared to previous studies (pH=5.2–5.7) (*8*,*26*).

Diversity of phaseolin patterns

By comparing the 2-DE phaseolin spot maps from different cultivars, changes among individual cultivars can be detected and quantified. This allowed identification of a total of 51 different spots matched across all cultivars and up to 35 of them achieving the same relative position on the master gel. Table 1 shows the number of major, minor and unique phaseolin spots detected in each cultivar. It can be seen that the number of spots was rather considerable within each cultivar and remarkably variable among cultivars: it ranged from 18 (S and B cultivars) to 28 (H cultivar) and averaged (±standard error, S.E.) 21.5±1.7. This number of phaseolin spots was far higher than the 6 spots on average reported previously (*26*,*37*). Therefore, the information regarding the amount of polypeptides that conforms to each one of phaseolin patterns is substantially increased. In addition, most differences among cultivars can be ascribed to major and unique spots. Thus, the number of major spots across cultivars ranged from 6 to 16, whereas that of minor spots only ranged from 10 to 12. The percentage of unique or specific spots of each cultivar also varied greatly among cultivars (from 0 to 16 %), with H and A cultivars showing the highest percentage of unique spots (10 and 16 %, respectively).

Table 1. Number of major, minor and unique 2-DE spots across different phaseolin types

Phaseolin type	Major spots	Minor spots	Unique spots	Percentage of unique spots*
S	6	12	0	0
B	6	12	0	0
C	13	11	0	0
T	11	12	1	2
H	16	12	5	10
A	8	10	8	16

*referred to the total amount of spots detected with PDQuest software

The degree of similarity of phaseolin patterns measured as the proportion of spots shared between pairs of phaseolin types was estimated through the F coefficient:

$$F=2n_{xy}/(n_x+n_y) \qquad /1/$$

where n_x and n_y are the total numbers of protein spots scored in phaseolin types x and y respectively, and n_{xy} is the number of spots shared by x and y (Fig. 4a) (*60*). The proteomic distance (D) between pairs of phaseolin patterns was computed as 1–F (*60*). Major and minor spots were considered separately in the analysis. A multidimensional scaling (MDS) analysis (*61*) from proteomic distance was carried out to construct a map showing the relationships between the different phaseolin patterns (Fig. 4b). XLSTAT software v. 1.01 (Addinsoft, Andemach, Germany) was used to perform MDS analysis and to generate the two-dimensional representation. The configuration produced by MDS showed that Mesoamerican and Andean phaseolin types are clearly differentiated either by major or minor spots. However, a clearer differentiation between phaseolin patterns was revealed from major spots. The map produced by MDS also showed that phaseolin type A was the most differentiated pattern. Nevertheless, a higher number of different Mesoamerican cultivars needs to be analyzed to achieve full validation.

It has been hypothesized that C-type phaseolin is a composite of T- and S-types on the basis of 1- and 2-DE phaseolin patterns because it exhibited polypeptides common to both types (*37*), which might imply that C-type is in an intermediate position between the Mesoamerican and the Andean pool. This is in disagreement with later studies that situated C cultivar within the Andean pool (*39*), and with phenotype data of seed and plant architecture of this cultivar that display characteristics that are clearly present in varieties of the Andean type. The 2-DE patterns identified here undoubtedly support the C-type as a phaseolin pattern belonging to the Andean pool, and moreover, closely related to the T-type (the percentage of shared spots was over 80 %). On the other hand, Boyacá 22 cultivar was used in the past to locate a

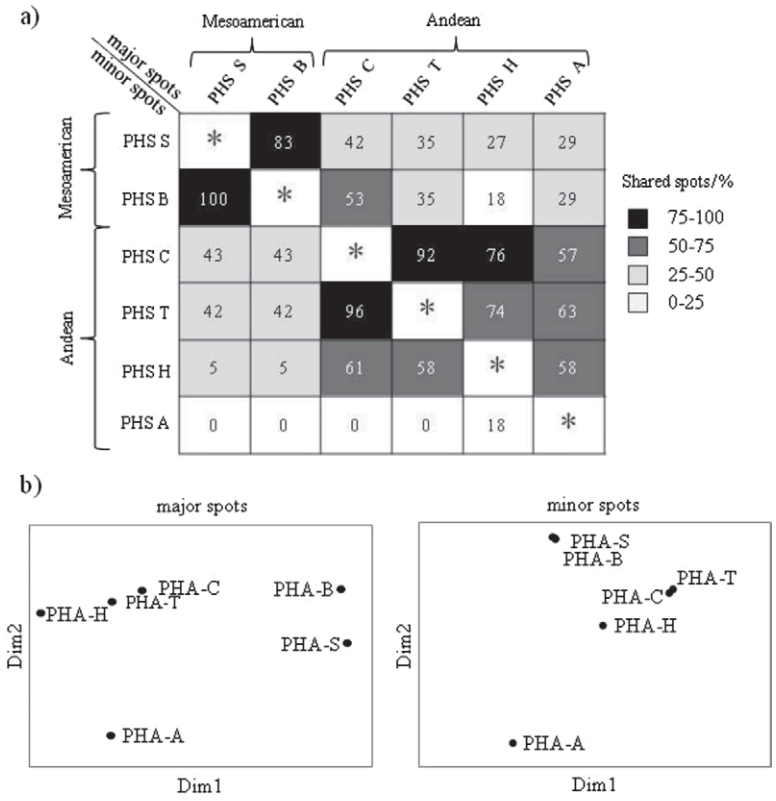

Fig. 4. Percentage of major (upper) and minor (bottom) spots shared between phaseolin cultivars as measured by F-index (a); non-metrical multidimensional scaling (MDS) from proteomic distance (D=1-F) between phaseolin types (b)

primary centre of domestication in Colombia, in addition to the Mesoamerican and the Andean centres (*9*). However, in the present analysis this cultivar showed a pattern very similar to the S-type, mainly in the minor spots (the percentage of shared minor spots was 100 %). This is not a surprising result because B and S types of phaseolin have been found in this cultivar (*9*). It cannot be excluded, however, that cultivars B and S were actually similar regarding phaseolin patterns. Important differentiation of H and A profiles from the other phaseolin patterns analyzed should also be highlighted, which is in agreement with initial studies of 2-DE (*9*).

Molecular interpretation of 2-DE phaseolin patterns

The above described 2-DE patterns can be interpreted taking into account the available information on the polypeptide composition of phaseolin together with its co- and post-translational modifications (see Introduction). The presence and distribution of α- and β-type polypeptides along strings of 2-DE phaseolin spots were assessed in cultivars S (Mesoamerican gene pool) and T (Andean gene pool) by MALDI-TOF and MALDI-TOF/TOF MS. Most selected spots (22 out of 24 spots) for MS analyses were unequivocally identified as α- or β-phaseolin polypeptides through PMF, MS/MS spectral data and distinctive features of their nucleotide sequences (see Materials and Methods section). The identification results are listed in Table 2. MS data revealed several salient features of phaseolin patterns. First, single isospot pairs contained only one type (α or β) of phaseolin polypeptide. Second, it was invariably found that isospot pairs with higher M_r (*e.g.* 22'/22 pair in cultivar S) were identified as α-type polypeptides, whereas those pairs with a more reduced M_r (*e.g.* 20'/20 spot pair in cultivar S) were β-type polypeptides. Higher M_r of α-type polypeptides becomes consistent with the fact that the most remark-

Table 2. Identification of the type of polypeptide (α or β) in 2-DE phaseolin spot patterns (cultivars S and T) by MALDI-TOF and MALDI-TOF/TOF MS*

Cultivar S		Cultivar T	
No. spot	Phaseolin polypeptide identity	No. spot	Phaseolin polypeptide identity
5'	α	10'	α
5	α	10	α
14'	α	15'	α
14	α	15	α
20'	β	18'	β
20	β	18	β
22'	α	19'	α
22	α	19	α
25'	β	22'	β
25	β	22	β
27'	α		
27	α		

*average values (±S.E.) of Mascot scores and percentages of the protein sequence for matched peptides were 833±24 and 60.3±1.6, respectively

able divergence distinguishing the coding regions of α- and β-type genes is due to the presence of different size direct repeats only into α-type genes (15-bp repeat in the fourth exon and 27-bp repeat in the sixth exon) (*18*,*19*, *23*,*24*,*56*). The prevalence of α-type polypeptides in phaseolin patterns (Fig. 3) is in agreement with the observation that the number of amino acid replacements appears to be higher in α-type polypeptides than in β-type polypeptides (*18*,*19*).

The occurrence of α- or β-phaseolin glycoforms could explain the differential M_r observed between isospots. In order to determine the degree of glycosylation of the phaseolin polypeptides identified by 2-DE, protein extracts from some of the cultivars studied in this work were incubated with the enzyme PNGase F to eliminate N-linked oligosaccharides. Note that after PNGase F digestion, glycoproteins change not only in M_r, but also in pI because asparagine residues are converted into aspartic acid. As shown in Fig. 5, spots were sensitive to partial and total PNGase F digestion. Firstly, samples partially treated with PNGase resulted in the emergence of additional strings of spots with a more reduced M_r than spots observed without PNGase digestion, indicating that isospots were glycosylated. Secondly, patterns of totally deglycosylated phaseolin revealed the existence of only two strings of spots with different M_r but higher electrophoretic mobility than spots of glycosylated phaseolin polypeptides, which suggests that each isospot pair contained either α- or β-type polypeptides with different degree of glycosylation. These results indicate, therefore, that up (*i.e.* 1', 2', *etc*.) and down (*i.e.* 1, 2, *etc*.) isospots observed in our 2-DE gels represent double and single glycosylated polypeptides, respectively. In addition, as shown in Fig. 3, double glycosylated up isospots of each pair seem to be generally more intensive than single glycosylated down isospots, which is in agreement with previous observations showing that α- and β-type polypeptides are found either once or twice glycosylated, but most are glycosylated twice (*29*). Finally, 2-DE images revealed the presence of pI shifts or repetitions of isospot pairs along the phaseolin patterns (*e.g.* 5'/5 and 8'/8 in S cultivar). These observations might be explained by several mutually non-exclusive causes. First, pI shifts might be explained by down- and up-regulated expression of different members of α- or β-gene subfamilies containing nucleotide changes in the coding sequence. Second, pI shifts are also a good indication of possible post-translational modifications of α- and β-phaseolin polypeptides, such as phosphorylations and acetylations (*62*,*63*). Unfortunately, the possible role of these post-translational modifications on the phaseolin polypeptides has barely been considered (*27*,*33*). Further studies are clearly needed to shed more light on this issue.

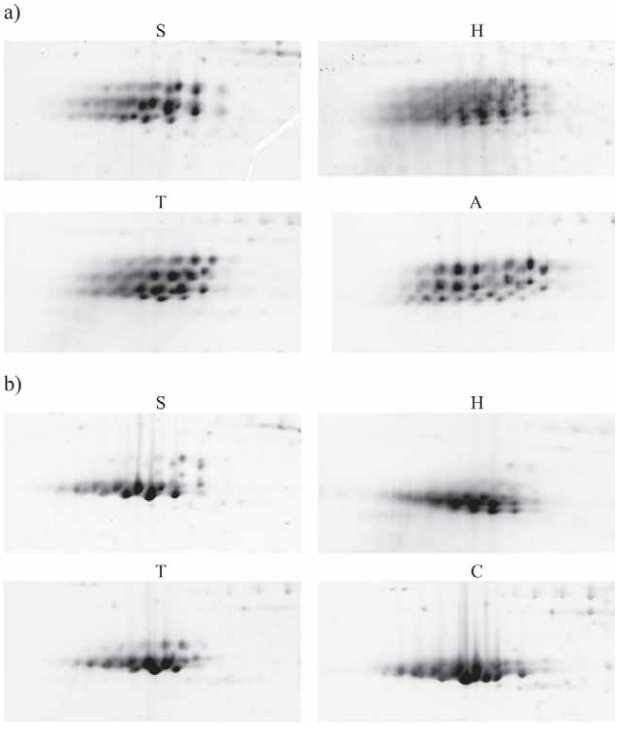

Fig. 5. 2-DE spot patterns of phaseolin types partially (a) and totally (b) deglycosylated with PNGase F. 2-DE was performed from 50 μg of total protein loaded on 24-cm long IPG strips, pH=4.7–5.9, and 10 % (by mass) polyacrylamide gels

Analysis of Phs (phaseolin) SCAR marker

The amplification by PCR of the *Phs* SCAR in the samples has rendered two major profiles across cultivars (Fig. 6). The first one consisted of two fragments of 249 (no repeats) and 270 bp (carrying 21-bp repeat); the second one contained three fragments of 249 (no repeats), 264 (15-bp repeat) and 285 bp (15- and 21-bp repeats). S, B and H samples presented the first profile, whilst T, C and A ones followed the second, although the A type profile lacked the smallest fragment.

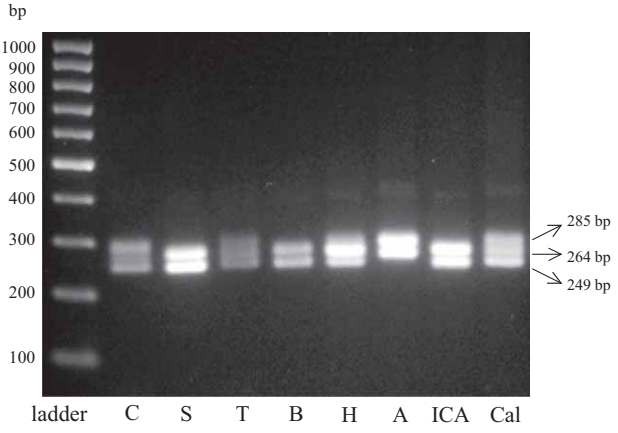

Fig. 6. Amplification by PCR of the SCAR marker of the *Phs* in the varieties Contender (C), Sanilac (S), Tendergreen (T), Boyacá 22 (B), Huevo de Huanchaco (H), Ayacucho (A), ICA Pijao (ICA) and Calima (Cal). Ladder range is 100 to 1000 base pairs (Fermentas). Arrows mark the expected size of PCR-amplified fragments for Tendergreen (*56*,*58*)

The coincidence between the SCAR profiles for S and B types as well as for T and C ones is in close agreement with the results obtained by MDS analysis from proteomic distances depicted in Fig. 4b. In addition, the SCAR profile for A type seems to be different from all the other profiles, which is in agreement with the high proteomic distance between the phaseolin A type and the other

types. This can be due to the primitive stage of domestication for A cultivar and to the presence of genetic divergence associated with environmental adaptation (*64, 65*). The primitive stage of the H type beans could also explain the different results obtained for this type from SCAR and by MDS analysis. This explanation also helps to understand the complex 2-DE profile for H type phaseolin because primitive beans (evolutionary closer to wild than to fully domesticated beans) maintain much of the genetic variability of wild beans in their genetic sequence as a result of low human selection pressures (*65*).

Conclusions

Application of current 2-DE technology revealed substantially more complex phaseolin patterns than previous studies. This high level of complexity is consistent with the fact that phaseolins are a family of proteins with variations in their polypeptide composition caused by changes in the nucleotide sequence of α- and β-gene subfamilies, as well as by co- and post-translational modifications. It can be concluded that the majority of the phaseolin spot patterns can be understood in terms of differentially glycosylated α- and β-type polypeptides, as shown by mass spectrometry and PNGase F digestion. However, the occurrence of down- and up-regulated expression of different members of α- or β-gene subfamilies and post--translational modifications such as phosphorylations and acetylations cannot be excluded, and therefore further research in this field is required. Overall, our observations show that 2-DE provides enhanced information on the polypeptide variations underlying the phaseolin protein, and that it will be useful for better understanding of phaseolin diversity and genetic differentiation of common bean populations.

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