Evidence for phosphorylation of the major seed storage protein of the common bean and its phosphorylation-dependent degradation during germination

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Abstract Phaseolin is the major seed storage protein of common bean, *Phaseolus vulgaris* L., accounting for up to 50 % of the total seed proteome. The regulatory mechanisms responsible for the synthesis, accumulation and degradation of phaseolin in the common bean seed are not yet sufficiently known. Here, we report on a systematic study in dormant and 4-day germinating bean seeds from cultivars Sanilac (S) and Tendergreen (T) to explore the presence and dynamics of phosphorylated phaseolin isoforms. High-resolution two-dimensional electrophoresis in combination with the phosphoprotein-specific Pro-Q Diamond phosphoprotein fluorescent stain and chemical dephosphorylation by hydrogen fluoride–pyridine enabled us to identify differentially phosphorylated phaseolin polypeptides in dormant and germinating seeds from cultivars S and T. Phosphorylated forms of the two subunits of type α and β that compose the phaseolin were identified by matrix-assisted laser desorption/ ionization time-of-flight (MALDI-TOF) mass spectrometry (MS) and MALDI-TOF/TOF tandem MS. In addition, we found that the levels of phosphorylation of the phaseolin changed remarkably in the seed transition from dormancy to early germination stage. Temporal changes in the extent of phosphorylation in response to physiological and metabolic variations suggest that phosphorylated phaseolin isoforms have functional significance. In particular, this prospective study supports the hypothesis that mobilization of the phaseolin in germinating seeds occurs through the degradation of highly phosphorylated isoforms. Taken together, our results indicate that post-translational phaseolin modifications through phosphorylations need to be taken into consideration for a better understanding of the molecular mechanisms underlying its regulation.

Keywords Phaseolin *Phaseolus vulgaris* Phosphoproteins Post-translational protein modification Seed phosphoproteome Phosphorylation-dependent regulation

Introduction

Phosphorylation is a ubiquitous and crucial post-translational protein modification (PTM) that contributes largely to the proteome complexity in all organisms. Changes in phosphorylation of proteins play a key regulatory role in the cellular mechanisms of signal transduction and in a diversity of cellular processes such as gene expression, metabolism, cell-cycle progression, differentiation, cytoskeletal regulation, environmental stress and hormone perception and responses. Plants have more than 1000 protein kinases, nearly twice as high as that in humans, and at least one-third of their proteins can be phosphorylated at any time (de la Fuente van Bentem and Hirt 2007; Kline-Jonakin et al. 2011). This evidence suggests that a complex network of phosphorylation events is involved in signalling pathways and regulatory processes in plants.

The role of the plant seed phosphoproteome on the spatiotemporal regulation of processes engaged during seed development and germination is only now being discovered (Agrawal and Thelen 2006; Kersten et al. 2009; Myernik and Hajduch 2011). Seed storage proteins (SSPs) play a pivotal role during seed germination and early seedling growth (Spencer 1984; Aberlenc-Bertossi et al. 2008; Myernik and Hajduch 2011). Thus, SSPs are rapidly accumulated during seed development and deposited in protein storage vacuoles or protein bodies. During germination and postgerminative growth, SSPs are mobilized to provide amino acids and reduced nitrogen necessary until the developing seedling and the assumption of autotrophy. Several recent large-scale identification and quantitative profilings of phosphoproteins

expressed during seed maturation in soybean (*Glycine max*), rapeseed (*Brassica napus*) and *Arabidopsis* (*Arabidopsis thaliana*) have substantially increased our knowledge on seed phosphoproteome (Agrawal and Thelen 2006; Meyer et al. 2012). A total of 2,001 phosphopeptides were identified spanning all three species, representing 1,026 unambiguous phosphorylation sites from 956 non-redundant phosphoproteins belonging to functionally diverse categories. In addition, evidence of phosphorylated SSPs such as cruciferins, albumins and cupins has also been reported (Agrawal and Thelen 2006; Irar et al. 2006; Wan et al. 2007; Meyer et al. 2012), but the potential functional role of these phosphorylated isoforms is yet unknown.

The common bean (Phaseolus vulgaris L.) is the most important grain legume for human consumption, mainly in South America and Africa (Gepts et al. 2008). In recent years, certain studies have addressed the characterization of common bean seed proteome (Lee et al. 2009; Mar- solais et al. 2010; Mensack et al. 2010; de la Fuente et al. 2011), but no systematic study has been conducted to date to unravel its phosphoproteome. However, the common bean has traditionally been recognized as a model for the study of SSPs (Vitale and Bollini 1995). The 7S globulin phaseolin is the major SSP of the common bean that may account for ca. 50 % of total seed protein (Emani and Hall 2008). The phaseolin has been studied for decades and many of the complicated aspects of its structure, genetic basis, expression, regulation and evolution are now well understood (for review, see Gepts 1998, 2008; Hall et al. 1999; Singh 2001; Emani and Hall 2008). It is a glycoprotein encoded by a gene family constituted by approx. 6-10 co-dominant genes per haploid genome, organized in a single

close cluster of related genes on chromosome 7. Members of the phaseolin gene family can be subdivided in two distinct gene types (or subfamilies) termed α and β encoding for α- and β-type polypeptides, respectively. Each α- and β-phaseolin polypeptide is co-translationally glycosylated in the lumen of the endoplasmic reticulum through Asn-linked N-glycans (Bollini et al. 1983; Lioi and Bollini 1984; Sturm et al. 1987).

The regulatory mechanisms involved in the phaseolin synthesis have been intensively studied at the transcriptional level. Thus, the phaseolin promotor of a member of the β-gene (*phas*) subfamily represents one of the best studied seed-specific promoters (Li et al. 1999; Hirayama and Shinozaki 2007; Ghelis et al. 2008; Ng and Hall 2008). It was found that different transcriptional activation mechanisms and various transcriptional factors are involved in the temporal and spatial regulation of the *phas* promotor (Ng and Hall 2008). In particular, the balance between the phytohormone abscisic acid (ABA) and the antagonist gibberellic acid (GA) plays a key regulator role in diverse seed physiological processes that include maturation, dormancy and germination (Finch-Savage and Leubner-Metzger 2006; Gutierrez et al. 2007). In addition, the reverse phosphorylation of ABA seems to play a crucial role in the regulation of the *phas* promoter because its transcription requires activation by an ABA- induced signal transduction cascade mediated by protein kinases and phosphatases (Hirayama and Shinozaki 2007; Ghelis et al. 2008). So far, however, there is no evidence about the existence of phosphorylated phaseolin isoforms.

Recently, high-resolution two-dimensional electrophoresis (2-DE) gels have

117 enabled the obtention of more informative reference maps of the protein
118 phaseolin than previous studies based on one-dimensional electrophoresis and
119 2-DE (de la Fuente et al. 2011). The analysis of high-resolution 2-DE phaseolin
120 patterns indicated that our understanding about the molecular characteristics of
121 the mature phaseolin protein is still far from being complete. By way of
122 illustration, a schematic representation of 2-DE phaseolin patterns in dry mature
123 seeds from widely grown domesticated cultivars (cvs.) of the common bean is
124 shown in Fig. 1. It can be seen that phaseolin patterns within each cv. are
125 organized in a horizontal string of multiple isospot pairs varying both in the first
126 dimension (p*I*, isoeletric point) and the second dimension (M_r, molecular mass).
127 Downstream analyses based on MALDI-TOF and MALDI-TOF/TOF MS and the
128 deglycosylation of the protein phaseolin revealed that each single isospot pair
129 contained only one type (i.e., α or β) of the two phaseolin polypeptides glyco-
130 sylated either once or twice. The observation of multiple α- and β-subunits
131 varying in p*I* along 2-DE phaseolin profiles suggests that they were more likely
132 the result of PTMs such as phosphorylations. This research aimed to survey the
133 presence and dynam- ics of phosphorylated phaseolin polypeptides by using a
134 wide range of complementary biochemical and proteomic approaches including
135 high-resolution 2-DE, staining with a phosphoprotein-specific Pro-Q Diamond
136 phosphoprotein fluorescence stain, enzymatic and chemical protein dephos-
137 phorylation treatments and MS methodologies. In particu- lar, this study aimed
138 to determine whether phosphorylation of the protein phaseolin should be taken
139 into consideration as a regulatory mechanism involved in its synthesis and
140 degradation.

Materials and methods

A schematic overview of the proteomics and biochemical experimental procedures used to characterize the status of phosphorylation of the protein phaseolin during different seed stages is shown in Fig 2.

Plant material

Dry mature seeds of common bean (*P. vulgaris*) cvs. from the Mesoamerican and Andean gene pools dis- playing different phaseolin types were provided by the United States Department of Agriculture: Sanilac (accession PI 549695) for S-type Mesoamerican phaseolin and Tendergreen (PI 549633) for T-type Andean phaseolin. Dry mature and 4-day germinated bean seeds from both cvs. were assayed. For germination, dry seeds were grown in a phytotron at 20–22 °C with a 16/8 h (light/ dark) cycle. Ten bean seeds at dormant stage and ten seeds at germinating stage from each cv. were lyophilized after previous grinding of the total seed tissue to a powder with liquid nitrogen using a pre-cooled mortar and pestle. The samples were stored at 80 °C until protein extraction

Seed protein extraction

Total seed protein was extracted by the phenol method (Saravanan and Rose 2004). This protein extraction method has been proved to give highly satisfactory results for the separation of common bean seed proteins by 2-DE and downstream identification by MS in comparison with other commonly used extraction methods (de la Fuente et al. 2011). The phenol method was carried out from 100 mg of sample suspended in extraction buffer (500 mM Tris– HCl, 500 mM EDTA, 700 mM sacarose, 100 mM KCl pH 8, 2 % DTT and 1 mM

PMSF). Phenol saturated with Tris–HCl (pH 6.6–7.9) was added and the phenolic phase was recovered after centrifuging (4,500 rpm at 4 °C). Proteins were precipitated (0.1 M ammonium acetate in MeOH), protein pellets were washed (0.1 M ammonium acetate, 10 mM DTT, 80 % acetone and 10 mM DTT) and re-suspended in lysis buffer (7 M urea; 2 M thiourea; 4 % CHAPS; 10 mM DTT, and 2 % Pharmalyte™ pH 3–10, GE Healthcare, Uppsala, Sweden). Protein quantification was assessed for each extraction using the commercial CB-X protein assay kit (G-Biosciences, St. Louis, MO, USA) following the instructions of the manufacturer for using a microplate reader.

DE phaseolin patterns

Optimized 2-DE protocol for high-resolution phaseolin pat- terns, including the use of low concentrations of total seed protein for loading into gels, zoom gels and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) at low polyacrylamide concentration, were used as previously described (de la Fuente et al. 2012). 2-DE was carried out from 50 µg of total seed protein extract dissolved in lysis and rehydration (7 M urea, 2 M thiourea, 4 % CHAPS, 0,002 % Bromophenol Blue) buffers (De La Fuente et al. 2011). Protein extracts were loaded on 24-cm-long ReadyStrip™ IPG Strips with a pH linear gradient of 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 iso- electric focusing (IEF) was carried out until reach 60 kVh after an initial rehydration step for 12 h at 50 V in a PRO- TEAN® 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 (SDS-PAGE), equilibrated strips were placed on 10 % (w/v) gels and they were run vertically 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. After the run, gels were stained with Sypro Ruby® stain (Lonza, Rockland, ME, USA) following the manufacturer's indications. The 2-DE gel images were acquired with a Gel Doc™ XR system (Bio-Rad Laboratories). Image analysis of digitalized gels was performed through PDQuest™ Advanced software v. 8.1. (Bio-Rad L aboratories). Spots were identified under a sensitivity of 1.1 and their p*I* and M_r were determined from their position on the IEF-strips and standard molecular mass markers ranging from 15 to 200 kDa (Fermen- tas, Ontario, Canada), respectively. When it was necessary, 2-DE gels were matched across replicates and volumes of each spot were quantitatively determined after normalization using total density in valid phaseolin spots. Four technical replicates arising from four independent protein extractions were carried out for each experiment. Using the optimized protocol, the 2-DE gel phaseolin patterns were highly reproducible

Dephosphorylation of phaseolin polypeptides

The protein phaseolin was treated with enzymatic and chemical dephosphorylation methods. Enzymatic dephosphorylation was carried out using calf intestinal alkaline phosphatase (New England, BioLabs, Inc, Massachusetts, USA). For alkaline phosphatase catalyzed dephosphorylation, 5 µL of the enzyme alkaline phosphatase (10,000 units/mL) were incubated with 50 µg of total protein extract in lysis and kit buffers (500 µL total volume) for 60 min at

37 °C. Chemical dephosphorylation of phaseolin phosphopeptides were performed according to the method of Kuyama et al. (2003) adapted to 2-DE by Kita et al. (2006). For chemical dephosphorylation, 1 mg total protein extract was dissolved in 250 µL of hydrofluoric acid (HF, 50 %) or hydrogen fluoride–pyridine (HF–P, 70 %), and left to stand in an ice bath for 3 and 1 h, respectively. The solution was then neutralized by NaOH 10 M, desalinated by using Amicon® Ultra-4 centrifugal filter devices (Millipore, Massachusetts, USA), and then eluted in 300 µL of lysis buffer. The Clean-up kit (GE Healthcare) was subsequently used for protein purification. The concentration of total protein was determined for each extraction using the commercial CB-X protein assay kit (G-Biosciences). The phaseolin changes induced by enzymatic and chemical dephosphorylations were identified by 2-DE as described above. Gel images were captured with the Gel Doc XR system and analyzed by PDQuest software.

Statistical analysis

The comparison of change in the volume of each phaseolin spot on 2-DE gels during the transition from dormancy to early germination stage was performed with the Mann–Whitney test. Fold change of the relative phosphorylation rate (*PR*) of spots from dormant to germinated seed stage was compared by Wilcoxon's signed-ranks test. The relationship between p*I*-values of spots and their relative *PR*- values was assessed by Spearman's correlation test. Statis- tical analyses were performed using the statistical software package IBM SPSS Statistics 20 (SPSS, Chicago, Il, USA).

Identification of phaseolin phosphopeptides by MS

Phaseolin spots were excised from the gels for subsequent in-gel protein

237 digestion with trypsin as described previously (*55*). The resulting peptide extracts
238 were pooled, concentrated in a SpeedVac® (Thermo Fisher Scientific, Waltham,
239 MA, USA) and stored at 20 °C. Identification of the type of phaseolin
240 polypeptide (α or β) of selected 2-DE spots was performed by the MALDI-TOF
241 and MALDI-TOF/TOF tandem MS according to the following procedure: Dried
242 samples were dissolved in 4 µL of 0.5 % HCOOH. Equal volumes (0.5 µL) of
243 peptide and matrix solution, consisting of 3 mg CHCA dissolved in 1 mL of 50 %
244 acetonitrile in 0.1 % TFA, were deposited onto a 384 Opti-TOF MALDI plate
245 (Applied Biosystems, Foster City, CA, USA) using the thin layer method. Mass
246 spectrometric data were obtained in an automated analysis loop using 4800
247 MALDI-TOF/TOF analyzer (Applied Biosystems). MS spectra were acquired in
248 positive-ion reflectron mode with a Nd:YAG, 355 nm wavelength laser, averaging
249 1,000 laser shots, and at least three trypsin autolysis peaks were used as internal
250 calibration. All MSMS spectra were per- formed by selecting the precursors with
251 a relative resolution of 300 (FWHM) and metastable suppression. Automated
252 analysis of mass data was achieved using the 4000 Series Explorer Software™
253 v. 3.5 (Applied Biosystems). Peptide mass fingerprinting (PMF) and peptide
254 fragmentation spectra data of each sample were combined through the GPS
255 Explorer Software v. 3.6 using Mascot software v. 2.1 (Matrix Science, Boston,
256 MA, USA) to search against a non-identical protein database (Swiss-Prot, Swiss
257 Institute of Bioinformatics, Lausanne, Switzerland), with 30 ppm precursor
258 tolerance, 0.35 Da MSMS fragment tolerance, carbamydomethyl cysteine
259 (CAM) as fixed modification oxidized methionine as variable modification and
260 permit- ting 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 CI (confidence interval) was above 98 %. In the case of MSMS 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 distinctive features of their nucleotide sequences (Slightom et al. 1983, 1985; Kami and Gepts 1994). Phosphorylation preliminary studies were performed allowing phosphoserine (PhosphoS), phosphotyrosine (PhosphoY) and phosphothreonine (PhosphoT) as variable modification to search against the Swiss-Prot database and using the spectra data previously obtained.

Results

Phosphorylated phaseolin polypeptides in dormant seeds

Reference (SYPRO Ruby stain) and phosphorylated (Pro-Q DPS) phaseolin patterns on 2-DE gels obtained from total protein extracts of dry mature seeds (cvs. S and T) are shown in Fig. 3a, b. There can be seen that Pro-Q DPS fluorescence signal was detected in all spots of the reference S- and T-phaseolin patterns. The PeppermintStick phosphoprotein molecular weight standards validated the specificity of the recognition of phosphoproteins by Pro-Q DPS under our experimental conditions. Thus, the phospho- proteins ovalbumin and β-casein gave fluorescence signal with Pro-Q DPS but not the unphosphorylated proteins in the marker mix (i.e., β-galactosidase, bovine serum albumin, avidin and lysozyme). Note that Fig. 3b shows only the

fluorescence signal of ovalbumin spots; the only PeppermintStick phosphoprotein marker that overlaps with phaseolin spots. It was found, therefore, that dormant common bean seeds from cvs. S and T contain phosphorylated phaseolin isoforms. In addition, the fact that phaseolin phosphopolypeptides are distributed over a wide range of p*I*-values on 2-DE gels indicates that they are differentially phosphorylated.

Enzymatic (alkaline phosphatase) and chemical (HF and HF-P) dephosphorylation methods were used for removal of phosphate moieties from phaseolin phosphopolypeptides. A comparative analysis among the three methods showed that HF-P gave more informative dephosphorylated phaseolin profiles on 2-DE gels (Supplemental Fig. 1). Representative 2-DE gel images of S- and T-phaseolin patterns after HF-P treatment are shown in Fig. 3c. It can be seen that highly expressed spots, or major spots, in reference patterns (i.e., 13′/13, 15′/15 and 17′/17 spot pairs of cv. S; and 11′/11, 12′/12 and 14′/14 spot pairs of cv. T) were detected on the same gel locations in dephosphorylated patterns, but with a lower relative volume. It follows that major spots in reference phaseolin patterns contained a mixture of phosphorylated/unphosphorylated polypeptides. The relative amount of phosphopolypeptides at each major spot was then assessed by PDQuest 2-DE gel analysis software, determining the difference in the corresponding gel-spot volume between control and treated samples. The relative phosphorylation rate (*PR*) at each spot was estimated as $PR = (X-Y)\,X100$, where X and Y denote the volume of a given spot in control and treated samples, respectively. Estimated mean (SE, standard error) volumes of the major spots

in control and treated samples and the corresponding mean (± SE) *PR*-values are shown in Supplemental Table 1. It was found that major spots of S- and T-phaseolin exhibited high *PR* scores: *PR*-values ranged from 49 to 81 % across replicates of cv. S and averaged 63+/-1.7 %, whereas for cv. T, they ranged from 13 to 82 % and averaged 46 +/- 3.9 %. In addition, *PR*- values tended to increase as p*I*s of major spots decreased. Figure 4 shows a plot of the relationship between p*I* and *PR* values over major spots in cvs. S and T. A statistically significant negative relationship between p*I* and *PR* was detected by the one-sided Spearman's nonparametric. correlation test ($r_S = -0.37$, $p < 0.05$, n = 24 for cv. S; r_S 0.62, $p < 0.01$, n 24 for cv. T). These results showed that a variety of differentially phosphorylated isoforms of the protein phaseolin were highly represented in dormant common bean seeds.

It is also expected that protein dephosphorylation causes predictable changes in the p*I* and M_r of the spots (Zhu et al. 2005; Irar et al. 2006). Protein dephosphorylation affects their p*I* by substituting the negatively charged phosphate groups on phosphoamino acid residues with neutral hydroxyl groups. As a result, dephosphorylation usually induces a basic shift on p*I* and multiple dephosphorylations may result in a significant p*I* increase. In addition, the loss of phosphate groups on phosphoserine, phospho- threonine and phosphotyrosine residues results in a mass decrease of 80 Da or multiples thereof in the case of multiple dephosphorylations. Accordingly, we found that some faintly stained spot pairs, or minor spots, located on the more acidic positions in reference patterns (i.e., spot numbers 3, 5′, 5 and 7 in cv. S; and spot numbers 1, 3′ and 3 in cv. T) were undetected after treatment with HF-P (Fig.

3c). These absent minor spots likely contained only phosphorylated phaseolin polypeptides that shifted to more basic positions on the gels after dephosphorylation. In fact, some novel spots found only in dephosphorylated patterns (i.e., one spot in cv. S; and three spots in cv. T) appeared toward more basic positions on 2-DE gels. Due to the loss of phosphate moieties, isospot pairs for the same type of polypeptide also showed a more uniform M_r than in control sample.

Phosphorylation changes of the protein phaseolin in germinating seeds

Temporal changes in phosphorylation status of the phaseolin protein were studied during the transition from dormant to 4-day germinated bean seeds in cvs. S and T (Fig. 5a–c). We found that reference 2-DE phaseolin patterns changed markedly during the shift from the seed dormant mode to the germination mode (cf. Figs. 3a, 5a). First of all, a clear difference can be seen in the spot volumes. Changes in the spot volume from seeds at dormant stage to that at germinating stage (four replicates each) were assessed by PDQuest software from a total number of 12 major spots in cvs. S and T (Supplemental Table 2). The fold change in the volume of each major spot was calculated as the ratio between the spot volume averaged across replicates in dormant and germinating seeds; fold changes less than one were represented as their negative reciprocal. Eleven out of twelve major spots exhibited a negative 1.2-fold or higher negative change in spot volume between the two sets matched in our study ($p < 0.01$, n 12, one-tailed Wil- coxon's signed-ranks test). For most (82 %) major spots with negative fold change, statistically significant quantita-

tive differences were detected by the Mann–Whitney test ($p < 0.05$). These results can be explained by proteolysis of the phaseolin occurring during postgerminative growth of the seed. Secondly, a substantial number of novel minor spots (i.e., 8 new spots in each cv.) appeared on the more acidic gel positions of reference phaseolin patterns when compared with reference phaseolin profiles in dormant seeds.

Analysis of phosphorylation levels of the phaseolin in germinating seeds was firstly performed using Pro-Q DPS (Fig. 5b). Unlike dormant seeds, phosphorylated polypeptide forms were no longer detectable on gel images after stain with Pro-Q DPS. These results, however, should be treated with caution. The absence of fluorescence signal in highly expressed major spots suggests that they do indeed contain phaseolin polypeptides, scarcely or not phosphorylated. However, the absence of fluorescence signal in faintly stained minor spots might be explained if their amounts of polypeptides have dropped back to undetectable levels for Pro-Q DPS. Our observations indeed showed that treatment with HF-P can be more effective for detecting low amounts of phosphorylated polypeptides on 2-DE gels than Pro-Q DPS. Figure 5c shows that S- and T-phaseolin patterns were sensitive to dephosphorylation with HF-P. Thus, a substantial number of minor spots on the more acidic gel positions of reference patterns (14 and 13 minor spots in cvs. S and T, respectively) were not detected after dephosphorylation. It follows that these spots contained only highly phosphorylated polypeptides. In addition, gel images from dephosphorylated samples disclosed the emergence of novel phaseolin spots on the right side of the pat- terns in comparison with untreated control samples,

which would correspond to phosphopolypeptides that underwent a basic shift on p*I* after dephosphorylation. Overall, these observations suggest that differentially phosphorylated polypeptides coexist with unphosphorylated forms in germinating bean seeds, but at a lower relative proportion than in dormant seeds because they were not detected by Pro-Q DPS. Accordingly, *PR*-values of phaseolin polypeptides decreased remarkably in germinating seeds when compared with dormant seeds (Supplemental Table 3). Most major spots (11 out of 12 spots of cvs. S and T) underwent a negative fold-change in *PR* from dormancy to early germination stage ($p < 0.05$, n 12, one-tailed Wilcoxon's signed-ranks test; Fig. 6; Supplemental Table 4). Interestingly, some minor spots toward more upstream locations of reference phaseolin patterns in germinating seeds exhibited the lowest M_r despite the fact that they contain highly phosphorylated polypeptides. This seemingly contradictory observation might be explained by assuming that these highly phosphorylated isoforms were actually intermediate degradation products, a result of the action of proteolytic enzymes.

Identity of phaseolin phosphopolypeptides

Twenty-four 2-DE gel spots of different p*I* and M_r along S- and T-phaseolin patterns of seeds at dormant stage were selected for MALDI-TOF and MALDI-TOF/TOF MS analyses (Table 1; Supplemental Table 5). Most selected spots (23 out of 24 spots) were unambiguously identified. Each single isospot pair contained only one type, α or β, of the two polypeptides that compose the phaseolin. Isospot pairs with higher and lower M_r corresponded to α- and β-type polypeptides, respectively, due to the presence of different size direct repeats

405 only into α-type genes: 15 bp repeat in the fourth exon and 27 bp repeat in the
406 sixth exon (Slightom et al. 1983, 1985; Kami and Gepts 1994). From the joint
407 analysis of MS data and the above-mentioned 2-DE-based information, it can be
408 concluded therefore that multiple isoforms of differentially phospohorylated α-
409 and β-type polypeptides are pre- sent in dormant seeds of the common bean. In
410 addition, spots of dephosphorylated T-phaseolin patterns on 2-DE gels were
411 identified by MS (Supplemental Fig. 2 and Supplemental Table 6). MS analyses
412 confirmed that, indeed, dephosphorylated phaseolin patterns contained only
413 phaseolin polypeptide

414 A preliminary study of phosphopeptide mapping was conducted from
415 MALDI-TOF spectral data. To detect a possible phosphopeptide,
416 phosphorylation was taken into consideration as a modification for the data
417 base search. In total, six different phosphopeptides were detected from α- and
418 β-polypeptide tryptic digests of cvs. S and T (Table 2). Each phosphopeptide
419 identified had two or more putative phosphorylation sites. For some spots,
420 unphosphorylated/ phosphorylated forms of the same peptide were identified in
421 accordance with 2-DE-based phosphorylation analyses. For instance, the major
422 spot 17′ of cv. S contained unphosphorylated/phosphorylated forms of the
423 peptide ATSNVN- FTGFGINANNNNR located at the position 336–354 of the β-
424 subunit sequence. Another interesting observation of MS analysis is that the
425 phosphopeptide TDNSLNVLISSIEM- KEGALFVPHYYSK (position 257–283 of
426 the β-subunit sequence) contained the X-T-D-X motif, a phosphorylation motif
427 largely represented in SSPs (Meyer et al. 2012). Enrichment of phosphorylated
428 polypeptides using titanium dioxide (TiO_2) column and immobilized metal-ion

affinity chromatography (IMAC) was performed for the specific capture of phosphopolypeptide spots. Unfortunately, these enrichment techniques combined with MSMS were unsuccessful in phosphopeptide identification (results not shown). Experiments in progress in our laboratories are trying to map the phosphopeptides and phosphorylation sites in α- and β-phaseolin subunits more precisely by using phosphopeptide enrichment strategies in combination with high-resolution MS.

Discussion

In this study, we report the first evidence of phosphorylated phaseolin isoforms in common bean seeds. More specifically, phosphorylated/unphosphorylated forms of both α- and β-type phaseolin polypeptides were found to coexist in dormant and germinating seeds from cvs. S and T. In addition, differentially phosphorylated α- and β-polypeptides were identified at relative high frequency in both cvs. of common bean. Therefore, the protein phaseolin adds to the growing catalog of phosphorylated SSPs found in several plant species (Schwenke et al. 2000; Agrawal and Thelen 2006; Irar et al. 2006; Meyer et al. 2012). Furthermore, these findings open new perspectives for understanding the regulatory mechanisms involved in the synthesis and degradation of the major SSP of the common bean. The pre- sent investigations suggest that phosphorylation-dependent regulation may play an important role in the synthesis and degradation of the protein phaseolin not only at the transcriptional level (see “Introduction” section) but also at the post-translational one.

Recent seed phosphoproteome studies have reported evidence for

quantitative changes in the profiling of phosphoproteins expressed at five sequential stages of seed development in *Arabidopsis*, rapeseed and soybean (Agrawal and Thelen 2006; Meyer et al. 2012). It was found that phosphoprotein expression profiles followed variable trends during seed filling depending on the protein functional categories. Different phosphoprotein sets seemed to be regulated in a stage-specific and coordinate manner, suggesting that protein phosphorylation might be involved in the regulation of SSP synthesis. The study of rapeseed phosphoproteome by Meyer et al. (2012) is particularly appropriate for comparison with our data because it represented all stages of seed development, including late maturation. It is noteworthy that a majority (74.5 %) of the phosphopeptides at different sequential stages of development in rapeseed were identified at the late maturation stage. Most fractions of phosphopeptides corresponded to proteins annotated as late-embryogenesis-abundant proteins as well as to proteins involved in cell wall structure or cell wall polysaccharide synthesis. In particular, the expression of some phosphorylated subunits of the SSP cruciferin was found to increase as seed development progressed, reaching higher levels during late maturation. Our investigations also showed that α- and β-type phaseolin phosphopolypeptides were abundantly expressed in dormant common bean seeds. Interestingly, about 25 % of the proteins containing the X-T-D-X phosphorylation motif in *Arabidopsis*, rapeseed and soybean were found to be SSPs (Meyer et al. 2012). Likewise, one of the phosphopeptides identified in our study contained the X-T-D-X motif, suggesting that this phosphorylation motif might be a key target of phosphorylation during seed filling. Overall, these studies indicate that phosphorylation of SSPs may

play a regulation role in seed transition to a quiescent state.

The dynamics of phosphoproteins during transition from seed dormancy to a germination state is as yet not very well known. In this study, we show that the levels of phosphorylation of the protein phaseolin are able to undergo dynamic changes in response to seed physiological and metabolic variations taking place from dormancy to early germination stage. Specifically, we found that the amount of α- and β-type phosphopolypeptides decreased considerably in germinating seeds as compared with dormant seeds. Previous observations in Scots pine (*Pinus sylvestris* L.) have also shown that patterns of SSP phosphorylation changed markedly in germinating seeds (Kovaleva et al. 2013). It has been recognized that changes in the extent of phosphorylation of proteins in response to a stimulus represent a useful way to identify phosphorylations with functional consequences (Agrawal and Thelen 2006; Myernik and Hajduch 2011). Unfortunately, changes in the amount of phosphorylated phaseolin do not necessarily prove their biological importance by themselves. That is to say, changes in phosphopeptide abundance can be the result of genuine phosphorylation changes, but also of altered protein abundance. In fact, the overall amount of phaseolin decreased noticeably when common bean seeds shifted from the dormant into the non-dormant state, reflecting utilization of SSPs during germination and early growth to provide the necessary biosynthetic intermediates until the developing of the seedling. However, we found a subset of phaseolin phosphopolypeptides with higher phosphorylation levels in germinating seeds than in dormant seeds, which cannot be explained merely by a decrease of the amount of phaseolin during germination. These changes in the

501 extent of phosphorylation of phaseolin in response to physiological and
502 metabolic variations suggest that they have functional significance. In addition to
503 this dynamic phosphorylation status, highly phosphorylated phaseolin isoforms
504 in germinating seeds were found to be partially degraded polypeptides. From
505 these findings, we suggest the hypothesis that the protein phaseolin is mobilized
506 through the degradation of highly phosphorylated isoforms by proteases.
507 Consider- able evidence supports the view that exposure of cells to a variety of
508 extracellular stimuli leads to the rapid phosphorylation of intracellular proteins
509 and their subsequent degradation by the ubiquitin-proteosome pathway (Karin
510 and Ben-Neriah 2000; Hellmann and Estelle 2002).

511 Protein phosphorylation is a transient and reversible event that can
512 change at very short intervals of time hindering its identification and
513 characterization (Schubert et al. 2006; Kersten et al. 2009). In this regard, many
514 aspects of the biology of seeds make them an excellent model system to assist
515 in identification of reverse phosphorylation events (Baud et al. 2002; Finch-
516 Savage and Leubner-Metzger 2006; Gutierrez et al. 2007). Seed dormancy is a
517 particularly interesting seed property because it entails a temporary metabolic
518 inactivity or minimal activity whereby germination is delayed as a mean to avoid
519 conditions unfavourable for seedling survival. Dormancy break and germination
520 can be experimentally induced and then any fast changes in the proteome
521 phosphorylation status from dormant seeds to early seedling growth can be more
522 easily monitored. In the present study, a high-resolution 2-DE-based
523 experimental strategy has been instrumental in unravelling the presence and
524 relative amounts of phosphorylated isoforms of phaseolin polypeptides as well

as their dynamic changes in dry-to-germinating seed transition. However, more prospective follow-up studies are clearly needed to address the phosphorylation-dependent regulation of the expression of the protein phaseolin at posttranslational level. Specifically, further research is required to pinpoint exact sites of phosphorylation, to determine the stoichiometry of phosphorylation at different stages of seed development and germination and linkage of phosphosites to specific kinases and phosphatases.

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Conflict of interest The authors declare that they have no conflict of interest.

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CAPTION TO FIGURES

Fig. 1 Schematic representation of 2-DE reference phaseolin patterns in dry mature common bean seeds. **a** Cv. Sanilac. **b** Cv. Tendergreen. Positions of the *spots* according to their isoelectric point (p*I*) and molecular mass (M*r*) are shown. *Up/down isospots* within each pair are distinguished as 1′/1, 2′/2, etc. *Isospot pairs* with higher and lower M*r* correspond to polypeptides α (*dark stripped circle*) and β (*light stripped circle*), respectively. *Up and down isospots* are phaseolin polypeptides with double (*DG*) and single (*SG*) glycosylations, respectively. *Intensive spots* (e.g., spot pair 15′/15 in cv. S) and *faint spots* (e.g., spot pair 3′/3 in cv. S) are designated as major and minor spots, respectively

Fig. 2 Schematic overview of biochemical and proteomic approaches used to unravel phaseolin isoforms on *2-DE* gels from total seed protein of dormant and 4-day germinating bean seeds (*cvs. S* and *T*). The steps followed to detect the presence of phosphorylated phaseolin polypeptides in dormant and germinating seeds as well as the changes in the extent of phosphorylation occurring during seed transition from dormancy to early germination stage are shown. Total seed proteins were separated by high-resolution *2-DE*. The spots were visualized by *SYPRO Ruby* staining and then scanned for image analysis to obtain reference *2-DE* phaseolin patterns. The phosphoprotein-specific *Pro-Q DPS* staining followed by *SYPRO Ruby* staining were used to identify phosphorylated phaseolin polypeptides

Fig. 3 Identification of phosphorylated phaseolin isoforms in dry mature bean seeds of cvs. S and T. **a** 2-DE images of gel sections showing reference phaseolin patterns of cvs. S (*left*) and T (*right*) obtained from untreated samples of total seed protein. Gels were stained with SYPRO Ruby protein gel stain. **b** Same gels stained with the phosphoprotein-specific fluorescent dye *Pro-Q DPS* to detect

Fig. 4 Plot of relative *PR* against p*I* for major isospot pairs of *S*- and *T-phaseolin* patterns in dry mature bean seeds. *Lines* connect mean values of *PR*-values across replicates for major isospot pairs

Fig. 5 Degradation and identification of phosphorylated phaseolin isoforms in germinating bean seeds of cvs. S and T. **a** 2-DE images of gel sections showing reference phaseolin patterns of cvs. S (*left*) and T (*right*) from untreated samples of total seed protein. *Asterisks* indicate spots absent in reference phaseolin patterns of dormant seeds as shown in Fig. 3. **b** Phosphorylated phaseolin patterns on 2-DE gels stained with *Pro-Q DPS*. **c** Dephosphorylated phaseolin patterns on 2-DE gels after HF-P treatment. *Arrows* show the signal of fluorescence of the phosphoprotein marker ovalbumin. The *dashed* and *closed circles* represent missing and newly arisen spots after dephosphorylation, respectively, as compared with reference patterns

714 **Fig. 6** Fold change of the relative *PR* for major phaseolin isospot pairs (cvs.
715 S and T) in the transition from dormant to germination. Gel position of assigned
716 *spots* is shown in Fig. 1

717

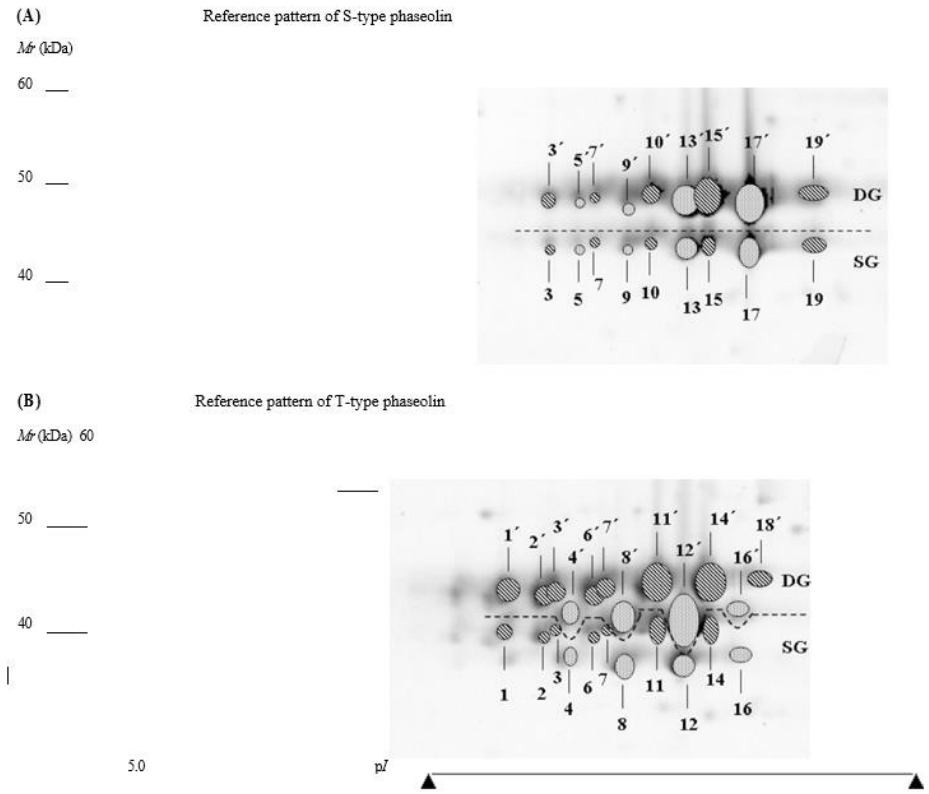
(A)
Reference pattern of S-type phaseolin
Mr (kDa)
60
50
40
3´ 5´ 7´ 9´ 10´ 13´ 15´ 17´ 19´
DG
SG
3 5 7 9 10 13 15 17 19
(B)
Reference pattern of T-type phaseolin
Mr (kDa) 60
50
40
1´ 2´ 3´ 4´ 6´ 7´ 8´ 11´ 12´ 14´ 16´ 18´
DG
SG
1 2 3 4 6 7 8 11 12 14 16
5.0
pI

Figure 1

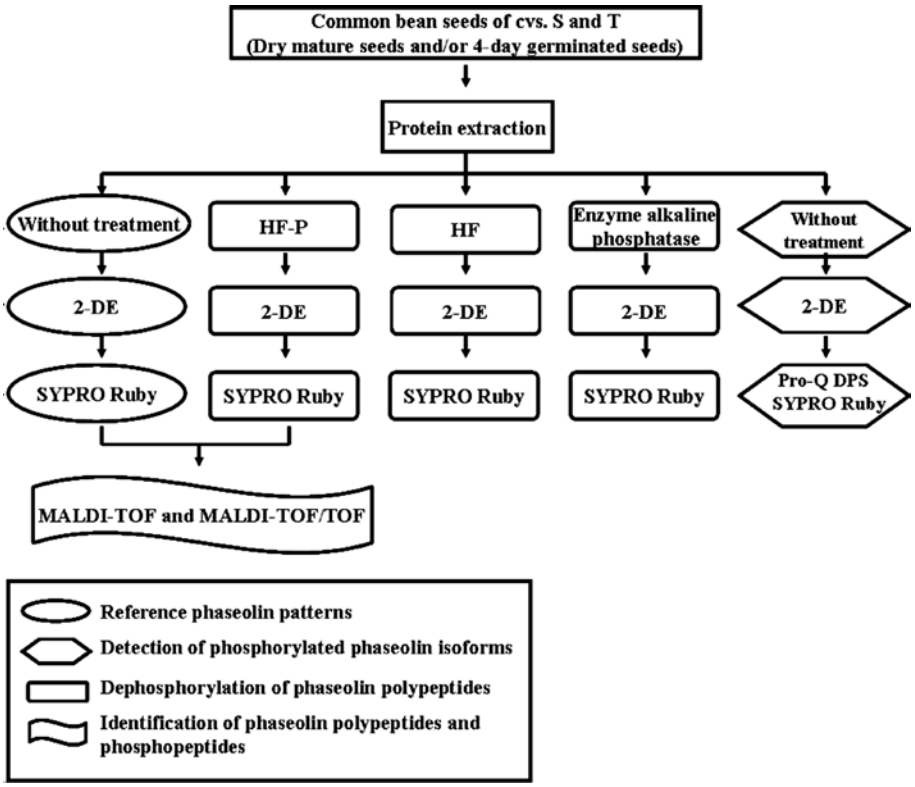
Common bean seeds of cvs. S and T
(Dry mature seeds and/or 4-day germinated seeds)
Protein extraction
Without treatment
HF-P
HF
Enzyme alkaline phosphatase
Without treatment
2-DE
2-DE
2-DE
2-DE
2-DE
SYPRO Ruby
SYPRO Ruby
SYPRO Ruby
SYPRO Ruby
Pro-Q DPS
SYPRO Ruby
MALDI-TOF and MALDI-TOF/TOF
Reference phaseolin patterns
Detection of phosphorylated phaseolin isoforms
Dephosphorylation of phaseolin polypeptides
Identification of phaseolin polypeptides and phosphopeptides

Figure 2

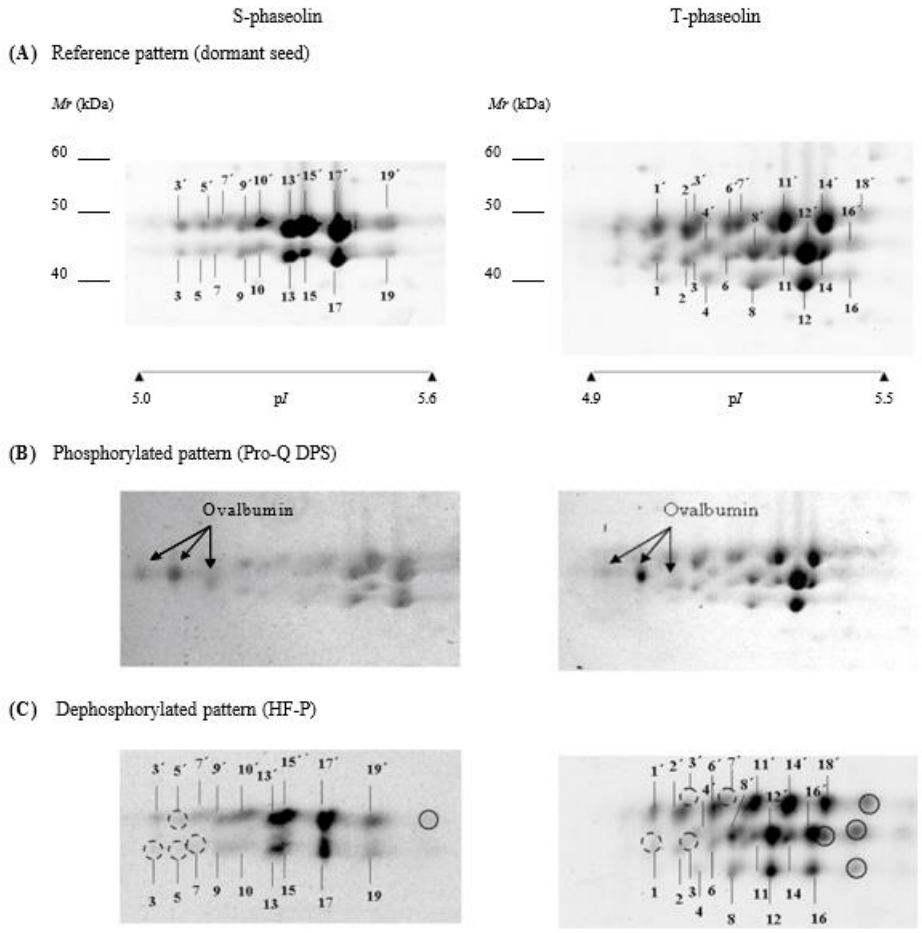
S-phaseolin
T-phaseolin
(A) Reference pattern (dormant seed)
Mr (kDa)
60
50
40
5.0
pI
5.6
4.9
5.5
(B) Phosphorylated pattern (Pro-Q DPS)
Ovalbumin
(C) Dephosphorylated pattern (HF-P)

Figure 3

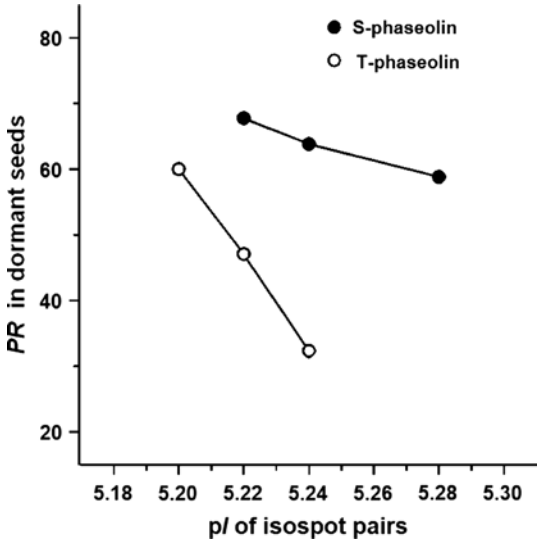
S-phaseolin
T-phaseolin
80
60
40
20
PR in dormant seeds
5.18
5.20
5.22
5.24
5.26
5.28
5.30
pI of isospot pairs

Figure 4

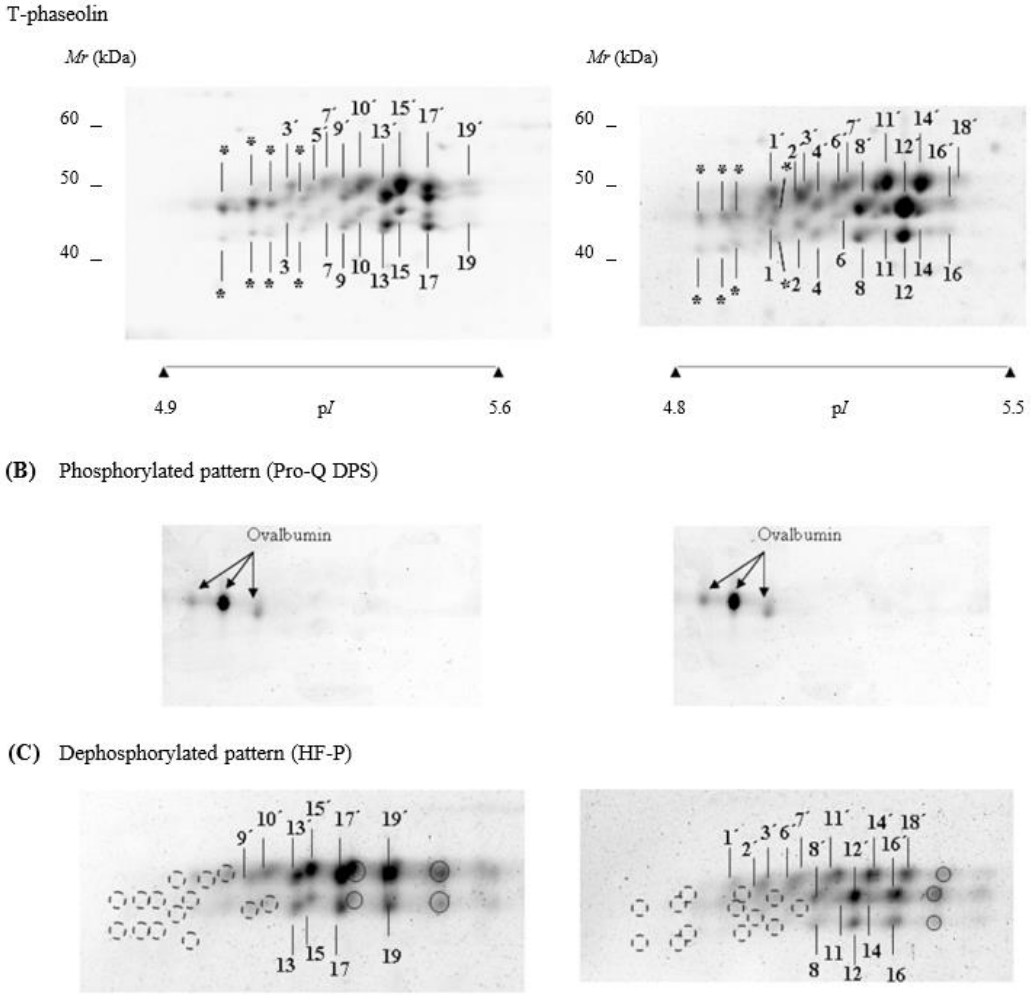
T-phaseolin
Mr (kDa)
60
50
40
4.9
pI
5.6
Mr (kDa)
60
50
40
4.8
pI
5.5
(B) Phosphorylated pattern (Pro-Q DPS)
Ovalbumin
Ovalbumin
(C) Dephosphorylated pattern (HF-P)

Figure 5

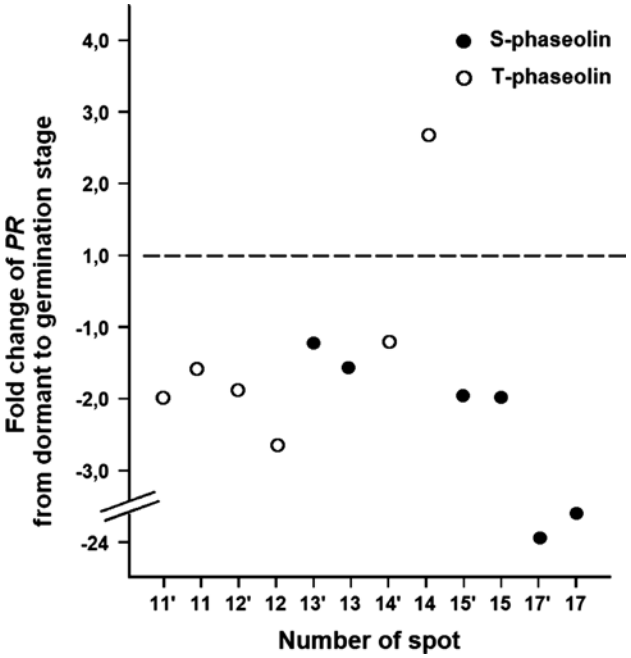
S-phaseolin
T-phaseolin
4,0
3,0
2,0
1,0
-1,0
-2,0
-3,0
-24
Fold change of PR
from dormant to germination stage
11' 11 12' 12 13' 13 14' 14 15' 15 17' 17
Number of spot

Figure 6

Table 1 Identification of the type of polypeptide (α or β) across 2-DE phaseolin spot patterns in dry mature common bean seeds of cvs. S and T by MALDI-TOF and MALDI-TOF/TOF MS

Cv. S					Cv. T				
Spot no.[a]	Exp. p*I*[b]	Mascot score	Match./ Cov. (%)[c]	Type of polypeptide	Spot no.	Exp. p*I*	Mascot score	Match./ Cov. (%)	Type of polypeptide
3′	5.09	774	24/62	α	7′	5.14	858	24/61	α
3	5.09	689	19/53	α	7	5.14	713	21/63	α
10′	5.19	895	21/58	α	11′	5.20	893	22/71	α
10	5.19	908	22/66	α	11	5.20	967	24/75	α
13′	5.22	890	21/57	β	12′	5.22	871	21/68	β
13	5.22	940	21/57	β	12	5.22	966	25/72	β
15′	5.24	940	24/64	α	14′	5.24	824	22/63	α
15	5.24	545	15/36	α	14	5.24	731	21/58	α
17′	5.28	935	21/64	β	16′	5.26	871	19/59	β
17	5.28	957	20/60	β	16	5.26	874	19/59	β
19′	5.33	577	19/53	α	18′	5.28	689	20/58	α

[a] Gel position of assigned spots is shown in Fig. 1

[b] Experimental p*I* value

[c] Matched peptides and percentage of the polypeptide sequence covered by matched peptides

Table 2 Phosphorylated peptides of the protein phaseolin in dry mature common bean seeds of cvs. S and T identified by MALDI-TOF

[a] Spot code numbers according to Fig. 1

[b] Experimental p*I* values

[c] Type of phaseolin polypeptide

[d] Tryptic peptide experimental masses (expressed in Da) of spots

[e] Theoretical masses (expressed in Da) resulting from the data base search

[f] Phosphopeptide sequence

[g] Position of the phosphopeptide in the polypeptide sequence

	Exp. p*I*[b]	Polypeptide[c]	Submitted mass [M + H]$^{+}$[d]	Theoretical mass [M + H]$^{+}$[e]	Phosphopeptide[f]	Position[g]
				Cultivar S		
3′	5.09	α	2,960.48	2,960.47	ARVPLLLLGILFLASLSASFATSLR	4–28
3′	5.09	α	1,932.98	1,932.95	HILEASFNSKFEEINR	189–204
10′	5.19	α	2,960.52	2,960.47	ARVPLLLLGILFLASLSASFATSLR	4–28
13′	5.22	β	3,230.37	3,230.46	TDNSLNVLISSIEMKEGALFVPHYYSK	257–283
15′	5.24	α	2,960.45	2,960.47	ARVPLLLLGILFLASLSASFATSLR	4–28
15	5.24	α	1,932.98	1,932.95	HILEASFNSKFEEINR	189–204
17′	5.28	β	3,230.38	3,230.46	TDNSLNVLISSIEMKEGALFVPHYYSK	257–283
17	5.28	β	2,103.92	2,103.91	ATSNVNFTGFGINANNNNR	336–354
				Cultivar T		
7′	5.14	α	1,932.95	1,932.95	HILEASFNSKFEEINR	189–204
12′	5.22	β	2,167.09	2,166.93	EYFFLTSDNPIFSDHQK	119–135
12′	5.22	β	2,296.05	2,296.05	DVLGLTFSGSGDEVMKLINK	376–395
12′	5.22	β	3,230.46	3,230.35	TDNSLNVLISSIEMKEGALFVPHYYSK	257–283
12′	5.22	β	1,991.95	1,991.83	HILEASFNSKFEEINR	189–204
12	5.22	β	1,932.95	1,932.95	HILEASFNSKFEEINR	189–204