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Identification and Mapping of Phosphorylated Isoforms of the Major Storage Protein of Potato Based on Two-Dimensional Electrophoresis

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Abstract

Protein phosphorylation plays a key role in the synthesis and degradation of dry seed storage proteins. In contrast, no evidence for phosphorylation has been reported to date in vegetative storage proteins (VSPs). The patatin multigene family encodes the major VSP of the potato, *Solanum tuberosum* L. This study addresses for the first time the identification and mapping of phosphorylated patatin forms based on high-resolution two-dimensional electrophoresis (2-DE) profiles. Patatin isoforms from mature tubers of cultivar Kennebec were separated by 2-DE and subsequently identified by tandem mass spectrometry. In-gel identification and mapping of phosphorylated isoforms were performed using the multiplex phosphoprotein-specific staining Pro-Q DPS. We found that phosphorylation is a ubiquitous post-translational protein modification associated with isoforms of patatin. In addition, protein dephosphorylation with hydrogen fluoride-pyridine coupled to 2-DE was used for quantitative profiling of phosphorylated patatin. This experimental approach showed that patatin comprises multiple isoforms with very different phosphorylation level. Thus, phosphorylation rates over isoforms ranged from 4.6 to 52.3%. Overall, the identification and mapping of differentially phosphorylated patatin opens up new exploratory ways to unravel the molecular mechanisms underlying its mobilization along the tuber life cycle.

Keywords: patatin, *Solanum tuberosum*, seed storage proteins, storage protein mobilization, tuber phosphoproteome, vegetative storage proteins

1. Introduction

Potato storage proteins provide necessary nutrients for the development of tuber, mature-to-sprouting tuber transition and successful plant growth [1–4]. The patatin is the major VSP of

S. tuberosum, accounting for up to 45% of the total soluble protein [1, 4–7]. However, the molecular mechanism that triggers the cleavage of the patatin along the tuber life cycle has not to date been identified. The regulatory mechanisms involved in the synthesis and degradation of storage proteins are better known in dry seeds [8–13]. Phosphorylation has proven to be a key regulator mechanism in the maturation, dormancy and germination of seed storage proteins (SSPs). Thus, reverse phosphorylation of the phytohormone abscisic acid (ABA) seems to play a crucial regulatory role in the synthesis of SSPs at the transcriptional level [11, 12]. More specifically, phosphoproteome studies in rapeseed and rice reported that cruciferins and cupins achieve higher levels of phosphorylation at the late maturation stage [9, 13]. In addition, mobilization of the major SSP in the common bean, phaseolin, was found to occur in germinating seeds through degradation of highly phosphorylated isoforms [10]. It suggests that degradation of SSPs in dry-to-germinating seed transition occurs through a phosphorylation-dependent regulatory mechanism.

The past few years have witnessed a steady discovery of phosphorylated SSPs such as cruciferins, napins, cupins, legumins and vicilins [8–10, 14, 15], but no evidence of phosphorylated isoforms has been reported to date in patatin or other VSPs such as sporamins and ocatins. Therefore, elucidating the question of whether patatin can be phosphorylated is a mandatory initial step in follow-up research concerning the molecular processes underlying its mobilization. First of all, the term patatin applies to a group of glycoproteins encoded by a gene family constituted by ~10–18 genes per haploid genome, most of them organized as a single gene cluster at the end of the long arm of chromosome 8 [16–18]. Patatin gene family members exhibit a very high degree of nucleotide sequence identity [19, 20]. In addition, patatin is a family of immunologically indistinguishable isoforms with similar structural properties and thermal conformational stability [21, 22]. Overall, extensive heterogeneity in molecular mass (40–45 kDa) and isoelectric point (4.5–5.2) seems to be the most salient differential molecular features among isoforms [21–25].

The 2-DE has provided the most complete information about the heterogeneity in molecular mass (M_r) and isoelectric point (pI) of the patatin [23–25]. Specifically, a total of 17–23 spots with variations in M_r and/or pI were detected in 2-DE patatin profiles obtained from different potato cultivars [25]. Variations in M_r seem to be mainly due to differential N-glycosylation at three specific asparagine residues of the amino acid sequence by N-linked oligosaccharide side chains or glycans [21, 22, 25]. Charge differences among isoforms could be explained by variations in positively and negatively charged amino acids [22]. However, variable phosphorylation has potential to change the pI of proteins by substituting hydroxyl groups on amino acid residues with negatively charged phosphate groups [26]. Therefore, phosphorylation could be a plausible but unexplored factor contributes to explain charge heterogeneity among patatin isoforms on 2-DE gels.

In this study, we undertook a proteomic approach addressed to the identification and mapping of phosphorylated isoforms of the patatin multigene family based on high-resolution 2-DE. First, relatively abundant tuber proteins were successfully separated from low-abundance proteins by loading low amounts of total protein sample into 2-DE

gels. Subsequently, high-abundance patatin proteins were identified and distinguished from other tuber abundant proteins on 2-DE gels using mass spectrometry (MS) techniques. Second, direct and rapid in-gel multiplex identification and mapping of phosphorylated isoforms of the patatin were achieved using the Pro-Q Diamond phosphoprotein stain (Pro-Q DPS), which specifically binds to the phosphate moieties of phosphoproteins [27]. Third, quantitative profiling of phosphorylated patatin isoforms was assessed by chemical dephosphorylation of phosphoproteins with hydrogen fluoride-pyridine (HF-P) [28–30]. For this purpose, the volume difference between phosphorylated and dephosphorylated 2-DE patatin spots was used to quantify protein phosphorylation levels. This experimental pipeline is a highly valuable top-down proteomic approach for the identification and mapping of phosphorylated isoforms of high-abundance storage proteins. It has been instrumental in unravelling the quantitative profiling of phosphorylated phaseolin isoforms in common bean seeds as well as their dynamic changes in dry-to-germinating seed transition [10]. Proteomic analyses were performed from total protein extracts of mature tubers of cultivar Kennebec. The obtained results will facilitate follow-up studies for better understanding of the regulatory mechanisms underlying patatin degradation and its biochemical status along the tuber life cycle.

2. Materials and methods

2.1. Plant material

Proteomic analyses were performed from mature potato tubers of cv. Kennebec ($2n = 4x = 48$). Larger pieces of lyophilized tuber were homogenized with a pre-cooled mortar and pestle. The samples were stored at -80°C until protein extraction. Four biological replicates were used for experiments.

2.2. Protein extraction and quantification

Total tuber proteins were extracted using the phenol extraction method. A 200 mg sample of lyophilized tuber was transferred to an extraction buffer (500 mM Tris-HCl, 500 mM EDTA, 700 mM sucrose, 100 mM KCl pH 8.0, 2% DTT and 1 mM PMSF). Tris-HCl (pH 6.6–7.9) saturated phenol was added and the phenol phase was collected using centrifuging (4500 rpm at 4°C). Protein precipitation solution of 0.1 M ammonium acetate in cold methanol was added. Protein pellet was washed with 0.1 M ammonium acetate and 10 mM DTT, and with 80% acetone and 10 mM DTT. The resuspended protein pellet was then diluted 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 concentration was evaluated using the commercial CB-X protein assay kit (G-Biosciences, St. Louis, MO, USA) according to the instructions of the manufacturer for interfering agent removal and use with a microplate reader. The bovine serum albumin (BSA) was used as standard protein to generate calibration curves.

2.3. Two-dimensional electrophoresis (2-DE)

High-resolution 2-DE profiles of patatin isoforms were obtained following the procedure described in López Pedrouso et al. [10]. Briefly, total protein samples (75 µg of protein) of each biological replicate were loaded into immobilized pH gradient (IPG) strips of 24-cm long and 4–7 pH linear gradient (Bio-Rad Laboratories, Hercules, CA, USA). First dimensional isoelectric focusing (IEF) was performed in a PROTEAN IEF Cell System (Bio-Rad Laboratories) after IPG strip rehydration for 12 h at 50 V. Rapid voltage ramping was subsequently applied to reach a total of 70 kVh. Equilibration of IEF strips was performed before running second dimension using equilibration buffers. The second dimension (SDS-PAGE) was performed on 10% polyacrylamide gels using an Ettan DALTsix large vertical electrophoresis system (GE Healthcare). Second-dimension gels were run using a constant electric current of 16 mA per gel for 15 h at 25°C.

2.4. Enzymatic deglycosylation of patatin

Patatin deglycosylation was performed with the enzyme protein-N-glycosidase F (PNGase F, New England Biolabs, Ipswich, MA, USA) according to the manufacturer specifications. A 75 µg sample of total protein extract from mature tuber was incubated with PNGase F (25 U/mL) and diluted in reaction buffer (New England Biolabs) until a final volume of 20 µL. The mixture was incubated for 12 h at 37°C. Patterns of deglycosylated patatin isoforms on 2-DE gels were obtained as described earlier.

2.5. Pro-Q staining for phosphoproteins

Pro-QDiamond phosphoprotein stain (Pro-QDPS, Molecular Probes, Leiden, The Netherlands) was used for the in-gel detection of phosphorylated patatin polypeptides, as described previously [31]. Briefly, gels were fixed with 50% methanol and 10% acetic acid for 60 min and washed twice for 15 min each with distilled water. The gels were subsequently incubated for 120 min with two-fold water-diluted Pro-Q DPS, destained four times (30 min per wash) with 50 mM sodium acetate and 20% ACN pH 4.0, and washed again twice with distilled water (5 min per wash). The PeppermintStick™ (Molecular Probes) phosphoprotein marker was added to tuber protein extracts before 2-DE. Phosphorylated (ovalbumin, 45.0 kDa; and β-casein, 23.6 kDa) and unphosphorylated (β-galactosidase, 116.25 kDa; bovine serum albumin, 66.2 kDa; avidin, 18.0 kDa; and lysozyme, 14.4) PeppermintStick proteins were used as positive and negative controls of phosphorylation, respectively.

2.6. Chemical dephosphorylation of patatin

The chemical dephosphorylation of patatin was performed with hydrogen fluoride-pyridine (HF-P) as previously described [28, 29], with some modification [10]. An amount of 1 mg of total protein extract from tuber of cv. Kennebec was dissolved in 250 µL of 70% HF-P and incubated on ice for 2 h. The mixture was neutralized by addition of 10 M sodium hydroxide solution. Proteins were desalinated using Amicon Ultra-4 centrifugal filter devices (Millipore, Billerica, MA, USA) and then eluted in 300 µL of lysis buffer. Prior to 2-DE, protein purification was performed using the Clean-up kit (GE Healthcare).

2.7. SYPRO Ruby staining for total protein

2-DE gels were stained with SYPRO Ruby fluorescent stain (Lonza, Rockland, ME, USA), for total protein density following the manufacturer's indications. Pro-Q DPS-stained gels were also post-stained with SYPRO Ruby to detect total protein.

2.8. Image analysis

The 2-DE images from gels stained with Pro-Q DPS or SYPRO Ruby fluorescent dyes were acquired using a Gel Doc XR+ system (Bio-Rad Laboratories). Digitalized gels were analyzed with PDQuest Advanced software v. 8.0.1 (Bio-Rad Laboratories). Gel matching, spot identification and quantification of spot volumes were performed after background subtraction and normalization based on total density in valid spots. Automatic matches were manually checked. Only the reproducibly detected patatin spots across replicates were selected for quantitative analyses. Experimental pI -values over spots were determined using the linear scale of IEF-strips as reference, whereas M_r -values were assessed using PeppermintStick (Molecular Probes) molecular weight markers and standard molecular mass markers ranging from 15 to 200 kDa (Fermentas, Ontario, Canada).

2.9. In-gel digests

Protein spots of interest were excised from polyacrylamide gels and subjected to in-gel digestion with trypsin as described previously [32]. Briefly, disulfide reduction and alkylation of the excised protein spots were performed with 10 mM DTT (Sigma-Aldrich, St. Louis, MO, USA) in 50 mM ammonium bicarbonate (Sigma-Aldrich) and 55 mM iodoacetamide (Sigma-Aldrich) in 50 mM ammonium bicarbonate, respectively. The gel pieces were washed with 50 mM ammonium bicarbonate in 50% methanol (HPLC grade, Scharlau, Barcelona, Spain), dehydrated with acetonitrile (ACN, HPLC grade) and subsequently dried in a SpeedVac (Thermo Fisher Scientific, Waltham, MA, USA). Dry gel pieces were incubated with modified porcine trypsin (Promega, Madison, WI, USA) at a concentration of 20 ng/ μ L in 20 mM ammonium bicarbonate, at 37°C for 16 h. After digestion, peptides were recovered by incubation (three times/20 min) in 40 μ L of 60% ACN in 0.5% formic acid. The resulting tryptic peptides were concentrated in a SpeedVac and stored at -20°C.

2.10. Mass spectrometry (MS)

Protein identification was performed by MALDI-TOF and MALDI-TOF/TOF MS as reported by López-Pedrouso et al. [10]. Peptides were dissolved in 4 μ L 0.5% formic acid and then were mixed with an equal volume (0.5 μ L) of matrix solution, containing 3 mg of α -Cyano-4-hydroxycinnamic acid (CHCA) dissolved in 1 mL of 50% ACN in 0.1% trifluoroacetic acid (TFA). The mixture was deposited using the thin layer method, onto a 384 Opti-TOF MALDI plate (Applied Biosystems, Foster City, CA, USA). Peptide MS and MS/MS data were acquired with a 4800 MALDI-TOF/TOF mass spectrometer (Applied Biosystems). MS spectra were acquired in positive-ion reflector mode with an Nd:YAG laser (355 nm wavelength) and an average number of 1000 laser shots. Each spectrum was internally calibrated with at least three trypsin autolysis products. All MS/MS spectra were performed by selecting

precursors ions with a relative resolution of 300 full width at half maximum (FWHM) and metastable suppression. The 4000 Series Explorer Software v. 3.5 (Applied Biosystems) was used for mass data analysis. Combined peptide mass fingerprinting (PMF) and MS/MS fragment-ion spectra were interpreted with GPS Explorer Software v. 3.6 using Mascot software v. 2.1 (Matrix Science, Boston, MA, USA) to search against the *S. tuberosum* UniProtKB/Swiss-Prot databases. Mascot database search parameters were: precursor mass tolerance of 50 ppm, MS/MS fragment tolerance of 0.6 Da, one missed cleavage allowed, carbamidomethyl cysteine (CAM) as fixed modification and oxidized methionine as variable modification. Identification of patatin phosphopeptides was also performed from spectrum data allowing phosphor-serine (PhosphoS), phosphor-tyrosine (PhosphoY) and phosphor-threonine (PhosphoT) residues as variable modification to search against the UniProtKB/Swiss-Prot databases. Analysis of phosphorylation sites was implemented using the Plant Protein Phosphorylation DataBase (P³DB) [33]. All identifications and spectra were manually checked for validation. Proteins with at least two matched peptides and statistically significant (p -value < 0.05) MASCOT scores were selected as positively identified.

2.11. Data analysis

The phosphorylation rate at each spot was quantified using the measure PR [10]. It is defined as $PR = [(T - D)/T] \times 100$, where T and D are the volumes of a given spot on 2-DE gels untreated (total protein volume) and treated (dephosphorylated protein volume) with HF-P, respectively. Non-parametric bootstrap confidence intervals (CIs) were obtained for mean values of PR across four biological replicates by the bias-corrected percentile method [34]. For each observed mean of PR , 2000 bootstrap samples of size $N = 4$ were drawn with replacement by applying a Monte Carlo algorithm. The 95 and 99% CIs for the observed mean of PR were constructed from distribution of 2000 bootstrap mean replications. The bootstrap estimate of bias was obtained from the proportion of bootstrap mean replications lower than the original estimate of the mean, and bias-corrected CIs were then calculated using the theoretical normal distribution as described by Efron [34]. PR data were clustered by using the unweighted pair-group method with arithmetic averaging (UPGMA). The UPGMA dendrogram derived from the matrix of pairwise PR -values was generated using NTSYSpc v. 2.1 software (Applied Biostatistics, Setauket, NY, USA). Descriptive statistics and Spearman's correlation test were calculated with the IBM SPSS Statistics 20 (SPSS, Chicago, IL, USA) statistical software package.

3. Results and discussion

3.1. Map of patatin isoforms based on 2-DE

Patatin isoforms in mature potato tuber of cv. Kennebec were first recognized on our 2-DE gels according to the previously reported studies on 2-DE patatin profiles [23–25]. We found that patatin profiles were constituted by a complex constellation of different spots showing large variations in M_r and/or pI (Figure 1). Specifically, 2-DE resolved a total of 20 spots distributed

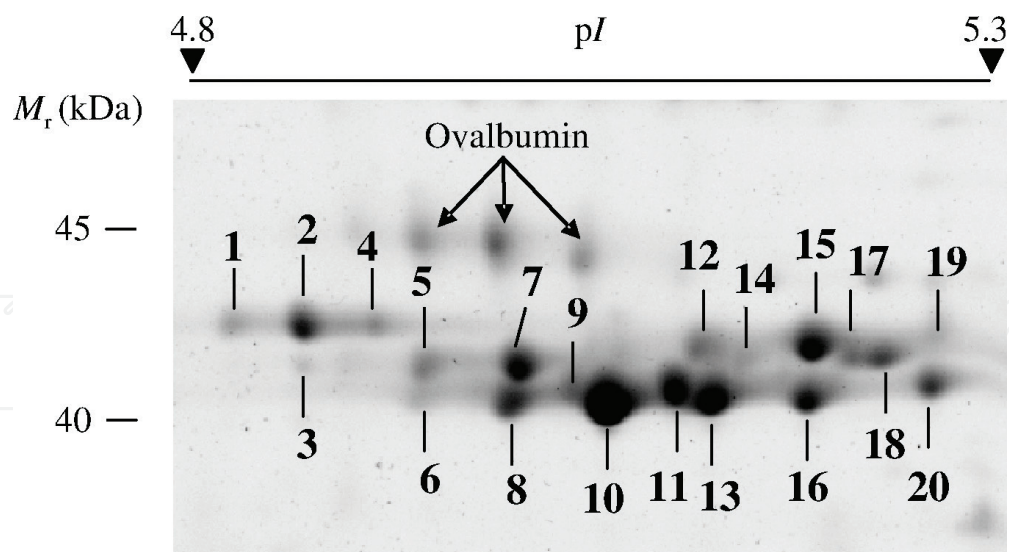


Figure 1. High-resolution 2-DE reference map of the patatin isoforms in mature tuber of cv. Kennebec. The enlarged gel image shows patatin spots consecutively numbered in the order of the lower to the higher *pI*. 2-DE was performed using a 24-cm long IPG strip of linear pH 4–7 gradient in the first dimension and SDS-PAGE (10% by mass) in the second. The protein loading was 75 μ g and the gel was stained with SYPRO Ruby fluorescent stain. The arrows indicate ovalbumin (45.0 kDa) marker position on the gel. The M_r of spots was assessed from ovalbumin and standard molecular mass markers ranging from 15 to 200 kDa and their *pI*s from strips of linear pH.

into three main levels with M_r between 40.1 and 43.0 kDa and *pI* range varying from 4.8 to 5.3. A total of 20 spots were excised from gel and identified by MALDI-TOF and MALDI-TOF/TOF MS. The identification results are listed in **Table 1**. All but one small and weakly stained spot (spot 3) were confidently identified. MS analyses confirmed that, indeed, those spots contained only patatin polypeptides. However, most of the identifications were ambiguous and compatible with the occurrence of different types of patatin. This uncertainty is a consequence of the well-known high degree of sequence homology (at least 90%) among isoforms [19, 20]. Only protein spots with higher M_r (spots 1, 2 and 4) were unambiguously identified as Patatin-3-Kuras 1 (PT3K1). It can be understood by considering that the *pt3k1* gene exhibits the most differentiated sequence from other patatin genes according to the phylogenetic tree inferred from cDNA sequence analysis [24].

The two-dimensional map of the patatin was implemented with the location of glycosylated isoforms using the enzyme PNGase F. It is an effective enzymatic method for removing almost all *N*-linked oligosaccharides (glycans) from glycoproteins through the hydrolysis of the glycosamide linkage between the terminal GlcNAc and the Asn amide nitrogen [35]. We found that the three main spot levels in M_r on 2-DE gels obtained from untreated samples merged to a single spot level after incubation with PNGase F, with an apparent decrease in M_r (not shown). It indicates that variable degrees of glycosylation are a major contributor to the M_r heterogeneity detected on 2-DE gels. High M_r difference among patatin isoforms has been explained by the presence of up to three potential *N*-glycosylation sites at Asn residues [22, 24, 25, 36]. The mapping of glycosylated isoforms on 2-DE gels can be useful in future studies investigating the functional role of this post-translational protein modification (PTM) of the patatin.

Spot no. ^a	Exp. pI ^b	Match./cov. (%) ^c	Mascot score	Protein name (type) ^d	No. phosphopeptides/ phosphosites
1	4.84	7/24	191	Patatin (PT3K1)	1/1
2	4.88	13/58	571	Patatin (PT3K1)	7/17
3	4.90	–	–	Unidentified	–
4	4.93	5/16	158	Patatin (PT3K1)	–
5	4.96	3/8	106	Patatin (various)	–
6	4.96	5/21	>60–187	Patatin (various)	–
7	5.02	11/40	>60–200	Patatin (various)	–
8	5.02	6/25	>60–297	Patatin (various)	–
9	5.05	7/28	>60–324	Patatin (various)	1/1
10	5.08	5/11	>60–199	Patatin (various)	1/1
11	5.12	9/32	>60–337	Patatin (various)	3/7
12	5.13	8/30	>60–298	Patatin (various)	–
13	5.14	9/31	>60–475	Patatin (various)	1/1
14	5.16	9/31	>60–267	Patatin (various)	–
15	5.20	2/11	>60–103	Patatin (various)	4/9
16	5.20	10/40	>60–440	Patatin (various)	4/10
17	5.23	5/25	>60–123	Patatin (various)	–
18	5.25	9/31	>60–259	Patatin (various)	5/9
19	5.29	3/9	>60–129	Patatin (various)	–
20	5.27	10/41	>60–550	Patatin (various)	4/8

^aGel position of assigned spots is shown in **Figure 1**.

^bExperimental pI value.

^cMatched peptides and percentage of the polypeptide sequence covered by matched peptides.

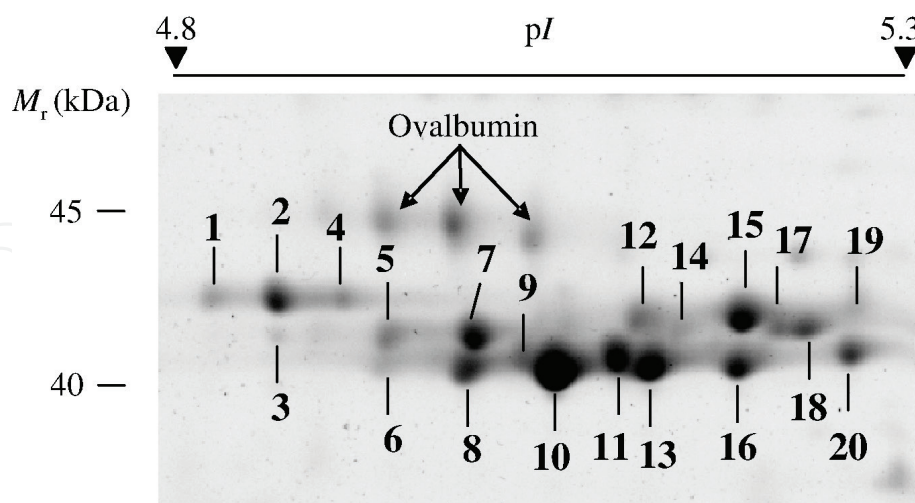
^dPT3K1, abbreviation for Patatin-3-Kuras 1.

Table 1. Protein, phosphopeptides and phosphosites along 2-DE patatin spots of cv. Kennebec, identified from MALDI-TOF and MALDI-TOF/TOF MS data.

3.2. In-gel identification of phosphorylated patatin isoforms

Pro-Q DPS was used for in-gel multiplex identification of phosphorylated patatin isoforms. Representative 2-DE images of patatin in mature tuber of cv. Kennebec on the same gel stained with Pro-Q DPS and post-stained with SYPRO Ruby are shown in **Figure 2**. The PeppermintStick markers used as positive and negative controls of protein phosphorylation validated the specificity of the recognition of phosphoproteins by Pro-Q DPS under our experimental conditions. It was found that all 20 patatin spots of the reference pattern exhibited Pro-Q DPS fluorescent signal. Similar result was obtained for patatins from mature tubers of cvs. Agria, Amanda and Ivory Russet (not shown). We can conclude, therefore, that phosphorylation is a ubiquitous PTM associated with isoforms of the patatin.

(a) 2-DE reference map of the patatin (dormant tuber)



(b) Phosphorylated patatin profile (dormant tuber)

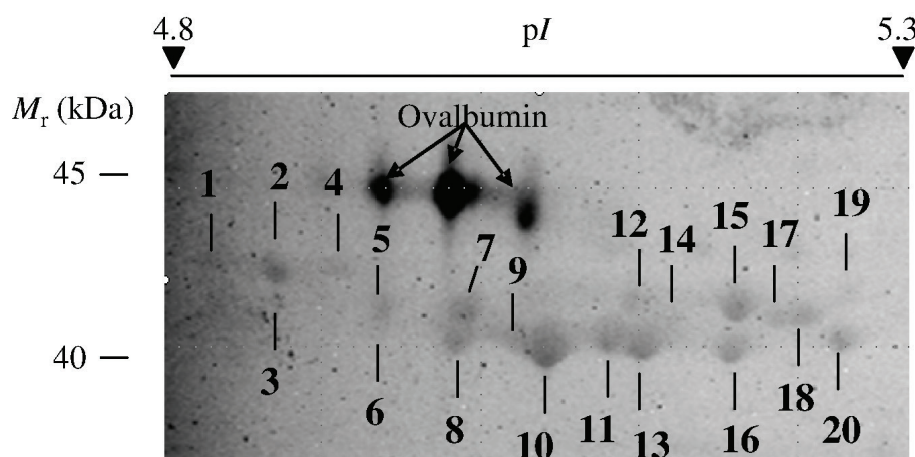


Figure 2. Mapping of phosphorylated patatin spots in mature tubers (cv. Kennebec) on 2-DE gel. (a) Reference profile of patatin spots on gel stained with the non-specific-protein SYPRO Ruby stain. (b) Profile of phosphorylated patatin spots from the same gel stained with the specific-phosphoprotein Pro-Q DPS fluorescent dye. The phosphoprotein ovalbumin was used as a marker of the reliability of Pro-Q DPS under our experimental conditions.

A prospective identification of phosphopeptides and phosphosites by MASCOT search using spectra data from MALDI-TOF and MALDI-TOF/TOF MS revealed 22 non-redundant patatin phosphopeptides containing 49 non-redundant phosphorylation sites (**Table 1**). Comparison with large-scale phosphoproteomic screens in other species using the Plant Protein Phosphorylation DataBase (P³DB) [33] suggests that most phosphorylation sites identified are novel to this study. Thus, no phosphorylated ortholog sites were identified in other plant phosphoproteomics data for *Arabidopsis thaliana*, *Brassica napus*, *Glycine max*, *Medicago truncatula*, *Oryza sativa* and *Zea mays*. It is noteworthy that enrichment methods of underrepresented phosphorylated proteins or peptides can be conducted prior to high-resolution MS analysis to precisely identify and map phosphorylation sites, but the amount of protein collected in a spot is often insufficient for downstream enrichment methods [37].

Consequently, it would be difficult to assign phosphorylation sites to the specific isoforms found along 2-DE patatin patterns. At the present time, the 2-DE map of phosphorylated isoforms appears to be more informative than the exact identification of phosphosites in order to evaluate their biological meaning. Regardless of this, phosphorylation site prediction analysis is an additional evidence for phosphorylation of patatin.

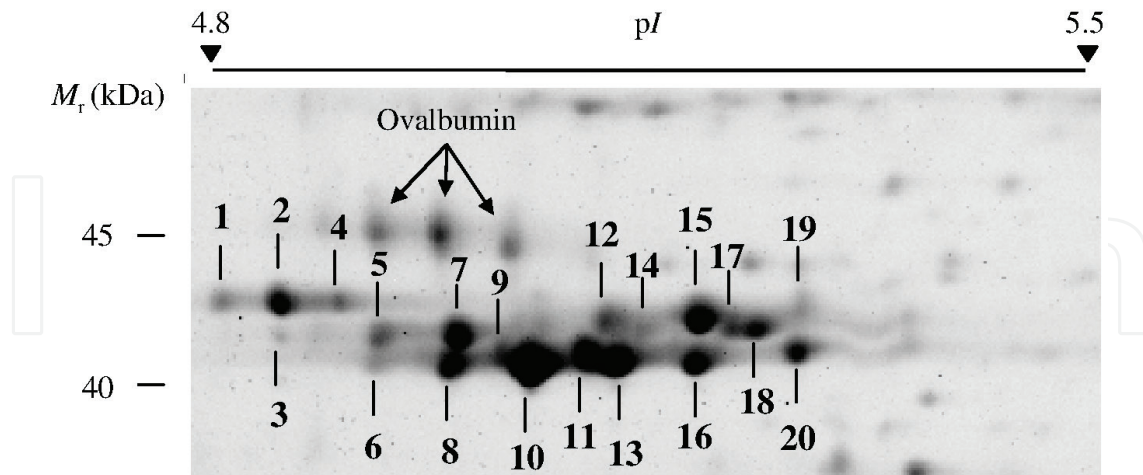
3.3. Quantitative profiling of phosphorylated patatin isoforms

Changes in the phosphorylation level across patatin spots of cv. Kennebec were assessed by chemical dephosphorylation of total tuber protein extracts with HF-P coupled to 2-DE. This experimental approach provides more efficient information than Pro-Q DPS to the identification and quantification of phosphorylated proteins on 2-DE gels [10]. The reason is that the Pro-Q DPS fluorescent signal of spots containing low-abundance phosphopeptides is seriously suppressed by abundant non-phosphorylated phosphopeptides. The chemical dephosphorylation method has the advantage of using SYPRO Ruby stain, which combines good sensitivity with excellent linearity [38].

Representative 2-DE gel images of the patatin pattern before and after HF-P treatment are shown in **Figure 3**. First of all, note that spots of the protein phosphorylation marker, ovalbumin, underwent a basic shift on *pI* after HF-P treatment. This indicates that HF-P had sufficient time to achieve a complete dephosphorylation of polypeptides. With respect to the 2-DE profiles of dephosphorylated patatin, we can highlight two important observations. First, all spots observed in untreated samples were also present after dephosphorylation, but with an apparent decrease in volume. This suggests that patatin spots contained a mixture of phosphorylated and unphosphorylated isoforms. Accordingly, other factors together with protein phosphorylation must be contributing to charge heterogeneity along 2-DE patatin patterns such as difference in charged amino acids over isoforms [22]. Second, newly arisen spots (spots 21–27) found in dephosphorylated patatin patterns appeared on more basic positions of 2-DE gels. MS analysis confirmed that these new spots contained patatin (data not shown), and thereby they are isoforms that underwent a basic shift on *pI* after dephosphorylation with HF-P.

The phosphorylation level of each spot was evaluated with the measure *PR* using volumes obtained by PDQuest software from phosphorylated and dephosphorylated profiles. Mean (\pm SE, standard error) values of *PR* for each spot together with bias-corrected 95 and 99% bootstrap CIs are shown in **Table 2**. Interestingly, we found that spots were not uniformly phosphorylated: mean *PR*-values across spots were in the range of 4.6–52.3% and averaged (\pm SE) $34.4 \pm 2.8\%$. The bootstrapped 95 and 99% CIs revealed statistically significant differences (p -value < 0.05) between many pairs of spots. Patatin spots were subsequently grouped into clusters from *PR*-values using a dendrogram UPGMA. The resulting dendrogram showed that spots cluster in three main groups with statistically significant mean differences in *PR* ($p < 0.01$) assessed by bias-corrected 99% bootstrap CIs (**Figure 4**). In particular, spots of the group 3 (spots 13 and 20) formed a well-separated cluster (mean *PR* = 8.3%) from the two remaining groups (the mean *PR* of groups 1 and 2 was 44.0 and 30.1%, respectively).

(a) 2-DE reference map of the patatin



(b) Dephosphorylated patatin profile

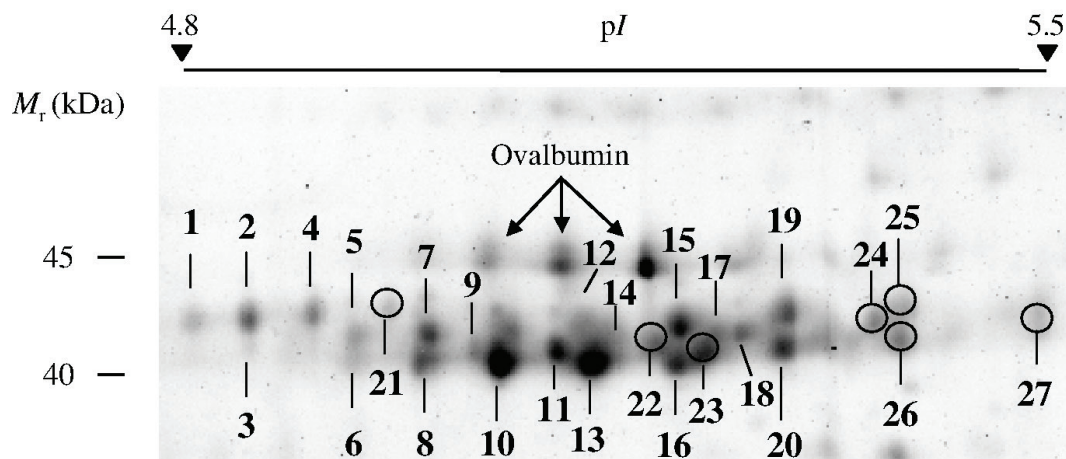


Figure 3. 2-DE profile of dephosphorylated patatin with HF-P in mature tuber (cv. Kennebec). (a) Reference profile of patatin without dephosphorylation treatment on gel stained with SYPRO Ruby stain. (b) Profile of patatin after chemical dephosphorylation on gel stained with SYPRO Ruby. Closed circles represent newly arisen spots on gel after dephosphorylation as compared to the reference profile. Identification of new spots as patatin was performed by MALDI-TOF and MALDI-TOF/TOF MS.

Elucidating whether changes in abundance of protein phosphorylation reflect either changes in phosphorylation status or changes in the abundance of the protein itself is a major challenge in the interpretation of quantitative phosphoproteomics studies [39, 40]. Thus, phosphopeptide enrichment methods prior to high-resolution MS permit the identification of low-abundance phosphoproteins but prevent joint quantitation of phosphorylation status and abundance of proteins [39]. However, our experimental approach allowed us to successfully tackle this problem. Thus, we have detected a statistically significant negative relationship between patatin spot volumes and their corresponding *PR*-values by Spearman's non-parametric correlation test ($r_s = -0.42$, $p < 0.001$, $n = 70$). In addition, *PR*-values were negatively correlated with spot pIs ($r_s = -0.31$, $p < 0.01$, $n = 70$). As expected under a differential phosphorylation

Spot no. ^a	pI	Mean (\pm SE) <i>PR</i>	P ($\hat{\theta}_B \leq \hat{\theta}$) ^b	95% bootstrap CI (CL, CU) ^c	99% bootstrap CI (CL, CU) ^c
1	4.84	39.75 \pm 2.53	0.53	35.6, 44.1	34.6, 44.3
2	4.88	41.21 \pm 6.07	0.57	31.9, 52.9	30.1, 54.5
3	4.90	43.05 \pm 2.88	0.55	37.5, 47.9	37.4, 49.3
4	4.93	28.11 \pm 4.35	0.76	23.8, 32.5	23.8, 32.5
5	4.96	42.83 \pm 2.02	0.56	40.1, 46.9	39.4, 48.3
6	4.96	52.34 \pm 4.10	0.57	46.4, 60.4	44.5, 61.8
7	5.02	39.01 \pm 4.11	0.51	32.4, 45.6	30.4, 45.7
8	5.02	27.19 \pm 5.61	0.52	16.9, 35.0	15.3, 35.0
9	5.05	32.48 \pm 3.30	0.51	26.7, 37.3	22.9, 38.0
10	5.12	30.48 \pm 4.72	0.57	23.3, 40.2	22.6, 42.4
11	5.12	25.13 \pm 4.31	0.57	18.8, 33.1	16.6, 34.1
12	5.13	51.39 \pm 5.52	0.55	41.2, 60.6	40.0, 62.1
13	5.14	4.60 \pm 2.04	0.75	2.6, 6.6	2.6, 6.6
14	5.16	44.34 \pm 9.37	0.52	25.7, 57.3	25.0, 59.0
15	5.20	41.87 \pm 3.80	0.52	35.7, 49.3	34.7, 51.2
16	5.20	26.99 \pm 8.37	0.58	18.1, 37.0	10.4, 37.0
17	5.23	35.96 \pm 2.42	0.53	32.9, 40.9	32.2, 42.8
18	5.25	34.16 \pm 4.09	0.53	26.2, 39.6	25.6, 40.0
19	5.29	N/A ^d	N/A	N/A	N/A
20	5.27	11.95 \pm 4.63	0.55	6.8, 20.9	5.6, 20.9

Volume of spots for untreated and dephosphorylated protein samples with HF-P were assessed by PDQuest software.

^aGel position of assigned spots is shown in **Figure 1**.

^bThe bootstrap distribution is median biased if the probability (P) of ($\hat{\theta}_B \leq \hat{\theta}$) \neq 0.50, which was calculated from 2000 bootstrap replicates; $\hat{\theta}_B$ and $\hat{\theta}$ are the bootstrap mean and the sample mean estimates, respectively.

^cCI—Confidence interval; CL—lower bound; CU—upper bound.

^dN/A = not available, weakly stained spot with a volume below the limit of detection.

Table 2. Mean (\pm SE) values of *PR* for patatin spots estimated from four replicates from dormant tubers of cv. Kennebec.

hypothesis, isoforms located on acidic gel positions tended to be more highly phosphorylated than those of basic positions. It is also noteworthy that phosphorylation levels were estimated using the measure *PR*, which takes into account the amount of protein at each spot. Therefore, differential phosphorylation along patatin spots seems to be genuine and cannot be explained only by changes in protein abundance.

The control of tuber sprouting is a major target in potato breeding because premature tuber sprouting during their lengthy storage leads to important quality and economic loss [41–43]. However, the molecular mechanisms controlling dormancy release and tuber sprouting are not yet sufficiently known [42–44]. The identification and mapping of phosphorylated isoforms

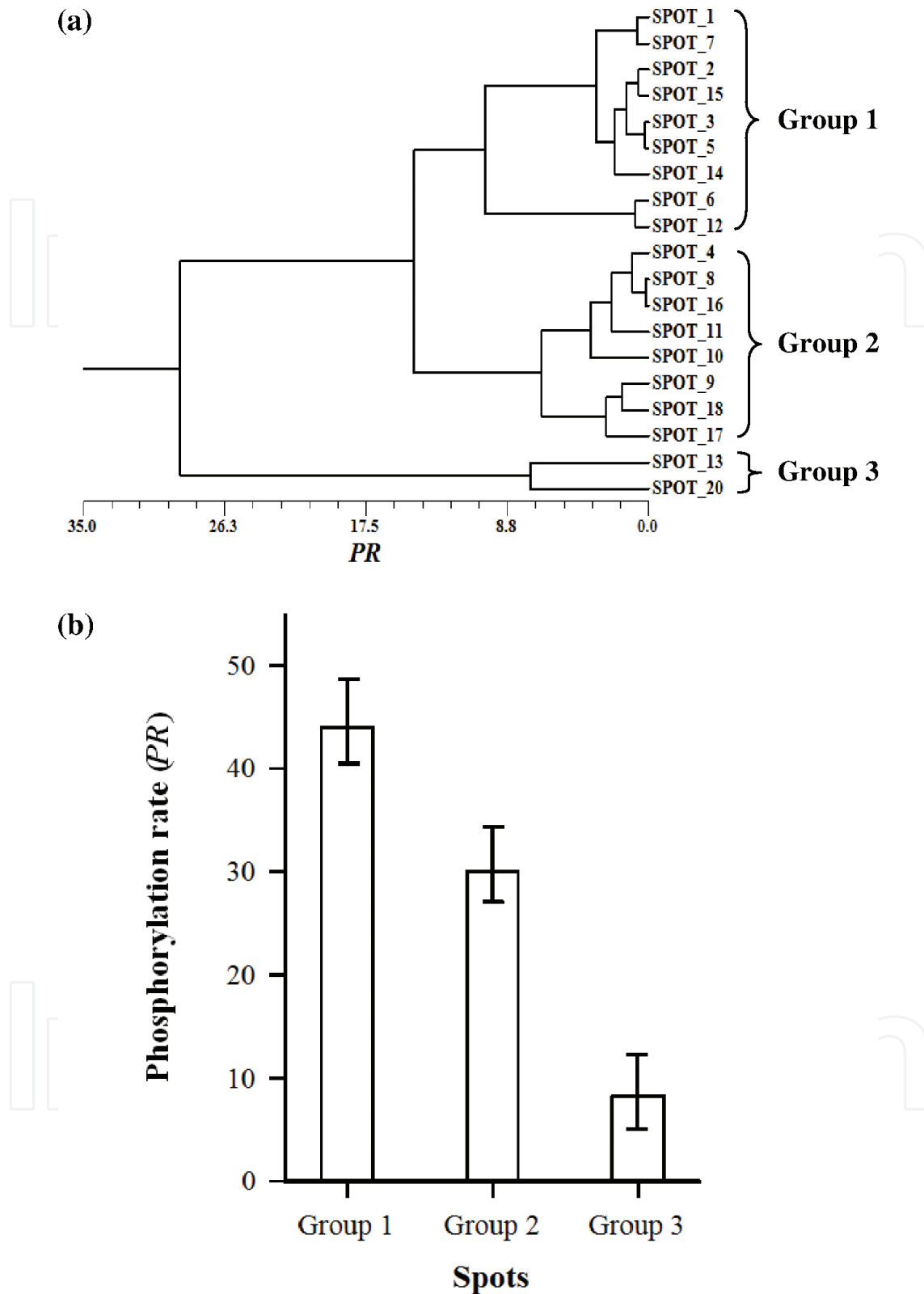


Figure 4. Evaluation of the differential *PR* along 2-DE patatin spots (mature tuber). (a) UPGMA dendrogram from the matrix of mean differences in *PR* between pairs of patatin spots. Spot numbers refer to numbers in **Figure 1**. (b) Mean *PR* values for each of the three main spot groups clustered by UPGMA. Bootstrapping (2000 replicates) was used to determine 99% CIs for mean *PR*-values at each group. *PR*-values over spots were calculated using the formula $PR = [(T - D)/T] \times 100$, where *T* and *D* represent the gel-spot volume in reference and dephosphorylated patatin profiles, respectively. Spot volumes over quadruplicate 2-DE gels were determined using the PDQuest software.

of the patatin opens up new exploratory ways to unravel the molecular mechanisms underlying mobilization of VSPs. The finding of differentially phosphorylated isoforms is particularly relevant because increase (or decrease) in phosphorylation status without a parallel change in the amount of protein has been considered to be a useful indicator for a specific functional change [39, 40, 45]. In this regard, systematic follow-up studies on VSPs will be needed to assess whether their degradation takes place through a phosphorylation-dependent regulatory mechanism, as it occurs in common bean during dry-to-germinating seed transition [10]. The establishment of a 2-DE-based reference map of patatin can be a very efficient tool to address this challenge in potato by monitoring changes in the phosphorylation status along the tuber life cycle.

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