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SHORT COMMUNICATION

Phosphorylated 11S globulins in sunflower seeds

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Abstract

Helianthinins are storage proteins present in *Helianthus annuus* seeds, belonging to the 11S globulin family. Here we describe that a fraction of the helianthinins is phosphorylated. This conclusion is supported by different criteria, including identification by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry of major protein bands revealed with a specific dye for phosphoproteins, anti-phosphoserine antibody and binding to a phosphoprotein affinity matrix. Moreover, we show that the phosphorylation status of helianthinins changes following germination.

Keywords: 11S globulins, germination, helianthinin, *Helianthus annuus*, phosphorylation, storage proteins

Introduction

Seed storage proteins accumulate in high amounts during the late stages of seed development and provide nitrogen for seed germination and early seedling growth. These proteins have been classified into albumins, globulins and glutelins, based on their solubility in water, saline and sodium hydroxide solutions, respectively (Osborne, 1924). Helianthinin, the main storage protein of sunflower seeds, belongs to the salt-soluble 11S globulin fraction (Raymond *et al.*, 1995). These globulins are composed of α and β subunits assembled as a hexamer (Shewry *et al.*, 1995). They are synthesized as a precursor in the rough endoplasmic reticulum and follow the secretory pathway to be delivered into storage vacuoles. Sequence analysis revealed that the gene encoding the sunflower 11S precursor (Helianthinin G3) contains a signal peptide responsible for its targeting to the secretory pathway, followed by the coding sequence for α and β chains (Vonder Haar *et al.*, 1988). In fact, the proglobulin containing the α and β polypeptides is proteolytically cleaved after disulphide bond formation and transported through the secretory system as intermediate trimers that further assemble into the mature hexameric form (Shewry *et al.*, 1995). On the other hand, helianthinins are polymorphic and their subunit composition varies among different sunflower cultivars. At least six types of subunits, with molecular masses ranging from 40 to 64 kDa have been described so far, consisting of different combinations of the large (32–40 kDa) acidic α polypeptide and the small (20–25 kDa) basic β polypeptide (Rahma and Narasinga Rao, 1979; Schwenke *et al.*, 1979).

Reversible protein phosphorylation is one of the most frequent post-translational modifications and its relevance is that it regulates diverse cellular processes. Protein phosphorylation in eukaryotes mostly occurs by the addition of a phosphate group on certain serine and threonine residues, whereas phosphorylation on tyrosine residues is less abundant. Post-translational modifications of a protein can determine its activity state, localization, turnover and interactions with other proteins (Zolnierowics and Bollen, 2000). Besides its well-known role in several signalling pathways activating and deactivating enzymes, phosphorylation participates in an expanding catalogue of physiological functions in plants (Kline-Jonakin *et al.*, 2011). Among them, it plays a key role during embryogenesis and seed germination (Fujii and Zhu, 2009; Nakashima *et al.*, 2009). Also, Wan *et al.* (2007) have shown that the 12S globulin cruciferin is the major phosphorylated protein in *Arabidopsis thaliana* seeds. A total of 20 phosphorylation sites were identified on this globulin,

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including serine, threonine and tyrosine residues (Wan *et al.*, 2007). Although a specific function was not assigned to this post-translational modification, cruciferin processing and mobilization were suggested. In this respect, Ghelis *et al.* (2008) detected changes in cruciferin tyrosine-phosphorylation in response to abscisic acid (ABA) treatment, suggesting this is induced by ABA to prevent cruciferin proteolysis. In a proteomic study of *Brassica napus*, Agrawal and Thelen (2006) identified phosphoproteins belonging to ten major functional categories, including storage proteins during seed filling. A recent analysis of seed maturation in *Arabidopsis*, rapeseed and soybean detected novel orthologues of seed storage proteins using a phosphoproteomic approach (Meyer *et al.*, 2012).

Taking into account these observations, the aim of this work was to elucidate whether phosphorylated storage proteins are present in sunflower, a phylogenetically distant species.

Materials and methods

Protein sample preparation and analysis

Helianthus annuus L. seeds (line 10 347 Advanta Semillas SAIC) were sterilized in 27.5 mg ml^{-1} sodium hypochlorite for 30 min, rinsed with sterile water and subjected to imbibition for the indicated times at 25°C. Petri dishes containing wet Whatman filter paper under sterile conditions were used. Dried seeds, germinating seeds (16 and 24 h of imbibition) and seedlings (48, 90 and 96 h of imbibition) were subjected to pulverization and extracted in the following buffer: 50 mM Tris-HCl pH 7.5, 2 mM dithiothreitol (DTT), 0.1 M EDTA, protease and phosphatase inhibitors (Martin *et al.*, 2007). Protein concentration was determined by the bicinchoninic acid method (Smith *et al.*, 1985) using bovine serum albumin as standard. Protein fractions (50 μg) were resolved in 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1970) and stained with Coomassie Brilliant Blue R-250 or Colloidal Coomassie Blue (Neuhoff *et al.*, 1988). Western blot assays were carried out by protein transfer on to nitrocellulose membranes. Rabbit anti-phosphoserine 1:500 (Invitrogen, Carlsbad, California, USA) or sunflower anti-11S globulin antibodies provided by Dr S. Petruccelli (1:1000) were used as primary antibodies and an anti-rabbit IgG coupled to alkaline phosphatase (Sigma, St. Louis, Missouri, USA) was used as secondary antibody (1:10,000).

Two-dimensional PAGE

Homogenates from imbibed seeds (100 μg protein) were subjected to chloroform–methanol precipitation and the pellet was suspended in 7 M urea, 2 M thiourea, 4% (w/v) 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulphonate (CHAPS), 100 mM DTT, 0.2% (v/v) ampholytes pH 3–10 (BioRad, Hercules, California, USA) and a trace of bromophenol blue. Samples were loaded on to pH 3–10 non-linear, immobilized pH gradient strips (7 cm, ReadyStrips, BioRad). Isoelectric focusing was performed applying the following conditions: for the rehydration step the voltage was maintained for 16 h at 50 V, and then the proteins were focused at 9000 V h^{-1} at 20°C. After isoelectric focusing the strips were equilibrated in 375 mM Tris-HCl pH 8.8, 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 2% (w/v) DTT, followed by 375 mM Tris-HCl pH 8.8, 6 M urea, 20% (v/v) glycerol, 2% (w/v) SDS, 2.5% (w/v) iodoacetamide. The second dimension was carried out according to Laemmli (1970) in 12% gels at 150 V and PeppermintStick phosphoprotein molecular mass standards (Invitrogen) were loaded in the first gel lane. After SDS-PAGE, gels were fixed with 50% (v/v) methanol and 10% (v/v) acetic acid for 30 min, washed with distilled water, stained with Pro-Q Diamond phosphoprotein stain (Life Technologies, Carlsbad, California, USA) according to the manufacturer's instructions and scanned with a FluorImager instrument (Amersham Biosciences, Piscataway, New Jersey, USA) using 514 nm laser as excitation source and a 570 nm band-pass emission filter. Then gels were stained with Sypro Ruby (Life Technologies) and scanned using a 488 laser as excitation source and a 610 band-pass emission filter.

Matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry

Protein bands and spots indicated in the text were automatically excised from one-dimensional (1D) and two-dimensional (2D) gels employing Investigator ProPic robotic workstation (Genomic Solutions, Huntingdon, Cambridgeshire, UK). Gel pieces were digested with trypsin according to standard protocols in a ProGest station (Genomic Solutions). Mass spectrometry (MS) analyses of peptides were performed in a 4700 Proteomics Station (Applied Biosystems, Carlsbad, California, USA) as previously described (Pérez-Reinado *et al.*, 2007). Protein identification was assigned by comparing the obtained peptide mass fingerprinting with the non-redundant plant database, using a Mascot 1.9 search engine (Matrixscience, London, UK) (Pinedo *et al.*, 2012).

Phosphoprotein purification

Phosphoprotein enrichment was performed employing phosphoprotein affinity columns (Qiagen, Leesburg,

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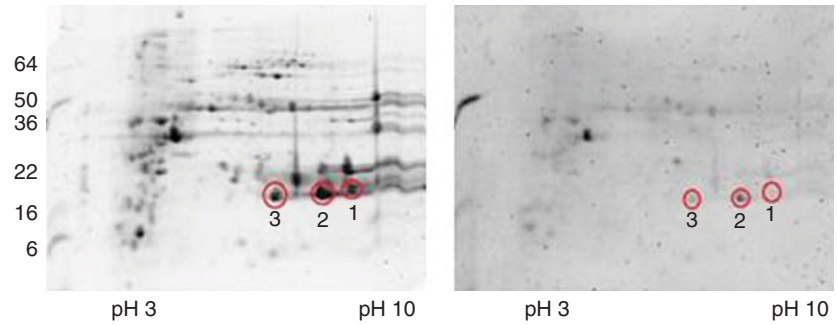

Figure 1. (colour online). Two-dimensional (2D) gel electrophoresis of protein extracts from 16-h-imbibed sunflower seeds. (A) Sypro Ruby staining, (B) Pro-Q Diamond staining. Location of phosphorylated molecular mass standards in kDa is indicated on the left. Circles indicate the spots identified by MALDI-TOF peptide mass fingerprinting.

Virginia, USA) according to the manufacturer's instructions and the previously described modifications adapted for plant extracts (Meimoun *et al.*, 2007). Seeds were decorticated and protein was extracted using 25 mM 2-(N-morpholino)ethanesulphonic acid (MES), 1 M NaCl, 0.25% (w/v) CHAPS, protease inhibitors and Benzonase Nuclease stock solution (Phosphoprotein Purification Kit, Qiagen). Protein extracts (5 mg) were loaded on to a Qiagen column and, after washing with loading buffer (8 ml), bound phosphoproteins were eluted with 3 ml of elution buffer (Qiagen) and submitted to MALDI-TOF for identification.

Results and discussion

As a first approach to detect putative phosphorylated proteins in sunflower seeds we performed 2D SDS-PAGE of 16-h-imbibed seed protein extracts and the resulting gels were sequentially stained for phosphoprotein detection (Pro-Q Diamond stain) and total protein (Sypro Ruby). Pro-Q Diamond phosphoprotein stain allows in-gel detection of phosphate groups attached to tyrosine, serine or threonine residues (Laugesen *et al.*, 2006; Chitteti and Peng, 2007; Oh *et al.*, 2009). Figure 1B shows that a limited number of spots were clearly detected upon Pro-Q Diamond staining. Among these, three spots that were consistently reproducible and could be major seed proteins were further analysed by peptide mass fingerprinting to identify them. They were digested with trypsin, submitted to MALDI-TOF and putatively identified using Mascot software on the GenBank database. Table 1 summarizes the data obtained. The three spots matched the 11S globulin seed storage protein G3 (Helianthinin G3) from *Helianthus annuus* (Uniprot accession number P19084). Also, good agreement was observed between the experimentally determined molecular mass of these spots and that predicted for the β subunit of Helianthinin G3 (20.98 kDa). Helianthinin G3 corresponds to the 493-amino-acid precursor protein, which, as described for other 11S globulins, is further cleaved into two chains: the acidic chain (α subunit) and the basic chain (β subunit) (Rahma and Narasinga Rao, 1979; Schwenke *et al.*, 1979; Vonder Haar *et al.*, 1988; Shewry *et al.*, 1995). Indeed, according to proteomic criteria, all the three spots detected with the phosphoprotein-specific stain can be assigned as 11S globulins. The phosphorylated spots between 35 and 40 kDa correlate with major spots in Fig. 1A (total proteins). One major spot (~35 kDa and acid isoelectric point) was clearly visible under Pro-Q Diamond staining; its molecular mass and isoelectric point are consistent with the α subunit. Even though these spots could not be identified by peptide mass fingerprinting, their features suggest that they correspond to α subunits of 11S globulins.

Supporting the suggestion that 11S globulin subunits are phosphorylated *in vivo*, a bioinformatic tool (NetPhos program, http://www.cbs.dtu.dk/services/NetPhos/) (Blom *et al.*, 1999) predicts 16 putative phosphorylation sites on the Helianthinin G3 precursor sequence. These correspond to 14 serines,

Table 1. Proteins identified in extracts from 16-h-imbibed *Helianthus annuus* seeds by peptide mass fingerprinting

Spot	Protein identification	Accession number UniProt	Molecular mass Exper/Theor (kDa)	Peptides matched	Coverage (%)	Score
1	11S globulin seed storage protein G3 precursor (α-β)	P19084	19.97/20.98 (β)	3	5.9	154
2	11S globulin seed storage protein G3 precursor (α-β)	P19084	19.97/20.98 (β)	15	27.6	113
3	11S globulin seed storage protein G3 precursor (α-β)	P19084	19.46/20.98 (β)	13	21.3	86

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MASKATLLLAFTLLFATCIARHQQRQQQQNQCQLQNIEALEPIEVIQAEAGVTEIWDAYD
QQFQCAWSILFDTGFNLVAFSCLPTSTPLFWPSSREGVILPGCRRTYEYSQEQQFSGEGG
RRGGGEGTFRTVIRKLENLKEGDVVAIPTGTAHWLHNDGNTELVVVFLDTQNHENQLDEN
QRRFFLAGNPQAQAQSQQQQQRQPRQQSPQRQRQRQRQGQGQNAGNIFNGFTPELIAQSF
NVDQETAQKLQGQNDQRGHIVNVGQDLQIVRPPQDRRSPRQQQEQATSPRQQQEQQQGRR
GGWSNGVEETICSMKFKVNIDNPSQADFVNPQAGSIANLNSFKFPILEHLRLSVERGELR
PNAIQSPHWTINAHNLLYVTEGALRVQIVDNQGNSVFDNELREGQVVVIPQNFAVIKRAN
EQGSRWVSFKTNDNAMIANLAGRVSASAASPLTLWANRYQLSREEAQQLKFSQRETVLFA
PSFSRGQGIRASR

Figure 2. Amino acid sequence of 11S globulin seed storage protein G3 from *Helianthus annuus* (UniProt accession number P19084). Putative phosphorylated residues were predicted using the NetPhos 2.0 program and appear underlined in the sequence. (S: 14, T: 1, Y: 1). The signal peptide is shown as a dark grey box; α and β subunits are in grey and white boxes, respectively.

1 threonine and 1 tyrosine (Fig. 2), covering both α and β subunits. To confirm this presumption experimentally, we used a strategy based on the purification of phosphoproteins by affinity chromatography; the robustness of this procedure has been reported (Laugesen *et al.*, 2006; Meimoun *et al.*, 2007). Total seed extracts were loaded on to a Qiagen affinity matrix and eluted proteins were fractionated by SDS-PAGE followed by MALDI-TOF analysis of the major proteins. Among them, four bands could be identified and all matched the 11S globulin precursor G3 (Table 2). Thus several criteria indicate that 11S globulins from imbibed sunflower seeds are phosphorylated: they are detected by a phosphoprotein-specific stain, they are retained in a specific affinity matrix and, in both cases, their identity can be confirmed by peptide mass fingerprinting.

In *Arabidopsis* the phosphorylation of cruciferins involves key residues allowing protein assembly, processing and mobilization (Wan *et al.*, 2007), and changes in phosphorylation are regulated by ABA treatment (Ghelis *et al.*, 2008). In this context, it can be hypothesized that globulins can vary in their phosphorylation status according to their physiological state. Thus, we have investigated whether phosphorylation of helianthinins can be regulated during seed germination and seedling growth. We have compared the total seed protein profile (Fig. 3A) and that of phosphoproteins detected with an anti-phosphoserine antibody (Fig. 3B). As observed in Fig. 3A, the expected protein pattern was observed in dry seeds (Raymond *et al.*, 1995; Serre *et al.*, 2001; Molina *et al.*, 2004) with major bands of around 32–40 and 20–25 kDa corresponding to 11S globulin α and β subunits, respectively. In addition, there were bands of around 60 kDa that could correspond to an αβ subunit (Molina *et al.*, 2004). The identity of these bands was also confirmed by immunoblotting because they were recognized by sunflower anti-11S globulin serum (not shown). The protein pattern in dry seeds does not change during the first 24 h of imbibition, but modification in the protein profile occurs thereafter (Fig. 3A). Particularly, bands around 40 kDa (α subunit) consistently decrease due to their mobilization, as previously reported (Shewry *et al.*, 1995). The 11S subunits appear to be differently phosphorylated during seedling growth, while the band of around 60 kDa retains the same phosphorylation status (Fig. 3B). Even if bands of ~22 kDa (β subunit) are barely detected by the anti-phosphoserine antibodies, subunits around 40 kDa are clearly visible from 16 to 48 h of seed imbibition, while they are almost undetectable in dried seeds and after 90–96 h. The absence of phosphorylated α subunits after 2 d could be attributed to their degradation, since they are also undetectable by Coomassie Blue staining (Fig. 3A). Dry seeds show a typical pattern of helianthinins but are barely detected by anti-phosphoserine antibodies.

In conclusion, the phosphorylation of helianthinins appears to be induced following the completion of

Table 2. Proteins identified in the eluted fraction of phosphoprotein affinity chromatography from *Helianthus annuus* seed extracts by peptide mass fingerprinting

Band	Protein identification	Accession number UniProt	Molecular mass Exper/Theor (kDa)	Peptides matched	Coverage (%)	Score
1	11S globulin seed storage protein G3 precursor (α-β)	P19084	29/20.98 (β)	10	18.5	34
2	11S globulin seed storage protein G3 precursor (α-β)	P19084	25/20.98 (β)	5	9.1	110
3	11S globulin seed storage protein G3 precursor (α-β)	P19084	24/20.98 (β)	18	26.4	290
4	11S globulin seed storage protein G3 precursor (α-β)	P19084	22/20.98 (β)	8	15	202

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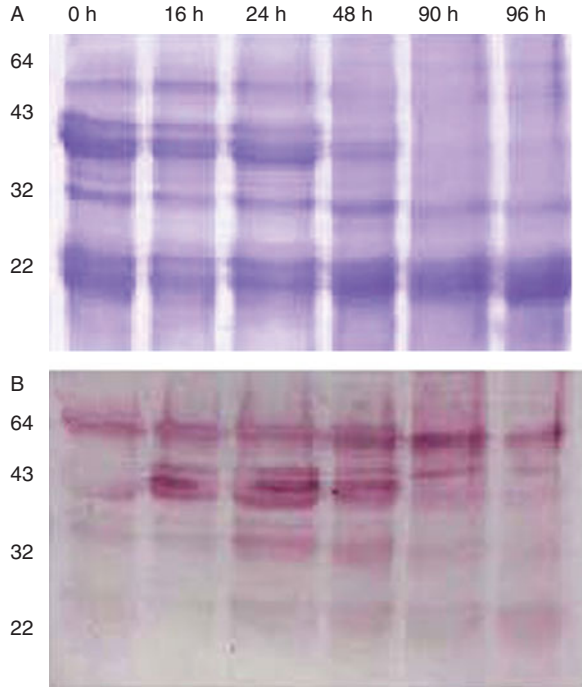

Figure 3. (colour online). Phosphoserine immunodetection of sunflower seed proteins at different times of germination and seedling growth: (A) 50 μg of total proteins extracted from dry seeds and after 16, 24, 48, 90 and 96 h of imbibition were fractionated by SDS-PAGE and stained with Coomassie Blue; (B) immunoblotting with anti-phosphoserine antibody of the same samples detailed in (A). A representative experiment of three replicates is shown. Molecular mass in kDa is indicated on the left.

germination. Even though the basis and role of this post-translational modification is unknown, our results are indicative of the necessity for future investigations to analyse the relationship between storage protein phosphorylation and mobilization during post-germinative growth.

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