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Sunflower (*Helianthus annuus* L.) seed development accompanies glycosylation and phosphorylation of total soluble and oil body membrane-associated proteins

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Abstract

Seed development is a complex process involving a series of physiological and metabolic events. During this process, glycosylation, a post-translational modification, plays a crucial role in protein targeting during cellular signaling, while protein phosphorylation acts as a molecular switch, activating proteins in the signaling cascade essential for development. Sunflower is an important oilseed crop in India and worldwide. Phosphoprotein analysis at three stages of sunflower seed development (20, 30 and 40 days after anthesis (DAA)) has revealed significant stage-specific differences in phosphoprotein and glycoprotein distribution. This analysis provides a metabolic framework for further characterizing the biochemical events associated with seed development. The present investigations will be instrumental in analysing the expression and modulation of glycosylation and phosphorylation of proteins during different phases of sunflower seed development. Additionally, this research offer insights into the regulatory roles phosphorylation and glycosylation as molecular switches in governing seed development in sunflower.

Keywords

desiccation; glycosylation; phosphorylation; seed development

Introduction

Glycoproteins (GPs) are proteins with enzymatic or non-enzymatic associations of carbohydrate residues. O-glycosylation is linked to various regulatory processes, including signaling, transcription, translation, stress response and glucose metabolism. N-glycosylation, on the other hand, is primarily associated with secretory proteins involved in processes such as cell -to-cell communication and stress tolerance (1). Extensive studies on GPs have been conducted in maize and legume plants (2-5). Proteins involved in carbohydrate metabolism with low glycosylation ratios may have enzymatic activities and functions regulated by glycosylation levels via the hexosamine pathway. Inhibition of protein glycosylation using tunicamycin, a glycosylation inhibitor, may lead to protein aggregation. Additionally, misfolding has been observed, highlighting the essential role of glycosylation in protein folding and membranal transport.

Protein phosphorylation is one of the most extensively studied post-translational modifications, enabling cells to transduce developmental and environmental signals, thereby regulating numerous biological processes across different organisms, including plants (6, 7). Extensive phosphoprotein analysis has been reported in rice plants, facilitating comparative studies with

integrated phosphoprotein data from *Arabidopsis* (8, 9). This analysis suggests that the phosphoproteome is among the most conserved proteomes across organisms, as orthologous proteins exhibit similar phosphorylation patterns despite significant phylogenetic distances (8-10). Protein phosphorylation is commonly associated with protein modification, signal transduction and kinase activity. Since phosphorylation often targets proteins involved in transcription factor (TF) activity, transcription regulator activity and transcriptional processes, it plays a crucial role in controlling gene expression (11).

The present study aims to elucidate the qualitative changes in glycosylation and phosphorylation of cytosolic and oil body membrane proteins during the three stages of sunflower seed development.

Materials and Methods

Electrophoretic detection of glycoproteins

***Sample preparation and electrophoresis*:** Five hundred milligrams of seeds from each stage of seed development (20, 30 and 40 DAA) were ground into a fine powder using liquid nitrogen. The powder was then transferred to Eppendorf tubes and incubated at 4°C for 30 min on a vortex shaker in an incubation medium containing 25 mM Tris-HCl (pH 7.5). After centrifugation at 10,000 g for 30 min, the supernatants were transferred to fresh Eppendorf tubes and protein was estimated according to Bradford method (12). Protein aliquots (10 µg per sample) were mixed with Laemmli buffer (1:1, V/V), boiled at 90°C for 2 min and loaded into the wells of a 5% stacking gel for separation on a 12.5% vertical SDS-PAGE (Mini-PROTEAN, Bio-Rad, USA). Electrophoresis was carried out at 75 V for 30 min, followed by 200 V for the remaining run time. For protein extraction from oil bodies, sodium bicarbonate washed oil bodies from the 20, 30 and 40 DAA seed development stages were processed using the same protocol. After protein estimation (13), 10 µg of protein was mixed with Laemmli buffer (1:1, V/V), boiled at 90°C for 2 min and loaded into a 5% stacking gel for separation on a 12.5% vertical SDS-PAGE under the previously mentioned conditions. Molecular weight markers used for electrophoretic gel analysis ranged from 12,000 to 18,000 kDa and were procured from BDH, England.

Staining for glycosylated proteins using Pro-Q® Emerald 300 protein gel stain

***Pro-Q® Emerald 300 stock solution preparation*:** The vial containing Pro-Q® Emerald stain was dissolved in 6 mL of dimethyl-sulphoxide (DMSO) and stored at -20°C for further use. Fixing solution prepared with 50% methanol and 5% acetic acid in distilled water. Wash solution: Freshly prepared with 3% glacial acetic acid in distilled water. Oxidizing solution prepared with a 250 mL of 3% acetic acid solution was added to a bottle containing periodic acid (provided in staining kit) and dissolved thoroughly. The solution was stored at 4°C for further use.

***Pro-Q® Emerald 300 staining*:** Gels obtained after electrophoresis were immersed in 80 mL of fixative solution for 30 min with gentle agitation on a rocker shaker at room temperature. The fixative was change twice to ensure complete removal of SDS from the gel. Next, gels were washed in 100 mL of wash solution for 20 min with gentle agitation on a rocker shaker at room temperature, with a single solution change. Gels were then immersed in 80 mL of oxidizing solution and gently agitated on a rocker shaker at room temperature for 30 min, followed by a 15- min wash in wash solution. For staining, the Pro-Q® Emerald stock solution was diluted by adding 500 µL of the stain to the staining buffer (ready to use, provided in the staining kit). Gels were incubated in 25 mL of stain solution for 120 min in dark at room temperature. This was followed by a 15 min washing in wash solution, performed in dark at room temperature with two solution changes (14).

***Visualization of gels for glycosylated proteins*:** Gels stained with Pro-Q® Emerald 300 were visualized in UV transilluminator (Gel Doc-It Imaging System, UVP, USA) at an excitation wavelength of 280 nm. Proteins bands were quantified using Vision Launch Software (UVP, USA) and differential protein expression was graphically represented based on band volume (arbitrary units).

Electrophoretic analysis of phosphoproteins

***Sample preparation and electrophoresis*:** A total of 500 mg of seeds from each seed developmental stage (20, 30 and 40 DAA) were ground into fine powder in liquid nitrogen. The resulting powder was transferred into Eppendorf tubes and incubated at 4°C for 30 min on a vortex shaker in an incubation medium containing 25 mM Tris-HCl (pH 7.5). Supernatants obtained after centrifugation at 10,000 g for 30 min were delipidated and desalted. For this, 150 µL from each sample was mixed with 600 µL of methanol and vortexed thoroughly. Then, 150 µL of chloroform was added and mixed well, followed by addition of 450 µL of ultrapure water and further mixing. The samples were centrifuged at 12,000 rpm for 5 min. The white disc formed between the upper and lower layers was collected, mixed with 450 µL of methanol and centrifuged again at 12,000 rpm for 5 min after thorough mixing. The supernatant was discarded and the resulting pellet was resuspended in Lammeli sample buffer. Protein aliquots equivalent to 10 µg from each sample were loaded into the wells of a 5% stacking gel and resolved in 12.5% vertical SDS-PAGE at 75 V (30 min), followed by 200 V for remaining run time. The molecular markers used in the electrophoretic gel analysis ranged from 12,000-18,000 kDa and were procured from BDH England.

Sample preparation for detection of protein

***phosphorylation in oil body membrane proteins*:** Oil body membrane proteins were extracted from sodium bicarbonate washed oil bodies from 20, 30 and 40 DAA stages of seed development by the previously mentioned protocol. After estimation of protein content, 10 µg of protein was mix with Laemmli buffer (1:1, V/V), boiled at 90° C for 2 min and were loaded into 5% stacking gel for separation on a 12.5% vertical SDS-PAGE under the previously mentioned conditions (13).

Staining for phosphorylated protein using Pro-Q® Daimond protein gel stain

***Preparation of stock solutions*:** Fixative solution: Freshly prepared solution containing 50% methanol and 10% acetic acid in distilled water. Wash solution*:* Distilled water. Staining solution: Ready to use Pro-Q® Daimond stain. Destining solution: Freshly prepared 20% acetonitrile in 50 mM sodium acetate buffer pH 4. Final washing solution: Distilled water.

Procedure for Pro-Q® Daimond staining: Gels were immersed in 100 mL fixation solution for 30 min with two solution changes. Fixation was followed by three washes in distilled water, each lasting in 10 min. Staining was performed in dark at room temperature at 60 mL Pro-Q® Daimond stain solution for 90 min with gentle agitation on a rocker shaker (14). After staining, gels were washed twice in distilled water for 5 min each.

Visualization of phosphorylated proteins: Gels stained with Pro-Q® Daimond were visualized using a Typhoon fluorescent scanner (GE Healthcare, UK) at an excitation wavelength of 555 nm and with a PMT intensity of 600 units. Protein bands showing differential expression were quantified through software analysis (Image Quant TL software, GE Healthcare, UK) to assess variations protein in phosphorylation among the samples. Differential protein expression was graphically represented based on band volume (arbitrary units).

Staining for total protein profile using SYPRO® Ruby protein gel stain

***Preparation of stock solutions*:** Fixative: Freshly prepared containing 50% methanol and 7% acetic acid in distilled water. Stain solution: Ready to use SYPRO® Ruby stain. Wash solution: Freshly prepared post-staining gel solution containing 10% methanol and 7% acetic acid in distilled water.

Procedure for SYPRO® Ruby staining: Gels obtained after electrophoresis were immersed in 80 mL of fixative solution for 30 min at room temperature. Fixation was immediately followed by staining with 80 mL SYPRO® Ruby (ready to use) stain in dark at 4°C overnight (14). After staining, gels were washed in wash solution for 30 min.

Visualization of gels for total proteins: Gels stained with SYPRO® Ruby were visualized using UV transilluminator at an excitation wavelength of 280 nm. Additionally, total protein profiles were analysed using a Typhoon fluorescent scanner at an excitation wavelength of 450 nm.

Results and Discussion

Seed desiccation phase (40 DAA) marks enhanced glycosylation of cytosolic proteins

SDS-PAGE analysis of glycoproteins in the total soluble proteins (10,000 g supernatant) from the three stages of developing seeds indicates that glycosylation is a common co-translational and post-translational modification across all the developmental stages. However, maximum glycosylation is evident at 40 DAA stage (Fig. 1 & 2). A

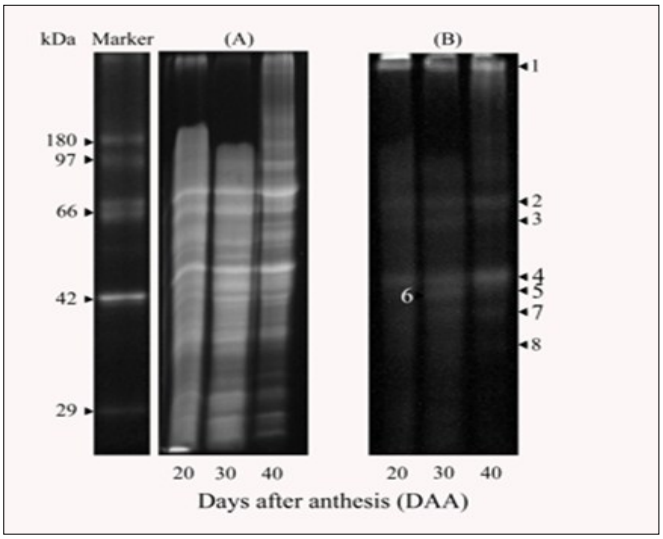

Fig. 1. Electrophoretic analysis of glycoproteins from total soluble proteins (10,000 g supernatant) obtained from three stages of developing seeds (20, 30 and 40 DAA). (A) Total protein profile as visualized with Sypro Ruby (B) Glycoprotein profile visualized following ProQ Emerald staining.

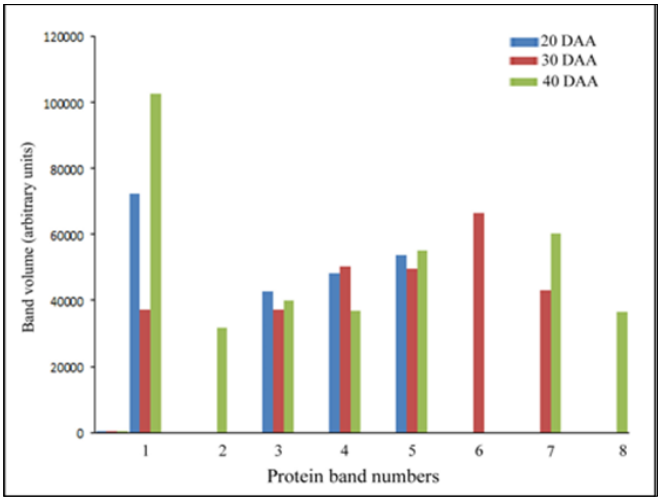

Fig. 2. Differential expression of glycoproteins from total soluble proteins in different developmental stages of sunflower seeds (20, 30 and 40 DAA). Histograms depict differential expression of glycoproteins in different protein bands represented in terms of band volume calculated using Image Quant TL software (GE Healthcare, UK).

notable increase in protein glycosylation levels was observed at 40 DAA stage. Similar findings have been in *Ricinus communis,* where glycoprotein analysis revealed increased glycosylation with advancing stages of seed development (15). At the 20 DAA stage, a greater proportion of high molecular weight proteins undergo glycosylation compared to low molecular weight. Two protein bands, with molecular weights of 74 and 35 kDa, are exclusively present in the 40 DAA stage and exhibited glycosylation. These proteins likely represent glycoproteins specific to seed maturation phase, which undergoes extreme desiccation. Additionally, 42 kDa protein band is exclusively present at the seed-filling stage (30 DAA), indicating its potential role in this phase of seed development.

Oil body maturation in developing seeds coincides with high glycosylation of oil body membrane proteins

Glycoproteins are one of the important bioactive molecules which have been localized in oil seed crop proteome, such as that of *Ricinus communis* (15) and soybean (3). Analysis of protein glycosylation in OB membrane proteins revealed the

presence of glycoproteins in all the three stages of development and showed seed development-associated quantitative expression patterns with greater levels of glycoproteins present in mature stage of seed development which is, 40 DAA (Fig. 3 & 4). This may account for the fact that 40 DAA stage witnesses maximum oil body maturation as seed prepares itself for maximum storage, which will be required during seed germination. Analysis of ricin, a glycoprotein in *Ricinus communis*, also shows development-associated increase with advancing stages of seed development (16). Number of glycoproteins present in the 30 DAA stage is lower than that in 20 and 40 DAA stages.

A correlation is evident between seed development and protein phosphorylation

Protein phosphorylation plays an important role in seed development and germination. Detailed proteomic analysis

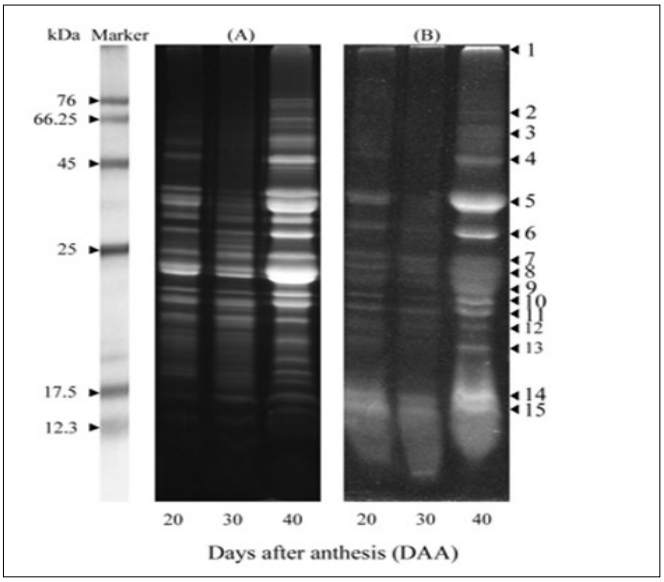

Fig. 3. Electrophoretic analysis of glycoproteins from oil bodies washed with 0.1 M sodium bicarbonate. Oil bodies were obtained from three stages of developing seeds (20, 30 and 40 DAA). (A) Total protein profile as visualized using Sypro Ruby (B) Glycoprotein profile visualized following ProQ Emerald staining.

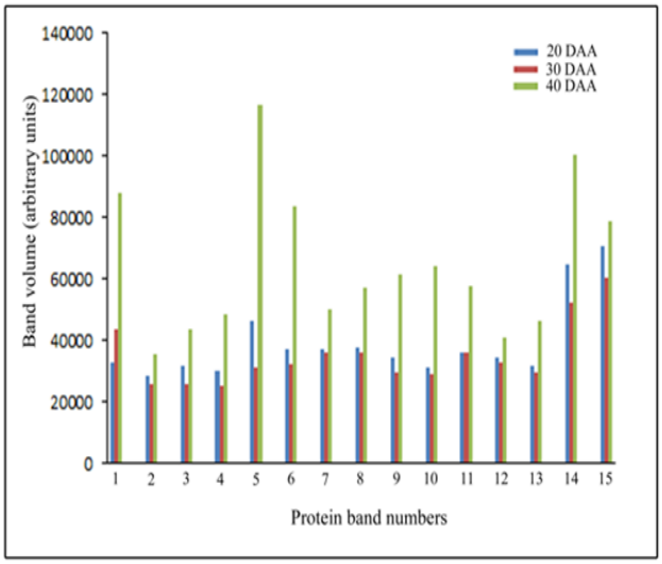

Fig. 4. Differential expression of glycoproteins in the oil body membranes. Oil body preparations were subjected to 0.1 M sodium bicarbonate washing. Histograms depict differential expression of glycoproteins in different protein bands represented in terms of band volume calculated using ImageQuant TL software (GE Healthcare, UK).

of phosphorylation events in the developmental processes has allowed the identification of the phosphoproteins involved and established the importance of phosphorylation in the specific steps of the various biochemical pathways (11, 15). Reports indicate that phosphorylation activates storage-associated enzymes, including phosphoenolpyruvate carboxylase (PEPC), underscoring its role in metabolic regulation during seed maturation (17). A calcium-dependent protein kinase (CDPK) isoform in rice seeds regulates the formation of storage reserves by phosphorylating sucrose synthase (18). Overexpression of CDPK2 in rice can prematurely stop seed development. While OsCPK23 is significantly upregulated in developing seeds (19). Similarly, during *Brassica napus* seed filling, a wide variety of functionally distinct phosphoproteins are expressed, further emphasizing the role of phosphorylation in seed metabolism (20). In *Arabidopsis* seeds, the glycoprotein 12S globulin cruciferin primarily functions as a phosphorylated storage protein (21). Additionally, lipid reserve mobilization events and storage protein accumulation are regulated by proteins such as tyrosine kinases and phosphatases (22). Phosphorylation can also exhibit enzymes such as legume sucrose phosphate synthase (SPS) in legume seeds, particularly when hexose and sucrose levels fluctuate (23). Furthermore, the leucine-rich repeat receptor kinase encoded by *IKU2 (HAIKU2)* targets SHB1, a positive regulator of seed development. Mutations in *IKU2* have been documented to result in reduced seed size and altered embryo and endosperm development (24).

Accumulation of seed storage proteins during later phase of seed development coincides with enhanced protein phosphorylation

Phosphoprotein analysis across three stages of sunflower seed development (20, 30 and 40 DAA) has revealed marked development-associated differences in the pattern of phosphoprotein distribution. Extensive phosphoprotein dynamics was undertaken in rapeseed highlighting the importance of phosphorylation involved in the seed developmental processes (21). In the present study, the seed maturation stage of sunflower (40 DAA) showed maximum quantitative presence of phosphoproteins from high to low molecular weight range (>90 to 75 kDa). This suggests that phosphoproteins play a key role in the desiccation phase of seed development where seeds maximally store reserves required later for seed germination (25). Similarly, an increase in phosphoprotein levels from two to six weeks after flowering in *Arabidopsis* indicated the regulation of storage proteins by phosphorylation, with maximum phosphorylation of storage proteins, such as cruciferins, occurring at later development stages (26). Most proteins showed quantitative differences in phosphorylation across the three developmental stages. Notably, high molecular weight phosphoproteins (>90-75 kDa) were exclusive to the 40 DAA stage (Fig. 5 & 6). A 21 kDa protein is phosphorylated exclusively at 20 DAA stage and phosphoprotein of 17.5 kDa is exclusive to 30 DAA stage of seed development. A 65 kDa phosphoprotein band, present in 20 and 40 DAA, is absent in 30 DAA stage and showed marked enhancement in phosphoprotein levels at 40 DAA stage. A 29 kDa polypeptide

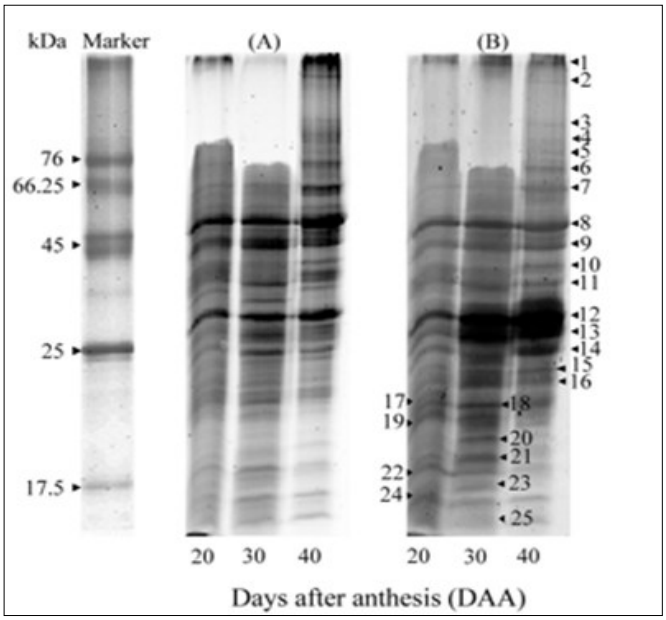

Fig. 5. Electrophoretic analysis of phosphoproteins from total soluble proteins (10,000 g supernatant) obtained from three stages of developing seeds (20, 30 and 40 DAA). (A) Total protein profile as visualized using Sypro Ruby (B) Phosphoprotein profile visualized following ProQ diamond staining.

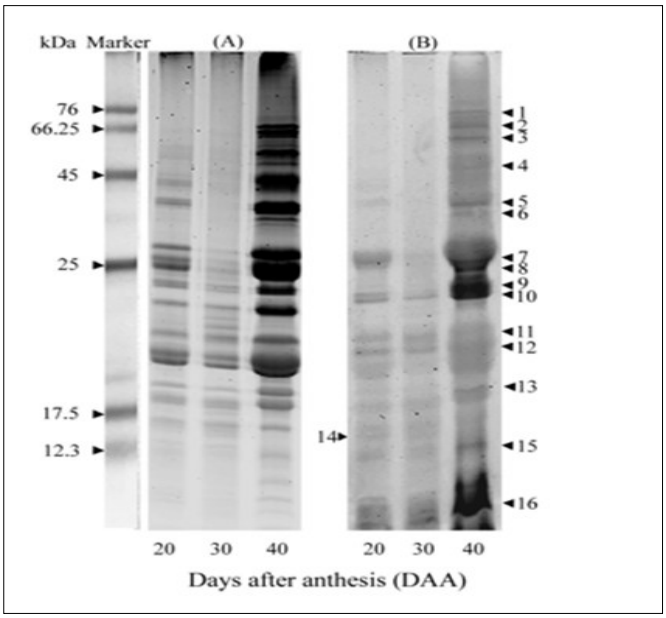

Fig. 7. Electrophoretic analysis of phosphoproteins from oil bodies washed with 0.1 M sodium bicarbonate. Oil bodies were obtained from three stages of developing seeds (20, 30 and 40 DAA). (A) Total protein profile as visualized using Sypro Ruby (B) Phosphoprotein profile visualized following ProQ diamond staining.

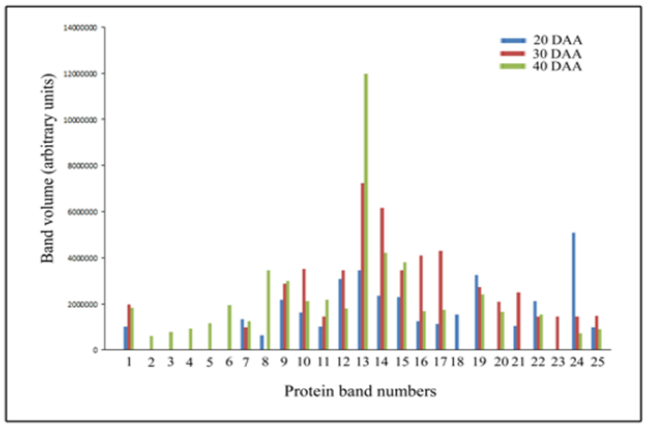

Fig. 6. Differential expression of phosphoproteins from total soluble proteins (10,000 g supernatant) in different developmental stages of sunflower seeds (20, 30 and 40 DAA). Histograms depict differential expression of phosphoproteins in different protein bands represented in terms of band volume calculated using Image Quant TL software (GE Healthcare, UK).

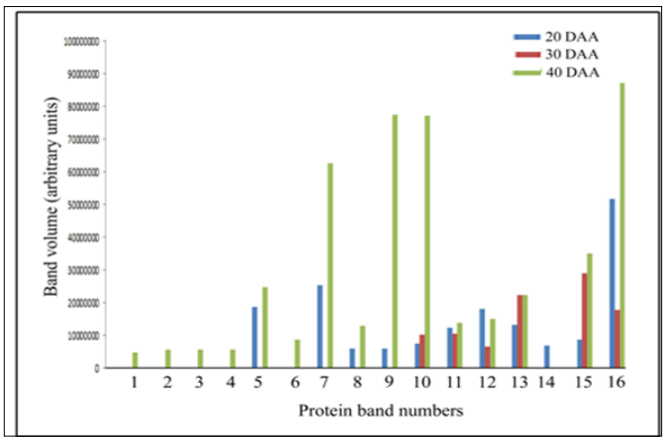

Fig. 8. Differential expression of phosphoproteins in the oil body membranes. Oil body preparations were subjected to 0.1 M sodium bicarbonate washing. Histograms depict differential expression of phosphoproteins in different protein bands represented in terms of band volume calculated using Image Quant TL software (GE Healthcare, UK).

showed highest level of phosphorylation at 40 DAA stage. The seed filling (30 DAA) and maturation (40 DAA) stages demonstrated a greater number of phosphoproteins across a range of molecular weights compared to the early developmental stage (20 DAA), further emphasizing the regulatory role of phosphorylation in seed maturation.

Analysis of phosphoproteins in oil body membrane proteins from developing seeds

Phosphoprotein analysis of the oil body membrane proteins at the three stages of seed development has revealed qualitative and quantitative changes in the expression patterns, with highest level of protein phosphorylation at 40 DAA stage. Polypeptides numbered 1, 2, 3, 4 and 6, with molecular weights ranging between 35 -76 kDa, were exclusively evident at 40 DAA stage, coinciding with the peak accumulation of well-developed oil bodies in the cells (Fig. 7 & 8). A higher distribution of phosphoproteins at 20 DAA, compared to phosphoprotein analysis of the total soluble proteins, was also observed. Oil body-associated phosphoproteins at 20 DAA displayed a broad molecular weight range of 8 kDa to 40 kDa, with 15.2 kDa protein band uniquely present at this stage of seed development. Notably, the presence of low-molecular weight phosphoproteins at 30 DAA was a significant finding in the phosphoprotein analysis of oil body membrane proteins extracted using 0.1 M sodium bicarbonate washings. These investigations highlight the dynamic nature of protein phosphorylation during seed maturation (27). Polypeptides with molecular weights of 40, 27, 24 and 23.5 kDa were absent at 30 DAA stage but they exhibited increased quantitative expression at 40 DAA compared to the 20 DAA stage.

Conclusion

Glycosylation is a well-established co-translational and post-translational modification that occurs throughout all stages of seed development, with highest level observed at 40 DAA. This may be attributed to the fact that 40 DAA stage marks peak oil body maturation, as seeds prepare for maximum oil storage,

which essential during germination. The seed maturation stage of sunflower (40 DAA) also shows the highest protein phosphorylation, particularly in polypeptides ranging from 90 to 75 kDa. This signifies the role of phosphoproteins in the desiccation phase of seed development, during which seeds accumulate reserves required for germination. The seed filling (30 DAA) and maturation (40 DAA) stages show a greater number of phosphoproteins with varying molecular weights compared to the 20 DAA stage. Analysis of phosphoproteins in oil body membrane proteins from developing seeds further indicates increased protein phosphorylation in the later stages of seed development. These finding suggest that protein glycosylation and phosphorylation play crucial metabolic roles during seed maturation.

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Authors' contributions

AT and GK conceived the idea and planned experiments. AT performed the experiments and collected the data. All authors analysed the research data and AT drafted the manuscript.

Compliance with ethical standards

Conflict of interest: Authors do not have any conflict of interest to declare.

Ethical issues: None

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