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Identification of Phosphorylated Proteins in Response to Salt Stress in Wheat Embryo and Endosperm during Seed Germination

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Seed germination is a new beginning for the crop life cycle, which is closely related to seed sprouting and subsequent plant growth and development, and ultimately affects grain yield and quality. Salt stress is one of the most important abiotic stress factors that restrict crop production. Therefore, it is highly important to improve crop salt tolerance and sufficient utilization of saline-alkali land. In this study, we identified the phosphorylated proteins involved in salt stress response by combining SEM, 2-DE, Pro-Q Diamond staining and tandem mass spectrometry. The results showed that salt stress significantly inhibited seed germination and starch degradation. In total, 14 phosphorylated protein spots (11 unique proteins) in the embryo and 6 phosphorylated protein spots (4 unique proteins) in the endosperm were identified, which mainly involved in stress/defense, protein metabolism and energy metabolism. The phosphorylation of some proteins such as cold regulated proteins, 27K protein, EF-1β and superoxide dismutase could play important roles in salt stress tolerance.

Keywords: wheat, embryo, endosperm, germination, salt stress, phosphoproteins

Introduction

Wheat (*Triticum aestivum* L.) is a widely planted food crop around the world. After a long period of development, wheat has owned the largest planted area with the highest total output and the largest amount of trade in the world (Yang and Qiao 2007). Seed germination is a new beginning of wheat life activities, which has important effects on subsequent plant growth and yield formation. Wheat seed consists of embryo and endosperm and both play important roles in seed germination. Meanwhile, seed germination is also susceptible to external abiotic stress factors such as salt, drought and low temperature etc. At present, the global saline-alkali land area has reached 954,000,000 hectares (Malcolm and Sumner 1998), and particularly China has about 99,133,000 hectares (Xing and Zhang 2006). Thus, to improve crop salt tolerance, it is critical to understand the molecular mechanisms of seed germination in response to salt stress.

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The endosperm is formed by the nuclear development of the fertilized polar nuclei. The majority of wheat seeds are composed of endosperm, so the chemical compositions and enrichment of endosperm determine the yield and quality. Wheat endosperm mainly contains starch, proteins and a small amount of fat and mineral elements, and provides nutrients for seed germination and seedling growth at the early stages. The embryo is formed by the development of the zygote and the fully developed embryo consists of the germ, radicle, hypocotyl and cotyledon, which will develop into a new plant body in the germination. Wheat seed germination begins with water absorption from a relatively quiescent state under adequate water and oxygen supply and suitable temperature conditions, and terminates in an elongation of the hypocotyl (Bewley and Black 1994). This process involves a complex series of physiological and biochemical changes (Han et al. 2017). Under salt stress, the cell membrane structure is difficult to repair, and the cells produce a large amount of reactive oxygen. Oxidative damage of the cellular components such as lipid, protein and DNA aggravates the destruction of biofilm structure and interferes with the normal physiological functions of cells (Ahmad and Prasad 2012). With the increase of salt concentration, seed germination is inhibited. Relative germination index and relatively simple activity index, seedling root length and plant height gradually decreased while leaf membrane permeability increased (Wu et al. 2009).

Protein phosphorylation is one of the protein posttranslational modifications (PTMs), which involves in the regulation of many biological processes such as signal transduction, cell division and differentiation and adverse stress responses. For example, phosphorylated modification can activate or passivate the enzyme, regulate the ionic balance in plants (Zhu 2003), and improve plant salt tolerance (Lv et al. 2014). In recent years, wheat phosphoproteome during plant growth and development and under various abiotic stresses have been investigated (Zhang et al. 2014). In particular, Pro-Q Diamond staining provides an effective and rapid method for phosphoprotein identification, which has been used to analyze the phosphorylated protein characterization during wheat grain germination and development (Guo et al. 2012; Dong et al. 2015) and under salt stress (Lv et al. 2016). These studies provided new evidence to understand the molecular mechanisms of protein phosphorylation regulating plant growth and development as well as various abiotic stress responses. However, the phosphorylated protein characterization in wheat embryo and endosperm in response to salt stress during seed germination and their potential functions are not clear.

This study used the Chinese elite bread wheat cultivar Zhengmai 366 and Pro-Q Diamond staining to identify the phosphorylated proteins in response to salt stress in wheat embryo and endosperm during seed germination. We aimed to explore the phosphorylated protein characterization and their potential roles in regulating wheat salt stress response during seed germination. Our results provide new evidence for further understanding the molecular mechanisms of salt tolerance and useful information for wheat cultivar improvement.

Materials and Methods

Wheat materials, seed germination and salt stress treatment

The elite Chinese bread wheat cultivar Zhengmai 366 (*Triticum aestivum* L., 2n = 6x = 42, AABBDD) was used as material, which was kindly provided by Dr. Zhengsheng Lei, Wheat Research Center of Henan Academy of Agricultural Science of China. This cultivar with high yield, superior quality and wide adaptability has been recently released and widely cultivated in the main wheat production areas of China.

Wheat seeds with equal size were selected and rinsed with sterilized water three times. Seed germination was performed on the filter paper in Petri dish in the artificial climate box with three biological replicates (each 1000 grains). Seed germination experiment included two groups: normal control group (CK) with sterilized water and salt stress group treated by 180 mM NaCl solutions in the dark conditions of temperature 23 °C and humidity 75%. Seed morphological features from germination 0 h and 24 h (emergence of radical) were recorded. Seed samples were collected and then stored in –80 °C prior to use.

Determination of relative water content

The aluminum boxes were pre-baked to constant weight. The mature and germinating 100 seeds in three biological replicates were weighed and then placed in aluminum boxes. After drying 72 h at 120 °C, the aluminum boxes and seed samples were weighed and recorded. Seed relative water content (RWC) was calculated by the formula: measured sample weight before drying – measured sample dry weight)/measured sample dry weight ×100%.

Seed ultrastructural observation by SEM

The germinated seeds were fixed in formalin-alcohol fluid overnight, and then treated by 70%, 80%, 90% and 100% ethanol for 20 min, respectively. Seed was dehydrated using isoamyl acetate and ethanol at 1:3, 1:1 and 3:1 for 20 min, respectively, and placed in the pure isoamyl acetate. Germinating seeds were broken along the groin of the grains, then the vacuum spraying gold was attached to the platform, and the ultrastructure of germinating seeds was observed with the S-4800 FESEM scanning electron microscope (SEM).

Embryo and endosperm separation and protein extraction

The embryo and endosperm from germinating seeds of three biological replicates were separated and proteins were extracted based on Gu et al. (2015). The extracted proteins were dried at room temperature and then store at –20 °C prior to use.

Two-dimensional electrophoresis (2-DE)

2-DE was performed according to Guo et al. (2012). After 2-DE, the gels were stained with 1% Coomassie Brilliant for 24 h and then decolorized with a solution containing 10% ethanol and 10% acetic acid. The gels were scanned using a GS-800 calibration densitometer (Bio-Rad, Dallas, Texas, USA).

Phosphorylated protein detection by Pro-Q Diamond staining

After 2-DE separation, the gels were stained with Pro-Q Diamond (GE Healthcare, USA) and the phosphorylated proteins were detected according to Agrawal and Thelen (2005). The staining and washing steps were performed on vortex (Forma Scientific 4520.USA) at 50 rpm. Gels stained by Pro-Q Diamond were imaged by using a 532 nm excitation laser and a 580 nm long pass filter on a Typhoon™ 9400 scanners (GE Health care, USA). The phosphorylated protein changes under salt stress were determined by ImageMaster 2D Platinum 7.0 (GE Healthcare, United States).

MALDI-TOF/TOF-MS

The phosphorylated protein spots detected by Pro-Q Diamond were collected from gels and digested by trypsin, and then used for further identified by matrix-assisted laser desorption/ionization time-of-flight/time-of-flight mass spectrometry (MALDI-TOF/TOF-MS) according to Guo et al. (2012).

Subcellular location prediction of the phosphorylated proteins

Subcellular locations of the identified phosphorylation proteins were predicted using WoLF PSORT (http://wolfpsort.org/) (Horton et al. 2006), Predotar (http://urgi.versailles.inra.fr/predotar/predotar.html) (Small et al. 2004) and UniprotKB (http://www.uniprot.org/) database programs.

Prediction of the phosphorylated sites

The phosphorylated sites among the identified phosphorylated proteins were predicted with NetPhos 2.0 software based on Blom et al. (1999).

Results

Seed morphological and ultrastructural changes during germination process under salt stress

Seed germination process begins upon imbibitions. As showed in Fig. 1A, the radicle and bud emerged and the radicle length reached 1–2 cm at 24 h under normal germination conditions, indicating the completion of seed germination and the beginning of seedling

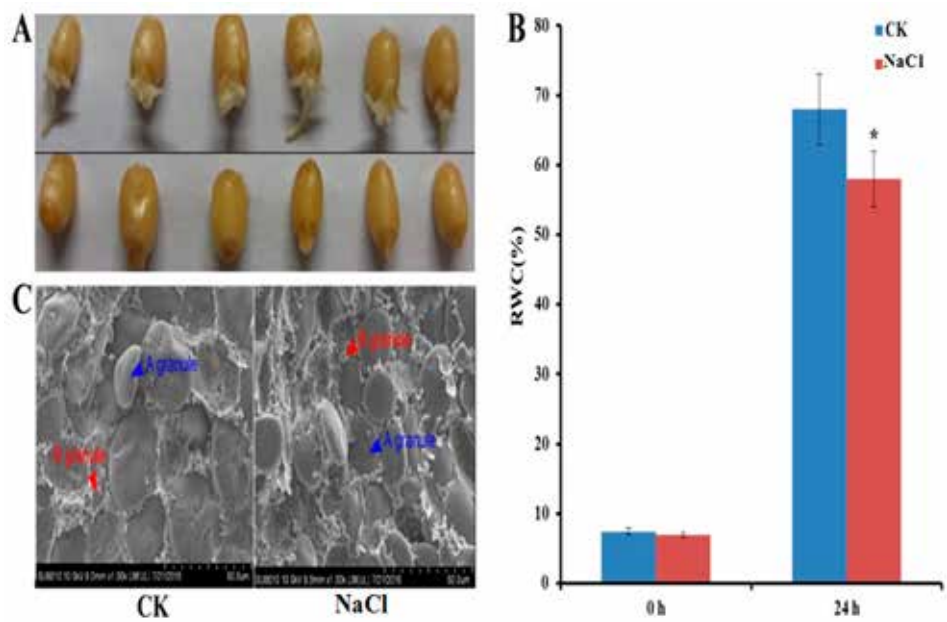

Figure 1. Grain morphology (A), relative water content (RWC) (B) and ultrastructure (C) changes of germinating seeds at 24 h in the elite Chinese bread wheat cultivar Zhengmai 366 under salt stress

growth. However, under salt stress (180 mM NaCl), the hypocotyl could break the seed coat at 24 h, but the radicle had no clear elongation compared to the normal germination. Meanwhile, the seed relative water content at 24 h under salt stress was significantly lower than the control group (Fig. 1B), indicating that the seed imbibitions was clearly inhibited by salt stress.

Further ultrastructural observation by SEM showed that the A-granules (> 10 μm in diameter) inflated and B-granule (5–10 μm in diameter) numbers were decreased due to imbibitions and starch degradation under the normal germination conditions. On the contrary, salt stress clearly inhibited seed imbibitions and starch degradation, resulting in the slight changes of starch granule sizes and numbers (Fig. 1C). These results indicated that salt stresses significantly restricted seed germination.

Phosphorylated protein analysis in the embryo and endosperm of germinating seeds under salt stress

The embryo and endosperm proteins from germinating seeds under normal conditions and salt stress treatment were separated by 2-DE, and then the phosphorylated proteins were detected by Pro-Q Diamond staining (Fig. 2). The stained protein spots were collected from 2-DE gels and digested by trypsin for further tandem mass spectrometry identification. Results of identified phosphorylated proteins present in embryo and en-

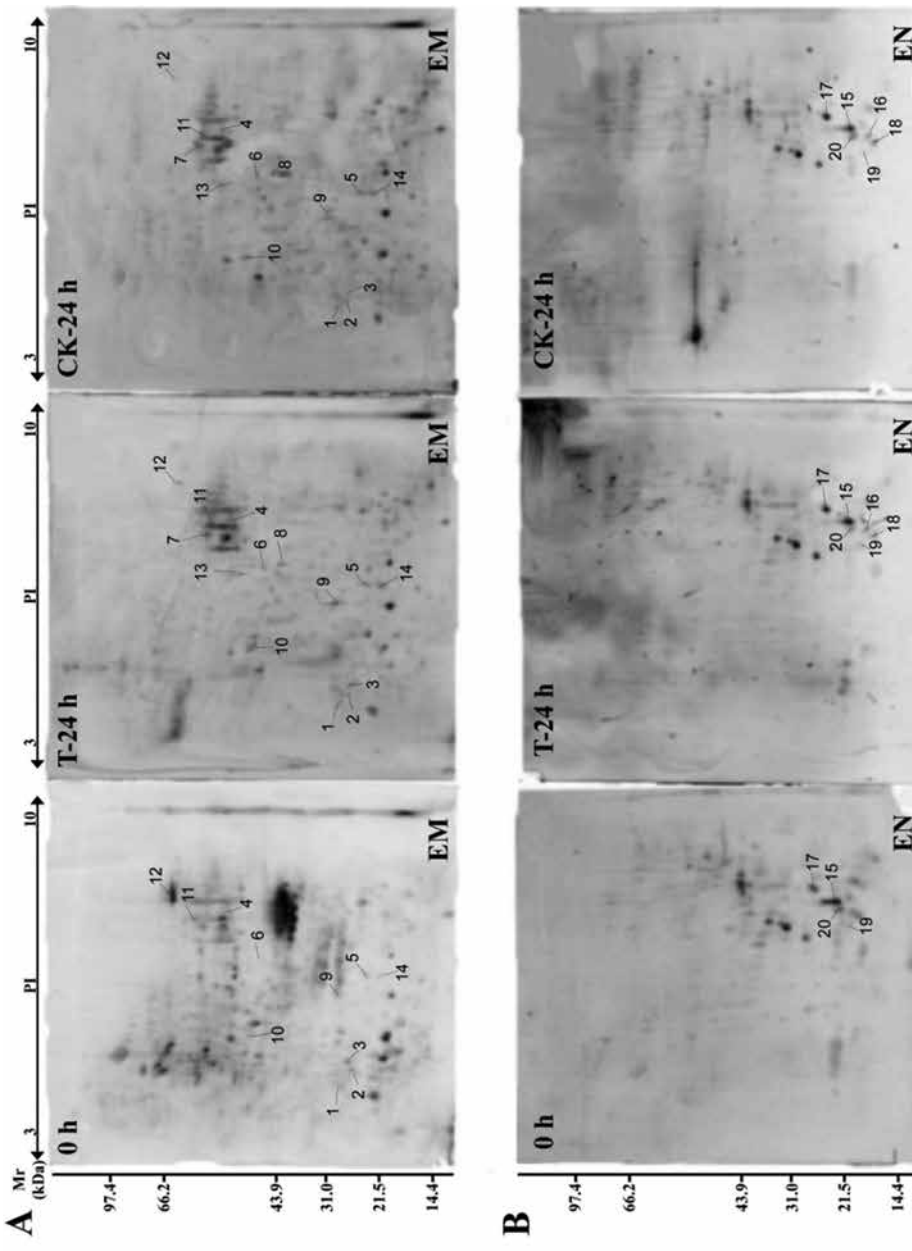

Figure 2. 2-DE images of the phosphorylated proteins during seed germination of Zhengmai 366 detected by 2-DE and Pro-Q Diamond staining. A. The stained phosphorylated proteins in the embryo. B. The stained phosphorylated proteins in the endosperm. The identified phosphorylated proteins were numbered in the 2-DE gels. CK: Control. T: Salt stress treatment. EM: Embryo. EN: Endosperm

dosperm were shown in Table 1. In the embryo, 14 phosphorylated protein spots (No. 1–14) corresponding to 11 unique proteins in response to salt stress were identified, which were mainly related to stress/defense, protein metabolism and energy metabolism. Among them, only elongation factor 1-beta was located in the nucleus and the other proteins were present in the cytoplasm (Fig. 2A and Table 1).

Fewer phosphorylated proteins were identified in the endosperm compared to the embryo (Fig. 2B and Table 1). Only 6 phosphorylated protein spots (No. 16–20) corresponding to 4 unique proteins were identified, which were mainly related to seed storage proteins**.** Subcellular location prediction showed that three globulin protein spots were located in vacuole, two alpha amylase inhibitor protein spots in chloroplast, and one superoxide dismutase in the cytoplasm (Table 1).

Compared with the control, ten phosphorylated protein spots (1, 2, 3, 5, 6, 9, 10, 11, 12 and 14) in the embryo were up-regulated in response to salt stress during grain germination, while four phosphorylated protein spots (4, 7, 8 and 13) were down-regulated (Fig. 2A and Table 1). In the endosperm, three protein spots 15, 17 and 19 were up-regulated while the remaining three protein spots 16, 18 and 20 were down-regulated (Fig. 2B and Table 1).

The isomers of the identified phosphorylated proteins were found in both embryo and endosperm (Fig. 2). For example, three protein spots 1, 2 and 3 were identified as cold regulated protein and both spots 5 and 6 were identified as 27K protein in the embryo. In the endosperm, protein spots 1, 2 and 3 were determined as globulin (Table 1). These isomers could be resulted from phosphorylated modification that led to molecular mass and isoelectronic point changes.

Prediction of phosphorylated sites in the identified phosphorylated proteins

The results from Pro-Q Diamond staining showed that phosphorylated modifications occurred in both embryo and endosperm of germinating seeds under salt stress, but the numbers and function groups of the phosphorylated proteins were distinct between embryo and endosperm (Fig. 3A).

To provide further information for supporting the results of phosphorylated proteins identified by Pro-Q Diamond staining and tandem mass spectrometry, the phosphorylated sites of all identified phosphorylated proteins in the embryo and endosperm were predicted by using the Netphones 2.0 tool. The results showed that all the identified phosphorylated proteins had multiple phosphorylated sites. Furthermore, the phosphorylated proteins involved in different function groups showed clearly different numbers of phosphorylated sites. For example, the energy related phosphorylated proteins in the embryo had much more phosphorylated sites (139) than those involved in stress defense (66) and protein metabolism (73) as shown in Fig. 3B. In the endosperm, phosphorylated storage proteins contained much more phosphorylated sites (143) than starch metabolism (17) and stress defense (110) related phosphorylated proteins (Fig. 3C). In total, the identified 14 phosphorylated protein spots in the embryo contained 278 phosphorylated sites, of which 155 (55.76%) occurred in the serine (Ser), 95 (34.17%) in the threonine (Thr) and

Table 1. The phosphoproteins identified in embryo and endosperm during seed germination under salt stress

Spot ID and phosphorylated level*	Accession No.	Protein name	Species	KOG number	MW(kDa)/*pI*	Protein score	Protein score C.I. %	Total ion score C. I. %	Pep. count	Ser/Thr/Tyr**	Subcellular location prediction
Stress/defense											
1, up-regulation	gi|26017213	Cold regulated protein (CORPS)	*T. aestivum*	NA	17.79/4.84	140	100	99.505	9	5/2/0	Cyto
2, up-regulation	gi|26017213	Cold regulated protein (CORPS)	*T. aestivum*	NA	17.79/4.84	210	100	100	6	5/2/0	Cyto
3, up-regulation	gi|26017213	Cold regulated protein (CORPS)	*T. aestivum*	NA	17.79/4.84	282	100	100	5	5/2/0	Cyto
4, down-regulation	gi|475492917	Aldose reductase	*Ae. tauschii*	KOG1577	35.63/6.51	1030	98	100	1	11/7/1	Cyto
7, down-regulation	gi|167113	Aldose reductase-related protein	*B. inermis*	KOG1577	35.42/6.28	216	100	100	24	14/10/2	Cyto
19, up-regulation	gi|226897529	Superoxide dismutase (SOD)	*T. aestivum*	KOG0441	15.30/5.71	217	100	100	6	4/6/0	Cyto
Protein metabolism											
6, up-regulation	gi|30793446	27K protein	*T. aestivum*	KOG3160	22.76/6.06	405	100	100	4	4/2/2	Cyto
5, up-regulation	gi|30793446	27K protein	*T. aestivum*	KOG3160	22.12/6.06	173	100	100	1	4/2/2	Cyto
8, down-regulation	gi|56315117	Serine-pyruvate aminotransferase	*O. sativa japonica*	NA	40.25/5.86	341	100	100	1	14/11/2	Cyto
9, up-regulation	gi|473753874	Elongation factor 1-beta (EF-1β)	*T. urartu*	KOG1668	22.81/6.2	159	100	100	5	19/9/2	Cyto
15, up-regulation	gi|474411419	Globulin-1S allele	*T. urartu*	NA	57.11/9.1	411	100	100	9	31/12/5	Vacu
16, down-regulation	gi|474411419	Globulin-1S allele	*T. urartu*	NA	57.11/9.1	434	100	100	10	31/12/5	Vacu
17, up-regulation	gi|390979705	Globulin-3A	*T. aestivum*	KOG2399	66.29/8.48	372	100	100	13	29/14/4	Vacu

Spot ID and phosphorylated level*	Accession No.	Protein name	Species	KOG number	MW(kDa)/*pI*	Protein score	Protein score C.I. %	Total ion score C. I. %	Pep. count	Ser/Thr/ Tyr**	Subcellular location prediction
Energy metabolism											
10, up-regulation	gi|11124572	Triosephosphat-isomerase	*T. aestivum*	KOG1643	27.01/5.38	170	100	99.859	12	11/6/2	Cyto
11, up-regulation	gi|475568723	Acetyl-CoA acetyltransferase	*Ae. tauschii*	KOG1390	46.944/8.47	86	100	100	2	9/10/1	Cyto
12, up-regulation	gi|119388731	Alcohol dehydrogenase ADH1A	*T. turgidum subsp. dicoccon*	KOG0022	41.73/6.15	220	100	100	11	30/9/6	Cyto
13, down-regulation	gi|32478662	Cytosolic glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	*T. aestivum*	KOG0657	18.17/6.34	537	100	100	12	12/11/3	Cyto
14, up-regulation	gi|475567072	Fructose-bisphosphate aldolase cytoplasmic isozyme	*Ae. tauschii*	KOG1557	39.19/6.85	310	100	100	9	12/12/5	Cyto
18, down-regulation	gi|38098487	Alpha-amylase inhibitor protein	*T. aestivum*	NA	18.21/7.44	284	100	100	2	9/2/1	Cyto
20, down-regulation	gi|66841026	Alpha-amylase inhibitor protein	*T. aestivum*	NA	12.77/6.86	317	100	100	2	2/1/2	Chlo

*The phosphorylated protein spots from 1 to 14 are from embryo and the others are from endosperm. **Number of phosphorylated sites prediction in serine (Ser), threonine (Thr) and tyrosine (Tyr).

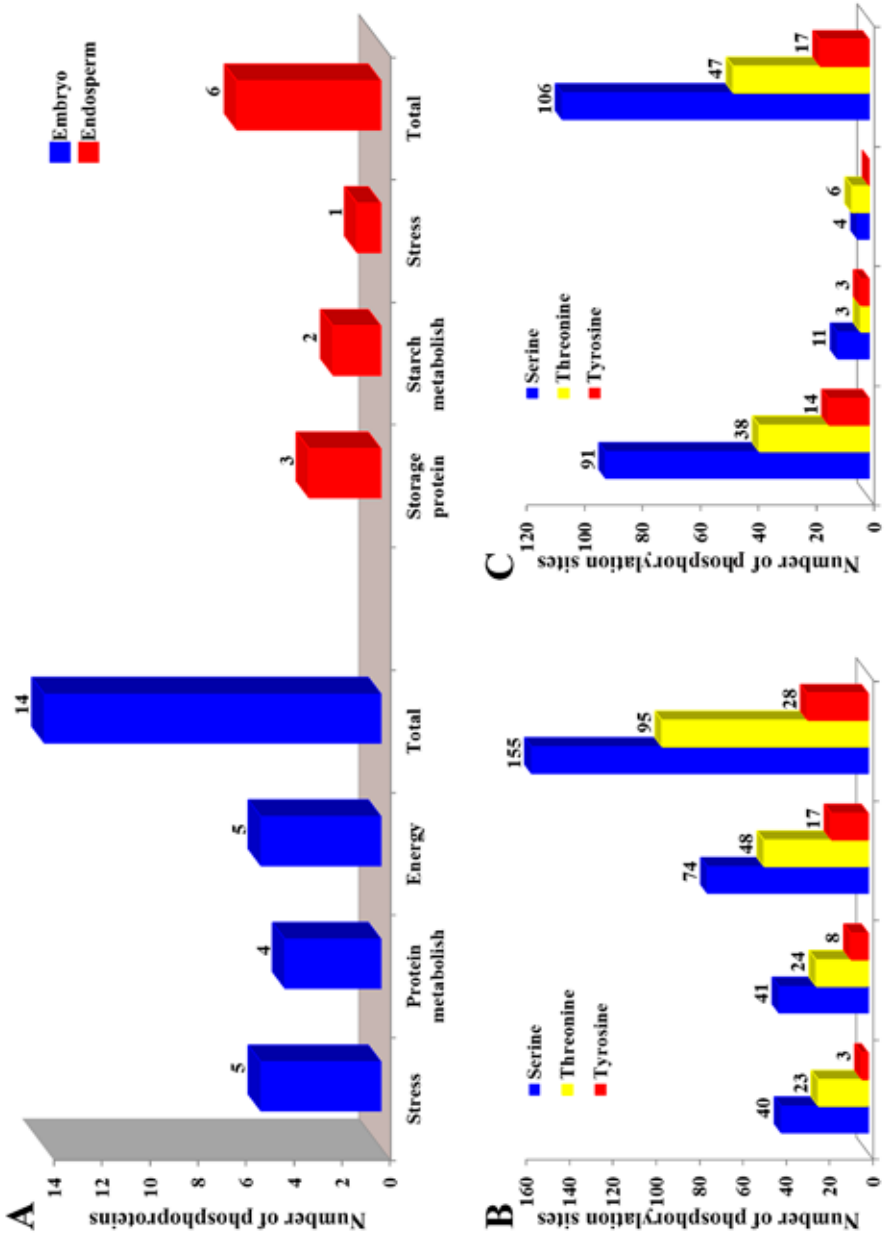

Figure 3. The identified phosphorylated proteins in the embryo and endosperm of germinating seeds in Zhengmai 366 under salt stress and phosphorylated sites predicted by Netphones 2.0. A. Distribution of the phosphorylated proteins in the embryo and endosperm. B. Distribution of the phosphorylated sites in Ser, Thr and Tyr in the embryo. C. Distribution of the phosphorylated sites in Ser, Thr and Tyr in the endosperm

28 (10.07%) in the tyrosine (Tyr). Meanwhile, 6 phosphorylated proteins identified in endosperm included 170 phosphorylated sites: 106 in the serine (62.35%), 47 (27.65%) in the threonine and 17 (10%) in the tyrosine. Therefore, most of the phosphorylated sites occurred at the serine in both embryo and endosperm.

Discussion

Protein phosphorylation is closely related to various biological processes, including metabolism, transcription and translation, protein degradation, homeostasis and stress response (Silva-Sanchez et al. 2015). Studies showed that many important proteins such as proteins of different isoforms, globulin 3, serpin, beta-amylase and AGPase were phosphorylated and they could play important roles in regulating wheat seed development and germination (Guo et al. 2012; Dong et al. 2015).

Protein phosphorylation in amyloplasts promoted starch branching enzyme activity and protein–protein interactions, and then enhanced starch biosynthesis (Tetlow et al. 2004). The phosphorylation of 12S globulin in *Arabidopsis thaliana* was involved in the processing, assembly and mobilization of proteins (Wang et al. 2007). Most nuclear phosphoproteins interacted with Sas10/Utp3 protein to mediate the early stage of rice seed germination while phosphorylation and dephosphorylation of nuclear proteins affected the germination of rice seeds (Li et al. 2015).

This study identified seven phosphorylated proteins with an up-regulation under salt stress in the embryo, including 27K protein, EF-1β and CORPS (cold regulated proteins) etc. (Table 1) and they could play important roles in salt tolerance during seed germination. It is known that the N-terminal SR domain of 27K protein plays an important role in the recognition and selection of the 3' and 5' splice sites of a given intron. It promotes the regulation of phosphorylation/dephosphorylation mediated by the pre-mRNA splicing, and may also potentially participate in SR protein-mediated protein/protein interactions (Fetzer et al. 1997). EF-1β can modulate EF-1α activity, cell growth, translation accuracy and protein synthesis (Carr-Schmid et al. 1999). This protein was also phosphorylated in the wheat shoots of seedlings (Vu et al. 2017). Thus, the phosphorylation of EF-1β could enhance embryo germination, seedling growth and salt tolerance. During cell dehydration, CORPS interacts with dehydration-sensitive proteins or fats to avoid excessive dehydration, and bind water molecules within the surface and three-dimensional structures of these macromolecules to increase cell dehydration tolerance, thereby maintaining the normal functions of the cells (Kazuoka and Deda 1992). In this study, three phosphorylated CORPS was up-regulated (Fig. 2A), which could be beneficial for reducing seed dehydration in response to salt stress during germination process. One stress/defense related phosphorylated protein superoxide dismutase (SOD) was identified in the endosperm (Table 1). Generally, salt stress induces redox in plants and produces large amounts of reactive oxygen species (Borsani et al. 2001). SOD cans disproportionate superoxide radical (O_2^-) into oxygen and hydrogen peroxide, catalase can catalyze the conversion of hydrogen peroxide to H_2O and O_2, and reduce the accumulation of ROS in

endosperm (Cheng et al. 2016). The phosphorylation could enhance the activity of SOD, expedite the conversion of hydrogen peroxide and alleviate the damage of ROS accumulation under salt stress.

Energy supply is necessary for seed germination, so energy metabolism is activated upon seed imbibition. Some energy metabolism related proteins were found to be phosphorylated such as alcohol dehydrogenase (ADH) in the embryo (spot 12), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (spot 13) and two alpha-amylase inhibitor proteins (spots 4 and 6) in the endosperm (Table 1). ADH is an up-regulated phosphorylated protein (Fig. 2A) in the embryo (Table 1). ADH is one of the members of the dehydrogenase/reductase protein gene superfamily. Under hypoxic conditions, ADH can convert acetaldehyde to ethanol, and at the same time generate NAD^+ and produce limited energy ATP (Zhang et al. 2015). Thus, it provides a part of the energy for seed germination. GAPDH catalyzes the conversion of glyceraldehyde-3-phosphate to 1, 3-diphosphoglycerate, providing energy and producing precursors of anabolic products such as amino acids and fatty acids (Andre et al. 2007). It also participates in a large number of cellular processes in mammalian cells (Sirover 2011). Alpha-amylases hydrolyze the starch in the endosperm into metabolizable sugars during seed germination, and provide energy for root and stem growth (Beck and Ziegler, 1989). Alpha amylase inhibitors (spots 4 and 6) were phosphorylated in the endosperm under salt stress (Fig. 2), which could reduce amylase activity and starch degradation and inhibit seed germination (Fig. 1).

In conclusion, we identified salt stress induced 11 and 4 phosphorylated proteins in the embryo and endosperm during seed germination, respectively. These proteins mainly involved in stress/defense, protein metabolism and energy metabolism. Among them, ten phosphorylated proteins were up-regulated under salt stress such as cold regulated proteins, 27K protein, EF-1β and superoxide dismutase, which could contribute to salt stress tolerance during wheat seed germination.

Acknowledgements

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