RESEARCH ARTICLE

Constructing the metabolic and regulatory pathways in germinating rice seeds through proteomic approach

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Construction of metabolic and regulatory pathways from proteomic data can contextualize the large-scale data within the overall physiological scheme of an organism. It is an efficient way to predict metabolic phenotype or regulatory style. We did protein profiling in the germinating rice seeds through 1-DE via LC MS/MS proteomic shotgun strategy. In total, 673 proteins were identified, and could be sorted into 14 functional groups. The largest group was metabolism related. The metabolic proteins were integrated into different metabolic pathways to show the style of reserves mobilization and precursor preparation during the germination. Analysis of the regulatory proteins indicated that regulation of redox homeostasis and gene expression also play important roles for the rice seed germination. Although transcription is unnecessary for the germination, it could ensure the rapidity and uniformity of germination. On the contrary, translation with the stored mRNA is required for the germination. This study will help us to further understand the metabolic style, regulation of redox homeostasis, and gene expression during rice seed germination.

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1 Introduction

Plant seeds provide staple food for the world population. In addition, they are also important for the plants life cycle. Most plants generate their progenies through seeds, which can help them to avoid the adverse or even extreme environmental conditions. There are two types of seeds: recalcitrant and orthodox seeds. The former one contains high water content and cannot be stored for a long time. On the contrary, the orthodox seeds are dry and physiological quiescent after maturation. They can keep their viability over a long period of storage. Seed germination is the beginning of the second round of plant life cycle. In physiology, germination is defined as the process commencing with water uptake and ending at the protrusion of radicle [1]. Seed germination could be divided into three phases, fast water uptake (phase I), metabolism reactivation (phase II), and radicle emergence (phase III) [1]. Among them, phase II is the most critical stage during which all the necessary metabolic pathways and physiological processes are reactivated, and hence decision is made to initiate the germination or not. In some species, seeds can easily germinate under favorable conditions. While in others, specific treatments are required to break the dormancy. Regulation of seed germination is one of the critical adaptive traits in plants in the long history of evolution. Unfortunately, the

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Abbreviations: ABA, abscisic acid; **GA,** gibberellin; **SOD,** superoxide dismutase; **TCA,** tricarboxylic acid; **Usp,** Universal stress protein

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mechanisms that control the germination are largely unknown.

Because of the great potential in agriculture, a large number of studies have been conducted to explore seed dormancy and germination mechanisms over the past several decades. Most of our knowledge about the dormancy release and germination initiation comes from the model system-*Arabidopsis thaliana* [2, 3]. It has been confirmed that a series of exogenous and endogenous factors such as light, temperature, circadian rhythm, abscisic acid (ABA), and gibberellin (GA) could regulate seed germination [2–6]. ABA insensitive3 (ABI3) plays a central role in the inhibition of germination by ABA [7]. In dormant seeds, imbibition can increase the expression of *NCED* and *ABA1* genes, both of which are involved in the ABA biosynthesis pathway [6, 8]. The dormancy-breaking treatment could increase the expression of *CYP707A2* gene that encodes an ABA catabolic enzyme [9]. By contrast, GA can promote the germination. This effect is realized by a GA-mediated degradation of DELLA proteins. It was reported that several members of DELLA proteins are necessary for seed dormancy [10–12]. Germination-promoting signals could enhance the GA biosynthesis and prevent its degradation [13, 14]. The environmental factors such as light, temperature, and circadian rhythm regulate the germination mainly through affecting the metabolism of GA and ABA [14–18].

In spite of the aforementioned knowledge, we are still far away from gaining a full understanding of seed germination mechanisms. Due to the great achievement in genomics, large-scale analysis of gene expression at both RNA and protein levels has been applied to uncover the features of seed dormancy and germination. Transcriptomic analysis in Arabidopsis showed that RNAs encoding proteins involved in translation, protein degradation, and cell wall modification increased rapidly along with the germination [19]. Similar results were also obtained through the proteomic studies of Arabidopsis seed germination [20–22]. For a comprehensive review, see Holdsworth et al. [23]. These studies showed us that the control of germination happened at different levels. Recently, rice is widely used as an alternative model system. The studies of ours and Kang's group in Korea have shown that enhancement of glycolysis and tricarboxylic acid (TCA) cycle provides the major energy for germination [24–26]. Meanwhile, these studies found that some antioxidation proteins might help to regulate the redox homeostasis in the cell, which was also reported in other plant species [27].

Previous studies have revealed that germination is a very complicated process during which a series of physiological and biochemical reactions including signal transduction, regulation of gene expression, reactivation of metabolism, and redox homeostasis regulation will happen. Since all these reactions are either catalyze or mediate by different proteins, it will be valuable to profile the protein constituents of the germinating seeds. Integrating the proteomic data into the network of metabolic or regulatory pathways will enable us to understand individual proteins in a systematic level and assess their roles in a broader range. Unfortunately, previous proteomic studies were solely focusing on the changes of the protein profiles and the differentially displayed proteins during the germination, which could not provide us the overall understanding about the metabolic and regulatory networks. Furthermore, 2-DE has limitation in solubilizing proteins, which might result in missing of some physiological important proteins. In the present study, the 1-DE via LC-MS/MS strategy was applied. Since the SDS buffer is much more powerful than the IEF buffer in solubilizing proteins, our study will undoubtedly provide more complementary information at the protein level and help us to further understand how the plants initiate germination through regulation of metabolism, redox homeostasis, and gene expression.

2 Materials and methods

2.1 Rice seeds germination

Rice (*Oryza sativa* "Nipponbare") seeds were dehulled and washed with distilled water three times, and then used for germination experiment. The dehulled seeds were imbibed in distilled water or 100 μM α-amanitin (Sigma, Saint Quentin Fallavier, France), or 100 mM cycloheximide (Sigma, Germany) [28], and incubated at 26°C with 100 mmol photons/m^2 s white light (12 h day/12 h night). The 24-h imbibed seeds were collected and stored at −80°C for protein extraction.

2.2 Periodic Acid Schiff staining

To monitor the cellular changes of embryos, the germinating seeds were fixed in 50% ethanol, 5% acetic acid (Sigma, Germany), and 10% formalin (Sigma, Germany), and then embedded in paraffin. The specimens were thin sliced (5–7μm) using a microtome (Leica RM2235, Japan), then mounted on a grid. After deparaffinized and hydrated to water, the sections were stained with Periodic Acid Schiff (PAS) solution: The stained specimens were observed with light microscopy (Nikon eclipse 80i, Japan).

2.3 Determination of aerobic respiration efficiency and in situ accumulation of reactive oxygen species

Seeds with total volume about 0.5 mL at different stages were used for respiration determination by warburg respiration analyzer. The efficiency of respiration was calculated as the volume of oxygen consumed by 1 g seed in 1 h (μL/g* h).

H_2O_2 was detected through TMB (Sigma) staining as described earlier [29] with a little modification. Briefly, rice seeds were collected at 0, 24, 48, and 72 h after imbibition

and soaked in a solution containing 0.42 mM TMB in Tris-acetate (pH 5.0) buffer for 1 h. Parallel sets of seeds at equivalent stages were treated with 1 M sodium pyruvate (Sigma) in Tris-acetate, pH 5.0, buffer for 30 min. The appearance of blue color was monitored to indicate the accumulation of H_2O_2. Superoxide anion was detected as described previously [30]. Seeds at different stage were incubated in 6 mM nitroblue tetrazolium (NBT) in 10 mM Tris-HCl buffer (pH 7.4) at room temperature for 1 h. The accumulation of superoxide anion was determined by the visualization of dark-blue color.

2.4 Protein extraction and 1-DE

Total proteins were extracted from rice seeds at 24 h after imbibition. A total of 0.25 g seed materials were homogenized with ice-cold buffer containing 20 mM Tris/HCl (pH 7.5), 250 mM sucrose, 10 mM EGTA, 1 mM PMSF, 1 mM DTT, and 1% Triton X-100 [24] and centrifuged at $15\,000 \times g$ (4°C) for 20 min. The supernatants were then incubated in 10% trichloroacetic acid (final concentration) in ice bath for more than 30 min. Centrifuge again, and wash the pellets with cold acetone three times. After centrifugation, the pellets were lyophilized. The proteins were solubilized into SDS sample buffer (40 mM Tris-HCl, pH 6.8, 2 mM DTT, 4% glycerol, 2% SDS, 0.1% Bromophenol blue) for electrophoresis.

The samples were resolved with 12% polyacrylamide gel. For each running, 150 μg proteins were loaded into one single well of the vertical gel. After the full-length run, the gel was stained with Coomassie Brilliant Blue (CBB) R-250 to visualize the proteins.

2.5 Trypsin digestion and LC-MS/MS

The visualized gel was cut into 20 slices, each of which was then subjected to trypsin digestion as follows: destained with destaining solution (50 mM NH_4HCO_3 in 50% v/v ACN) for 1 h at 40°C. The destaining step was repeated until the gel was colorless. After washed with HPLC-grade water and lyophilized, the gel pieces were rehydrated in 25 mM NH_4HCO_3 with 10 ng sequencing grade-modified trypsin (Promega, Madison, WI, USA) at 37°C overnight. After digestion, the protein peptides were collected, and the gels were washed with 0.1% TFA in 50% ACN three times to collect the remaining peptides.

LC-MS/MS was conducted as reported previously [31]. Briefly, a Waters nano-Acquity Sample Manager (www.waters.com) was used to inject the extracted peptides. The sample was loaded onto a Waters Symmetry C18 peptide trap (5 mm, 180 mm × 20 mm) at 4 mL/min in 2% ACN/0.1% formic acid. The bound peptides were then eluted onto a Waters BEH C18 nano-Acquity column (1.7 mm, 100 mm × 100 mm) and then eluted over 30 min with a gradient of 5–90% buffer B (99.9% ACN/0.1% formic acid) using a Waters nano-Acquity UPLC system. (buffer A: 99.9% water/0.1% formic acid) into a ThermoFisher LTQ-FTICR mass spectrometer (www.thermo.com) at a flow rate of 300 nL/min. Survey scans were taken in the Fourier transformation at 25 000 resolution at a mass-to-charge ratio of 400, and the top ten ions in each survey scan were then subjected to automatic low-energy collision-induced dissociation in the LTQ-FTICR mass spectrometer. The resulting MS/MS spectra were converted to peak lists using SEQUEST program in the BioWorks Browser version 3.2 (ThermoFisher) with default parameters and searched against the rice genome database in NCBI. The false-positive rate (FPR) was kept $<5\%$ as the filtration criterion. The minimum cross-correlation (Xcorr) for the peptides with a $+1$, $+2$, or $+3$ charge state were 1.9, 2.2, and 3.75, respectively, and the delta CN was more than 0.1. The searching parameters were as follows: trypsin specificity, one missed cleavage, carbamidomethylation of Cys and oxidation of Met, and 0.1 Da mass tolerance. The output was then analyzed using Scaffold (www.proteomesoftware.com) to probabilistically validate protein identifications using the ProteinProphet [32] computer algorithm. All the identified proteins should have at least one matched peptide with a probability more than 95%.

3 Results and discussion

3.1 Proteome profiling of germinating rice seed

Generally, seed germination could be divided into three phases based on the style of water up-taking [1]. Phase II was regarded as the most important stage in which all the germination required metabolic reactions are reactivated. During rice seed germination, the time slot between 20 and 50 h after imbibition was determined as phase II [24]. To acquire a comprehensive knowledge of the reactivated processes at the early stage of germination, we did protein profiling for the germinating rice seeds at this stage. Proteins were extracted from the germinating seeds at 24 h after imbibition. In all, 150 μg proteins were loaded for 1-DE (Fig. 1A). After electrophoresis, the sample lane was cut into 20 slices (Fig. 1A). All the slices were subjected to in-gel digestion and LC-MS/MS analysis to identify the proteins.

Totally, 673 different proteins were identified with high probability in this study. Up to now, this is the most comprehensive protein profile for the germinating rice seeds. All the proteins could be sorted into 14 functional categories (Fig. 1B) according to the MapMan ontology which was defined by Thimm et al. [33]. The most abundant group was occupied by the proteins involved in metabolism. This group contained 203 proteins accounting for about 37.6% of the total (Table 1, Fig. 1B). The second abundant group was development-related proteins including those storage proteins. Although there were just 40 of them

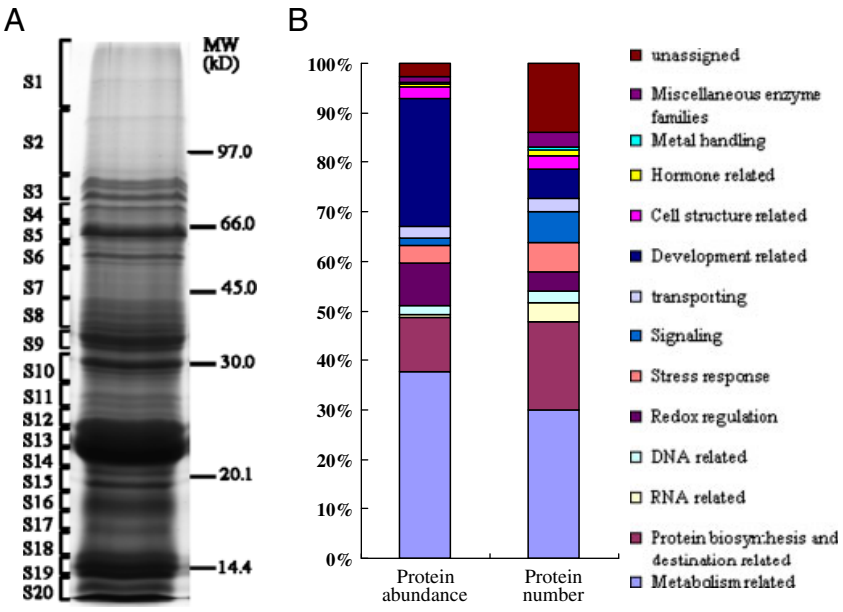

Figure 1. 1-DE via LC-MS/MS identification of the proteins in germinating rice seeds. (A) 1-DE gel of the 24-h imbibied rice seeds proteins. 150 μg proteins were loaded for each sample. After CBB staining, the gel was cut into 20 slices (S1–S20) each of which was then subjected to in-gel digestion with trypsin and LC-MS/MS analysis. (B) Functional categorization of the identified proteins. The histogram shows the percentage of different categories in terms of protein abundance (left) and proteins number (right).

identified, they accounted for ~25.9% of the total proteins (Fig. 1B). This is reasonable since rice seeds accumulated large number of storage proteins (e.g. glutelin) during the maturation. The third abundant groups consisted of 120 proteins involved in protein biosynthesis, modification, degradation, folding, and destination (Table 2). Other functional groups included transport (18), DNA (17), RNA (27), signaling (42), Redox regulation (25), stress response (41), Miscellaneous enzymes (20), Hormones (9), Cell (18), and Metal handling (4) (Supporting Information Table 1). The 94 proteins with either unassigned functional groups or unknown functions accounted <3% of the total proteins (Supporting Information Table 2). Among all the identified proteins, three could be sorted into three different groups and 17 into two different groups.

3.2 Carbohydrate metabolic pathways

The 203 metabolism-related proteins could be further categorized into 20 subgroups (Table 1, Fig. 2A). Among them, 105 are involved in carbohydrate metabolic pathways including photosynthesis, major carbohydrates metabolism, minor carbohydrates metabolism, glycolysis, TCA cycle, fermentation, gluconeogenesis and glyoxylate cycle, and pentose phosphate pathway.

3.2.1 Starch catabolism, glycolysis, fermentation, TCA cycle, and other carbohydrate metabolic pathways

Starch is the major reserves in mature seed of cereals. All the enzymes involved in starch degradation to hexose phosphate were identified (Table 1, Fig. 2B). Except for α-amylase (♯1) which is upregulated during the germination [24], all the other enzymes are constant during germination [24–26]. Upon imbibition, the starch granules are first attacked by α-amylase (♯1) in the endosperm, then the large-branched glucan are catalyzed by debranching enzymes (♯2, 3) to form into linear glucan. There are three ways for the degradation of linear glucan. First, β-amylase (♯4) degrades it into maltose which could be further degraded into glucose by α-glucosidase (♯7, 8); second, it could be degraded into shorter glucans and then into glucose (♯5–8); third, α-1,4 glucan phosphorylase (♯9) catalyze into glucose-1-phosphate. Through the synthesis and degradation of sucrose (♯10–13), the glucose could be transferred into glucose phosphate and fructose phosphate. The detail information of the degradation of starch in the germinating rice seeds are shown in Fig. 2B.

The phosphate glucose from either the degradation of starch or the phosphorylation of glucose which catalyze by hexokinase (♯24) will then experience glycolysis and TCA cycle. Totally, 22 enzymes that catalyze all the steps in the glycolysis pathway were detected in this experiment. Most of them were upregulated upon the imbibition [24]. The final product of glycolysis is pyruvate that could be transferred into mitochondria, and used as the substrate for TCA cycle. We identified eight mitochondrial electron transport/ATP synthesis proteins (♯67–74) and most of the enzymes in the TCA cycle, such as aconitase (♯52, 53), isocitrate dehydrogenase (♯54, 55), Succinyl-CoA ligase (♯56), Fumarate hydratase (♯57), malate dehydrogenase (♯58–61). But the citrate synthase and succinate dehydrogenase in this pathway were not detected in this experiment. Kim et al. [26] have shown that succinyl-CoA ligase and cytoplasmic malate dehydrogenase were stably accumulated during germination. They then concluded that TCA cycle might, along with glycolysis, provided the main energy for germination. In

Table 1. Proteins involved in different metabolic pathways

Protein ID	Accession no.	Description	MW (kDa)	NUMP[a)]
Major carbohydrates (23)				
Starch degradation				
1	NP_001062023	α-Amylase isozyme 3E precursor	48.6	11
2	NP_001062271	Isoamylase	81.6	9
3	NP_001052129	Starch debranching enzyme	69.4	40
4	NP_001060573	Putative β-amylase	56.6	1
5	NP_001057611	Glycoside hydrolase, family 13	103.7	5
6	NP_001059480	Glycoside hydrolase, family 31 protein	118.9	2
7	NP_001058347	High p/ α-glucosidase	96.2	3
8	NP_001058353	High p/ α-glucosidase	96.4	6
9	NP_001051330	α-1,4 Glucan phosphorylase	71.7	6
10	NP_001050064	Sucrose synthase	92.1	14
11	NP_001057047	Sucrose synthase 1	92	3
12	NP_001050319	Sucrose synthase 2	92.8	11
13	NP_001060278	Sucrose synthase 3	93	12
14	NP_001060837	Fructokinase 2	35.4	19
Starch biosynthesis				
15	NP_001043654	ADP-glucose pyrophosphorylase large subunit	57.4	8
16	NP_001056424	Glucose-1-phosphate adenylyltransferase large subunit 1	58	12
17	NP_001062808	ADP-glucose pyrophosphorylase small subunit	54.7	9
18	NP_001056881	Starch synthase I, chloroplast precursor	70.8	1
19	NP_001057216	Starch synthase isoform zSTSII-1	88.2	3
20	NP_001058629	Starch-branching enzyme I	93.1	29
21	NP_001047009	Branching enzyme-3 precursor	92.6	19
22	NP_001056704	Granule-bound starch synthase I	66.3	28
23	NP_001064678	Granule-bound starch synthase II, chloroplast precursor	83.2	2
Glycolysis (22)				
24	NP_001044214	Hexokinase	55	1
25	NP_001063879	UTP – glucose-1-phosphate uridylyltransferase	51.6	11
26	NP_001051066	Phosphoglucomutase	62.8	10
27	NP_001064271	Phosphoglucomutase family protein	66	1
28	NP_001062053	Phosphoglucose isomerase (PGI) family protein	67.1	6
29	NP_001057284	Pyrophosphate-dependent 6-phosphofructose-1-kinase (PPi-PFK)	61.1	13
30	NP_001055494	Fructose-bisphosphate aldolase Aldolase C-1	36.1	18
31	NP_001042016	Triosephosphate isomerase, cytosolic	26.9	16
32	NP_001042016	Putative triosephosphate isomerase	26.9	16
33	NP_001047348	Glyceraldehyde-3-phosphate dehydrogenase, cytosolic 3	36.4	31
34	NP_001060897	Glyceraldehyde-3-phosphate dehydrogenase	36.3	10
35	NP_001058309	Glyceraldehyde-3-phosphate dehydrogenase precursor	43.4	2
36	NP_001058317	Phosphoglycerate kinase, cytosolic	42.1	18
37	NP_001046020	Phosphoglycerate kinase, cytosolic	42	15
38	NP_001055937	3-Phosphoglycerate kinase	18.4	5
39	NP_001055868	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase	60.7	13
40	NP_001044625	2,3-Bisphosphoglycerate-independent phosphoglycerate mutase	60.7	16
41	BAF23843	D-3-Phosphoglycerate dehydrogenase	23.7	2
42	NP_001049556	Enolase 2	47.8	10
43	NP_001064223	Enolase 2	47.8	8
44	NP_001054265	Pyruvate kinase, cytosolic isozyme	55.1	2
45	NP_001048068	Pyruvate dehydrogenase E1 α subunit	42.6	5
Fermentation (6)				
46	NP_001049811	Pyruvate decarboxylase isozyme 2	61.4	1
47	NP_001055803	Pyruvate decarboxylase isozyme 2	64.6	6
48	NP_001067485	Alcohol dehydrogenase 2	40.7	7
49	NP_001063281	Aldehyde dehydrogenase family 7 member A1	54.4	2
50	NP_001048010	Mitochondrial aldehyde dehydrogenase ALDH2a	58.8	3
51	NP_001057358	T cytoplasm male sterility restorer factor 2	59.2	6
61[b)]	NP_001060357	Lactate/malate dehydrogenase family protein	42.1	4

Table 1. Continued.

Protein ID	Accession no.	Description	MW (kDa)	NUMP[a)]
TCA cycle (15)				
52	NP_001048898	Aconitate hydratase, cytoplasmic	106.2	11
53	NP_001045743	Cis-homoaconitase family protein	55.4	4
54	NP_001047314	NAD-dependent isocitrate dehydrogenase precursor	40.5	3
55	NP_001042583	NADP-dependent isocitrate dehydrogenase	46.4	3
56	NP_001060091	Succinyl-CoA ligase	34.1	4
57	NP_001050052	Fumarate hydratase 2, chloroplast precursor	49.9	2
58[b)]	NP_001043717	Malate dehydrogenase	35.3	11
59[b)]	NP_001056389	Malate dehydrogenase	35.3	24
60[b)]	NP_001044691	Malate dehydrogenase precursor	41.7	2
61[b)]	NP_001060357	Lactate/malate dehydrogenase family protein	42.1	4
62[b)]	NP_001044219	NADP-dependent malic enzyme	64.1	2
63[b)]	NP_001044107	Malic oxidoreductase family protein	62.3	7
64[b)]	NP_001064860	Cytosolic malate dehydrogenase	35.4	16
65	NP_001046417	Phosphoenolpyruvate carboxylase 1	109.8	5
66	NP_001042817	ATP:citrate lyase	65.9	3
Mitochondrial electron transport/ATP synthesis (8)				
67	NP_001056261	ATP synthase β chain, mitochondrial precursor	58.8	24
68	NP_001065212	CcmE/CycJ protein family protein	28.6	1
69	NP_001051072	NADH-ubiquinone oxidoreductase 75 kDa subunit, mitochondrial precursor	81	8
70	NP_001055136	Cytochrome c1	33.2	1
71	NP_001059694	ATP synthase delta′ chain, mitochondrial precursor	21.1	4
72	NP_001060247	Cytochrome *c* and b562 family protein	26.4	10
73	NP_001051412	Cytochrome *c* oxidase, subunit VIa family protein	10.7	1
74	EEE53982	ATP synthase F1 subunit α	11.4	1
Photosynthesis (13)				
75	NP_001045917	Ribulose bisphosphate carboxylase	20.5	1
76	NP_001049096	Ribulose-phosphate 3-epimerase, chloroplast precursor	28.9	6
77	NP_001049664	Fructose-1,6-bisphosphatase, chloroplast precursor	43.5	1
78	NP_001047825	Phosphoribulokinase, chloroplast precursor	44.7	2
79	NP_001046026	Glycine cleavage system H protein, mitochondrial, putative	16.9	1
80	NP_001058062	Glycine cleavage system H protein, mitochondrial, putative	96.8	20
81	NP_001056304	Photosystem I reaction center subunit VI	14.9	3
82[b)]	NP_001049115	Alanine:glyoxylate aminotransferase-like protein	52.6	2
83[b)]	NP_001056711	Transketolase, chloroplast precursor	73.5	23
84[b)]	NP_001052335	Transketolase 2	77.5	3
85[c)]	NP_001066640	Serine hydroxymethyltransferase	50.7	4
86[c)]	NP_001067846	Glycine hydroxymethyltransferase	51.3	12
87[c)]	NP_001051211	Serine hydroxymethyltransferase, mitochondrial precursor	56.3	17
88	NP_001051476	Ferredoxin – NADP reductase	41.7	3
Minor carbohydrates (6)				
89	NP_001055826	Aldose reductase	35.5	3
90	NP_001062412	NAD-dependent sorbitol dehydrogenase	39.2	9
91	NP_001060421	Carbohydrate kinase, FGGY family protein	61.7	2
92	NP_001045735	NADPH-dependent mannose 6-phosphate reductase	41.7	2
93	NP_001052977	Aldose 1-epimerase	40.3	1
94	NP_001066401	2-Dehydro-3-deoxyphosphooctonate aldolase	31.4	1
Gluconeogenesis/glyoxylate cycle (5)				
95	NP_001052622	Isocitrate lyase	41.2	2
96	CAA06247	Cytosolic pyruvate orthophosphate dikinase	97.2	7
97	CAD41322	Malate synthase	49	1
98	NP_001067346	Malate dehydrogenase, glyoxysomal precursor	37.3	1
99	NP_001055507	Orthophosphate dikinase precursor	102.7	33
100	NP_001050430	Pyruvate, phosphate dikinase, chloroplast precursor	86.9	17
Phosphate pentose pathway (6)				
101	NP_001066705	UDP-glucose 6-dehydrogenase	52.7	6

Table 1. Continued.

Protein ID	Accession no.	Description	MW (kDa)	NUMP[a)]
102	NP_001056586	Cytosolic 6-phosphogluconate dehydrogenase	52.6	6
103	NP_001063604	Ribulose-phosphate 3-epimerase family protein	21.7	1
104	NP_001045261	Transaldolase	46.3	4
105	NP_001063741	Glucosamine/galactosamine-6-phosphate isomerase	28.9	3
83[b)]	NP_001056711	Transketolase, chloroplast precursor	73.5	23
84[b)]	NP_001052335	Transketolase 2	77.5	3
Lipid metabolism (16)				
106	NP_001042854	Phospholipase D p1	78.6	1
107	NP_001047506	Lipase, class 3 family protein	202	4
108	NP_001056487	Esterase/lipase/thioesterase	31.9	1
109	NP_001047179	Esterase/lipase/thioesterase	33.6	2
110	NP_001057131	Esterase/lipase/thioesterase	33.9	2
111	NP_001060129	Esterase precursor (EC 3.1.1.-) (Early nodule-specific protein homolog)	42.4	1
112	NP_001057786	Lipolytic enzyme	42.7	2
113	NP_001042924	Lipolytic enzyme, putative early nodule-specific protein ENOD8	45.6	1
114	NP_001057978	Enoyl-CoA hydratase	46.8	4
115	NP_001046536	Peroxisomal fatty acid β-oxidation multifunctional protein (MFP)	78.3	4
116	NP_001062685	Acetyl-CoA C-acetyltransferase	44.1	10
117[b)]	NP_001048523	Acetyl-CoA acyltransferase (3-ketoacyl-coa thiolase)	46.7	3
118	NP_001045215	Acyl-[acyl-carrier-protein] desaturase, chloroplast precursor	45.2	2
119	NP_001061557	Enoyl-[acyl-carrier-protein] reductase	39	3
120	NP_001049837	Malonyl-CoA:ACP transacylase	40.3	5
121	NP_001042870	2-Oxoacid dehydrogenases acyltransferase	56.5	2
Amino acid metabolism (47)				
Central amino acid metabolism				
122	NP_001046388	Aspartate aminotransferase	48	5
123	NP_001057825	Mitochondrial aspartate aminotransferase precursor	51.2	3
124	NP_001053863	Viroid RNA-binding protein	56.3	4
125	NP_001044317	Aspartate aminotransferase, cytoplasmic	49.8	10
126	NP_001049852	Aspartate aminotransferase	49.7	2
127	NP_001060284	Alanine aminotransferase	36.3	3
128	NP_001064504	Alanine aminotransferase	52.5	11
85[b)]	NP_001049115	Alanine:glyoxylate aminotransferase-like protein	52.6	4
Aspartate, lysine, methionine, alanine and threonine				
129	NP_001054575	*S*-adenosylmethionine synthetase 1	43.1	17
130	NP_001042794	*S*-adenosylmethionine synthetase 1	43.2	6
131	P93438	*S*-adenosylmethionine synthetase 2	42.6	4
132	BAD37853	Putative *O*-succinylhomoserine (Thiol)-lyase	46.6	5
133	NP_001067845	Adenosylhomocysteinase	51.5	10
134	NP_001067060	Diaminopimelate epimerase	37.7	6
135	NP_001048324	Lysine-ketoglutarate reductase/saccharopine dehydrogenase bifunctional enzyme	65.5	1
136	BAD19288	Lysine-ketoglutarate reductase/saccharopine dehydrogenase bifunctional enzyme	58.5	1
137	NP_001061172	Glyoxalase I family protein	32.4	11
138	NP_001049720	Glyoxalase (threonine degradation)	14.9	3
Branched chain amino acid				
139	NP_001059082	Methylmalonate-semialdehyde dehydrogenase	57.1	12
140	NP_001047389	Acetolactate synthase, small subunit family protein	35.3	1
141	NP_001046931	Acetohydroxyacid synthase	69.3	3
142	NP_001043738	Ketol-acid reductoisomerase	59.6	7
143	NP_001056384	Ketol-acid reductoisomerase	62.2	12
144	ABA91408	2-Isopropylmalate synthase B	68.6	5
145	NP_001066116	2-Isopropylmalate synthase	68.3	9
146	NP_001050807	3-Isopropylmalate dehydrogenase, chloroplast precursor	41.1	3
117[b)]	NP_001048523	Acetyl-CoA acyltransferase (3-ketoacyl-coa-thiolase)	46.7	3

Table 1. Continued.

Protein ID	Accession no.	Description	MW (kDa)	NUMP[a)]
Serine, glycine, cysteine				
85[c)]	NP_001066640	Serine hydroxymethyltransferase	50.7	4
86[c)]	NP_001067846	Glycine hydroxymethyltransferase	51.3	12
87[c)]	NP_001051211	Serine hydroxymethyltransferase, mitochondrial precursor	56.3	17
147	NP_001054094	Phosphoglycerate dehydrogenase	63.8	4
148	AAM51827	Phosphoserine aminotransferase	44.8	4
149	NP_001063566	Sarcosine oxidase	43.8	3
150	NP_001066961	Potential sarcosine oxidase	43.9	2
151	NP_001045577	Cysteine synthase, mitochondrial precursor	41.7	8
152	NP_001051262	Cysteine synthase	34.2	10
Aromatic amino acid				
153	NP_001046195	Fumarylacetoacetase	47	7
154	NP_001051779	Fumarylacetoacetate (FAA) hydrolase family protein	23.9	1
155	NP_001046502	Phosphoribosylanthranilate isomerase	33.8	1
156	NP_001062693	Indole-3-glycerol phosphate synthase	41.3	1
157	NP_001061528	Indole-3-glycerol phosphate synthase, chloroplast precursor	43.5	1
158	BAD37798	Putative Tryptophan synthase β-chain	52.9	3
Arginine and Glutamate				
159	NP_001050668	*N*-acetyl-γ-glutamyl-phosphate reductase	44.7	2
160[b)]	NP_001047880	Carbamoyl-phosphate synthase small chain	44.1	3
161	NP_001047862	Ornithine carbamoyltransferase OOCT1	39.8	3
162	NP_001066459	Argininosuccinate synthase	52.1	2
163	NP_001049883	Argininosuccinate lyase	57.3	1
Nucleotide metabolism (15)				
Degradation				
164	NP_001044897	Uricase	34.4	3
165	NP_001050402	Inosine/uridine-preferring nucleoside hydrolase	34.4	2
166	NP_001066462	Adenylate kinase A	26.3	8
Biosynthesis				
167	NP_001049128	Adenylosuccinate synthetase	52	3
168	EEE62020	Phosphoribosylformylglycinamidine synthase	154.1	3
169	NP_001066971	Nucleoside diphosphate kinase family protein	23.3	3
170	NP_001065404	Nucleoside diphosphate kinase family protein	16.6	9
171	NP_001059682	Nucleoside diphosphate kinase I	16.7	10
172	NP_001048053	Dihydroorotate dehydrogenase family protein	45.2	5
173[b)]	NP_001047880	Carbamoyl-phosphate synthase small chain	44.1	4
174[b)]	NP_001047479	Adenosine kinase 2	36.9	3
Phosphotransfer and pyrophosphatases				
175	NP_001047863	Inorganic pyrophosphatase-like protein	24	1
176	NP_001054331	Soluble inorganic pyrophosphatase	24	2
177	NP_001064561	Soluble inorganic pyrophosphatase	23.2	4
178	NP_001067759	Adenylate kinase B	26.5	3
Cell wall (8)				
179	NP_001042921	Putative polygalacturonase	77.6	2
180	NP_001051275	Exoglucanase precursor	67.6	6
181	NP_001054295	Phosphomannomutase 2	28.1	2
182	NP_001058235	GDP-4-keto-6-deoxy-D-mannose-3, 5-epimerase-4-reductaseGER1	35.6	1
183	NP_001050617	UDP-glucose:protein transglucosylase 1	41.2	12
184	NP_001054143	Reversibly glycosylated polypeptide	38.8	6
185	NP_001065351	β-Expansin precursor	29.1	2
186	NP_001045523	dTDP-glucose 4,6-dehydratase	44.2	1
Secondary metabolism (7)				
isoprenoids				
187	NP_001048623	3-Hydroxy-3-methylglutaryl-coenzyme A reductase 2	37.7	4
188	NP_001059919	Isopentenyl pyrophosphate: dimethyllallyl pyrophosphate isomerase	27.2	3

Table 1. Continued.

Protein ID	Accession no.	Description	MW (kDa)	NUMP[a)]
Lignin biosynthesis				
189	NP_001062143	Caffeoyl-CoA *O*-methyltransferase	31.9	1
190	NP_001047481	Phenylalanine ammonia-lyase	75.4	11
Flavonoid				
191[b)]	NP_001056105	UDP-glucuronosyl/UDP-glucosyltransferase family protein	52.2	1
192	NP_001064043	NADPH-dependent codeinone reductase	35.6	3
193	NP_001041776	Isoflavone reductase-like protein	33.4	3
N-metabolism (1)				
194	NP_001047589	Glutamate dehydrogenase 2	44.3	2
S-assimilation (1)				
195	NP_001051234	ATP sulfurylase	52.3	1
Co-factor and vitamine metabolism (2)				
196	NP_001049588	Chorismate synthase 1, chloroplast precursor	46.8	3
197	NP_001067120	Chorismate mutase, chloroplast precursor	36.3	3
Tetrapyrrole synthesis (2)				
198	NP_001058510	Delta-aminolevulinic acid dehydratase	46.2	3
199	ABF95686	Magnesium-chelatase subunit H family protein	153.5	3
Poly-amine metabolism (2)				
200	NP_001047375	Quinoprotein amine dehydrogenase	53.4	1
201	NP_001059438	Spermidine synthase 1	35	1
C1 metabolism (3)				
85[c)]	NP_001066640	Serine hydroxymethyltransferase	50.7	4
86[c)]	NP_001067846	Glycine hydroxymethyltransferase	51.3	12
87[c)]	NP_001051211	Serine hydroxymethyltransferase, mitochondrial precursor	56.3	17
202	NP_001057666	Formate dehydrogenase, mitochondrial precursor	41.2	9
203	NP_001063310	Formate-tetrahydrofolate ligase	75.4	5

a) NUMP is the abbreviation of number of unique matched peptides.
b) Indicating that proteins could be sorted into two different functional groups.
c) Indicating that proteins could be sorted into three different functional groups.

order to make it clear, we measured the aerobic respiration efficiency of the rice seeds during germination. The results showed that aerobic respiration increased slowly during the first 48 h imbibition, and increased sharply during 48–72 h (Fig. 2C). At the late stage of germination, cell wall loosening and cell expansion made it possible to absorb more oxygen into the cells, which might be the reason for the sharp increase of the aerobic respiration. This result also indicated the existence of anaerobic respiration pathway, such as fermentation, which was supported by the identification of lactate dehydrogenase (LDH, ♯61), pyruvate decarboxylase (♯46, 47) and alcohol dehydrogenase (♯48). Lactate dehydrogenase (♯61) catalyzes the reaction from pyruvate to lactate, whereas pyruvate decarboxylase (♯46, 47) and alcohol dehydrogenase (♯48) catalyze the two-step reaction alcoholic fermentation pathway to produce ethanol. When oxygen is not enough, TCA cycle will be negatively affected. Fermentative pathway may help to provide supplementary ATPs. The pentose phosphate pathway (PPP) may also exist in the germinating seeds since most of the enzymes (♯83, 84, 101–105) involved in it were detected here. This pathway can provide not only pentose phosphate for nucleotide metabolism but also NADH for different biosynthesis reactions.

Besides the above-mentioned enzymes that involved in the major carbohydrate metabolism, many enzymes involved in other carbohydrate metabolisms were also identified in the germinating rice seeds. Aldose reductase (♯88) and sorbitol dehydrogenase (♯89) are the enzymes that catalyze the reactions from glucose to fructose through the sorbitol pathway (Fig. 2B). The degradation of mannitol may also happen during the germination of rice seed (Fig. 2B). Along with all these catabolic process, large amount of energy and different kinds of substrates will be produced. Since the starch is the major reserves in cereal seeds, its degradation pathway and the regulation of related enzymes are resembled in the germination of different cereal seeds [34, 35].

3.2.2 Gluconeogenesis and starch biosynthesis

All the enzymes except 6-phosphofructose-1-kinase (♯29) involved in glycolysis also function in the gluconeogenesis.

Table 2. Proteins involved in protein biosynthesis, modification, targeting, folding, and degradation

Protein ID	Accession no.	Description	MW (kDa)	NUMP[a)]
Biosynthesis				
Ribosomal protein				
204	NP_001045624	40S ribosomal protein S4	29.7	1
205	NP_001041738	40S ribosomal protein S5-1	22.1	2
206	NP_001051645	40S ribosomal protein S5	29.8	1
207	NP_001060313	Ribosomal protein s6 RPS6-2	28.3	1
208	NP_001049835	40S ribosomal protein S7	22.1	2
209	NP_001049004	40S ribosomal protein S9	22.5	1
210	NP_001060861	40S ribosomal protein S13	17	2
211	NP_001046846	40S ribosomal protein S15a	14.7	1
212	NP_001064585	40S ribosomal protein S17	16.3	3
213	NP_001050855	40S ribosomal protein S21	13	2
214	NP_001062500	40S ribosomal protein S25 (RPS25B)	11.9	2
215	NP_001044658	40S ribosomal protein S26	14.9	2
216	NP_001055840	Ribosomal protein S26E family protein	15	1
216	NP_001060277	40S ribosomal protein SA (p40)	32.8	3
217	NP_001060923	60S acidic ribosomal protein P0	34.2	4
218	NP_001065684	60S acidic ribosomal protein P0	34.3	4
219	NP_001067062	60S ribosomal protein L2	28.1	4
220	NP_001065844	Ribosomal protein L3	44.4	2
221	NP_001059041	Ribosomal protein L4	44.6	7
222	NP_001045083	60S ribosomal protein L5	34.6	3
223	NP_001047288	60S ribosomal protein L6	24.1	2
224	NP_001059229	Ribosomal protein L7Ae/L30e/S12e	14.6	4
225	NP_001063520	60S ribosomal protein L9	21.2	4
226	NP_001060854	60S acidic ribosomal protein P1 (L12)	11	6
227	NP_001050537	Ribosomal protein large subunit 13	23.9	2
228	NP_001051314	Putative ribosomal protein L13a	23.5	1
229	NP_001055072	60S ribosomal protein L15	23.1	1
230	NP_001050069	60S ribosomal protein L18	20.9	2
231	NP_001050051	60S ribosomal protein L19	24	3
232	NP_001047935	Ribosomal protein L31e domain containing protein	13.9	1
233	NP_001055683	60S acidic ribosomal protein P2B	11.5	7
234	EEE61273	60s ribosomal protein l23	16.4	1
Initiation/elongation				
235	NP_001065446	Translation initiation factor eIF-3b	83.1	2
236	NP_001052582	eIF-3 γ	39	1
237	NP_001045733	Eukaryotic translation initiation factor 3 subunit 8 (eIF3 p110)	111.8	1
238	NP_001049175	Eukaryotic translation initiation factor 3 subunit 11	30.4	2
239	NP_001058481	Eukaryotic initiation factor 4A (eIF4A)	47	12
240	NP_001053219	Initiation factor eIF-4 γ	88.5	3
241	NP_001060417	Translation initiation factor IF6 family protein	26.3	1
242	NP_001049145	EF-1 α	49.2	13
243	NP_001060543	Elongation factor 1-β	23.7	2
244	NP_001060264	Elongation factor 1-delta 1	24.7	2
245	NP_001050333	Elongation factor 1-delta 2	24.5	3
246	NP_001046972	Elongation factor EF-2	93.9	16
247	NP_001055369	Nascent polypeptide-associated complex α polypeptide	24.9	3
248	NP_001148944	Nascent polypeptide-associated complex α subunit-like protein (*Zea mays*)	22.3	3
AA activation				
249	NP_001064254	Alanyl-tRNA synthetase	109.3	3
250	NP_001050572	Lysyl-tRNA synthetase	68.3	1
Modification				
251	NP_001047021	Poly(ADP-ribose) polymerase	92.3	3
252	NP_001049967	14 kDa zinc-binding protein (protein kinase C inhibitor), HIT family protein	14.1	1

Table 2. Continued.

Protein ID	Accession no.	Description	MW (kDa)	NUMP[a)]
Targeting				
253	NP_001052270	Sec1-like protein family protein	67.6	1
254	NP_001063744	EAP30/Vps36 family protein	22.1	1
255	NP_001049689	Chloroplastic outer envelope membrane protein (OEP75) precursor	87.6	14
256	NP_001049508	Chloroplast protein import component Toc34 family protein	36.1	1
257	NP_001049884	Mitochondrial import inner membrane translocase	18.2	2
258	NP_001042833	Mitochondrial import inner membrane translocase, subunit Tim17/22 family protein	9.84	1
Folding				
259	NP_001056601	RuBisCO subunit-binding protein β subunit (60 kDa chaperonin β subunit) (CPN-60 β)	64	11
260	NP_001066567	RuBisCO subunit binding protein α subunit (60 kDa chaperonin α subunit) (CPN-60 α)	61	21
261	NP_001053500	TCP-1/cpn60 chaperonin family	59.1	3
262	NP_001057796	Chaperonin Cpn60/TCP-1 family protein	60.8	3
263	NP_001051968	CPN-60 α	32.9	3
264	NP_001064784	Chaperonin CPN60-1, mitochondrial precursor	60.7	3
265	NP_001056181	Chaperonin CPN60-2, mitochondrial precursor	61.5	3
266	NP_001051497	DnaJ protein homolog (DNAJ-1)	46.6	2
267	NP_001068540	DnaK-type molecular chaperone HSP70	71.1	4
268	NP_001058535	Cyclophilin	23.4	2
269	NP_001065054	T-complex protein 1 delta subunit	58	5
270	NP_001046725	T-complex protein 1 delta subunit	57.6	3
271	NP_001050410	ClpB protein	108.9	3
272	NP_001054392	Peptidyl-prolyl cis-trans isomerase TLP20	26.5	2
273	NP_001045717	Peptidyl-prolyl cis-trans isomerase	18.2	3
274	NP_001063270	20 kDa chaperonin, chloroplast precursor	25.4	5
275	NP_001057057	20 kDa chaperonin, chloroplast precursor	45.6	2
276	NP_001052822	GrpE protein family protein	31.4	3
277	NP_001058590	Endoplasmin homolog precursor (GRP94 homolog)	92.7	7
278	NP_001045675	Endosperm lumenal binding protein	73.3	18
279	NP_001051724	Cell-autonomous heat shock cognate protein 70	71.2	7
Degradation				
280	NP_001047516	Proteasome subunit α type 2	25.7	3
281	NP_001056953	Proteasome subunit α type 4	26.9	2
282	NP_001045808	20S proteasome α 6 subunit	29.5	2
283	NP_001049162	Proteasome subunit α type 6	27.4	6
284	NP_001046700	26S protease regulatory subunit 6B homolog	46.3	4
285	NP_001062437	Proteasome subunit α type 7	27.2	3
286	NP_001048247	β 1 subunit of 20S proteasome	26.1	5
287	BAB78487	26S proteasome regulatory particle non-ATPase subunit 8	34.9	3
288	NP_001047302	Putative ubiquitin-associated (UBA) protein	92.6	1
289	EEE69940	polyubiquitin-like protein	95.3	1
290	NP_001043299	Ubiquitin-specific protease 6	55	3
291	NP_001065539	Ubiquitin-activating enzyme family protein	117	8
292	NP_001066793	Ubiquitin-specific protease 12	64	2
293	NP_001042185	Ubiquitin-specific protease 14	88.5	1
294	NP_001042272	Mitochondrial processing peptidase	53.8	11
295	NP_001049888	Mitochondrial ATP-dependent protease Lon precursor	103.3	1
296	NP_001049357	Cytochrome *c* reductase-processing peptidase subunit I	58.6	8
297	NP_001045333	Peptidase aspartic family protein	41.7	1
298	NP_001059824	Peptidase C48, SUMO/Sentrin/Ubl1 family protein	173.9	2
299	NP_001051710	Peptidase M1, membrane alanine aminopeptidase	67.7	2
300	NP_001047794	Peptidase M20 family protein	48.5	1
301	NP_001055274	Peptidase M24 family protein	43.1	5
302	NP_001052181	Peptidase S8 and S53, subtilisin, kexin, sedolisin domain containing protein	78.5	1

Table 2. Continued.

Protein ID	Accession no.	Description	MW (kDa)	NUMP[a)]
303	NP_001063483	Peptidase S8 and S53, subtilisin, kexin, sedolisin domain containing protein	65.6	1
304	NP_001044360	Lysosomal Pro-X carboxypeptidase	57.3	1
305	NP_001047562	C13 endopeptidase NP1	49.6	3
306	NP_001052440	Cysteine proteinase 1 precursor	41.1	2
307	NP_001042702	Cysteine proteinase inhibitor	27.1	3
308	NP_001055527	Cysteine proteinase inhibitor 3	19.5	1
309	NP_001045138	Cysteine endopeptidase	40.6	3
310	NP_001055050	Serine carboxypeptidase II-2 precursor	52.9	6
311	NP_001053358	ClpP2	32.9	1
312	NP_001047512	ATP-dependent Clp protease	31.8	6
313	NP_001066442	ATP-dependent Clp protease ATP-binding subunit clpA homolog, chloroplast precursor	101.9	20
314	NP_001043786	Aspartic protease	55.7	10
315	NP_001056348	Aspartic proteinase oryzasin 1 precursor	53.9	8
316	NP_001053428	C13 endopeptidase NP1	54.8	3
317	NP_001053083	TA5 protein	94.3	2
318	EEE64346	Proline iminopeptidase	33.5	2
319	ABF97577	Serpin-ZXA	42.1	1
320	NP_001060680	Proteinase inhibitor I9	81.2	1
321[b)]	NP_001050419	Proteinase inhibitor I25, cystatin family protein	12.7	3
322	NP_001060934	Similar to Tpv2-1c protein	55.5	1
323	BAD35625	Ulp1 protease-like	19	2

a) NUMP is the abbreviation of number of unique matched peptides.
b) Indicating that proteins could be sorted into two different functional groups.

Besides, the enzymes (♯62–65) that catalyze the malate to form into pyruvate in the cytosol were detected. This indicated that the de novo biosynthesis of carbohydrates might also happen in the germinating rice seeds. This hypothesis is supported by the fact that nine enzymes that catalyze the biosynthesis of starch were also identified in the germinating rice seeds (Table 1, Fig. 2B). These anabolic enzymes are not necessary to be degraded during the germination based on our previous study [24]. It has been reported that there were starch biosynthesized around the vascular cells in rice embryo during the germination [36]. In consistent with this report, we observed gradual accumulation of the starch granules in the embryonic cells of rice seeds during the germination (Fig. 3). In the dry seeds, there were very few starch granules in the cells around the vascular tissue (Fig. 3), whereas the same cells in 24, 48, and 72 imbibed seeds were filled with starch granules (Fig. 3). Starch is the major reserve in cereal seeds. During the germination, the stocking starch should be mobilized and degraded, which can provide enough substrates and ATPs for the following seedling establishment. The soluble sugars will be transported into the cells around the vascular tissue where they should be transferred into starch before the protrusion is completed [36]. This is supported by the fact that all the starch biosynthesis required enzymes existed in the germinating rice seeds and were not degraded during this process. We also observed that the cells of the 48 imbibed seeds contain the most intensive starch granules, which indicated that the accumulation of starches was weakened after the radicle protrusion. We also identified ATP:citrate lyase (♯66) the enzyme that catalyzes the citrate to produce acetyl-CoA. This reaction is a critical step in the biosynthesis of fatty acid.

3.3 Amino acid metabolism

Forty-seven amino acid metabolism-related proteins were identified. They represented 23.5% of the total metabolism proteins detected in the germinating rice seeds (Table 1). The enzymes included eight central amino acid metabolism-related proteins, ten for Aspartate family amino acids, nine for Branched chain amino acid, nine for Serine, glycine, and cysteine, six for aromatic amino acid, three for arginine, and two for glutamate (Table 1). Totally, biosynthetic pathways of 13 amino acids including glycine, serine, cysteine, methionine, threonine, lysine, valine, leucine, isoleucine, tryptophan, arginine, aspartate, and glutamate could be constructed (Fig. 2B). As for the rest seven amino acids, the request for them might be satisfied by the degradation of stocking and other proteins.

Among the six aspartate family amino acid biosynthesis-related enzymes, five (♯130–134) were involved in methionine biosynthesis. Diaminopimelate epimerase (♯135) is the

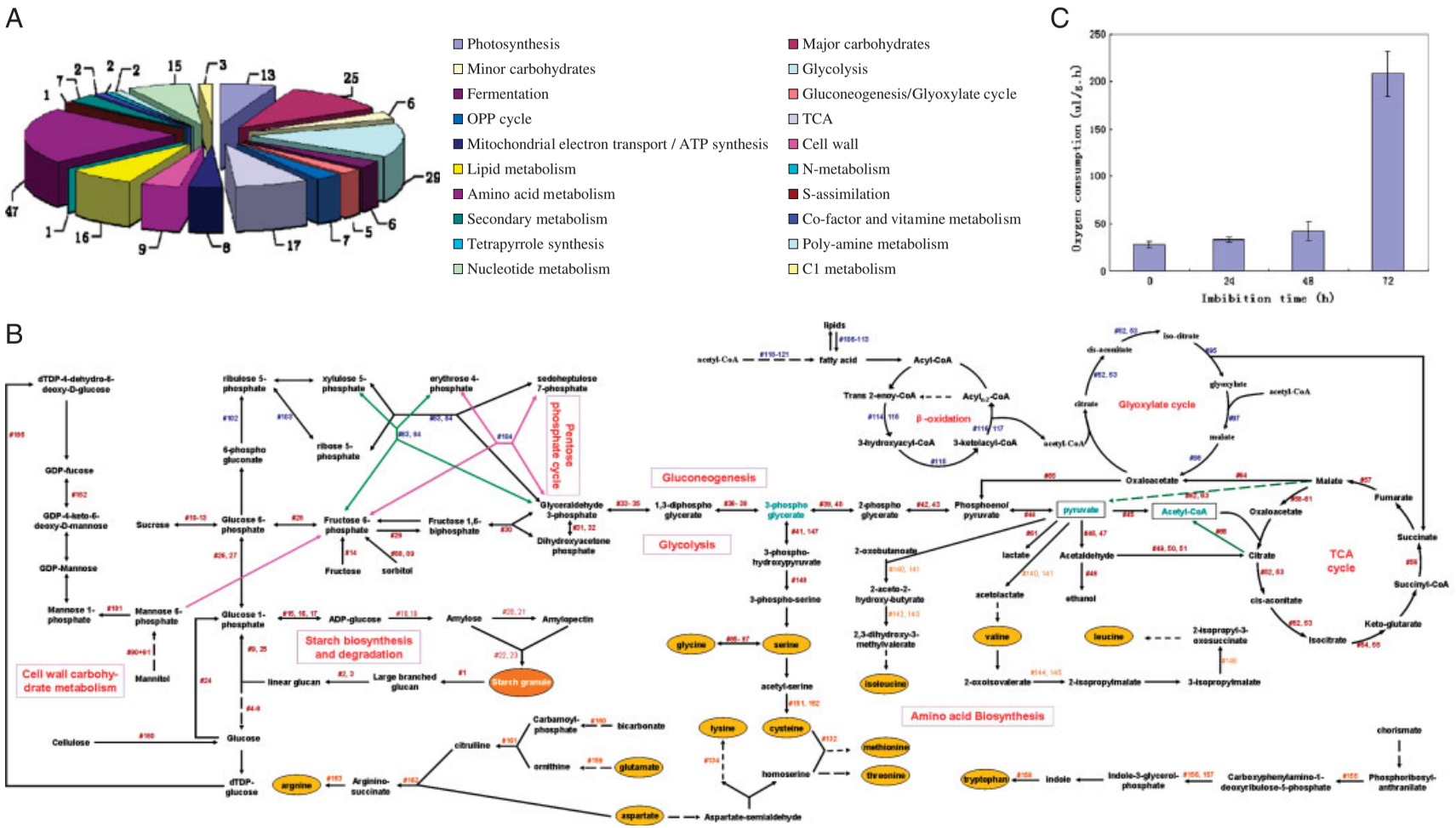

Figure 2. Metabolic pathways in germinating rice seeds. (A) Functional categorization of the metabolism-related proteins. The digital number indicates the number of proteins in each subgroup. (B) The metabolic pathways that were constructed based on the proteomic data. The digital numbers are the protein ID and the broken arrows indicate that there are multisteps between the two compounds. (C) The histogram shows the increase of aerobic respiration efficiency during germination. The *x*-axis stands for the imbibition time, and *y*-axis stands for the consumption of oxygen.

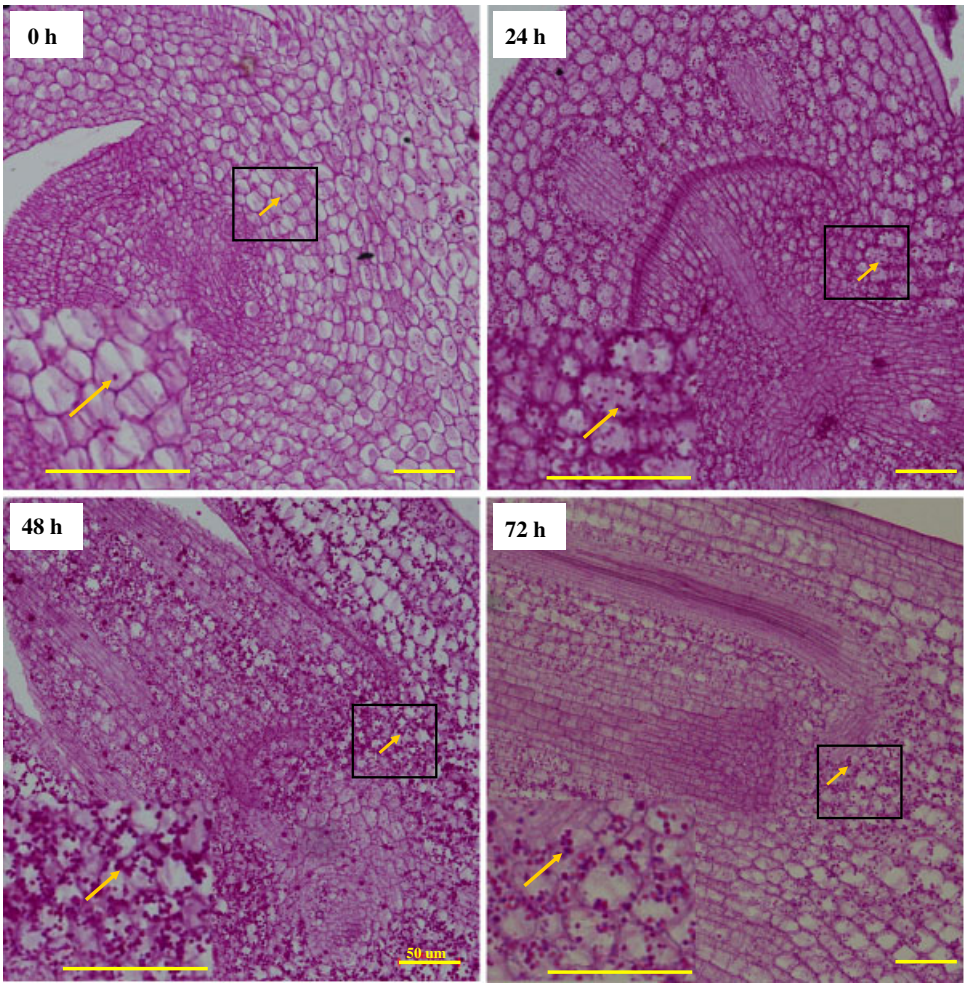

Figure 3. Periodic Acid Schiff-stained sections of the rice embryos showing the accumulation of starch granules in the cells around the vascular tissue during the germination. The rectangle areas were enlarged to show the starch granules (indicated with arrows) and attached at the corner of each image. The most intensive starch granules could be found in the embryonic cells of the 48 h imbibed seeds. Arrows indicate the starch granules (Bars = 50 μm).

enzyme that participates in the biosynthesis of lysine. It catalyzes LL-2,6-diaminoheptanedioate to form into meso-diaminoheptanedioate which is the immediate precursor of lysine (Fig. 2B). Interestingly, we also detected the lysine catabolism initiation enzymes lysine-ketoglutarate reductase/saccharopine dehydrogenase bifunctional enzyme (♯136, 137). Since lysine is one of the abundant amino acids that exists in rice seed, its degradation could also provide energy or substrate for the following seedling growth.

Seven enzymes involved in branched chain amino acids biosynthesis were identified. They covered most of the steps in leucine and isoleucine biosynthesis pathway (Fig. 2B). Except for the serine acetyl transferase, all the other enzymes required for the biosynthesis of serine, glycine, and cysteine were detected (Table 1, Fig. 2B). The intermediate of glycolysis 3-phosphoglycerate could also be used as the substrate of 3-phosphoglycerate dehydrogenase (♯41) for the biosynthesis of serine. Some enzymes required for tryptophan and arginine biosynthesis were also detected (Table 1, Fig. 2B). During grain filling and germination, the amino acid biosynthesis-related proteins are stable in their abundance [24, 26, 37].

3.4 Lipids metabolism-related pathways

Most of the enzymes required for lipids degradation were detected. Lipase and lipolytic enzymes (♯105–112) catalyze the hydrolysis of triacylglycerol (TAG) into glycerol and fatty acid. β-Oxidation and glyoxylate cycle are the two major pathways through which fatty acids are degraded. Enoyl-CoA hydratase (♯113), fatty acid β-oxidation multifunctional protein (MFP) (♯114), and Acetyl-CoA acyltransferase (♯115, 116) were the enzymes that involved in the β-oxidation of fatty acid. In addition, the glyoxylate cycle required enzymes aconitate hydratase (♯52, 53), Isocitrate lyase (♯95), Malate synthase (♯97), and Malate dehydrogenase (♯98) were also detected. These enzymes will help to mobilize the stocking lipids during the germination (Fig. 2B). Although the lipids are not the major reserves, the storage lipids do accumulate during the grain filling [38] and stored in aleurone cells of the cereal seeds [39]. Besides this, the endosperm cells are dead cells that will be decomposed during the germination and seedling growth. The existence of the lipids degradation enzymes might help to utilize not only those stocking lipids but also the plasma membrane lipids of the endosperm cells.

3.5 Nucleotides, cell wall, secondary, and other metabolism

Fifteen nucleotides metabolism-related proteins were detected (Table 1). These proteins were involved in nucleotide degradation, biosynthesis, and phosphor transferring. But most of the enzymes required for nucleotide metabolism did not detected here, which indicate that the metabolism of nucleotides is not active at this stage. Eight cell-wall polysaccharide biosynthesis and degradation-related proteins were detected. Except for polygalacturonase (♯179), the other seven proteins were all involved in the cell-wall biosynthesis. There were seven secondary metabolism-related proteins identified. 3-Hydroxy-3-methylglutaryl-coenzyme A reductase (♯187) and isopentenyl pyrophosphate: dimethyllallyl pyrophosphate isomerase (♯188) are two enzymes that required for the mevalonate pathway of isoprenoid biosynthesis. Caffeoyl-CoA *O*-methyltransferase (♯189) and phenylalanine ammonia-lyase (♯190) were the enzymes catalyze the reactions in the lignin biosynthesis pathway. UDP-glucosyltransferase, NADPH-dependent codeinone reductase, and Isoflavone reductase-like protein are involved in the flavonoid metabolic pathway. Besides these proteins, we also detected the proteins involved in other metabolisms including N metabolism, S assimilation, Co-factor, and vitamin metabolism, Tetrapyrrole synthesis, Poly-amine metabolism, and C1 metabolism.

3.6 Protein biosynthesis, modification, targeting, folding, and degradation

In total, 120 proteins that related to the protein biosynthesis, modification, targeting, folding, and degradation were identified in this study. Among them, 47 were protein biosynthesis related, including 31 ribosomal proteins, 14 translation initiation or elongation factors, 2 amino acid activation proteins. All of them are basic components for protein biosynthesis. Among the 31 ribosomal proteins, 13 are constitutes of the small subunit, and 18 belong to the large subunit. The two amino acid activation proteins were Alanyl-tRNA synthetase (♯249) and Lysyl-tRNA synthetase (♯250). There were six that play roles in protein targeting. Sec1-like protein (♯253) and Vps36 (♯254) are involved in the trafficking and secretion of proteins to plasma membrane or cell wall; OEP75 (♯255) and Toc34 (♯256) help to import the nuclear-encoded proteins into chloroplast; and the rest two (♯257, 258) function in importing proteins into mitochondria. Twenty-one were identified as protein folding-related proteins (Table 2) most of which were chaperonin.

In addition, there were 44 related to protein degradation, nearly the same number with that related to protein biosynthesis. Among the degradation-related proteins, there were only eight proteins belong to proteasomal system. During seed maturation, large amounts of storage proteins were accumulated. They will be used as the primary source of amino acids and reduced nitrogen for the seed germination and seedling growth. Although, degradation of the stocking proteins is one of the energy and substrate sources for the germination, it may also be helpful to remove some inhibitory proteins [24]. Transcriptomic and proteomic analysis have shown that both the protein degradation-related proteins and the mRNA encoding these proteins were accumulated in the germinating seeds [19, 20, 24]. Most of the protein degradation-related proteins were different kinds of protease or proteinase inhibitors, which implied that the proteases might play major roles in the protein degradation during rice seed germination.

3.7 Transcriptions regulation

Twenty-seven RNA-associated proteins were detected in this study (Table 3). They were mainly transcription factors. Zinc finger protein (♯324–327) is a universal transcription factor that involved in most of the house-keeping gene expression, whereas MYB (♯332, 333) and GRAS (♯334) are specific transcription factors that play regulatory roles. Besides these, there were also some RNA-binding proteins that might function at either the transcription level or the post-transcription level.

It has been reported that all the mRNAs required for germination have been synthesized and stocked during seed maturation in Arabidopsis [21, 40]. Although germination of wheat seeds was strongly impeded by the transcriptional inhibitor [41], many studies supported the idea that transcription is not required for the completion of germination [21, 42, 43]. In order to see if newly synthesized mRNAs are necessary or not for the rice seed germination, we inhibited the transcription with α-amanitin. Under the treatment of α-amanitin, the seed germinated well, but the germination is more slowly than the control. Moreover, the protruded seeds could not enter into the seedling establishment stage as the mock did (Fig. 4). Blocking the protein biosynthesis with cycloheximide resulted in total inhibition of the germination (data not shown). This result is consistent with the other studies in Arabidopsis and wheat [21, 41]. It has been reported that mature dry seeds contain large amount of stored mRNAs called long-lived mRNAs [19] and the de novo synthesis of proteins occurs with the long-lived mRNA as template [42, 43]. All these data indicate that the potential of germination has been programmed during the seed maturation.

3.8 Redox regulation proteins

ROS can be produced in all living organisms as signal molecules or by-products. Upon the imbibition, the contents of superoxide anion and H_2O_2 were gradually increased (Fig. 5). The accumulation of H_2O_2 was later than that of superoxide anion (Fig. 5). Overaccumulation of ROS can

Table 3. RNA (regulation of transcription)

Protein ID	Accession no.	Description	MW (kDa)	NUMP[a)]
324	NP_001049734	Zinc finger DNA-binding protein	19.5	1
325	NP_001067878	Zn-finger, C-x8-C-x5-C-x3-H type domain containing protein	49.7	1
326	NP_001042637	Zn-finger, C-x8-C-x5-C-x3-H type domain containing protein	49.7	1
327	NP_001050819	Zn-finger, CCHC type domain containing protein	76.3	1
328	NP_001057860	Leucine-rich repeat, typical subtype containing protein	58.3	3
329	NP_001055971	Leucine-rich repeat protein	77.4	1
330	NP_001064690	Transcription factor DP	36.8	1
331	NP_001064883	Transcription factor homolog BTF3-like protein	17.5	2
332	NP_001059708	MYB transcription factor	41.5	2
333	NP_001055902	OsMyb5	45	1
334	NP_001047748	GRAS family transcription factor	64.1	1
335	NP_001061165	RNA-binding region RNP-1 (RNA recognition motif)domain containing protein	52.7	1
336	NP_001053941	RNA-binding protein 45	46	6
337	NP_001042645	PUR-α/β/γ, DNA/RNA-binding family protein	42.5	1
338	NP_001054436	RNA-binding protein PufA	95.1	3
339	NP_001067344	Glycine-rich RNA-binding protein GRP1A	16	4
340	NP_001056523	Nucleolar histone deacetylase HD2-p39	32.4	2
341	NP_001068221	DEAD-box protein 3	65.9	1
342	NP_001054997	BED finger domain containing protein	80.7	1
343	NP_001055231	Reverse transcriptase	181.7	3
344	NP_001045988	Reverse transcriptase	143.1	2
345	NP_001062583	Poly(A)-binding protein	71.5	3
346	NP_001056321	DDT domain containing protein	156.7	3
347	NP_001046985	110 kDa 4SNc-Tudor domain protein	108	5
348[b)]	NP_001044036	X1 protein	50	4
349	NP_001048736	GT-2 factor	76.9	1
350[b)]	NP_001052524	Response regulator receiver domain containing protein	41.8	2

a) NUMP is the abbreviation of number of unique matched peptides.
b) Indicating that proteins could be sorted into two different functional groups.

result in oxidative stress, especially the oxidization of some functional important proteins [44, 45], and hence impede germination [46]. To keep a balance of the redox homeostasis and protect themselves from oxidative damages, plants have evolved high-efficient antioxidant systems among which antioxidant enzymes might be the most efficient one [47]. Scavenging the ROS by the antioxidant enzymes including superoxide dismutases (SODs), glutathione *S*-transferase (GST), catalase, peroxidases, and enzymes in the ascorbate–glutathione cycle is an efficient way. In this study, 25 redox regulation proteins were identified (Table 4). Among them, four were SOD including three Cu-Zn SOD (Table 4), and one Mn SOD (Table 4). SODs catalyze the reaction from superoxide and water to H_2O_2 and O_2. Manganese (Mn) SODs localize in mitochondria and Cu-Zn SODs localize in both cytosol and plastids. These two types of SODs were identified, indicating that the ROS might be produced not only in cytosol but also in plastids and mitochondria during seed germination. Four enzymes in ascorbate–glutathione cycle system were identified. They were thylakoid-bound ascorbate peroxidase, ascorbate peroxidase (APX), dehydroascorbate reductase, and monodehydroascorbate reductase. They can scavenge both superoxide and H_2O_2. Catalase and peroxidase can also scavenge H_2O_2.

Besides the scavenging by antioxidant enzymes, another critical way in keeping redox homeostasis is reducing the oxidized proteins. Some enzymes that help to reduce the oxidized proteins were identified. Among them, there were three GST, two protein disulfide-isomerase (PDI), four thioredoxin, and three peroxiredoxin thioredoxins (Trxs) are small and powerful disulfide reductases which reduce protein targets involved in different cellular processes, such as metabolism, gene expression, and seed germination [48, 49]. They play this function through two close and reactive cysteine residues in a conserved motif: WCG/PPC [50, 51]. They can also regenerate of peroxiredoxins, and hence repair proteins and protect cell from oxidative damages [52]. Protein disulfide-isomerase can also help to keep protein in a correct redox status.

3.9 Stress response and signaling proteins

There were 41 stress-responsive proteins (Table 4) in this study. Among them, 28 were response to abiotic stress. HSPs were the largest group responding to the abiotic stress. They included five HSP70, three small HSPs, and four other HSPs. Besides, there were five Universal stress proteins (Usp), three dehydrins and four DNA J family

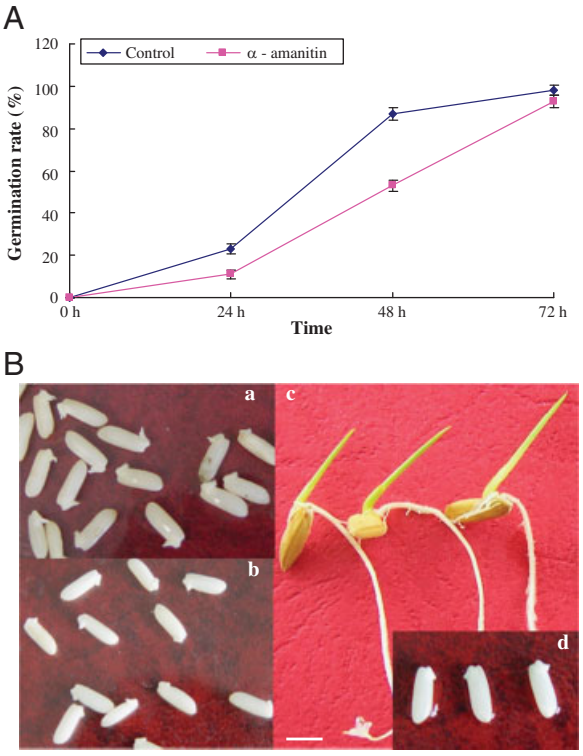

Figure 4. Germination assay of the rice seeds with or without α-amanitin. (A) Germination rate of rice seeds with or without α-amanitin. Values are means of three replicates ± SD. (B) (a) Seeds after 48 h imbibition with water, (b) seeds after 48 h imbibition with 100 μM α-amanitin, (c) seedlings after 5-day germination with water; (d) seeds after 5 days imbibition with α-amanitin. (Bar = 0.5 cm).

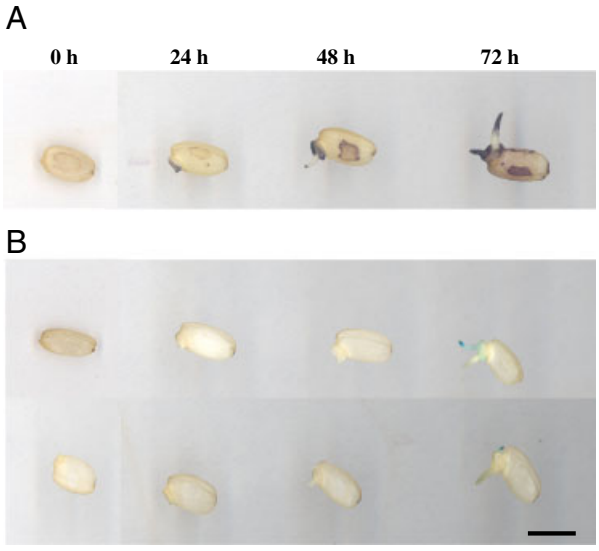

Figure 5. Accumulation of ROS in the germinating rice seeds. (A) Superoxide anion; (B) H_2O_2, the upper panel shows TMB-staining results indicating the accumulation of H_2O_2, and the lower panel shows the results that the seeds were treated with sodium pyruvate prior to TMB staining.

proteins. Nine late embryogenesis abundant (LEA) proteins in the developmental group could also be regarded as stress responsive. Along with the maturation of rice seeds, desiccation happened. To survive from dry status, seeds accumulate different kinds of stress responsive proteins, especially those related to drought stress. Dehydrin, osmotin and late embryo-abundant proteins (LEA) are all reported to be related to the desiccation response [53, 54]. Our earlier studies have shown that LEAs were gradually degraded during germination [24]. Besides these proteins, Usps, and HSPs (mainly including small HSP and HSP70) may also play important roles in the dry seeds. Interestingly, 13 biotic stress-related proteins were also identified in the germinating rice seeds. Kim et al. [26] reported that Probenazole-inducible protein PBZ1 (♯414) was upregulated by the imbibition in rice seeds. Preexistence of these biotic stress-related proteins might help the young seedlings to cope with different potential attacks.

Half of the 42 signaling proteins were protein kinase. Besides the universal one, there were five serine/threonine-specific protein kinase, two receptor protein kinase, and two kinase involved in MAPK signaling pathway. Others included three 14-3-3 proteins and five calcium signaling proteins. How these signaling proteins contribute for the germination is still elusive.

4 Concluding remarks

Investigators are now able to study living systems on an unprecedented large scale because of the availability of genome sequence information. Network discovery is defined as the effort of elucidating the natural relationships between molecules and associated properties that exist in a biological system [55]. Aimed at characterizing the novel metabolic style, metabolic network constructions have attracted increasing attention in recent years. Unfortunately, there is are existing data that can provide us a full view of all the biochemical reactions that happen in cells or tissues. It is even worse in plant kingdom. Currently, the studies in plant kingdom mainly focus on specific metabolic pathways, such as fatty acid and amino acid metabolism [56, 57], and transcriptional regulatory networks of specific developmental stages, such as flower and root development [58], photomorphogenesis [59, 60]. Because of the importance of proteins in all biochemical reactions, systematic analysis of proteins localizations or proteins profiling at specific stages is essential to accurately understand metabolic and regulatory networks. Two pioneer projects have been launched and provided valuable information [61, 62]. In the current study, we constructed the metabolic and regulatory pathways in the germinating rice seeds through protein profiling strategy. Our results suggest that mobilization of the reserves including starch, storage proteins, and lipids. Besides the central carbon metabolism pathways (glycolysis, TCA cycle), fermentation can also provide ATP for the

Table 4. Proteins involved in redox regulation, stress response, and signaling.

Protein ID	Accession no.	Description	MW (kDa)	NUMP[a)]
Redox regulation				
351	NP_001050118	SOD [Cu-Zn] 1	15.1	8
352	NP_001060564	SOD [Cu-Zn] 2	15	13
353	NP_001062514	SOD [Cu-Zn], chloroplast precursor	21.2	4
354	NP_001055195	Manganese- SOD precursor	24.9	12
355	NP_001066305	Thylakoid-bound ascorbate peroxidase	33.4	1
356	NP_001049769	Ascorbate peroxidase	27	5
357	NP_001054470	Glutathione-dependent dehydroascorbate reductase	23.4	9
358	NP_001062486	Monodehydroascorbate reductase	46.6	6
359	NP_001044339	GST II	23.8	7
360	NP_001043071	Glutathione transferase	24.1	1
361	NP_001065123	Tau class GST protein 4	25.4	4
362	NP_001055996	Thioredoxin H	13	1
363	NP_001059069	Thioredoxin H-type	13	3
364	NP_001053968	Thioredoxin-like 4	14.5	1
365	NP_001046165	Thioredoxin peroxidase	23	3
366	NP_001043845	Peroxiredoxin	17.2	5
367	NP_001060407	Peroxiredoxin 6	23.9	21
368	NP_001052764	2-Cys peroxiredoxin BAS1, chloroplast precursor	16.6	3
369	NP_001045979	Peroxidase precursor	36	1
370	BAC45154	Class III peroxidase 103	35.9	1
371	NP_001042918	Ferric leghemoglobin reductase	52.5	3
372	NP_001058635	Catalase isozyme B	56.5	5
373	NP_001050331	PDI-like protein	63.2	9
374	NP_001067436	Protein disulfide-isomerase precursor	56.7	27
375	NP_001053270	Glutaredoxin	11.6	2
Stress response				
Abiotic stress				
376	NP_001055972	Usp family protein	28.2	1
377	NP_001066983	Usp family protein	18	2
378	NP_001055716	Usp family protein	17.8	5
379	NP_001044454	Usp family protein	27.4	2
380	NP_001044810	Usp family protein	17.9	1
381	NP_001061194	Luminal binding protein 5 precursor (BiP 5)	73.4	2
382	NP_001067838	Dehydrin 9	31.1	13
383	NP_001067843	Dehydrin RAB 16B	16.4	2
384	NP_001067844	Dehydrin family protein	17.2	2
385	NP_001044756	Osmotin-like protein precursor	26.1	1
386	NP_001048317	Small heat stress protein class CIII	18.5	1
387	NP_001042231	17.5 kDa class II HSP	17.9	1
388	NP_001041955	17.7 kDa HSP	16.8	3
389	NP_001049541	HSP26	26.5	1
390	NP_001055754	HSP70	70.7	9
391	NP_001055140	Stromal 70 kDa heat shock-related protein	48.6	17
392	NP_001066486	Heat shock 70 protein	74	13
393	NP_001042210	Hsp70 family protein	93	4
394	NP_001054812	Hsp70 family protein	94	1
395	NP_001063504	HSP82	64.4	14
396	NP_001056066	101 kDa HSP	100.8	6
397	NP_001062103	HSP precursor	88.2	3
398	NP_001047563	HSP STI (stress inducible protein)	64.8	8
399	NP_001055227	HSP DnaJ family protein	48.1	2
400	NP_001056320	DnaJ-like protein	38.5	1
401	ABA99886	DnaJ protein	49.4	1
402	NP_001049808	DnaJ domain containing protein	48.9	3
403	NP_001055802	Cold-regulated protein	17.5	3
Biotic stress				
321[b)]	NP_001050419	Proteinase inhibitor I25, cystatin family protein	12.7	3
404	ABG66007	Zinc knuckle family protein	102.3	11

Table 4. Continued.

Protein ID	Accession no.	Description	MW (kDa)	NUMP[a)]
405	NP_001062402	Disease-resistance protein	92.1	2
406	NP_001066554	Disease-resistance protein family protein	86.3	1
407	NP_001057389	Disease-resistance protein family protein	109.3	1
408	NP_001046239	Disease-resistance protein family protein	135.3	1
409	NP_001043016	Powdery mildew resistance protein PM3b	126.9	1
410	NP_001049857	Pathogen-related protein	17	3
411	NP_001052704	Bipartite-response regulator	32.1	2
412	NP_001053363	α-Amylase/subtilisin inhibitor (RASI)	21.3	5
413	NP_001068520	Class III chitinase homologue	31.5	4
414	NP_001066998	Probenazole-inducible protein PBZ1	16.6	3
415	NP_001049995	Putative r40c1 protein	38.7	2
Signaling				
416	NP_001062152	COP9 complex subunit 6	35.9	1
417	NP_001056747	Acid phosphatase-1(1)	32.2	5
418	NP_001049806	Ran-binding protein 1 homolog	23.3	3
419	NP_001068067	14-3-3 Protein	29.5	3
420	NP_001061856	14-3-3-Like protein GF14 nu	28.7	4
421	NP_001053003	14-3-3-Like protein GF14-6	29.7	1
422	NP_001042615	NTGB2	21.8	1
423	NP_001043910	Guanine nucleotide-binding protein β subunit-like protein (GPB-LR)	36.1	8
424	ABA98253	Protein kinase	86.5	3
425	NP_001052410	Protein kinase	87.4	4
426	NP_001045202	Protein kinase	141.2	1
427	NP_001063652	Pto kinase interactor 1	47.9	1
428	NP_001060256	Protein kinase APK1B	51.1	1
429	NP_001045751	Protein kinase CPK1	58.4	5
430	NP_001052560	Protein kinase domain containing protein	83.6	1
431	NP_001048858	Protein kinase domain containing protein	41.9	2
432	NP_001067422	Protein kinase domain containing protein	73.1	1
433	NP_001047429	Protein kinase domain containing protein	110.2	1
434	NP_001044045	Protein kinase domain containing protein	70.4	1
435	NP_001065874	Protein kinase domain containing protein	110.4	1
436	NP_001067102	Serine/Threonine protein kinases	74.5	2
437	NP_001047579	Putative serine/threonine-specific protein kinase	38.6	1
438	NP_001052827	Serine/threonine-protein kinase SAPK7	41.2	1
439	BAF14564	Serine/threonine-protein kinase	49.8	3
440	NP_001047579	serine/threonine-specific protein kinase	38.4	3
441	NP_001050364	MAP3K protein kinase-like protein	38.7	1
442	BAF46959	MAP kinase phosphatase	82.9	1
443	NP_001059871	Receptor protein kinase	71.9	1
444	NP_001052282	Receptor-like protein kinase 4	90.8	3
445	NP_001061532	β-Ig-H3	43.5	3
446	NP_001062168	β-Ig-H3	28	3
447	NP_001055907	Calmodulin	16.7	2
448	NP_001059711	Calreticulin-interacted protein	100.8	5
449	NP_001056551	calmodulin-binding region domain containing protein	35.8	3
450	NP_001056849	IQ calmodulin-binding region domain containing protein	39.3	1
451	NP_001047417	Calcium-binding EF-hand domain containing protein	54.7	3
452	NP_001065908	Ankyrin repeat containing protein	64.8	4
453	AAX95653	Phosphatidylinositol 3- and 4-kinase	215	1
454	NP_001065485	Inositol phosphate kinase	39.4	1
455	NP_201203	3′(2′),5′-Bisphosphate nucleotidase/inositol or phosphatidylinositol phosphatase	43.4	1
456	NP_001060934	Retroelement pol polyprotein-like	57	1
457	AAL58182	putative far-red impaired response protein	63	2

a) NUMP is the abbreviation of number of unique matched peptides.
b) Indicating that proteins could be sorted into two different functional groups.

germination and following seedling growth. Up-to-date, the metabolic pathway that has been constructed here is the most comprehensive one. Moreover, the regulatory networks will help us to know much better about how the cells regulate their gene expression, keep their redox homeostasis with antioxidant enzymes, and respond to environmental stimuli.

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