Plant Science 181 (2011) 660–666

Contents lists available at ScienceDirect

Plant Science

journal homepage: www.elsevier.com/locate/plantsci

The differential proteome of endosperm and embryo from mature seed of *Jatropha curcas*

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ARTICLE INFO

Article history:
Received 12 August 2010
Received in revised form 24 January 2011
Accepted 18 March 2011
Available online 1 April 2011

Keywords:
Jatropha curcas
Dormant seed
Endosperm
Embryo
Proteomics

ABSTRACT

Jatrpha curcas L., a non-model woody plant belonging to Euphorbiaceae family, is a promising economic plant due to the high oil content in seed and high tolerance to drought and salt stress. The embryo and endosperm of *J. curcas* seed differ in morphology, function and ploidy. To characterize the protein profiles of these two tissues, we have performed proteomic analysis with the dry mature *J. curcas* seeds. The data showed that the 2-DE profiles of endosperm and embryo were similar to each other. There are 66 differential proteins between the two seed tissues, in which 28 proteins distributed in 9 distinct functional classes, have been identified successfully in endosperm or embryo. The major groups of differential proteins are associated with metabolism (25%) and disease/defence (18%). Our results demonstrated that in the dry mature *J. curcas* seeds, the proteins involved in oil mobilization, signal transduction, transcription, protein synthesis, and cell cycle which are essential for the seed germination have occurred in endosperm and embryo, reflecting the fact that proteins required for germination are already present in the dry mature seed.

1. Introduction

Jatropha curcas L., also known as the Barbados or Physic nut, is a perennial woody plant of the Euphorbiaceae family. It is considered as a potential feedstock for biofuel production since it can grow in marginal and semi-marginal lands such as salty and arid areas, and as such it does not compete with food crops [1–4]. What's more, the seeds of *J. curcas* contain 30–40% oil (by dry weight) with more than 70% of unsaturated fatty acids that can be used in a standard diesel engine [3]. The by-products (press cake) can be also used as feedstock to power electricity or used as a good organic fertilizer [2]. High oil contents of 50–60% occur in the kernel, in which the thin embryo embedded in thick endosperm that is nearly 12 times heavier than embryo [5,6].

The embryo and the endosperm tissues have different ploidies and there exist interactions between the embryo and the endosperm in a coordination way to regulate seed development. Functioning as sources of nitrogen, sulfur, minerals, and energy, endosperm is well known to provide the embryo with nourishment [7,8]. Beyond simple nutrient delivery to the embryo, the endosperm also plays important roles in maintaining high osmotic potential around the embryo, giving mechanical support during early embryo growth [9] and providing hormones such as cytokines which regulate embryonic organ formation [10,11]. In addition, endosperm was reported to be the source of signals involved in embryogenesis [12,13], altering the embryo size and regulating embryo development [14]. A recent report indicated that the zygotic embryo can control the endosperm growth during Arabidopsis seed development [10].

Following the maturity, plant seeds may enter in a special developmental phase in which a viable seed fails to germinate even under favorable conditions. This phase is known as seed dormancy, also called the after-ripening phase [5,15]. Seed dormancy is referred to as embryo dormancy or internal dormancy, and is caused by endogenous characteristics of the embryo that prevents germination [16]. Therefore seed dormancy is regarded as an inherited quality of seed that keeps seeds less active or inactive until the environmental conditions are right for seed germination. The plant hormone balance theory suggested that the induction, maintenance and disappearance of seed dormancy are believably regulated by the simultaneous action of the dormancy promoting plant hormone abscisic acid (ABA) and the germination promoting hormone gibberellin (GA) [17,18]. It is well established that resumption of cell cycle activity is a prerequisite for growth and organ formation [19] and a specific feature of early germination [20–22]. Microarray analysis has revealed that mRNAs required for germination actually occurred in the dry mature seed [23–25]. However, mRNA levels are not necessarily often reflective of protein levels or enzyme activities, and whether their protein products are established or not and

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doi:10.1016/j.plantsci.2011.03.012

what kinds of proteins exist in the dormant seeds remain to be clarified.

In our previous preliminary work, 14 identical proteins were found in both of the endosperm and embryo tissues in dry mature *J. curcas* seeds [6]. To make further and clear comparison of the endosperm with embryo, we have in the present work investigated the protein profiles between these two tissues from the dry mature *J. curcas* seeds by using proteome which is a very useful tool and widespread used to investigate and track the biological process [26,27], such as seed development [25,28–32], seed dormancy [33] and seed germination [19,34–36]. Our proteomic results indicated that there are 66 differential proteins in endosperm and embryo and these proteins are associated with 9 functional groups, and some proteins required for seed germination are already present in both endosperm and embryo of *J. curcas* dormant seeds. The data is valuable for us to understand the different functions of embryo and endosperm of *J. curcas* seeds.

2. Materials and methods

2.1. Plant materials

Mature *J. curcas* seeds were collected from the city of Panzhihua, in Sichuan Province of China. The full seeds were selected and the dry seed capsules were taken off manually. The endosperm and embryo were separated with scalpel manually and each of them with around 0.5 g was packaged with silver paper and stored at −80 °C for further use.

2.2. Protein extraction and 2-DE

Proteins of the embryo and endosperm from *J. curcas* seeds were extracted as following Shen et al. [37]. A package of seed parts (embryo and endosperm) were ground into fine powder, and then homogenized in 2 mL homogenate buffer containing 20 mM Tris–HCl (pH 7.5), 250 mM sucrose, 10 mM ethylene glycol tetraacetic acid, 1 mM phenylmethylaufonyl fluoride, 1 mM dithiothreitol (DTT) and 1% Triton X-100. The homogenate was transferred into Eppendorf tube and centrifuged at 10,000 × *g* for 10 min at 4 °C. The supernatant was collected and mixed with 1/4 volume of 50% trichloroacetic acid and kept in at 4 °C for more than 30 min. The mixture was followed by a centrifugation at 15,000 × *g* for 10 min at 4 °C. The precipitate was washed with acetone containing 1% DTT three times, and after that it was centrifuged and natural-dried at room temperature. The dried pellet was solubilized in sample buffer containing 7 M urea, 2 M thiourea, 6.5 mM DTT, 4% 3[(cholamidopropyl)demethylammonio]-1-propane sulphonate, 2% ampholine pH 3.5–10 (GE Healthcare Bio-Science, UK Ltd., UK). The two-dimensional electrophoresis (2-DE) was carried out according to the method of Yang et al. [36]. After electrophoresis, the gels were stained with Coomassie Brilliant Blue (CBB) R-250.

2.3. Image and data analysis

The stained gels were scanned by UMAX Power Look 2100XL scanner (UMAX Technologies Inc., Taiwan) to obtain the 2-DE images of endosperm and embryo. The image analysis was performed by Image Master 2D-platinum version 5.0 software (GE Healthcare Bio-Science, UK Ltd., UK). For each tissue, three images represent three independent biological replicates and were grouped as a class to calculate the averaged volume of all the individual protein spots. The abundance of each detected spots was calculated as relative intensity (each spot volume value was divided by the sum of total spot volume values to obtain individual relative spot volumes). Only the spots abundance which change more than 2-fold between embryo and endosperm and pass the Independent-Samples T Test by employing SPSS software (SPSS Inc., USA) ($p < 0.05$) were selected for protein identification.

2.4. Protein identification

Differential protein spots were excised from the 2-DE gels manually and incised into small pieces. Protein digestion was performed basing on Shen et al. [37] with minor modification. Each small protein gel piece was discolored twice with 50 mM NH_4HCO_3 in 50% (v/v) methanol for 1 h at 40 °C. And then was reduced with 10 mM ethylenediaminetetraacetic acid (EDTA), 10 mM DTT in 100 mM NH_4HCO_3 for 1 h at 60 °C and incubated with 10 mM EDTA, 40 mM iodacetamide in 100 mM NH_4HCO_3 for 30 min at room temperature in the dark. The resulting gel pieces were minced, lyophilized and digested in 25 mM NH_4HCO_3 with 10 ng of sequencing grade modified trypsin (Promega, Madison, USA) at 37 °C overnight. After digestion, the protein peptides were collected, followed the digested gels were washed three times with 0.1% trifluoroacetic acid (TFA) in 50% acetonitrile to collect the remaining peptides. The peptides were desalted by ZipTipC 18 pipet tips (Millipore, Bedford, USA) and cocrystallized with 1 vol of saturated R-cyano-4-hydroxycinnamic acid in 50% (v/v) acetonitrile containing 1% TFA.

The desalted protein samples were subject to LTQ-ESI-MS/MS (Thermo-Finnigan, San Jose, USA), using a surveyor high performance liquid chromatography (HPLC) system. LC–MS/MS analysis was performed according to Yang et al. [36].

As Yang et al. described previously, by using the Turbo SEQUEST program in the BioWork 3.1 software suite (Thermo-Finnigan, San Jose, USA) and searching the NCBInr database (as of April 2010), the initial results of differential proteins can be obtained. Peptide hits were accepted when singly, doubly and triply charged peptides with Xcorr > 1.9, 2.2 and 3.75, respectively; and deltaCn > 0.1 in all cases. After the peptide sequence raw data was searched using SEQUEST, a number of other criteria were considered in the final assignment of peptide and protein identifications: the number of matching peptides, the coverage, the Xcorr, and the molecular weight (MW) and isoelectric point (pI) of the protein.

3. Results

3.1. Protein profiles in endosperm and embryo

To compare the difference between endosperm and embryo of dry mature *J. curcas* seed, 2-DE of endosperm and embryo were performed respectively. By staining with CBB and through scanning with UMAX scanner, general protein patterns from 2-DE gels in the range of pHs 4–7 were built for each tissue (Fig. 1). As can be seen from Fig. 1, the spot pattern of embryo was similar to that of endosperm according to the protein distribution, MW and pI. Taking the 2-DE profiles of endosperm as a reference, individual gel spot was compared by ImageMaster 2D Platinum software. It is found that, in the range of 97–12 kDa, there were obviously differential 66 protein spots with varying abundance between endosperm and embryo of dry mature *J. curcas* seeds.

3.2. Protein identification and functional categorization

28 spots were successfully identified from 66 selected spots through LTQ-ESI-MS/MS and NCBI database searching (Table 1, Fig. 1). According to the protein function [38], these differential proteins can be categorized into 9 classes: metabolism (25%), energy (7%), transcription (7%), protein synthesis (7%), cell structure (11%), signal transduction (4%), disease/defence (18%), secondary metabolism (7%), and unclassified (14%) (Table 1, Fig. 2). The per-

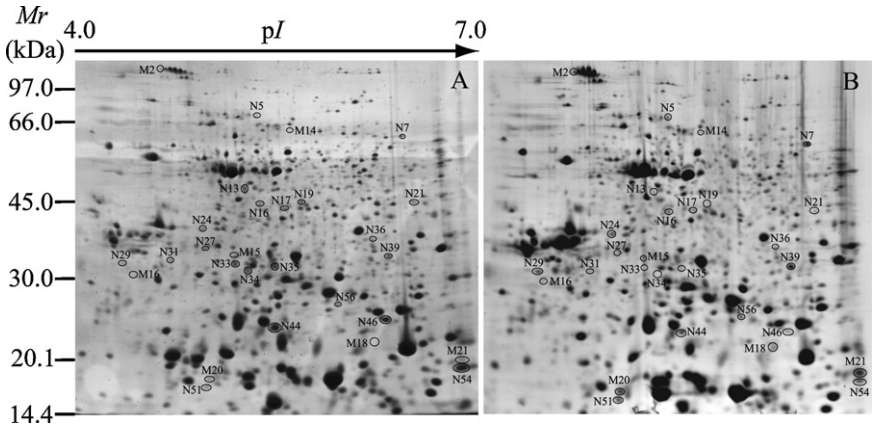

Fig. 1. The 2-DE maps of endosperm and embryo from dry mature *J. curcas* seeds. (A) Endosperm; (B) Embryo. Circles indicate the 28 proteins identified by LC–MS/MS, which changed in abundance more than 2-fold between endosperm and embryo. N stands for the differential proteins detected in endosperm; M stands for the differential proteins detected in embryo. This is a representative figure from three biological replicates.

centage in parenthesis represents the proportion of each protein category.

3.3. Protein abundance in endosperm and embryo

There are protein spots (N13, N17, N19, N21, N27, N31, N33, N34, N35, N36, N44, N46 and N54) with higher abundance in the endosperm than in embryo while there are 15 protein spots (N5, N7, N16, N24, N29, N39, N51, N56, M2, M14, M15, M16, M18, M20, M21) having higher abundance in the embryo (Figs. 3 and 4). According to the functional classification, the proteins related to energy (spot N24, M14), transcription (spot M18, M20), signal transduction (spot M16) and secondary metabolism (spot N7, N39) have higher abundance in embryo, whereas cell structure related proteins (spot N13, N27, N35) have higher abundance levels in endosperm (Table 1, Figs. 3 and 4).

4. Discussion

J. curcas is an oil plant as Arabidopsis and Brassica. However, little is known about the protein files in *J. curcas* seeds, especially the types of proteins in the endosperm and embryo tissues. In tomato, the 2-DE profiles of endosperm and embryo of dry mature seeds were similar to each other [34]. In this study, the endosperm of *J. curcas* dry mature seed also displayed similar 2-DE profiles to the embryo, indicating the similar protein types and distributions for the two tissues. In *J. curcas*, embryo and endosperm are formed from individual fertilization events of double fertilization, a key process of sexual reproduction in higher plants. Embryo is produced when one sperm nucleus fuses with the egg, while endosperm is formed when a second sperm nucleus fuses with two polar nuclei [9]. In this case, our results may reflect that the origin of endosperm was evolved from an altruistic twin embryo in *J. curcas* seeds, as other oil plant seeds [7]. Besides, our results may suggest the fact that functions of endosperm and embryo are only related to the ploidies instead of morphology.

At the late seed development, different sets of mRNAs can accumulate during seed desiccation and dormancy that are essential for seed viability and germination [39], but the actual functions of their protein products are not always confirmed [40]. In the mature embryo of *J. curcas*, the proteins associated with transcription and protein synthesis are identified as regulator of ribonuclease-

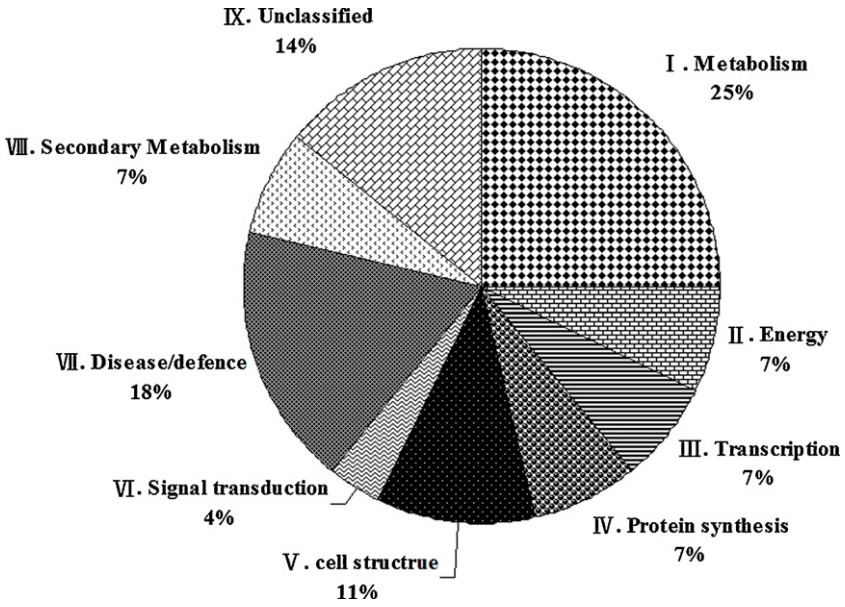

Fig. 2. The functional proportion of identified proteins from endosperm and embryo of dry mature *J. curcas* seed. A total of 28 identified differential proteins were assigned to the functional categories. The Roman number in each category is corresponding to the functional category described in Table 1. The percentage represents the proportion of each category.

Table 1
Differential proteins between endosperm and embryo from dry mature *J. curcas* seeds.

Spot no.	Mr(Da)/pI	*P*	SC (%)	Xcorr	Description	Functional categories	Accession no.	Peptide identified by LTQ-ESI-MS/MS
N16↑	46846.56/6.37	5	15.49	4.5066	Dihydroorotate dehydrogenase family protein	I	NP_188408	R.AFDEGWGAVIAK.T 2
N17↓	45237.39/6.29	2	6.08	3.4312	Stearoyl-acyl carrier protein desaturase	I	NP_197128	R.ATFISHGNTAR.L 2
N19↓	50396.25/7.02	3	6.72	3.4811	LL-diaminopimelate aminotransferase, chloroplastic	I	NP_567934	R.EQLTQLVEFAK.K 2
N21↓	45552.68/5.92	2	7.60	4.9157	Beta-ureidopropionase	I	NP_201242	R.YKDGLLISDMDLNLCR.Q 2
N31↓	31928.4/5.19	1	4.24	4.4989	Lactoylglutathione lyase/metal ion binding	I	NP_172648	K.DPDGYTFELIQR.G 2
N34↓	39141.83/8.74	1	5.82	2.7035	Pectinesterase	I	NP_177152	K.GLDPALVAAEAAPRIINVNPK.G 2
M15↑	32028.2/5.93	2	7.42	2.7497	Phytanoyl-CoA dioxygenase	I	NP_565262	K.ISFFFEEK.A 2
N24↑	39176.03/5.67	2	8.82	3.9314	Pyruvate dehydrogenase E1 component subunit beta, mitochondrial	II	NP_199898	K.SNYM*SAGQINVPIVFR.G 2
M14↑	47719.17/5.54	3	8.56	4.8536	Enolase	II	NP_181192	K.YGQDATNVGDEGGFAPNIQENK.E 2
M18↑	17566.88/5.34	1	5.42	3.3814	Regulator of ribonuclease-like protein 3	III	NP_200437	K.VFEDNVLVR.N 2
M20↑	17566.88/5.34	1	5.42	3.6639	Regulator of ribonuclease-like protein 3	III	NP_200437	K.VFEDNVLVR.N 2
N44↓	25728.54/4.92	2	7.08	3.1339	Eukaryotic translation initiation factor subunit K	IV	NP_195051	R.LYQFEPER.M 2
M2↑	99377.37/7.53	1	0.68	2.0917	Polynucleotide adenylyltransferase family protein	IV	NP_190452	K.YNLAEK.L 1
N13↓	49800.19/4.92	3	12.00	5.1201	Tubulin alpha-1 chain	V	NP_176654	R.FDGAINVDITEFQTNLVPYPR.I 2
N27↓	49654.22/4.95	3	10.89	5.4265	Tubulin alpha-3/alpha-5 chain	V	NP_197478	R.FDGAINVDITEFQTNLVPYPR.I 2
N35↓	41735.83/5.31	4	14.06	3.7866	Actin-7	V	NP_196543	K.NYELPDGQVITIGAER.F 2
M16↑	29161.83/4.71	6	22.78	4.5256	14-3-3-like protein	VI	NP_565176	R.YEEMVEFMEK.V 2
N5↑	68356.61/5.22	2	4.38	4.4778	Early-responsive to dehydration 2	VII	NP_176036	K.ATAGDTHLGGEDFDNR.M 2
N36↓	36036.25/4.69	4	14.46	4.2659	Late embryogenesis abundant family protein	VII	NP_181934	K.VDLIVDVPVLGR.L 2
N46↓	22915.04/6.06	2	7.80	4.9711	IgE-binding protein MnSOD	VII	CAC13961.1	R.LVVETTANQDPLVTK.G 2
N51↑	16746.99/4.94	3	10.13	3.2554	Disease resistance response protein	VII	P14710	K.SIEIVEGNGGAGTIKK.L 2
N56↑	26891.6/6.25	1	6.22	5.0265	Superoxide dismutase	VII	NP_191194	R.LVVETTANQDPLVTK.G 2
N7↑	61773.02/6.26	2	5.04	2.7483	Delta-1-pyrroline-5-carboxylate dehydrogenase, mitochondrial	VIII	NP_568955	R.GAGIGTPEAIK.L 2
N39↑	33216.32/5.79	7	22.01	5.1171	Pyridoxal biosynthesis protein	VIII	NP_195761	R.IAEEAGACAVMALER.V 2
N29↑	33655.46/5.67	1	3.55	3.7838	Probable plastid-lipid-associated protein 2, chloroplastic	IX	NP_193955	R.GDGGSVFVLIK.E 2
N33↓	32643.1/5.46	2	7.56	2.589	Hypothetical protein	IX	CAB09799.1	R.GPTPEPLCQVM*LR.V 2
N54↓	25085.8/7.51	1	5.94	2.4737	Uncharacterized protein	IX	Q9M2A4	-.MVNPTISDDLLAK.I 2
M21↑	14752.74/8.21	1	5.43	2.0289	Protein yippee-like	IX	NP_566389	K.YEFAFEK.N 1

N: The protein spots are selected from endosperm; M: The protein spots are selected from embryo; The arrows indicate the protein spots in embryo either increase↑ or decrease↓ in abundance; The MW and pI are theoretical; P: number of unique matched peptides; SC: sequence coverage; Xcorr: SEQUEST cross-correlation score of the peptide (for each protein, only the peptide with the highest Xcorr is presented); Functional categories were decided by the system in Bevan et al. [38]. The Roman Number represents the protein function category respectively: (I) Metabolism; (II) Energy; (III) Transcription; (IV) Protein synthesis; (V) Cell structure; (VI) Signal transduction; (VII) Disease/defence; (VIII) Secondary metabolism; (IX) Unclassified; Numbers (1–3) after peptide sequence in the column "Peptide identified by LTQ-ESI-MS/MS" means singly, doubly, triply charged state, respectively; All the protein identities were from searching in NCBInr database.

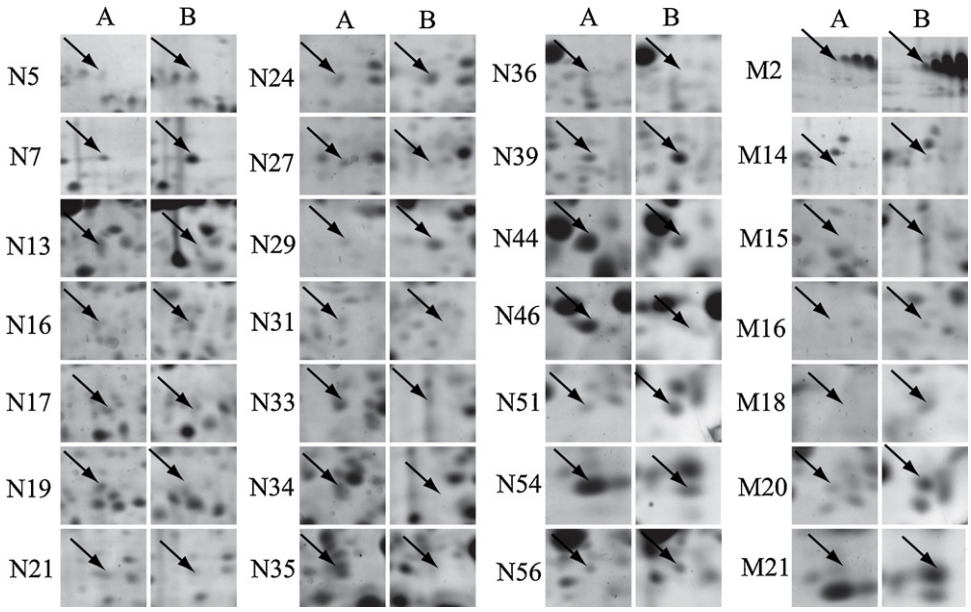

Fig. 3. Enlarged area showing distribution of the 28 differentially protein spots in Fig. 1. (A) Representatives of the spots from endosperm; (B) representatives of the spots from embryo.

like protein (spot M18, M20), polynucleotide adenylyltransferase family protein (spot M2), and a subunit K of eukaryotic translation initiation factor (spot N44). Protein spot M2 belongs to polynucleotide adenylyltransferase family and is favorable for the translation. Spots M2, M18 and M20 are few in the endosperm (Figs. 1 and 3) while N44 has an obvious abundance in the endosperm (Table 1, Figs. 3 and 4). This is in good agreement with the higher levels of transcription in embryo. Signal transduction related protein 14-3-3 (spot M16) has significant abundance in embryo (Table 1, Figs. 3 and 4). The 14-3-3 proteins are large family of approximately 30 kDa acidic proteins which are found in all eukaryotic cells where they function as conserved regulators of diverse signaling proteins including kinases, phosphatases, and transmembrance receptors [41,42]. In this respective, our results may suggest that the embryo from *J. curcas* has the ability to carry out signal transduction for seed germination in response to environment stimulus. In addition, the energy related proteins pyruvate dehydrogenase E1 component subunit beta (spot N24) and enolase (spot M14), which are previously shown to involve in citric acid cycle and play an important role in oil mobilization during the seed germination and post germination [36], also have been detected in endosperm and embryo of dry mature *J. curcas* seeds. Moreover, delta-1-pyrroline-5-carboxylate dehydrogenase (spot N7) and pyridoxal biosynthesis protein (spot N39) are related to the secondary metabolism, and both of them have higher abundance in embryo. Taken together, all of the results described above suggested that some proteins required for the seed germination have already been present in the endosperm and embryo of the dry mature *J. curcas* seeds.

In the seed dry state, the cell cycle in G1 phase is arrested, leading to the suppression of most metabolic activities in the seed [43]. In plants, tubulin and actin as the main components of micro-

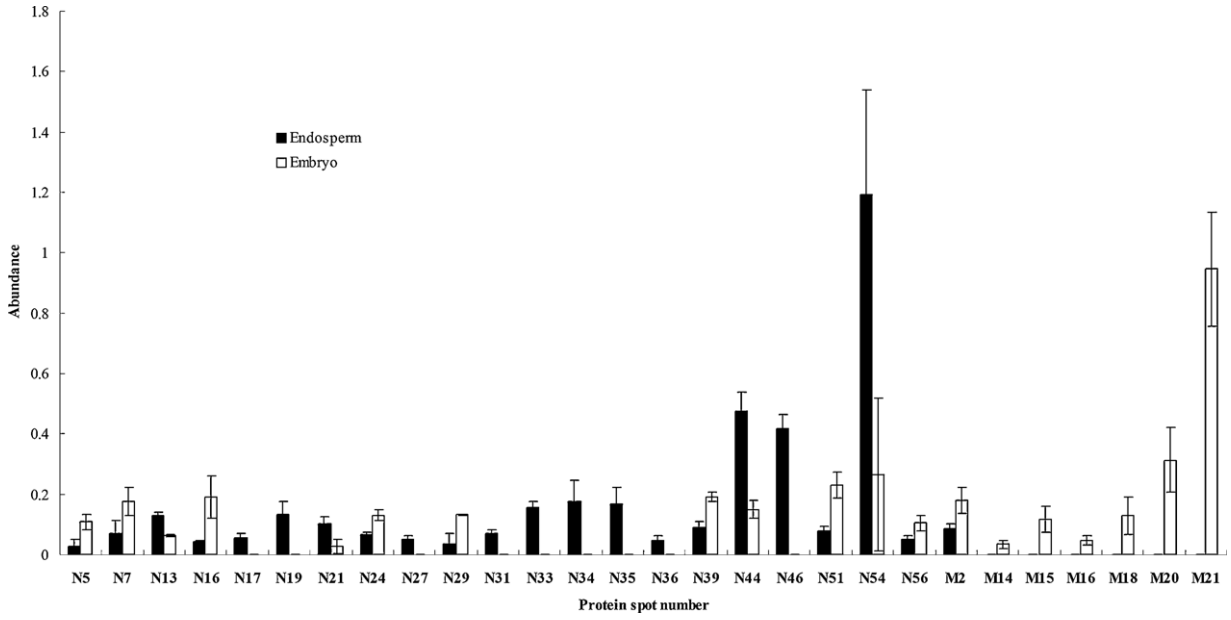

Fig. 4. Abundance of the 28 differential proteins in Fig. 1. For each spot, each value is from an average of three independent biological replicates (±SD). Spot numbers are corresponding to the number described in Table 1, Figs. 1 and 3.

tubules are associated with the most embryonic radicle cell in the G1 phase of cell cycle [44,45]. The comparative proteomic analysis of dormant with non-dormant dry Arabidopsis seeds revealed that accumulation of tubulin proteins in non-dormant germinating seeds is an important determinant of completion of germination and growth initiation [33]. In the present work, there are 3 differential proteins identified as α-tubulin (spots N13, N27) and actin (spot N35) in endosperm and embryo, most interesting, all these three proteins have a significant higher abundance in endosperm (Figs. 1, 3 and 4). Endosperm has been reported to be an important tissue as nutrient reserve for next generation for seed germination and promoting early growth of the embryo until sufficient reserves are stored in the cotyledons [7]. Ultrastructure of endosperm cell of *J. curcas* in the germination process revealed that the endosperm played the nutrient role and was broken down eventually [36]. In the case of an economic system in plant, cell division related to proteins may be useful for the growth of embryo. Therefore, our results suggest that the proteins which are necessary for the mitotic events and cell divisions have existed in dry mature *J. curcas* seeds. Besides, as the main tissue for seed germination, the endosperm has prepared more proteins for the mitotic events and cell divisions in dry mature seeds and these proteins might be transferred to embryo for growth in the seed germination of *J. curcas*. However, the transfer mechanism is still unknown.

The stress-related proteins did not distribute evenly throughout the seed tissues. Proteins of late embryogenesis abundant (LEA) (spot N36) and IgE-binding protein manganese superoxide dismutase (MnSOD) (spot N46) have higher abundance in endosperm while proteins of early-responsive to dehydration (ERD) (spot N5), disease resistance response (spot N51) and superoxide dismutase (SOD) (spot N56) have higher abundance in embryo. Different with the abundance of N5, N51 and N56 in endosperm, the abundance of N36 and N46 are nearly bare in embryo while are obvious in endosperm (Figs. 1, 3 and 4). LEA proteins belong to the dehydrin superfamily and have been shown to be located in many cell types at variable concertrations [46]. The functions of these proteins are involved in abiotic stress including cold, heat, salt and drought tolerance in many plant species [47–49]. ERD proteins are members of the dehydrin family that response to abiotic stresses related to dehydration, such as drought, high salinity and low temperature in which these proteins protect cells against the consequences of water stress [50–52]. In Arabidopsis, ERD protein is proposed as a novel mediator of stress-related ABA signaling in Arabidopsis [50]. SODs are found with different metal cofactors used for catalysis in all subcellular locations [53]. Within a cell, SODs constitute the first line of defence against reactive O_2 species (ROS) [54], of which MnSOD is the principal antioxidant enzyme [55]. By this token, our results may suggest that both of the embryo and endosperm of *J. curcas* seeds have the mechanism in response to abiotic stresses and endosperm have accumulated more LEA proteins in the seed desiccation to sense dehydration.

Storage proteins are accumulated in seeds during the late stage of seed development [18]. However, few storage proteins were identified from endosperm and embryo of dry mature *J. curcas* seeds in this study. One of reasons could be the limitation of MS identification. Because the genomic database of *J. curcas* is not currently available in the public, we generally use genomic databases of other oil or woody plant such as Arabidopsis, *Ricinus communnis* and Populus to search proteins for *J. curcas* analysis. As a result, we identified fewer proteins than expected. For example, protein spot N54 has significant abundance for protein identification, but it is still identified as an unknown protein for *J. curcas* seeds. Another reason could be that there are little differences of the contents of storage protein with the same MW between these two tissues, though the endosperm and embryo of *J. curcas* dry mature seed have great contrast based on the morphology and weight.

In conclusion, we have shown by proteomic analysis that 66 differential proteins occurred between the two seed tissues, of which 28 proteins were successfully identified. These proteins are functioned as metabolism, transcription, cell structure, signal transduction and so on. They are essential for the seed germination and have occurred in endosperm and embryo, reflecting the fact that some proteins required for germination are already present in the dry mature seed.

Acknowledgements

This work was supported by the Knowledge Innovation Program of the Chinese Academy of Sciences (KSCX2-YW-G-035, KSCX2-YW-G-027-2) and the National Natural Science Foundation of China (U0733005, 30972333).

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