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Proteomic analysis of embryonic axis of *Pisum sativum* seeds during germination and identification of proteins associated with loss of desiccation tolerance

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ABSTRACT

Seed germination is an important stage in life cycle of higher plants. The germination processes and its associated loss of desiccation tolerance, however, are still poorly understood. In present study, pea seeds were used to study changes in embryonic axis proteome during germination by 2-DE and mass spectrometry. We identified a total of 139 protein spots showing a significant (>2-fold) change during germination. The results show that seed germination is not only the activation of a series of metabolic processes, but also involves reorganization of cellular structure and activation of protective systems. To uncouple the physiological processes of germination and its associated loss of desiccation tolerance, we used the fact that pea seeds have different desiccation tolerance when imbibed in water, $CaCl_2$ and methylviologen at the same germination stage. We compared the proteome amongst these seeds to identify the candidate proteins associated with the loss of desiccation tolerance and found a total of seven proteins – tubulin alpha-1 chain, seed biotin-containing protein SBP65, P54 protein, vicilin, vicilin-like antimicrobial peptides 2–3, convicilin and TCP-1/cpn60 chaperonin family protein. The metabolic function of these proteins indicates that seed desiccation tolerance is related to pathogen defense, protein conformation conservation and cell structure stabilization.

1. Introduction

The seed occupies a central position in the life cycle of higher plants, where seed formation represents the beginning of the next generation. After maturation, the orthodox seed dries to a water content of 5–15% depending on the environmental humidity. For germination, the seed needs to take up water to initiate a series of complex metabolic processes. The water uptake is triphasic, including an initial rapid period (phase I), followed by a plateau phase with a little water uptake (phase II), and a further increase in water content (phase III) coincident with protrusion of radicle and seedling growth [1]. Germination during phases I and II of water uptake is commonly considered as germination in the strict sense, i.e. germination *sensu stricto* [2–4]. Optimal seed germination is a prerequisite for successful seedling establishment and plant vigor [5,6].

Seed germination is accompanied by loss of desiccation tolerance. Desiccation tolerance refers to the ability of organisms to survive loss of 80 to 90% of protoplasmic water to reach <0.3 g H_2O g^{-1} dry matter [7,8]. Desiccation tolerance is acquired during seed maturation, approximately halfway through development [9,10]. This property ensures that the seed passes unharmed through maturation drying and retains viability in the dry state for long periods of time under natural conditions. When mature

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seeds are imbibed and subsequently dehydrated, survival decreases gradually with increasing imbibition time, that is, desiccation tolerance is lost during seed germination [11–15]. In nature, seeds may experience several imbibition–desiccation cycles during germination due to fluctuations in soil moisture. Before the point at which imbibition has lead to a loss of desiccation tolerance, these cycles can give a natural priming effect to enhance seed germination, seedling establishment and growth, in a manner similar to the well-studied commercial process of seed priming in which seeds are hydrated and then dehydrated to standardize their germination characteristics [6,16,17]. However, after the point at which desiccation tolerance is lost, the imbibition–desiccation cycles will decrease seed germination and seedling formation. Thus, understanding seed germination and its associated loss of desiccation tolerance will be important for optimizing crop yield.

As the availability of genomic sequence data and EST sequences increases for many plant species and with the significant improvements in protein isolation and separation methods and in instrumental sensitivity and accuracy, proteomic approaches have opened up enormous possibilities to analyze complex functions in plant biology like seed dormancy, germination and vigor [18,19]. Proteomic analyses have been conducted on germinating seeds from a number of species, such as *Arabidopsis thaliana* [20,21], barley [22,23], tomato [24], rice [25], sugar beet [26], alfalfa [27] and maize [28]. Many key proteins have been identified in these studies, most of which correspond to storage proteins, carbohydrate catabolism and biosynthesis, and stress-related proteins. However, most of these studies have been done on intact seeds, and the proteome of the germinating embryonic axis has not been targeted previously.

Although loss of desiccation tolerance during seed germination is a well-known phenomenon, it is not easy to distinguish these two events, germination *per se* and loss of desiccation tolerance, both occurring in parallel during germination. This is the major difficulty for the application of 'omics' methods to identify possible genes and proteins related to desiccation tolerance [15,29]. Some physiological models have been developed to overcome this difficulty. Re-establishment of desiccation tolerance in germinating seeds by application of a mild osmotic stress with PEG solution to the imbibed seeds is one such model. This was first reported for *Cucumis sativus* and *Impatiens walleriana* seeds by Bruggink and van der Toorn [30], and it has since been successfully applied to many species, like *Medicago truncatula* [15] and *A. thaliana* [31]. Recently, we found that pea (*Pisum sativum*) seeds imbibed in $CaCl_2$ or methylviologen (MV, an oxidative stress-inducing chemical) had higher and lower desiccation tolerance, respectively, at the same imbibition periods of time compared to seeds imbibed in distilled water [32]. Using this method, it is possible to obtain the seeds with different desiccation tolerance at the same germination stage. In other words, germination and desiccation tolerance events can be uncoupled.

Pea is one of the most cultivated crop species in world, and the seed of this species is so large that their embryonic axis can easily be excised. The completion of genome sequencing of the legume model species *M. truncatula* (www.medicago.org) and access to large amounts of EST data for *P. sativum* (21837 in NCBI database in April, 2012) make the present study of the pea seed proteome feasible. Bourgeois et al. [33] presented the first proteome reference map for mature pea seeds. Recently, a proteomic study was conducted to understand the role of H_2O_2 during pea seed germination [34]. In the present study, we investigated first the changes in the embryonic axis proteome during seed germination and then compared the axis proteomic difference of seeds imbibed in distilled water to $CaCl_2$ and to MV by two-dimensional electrophoresis (2-DE) and mass spectrometry (MS). It was the aim to identify proteins related to seed germination and its associated loss of desiccation tolerance in embryonic axis of pea seed.

2. Materials and methods

2.1. Plant material

Pea (*P. sativum* L. cv. Jizhuang) seeds were obtained from Guangling Seed Company (Datong, Shanxi, China) in September, 2009, and were kept in plastic bags at 5 °C until used. The water content of the seeds was about 0.10 g H_2O (g dry weight)$^{-1}$.

2.2. Seed germination, imbibition, dehydration and survival

Three replicates of 25 pea seeds each were placed on two layers of filter paper and incubated in 40 ml distilled water in closed 15-cm diameter Petri dishes at 20 °C in darkness for up to 48 h. Radicle protrusion of 2 mm was used as the criterion of germination, and the number of germinated seeds was counted regularly throughout the test period.

Pea seeds were imbibed in distilled water for 18, 25 and 36 h or in treatment solution (20 mM $CaCl_2$ or 2 mM MV) for 25 h at 20 °C in darkness, and then dehydrated by burying them in activated silica gel (1:8 (v:v) seeds : silica gel) within a closed plastic box. Seeds imbibed for 18 h and 36 h were dehydrated for 24 and 36 h, respectively. Seeds imbibed in distilled water and different treatment solutions for 25 h were dehydrated for 0, 12, 22, 30 and 36 h to determine whether the drying rate affects desiccation tolerance. Dry un-imbibed seeds were used as control (CK). These seeds were germinated on two layers of filter paper moistened with 40 ml of distilled water in Petri dishes (15-cm diameter) at 20 °C in darkness for 5 days to measure seed survival and seedling weight. Seeds showing a normal epicotyl were considered as being alive.

2.3. Determination of water content

The water content of seeds was determined according to the method of International Seed Testing Association [35], and expressed on a dry mass basis (g H_2O (g dry weight)$^{-1}$, g g^{-1}).

2.4. Preparation of protein samples

The total soluble proteins were extracted using a phenol extraction procedure according to Hurkman and Tanaka [36]. Three replicates of 50 embryonic axes each were homogenized in 1.5 ml precooled extraction buffer composed of 50 mM Tris, 0.9 M sucrose, 10 mM ethylene glycol-bis(b-aminoethylether)-*N*, *N*,*N*′,*N*′-tetraacetic acid (EGTA), 1 mM phenylmethanesulfonyl fluoride, 1 mM dithiothreitol (DTT), and 1% (v/v) Triton X-100, adjusted to pH 7.5 with HCl. After washing the mortar with 0.5 ml extraction buffer, the total 2 ml homogenate was first

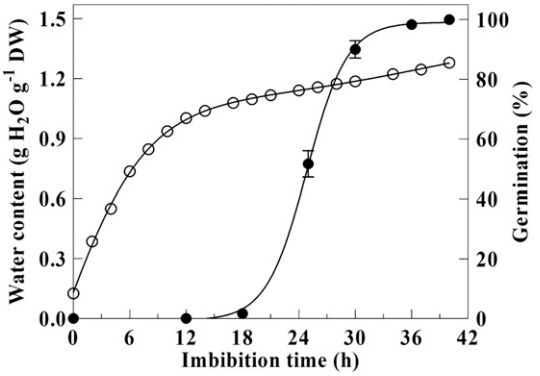

Fig. 1 – Water uptake and seed germination during imbibition. Pea seeds were imbibed at 20 °C in darkness and were taken out every 2–3 h to determine the seed water content. Open circle, water content; filled circle, seed germination. Data are means ± SE (n = 3). DW, dry weight.

centrifuged at 16 000 *g* for 10 min, and the pellet was discarded. The supernatant was centrifuged at 32 000 *g* for 20 min at 4 °C, and the supernatant mixed with two volumes of ice-cold Tris–HCl (pH 7.5) saturated with phenol and placed on ice on a shaker for 30 min. After centrifugation at 16 000 *g* for 20 min, the phenol phase was collected and 5-volume precooled NH_4SO_4-saturated methanol added and left to precipitate overnight at –20 °C. The pellets were rinsed four times with ice-cold acetone containing 13 mM DTT and then lyophilized. The lyophilized powder was stored –20 °C until use.

2.5. 2-DE

Isoelectrofocusing (IEF) was performed using a Multiphor II horizontal electrophoresis system (Bio-Rad Hercules, CA, USA) and 17 cm Immobiline Dry Strips with a linear pH gradient of 5–8 (Bio-Rad). Protein samples were loaded onto the strips and soaked in re-hydration solution, containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 20 mM DTT, and 0.5% (v/v) immobilized pH gradient (IPG) buffer (pH 5–8) for 16 h at 20 °C. IEF was then performed by applying a voltage of 250 V for 1 h, ramping to 500 V over 1 h, 2000 V for 2 h, 10 000 V for 4 h and holding at 10 000 V until a total of 60 kVh was reached. Prior to the second dimension, the gel strips were equilibrated for 15 min in equilibration buffer containing 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, 50 mM Tris–HCl (pH 8.8), 0.01% (w/v) bromophenol blue with 1% DTT or 2.5% (w/v) iodoacetamide, respectively. After equilibration, the strips were applied to vertical SDS-polyacrylamide gels (12% resolving and 5% stacking) and sealed with 0.5% (w/v) low-melting agarose in SDS buffer containing 0.01% bromophenol blue. Low molecular range markers (Bio-Rad) were loaded beside the strips before sealing. Electrophoresis was performed at 15 °C in SDS electrophoresis buffer (pH 8.3), containing 25 mM Tris base, 192 mM glycine and 1% (w/v) SDS, for 30 min at 25 mA and for 4 h at 40 mA. Gels were stained overnight with 0.25% (w/v) Coomassie brilliant blue R-250 (CBB) in 5:1:4 (v/v) methanol: acetic acid: water and destained with 2:1:7 (v/v) methanol: acetic acid: water solution with several changes, until a colorless background was achieved.

2.6. Image analysis, in-gel digestion with trypsin, and protein identification by MALDI-TOF-TOF mass spectrometry

The 2-DE gels were scanned at a 300 dpi resolution with a UMAX Power Look 2100XL scanner (Maxium Tech., Taipei, China). Spot detection and gel comparison were made with ImageMaster 2-D Platinum, version 5.01 (GE Healthcare Bio-Science, Little Chalfont, UK).

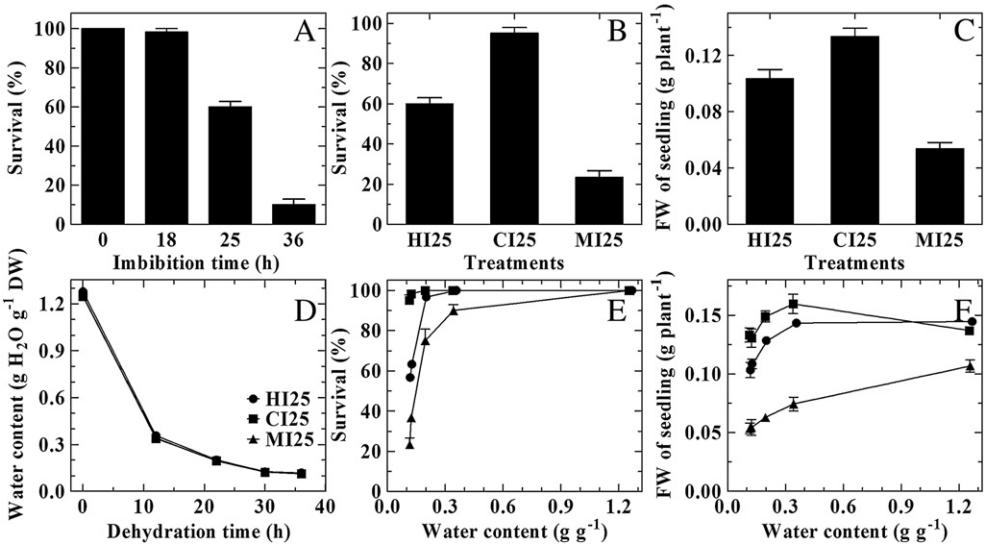

Fig. 2 – Change in survival of imbibed seeds after dehydration and in seed water content, survival and fresh weight (FW) of seedling during dehydration. A, survival of seeds that were imbibed in distilled water for 0, 18, 25 and 36 h and then dehydrated. B and C, survival (B) and FW of seedling (C) of seeds imbibed in distilled water (HI25), $CaCl_2$ (CI25) and MV (MI25) solution for 25 h and then dehydrated. D, E and F, seeds imbibed in distilled water (HI25), $CaCl_2$ (CI25) and MV (MI25) solution for 25 h were dehydrated for 0, 12, 22, 30 and 36 h. Seed water content (D), survival (E) and FW of seedling (F) were determined for each dehydration time. Seed survival and FW of seedling were tested at 20 °C after 5 days. Data are means ± SE (n = 3).

Most (90%) of the differentially accumulated protein spots were excised from stained gels of HI36 seeds, the remaining 10% from other gels. In-gel digestions and tryptic peptide extractions was performed according to Shevchenko et al. [37] with a little modification. Each small gel piece with protein was washed and destained using a series of washes consisting of 50 μl of deionized water, 50 μl of 50% acetonitrile (Fisher Scientific, Fair Lawn, NJ, USA) and 50 μl of 100% acetonitrile at room temperature. The protein in the gel piece was reduced with 10 mM DTT in 100 mM NH_4HCO_3 for 45 min at 56 °C, and then incubated with 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min at room temperature in darkness. After a second series of washes mentioned above, the gel pieces were rehydrated in 50 mM NH_4HCO_3 with 5 ng sequencing grade modified trypsin (Promega, Madison, WI, USA) on ice for 45 min and then incubated overnight at 37 °C. After digestion, the supernatant from each sample was recovered and the remaining peptides were then sequentially extracted using 5 μl of 5% trifluoroacetic acid (TFA) followed by 50 μl of 50% acetonitrile with 2.5% TFA. Each sample was sonicated for 5 min before removing the supernatant. All supernatants were combined from each extraction. Samples were spotted on a MALDI target plate and immediately spotted on top with 0.5 μl of saturated matrix [10 mg ml^{-1} recrystallized α-CHCA (α-cyano-4-hydroxy cinnamic acid, Sigma) in 70% (v/v) acetonitrile with 0.1% (v/v) TFA] and dried completely. Samples were then subjected to MALDI MS/MS analysis using autoflex III smartbeam (Bruker, Germany).

The peptide mass fingerprints (PMFs) obtained were searched against plant data in the Swissport or NCBInr database using MASCOT software (Matrix Science, London). The following search parameters were applied: The protein sequence database used was Swissprot or NCBI; a mass tolerance of 70 ppm and one incomplete cleavage were allowed; acetylation of the N-terminus, alkylation of cysteine by carbamidomethylation, and oxidation of methionine were considered as possible modifications.

2.7. *Statistics*

Three biological replicates for each treatment were run separately on 2-D gels. Gel maps were compared among seeds imbibed in distilled water for different periods of time and between seeds imbibed in $CaCl_2$ or MV and in distilled water using Image Master 2-D platinum. Spots were considered reproducible when they were well resolved in the three biological replicates or at least in two of them. The normalized volume of each spot was assumed to represent its expression abundance. Differentially accumulated proteins were chosen based upon the criterion that the average volume of the protein spots showed a significant change ($P<0.05$) of more than 2-fold in at least one treatment as evaluated by Image Master 2-D platinum using the Student's t-test.

3. Results

3.1. *Seed germination during imbibition*

The seed water content increased rapidly during the first 12 h of imbibition (phase I), it then entered a phase of slower water uptake (phase II), and finally the rate of water uptake started increasing very slightly again around 30 h (Fig. 1, open circles). There was no distinct quick increase in water uptake (phase III, see [1]). The first pea seeds germinated after 18 h of imbibition and all the seeds had germinated after 36 h (Fig. 1, filled circles).

3.2. *Seed desiccation tolerance during imbibition and the effect of $CaCl_2$ and MV*

When imbibed pea seeds were dehydrated, survival was 100% until 18 h of imbibition, but after that time seed survival decreased rapidly with increasing imbibition time. Only about 10% of the seeds survived dehydration after 36 h imbibition (Fig. 2A). The survival increased to 95% and decreased to 20% when the seeds were imbibed for 25 h in $CaCl_2$ (CI25) and MV (MI25), respectively, compared to the 60% survival observed after imbibition in water for 25 h (HI25) (Fig. 2B). At the same time, the growth of seedling was quicker and slower in CI25 and MI25 seeds than in HI25 seeds, respectively (Fig. 2C).

Seeds imbibed in distilled water, $CaCl_2$ or MV solution for 25 h showed identical changes in water content over the 36 h dehydration treatment (Fig. 2D). Thus, the difference in desiccation tolerance among HI25, CI25 and MI25 seeds (Fig. 2B and C) did not result from differences in the dehydration rate.

When CI25 and MI25 seeds were moved to distilled water for testing survival and seedling growth before dehydration, they both showed 100% survival (Fig. 2E), but in MI25 seeds the fresh weight of seedling was decreased (Fig. 2F). Thus, imbibition in

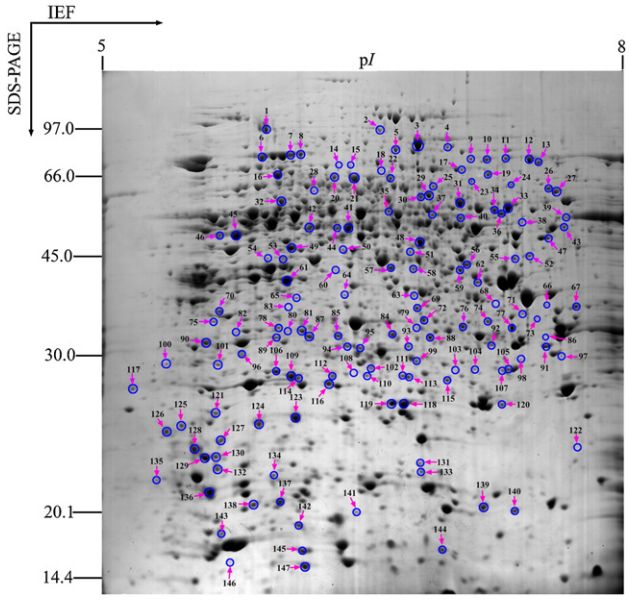

Fig. 3 – Gel map of identified differentially accumulated proteins. This 2-D gel is representative image of the Commassie Brillant Blue (CBB) stained gel from HI36 embryonic axis. A total of 600 μg of proteins were extracted and separated by 2-DE, and visualized with CBB R-250. The circled spots changed in volume by at least 2 fold during imbibition and/or with treatment (CI25 and MI25). The spots were excised, proteins were extracted and finally identified by MS/MS.

Table 1 – Differentially expressed proteins in germinating pea embryonic axis identified in spots on 2D gels (Fig. 3 and Supplemental Fig. S1). The selected spots changed their spot volume significantly ($P<0.05$) by at least a factor two during imbibition in distilled water, $CaCl_2$ or MV solutions.

Biological processes	Spot ID	Identified protein	Accession no.	Mascot score	Sequence coverage (%)	No. of sequenced/ matched peptides	Experimental protein mass (kDa)/pI	Theoretical protein mass (kDa)/pI
Metabolism								
Amino acid	3	Methionine synthase	XP_003624679	863	19	6/24	77/6.8	83.418/5.87
	23	Branched-chain amino acid aminotransferase-like protein	CAD70620	170	13	2/11	66/7.1	62.267/7.23
	27	Threonine synthase	XP_003597553	783	16	6/21	64/7.6	59.178/8.42
	33	Serine hydroxymethyltransferase	XP_003593836	826	38	7/23	60/7.3	52.049/6.84
	34	Serine hydroxymethyltransferase	XP_003593836	228	18	2/12	59/7.3	52.049/6.84
	47	Ornithine aminotransferase	ABZ10818	536	36	5/18	54/7.6	51.664/7.90
	49	S-adenosylmethionine synthase	A4ULF8	827	31	6/15	51/6.1	43.588/5.77
	50	Aminomethyltransferase, putative	XP_002509611	82	4	1/3	51/6.4	43.563/5.82
	52	Chorismate synthase, chloroplastic-like	XP_003556230	230	14	2/6	50/7.5	47.501/7.63
	72	Cysteine synthase	XP_003591036	79	14	1/4	39/6.8	26.371/6.02
	76	Cysteine synthase	XP_003608087	251	15	3/9	38/7.1	39.359/8.06
Lipid	56	Acetyl-CoA acetyltransferase, cytosolic 1-like	XP_003545555	460	24	2/9	48/7.1	41.93/6.16
	69	Cycloartenol-C-24-methyltransferase	XP_003599380	441	20	4/11	40/6.8	38.723/6.04
Nitrogen and sulphur	57	Glutamine synthetase root isozyme A	P07694	1160	58	8/24	47/6.6	39.435/6.12
	75	Mercaptopyruvate sulfurtransferase-like protein	AES84881	293	18	4/14	38/5.6	42.081/6.37
Sugars	2	Sucrose synthase	CAA09910	628	21	7/20	81/6.6	92.843/5.85
	10	Probable rhamnose biosynthetic enzyme 3	Q9LH76	302	19	3/16	72/7.2	75.437/6.11
	11	Probable rhamnose biosynthetic enzyme 1	Q9SYM5	459	16	5/14	72/7.3	75.837/6.84
	12	Probable rhamnose biosynthetic enzyme 1	Q9SYM5	552	19	5/15	72/7.5	75.837/6.84
	59	GDP-mannose 4, 6-dehydratase	XP_003612141	665	36	5/17	47/7.0	41.392/6.54
Cofactors	81	Pyridoxal biosynthesis protein PDX1.3	Q8L940	616	30	7/19	37/6.1	33.48/5.79
Secondary metabolism	58	Cinnamyl alcohol dehydrogenase	XP_003612983	268	22	4/15	47/6.8	85.394/6.33
	117	Chalcone isomerase 4B	AAT94363	121	32	2/5	29/5.2	15.641/4.64
Energy								
Glycolysis	13	Pyrophosphate-fructose 6-phosphate 1-phosphotransferase alpha subunit	XP_003589697	605	40	6/23	72/7.5	65.692/6.22
	16	2,3-bisphosphoglycerate-independent phosphoglycerate mutase	XP_003623692	633	43	4/15	68/6.0	42.644/5.52
	30	UDP-D-glucose dehydrogenase	XP_003614035	533	39	5/20	62/6.8	53.556/6.76
	32	Putative uridine diphosphate glucose dehydrogenase	ADT91651	713	34	5/17	61/6.0	53.52/5.70
	62	Fructose-bisphosphate aldolase, cytoplasmic isozyme 2	P46257	451	42	3/12	45/7.2	38.638/6.77
	68	Fructose-bisphosphate aldolase, cytoplasmic isozyme 2	P46257	408	50	4/16	41/7.3	38.638/6.77
	109	Triose-phosphate isomerase	CAI43251	188	23	1/5	31/6.1	27.411/5.87
Pentose-phosphate pathway	5	Transketolase	XP_003605140	283	19	3/13	75/6.7	80.200/6.44
Respiration	9	ATP synthase beta subunit	CBY80071	92	7	1/4	72/7.1	50.041/5.09
	40	ATP synthase subunit alpha, mitochondrial	P05493	261	24	2/13	57/7.1	55.296/6.01

Tricarboxylic acid pathway	29	Dihydrolipoyl dehydrogenase, mitochondrial	P31023	971	50	6/27	63/6.9	53.562/6.69
	48	Isocitrate dehydrogenase [NADP], chloroplastic (Fragment)	Q40345	544	28	4/13	52/6.8	48.695/6.15
Transcription								
	4	YTH domain family protein	XP_003629923	100	6	1/4	76/7.0	74.458/6.18
	24	RNA Binding Protein	XP_003608019	114	10	2/7	65/7.4	50.101/6.72
	26	RNA Binding Protein	XP_003608019	123	10	2/7	65/7.6	50.101/6.72
	43	Putative heterogeneous nuclear ribonucleoprotein	ABY56086	110	20	1/2	56/7.7	8.424/5.52
	55 [a]	Uncharacterized RNA-binding protein C660.15-like	XP_003554766	386	24	3/12	49/7.4	39.282/7.11
	93	Transcription factor APFI-like protein	XP_003612060	140	8	2/4	35/6.8	29.391/6.06
	131	Hypothetical protein MTR_2g014360	XP_003593631	311	44	3/9	22/6.8	16.657/6.62
Protein synthesis and destination								
Protein synthesis	19	Poly(A)-binding protein	XP_003603074	215	12	2/8	69/7.2	68.655/6.18
	64	Eukaryotic translation initiation factor 2 subunit beta	P55871	285	16	3/10	43/6.4	30.666/5.55
	67	Eukaryotic translation initiation factor 3 subunit I	Q38884	187	12	2/5	41/7.8	36.651/6.50
	71	50 S ribosomal protein L1, chloroplastic	P49208	48	4	1/4	40/7.4	23.481/10.23
	138	Eukaryotic translation initiation factor 5A-3	Q9AXQ4	450	26	5/11	19/5.9	17.532/5.47
Protein folding	22	TCP-1/cpn60 chaperonin family protein	NP_197111	274	11	2/9	68/6.7	59.347/5.88
Proteolysis	91	Proteasome subunit alpha type-7	Q9SXU1	663	32	4/12	35/7.6	27.194/6.86
	121	Proteasome subunit beta type	XP_003624868	821	50	6/17	27/5.6	23.079/5.31
Storage protein								
	6	Convicilin	CAB82855	540	24	6/21	73/5.9	72.134/5.50
	7	Convicilin	CAB82855	659	31	7/23	74/6.1	72.134/5.50
	8	Convicilin	CAB82855	486	24	5/21	74/6.1	72.134/5.50
	14	Convicilin	P13915	280	20	4/13	71/6.4	67.063/6.29
	15	Convicilin	P13915	238	11	3/9	71/6.4	67.063/6.29
	17	Convicilin	CAB82855	251	12	4/11	70/7.1	72.134/5.50
	18	Convicilin	P13915	546	28	6/22	69/6.6	67.063/6.29
	28	Convicilin (Fragment)	P13919	197	19	3/8	64/6.2	46.368/5.32
	35	Convicilin	CAB82855	352	22	4/16	59/6.6	72.134/5.50
	37	Convicilin	CAB82855	389	19	4/14	58/6.9	72.134/5.50
	38	Convicilin	P13915	208	14	4/10	56/7.4	67.063/6.29
	39	Convicilin	P13915	456	28	5/20	58/7.7	67.063/6.29
	41	Provicilin (Fragment)	P02855	696	42	5/14	56/6.4	31.521/5.57
	42	Convicilin	CAB82855	326	24	5/21	56/6.2	72.134/5.50
	44	Provicilin (Fragment)	P02855	646	49	6/17	56/6.4	31.521/5.57
	45 [a]	P54 protein	CAA72090	971	28	9/25	54/5.8	55.027/6.05
	46 [a]	P54 protein	CAA72090	831	28	8/23	54/5.7	55.027/6.05
	53	P54 protein	CAA72090	325	17	4/11	49/6.0	55.027/6.05
	54 [a]	P54 protein	CAA72090	1030	28	9/25	49/5.9	55.027/6.05
	60	Convicilin	P13915	305	16	4/13	47/6.3	67.063/6.29
	61	Legumin A2	P15838	430	14	4/11	45/6.1	59.633/6.21
	65	Vicilin	P13918	561	13	6/13	42/6.1	52.257/5.39
	66	cvc, partial	CAP06315	174	14	3/11	41/7.6	62.163/5.67

(continued on next page)

Table 1 *(continued)*

Biological processes	Spot ID	Identified protein	Accession no.	Mascot score	Sequence coverage (%)	No. of sequenced/ matched peptides	Experimental protein mass (kDa)/pI	Theoretical protein mass (kDa)/pI
	70[a]	P54 protein	CAA72090	481	20	5/18	40/5.7	55.027/6.05
	73	Vicilin	P13918	234	16	2/9	39/7.5	52.257/5.39
	74	Vicilin	P13918	365	13	4/11	38/7.2	52.257/5.39
	78	Legumin A2	P15838	273	10	3/8	37/6.0	59.633/6.21
	79	Convicilin (Fragment)	P13919	81	4	2/4	37/6.8	46.368/5.32
	80	Legumin A2	P15838	449	26	3/16	37/6.1	59.633/6.21
	82	Legumin A2	P15838	517	16	6/13	37/5.8	59.633/6.21
	83	Vicilin	P13918	355	16	4/11	40/6.1	52.257/5.39
	85	Legumin A2	P15838	455	14	5/13	36/6.4	59.633/6.21
	87	Legumin A2	P15838	467	14	4/12	36/6.2	59.633/6.21
	89	Legumin A2	P15838	311	12	4/9	36/6.0	59.633/6.21
	94	Legumin A2	P15838	98	7	1/5	35/6.4	59.633/6.21
	95	Provicilin (Fragment)	P02855	550	38	5/14	34/6.5	31.521/5.57
	96	Legumin A2	P15838	368	16	4/12	34/5.8	59.633/6.21
	97	Convicilin	P13915	719	23	7/21	33/7.7	67.063/6.29
	98	Convicilin (Fragment)	P13919	310	25	4/15	33/7.4	46.368/5.32
	100	Vicilin	P13918	272	28	2/8	32/5.4	52.257/5.39
	101	Vicilin	P13918	512	23	5/14	32/5.7	52.257/5.39
	102	Convicilin (Fragment)	P13919	568	24	5/17	32/6.5	46.368/5.32
	103	Convicilin	P13915	202	12	3/10	32/7.0	67.063/6.29
	104	Convicilin	P13915	75	7	1/6	32/7.1	67.063/6.29
	105	Convicilin	P13915	552	26	5/20	32/7.3	67.063/6.29
	107	Convicilin (Fragment)	P13919	421	31	4/19	32/7.3	46.368/5.32
	108	Convicilin (Fragment)	P13919	489	29	4/19	31/6.4	46.368/5.32
	112	Convicilin (Fragment)	P13919	315	25	3/16	31/6.3	46.368/5.32
	113	Convicilin (Fragment)	P13919	182	22	2/10	31/6.8	46.368/5.32
	115	Convicilin (Fragment)	P13919	452	23	4/16	30/7.0	46.368/5.32
	116	Convicilin (Fragment)	P13919	308	25	3/15	30/6.3	46.368/5.32
	120	Convicilin (Fragment)	P13919	552	26	5/18	28/7.3	46.368/5.32
	123	Convicilin	CAB82855	385	24	4/15	26/6.1	72.134/5.50
	124	Convicilin	CAB82855	494	17	4/12	26/5.9	72.134/5.50
	125[a]	P54 protein	CAA72090	574	26	6/22	26/5.4	55.027/6.05
	126[a]	P54 protein	CAA72090	548	26	6/22	25/5.4	55.027/6.05
	128	Convicilin	CAB82855	468	17	4/12	23/5.5	72.134/5.50
	129	Legumin A2	P15838	274	7	3/6	22/5.6	59.633/6.21
	132	Convicilin (Fragment)	P13919	402	29	4/18	21/5.7	46.368/5.32

	133	Convicilin (Fragment)	P13919	394	27	4/18	21/6.8	46.368/5.32
	134	Vicilin	P13918	258	11	3/9	21/6.0	52.257/5.39
	135	Vicilin	P13918	360	11	4/10	20/5.3	52.257/5.39
	136	Vicilin	P13918	293	14	4/10	19/5.6	52.257/5.39
	139	Provicilin (Fragment)	P02854	346	20	5/15	18/7.2	46.414/5.39
	143	Convicilin (Fragment)	P13919	258	16	3/8	17/5.7	46.368/5.32
	147	Provicilin (Fragment)	P02854	657	14	5/12	15/6.2	46.414/5.39
Cell growth and structure								
Cytoskeleton	20	Tubulin alpha-1 chain	P09204	54	9	1/2	68/6.3	50.182/5.01
	144	Actin depolymerizing factor-like protein	XP_003629907	164	16	1/2	16/6.9	16.186/6.74
Growth regulators	84	1-aminocyclopropane-1-carboxylate oxidase	XP_003620934	303	17	2/6	36/6.7	34.693/5.70
	88	1-aminocyclopropane-1-carboxylate oxidase	XP_003620934	288	22	2/7	36/6.9	34.693/5.70
Cell defense and rescue								
Defense-related	1	Vicilin-like antimicrobial peptides 2-3	XP_003611961	80	3	1/3	82/6.0	86.755/5.36
	86	Endochitinase A2	P21226	153	17	2/5	36/7.6	35.739/7.33
	110	Momilactone A synthase	XP_003626028	148	14	2/5	31/6.5	29.946/5.33
	119	Cysteine proteinase inhibitor precursor	NP_001237734	171	19	2/6	28/6.7	27.659/7.27
Detoxification	31	Aldehyde dehydrogenase, partial	AAL77005	93	13	1/4	61/7.1	25.666/7.75
	36	Glutathione reductase, cytosolic	Q43621	1110	45	7/25	58/7.3	54.205/6.59
	106	L-ascorbate peroxidase, cytosolic	P48534	721	44	5/16	32/6.0	27.233/5.52
	114	Ferritin-1, chloroplastic	P19975	864	34	7/16	31/6.1	28.773/6.14
	118	Superoxide dismutase [Mn], mitochondrial	P27084	270	36	2/8	28/6.8	25.863/7.16
	137	Glutathione peroxidase	XP_003630522	216	20	2/5	19/6.0	21.706/6.82
Stress response	21	Seed biotin-containing protein SBP65	Q41060	665	24	5/15	67/6.5	59.632/5.93
	51	Activator of 90 kDa heat shock protein ATPase homolog 1-like	XP_003521222	258	16	3/6	50/6.8	40.033/5.73
	77	Annexin D1-like	XP_003542418	317	14	3/9	37/7.4	36.072/6.48
	99	Dehydrin DHN3	P28641	512	23	3/6	33/6.8	23.909/6.03
	122	Dehydrin 2	AAB51380	290	12	2/4	23/7.8	27.129/6.52
	130	Lactoylglutathione lyase	O49818	468	67	5/16	22/5.6	21.131/5.26
	140	17.5 kDa class I heat shock protein-like	XP_003523204	75	26	1/6	18/7.4	15.904/8.86
	145	Late embryogenesis abundant protein D-19	XP_003604723	194	26	2/6	15/6.1	10.734/5.93
	146	Desiccation protectant protein LEA14	P46519	110	9	1/2	15/5.7	16.542/4.94
Unknown								
	63	UPF0510 protein INM02-like	XP_003534501	96	7	1/3	43/6.8	32.669/6.00
	141	Unknown	ACJ83999	87	14	1/3	18/6.5	18.112/5.53
Others [b] — two proteins found — see Supplemental Table S3 (spots 25, 90, 92, 111, 127, 142)								

[a] The protein spot has a significant match to more than one protein but the proteins show a high degree of similarity (more than 60% identify). The spot is considered to contain only one protein, the protein with the highest mascot score and largest number of sequenced and matched peptides. This protein includes all the matched and sequenced peptides for the other matched proteins in the spot.
[b] The protein spot has significant match to more than one protein and is considered to contain two proteins based upon their lack of sequence homology.

$CaCl_2$ and MV for 25 h did not in itself affect seed germination and formation of seedling, but would affect the tolerance of seed to desiccation and the growth of seedling. Monitoring the survival during dehydration showed that $CaCl_2$ decreased and MV increased the critical water content at which the seeds became sensitive to desiccation (decreased survival and seedling weight), which was about 0.35 g g^{-1} for MV and 0.12 g g^{-1} for $CaCl_2$ in comparison to about 0.20 g g^{-1} in distilled water (Fig. 2E and F). In CI25 seeds, a slight desiccation could also promote the growth of seedling (Fig. 2F).

3.3. *Identification of differentially accumulated proteins*

Embryonic axes of seeds imbibed in distilled water for 0, 18, 25 and 36 h (CK, HI18, HI25 and HI36), and in $CaCl_2$ and MV solution for 25 h (CI25 and MI25) were excised, and the total soluble proteins were extracted and analyzed by 2-DE (Supplemental Fig. S1). After CBB staining, about 1400 protein spots were detected in each gel (Fig. 3 and Supplemental Fig. S2). Gel maps of CK, HI18, HI25 and HI36 treatments were compared to each other, and CI25 and MI25 treatments were compared to HI25 treatment using Image Master 2-D platinum. These comparisons revealed a total of 160 protein spots whose volume showed a significant change of more than 2-fold ($P<0.05$) in at least one treatment. All of these protein spots were excised and analyzed by MALDI MS/MS. Proteins were identified by first searching in the Swissprot database and, if that showed no matches, then in the NCBI database.

Proteins were identified in 147 spots (92%, Fig. 3, Table 1, Supplemental Tables S1, S2 and S3). For the majority of protein spots (134 spots), only one significant protein match was found when searching in the NCBI database. For 13 protein spots, a significant match to more than one protein was found. Out of these, spots 45, 46, 54, 70, 125 and 126 all showed significant matches to three homologous proteins, P54 protein, vicilin-like antimicrobial peptides 2–2 and putative sucrose binding protein. However, in all seven spots the match to the P54 protein had highest mascot score and the highest number of sequenced and matched peptides. In addition, no unique peptides deriving from vicilin-like antimicrobial peptides 2–2 or putative sucrose binding protein were found. We therefore conclude that these seven spots all contained only P54 protein (Table 1). A similar problem of match was seen for spot 55, which matched three homologous proteins, uncharacterized RNA-binding protein C660.15-like, nuclear pore membrane glycoprotein 210-like and heterogeneous nuclear ribonucleoprotein A2 homolog 2-like. Spot 55 was considered to contain only uncharacterized RNA-binding protein C660.15-like (Table 1). Thus, we had a total of 141 (134+7) spots containing one protein each, which changed significantly during germination. The two proteins identified in each of the six spots 25, 90, 92, 111, 127 or 142 had little similarity and no matching peptides, and these six spots were considered to contain two proteins (Supplemental Table S3). Since we do not know which of the two proteins present in each of the six spots changed in amount during germination, they were not considered in the search for proteins involved in desiccation tolerance (see below).

We subjected the proteins from the 141 spots to a functional and clustering analysis. The proteins matching 78 unique genes were classified into seven functional groups: 47% belong to storage proteins, 16% to metabolism, 13% to cell defense and rescue, 9% to energy, 6% to protein synthesis and destination, 5% to transcription, and 3% to cell growth and structure (Fig. 4). Out of these proteins, 139 varied significantly during the time course of water imbibition (Table 2 and Supplemental Table S2), while two changed specifically as a result of $CaCl_2$ and/or MV treatment (spots 114 and 140; Suppelmental Tables S2 and 3).

3.4. *Proteins differentially accumulated in embryonic axes during seed imbibition*

The differentially accumulated proteins during imbibition were grouped into three expression types (Table 2). Type 1 and Type 3 correspond to the proteins whose abundance increased and decreased significantly ($P<0.05$) during imbibition, respectively, while Type 2 showed an expression pattern of an initial significant increase ($P<0.05$) followed by a significant decrease ($P<0.05$) during imbibition. Approximately 50% (68 spots) of the proteins showed Type 1 expression pattern. The most abundant proteins were metabolic (20 spots) and storage proteins (17 spots), but proteins following Type 1 expression pattern were found in all functional groups (Table 2). The Type 3 expression pattern included 45 proteins (32%), out of which 27 were storage proteins and 11 involved in cell defense and rescue (Table 2). Relatively few proteins showed Type 2 expression pattern (18%, 26 spots), and the majority of those (22) were storage proteins (Table 2).

3.5. *Proteins differentially accumulated in embryonic axes of seeds imbibed in $CaCl_2$ or MV*

A total of 22 and 23 proteins had differential expression in CI25 and MI25 seeds in comparison to HI25 seeds, respectively (Table 3). Out of these differentially accumulated proteins, 18 and 13 proteins were increased and 4 and 10 decreased when seeds imbibed in $CaCl_2$ and MV in comparison to in distilled water, respectively (Table 3).

By comparing the relative protein expression pattern in the germinating seeds (Tables 2 and 3) with their widely different desiccation tolerances (Fig. 2A, B and C), we can identify the proteins that are potentially related to the loss of desiccation tolerance during pea seed germination. The criteria are: (1) a decreasing amount between 18 and 36 h of imbibition (Table 2), to correlate with the change in desiccation tolerance, which was lost during this period (Fig. 2); (2) A higher and/or lower abundance in CI25 and MI25 seeds in comparison to HI25 seeds to correlate with the effect of these treatments on desiccation tolerance (Table 3). Seven proteins fulfilled these criteria (Fig. 5), four of which increased in CI25 seeds, tubulin alpha-1 chain, seed biotin-containing protein SBP65, P54 protein and vicilin; and three of which decreased in MI25 seeds, vicilin-like antimicrobial peptides 2–3, convicilin (four spots) and TCP-1/cpn60 chaperonin family protein. No protein both increased in amount in CI25 seeds and decreased in MI25 seeds. We also searched for proteins fitting the inverse criteria, which would give them a potential role as desiccation intolerance mediators, but we did not find any.

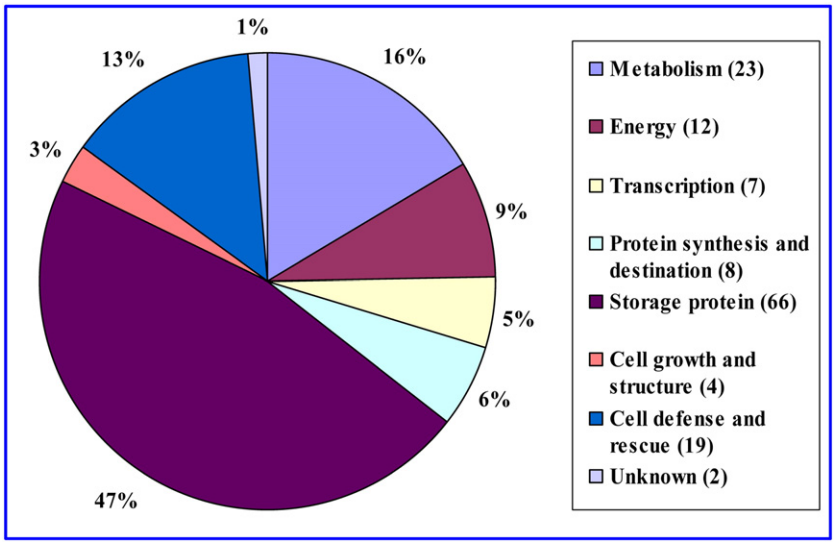

Fig. 4 – Functional classification and distribution of the 141 identified proteins with only one protein match. The number of protein spots is shown in brackets for each category.

4. Discussion

4.1. *Seed germination*

Germination of pea seeds started at 18 h, was 50% complete at 25 h and virtually complete at 36 h. The pea seeds showed only a very weak phase III of water uptake (Fig. 1). We found that 139 protein spots each containing only one protein changed at least 2-fold during the germination process. These proteins were clustered into seven functional groups (Fig. 4) and three expression patterns: Type 1 — increased, Type 2 — increased and then decreased, and Type 3 — decreased (Table 2). While Type 1 clearly includes the proteins important for seed germination, the other expression patterns can also be linked to germination, like changes in storage protein (see later). The clusters for changing proteins show that the mobilization of storage protein, de novo synthesis of proteins, production of energy, biosynthesis of primary and secondary metabolic compounds like amino acids, lipids and sugars and of signal molecules like ethylene, reorganization of cellular structure and activation of protective mechanism against reactive oxygen species (ROS) damage are the major events occurring in embryonic axes of pea seeds during germination. These physiological processes can supply the nutrition, energy, newly synthesized proteins, signal molecules, complete and flexible cellular structure and also protection for seed germination (Fig. 6).

4.1.1. *Mobilization of storage protein*

Storage proteins are the most abundant proteins in seeds, accounting for as much as 60% of the total protein content [38]. In dicotyledonous plants, the major seed reserves are storage proteins like legumins and vicilins. These proteins are degraded to provide the necessary nutrition for seed germination and seedling growth [39,40]. Thus, storage protein commonly account for a high percentage of the proteins changed during seed germination in many species such as Arabidopsis [20], tomato [24], barley [23], rice [25], alfalfa [27] and maize [28]. In pea embryonic axis, the storage proteins including convicilin, provicilin, vicilin, P54 protein and legume A2 changed during germination (Tables 1 and 2). As a result of mobilization of these storage proteins, their spot abundance can change in several ways with germination. Obviously, breakdown reduces the abundance of a storage protein (Type 3), but the larger products will be detectable on 2-D gels at lower molecular mass, where they present an increasing trend (Type 1) or even first an increasing and then a decreasing trend (Type 2) due to further degradation. Thus, many spots with different experimental mass are the product of the same storage protein gene. For example, spots 7–8 and 123–124 with experimental mass of about 74 and 26 kDa were all identified to be convicilin, which has a theoretical mass of 72.134 kDa (Fig. 3 and Table 1). This is consistent with results from studies of seed germination in other species [20,25,28]. Proteasome subunits were identified in the embryonic axes of pea seeds (Table 2 and Supplement Table S3) and increased during germination (Table 2), and are therefore likely participants in the degradation of the storage proteins.

4.1.2. *De novo synthesis of proteins*

Initiation of de novo protein synthesis is necessary for the completion of seed germination [4,41], as inferred from the observation that the protrusion of radicle in Arabidopsis seeds can be totally blocked in the presence the translation inhibitor cycloheximide [42]. In pea seeds, several proteins responsible for protein synthesis, such as RNA binding protein (spot 19), ribosome (spot 71) and translation initiation factors (spots 64, 67, 138) were present in dry seeds and increased during germination (Table 2). In eukaryotes, proteins are synthesized through cap-dependent and -independent translation initiation mechanisms [43]. A proteomic investigation of seed vigor of sugar beet showed that translation initiation could occur

Table 2 – Expression patterns of differentially accumulated axis proteins during seed imbibition.
Types 1 and 3 correspond to proteins whose abundance increased and decreased significantly ($P<0.05$) by at least two-fold at the end of germination (at 36 h), respectively. Type 2 includes proteins showing an initial increase in amount followed by a significant decrease during imbibition.

Protein function	Expression pattern					
	Type 1: increased		Type 2: increased and then decreased		Type 3: decreased	
	No.	Spot ID	No.	Spot ID	No.	Spot ID
Metabolism	**20**		**1**		**2**	
Amino acid	10	3, 23, 27, 33, 34, 47, 49, 52, 72, 76	0		1	50
Lipid	2	69, 56	0		0	
Nitrogen and sulphur	1	57	1	75	0	
Sugars	5	2, 10, 11, 12, 59	0		0	
Cofactors	1	81	0		0	
Secondary metabolism	1	58	0	1		117
Energy	**8**		**0**		**4**	
Glycolysis	5	13, 16, 30, 32, 109	0	2		62, 68*
Pentose-phosphate pathway	1	5	0		0	
Respiration	0	0	2			9*, 40*
Tricarboxylic acid pathway	2	29, 48	0		0	
Transcription	**6**	4, 24, 26, 43, 55, 131	**1**	93	**0**	
Protein synthesis and destination	**5**		**1**		**2**	
Protein synthesis	4	19, 67, 71, 138	0		1	64
Protein folding	0		0		1	22*
Proteolysis	1	91	1	121	0	
Storage protein	**17**	65, 78, 89, 94, 96, 101, 112, 113, 116, 120, 128, 129, 133, 136, 139, 143, 147	**22**	41, 42, 44, 70, 80, 83, 85, 87, 95, 98, 100, 102, 103, 105, 107, 108, 115, 123, 124, 125, 126, 134	**27**	6*, 7*, 8*, 14*, 15*, 17*, 18*, 28*, 35*, 37*, 38, 39, 45*, 46*, 53*, 54*, 60*, 61*, 66, 73, 74*, 79*, 82*, 97, 104, 132, 135
Cell growth and structure	**3**	84, 88, 144	**0**		**1**	20*
Cell defense and rescue	**7**		**1**		**9**	
Defense-related	1	110	0		3	1*, 86*, 119*
Detoxification	4	31, 36, 106, 137	1	118	0	
Stress response	2	51, 130	0		6	21*, 77, 99*, 122, 145*, 146*
Unknown	**2**	63, 141	**0**		**0**	
Total	**68**		**26**		**45**	

* The protein spot decreases significantly in volume from 18 h to 36 h of imbibition.

through both mechanisms [26]. During pea seed germination, only the cap-dependent initiation factors, poly(A)-binding protein (spot 19), eukaryotic translation initiation factor 3 (spot 67) and eukaryotic translation initiation factor 5A-3 (spot 138) were observed increase in abundance (Table 1). The poly(A)-binding protein binding to the 3′ mRNA poly(A) tail and eIF4G can trigger the circularization of mRNA and stimulate the translation initiation [43].

Amino acids are the main substrates for protein synthesis. Degradation of storage proteins supplies some of the amino acids, but de novo amino acid biosynthesis is also required. We observed that proteins related directly or indirectly to biosynthesis of methionine (spot 3), leucine (spot 23), threonine (spot 27), serine (spots 33, 34), proline (spot 47), cysteine (spots 72, 76) and aromatic amino acids (spot 52) accumulated in pea embryonic axis during imbibition (Table 2). Rajjou et al. [6] proposed that methionine metabolism plays a central role in seed germination. The methionine is not only used as substrate for protein synthesis, but also as precursor for S-adenosylmethionine (Adomet), which is the methyl group donor in the biosynthesis of polyamines, ethylene and biotin [44]. In Arabidopsis, methionine synthase and Adomet synthetase accumulate differentially during seed germination, and the specific inhibitor of methionine biosynthesis, D,L-propargylglycine, strongly inhibits seed germination and seedling establishment [45]. In alfalfa seeds, methionine synthase and cysteine synthase are up-regulated during germination [27]. In addition, several methionine metabolic proteins, such as Adomet synthetase, methionine synthase, cysteine synthase are related to seed vigor of sugar beet during germination [26]. The increasing accumulation of methionine synthase, serine hydroxymethyltransferase, Adomet synthase

Table 3 – Protein spots that changed significantly ($P<0.05$) at least 2-fold in volume in the embryonic axis of pea seeds imbibed in $CaCl_2$ (CI25) or methylviologen (MI25) compared to pea seeds imbibed in distilled water (HI25).

Functional groups	CI25/HI25				MI25/HI25			
	Increased		Decreased		Increased		Decreased	
	No.	Spot ID	No.	Spot ID	No.	Spot ID	No.	Spot ID
Metabolism								
Amino acid	**1**	56					**1**	76
Sugars	**1**	12						
Nitrogen and sulphur			**1**	75				
Cofactors								
Secondary metabolism	**1**	117			1	117		
Energy/Glycolysis			**1**	62			**2**	32, 62
Transcription	**2**	24, 55						
Protein synthesis and destination								
Protein folding							**1**	22
Proteolysis					**1**	121		
Storage protein	**7**	54, 74, 78, 96, 97, 100, 113, 133			**8**	65, 70, 78, 112, 120, 129, 132, 133	**5**	7, 8, 15, 18, 73
Cell growth and structure	**2**	20, 88						
Cell defense and rescue								
Defense-related					**1**	119	**1**	1
Detoxification	**2**	31, 114*	**2**	106, 118	**1**	114*		
Stress response	**1**	21			**1**	140*		
Total	**17**		**4**		**13**		**10**	

* Proteins changed <2-fold during imbibition but more than 2-fold and significantly comparing CI25 or MI25 to HI25 seeds.

and cysteine synthase during pea seed germination (Tables 1 and 2) agrees with the essential role of methionine metabolism in seed germination.

Dry seeds contain mRNA, which is synthesized and then stored during seed maturation [46–48]. This stored mRNA supports protein synthesis during early seed germination, but new DNA transcription is also a necessary physiological process, as shown in pea seeds where proteins involved in the transcription process accumulated during germination (Table 2).

4.1.3. *Energy production*

The physiological processes occurring during seed germination, such as transcription, proteolysis and protein biosynthesis, require energy. Energy production occurs immediately upon imbibition and seed respiration can be detected within minutes [1]. A number of proteomic studies have reported the differential accumulation of enzymes involved in energy metabolism during seed germination [20,23,25,27]. The glycolytic enzymes are commonly observed proteins associated with the progression of seed germination, but differential accumulation of enzymes associated with the pentose phosphate pathway and TCA cycle are also found in some species, like Arabidopsis [20], barley [23] and alfalfa [27] seeds. In pea seed, we observed that triose phosphate isomerase (spot 109), a protein involved in glycolysis increased its abundance, and several glycolysis (spots 62, 68) and respiration proteins (spots 9, 40) were present at relatively high levels in dry seeds and during the first 18 h of imbibition (Table 2 and Supplemental Table S1). These proteins may be important for energy production during early seed germination. After that time, other proteins for energy production, including enzymes of the glycolysis, pentose phosphate pathway and TCA cycle (Table 2 and Supplemental S1) increased to supply the energy requirement during the later stages of germination. These results at the proteomic level are consistent with the significant increase in respiration observed in mitochondria of pea embryonic axis during germination [32].

4.1.4. *Biosynthesis of primary and secondary metabolites and signal molecules*

Not only biosynthesis of amino acid, but also that of a large number of other compounds in primary and secondary metabolism is initiated during pea seed germination (Table 2). In embryonic axis of pea seeds, acetyl-CoA acetyltransferase (spot 56) involved in biosynthesis of fatty acids and cycloartenol-C-24-methyltransferase (spot 69) involved in sterol biosynthesis in plant [49,50] both increased during germination (Table 2). Fatty acids are a major component of cellular membranes, while sterols play crucial roles in the fluidity and permeability of membranes [49], and they also act as precursors for biosynthesis of steroid growth regulators such as brassinosteroids [51]. In rice, enzymes involved in fructose and sucrose biosynthesis increased during late germination [25], while in pea seeds, it was the enzymes responsible for sucrose, rhamnose and mannose biosynthesis that increased (Table 2). Sugars are not only metabolic substrate, they also have hormone-like regulatory effects on seed embryogenesis, germination and seedling growth [52,53].

Ethylene can act as a signal molecule inhibiting ABA signals to promote seed germination and seedling development [54]. Petruzzelli et al. [55] showed that one of the key ethylene biosynthesis enzymes, 1-aminocyclopropane-1-carboxylate (ACC) oxidase activity increased during pea seed germination and reached maximum activity when germination was complete. Consistent with this, we found

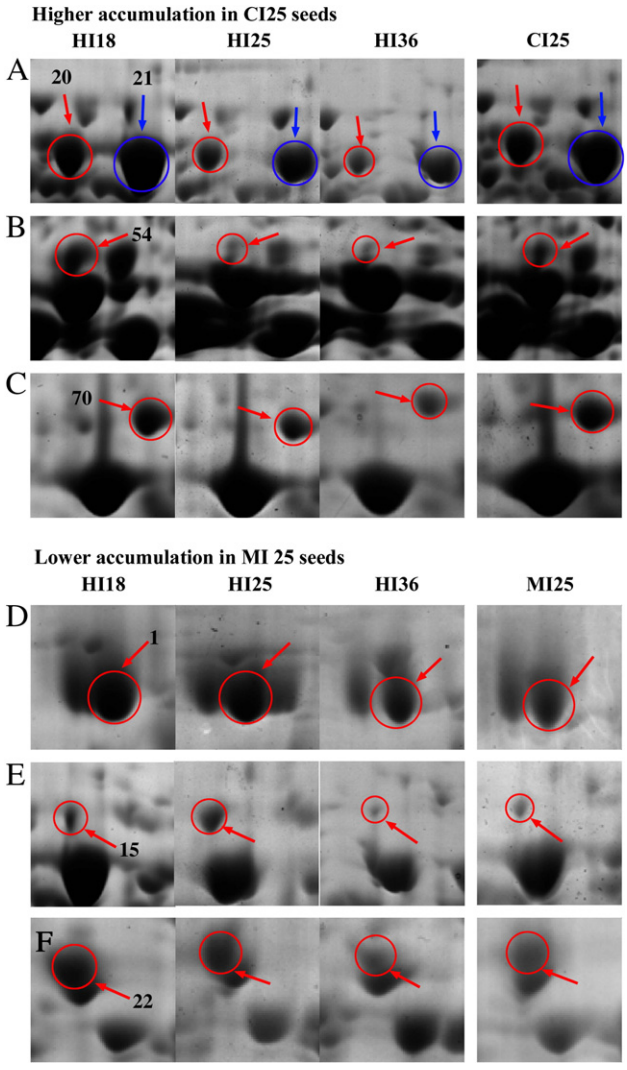

Fig. 5 – Differentially accumulated proteins associated with desiccation tolerance increased and decreased by imbibition with $CaCl_2$ or methylviologen, respectively. All the selected protein spots decreased in volume between 18 h and 36 h of imbibition in water in parallel with loss of desiccation tolerance (Tables 2 and 3). A, spot 20, tubulin alpha-1 chain, spot 21, seed biotin-containing protein SBP65; B, spot 54, P54 protein; C, spot 74, vicilin; D, spot 1, vicilin-like antimicrobial peptides 2–3; E, representative spots of convicilin (representing spots 7, 8, 15 and 18); F, spot 22, TCP-1/cpn60 chaperonin family protein.

an increase in the amount of ACC oxidase (spots 84 and 88) during germination (Table 2). In addition, we observed that germination was accompanied by an increase in Adomet synthase (spot 49), which catalyzes the formation of Adomet, the precursor for ethylene biosynthesis in plants [44] (Table 2). All these results are consistent with a role for ethylene synthesis in pea seed germination.

4.1.5. *Reorganization of cellular structure*

Actin is a central component of the cytoskeleton. Temporal and spatial alternations of actin cytoskeleton are necessary for many cellular processes, such as cell division, cell elongation, and tip growth [56]. Actin depolymerizing factor is one of the modulator of actin reorganization [57,58]. In pea seeds, germination may be accompanied by a reorganization of cell structure, as indicated by the significant increase in actin depolymerizing factor-like protein (spot 144) during pea seed germination (Table 2). This reorganization will be important for cell elongation or division during radicle protrusion and seedling growth. Our results together with the observation of another actin depolymerizing factor-like protein, profilin in tomato seed [24] imply that seed germination requires not simply the establishment of cellular structures, but also their reorganization.

4.1.6. *Activation of protective mechanisms*

Seed germination relies also on a range of protective mechanisms against stress damage, especially oxidative damage. The reactivation of metabolism during germination is a main source of ROS. In soybean [59], radish [60], sunflower [61] and tomato [62] seeds, H_2O_2 is produced during early germination, and the production of hydroxyl radicals and superoxide radicals [60] has been observed in seeds from other species. ROS can damage cellular components, like lipids, proteins and DNA [63]. ROS removal is therefore a necessary process in seed germination. ROS-scavenging enzymes like glutathione reductase (GR) and catalase (CAT) have been indicated to play this role in various species [64]. It appears that ROS removal is essential for pea seed germination, and several ROS scavenging enzymes, including aldehyde dehydrogenase (spot 31), GR (spot 36), L-ascorbate peroxidase (APX, spot 106) and glutathione peroxidase (GPX, spot 137) increased significantly during germination (Table 2). APX and GR are part of the ascorbate-glutathione cycle of ROS-scavenging enzymes, GPX is involved in NADPH-dependent ROS scavenging pathway [65], while aldehyde dehydrogenase can detoxify reactive aldehydes derived from lipid peroxidation [66].

4.2. *Desiccation tolerance*

4.2.1. *The method*

Pea seeds gradually lost desiccation tolerance during imbibition from 18 to 36 h (Fig. 2). Type 2 proteins, especially those showing a significant decrease in amount between 18 and 36 h of imbibition could be involved in giving desiccation tolerance, but their decrease could also be the consequence of another physiological process of seed germination. Thus, we require a method to separate these two processes. We used the fact that pea seeds imbibed in $CaCl_2$ or MV had very different desiccation tolerance at the same germination stage compared to pea seeds imbibed in water (Fig. 2). We found that four and three proteins that decreased in their abundance between imbibition from 18 to 36 h had higher and lower expression in CI25 and MI25 seeds than in HI25 seeds, respectively (Table 3). This indicates that these seven proteins are potentially important for maintaining desiccation tolerance during seed germination. We did not find any protein that both increased in CI25 and decreased in MI25 seeds in comparison to HI25 seeds. Many mechanisms have been implicated in desiccation tolerance [67], and Ca^{2+} and ROS may have independent mechanisms in affecting desiccation tolerance.

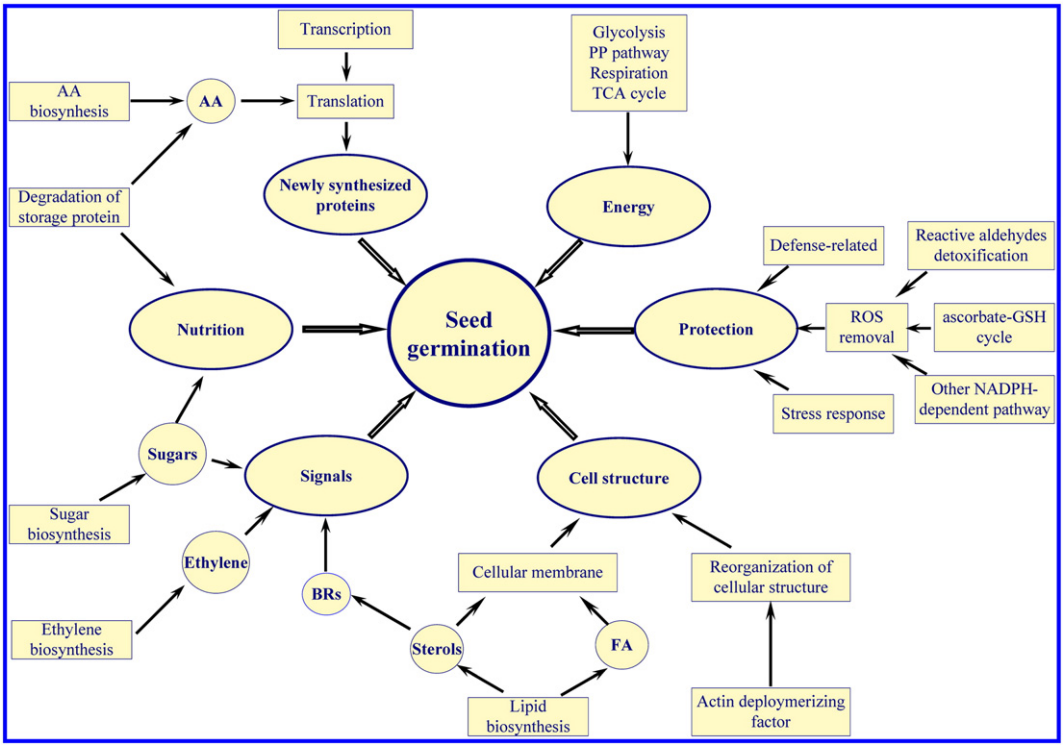

Fig. 6 – Major physiological processes involved in pea seed germination. AA, amino acid; PP pathway, pentose-phosphate pathway; TCA cycle, tricarboxylic acid cycle; BRs, brassinosteroids; FA, fatty acids; ROS, reactive oxygen species.

4.2.2. *Storage proteins*

Storage proteins are normally thought to be storage reserves for seed germination, but there is evidence that they may have additional roles, one of which could be improving desiccation tolerance. The amino acid sequence and structural analysis of seed legumin- and vicilin-like seed storage globulins genes revealed conserved structural motifs between the α and β-subunits of 11-S globulins and the germination-specific wheat germins, which are thought to have a role in embryo-specific hydration processes during germination; as well as the spherulation-specific spherulins, which are thought to be involved in tissue desiccation or hydration [68]. Therefore, it was suggested that seed globulins have evolved from a group of ancient proteins involved in cellular desiccation/hydration processes [68].

Transcriptome analysis of *M. truncatula* [69] and *A. thaliana* [31] seeds showed that a large amount of storage protein genes are deregulated after reestablishment of desiccation tolerance by PEG treatment. Huang et al. [28] found that the amount of a globulin 2 storage proteins in maize embryos increased during development, decreased after germination and increased in desiccation-tolerant embryos during desiccation, indicating a role in seed desiccation tolerance. It has been reported that β-subunits of 11-S globulins are abundant in primed sugar beet seeds [70], and are positively correlated with the advancement of seed germination by priming treatment whether hydro- or osmo-priming [71]. This indicates that the globulin subunits can play a role in control of germination performance. In pea seeds, many storage proteins decreased throughout germination (Table 2), and two storage proteins, P54 (spot 54) and vicilin (spot 70), showed increased accumulation while convicilin (spots 7, 8, 15 and 18) showed decreased accumulation when desiccation tolerance was improved and decreased by imbibing seeds in $CaCl_2$ and MV solution, respectively (Fig. 5). In fact, the imbibition–dehydration process in this research is to some extent similar to the hydropriming process, the well-studied commercial technique to improve seed vigor [6,17]. The increased and decreased accumulation pattern of storage proteins in CI25 and MI25 seeds may imply the inverse effects of Ca^{2+} and MV on the hydropriming. Storage proteins may promote desiccation tolerance by defending against pathogens. Several reports have indicated that storage protein can be involved in defense response due to their properties as insecticidal and antimicrobial proteins [72–75]. For example, vicilins from cowpea and other legume seeds can bind to chitin, the major component of cell walls in many fungi, and thus inhibit fungal growth [72,76].

4.2.3. *Pathogen defense*

Defense-related proteins, vicilin-like antimicrobial peptides 2–3 (spot 1), endochitinase A2 (spot 86) and cysteine proteinase inhibitor precursor (spot 119) have decreased significantly between 18 and 36 h of imbibition (Table 2). They are all involved in pathogen defense. Vicilin-like antimicrobial peptides 2–3 is a processing product of 7S globulin precursor found in *Macadamia integrifolia* kernels with antimicrobial activity [73]. Endochitinase A2 belongs to the chitinase protein group, which is one of the most important antifungal enzymes in plant [74]. Chitinases inhibit fungal growth through the hydrolysis of chitin [77,74]. Transformation of tobacco or other plant species with a proteinase inhibitor can improve resistance against pathogens and insects that possess cysteine proteinases

[78–80]. We did not detect the difference in endochitinase A2 and cysteine proteinase inhibitor precursor in CI25 or MI25 seeds in comparison to HI25 seeds, while the expression pattern of several storage proteins and vicilin-like antimicrobial peptides 2–3 during seed germination and after $CaCl_2$ or MV treatment all indicate a positive role of pathogen defense in seed desiccation tolerance (Fig. 5).

4.2.4. *LEA proteins*

In pea seed embryonic axis, Type 2 stress response proteins including seed biotin-containing protein SBP65 (spot 21), dehydrin DHN3 (spot 99), dehydrin 2 (spot 122), late embryogenesis abundant (LEA) protein D-19 (spot 145) and desiccation protectant protein LEA 14 (spot 146) (Table 2), all belong to LEA protein group. As the name implies, LEA proteins accumulate to high levels late in seed maturation and play a protective role during maturation drying [81,82]. Disappearance of different members of LEA protein has been linked to loss of desiccation tolerance during seed germination in plant species [15,20,28,83,84]. Using imbibition in hypoosmolar PEG solutions to uncouple desiccation tolerance from seed germination, Boudet et al. [15] identified six LEA proteins, MtEm6, DHN3, MtPM25, MP2, PM18 and SBP65, associated with loss of desiccation tolerance during germination of *M. truncatula* seeds. Our uncoupling strategy with $CaCl_2$ treatment identified one of the same LEA protein, SBP65, to be associated with pea seed desiccation tolerance (Fig. 5, Table 3). In sugar beet seeds, the disappearance of SBP65 correlated with loss of germination performance during priming [71]. All of these findings show the importance of the SBP65 protein for seed desiccation tolerance.

The SBP65 protein is not only an LEA-like protein with a protective role during seed germination. It can also be biotinylated and involved in regulating biotin levels in plant seeds [85–87]. The SBP65 protein has been shown to accumulate during the later stages of seed development and to be degraded during germination [71,86,88]. Therefore, changes in the accumulation of the SBP65 protein can help fine-tune the metabolic activity of imbibed seeds [6]. Whether the role of the SBP65 protein in desiccation tolerance is protection against desiccation damage or regulation of metabolic activity remains to be established.

There is a great deal of similarity between the identified LEA proteins with pattern of decreasing accumulation during germination in pea seeds and those identified in *M. truncatula* [15]. The desiccation protectant protein LEA 14 (Table 1) and PM25 [15] both belong to group 5, LEA protein D-19 (Table 1) and EM6 [15] to group 1, MP2, PM18 [15] and SBP65 (Table 1, [15]) to group 3, and dehydrin 2 (Table 1) and DHN 3 [15] to group 2 LEA protein. Thus, the protective mechanism in desiccation tolerance in legume species appears to be similar. There are two major differences between the methods used by Boudet et al. [15] and the present study. Boudet et al. [15] used PEG treatment and analyzed only heat-soluble proteins, whereas we used Ca^{2+} treatment and analyzed total protein. This may be the reason that we only identified one of these LEA proteins (SBP65) as involved in desiccation tolerance.

4.2.5. *Stabilization of cell structure*

Chaperonins take part in protein folding and subsequent assembly into oligomers. They are required for normal cell growth and are stress-induced, acting to stabilize or protect partially denatured or disassembled polypeptides under stress conditions [89]. Members of the TCP-1 chaperonin family act as molecular chaperones for cytoskeleton proteins of tubulin and actin and probably also other proteins [90,91]. In pea seeds, the TCP-1/cpn60 chaperonin family protein (spot 22) accumulated to a high level in dry seeds and decreased in abundance during seed germination, while it decreased even further when imbibed with MV (Table 2). In relative desiccation-tolerant CI25 seeds, tubulin alpha-1 chain (spot 20) had higher accumulation than in HI25 seeds. We found that the tubulin alpha-1 chain and actin depolymerizing factor-like protein, both important for cellular structure, changed their abundance mainly after 18 h of imbibition, when pea seeds started to germinate (Fig. 1) and lose desiccation tolerance (Fig. 2). We speculate that the time at which the seeds reorganize their cellular structure for cell elongation or division during germination is the stage most sensitive to desiccation stress. All in all, the change of chaperone and tubulin both indicate that desiccation tolerance in pea seeds is tightly coupled to stabilization of cell structure.

Huang et al. [28] applied a similar proteomic approach to identify the candidate proteins for acquisition of desiccation tolerance during development and loss of it during germination in maize embryos. A total of nine proteins were implicated in desiccation tolerance of maize embryos, most of which were stress-related. Several of the seven candidate proteins found in pea axes have similar functions. EMB564 in maize embryos and SBP65 in pea axes both belong to the LEA protein family. Proteins involved in pathogen defense are found both in maize embryos and pea axes, vicilin-like antimicrobial peptides 2–3 and major allergen Bet v 1.01 C, respectively. In addition, storage proteins are also thought to have a role in desiccation tolerance in these two species. An important difference between pea axes and maize embryos is that several proteins involved in the stabilization of cell structure are involved in desiccation tolerance of pea axes, which was not indicated in maize embryos [28].

4.2.6. *The role of Ca^{2+} and ROS in desiccation tolerance*

Ca^{2+} acting as a signal molecule is involved in a range of abiotic and biotic stresses [92,93], but its role in seed desiccation tolerance is still unclear. The Ca^{2+} signal can mediate a series of phosphorylation events or regulate the gene expression in response to the stresses [93,94]. Increased accumulation of several proteins in pea seeds imbibed in $CaCl_2$ solution (Table 3) may point at Ca^{2+}-regulated transcription during seed germination. Some of these proteins correlated with loss of desiccation tolerance during seed germination since their abundance decreased significantly during germination, but increased in CI25 seeds. However, other proteins may also be important for Ca^{2+} signaling in response to desiccation.

Ethylene is involved in mediating the cross-talk between Ca^{2+}-dependent protein kinases and mitogen-activated protein kinase (MAPK) signaling in controlling stress responses in plants [95]. The higher accumulation of ACC oxidase (spot 88) in CI25 than in HI25 seeds is consistent with the involvement of ethylene in mediating Ca^{2+} phosphorylation response to desiccation. Ca^{2+} and ROS signals can interact with each other in response to various stresses [96]. We observed that aldehyde dehydrogenase (spot 31) and ferritin-1 (spot 114), both involved in ROS

detoxification and accumulated more in CI25 seeds, and that ferritin-1 also increased more in MI25 seeds compared to HI25 seeds (Table 3). Barba-Espin et al. [34] showed that treatment of pea seeds with H_2O_2 increased the germination performance and seedling growth. They also found that two MAPK genes are activated by H_2O_2. Therefore, the opposite effect of Ca^{2+} and MV may reflect the dual role of ROS in the hydropriming and desiccation tolerance, i.e., the lower amount as signal molecules to induce protective mechanism, and the higher amount as toxic molecules to incur damage [63].

Production of ROS during dehydration is considered to be one of the reasons for desiccation sensitivity [64,67]. Imbibition with MV would be expected to increase ROS in germination pea seeds, but only one of the ROS-detoxifying proteins, ferritin-1, increased in amount (Table 3). Additionally, this treatment decreased the accumulation of cysteine synthase (spot 76, Table 3), which is responsible for biosynthesis of cysteine, a precursor of glutathione, one of the major antioxidants [44]. For this reason, MI25 seeds may not have the ability to remove relatively high amounts of ROS, which would inhibit their seedling growth and decrease their tolerance to desiccation compared to HI25 seed (Fig. 2).

Although cysteine synthase increased during seed germination (Table 2 and Supplement Table S1) and therefore does not appear to be related to the loss of desiccation tolerance, the accumulation of this protein was decreased in MI25 in comparison to HI25 seeds (Table 3). In addition to participating in the biosynthesis of glutathione, cysteine synthase also plays an important role in biosynthesis of methionine, which is an essential amino acid during seed germination and in stability of proteins [6,44]. The cysteine synthase has been identified as one of the proteins whose accumulation varied with sugar beet seed vigor [26]. Thus, cysteine synthase may be one of the major factors in the intolerance of MI25 seeds to desiccation.

5. Conclusions

In the present work, we investigated the changes in the embryonic axis proteome in germinating pea seeds. Our results are generally consistent with proteomic studies in other species, but a number of new proteins were also found during pea seed germination, such as sterol biosynthetic enzyme, cycloartenol-C-24-methyltransferase, ethylene biosynthetic enzyme, ACC oxidase, actin depolymerizing factor-like protein, the ROS detoxification enzyme, GPX, and actin reorganization factor. In pea seeds, germination is not simply the activation of a series of metabolic processes, including the degradation of storage protein, de novo synthesis of proteins, production of energy, biosynthesis of primary and secondary metabolic compounds and signal molecules, but also involves reorganization of cellular structure and activation of protective systems against stress damage, especially oxidative damage.

Seed germination is usually associated with loss of desiccation tolerance. Due to the difficulty in differentiating desiccation tolerance and germination, the reason for the loss of desiccation tolerance is still unclear. In the present study, we used $CaCl_2$ and MV treatments to acquire the seeds with different desiccation tolerance at the same stage of germination and identify proteins affected by these two treatments. We found a total of seven proteins, including storage protein and proteins associated with cell defense, stress response, protein folding and cellular structure, which have a potential role in maintaining seed desiccation tolerance during germination. We therefore propose that seed desiccation tolerance relies on mechanism of pathogen defense, prevention of protein conformational changes and stabilization of cell structure.

Seed germination is a complex process. We cannot expect to fully understand this process based on proteomics only, but the new information acquired here can support a platform to apply molecular methods to explore new genes for seed germination. Present work identified the proteins possibly related to loss of desiccation tolerance during seed germination, but how these proteins changed during desiccation and subsequent rehydration process are not clear. In addition, it is very likely that other unidentified proteins play a role during dehydration and/or rehydration. Thus, further investigations of the seed proteome during the dehydration and rehydration process are necessary.

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