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# Proteomics of desiccation tolerance during development and germination of maize embryos

Hui Huang<sup>a,b</sup>, Ian Max Møller<sup>c</sup>, Song-Quan Song<sup>a,\*</sup>

<sup>a</sup>Institute of Botany, Chinese Academy of Sciences, Beijing 100093, China

<sup>b</sup>Kunming Institutes of Botany, Chinese Academy of Sciences, Kunming, Yunnan 650204, China

<sup>c</sup>Department of Molecular Biology and Biotechnology, Aarhus University, Flakkebjerg, DK-4200 Slagelse, Denmark

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## ABSTRACT

Maize seeds were used to identify the key embryo proteins involved in desiccation tolerance during development and germination. Immature maize embryos (28N) during development and mature embryos imbibed for 72 h (72HN) are desiccation sensitive. Mature maize embryos (52N) during development are desiccation tolerant. Thiobarbituric acid reactive substance and hydrogen peroxide contents decreased and increased with acquisition and loss of desiccation tolerance, respectively. A total of 111 protein spots changed significantly (1.5 fold increase/decrease) in desiccation-tolerant and -sensitive embryos before (28N, 52N and 72HN) and after (28D, 52D and 72HD) dehydration. Nine pre-dominantly proteins, 17.4 kDa Class I heat shock protein 3, late embryogenesis abundant protein EMB564, outer membrane protein, globulin 2, TPA:putative cystatin, NBS-LRR resistance-like protein RGC456, stress responsive protein, major allergen Bet v 1.01C and proteasome subunit alpha type 1, accumulated during embryo maturation, decreased during germination and increased in desiccation-tolerant embryos during desiccation. Two proteins, Rhd6-like 2 and low-molecular-weight heat shock protein precursor, showed the inverse pattern. We infer that these eleven proteins are involved in seed desiccation tolerance. We conclude that desiccation-tolerant embryos make more economical use of their resources to accumulate protective molecules and antioxidant systems to deal with maturation drying and desiccation treatment.

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## 1. Introduction

Orthodox seeds undergo maturation drying on the mother plant and enter a state of quiescence and acquire desiccation tolerance [1], thereby permitting their storage with low water content and survival under various environmental conditions [2]. Under these conditions, tolerance to desiccation may last for many years. During germination, tolerance to desiccation

is rapidly lost, often after only a few hours of imbibition and prior to [3] or after [4] the emergence of the radicle depending on the species. Recalcitrant seeds are characterized by the absence of mature drying, and they are shed from their mother plant with a high moisture content and an active metabolism. They are sensitive to desiccation and low temperature, and lose viability rapidly during storage [5]. Seeds of many important economical plants are recalcitrant and it is difficult to

**Abbreviations:** 2-DE, two-dimensional electrophoresis; ABA, abscisic acid; ACN, acetonitrile; CBB, coomassie brilliant blue; DAP, days after pollination; DTT, dithiothreitol; DW, dry weight; HSP, heat shock protein; IEF, isoelectrofocusing; LEA, late embryogenesis abundant protein; MS, mass spectrometry; MW, molecular weight; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substance; TCA, trichloroacetic acid; TFA, trifluoroacetic acid.

\* Corresponding author. Tel.: +86 01062836484.

E-mail address: [sqsong@ibcas.ac.cn](mailto:sqsong@ibcas.ac.cn) (S.-Q. Song).

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find a suitable conservation strategy. Thus, it is essential to understand the mechanism of desiccation tolerance in seeds, which is largely unknown.

A number of processes or mechanisms have been suggested to confer, or contribute to, desiccation tolerance, including accumulation of putatively protective molecules, e.g. late embryogenesis abundant (LEA) proteins and dehydrins [1], presence and efficient operation of antioxidant system [6], intracellular de-differentiation [7], metabolic 'switching off' [8], and the presence and operation of repair systems during rehydration of seeds [9–11].

In combination with the availability of genome sequence data, proteomics has opened up enormous possibilities for identifying the total set of expressed proteins [12], and is having an increasing impact on the study of the seed proteome [13]. On the one hand, seed proteomics mainly focus on identifying as many proteins as possible to create reference proteome maps at specific stages [14,15]. On the other hand, comparative proteomics analysis has become a basic method for the large-scale analysis of proteins in many fields of plant biology, which would give insight into the functional gene products and how their expression is modulated in seeds during important life processes [16–18]. New insights have been obtained into desiccation tolerance by comparative proteome studies. Cumming [19] and Buitink et al. [20] found that the presence of LEA proteins correlated well with desiccation tolerance. LEA proteins accumulated to high levels in developing seeds during late maturation and in dehydrating vegetative tissues of resurrection plants [21]. Grelet et al. [22] identified a LEA protein of group 3 (PsLEAm) in the matrix space of pea (*Pisum sativum*) seed mitochondria which was shown to protect two mitochondrial matrix enzymes during drying. Boucher et al. [23] showed that MtPM25 helps prevent and dissolve protein aggregates formed during various types of stress including desiccation. The content of Parab21 protein belonging to LED11 in almond embryos and Cor14b protein in barley are also closely correlated with desiccation tolerance [24,25]. Correlations between the disappearance of various LEA proteins and loss of desiccation tolerance during germination have also been reported [26]. However, some dehydrins appear to play no role in desiccation tolerance, but instead promote seed longevity [27,28].

Previous studies on desiccation tolerance have been conducted on orthodox or recalcitrant seeds, respectively [3,29–34]. In fact, comparing their different responses to desiccation is an essential way to understand the mechanism of desiccation tolerance. However, the comparative analysis of desiccation tolerance between the seeds from two different species is difficult due to their different genetic background. So it is important to get desiccation-tolerant and -sensitive seeds with identical genetic background for comparison. Imbibed orthodox seeds are sometime studied instead of recalcitrant seeds because they resemble each other by being desiccation sensitive, and by having a high degree of subcellular development and high metabolic activity [11]. Therefore, comparison of desiccation tolerance in orthodox and recalcitrant seeds with identical genetic background by a combination of development with germination is necessary for understanding the mechanism of desiccation tolerance. As far as we know, it has not been done as yet.

It is the purpose of the present study to compare the proteome profile of desiccation-tolerant and -sensitive maize seeds

by two-dimensional electrophoresis (2-DE) and mass spectrometry (MS). The intention was to identify potential candidate proteins (e.g. stress-related proteins) involved in conferring desiccation tolerance in maize seeds. We identify nine proteins that increase in amount during acquisition of desiccation tolerance and decrease during loss of desiccation tolerance.

## 2. Materials and methods

### 2.1. Plant material

To analyze acquisition of desiccation tolerance during development of maize embryos, maize (*Zea mays* L. cv. Nongda 108) seeds were manually collected at different days after pollination (DAP) from plants growing in Xishuangbanna Tropical Botany Garden in July 2005. Immediately after excision from seeds, some fresh embryos were frozen rapidly in liquid nitrogen for protein extraction. Other fresh embryos were dehydrated to a water content of  $0.07 \pm 0.01$  [g H<sub>2</sub>O (g DW)<sup>-1</sup>, g g<sup>-1</sup>], and either frozen rapidly in liquid nitrogen for protein extraction or germinated for measuring survival.

For analysis of loss of desiccation tolerance during germination of maize embryos, mature dry maize (*Zea mays* L. cv. Nongda 108) seeds were surface sterilized with 0.1% (w/v) HgCl<sub>2</sub> for 3 min, and then rinsed three times in sterilized water. After pre-imbibition of the seeds in water at 25 °C in the dark for different periods of time, the embryos were excised from the seeds. Some fresh embryos were frozen rapidly in liquid nitrogen for protein extraction. Other embryos were dehydrated to a water content of  $(0.07 \pm 0.01)$  g g<sup>-1</sup>, and either frozen rapidly in liquid nitrogen for protein extraction or germinated for measuring survival.

All samples were assayed for thiobarbituric acid reactive substance (TBARS), H<sub>2</sub>O<sub>2</sub> and proteomic analyses.

### 2.2. Dehydration of embryos

Dehydration of embryos was rapidly achieved by placing them on the filter paper, and then the filter paper was placed over activated silica gel for different times to a water content of  $(0.07 \pm 0.01)$  g g<sup>-1</sup> within closed desiccators at 25 °C (Supplemental Table S1).

### 2.3. Determination of water content

Water content of maize embryos was determined gravimetrically before and after drying at 80 °C for 48 h. Water content of embryos is expressed on a dry mass basis (g g<sup>-1</sup>).

### 2.4. Assessment of maize embryo survival

Batches of twenty embryos were germinated on moist filter paper in closed Petri dishes in the dark at 25 °C for 5 days. The embryos showing measureable increase in length and volume and appearing light green were counted as surviving. The embryos showing no increase in length and volume and appearing dark brown were counted as dead. The lengths of shoot and radicle were determined on day 5 of germination.

## 2.5. Determination of TBARS and H<sub>2</sub>O<sub>2</sub> contents

The content of TBARS was determined as described by Cho and Seo [35], and is expressed as  $\mu\text{mol g}^{-1}$  DW.

The H<sub>2</sub>O<sub>2</sub> content was assayed based on the method of Brennan and Frenkel [36], but modified as follows: twenty embryos were homogenized in 5 ml of cold acetone ( $-20^{\circ}\text{C}$ ). A titanium reagent (titanic tetrachloride in concentrated HCl), NH<sub>4</sub>OH and H<sub>2</sub>SO<sub>4</sub> were added to the homogenate. After centrifugation at 15000 *g* for 15 min at  $4^{\circ}\text{C}$ , the absorbance of the supernatant was determined immediately at 415 nm. The H<sub>2</sub>O<sub>2</sub> content is expressed as  $\mu\text{mol g}^{-1}$  DW.

## 2.6. Preparation of protein samples

Embryos were ground to a fine powder in liquid nitrogen, and the powder was then suspended in cold acetone containing 10% (w/v) trichloroacetic acid (TCA) and 1% (w/v) dithiothreitol (DTT), vortexed and kept at  $-20^{\circ}\text{C}$  for 2 h (or overnight). Each sample was centrifuged at 30000 *g* for 20 min at  $4^{\circ}\text{C}$ . The resulting pellet was washed twice by re-suspending in cold acetone containing 1% DTT, for 1 h each at  $-20^{\circ}\text{C}$ , before further centrifugation at 30000 *g* for 20 min at  $4^{\circ}\text{C}$ . The resulting pellet was vacuum dried, solubilized in freshly prepared lysis buffer containing 8 M urea, 4% (w/v) CHAPS, 10 mM DTT and 1% (v/v) ampholyte (pH 3–10), and then centrifuged at 30000 *g* for 20 min. The supernatant was submitted to a second clarifying centrifugation at 30000 *g* for 20 min, and the resulting supernatant was used to assay for protein content

and for 2-DE. The protein concentration was determined by Bradford [37] using bovine serum albumin as the standard.

## 2.7. 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 4–7 (Bio-Rad; Hercules, CA, USA). Protein sample was loaded onto the strip, which was soaked in re-hydration solution, containing 8 M urea, 2% (w/v) CHAPS, 20 mM DTT, 2% (v/v) immobilized pH gradient (IPG) buffer, pH 3–10, and 0.002% (w/v) bromophenol blue for 16 h at  $22^{\circ}\text{C}$ . 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, and holding at 8000 V until a total of 75 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 2% (w/v) iodoacetamide. After equilibration, the strips were applied to vertical 20 cm SDS-polyacrylamide gels (12% resolving and 5% stacking) and sealed with 0.5% (w/v) low-melting agarose in SDS buffer containing bromophenol blue. Low molecular range markers (Bio-Rad) were loaded at either end of the strips before sealing. After solidification of the gel, electrophoresis was performed at  $10^{\circ}\text{C}$  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 6 h at 40 mA. Gels were stained overnight with 0.25% (w/v) coomassie brilliant

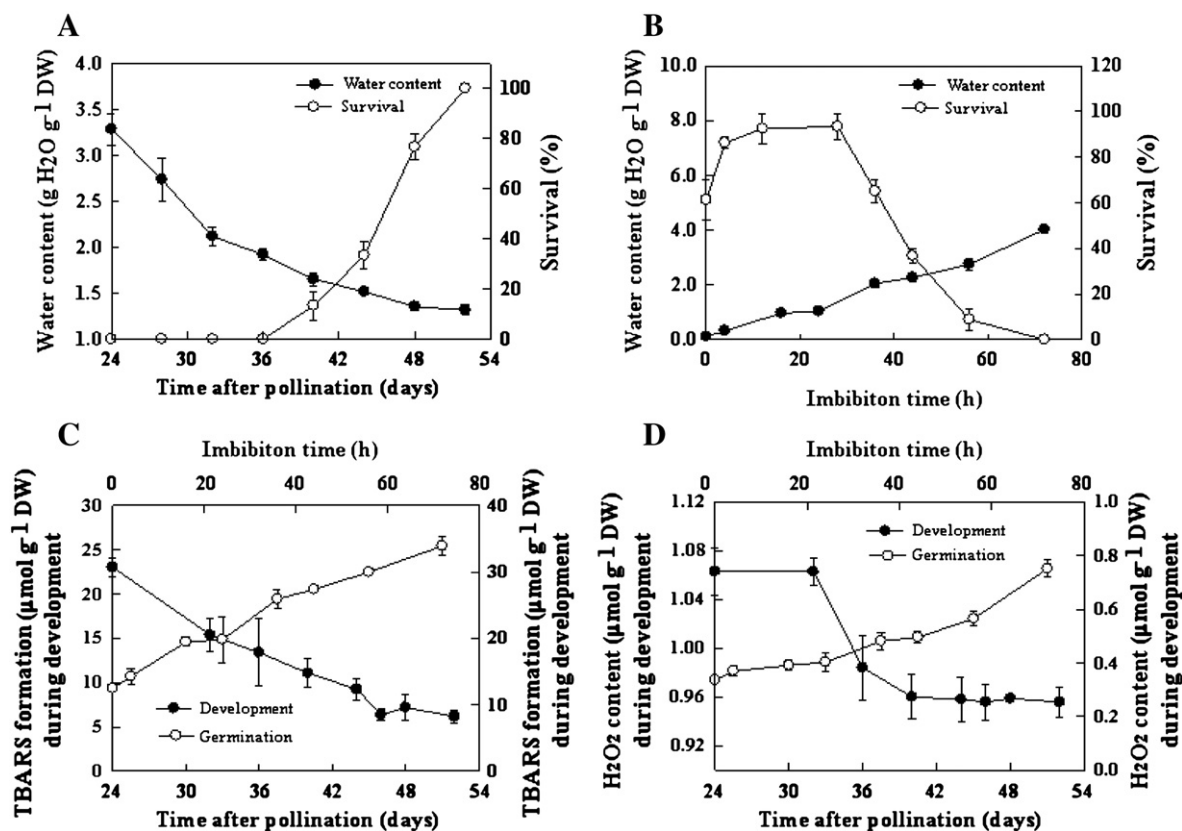


Fig. 1 – Changes in water content, survival (A, B), TBARS formation (C) and H<sub>2</sub>O<sub>2</sub> content (D) of maize embryos after dehydration during development and germination.

blue (CBB) R-250 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.

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 2D Platinum, version 5.01 (GE Healthcare Bio-Science, Little Chalfont, UK). After automated detection and matching, manual editing was carried out to correct the mismatched and unmatched spots.

Three well-separated gels of treatment were used to create "replicate groups". Spots were considered reproducible when they were well resolved in the three biological replicates or at least two of them. For each matched spot, a measurement was carried out for each biological replicate. The normalized volume of each spot was assumed to represent its expression abundance. A criterion of at least 1.5-fold was used to define significant differences when comparing spot size between groups.

## 2.8. In-gel digestion with trypsin, image analysis and protein identification by MALDI-TOF-TOF MS

Differentially expressed protein spots were excised from the stained gels. In-gel digestion and tryptic peptide extraction were performed according to Yang et al. [38] with little modification. Each small gel piece with protein was washed and destained using a series of washes consisting of 50  $\mu$ l of deionized water, 50  $\mu$ l of 50% acetonitrile (ACN) (Fisher Scientific; Fair Lawn, NJ, USA)/50% 50 mM  $\text{NH}_4\text{HCO}_3$  (Sigma) (pH 7.8),

and 50  $\mu$ l of 100% ACN at room temperature. The protein in the gel piece was reduced with 10 mM DTT in 100 mM  $\text{NH}_4\text{HCO}_3$  for 1 h at 60  $^\circ\text{C}$ , and then incubated with 40 mM iodoacetamide in 100 mM  $\text{NH}_4\text{HCO}_3$  for 30 min at room temperature in darkness. The gel pieces were minced and lyophilized, then rehydrated in 25 mM  $\text{NH}_4\text{HCO}_3$  with 10 ng sequencing grade modified trypsin (Promega, Madison, WI, USA). Samples were left on ice for 15 min and incubated overnight at 37  $^\circ\text{C}$ . After digestion, the supernatant from each sample was recovered and the remaining peptides were then sequentially extracted using 5  $\mu$ l of 5% trifluoroacetic acid (TFA) followed by 50  $\mu$ l of 50% ACN 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  $\mu$ l of  $\alpha$ -cyano-4-hydroxy cinnamic acid (CHCA, Sigma) saturated matrix (5 mg/ml in 0.1% TFA/75% ACN) and dried completely. Samples were then subjected to MALDI MS/MS analysis using autoflex III smartbeam (Bruker, Germany).

The peptide mass fingerprints obtained were searched against maize data and plant data in the NCBI database using MASCOT software (Matrix Science, London, UK). Peptides were selected in the mass range between 500 and 3000 Da. The following search parameters were applied: NCBI was used as the protein sequence database; a mass tolerance of 100 ppm and one incomplete cleavage were allowed; acetylation of the N-terminus, alkylation of cysteine by carbamidomethylation, oxidation of methionine, and the pyroGlu formation of N-terminal Gln were considered as possible modifications.

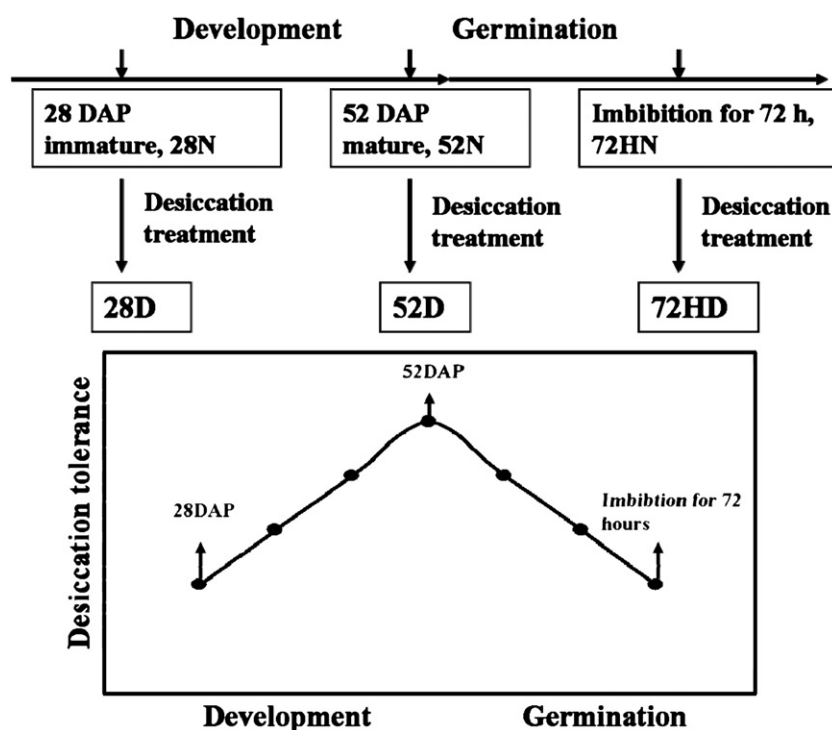


Fig. 2 – Schematic drawing of the treatments compared in the proteomic analyses of maize embryos (top) and the changes of desiccation tolerance during development and germination (bottom).

### 3. Results and discussion

#### 3.1. Acquisition and loss of desiccation tolerance of maize seed during development and germination

During development, the water content of maize embryos decreased approximately linearly from  $3.28 \text{ g g}^{-1}$  to  $1.32 \text{ g g}^{-1}$  within 28 days. The maize embryos began to acquire desiccation tolerance at 35–40 DAP (Fig. 1A). Their desiccation tolerance (% survival) increased in parallel with the moisture content loss from 0% at 24 DAP to 100% survival at 52 DAP. Thus, 28 DAP maize embryos were desiccation sensitive and 52 DAP maize embryos were desiccation tolerant.

During imbibition of mature embryos, the water content rapidly increased during the early phase of imbibition until 16 h, followed by a short period with a slower increase. Then, there was a further more rapid increase to  $4.02 \text{ g g}^{-1}$  after imbibition for 72 h (Fig. 1B). During the initial stage of imbibition, maize embryos were desiccation tolerant with an increasing survival from 61% (control) to 93% (imbibed for 28 h) (Fig. 1B). After 28 h of imbibition (radicle emergence), maize embryos became vulnerable to desiccation. Their survival ability decreased rapidly to 0% after 72 h of imbibition, at which point the embryos were therefore desiccation sensitive. Thus, in agreement with previous studies [7,29,39], the maize seeds gradually acquired tolerance to dehydration during their development and lost desiccation tolerance with increasing germination activity.

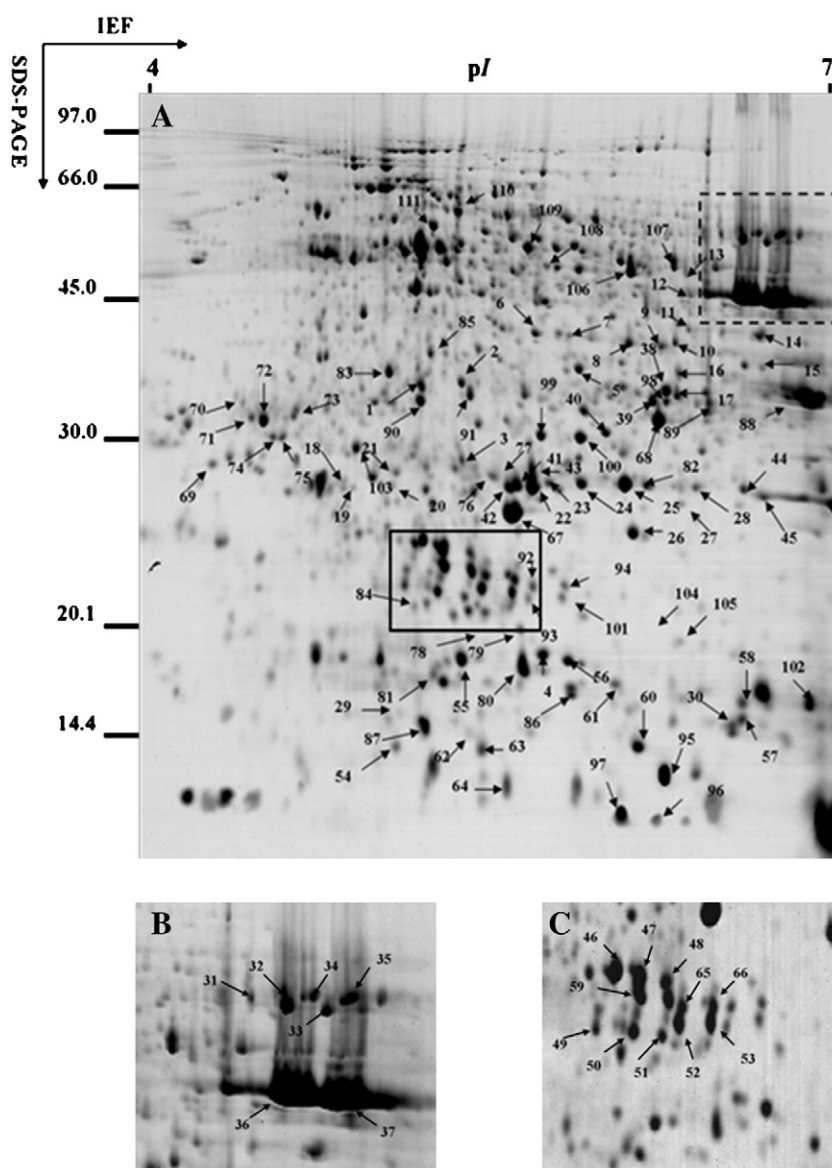


**Fig. 3 – 2-DE analysis of maize embryo proteins during acquisition and loss of desiccation tolerance.** A total of 400  $\mu\text{g}$  of proteins were extracted and separated by 2-DE, as described in Materials and methods, and visualized with CBB R-250. A, the reference gel with 111 differentially expressed proteins; B (amplificatory areas with broken line) and C (amplificatory areas with continuous line) show proteins with molecular weight of 20.1–30.0 and 45.0–66.0 kDa, which were more abundant in 52N and 52D than in 28N, 28D, 72HN and 72HD. Differentially expressed protein spots in different treatments are indicated by arrows and description as in Table 1.

**Table 1 – Proteins changed in abundance between non-desiccated maize embryos (28N, 52N and 72HN) and desiccated embryos (28D, 52D and 72HD) and differentially expressed proteins between 28N and 52N and between 52N and 72HN identified by MALDI-TOF or TOF-TOF MS. Only protein spots that changed in abundant at least 1.5 fold in at least two of three replicates are included. Some fold changes are between –1.5 and +1.5, because of there is a change of at least 1.5 fold in one of the other treatment. \*The part of Fig. 3, where the position of the spot is shown, is given in brackets.**

| Spot no.* <sup>a</sup> | Exp. MW (kDa)/pI | Description                                       | Accession number | C (%) <sup>b</sup> | S <sup>c</sup> | M <sup>d</sup> | Fold change in protein amount going from (+ increased, – decreased) |            |                |            |             |
|------------------------|------------------|---------------------------------------------------|------------------|--------------------|----------------|----------------|---------------------------------------------------------------------|------------|----------------|------------|-------------|
|                        |                  |                                                   |                  |                    |                |                | 28N to 28D                                                          | 52N to 52D | 72HN to 72HD   | 28N to 52N | 52N to 72HN |
| 1 (3A)                 | 36/5.41          | Enoyl-[acyl-carrier-protein] reductase [NADH]     | ACG35839         | 14                 | 85             | 5/34           | +2.49                                                               | +1.17      | +2.23          | –1.30      | –5.66       |
| 2 (3A)                 | 36/5.6           | Fructokinase-2                                    | NP_001105211     | 48                 | 225            | 20/54          | –1.02                                                               | +1.17      | –2.20          | –4.40      | +2.11       |
| 3* (3A)                | 29/5.59          | Triosephosphate isomerase                         | NP_001147215     | 30                 | 123            | 8/22           | –1.50                                                               | –1.05      | +1.46          | –4.71      | –1.24       |
| 4 (3A)                 | 18/5.87          | Putative Pto-like serine/threonine kinase         | AAK82717         | 27                 | 128            | 4/12           | –1.06                                                               | +1.83      | –1.82          | –1.05      | –1.29       |
| 5* (3A)                | 39/6.00          | Malate dehydrogenase                              | NP_001105603     | 38                 | 137            | 12/32          | –1.77                                                               | –1.10      | –2.71          | –2.95      | +3.32       |
| 6 (3A)                 | 42/5.86          | Hypothetical protein LOC100272834                 | NP_001140759     | 19                 | 82             | 8/21           | +1.18                                                               | +1.82      | +1.59          | –2.30      | –1.66       |
| 7 (3A)                 | 42/5.96          | Hypothetical protein LOC100272834                 | NP_001140759     | 24                 | 124            | 13/38          | +1.08                                                               | +1.27      | +1.69          | –4.06      | –1.19       |
| 8 (3A)                 | 42/6.15          | Putative stearoyl-acyl-carrier protein desaturase | ABA40392         | 22                 | 82             | 9/23           | –1.35                                                               | +1.33      | –3.43          | –2.42      | –1.48       |
| 9 (3A)                 | 42/6.27          | Putative stearoyl-acyl-carrier protein desaturase | ABA40392         | 35                 | 138            | 13/25          | –1.62                                                               | +2.10      | 0              | –9.05      | D           |
| 10 (3A)                | 43/6.34          | Hypothetical protein LELG_01806                   | XP_001526977     | 24                 | 99             | 5/24           | –1.22                                                               | –3.25      | D <sup>e</sup> | +3.12      | –2.93       |
| 11* (3A)               | 44/6.37          | Acetyl-CoA acetyltransferase                      | ACG34735         | 43                 | 95             | 15/45          | –1.13                                                               | +1.37      | A <sup>f</sup> | –1.96      | D           |
| 12 (3A)                | 50/6.54          | Predicted methyltransferase                       | CAL58292         | 14                 | 93             | 4/16           | –1.91                                                               | +1.27      | +4.36          | +2.04      | –3.26       |
| 13* (3A)               | 53/6.57          | Globulin 2                                        | 1802402A         | 18                 | 173            | 8/13           | +1.17                                                               | +1.08      | +1.67          | +4.85      | –5.29       |
| 14 (3A)                | 43/6.63          | Prpol                                             | AAD11615         | 28                 | 78             | 8/19           | +2.52                                                               | –1.19      | D              | +2.09      | –6.69       |
| 15 (3A)                | 38/6.63          | Malate dehydrogenase                              | ACG36184         | 14                 | 98             | 7/21           | –1.47                                                               | –1.57      | D              | –2.24      | –1.49       |
| 16 (3A)                | 38/6.35          | Malate dehydrogenase                              | ACG36184         | 15                 | 107            | 8/29           | –1.70                                                               | –1.35      | –3.11          | –1.97      | +1.88       |
| 17 (3A)                | 35/6.34          | Legumin-like protein                              | ACG36012         | 39                 | 146            | 11/25          | –1.07                                                               | +1.55      | –1.45          | –1.50      | –1.47       |
| 18 (3A)                | 28/5.14          | Sugar metabolism transcriptional regulator        | YP_438003        | 23                 | 103            | 4/18           | –1.29                                                               | –1.63      | +3.39          | –1.83      | –1.06       |
| 19 (3A)                | 28/5.14          | Zinc-binding alcohol dehydrogenase                | YP_617174        | 16                 | 78             | 4/12           | –1.77                                                               | –1.04      | +1.75          | –3.83      | +1.58       |
| 20 (3A)                | 27/5.28          | Short chain dehydrogenase                         | YP_881652        | 25                 | 111            | 6/24           | –1.92                                                               | –1.32      | +1.31          | –4.04      | –1.07       |
| 21* (3A)               | 28/5.31          | Triosephosphate isomerase                         | P12863           | 37                 | 94             | 12/35          | –1.26                                                               | +1.02      | –1.50          | –1.81      | –1.16       |
| 22* (3A)               | 27/5.81          | Triosephosphate isomerase                         | P12863           | 44                 | 143            | 7/16           | –1.11                                                               | +3.02      | –3.04          | –2.32      | +2.30       |
| 23 (3A)                | 27/5.9           | Ascorbate peroxidase                              | NP_001170482     | 25                 | 72             | 5/12           | –1.26                                                               | –1.12      | –2.17          | –2.48      | +3.07       |
| 24 (3A)                | 28/6.02          | Carbomoylphosphate synthase                       | ABZ91363         | 23                 | 83             | 5/33           | –1.37                                                               | +1.18      | –1.73          | –2.19      | +1.35       |
| 25 (3A)                | 28/6.14          | Ferritin                                          | CAA43663         | 24                 | 75             | 6/27           | –1.07                                                               | +1.50      | –1.37          | +1.29      | –1.13       |
| 26 (3A)                | 25/6.18          | Superoxide dismutase [Mn]                         | P41979           | 25                 | 72             | 6/34           | –1.02                                                               | +1.11      | –1.62          | +1.10      | +1.22       |
| 27 (3A)                | 27/6.34          | Maize 20S proteasome alpha subunit                | NP_001105138     | 24                 | 80             | 6/13           | –3.81                                                               | –2.46      | –2.48          | –1.96      | +1.16       |
| 28 (3A)                | 28/6.39          | Maize 20S proteasome alpha subunit                | NP_001105138     | 26                 | 80             | 6/12           | –1.27                                                               | +1.19      | +1.69          | –1.76      | +1.22       |
| 29 (3A)                | 17/5.46          | Rhd6-like 2                                       | ABO84931         | 34                 | 73             | 5/14           | –4.04                                                               | –1.64      | –1.42          | –5.46      | +18.8       |
| 30 (3A)                | 15/6.51          | Peroxidase 51                                     | NP_001152260     | 16                 | 137            | 4/9            | –1.16                                                               | –2.44      | –1.22          | +1.16      | +3.48       |
| 31* (3B)               | 62/6.57          | Vicilin-like embryo storage protein               | CAA41809         | 25                 | 106            | 14/55          | +2.96                                                               | –2.50      | 0              | +3.90      | D           |
| 32 (3B)                | 62/6.61          | Vicilin-like embryo storage protein               | CAA41809         | 30                 | 138            | 18/51          | +1.73                                                               | –2.87      | 0              | +10.9      | D           |
| 33 (3B)                | 62/6.64          | Vicilin-like embryo storage protein               | CAA41809         | 23                 | 91             | 13/51          | +1.18                                                               | –1.16      | 0              | +2.80      | D           |
| 34 (3B)                | 62/6.67          | Vicilin-like embryo storage protein               | CAA41809         | 32                 | 134            | 17/47          | +2.80                                                               | –6.01      | 0              | +32.6      | D           |
| 35 (3B)                | 62/6.75          | Vicilin-like embryo storage protein               | CAA41809         | 34                 | 146            | 17/41          | +1.63                                                               | –3.09      | 0              | +21.5      | D           |
| 36* (3B)               | 50/6.58          | Globulin 2                                        | 1802402A         | 33                 | 143            | 20/60          | +1.89                                                               | +1.16      | 0              | +26.4      | D           |
| 37* (3B)               | 50/6.66          | Globulin 2                                        | 1802402A         | 34                 | 173            | 19/58          | +1.16                                                               | –1.05      | 0              | +17.5      | D           |

|         |         |                                                     |              |    |     |       |       |       |       |       |       |
|---------|---------|-----------------------------------------------------|--------------|----|-----|-------|-------|-------|-------|-------|-------|
| 38 (3A) | 36/6.28 | Proliferation-associated protein 2G4                | NP_001149216 | 11 | 86  | 5/17  | +1.36 | +1.19 | +2.14 | +3.68 | -1.33 |
| 39 (3A) | 35/6.24 | Protein kinase APK18                                | ACG32116     | 25 | 104 | 11/79 | +1.33 | -1.34 | +2.39 | +8.22 | -2.70 |
| 40 (3A) | 32/6.1  | Transcriptional regulator                           | NP_103002    | 28 | 93  | 5/38  | -1.17 | +1.02 | -2.11 | +1.55 | +1.24 |
| 41 (3A) | 28/5.79 | Brassinolide responsible protein-like protein       | BAC20042     | 39 | 75  | 6/42  | -1.55 | -1.31 | -2.03 | +1.31 | -2.39 |
| 42 (3A) | 27/5.77 | Hypothetical protein HCH_04318                      | YP_435450    | 42 | 97  | 7/33  | -3.17 | +1.10 | -1.09 | +1.13 | -7.07 |
| 43*(3A) | 29/5.58 | Glutathione S-transferase                           | AAA33469     | 37 | 134 | 9/32  | -1.53 | +1.54 | -1.00 | -1.18 | -1.07 |
| 44 (3A) | 28/6.55 | Glutathione transferase 5                           | NP_001105714 | 19 | 71  | 8/29  | +1.25 | -1.07 | -1.57 | +3.94 | +2.67 |
| 45*(3A) | 27/6.61 | 1-Cys peroxiredoxin PER1                            | NP_001105998 | 46 | 128 | 11/23 | -1.52 | -1.88 | +1.36 | +3.99 | -2.27 |
| 46*(3C) | 23/5.45 | Vicilin-like embryo storage protein                 | CAA41809     | 32 | 82  | 17/47 | +1.15 | -1.89 | +5.22 | +6.94 | -11.5 |
| 47 (3C) | 23/5.53 | Globulin 2                                          | 1802402A     | 18 | 75  | 9/42  | +1.04 | -1.17 | +4.03 | +14.5 | -3.37 |
| 48 (3C) | 23/5.63 | ADP-glucose pyrophosphorylase small subunit         | AAK69628     | 16 | 71  | 8/32  | -1.32 | -1.48 | +2.07 | +8.96 | -6.37 |
| 49*(3C) | 21/5.38 | Globulin 2                                          | 1802402A     | 12 | 69  | 7/25  | -1.07 | -1.03 | -1.58 | +1.69 | -1.54 |
| 50*(3C) | 21/5.5  | Globulin 2                                          | 1802402A     | 18 | 181 | 11/36 | -1.46 | -1.18 | +1.24 | +2.34 | -5.13 |
| 51 (3C) | 21/5.62 | Globulin 2                                          | 1802402A     | 25 | 114 | 8/32  | +1.26 | -1.62 | 0     | +2.56 | D     |
| 52*(3C) | 21/5.68 | Globulin 2                                          | 1802402A     | 18 | 154 | 9/42  | -1.10 | -1.47 | -1.57 | +2.61 | +1.92 |
| 53*(3C) | 21/5.8  | Globulin 2                                          | 1802402A     | 20 | 75  | 11/49 | -1.25 | -1.08 | -1.33 | +1.53 | +2.10 |
| 54 (3A) | 14/5.32 | ATP phosphoribosyltransferase catalytic subunit     | YP_508889    | 28 | 97  | 8/27  | +1.18 | -1.58 | -1.56 | +2.46 | -1.20 |
| 55 (3A) | 18/5.59 | 17.4 kDa Class I heat shock protein 3               | ACG35098     | 30 | 112 | 5/19  | -1.02 | +1.90 | +1.31 | +1.65 | -1.68 |
| 56 (3A) | 18/5.98 | Translation initiation factor 5A                    | NP_001105606 | 31 | 77  | 5/10  | -1.58 | -1.08 | 0     | -1.44 | D     |
| 57*(3A) | 16/6.49 | Late embryogenesis abundant protein EMB564          | NP_001105429 | 58 | 88  | 5/26  | +1.22 | +2.40 | +1.58 | +2.04 | -1.54 |
| 58 (3A) | 17/6.57 | OmpA/MotB family outer membrane protein             | YP_464091    | 37 | 72  | 7/32  | +1.17 | +2.03 | +1.62 | +2.40 | -2.47 |
| 59*(3C) | 22/5.55 | Vicilin-like embryo storage protein                 | CAA41809     | 32 | 155 | 17/47 | +1.51 | +1.18 | +1.76 | +11.3 | -10.1 |
| 60 (3A) | 15/6.19 | Glycine-rich protein1                               | NP_001105707 | 47 | 84  | 6/28  | +3.20 | -1.01 | -1.50 | +2.10 | +2.63 |
| 61 (3A) | 17/6.14 | Predicted protein                                   | XP_001419985 | 45 | 83  | 5/16  | -1.30 | -1.22 | -2.27 | +1.26 | +3.15 |
| 62 (3A) | 15/5.59 | 3-hydroxymyristoyl-acyl carrier protein dehydratase | ZP_01046404  | 31 | 109 | 5/14  | -1.10 | -3.48 | -1.03 | +2.32 | +1.38 |
| 63 (3A) | 14/5.66 | Transcriptional regulator, TetR family              | ZP_01311648  | 24 | 97  | 5/11  | +1.51 | -1.14 | -1.26 | +1.53 | +2.05 |
| 64 (3A) | 13/5.67 | Disease resistance gene analog PIC18                | AAC83566     | 20 | 144 | 4/10  | +1.10 | +1.05 | -1.16 | +2.68 | -2.25 |
| 65*(3C) | 22/5.69 | Globulin 2                                          | 1802402A     | 23 | 106 | 12/49 | -1.03 | -1.42 | -1.09 | +11.5 | +1.31 |
| 66 (3C) | 22/5.77 | Globulin 2                                          | 1802402A     | 20 | 104 | 10/32 | D     | +1.51 | +2.15 | +12.3 | -1.57 |
| 67 (3A) | 26/5.77 | Hypothetical protein LOC100273125                   | NP_001141044 | 34 | 87  | 4/36  | -1.18 | +1.77 | +1.22 | +1.32 | -1.93 |
| 68 (3A) | 33/6.27 | Predicted protein                                   | XP_001416927 | 21 | 94  | 12/47 | -1.58 | +2.02 | +1.07 | +1.32 | -1.53 |
| 69 (3A) | 29/4.56 | Predicted protein                                   | XP_001763391 | 15 | 74  | 5/14  | +3.94 | -1.14 | +1.53 | +2.72 | -2.63 |
| 70 (3A) | 34/4.66 | Proliferating cell nuclear antigen                  | NP_001105461 | 22 | 89  | 6/6   | +1.16 | -1.67 | +1.00 | -1.64 | -1.06 |
| 71*(3A) | 32/4.68 | 14-3-3-like protein GF14-12                         | Q01526       | 19 | 170 | 7/21  | +1.15 | +2.15 | +3.04 | -4.24 | +1.55 |
| 72 (3A) | 32/4.74 | 14-3-3-like protein GF14-6                          | P49106       | 38 | 81  | 9/42  | +2.22 | +3.07 | +1.21 | -1.89 | +2.03 |
| 73 (3A) | 32/4.88 | CHY1                                                | NP_001150059 | 27 | 104 | 12/38 | +2.08 | +1.29 | +1.84 | -1.20 | +1.19 |
| 74 (3A) | 31/4.82 | Hypothetical protein LOC100276029                   | NP_001143391 | 22 | 81  | 7/28  | +1.42 | +2.93 | -2.05 | -2.42 | +1.04 |
| 75 (3A) | 31/4.9  | Hypothetical protein LOC100501647                   | NP_001183256 | 20 | 72  | 8/23  | +1.64 | +1.18 | +4.15 | -1.13 | -3.42 |
| 76 (3A) | 28/5.62 | Ascorbate peroxidase 2                              | NP_001105500 | 25 | 208 | 7/32  | -2.10 | -1.14 | -1.51 | -2.24 | +2.04 |
| 77 (3A) | 28/5.69 | GTP cyclohydrolase                                  | YP_527565    | 13 | 98  | 4/5   | -1.04 | +1.03 | -5.14 | -3.56 | +5.57 |
| 78 (3A) | 20/5.54 | Low molecular weight heat shock protein precursor   | NP_001105607 | 25 | 99  | 4/7   | -1.10 | -1.82 | +1.04 | -3.51 | +2.36 |
| 79 (3A) | 20/5.73 | Transcriptional regulator                           | YP_437483    | 34 | 83  | 4/8   | A     | +1.02 | 0     | A     | D     |
| 80 (3A) | 18/5.79 | Methyl coenzyme M reductase II D protein            | AAA73437     | 41 | 84  | 9/51  | +1.37 | +1.90 | +1.82 | +1.15 | -2.97 |
| 81 (3A) | 17/5.43 | Actin depolymerizing factor                         | NP_001105474 | 34 | 94  | 10/23 | -1.62 | -1.37 | -1.75 | -1.50 | -2.0  |
| 82*(3A) | 28/6.21 | TPA: putative cystatin                              | CAG29024     | 38 | 126 | 9/22  | +1.39 | +1.97 | +4.06 | +1.63 | -2.09 |
| 83*(3A) | 36/5.26 | Thiazole biosynthetic enzyme 1-2                    | Q41739       | 40 | 147 | 13/23 | +1.87 | +1.55 | +2.42 | -1.52 | -2.44 |

(continued on next page)

Table 1 (continued)

| Spot no.* <sup>a</sup> | Exp. MW (kDa)/pI | Description                                     | Accession number | C (%) <sup>b</sup> | S <sup>c</sup> | M <sup>d</sup> | Fold change in protein amount going from (+ increased, – decreased) |            |              |            |             |
|------------------------|------------------|-------------------------------------------------|------------------|--------------------|----------------|----------------|---------------------------------------------------------------------|------------|--------------|------------|-------------|
|                        |                  |                                                 |                  |                    |                |                | 28N to 28D                                                          | 52N to 52D | 72HN to 72HD | 28N to 52N | 52N to 72HN |
| 84 (3A)                | 20/5.45          | Metal dependent phosphohydrolase                | ZP_02847397      | 29                 | 82             | 4/14           | +1.24                                                               | +1.07      | +1.23        | +1.57      | –1.08       |
| 85 (3A)                | 37/5.42          | Acyl-CoA dehydrogenase                          | YP_586561        | 18                 | 96             | 5/13           | –1.37                                                               | –1.14      | +1.12        | +1.39      | –2.35       |
| 86*(3A)                | 17/5.99          | NBS-LRR resistance-like protein RGC456          | ABQ57768         | 35                 | 100            | 4/27           | 0                                                                   | +19.0      | A            | A          | D           |
| 87 (3A)                | 15/5.43          | Embryo specific protein5                        | NP_001105349     | 69                 | 99             | 10/64          | +1.58                                                               | –1.17      | –1.22        | +1.79      | –1.08       |
| 88*(3A)                | 36/6.78          | Stress responsive protein                       | NP_001149550     | 28                 | 80             | 10/48          | +10.2                                                               | +1.6       | –1.8         | +6.69      | –4.56       |
| 89 (3A)                | 35/6.45          | Eukaryotic translation initiation factor 3c     | ABW96015         | 14                 | 79             | 4/9            | –5.44                                                               | –1.57      | +1.30        | –1.47      | –1.59       |
| 90 (3A)                | 33/5.39          | Rice blast resistance protein                   | ABC59043         | 18                 | 119            | 6/18           | +2.60                                                               | –1.04      | –1.37        | +9.37      | –2.53       |
| 91*(3A)                | 34/5.62          | Thiazole biosynthetic enzyme 1-2                | Q41739           | 18                 | 100            | 10/23          | +1.51                                                               | +1.11      | A            | –1.09      | D           |
| 92*(3A)                | 22/5.89          | Globulin 2                                      | 1802402A         | 17                 | 94             | 11/40          | 0                                                                   | –1.04      | +2.69        | A          | –3.78       |
| 93 (3A)                | 21/5.84          | Globulin 2                                      | 1802402A         | 12                 | 74             | 7/15           | 0                                                                   | –1.45      | 0            | A          | D           |
| 94 (3A)                | 22/5.94          | Globulin 2                                      | 1802402A         | 17                 | 78             | 7/20           | A                                                                   | –1.21      | +5.13        | A          | –1.32       |
| 95 (3A)                | 14/6.31          | Hypothetical proteins LOC100502442              | NP_001183849     | 31                 | 90             | 6/40           | A                                                                   | +1.19      | +15.2        | A          | –3.96       |
| 96 (3A)                | 12/6.28          | Major allergen Bet v 1.01C                      | ABC41583         | 26                 | 132            | 4/24           | A                                                                   | +3.91      | A            | A          | D           |
| 97 (3A)                | 12/6.14          | Proteasome subunit alpha type 1                 | NP_001149085     | 35                 | 110            | 5/38           | A                                                                   | +2.27      | A            | A          | D           |
| 98 (3A)                | 35/6.31          | DNA binding protein                             | CBG27180         | 32                 | 92             | 10/70          | +1.35                                                               | +1.95      | –1.87        | +3.86      | –1.47       |
| 99 (3A)                | 31/5.86          | Glyoxalase I                                    | NP_001105217     | 51                 | 90             | 18/73          | –1.02                                                               | +1.22      | –3.61        | –1.40      | +2.45       |
| 100(3A)                | 31/5.99          | Putative calciumodilin dependent protein kinase | NP_001105906     | 22                 | 79             | 8/31           | –1.25                                                               | +1.35      | –5.79        | –1.41      | +1.70       |
| 101(3A)                | 21/5.93          | Globulin 2                                      | 1802402A         | 20                 | 119            | 8/20           | 0                                                                   | –1.53      | 0            | A          | D           |
| 102(3A)                | 17/6.79          | 16.9 kDa class I heat shock protein 1           | NP_001152609     | 70                 | 125            | 12/38          | –1.20                                                               | –1.52      | –1.26        | +5.27      | –1.53       |
| 103(3A)                | 30/5.08          | Unknown protein                                 | ACN33806         | 60                 | 79             | 14/36          | –1.09                                                               | –1.02      | +1.34        | –1.32      | –1.58       |
| 104(3A)                | 20/6.25          | RAB17 protein                                   | CAM56274         | 43                 | 90             | 11/41          | 0                                                                   | 0          | A            | 0          | 0           |
| 105(3A)                | 20/6.38          | RAB17 protein                                   | CAM56274         | 39                 | 80             | 10/46          | 0                                                                   | –1.04      | A            | A          | D           |
| 106(3A)                | 57/6.08          | ATPase subunit 1                                | YP_588408        | 55                 | 229            | 24/62          | –1.27                                                               | +1.40      | –2.44        | –1.37      | –1.58       |
| 107(3A)                | 60/6.21          | Mitochondrial aldehyde dehydrogenase 2          | NP_001105891     | 42                 | 120            | 16/67          | +1.27                                                               | +1.84      | +1.30        | –1.60      | –1.59       |
| 108(3A)                | 57/5.87          | Succinate semialdehyde dehydrogenase            | NP_001147173     | 45                 | 144            | 14/35          | –1.01                                                               | +1.06      | –1.68        | +2.29      | –2.32       |
| 109(3A)                | 61/5.82          | Enolase                                         | ACG31732         | 50                 | 200            | 17/36          | –1.07                                                               | –1.10      | –1.58        | –1.30      | +1.04       |
| 110(3A)                | 68/5.58          | Vicilin-like embryo storage protein             | CAA41809         | 47                 | 212            | 22/62          | –2.01                                                               | –1.06      | –1.29        | –2.14      | –1.06       |
| 111(3A)                | 64/5.44          | Chaperonin CPN60-1, mitochondrial precursor     | NP_001105716     | 40                 | 198            | 23/48          | –1.43                                                               | –1.11      | –3.27        | +1.10      | –1.04       |

<sup>a</sup> Protein identified by MALDI-TOF and MALDI-TOF/TOF mass spectrometry and the others identified by MALDI-TOF mass spectrometry.

<sup>b</sup> Sequence coverage.

<sup>c</sup> Protein score and Ions score. Protein score and Ions score are  $-10 \cdot \log(P)$ , where P is the probability that the observed match is a random event.

<sup>d</sup> Matched peptides/Searching peptides.

<sup>e</sup> Disappeared.

<sup>f</sup> Appeared.

Dehydration is a stress to maize embryos due to reactive oxygen species (ROS) production by autoxidation and Fenton reactions [40]. ROS and metal ions are regarded as the initiators of oxidative cell damage [41–43]. Fig. 1C shows that TBARS content, a crude measure of ROS production, was high in immature embryos and decreased gradually with embryo development to reach  $6.15 \mu\text{mol g}^{-1}$  DW at 52 DAP after desiccation. However, a greater accumulation of TBARS in the embryos was observed during seed imbibition. TBARS continued to increase during the 72 h of imbibition to reach a value 2.72 times that of control embryos.

Another type of ROS,  $\text{H}_2\text{O}_2$ , was also investigated.  $\text{H}_2\text{O}_2$  contents of dehydrated embryos decreased slightly from  $1.06 \mu\text{mol g}^{-1}$  DW during development in parallel with acquisition of desiccation tolerance, and increased with loss of desiccation tolerance during germination from 0.34 to  $0.75 \mu\text{mol g}^{-1}$  DW at 72 h (Fig. 1D).  $\text{H}_2\text{O}_2$  appears to play a dual role in seed physiology behavior, on the one hand, as a messenger in cellular signaling pathways at low concentrations [44,45] and, on the other hand, as toxic products that accumulate under stress conditions at high concentrations [46,47]. Seed desiccation and seed aging occurring in seeds appear as being tightly linked to the deleterious role of ROS [48]. The  $\text{H}_2\text{O}_2$  content varies dramatically depending on the physiological state of the seed. It is quite high at the beginning of seed development and during germination, probably because the moisture content is high enough to allow metabolic activities, but subsequently decreases during seed desiccation [48,49].

With development seeds pass into a metabolically inactive or quiescent state and become desiccation-tolerant, in parallel both TBARS production and  $\text{H}_2\text{O}_2$  contents decreased (Fig. 1). Germinating tissues are particularly vulnerable to damage from desiccation caused by rapidly increasing respiratory rate upon imbibition of quiescent dry seeds, thereby resulting in an increase in ROS production and lipid peroxidation [3,50,51]. The observed increase in TBARS production and  $\text{H}_2\text{O}_2$  contents in maize embryos with increasing imbibition time and loss of desiccation tolerance (Fig. 1) was consistent with this model (Fig. 2).

In the following experiment we compared the proteomes of 28N — non-dehydrated embryos at 28 DAP (desiccation

sensitive); 52N — non-dehydrated embryos at 52 DAP (desiccation tolerant); 72HN — non-dehydrated embryos from mature seeds imbibed for 72 h (desiccation sensitive); 28D — dehydrated 28N; 52D — dehydrated 52N; 72HD — dehydrated 72HN (Fig. 2).

### 3.2. The proteome profiles, identification and functional classification of the differentially expressed proteins

After CBB R-250 staining, each gel contained approximately 1000 protein spots (Fig. 3A and Supplemental Fig. S1). A total of 111 differentially expressed protein spots with intensities changed by more than 1.5-fold were analyzed by MALDI-TOF and TOF/TOF MS. Of these, 99 spots (89%) were successfully identified by peptide mass fingerprinting, whereas 12 spots were annotated as either unknown or hypothetical protein. Eight of the unknown proteins were matched with functional domains in NCBI and their biological functions were inferred. 89 of differentially expressed protein spots were searched and matched against maize data in the NCBI database. In the following we will often use the terms “up-/down-regulated” to mean 50% increase/decrease in amount of protein in a spot without implying a change in protein synthesis and/or degradation. In fact, posttranslational modifications can change the pI and/or the apparent size of a protein and form new spots. In this way necklaces of spots all containing the same gene product but with different pI are formed (e.g. vicilin-like embryo storage protein, spots 31–35, Table 1, Fig. 3B).

Taken together, the 99 identified proteins represent 67 unique proteins. Based on the metabolic and functional features of maize embryos [52,53], these proteins have been classified into 7 major categories (Fig. 4). The majority of the identified proteins belong to the three functional groups protein metabolism (26%), stress response (21%) and carbohydrate and energy metabolism (17%).

Comparative analysis of differentially expressed proteins among non-dehydrated maize embryos showed that during development and mature drying (from 28N to 52N), 86 protein spots increased or decreased significantly (Table 2). Of the 54

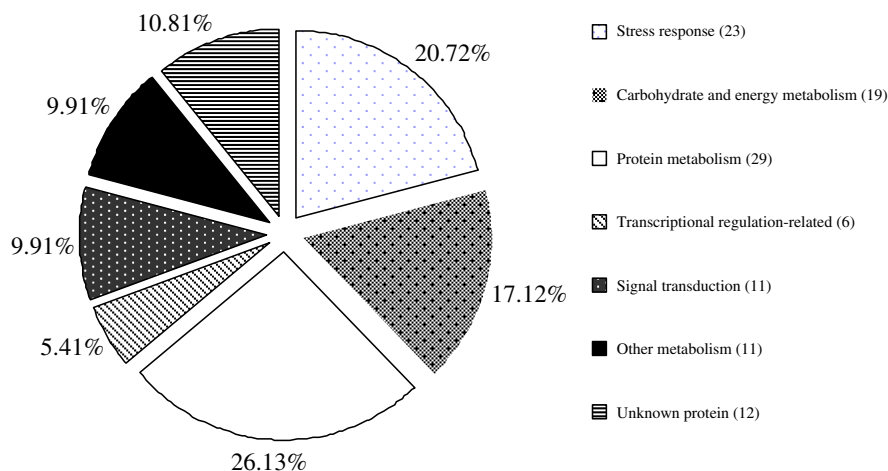


**Fig. 4 – Functional classification and distribution of all 111 identified proteins. Proteins were categorized into six classes (plus Unknown proteins) according to Bevan et al. (1998) and Schiltz et al. (2004).**

**Table 2 – The number and percentage of differentially expressed protein spots in maize embryos 28N, 52N and 72HN. The total number of spots affected is larger than 111 because the same spot often changed both in response to development (28N to 52N) and in response to germination (52N to 72HN).**

| Function groups                    | Change from 28N to 52N |       |                     |       | Change from 52N to 72HN |       |                     |       |
|------------------------------------|------------------------|-------|---------------------|-------|-------------------------|-------|---------------------|-------|
|                                    | Upregulated spots      |       | Downregulated spots |       | Upregulated spots       |       | Downregulated spots |       |
|                                    | No.                    | %     | No.                 | %     | No.                     | %     | No.                 | %     |
| Stress response                    | 13                     | 24.1% | 3                   | 9.4%  | 6                       | 28.6% | 11                  | 18.6% |
| Carbohydrate and energy metabolism | 2                      | 3.7%  | 13                  | 40.6% | 6                       | 28.6% | 7                   | 11.9% |
| Protein metabolism                 | 24                     | 44.4% | 5                   | 15.6% | 2                       | 9.5%  | 19                  | 32.2% |
| Signal transduction                | 1                      | 1.9%  | 3                   | 9.4%  | 3                       | 14.3% | 2                   | 3.4%  |
| Transcriptional regulation-related | 5                      | 9.3%  | 2                   | 6.3%  | 2                       | 9.5%  | 6                   | 10.2% |
| Other metabolism                   | 6                      | 11.1% | 3                   | 9.4%  | 1                       | 4.8%  | 5                   | 8.5%  |
| Unknown protein                    | 3                      | 5.6%  | 3                   | 9.4%  | 1                       | 4.8%  | 9                   | 15.3% |
| Total                              | 54                     | 100   | 32                  | 100   | 21                      | 100   | 59                  | 100   |

up-regulated spots, 24 spots (44%) represented proteins involved in protein metabolism, such as globulin and vicilin-like embryo storage protein, which was expected because they are known to be synthesized during seed filling [15,26]. At maturity, the maize seed accumulates large amounts of starch and storage proteins [54]. However, also proteins involved in stress response (24%) were often up-regulated during seed maturation. Of the 32 down-regulated spots from 28N to 52N, the best represented functional group was carbohydrate and energy metabolism (41%) (Table 2).

A total of 80 protein spots changed significantly during the first 72 h of germination (from 52N to 72HN) (Table 2). Half of the 21 up-regulated proteins were involved in stress response or carbohydrate and energy metabolism. About a third of the down-regulated proteins belonged to the functional group

protein metabolism, which was unsurprising since storage proteins belong to that group (Tables 1 and 2). Mobilization of protein reserves plays a crucial role in seed germination [50,55]. Indeed, germinating seeds rely exclusively on reserve mobilization for energy and biosynthesis until photosynthesis becomes active [55,56].

The effect of desiccation on protein expression was evaluated by comparing the proteome of desiccated maize embryos 28D, 52D and 72HD with non-dehydrated embryos 28N, 52N and 72HN, respectively (Fig. 5, Table 1). Proteins involved in protein metabolism, stress responses, and carbohydrate and energy metabolism changed most often during desiccation (Fig. 5). However, the pattern of change was not the same for desiccation-tolerant and -sensitive embryos. During drying of desiccation-tolerant embryos (52N and 52D), the largest up-

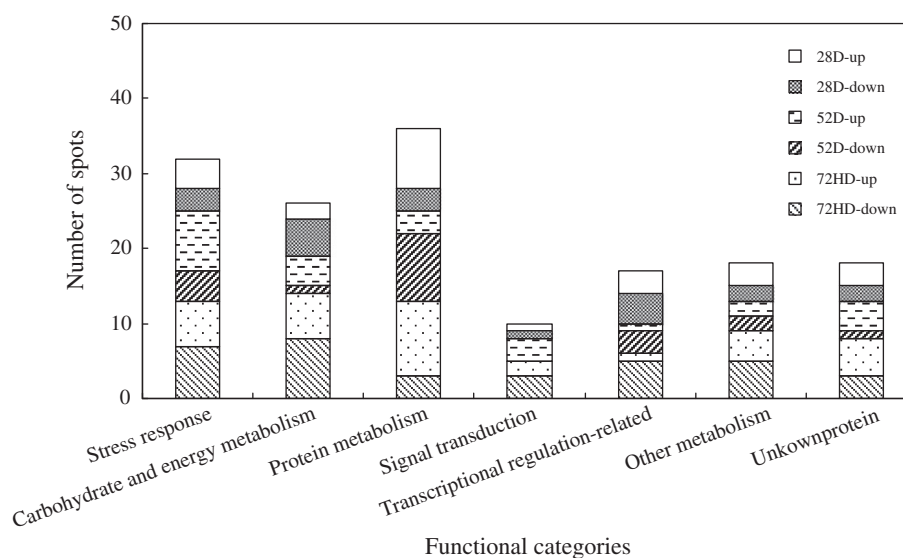


**Fig. 5 – The number and functional classification of identified proteins changed in abundance during desiccation treatment in desiccation-tolerant and -sensitive maize embryos. The distribution of changed proteins increased or decreased after desiccation treatment in maize embryos are given for each category. 28D-up, the number of proteins increased after desiccation in 28D compared with 28N; 28D-down, the number of proteins decreased after desiccation in 28D compared with 28N; 52D-up, the number of proteins increased after desiccation in 52D compared with 52N; 52D-down, the number of proteins decreased after desiccation in 52D compared with 52N; 72HD-up, the number of proteins increased after desiccation in 72HD compared with 72HN; 72HD-down, the number of proteins decreased after desiccation in 72HD compared with 72HN.**

regulated functional group were stress-related proteins (8 proteins up-regulated), and the largest down-regulated functional group was protein metabolism (9 proteins) (Fig. 5). During drying of immature embryos (28N and 28D) and germinating embryos (72HD and 72HN), the largest increasing functional group was protein metabolism and the largest decreasing function group was carbohydrate and energy metabolism.

Immature (28D) and germinating maize embryos (72HD) appeared to invest most of their energy in protein metabolism and energy metabolism during desiccation stress, which they did not tolerate well (Fig. 1). In contrast, the desiccation-tolerant embryos (52D) accumulated many stress response proteins including pathogenesis-resistant proteins, antioxidant systems proteins and protective proteins both during development (Table 2) and during desiccation (Fig. 5). Those proteins are proposed to be involved in conferring desiccation tolerance. The presence and efficient operation of antioxidant systems is considered as one of the major factors associated with desiccation tolerance of orthodox seeds [9,57]. Bewley and Black [1] indicated that LEA proteins and dehydrins protect plants efficiently from desiccation damage.

### 3.3. Identification of target proteins

To further identify proteins that are involved in making the embryos desiccation tolerant, we looked for proteins that were up-regulated (>1.5 fold increase) during seed maturation and acquisition of desiccation tolerance (52N vs 28N) and down-regulated (1.5 fold decrease) again during imbibition and loss of desiccation tolerance (72HN vs 52N) especially the proteins that increased further (>1.5-fold) during desiccation of desiccation-tolerant embryos (52D vs 52N). A total of nine proteins fulfilled these criteria — 17.4 kDa Class I heat shock protein 3 (spot 55), late embryogenesis abundant protein EMB564 (spot 57), OmpA/MotB family outer membrane protein (spot 58), globulin 2 (spot 66), TPA: putative cystatin (spot 82), NBS-LRR resistance-like protein RGC456 (spot 86), stress responsive protein (spot 88), major allergen Bet v 1.01C (spot 96) and proteasome subunit alpha type1 (spot 97) (Table 1). Close-up views of these differentially expressed proteins are shown in Fig. 6. We infer that these “target proteins” probably have a very close relationship with desiccation tolerance of maize embryos.

We also speculated that the presence of a specific protein could be an indicator of desiccation sensitivity. Thus, we looked for the inverse pattern — decrease during seed maturation, increase during imbibition and decrease during desiccation of mature desiccation-tolerant seeds. Only two proteins (spot 29 — Rhd6-like 2 and spot 78 — low-molecular-weight heat shock protein precursor) showed this pattern.

Finally, it is possible that the storage proteins accumulating in large amounts during seed maturation and disappearing during seed germination have a function in desiccation tolerance in addition to their storage role [1,4,58].

In the following we will consider how these target proteins might be involved in conferring desiccation tolerance.

#### 3.3.1. Stress response proteins

Six out of the nine target proteins belong to the functional category “stress-related”. ROS production increases under stress

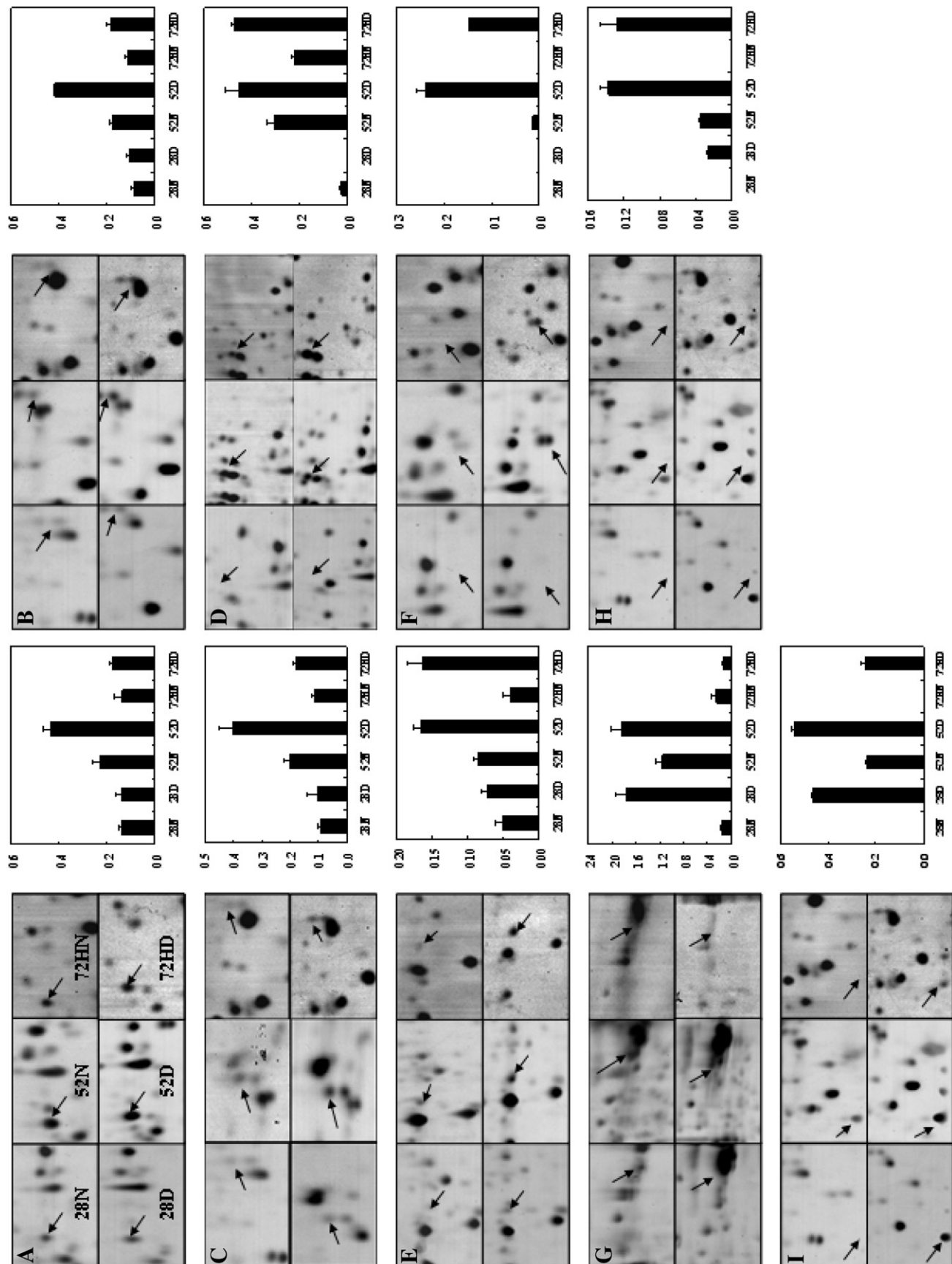
conditions and causes damage to cellular components [42,43]. To protect against oxidative damage, cells have developed a wide range of antioxidant systems that scavenge excessive ROS or ROS-induced toxic substances [59]. Under stress conditions, stress-related proteins are induced and play an important role to protect cell against damage [60]. These processes are clearly of importance for the adaptation of maize embryos to desiccation.

After maturation drying and desiccation treatment in desiccation-tolerant embryos, 17.4 kDa Class I heat shock protein 3 (HSPs) (spot 55) participate in many kinds of stress response. HSPs and small HSP act as molecular chaperones and are thought to repair and aid in the renaturation of stress-damaged proteins, and perhaps in that way help protecting cells against the effects of stress [61–63]. The drought-responsive element-binding factor 2 (DREB2) and heat shock factor A9 (HaHSFA9) are involved in transcriptional activation of additional small HSP promoters, and thus contribute to the HaHSFA9-mediated enhancement of longevity and basal thermo-tolerance of seeds [64].

The low-molecular-weight HSP precursor (spot 78) showed the inverse pattern to the 17.4 kDa Class I heat shock protein 3 (spot 55), which makes excellent sense if its mature form is involved in conferring desiccation tolerance. However, we did not identify the mature form in any of the spots analyzed.

Several stress response proteins increased in response to dehydration stress in desiccation-tolerant embryos and may have close relationship with desiccation tolerance. The late embryogenesis abundant protein EMB564 (spot 57) is a very hydrophilic protein that is a member of a class of highly conserved proteins, typified also by the Em protein of wheat and the LEA D19 protein of cotton, which may play an essential role in seed survival and in controlling water exchange during seed desiccation and imbibition [65,66]. The presence and increased level of LEAs correlated well with desiccation tolerance [19,20]. LEA proteins accumulate to high levels in developing seeds during late maturation and in dehydrating vegetative tissues of resurrection plants [21]. On the other hand, an RNA interference (RNAi)-construct against the seed-expressed dehydrin of *Arabidopsis thaliana* LEA 14 (At2g21490) as well as two other seed-expressed dehydrin homologues, XERO1 (At3g50980) and RAB 18 (responsive to abscisic acid 18, At5g66400) showed that these dehydrins play no important role in desiccation tolerance and relate to seed longevity [27]. These results could indicate that the abundance of some, but not all, LEAs are associated with desiccation tolerance [27,28]. For example, in our results RAB 17 (spot 104 and 105) is a dehydrin homologue and an abscisic acid-responsive protein [67], and was not included among the eleven identified stress-response target proteins in maize embryos during desiccation stress. However, it is worth keeping in mind that the results to some extent depends on the species studied, the experimental conditions chosen and the specific group of LEA proteins monitored.

TPA: putative cystatin (spot 82) and stress responsive protein (spot 88) could be induced by ABA and overexpressed in seed during the mature drying phase, and are degraded when the seed germinates [68]. A group of pathogenesis-resistant proteins appeared and increased in level in desiccation-tolerant embryos after maturation drying and desiccation treatment.



The disease-resistant NBS-LRR protein (spot 86) functions as a molecular switch in plant defense systems [69]. McHale et al. [70] reported that plant NBS-LRR proteins act through a network of signaling pathways and induce a series of plant defense responses. The major allergen Bet v 1.01C (spot 96) present in dehydrated embryos is a pathogenesis-related protein and is related to stress-related [71].

Some of the stress-related proteins increased during maturation drying while others were induced during desiccation treatment. This suggests that they play different roles in the protection against desiccation damage. For instance, glutathione transferase 5 (spot 44) and 1-Cys peroxiredoxin PER1 (spot 45) increased during mature drying and decreased during the following desiccation. These stress-related proteins apparently work during slight desiccation but are degraded during severe desiccation. In contrast, NBS-LRR resistance-like protein RGC456 (spot 86) appeared during mature drying, and increased markedly during desiccation treatment. Such proteins may mainly have a role under severe desiccation. The results reflected the importance of protection against desiccation-induced injury due to ROS produced during seed development and germination.

Some stress-related proteins were found to decrease in response to dehydration stress. The decrease in the amount of peroxidase 51 (spot 30), upon exposure to dehydration stress, suggests that it is damaged by dehydration and therefore cannot detoxify ROS-induced lipid peroxidation products [72]. This is supported by the observation that ascorbate peroxidase decreased at the protein and transcript levels in response to water stress [73] or heat stress [74].

### 3.3.2. Protein metabolism

In dicotyledonous plants, the major seed reserves are storage proteins like globulins, legumins and vicilins [15]. We found that vicilin-like embryo storage protein and globulin constituted a very high proportion (79%) of the identified storage proteins. A number of proteins with molecular weight (MW) of 20.1–30.0 and 45.0–66.0 kDa (Fig. 3B and C) were identified as vicilin-like embryo storage protein (spots 31–35, 46, 59 and 110) and globulin (spots 13, 36, 37, 47, 49–53, 65, 66, 92–94 and 101). The storage proteins, lipids, and polysaccharides accumulate in seeds during seed maturation [75] and this is accompanied by an increase in seed desiccation tolerance [1]. Vicilins belong to the cupin superfamily of proteins, and they have multiple functions in addition to simple storage molecules [26,76]. Globulin with a high content of sulfur-containing amino acids is utilized during seed germination [77]. They accumulated gradually during development and disappeared during germination. The high frequency of identified proteins involved in protein metabolism in mature

embryos confirms results of a previous proteomic study and physiological study [54]. These proteins are degraded during germination, and used by germinating seedlings as a source of nitrogen and carbon skeleton [78].

Globulin 2 (spot 66) increased after maturation drying in 52N, decreased during germination and accumulated during desiccation treatment in 52D. Storage proteins were observed to accumulate during seed filling, which is concomitant with the acquisition of both seed vigor and seedling vigor [76]. Boudet et al. [4] identified a pea (*Pisum sativum*) storage proteins legumin precursor homolog in *Medicago truncatula* radicles associated with desiccation tolerance. We identified three forms of globulin of 21, 22 and 50 kDa, but with identical pI values. Storage protein heterogeneity has been also reported in other plant species and their presence has been attributed to: (i) expression of multigene families encoding for different primary sequences; (ii) differential proteolytic processing of expressed genes; and (iii) differential protein glycosylation [79,80]. A genomic study in castor plant seed reveals that those 11S globulins are encoded by two diverged subfamilies comprising a total of nine genes and two putative pseudogenes. The predicted sizes for  $\alpha$  subunit isoforms were 28.6–32.6 kDa and 20.2–21.6 kDa for  $\beta$ -subunit, having a wide variation in pI of 5.41–10.21 [79]. Our data spot 66 was identified as globulin 2 with an experimental MW of 22 kDa are within these ranges.

Proteasome subunit alpha type 1 (spot 97) increased after maturation drying in 52N, decreased during germination and accumulated during desiccation treatment in 52D. A number of reports have shown that cells exhibit increased rates of proteolysis following exposure to oxidative stress-inducing agents [81,82]. The proteasome is a multicatalytic proteinase complex that is involved in ATP/ubiquitin-dependent proteolytic pathways [83] and can degrade unneeded or damaged proteins to protect the seed from damage [81,84]. The constant increasing of proteasome during mature drying and the following desiccation treatment might imply that intracellular proteins are oxidatively modified by free radicals and/or related oxidant, and that these modified proteins are selectively recognized and preferentially degraded by intracellular proteolytic enzymes. Modulation of the dehydration stress response is known to be dependent on the cellular control of degradation and the maintenance of quality of proteomes by the ubiquitin-proteome system [85].

## 4. Conclusions and perspectives

This study has for the first time shed light on changes in proteome profile of maize embryos during acquisition and loss of

**Fig. 6 – Close-up views of the proteins differentially up-regulated in response to desiccation. The proteins were selected by the following criteria: An up-regulation in response to acquisition of desiccation tolerance (increased >1.5 fold between 28N and 52N), an up-regulation in response to desiccation (increased >1.5 fold between 52N and 52D) and a down-regulation in response to loss of desiccation tolerance (decreased >1.5 fold between 52N and 72HN). A, 17.4 kDa Class I heat shock protein 3, ACG35098 (spot 55); B, Late embryogenesis abundant protein EMB564, NP\_001105429 (spot 57); C, OmpA/MotB family outer membrane protein, YP\_464091 (spot 58); D, Globulin2, 1802402A (spot 66); E, TPA: putative cystatin, CAG29024 (spot 82); F, NBS-LRR resistance-like protein RGC456, ABQ57768 (spot 86); G, Stress responsive protein, NP\_001149550 (spot 88); H, Major allergen Bet v 1.01C, ABC41583 (spot 96); I, Proteasome subunit alpha type1, NP\_001149085 (spot 97). Data in histogram presented as mean  $\pm$  SD (n=3).**

desiccation tolerance. The physiological and proteomic data of desiccation-tolerant and -sensitive embryos with identical genetic background were analyzed. The desiccation-sensitive embryos used in our study were immature and imbibed seeds which can be used as a model system for recalcitrant seeds to find the cause of their sensitivity to dehydration.

When maize embryos are exposed to dehydration, ROS is readily produced, lipid peroxidation occurs and TBARS accumulates. Based on our data, an oxidative stress-responsive protein network is proposed. A number of stress-response proteins increase strongly during dehydration of desiccation-tolerant mature embryos, but these embryos also markedly restrained the expression of metabolism-related proteins and carbohydrate metabolism-associated proteins. This did not happen in desiccation-sensitive embryos and this may be the reason that they do not respond as efficiently to dehydration stress. Plants are known to display “cross-talk” between different abiotic stresses. Maturation drying warns mature embryos and gives them sufficient time to respond to the ensuing desiccation. Immature maize embryos resemble recalcitrant seeds in that both maintain an active metabolism while being intolerant to dehydration.

In general, if the embryos fail to respond to dehydration in time and the appropriate defenses are activated too late, as perhaps happened in immature and germinating maize embryos, and may also happen in recalcitrant seeds, the seeds are adversely affected by the stress; whereas the maturation dehydration activates the appropriate defenses beforehand resulting in better stress tolerance.

We identified 11 proteins that are potentially involved in conferring desiccation tolerance. Most of these proteins are stress-related. It is our aim to study the expression of these proteins more thoroughly to establish how they are involved in desiccation tolerance with a view to using this knowledge in plant breeding, but also in the hope that they can help us understand recalcitrant seeds.

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