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Proteomic analysis of the enhancement of seed vigour in osmoprimed alfalfa seeds germinated under salinity stress

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Abstract

Alfalfa (*Medicago sativa* L.) yield is severely compromised by soil salinity, especially at the level of seedling establishment. This question was addressed by proteomics to decipher whether specific changes in protein accumulation correlate with germination performance of alfalfa seeds submitted to a salinity stress as obtained by imbibing seeds in the presence of NaCl. This study used alfalfa seeds submitted to an osmopriming invigoration treatment that proved very efficient in counteracting the negative effect of salinity stress on germination performance. Comparative proteomic analyses disclosed 94 proteins commonly characterizing the response of both the untreated control and osmoprimed seeds to the experimental salinity stress. Remarkably, many of them, representing 84 proteins, showed contrasting accumulation patterns when comparing the untreated control and osmoprimed seeds submitted to the same salt stress. Thus numerous changes observed in the proteome of the untreated control seeds imbibed in the presence of salt, and presumably accounting for the loss in seed vigour associated with salinity stress, can be substantially reversed in osmoprimed seeds undergoing this stress. These data therefore provide a biochemical understanding of the increase in seed vigour generally observed with primed seeds.

Keywords: alfalfa, osmopriming, proteomics, salt stress, seed germination, vigour

Introduction

Salinity, one of the main factors limiting plant productivity, affects nearly 20% of the world's cultivated area and half of the world's irrigated lands (Bohnert *et al.*, 1995). In Tunisia nearly 1.5 million hectares, corresponding to nearly 10% of the total area of the country and about 30% of cultivated lands, are affected by this stress (Hachicha *et al.*, 1994; Hachicha, 2007). Alfalfa (*Medicago sativa* L.) is the most cultivated forage legume in Tunisia and in the world, being an important fodder plant due to its high nutritive value and growth potential. Its cultivation requires irrigation, which may be provided by salt-laden water (Boughanmi *et al.*, 2005). However, germination and seedling establishment of this species, which are considered as being the most vulnerable stages of plant development (Rajjou *et al.*, 2012), are highly sensitive to salt stress (Peel *et al.*, 2004). The production of high-vigour, salt-tolerant alfalfa seeds is therefore an important agronomic objective. Unfortunately, in alfalfa the most salt-tolerant species might not be the most productive or desirable (Peel *et al.*, 2004), and therefore alternative strategies should be considered. One way to address this question is to develop seed technologies aimed at invigorating low-vigour seedlots; for example, by conditioning of seeds in osmotics. During osmopriming, seeds are exposed to an external water potential that is low enough to prevent germination but allows some pre-germinative physiological and biochemical processes to take place (Heydecker and Coolbear, 1977; Bradford, 1986; Ashraf and Foolad, 2005). For storage purpose, a drying of the treated seeds following this controlled hydration is permitted because imbibed seeds generally remain desiccation tolerant up to radicle emergence (Boudet *et al.*, 2006). In this way, osmoprimed alfalfa seeds were shown to display better performance than untreated seeds under salinity stress (Amooaghaie, 2011; Yacoubi *et al.*, 2011).

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Despite the wide use of these invigoration treatments, their optimization currently rests on carrying out germination assays, and the mechanisms underlying the improvement in seed vigour are largely unknown. To fill this gap, in recent work we used proteomics to analyse protein patterns from untreated control and osmoprimed alfalfa seeds, both in their respective dry seed state and during germination in optimal conditions. The results unveiled the unexpected finding that osmopriming cannot simply be viewed as an advance of germination-related processes but involves other mechanisms such as the mounting of defence mechanisms, enabling osmoprimed seeds to surmount environmental stresses potentially occurring during germination (Yacoubi *et al.*, 2011). In the present work we extend this previous proteomic analysis to characterize the impact of salinity stress on untreated control and osmoprimed alfalfa seed germination. The data disclosed proteomic signatures allowing a better understanding of how osmopriming effectively enabled the invigorated seeds to surmount the deleterious effects of the salinity stress.

Materials and methods

Plant material and germination experiments

Alfalfa seeds (cv. Gabès) were used in all experiments. Osmoprimed seeds were prepared as described (Yacoubi *et al.*, 2011) by incubating dry mature seeds in a −1.0 MPa polyethylene glycol (PEG 8000) solution (290 g l^{-1}) for 24 h at 25°C under dark conditions. Then seeds were briefly rinsed in distilled water and dried back to their original moisture level (10%) at 20°C. Germination assays were carried out at 25°C, in covered plastic boxes where seeds (100 seeds per box; three replicates for each condition) were placed on three sheets of absorbent paper wetted with 6 ml of NaCl solution (10 g l^{-1}). A seed was regarded as germinated when the radicle protruded through the seed coat. The Seed Calculator software (Plant Research International B.V., Wageningen, The Netherlands) was used to estimate the germination parameters.

Preparation of protein extracts

Total water-soluble protein extracts (albumins) were prepared as described (Yacoubi *et al.*, 2011) from seeds collected by the end of germination *sensu stricto*, namely at imbibition time T_1 corresponding to achievement of 1% germination with the various seed samples. Following grinding of seeds (100 mg) using a mortar and pestle in liquid nitrogen, albumins were extracted at 4°C in 8.0 ml of water containing the protease inhibitor cocktail 'complete Mini' from Roche Diagnostics (Meylan, France), 64 U DNase I (Roche Diagnostics) and 8 U RNase A (Sigma, Lyon, France). After 10 min at 4°C, 20 mM dithiothreitol was added and the protein extracts were stirred for 20 min at 4°C then centrifuged (15,000 *g* for 15 min at 4°C). Final supernatants corresponded to the soluble albumin extracts. Protein concentrations were measured using bovine serum albumin as a standard (Bradford, 1976).

Two-dimensional polyacrylamide gel electrophoresis, protein staining and gel analyses

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) analyses were carried out as described (Yacoubi *et al.*, 2011), using protein samples corresponding to about 100 μg of protein. For each condition analysed, 2D gels were made in triplicate and from two independent protein extractions. Following protein staining with silver nitrate, image analysis of the scanned 2D gels was carried out with the Image Master 2D Elite software (Amersham Biosciences, Orsay, France) (Yacoubi *et al.*, 2011). Only spots with an average standardized abundance that varied by a minimum of 20% ($P \leq 0.05$; Student's *t*-test) were considered as varying spots.

In-gel digestion, mass spectrometry and database searching

Silver-stained protein spots of interest were excised from 2D-PAGE gels, treated with trypsin, and peptide fragments were analysed by tandem mass spectrometry (MSMS) and identified as described previously (Yacoubi *et al.*, 2011). MSMS raw data were processed (smooth 3/2 Savitzky Golay and no deisotoping) using the ProteinLynx Global Server 2.05 software (Waters; http://waters.com/waters/nav.htm?cid=10008600&locale=fr_FR) and peak lists were exported in the micromass pkl format. Peak lists of precursor and fragment ions were matched automatically to both proteins in the *Medicago truncatula* genome assembly MT3 (release 3, www.medicago.org; 53,423 sequences, 12,992,982 residues) and TIGR *M. truncatula* and *M. sativa* transcript assemblies (TA) (357,600 sequences; 78,133,384 residues) (ftp://ftp.tigr.org/pub/data/plantta/), using a local Mascot version 2.3 program (Matrix Science, London, http://www.matrixscience.com). If no match was obtained, a final search in the National Center for Biotechnology Information (NCBI) non-redundant protein databank (NCBInr 20101115, taxonomy viridiplantae, 844,562 sequences) was completed. Mascot searches were performed with the following parameters: trypsin specificity, two missed cleavages, variable carbamidomethyl cysteine and oxidation of

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methionine, 0.2 Da mass tolerance on both precursor and fragment ions, and the possibility to pick the $^{13}C_2$ peak for precursor ion mass ($\neq {}^{13}C = 2$). To validate protein identification, only matches with individual ion scores above 47, 55 and 60 (for the *Medicago* MT3 database, TIGR TA database and NCBI viridiplantae database, respectively), a threshold value corresponding to $P < 0.005$ and calculated by the Mascot algorithm with our databases were considered. Moreover, among the positive matches, only protein identifications based on at least three different peptide sequences of more than six amino acids with an individual ion score above 20 were accepted. These additional validation criteria are a good compromise to limit the number of false-positive matches without missing real proteins of interest (Waanders *et al.*, 2009).

Results

Seed samples

Alfalfa seeds were very sensitive to salinity stress, exhibiting T_1 and T_{50} values that correspond, respectively, to the imbibition time at which 1% and 50% of seeds germinated, of 18 h and 49 h, as compared to 11 h and 16 h of the untreated control seeds germinated on water, and a final germination percentage (G_{max}) of only 59% as compared to 98% for the untreated control seeds germinated on water (Table 1). In contrast, osmoprimed seeds submitted to the same salinity stress showed a T_1 of 8 h and a T_{50} of 21 h together with a G_{max} of 88%, indicating that osmopriming (see Materials and methods) entailed increased seed vigour (Yacoubi *et al.*, 2011) (Table 1).

Table 1. Germination parameters for the untreated control seeds and the osmoprimed alfalfa seeds imbibed on water or on NaCl. Germination in water of untreated control (Untreated-H_2O) and osmoprimed (OP-H_2O) alfalfa seeds was conducted as described in Materials and methods at a temperature of 25°C. Germination experiments were also conducted in the presence of NaCl with the untreated control (Untreated-NaCl) and the osmoprimed alfalfa seeds (OP-NaCl). The data correspond to germination experiments conducted in triplicate (3 × 100 seeds). T_1 and T_{50} correspond respectively to the times to reach 1% and 50% of germination following imbibition. G_{max} corresponds to the maximal germination percentage in the different conditions. These parameters were calculated with the Seed Calculator software as indicated in Materials and methods

Seed sample	$T_1 \pm$ SD (h)	$T_{50} \pm$ SD (h)	$G_{max} \pm$ SD (%)
Untreated-H_2O	10.9 ± 1.2	16.1 ± 1.4	98.5 ± 1.0
Untreated-NaCl	18.2 ± 0.9	49.2 ± 4.7	59.5 ± 6.7
OP-H_2O	4.1 ± 0.8	9.5 ± 1.2	99.5 ± 0.5
OP-NaCl	8.5 ± 1.1	20.7 ± 2.3	87.7 ± 1.9

Proteomics analyses and comparison of seed proteins samples

Soluble protein extracts corresponding to the albumin fraction prepared from the untreated control and osmoprimed seeds collected at their respective imbibition time T_1 following germination under salt conditions were analysed by 2D-PAGE (Fig. 1). The results were compared to those previously obtained from untreated control and osmoprimed alfalfa seeds imbibed (T_1) on water (Yacoubi *et al.*, 2011; Fig. 1). This comparison should allow us to decipher whether the osmopriming treatment entails modifications in the seed proteome that are correlated with the observed improvement in seed vigour (Table 1).

The 2D-gels obtained from the four seed samples, untreated control seeds imbibed in the presence of water, untreated control seeds imbibed in the presence of NaCl, osmoprimed seeds imbibed in the presence of water, and osmoprimed seeds imbibed in the presence of NaCl, disclosed very similar protein patterns. However, statistical image analyses revealed protein spots whose volume varied, considering a variation in spot volume of at least 1.2 (up and down) and $P < 0.05$. There were 110 and 115 spots fulfilling these two criteria for the imbibed untreated control and osmoprimed seeds in the presence or absence of NaCl, respectively (see Tables S1 and S2, available online). Proteins from varying spots were identified by liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) analyses (see Tables S1 and S2, available online) and from alfalfa seed proteome reference maps reported previously (Yacoubi *et al.*, 2011). This new analysis led to the identification of 44 new proteins from alfalfa seeds (see Tables S1 and S2, available online). For the untreated control seeds (110 spots), two of them contained four proteins, three contained three proteins, 11 contained two proteins and 94 contained a single protein, for a total of 133 proteins (see Table S1, available online). For the osmoprimed seeds (115 spots), two of them contained four proteins, three contained three proteins, 12 contained two proteins and 98 contained a single protein, for a total of 139 proteins (see Table S2, available online). Spots with protein mixtures were excluded since it was not possible to determine which of the proteins were changing in abundance in response to the salinity stress. A further comparative analysis of the 2D-gels showed that 94 varying spots containing a unique protein were common for the imbibed untreated control and osmoprimed seeds in the presence or absence of NaCl (see Tables S1 and S2; Fig. S1, available online).

That osmopriming reversed the proteomic changes observed with the untreated control seeds that were imbibed under salinity conditions is further

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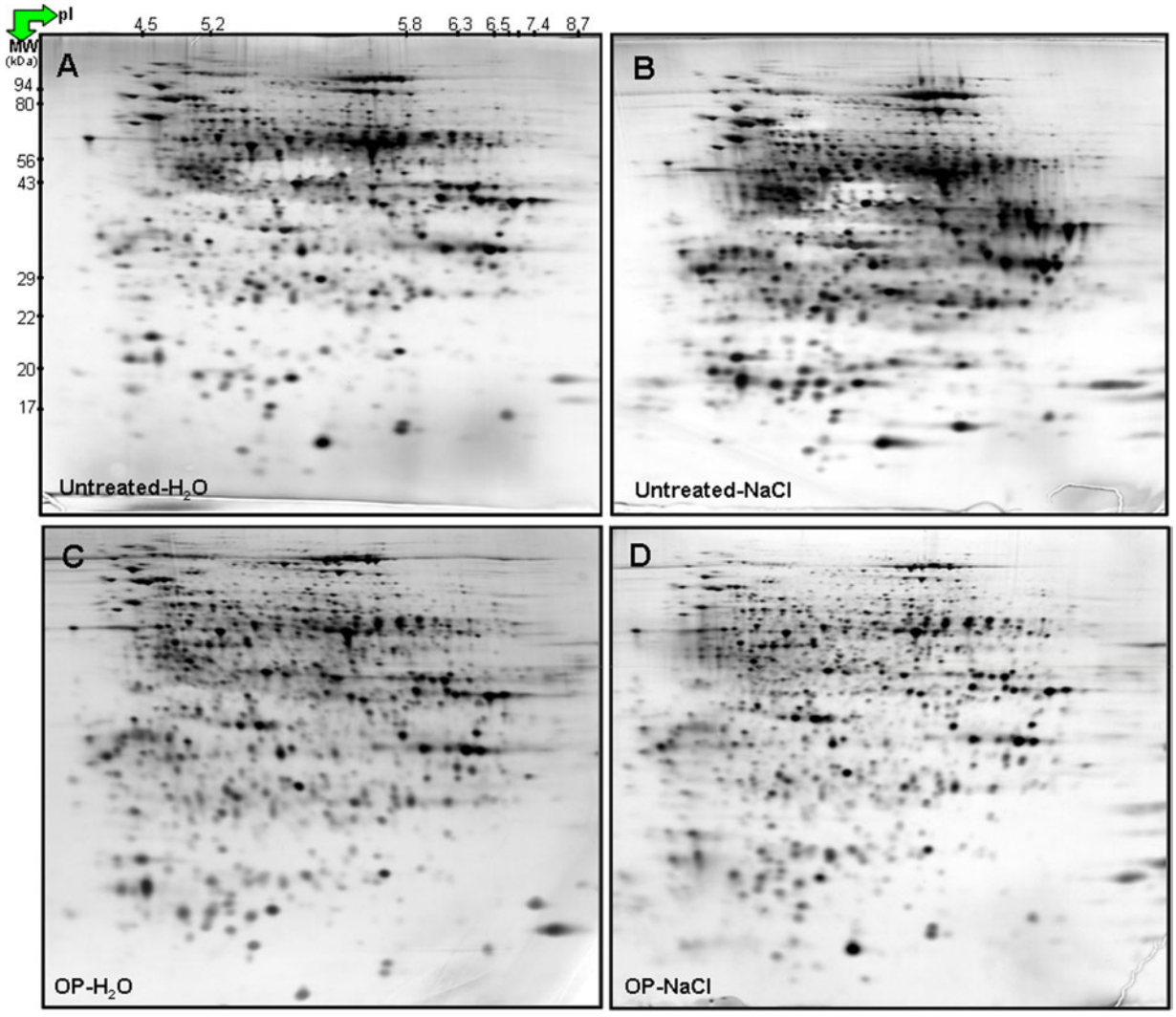

Figure 1. Influence of seed vigour on the soluble proteome of alfalfa seeds submitted to a salinity stress during imbibition. Silver-stained 2D-gel profiles of water-soluble albumin proteins from untreated control seeds imbibed on water (A) or NaCl (B) and from osmoprimed seeds imbibed on water (C) or NaCl (D). Seed samples were collected at their respective T_1 imbibition time values, T_1 being the time to reach 1% germination (see Table 1). An equal amount (100 μg) of the albumin protein extracts were loaded in each gel. MW, molecular weight (kDa); pI, isoelectric point.

supported by the results in Fig. 2. Thus, among the 94 common varying proteins present in unique spots, a number of them representing 76 proteins (81% of total) showed contrasting accumulation behaviour (e.g. up- versus down-, constant versus up- or constant- versus down-regulation) when comparing the untreated control and osmoprimed seeds (Fig. 2; compare Tables S1 and S2, available online; Table 2). Furthermore, out of the remaining 18 spots (19%) showing similar patterns of accumulation (e.g. up- versus up- or down- versus down-regulation) when comparing the untreated control and osmoprimed seeds imbibed in the presence or absence of NaCl, eight of them (spot nos 35, 66, 72, 103, 319, 357, 386 and 387) showed accumulation ratios differing by a factor of at least 1.5 when comparing the two protein lists (Table 2, Tables S1 and S2, available online). Thus, out of the 94 varying unique spots common to the two protein lists, in total 84 (76+8) spots (89% of total) exhibited contrasting accumulation behaviour when comparing the untreated control and osmoprimed seeds (Table 2, Tables S1 and S2, available online).

Functional classes of evidenced proteins

According to the functional classes of Bevan *et al.* (1998), the most represented functional groups for these 84 proteins were 'Protein destination and storage' (30 proteins; 36%) such as seed storage proteins (spot nos 297, 298, 305, 334, 345, 411 and 419) and small heat-shock proteins (HSPs) (spot nos 431 and 433); 'Cell growth/Division' (13 proteins; 15%)

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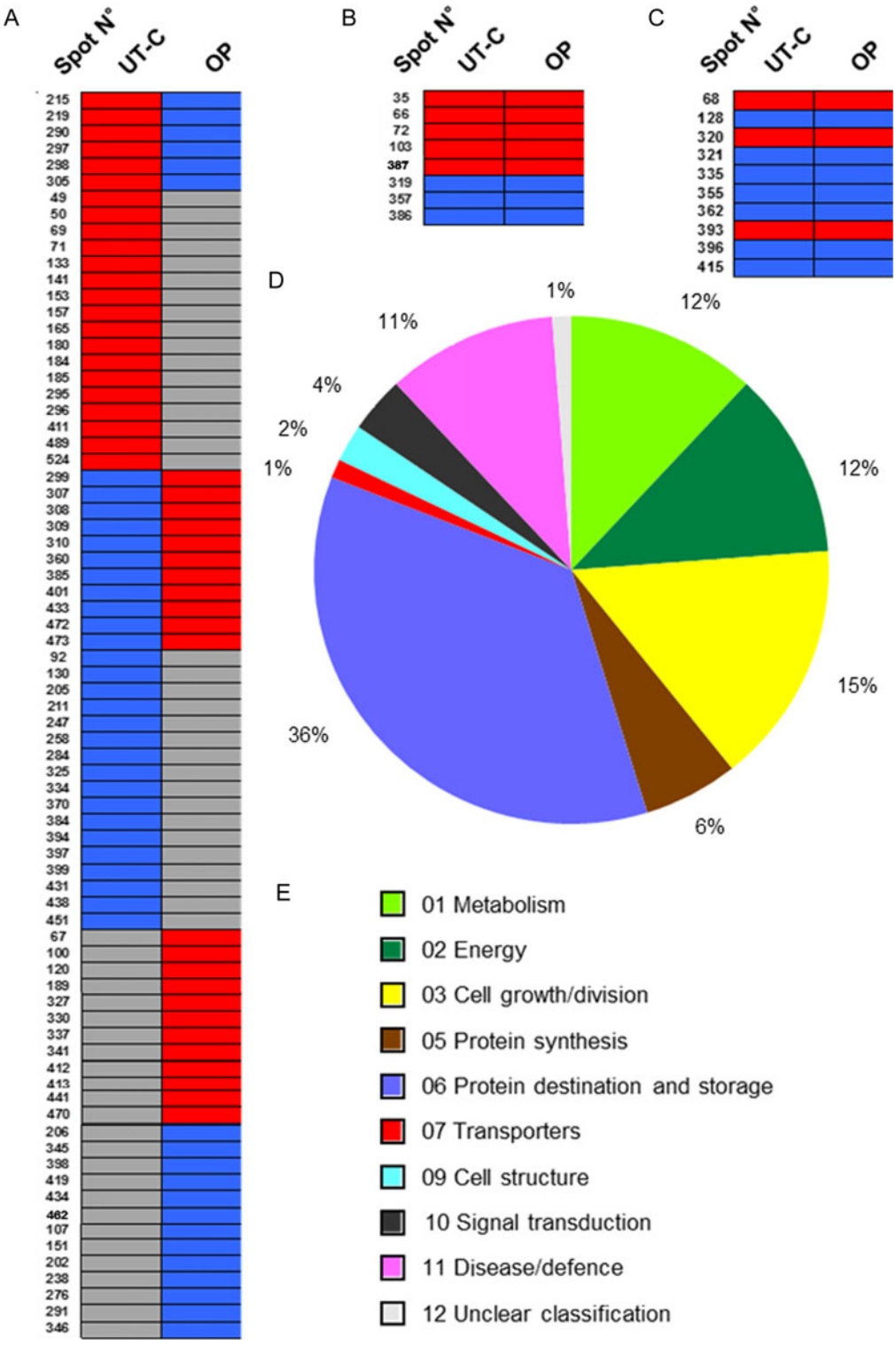

Figure 2. Schematic representation of the accumulation patterns of varying proteins during imbibition of untreated control (UT-C) or osmoprimed (OP) alfalfa seeds submitted to a salt stress. Blue, up-regulation; red, down-regulation; grey, constant accumulation. The figure shows 76 unique proteins showing contrasted accumulation patterns (A), eight proteins showing similar patterns of accumulation, yet differing by at least a factor 1.5 (B), and ten proteins showing strictly similar accumulation patterns of accumulation (C). Pie chart of the 84 (76 + 8) proteins showing contrasting patterns of accumulation divided into functional categories (D). Functional categories (E). See Table 2 for further details on the function of the proteins. Spot N°, spot number (see Fig. S1 and Tables S1 and S2, available online); UT-C, untreated control seeds imbibed on NaCl or on water; OP, osmoprimed seeds imbibed on NaCl or on water) (a colour version of this figure can be found online at http://www.journals.cambridge.org/ssr).

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Table 2. List of common proteins between the two protein sets presented in Tables S1 and S2 (available online). Protein accumulation patterns are depicted by the colour code used in Fig. 2. Blue, up-accumulated proteins; red, down-accumulated proteins; grey, constant proteins. Legend: N° spot: spot number. Proteins per spot: number of identifications per spot. Protein name; Organism: protein name and organism in which the protein has been identified. Pattern UTC-NaCl/UTC-H20 (T1): protein accumulation patterns from alfalfa untreated control seeds imbibed in the presence of NaCl (UTC-NaCl) compared to alfalfa untreated control seeds imbibed on water (UTC-H20); seeds were collected at their respective T_1 values, corresponding to the time to reach 1% germination; U, up-accumulated proteins; D, down-accumulated proteins; C, constant proteins (see Table S1, available online). Pattern OP-NaCl/OP-H20 (T_1): protein accumulation patterns from alfalfa osmoprimed seeds imbibed in the presence of NaCl (OP-NaCl) compared to alfalfa osmoprimed seeds imbibed on water (OP-H20); seeds were collected at their respective T_1 values, corresponding to the time to reach 1% germination; U, up-accumulated proteins; D, down-accumulated proteins; C, constant proteins (see Table S2, available online). Accession number: accession number from MT3, TIGR TA or NCBI databases. Function category and Function description: functional categories defined according to the ontological classification of Bevan *et al.* (1998). Common proteins: proteins from varying single protein spots commonly present in the two protein lists shown in Tables S1 and S2, available online; the volumes of the spots corresponding to these proteins varied upon imbibition of both UTC and OP seeds in NaCl compared to water. Protein accumulation patterns: SPP, similar protein accumulation patterns (brown), including eight proteins (pink; spot nos 35, 66, 72, 103, 319, 357, 386 and 387) showing ratios of normalized volumes differing by a factor of at least 1.5 in the untreated control and osmoprimed seed samples (see Tables S1 and S2, available online); CPP, contrasted accumulation protein patterns (orange) (a colour version of this table can be found online at http://www.journals.cambridge.org/ssr)

N° spot	Proteins per spot	Accumulation Pattern UTC-NaCl/UTC-H20 (T1)	Accumulation Pattern OP-NaCl/OP-H20 (T1)	Protein Name	Organism	Accession Number	Function category	Function description	Common Proteins	Protein Patterns
215	1	D	U	Convicilin	*Medicago truncatula*	IMGAIMedtr7g089490.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
219	1	D	U	RNA binding protein	*Medicago truncatula*	IMGAIMedtr8g146650.1	05 Protein synthesis	05.99 Others	CP	CPP
290	1	D	U	Annexin	*Medicago truncatula*	IMGAIMedtr5g072570.1	09 Cell structure	09.04 Cytoskeleton	CP	CPP
297	1	D	U	Convicilin	*Medicago truncatula*	IMGAIMedtr7g089440.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
298	1	D	U	Convicilin	*Medicago truncatula*	IMGAIMedtr7g089440.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
305	1	D	U	Convicilin	*Medicago truncatula*	IMGAIMedtr7g089440.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
49	1	D	C	methionine synthase	*Medicago truncatula*	IMGAIMedtr7g103050.2	01 Metabolism	01.01 Amino Acid	CP	CPP
50	1	D	C	methionine synthase	*Medicago truncatula*	IMGAIMedtr7g103050.2	01 Metabolism	01.01 Amino Acid	CP	CPP
69	1	D	C	protein disulfide-isomerase	*Medicago sativa*	TA1878_3879	06 Protein destination and storage	06.01 Folding and stability	CP	CPP
71	1	D	C	heat shock protein Hsp70	*Arabidopsis thaliana*	TA20020_3880	06 Protein destination and storage	06.01 Folding and stability	CP	CPP
133	1	D	C	betaine aldehyde dehydrogenase	*Medicago truncatula*	IMGAIMedtr8g125020.1	11 Disease/defence	11.05 Stress responses	CP	CPP
141	1	D	C	phosphoglucosamine mutase	*Medicago truncatula*	IMGAIMedtr1g120920.1	01 Metabolism	01.05 Sugars and polysaccharides	CP	CPP
153	1	D	C	Calreticulin	*Medicago truncatula*	IMGAIMedtr1g100460.1	10 Signal transduction	10.99 Others	CP	CPP
157	1	D	C	viciln (sucrose binding protein)	*Medicago truncatula*	IMGAIMedtr1g084050.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
165	1	D	C	UTP-glucose-1-phosphate uridylyltransferase	*Medicago truncatula*	IMGAIMedtr5g084880.2	01 Metabolism	01.05 Sugars and polysaccharides	CP	CPP
180	1	D	C	orotidine 5-phosphate decarboxylase	*Medicago truncatula*	IMGAIMedtr3g020170.1	01 Metabolism	01.03 Nucleotides	CP	CPP
184	1	D	C	Enolase	*Medicago truncatula*	IMGAIMedtr6g069700.1	02 Energy	02.01 Glycolysis	CP	CPP
185	1	D	C	ribulose bisphosphate carboxylase, large chain	*Medicago truncatula*	IMGAIMedtr7g145780.1	02 Energy	02.30 Photosynthesis	CP	CPP
295	1	D	C	cysteine synthase	*Medicago truncatula*	IMGAIMedtr5g006410.1	01 Metabolism	01.01 Amino Acid	CP	CPP
296	1	D	C	proteasome subunit alpha	*Glycine max*	TA21063_3880	06 Protein destination and storage	06.13 Proteolysis	CP	CPP
411	1	D	C	allergen Gly m Bd (vicilin)	*Glycine max*	BAB21619.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
489	1	D	C	phosphoglycerate kinase	*Medicago truncatula*	IMGAIMedtr2g077510.1	02 Energy	02.01 Glycolysis	CP	CPP
524	1	D	C	vacuolar ATP synthase subunit A	*Mesembryanthemum crystallinum*	CAC33578	07 Transporters	07.22 Transport ATPases	CP	CPP
299	1	U	D	alcohol dehydrogenase	*Medicago truncatula*	IMGAIMedtr2g014170.1	02 Energy	02.16 Fermentation	CP	CPP
307	1	U	D	late embryogenesis abundant protein	*Medicago truncatula*	IMGAIMedtr2g017540.1	03 Cell growth/division	03.30 Seed maturation	CP	CPP
308	1	U	D	late embryogenesis abundant protein D-34	*Medicago truncatula*	IMGAIMedtr1g086190.1	03 Cell growth/division	03.30 Seed maturation	CP	CPP
309	1	U	D	late embryogenesis abundant protein D-34	*Medicago truncatula*	IMGAIMedtr1g086190.1	03 Cell growth/division	03.30 Seed maturation	CP	CPP
310	1	U	D	DNA-damage-repair/toleration protein DRT102	*Medicago truncatula*	IMGAIMedtr7g140600.1	03 Cell growth/division	03.19 Recombination/Repair	CP	CPP
360	1	U	D	carbonic anhydrase	*Glycine max*	TA1731_3879	12 Unclear classification	12 Unclear classification	CP	CPP
385	1	U	D	glutathione S-transferase GST 9	*Glycine max*	AAG34799	11 Disease/defence	11.06 Detoxification	CP	CPP
401	1	U	D	manganese superoxide dismutase	*Pisum sativum*	CAA42737.1	11 Disease/defence	11.06 Detoxification	CP	CPP
433	1	U	D	18.2 kDa class I heat shock protein	*Medicago truncatula*	IMGAIMedtr5g088740.1	06 Protein destination and storage	06.01 Folding and stability	CP	CPP
472	1	U	D	seed maturation protein PM22; late embryogenesis abundant protein	*Glycine max*	AAD25354.1	03 Cell growth/division	03.30 Seed maturation	CP	CPP
473	1	U	D	seed maturation protein PM22; late embryogenesis abundant protein	*Glycine max*	AAD25354.1	03 Cell growth/division	03.30 Seed maturation	CP	CPP

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Table 2. (*Continued*)

92	1	U	C	NADP-dependent malic enzyme	*Medicago truncatula*	IMGAIMedtr4g159740.1	02 Energy	02.10 TCA pathway	CP	CPP
130	1	U	C	GroEL-like chaperone, ATPase	*Medicago truncatula*	IMGAIMedtr3g102720.1	06 Protein destination and storage	06.01 Folding and stability	CP	CPP
205	1	U	C	Convicilin	*Medicago truncatula*	IMGAIMedtr7g089460.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
211	1	U	C	elongation factor 1-gamma	*Medicago truncatula*	IMGAIMedtr2g005400.2	05 Protein synthesis	05.04 Translation factors	CP	CPP
247	1	U	C	alpha-1,4-glucan-protein synthase (UDP-forming)	*Medicago truncatula*	IMGAIMedtr5g048590.1	09 Cell structure	09.01 Cell wall	CP	CPP
258	1	U	C	fructose-bisphosphate aldolase	*Medicago truncatula*	IMGAIMedtr5g077730.1	02 Energy	02.01 Glycolysis	CP	CPP
284	1	U	C	malate dehydrogenase	*Medicago sativa*	AF020273	02 Energy	02.10 TCA pathway	CP	CPP
325	1	U	C	glucose and ribitol dehydrogenase (seed maturation protein PM34)	*Medicago truncatula*	IMGAIMedtr7g139420.1	03 Cell growth/division	03.30 Seed maturation	CP	CPP
334	1	U	C	Convicilin	*Medicago truncatula*	IMGAIMedtr7g089440.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
370	1	U	C	1-cys peroxiredoxin	*Medicago truncatula*	TA26514_3880	11 Disease/defence	11.06 Detoxification	CP	CPP
384	1	U	C	1-cys peroxiredoxin	*Medicago truncatula*	TA26514_3881	11 Disease/defence	11.06 Detoxification	CP	CPP
394	1	U	C	allergen Gly m Bd (vicilin)	*Medicago truncatula*	IMGAIMedtr4g080550.2	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
397	1	U	C	2-cys peroxiredoxin BAS1	*Medicago truncatula*	IMGAIAC146630_2.1	11 Disease/defence	11.06 Detoxification	CP	CPP
399	1	U	C	manganese superoxide dismutase	*Medicago sativa*	AAN34501	11 Disease/defence	11.06 Detoxification	CP	CPP
431	1	U	C	heat shock protein 18.2 kDa class I	*Medicago truncatula*	IMGAIMedtr5g088740.1	06 Protein destination and storage	06.01 Folding and stability	CP	CPP
438	1	U	C	40S ribosomal protein S12	*Medicago truncatula*	IMGAIMedtr4g142880.1	05 Protein synthesis	05.01 Ribosomal proteins	CP	CPP
451	1	U	C	phosphatidylinositol/phosphatidyl glycerol transfer protein	*Medicago truncatula*	IMGAIMedtr5g093210.1	01 Metabolism	01.06 Lipid and sterol	CP	CPP
67	1	C	D	heat shock protein Hsp70	*Medicago truncatula*	IMGAIMedtr8g145020.1	06 Protein destination and storage	06.01 Folding and stability	CP	CPP
100	1	C	D	protein disulfide isomerase	*Medicago sativa*	TA1878_3879	06 Protein destination and storage	06.01 Folding and stability	CP	CPP
120	1	C	D	chaperonin CPN60-like protein	*Medicago truncatula*	IMGAIMedtr6g030660.1	06 Protein destination and storage	06.01 Folding and stability	CP	CPP
189	1	C	D	UDP-glucuronosyl/UDP-glucosyltransferase	*Medicago truncatula*	IMGAIMedtr5g016580.1	01 Metabolism	01.05 Sugars and polysaccharides	CP	CPP
327	1	C	D	glucose and ribitol dehydrogenase (seed maturation protein PM34)	*Medicago truncatula*	IMGAIMedtr1g099380.1	03 Cell growth/division	03.30 Seed maturation	CP	CPP
330	1	C	D	glucose and ribitol dehydrogenase (seed maturation protein PM34)	*Medicago truncatula*	IMGAIMedtr7g139420.1	03 Cell growth/division	03.30 Seed maturation	CP	CPP
337	1	C	D	glucose and ribitol dehydrogenase (seed maturation protein PM34)	*Medicago truncatula*	IMGAIMedtr7g139420.1	03 Cell growth/division	03.30 Seed maturation	CP	CPP
341	1	C	D	late embryogenesis abundant protein D-34	*Glycine max*	TA27168_3880	03 Cell growth/division	03.30 Seed maturation	CP	CPP
412	1	C	D	translationally-controlled tumor protein homolog	*Medicago sativa*	BQ146117	10 Signal transduction	10.04.10 G proteins	CP	CPP
413	1	C	D	phosphatidylethanolamine binding protein, putative	*Ricinus communis*	XP_002530493.1	03 Cell growth/division	03.99 Others	CP	CPP
441	1	C	D	Conglutin	*Medicago truncatula*	IMGAIMedtr2g097090.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
470	1	C	D	glucose and ribitol dehydrogenase (seed maturation protein PM34)	*Medicago truncatula*	IMGAIMedtr7g139420.1	03 Cell growth/division	03.30 Seed maturation	CP	CPP
107	1	C	U	chaperonin CPN60-like protein	*Pisum sativum*	TA20056_3880	06 Protein destination and storage	06.01 Folding and stability	CP	CPP
151	1	C	U	viciln (sucrose binding protein)	*Medicago truncatula*	IMGAIMedtr1g084050.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
202	1	C	U	Convicilin	*Medicago truncatula*	IMGAIMedtr7g089490.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
206	1	C	U	elongation factor 1-gamma	*Medicago truncatula*	IMGAIMedtr2g005400.1	05 Protein synthesis	05.04 Translation factors	CP	CPP
238	1	C	U	glyceraldehyde-3-phosphate dehydrogenase	*Medicago truncatula*	IMGAIMedtr3g160060.1	02 Energy	02.01 Glycolysis	CP	CPP
276	1	C	U	fructose-bisphosphate aldolase	*Medicago truncatula*	IMGAIMedtr4g097300.1	02 Energy	02.01 Glycolysis	CP	CPP
291	1	C	U	cysteine synthase	*Medicago truncatula*	IMGAIMedtr5g006410.1	01 Metabolism	01.01 Amino Acid	CP	CPP
345	1	C	U	Convicilin	*Medicago truncatula*	IMGAIMedtr7g089440.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
346	1	C	U	elongation factor 1-beta	*Medicago truncatula*	*08/01/2012*	05 Protein synthesis	05.04 Translation factors	CP	CPP
398	1	C	U	proteasome subunit beta type	*Medicago truncatula*	IMGAIMedtr5g007000.1	06 Protein destination and storage	06.13 Proteolysis	CP	CPP
419	1	C	U	Convicilin	*Medicago truncatula*	IMGAIMedtr7g089440.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
434	1	C	U	Conglutin	*Medicago truncatula*	IMGAIMedtr2g097130.1	06 Protein destination and storage	06.20 Storage proteins	CP	CPP
462	1	C	U	Enolase	*Medicago truncatula*	IMGAIMedtr6g069700.1	02 Energy	02.01 Glycolysis	CP	CPP
35	1	D	D	lipoxygenase 2	*Medicago truncatula*	IMGAIMedtr2g120730.1	11 Disease/defence	11.05 Stress responses	CP	SPP
66	1	D	D	heat shock protein Hsp70	*Medicago truncatula*	IMGAIMedtr8g145020.1	06 Protein destination and storage	06.01 Folding and stability	CP	SPP
72	1	D	D	heat shock protein Hsp70	*Medicago truncatula*	IMGAIMedtr7g025840.1	06 Protein destination and storage	06.01 Folding and stability	CP	SPP
103	1	D	D	protein disulfide isomerase	*Medicago sativa*	TA1878_3879	06 Protein destination and storage	06.01 Folding and stability	CP	SPP
319	1	U	U	14-3-3 protein	*Medicago truncatula*	IMGAIMedtr5g073680.1	10 Signal transduction	10.04 Mediators	CP	SPP
357	1	U	U	heme oxygenase (heme degradation)	*Medicago truncatula*	IMGAIMedtr8g137720.1	01 Metabolism	01.07 Cofactors	CP	SPP
386	1	U	U	glutathione S-transferase	*Medicago truncatula*	IMGAIMedtr5g098100.1	11 Disease/defence	11.06 Detoxification	CP	SPP

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Table 2. (*Continued*)

387	1	D	D	proteasome subunit beta type	*Medicago truncatula*	IMGAIMedtr3g117480.1	06 Protein destination and storage	06.13 Proteolysis	CP	SPP
68	1	D	D	heat shock protein Hsp70	*Medicago truncatula*	IMGAIMedtr8g145020.1	06 Protein destination and storage	06.01 Folding and stability	CP	SPP
128	1	U	U	oxalyl-CoA decarboxylase	*Arabidopsis thaliana*	XM_002265968.1	11 Disease/defence	11.06 Detoxification	CP	SPP
320	1	D	D	Convicilin	*Medicago truncatula*	IMGAIMedtr7g089440.1	06 Protein destination and storage	06.20 Storage proteins	CP	SPP
321	1	U	U	glucose and ribitol dehydrogenase (seed maturation protein PM34)	*Medicago truncatula*	IMGAIMedtr1g099380.1	03 Cell growth/division	03.30 Seed maturation	CP	SPP
335	1	U	U	lactoylglutathione lyase	*Medicago truncatula*	IMGAIMedtr8g146940.1	11 Disease/defence	11.06 Detoxification	CP	SPP
355	1	U	U	proteasome alpha-subunit	*Medicago truncatula*	IMGAIMedtr2g071490.1	06 Protein destination and storage	06.13 Proteolysis	CP	SPP
362	1	U	U	Lectin	*Medicago sativa*	AAA82737	11 Disease/defence	11.02 Defense-related	CP	SPP
393	1	D	D	allergen Gly m Bd (vicilin)	*Medicago truncatula*	IMGAIMedtr4g080550.2	06 Protein destination and storage	06.20 Storage proteins	CP	SPP
396	1	U	U	proteasome subunit beta type	*Medicago truncatula*	IMGAIMedtr7g105310.1	06 Protein destination and storage	06.13 Proteolysis	CP	SPP
415	1	U	U	peroxiredoxin (mitochondrial)	*Pisum sativum*	TA23206_3880	11 Disease/defence	11.06 Detoxification	CP	SPP

such as late embryogenesis abundant (LEA) proteins (spot nos 307, 308, 309 and 341) and seed maturation proteins (SMPs) (spot nos 472, 473, 321, 325, 327, 330, 337 and 470); 'Metabolism' (10 proteins; 12%) such as methionine synthase (spot nos 49 and 50), cysteine synthase (spot nos 291 and 295) and haem oxygenase (spot no. 357); 'Energy' (10 proteins; 12%), and 'Disease and defence' (9 proteins; 11%) such as glutathione S-transferase (spot no. 385). Also among evidenced proteins, there were proteins related to 'Cell structure' such as annexin (spot no. 290) and proteins related to 'Protein synthesis' such as RNA binding protein (spot no. 219) (see Tables S1 and S2, available online; Fig. 2).

Discussion

In accordance with previous studies (Amooaghaie, 2011; Yacoubi *et al.*, 2011), an osmopriming treatment of alfalfa seeds increased their vigour substantially, especially in salt stress conditions. Moreover, in our previous study, we established proteomic reference maps for the dry mature alfalfa seeds, as well as for the untreated control and osmoprimed seeds during germination on water and harvested at the same stage after sowing, namely at imbibition time T_1, at which 1% of the seeds have germinated and that provides a good estimate of the achievement of germination *sensu stricto* (Yacoubi *et al.*, 2011). Based on these previous findings, the objective of the current work was twofold: (1) characterize the proteome of the untreated control seeds subjected to salt stress during imbibition; and (2) test the hypothesis that the osmopriming treatment could reverse the proteome changes observed with the untreated control seeds during imbibition in salt stress conditions. If this were the case, proteins showing contrasting levels of accumulation with the untreated control and osmoprimed seeds would provide potential markers of germination vigour. To this end, and using the data previously reported for proteins whose accumulation varied during imbibition (T_1) of untreated control and osmoprimed seeds in water (Yacoubi *et al.*, 2011), we presently compared the four following germinated (T_1) seed samples: i) the untreated control seeds imbibed on NaCl or on water and ii) the two corresponding osmoprimed seed samples. By this approach, we observed that 94 proteins could characterize the response of both the untreated control and osmoprimed seeds to salt stress (Fig. 2; Table 2). Remarkably, a large number of them (84 proteins, 89%) displayed contrasting levels of accumulation in untreated control and osmoprimed seeds (Fig. 2; Table 2). This indicates that numerous changes observed in the proteome of untreated control seeds imbibed in the presence of salt and presumably accounting for the loss in seed vigour associated with salinity stress, could be substantially reversed in osmoprimed seeds undergoing the same salt stress. Since the osmoprimed seeds displayed higher germination vigour in salinity conditions, it seems reasonable to propose that these proteins showing contrasting accumulations in both types of untreated control and osmoprimed seeds are potential markers of seed vigour in alfalfa, notably under salt stress conditions. Below we discuss the role of some of these proteins in seed vigour.

Several spots assigned to alfalfa seed storage proteins corresponded to proteolytic fragments of the native proteins (spot nos 297, 298, 305, 334, 345, 411 and 419; Table S2, available online). Since seed storage proteins are used as energy and nitrogen resources during seedling growth, the increased accumulation of storage protein fragments in osmoprimed seeds but not in untreated control seeds most presumably reflects an increased initiation of seed storage mobilization during early germination of the osmoprimed seeds under salinity stress. In agreement with this, the initial mobilization of seed storage proteins during early germination is considered as a vigour marker in other species, such as soybean

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(Alam *et al.*, 2011), sugarbeet (Job *et al.*, 1997; Catusse *et al.*, 2011) and *Arabidopsis* (Gallardo *et al.*, 2001).

The small HSPs are molecular chaperones that are abundant in mature dry seeds and disappear during germination (Wehmeyer and Vierling, 2000). Here, two small HSPs (spot nos 431 and 433) were evidenced showing increased accumulation behaviour (3 times) during germination of the untreated control seeds under NaCl stress (Table S1, available online). This accumulation suggests a defect in the assembly and correct folding of proteins during this stress. This pattern of accumulation was reversed during imbibition of the osmoprimed seeds under salinity stress (supplementary Table S2). Thus the osmopriming treatment allowed overcoming of the stress defect encountered by the seeds germinated in salinity conditions. Small HSPs are also considered as seed vigour markers in sugarbeet (Catusse *et al.*, 2011), *M. truncatula* (Boudet *et al.*, 2006) and *Arabidopsis* (Gallardo *et al.*, 2001).

Methionine is essential in all organisms as a building block of proteins and as a component of the universal activated methyl donor *S*-adenosylmethionine (AdoMet). Recycling of the homocysteinyl moiety and regeneration of Met, a set of reactions designated as the activated methyl cycle, accompany utilization of the methyl group of AdoMet in transmethylation reactions (Ravanel *et al.*, 1998). Furthermore, in plants AdoMet is the precursor for ethylene, polyamine and biotin biosyntheses (Ravanel *et al.*, 1998). Previous work documented the role of the methyl cycle in *Arabidopsis* seed germination, as inferred notably by the fact that germination was strongly delayed in the presence of DL-propargylglycine, a specific inhibitor of Met synthesis (Gallardo *et al.*, 2002). In this context, it is therefore interesting to observe the decreased accumulation of two spots containing Met synthase (spot nos 49 and 50), the enzyme responsible for Met synthesis in plants, during imbibition of the untreated control seeds in the presence of NaCl (Table S1, available online). This decreased accumulation most presumably accounts for the decreased seed vigour observed under salinity conditions, as it would mimic the Met biosyntheis inhibitor DL-propargylglycine. It is noted that this decrease was not observed when comparing the osmoprimed seeds imbibed under salinity or control (water) conditions, which paralleled an increased seed vigour afforded by the osmopriming treatment. Cysteine synthase is also involved in the methyl cycle, as Cys is a precursor for Met biosynthesis (Ravanel *et al.*, 1998). Cys also serves as a precursor for the synthesis of glutathione, a major antioxidant (Noctor and Foyer, 1998). The present study disclosed two Cys synthase containing spots (spot nos 291 and 295), exhibiting similar patterns of accumulation as Met synthase containing spots (spot nos 49 and 50) when comparing the untreated control and osmoprimed alfalfa seeds germinated in salinity conditions (compare Tables S1 and S2, available online; Table 2). Altogether, these observations confirm the importance of the sulphur amino acid biosynthesis pathways in seed vigour, in agreement with results reported for sugarbeet (Catusse *et al.*, 2011) and *Arabidopsis* (Rajjou *et al.*, 2008, 2012).

Several water stress-related proteins were also identified, collectively referred to as late embryogenesis abundant (LEA) proteins. These proteins accumulate late during embryogenesis, coincident with acquisition of desiccation tolerance of the developing seeds, and disappear during germination. They are presumed to be involved in binding or replacement of water, in sequestering ions that will accumulate under dehydration conditions, or in maintaining protein and membrane structure (Dure, 1993). Furthermore, expression of the barley *HVAI* LEA protein gene confers tolerance to salt stress in transgenic rice (Xu *et al.*, 1996). Four LEA protein spots (spot nos 307, 308, 309 and 341) were strongly accumulated in the untreated control seeds submitted to the salinity stress (Table S1, available online), presumably in response to the dehydration and ionic stresses imposed by NaCl during germination. This increased accumulation was abolished in the osmoprimed seeds challenged by the NaCl stress (Table S2, available online; Table 2), supporting the finding that LEA proteins constitute seed vigour markers in alfalfa, as proposed for soybean (Cheng *et al.*, 2010), beech (Kalemba and Pukacka, 2008) and sugarbeet (Catusse *et al.*, 2011).

Another group of proteins strongly accumulating during late seed maturation corresponds to the group of seed maturation proteins (SMPs). Some of them would play similar roles as the LEA proteins during seed development (Hundertmark and Hincha, 2008), but this might not be the case for all SMPs. Two spots containing the PM22 SMP (spot nos 472 and 473) were identified (Tables S1 and S2, available online). This protein exhibits considerable sequence homology with the drought-induced soybean protein desiccation protectant protein LEA14 homologue (Maitra and Cushman, 1994). Therefore, it is not surprising to observe that the alfalfa PM22 spots displayed accumulation patterns identical to those for the above-discussed LEA protein spots. Thus these two PM22 spots were up-regulated during imbibition of the untreated control seeds submitted to salinity stress whereas they were down-regulated during imbibition of the osmoprimed seeds submitted to the same stress (compare Tables S1 and S2, available online; Table 2), thereby supporting a role of PM22 in alfalfa seed vigour. Another SMP detected in the present study corresponds to the short-chain dehydrogenase glucose and ribitol dehydrogenase (spot nos 321, 325, 327, 330, 337 and 470), an enzyme that catalyses the oxidation of D-glucose using NAD (nicotinamide adenine dinucleotide) as co-substrate (Jörnvall *et al.*, 1984; Alexander *et al.*, 1994). Most spots

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containing this enzyme (spot nos 325, 327, 330, 337 and 470) showed contrasting accumulation behaviour when comparing the untreated control and osmoprimed seeds imbibed under salinity conditions (compare Tables S1 and S2, available online; Table 2). Interestingly, barley lines tolerant to saline stress during germination express a higher level of glucose and ribitol dehydrogenase compared to less-tolerant lines (Witzel *et al.*, 2010). Also, this protein was proposed to correspond to a potential seed vigour marker in sugarbeet (Catusse *et al.*, 2011). Altogether, these observations are in favour of a role of this enzyme in seed vigour.

Annexins are multifunctional proteins characterized by their capacity to bind calcium ions and negatively charged lipids. Transgenic *Arabidopsis* seeds ectopically expressing a sacred lotus (*Nelumbo nucifera*) annexin exhibited improved resistance to accelerated ageing treatment used for assessing seed vigour (Chu *et al.*, 2012). Also, the expression of most of the *Arabidopsis* annexin genes is differentially regulated by exposure to salt, drought, and high- and low-temperature conditions, indicating a likely role for members of this gene family in stress responses (Cantero *et al.*, 2006; Huh *et al.*, 2010). In agreement, proteomic analyses revealed differential accumulation of an annexin isoform (AnnAt1) during *Arabidopsis* germination and early seedling growth in response to salinity stress (Lee *et al.*, 2004). Consistent with the finding that annexins could represent a potential seed vigour marker, an annexin was identified in alfalfa seeds in the present study (spot no. 290), which displayed decreased accumulation (2.6-fold) and increased accumulation (1.7-fold) in untreated control and osmoprimed seeds, respectively (compare supplementary Tables S1 and S2; Table 2).

Among the proteins exhibiting contrasting accumulation behaviour when comparing the untreated control and osmoprimed seeds submitted to salinity stress, spot no. 385 was identified as glutathione *S*-transferase (GST) 9. During imbibition under salt stress the accumulation level of this GST increased by 2.6-fold for the untreated control seeds and decreased by 1.5-fold for the osmoprimed seeds (compare supplementary Tables S1 and S2; Table 2). GSTs have been suggested to be responsible for tolerance to various stresses, such as cold, salt and drought, by detoxification of xenobiotic compounds and reactive oxygen species. Thus manipulation of GST levels in transgenic plants was shown to improve seed germination and seedling growth under salt stress (Roxas *et al.*, 2000). Consistent with our observations, GST9 was also identified by proteomics as a potential seed vigour marker in soybean cultivars exhibiting different sensitivities towards salinity stress (X.Y. Xu *et al.*, 2011).

A haem oxygenase (spot no. 357) was identified in the present study, whose accumulation level increased sharply (5.7-fold) for the untreated control seeds imbibed in the presence of NaCl, while its accumulation level increased much more weakly (1.4-fold) for the osmoprimed seeds imbibed under same stress conditions (compare Tables S1 and S2, available online). Haem oxygenase catalyses the oxidative conversion of haem to biliverdin with a concomitant release of carbon monoxide (CO) and free iron (Fe^{2+}) (Otterbein *et al.*, 2003). Recent results revealed that CO plays an important role in a number of physiological processes, such as growth and developmental regulation, stomatal closure and adaptation responses to environmental stresses (Liu *et al.*, 2010). In addition, CO behaves as an important positive regulator of seed germination, since the application of haematin as an exogenous haem oxygenase inducer and a CO aqueous solution alleviated the inhibition of rice seed germination and seedling growth encountered under salt stress, both of which were partially due to the induction of antioxidant metabolism as well as the degradation of storage reserve (Liu *et al.*, 2007). Similarly, pre-soaking with haemin, another haem oxygenase-1 inducer, proved to improve salinity tolerance during wheat seed germination (S. Xu *et al.*, 2011). Therefore the observed increase in haem oxygenase in the present study, with the untreated control seeds imbibed in the presence of NaCl (Table S1, available online), can be viewed as a defence response of the alfalfa seeds to counteract the negative effects of the salinity stress (Table 1). In turn, the much lower increase in this enzyme level observed with the osmoprimed seeds imbibed in the presence of NaCl (Table S2, available online) is an indication of the increased seed vigour afforded by the osmopriming treatment (Table 1).

A protein called RNA binding protein (spot no. 219) was also detected in the current study whose level of accumulation strongly increased (tenfold) in osmoprimed seeds during imbibition in salt stress conditions, and severely decreased (2.3-fold) during imbibition of the untreated control seed subjected to the same stress (compare Tables S1 and S2, available online; Table 2). RNA-binding proteins appear to govern many aspects of RNA metabolism, including pre-mRNA processing, transport, stability/decay and translation, and are emerging as a novel class of proteins involved in a wide range of post-transcriptional regulatory events that are important in providing plants with the ability to respond rapidly to changes in environmental conditions (Lorkovic, 2009; Ambrosone *et al.*, 2012). Our present results are in good agreement with this finding.

Conclusion

In conclusion, seed priming has long been known to enable seeds to overcome biotic and abiotic stresses

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(Soeda *et al.*, 2005). The present proteomic study contributes to the understanding of osmopriming physiology, and its association with post-priming salinity stress tolerance during germination. Some of the presently identified proteins had previously been shown to play a role in salt stress tolerance in several plant species, a finding that underlines the robustness of such protein markers and the usefulness of proteomics to unravelling them. This concerned small HSPs, water stress-related proteins such as the LEA proteins or the detoxification enzyme glutathione *S*-transferase. Besides, the present approach also revealed new proteins associated with salinity stress in alfalfa (e.g. a haem oxygenase or an RNA binding protein). Future studies will be directed toward the function of the identified proteins in the salt response.

Supplementary material

To view supplementary material for this article, please visit http://dx.doi.org/10.1017/S0960258513000093

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References

Alam, I., Sharmin, S.A., Kim, K.H., Kim, Y.G., Lee, J.J., Bahk, J.D. and Lee, B.H. (2011) Comparative proteomic approach to identify proteins involved in flooding combined with salinity stress in soybean. *Plant Soil* **346**, 45–62.

Alexander, R., Alamilla, J.M., Salamini, F. and Bartels, D. (1994) A novel embryo-specific barley cDNA clone encodes a protein with homologies to bacterial glucose and ribitol dehydrogenase. *Planta* **192**, 519–525.

Ambrosone, A., Costa, A., Leone, A. and Grillo, S. (2012) Beyond transcription: RNA-binding proteins as emerging regulators of plant response to environmental constraints. *Plant Science* **182**, 12–18.

Amooaghaie, R. (2011) The effect of hydro and osmopriming on alfalfa seed germination and antioxidant defenses under salt stress. *African Journal of Biotechnology* **10**, 6268–6275.

Ashraf, M. and Foolad, M.R. (2005) Pre-sowing seed treatment – a shotgun approach to improve germination, plant growth, and crop yield under saline and non-saline conditions. *Advances in Agronomy* **68**, 223–271.

Bevan, M., Bancroft, I., Bent, E., Love, K., Goodman, H., Dean, C., Bergkamp, R., Dirkse, W., Van Staveren, M., Stiekema, W., *et al.* (1998) Analysis of 1.9 Mb of contiguous sequence from chromosome 4 of *Arabidopsis thaliana*. *Nature* **391**, 485–488.

Bohnert, H.J., Nelson, D.E. and Jensen, R.G. (1995) Adaptations to environmental stresses. *The Plant Cell* **7**, 1099–1111.

Boudet, J., Buitink, J., Hoekstra, F.A., Rogniaux, H., Larré, C., Satour, P. and Leprince, O. (2006) Comparative analysis of the heat stable proteome of radicles of *Medicago truncatula* seeds during germination identifies late embryogenesis abundant proteins associated with desiccation tolerance. *Plant Physiology* **140**, 1418–1436.

Boughanmi, N., Michonneau, P., Daghfous, D. and Fleurat-Lessard, P. (2005) Adaptation of *Medicago sativa* cv. Gabès to long-term NaCl stress. *Journal of Plant Nutrition and Soil Science* **168**, 262–268.

Bradford, K.J. (1986) Manipulation of seed water relations via osmotic priming to improve germination under stress conditions. *HortScience* **21**, 1105–1112.

Bradford, M. (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principal of protein-dye binding. *Analytical Biochemistry* **72**, 248–254.

Cantero, A., Barthakur, S., Bushart, T.J., Chou, S., Morgan, R.O., Fernandez, M.P., Clark, G.B. and Roux, S.J. (2006) Expression profiling of the *Arabidopsis* annexin gene family during germination, de-etiolation and abiotic stress. *Plant Physiology and Biochemistry* **44**, 13–24.

Catusse, J., Meinhard, J., Job, C., Strub, J.M., Fischer, U., Pestsova, E., Westhoff, P., Van Dorsselaer, A. and Job, D. (2011) Proteomics reveals potential biomarkers of seed vigor in sugarbeet. *Proteomics* **11**, 1569–1580.

Cheng, L.B., Gao, X., Li, S.Y., Shi, M.J., Javeed, H., Jing, X.M., Yang, G.X. and He, G.Y. (2010) Proteomic analysis of soybean [*Glycine max* (L.) Meer.] seeds during imbibition at chilling temperature. *Molecular Breeding* **26**, 1–17.

Chu, P., Chen, H., Zhou, Y., Li, Y., Ding, Y., Jiang, L., Tsang, E.W., Wu, K. and Huang, S. (2012) Proteomic and functional analyses of *Nelumbo nucifera* annexins involved in seed thermotolerance and germination vigor. *Planta* **235**, 1271–1288.

Dure, L. 3rd (1993) A repeating 11-mer amino acid motif and plant desiccation. *The Plant Journal* **3**, 363–369.

Gallardo, K., Job, C., Groot, S.P.C., Puype, M., Demol, H., Vandekerckhove, J. and Job, D. (2001) Proteomic analysis of *Arabidopsis* seed germination and priming. *Plant Physiology* **126**, 835–848.

Gallardo, K., Job, C., Groot, S.P.C., Puype, M., Demol, H., Vandekerckhove, J. and Job, D. (2002) Importance of methionine biosynthesis for *Arabidopsis* seed germination and seedling growth. *Physiologia Plantarum* **116**, 238–247.

Hachicha, M. (2007) Les sols salés et leur mise en valeur en Tunisie. *Sécheresse* **18**, 45–50.

Hachicha, M., Job, J.O. and Mtimet, A. (1994) Les sols salés et la salinisation en Tunisie. *Sols de Tunisie* **5**, 271–341.

Heydecker, W. and Coolbear, P. (1977) Seed treatments for improved performance – survey and attempted prognosis. *Seed Science and Technology* **5**, 353–425.

Huh, S.M., Noh, E.K., Kim, H.G., Jeon, B.W., Bae, K., Hu, H.C., Kwak, J.M. and Park, O.K. (2010) *Arabidopsis* annexins AnnAt1 and AnnAt4 interact with each other and regulate drought and salt stress responses. *Plant and Cell Physiology* **51**, 1499–1514.

Hundertmark, M. and Hincha, D.K. (2008) LEA (Late Embryogenesis Abundant) proteins and their encoding genes in *Arabidopsis thaliana*. *BMC Genomics* **9**, 118.

Job, C., Kersulec, A., Ravasio, L., Chareyre, S., Pépin, R. and Job, D. (1997) The solubilization of the basic subunit of sugarbeet seed 11-S globulin during priming and early germination. *Seed Science Research* **7**, 225–243.

Jörnvall, H., von Bahr-Lindström, H., Jany, K.D., Ulmer, W. and Fröschle, M. (1984) Extended superfamily of short alcohol-polyol-sugar dehydrogenases: structural similarities between glucose and ribitol dehydrogenases. *FEBS Letters* **165**, 190–196.

Kalemba, E.M. and Pukacka, S. (2008) Changes in late embryogenesis abundant proteins and a small heat shock protein during storage of beech (*Fagus sylvatica* L.) seeds. *Environmental and Experimental Botany* **63**, 274–280.

Lee, S., Lee, E.J., Yang, E.J., Lee, J.E., Park, A.R., Song, W.H. and Park, O.K. (2004) Proteomic identification of annexins, calcium-dependent membrane binding proteins that mediate osmotic stress and abscisic acid signal transduction in *Arabidopsis*. *The Plant Cell* **16**, 1378–1391.

Liu, K.L., Xu, S., Xuan, W., Ling, T.F., Cao, Z., Huang, B.K., Sun, Y.G., Fang, L., Liu, Z.Y., Zhao, N. and Shen, W.B. (2007) Carbon monoxide counteracts the inhibition of seed germination and alleviates oxidative damage caused by salt stress in *Oryza sativa*. *Plant Science* **172**, 544–555.

Liu, Y., Xu, S., Ling, T., Xu, L. and Shen, W. (2010) Heme oxygenase/carbon monoxide system participates in regulating wheat seed germination under osmotic stress involving the nitric oxide pathway. *Journal of Plant Physiology* **167**, 1371–1379.

Lorkovic, Z.J. (2009) Role of plant RNA-binding proteins in development, stress response and genome organization. *Trends in Plant Science* **14**, 229–236.

Maitra, N. and Cushman, J.C. (1994) Isolation and characterization of a drought-induced soybean cDNA encoding a D95 family late-embryogenesis-abundant protein. *Plant Physiology* **106**, 805–806.

Noctor, G. and Foyer, C.H. (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annual Review of Plant Physiology and Plant Molecular Biology* **49**, 249–279.

Otterbein, L.E., Soares, M.P., Yamashita, K. and Bach, F.H. (2003) Heme oxygenase-1: unleashing the protective properties of heme. *Trends in Immunology* **24**, 449–455.

Peel, M.D., Waldron, B.L., Jensen, K.B., Chatterton, N.J., Horton, H. and Dudley, L.M. (2004) Screening for salinity tolerance in alfalfa: a repeatable method. *Crop Science* **44**, 2049–2053.

Rajjou, L., Lovigny, Y., Groot, S.P.C., Belghazi, M., Job, C. and Job, D. (2008) Proteome-wide characterization of seed aging in *Arabidopsis*: a comparison between artificial and natural aging protocols. *Plant Physiology* **148**, 620–641.

Rajjou, L., Duval, M., Gallardo, K., Catusse, J., Bally, J., Job, C. and Job, D. (2012) Seed germination and vigor. *Annual Review of Plant Biology* **63**, 507–533.

Ravanel, S., Gakière, B., Job, D. and Douce, R. (1998) The specific features of methionine biosynthesis and metabolism in plants. *Proceedings of the National Academy of Sciences, USA* **95**, 7805–7812.

Roxas, V.P., Lodhi, S.A., Garrett, D.K., Mahan, J.R. and Allen, R.D. (2000) Stress tolerance in transgenic tobacco seedlings that overexpress glutathione *S*-transferase/glutathione peroxidase. *Plant and Cell Physiology* **41**, 1229–1234.

Soeda, Y., Konings, M.C.J.M., Vorst, O., van Houwelingen, A.M.M.L., Stoopen, G.M., Maliepaard, C.A., Kodde, J., Bino, R.J., Groot, S.P.C. and van der Geest, A.H.M. (2005) Gene expression programs during *Brassica oleracea* seed maturation, osmopriming, and germination are indicators of progression of the germination process and the stress tolerance level. *Plant Physiology* **137**, 354–368.

Waanders, L.F., Chwalek, K., Monetti, M., Kumar, C., Lammert, E. and Mann, M. (2009) Quantitative proteomic analysis of single pancreatic islets. *Proceedings of the National Academy of Sciences, USA* **106**, 18902–18907.

Wehmeyer, N. and Vierling, E. (2000) The expression of small heat shock proteins in seeds responds to discrete developmental signals and suggests a general protective role in desiccation tolerance. *Plant Physiology* **122**, 1099–1108.

Witzel, K., Weidner, A., Surabhi, G.K., Varshney, R.K., Kunze, G., Buck-Sorlin, G.H., Börner, A. and Mock, H.P. (2010) Comparative analysis of the grain proteome fraction in barley genotypes with contrasting salinity tolerance during germination. *Plant, Cell and Environment* **33**, 211–222.

Xu, D., Duan, X., Wang, B., Hong, B., Ho, T.H.D. and Wu, R. (1996) Expression of a late embryogenesis abundant protein gene, *HVAI*, from barley confers tolerance to water deficit and salt stress in transgenic rice. *Plant Physiology* **110**, 249–257.

Xu, S., Lou, T.L., Zhao, N., Gao, Y., Dong, L.H., Jiang, D.J., Shen, W.B., Huang, L.Q. and Wang, R. (2011) Presoaking with hemin improves salinity tolerance during wheat seed germination. *Acta Physiologiae Plantarum* **33**, 1173–1183.

Xu, X.Y., Fan, R., Zheng, R., Li, C.M. and Yu, D.Y. (2011) Proteomic analysis of seed germination under salt stress in soybeans. *Journal of Zhejiang University, Science B* **12**, 507–517.

Yacoubi, R., Job, C., Belghazi, M., Chaibi, W. and Job, D. (2011) Toward characterizing seed vigor in alfalfa through proteomic analysis of germination and priming. *Journal of Proteome Research* **10**, 3891–3903.