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RESEARCH ARTICLE

Analysis of desiccation-induced candidate phosphoproteins from *Craterostigma plantagineum* isolated with a modified metal oxide affinity chromatography procedure

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Reversible protein phosphorylation/dephosphorylation is crucial for regulation of many cellular events, and increasing evidence indicates that this post-translational modification is also involved in the complex process of acquisition of desiccation tolerance. To analyze the phosphoproteome of the desiccation tolerant resurrection plant *Craterostigma plantagineum*, MOAC-enriched proteins from leaves at different stages of a de-/rehydration cycle were separated by 2-D PAGE and detected by phosphoprotein-specific staining. Using this strategy 20 putative phosphoproteins were identified by MALDI-TOF MS and MS/MS, which were not detected when total proteins were analyzed. The characterized desiccation-related phosphoproteins CDeT11-24 and CDeT6-19 were used as internal markers to validate the specificity of the analyses. For 16 of the identified proteins published evidence suggests that they are phosphoproteins. Comparative analysis of the 2-D gels showed that spot intensities of most identified putative phosphoproteins change during the de-/rehydration cycle. This suggests an involvement of these proteins in desiccation tolerance. Nearly all changes in the phosphoproteome of *C. plantagineum*, which are triggered by dehydration, are reversed within 4 days of rehydration, which is in agreement with physiological observations. Possible functions of selected proteins are discussed in the context of the de-/rehydration cycle.

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1 Introduction

Reversible protein phosphorylation/dephosphorylation is one of the main regulatory mechanisms of the cell. Enzymatic addition or removal of phosphate groups modulates protein function, increases or decreases the activities of enzymes, redirects the sub-cellular localization of proteins, promotes or disrupts protein-protein interactions or labels proteins for degradation [1, 2].

Increasing evidence indicates that protein phosphorylation also plays an important role in plant responses to water stress. Different protein kinases and phosphatase are up-regulated upon dehydration [3] and several late embryogenesis abundant (LEA) proteins or LEA-like proteins, which accumulate during seed maturation, have been shown to be phosphoproteins [4–6]. We have recently reported dehydration–induced protein phosphorylation in the desiccation tol-

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Abbreviations: **LEA,** late embryogenesis abundant; **MOAC**, metal oxide affinity chromatography; **TCA,** trichloroacetic acid

erant resurrection plant *Craterostigma plantagineum* [7]. Resurrection plants are a small group of vascular plants, which have evolved the unique feature of tolerance to nearly complete water loss in vegetative tissues. In the desiccated state resurrection plants can survive for several months, and upon re-watering they resume full physiological activities within several hours [8]. Desiccation of *C. plantagineum* induces dynamic changes in levels of phosphoproteins. Two such phosphoproteins were previously identified as the dehydration and abscisic-acid (ABA)-responsive protein CDeT11-24 and the group 2 LEA protein CDeT6-19 [7]. Several phosphorylation sites were identified on the CDeT11-24 protein. CDeT6-19 contains a serine-rich sequence motif with strong evidence that most if not all of the continuous serines are phosphorylated [7, 9]. Although CDeT11-24 and CDeT6-19 accumulate with similar kinetics in response to dehydration, their phosphorylation patterns differ. The kinetics of CDeT6-19 phosphorylation correlates with the protein accumulation levels. In contrast, phosphorylated CDeT11-24 protein was detected primarily in dried tissues, although the non-phosphorylated form is induced early during water depletion and is detected several days after re-watering of dried plants. Both proteins were identified in 2-D gels using a phosphoprotein-specific fluorescent dye and MS. The previous analysis of the desiccation phosphoproteome of *C. plantagineum* was restricted to these abundant phosphoproteins, since no other phosphoproteins were present in sufficient quantities for identification.

Since phosphoproteins are often present in relatively low amounts, and since the stoichiometry of phosphorylation is generally low, the identification of phosphoproteins would profit from an efficient enrichment step. Recently, the identification of *Arabidopsis* seed phosphoproteins was described using an enrichment strategy that combines heat-stability and phosphoaffinity chromatography [6]. This procedure led to the identification of several phosphorylated LEA, LEA-like and seed storage proteins, which accumulate abundantly during seed maturation and whose function is not yet known. Yet this analysis was limited to the heat-stable fraction of the phosphoproteome, making the technique unsuitable as a general approach to identify phosphoproteins.

Metal oxide affinity chromatography (MOAC) is a promising procedure for the enrichment of phosphoproteins [10, 11]. MOAC makes use of aluminum hydroxide, which has a high affinity to phosphate groups and is nearly insoluble in aqueous solutions between pH 4 and pH 9.5. It has been demonstrated that this technique is suitable for enrichment of *Arabidopsis* leaf phosphoproteins by 1-D SDS-PAGE and MS [11]. It is possible to use phenol-extracted proteins in a denaturing buffer system for MOAC, which should allow the enrichment of phosphoproteins from various plant tissues, which have, yet, proved difficult to analyze. Furthermore, this technique makes expensive protease and phosphatase inhibitors unnecessary. Nevertheless, no 2-D PAGE analysis of MOAC-enriched phosphoproteins has yet been reported, although such a method would represent an important core technique for the comprehensive analysis of plant phosphoproteomes.

Here, we describe a modified MOAC protocol, which is compatible with 2-D PAGE. Using a combination of MOAC, 2-D PAGE, phosphoprotein-specific staining and MS we have analyzed the desiccation phosphoproteome of *C. plantagineum* leaves and identified several candidate phosphoproteins, which may be involved in the establishment of desiccation tolerance.

2 Materials and methods

2.1 Plant material, growth conditions and treatments

Cultivation and de-/rehydration treatment of *C. plantagineum* Hochst. plants were performed as previously described [7]. Fully hydrated plants (F), desiccated plants (D) and desiccated plants re-hydrated for 2 (R2) or 4 days (R4) were used for the analyses [7]. The relative water content (RWC) was determined as previously described [12].

2.2 Extraction of denatured proteins

Total proteins were extracted essentially as described [7] with some modifications. For each stage of a de-/rehydration cycle proteins were isolated from leaves of independent biological replicate pools (at least four plants). PVP was added to the leaves (10% for plant material F, R2, R4, 100% for plant material D) and the samples were ground to a fine powder under liquid nitrogen. The powder was transferred to 14-mL tubes (a volume corresponding to 3.5 mL for F, R2 and R4; a volume of 1 mL for D) and resuspended in 10 mL ice-cold acetone. The suspension was vortexed vigorously and centrifuged (10 min at $4000 \times g$). After an additional washing step with acetone, the pellet was resuspended in 10% w/v trichloroacetic acid (TCA) in acetone and sonicated in a sonication bath (10 min). The pellets were washed three times with 10% w/v TCA in acetone and then resuspended in 20% w/v TCA. After centrifugation, the pellets were washed three times with 80% v/v acetone and air-dried. Dried pellets were resuspended in 8 mL dense SDS buffer (30% w/v sucrose, 2% w/v SDS, 0.1 M Tris-HCl pH 8.0, 5% v/v 2-mercaptoethanol), and the solution was divided into two tubes. After addition of 4 mL Tris-buffered phenol (Biomol, Hamburg, Germany) to each tube, the mixtures were vortexed, and the phenol phases were separated by centrifugation (10 min at $6000 \times g$). Proteins were recovered from the phenol phase by precipitation with five volumes of 0.1 M ammonium acetate in methanol and incubation at −20°C (60 min), followed by centrifugation and subsequent washes of the pellet twice with 0.1 M ammonium acetate in methanol and twice with 80% v/v acetone. The resulting pellet was air-dried and stored at −80°C. A separately precipitated aliquot of the phenol phase was dissolved in 8 M

urea and used for the protein quantification using the Bradford assay (Bio-Rad, München, Germany) with BSA as standard.

2.3 Enrichment of phosphoproteins

Unless otherwise indicated the following protocol was used for MOAC. Total proteins (7 mg) were resuspended in 12 mL of incubation buffer (IB: 30 mM MES, pH6.1, 0.1 M sodium L-glutamic acid, potassium L-aspartic acid, 0.25% CHAPS and 8 M urea) and re-solubilized by sonication in a water bath (5 min) and subsequently incubated overnight at 10°C. After centrifugation at 12 000 × *g*, the clear supernatant was added to 700 mg $Al(OH)_3$, which had been pre-washed twice with IB. Binding of phosphorylated proteins to the aluminum matrix was achieved by incubating the mixture on a rotator at 10°C for 60 min. The matrix with retained proteins was recovered by centrifugation and washed twice with IB and four times with wash buffer (WB, similar to IB except for the amino acid concentration, which is 0.15 M). Finally, bound putative phosphoproteins were eluted by incubation with 7 mL elution buffer (EB: 200 mM potassium pyrophosphate, 8 M urea, pH 9) on a rotator at room temperature. After centrifugation (10 min, 6000 × *g*, room temperature) the supernatant was removed from the matrix and re-centrifuged to avoid matrix carryover. To the eluted proteins first 0.01 volume of 2% w/v DOC was added and the solution was vortexed. Then, 0.1 volume of 100% w/v TCA was added, the solution was mixed and placed on ice for 1 h. All following steps were performed at 4°C with ice-cold solutions. Precipitated proteins were successively collected by centrifugation (14 000 × *g* for 10 min.) in one 1.5-mL microtube. Following removal of the supernatant, the next aliquot of eluted phosphoprotein candidates was centrifuged in the same microtube. This procedure was repeated until all proteins were pelleted. The final pellet was washed with 25% w/v TCA, resuspended in 10 mM Tris-HCl pH 7.5 in 80% v/v acetone by vortexing and sonication treatment and washed with acetone after centrifugation. After air drying the protein pellet was stored at −80°C. The typical yields of enriched phosphoproteins from 7 mg of total proteins varied between 300 and 500 μg.

2.4 Gel electrophoresis, staining of gels and immunoblot analyses

The 1-D and 2-D PAGE were performed as described [7]. For 1-D PAGE, NuPAGE 4–12% Bis(2-hydroxyethyl)imino(Bis)-Tris gels with MES-SDS running buffer were used (Invitrogen, Karlsruhe, Germany). IEF was performed with 7-cm IPG strips, pH 3–10 (GE Healthcare, München, Germany) and the second dimension was run on NuPAGE 4–12% Bis-Tris Zoom gels (Invitrogen, Karlsruhe, Germany). In general, 30–50% of the eluted phosphoproteins were used for one 2-D gel (corresponding to approximately 2–3.5 mg of total proteins). After the run, phosphoproteins were stained in the gels with Pro-Q Diamond fluorescent gel stain and total proteins were stained with SYPRO Ruby (Invitrogen) according to the protocols of the manufacturer. Fluorescent dyes were visualized using a Fuji FLA 3000 Fluorescence Laser Scanner (Fuji, Photofilm, Tokyo, Japan) equipped with lasers that emit at 473 and 532 nm, respectively. Immunoblot analyses were performed as described [7]. Polyclonal antisera raised against *C. plantagineum* CDeT11-24 [7], cytosolic fructose bisphosphatase (FBPase) and light harvesting chlorophyll a/b binding protein 1 (Lhcb1), both from *Arabidopsis thaliana* (Agrisera, Vännäs, Sweden), were used at a dilution 1:5000.

2.5 In-gel tryptic digest, sequence data and protein identification by MS

Protein spots were picked from gels stained with Pro Q Diamond fluorescent dye by a *Proteineer* spII spot picker and passed to a *Proteineer* dp robot (Bruker Daltonics, Bremen, Germany) for automated tryptic digestion and target preparation. Samples were spotted onto Anchorchip targets (Bruker) using a thin-layer protocol with CHCA matrix [13]. MALDI MS and MS/MS analyses were performed on an Ultraflex III system (Bruker) according to the following workflow. PMFs were collected on the thin-layer samples and post-calibrated using tryptic autodigestion masses, when present. These spectra were used for database searches using MASCOT 2.2 (Matrix Science, London. UK) against a small database of cDNA sequences from *C. plantagineum* (cDNA clones constructed from poly $(A)^+$ RNA of dehydrated *C. plantagineum* leaves, unpublished data) and against the NCBInr database without taxonomic restrictions and with a 20 ppm mass tolerance. Non-post-calibrated spectra, which failed to return identifications in this round of searching, were then used to search with a 100 ppm mass tolerance. For each sample spot, precursor ions of sufficient intensity and quality were flagged automatically for fragmentation according to the following strategy: where a PMF-based identification was successful, two assigned precursors were selected (if present) for validation by MS/MS. Then up to five further unassigned precursors were flagged for fragmentation. In cases where PMF-based identification failed, five precursors were selected based on signal quality. All samples on the target were re-crystallized in preparation for MS/MS collection. The MS/MS spectra were also used to perform MASCOT MS/MS searches against the databases mentioned above.

Search results were evaluated based on the following criteria: MASCOT scores above corresponding significance thresholds (for the cDNA database PMF: 50, PFF: 20; for the NCBInr database PMF 79, PFF: 47), a low mass error, a continuous ORF (in the case of the cDNA database). Marginal hits were accepted if both MS and MS/MS searches returned sub-threshold scores for the same protein. Since the number of sequences known from *C. plantagineum* is low, the reported identifications are mostly homologues from sequenced organisms arising either from local homology recognized in

MS/MS searches, or from BLAST annotation of cDNA sequences in the *C. plantagineum* database. The agreement between the observed and predicted MW and p*I* of the complete homologous sequence was used as a validation criterion.

2.6 Phosphatase treatment of phosphoproteins

Phosphoproteins (enriched from 2 mg of total proteins) were separated by NuPAGE 4–12% Bis-Tris gels (Invitrogen, Karlsruhe, Germany) and transferred to a Sequi-Blot PVDF membrane (0.2 μm, for protein sequencing; Bio-Rad, München, Germany). The membrane was incubated with TBS containing 0.1% v/v Triton X-100 and 1% w/v BSA for 1 h to block unspecific binding. Phosphatase treatment of membrane-bound proteins was performed with 800 U Lambda protein phosphatase (New England Biolabs, Frankfurt am Main, Germany) *per* 1 mL TBS containing 0.1% v/v Triton X-100, 1% w/v BSA and 2 mM $MnCl_2$. The incubation was carried out on a shaker for 4 h at 25°C. After phosphatase treatment, the membrane was washed once with PBS and five times with H_2O (5 min each). Then the membrane was air-dried, shortly immersed in methanol, subsequently treated with 7% acetic acid/10% methanol (15 min) and finally washed four times with H_2O (5 min each). To detect phosphoproteins the membrane was immersed for 15 min in Pro-Q Diamond Phosphoprotein Blot Stain (a 1000-fold dilution of Pro-Q Diamond blot stain reagent in Pro-Q Diamond blot stain buffer). Excess of destaining solution was washed off four times with 30 mL 50 mM sodium acetate, pH 4, 20% v/v ACN (10 min each wash). After destaining, the membrane was air-dried and phosphoproteins were visualized using an excitation wavelength of 473 nm and measuring emission at 580 nm. To stain total proteins the membrane was immersed for 2 min in PageBlue (Fermentas, Vilnius, Lithuania) and then destained with 30% v/v ethanol.

3 Results

3.1 MOAC-enriched putative phosphoproteins from *C. plantagineum* and 2-D PAGE

For the analyses of the *C. plantagineum* phosphoproteome, leaf tissues were used from untreated (F), desiccated plants (D), as well as plants rehydrated for 2 (R2) or 4 days (R4). Total proteins were extracted and candidate phosphoproteins were initially enriched from the denatured proteins using MOAC according to the original protocol [10, 11]. Following this procedure, we were not able to perform 2-D PAGE of the enriched proteins, probably due to traces of $Al(OH)_3$ in the samples, which interfere with IEF (data not shown). Therefore, several modifications of the original protocol were tested for phosphoprotein enrichment and compatibility with 2-D PAGE.

After the elution of candidate phosphoproteins from the $Al(OH)_3$ matrix, these proteins were precipitated in the original protocol [10] with chloroform and methanol. This procedure can lead to a carryover and co-precipitation of the aluminum hydroxide matrix with the proteins. Since the solubility of aluminum hydroxide increases at acidic pH, we performed a TCA precipitation of putative phosphoproteins followed by washing protein pellets with TCA to get rid of residual $Al(OH)_3$. This method was used to precipitate putative phosphoproteins enriched by MOAC and was compared to the chloroform/methanol method by PAGE analysis. Staining of the gels with the phosphoprotein specific fluorescent dye Pro-Q Diamond demonstrates that MOAC efficiently enriches putative phosphoproteins from total proteins (Fig. 1). Both precipitation methods show similar efficiency of phosphoprotein recovery from the eluate solution but with the significant difference that TCA-precipitated phosphoproteins could be separated by IEF. Therefore, this method was used for all further experiments.

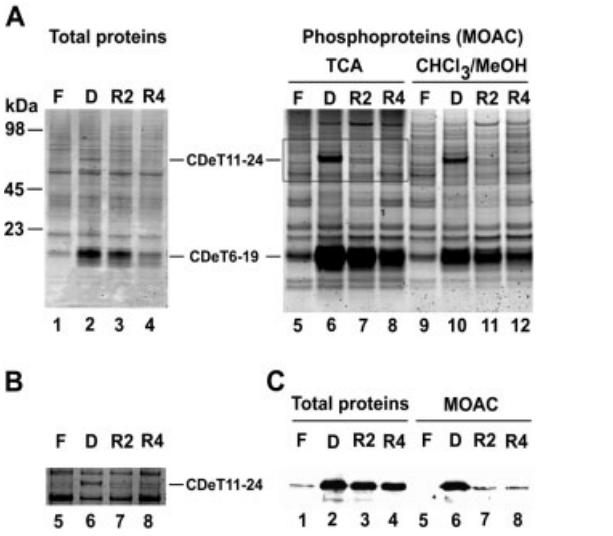

Figure 1. Analysis of putative phosphoproteins enriched from *C. plantagineum* at different stages of a de-/rehydration cycle. Total proteins (lanes 1–4, 1 μg each lane) were isolated from leaves of untreated (F), desiccated (D) as well as for 2 and 4 days rehydrated (R2, R4) *C. plantagineum* plants and used for the enrichment of putative phosphoproteins with MOAC (lanes 5–12, corresponding to 0.5 mg of total proteins each lane). After elution from the aluminum hydroxide matrix putative phosphoproteins were precipitated either with TCA (lanes 5–8) or with $CHCl_3$/MeOH (Lanes 9–12). Proteins were separated on NuPAGE 4–12% Bis-Tris gels. (A) The gels were stained with Pro-Q Diamond fluorescent gel stain to visualize phosphoproteins. The CDeT11-24 and CDeT6-19 proteins are indicated. (B) Region of the gel indicated in (A) showing staining with SYPRO Ruby for total proteins. (C) Immunoblot analysis of corresponding protein samples shown in (A). Proteins were separated on a NuPAGE gel and transferred to an NC membrane. Polyclonal antiserum raised against CDeT11-24 was used to detect the protein.

3.2 Refinement of MOAC

For the analysis of the *C. plantagineum* desiccation phosphoproteome, the characterized phosphoproteins CDeT6-19 and CDeT11-24 [7] were used as reference proteins. In the original MOAC protocol, proteins were eluted by incubation of the $Al(OH)_3$ matrix with 100 mM potassium pyrophosphate containing 8 M urea, pH 9.0 [10]. Using this buffer for elution, we observed that significant amounts of potentially phosphorylated proteins remained bound to the matrix (data not shown), so that the concentration of potassium pyrophosphate was increased to 200 mM. Figure 1 shows that the CDeT6-19 protein is successfully enriched, indicating that polyphosphorylated proteins can be efficiently eluted from the matrix at higher buffer molarities. This is an important criterion since a correlation between the degree of phosphorylation and binding strength to the $AL(OH)_3$ matrix has been reported [10].

In addition, co-purification of acidic proteins is often a problem when using metal affinity capture methods due to the interaction of carboxylic side chains with the charged matrix. Figure 1 shows that the acidic protein CDeT11-24 (p*I* 4.7) was enriched from leaves of fully dehydrated plants (D) but not from leaves of plants which were rehydrated for 2 or 4 days (R2, R4), although the protein level is similar in these samples [7]. Since the phosphorylated form of CDeT11-24 occurs primarily in desiccated plants, this demonstrates the selectivity of MOAC for the phosphate group of this protein, rather than its high content of acidic residues.

Furthermore, the influence of aspartate and glutamate concentrations was tested on the selectivity of MOAC and the yield of putative phosphoproteins (Fig. 2). These amino acids were used to compete against unspecific interactions between acidic amino acids in the proteins and the aluminum matrix [10]. In addition to the originally described concentration of 200 mM acidic amino acids in the incubation and washing buffer, the enrichment of potentially phosphorylated proteins in the presence of 50 and 10 mM acidic amino acids was also tested. The *C. plantagineum* protein extracts before (TP) and the supernatant after incubation with the $Al(OH)_3$ matrix (SN), together with the eluted fractions (EF) were then analyzed by PAGE. Staining of the gel for phosphoproteins showed that $Al(OH)_3$ binds putative phosphoproteins, which then can be eluted from the matrix (Fig. 2). In the presence of 200 mM amino acids, the amount of total proteins is not affected by incubation with the matrix (lanes 1 and 2), indicating low unspecific binding. Nevertheless, the amount of eluted protein is also low. Using 10 mM acidic amino acids as competitor leads to a strong reduction of total proteins in the supernatant (lane 8), indicating increased unspecific binding of non-phosphorylated proteins and results in large amounts of protein in the eluted fraction (lane 9). Based on these findings we developed a protocol, which strikes a balance between yield and selectivity of putative phosphoproteins. Candidate phosphoproteins are bound to the aluminum matrix in the presence of 100 mM acidic amino acids followed by different washing steps with higher stringency (150 mM acidic amino acids). This protocol yielded the best enrichment of *C. plantagineum* candidate phosphoproteins with MOAC.

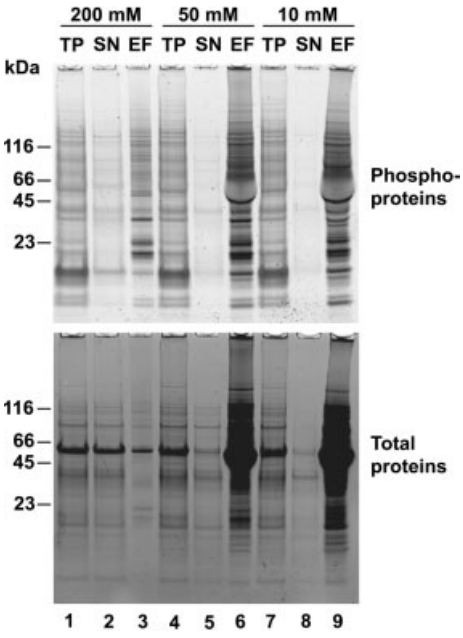

Figure 2. The efficiency and selectivity of phosphoprotein enrichment with MOAC depends on the concentrations of acidic amino acids. Total proteins extracted from leaves of fully hydrated *C. plantagineum* plants were used for the enrichment of putative phosphoproteins. Binding of phosphoproteins to the aluminum hydroxide matrix as well as washing of the matrix bound phosphoproteins was performed with incubation and washing buffers containing 200, 50 or 10 mM for each acidic amino acid (glutamic acid, aspartic acid). Proteins (5 μg) in incubation buffer before (TP), and after incubation with the aluminum hydroxide matrix (SN) together with TCA precipitated candidate phosphoproteins from the eluted fraction (EF, corresponding to 0.5 mg of total proteins each lane) were analyzed by NuPAGE 4–12% Bis-Tris gels. Gels were stained with Pro-Q Diamond to detect phosphoproteins, and subsequently with SYPRO Ruby to visualize total proteins.

To further validate the selectivity of MOAC for phosphoproteins, these proteins were treated with phosphatase. Therefore, MOAC-enriched proteins from leaves of untreated *C. plantagineum* plants were separated by PAGE, transferred to a PVDF membrane and used for phosphatase treatment with Lambda phosphatase. Staining of the membrane-bound potentially phosphorylated proteins with Pro-Q Diamond and subsequent staining with PageBlue for total proteins demonstrated that the phosphatase reduces the signal intensities of the phosphorylated marker protein ovalbumin (OVA) and the MOAC-enriched proteins (F) in comparison to the control (Fig. 3). This result, together with the similarity of the patterns for candidate phosphoproteins and total proteins provides further evidence that the MOAC-enriched fraction contains mainly phosphorylated proteins.

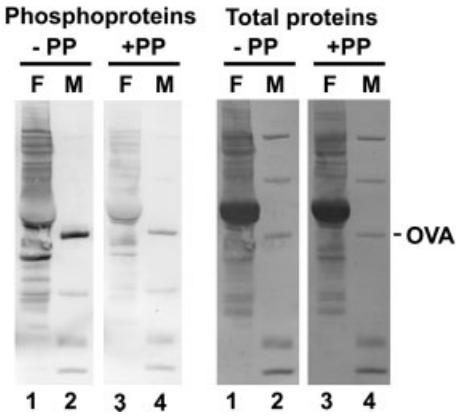

Figure 3. Dephosphorylation of MOAC-enriched candidate phosphoproteins with phosphatase. Putative phosphoproteins (corresponding to 2 mg of total proteins) from untreated leaves (F; lanes 1, 3) and marker proteins (M : 116, 66, 45, 18, and 14 kDa; lanes 2, 4) were separated on a NuPAGE gel and transferred to a PVDF membrane. The position of the phosphorylated marker protein ovalbumin (OVA, 45 kDa) is indicated. One part of the blot was treated with Lambda Phosphatase (+PP; lanes 3, 4), the other part was used as control (−PP; lanes 1, 2). After staining the blots with Pro-Q Diamond for the detection of phosphoproteins (left hand side) the blots were re-stained with PageBlue for total proteins (right hand side).

3.3 Comparative 2-D gel analysis of the *C. plantagineum* putative desiccation phosphoproteome

The MOAC-enriched putative phosphoproteins from different stages of a de-/rehydration cycle were separated by 2-D PAGE and stained with Pro-Q Diamond for phosphoproteins or SYPRO Ruby for total proteins (Fig. 4). Proteins CDeT11-24 and CDeT6-19 were always used to validate individual experiments. Comparison of the 2-D gels stained with Pro-Q Diamond showed that the potentially phosphorylated proteins are spread across the pH range of 3–10, although more protein spots are located in the acidic region. Some spots show a "string of pearls" appearance, which is typical for proteins with multiple phosphorylation states. When several different proteins with similar molecular mass and p*I* were resolved, a horizontal streaking of the fluorescent signal was observed. Besides CDeT11-24 and CDeT6-19 several other proteins, which were not detectable previously, appear in the 2-D gels after staining with Pro-Q Diamond [7].

A comparison of the different 2-D gels shows that most spots of potentially phosphorylated proteins change their intensities during the de-/rehydration cycle (compare F to D, R2 and R4, stained for phosphoproteins in Figs. 4 and 5). Besides the abundant induction of phosphorylated CDeT11-24 and CDeT6-19 (D, R2 in Fig. 4) some spots not detected in untreated plants (F) appear in the sample of fully dehydrated plants (D, spots 11, 17, 28) or re-hydrated plants (R2, R4; spot 10). Most spots of potentially phosphorylated proteins are reduced or disappear in response to dehydration (spots 1–9, 12–16, 18–20, 22, 23). Notably, nearly all changes in the putative phosphoproteome of *C. plantagineum*, which are triggered by desiccation, are reversed after 4 days of rehydration (Figs. 4 and 5).

3.4 Identification of candidate phosphoproteins by MALDI MS/MS

To identify putative phosphoproteins, spots were picked from the ProQ Diamond-stained 2-D gels, the proteins were digested in gel with trypsin and analyzed by MALDI MS and MS/MS. Collected mass spectral data were used to search a database of cDNA clones from *C. plantagineum* (unpublished data) and the NCBI non-redundant protein database using MASCOT software. The average identification rate for the analyzed spots was 42%, reflecting both the low quantity of proteins present (due to the sensitivity of Pro-Q Diamond) and the relative lack of sequence information for *C. plantagineum*. Twenty potentially phosphorylated proteins were, however, identified (Table 1).

The identified proteins can be classified into the following functional categories: desiccation-related proteins, RNA/DNA-binding proteins, proteins involved in translation, photosynthesis-related proteins, enzymes involved in carbohydrate metabolism, molecular chaperones and other enzymes. Most of these proteins or homologues have been reported to be phosphoproteins (Table 2). Since most spots did not separate well due to the existence of phosphoprotein isoforms at different p*I* and the high sensitivity of the phosphoprotein-specific fluorescent dye, this made the use of quantification software impracticable. Therefore, protein abundance was scored visually and the comparison was kept semi-quantitative. Protein spot intensities were compared in all four physiological stages of the re-/dehydration cycle and the results are reported in Table 2.

3.5 Expression analysis of fructose bisphosphatase and light harvesting chlorophyll a/b-binding protein

To corroborate the quantitative variations of putative phosphoproteins described in Fig. 4 and Table 2, two proteins were selected and analyzed immunologically. We used antibodies raised against cytosolic fructose-1,6-bisphosphatase (FBPase) to analyze the expression of this protein. The 1-D immunoblot analysis of total proteins showed no differences in expression of FBPase during the de-/rehydration cycle (Fig. 6A). However, on 2-D immunoblots, the signal for FBPase is separated in the first dimension into a main signal with a p*I* of approximately 5–6 (Fig. 6B; spot 1) and two weaker signals with more acidic p*I* (Fig. 6B; spots 2, 3). The main signal represents cytosolic FBPase (spot 1), whereas the protein spot 2 was observed in the 2-D immunoblot at the same position as the MOAC-enriched FBPase (Fig. 4), indicating that this may represent a potentially phosphorylated

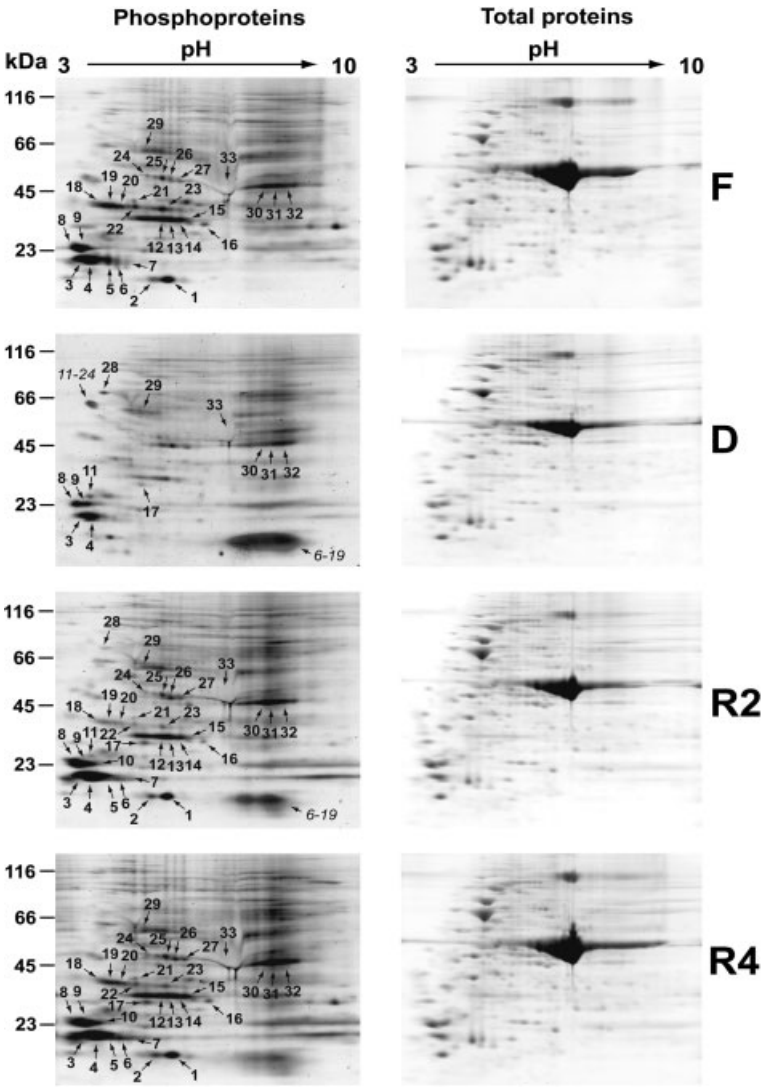

Figure 4. The 2-D gel analysis of putative phosphoproteins from *C. plantagineum* leaves at different stages of a de-/rehydration cycle. Potentially phosphorylated proteins (corresponding to 3.5 mg of total proteins) were enriched from leaves of untreated (F), fully dehydrated (D) as well as re-hydrated (RH4, R4) *C. plantagineum* plants and analyzed by 2-D PAGE. IEF in the first dimension was performed over the pH range 3–10, in the second dimension on NuPAGE 4–12% Bis-Tris gels. The gels were stained with Pro-Q Diamond or with SYPRO Ruby fluorescent gel stains to detect putative phosphoproteins and total proteins, respectively. Identified spots are numbered and spots corresponding to CDeT11-24 and CDeT6-19 are indicated in D and R2. Representative gels of three replicates with independent biological material are shown.

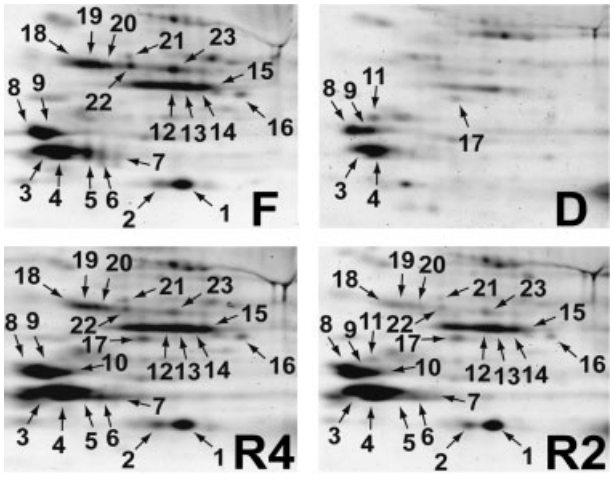

Figure 5. Enlarged sections of 2-D gels stained with Pro-Q Diamond for detection of candidate phosphoproteins (see Fig. 3).

chloroplastic isoform of this enzyme which is also recognized by the antibody. In addition, protein spot 3 (Fig. 6B) may represent the corresponding precursor protein with signal peptide sequence because it shows a slightly higher molecular weight. Nevertheless, immunoblot analysis shows that this FBPase isoform has the same expression pattern during the de-/rehydration cycle on the immunoblot of total proteins as observed for the MOAC-enriched putative phosphoprotein (Fig. 4, Table 2).

To analyze the expression of the chlorophyll a/b-binding proteins we used antibodies against Lhcb1 from *A. thaliana* (BLAST search showed a high homology between the *A. thaliana* and *C. plantagineum* proteins, data not shown). The observed spot pattern in the immunoblots corresponds to the pattern observed with MOAC enriched chlorophyll a/b-binding proteins (Fig. 4, spots 5–7). Since the signals for Lhcb1 on the immunoblots were reduced in desiccated leaves of *C. plantagineum* in

Table 1. Identification of putative phosphoproteins from *C. plantagineum* leaf-protein extracts at different stages of a de-/rehydration cycle

Spot[a]	Protein[b]	Organism[c]	Accession no.[d]	BLAST[e]		PMF[f]			MS/MS[g]			Predicted M_r/pI[h]
				Score (Bits)	E-value	Score	Coverage (%)	NP	Score	Coverage (%)	NP	
1	Eukaryotic initiation factor 5A (3)	*S. lycopersicum*	GI: 20138706			**125**/51	73	8	**154**	26	3	17.4/5.7
2	Eukaryotic initiation factor 5A (3)	*S. lycopersicum*	GI: 20138706			70/58	26	4	**53**	14	2	17.4/5.7
3	Ribonucleoprotein A (CP29A)	*C. plantagineum*	AM992040			**64**/28	38	9	**235**	12	2	29.7/4.8
		N. sylvestris	GI: 12230584	286	1.31e-076							
4	Ribonucleoprotein A (CP29A)	*C. plantagineum*	AM992040			**72**/29	39	10	**104**	9	1	29.7/4.8
		N. sylvestris	GI: 12230584	286	1.31e-076							
5	Chlorophyll a/b binding protein	*Lemna gibba*	GI: 482445						**117**	10	3	28.1/5.2
6	Chlorophyll a/b binding protein	*Lemna gibba*	GI: 482445						**114**	10	3	28.1/5.2
7	Chlorophyll a/b binding protein	*C. japonica*	GI: 3417451						**167**	5	2	28.1/5.2
8	Elongation factor 1 beta 2	*A. thaliana*	GI: 13124717						**83**	4	1	24.6/4.2
9	Elongation factor 1 beta 2	*O. sativa*	GI: 53370742						**55**	4	1	24.6/4.2
10	14-3-3 protein GF14 omega	*A. thaliana*	GI: 21618266						**204**	13	2	29.1/4.6
11	Chloroplast drought-induced stress protein (CDSP34)	*C. sativus*	GI: 62899808						**51**	4	1	35.6/5.1
									139*	12*	3*	
12	60S acid ribosomal protein P0	*A. thaliana*	GI: 2088654						**91**	4	2	33.6/5.0
13	Hydroxymethylbilane synthase, putative	*A. thaliana*	GI: 21689853						**81**	3	1	41.0/9.7
14	60S acid ribosomal protein P0	*A. thaliana*	GI: 2088654						**78**	4	1	33.6/5.0
15	Pyridoxine biosynthesis protein putative	*C. plantagineum*	AM992041			**117**/38	30	13				33.1/5.9
		N. tabacum	GI: 46399269	443	6.83e-124							
16	Pyridoxine biosynthesis protein putative	*C. plantagineum*	AM992041			**126**/42	34	14	**55**	9	3	33.1/5.9
		N. tabacum	GI: 46399269	443	6.83e-124							
17	Thiazole biosynthetic enzyme	*A. thaliana*	GI: 23296288						**100**	7	2	37.6/5.4
18	Fructose bisphosphatase	*A. thaliana*	GI: 23397203						**91**	6	2	45.1/5.1
19	Fructose bisphosphatase	*P. sativum*	GI: 20717						**130**	6	2	45.1/5.1
20	30S ribosomal protein S1 (CS1)	*C. plantagineum*	AM992042			**146**/29	42	22	**184**	8	2	44.8/5.4
		S. oleracea	GI: 133872	365	2.00e-100							31.1/5.8
21	Phosphoribulokinase	*C. plantagineum*	AM992043			**76**/35	42	9	**52**	4	2	44.1/6.0
		M. crystallinum	GI: 125578	436	8.07e-122							
22	Plastidic aldolase	*C. plantagineum*	AM992044			**139**/26	48	14	**133**	5	2	42.8/6.4
		N. paniculata	GI: 4827253	554	4.43e-157							
23	Plastidic aldolase	*C. plantagineum*	AM992044			**211**/31	61	20	**175**	5	2	42.8/6.4
		N. paniculata	GI: 4827253	554	4.43e-157							
24	Enolase	*G. barbadense*	GI: 33415263						**222**	9	2	47.9/6.2
25	Enolase	*G. max*	GI: 42521309						**550**	11	4	47.8/5.5
26	Enolase	*C. plantagineum*	AM992045			**52**/32	25	6				47.8/5.5
		H. brasiliensis	GI: 14423687	384	3.25e-106							
26	Enolase	*G. barbadense*	GI: 33415263						**265**	11	3	47.9/6.2
26	DEAD BOX RNA helicase	*O. sativa*	GI: 34913862						**171**	10	3	48.1/5.6

Table 1. Continued

Spot[a]	Protein[b]	Organism[c]	Accession no.[d]	BLAST[e]		PMF[f]			MS/MS[g]			Predicted M_r/p*I*[h]
				Score (Bits)	E-value	Score	Coverage (%)	NP	Score	Coverage (%)	NP	
27	Enolase	*C. plantagineum*	AM992045			**74**/37	44	11				47.8/5.5
		H. brasiliensis	GI: 9581744	384	3.25e-106				**309**	7	2	
27	DEAD BOX RNA helicase	*O. sativa*	GI: 34913862						**280**	10	3	48.1/5.6
28	Heat shock 70 protein	*S. oleracea*	GI: 2654208						**296**	9	4	76.1/5.0
29	Chaperonin 60 beta precursor	*C. plantagineum*	AM992046						**87**	7	2	63.8/6.2
		A. thaliana	GI: 9280299	506	1.0 e-141							
29	Chaperonin 60 beta precursor	*A. thaliana*	GI: 81601						**115**	3	1	63.8/6.2
30	EBP1	*C. plantagineum*	AM992047			**53**/35	25	7	**100**	9	2	42.8/6.3
		S tuberosum	GI: 116292768	472	1.75e-132							
31	EBP1	*C. plantagineum*	AM992047			**56**/30	37	9	**234**	15	3	42.8/6.3
		S tuberosum	GI: 116292768	472	1.75e-132							
32	EBP1	*C. plantagineum*	AM992047			48/36	29	9	**134**	9	2	42.8/6.3
		S tuberosum	GI: 116292768	472	1.75e-132							
33	Rubisco	*I. purpurea*	GI: 58380						**289**	9	4	53.4/6.4

a) Spot number corresponding to spots in Fig. 3.
b) Protein annotation.
c) Plant species from which the protein was identified.
d) NCBI database accession number of identified protein or EMBL database accession number of identified *C. plantagineum* cDNA clone used for BLAST search.
e) Bit score and E-value of a blastp search used to annotate cDNA identified by PMF. When blank, then the given NCBI accession number was identified directly.
f) The MASCOT score, sequence coverage, and number of matched peptide masses where PMF contributed to the identification. The MASCOT score is presented as the ratio of the actual mascot score and the decoy score. MASCOT scores printed in boldface exceed the significance value for the corresponding database (see www.matrixscience.com for an explanation of score significance and decoy).
g) MASCOT score, sequence coverage and number of peptides used for identification of proteins by MALDI MS/MS in the NCBI database. Scores exceeding the significance threshold are printed in boldface. The numbers marked with asterisks represent the results from error tolerant searches (see http://www.matrixscience.com/help/error_tolerant_help.html). In many cases, a second round of error tolerant searching revealed additional homologous peptides corresponding to initially unassigned MS/MS spectra. The sequences of matching peptides are provided in the Supporting Information.
h) Predicted molecular mass (kDa) and p*I* of the identified proteins.

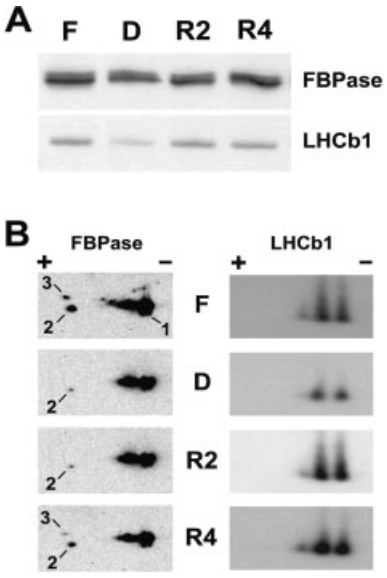

Figure 6. Expression analysis of fructose bisphosphatase (FBPase) and light harvesting chlorophyll a/b binding protein 1 (LHCb1) during a de-/rehydration cycle of *C. plantagineum*. Total proteins from leaves of untreated (F), fully dehydrated (D) as well as re-hydrated (R2, R4) *C. plantagineum* plants were separated by 1-D PAGE and 2-D PAGE and analyzed immunologically for FBPase and Lhcb1. (A) Proteins were separated on a NuPAGE 4–12% Bis-Tris gel and transferred to a nitrocellulose membrane. Antisera raised against FBPase and LHCb1 proteins were used to detect the respective proteins. (B) The 2-D immunoblot analysis of FBPase and LHCb1. The cytoplasmic (1), putative chloroplastic isoform (2) as well as the putative chloroplastic precursor protein (3) are indicated.

comparison to untreated and rehydrated tissues, this finding also correlates with the results obtained in the MOAC samples.

The observed differences for FBPase and Lhcb are more likely due to differences in protein abundance and not to changes of the phosphorylation states, since we could not observe a shift in the p*I* of either protein during the de-/rehydration cycle.

4 Discussion

We describe a strategy for the analysis of putative phosphoproteins, which is generally applicable to different plant tissues as demonstrated for hydrated and desiccated *C. plantagineum* leaves. The method incorporates purification of denatured proteins by a phenol-based extraction, enrichment of phosphoproteins by MOAC, 2-D PAGE, phosphoprotein-specific staining and MS analysis. Although MOAC has already been described as a convenient method for the enrichment of phosphoproteins [10, 11], here we report a refined method which is compatible with the subsequent separation of putative phosphoprotein fractions by 2-D PAGE. This methodology enables a large-scale analysis of *in vivo* phosphorylation/dephosphorylation processes of plant proteins.

To establish this strategy we have used *C. plantagineum* for two reasons. First, the characterized phosphoproteins CDeT6-19 and CDeT11-24 can be used as reference proteins to monitor and validate the efficiency and selectivity of our strategy. Secondly, *C. plantagineum* is a model system for vegetative desiccation tolerance and all processes induced by water stress are reversed within several days after re-watering [31]. Our data show that this reversibility is also reflected in the putative phosphoproteome during the de-/rehydration cycle.

We have identified 20 proteins, for most of which (16 proteins) published evidence suggests that they exist as phosphoproteins. Several proteins were identified in more than one spot, indicating multiple phosphorylation states of the same protein. In contrast to the protein corresponding to the plant-growth regulator EBP 1 (spots 30, 31, and 32), which seems to be constitutively present during the de-/rehydration cycle, spot intensities of most candidate phosphoproteins change upon desiccation. This may be explained by phosphorylation/dephosphorylation of constitutively expressed proteins and/or desiccation-induced synthesis or degradation of phosphoproteins.

One protein identified in this study is fructose-1,6-bisphosphatase (FBPase, spots 18, 19). BLAST searches of the amino acid sequences from the *A. thaliana* and *Pisum sativum* homologs identified indicated that this is the chloroplastic isoform of the enzyme (data not shown). Together with the plastidic aldolase (spots 22, 23), which was also identified during this study, chloroplastic FBPase is responsible for the synthesis of fructose-6-phosphate from triose-phosphates in two steps. This is an important reaction in chloroplasts leading to the formation of starch from photosynthetic metabolites during the light period. Our data show that nearly no phosphoprotein signals could be detected for FBPase and aldolase in desiccated leaves, but both proteins were present in the candidate phosphoprotein fractions of untreated and re-hydrated leaves. Immunoblot analyses of total proteins indicated that for FBPase this effect is more likely due to desiccation-induced degradation and rehydration-triggered synthesis of the protein, rather than to a change in the phosphorylation status. This may also be the case for plastidic aldolase, since expression of genes encoding this enzyme have already been shown to be responsive to salt stress in *Nicotiana* plants [32] and a down-regulation of the corresponding mRNA was observed in *C. plantagineum* leaves during desiccation (C. Meesters, personal communication). Together, these data indicate that starch synthesis is reduced upon water stress, which is in agreement with previous studies [33].

An important process requiring a tight regulation during water stress is photosynthesis. During the de-/rehydration cycle of *C. plantagineum* leaves, we observed a reversible regulation of the chlorophyll a/b binding protein (spots 5–7). The chlorophyll a/b protein is the major phosphoprotein

Table 2. Change in abundance of the identified phosphoprotein candidates during the de-/rehydration cycle of *C. plantagineum*

Spot[a)]	Protein[b)]	Reference for phosphorylation[c)]	Regulation[d)]			
			F	D	R2	R4
1	Eukaryotic initiation factor 5A (3)	[14]	++	−	++	++
2	Eukaryotic initiation factor 5A (3)	[14]	+	−	+	+
3, 4	Ribonucleoprotein A (CP29A)	[15, 16]	++	↓	++	++
5	Chlorophyll a/b binding protein	[17]	++	−	++	++
6, 7	Chlorophyll a/b binding protein	[17]	+	−	+	+
8, 9	Elongation factor 1 beta 2	[18, 19]	++	↓	++	++
10	14-3-3 Protein GF14 omega	[20]	−	−	+	+
11	Chloroplast drought-induced stress protein (CDSP34)		−	+	(+)	−
12	60S acid ribosomal protein P0	[21]	+	↓	+	+
13	Hydroxymethylbilane synthase, putative		+	↓	+	+
14	60S acid ribosomal protein P0	[21]	+	↓	+	+
15	Pyridoxine biosynthesis protein, putative		+	↓	+	+
16	Pyridoxine biosynthesis protein, putative		+	−	(+)	+
17	Thiazole biosynthetic enzyme		−	(+)	+	+
18, 19	Fructose bisphosphatase	[22]	+	−	(+)	+
20	30S ribosomal protein S1 (CS1)	[23]	+	−	(+)	+
21	Phosphoribulokinase	[24]	+	nw	+	+
22, 23	Plastidic Aldolase	[24]	+	−	+	+
24, 25,26,27	Enolase	[25]	nw	nw	nw	nw
26, 27	DEAD BOX RNA helicase	[26]	nw	nw	nw	nw
28	Heat shock 70 protein	[27]	−	+	(+)	−
29	Chaperonin 60 beta precursor	[24]	+	nw	nw	+
30, 31, 32	EBP1	[28]	+	+	+	+
33	Rubisco	[29, 30]	nw	nw	nw	nw

a) Spot number corresponding to numbers in Fig. 3.
b) Protein name (NCBI).
c) Literature supporting that the identified protein or a member of the same protein family is phosphorylated.
d) Visual assessment of phosphoprotein regulation. Spot intensities of phosphoproteins stained with Pro-Q Diamond in Fig. 3 were rated strong ++, medium +, weak (+) or no signal −, respectively); ↓ signal down-regulation during the de-/rehydration cycle; nw, no weighting of signal intensities.

component of the light harvesting chlorophyll a/b complex (LHC). By using antibodies against Lhcb1 from *A. thaliana,* we could show that the observed quantitative changes in MOAC-enriched protein signal intensities are correlated with a decrease in protein abundance and are not due to changes in the phosphorylation states. It is known that several other thylakoid proteins are phosphorylated [34] but Lhcb1 protein was the only one found among the 20 identified candidate phosphoproteins. The reason for this may be that membrane thylakoid proteins could not resolubilized with the urea buffer used for the MOAC-enrichment procedure.

Another photosynthesis-related protein is the large subunit of ribulose-1,5-bisphosphate carboxylase (Rubisco). Rubisco is enriched by MOAC from *C. plantagineum* leaves and although it has been shown that this is a phosphoprotein [29] it was not stained with Pro-Q Diamond in the gels shown here. This effect may arise from negative staining of this highly abundant protein due to signal overloading, since lower amounts of Rubisco could be stained with Pro-Q Diamond (data not shown). Rubisco is less efficiently enriched from D and RH2 plants than from F and RH4 plants, although present in equal amounts in the total protein extracts (data not shown). This indicates some water stress-triggered de-phosphorylation and re-phosphorylation of Rubisco without a change in total protein level during the de-/rehydration cycle. In this context it has been reported that phosphorylation of Rubisco may play a role in cold stress tolerance of rice [30].

Since a low cellular relative water content (RWC) impairs the structures of many proteins, the observed accumulation of molecular chaperones upon water depletion is explained [3]. We also detected the transient accumulation of Hsp70 during the de-/rehydration cycle (spot 28). It is well established that Hsp70 acts as molecular chaperone during the translocation of precursor proteins across the ER of plant cells and it has been speculated that this chaperone is regulated by phosphorylation [27].

Another identified protein is homologous to the 14-3-3 protein GF14 omega from *Arabidopsis*. The 14-3-3 proteins are a class of highly conserved phosphoproteins that exhibit different functions in all eukaryotic cells [35]. The *Arabidopsis* GF14 omega was originally described as associating with DNA-protein complexes. In addition, the protein has bio-

chemical properties consistent with a potential signaling role in plants [20]. In our approach, we have identified a spot of potentially phosphorylated GF14 omega in rehydrated but not in untreated and dehydrated plants (spot 10). Thus, GF14 omega may be involved in signaling events, which are required during rehydration.

Several proteins involved in translation or RNA-binding were identified in our analysis, indicating a stress-responsive regulation at the post-transcriptional level. A homologue of the hypusine-containing eukaryotic translation initiation factor 5A (eIF5A; spot 1, 2) shows a water stress-dependent reversible de-phosphorylation or degradation of the protein. Translation initiation factor 5A is a small, highly conserved protein that undergoes a PTM at one of its lysine residues to form hypusine. It was shown that eIF5A is phosphorylated on a serine residue in *Saccharomyces cerevisiae* [36]. Although the precise function is still not known, *in vivo* data indicated that eIF5A may be involved in mRNA turnover [37] and it has been reported that genes encoding eIF5A are involved in developmental and environmental responses in rice [38]. Desiccation-dependent down-regulation of the spot intensity was also observed for the potentially phosphorylated ribonucleoprotein A, which is a chloroplastic RNA-binding protein (spots 3, 4). Many proteins of this class have been shown to be phosphorylated [39]. Notably, a chloroplastic RNA-binding protein from spinach changes its affinity to RNA upon phosphorylation [16]. Additionally, desiccation-reduced amounts of putative phosphoproteins were also detected for the elongation factor 1 beta 2 (spots 8, 9), 60S acidic ribosomal protein P0 (spots 12, 14) and 30S ribosomal protein S1 (CS1; spot 20). For elongation factor 1 beta it has been shown that phosphorylation of a serine residue negatively modulates its ability to exchange nucleotides, which could have important biological consequences for the regulation of translation [18]. Protein P0 is an essential component of the ribosomal stalk and it has been shown that phosphorylation affects expression of specific proteins involved in metabolic processes such as osmo-regulation [21]. The ribosomal protein CS1 is believed to be involved in the process of ribosome binding to mRNA during translation. This chloroplastic protein is the homologue of bacterial ribosomal protein S1 for which it was demonstrated that phosphorylation is involved in the control of translation [23].

In conclusion, we have been able to detect and identify several phosphoprotein candidates with a modified MOAC enrichment strategy and subsequent two-dimensional separation of the proteins. A drawback of the analysis of *C. plantagineum* leaves is the massive occurrence of phosphorylated Rubisco in our enriched fractions that reduces the sensitivity for detection of other less abundant phosphoproteins. Preliminary data with callus material showed that the sensitivity for detection of phosphoproteins is increased by using non-green plant material (F. Facchinelli, unpublished results). Nevertheless, we were able to identify 20 putative phosphoproteins in leaves, which could not be detected during the analysis of total proteins with phosphoprotein-specific stains alone [7]. We cannot exclude identification of some false-positives (non-phosphorylated proteins) with certainty but the approach presented here incorporates two levels of specificity, enrichment and specific staining of phosphoproteins, as demonstrated by identification of CDeT11-24 and CDeT6-19 and other known phosphoproteins. In addition, the phospho-selectivity of the methodology has been validated by phosphatase treatment of the enriched fraction. Most putative phosphoproteins identified during this study were responsive to desiccation in *C. plantagineum*, implying a functional involvement of these proteins in the molecular process of desiccation tolerance. Both the identity and the dynamics of several of these proteins are consistent with our knowledge of processes involved in dehydration-stress both in the *C. plantagineum* and in other plants. The results of this work indicate that MOAC-based phosphoprotein enrichment can effectively enhance the sensitivity of both phosphoprotein visualization and MS analysis. The specificity it confers, combined with 2-D gel separation and phospho-specific staining in the presented framework facilitates rapid, large-scale MS-based analysis of plant phosphoproteomes.

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