Proteome analysis of leaves of the desiccation-tolerant grass, Sporobolus stapfianus, in response to dehydration

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ARTICLE INFO
Article history:
Available online 23 November 2010

Keywords:
Dehydration
Drought stress
Two-dimensional gel
Difference Gel Electrophoresis

ABSTRACT
Drought and its affects on agricultural production is a serious issue facing global efforts to increase food supplies and ensure food security for the growing world population. Understanding how plants respond to dehydration is an important prerequisite for developing strategies for crop improvement in drought tolerance. This has proved to be a difficult task as all of the current research plant models do not tolerate cellular dehydration well and, like all crops, they succumb to the effects of a relatively small water deficit of −4 MPa or less. For these reasons many researchers have started to investigate the usefulness of resurrection plants, plants that can survive extremes of dehydration to the point of desiccation, to provide answers as to how plants tolerate water loss. We have chosen to investigate the leaf proteome response of the desiccation-tolerant grass Sporobolus stapfianus Gandoger to dehydration to a water content that encompasses the initiation of the cellular protection response evident in these plants. We used a combination of two-dimensional Difference Gel Electrophoresis (2D-DIGE) and liquid chromatography–tandem-mass spectrometry to compare the proteomes of young leaves from hydrated plants to those dehydrated to approximately 30% relative water content. High-resolution 2D-DIGE revealed 96 significantly different proteins and 82 of these spots yielded high-quality protein assignments by tandem-mass spectrometry. Inferences from the bioinformatic annotations of these proteins revealed the possible involvement of protein kinase-based signaling cascades and brassinosteroid involvement in the regulation of the cellular protection response. Enzymes of glycolysis, both cytoplasmic and plastidic, as well as five enzymes of the Calvin cycle increased in abundance. However, the RuBisCO large subunit and associated proteins were reduced, indicating a loss of carbon fixation but a continued need to supply the necessary carbon skeletons for the constituents involved in cell protection. Changes in abundance of several proteins that appear to have a function in chromatin structure and function indicate that these structures undergo significant changes as a result of dehydration. These observations give a unique “snap-shot” of the proteome of Sp. stapfianus at a critical point in the passage towards desiccation.

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1. Introduction
An understanding of plant responses to dehydration has important consequences not only for plant biology in general but also more directly for agriculture. Drought is the leading cause of agricultural productivity losses in the US (Boyer, 1982; Kramer and Boyer, 1995) such that in 2000 crop losses due to drought (combination of heat and water stress) were estimated to exceed 4 billion USD (Mittler, 2006). These figures are expected to rise with emerging patterns of global climate change (Tubiello et al., 2007) and will have a disproportionate impact on the poorest sectors of the global economy (Hyman et al., 2008). Thus, improving drought tolerance is a critical priority area for agricultural research agencies. Understanding how plant cells tolerate water loss is a vital prerequisite for developing strategies for improving drought tolerance and maintaining biomass and yields.

All of the current crops and research plant models, e.g., maize and Arabidopsis, are sensitive to water deficits. Maize cannot support photosynthesis at water deficits of −1.8 MPa and leaves start to senesce at −2.0 MPa (Boyer, 1976), and mature Arabidopsis plants experience 95% mortality if leaf water potentials reach
–2.8 MPa (Yang et al., 2005). These plants are thus difficult to use and generally unsuitable as research models for research into mechanisms, genes, and proteins that relate to dehydration tolerance in plants. However, there is a group of plants that are suitable and well capable of tolerating dehydration, the so-called “resurrection” plants, which are rapidly becoming the focus of an expanding interest in dehydration tolerance biology. Resurrection plants exhibit vegetative desiccation tolerance, dehydration to equilibrium with the surrounding atmosphere, generally below 50% relative humidity or –100 MPa and often exceeding –250 MPa (Alpert and Oliver, 2002; Oliver et al., 2010). One of the most agriculturally relevant of the resurrection plant models is the African grass Sporobolus stapfianus Gandoger, which can not only serve directly as a forage species (Sutaryono and Gaff, 1993) but as a Poacea member it offers a more direct link to economically important cereal crops. As a result of these considerations there is a growing body of work that provides useful insights into the morphological, genetic, metabolic, and physiological aspects of desiccation tolerance in S. stapfianus (Gaff et al., 2009; Blomstedt et al., 2010).

Intact leaves of S. stapfianus that dry on the plant can survive equilibration with air at 2% relative humidity (RH), equivalent to a leaf water potential of –540 MPa (Gaff, 1971; Gaff and Ellis, 1974) but generally rarely experience drying atmospheres much below 30–40% RH (–120 to –150 MPa) in their natural habitat (Gaff, 1971, 1977). During dehydration that leads to the desiccated state, S. stapfianus, like all desiccation tolerant angiosperms, initiates a cellular program that protects the cells from the rigors of drying and can maintain them for an extended period of time in the dried state. This dehydration induced cellular protection program involves a complex and highly regulated and coordinated build up of osmolytes, protective compounds, and proteins that includes sugars, such as sucrose, amino acids, antioxidants such as glutathione, and hydrophilin proteins that include members of the late embryogenesis proteins (LEAs) and chaperonins (for review, Gaff et al., 2009). The establishment of a cellular protection mechanism for desiccation tolerance also requires a tightly regulated alteration in gene expression that is induced by water loss from the tissues of the plant and this can be observed in changes in the protein profiles of leaf tissues as they dry (Kuang, 1995; Kuang et al., 1995). Kuang et al. (1995), using 2D-gel electrophoresis, observed that the pattern of protein synthesis was altered at two distinct stages. An early stage that is characterized by the appearance of 10 novel proteins and spans the dehydration process from 85% to 50% relative water content (RWC) and a late stage characterized by the appearance of 15 novel proteins that spans the dehydration process from 35% RWC to dryness at 5% RWC. This later stage appears to be when the leaf cells directly begin to lose water and the protective metabolites start to accumulate at a higher rate and to higher levels (Whittaker et al., 2001, 2004; Martinelli et al., 2007). It is also at this level of dehydration that there is a peak in the endogenous level of the plant hormone abscisic acid (ABA) in Sporobolus leaves (Gaff and Loveys, 1993); ABA has long been associated with the regulation of plant responses to dehydration (Shinozaki and Yamaguchi-Shinozaki, 1996; Bray, 2002). Thus, when the leaves of S. stapfianus reach a RWC of approximately 35% those components or processes critical to the initiation of the establishment of a cellular protective state, capable of withstanding desiccation, are fully engaged.

In this study, we have targeted 30% RWC to investigate alterations in the proteome in the desiccation tolerant young leaves of S. stapfianus during the preliminary stages of the key dehydration stage that occurs between 35% and 5% RWC. To identify those proteins that accumulate or decline in amount during this critical period we have used two-dimensional Difference Gel Electrophoresis (2D-DIGE) to compare the proteomes of young fully hydrated leaves to leaves of plants that have been dehydrated to approximately 30% RWC. Those proteins that exhibited a statistically significant difference between the levels of accumulation in hydrated or dehydrated samples, in either a positive or negative manner, were then isolated from a silver-stained gel and subjected to trypsin digestion for protein identification using liquid chromatography–tandem mass spectrometry (LC–MS/MS). We were able to isolate and identify a total of 82 spots corresponding to 108 proteins (some spots yielded multiple statistically significant protein matches) that have altered levels of accumulation in the dehydrated compared to hydrated leaf tissues: 54 that have elevated levels in the dehydrated leaves and 54 whose levels diminish. The identities of these proteins reveal the full cellular nature of the dehydration response and the large number of cellular processes that are altered as cells prepare for the desiccated state. In particular, the data point to the need for the production of the basic carbon skeletons required for the accumulation of protective compounds. These findings help us to understand how plant cells prepare for dehydration and ultimately how plants survive desiccation of their vegetative tissues.

2. Results and discussion

2.1. Differential protein expression analysis

Extractable soluble leaf proteins were obtained for each of the six samples, three replicates for each of the two treatments, hydrated and dehydrated (31% RWC). From these six protein extracts, protein mixes were prepared in the following sequence for the 2D-DIGE analysis. Gel 1: Cy3 hydrated replicate 1 plus Cy5 dehydrated replicate 1, Gel 2: Cy3 dehydrated replicate 1 plus Cy5 hydrated replicate 1, Gel 3: Cy3 hydrated replicate 2 plus Cy5 dehydrated replicate 2, Gel 4: Cy3 dehydrated replicate 2 plus Cy5 hydrated replicate 2, Gel 5: Cy3 hydrated replicate 3 plus Cy5 dehydrated replicate 3, Gel 6: Cy3 dehydrated replicate 3 plus Cy5 hydrated replicate 3. The dye swap strategy allowed for the determination and exclusion of non-specific labeling or variation in labeling efficiencies between dyes. Each sample contained an equal amount of a Cy2 labeled internal standard protein mix derived from pooling an equal amount of protein from each of the six extracts to allow correction of gel-to-gel variation. The six, high resolution 2D-gels derived from these samples, silver stained after quantification of the dye signals, are shown in Fig. 1 (Gels 1 through 6). With the use of the CyDye labeling method, we were able to detect approximately 900 2D protein spots from S. stapfianus young leaf tissues. It is clear from these images that the large subunit of ribulose 1,5-bisphosphate carboxylase/oxygenase (RuBisCO), represented by spots 842–846 (Fig. 2) is not the predominant protein in the soluble protein extracts from the leaves of S. stapfianus. This is probably a reflection of the fact that only young leaves of the grass were taken for analysis, since these are the only leaves that are desiccation tolerant (Gaff et al., 2009). This was fortunate in that to follow the changes in abundance of proteins in leaf tissues it is sometimes necessary to remove the large subunit of RubisCO prior to running the protein as it is often so abundant that it masks the response of other proteins when equal amounts of proteins are separated for each sample. Also, as is the case in this analysis, if the level of RuBisCO changes significantly it could lead to all proteins appearing to change in abundance in an equal protein loading strategy. In this study, the abundance of large subunit of RubisCO does change significantly in a negative manner with dehydration. However the drop in this protein does not appear to generate an apparent increase in all proteins separated in the 2D analysis as many do not change in abundance at all and those that do change in abundance, do so in both a positive and negative manner. This would not be the case if RuBisCO was the predominant protein in the extracts.
Image and spot analysis enabled the identification of 96 protein spots that exhibit a significant difference in accumulation, either positive or negative, in the 30% RWC leaf samples versus the hydrated leaf samples. These protein spots are highlighted in Fig. 2 along with the numerical designation assigned to them by DeCyder™ Differential Analysis software. Quantification of spot volume, coupled with the use of three replicates and a Cy2 labeled internal standard, enabled us to chose the 96 protein targets on the basis of a significant difference in accumulation between the two treatments at the $p \leq 0.05$ confidence level. The DeCyder™ software depicts this analysis graphically and with a 3D peak representation of relative abundance, as shown for spot 705 (Fig. 3). Quantitative analysis of the differential accumulation patterns of proteins from the two treatments, hydrated and dehydrated to 30% RWC, did not detect polypeptides unique to either treatment. This was unexpected as Kuang et al. (1995) report the appearance of at least 25 novel proteins during the 37–5% RWC dehydration step and the loss of seven in their analysis of Sporobolus leaf proteins during drying. However, Kuang et al., were only able to visually assess the 2D-gels they produced and although silver staining is a sensitive method of detection the use of CyDye labeling offers much greater sensitivity: 0.25 ng (Cy dyes) compared to 1 ng for silver staining (Marouga et al., 2005). With the increase in sensitivity coupled with the use of a scanning CCD camera capable of...
detecting low levels of fluorescence it is reasonable to assume that the DIGE approach used in this study would detect less abundant proteins that would not be seen by silver staining. In a comparative study, this could lead to the conclusion that a protein was missing in one of the treatments and that the appearance of a protein spot in a different treatment would be a novel protein. In the DIGE analysis presented here the novel proteins would simply be assessed as proteins that are significantly enhanced or decreased in abundance (the “lost” proteins).

A comparison of the 2D-DIGE reference map with silver-stained gels (for spot-picking) resulted in one or more assignments for 82 of the 96 differential spots; 9 2D-DIGE spots could not be detected by silver staining due to reduced sensitivity. All 96 differential spots were excised from the silver-stained gels and subjected to trypsin digestion prior to LC–MS/MS. Raw LC–MS/MS files for each spot were excised from the silver-stained gels and subjected to trypsin digestion prior to LC–MS/MS. Raw LC–MS/MS files for each spot were excised from the silver-stained gels and subjected to tryptic digestion. The identification of peptides was conducted using the SEQUEST algorithm. The O. sativa non-redundant database was chosen because in the analysis of S. stapfianus transcriptome (Oliver, unpublished data) it became evident that transcript sequences from this grass shared a higher similarity to the O. sativa genome sequence than for any other member of the grass family in the NCBI databases. After applying statistical thresholds for protein assignment, the 82 protein spots resulted in 108 high-confidence assignments presented in Tables 1 and 2 (and in Supplemental Table 1). One protein spot did not yield peptides that could be reliably used for annotation of the parent protein and eighteen of the protein spots revealed peptides mapping to more than one protein. In the majority of cases more than one peptide sequence were used in the annotation but where only one peptide was available for sequence comparisons to the database, the high Xcorr values coupled with low p-values for the peptide match allowed for high confidence that the annotation was valid. The 54 proteins that have a significant drop in accumulation resulting from the dehydration event are presented in Table 1, and 54 that exhibit a significant elevation in accumulation as a result of dehydration are presented in Table 2.

It is notable that we did not register a LEA protein in the list of proteins that exhibit an increase in accumulation. These proteins have been suggested to accumulate in response to desiccation in S. stapfianus based on the appearance of transcripts that encode them during drying (Blomstedt et al., 1998a,b). The transcripts for several proteins start to accumulate between 59% and 40% peaking at RWCs below 20%, as quantified by northern blot analysis to generate expression level estimates based on a comparison to extractable rRNA (Blomstedt et al., 1998; Neale et al., 2000) including a group 3 LEA and a dehydrin. Similar data have been attained with microarray based analyses of gene expression during drying in S. stapfianus (Oliver, unpublished data). Increases in transcript abundance for LEA proteins, primarily group 3 and group 2 (dehydrins), are a commonly reported response to dehydration for desiccation tolerant plants (Illing et al., 2005; Bartels, 2005; Oliver et al., 2010). The fact that we do not detect an increase in a dehydrin or LEA-like proteins in this proteomic study suggests that transcript abundance levels are not an accurate predictor of the time of appearance of the proteins they encode, especially given the transcript abundance measurements used in the Sporobolus studies so far have only been semi-quantitative. It is also possible that the dehydrins of S. stapfianus that respond to dehydration have pl values beyond the pH range of the 2D gel separation, pH 4–7, used in the analysis. In barley, the dehydrins that respond to water deficits and ABA all have predicted pl values ranging from 9.02 to 10.58, only those that respond to cold have acidic pl values below 7.0 (Choi et al., 1999). The lack of the inclusion of a LEA protein in the protein isolations may also indicate that we have achieved the goal of targeting the level of dehydration that precedes the expected build up of protein protectants that would tend to mask the critical events that initiate or fuel the cellular protection process.

2.2. Proteome response at the onset of the cellular protection program associated with desiccation

The relatively diverse nature of the differential proteins, as illustrated in the detailed analysis of some of the key examples (Fig. 4), is indicative of the full impact on cellular function that dehydration delivers even at the still relatively hydrated level of 30% RWC. This is to be expected given the nature of the stress and as evidenced by many studies that have detailed the structural and physical effects of dehydration, and or desiccation, on the cells of several desiccation tolerant species (Oliver and Bewley, 1984; Platt et al., 1994; Vicré et al., 2004; Farrant et al., 2007). Within this diverse set of differentially expressed proteins, sub-sets of proteins whose functions point to particular metabolic processes that reveal insights into and novel possibilities for an understanding of the mechanisms by which leaf cells of S. stapfianus survive desiccation.

2.2.1. Cell signaling

Targeting of the dehydration stage that appears to precede the dehydration induced build up of cellular protectants offered the possibility that the abundance of proteins involved in stress signaling pathways or the production of regulatory compounds such as plant hormones would be altered sufficiently enough for detection...
using the sensitive 2D-DIGE methodology. The results appear to support this possibility.

Two proteins that are elevated in the 30% RWC dehydrated leaves appear to play a role in kinase-dependent signal transduction pathways in plants (Ferl, 2004). The 14–3–3 GF14-delta (spots 1429 and 1425) is a dimeric protein that has a binding affinity for phosphorylated proteins. In rice 14–3–3 GF14-delta has been demonstrated to bind directly to phosphorylated receptor-like protein kinases (Rohila et al., 2006). Spot 1137 was identified as a TGF-β-receptor interacting protein 1, a cytoplasmic target protein for a transmembrane serine/threonine kinase (TGF-β type-II). In Arabidopsis an ortholog of this protein, TRIP-1, is phosphorylated by the Brassinosteroid-sensitive 1 (BRI-1) protein which is a receptor serine/threonine kinase that is essential for brassinosteroid hormone perception and signal transduction (Ehsan et al., 2005). These two proteins were also shown to interact in vivo.

The increase in abundance of a TGF-β-receptor interacting protein 1 is intriguing because if it has a similar role as the RIP-1 protein of Arabidopsis it would suggest that brassinosteroid hormones may play a role in the establishment of the cellular protection aspect of desiccation tolerance in *S. stapfianus* pectin. This suggestion is strengthened by the increased accumulation of the protein product of the *TASSELSEED2* gene (spot 1869), a 3β[17]-hydroxysteroid dehydrogenase an enzyme that is believed to be involved in the
brassinosteroid biosynthesis pathway (Wu et al., 2007). At this point only the hormone abscisic acid (ABA) has been implicated as a major component in the hormonal control of the acquisition of desiccation tolerance in *S. stapfianus* (Gaff et al., 2009) as in other resurrection species (Oliver et al., 2010). However, in both *Craterostigma plantagineum* and *S. stapfianus* “non-ABA” regulatory pathways have been implicated in the response to desiccation (Gaff et al., 2009; Bartels, 2005). In *C. plantagineum* the “non-ABA” regulatory pathway has been shown to involve both a double stranded 21-bp short interfering RNA (siRNA), the product of the *CDT-1* gene (Hilbricht et al., 2008), and the phospholipid based and calcium linked signal transduction pathways (Bartels, 2005).

In *S. stapfianus*, the involvement of calcium signaling, in at least this part of the dehydration process, is not likely given the reduction in the abundance of a *Sporobolus* calcium-dependent protein kinase (1468). The “non-ABA” regulatory pathway involved in desiccation tolerance in *S. stapfianus* is at present unknown but the possibility that it involves brassinosteroid-directed regulatory pathways is intriguing and not implausible as brassinosteroids have often been linked to abiotic stress responses including the response to water deficits (Jager et al., 2008).

The possibility of the involvement of other hormones in the response of *S. stapfianus* to dehydration was first put forward by Ghasempour et al. (1998). Using suspension cells derived from...
fully expanded leaves, which are not capable of surviving dehydration, Ghasempour et al. (1998) were able to demonstrate a small improvement in dehydration tolerance by exogenous application of a brassinosteroid or methyl jasmonate. A combination of the two hormones did not further improve the ability of the cells to tolerate dehydration (Ghasempour et al., 2001). Although these studies indicate that a brassinosteroid or methyl jasmonate may alter the dehydration tolerance of leaf cells there is no indication, until now, that these hormones are involved in the desiccation tolerance properties of *S. stapfianus* in the intact plant.

At the 30% RWC level of dehydration there appears to be relatively concerted decrease in the level of several Hsp70 proteins, both cytoplasmic (spots 566, 577, 534, 535) and mitochondrial (spot 572). This drop in the Hsp70 chaperonins indicates a general decrease in the import of proteins across membranes, a major function of this class of heat shock proteins (Wang et al., 2004) which would reflect an overall suspension of energy-dependent cellular trafficking one would expect as cells dehydrate. However, it is also possible that the decrease in abundance of the Hsp70 proteins may serve in altering signaling pathways in the cell that would allow for the synthesis of other chaperonins that are more directed towards general protein folding/unfolding processes associated with dehydration. Members of the Hsp70 protein family have been demonstrated to inhibit the synthesis of other heat shock proteins by direct interaction with the heat shock transcription factor (HSF) to block its binding to the heat shock elements (HSE) that control heat shock protein gene activation (Kim and Schoffl, 2002). It is possible that the decrease in abundance of HSP70 proteins we see in *S. stapfianus* at 30% RWC could trigger the start of the synthesis of small heat shock proteins. The small heat shock proteins (below 22 kDa) have been strongly linked to the acquisition of desiccation tolerance in seeds (Wehmeyer and Vierling, 2000; Prieto-Dapena et al., 2008) and in the desiccation tolerant plant *C. plantagineum* (Alamillo et al., 1995). In addition, we have some evidence that an 18 kDa small heat shock protein transcript accumulates during dehydration in *S. stapfianus* leaves (Oliver and Payton, unpublished observation).

### 2.2.2. Energy metabolism

At 30% RWC the young leaves of *S. stapfianus* appear to have a metabolic focus that is aimed at maintaining and conserving energy in the form of ATP and NAD(P)H. There is a significant accumulation of several of the enzymes associated with glycolysis, both the cytoplasmic and plastid pathways, that would generate ATP, both NADH and NADPH and pyruvate in both compartments (Plaxton, 1996). In the chloroplast these enzymes include aldolase (spots 1162, 1173, 1187) and phosphoglycerate kinase (spots 1010, 1045, 1047), and in the cytoplasm an aldolase (spot 1137), phosphoglycerate kinase (spots 954, 974, 1149), glyceraldehyde-3-phosphate dehydrogenase (spots 974, 1163). The production of reducing power and ATP is critical at this stage of the dehydration phase as it is at this point that the large increases in osmolytes (sugars and amino acids) initiates (Martinelli et al., 2007; Whittaker et al., 2007) and this would require a large expenditure of both NAD(P)H and ATP to accomplish. The presumed increase in pyruvate could also serve as a source of the carbon skeletons
required for the increase in these compounds. Of the glycolytic enzymes we have demonstrated to increase in abundance in *S. stapfianus* leaves at 30% RWC only aldolase has been demonstrated to increase in activity as dehydration progresses (Whittaker et al., 2004). Increased synthesis of ATP at 30% RWC is also implied by the increase in abundance of the chloroplastic ATP synthase, both the CF1 alpha subunit (spots 677, 681, 682, 690, 696, 705) and the beta subunit (spots 815, 817, 817C), and the mitochondrial F1-ATPase (spot 815C). *S. stapfianus* retains between 13% and 67% of its ATP in the dried state, presumably for use when the plant rehydrates (Gaff and Ziegler, 1989; Whittaker et al., 2004). Jiang et al. (2007) suggest, from a small proteomic analysis, that the resurrection dicot *Boea hygrometrica* may maintain ATP levels by elevating levels of a vacuolar H+-ATPase and an ABC transporter ATPase. Although the transcript for similar proteins have been seen to increase in *S. stapfianus* leaves during dehydration (Blomstedt et al., 1998; Oliver and Payton, unpublished observations), in this study we do not detect an increase in an ABC transporter ATPase, and we detect a significant decrease in abundance for the chloroplastic ATP synthase.

The increase in the abundance of these enzymes associated with photosynthesis in *S. stapfianus* leaves (Blomstedt et al., 1998a,b; Neale et al., 2000; Le et al., 2007). The possibility that the conformation of chromatins is altered during dehydration to 30% RWC may also be indicated by the increase in abundance of the SNAP2 protein (spot 1951), a helix-case-related protein involved in ATP dependent chromatin remodeling (Owen-Hughes, 2003).

**2.2.3. Photosynthesis**

Photosynthesis steadily declines as the leaves of *S. stapfianus* dry such that at approximately 45% RWC, carbon fixation ceases (Di Blasi et al., 1998). Photosynthesis is one of the more sensitive cellular processes to dehydration and its decline during drying is a common feature of desiccation tolerant plants (Bewley, 1979; Alpert and Oliver, 2002). At 30% RWC carbon fixation has ceased and the levels of the large subunit of Rubisco have declined significantly (spots 842, 843, 844, 846). In addition, the Rubisco subunit binding proteins alpha (spot 647, 648, 656, 897) and beta (spots 392, 656C) have also declined by significant amounts. Whether or not this is the cause (or consequence) for the drop in photosynthesis as a result of drying in *S. stapfianus* is not known but others have suggested that it is the relative sensitivity of the oxygen evolving complex associated with photosystem II that effects the reduction in photosynthesis in desiccation tolerant plants (Jiang et al., 2007; Ingle et al., 2007).

Other Calvin cycle enzymes of the carbon fixation are also affected by dehydration and at 30% RWC there is a notable alteration in the relative abundance of these enzymes. The majority of the enzymes exhibit a significant increase in abundance including chloroplastic phosphoglycerate kinase (spots 1010, 1045, 1047), glyceraldehyde-3-phosphate dehydrogenase (spots 974, 1163), chloroplastic aldolase (spots 1162, 1173, 1187), sedoheptulose 1,7-bisphosphatase (spot 1143), and phosphoribulose kinase (spot 1045). The increase in the abundance of these enzymes associated with dehydration of desiccation tolerant plants is a novel observation and may indicate an important role for a partial Calvin cycle in the establishment of desiccation tolerance, at least for *S. stapfianus*. However, not all of the Calvin cycle enzymes exhibit an increase in abundance. The abundance of the transketolase that catalyzes the regeneration of ribulose 5-phosphate from sedoheptulose is significantly reduced as a result of dehydration to 30% RWC. The susceptibility of the chloroplastic transketolase to dehydration has been noted for the monocotyledonous resurrection plant *Xerophyta viscosa* (Ingle et al., 2007) but in *C. plantagineum* the transcript for transketolase is constitutive, however there are two isoforms of the transketolase that increase in abundance during rehydration of the desiccated plant (Bernacchia et al., 1995).

The role that the Calvin cycle may play in the dehydration response prior to the establishment of the cellular protective state is enigmatic at this time. It is also difficult to determine if the increase in abundance of some of these enzymes, those that have a dual role in glycolysis (as the plant shifts from energy-generating (autotrophic) to energy-storing (heterotrophic)) is indicative that the Calvin cycle is indeed still active at this level of dehydration. However, it is a distinct possibility that Calvin cycle activity is required in order to supply carbon skeletons, perhaps as 3-phosphoglycerate, for the large increase in sugars and amino acids associated with entry into the dried state (Martinelli et al., 2007; Whittaker et al., 2007). This would occur in the absence of carbon fixation so presumably stored complex carbohydrates, such as starch, would supply the Calvin cycle with the necessary intermediates in some fashion. This is purely speculative at this point but does offer a testable hypothesis.

**2.2.4. DNA related processes**

Several protein spots were annotated as Epstein–Barr virus (EBNA-1 like proteins, spots 1782 and 2110 increase in abundance and spots 1228, 641C, 944, 967, 1243, 1463 decrease in abundance. Epstein–Barr virus (EBNA-1 like proteins are involved in the replication and transcriptional activation of the Epstein–Barr virus in mammalian cells (Leight and Sugden, 2000). The latter aspect is intriguing, as it has been clearly demonstrated that there is selective transcriptional activity during dehydration of *S. stapfianus* leaves (Blomstedt et al., 1998a,b; Neale et al., 2000; Le et al., 2007). The possibility that the conformation of chromatins is altered during dehydration to 30% RWC may also be indicated by the increase in abundance of the SNF2P protein (spot 1951), a helix-case-related protein involved in ATP dependent chromatin remodeling (Owen-Hughes, 2003).

**2.2.5. Oxidative metabolism**

The proteome analysis is marked by both the absence of and increase in abundance of proteins associated with oxidative metabolism, in particular those enzymes involved in the protection of cells from reactive oxygen species (ROS). Increases in the activity of enzymes and compounds that reduce the effects of ROS are a common feature of metabolism of resurrection species as they dry, including *S. stapfianus* (Kranner and Birtic, 2005; Farrant et al., 2007; France et al., 2007). The absence of a change in an antioxidant associated protein is even more surprising as we do observe a decrease in abundance of a superoxide dismutase (spot 1959) and a cytosolic monohydoacorbate reductase (spots 992, 1026), both of which are involved in cellular protection strategies for limiting damage from ROS. However, it is clear from earlier work that *S. stapfianus* relies more heavily on the glutathione and tocopherol as antioxidants than it does on ascorbate (Navari-Izzo et al., 1997; Oliver et al., unpublished data). Thus, it appears that at 30% RWC dehydration is probably insufficient to trigger a need for an increase in abundance of the enzymes involved in glutathione or tocopherol biosynthesis or there is sufficient protein to accommodate a need for these antioxidants.

**2.2.6. Other cellular processes**

The protein analysis revealed the responses of other proteins to dehydration with regards to their relative abundance that do not appear to be easily associated with a group of proteins of similar or collective function. These include two proteins that are reduced in abundance by dehydration and that have disparate roles in the structure of plant cells. These are actin (spot 505), a protein...
involved in the cytoskeleton and a plastid division ftsZ1 protein (spot 1102) that plays a role in chloroplast biogenesis and division. A protein involved in protein degradation, the 20S proteosome alpha 6 subunit (spot 1323) is reduced in abundance, which may indicate an inhibition of this process by dehydration. We also see a reduction in the abundance of malate dehydrogenase (spot 1099) that may inhibit the movement of carbon from pyruvate into respiration via oxaloacetate synthesis. It is also intriguing to note that there is a reduction in the abundance of an ethylene-responsive mitochondrial methionine synthase (spots 508, 519) as methionine is one of the more difficult amino acids to detect in mitochondrial methionine synthase (spots 508, 519) as methionine is one of the more difficult amino acids to detect in S. stapfianus (Martinelli et al., 2007; Oliver et al., unpublished data). This may signify that the synthesis of the sulfur amino acids is sensitive to dehydration.

3. Conclusions

The sensitivity of 2D-DIGE, coupled with accurate protein identification using LC–MS/MS has allowed for a detailed “snap-shot” of the dynamic proteome of young leaves of S. stapfianus at a critical dehydration stage, 30% RWC, leading to desiccation (Fig. 5). The 30% RWC dehydration level is important as it is at this point that the leaf cells begin to amass the cellular constituents necessary to protect the cells from the rigors of desiccation (as derived from Whittaker et al. (2004), Martinelli et al. (2007) and Oliver et al. (unpublished data). Proteome analysis revealed the possibility that protein kinase-based signaling cascades and the brassinos-teroid hormones may play a role in the establishment of the cellular protection process. Proteins involved in glycolysis, cytoplasmic and plastid, and the Calvin cycle were altered in abundance in such a way as to suggest that both pathways are involved in supplying carbon skeletons to the metabolic processes involved in the accumulation of sugars and amino acids associated with the final stages in the preparation for desiccation. To drive these processes it is clear that the leaf cells are focused on the synthesis and maintenance of NAD(P)H and ATP as evidenced by the increase in abundance of ATPase subunits and the enzymes involved in glycolysis. Changes in proteins associated with chromatin structure and function also indicate that the nucleus undergoes important changes during dehydration which may involve some remodeling of chromatin as indicated by an increase in abundance of the SNF2P protein. These results indicate the complete alteration of cellular function during dehydration and point towards some important processes involved in the establishment of the cellular protection process and its regulation in S. stapfianus. These results give us a unique insight into the process of cellular protection as it relates to the complex phenotype of desiccation tolerance in plants.

4. Experimental

4.1. Plant material

Young leaves, the innermost leaves of individual tillers (Martinelli et al., 2007), of 12 mature pre-flowering S. stapfianus

Fig. 5. Summary cell diagram depicting the differential proteome of Sporobolus stapfianus leaves in response to dehydration to 30% RWC. Each differential protein (denoted by spot number) annotated and verified as exhibiting a statistically significant change in abundance in the proteome analysis is positioned within a depiction of the major processes in the cellular milieu. Yellow squares signify proteins whose abundance is significantly reduced at 30% RWC, blue squares signify proteins whose abundance is significantly elevated at 30% RWC.
Gandoger (original provenance: Verena, Transvaal, South Africa) plants (approximately 6 months post germination), grown in 1-gallon pots under greenhouse conditions (16-h light and day/night temperatures of 28 °C/19 °C), were sampled (hydrated) as three replicates with each replicate consisting of bulked tissue from four plants. Irrigation was suspended and the relative water content (RWC) was monitored during dehydration. Full dehydration (5% RWC) was achieved within 15 days under these conditions. Once the plants had reached approximately 30% RWC (dehydrated) three replicate leaf samples, each dehydrated sample paired with the hydrated sample from the same four plant replicate, were taken and frozen in liquid nitrogen. The mean dehydration level for the three replicates, measured in duplicate samples from the same plants, was 31% RWC representing a dehydration level for the three replicates, measured in duplicate.

4.2. Protein isolation and Difference Gel Electrophoresis

Leaf protein extracts were obtained according to the phenol-based method proposed by Hurkman and Tanaka (1986) and modified by Mooney and Thelen (2004). After precipitation, protein pellet was reconstituted in lysis buffer (30 mM Tris–HCl, pH 8.5, 7 M urea, 2 M thiourea, and 4% (w/v) CHAPS) with gentle mixing for 30 min at room temperature, followed by centrifugation for 15 min at 14,000 rpm to remove insoluble material. Protein was then quantified using the Coomassie dye binding assay (BioRad, Hercules, CA) assay against standard curve of BSA. For Difference Gel Electrophoresis (DIGE) analysis, 50 μg of protein was taken and adjusted to final volume of 10 μL with lysis buffer. One micro-liter of CyDye (200 pmol) was added and incubated on ice for 30 min in the dark. Samples from dehydrated plants were labeled with Cy3 and those from hydrated were labeled with Cy5. The labeling reaction was terminated with 1 μL of 10 mM lysine on ice for an additional 10 min in the dark. Prior to isoelectric focusing (IEF), labeled samples were mixed with equal volume of 2x sample buffer (8 M urea, 130 mM DTT, and 4% (w/v) CHAPS), incubated 10 min on ice, mixed with 2.25 μL of IEF buffer (GE Healthcare, Piscataway, NJ), and adjusted to total volume of 450 μL with sample buffer. IEF was carried out in Immobiline Dry Strip gels pH 4–7 (24 cm) for a total of 80,000 Vh at 15 °C. After IEF, gel strips were equilibrated for 15 min in 8 M urea, 100 mM Tris–HCl (pH 6.8), 30% (w/v) glycero1, 1% (w/v) SDS and 2.0% (w/v) DTT to reduce cysteine residues and 15 min in 8 M urea, 100 mM Tris–HCl (pH 6.8), 30% glycero1, 1% SDS and 2.5% (w/v) iodoacetamide for carboxamidomethylatino of proteins. The equilibrated strips were then placed on a top of 1.0 mm thick SDS–polyacrylamide gel (12%) on low fluorescent glass plates and separation was performed on an EttanMDALT 12 apparatus at 2 W/gel until bromophenol blue front reached the bottom of the gel.

4.3. DIGE image analysis

DIGE gels were scanned using an Ettan DIGE Imager™ (GE Healthcare) using standard settings (exposure times set for Cy2, Cy3, Cy5 were 0.8, 0.3, 0.5 s, respectively). All gels were scanned at 100 μm resolution. Images were cropped prior to DeCyder™ analysis using ImageQuanttl (v2005, GE Healthcare). The images were loaded to DeCyder with the image loader and were processed using batch processor. The resulting images were analyzed using DeCyder™ Differential Analysis software version 6.5 (GE Healthcare) designed specifically for 2-D DIGE image analysis. The DIA (differential in-gel analysis) module of this software was used, allowing co-detection and differential quantification of protein spots from Cy2 pooled standard and Cy3/Cy5 labeled sample images. Biological variation analysis module was also utilized to analyze gel to gel matching of standard spot maps and provide statistical analysis of protein abundance changes between the samples. To calculate abundance changes on each matched spot pair, one-way ANOVA and paired student’s t-test was performed. Spots showing significant difference (p < 0.05) were assigned as protein spots of interest.

4.4. Silver staining and in-gel tryptic digestion

Prior to protein identification, DIGE gels were silver stained according to Shevchenko et al. (1996). Then, proteins spots of interest were manually excised and destained in a solution containing 15 mM K3 (FeCN)6 and 50 mM Na2S2O3 for 10 min. After two washing steps in 18 MΩ water for 15 min each, destained spots were incubated in 25 mM ammonium bicarbonate, 50% acetonitrile (v/v) twice for 30 min. Gel pieces were then dried for 10 min under vacuum and 10-20 μL of trypsin (Promega, Madison, WI) containing 25 mM ammonium bicarbonate were added until all gel pieces were completely covered. Digestion was performed overnight at 37 °C and the peptides were eluted by vortexing in 50 μL acetonitrile, 0.5% (v/v) trifluoroacetic acid.

4.5. LC–MS/MS analyses

Dried peptides were reconstituted in 40 μL of 0.1% (v/v) formic acid (FA), from which 15 μL were used for mass spectrometry analysis on an LTQ ProteomeX linear ion trap LC–MS/MS (ThermoFisher, San Jose, CA). Briefly, on-line capillary LC consisted of two polymeric PSDVB-based peptide traps (2 μg capacity each; MicroBiosource) and a fused-silica capillary emitter packed in-house with magic C18 matrix (magic C18, 5 μm, 100 Å, 150 μm ID × 10.5 cm, Michrom Biosources, Inc., Auburn, CA). The method alternated between loading/equilibration and elution using the two peptide traps to reduce the time required for sample analysis on LC–MS/MS. Sample was loaded onto peptide traps for concentration and desalting prior to final separation by C18 capillary column using an acetonitrile gradient (0–95%) solvent B in solvent A for a duration of 35 min, Solvent A = 0.1% (v/v) FA in water; Solvent B = 100% acetonitrile containing 0.1% FA). The peptide trap and C18 capillary column were then re-set for 2 min and re-equilibrated for 13 min with 100% of solution A before the sample already loaded onto the second trap was eluted. The mass/charge (m/z) ratios of eluted peptides and fragmented ions from fused-silica capillary emitter were analyzed in the data-dependent positive acquisition mode on LC–MS/MS. Following each full scan (400–2000 m/z), two data-dependent triggered MS/MS scans [isolation width 1.5 amu, 35% normalized collision energy, minimum signal threshold 500 counts, dynamic exclusion (repeat count, 2; repeat duration, 30 s; exclusion duration 180 s)] for the two most intense parent ions were acquired. The heated fused-silica capillary emitter was held at ion sprays 2.5 kV and a flow rate 250 nL/min.

4.6. Database search

The National Center for Biotechnology Information (NCBI; ftp://ftp.ncbi.nih.gov/blast/ desired O. sativa non-redundant database (March 04, 2007; total entries 132294) was used for querying all data. For false discovery rate determination, the rice database was randomized and concatenated to the forward database using “DecoyDBCreator” available at www.p3db.org. The FASTA database utilities and index of the BioWorks 3.2SR1 software allowed us to index the database against trypsin enzyme. Cysteine (carboxamidomethylation; +57 Da) and methionine (oxidation; +16 Da) were selected as static and differential modifications, respectively. Acquired data were searched against the indexed database using
the SEQUEST algorithm as part of the BioWorks 3.2SR1 software suite. The search parameters for this database were set as follows: enzyme: trypsin; number of internal cleavage sites: 2; mass range: 400–4000; threshold: 500; minimum ion count: 35; and peptide mass tolerance: 1.5. Matching peptides were filtered for non-redundant and non-overlapping peptides, as well as correlation scores (XCorr) at least 1.5, 2.0, and 2.5 for +1, +2, and +3 charged ions, respectively, and peptide probability (BioWorks 3.2) higher than 0.05 were required for confident identification. The BioWorks peptide probability score is based on the probability that the peptide is a random match to the spectral data. This probability algo-
rithm uses sequences identified by SEQUEST and then calculates the probability values by comparing these sequences against the spectral data in the .dta file using a proprietary algorithm. For some protein spots more than one protein met these assignment thresh-
olds. All proteins meeting these assignment criteria are included and represented equally in discussions about differential expression.

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