Proteomic analysis of leaf proteins during dehydration of the resurrection plant *Xerophyta viscosa*

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**ABSTRACT**

The desiccation-tolerant phenotype of angiosperm resurrection plants is thought to rely on the induction of protective mechanisms that maintain cellular integrity during water loss. Two-dimensional (2D) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis of the *Xerophyta viscosa* Baker proteome was carried out during dehydration to identify proteins that may play a role in such mechanisms. Quantitative analysis revealed a greater number of changes in protein expression levels at 35% than at 65% relative water content (RWC) compared to fully hydrated plants, and 17 dehydration-responsive proteins were identified by tandem mass spectrometry (MS). Proteins showing increased abundance during drying included an RNA-binding protein, plastid FisH protease, glycolytic enzymes and antioxidants. A number of photosynthetic proteins declined sharply in abundance in *X. viscosa* at RWC below 65%, including four components of photosystem II (PSII), and Western blot analysis confirmed that two of these (psbP and Lhcb2) were not detectable at 30% RWC. These data confirm that poikilo- chlorophyllous species in *X. viscosa* involves the breakdown of photosynthetic proteins during dismantling of the thylakoid membranes. In contrast, levels of these photosynthetic proteins were largely maintained during dehydration in the homoiochlorophyllous species *Craterostigma plantagineum* Hochst., which does not dismantle thylakoid membranes on drying.

**Key-words:** desiccation tolerance; photosynthesis; poikilo-chlorophyll; proteome.

**INTRODUCTION**

Desiccation tolerance describes the ability of an organism to survive drying to equilibrium with the relative humidity of air, reviving when water again becomes available (Illing et al. 2005; Alpert 2006). The majority of higher plants produce desiccation-tolerant seeds and pollen, but it is a rare phenomenon in vegetative tissues (Vicré, Farrant & Driouich 2004a; Illing et al. 2005). However, a small group of taxonomically diverse plants known as resurrection plants are able to tolerate severe (> 95%) water loss from their vegetative tissues for prolonged periods, and recover full physiological activity upon rehydration (Gaff 1971; Ingram & Bartels 1996; Vicré et al. 2004a). *Xerophyta viscosa* (Velloziaceae) is one such species, and is able to survive drying to 5% relative water content (RWC) and resumes full physiological activity within 80 h of rewatering (Sherwin & Farrant 1998). The origin of desiccation tolerance in vegetative tissues is polyphyletic (Oliver, Tuba & Mishler 2000) and it has been suggested that it arose via the activation of seed-specific protection mechanisms in vegetative tissue (Oliver et al. 2000; Illing et al. 2005). The observation that homologs of two seed-specific genes in *Arabidopsis thaliana*, LEA6 and a 1-Cys peroxiredoxin are expressed in vegetative tissues of the resurrection plants *Xerophyta humilis* and *Xerophyta viscosa*, respectively, at RWC < 65% (Mowla et al. 2002; Illing et al. 2005) lends some support to this hypothesis.

Desiccation tolerance in plants is thought to rely on two strategies: the protection of cellular integrity and the repair of dehydration- or rehydration-induced damage (Cooper & Farrant 2002). Lower-order plants such as the moss *Tortula ruralis* which desiccates rapidly (in as little as 1 h) rely on constitutive damage repair mechanisms during rehydration, and are classified as full desiccation-tolerant plants as tolerance is unaffected by the rate of drying (Oliver & Bewley 1997). In contrast, higher-order plants rely more on the induction of mechanisms to protect cellular integrity during water loss, and are classified as modified desiccation-tolerant plants, as a certain amount of time is required for the induction of tolerance (Oliver & Bewley 1997). To tolerate desiccation, resurrection plants must be able to combat three major subcellular stresses; mechanical stress, e.g. shrinkage of the plasma membrane away from the cell wall and subsequent cytorhesis, oxidative stress caused by the production of reactive oxygen species (ROS) and damage to macromolecules such as DNA and proteins (Farrant 2000; Alpert 2006). Mechanisms employed by plants to prevent mechanical damage include changes in cell wall composition to allow folding (Vicré et al. 2004b; Moore et al. 2006), cytoplasmic packaging with vacuoles in which water is replaced with compatible solutes (Farrant 2000; Vander Willigen et al. 2004) and changes in membrane
lipid composition to increase fluidity (Moore et al. 2005). The inability to utilize light energy in photosynthesis during water stress can lead to the elevated production of ROS (Smirnoff 1993). Resurrection plants carry out a controlled shutdown of photosynthesis early on during drying (at RWC > 50%) to avoid the generation of ROS, and also employ a range of antioxidant systems to limit damage (Sherwin & Farrant 1998; Farrant 2000). Poikilochlorophyllous species such as X. viscosa dismantle thylakoid membranes and degrade chlorophyll, while homoiochlorophyllous species retain the majority of their chlorophyll and rely on mechanisms such as anthocyanin production and leaf folding to prevent chlorophyll–light interactions (Sherwin & Farrant 1998). Strategies to protect the integrity of macromolecules include the synthesis of compatible solutes such as sucrose and trehalose (Müller, Boller & Wiemken 1995; Ingram et al. 1997; Whittaker et al. 2001) and the synthesis of late embryogenesis abundant (LEAs) and chaperone proteins (Bernacchia & Furini 2004).

To identify genes that may underlie such desiccation tolerance mechanisms, a number of studies have examined changes that occur during dehydration and/or rehydration in the transcriptome of several resurrection plants. These include X. humilis (Collett et al. 2003, 2004), Craterostigma plantagineum (Bartels et al. 1990; Bernacchia, Salamini & Bartels 1996; (Bockel, Salamini & Bartels 1998) and Sporobolus stapfianus (Bloomstedt et al. 1998; Neale et al. 2000) and the moss T. ruralis (Wood, Duff & Oliver 1999; Oliver et al. 2004). However, there has been little corresponding study of the proteome of any resurrection plant. De novo protein synthesis during rehydration has previously been studied in both T. ruralis and C. plantagineum using two-dimensional (2D) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) (Oliver & Bewley 1984; Bernacchia et al. 1996), but the identity of the newly synthesized proteins was not determined. In addition, several dehydration-induced proteins were recently shown to be transiently phosphorylated in C. plantagineum during a dehydration using 2D SDS–PAGE coupled with a phosphoprotein specific stain (Röhrig et al. 2006). Mass spectrometry (MS) analysis in conjunction with the increasing amount of protein sequence data [over 250 000 sequences in the National Center for Biotechnology Information (NCBI) Viridiplanta database] now offers the opportunity to identify homologous proteins in non-model plant species such as X. viscosa. A major advantage of proteomics over transcriptomics is that it focuses on the actively translated portion of the genome. The importance of post-transcriptional regulation has been demonstrated by several studies revealing a weak or moderate correlation between mRNA and protein levels, except for very abundant proteins in yeast (Gygi et al. 1999; Ideker et al. 2001). A further consideration in the case of resurrection plants is that mRNAs appear to be stored during drying and only translated during rehydration (Duce et al. 1998; Collett et al. 2003), thus there may be significant differences between mRNA and protein levels during dehydration. A study of the desert legume Retama raetam provides support for this hypothesis. This species shuts down photosynthesis in its upper stems during the dry season (Mittler et al. 2001). However, while protein levels of psbA (the D1 polypeptide) and large and small subunits of ribulose 1,5-bisphosphate carboxylase/oxygenase (Rubisco) are much reduced in the upper stems compared to the lower stems, there is no corresponding difference in mRNA levels. Protein levels recovered within 6–24 h after watering suggesting that this species may also store mRNAs during dry periods (Mittler et al. 2001). The aim of the present work was thus to investigate changes in the proteome that occur in X. viscosa during dehydration to identify proteins that may play a role in desiccation tolerance mechanisms in this species.

MATERIALS AND METHODS

Plant material and culture

Xerophyta viscosa Baker plants were collected from Cathedral Peak, Kwa-Zulu Natal, and C. plantagineum Hochst plants from the Pilanesberg Nature Reserve, Northwest Province, South Africa. The plants were potted and grown under glasshouse conditions as described by Sherwin & Farrant (1996). Prior to this study, the plants were transferred to a controlled environment room with a photosynthetic flux of 350 μmol m−2 s−1 under a 16 h light/8 h dark cycle at 25 °C and allowed to acclimate for 2 weeks before dehydration stress was imposed.

Dehydration treatment and determination of RWC

Three independent biological replicate groups of X. viscosa plants, each consisting of three individual plants that were dried down by withholding water. Tissue samples were harvested from these plants prior to dehydration (100% RWC) and at two points during dehydration (65 and 35% RWC). All samples were collected at noon to avoid apparent differences in protein abundance caused by circadian or light–dark regulation. Fresh and dry (after oven drying at 70 °C for 48 h) biomass was determined gravimetrically for each leaf sample harvested (10 per plant), and absolute water content (AWC) calculated using the following formula: (fresh biomass – dry biomass)/dry biomass. RWC was calculated using the following formula: (AWC × 100)/AWC at full turgor (determined by bagging plants overnight after watering).

Isolation of total protein

Total protein for one-dimensional (1D) and 2D SDS–PAGE was isolated from 1 g of leaf tissue as previously described (Ingle, Smith & Sweetlove 2005).

Two dimensional gel electrophoresis

Total soluble protein (800 μg) was separated by isoelectric focusing on 18-cm-long immobilized non-linear pH 3.0–10.0
Graduation was performed at a collage energy of 1 kV, and a collision acquisition list for the MS/MS spectra. Peptide fragmentation of spots that met the threshold criteria were included in the positive reflector mode and were generated by accumulating 192-well MALDI target plate. MS analyses were performed on a 4700 Proteomics Analyser MALDI time-of-flight (TOF)/TOF system (Applied Biosystems, Framingham, MA, USA). The instrument was equipped with a Nd : YAG laser operating at 200 Hz. All mass spectra were recorded in positive reflector mode and were generated by accumulating data from 5000 laser pulses. Up to 5 spectral peaks per spot that met the threshold criteria were included in the acquisition list for the MS/MS spectra. Peptide fragmentation was performed at collision energy of 1 kV, and a collision gas pressure of approximately 1.5 × 10⁻⁶ Torr. During MS/MS data acquisition, a minimum of 3000 laser pulses and a maximum of 6000 laser pulses were allowed for each spectrum. Global Proteomics Server (GPS) Explorer Software (Applied Biosystems) was used for submitting MS data for database searching with the Mascot search engine (www.matrix-science.com). All searches were performed against the Viridiplanta NCBI database with the following settings: maximum number of missed cleavages, 1; peptide tolerance, ± 50 ppm; MS/MS tolerance, ± 0.2 Da. Carbamoylation of cysteine was set as fixed modification and oxidation of methionine were selected as variable modification. The criteria for considering a protein to be successfully identified were: (1) at least two peptides were matched to the sequence of the homolog in the NCBI database and (2) the overall protein MOWSE score was significant at P < 0.05. The molecular weight/isoelectric point (MW/pl) predicted for the homologs in the NCBI database was in good agreement with that estimated from the SDS–PAGE gels.

## Western analysis

Total protein (30 μg) was separated on 12% SDS–PAGE gels and transferred to nitrocellulose membrane overnight. Equal loading of protein was confirmed by Ponceau staining of the membrane. Membranes were blocked for 2 h or overnight at 4 °C in 1 × Tris-buffered saline Tween-20 (TBST) [except psbP where 1 × phosphate-buffered saline Tween-20 (PBST) was used] containing 10% w/v non-fat milk powder. Primary antibodies were diluted in 1 × TBST [2-Cys peroxiredoxin (2-Cys-Prx) 1:2000, FtsH 1:1000, HCF136 1:1000, Lhcb2 1:2000 and psbS 1:2000] or 1 × PBST (psbP 1:300) containing 10% w/v non-fat milk powder. Blots were incubated with primary antibody for 1.5 h, followed by 3 × 5 min washes in 1 × TBST or 1 × PBST (psbP) and incubation with secondary antibody (IgG HRP, 1:5000 dilution) for 1 h. Bands were detected using chemiluminescence, and sized by comparison to a prestained protein ladder.

## Measurement of ascorbate content

The total ascorbate content was determined from 0.15 g of tissue according to the method of Wang, Jiao & Faust (1991), except that 0.35% (w/v) iodoacetamide rather than N-ethylmaleimide was used to alkylate excess dithiothreitol (DTT) after reduction of dehydroascorbate to ascorbate.

## RESULTS AND DISCUSSION

Leaf samples were taken from X. viscosa plants at full turgor (100% RWC) and during drying at 65 and 35% RWC, and total soluble protein extracted and analysed by 2D SDS–PAGE. These two RWCs represent two distinct phases of the dehydration process where induction of putative ‘early’ and ‘late’ protection mechanisms is initiated (Mundree & Farrant 2000; Illing et al. 2005). At 65% RWC, Xerophyta species are still green and photosynthetically active although at a reduced rate (Farrant 2000), while at 35% RWC, photosynthesis has ceased and chlorophyll has
been degraded. Of the approximately 430 protein spots detected on the gels, quantitative analysis using PDQuest identified 20 that were significantly increased in abundance (paired Student's *t*-test, *P* < 0.05) during drying, while 13 showed a significant decrease compared to levels at 100% RWC. In addition, Boolean analysis identified 21 protein spots present in all three biological replicates at 65% and/or 35% RWC (referred to here as 'de novo' proteins) that could not be detected in soluble protein extracts from fully hydrated plants (Fig. 1). These may represent proteins that are only produced, or are only present at detectable levels during dehydration. However, the possibility that post-translational modification, for example, phosphorylation has altered the MW/pI of these proteins during dehydration cannot be ruled out. In all three classes (increased, decreased and 'de novo' proteins), there was a greater number of changes at 35% RWC, particularly of proteins showing decreased expression. The 54 proteins could be divided into three groups based on their expression patterns. Eight proteins were early-dehydration responsive (significant change only at 65% RWC), suggesting a possible role during the initial stages of drying, while 24 were late-dehydration responsive (significant change only at 35% RWC), perhaps indicating a role in the latter stages of drying. The remaining 22 proteins were full-dehydration responsive, with altered levels of expression at both 65 and 35% RWC (Fig. 1).

These 54 protein spots were cut from the gels for MS identification. To determine whether peptide mass fingerprinting (PMF) was a viable option for protein identification, eight of these spots, plus spots corresponding to the large subunit of Rubisco and the β-subunit of the F1 ATPase (based on size and relative position on the gels), were subjected to MALDI-TOF analysis. These two highly conserved proteins were successfully identified by PMF; however, a significant score for protein ID was only obtained for one of the eight experimental samples after searching of the NCBI databases with the Mascot search engine. This protein corresponded to the 33 kDa oxygen-evolving complex of photosystem II (PSII) (psbO) and showed reduced expression in *X. viscosa* at 35% RWC. To achieve a higher identification rate, trypsin digest fragments were subjected to MALDI-TOF/TOF analysis. This resulted in a significant score for protein ID being obtained for a further 16 of the remaining 53 samples (an ID rate of 30%). The putative MS/MS identifications of these proteins are shown in Table 1, amino acid sequences of the matched peptides in Table 2 and representative gels at 100, 65 and 35% RWC in Fig. 2. To validate the protein expression data, antibodies were obtained to four of the proteins identified by MS/MS: psbP, HCF136 (down-regulated), 2-Cys-Prx and FtsH (up-regulated), and Western blotting was carried out. The expression patterns determined by immunodetection were in good agreement with those determined by 2D SDS–PAGE (Fig. 3).

Changes in expression of photosynthetic proteins during drying in *X. viscosa*

The abundance of five chloroplast proteins involved in photosynthesis was significantly decreased at 35% RWC: psbO and psbP, two components of the luminal oxygen evolving complex (OEC) of PSII, the PSII stability factor HCF136, the α-subunit of the F-ATPase and the Calvin cycle enzyme transketolase (Table 1). Of these, only HCF136 was also significantly lower at 65% RWC, which correlates with the reported decline of the chlorophyll fluorescence parameter ratio of variable/maximum fluorescence (*Fv*/Fm) indicating a reduced rate of electron transport through PSII (Farrant 2000; Mundree & Farrant 2000). HCF136 is a thylakoid luminal protein required for PSII stability and assembly; Arabidopsis hcf136 mutants lack PSII activity and do not accumulate PSII subunits (Meurer et al. 1998). Reduced levels of HCF136 protein in *X. viscosa* during drying may be one component of the shutdown of photosynthesis if this served to reduce the rate of stable PSII formation. It has recently been reported that the OEC may be the primary site of photoinhibition of PSII, with oxidative damage to the D1 polypeptide occurring secondarily once the OEC is unable to supply electrons to reduce oxidized P680+ (Hakala et al. 2005; Ohnishi et al. 2005). Thus, the decrease in psbO and psbP protein levels in *X. viscosa* during drying could represent either down-regulation or degradation caused by damage, or both. The observation that psbP and psbO mRNA levels also decline in the related species *Xerophyta humilis* at RWCs below 50% suggests that at least some of the observed decrease in protein levels is likely caused by down-regulation of gene expression (Collett et al. 2003, 2004). Finally, a twofold decrease in levels of glutamate:glyoxylate aminotransferase I (GGT1) was
<table>
<thead>
<tr>
<th>Putative identification</th>
<th>Species</th>
<th>Accession number</th>
<th>Measured MW/pI</th>
<th>Predicted MW/pI</th>
<th>Response</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Decreased abundance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 Transketolase</td>
<td>Solanum tuberosum</td>
<td>CA90427</td>
<td>78/5.4</td>
<td>80/5.9</td>
<td>65% 35%</td>
</tr>
<tr>
<td>2 F-ATPase (α subunit)</td>
<td>Ranunculus macranthus</td>
<td>AAZ03784</td>
<td>59/5.2</td>
<td>55/5.3</td>
<td>nsd 0.41 ± 0.13</td>
</tr>
<tr>
<td>3 Gluconolactonase</td>
<td>Arabidopsis thaliana</td>
<td>AAN62332</td>
<td>52/6.5</td>
<td>54/6.5</td>
<td>0.47 ± 0.06</td>
</tr>
<tr>
<td>4 PSII stability factor</td>
<td>Oryza sativa</td>
<td>BAP06215</td>
<td>40/5.2</td>
<td>45/9.0</td>
<td>0.44 ± 0.1 0.58 ± 0.15</td>
</tr>
<tr>
<td>5 Ascorbate peroxidase</td>
<td>A. thaliana</td>
<td>CA69925</td>
<td>32/5.0</td>
<td>28/5.9</td>
<td>nsd 0.11 ± 0.01</td>
</tr>
<tr>
<td>6 PsbO</td>
<td>A. thaliana</td>
<td>CA36675</td>
<td>30/5.6</td>
<td>35/5.7</td>
<td>nsd 0.34 ± 0.06</td>
</tr>
<tr>
<td>7 PsbP</td>
<td>Xerophyta humilis</td>
<td>AAN77240</td>
<td>26/7.0</td>
<td>26/6.9</td>
<td>nsd 0.14 ± 0.04</td>
</tr>
<tr>
<td><strong>Increased abundance</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8 Chloroplast FtsH protease</td>
<td>A. thaliana</td>
<td>CA68141</td>
<td>72/5.2</td>
<td>76/5.8</td>
<td>3.14 ± 0.77 3.30 ± 1.50</td>
</tr>
<tr>
<td>9 GDP-mannose-3',5'-epimerase</td>
<td>O. sativa</td>
<td>Q2RIV8</td>
<td>47/6.2</td>
<td>43/5.8</td>
<td>2.18 ± 0.30 2.67 ± 0.52</td>
</tr>
<tr>
<td>10 Alcohol dehydrogenase</td>
<td>Citrus × paradisi</td>
<td>AAY86033</td>
<td>46/6.4</td>
<td>42/5.8</td>
<td>nsd 2.22 ± 0.16</td>
</tr>
<tr>
<td>11 Protein phosphatase type 2C</td>
<td>A. thaliana</td>
<td>CAB79642</td>
<td>33/6.0</td>
<td>30/6.4</td>
<td>4.20 ± 0.36 3.92 ± 0.07</td>
</tr>
<tr>
<td>12 VDAC1.1</td>
<td>Lotus corniculatus</td>
<td>AAQ07019</td>
<td>28/6.5</td>
<td>30/8.6</td>
<td>nsd 1.81 ± 0.20</td>
</tr>
<tr>
<td>13 2-Cys peroxiredoxin</td>
<td>O. sativa</td>
<td>CAJ01693</td>
<td>26/5.0</td>
<td>28/5.7</td>
<td>nsd 1.72 ± 0.52</td>
</tr>
<tr>
<td><strong>De novo proteins</strong></td>
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<td></td>
</tr>
<tr>
<td>14 dnaK-type molecular chaperone</td>
<td>O. sativa</td>
<td>NP_001048724</td>
<td>75/5.3</td>
<td>73/5.5</td>
<td>Present Present</td>
</tr>
<tr>
<td>15 RNA-binding protein</td>
<td>Daucus carota</td>
<td>AAK30205</td>
<td>76/6.3</td>
<td>72/6.9</td>
<td>Absent Present</td>
</tr>
<tr>
<td>16 Phosphoryruvate</td>
<td>Zea mays</td>
<td>P26301</td>
<td>50/6.0</td>
<td>48/5.2</td>
<td>Present Present</td>
</tr>
<tr>
<td>17 Desiccation-related protein</td>
<td>Craterostigma</td>
<td>AAA63616</td>
<td>33/5.0</td>
<td>34/5.8</td>
<td>Absent Present</td>
</tr>
</tbody>
</table>

Putative protein identification and accession number of the closest match in the National Center for Biotechnology Information (NCBI) database are indicated. Approximate molecular weight (MW) (kDa) and isoelectric point (pI) values as estimated from the gels are indicated, along with the predicted values of the closest match. The mean expression level (± SD, n = 3) at 65 and 35% relative water content (RWC) compared to fully hydrated plants is indicated for proteins that exhibited significant changes in abundance (paired t-test; P < 0.05); nsd indicates no significant difference in expression. For proteins not detectable in fully hydrated plants (de novo proteins), detection at 65 or 35% RWC is indicated by present (detected) or absent (not detected).
Table 2. Peptide fragments identified by matrix-assisted laser desorption/ionization (MALDI) time-of-flight (TOF)/TOF analysis

<table>
<thead>
<tr>
<th>Putative identification</th>
<th>Peptides matched</th>
<th>Score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 Transketalose</td>
<td>ALPTYTPESPADATR (82); KTSPILALSR (28)</td>
<td>103</td>
</tr>
<tr>
<td>2 F-ATPase (α subunit)</td>
<td>LIESPAHISRR (72); LIESPAHISRR (64); IAQIPVSEDYLGRR (87); VINALAKPIDGRGEOASER (70)</td>
<td>292</td>
</tr>
<tr>
<td>3 Glutaryl-oxylate aminotransferase I</td>
<td>DYGPSDPPLFILTDGASK (98); HYLSLTSGGGLAYSDR (104); IITNVSNHNLQGKPLFPR (38); AKHSLTSQGLGAYSDR (119)</td>
<td>358</td>
</tr>
<tr>
<td>4 PSII stability factor HCF136</td>
<td>GFGLDVLYGR (42); AAVQETVSATLNR (43)</td>
<td>85</td>
</tr>
<tr>
<td>5 Ascorbate peroxidase</td>
<td>TGGPGTIR (65); QELAHANDNGLILDW (54)</td>
<td>101</td>
</tr>
<tr>
<td>6 psbO</td>
<td>GSSFLDPKGR (44); RLTYDIEQSK (67)</td>
<td>111</td>
</tr>
<tr>
<td>7 psbP</td>
<td>EVEYPGQVLR (72); SITDGSPEEFLSKVYLLGK (16)</td>
<td>88</td>
</tr>
<tr>
<td>8 Chloroplast FtsH protease</td>
<td>VKILOVHSR (20); SYLENQAMAVGLGRR (36); AOGPGGGPGLGGPPGFGR (58)</td>
<td>114</td>
</tr>
<tr>
<td>9 GDP-mannose-3′,5′-epimerase</td>
<td>QLPPIHHPPEGVR (70); QLPPIHHPGEPVRGR (56)</td>
<td>126</td>
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<tr>
<td>10 Alcohol dehydrogenase</td>
<td>GTFFGNYKPR (60); TLLGTFFGNYKPR (84); IGVDLNPJMR (38)</td>
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<tr>
<td>11 Protein phosphatase type 2C</td>
<td>GGFVSNIPGDVPR (39); GGFVSNIPGDVPRVDGQLAVAR (58)</td>
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<td>12 VDAC1.1</td>
<td>SFPTISGEVDTK (84); GPGLYTDIGKK (88)</td>
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<td>13 2-Cys peroxiredoxin</td>
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<td>14 dnaK-type molecular chaperone</td>
<td>IAGLDVQR (39); EVDEVLLGGGTR (19); AVITVPAYFNDQR (31)</td>
<td>88</td>
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<td>15 RNA-binding protein</td>
<td>GSFVAFSTPEEASR (112); VYVGVFLR (20)</td>
<td>124</td>
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<td>16 Phosphopyruvate hydratase</td>
<td>FRAPEFY (41); LAKYNNLKL (25); VQIVGDVILTNPTR (45)</td>
<td>111</td>
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<tr>
<td>17 Desiccation-related protein</td>
<td>KLVAGLAVEQDAQDR (35); VEPYITVAETF (33)</td>
<td>68</td>
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</tbody>
</table>

The number and amino acid sequences of the peptides matched to proteins in the National Center for Biotechnology Information (NCBI) database are shown. Probability-based MOWSE scores for the individual peptides (ion scores) and an overall score for the protein (derived from the ion scores) provided. Ion scores > 35 and protein scores > 67 indicate identity or extensive homology at P < 0.05. M indicates an oxidized Met residue.

Figure 2. (a) Changes in protein abundance in leaf tissue of Xerophyta viscosa during dehydration from 100 to 65 and 35% relative water content (RWC). Total protein was isolated, separated by isoelectric focusing (IEF) (non-linear pH 3.0–10.0 gradient) followed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) and analysed using PDQuest software. Three replicate gels, each with protein isolated from an independent biological replicate consisting of tissue pooled from three plants, were run for each RWC. Dehydration-responsive proteins that were successfully identified by mass spectrometry (MS) analysis are highlighted on representative gels: increase (square), decrease (circle), de novo (triangle). (b) Enlargement of representative gels showing examples of two late dehydration-responsive proteins, psbP (decreased expression) and alcohol dehydrogenase (ADH) (increased expression). The increase in ADH expression was only statistically significant at 35% RWC.

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observed at 35% RWC. GGT1 is localized to the peroxisome where it catalyses the conversion of glyoxylate to glycine during photorespiration (Liepman & Olsen 2003). As photosynthesis has ceased in *X. viscosa* at this stage in drying, it is probable that flux through the photorespiratory pathway had also stopped.

In conjunction with the decreased abundance of the five chloroplastic proteins, there was an increase in levels of a chloroplast FtsH protease at both 65 and 35% RWC. These proteins constitute a small family of membrane-bound zinc metalloproteases containing an ATPase domain. In *Arabidopsis*, two of the nine chloroplast-targeted FtsH proteases have been shown to play a role in the repair of PSII after oxidative damage. *FtsH2* and to a lesser extent *fsh5* mutants display elevated photoinhibition because of a reduced turn-over of the D1 polypeptide (Bailey et al. 2002; Sakamoto et al. 2003), and it has been shown that FtsH1, 2, 5, and 8 form heteromeric complexes, with functional redundancy between FtsH1 and 5, and between FtsH2 and 8 (Yu, Park & Rodermel 2004; Zaltsman, Ori & Adam 2005). MS/MS analysis putatively identified the FtsH in the present study as FtsH1, but the antibody used in Western analysis (Fig. 3) recognizes all plant FtsH proteins, and so cannot be used to differentiate between the FtsH isoforms in *X. viscosa*. *In vitro* studies have suggested that the FtsH protease complex may also degrade an unassembled Rieske Fe–S thylakoid protein (Ostersetzer & Adam 1997), but to date no further targets have been identified. An increase in FtsH protease activity in *X. viscosa* might serve to repair damage to the D1 polypeptide during the early stages of drying when photosynthesis is still active, but would not be required at lower RWC. In line with the observation that thylakoid membranes are disassembled during drying (Sherwin & Farrant 1998; Mundree & Farrant 2000), it is possible that other components of PSII (and other chloroplast proteins) might be targeted for degradation by FtsH proteases to halt photosynthesis and prevent ROS formation during water deficit. Alternatively, the observation that the *fsh2* and *fsh5* mutants are variegated suggests that FtsH proteases may play a role in thylakoid formation (Zaltsman et al. 2005). Thus, the increase in the FtsH protease observed might represent synthesis and storage of this protein to allow rapid reassembly of the thylakoid membranes when water becomes available again.

**Comparison of photosynthetic protein levels between poikilochlorophyllous and homiochlorophyllous resurrection plants during drying**

*Xerophyta viscosa* is poikilochlorophyllous and dismantles thylakoid membranes during drying such that only vesiculated remnants of thylakoids are present in dry leaves (Sherwin & Farrant 1998; Mundree & Farrant 2000). 2D SDS–PAGE analysis during drying suggested that these vesicles contain reduced levels of photosynthetic proteins (Fig. 2; Table 1), providing an explanation for the results of earlier inhibitor studies showing that *de novo* translation is required for resumption of PSII activity during rehydration (Dace et al. 1998). In contrast, *Craterostigma* spp. are homiochlorophyllous, maintaining the integrity of thylakoid membranes during drying, and *Craterostigma wilmsii* has been shown to recover PSII activity upon rehydration without the need for *de novo* transcription or translation (Cooper & Farrant 2002). This observation suggests that unlike *Xerophyta* spp., *Craterostigma* spp. must maintain and protect PSII proteins during dehydration and in the desiccated state. To determine whether this is the case, expression levels of three photosynthetic proteins (*Lhcb2*, *psbP* and *psbS*) were monitored during drying in *X. viscosa* and *C. plantagineum*. *Lhcb2* is a component of the light-harvesting antennae of both photosystem I (PSI) and PSII (Jansson 1999), while *psbS* is a component of PSII that plays a role in non-photochemical quenching (Niyogi et al. 2005). In *X. viscosa*, levels of all three proteins were reduced at 55% RWC, and by 30% RWC *psbP* and *Lhcb2* were no longer detectable (Fig. 4). Thus, *de novo* synthesis of these two proteins at least would likely be required during rehydration for resumption of PSII activity. In contrast, *psbP* and *psbS* expression remained constant throughout dehydration in *C. plantagineum*, while *Lhcb2* levels decreased but were still detectable at 11% RWC (Fig. 4). Thus, poikilochlorophyllous in *X. viscosa* involves the breakdown of photosynthetic proteins during dismantling of the thylakoid membranes, while in the homiochlorophyllous *C. plantagineum* PSII protein levels appear to be largely

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maintained during drying. In light of this difference, analysis of FtsH protein levels was also carried out. In *X. viscosa*, FtsH protein levels again increased early on during drying (at 70% RWC) and remained elevated throughout, while in *C. plantagineum* FtsH levels remained unchanged until 11% RWC (Fig. 4). Whether increased FtsH levels in *X. viscosa* are causally related to the increased degradation of chloroplastic proteins during drying is unknown. Identification of the proteins targeted for FtsH-mediated degradation in *X. viscosa* would assist in determining whether this protease complex plays a role in the controlled shutdown of photosynthesis that occurs during drying in this species.

**Proteins involved in protection against oxidative stress**

Three proteins with a potential role in protection against oxidative damage changed in abundance in *X. viscosa* during drying: 2-Cys-Prx and GDP-mannose-3′, 5′-epimerase increased and ascorbate peroxidase (APX) declined during drying (Table 1). 2-Cys-Prxs are chloroplast-targeted proteins that reduce peroxide substrates to the corresponding alcohol. It is thought that they function to protect chloroplasts against ROS generated during photosynthesis, as antisense *Arabidopsis* plants displayed increased levels of photoinhibition (Baier & Dietz 1999). 2-Cys-Prx protein levels increase moderately in high light in *Arabidopsis*, in response to a redox signal correlating with the availability of electron acceptors downstream of PSI (Horling et al. 2003; Baier, Stroher & Dietz 2004). Similarly, increased electron pressure in the electron transport chain caused by inhibition of photosynthesis during water stress might result in an increase in 2-Cys-Prx expression in *X. viscosa*, and may help to protect against oxidative damage to the chloroplast.

Ascorbate acts as a scavenger of H\(_2\)O\(_2\) and is converted to monodehydroascorbate in a reaction catalysed by APX. Increased activities of the antioxidant enzymes APX, glutathione reductase and superoxide dismutase have been reported in several resurrection plants including *X. viscosa* during drying (Sherwin & Farrant 1998; Farrant 2000). In the present study, a ninefold decrease in the abundance of a cytosolic isofrom of APX was detected at 35% RWC. APX constitutes a multigene family, with eight members in *Arabidopsis* (Panchuk, Zentgraf & Volkov 2005), and it is possible that differential regulation of the various isoforms may occur during drying in *X. viscosa*. However, it has been suggested that while APX plays a role in abiotic stress tolerance in vegetative tissues, it may not be involved in the acquisition of desiccation tolerance in seeds (Bailly 2004). Because the mechanism of desiccation tolerance in *Xerophyta* species has been proposed to involve the induction of ‘seed-specific’ genes in vegetative tissues during the latter stages of drying (Ilign et al. 2005), it is possible that APX is not involved in ROS scavenging at low water contents in leaf tissue of *X. viscosa*.

An indication of a possible increase in ascorbate synthesis in *X. viscosa* during the early stages of drying was the approximately twofold increase in ascorbate, GDP-mannose-3′, 5′-epimerase observed at 65% (and 35%) RWC. This enzyme catalyses the conversion of GDP-mannose to GDP-1-galactose or GDP-1-gulose, and represents the first step in the de novo synthesis of ascorbate (Wolucka et al. 2001). It has recently been demonstrated that application of methyl jasmonate (a signalling molecule involved in responses to biotic and abiotic stress) leads to transcriptional up-regulation of the gene encoding GDP-mannose-3′, 5′-epimerase and increased ascorbate biosynthesis in tobacco cell cultures (Wolucka, Goossens & Inze 2005). Correspondingly, measurement of total ascorbate content revealed a 25% increase in ascorbate levels in *X. viscosa* plants at 65% RWC compared to 100% RWC (Fig. 5). However, this increase in the ascorbate pool was transient, because at 35% RWC ascorbate content was not significantly different to that observed at 100% RWC, although GDP-mannose-3′, 5′-epimerase protein levels did not decline. This may indicate that increased ascorbate production is an ‘early’ response similar to that previously reported in vegetative tissues of non-resurrection plants in response to a variety of biotic and abiotic stresses (Wolucka et al. 2005). In *Arabidopsis* GDP-mannose-3′, 5′-epimerase copurifies with a HSP70 protein, which is believed to be involved in folding or regulation of the enzyme (Wolucka & Van Montagu 2003). Interestingly, a dnaK-type HSP70 was

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**Figure 4.** Differential expression of photosynthetic proteins between *Xerophyta viscosa* and *Craterostigma plantagineum* during dehydration. Plants were dried by withholding water, and samples were collected at various relative water content (RWC). Total protein (30 μg) was separated by one-dimensional (1D) sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE), transferred to nitrocellulose membrane and probed with antibodies to psbP, psbS and Lhcb2. Equal loading of the gel was verified by Ponceau staining of the membrane after protein transfer. The results shown are from one experiment representative of three carried out on independent biological replicates.

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The ascorbate pool of Xerophyta viscosa increases transiently during dehydration. Total ascorbate content was measured after reduction of dehydroascorbate to ascorbate by dithiothreitol (DTT). Mean ascorbate content from three independent biological replicates (μmol g⁻¹ dry biomass) ± SD is indicated. The * indicates an ascorbate content significantly different (t-test, P < 0.05) to that of fully hydrated plants.

**Levels of two glycolytic enzymes increase during drying**

Two glycolytic enzymes increased in abundance during drying of X. viscosa: phosphopyruvate hydratase and alcohol dehydrogenase (ADH). Carbohydrate metabolism is modulated in X. viscosa and other resurrection plants during drying, particularly towards the synthesis of sucrose (Bianchi et al. 1991; Whittaker et al. 2001), and possibly towards the synthesis of compatible solutes such as sorbitol (Mundree et al. 2000). The accumulation of sucrose is independent of photosynthetic activity because it occurs predominately at lower RWC after the cessation of photosynthesis (Cooper & Farrant 2002). The function of sucrose accumulation remains unclear, but it has been suggested that it may act as a water replacement molecule to stabilize membrane and protein structure or in vitrification of the cytoplasm in desiccated tissue, and as an energy source during rehydration (Hoekstra et al. 2001; Buïttink & Leprince 2004; Vicré et al. 2004a). Phosphopyruvate hydratase (enolase) catalyses the conversion of 2-phosphoglycerate to phosphoenolpyruvate during glycolysis, and catalyses the reverse reaction in gluconeogenesis. An increase in flux through the gluconeogenic pathway during drying would provide an increased pool of hexose phosphate substrates required for both sucrose and sorbitol synthesis. ADH catalyses the conversion of ethanol to ethanol in anaerobic glycolysis, and the ADH gene in Arabidopsis is up-regulated in abscisic acid (ABA)-dependent manner in response to dehydration (de Bruxelles et al. 1996).

However, the function of this enzyme is unclear except under anoxic conditions such as flooding.

**Detection of an RNA-binding protein at 35% RWC**

An RNA-binding protein was detected in soluble protein extracts from X. viscosa at 35% RWC but not at 65 or 100% RWC. The subcellular localization of the closest match (AAK30205) is not known, but a putative chloroplast transit peptide is predicted by ChloroP analysis (Emmanuelsson, Nielsen & von Heijne 1999). Messenger RNAs required for the repair of dehydration-induced damage are stored in desiccated T. ruralis plants (Dhindsa & Bewley 1978), and there is evidence from inhibitor studies to suggest that mRNAs may also be stored in Xerophyta species during desiccation (Dace et al. 1998). In addition, the maintenance of two mRNAs encoding components of PSI: psbA (D1 polypeptide) and psbP has been demonstrated in desiccated X. humilis leaf tissue (Collett et al. 2003). Microarray analysis has previously identified a chloroplast ribonucleoprotein (cpRNPs) homologous to cp29A from tobacco as up-regulated in X. humilis at RWCs below 50% (Collett et al. 2004). CpRNPs have been shown to bind and stabilize psbA mRNA in tobacco stromal extracts (Nakamura et al. 2001), and it is possible that RNA-binding proteins may serve to stabilize specific mRNA transcripts, for example, mRNAs for PSII proteins during desiccation in Xerophyta species. RNA co-immunoprecipitation assays on samples collected from Xerophyta plants at different stages of drying would allow the complement of RNAs bound by these proteins at different RWC to be examined.

**Increased expression of a phosphatase implicated in ABA-signalling**

The expression of a type 2C phosphatase increased approximately fourfold at both 65 and 35% RWC. These proteins act as negative regulators of signalling pathways by opposing the action of protein kinases, and have been implicated as negative regulators of the ABA signalling. ABA is one of the major signalling molecules involved in response to changes in water availability in plants (Himmelbach, Yang & Grill 2003). ABA concentrations increase in response to osmotic stress, leading to the modulation of gene expression through ABA-response elements found in the promoters of many dehydration-induced genes in a wide range of species including resurrection plants, as well as regulating translation and protein turnover (Himmelbach et al. 2003). In Arabidopsis, the type 2C phosphatases ABI1 and 2 are negative regulators of ABA signalling and are up-regulated by ABA at the mRNA level (Gosti et al. 1999). It is thought that such a negative feedback loop allows the plant to reset the ABA signalling pathway in order to continually monitor the presence or absence of ABA (Gosti et al. 1999).

**CONCLUSIONS**

In summary, analysis of the proteome of X. viscosa identified a number of dehydration-responsive proteins that may
play a role in desiccation tolerance in this species, including antioxidants, an RNA-binding protein and a number of proteins involved in photosynthesis. These proteins could be grouped into three classes based on their expression pattern: early-dehydration, late-dehydration and full-dehydration responsive. Proteins that are late-dehydration responsive may be of greatest interest in uncovering the molecular basis of desiccation tolerance, because such changes in expression are likely unique to resurrection plants; non-desiccation tolerant species such as Arabidopsis cannot survive at such low RWC. This study also demonstrated that protein sequence information in the NCBI database could be used to identify proteins from plant species that are phylogenetically distant from model plants, albeit with a relatively modest success rate (approximately 30%). Identification of these proteins provides a foundation for further functional studies to determine their precise biochemical roles in desiccation tolerance. Proteomic analysis has several advantages over transcriptomics in that it provides a view of the end-point of gene expression, i.e. the actively translated component of the mRNA pool, and allows the investigation of post-transcriptional and post-translational regulatory events. However, it should be noted that the 2D SDS–PAGE approach used here has limitations, as it allows the detection and analysis of only a subset of relatively abundant and soluble proteins. One possible method to increase the number of proteins that can be studied by 2D SDS–PAGE is the fractionation of the protein into subcellular fractions. Analysis of the X. viscosa chloroplast proteome during dehydration would be of particular interest given that chloroplast proteins constituted 8 of the 17 dehydration-responsive proteins identified by tandem MS, and that reduction of PSI proteins during drying seems to be a component of poikilochlorophyll. Alternatively, the gel-free iTRAQ MS system could be employed to greatly increase the number of proteins that can be analysed, with the additional benefit that hydrophobic proteins lost during isoelectric focusing (IEF) can be analysed, with the additional benefit that hydrophobic proteins lost during isoelectric focusing (IEF) can be employed to greatly increase the number of proteins that can be analysed, with the additional benefit that hydrophobic proteins lost during isoelectric focusing (IEF) can be analysed, with the additional benefit that hydrophobic proteins lost during isoelectric focusing (IEF) can be identified by Suzuki, Simon & Slabas 2006). A systems biology approach combining proteomics, metabolomics and transcriptomics offers the possibility to better understand the ability of resurrection plants to tolerate desiccation, and to determine at which level of control these changes are effected.

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