Desiccation tolerance in the vegetative tissues of the fern *Mohria caffrorum* is seasonally regulated

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Summary

As there is limited information on the mechanisms of vegetative desiccation tolerance in pteridophytes, we undertook a comprehensive anatomical, ultrastructural, physiological and biochemical study on the fern *Mohria caffrorum*. Our data show that this species is desiccation-tolerant during the dry season, and desiccation-sensitive in the rainy season. This system allows the verification of protection mechanisms by comparison of tolerant and sensitive tissues of the same species at the same developmental age. Tolerant fronds acquire protection mechanisms during drying that are mostly similar to those reported for angiosperms. These include: (i) chlorophyll masking by abaxial scales and frond curling; (ii) increased antioxidant capacity that is maintained in dry tissues; (iii) mechanical stabilization of vacuoles in the dry state; (iv) *de novo* production of heat stable proteins (at least one identified as a putative chaperonin); (v) accumulation of protective carbohydrates (sucrose, raffinose family oligosaccharides and cyclitols). This study has implications for the biotechnological production of drought-tolerant crops, and allows speculation on the evolution of vegetative desiccation tolerance.

Keywords: desiccation-tolerant, desiccation-sensitive, fern, seasonal regulation, protection.

Introduction

The vegetative tissues of most plants are sensitive to water deficit and cannot persist in times of low water availability. However, there are a few species, referred to as ‘resurrection plants’ that tolerate desiccation to an extent where almost all protoplasmic water is lost, and, upon re-wetting, regain full physiological functionality in existing tissues (Farrant, 2007; Illing *et al.*, 2005; Oliver *et al.*, 1998, 2004). Desiccation tolerance is relatively common among bryophytes and lichens, but is rare in pteridophytes and angiosperms, occurring in only 0.02% of vascular plants (Alpert and Oliver, 2002).

In bryophytes and lichens, desiccation occurs rapidly, allowing little time for protection to accrue. Protection is constitutive but minimal, and survival is thought to be based largely on rehydration-induced repair processes (Oliver *et al.*, 1998, 2004). In the moss *Tortula ruralis*, for example, sucrose is constitutively held at 10% of gametophyte dry mass, and does not increase during desiccation (Bewley *et al.*, 1978). No novel transcripts are made in response to desiccation, but there is an alteration in the nature of proteins synthesized on rehydration, this being largely under translational control (Oliver, 1991). Rehydration-associated transcripts include enzymes associated with oxidative stress metabolism and late embryogenesis abundant (LEA) proteins, which are classic putative protectants that are also upregulated during drying in angiosperms (Oliver *et al.*, 2004).

In angiosperms, considerable and complex protection mechanisms are laid down during drying, minimizing the need for extensive repair on rehydration (Alpert and Oliver, 2002; Farrant, 2000, 2007; Illing *et al.*, 2005; Vicre *et al.*, 2004a). Purported protection mechanisms include: (i) controlled loss of chlorophyll and dismantling of thylakoid membranes (poikilochlorophyllous), or retention but masking of chlorophyll from light (homoiochlorophyllous), thought to minimize the reactive oxygen species (ROS) produced during photosynthesis (Farrant *et al.*, 2003; Sherwin and Farrant, 1998; Tuba *et al.*, 1994); (ii) accumulation and upregulation of antioxidants to quench the ROS that do form (e.g. Farrant, 2000, 2007; Illing *et al.*, 2005; Kranner and Birtić, 2005; Kranner *et al.*, 2002; Sgherri *et al.*, 1994;
Sherwin and Farrant, 1998; Smirnoff, 1993); (iii) accumulation of sucrose, together with some raffinose family oligosaccharides and cyclitols (e.g. Farrant, 2007; Illing et al., 2005; Norwood et al., 2000; Peters et al., 2007; Scott, 2000; Whittaker et al., 2001); (iv) stress-associated proteins, including LEAs and small heat-shock proteins (sHSPs) (Cuming, 1999; Farrant, 2007; Illing et al., 2005; Mtewisha et al., 2006) believed to act together to maintain macromolecular and subcellular integrity (Berjak et al., 2007); (v) many also undergo structural alterations, in the form of regulated wall folding (e.g. Moore et al., 2006; Vicre et al., 1999, 2004a,b) or additional vacuole formation with substitution of compatible solutes for vacuolar water content (Farrant, 2000, 2007; Moore et al., 2006; Vander Willigen et al., 2004), proposed to prevent the mechanical stress associated with plasmolysis and cytorhesis. Most angiosperms appear to use a combination of these mechanisms, and they are not mutually exclusive. Transcriptome studies have demonstrated the upregulation of numerous genes in response to drying, many corresponding to metabolic pathways involved in the putative protection mechanisms outlined above, and most are yet to be functionally characterized (Bartels and Salamini, 2001; Collett et al., 2004; Illing et al., 2005; Le et al., 2007). Whereas these putative protection mechanisms are compelling, there have been no studies in which they have been verified by direct comparison of desiccation-tolerant (DT) and desiccation-sensitive (DS) tissues of a similar developmental age within a single species. Studies attempting to do so have compared whole plants (DT) with detached (DS) (e.g. Le et al., 2007) or senescent (DS) leaves (e.g. Vander Willigen et al., 2004), or have compared species within a genus that includes both DS and DT species (Balsamo et al., 2006; Illing et al., 2005).

Although it is well documented that there are several species of pteridophytes with vegetative DT (Alpert and Oliver, 2002; Gaff, 1977; Helseth and Fischer, 2005; Lebkuecher and Eickmeier, 1993; Muslin and Homann, 1992; Pessin, 1924; Reynolds and Bewley, 1993), little has been reported on the mechanisms used to survive cellular desiccation. It has been inferred by Oliver et al. (1998) that as the rate of drying in pteridophytes is slow, they are likely to, like angiosperms, rely more on the de novo accumulation of protection during drying than on the constitutive protection coupled with rehydration-associated repair that is characteristic of bryophytes. Studies on Polypodium polyphyloides have reported frond curling and wall folding to occur during drying as potential mechanisms to prevent light and mechanical stress (Helseth and Fischer, 2005; Muslin and Homann, 1992), suggesting at least some mechanisms in common with angiosperms. However, characterization of changes in protein synthesis during drying and rehydration of Polypodium virginianum have shown that only a few proteins, none unique to desiccation tolerance, are synthesized in response to drying and ABA treatment, whereas unique rehydration-specific polypeptides are produced during recovery. This suggests that this species has some mechanisms in common with bryophytes (Reynolds and Bewley, 1993).

In order to further characterize mechanisms of DT in ferns, we undertook a study on some of the anatomical, ultrastructural, physiological, and biochemical changes associated with drying and rehydration in the resurrection fern Mohria caffrorum (L.) Desv. (Gaff, 1977). This species is common in Southern Africa and grows in semi- to fully-exposed habitats on forest margins (Roux, 1979). Plants were collected in both dry and rainy seasons, and our data show that the plants are DT in the dry season but DS in the rainy season. When DT, fronds acquire protection mechanisms during drying that are most similar to those accumulated in angiosperms. These are not acquired when DS fronds are dried.

Results

Viability assessment and microscopy

Plants collected in the dry season dried more slowly than those from the rainy season, and rehydrated fully following desiccation to the air-dry state (8% relative water content, RWC). Rainy season plants were never able to rehydrate to more than 20% of their RWC (Figure 1). Fronds from dry-season plants had little electrolyte leakage throughout the drying and rehydration time course (Figure 2), suggesting that the membranes were not damaged by desiccation, and that these plants were thus typically DT. Conversely, fronds from plants collected in the rainy season were characteristically DS, as electrolyte leakage increased markedly once the RWC declined below 60%, and also increased upon

![Figure 1. Dehydration and rehydration time course of Mohria caffrorum fronds. The relative water content (RWC) of fronds from plants collected during the dry season (■), and of those collected in the rainy season (○), is plotted over the course of dehydration and rehydration. The drying rate of the fronds was similar whether dried on the plant (once the soil water content had reached an air-dry equilibrium) or dried once detached from the plant. Data from both experimental procedures are combined here. When no error bar is visible, the SD is less than the symbol.](image)
rehydration (Figure 2). Images of fronds from time-lapse photography (see Video clip S1) during rehydration showed that fronds from plants collected in the dry season were tightly curled in the dry state, but were able to unfurl during rehydration, whereas dry fronds from rainy season plants appeared wilted when dry, and remained so after 8 h of immersion in water (Figure 3). From these experiments, we considered plants collected in the dry seasons to be DT, and those collected in the rainy season to be DS, and hereafter referred to the plants as either DT or DS.

The specific leaf area of DS fronds was significantly ($P < 0.001$) larger ($450 \pm 150 \text{ cm}^2 \text{ g dm}^{-1}$) than that of DT fronds ($100 \pm 50 \text{ cm}^2 \text{ g dm}^{-1}$), thereby suggesting more efficient photosynthesis in the DS leaves than in the DT leaves (Agustí et al., 1994). Microscopical examination revealed that the abaxial surfaces (pinnae and stipe) of the DT fronds were covered by orange-coloured scales (Figure 4a), which were absent from abaxial surfaces of DS fronds (Figure 4b). On dehydration, the DT fronds curled such that the adaxial leaf surfaces were hidden, and the abaxial scales masked the chlorophyll contained in those surfaces (Figure 4c, d). Chlorophyll shading and masking is characteristic of homoiochlorophyllous angiosperm resurrection plants (Farrant et al., 2003; Sherwin and Farrant, 1998), and frond curling has also been noted in the DT fern Polypodium polypodioides and fern ally Selaginella lepidophylla (Helseth and Fischer, 2005; Lebkuecher and Eickmeier, 1993), features that are thought to minimize photosynthetic ROS production during drying (Farrant, 2000, 2007; Oliver et al., 1998; Smirnoff, 1993; Vicre et al., 2004a). Leaf and stem curling did not occur in DS fronds (Figure 3), and, with few leaf scales present (Figure 4b), there was no apparent mechanism of prevention of light–chlorophyll interaction during desiccation.

**Figure 2.** Electrolyte leakage of Mohria caffrorum. Rate of electrolyte leakage, measured as $\mu$S per gram dry mass per second, during the time course of drying and rehydration from fronds of Mohria caffrorum collected in the dry season (■) and in the rainy season (□).

**Figure 3.** Rehydration of Mohria caffrorum. Images are taken from time-lapse photography during the rehydration of dessication-tolerant (DT, a) and dessication-sensitive (DS, b) fronds of Mohria caffrorum.
The ultrastructural organization of mesophyll cells from hydrated DT and DS fronds was similar and typical of hydrated tissue (Figure 5a). Cytoplasm was restricted to the cell periphery, and was comprised mainly of chloroplasts with well-defined thylakoid membranes and starch. Mitochondria had darkly stained cristae, indicative of a high level of metabolic activity. In many cells, the central vacuoles contained spherical osmophilic polyphenol bodies (insert, Figure 5a). Drying of *M. caffrorum* DS plants resulted in the rupture of both the plasmalemma and tonoplast membranes, with diffuse polyphenolic material visible within the ruptured vacuoles (Figure 5b). Mesophyll cells from dry DT fronds showed no apparent subcellular damage. There was no plasmalemma withdrawal, and the peripheral location of cytoplasm around a central vacuole was maintained (Figure 5c). Phenolic inclusions lined the periphery of the vacuole, possibly acting in a stabilizing capacity, as was shown to occur in the angiosperm resurrection plant *Myrothamnus flabellifolius* (Moore et al., 2004). Several smaller vacuoles had also formed in the peripheral cytoplasm, similar to those described for angiosperm resurrection plants in which vacuolation and organelle packaging is proposed to act as a mechanism of mechanical stabilization (Farrant, 2000, 2007; Vander Willigen et al., 2004). There are no reports on mechanisms of mechanical stabilization in other pteridophytes, but in DT bryophytes, there are no apparent mechanisms of mechanical stabilization. The central vacuole is lost as water is withdrawn, and walls collapse inwards, with repair occurring on rehydration (Bewley and Krochko, 1982).

Chloroplasts from dry DS mesophyll cells had indistinct and sometimes swollen or vesiculated thylakoids, and some starch was also evident (Figure 5b). Those from dry DT tissues had retained thylakoid organization, although numerous plastoglobuli were observed, but no starch was evident. The retention of chloroplast ultrastructure, presence of plastoglobuli (in some, but not all) and loss of starch is typical of homiochlorophyllous resurrection angiosperms (Farrant, 2000, 2007; Sherwin and Farrant, 1998). Bryophytes also retain thylakoids, but starch can be retained if drying is rapid (Oliver and Bewley, 1984). There are no reports on chloroplast ultrastructure following the drying of homiochlorophyllous DT pteridophytes.

**Physiological and biochemical studies**

Chlorophyll was retained during drying in both DT and DS forms of the plant (Figure 6a), confirming that this species is homiochlorophyllous. There was some chlorophyll degradation during the rehydration of DS fronds, probably
because of desiccation-induced damage rather than poikilochlorophyll, but DT fronds retained chlorophyll during rehydration.

The quantum efficiency of photosystem II (Fv/Fm) of DT fronds declined once the water level dropped below 70% RWC, but it was recovered to pre-desiccation levels upon rehydration, suggesting that there was little damage (or there was rapid repair) to the photosynthetic apparatus in those tissues (Figure 6b). The prevention of photosystem-II (PS-II) activity at high RWC is also typical of angiosperm resurrection plants (Farrant, 2007; Mundree et al., 2002), and this observation supports our proposal that the presence of pigmented abaxial scales and frond curling upon drying might prevent the light–chlorophyll interaction in *M. caffrorum*, causing early shutdown of Fv/Fm and, in turn, the minimization of ROS production. That Fv/Fm increased again after 6 h of rehydration, when DT fronds had unfurled sufficiently for light interception (Figure 3), further confirms the role of abaxial scales and frond curling. DS fronds initially maintained higher values of PS-II activity (Fv/Fm) than DT fronds until the RWC declined below 46%, after which Fv/Fm declined and was never recovered during rehydration (Figure 6b), suggesting irreversible damage to the photosynthetic apparatus, probably because of ROS activity. Coincident increases in electrolyte leakage (Figure 2) and damaged chloroplasts (Figure 5b) are further indicators of potential ROS activity, these being noted targets of ROS damage (Reddy et al., 2004; Seel et al., 1992; Smirnoff, 1993).

Hydrated fronds from DT plants had higher levels of activity of the antioxidant enzymes catalase (CAT), glutathione reductase (GR) and superoxide dismutase (SOD) than were measured in hydrated DS fronds (Figure 7), suggesting some level of constitutive protection in DT plants. Enzymes were still active, although at a lower level, in extracts from dry DT fronds, but no activity was recorded in extracts from dry DS fronds. The ability to prevent desiccation-induced denaturation of antioxidant enzymes during drying is a key difference between DT and DS angiosperm species, and has been proposed to play an important role in the prevention of ROS damage during the late stages of drying, and also upon rehydration (Farrant, 2007; Illing et al., 2008; Kraner et al., 2002). Thus, in this regard, *M. caffrorum* yet again shows protection mechanisms that are characteristic of
angiosperms, and this study confirms that prevention of antioxidant enzyme denaturation is important for the survival of and recovery from desiccation. There is little information on antioxidant control in pteridophytes, but in bryophytes such as *T. ruralis*, antioxidant enzymes are primarily transcribed and translated on rehydration (Oliver et al., 2004).

Figure 8 shows the heat-stable, and thus potential LEA-like, and shSP proteins from hydrated and dry, DS and DT, fronds of *M. caffrorum*. The total protein profile of hydrated DS and DT fronds was similar. With the exception of an ~25-kDa heat-stable protein present only in DS fronds, and two proteins with molecular weights of approximately 70 and 75 kDa in both DS and DT, hydrated tissues had few heat-stable proteins. During drying, the protein content of DS fronds was considerably reduced, probably as a result of desiccation-related damage. Dehydration of DT fronds resulted in an increase in concentration of several proteins and in the appearance of new proteins, most of which were heat stable (arrowed) and which disappeared on rehydration (not shown). Only three of these proteins (Table 1) gave sequence information that had reasonably significant homology to proteins within the database. One of these is a putative chaperonin from *Oryza sativa*, from a class of proteins known for their role in protection against heat and desiccation stress (reviewed in Oliver et al., 2002 and Mtwisha et al., 2006). The functions of the other two proteins, also from *O. sativa*, have not yet been elucidated. The unknown status of the remaining heat-stable proteins upregulated during the drying of DT fronds is probably largely a consequence of the small size of databases from non-model organisms, especially of those exhibiting the rare property of vegetative DT. The de novo production of such heat-stable protectants during desiccation is again a typical feature of DT in angiosperm tissues (Boudet et al., 2006; Buitink et al., 2002; Collett et al., 2004; Cuming, 1999; Illing et al., 2005; Mtwisha et al., 2006), whereas such proteins are mostly induced on rehydration in ferns (Reynolds and Bewley, 1993) and bryophytes (Oliver et al., 2004).

Since the accumulation of soluble carbohydrates are proposed to play a significant role in the DT of angiosperms (Berjak et al., 2007; Farrant, 2007), but not in bryophytes (Bewley et al., 1978; Smirnoff, 1992), we examined changes in these during the drying of DT and DS fronds. Figure 9 shows carbohydrates that only differed significantly ($P < 0.05$) between DT and DS plants. Hydrated DT fronds had greater quantities of 1,6-anhydroglucose, maltose, galactinol, raffinose and melezitose than hydrated DS fronds. The drying of DS fronds resulted in a significant reduction in 1,6-anhydroglucose, fructose, glucose, galactinol and sucrose, and in increases in arabinose, maltose and trehalose. Dehydration of DT fronds resulted in decreased fructose, glucose, maltose and galactinol, and no change in trehalose content. There were considerably increased levels of quebrachitol, monohexadecanoglycerol, glycerol, arabinose, sucrose, 1,6-anhydroglucose, melezitose and raffinose. Thus, as has been reported for angiosperm resurrection plants (reviewed in Oliver et al., 1998; Scott, 2000; Vicre et al., 2004a; Farrant, 2007), the drying of DT fronds of *M. caffrorum* resulted in significant increases in the ubiquitous protectants (sucrose, raffinose family oligosaccharides and cyclitols), and in several others that might also perform a protective role, as well as in the depletion of the potentially damaging reducing sugars, fructose and glucose. The total sucrose content of dry DT fronds was 120 $\mu$mol g dw$^{-1}$, which is well within the range reported for angiosperm resurrection plants: viz. 44 $\mu$mol g dw$^{-1}$ in the desiccated leaves of *Borya constricta* and 400 $\mu$mol g dw$^{-1}$ in *Craterostigma wilmsii* (reviewed in Farrant, 2007).

Figure 7. Activities of the antioxidant enzymes in desiccation-tolerant (DT) and desiccation-sensitive (DS) fronds of *Mohria caffrorum*. (a) catalase (CAT); (b) glutathione reductase (GR); and (c) superoxide dismutase (SOD). Activities were only recorded in wet and dry frond tissues.

![Figure 7](image-url)
Sucrose, together with the raffinose family oligosaccharides, cyclitols and LEA proteins, have been proposed to form intracellular glasses that confer stability on macromolecules and membranes, and restrict molecular mobility, particularly that of damaging free radicals (Berjak et al., 2007; Koster and Bryant, 2005; Wolkers et al., 2001). That the DT fronds of *Mohria caffrorum* do indeed have increased levels of appropriate sugars and newly produced heat-stable proteins, whereas the DS fronds do not, supports the contention that these ingredients are necessary for DT, albeit that we cannot comment yet on whether they are present in *Mohria caffrorum* in appropriate quantities or ratios for glass formation. Nevertheless, we propose that vitrification does indeed occur in DT plants of *Mohria caffrorum*, and that in particular it enables the maintenance of membrane integrity (Figure 2 and 5c), prevents the denaturation of macromolecules such as the antioxidant enzymes (Figure 6) during drying, and allows the resumption of their activity upon rehydration. Sugars and the *de novo* production of protectant proteins such as LEAs are apparently not accumulated upon drying in lower order species (Bewley *et al*., 1978; Smirnoff, 1992), and in this regard, *Mohria caffrorum* is again more angiosperm-like in its response to desiccation.

In angiosperms, photosynthesis declines early in the drying time course, and thus cannot account for the levels of sucrose accumulated in the desiccated state. This is probably also true of *Mohria caffrorum*, as PS-II activity in DT fronds shuts down below 70% RWC (Figure 6b). In angiosperms, conversion of starch (Ghasempour *et al*., 1998; Whittaker *et al*., 2001), and/or carbohydrates such as octulose and stachyose (Norwood *et al*., 2000, 2003; Peters *et al*., 2007), into sucrose has been reported to occur. Starch disappears (Figure 5a, c), and fructose, glucose, maltose and galactinol decline (Figure 9) during the drying of DT fronds of *Mohria caffrorum*: the breakdown of these molecules possibly provides the carbon source for increased levels of protective sugars in DT fronds (Figure 9). Moreover, the higher levels of maltose and galactinol in wet DT compared with DS leaves might infer a constitutive mechanism of carbon storage for transfer to protectants on drying. Starch did Table 1 Mass spectrometry identification of heat stable proteins upregulated, or produced *de novo*, during the drying of desiccation-tolerant fronds of *Mohria caffrorum*

<table>
<thead>
<tr>
<th>Homologous protein</th>
<th>Organism</th>
<th>Accession number</th>
<th>Theoretical MW (kDa)</th>
<th>Protein score</th>
</tr>
</thead>
<tbody>
<tr>
<td>P0020E09.6</td>
<td>Oryza sativa</td>
<td>gi/34906092</td>
<td>114.19</td>
<td>46</td>
</tr>
<tr>
<td>OSJNBa0074L08.8</td>
<td>Oryza sativa</td>
<td>gi/36346617</td>
<td>26.85</td>
<td>42</td>
</tr>
<tr>
<td>Putative chaperonin</td>
<td>Oryza sativa</td>
<td>gi/55908889</td>
<td>61.89</td>
<td>36</td>
</tr>
</tbody>
</table>

Putative protein identification and accession numbers of the closest matches in the National Centre for Biotechnology Information (NCBI) data base are indicated. Approximate molecular weights (MWs, in kDa) are given. These might differ from the apparent MWs in Figure 8 because of post-translational modifications. Only proteins with reasonably significant ion and protein scores (> 35) against proteins of known homology in the databases are shown. The remaining eight proteins were either listed as ‘unknown’ or had scores <35.

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not decline entirely, and there was an increase in maltose in dry DS fronds (Figures 6b and 9, respectively). Leaf starch is thought to be degraded by β-amylases together with a disproportionating enzyme and isoamylase, with maltose being the first intermediate from this process (Edner et al., 2007; Zeeman et al., 2007). It is possible that in DS plants there is an inability to further utilize the maltose for downstream production of sucrose and other putative protectants.

The significant -fold increases in glycerol and monohexadecanoglycerol in DT fronds is interesting, as although they are cytotoxic in plants in large quantities (Fahy, 1986), glycerol accumulates as an osmoprotectant in yeast (Brown et al., 1991; Volk and Walters, 2006). It is possible that these compounds act in concert with other sugars to form a vitrifying agent protecting against ice-crystal damage (Sakai et al., 1991; Volk and Walters, 2006). Alternatively, and particularly considering the putative cytotoxic nature of glycerol, it is possible that they act more as water replacement substances in the vacuoles (Figure 5b), facilitating mechanical stabilization (discussed above), as has been suggested for angiosperms (Farrant, 2000, 2007).

The increased level of trehalose in DS but not in DT fronds is similarly intriguing. This sugar has been claimed as one of the most important protectants against desiccation damage in animal tissues (Crowe et al., 1996; Kaushik and Baht, 2003), but rarely occurs in higher plants (Paul, 2007), including most DT angiosperms (Farrant, 2007). Trehalose does, however, accumulate in relatively significant quantities during the drying of the DT fern allies S. lepidophylla (291.42 µmol g dw⁻¹) and Selaginella sartorii (230.9 µmol g dw⁻¹) (Iturriaga et al., 2000). Despite the absence of trehalose from higher plants, the enzymes of its metabolism are ubiquitous in higher plants (Paul, 2007). Their gene families are ancient, pre-dating the divergence of streptophyte and chlorophyte lineages (Lunn, 2007), and trehalose metabolism is thought to still play an important function in higher plants, predominantly in sugar signalling via the intermediate trehalose-6-phosphate (Eastmond et al., 2003; Paul, 2007). Although speculative, it is possible that trehalose was once necessary for DT in the primitive ferns that colonized the land (and is still utilized in some extant ferns), but this has been replaced by sucrose in DT angiosperms as the predominant protectant sugar. In this process, we postulate that the intermediate metabolite trehalose-6-phosphate was retained as a signalling molecule for sucrose production in seeds and vegetative tissues of higher DT plants. The methodology used here did not allow the detection of trehalose-6-phosphate, but we propose that in DS fronds of M. caffrorum, trehalose-6-phosphate is utilized for trehalose production, hence explaining the elevated levels of trehalose in dry fronds. In DT fronds, we postulate that it might act as a signal for alternative routes of sugar metabolism, which in turn provides protection via vitrification (inter alia). We are now testing this hypothesis. If we are correct, there is potential for an application in the induction of drought tolerance in crops.

**Desiccation tolerance of reproductive structures**

The spores of M. caffrorum are dry and are DT on shedding (Table 2). Drying to 4% RWC still allowed 88% germination, and levels of sucrose, glucose and fructose, and activities of the antioxidant enzymes GR, CAT and SOD, were similar to those reported above for dry DT fronds.

**Discussion**

*Mohria caffrorum* is a pteridophyte capable of vegetative DT that is apparently seasonally regulated: the plants being DS in the wet season and DT in the dry season. This phenomenon has not been reported to date. Comparison of DT and DS vegetative states has enabled the evaluation of the putative mechanisms of DT widely reported in the literature. Significantly, this evaluation (and verification) of mechanisms of DT has been achieved on intact plants of the same

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**Figure 9. Differences in carbohydrates of desiccation-tolerant (DT) and desiccation-sensitive (DS) fronds from *Mohria caffrorum*, as measured by high-throughput GC-MS. Black bars indicate the -fold differences between hydrated DT and DS fronds. Where the grey bar is greater than 1, it indicates higher constitutive levels in the DT fronds. White bars indicate the -fold differences in dry-to-hydrated DT fronds. A value of 1 indicates no difference in the levels of the carbohydrate.**
developmental age within the same species. In addition, characterization of the mechanisms of DT and their role within the lifecycle of the fern may give insight to the evolutionary aspects of vegetative DT in land plants.

As outlined throughout the results section, the responses of the DT fronds of *Mohria caffrorum* to desiccation display greater similarity to those reported for angiosperms than to those of lower order resurrection plants, including other ferns reported on to date. The rate of drying was slow, and with the exception of the constitutive presence of abaxial scales, higher levels of some sugars and enhanced antioxidant enzyme activities, protection was largely accrued de novo during drying, and not upon rehydration. Features that are particularly typical of angiosperm DT, and that we confirm as mechanisms of DT in our study, by their absence in DS tissues are as follows:

(i) Chlorophyll masking, to reduce photosynthetically produced free radicals, achieved in this species by the presence of abaxial scales and frond curling (Farrant, 2000, 2007; Lebkuecher and Eickmeier, 1993; Sherwin and Farrant, 1998; Smirnoff, 1993).

(ii) Maintenance of antioxidant potential during drying and in the dry state (as proposed by Illing et al., 2005; Farrant, 2007).

(iii) Prevention of mechanical stress by maintenance of vacuolar volume through the replacement of water with solutes (as proposed by Farrant, 2000; Moore et al., 2004; Vander Willigen et al., 2004), with the likely candidates in *Mohria caffrorum* being polyphenols, glycerol and also possibly other carbohydrates.

(iv) The *de novo* production of heat stable LEA/shSHP-like proteins (one being identified as a putative chaperonin in this study), which are commonly cited as being protectants (e.g. Cuming, 1999; Buitink et al., 2002; Illing et al., 2005; Boudet et al., 2006; Mtwisha et al., 2006; Berjak et al., 2007; Farrant, 2007).

(v) Accumulation of sucrose in particular, but also of raffinose family oligosaccharides (arabinose, raffinose and melezitose) and cyclitols (quebrachitol) that are believed to protect independently (e.g. Crowe et al., 1996; Scott, 2000), but are more likely, probably together with proteins, to stabilize the subcellular milieu by vitrification (Berjak et al., 2007; Koster and Bryant, 2005; Wolkers et al., 2001).

*Mohria caffrorum* occurs predominantly in the forest margins, where it is exposed to desiccating conditions only during the dry season. We propose a life cycle (Figure 10) that is ideally adapted to this environment, allowing it considerable ecological advantage over co-occurring annual DS plants. In the dry season it is DT (1), the tissues are desiccation-tolerant. In the dry season, these fronds produce sporangia (5, 6) that rupture during the rainy season (4) are DS, with a larger specific leaf area, enabling more efficient photosynthesis (Agustı´ et al., 1994) and rapid growth, again allowing *Mohria caffrorum* to compete successfully with cohabiting species. At the completion of the wet season, these fronds produce sporangia (5, 6) that rupture (see insert) to release DT spores that enable the production of new DT plants (1) at the onset of the dry season.

Finally, the characterization of the mechanisms of DT, and of their roles within the life cycle of this fern, allows us to speculate on the evolutionary aspects of vegetative DT in land plants. This is an extinct candidate that could typify one of the ancestors (Oliver et al., 2000 postulated at least eight independent cases of evolution, or re-evolution) of DT angiosperms. In its DT stage, this species exhibits many of the mechanisms of protection identified in angiosperms. Unlike *Mohria caffrorum*, however, most present-day angiosperm resurrection plants occur in more arid niches, typically in shallow soils on rocky outcrops, in which they

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**Table 2** Water content, percentage germination, sugar (glucose, fructose and sucrose) and antioxidant enzyme activities of newly shed spores, and after drying over silica gel.

<table>
<thead>
<tr>
<th></th>
<th>Newly shed spores</th>
<th>Dried spores</th>
<th>Dry DT fronds</th>
</tr>
</thead>
<tbody>
<tr>
<td>Absolute water content (g H2O g dm(^{-1}))</td>
<td>0.06</td>
<td>0.04</td>
<td>0.05</td>
</tr>
<tr>
<td>Spore germination (%)</td>
<td>75</td>
<td>88</td>
<td></td>
</tr>
<tr>
<td>Sucrose (μmol g dm(^{-1}))</td>
<td>125 ± 8.8</td>
<td>nd</td>
<td>120 ± 13</td>
</tr>
<tr>
<td>Glucose (μmol g dm(^{-1}))</td>
<td>1.3 ± 0.01</td>
<td>nd</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>Fructose (μmol g dm(^{-1}))</td>
<td>4.1 ± 0.05</td>
<td>nd</td>
<td>5 ± 0.2</td>
</tr>
<tr>
<td>GR (μmol NADPH elim. min(^{-1}) mg prot(^{-1}))</td>
<td>18.2 ± 0.12</td>
<td>nd</td>
<td>17.8 ± 0.1</td>
</tr>
<tr>
<td>CAT (mmol H2O2 elim. min(^{-1}) mg prot(^{-1}))</td>
<td>30.04 ± 5.25</td>
<td>nd</td>
<td>26.18 ± 7.4</td>
</tr>
<tr>
<td>SOD (units mg prot(^{-1}))</td>
<td>60.1 ± 12.0</td>
<td>nd</td>
<td>20.3 ± 7.5</td>
</tr>
</tbody>
</table>

CAT, catalase; GR, glutathione reductase; nd, not determined; SOD, super oxide dismutase; DT, desiccation-tolerant.

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experience frequent spells of desiccation, even within the rainy season (Alpert and Oliver, 2002; Farrant, 2007; Porembski and Barthlott, 2000). Having a DS vegetative stage during the rainy season would be disadvantageous to such plants. Thus, we postulate that if vegetative DT in angiosperms did indeed evolve from an ancestor such as *Mohria caffrorum*, the DS stage of the life cycle was lost, leaving the ancestral angiosperms with only facultative DT in the vegetative tissues. It has also been argued that DT in angiosperm vegetative tissues might have evolved from an appropriation of the programme of gene expression in seeds into vegetative tissues, entrained to environmental rather than developmental cues (Illing *et al.*, 2005; Oliver *et al.*, 2005). This does not necessarily contradict our proposal. DT could have first arisen in the spores of the proposed fern ancestor, and, as we have proposed for *Mohria caffrorum* (Figure 10), such spores gave rise to the DT vegetative state.

Our discovery of this unique mechanism of DT in *Mohria caffrorum* opens up opportunities for future biotechnological applications. As the mechanisms of DT present in *Mohria caffrorum* are so similar to those present in most angiosperms, it is possible that the regulation of their induction in response to water deficit is also similar. We have used differential screening and microarray technology to identify genes upregulated during drying in angiosperm vegetative tissues of the resurrection plants *Xerophyta viscosa* and *Xerophyta humilis* (Collett *et al.*, 2004; Mundree and Farrant, 2000), and are in the process of inserting such genes into crops for improved drought stress (Mundree *et al.*, 2002; Garwe *et al.*, 2003; Iyer *et al.*, 2007). Furthermore, we have recently found considerable similarity in genes induced during the acquisition of DT in seeds, and in vegetative tissues of *Xerophyta humilis* (Mowla *et al.*, 2002; Mulako *et al.*, 2008; Walford *et al.*, 2007), thereby supporting the hypothesis that DT in vegetative tissue is a consequence of the activation of seed-specific genes in roots and leaves upon dehydration, these being induced in response to environmental rather than developmental cues (Illing *et al.*, 2005). An understanding of the environmental signals that induce DT in vegetative tissue, and the implementation of them in crops, could facilitate the induction of drought-tolerant crops without genetic modification. The *Mohria caffrorum* system has potential for expediting this process. The identification and understanding of the environmental and biological signals that induce such DT-related gene expression in DT plants, by their absence or lack of similar responses in DS plants, could ultimately enable us to utilize them for the induction of drought tolerance in the vegetative tissues of crop species.

**Experimental procedures**

**Plant material**

Plants were collected during rainy and dry seasons (in 2006 and 2007, inclusive) from Table Mountain in the Western Cape, South Africa. For some of the analyses outlined below, plants were used immediately upon collection. The remainder of the plants were transported to the glasshouse at the University of Cape Town, where they were potted in small pots (width, 10 cm; depth, 15 cm) with as much soil from the site of collection as possible, and were then topped up with potting soil. They were maintained under natural conditions of light, temperature and humidity typical of the season. In winter, these were 200–800 μmol m⁻² sec⁻¹, 15–20°C, 50–70%, respectively, and in summer they were 400–1000 μmol m⁻² sec⁻¹, 20–30°C, 50–70%, respectively. Plants were watered regularly, to field capacity, and were allowed to acclimate for at least 1 month.
before experimentation. All of the experiments described below were performed on fronds from both rainy and dry season plants.

Dehydration, rehydration and determination of water content

Both whole plants and excised fronds were subject to drying and rehydration. Whole plants were dried by withholding water from the soil until the plants had reached an air-dry state, after which they were left for a minimum of 1 week before rehydration. Rehydration was achieved by overhead irrigation, simulating rainfall. The water content of pinnate (10 were randomly selected from different plants) was measured at regular intervals during the drying and rehydration time courses. Excised fronds were dried by placing them on a bench top under laboratory conditions (20–25°C; 45–55% relative humidity) until they were air-dry, and were then rehydrated by placing the base such that the bottom two pinnate of each frond were submerged into water (Figure 3). Fronds were weighed at the start and at regular intervals during the drying time course, and the water contents were determined at the end of the experiment on a subset of dry and rehydrated fronds. Water contents were gravimetrically determined by oven drying at 70°C for 48 h, as previously described (Farrant, 2000). The RWC was calculated as the absolute water content at any stage in the drying/rehydration time course, relative to the absolute water content of fully hydrated fronds prior to the start of experimentation.

Viability assessment

The viability of fronds was assessed by the degree of electrolyte leakage from membranes (indicating membrane damage). This was determined on fronds sampled at various stages during drying and rehydration using a portable, Autoranging Microprocessor Conductivity/Total Dissolved Solutes meter (Model HI 9835; Hanna Instruments, http://www.hannainst.com), as previously described in Farrant et al. (1999). The leakage rate was calculated as the slope of the line generated from the time course of leakage, and was corrected by leaf dry mass. The ability to recover and resume growth was illustrated by time-lapse photography. Excised fronds were individually photographed before and after dehydration, and during rehydration: the images were the captured at 10-s intervals using a Canon Powershot S2 IS digital camera (Canon, http://www.canon.com). A time-lapse sequence was generated using Adobe Premiere Pro 2.0 (Adobe, http://www.adobe.com). Representative images were selected to illustrate the rehydration time lapse.

Spores were released from mature sporangia and the RWC was determined for 10 replicates of 0.05 g, as described above. The DT of mature spores was determined by placing them in open Petri dishes over activated silica gel for 24 h, after which the RWC and viability (eight plates containing randomly dispersed spores) was determined by the ability to germinate on nutrient agar (Dyer, 1979).

Microscopical and ultrastructural studies

Light microscopy was used to investigate the morphological differences, and scanning electron microscopy was used to investigate the fine surface details between the DT and DS fronds in both the dehydrated and hydrated state. Transmission electron microscopy was used to examine the subcellular organization of cells from hydrated and dehydrated fronds of DS and DT plants.

For light microscopy, unfixed intact fronds or hand-sectioned tissue slices were examined using a Nikon SMZ1500 Stereoscopic Zoom Microscope (Nikon, http://www.nikon.com). Samples for scanning electron microscopy were fixed in 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2). Following alcohol dehydration, the samples were critical-point dried, mounted on aluminium stubs, sputter coated with gold palladium and were viewed using an LEO fully analytical S440 scanning electron microscope (Zeiss, http://www.smt.zeiss.com/nts). Samples for transmission electron microscopy were processed as previously described for leaf tissue from angiosperm resurrection plants (Sherwin and Farrant, 1998). Tissues were sectioned using a Reichert Ultracut-S microtome (Reichert, http://www.reichert.com), stained with uranyl acetate and lead citrate (Reynolds, 1983), and were viewed using an LE0912 transmission electron microscope.

Calculation of specific leaf area

Specific leaf area, an estimation of leaf thickness, was determined dividing the frond area by dry mass (Vile et al., 2005). These measurements were calculated from microscopic images captured as described above. An analysis of variance (ANOVA) was performed to determine whether there was a significant difference in specific-leaf areas between DT and DS fronds.

Chlorophyll content and chlorophyll fluorescence studies

Chlorophyll was extracted from fronds (n = 5) at various stages of dehydration and rehydration in the dark in 100% acetone, and the absorbance was measured using a Shimadzu UV 2201 light/UV spectrophotometer (Shimadzu Scientific Instruments, http://www.shimadzu.com). The chlorophyll content was calculated using the adjusted extinction coefficients, according to the method described by Lichtenthaler (1987).

The effect of drying and rehydration on the quantum efficiency (Fv/Fm) of PS II was determined using a portable fluorometer (OS 500; Optiscience, http://www.optisci.com), as previously reported (Sherwin and Farrant, 1998). A minimum of three replicates on three separate plants were measured at each sampling point. The DT fronds curled during drying. We were unable to uncurl dry fronds without causing damage, so for DT plants Fv/Fm was recorded on intact but curled fronds.

Antioxidant enzyme assays

The activity of the antioxidant enzymes CAT, GR and SOD was measured in hydrated and dry DS and DT fronds, and in newly released spores. Extraction was performed on three independent replicates of 150 mg of tissue, as described in Farrant et al. (2004). The activities of SOD (EC 1.15.11) and CAT (EC 1.11.16) within the supernatants were determined at 25°C, according to the method described by Bailly et al. (1996). The results are expressed as units mg⁻¹ protein (SOD) and nmol H₂O₂ decomposed min⁻¹ mg⁻¹ protein (catalase). The activity of GR (EC 1.6.4.2) was determined at 25°C, as described by (Estebauer and Grill, 1978). Protein contents of the extracts were determined using the Bio-Rad protein assay kit with bovine serum albumin as calibration standard (Bio-Rad, http://www.bio-rad.com).

Identification of heat-stable proteins

Both LEAs and sHSPs are characteristically heat stable (Cuming, 1999), and we thus used heat stability as an indicator of the presence of potential protectant proteins in hydrated and dry, DT and DS,
fronds. Total proteins were extracted from three independent replicates of 200 mg of tissue, and the heat stable fraction was obtained as previously described in Farrant et al. (2004). Proteins were quantified using the Bio-Rad assay kit with bovine serum albumin as standard, and 10 µl (or up to 30 µl to test for low abundance proteins) was loaded onto a 12% acrylamide running gel (with 4% acrylamide stacking gel), and was then separated by SDS-PAGE at 90 V for 90 min. Proteins were stained with Coomassie Blue according to the manufacturer’s instructions. Heat-stable proteins from dry DT leaves were excised, digested as described in (Ingle et al., 2007) and proteins were analyzed on an ABI 4800 MALDI TOF/TOF system (Centre for Proteomic and Genomic Research, http://www.cpgr.org.za). The instrument was equipped with a 355 nm Nd:YAG laser operating at 200 Hz. All mass spectra were recorded in positive reflector mode, and were generated by accumulating data from 1800 laser pulses. Bovine trypsin autolytic fragments were used for internal calibration. Up to five spectral peaks per band that met the threshold criteria were included in the acquisition list for the MS/MS spectra. Peptide fragmentation was performed at a collision energy of 1 kV, and a collision gas pressure of approximately 1.5 × 10⁻⁶ Torr. During MS/MS data acquisition, 4000 laser pulses were allowed for each spectrum. The Global Proteomics Server (GPS) EXPLORER software (Applied Biosystems, http://www.appliedbiosystems.com) was used for submitting MS data for database searching with the Mascot search engine (http://www.matrix-science.com). All searches were performed against the Viridiplanta NCBI database with the following settings: missed cleavages, 0; peptide tolerance ± 2 Da; bovine trypsin autolytic fragments, as well as matrix clusters, were excluded from the database search; MS/MS tolerance ± 0.2 Da. Carboxymidomethyl­thylation of cysteine was set as a fixed modification, and the oxida­tion of methionine and pyroglutamic acid of N-terminal glutamic acid were selected as variable modifications. The molecular weight predicted for the homologues in the NCBI database was in good agreement with that estimated from the SDS-PAGE gels.

**Determination of soluble carbohydrate content**

Changes in soluble carbohydrates were analysed using a high­throughput GC-MS system. Extraction, derivatization and analysis was carried out according to the method outlined in Roessner et al. (2006), with some variation. TriPLICATE 20 mg samples of frond tissue from each treatment were homogenized under liquid nitrogen, and were extracted in 350 µl of methanol with 20 µl of polar internal standard (0.2 mg ml⁻¹ Ribitol in water), as a quantification standard. The mixture was extracted for 15 min at 70°C and, following centrifugation at 2200 g, non-polar metabolites were removed by the addition of chloroform, and the supernatant was dried in vacuo until derivatization with TMS. A 5-µl volume of the retention time standard mixture [0.029% (v/v) n-dodecane, n-pentadecane, n-non­adecane, n-docosane, n-octacosane, n-dotriacontane and n-hexatria­contane dissolved in pyridine)] was added prior to derivatization. Sample volumes of 1 µl were injected into the GC-MS system, which comprised an AS 3000 autosampler, a Trace gas chromatograph Ultra and a DSQ quadrupole mass spectrometer (Thermo Electron Corporation, http://www.thermo.com). Gas chromatography was performed on a 30-m VF-SMS column with a 0.2 µm film thickness and a 10 m long Integra guard column (Varian, Inc., http://www.varianinc.com) under conditions described by Roessner et al. (2006). Mass spectra were recorded at a rate of 2 scan sec⁻¹ with a scanning range of m/z 70-600. Chromatograms and mass spectra were evaluated using the XCALIBUR program (Thermo Electron Corporation). Mass spectra of eluting TMS compounds were identified using the commercial mass spectra library NIST (http://www.nist.gov), and the public domain mass spectra library of the Max-Planck-Institute for Plant Physiology (http://csdbb.mpimp­golm.mpg.de/csdbdb/dbma/msri.html). All matching mass spectra were additionally verified by determining the retention time by the analysis of authentic standard substances. The resulting relative response ratios were corrected by fresh masses of tissue extracted. Data of selected carbohydrates only are presented as -fold changes in (i) hydrated DT compared with hydrated DS fronds; (ii) hydrated DS compared with dry DS fronds; and (iii) hydrated DT compared with dry DT fronds. The data shown are a combination of two independent treatments. Samples were considered to be signifi­cantly different at the P < 0.05 level according to the Student’s t-test algorithm incorporated into Microsoft EXCEL (Microsoft, http://www.microsoft.com).

The succrose, glucose and fructose contents of fronds and spores were quantitated using a o-glucose/o-fructose sugar assay kit (Boehringer Mannheim, Roche, http://www.roche.com), as described in Illing et al. (2005).

**Statistical analyses**

For physiological and biochemical data, an analysis of variance (ANOVA) was performed to investigate whether there was a signifi­cant difference between the samples, and, if a significant difference was found, a post-hoc Tukey’s honestly significantly different (HSD) test was also performed to tell which samples were responsible for the significant differences. Standard deviations are shown in all such figures.

**Acknowledgements**

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**Supporting Information**

Additional Supporting Information may be found in the online version of this article:

**Video clip S1.** Time-lapse sequence showing rehydration, following drying to the air-dry state, of desiccation-sensitive (DS, left) and desiccation-tolerant (DT, right) fronds of *Mohria caffrorum*. Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.

**References**


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