A proteomic investigation of the rhizomes of the resurrection fern *Mohria caffrorum* L. (Desv.) in response to desiccation

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“Life has always seemed to me like a plant that lives on its rhizome. Its true life is invisible, hidden in the rhizome. The part that appears above ground lasts only a single summer. Then it withers away—an ephemeral apparition. When we think of the unending growth and decay of life and civilizations, we cannot escape the impression of absolute nullity. Yet I have never lost a sense of something that lives and endures underneath the eternal flux. What we see is the blossom, which passes. The rhizome remains.”

— C.G. Jung, Memories, Dreams, Reflections

Contents

Abstract

As there is limited information on the mechanisms of vegetative desiccation-tolerance in resurrection plant rhizomes, this work was undertaken to study the mechanisms of desiccation-tolerance in *Mohria caffrorum* rhizomes. Fronds of this plant have been previously characterized as being desiccation-tolerant in summer and desiccation-sensitive in winter. Since fern rhizomes are perennial organs, it was of interest to establish whether these organs are also perennially desiccation-tolerant and, whether or not the rhizomes regulate desiccation-tolerance in the fronds. Ultra-structural evidence using transmission electron microscopy and viability studies using electrolyte leakage analysis showed that the rhizomes were desiccation-tolerant throughout the seasons. Quantitative proteomics analysis using isobaric tags for relative and absolute quantification was employed to investigate molecular mechanisms of desiccation-tolerance in the rhizomes of this plant. Using a custom fern rhizome specific peptide sequence database, 236 proteins were identified. Of these, 16 proteins increased in abundance while 14 declined, in the summer collected rhizomes. On the other hand, 16 proteins increased in abundance and 20 declined in the winter form. Western blot analysis confirmed the expression trends of heat shock protein 70-2 and superoxide dismutase-[Cu-Zn], which were among the differentially expressed proteins. Bioinformatics analysis of the differentially expressed proteins was carried out using network enrichment tools, to identify key molecular processes and pathways involved in the rhizome response to desiccation stress. Results indicate that the rhizomes use different molecular mechanisms to achieve desiccation-tolerance in winter and summer. Potential cross-talks and cross-tolerances were identified in which mechanisms protecting the rhizomes against desiccation-tolerance appeared to also protect them against heat stress, and in winter an apparent cross-talk against desiccation and pathogen stresses was also identified. This study is the first report of evidence that *M. caffrorum* rhizomes are the 'master-regulator organs' responsible for regulating desiccation-tolerance in the fronds. This role was inferred from the rhizome's predicted up-/down-regulation of biological processes and pathways that relate to leaf senescence, shoot system morphogenesis and gametophyte development, among others.

List of abbreviations

ABA	Abscisic acid
ACN	Acetonitrile
AUX	Auxin
BLAST	Basic local alignment search tool
CAT	Catalase
DS	Desiccation-sensitive
DT	Desiccation-tolerant
EL	Electrolyte leakage
ER	Endoplasmic reticulum
EST	Expressed sequence tags
ET	Ethylene
FA	Formic acid
FDR	False discovery rate
FASP	Filter assisted sample preparation procedure
FDT	Fully desiccation-tolerant
GeneMANIA	Gene Multiple Association Network Integration Algorithm
GO	Gene ontology annotation
GOA	Gene ontology
GR	Glutathione reductase
HPLC	High pressure liquid chromatography
HSP	Heat shock proteins
IAA	Indole acetic acid
IPG	Immobiline DryStrip gel
iTRAQ	Isobaric tags for absolute and relative quantification
JA	Jasmonic acid
LEA	Late embryogenesis abundant
MDT	Modified desiccation-tolerance
MMTS	Methyl methanethiosulfonate
MS	Mass spectrometry

ORF	Open reading frames
PCD	Programmed cell death
PEPC	Protein phosphoenolpyruvate carboxylase
PPI	Protein-protein interactions
PTM	Post-transcriptional modifications
REViGO	Reduce and visualize gene ontology
ROS	Reactive oxygen species
RWC	Relative water content
SA	Salicylic acid
SOD	Superoxide dismutase
SDS	Sodium dodecyl sulphate
SWC	Soil volumetric water contents
TAIR	The Arabidopsis Information Resource
TBS	Tris-buffered saline
TCA	Tricarboxylic acid
TCEP	Tris (2-carboxyethyl)phosphine
TEAB	Triethylammonium bicarbonate
TFA	Trifluoroacetic acid
TEM	Transmission electron microscopy
UPS	Ubiquitin-proteasome system

List of figures

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DECLARATION

I, Ryman Shoko, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other university. I authorize the University to reproduce for the purpose of research either the whole or any portion of the contents in any manner whatsoever.

Signed by candidate

Signature: Date: **16/02/2015**

Chapter 1: Introduction

1.1 Resurrection plants

Resurrection plant tissues can survive loss of up to 95% of their water content, remain in the dry state for prolonged periods of time and upon watering, rapidly restore their normal metabolism (reviewed *inter alia* in Gaff, 1989; Oliver *et al*., 1998; Farrant, 2007; Farrant *et al*., 2012). In the desiccated state, growth and reproduction processes are prevented in resurrection plants until rehydration. The tracheophytes (vascular plants) are generally found in shallow soils or on rocky outcrops in semi-tropical and tropical regions (Porembski and Barthlott, 2000). Under these conditions, they are subjected to frequent cycles of drying and rehydration throughout the year and thus tolerate being dry under both hot and cold environmental conditions. The longest periods of desiccation, however, will naturally be during the non-rainy seasons (Farrant, 2007). Since vegetative tissues of resurrection plants can survive extended desiccation and be able to recover fully upon rehydration, they are described as being desiccation-tolerant (DT) (Gaff, 1971; Farrant, 2000; Farrant *et al*., 2007). While virtually all plants exhibit some degree of desiccation-tolerance at some stage in their life cycle (seeds and spores), resurrection plants are unique in being able to maintain tolerance in vegetative tissues such as leaves, roots and rhizomes. In contrast, vegetative tissues of desiccation-sensitive (DS) plants die when they lose approximately 41 to 70% (depending on the species) of total water content at full turgor (Höfler *et al*., 1941).

The term ‘fully desiccation-tolerant’ (FDT) plants has been used to describe resurrection species such as algae, lichens, and mosses which are capable of withstanding rapid, complete loss of free protoplasmic water (Oliver *et al*., 1998). These plants do not possess complex morphological or physiological adaptations to retain water and while some constitutive protection mechanisms exist, these plants survive desiccation by having efficient repair systems. This indicates that inducible protection mechanisms are not essential for survival in FDT plants (Oliver *et al*., 2000). Pteridophyte and angiosperm resurrection plants are described as having ‘modified desiccation-tolerance’ (MDT) (Oliver *et al*., 1998). There are no extanct gymnosperms that display vegetative desiccation-

tolerance (Bewley, 1979; Farrant, 2007). Compared to fully DT plants, they dry more slowly because they employ as adaptive features, a range of morphological, physiological and biochemical mechanisms to reduce initial water loss and activate cell protection mechanisms in the course of dehydration and repair mechanisms during rehydration (Dace *et al*., 1998; Oliver *et al*., 1998). In this review, only mechanisms reported for MDT plants will be reviewed since this thesis involves examination of a pteridophyte. However, given the evolutionary status of ferns, it is possible that some primitive features displayed in fully DT (FDT) plants might be present.

MDT plants can also be divided into two classes based on the degree of retention of chlorophyll and maintenance of the photosynthetic apparatus during desiccation. Poikilochlorophyllous plants such as the *Xerophyta* species lose their chlorophyll and dismantle thylakoid membranes during drying and re-synthesize them following rehydration (Sherwin and Farrant, 1996; Tuba *et al*., 1996; Sherwin and Farrant, 1998; Tuba, 2008). This strategy achieves significant minimization of photo-oxidative damage and reduces energy costs associated with maintaining the chloroplast structure during desiccation stress (Sherwin and Farrant, 1998; Oliver *et al*., 2000; Tuba, 2008). Consequently, these plants need a longer recovery time (about 80 hours in *X. viscosa*) on rehydration to restore their photosynthetic activities (Walford *et al*., 2004; Farrant, 2007).

Homoiochlorophyllous plants such as the *Craterostigma* species and *Myrothanmus flabellifolius* retain chlorophyll and chloroplast structure during desiccation (Sherwin and Farrant, 1996; 1998; Dace *et al*., 1998; Farrant, 2000; Farrant *et al*., 2003). To preserve chloroplast integrity, these plants rely on mechanisms such as anthocyanin production to neutralize reactive oxygen species (ROS) and leaf folding to prevent chlorophyll-light interactions (Sherwin and Farrant, 1998; Ingle *et al*., 2007). These plants recover rapidly after desiccation and can restore photosynthetic activities within 24 hours of rehydration (Bernacchia *et al*., 1996; Sherwin and Farrant, 1998; Farrant, 2000; Bartels, 2005).

1.2 *Mohria caffrorum*

The resurrection fern *M. caffrorum* (L.) Desv. is unique in that it has been reported to be DT in summer (dry season) and DS in winter (rainy season) (Farrant *et al*., 2009). This fern is endemic to the winter rainfall areas of South Africa, growing in habitats that are partially or fully exposed, mainly in forest margins (Roux, 1979). Fig 1.1 shows the seasonal cycle proposed for this fern, based on observation of responses of aerial fronds to desiccation.

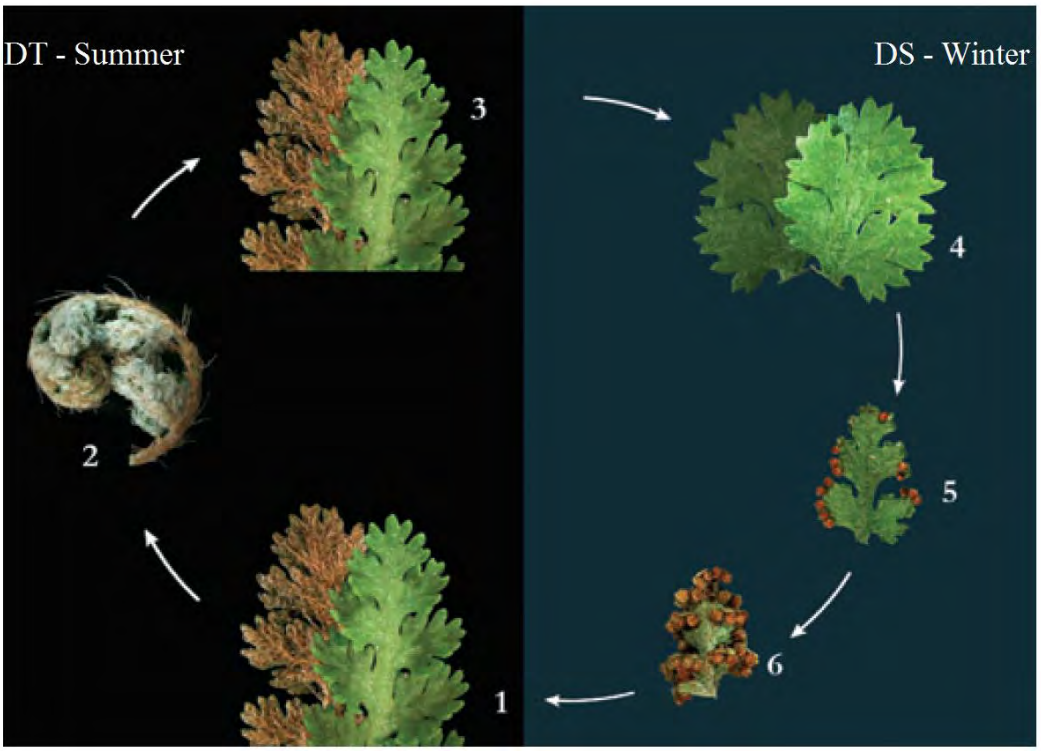


Fig 1.1: Seasonal cycle of *M. caffrorum* showing the transitions between DT and DS forms of the plant (adapted from Farrant *et al*., 2009).

In summer (1, 2 and 3 in Fig 1.1), fronds are DT employing protection features similar to those described in angiosperms. Some of the mechanisms observed in the dry state include; frond curling and presence of reflective hairs to facilitate chlorophyll masking and minimize photosynthetic ROS formation, high anti-oxidant capacity, mechanical stabilization by presence of constitutively plasticized walls (Moore *et al*., 2012) and replacement of water in central vacuoles, *de-novo* production of heat stable proteins,

including a putative chaperonin and accumulation of protective sugars such as sucrose and raffinose family oligosaccharides. During winter (4, 5 and 6 in Fig 1.1), fronds sporulate and the resultant DT spores being released at the end of that season. During this period, fronds are DS and the mechanisms described above for DT summer types are absent or not induced on drying (Farrant *et al*., 2009).

The unique characteristic of displaying seasonal desiccation-tolerance is probably an energy saving mechanism in winter (DT is metabolically expensive to induce and maintain) since, unlike in fully DT species, protection is generally not constitutive. To date, *M. caffrorum* is the only resurrection plant reported to display seasonal desiccation-tolerance. This characteristic makes it an ideal model for assessing the differences between DT and DS states in terms of potential protective mechanisms and signalling involved in regulating tolerance. The fact the fern rhizomes are perennial organs leads to the questions: Do the *M. caffrorum* rhizomes retain tolerance throughout the year, sending up fronds of different tolerances during summer *vs* winter? Or alternatively: is desiccation-tolerance in the rhizomes seasonally regulated as is the case for the fronds? To date, there is little information concerning potential protection mechanisms present in rhizomes during desiccation. Therefore, the present work seeks to carry out physiological and ultra-structural work to establish whether the rhizomes maintain tolerance throughout the seasons. Furthermore, proteomics techniques will be employed to investigate and compare rhizome proteomes of plants desiccated during summer (when fronds are DT) with those desiccated in winter (when fronds are DS). In particular, it is of interest to determine whether the rhizomes act as regulator organs of the seasonal DT observed in fronds.

1.3 Mechanisms of desiccation-tolerance

Desiccation can cause extensive sub-cellular damage, including injury incurred from mechanical stress caused by loss of cell turgor, destabilization or loss of macromolecular integrity from dehydration, and oxidative damage from reactive oxygen species and the associated free radical mediated processes (Figure 1.2; reviewed by Vertucci and Farrant, 1995; Oliver and Bewley, 1997; Walters *et al*., 2002; Berjak *et al*., 2007; Farrant *et al*., 2012). Therefore, there are numerous processes that must be protected in resurrection

plants to withstand this damage. Protection mechanisms employed by MDT resurrection plants to achieve DT consist of very complex processes. Fig 1.2 below presents a simplified outline of the damage caused by desiccation stress in resurrection plants and seeds, and some of the mechanisms of tolerance proposed to offset the effects of the damage.

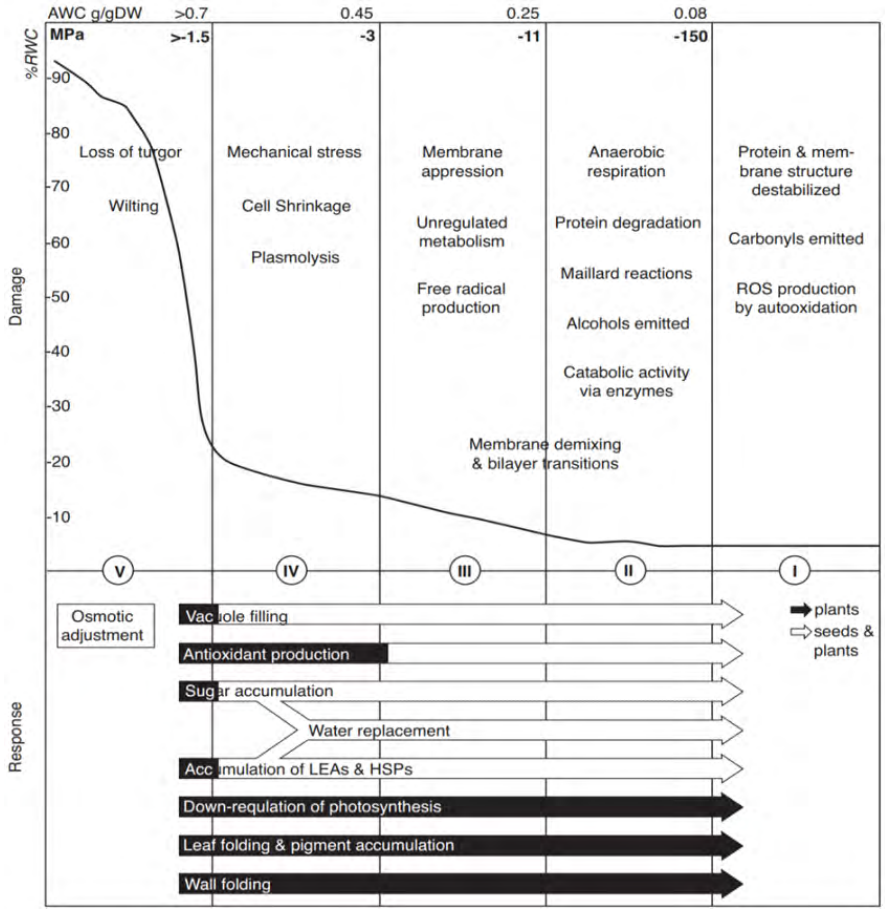


Fig 1.2: Summary of damages caused by desiccation stress in resurrection plants and seeds and mechanisms of osmotic adjustments to offset the effects of the damage. The appropriate protection mechanisms employed in response to water-deficit in both seeds and plants are shown by the white arrows while the black arrows show those present in resurrection plants only. The bold black line represents a typical decline in water content ($gH_2O\ gDW^{-1}$) as a function of relative water content (RWC) in many resurrection plants. (from Farrant and Moore, 2011).

As Fig 1.2 shows, some of the protection mechanisms laid down as a consequence of metabolic stress in response to water-deficit in resurrection plants include the production

and accumulation of non-reducing sugars, an increase in production of anti-oxidants, and the accumulation of stress-related proteins (Farrant, 2000; Scott, 2000; Vicre *et al*., 2004; Illing *et al*., 2005; Farrant *et al*., 2007). Some of the physiological and morphological changes that occur in resurrection plants are cell wall folding, leaf folding and pigment accumulation as well as the down-regulation of photosynthesis (Farrant, 2007).

As shown in Fig 1.2, some of the protection mechanisms e.g. vacuole filling, anti-oxidant production, accumulation of sugar and accumulation of late embryogenesis abundant (LEA) and heat shock proteins (HSPs), known to occur in resurrection plants are also found in seeds particularly during the early stages of drying. The similarity supports the idea that desiccation-tolerance in vegetative tissues of some resurrection plants may have been derived from mechanisms developmentally controlled in seeds during the course of evolution (Bewley and Oliver, 1992; Illing *et al*., 2005; Oliver *et al*., 2005).

From Fig 1.2, it is clear that the protection mechanisms used by resurrection plants to protect against desiccation stress are many and complex. Here, only a few of the important commonly reported mechanisms will be reviewed, *viz,* the desiccation-induced accumulation of: i) reducing sugars, ii) stress associated proteins and, iii) anti-oxidants. For in-depth discussions on induction of specialised protection mechanisms during dehydration to minimize the damage associated with sub-cellular water loss in angiosperm resurrection plants, see reviews by Oliver *et al*., (1998); Vicre *et al*., (2004); Farrant, (2007); Moore *et al*., (2009) and, Dinakar and Bartels, (2013).

1.3.1 Reducing sugars

One of the most important molecular changes in response to desiccation in resurrection plants is the re-routing of normal carbohydrate metabolism (Velasco *et al*., 1994). Enzymes of central carbohydrate metabolism, such as hexokinase (Whittaker *et al*., 2001), and sucrose phosphate synthase (Whittaker *et al*., 2004; Whittaker *et al*., 2007), increase in activity upon desiccation, which leads to the re-direction of carbon flow from reserve substances such as starch or octulose (Scott, 2000) to soluble saccharides. Sucrose and short-chain oligosaccharides such as raffinose and stachyose have been observed to

accumulate during dehydration in resurrection plants and seeds during desiccation (Bianchi *et al*., 1991; Horbowicz and Obendorf, 1994; Hoekstra *et al*., 1997; Norwood *et al*., 2000; Peters *et al*., 2007; Farrant *et al*., 2009; 2012; Ghasempour *et al*., 1998; Cooper and Farrant, 2002; Whittaker *et al*., 2007; Phillips *et al*., 2008; Toldi *et al*., 2009; Oliver *et al*., 2011). The importance of sucrose in the acquisition of desiccation-tolerance was demonstrated in a comparative metabolic analysis of the DT *Eragrostis nindensis vs* the DS *Eragrostis* species (*E. curvula* and *E. teff*). While desiccated leaves of *E. nindensis* accumulated sucrose in response to desiccation, the DS species of *Eragrostis* did not accumulate sucrose (Illing *et al*., 2005). Although trehalose is a vital key compound of some DT animals (e.g. brine shrimp cysts and nematodes), it is a minor component in a few DT angiosperm species (Clegg, 1965; Behm, 1997; Pereira *et al*., 2004; Ghasempour *et al*., 1998).

The functions of sugars that accumulate during dehydration in resurrection plants, are proposed to be mainly protective, e.g. (i) suppression of crystallisation of protoplasmic constituents, (ii) prevention of fusion between membrane systems (iii) prevention of protein denaturation (Hoekstra *et al*., 2001) and, (iv) the promotion of glass formation (Crowe *et al*., 1998; Hoekstra *et al*., 2001). In the glassy state, a liquid has a high viscosity resulting in the slowing of chemical reactions and prevention of interactions between cell components. Thus, a glassy state is very stable and ideal to survive desiccation (Hoekstra *et al*., 2001). Sucrose and trehalose are believed to serve as osmoprotectants for biomembranes and stabilization of biomolecules such as proteins (Hoekstra *et al*., 2001). According to the 'water replacement hypothesis', in highly desiccated cells, these sugars can substitute water in the glassy cytoplasm by binding the proteins and lipids *via* hydrogen interactions (Hoekstra *et al*., 2001).

1.3.2 Stress associated proteins

Dehydration in plants may induce the accumulation of putative structure-stabilizing molecules, such as the LEA proteins (Dure, 1993; Ditzer *et al*., 2001; Collett *et al*., 2004; Farrant *et al*., 2004; Vicre *et al*., 2004; Layton *et al*., 2010). Several specific protective functions have been proposed for LEA proteins, including the protection of DNA,

stabilization of cytoskeletal filaments, acting as molecular chaperones and functioning as anti-aggregants against desiccation-induced protein aggregation in DT organisms (Wise and Tunnacliffe, 2004; Chakrabortee *et al*., 2007). It has been demonstrated that LEA proteins can act synergistically with such sugars as trehalose, to prevent aggregation of proteins during desiccation *in vitro* (Goyal *et al*., 2005). Also, LEA proteins have been shown to form stabilizing structures in the vicinity of membranes (Tunnacliffe and Wise, 2007) and may be involved in the resistance to mechanical failure of the cell wall (Layton *et al*., 2010).

HSPs also play an important role in DT as they tend to accumulate universally in resurrection plants and seeds during desiccation (Ingram and Bartels, 1996; Mtwisha *et al*., 2006; Farrant *et al*., 2007), suggesting a possible cross-tolerance against high temperature stress. Gupta *et al*., (2010) placed the plant HSPs into the following families according to their molecular weights, functions, and amino acid sequence homologies: HSP100, HSP90, HSP70, HSP60, and the small HSPs. The small HSPs, which are usually undetectable in plant cells under normal physiological conditions, are induced upon stress and have been associated with plant tolerance to stress, including desiccation (Wehmeyer and Vierling, 2000; Sun *et al*., 2002; Prieto-Dapena *et al*., 2008). The HSPs are thought to act *inter alia* as molecular chaperones for other proteins, to prevent them from aggregating and denaturing (reviewed by Al-Whaibi, 2011).

The stress protein ubiquitin acts as a tag for denatured, or mis-folded proteins destined for proteolytic degradation *via* the 26S proteasome (Kurepa and Smalle, 2008). When the HSP chaperone system fails to correctly fold a denatured protein, the mis-folded protein is polyubiquitinated and forwarded to the proteasome catabolic process. It is thought that protein degradation *via* the ubiquitin-proteasome system (UPS) plays a key role in controlling cellular processes, including transcriptional control in eukaryotic cells (Hershko and Ciechanover, 1998). Since desiccation stress in tissues is likely to result in down-regulation of many pathways in which continued operation might cause damage in the face of water-deficit stress, proteins associated with the down-regulated pathways need to be removed, probably *via* the UPS. Amino acids from the degraded proteins are

subsequently redistributed and incorporated in the biosynthesis of novel protective proteins (Farrant *et al*., 2012).

1.3.3 Anti-oxidants

ROS can potentially damage proteins, nucleic acids, and lipids. Most of the desiccation-induced cellular damage in plant tissues occurs through the activity of ROS (Smirnoff, 1993). In lichens, the recovery of resurrection plants from the desiccated state correlates with its capacity to establish several anti-oxidant protective mechanisms during dehydration, and to maintain these systems on rehydration (Kranner *et al*., 2002). An important protective mechanism in resurrection plants is the induction of free radical-scavenging enzymes such as superoxide dismutase (SOD), glutathione reductase (GR), and ascorbate peroxidase (APX) to detoxify the ROS (Sgherri *et al*., 1994; Sherwin and Farrant, 1998). These proteins are up-regulated by both DT as well as DS plants to remove ROS upon dehydration, and are considered to be general 'house-keeping' protectants (Illing *et al*., 2005). The major difference between DT plants and DS plants appears to be the ability to sustain these anti-oxidants at elevated levels during the later stages of desiccation where oxidative stress prevails (Mowla *et al*., 2002; Illing *et al*., 2005). The ability of resurrection plants to sustain high levels of anti-oxidants during drying may be a consequence of protecting mechanisms that maintain the anti-oxidant enzymes in their native states, which may result in increased activity and/or half-life of the enzymes, rather than a unique DT mechanism (Illing *et al*., 2005). The preservation of anti-oxidants during drying is also thought to be a strategy to ensure their availability during recovery following rehydration (Farrant, 2007; Moore *et al*., 2009).

Besides the common anti-oxidants mentioned above, there is a diverse range of other anti-oxidants in different resurrection plants. In *Myrothanmus flabellifolia*, polyphenols (e.g. 3,4,5-tri-O-galloylquinic acid) accumulated in response to desiccation. Phenylpropanoids (e.g. coniferylalcohol), phenolics (e.g. caffeate), and flavonols (e.g. apigenin and naringenin) accumulated in the desiccated tissues of *S. lepidophylla* (Yobi *et al*., 2013). Support for the anti-oxidant capacity of polyphenols and phenolic anti-oxidant enzymes has been gained from studies on the resurrection plant *Ramonda serbica* (Sgherri *et al*.,

2004; Veljovic-Jovanovic *et al*., 2006; Veljovic-Jovanovic *et al*., 2008). The seed-specific anti-oxidants (such as 1-cys peroxiredoxin) have been found in *X. humilis* and *X. viscosa* (Ndima *et al*., 2001; Mowla *et al*., 2002; Moore *et al*., 2005), while in *T. ruralis*, 1-cys peroxiredoxin is expressed both during dehydration and rehydration (Oliver, 1996). Several members of the aldehyde dehydrogenase (ALDH) family have been identified in dehydrated vegetative tissues of resurrection plants (Velasco *et al*., 1994; Kirch *et al*., 2001; Abdalla and Rafudeen, 2012). In *Tortula ruralis*, the ALDH21A1 gene (AI305018) is thought to have a critical role in the detoxification of aldehydes generated in response to salinity and desiccation stress (Chen *et al*., 2002).

1.4 Desiccation-tolerance in underground organs

While research on protection mechanisms present in leaves and fronds of resurrection plants has progressed steadily (*inter alia* Sherwin and Farrant, 1998; Farrant, 2000; Mowla *et al*., 2002; Balsamo *et al*., 2005; Farrant *et al*., 2007; Ingle *et al*., 2007; Moore *et al*., 2007; Farrant *et al*., 2009; Abdalla and Rafudeen, 2012), little is known about protection mechanisms within underground organs such as roots and rhizomes. This is because, when excavating these organs from the soil, there is a risk of damaging the finer structures. Also, there is a high risk of microbial contamination due to fine soil particles adhering to the roots, since washing off the soil can alter tissue water contents. All these factors have deterred many researchers from pursuing studies on the underground organs. However, understanding protection mechanisms employed by underground systems is important because they are the first to perceive the soil moisture deficit by virtue of their being in direct contact with the soil.

Here, a summary of the main findings in some underground systems is presented. Among pteridophytes, only *Polypodium vulgare* rhizomes have had their rhizomes meaningfully studied at a physiological and anatomical level to observe the response to desiccation (Zenkteler and Bagniewska-Zadworna, 2005; Bagniewska-Zadworna and Zenkteler, 2006; Bagniewska-Zadworna *et al*., 2007; Bagniewska-Zadworna *et al*, 2008). The main findings from these investigations will be outlined in Chapter 2, where similar work conducted on *M. caffrorum* is reported. Currently, there is no large scale study reported on the

characterization of the molecular mechanisms of desiccation-tolerance in pteridophyte rhizomes.

Kamies *et al*., (2010) designed an aeroponics system which they used to investigate anti-oxidant activity in the roots of *X. viscosa* in response to desiccation. Using the system, it was reported that *X. viscosa* roots retained anti-oxidant (SOD, catalase (CAT) and APX) potential during drying, just as has been reported in leaf tissues of this and other resurrection plants (Kamies *et al*., 2010).

Taking advantage of the innovative aeroponic system, Kamies, (2011) carried out a proteomics investigation of *X. viscosa* roots using off-gel fractionation followed by liquid chromatography tandem mass spectrometry (LC-MS/MS). While the study succeeded in isolating proteins from hydrated roots, methodological difficulties in working with dry roots precluded the author from doing comparative proteome studies on dehydrating root tissues. However, it was reported that there was significant expression of 80 proteins belonging to several functional classes, *viz* carbohydrate metabolism, energy metabolism, protein metabolism, anti-oxidant mechanism, stress response, apoptosis, protein transport and signalling and nucleosome assembly. As is the case for non-model organisms in which genomes have not yet been sequenced, there were also several unknown proteins found.

A few isolated studies have also been reported on the mechanism of DT in geophytes (bulbs, tubers and corms). In the tuberous roots of *Ranunculus asiaticus*; light microscopy, histochemical analysis, and protein analyses by one dimensional SDS-PAGE showed that during growth and maturation of the roots, cortical cells increased in size with their cell walls accumulating pectins in primary walls and, the starch granules and protein bodies increased in number (Kamenetsky *et al*., 2005). In the same study, it was observed that after dehydration and quiescence and rehydration of the roots, cell walls decreased in thickness, and a significant quantity of starch granules and proteins were lost from the cells (Kamenetsky *et al*., 2005). The authors proposed that these underground organs store nitrogen and carbon reserves within their cells, and accumulate pectin within the cell walls to support plant growth following summer quiescence and rehydration.

Desiccation-tolerance is also known to exist in the *Anemone coronaria* bulbs, but there seems to be little systematic research to determine whether these are truly DT (Antipov and Romanyak, 1983; Proctor and Pence, 2002). *Poa bulbosa* has a rare characteristic of DT in which its bulbs are believed to be capable of undergoing periods of summer dormancy (Lazarides, 1992; Volaire and Norton, 2006). While some dormancy associated genes have been identified in resurrection plant tissues, it is unlikely that resurrection plants can undergo periods of dormancy (Volaire and Norton, 2006; Farrant, pers. com.). Indeed, it is highly contested that *P. bulbosa* is a true resurrection plant (Lazarides, 1992; Volaire and Norton, 2006).

1.5 Tools for studying resurrection plants

DT in resurrection plants is usually studied *via* several approaches, including; physiological, ultra-structural, biochemical, molecular and the 'omics' technologies. Since physiology/ultra-structural analysis and proteomics were the major tools used in the experimental investigations of *M. caffrorum* in this study; a brief review of the status of these approaches in resurrection plants is presented below.

1.5.1 Physiology and anatomy

Since extremes of water-deficit stress disrupt normal physiological activities and may result in cellular damage or cell death, much work has been directed at understanding the physiological and structural aspects of desiccation-tolerance (Vander Willigen *et al*., 2001; Cooper and Farrant, 2002; Vicre *et al.*, 2004; Farrant *et al*., 2009; Ginbot and Farrant, 2013).

Ultra-structural evidence is an essential tool in the understanding of desiccation-tolerance of plant tissues. The ability to observe structural changes in tissues under water-deficit stress has contributed considerably towards explaining and confirming the physiological, biochemical and molecular aspects that have been analysed during both dehydration and rehydration in DT plants (Hallam, 1976; Gaff, 1980; Platt *et al.,* 1997; Sherwin and

Farrant, 1998, *inter alia*). Electron microscopy provides the means to examine tissues at an ultra-structural level.

Desiccation causes changes in membrane structure and composition, and may cause significant cell membrane injuries and ion leakage from plant tissues (Blum, 1988). It has been proposed that electrolyte leakage (EL) exceeding 50% of that displayed in hydrated tissues indicates considerable disruption of membrane structure and is a measure of cell death (Bewley, 1979). Therefore the determination of EL is an important test for cell viability.

Besides studies on *P. vulgare* already mentioned above, there is very little in the literature concerning the physiological and ultra-structural response of roots and rhizome to desiccation. The reasons for that have already been mentioned. In the present study, the focus of the application of physiological and ultra-structural investigation will be directed at determining whether or not the *M. caffrorum* rhizome is constantly DT. Ultra-structural and viability studies would make it possible to assess the integrity of cellular or organelle systems in response to desiccation.

1.5.2 Proteomics

Advances in the"omics" technologies (transcriptiomics, proteomics and metabolomics, etc) have made it possible to quantitatively analyse changes in the abundance of biological molecules in a high-throughput manner. This has made it possible to compare levels of molecules between DT and DS species, or to observe changes in the molecular profiles of the plant at different hydration states. The objective of the 'omics' techniques in resurrection plants is to capture the maximum information possible on the alteration of the transcripts/proteome/metabolome profiles in response to desiccation and/or rehydration (Dinakar and Bartels, 2013). A successful 'omics' experiment is capable of providing an outline of the molecular situation in the cell at a specific level of hydration. For a detailed discussion of outputs from these 'omics' technologies in resurrection plants, see the review by Dinakar and Bartels (2013). Here, only the application of proteomics in resurrection plants research will be emphasized.

The identification of the abundant transcripts gives an indication as to which molecular processes may be important at different physiological conditions. Thus, useful insights have been obtained from transcript analysis on the mechanisms of desiccation-tolerance in resurrection plants (Gaff *et al*., 1997; Blomstedt *et al*., 1998; Collett *et al*., 2004, Rodriguez *et al*., 2010; Gechev *et al*., 2013). While transcript level analysis of gene expression is a powerful technique to understand desiccation-tolerance, the weakness of the approach lies in that mRNA abundances may only represent putative function as mRNA levels do not necessarily correlate with protein expression patterns (Bernacchia *et al*., 1995; Gygi *et al*., 1999). This is mainly due to the instability of the transcript or due to post-transcriptional modifications (PTMs). On the other hand, proteomics provides a snapshot of the molecular processes by identifying and quantifying the actual proteins performing the enzymatic, regulatory, and structural functions encoded by the genome and expressed by the transcriptome at a given point in time. Thus, proteomics provides information on the end-point of gene expression.

Several proteomics studies have been reported for leaves of angiosperm resurrection plants during desiccation (Röhrig *et al*., 2006; Ingle *et al*., 2007; Jiang *et al*., 2007; Abdalla *et al*., 2010; Wang *et al*., 2010; Oliver *et al*., 2011; Abdalla and Rafudeen, 2012) but there are few reports on the proteomics of underground organs. The thesis by Kamies (2011) elegantly describes the problems associated with conventional means of protein extraction and separation from root tissues.

For those plants that have them, rhizomes are diageotropic subterranean stems of vital importance to plant growth, competitiveness, propagation and persistence (Hu *et al*., 2003; Jang *et al*., 2009). Despite this, the first large scale plant rhizome proteomics project was reported only recently (Balbuena *et al*., 2012). The investigation reported on the proteomic characterization of the fern *E. hyemale*. Though *E. hyemale* is a DS plant, this study provided useful insights on the molecular processes underlying fern rhizomes. In the present work, proteomics were employed in the context of understanding protection mechanisms of *M. caffrorum* rhizomes against desiccation, and to establish whether rhizomes are master regulatory organs of the ferns which send molecular signals to the fronds to switch to the DS or DT state.

1.6 Objectives of this study

The first aim of this study was to determine whether or not *M. caffrorum* rhizomes remain permanently DT during all seasons. To achieve this aim, ultra-structural and viability analysis (EL) were performed on fully hydrated and dehydrated (<15% RWC) rhizomes from plants collected in winter (DS fronds) *vs* those collected in the summer (DT fronds). The second aim was to observe and quantify the differential protein expression profiles in the rhizomes of such plants in response to moisture deficit stress. To achieve this aim, isobaric tags for relative and absolute quantification (iTRAQ) analysis was employed to compare the protein profiles of rhizomes of fully hydrated *vs* fully dehydrated states. Western blotting analysis was used to validate iTRAQ quantification and protein identification. Bioinformatics pipelines were used to predict molecular mechanisms of desiccation-tolerance.

Chapter 2: Physiology and ultra-structural characterization of *M. caffrorum* rhizomes in response to desiccation stress

2.1 Introduction

The damage to cell structure in tissues, subjected to different levels of dehydration has been reviewed in certain DT species (Farrant, 2000; Brighina *et al.*, 2002; Farrant *et al*, 2007). Among DT pteridophytes, only *P. vulgare* has been meaningfully studied in relation to ultra-structural changes in rhizomes during desiccation stress. Briefly, studies in *P. vulgare* showed that the rhizomes cope with dehydration through cytological and osmotic adjustments (Zenkteler and Bagniewska-Zadworna, 2005; Bagniewska-Zadworna and Zenkteler, 2006; Bagniewska-Zadworna *et al*., 2007). Microscopic observations showed that the rhizomes have several structural features that allow them to protect themselves against effects of drying e.g. maintaining water inside the stele (Bagniewska-Zadworna and Zenkteler, 2006). Other reported features include possession of Casparian strips on the endodermal walls, thicker cell walls of cortical parenchyma cells bordered by endodermal cells. In parenchymatous cells of the pericycle and vascular bundle, the cell ultra-structural changes observed after dehydration included slightly condensed chromatin in the nuclei, smaller starch grains and vesicle formation in the cytoplasm (Bagniewska-Zadworna and Zenkteler, 2006). Also, numerous electron-dense vesicles were formed in the endodermal cells in dehydrated rhizomes (Bagniewska-Zadworna and Zenkteler, 2006).

Dehydrated leaf tissues of DT plants display specific ultra-structural features which indicate the establishment of protective mechanisms (Farrant and Sherwin, 1998; Dace *et al*. 1998; Farrant, 2000). These include vacuoles filled with electron dense bodies and sub-division and maintenance of membrane integrity. In contrast, sensitive tissues show damage to organelles and membranes which is exacerbated upon rehydration (Dace *et al*. 1998; Farrant, 2000).

This chapter presents physiological, viability and ultra-structural responses to dehydration in *M. caffrorum* rhizomes collected in winter *vs* those collected in summer. The relative tolerance to water-deficit was evaluated based on changes in EL (to provide an indication of membrane damage) and ultra-structural organization. The ultimate aim of this chapter was to determine whether *M. caffrorum* rhizomes were perennially DT across all seasons.

2.2 Methods

2.2.1 Plant collection, management and stress treatment

Adult ferns were collected from their natural habitat on the Table Mountain, (33°57'16.6"S 18°27'39.3"E) adjacent to the University of Cape Town Upper Campus (South Africa) during winter and during summer. Fig 2.1 below shows the aerial picture of the site where the plants were collected.

Fig 2.1: Aerial photograph of the area where the ferns were collected on Table Mountain, (33°57'16.6"S 18°27'39.3"E). The specific site is indicated by a star.

Only mature, healthy plants (with no visible stress symptoms) of uniform size (apical height of 180-220 mm) were used for the experiments described in this thesis. Further, only plants with about five fronds and with relatively similar rhizome length and diameter were selected. These were assumed to be at the same growth and developmental stage. The ferns were transported to the laboratory where they were potted in well-drained plastic trays (length = 45 cm, width = 30 cm and depth = 7 cm), using soil collected from their natural environment. In each tray, 10 plants were randomly planted at a depth of between 1.5 cm and 2 cm and, the plants were allowed to acclimatize for two weeks in a growth chamber (Percival, USA) under controlled environmental conditions before experimentation proceeded. Plants collected in winter were maintained at 12 - 20^{O}C; with a photoperiod of 11 hours day and a 13 hour night. Plants collected in summer were

maintained at 24^{o}C - 28^{o}C and the day/night durations were fixed at 16/10 hours respectively. A data logger was used to monitor and verify the maintenance of the set conditions. Fresh collections were made at least twice *per* season, the work being repeated over 2 years.

Once in environmental chambers, plants were watered with tap water regularly to maintain field capacity during the two week period of acclimation. In order to verify the absence of a moisture gradient between trays, soil volumetric water contents (SWC) were monitored regularly using a five pronged Thetaprobe moisture sensor (Delta-T Devices). Moisture stress was initiated by cessation of watering and plants were allowed to dry in the growth chamber under the defined growth chamber conditions. Rhizome water status was determined gravimetrically by oven drying at 70^{o}C for 48 hours and was calculated as both absolute water content (AWC) (g H_2O.g dry mass^{-1}) and 'normalized' RWC (Sherwin and Farrant, 1996). Our use of the 'normalized' RWC compares the water status of tissues relative to that at full turgor. For full turgor determination, rhizome samples were placed for 24 hours in distilled water and water content gravimetrically determined thereafter. Rhizomes were sampled at regular intervals during dehydration for the experiments outlined below. Dehydration time courses were performed over 2 successive years.

2.2.2 Gross rhizome anatomy

Wet and dry rhizomes collected in winter and summer were hand sectioned using a single edge, carbon steel razor blade and observed under a Nikon SMZ1500 Stereoscopic Zoom Microscope fitted with a Nikon DS-5M digital camera (www.nikon.com).

2.2.3 Electrolyte leakage

Membrane integrity of rhizome tissues was measured using a CM100 multiple cell conductivity meter (Reid and Associates). At each time point, rhizome segments were placed in purified deionised water (Millipore, milli-Q water system) within a 100 well

plate. The initial leakage was measured as the change in conductivity over a 1 hour period, with measurements taken every 60 seconds. The maximum leakage was obtained after repeated snap freezing in liquid N_2 and thawing of the rhizome samples. The EL was presented as a percentage of the maximum leakage. The experiment was conducted in triplicate for each time point.

2.2.4 Transmission electron microscopy (TEM)

To observe the possible differences in the cellular organization of rhizomes collected in summer *vs* those collected in winter and thus postulate possible mechanisms of desiccation-tolerance in the two forms, the ultra-structure of cortical cells (non-vascular tissue) were examined. TEM was carried out in accordance with the method described by Sherwin and Farrant (1996), with modifications. Small cortical rhizome pieces (less than $3mm^3$) were fixed in 2.5% glutaraldehyde with 0.5% caffeine in 0.1M phosphate buffer (pH 7.4) for a week and post-fixed with 1% osmium tetroxide (OsO4) for 2 h. Specimens were washed three times in phosphate buffer solution for a total of 15 minutes. Tissues were then dehydrated using an ethanol gradient followed by gradual infiltration with epoxy resin (Spurr, 1969) over 14 days. Polymerization was achieved by keeping the resin impregnated samples at 60°C for at least 16 hours. Specimens were sectioned using a Reichart Ultracut-S (Leica) microtome and stained with 1% uranyl acetate and 1% lead citrate (Reynolds, 1963) for 10 min each. Rhizome cortex sections were then viewed with a LEO912 transmission electron microscope (Zeiss, Germany). On average, 5 samples from at least three different rhizomes were investigated.

2.3 Results and discussion

2.3.1 Rhizome dehydration

Since the plants were dried under different environmental conditions (winter *vs* summer conditions), the rhizome RWC decreased at different rates (Figs 2.2). At full turgor, the absolute water contents of the rhizomes collected in summer and winter were 4.25±0.41 and 4.88±0.65 g H_2O.g dry mass^{-1} respectively. The RWC of rhizomes from the plants dried in summer conditions showed a notable decrease after about eight days and reached a RWC of less than 15% after 20 days. The RWC of rhizomes from the winter plants equilibrated at 15% RWC after 34 days. The longer drying time of rhizomes from the winter plants can be attributed to the cooler temperatures which may lead to reduce transpiration and slow water loss.

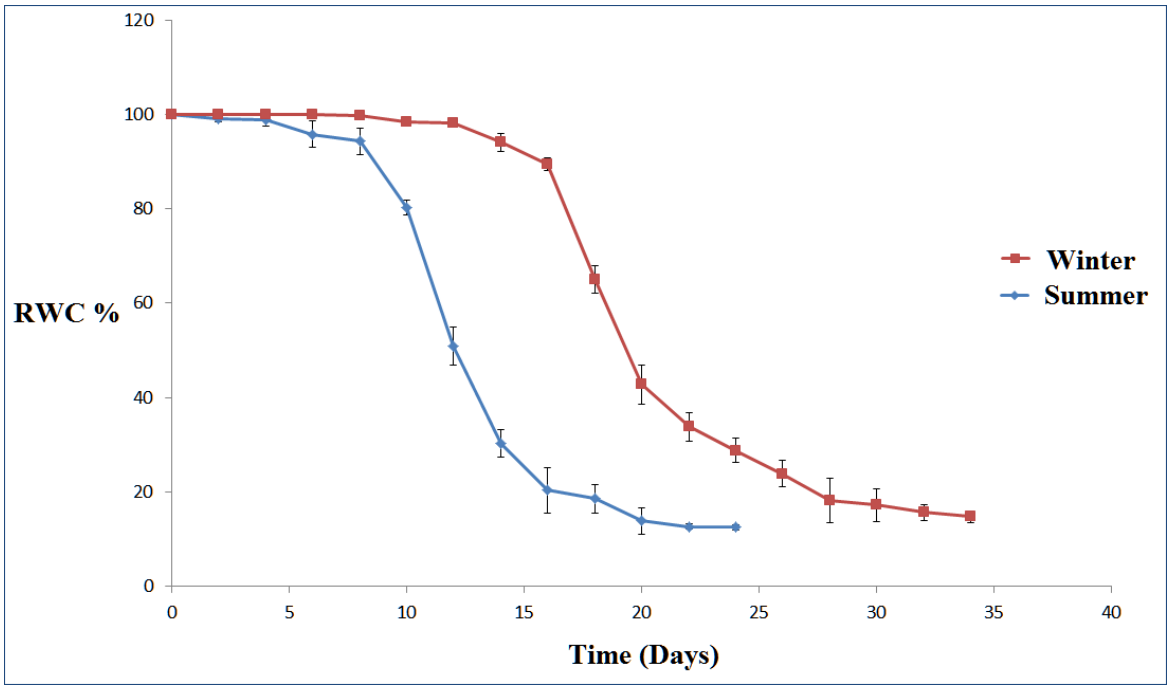
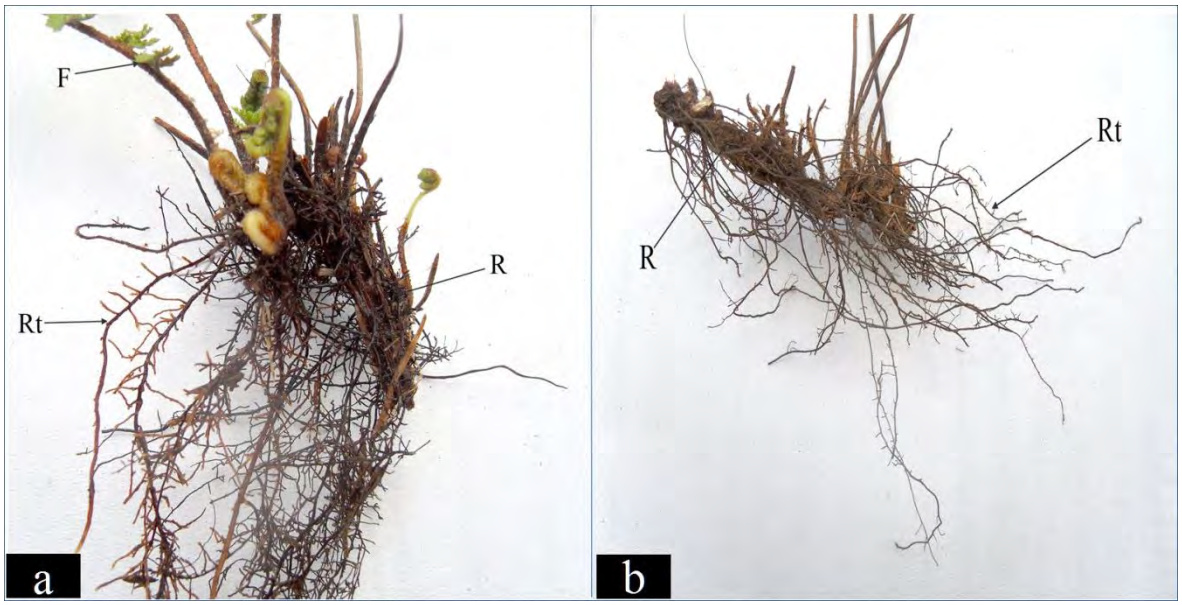


Fig 2.2: Time course of the changes in normalized RWC during dehydration of *M. caffrorum* rhizomes dried under summer conditions. Error bars represent standard errors.

As Fig 2.2 shows, the RWC of rhizomes equilibrate at RWC of slightly below 15%. Therefore, for the rest of this thesis, dry rhizomes shall refer to rhizomes of RWC ≤15% while rhizomes are considered to be fully hydrated when RWC ≥95%.

2.3.2 *M. caffrorum* rhizome

Wet and dry rhizomes collected in both summer and in winter did not show any visible differences when examined by the naked eye and only those from the summer collected plants are shown in Figs 2.3a and b.

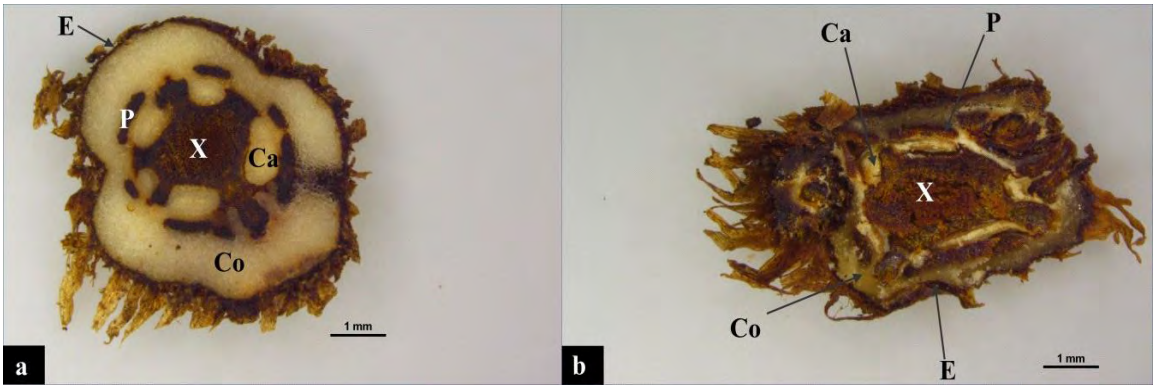


Figs 2.3a and b: Photographs of a typical hydrated (left) and dehydrated (right) *M. caffrorum* rhizomes collected in summer. F = frond, Rt = root and R = rhizome.

As shown in Fig 2.3, the rhizomes contain an extensive network of roots and fronds. Since the work described in this thesis was conducted on rhizome tissue only, all root and frond tissues were carefully removed prior to further to experimentation.

2.3.3 Internal structure of *M. caffrorum* rhizome

Figure 2.4 shows cross-sections from hydrated (a) and dry (b) *M. caffrorum* rhizomes from plants collected in winter. Again, there were no differences in structure between summer and winter rhizomes (data not shown). On dehydration, there was considerable shrinkage of the tissue as water was lost.



Figs 2.4a and b: Photomicrographs showing cross-sections of hydrated (left) and dehydrated (right) *M. caffrorum* rhizomes collected in winter. E = endodermis, Co = cortex, Ca = cambium, P = phloem and X = xylem.

As shown in Fig 2.4, on dehydration, there was considerable shrinkage of the tissues as water was lost. The rhizomes have an internal structure resembling that of a dicotyledonous root. The epidermis, which is the outer layer of the rhizomes, protects the rhizomes against physical damage. The cortex functions as a store for carbohydrates and other substances such as resins, latex, oils, and tannins. The vascular bundles (phloem and xylem) are arranged as a central cylinder in the rhizome. The xylem system carries water and nutrients throughout the plant.

2.3.4 Electrolyte leakage

EL analysis results for both the summer rhizomes and winter rhizomes did not show any significant difference. In both cases, there was not much change in the EL between the dehydrated rhizomes relative to the fully hydrated controls (Fig 2.4). This suggests that the

rhizomes are able to maintain their membranes and cellular integrity during desiccation stress.

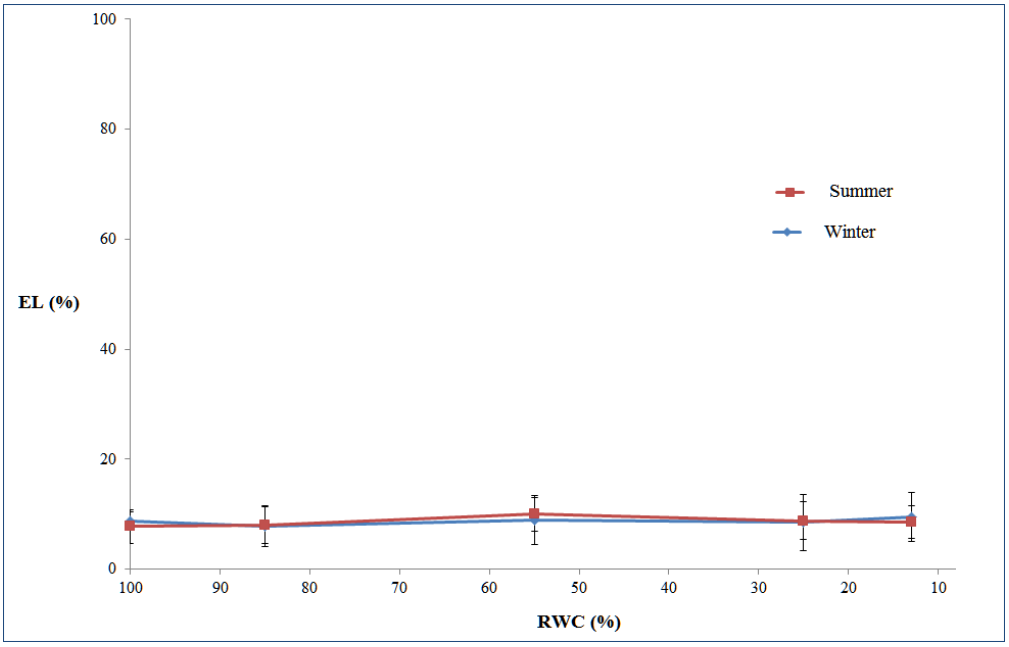


Fig 2.5: EL expressed as a percentage of maximum leakage from dead tissues, of rhizome cortical cells from the summer (red line) and winter (blue line) ferns. The error bars represent the standard error.

Maintenance of membranes and overall cellular integrity by the rhizomes shows that *M. caffrorum* rhizomes are well adapted to stress conditions and can maintain viability throughout the year.

2.3.5 Ultra-structure of *M. caffrorum* rhizomes (summer *vs* winter)

To observe the possible differences in the cellular organization of rhizomes collected in summer *vs* those collected in winter and thus infer possible mechanisms of desiccation-tolerance in the two forms, the cortical cells (non-vascular tissue) were examined using TEM micrographs of hydrated tissues from both summer and winter collected rhizomes is

given in Figs 2.6a and 2.6b and that of dehydrated rhizomes are shown in Figs 2.7a and 2.7b.

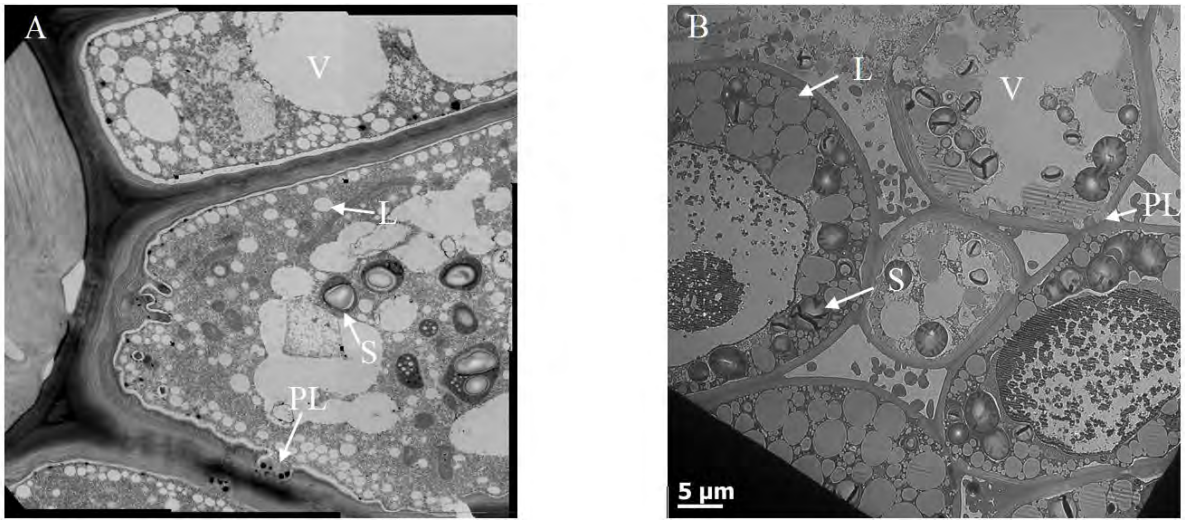
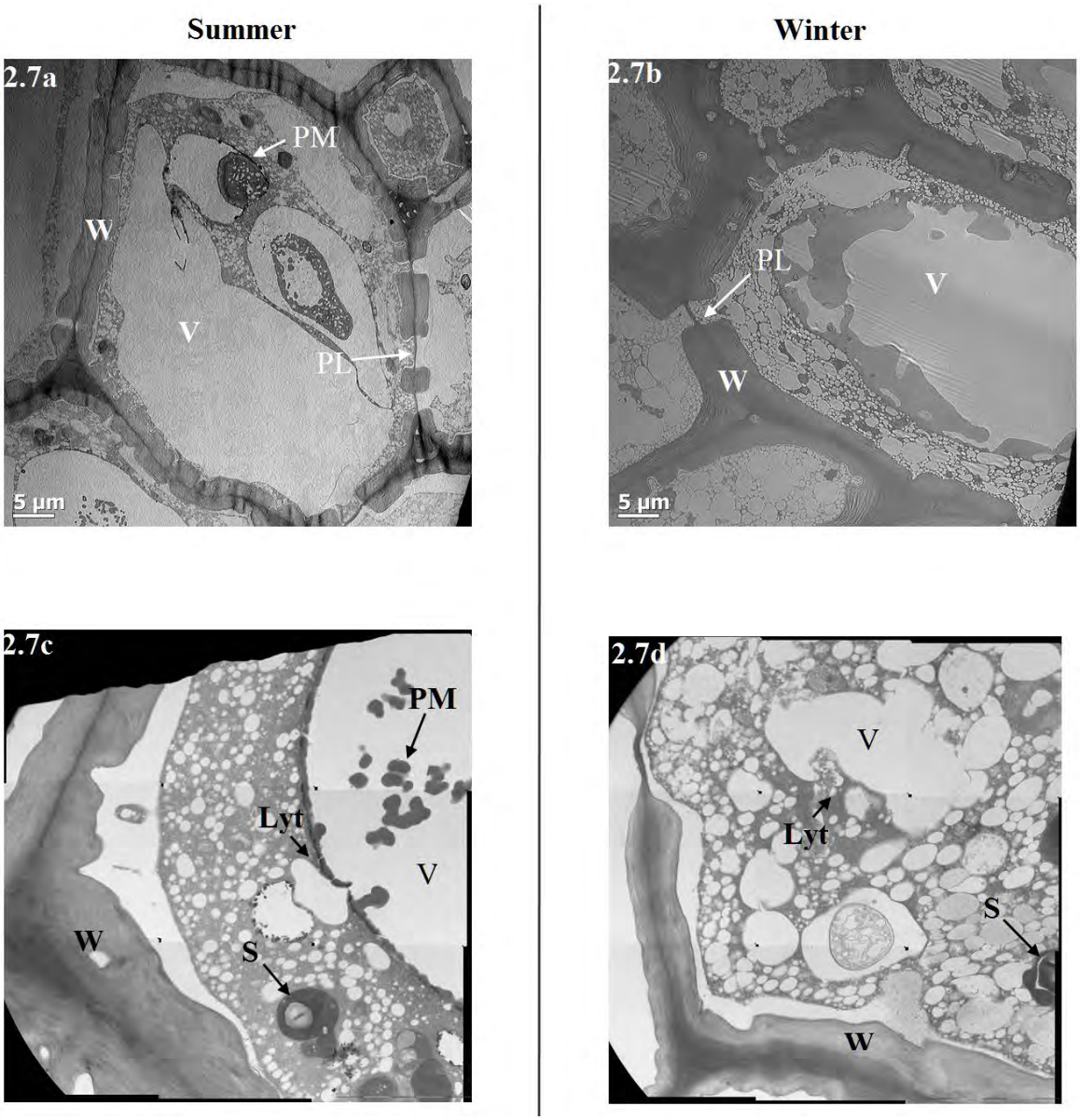


Fig 2.6a and b: Representative electron micrographs of hydrated summer (left) and winter (right) cortical cells. PL = plasmodesmata, L =lipid, S = starch, V = vacuole.

As shown in Figs 2.6a and b, in the hydrated state, both summer and winter collected rhizomes had a sub-cellular organization typical of well hydrated, generally unstressed cells. Cell walls were clearly transversed by plasmodesmata suggesting cell-to-cell communication. The tissues were vacuolated as typical of the hydrated condition, with small amounts of dense materials that we propose to be polyphenols as evidenced by dense electron bodies. The cytoplasm contained considerable storage reserves in the form of lipid bodies and starch granules.

The electronmicrographs of the dehydrated rhizome cortical cells from the summer and winter plants are shown in the Figures 2.7a, b, c and d below.



Figures 2.7a, b, c and d: Representative electron micrographs of dehydrated summer (left) and winter (right) cortical cells. PM = polyphenolic material, PL = plasmodesmata, S = starch, W = cell wall, L = lipid, V = vacuole, Lyt = lytic activity.

Generally; rhizome cortical cells from both the summer and winter dried plants appeared to be well preserved after the desiccation treatment, as shown in the Figures 2.7a-d above. While some detachment of the plasmalemma from the cell wall was evident in both the summer and winter tissues, there was no membrane damage observed. The detachment is likely to be a consequence of fixation rather than desiccation-induced damage. The preservation of membrane integrity agrees with the EL results. Cell walls were well

preserved and appeared to be more punctuated with large plasmodesmata conduits (arrowed on the Figure 2.7a and b), suggesting increased cytoplasmic communication between cells. The vacuolar contents were largely electrontransparent although osmophilic bodies were observed in some vacuoles. It is unlikely that the vacuoles contained water, as the tissue moisture content was <15%. It is proposed that these vacuoles contain compatible solutes, as proposed for frond tissues in this species (Farrant *et al*., 2009) and leaf tissues of many angiosperm resurrection plants examined (Farrant, 2000; 2007). The vacuoles showed signs of lytic activity, which suggests that some reserve mobilization and sub-cellular re-organization took place during desiccation in both summer and winter collected rhizomes. In both the dried summer and winter rhizome tissues, in most cells examined, there was little evidence of lipid reserves observed in the dehydrated tissues. There were very rare starch granules, only present in a few cells in both the summer and winter dried tissues. Depletion of starch in rhizomes subjected to desiccation was also reported in *P. vulgare* rhizomes (Bagniewska-Zadworna, 2007). It has been proposed that carbon, required for sucrose synthesis, might originate from starch breakdown (Veramendi *et al*., 1999; Whittaker *et al*., 2001). Therefore, it is possible that starch hydrolysis products could be a source for sucrose synthesis, which is an important osmolyte promoting osmotic adjustment during plant drying (Scott, 2000). Sucrose can function in water replacement for protection of membranes and vitrification (the formation of a stable glass to put a stasis on metabolism, as described in Chapter 1), which is important for protection. Large amounts of probable polyphenols were observed in the cells examined in the summer rhizomes compared to those from the winter plants. The presence of polyphenols in the cortical cells is not surprising since rhizomes are known to contain higher levels of polyphenolic compounds (caffeic and glucocaffeic acids, catechins, flavonoids, methyl salicylate, phloroglucin, lophenol) (Duke, 2000). As mentioned in Chapter 1, polyphenols may have anti-oxidant capacity which can be beneficial to the cells in quenching desiccation-induced ROS. The apparent high content of polyphenols in summer-dried rhizomes compared to the winter tissues is probably related to the harsher conditions (high temperature) characteristic of summer.

2.4 Concluding remarks

Ultra-structural analysis and EL results suggest that the rhizomes are indeed DT throughout the year. These results differ from the findings in the frond tissues where it was established that cellular integrity of fronds from winter plants was compromised by desiccation. Therefore, observations from this chapter imply the rhizomes act as the principal organ which determines whether new fronds are DT or DS depending on the season. For the purpose of this thesis, the summer rhizomes shall be termed ‘DT rhizomes’ while winter rhizomes shall be termed ‘DS rhizomes’.

While these results may have value ultimately in helping to define the pivotal role of the rhizomes in determining tolerance of fronds, they do not supply any insights into distinctive mechanisms by which this regulation is achieved. Thus, the following chapters aim to identify the molecular mechanisms by which the rhizomes achieve tolerance and identify possible communication between rhizomes and fronds.

Chapter 3: The quantitative proteomic characterization of *Mohria caffrorum* rhizomes in response to desiccation

3.1 Introduction

Since the *M. caffrorum* rhizome appears to retain the ability to tolerate desiccation perennially (Chapter 2), and since it is this stem-like organ that gives rise to DT fronds in summer and DS in winter, it is likely that the rhizome regulates, to some degree, the nature of tolerance displayed in the above ground tissues. The aim of the remainder of this thesis was thus to investigate the rhizome proteins from the DT and DS forms of the plant in the fully hydrated and fully dehydrated states. It was hoped that such an approach would enable identification of proteins that might give insight not only into mechanisms of desiccation-tolerance in this organ, but also into potential involvement in the seasonal production of DT or DS fronds.

In the last few decades, many proteomics platforms have been developed for the qualitative and quantitative analysis of protein mixtures and post-translational modifications, e.g. a combination of 2D-PAGE and LC-MS/MS (geLC-MS/MS) (Kubota *et al*., 2009), and iTRAQ (Ross *et al.*, 2004). The advantage of the iTRAQ technique is that it enables the parallel identification and quantification of up to eight protein samples using labelled peptides identified by sensitive mass spectrometers (Evans *et al*, 2012). In the present study, iTRAQ labelling was successfully employed, coupled to off-gel electrophoresis and electrospray ionisation quadrupole time-of-flight tandem mass spectrometry (ESI-Q-TOF-MS/MS), to assess proteome changes in *M. caffrorum* rhizomes in response to desiccation. The iTRAQ technique involves the isobaric labelling using isobaric tags (identifiable stable isotopic molecules covalently linked to all amines, n-terminus and side chains, in a protein) – allowing quantification of peptides in a mass spectrometer. We took advantage of the multiplexing ability of the iTRAQ technique to perform a simultaneous characterization of the DT and DS rhizome proteomes in response to desiccation.

Proteomics research depends heavily on the use of MS/MS that produces thousands of MS/MS datasets consisting of individual mass spectra. Protein identification pipelines

usually rely on search algorithms that compare the MS/MS spectra to reference databases to find exact matches. Therefore, the availability of a relevant sequence database is a critical requirement for the fast, accurate and unsupervised interrogation of these spectra to provide accurate identifications (Balbuena *et al*., 2012; Romero-Rodríguez *et al*., 2014). The chosen database determines, to a large extent, the number of identifications obtained and the quality of the identifications.

The major limitation of proteomics approaches in resurrection plants is the identification of an appropriate database due to the absence of genome sequence information on these plants. The lack of genome information has resulted in many researchers reporting identifications of unknown proteins or proteins of unknown functions. For example in the fern-ally *Selaginella tamariscina*, using 2DE, 138 dehydration-responsive protein spots were identified of which 35 were unknown (Wang *et al*., 2010). Protein identification in plants whose genomes have not been sequenced (orphan plants) commonly rely on cross-species identifications against the Viridiplantae database (from NCBI and UniProt/Swiss-Prot) or *A. thaliana* to identify closest homologs. The disadvantage of using such an approach is that the proteins from orphan plants are often under-represented in the database, thereby resulting in poor identification rates or poor quality identifications. The under-representation of proteins from orphan plants in such databases can be explained by the fact that either some species-specific proteins will not be present in the databases or homologous proteins will show small evolutionary differences in their sequences.

In order to improve protein identification rates and quality in orphan plants, custom databases can be constructed from species-specific RNA sequences or publicly available RNA sequences of closely related species (such as expressed sequence tags (ESTs) available in public repositories). Such databases have been shown to increase the number of identified proteins together with better specificity and confidence of protein identification (Lippert *et al*., 2009; Lei *et al*., 2011; Valledor *et al.*, 2012; Romero-Rodríguez *et al*., 2014). When such custom databases are also organ-specific, the accuracy of protein identifications is further increased (Balbuena *et al*., 2012; He *et al*., 2014).

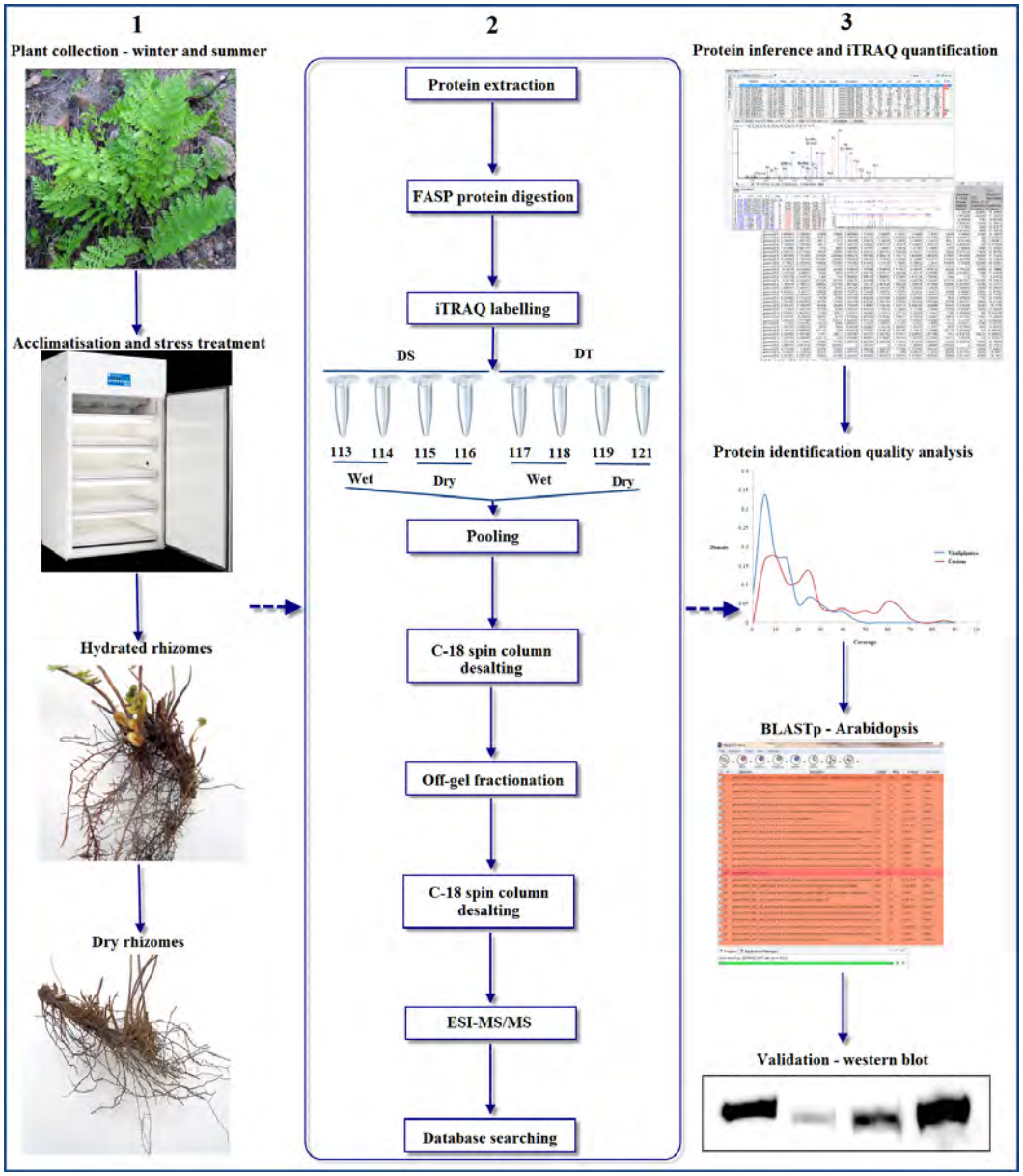
In the present study, a fern rhizome-specific custom database generated from a 6-frame translation of *Equestrem hymale* rhizome EST database available in the public domain

(http://www.plantrhizome.org/download/) was used to identify proteins from *M. caffrorum* rhizomes. A major problem associated with the use of custom databases constructed from ESTs of orphan organisms to identify proteins is obtaining annotations compatible with bioinformatics analyses. *A. thaliana* is the most popularly used model organism, both in classical genetics and in molecular biology. This is because of its small size, great natural variation, and a short life cycle. The availability of the Arabidopsis genome sequence (The Arabidopsis genome initiative, 2000) marked the beginning of a new paradigm in plant molecular biology and the "omics" biology including proteomics (Koornneef and Meinke, 2010). To carry out bioinformatics analysis of the data obtained from the present study, all protein identifications were converted into *A. thaliana* orthologs thereby making it possible to benefit from the rich bioinformatics resources developed for this species. However it must be borne in mind that the use of *A. thaliana* gene annotations has limitations as some processes and features are unique and cannot be approached *via* the use of a model plant. For example, unlike *A. thaliana*, some plants are perennials with a long life cycle and have evolved special features to survive harsh environmental conditions (Carpentier *et al*., 2008).

This work represents the first investigation into proteomic expression profiles of *M. caffrorum* rhizomes, using iTRAQ. Results from this study have the potential to provide useful insights into important molecular processes that are the major key differentiators between desiccation-tolerance and sensitivity in resurrection plants and guide new directions in future research in the field. The use of an organ specific custom protein database from a relatively close species to raise identification rates and quality provides an example for this kind of work in future.

3.2 Methods and materials

Figure 3.1 gives a diagrammatic representation of the steps used in isolation, separation and analysis of rhizome proteins from *M. caffrorum*.



(see next page for caption)

Fig 3.1: Outline of the approach used to compare the differential proteome profile of DT and DS rhizomes of *M. caffrorum* rhizomes in response to desiccation. 1) DT and DS plants collected from their natural habitats were acclimatized in the growth chamber and wet (control) and dry rhizomes were excavated. 2) Proteins were extracted from the wet and dry rhizomes followed by peptide digestion, iTRAQ tagging and MS/MS analysis 3) Protein identification and iTRAQ quantification was followed by data analysis and validation (see text for details).

3.2.1 Plant management and sampling

Plants were collected and maintained as described in Chapter 2. For each of the two plant phenotypes, 4 trays (length = 45 cm, width = 30 cm and depth = 7 cm) with ten plants each were allowed to acclimate for two weeks, with regular watering to maintain the SWC at about 35%. After the acclimation, plants from a quarter section of each tray were harvested and the rhizomes from these plants were deemed to be the controls (fully hydrated). For each phenotype, harvested rhizomes were pooled, flash frozen and stored at -70^{o}C until needed for protein extraction. The stress treatment was imposed by the cessation of watering and rhizomes were considered to be dried when the rhizome RWC had reached 15% (when tissues reach equilibrium with the surrounding environment - see Chapter 2). Dry rhizomes were harvested, pooled (to reduce variability) and stored as described for the control rhizomes. These two sampling points were selected to enable the observation of proteome changes as a result of desiccation and therefore obtain a snapshot of potentially important protectants. Sampling for RWC determination was carried out as described in Chapter 2.

3.2.2 Protein extraction

Proteins were extracted from the fully hydrated and dry rhizomes of DT and DS plants using a method based on phenol-ammonium acetate, modified and adapted from Isaacson *et al*., (2006). Briefly, rhizome pieces were ground under liquid nitrogen into a thin powder using a pestle and mortar. The powder was then aliquoted into 2 ml micro-centrifuge tubes up to the 0.5 ml mark and mixed with 1ml phenol extraction buffer (0.5M Tris pH 7.5, 10 mM EDTA, 1% (v/v) triton, 2% mercaptoethanol, 1% PVPP and one Roche Complete

Mini protease inhibitor tablet per 50ml buffer) for 10 minutes. Tubes were spun in an Eppendorf 5415C centrifuge at 12,000 rpm for 10 minutes and the supernatant retained. Four volumes of Tris-buffered phenol (pH 8.0) were added and after shaking briefly, the mixture was centrifuged for 10 minutes. The phenolic phase was collected and mixed with four volumes of 0.1M ammonium acetate in methanol and proteins were allowed to precipitate overnight at -20^{o}C. After centrifugation, pellets were washed once with 80% acetone and air-dried in the fume-hood. The dried pellets were re-suspended in urea lysis buffer (ULB) (7M urea, 2M thiourea and 2% CHAPS). Protein concentration was estimated using Bradford Reagent (Biorad) with bovine serum albumin as a protein standard. A volume corresponding to 200 µg proteins was precipitated in 80% acetone for 30 minutes at -20^{o}C, followed by centrifugation and air drying. The dried pellets were re-suspended in 200 µl of reducing solution (8 M urea, 3% SDS and 200 mM Tris (2-carboxylethyl)-phosphine hydrochloride [TCEP]) followed by incubation for one hour in a heating block at 60^{o}C. Thereafter, proteins were subjected to filter assisted sample preparation procedure (FASP) (Wiśniewski *et al*., 2009) and described below.

3.2.3 FASP

Reduced proteins were concentrated by centrifugation for 15 minutes in an Amicon filter device with a 30 kDa cut-off point. TCEP and sodium dodecyl sulphate (SDS) concentrations were reduced by diluting with 200 µl of buffer (8 M urea in 1 M triethylammonium bicarbonate [TEAB], pH 8.5) followed by centrifugation for 15 minutes. This was repeated twice and the recovered proteins were cysteine-blocked with 20 mM methyl methanethiosulfonate (MMTS) in 8 M urea for 15 minutes at room temperature. The preparations were centrifuged and the concentrates diluted with 200 µl of 8 M urea in 1 M TEAB, pH 8.5. After repeating the latter step twice, modified trypsin (Trypsin Gold, MS grade, Promega, Madison, WI, USA) in 30 µl of TEAB was added at an enzyme/protein ratio of 1:20. Protein digestion was completed by overnight incubation of the mixture at 37^{o}C. Finally, peptides were collected by centrifugation of the filter units for 40 minutes at room temperature.

3.2.4 iTRAQ labelling

Briefly, 60 µl of recovered peptide digests were obtained from each sample and concentrated to 20 µl using a Savant SC110 Speed-Vac (Thermo Scientific) followed by labelling with iTRAQ 8-plex reagents (113,114,115,116,117,118, 119, and 121) (ABSciex) according to the manufacturer's instructions. Briefly; the tags were first reconstituted with proteomics grade isopropanol (Sigma-Aldrich) and added to each respective sample. The labels and samples were then mixed by vortexing, and incubated at room temperature for 2 hours. After labelling, the samples were pooled. The iTRAQ tags used for each respective treatment are shown in Fig 3.1. Due to financial constraints, the iTRAQ experiment reported here consisted of two technical replicates *per* sample and one biological replicate.

After concentrating the pooled samples to approximately 30 µl, peptides were reconstituted in 5% (v/v) acetonitrile (ACN) and 0.5% (v/v) Trifluoroacetic acid (TFA) before de-salting in C-18 Spin Columns (Pierce, USA) according to the manufacturer's instructions. Peptides were eluted in 70% ACN (v/v) with 0.1% (v/v) formic acid (FA) and then dried in the speed-vac before off-gel fractionation. Desalting *via* C-18 column clean-up was necessary to ensure the removal of unused iTRAQ labels and other contaminants that may interfere with subsequent steps such as off-gel fractionation.

3.2.5 Peptide off-gel fractionation

Peptide off-gel fractionation was carried out to ensure the effective separation of peptides into fractions and therefore ensure the identification of many proteins. Tryptic peptides were re-suspended in 1.8 ml of off-gel sample buffer (3% (v/v) of glycerol and 1% (v/v) ampholytes). The 3100 OFF-GEL Fractionator (Agilent Technologies) with a 12-well frame was used for pI - based peptide separation. After assembling frames according to the manufacturer's instructions; 13 cm immobiline IPG gel strips (GE Healthcare) with a 3-10 linear pH range were rehydrated for 15 minutes with 40 µl of 1% glycerol in each well. Then, 150 µl of the peptide sample was loaded in each of the 12 wells. Peptide electro-focusing was performed up to a total of 20 kVh. After electro-focusing, all the 12 peptide

fractions were withdrawn and then desalted as described in 3.2.4, dried and stored at -20°C for LC-MS/MS analysis.

3.2.6 Mass spectrometry (ESI- Q-TOF -MS/MS) analysis

MS/MS analysis was carried out using an Agilent 6530 quadrupole-time-of-flight (Q-TOF) mass spectrometer fitted with a Polaris HR 3 µm C18 high pressure liquid chromatography (HPLC)-Chip Cube source (Agilent Technologies). The chip was equipped with a 75 µm x 150 mm analytical column, 3 µm particle size and a 360 nl Zorbax enrichment column connected online to the 1200 Series nanoflow HPLC *via* an orthogonal spray HPLC-Chip/MS interface (Agilent Technologies). Both systems were controlled by MassHunter Workstation Data Acquisition for Q-TOF (Agilent Technologies).

Approximately 2 µg peptides were re-suspended in 3% (v/v) ACN and 0.1% (v/v) FA loaded onto the trapping column at 1.6 µL/min with the chip switched to enrichment and using the capillary pump. After loading, the chip was then switched to separation mode and peptides were eluted from the enrichment column and run through the separation column during a 1 h gradient (from 3% (v/v) acetonitrile, 0.1% (v/v) FA to 50% (v/v) acetonitrile, 0.1% FA) directly into the mass spectrometer. The mass spectrometer was run in positive ion mode, with MS scan range of m/z 200 to 1700 at a rate of seven spectra/second. The MS/MS scan range was m/z 90 to 1700 at a scan rate of 2.50 spectra/second and a narrow (~1.3 amu) isolation width. Precursor ions were selected for auto MS/MS at an absolute threshold of 1000 or a relative threshold of 0.01, with a maximum of ten precursors per cycle, and active exclusion set at one spectrum and released after 0.6 min. Precursor charge-state selection and preference were set to 2+, 3+, and >3+, and precursors were sorted by abundance only.

3.2.7 Spectra data preparation

Raw spectral data files (.d format) from the Agilent MassHunter software were first converted to .mzML and then to .mgf file formats, using MSConvert, a part of the

Proteowizard v1.6.0 package (Kessner *et al*., 2008) with default settings. The .mgf files were fed into PEAKS Studio v6.0 (BSI) program. Considering the fact that a mass spectrometer may acquire an MS/MS scan of the same peptide multiple times, replicate spectra were merged using the PEAKS 'data refine' tool with default parameters (parent ion m/z tolerance at 0.1, retention time tolerance window of 30s and with precursor charge correction). Data refinement was carried out with no merged scans, and with no filtering. Refinement was necessary to achieve a more complete fragmentation pattern, low signal-to-noise ratio and a more representative intensity value for each reporter ion, before database search.

3.2.8 Protein database searches

PEAKS integrates *de-novo* sequencing with database based protein identification (Zhang *et al*., 2012). Since the database choice determines the quantity and quality of identifications obtained from an MS/MS scan, a comparison of outputs from the custom database *vs.* the UniProtKB/Swiss-Prot database was made so as to decide which database to use for subsequent steps. The Viridiplantae (green plants) UniProtKB/Swiss-Prot database, which contains non-redundant manually curated and reviewed sequences, was downloaded from www.uniprot.org on 30 August 2013. The custom protein database was constructed from the *E. hyemale* rhizome (apical tip and elongation zone tissues) EST as described by Balbuena *et al*., (2012). Briefly, the nucleotide sequences were downloaded from http://www.plantrhizome.org/download/ and translated into peptide sequences using the Virtual Ribosome v1.1 software (Wernersson, 2006) by scanning the open reading frames (ORFs) and compiling the longest ORFs from all sequences into a FASTA database file.

De-novo peptide sequencing was performed with a precursor mass tolerance of 20 ppm for the parent ion and 0.1 for the fragment ion, with trypsin specified as the cleavage enzyme. Database searches were conducted using the following parameters; parent mass error tolerance: 20.0 ppm, fragment mass error tolerance: 0.1 Da, precursor mass search type - monoisotopic, enzyme- trypsin, max missed cleavages - one, non-specific cleavage - one, fixed modifications- iTRAQ 8plex (K, N-term) and beta-methylthiolation, variable modifications- iTRAQ 8plex (Y) and oxidation (M), max variable PTM *per* peptide - 3.

The decoy database was automatically generated by PEAKS. The *de-novo* score and peptide hit (-10logP) thresholds were set at 30 while the FDR was set at 1%. An identified protein was considered valid if its probability score (-10logP) was greater than 30 with a minimum of two unique peptides *per* protein.

To increase the number of identified proteins, the PEAKS PTM search (Han *et al*., 2011) and Sequence Tag Homology Search (SPIDER) (Han *et al*., 2005) were employed. The SPIDER algorithm detects peptide mutations and performs cross-species homology searches. Briefly, the PEAKS SPIDER algorithm tries to match the *de-novo* amino acid sequences (sequence tags) with the database proteins. The algorithm computes one or more possible sequence tags for each MS/MS spectrum. When a significant similarity is established, the algorithm uses both *de-novo* sequencing errors and homology mutations to account for the differences. Specifically, SPIDER reconstructs a "real" sequence to reduce the sum of; (1) *de-novo* errors between the *de-novo* sequence and the real sequence, and (2) homology mutations between the database sequence and the real sequence (Han *et al*., 2005). In the present study, the ultimate search workflow was: *de-novo* + PEAKS DB + PEAKS PTM + SPIDER. For the execution of the whole identification pipeline, additional parameters used were: *de-novo* score (ALC%) threshold - 30, maximum number of PTMs - 3, and peptide hit threshold (-logP) - 30.

Proteins with similar peptides that could not be differentiated based on MS/MS analyses alone were automatically grouped together by the PEAKS program. To satisfy the principle of parsimony (Occam's razor), for each group, the protein with the highest score (-10logP) and/or the highest number of unique peptides was selected as the 'correct' identity.

3.2.9 iTRAQ quantification

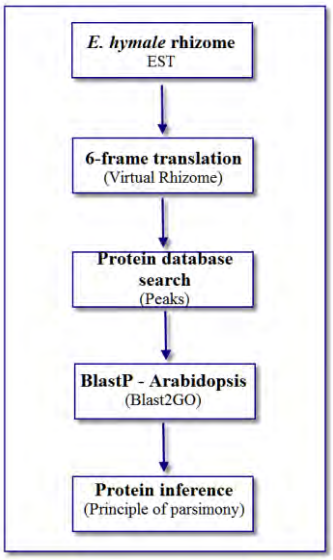
For protein quantitation, the PEAKS Q module integrated into PEAKS Studio v6.0 was used with default settings for iTRAQ 8-plex. The PEAKS Q program identifies supporting spectra for each peptide match to calculate the relative abundance of proteins. The program searches through each spectrum in the supporting spectra set to calculate the intensity for

each reporter ion. Peptide match ratios are calculated from reporter ion intensities and outliers are automatically removed. For each protein, all supporting peptides have their weights normalized to 1 and then weighted averages are calculated.

Data from the PEAKS Q package were exported into an MS Excel file for further analysis. Only proteins with all eight iTRAQ reporter channels present were considered noteworthy for further analysis. To compensate for the possible variations in the starting amounts, all reporter ion intensities were $\log_2$-transformed and then quantile normalized across all proteins using the CLC Genomics Workbench v6.0 software package (CLC bio). Fold changes were calculated using average normalized $\log_2$ ratios of the hydrated (also termed control) and the desiccated treatments for both DT and DS rhizomes. Data were subjected to two-tailed Student's t-test analysis using GraphPad Prism 5 software. Changes in protein expression were considered significant only when $p \leq 0.05$.

3.2.10 Protein annotation of proteins identified from the custom database

In addition to the procedures described above, additional steps were necessary to obtain annotations for proteins identified in the rhizome-specific custom database. Fig 3.2 summarizes the pipeline used for protein inference for identifications from the rhizome custom database.



(see next page for caption)

Fig 3.2: Workflow used in protein identification using the custom database. *E. hymale* rhizome EST sequences were translated using the Virtual Ribosome software to construct a rhizome-specific protein database. The resulting database was searched to identify *M. caffrorum* rhizome homologs using the PEAKS Studio software. Protein annotations were obtained from the Arabidopsis database using the Blast2GO Blastp algorithm.

Since sequences from the *E. hymale* rhizome database were deployed in the public domain without standard descriptions and annotations to date, database searches match amino acid sequences identified only by a unique entry number. In order to get protein descriptions compatible with bioinformatics analysis tools, sequences of identified proteins were batch retrieved from the *E. hymale* amino acid sequence database using the FASTA Sequence Extractor tool from the FaBox toolbox (v1.41) software package (<http://users-birc.au.dk/biopv/php/fabox/>) (Villesen, 2007). Blast2GO v2.1 (Conesa *et al*., 2005) was used to conduct an automatic and unsupervised assignment of protein descriptions.

Blast2GO was fed with the FASTA file exported from FaBox and set to perform a BLASTp search against the NCBI Plants/Arabidopsis database using the following settings; BLAST Expect Value: 1.0E-3 and number of BLAST hits: 20. These settings were selected for their high stringency to ensure high confidence in protein annotation. With these settings, two peptide sequences could not be aligned to any known protein. These sequences were then identified as protein CER1-like 1 and DNA topoisomerase 1 beta after a liberal BLASTp alignment with an Expect Value cut-off of 1. For all identified proteins, identifiers from the NCBI Reference Sequence (RefSeq) from Blat2GO were converted to The Arabidopsis Information Resource (TAIR) identifiers using the gProfiler program (Reimand *et al*., 2007; 2011). Converting to TAIR identifiers was necessary because some software packages used in this work could not recognise RefSeq identifiers. For protein classification, the MapMan classification scheme from the Browser-based Functional Classification SuperViewer for Arabidopsis Genomics (http://bbc.botany.utoronto.ca/ntools/cgi-bin/ntools_classification_superviewer.cgi) (Provart and Zhu, 2003) was used. Proteins without functional assignments in the MapMan system were classified based on the PANTHER system (http://www.pantherdb.org/) (Mi *et al*., 2013).

3.2.11 Western blot analysis

Western blot analysis was used to confirm both the protein identification and quantification results. Two proteins, HSP 70-2 and SOD-[Cu-Zn], whose abundance changed in response to desiccation in the DT and DS rhizome forms were selected for this confirmatory process. Total proteins were extracted as described in 3.2.2, except that after quantification, volumes corresponding to 20 µg proteins for SOD and 50 µg for HSP 70 were mixed with four volumes of cold acetone and incubated at -20^{o}C for 20 minutes. Thereafter, precipitates were pelleted by centrifugation and washed once with cold acetone. After drying in the fume-hood, pellets were re-suspended in 20 µl of sample buffer (63 mM Tris-HCl pH 6.8, 10% glycerol, 2% SDS, 0.0025% bromophenol blue and 100 mM dithiothreitol) and, after heating at 65^{o}C in the heating block for 10 minutes, proteins were separated on sodium dodecyl sulphate polyacrylamide gels. Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was conducted at 100V using a Mini-Protean Tetra (Biorad Lab) apparatus at room temperature. Proteins were then transferred onto nitrocellulose membranes using Mini-Transblot apparatus (Biorad Lab) at 4^{o}C for one hour. Non-specific proteins were blocked for one hour at room temperature with 5% non-fat dry milk in Tris-buffered saline (TBS) (50 mM Tris-HCl, pH 7.4, 150 mM NaCl) containing 0.05% (v/v) Tween-20. Membranes were incubated overnight at 4^{o}C (with gentle shaking) in primary antibodies in TBS containing 0.05% (v/v) Tween-20 and 5% non-fat dry milk. The rabbit raised primary antibodies against SOD-Cu/Zn and HSP-70; chloroplastic (Agrisera) were used at a concentration of 1:10000. After washing three times with TBS buffer containing 0.05% (v/v) Tween-20, the membranes were incubated with goat anti-rabbit secondary antibodies (LPL), at a concentration of 1:3000 for 2 hours at RT. Membranes were then washed twice in TBS containing 0.05% (v/v) Tween-20 and once with TBS followed by detection using the WesternBright ECL HRP chemiluminescent detection kit (Advansta). Images were acquired using the ChemiDoc XRS imager (Biorad) and analysed using the ImageLab v4.1 software (Biorad). To determine relative expression; fold changes were calculated for each treatment blot relative to the control. In order to calculate fold changes, the expression level of the control was set at 1 and the expression level in the dry rhizomes is the fold change from the control (i.e., either greater or larger than 1). Three biological repeats were conducted and statistical significance was established if $p \leq 0.05$.

3.3 Results and preliminary discussion

3.3.1 Preamble

The first objective of this chapter was to evaluate the impact of using a custom database that is both rhizome and phylum specific *vs.* the standard Viridiplantae database for *M. caffrorum* rhizome protein identification. It was demonstrated that the custom database constructed through a 6-frame translation of publicly available *E. hymale* rhizomes EST sequences performed better in terms of number of identified proteins and the quality of identifications. The second objective was to determine the changes in protein expression patterns in the DT and DS rhizomes in response to desiccation. Using proteins identified in the custom database, iTRAQ analysis was used to assess protein expression changes between proteomes present in dry rhizome tissues relative to the fully hydrated controls for both DT and DS plant phenotypes. Western blot analysis confirmed the identities and expression tendencies of two proteins observed in the iTRAQ analysis.

This chapter was mainly concerned with the methodological aspects involved in protein identification and quantification, to provide a background for further bioinformatics/functional analysis (Chapter 4). However, a brief outline of major similarities and differences in protein expression profiles between DT and DS is presented.

3.3.2 Protein identification rates and quality

In order to decide which database to use for protein annotations and functional analysis, identification rates were compared between the Viridiplantae database and the rhizome database against the same MS/MS spectral file. Table 3.1 below presents results on the protein identification rates for the two databases.

Table 3.1: Comparison of identification rates obtained from using the custom *E. hymale* and Viridiplantae databases. Identifications were carried out using PEAKS software as described in section 3.2.8.

	Database	
	Swiss/Prot	**Custom**
Number of MS/MS scans	69379	69379
Peptide-spectrum matches	7827	9127
Peptide sequences	2673	3405
Proteins (non-redundant)	171	236

As shown in Table 3.1, results indicated that compared to the Viridiplantae database, given the same spectra, higher identification rates were obtained in the custom database. In total 236 unique proteins were identified in the custom database *vs.* 171 identified in the Viridiplantae database. In addition to comparing the number of identified proteins, the quality of the identifications was also examined. The results of the comparison are shown in Figs 3.3 and 3.4.

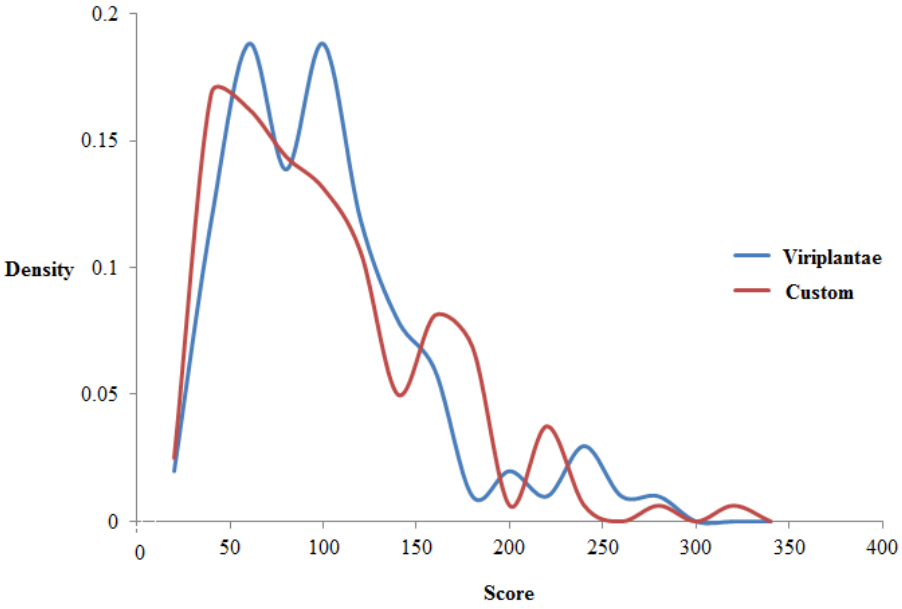


Fig 3.3: Distribution of protein identification scores for proteins identified in custom database *vs.* Viridiplantae. Density is defined as the relative distribution of frequencies (number) of proteins expressed as a fraction of all proteins for each score. Frequency distribution data was calculated by GraphPad and exported for visualization in Microsoft Excel 2010.

The results (Fig 3.3) show that there were no major differences between identifications from the two databases in terms of protein identification score; though between the score 300 to 350 only proteins identified in the custom database were found, albeit with very low densities. In all databases, protein identification scores were biased towards the low score with the highest density observed at scores between 50 and 150.

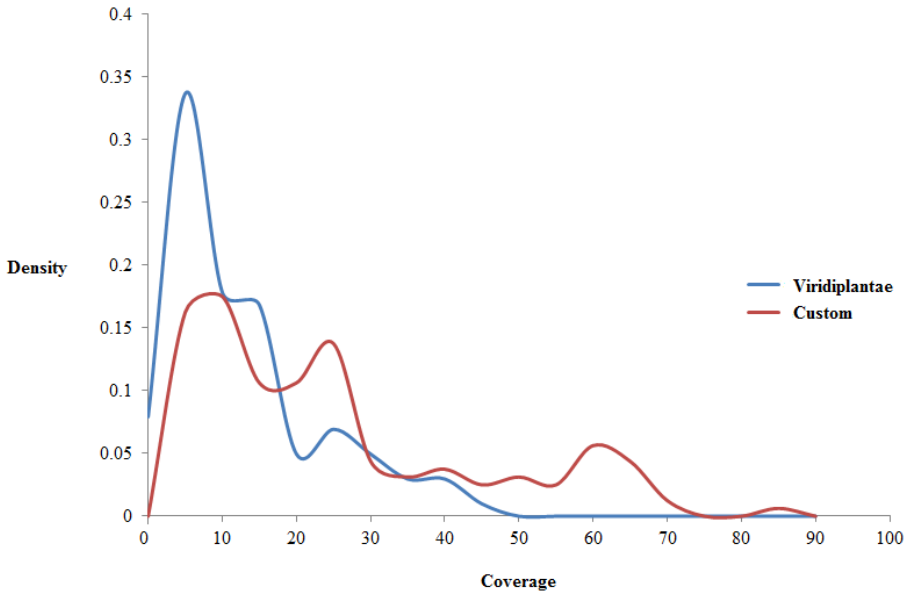


Fig 3.4: Density map of peptide coverage of identified proteins. Density is defined as the relative distribution of frequencies (number) of proteins expressed as a fraction of all proteins falling in the given amino acid coverage range. Frequency distribution data was computed by GraphPad and the results were exported and visualized in Microsoft Excel 2010.

As shown in Fig 3.4, peptide coverages of proteins identified in the Viridiplantae database were less comprehensive relative to those identified using the custom database. In the Viridiplantae database there is a spike of high density at proteins identified with peptide coverage of only around ten amino acids. All identifications made from the Viridiplantae database were based on protein coverages of less than 50 amino acids while 25 proteins had coverage greater than 50 amino acids in the custom database.

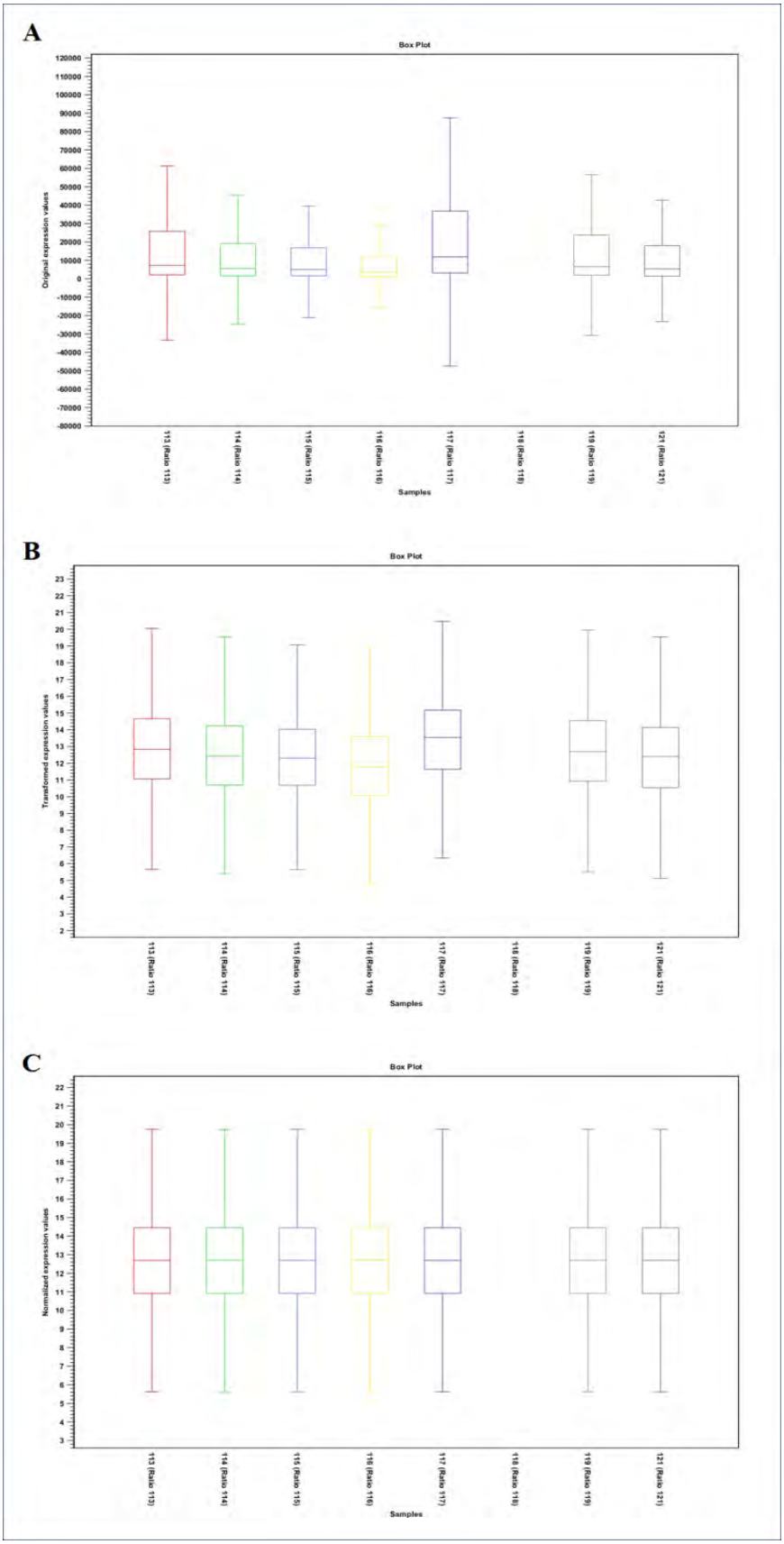
The overall improvement in terms of number of proteins identified and quality of the custom database over the Viridiplantae can be explained by the fact that the custom database employed in this study was organ (rhizome) specific and phylum specific and thus evolutionarily closer than other plant genera and species in the Viridiplantae database.

The poor performance normally observed in protein identifications in orphan plants is, at least in part, due to evolutionary differences that tend to accumulate as organisms get more distantly related. The results of the present work supports the observation that protein identification based on homology depends on perfect matches between spectra and the database to achieve a high number of identifications (Balbuena *et al*., 2012). It also supports the observation that a high confidence in protein identifications depends on the score of the length and composition of the amino acid between the homologous proteins (Romero-Rodríguez *et al*., 2014). The data further reaffirmed that the use of custom databases increases the number of identified proteins and confidence of the identified proteins since an MS/MS spectra would have more matches in the database than can be achieved with non-specific (standard) databases (Liska and Shevchenko, 2003; Romero-Rodríguez *et al*., 2014). This work has shown that there is a clear benefit to the use of custom protein database for the interpretation of proteomics data even for related species not directly represented in the database itself. Since custom databases are easy to construct with minimum resources and, until well-developed molecular resources for resurrection plants become publicly available, the approach employed in the present study can be recommended to other researchers working in the field of resurrection plant proteomics.

Due to the custom database providing a higher identification rate and better quality scores compared to the Viridiplantae database, the rest of the analyses discussed in this chapter and the rest of this thesis shall be based on identifications made in the rhizome-specific database. For the interested reader, Table S2 in Supplementary Materials provides a list of all proteins identified in the Viridiplantae database. These proteins were not analysed any further in the context of this thesis as they provided no additional information or qualitative value to the study.

3.3.3 Proteins showing changes in abundance

iTRAQ tagging was used to identify proteins that changed in abundance in the DT and DS rhizomes in response to desiccation. To carry out protein quantification, iTRAQ expression data were first $\log_2$-transformed and normalized as described in section 3.2.9. Fig 3.5 below summarizes the data handling stages employed.



(see the next page for caption).

Fig 3.5: Box plots showing data processing stages for the 8 iTRAQ channels. Original expression values (A) were first log_2-transformed (B) and quantile normalized (C) using CLC Workbench software.

Since the data were normalized (Fig 3.5) before statistical analysis, to eliminate major sources of bias, any statistically significant quantitative differences observed in the differential expression data was attributed only to be a consequence of the desiccation treatment. Lists of proteins that showed abundance changes are provided in Tables 3.2 to 3.5 below. In the DT rhizomes 16 proteins increased in abundance and 14 declined in abundance. On the other hand, 16 proteins increased and 20 decreased in abundance, respectively, in the DS form. A list of all proteins identified in the custom database with at least two unique peptides and meeting all the requirements for quantification as described in the methods section, but did not change in abundance, is provided in the Supplementary Materials (Table S1).

Table 3.2: List of proteins that significantly increased in abundance during desiccation of rhizomes from DT *M. caffrorum.**

D/base entry	Description	Fold	TAIR	Function
EhRi_032915	serine decarboxylase 1	1.25	AT1G43710	amino acid metabolism/ degradation
EhRi_051576	protein CER1-like 1	3.16	AT1G02190	secondary metabolism (wax)
EhRi_035533	DNA topoisomerase 1 beta	1.36	AT5G55310	DNA synthesis/chromatin structure
EhRi_046794	pyruvate kinase	0.59	AT3G52990	glycolysis
EhRi_047159	UTP--glucose-1-phosphate uridylyltransferase 1	1.24	AT5G17310	glycolysis
EhRi_057735	allene oxide synthase	0.41	AT5G42650	hormone metabolism
EhRi_062341	ATPase subunit 1	0.53	ATMG01190	ATP synthesis
EhRi_046663	SNF1-related protein kinase catalytic subunit alpha KIN10	0.58	AT3G01090	signalling

Table 3.2 continued

EhRi_052838	20S proteasome alpha subunit G1	0.61	AT2G27020	protein degradation
EhRi_033037	probable 26S proteasome non-ATPase regulatory subunit 8A	1.25	AT5G05780	protein degradation
EhRi_032638	60S ribosomal protein L11-1/L16A	0.67	AT2G42740	Protein synthesis
EhRi_020182	Importin subunit alpha-1	1.22	AT3G06720	protein targeting
EhRi_054509	chalcone synthase	0.74	AT5G13930	flavonoids, chalcones, etc.
EhRi_040776	GTP-binding nuclear protein Ran-3	2.74	AT5G55190	signalling
EhRi_049303	heat shock protein 70-2	0.24	AT5G49910	heat stress
EhRi_052878	heat shock protein 91	0.84	AT1G79930	heat stress

*The D/base entry is the unique ID assigned to the sequence in the custom *E. hymale* database. Protein descriptions were obtained from the BLAST2GO annotations as described in 3.2.10. Protein functional assignments were obtained from the MapMan classification scheme using the Classification SuperViewer tool or the PANTHER system as mentioned in 3.2.10.

As Table 3.2 shows, iTRAQ quantification has identified some proteins that have been widely reported to be important in the desiccation stress response as changing in abundance in the DT rhizomes in response to desiccation. These include members of the HSP family (heat shock protein 70-2, and HSP 91) and some proteins of the UPS (20S proteasome alpha subunit G1, and probable 26S proteasome non-ATPase regulatory subunit 8A). The importance of proteins in the HSP and UPS families in resurrection plants was reviewed in Chapter 1.

Table 3.3: List of proteins that significantly increased in abundance during desiccation of rhizomes from DS *M. caffrorum*.*

D/base entry	**Description**	**Fold**	**TAIR**	**Function**
EhRi_057924	tubulin beta-8 chain	0.85	AT5G23860	cell organization
EhRi_033207	putative phosphoglucomutase	0.66	AT1G23190	glycolysis
EhRi_046794	pyruvate kinase	0.42	AT3G52990	glycolysis

Table 3.3 continued

EhRi_047159	UTP--glucose-1-phosphate uridylyltransferase 1	1.98	AT5G17310	glycolysis
EhRi_039615	allene oxide synthase	1.12	AT5G42650	hormone metabolism
EhRi_062341	ATPase subunit 1	1.66	ATMG01190	ATP synthesis
EhRi_035071	ubiquitin-specific protease family C19-related protein	2.55	AT1G16860	Uncharacterized membrane protein
EhRi_057043	heat shock cognate protein 70-1	1.55	AT5G02500	protein folding
EhRi_057122	superoxide dismutase [Cu-Zn]	0.84	AT1G08830	redox
EhRi_031175	monodehydroascorbate reductase (NADH)	0.42	AT3G52880	redox
EhRi_040999	phenylalanine ammonia-lyase 1	0.62	AT2G37040	phenylpropanoid, lignin biosynthesis *etc*.
EhRi_058134	brefeldin A-inhibited guanine nucleotide-exchange protein 5	0.66	AT3G43300	signalling
EhRi_033074	calcium-dependent protein kinase 2	2.23	AT3G10660	signalling
EhRi_060199	general regulatory factor 11	0.96	AT1G34760	signalling
EhRi_042919	carbonic anhydrase 1	3.13	AT3G01500	tricarboxylic acid (TCA)
EhRi_044215	ethylene-insensitive protein 2	1.35	AT5G03280	metal transport

*The D/base entry is the unique ID assigned to the sequence in the custom *E. hymale* database. Protein descriptions were obtained from the BLAST2GO annotations as described in 3.2.10. Protein functional assignments were obtained from the MapMan classification scheme using the Classification SuperViewer tool or the PANTHER system as mentioned in 3.2.10.

As shown in Table 3.3, some proteins identified by iTRAQ to have increased in abundance in the DS rhizomes are heat shock cognate protein 70-1 and SOD-[Cu-Zn]. Heat shock cognate protein 70-1 belongs to the HSP family of proteins, whose roles have been

reviewed in Chapter 1. The enzyme monodehydroascorbate reductase (NADH) is involved in the formation of ascorbate. The role of ascorbate as an anti-oxidant in resurrection plants has been reported in several plants e.g. *M. flabellifolia* (Kranner *et al*., 2002) and *R. serbica* (Živkovic´ *et al*., 2002).

Table 3.4: List of proteins that significantly decreased in abundance during desiccation of rhizomes from DT *M. caffrorum.**

D/base entry	Description	Fold	TAIR	Function
EhRi_050337	lactoylglutathione lyase family protein	-3.13	AT1G08110	Biodegradation of Xenobiotics
EhRi_070972	peptidyl-prolyl cis-trans isomerase CYP19-2	-4.35	AT2G21130	cell cycle
EhRi_070716	microtubule organization 1 protein	-0.31	AT2G35630	cell organization
EhRi_047010	phospholipase D alpha 1	-0.29	AT3G15730	lipid metabolism
EhRi_052281	2-alkenal reductase	-0.94	AT5G16970	oxidoreductase activity
EhRi_049876	short-chain dehydrogenase-reductase B	-0.46	AT3G12800	oxidoreductase activity
EhRi_060769	tetratricopeptide repeat-containing protein	-1.23	AT1G80410	acetyltransferase activity
EhRi_059572	6-phosphogluconolactonase 1	-6.67	AT1G13700	pentose phosphate pathway
EhRi_046311	calcium-independent ABA-activated protein kinase	-0.4	AT4G33950	protein kinase activity
EhRi_058952	L-ascorbate peroxidase	-0.7	AT4G35000	redox
EhRi_038934	peroxiredoxin-2E	-1.43	AT3G52960	redox
EhRi_070909	trans-cinnamate 4-monooxygenase	-1.67	AT2G30490	phenylpropanoids, lignin biosynthesis, *etc.*
EhRi_059505	14-3-3-like protein GF14 nu	-0.31	AT3G02520	signalling
EhRi_040719	clathrin, heavy chain	-0.8	AT3G11130	vesicle transport

*The D/base entry is the unique ID assigned to the sequence in the custom *E. hymale* database. Protein descriptions were obtained from the BLAST2GO annotations as described in 3.2.10. Protein functional assignments were obtained from the MapMan classification scheme using the Classification SuperViewer tool or the PANTHER system as mentioned in 3.2.10.

As Table 3.4 shows, one of the main features among the proteins that reduced abundance in the DT rhizomes is the decrease of proteins involved in anti-oxidant activities, i.e peroxiredoxin-2E and L-ascorbate peroxidase in response to desiccation stress. As mentioned in Chapter 1, anti-oxidants are normally up-regulated in resurrection plants as a consequence of desiccation.

Table 3.5: List of proteins that significantly decreased in abundance during desiccation of rhizomes of DS *M. caffrorum.**

D/base entry	Description	Fold	TAIR	Function
EhRi_045723	glycyl-tRNA synthetase / glycine--tRNA ligase	-0.51	AT1G29880	Amino acid activation
EhRi_040066	histone H2B	-0.59	AT2G28720	DNA synthesis/ chromatin structure
EhRi_059080	nucleosome assembly protein 1;3	-0.52	AT5G56950	DNA synthesis/ chromatin structure
EhRi_065602	L-lactate dehydrogenase	-0.81	AT4G17260	Fermentation/ dehydrogenase
EhRi_039827	phosphoenolpyruvate carboxylase 4	-1.75	AT1G68750	glycolysis
EhRi_042344	alpha-glucan phosphorylase isozyme H	-1.82	AT3G46970	major carbohydrate metabolism
EhRi_061355	fructose-1,6-bisphosphatase	-1.82	AT1G43670	major CHO metabolism
EhRi_040952	alcohol dehydrogenase class-3	-0.33	AT5G43940	oxidoreductase activity
EhRi_059730	regulatory particle triple-A ATPase 3	-2.08	AT5G58290	protein degradation
EhRi_045106	40S ribosomal protein Sa-2	-1.06	AT3G04770	protein synthesis
EhRi_040836	60S ribosomal protein L24-2	-0.51	AT3G53020	protein synthesis
EhRi_048268	elongation factor 1-alpha 4	-0.47	AT5G60390	protein synthesis
EhRi_056224	elongation factor EF-2	-1.19	AT1G56070	protein synthesis

Table 3.5 continued

EhRi_054645	outer envelope pore protein 16-2	-1.01	AT4G16160	protein targeting
EhRi_031721	2-Cys peroxiredoxin BAS1	-0.6	AT3G11630	redox
EhRi_038773	chalcone synthase	-0.5	AT5G13930	flavonoids, chalcones, etc.
EhRi_031846	14-3-3-like protein GF14 omega	-2.56	AT1G78300	signalling
EhRi_049303	heat shock protein 70-2	-0.11	AT5G49910	heat stress
EhRi_048154	Isocitrate dehydrogenase [NAD] regulatory subunit 1	-0.86	AT4G35260	tricarboxylic acid (TCA)
EhRi_053908	WEB family protein	-0.5	AT4G27595	unknown

*The D/base entry is the unique ID assigned to the sequence in the custom *E. hymale* database. Protein descriptions were obtained from the BLAST2GO annotations as described in 3.2.10. Protein functional assignments were obtained from the MapMan classification scheme using the Classification SuperViewer tool or the PANTHER system as mentioned in 3.2.10.

Results in Table 3.5 above shows the complexity of the desiccation stress response in this plant. Whereas the abundance of heat shock cognate protein 70-1 from the HSP family increased (Table 3.3) in the DS rhizomes; the abundance of a related protein, heat shock protein 70-2 declined in the same rhizome tissues. Futher, whereas the abundance of the anti-oxidant 2-Cys peroxiredoxin BAS1 declined; the abundance of two anti-oxidants, L-ascorbate peroxidase and peroxiredoxin-2E increased in the same DS rhizomes (Table 3.3). Thus, these results demonstrates that it is difficult to speculate on the overall molecular status of the rhizomes by merely identifying individual proteins that are differentially expressed. The results demonstrates the importance of conducting further experimental analysis or alternatively, bioinformatics analysis to fully understand the molecular mechanisms involved in the response of the *M. caffrorum* rhizomes against desiccation stress.

3.3.4 Comparison of profiles of proteins whose abundance were significantly altered.

The Venn diagram below (Fig 3.6) summarizes the distribution of the proteins that changed in abundance in response to rhizome desiccation.

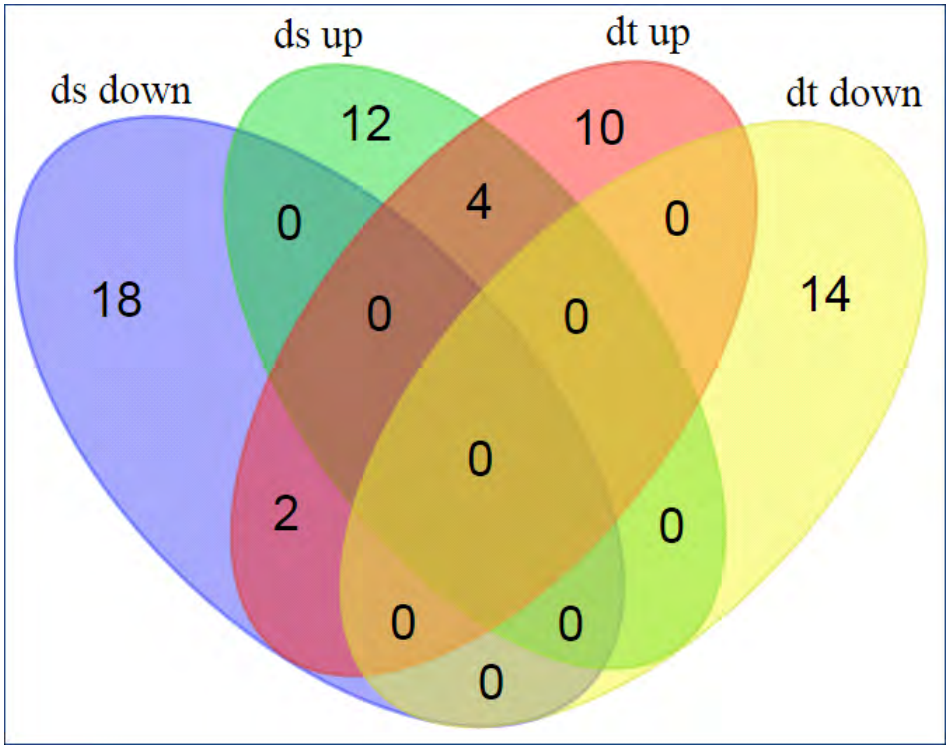


Fig 3.6: DT and DS rhizomes proteins that changed in abundance in response to desiccation stress. 'UP' refer to increase in abundance while 'Down' refers to decrease in abundance.

It was observed that certain proteins showed similar patterns in abundance changes across the DT and DS phenotypes while some protein abundance changes were specific to one rhizome phenotype (Tables 3.2 to 3.5 and Fig 3.6). Four proteins (Pyruvate kinase, UTP--glucose-1-phosphate uridylyltransferase 1, allene oxide synthase and, ATPase subunit 1) were increased in common in rhizomes of both the DT and DS phenotypes. Since the abundance these proteins increased in both the DT and DS rhizomes regardless of the environmental conditions, it is possible that these proteins are important in the attainment of desiccation-tolerance. The abundance of the proteins chalcone synthase and heat shock

protein 70-2 were increased in the DT but declined in the DS rhizomes, in response to desiccation stress. This difference may be a reflection of the environmental conditions in which the two plant types were grown and desiccated. There were no proteins that decreased in abundance commonly in the two rhizome phenotypes.

In this chapter (sections 3.3.4.1 and 3.3.4.2 below), only the individual proteins that showed a common pattern in abundance changes and those showing contrasting change patterns between the DT and DS rhizome phenotypes will be discussed. A detailed analysis of protein abundance changes in the two rhizome phenotypes (DT *vs* DS) in response to desiccation will be presented using more advanced bioinformatics tools in Chapter 4.

3.3.4.1 Proteins that increased in abundance in both the DT and DS rhizomes

Allene oxide synthase is involved in the biosynthesis of the phytohormone jasmonic acid (JA). Analysis of both leaves and roots of the resurrection plant *Haberlea rhodopensis* revealed that JA appeared to serve as a signal that triggers the plant's response to desiccation (Djilianov *et al*., 2013). In *Agropyron cristatum*, it was suggested that the water-deficit stress-induced JA production may function as a signal that leads to the regulation of glutathione and ascorbate metabolism and may have a crucial role in the acquisition of water-deficit stress tolerance in that plant (Shan and Liang, 2010). Therefore, the increase in the abundance of allene oxide synthase in response to desiccation in the rhizomes of *M. caffrorum* may be related to the synthesis of JA which in turn may play a key function in the acquisition of DT.

The increase in the abundance of pyruvate kinase and UTP--glucose-1-phosphate uridylyltransferase 1 which are glycolysis intermediates suggests that they are needed for the production of energy and synthesis of downstream products required for the acquisition of desiccation-tolerance. This agrees with ultra-structural findings (Chapter 2) where it was established that energy reserves in the form of lipids and starch were diminished in both the DT and DS rhizomes, in response to desiccation. The abundance of another protein involved in energy metabolism, ATP synthase subunit 1, was also increased providing further support to the idea that water stressed caused an increase in energy metabolism in

the rhizomes of this plant. The abundance of two proteins matching to ATP synthases (ATPases) to increase in response to dehydration in leaves of the resurrection plant *Boea hydrometrica* (Jiang *et al*., 2007) and, the abundance of a vacuolar ATPase declined during desiccation in the resurrection plant *X. viscosa* (Mundree and Farrant, 2000). See also Oliver *et al*., (2010) for studies in *S. stapfianus*. It has been proposed by the latter authors that vacuolar ATPases are likely to play a role in the filling of vacuoles with solutes that ultimately enable maintenance of mechanical stabilization in dry tissues. As water replacement in vacuoles appears to be a mechanism utilised, at least in cortical cells of the rhizome (Chapter 2), it is possible that the ATPases identified in the current study facilitate this process. Whether this particular function is true or not, results from the present work suggest that, in corroboration with other studies on DT in vegetative tissues, ATPases may have an important role to play in the attainment of desiccation-tolerance by *inter alia* providing energy for protection and repair in the desiccated state or may help in the preparation for rehydration.

It is worth noting that in the DS rhizomes there is an apparent contradiction where the abundance of one protein (phosphoenolpyruvate carboxylase 4) involved in glycolysis was reduced. This could be explained by the fact that there are two alternative pathways for glycolysis that are utilised under different physiological conditions (Plaxton, 1996; Sweetman *et al*., 2009; van Dongen *et al*., 2011; Tcherkez *et al*., 2012). The 'normal' glycolysis pathway is regulated by pyruvate kinase (which increased in abundance in both DT and DS) which catalyses the conversion of phosphoenolpyruvate to pyruvate and is thus an important regulator of glycolysis (Plaxton 1996; Carroll *et al*., 2008; van Dongen *et al*., 2011). The alternative pathway of glycolysis goes *via* the protein phosphoenolpyruvate carboxylase (PEPC) - which declined in abundance in the dehydrated DS rhizomes. The PEPC pathway of glycolysis is assumed to be more efficient when plants are ADP limited during stress (Plaxton, 1996; O'Leary *et al*., 2011). PEPC is also important for the replenishment of metabolites to the tricarboxylic acid cycle (TCA) cycle that are used for biosynthetic processes (van Dongen *et al*., 2011; Tcherkez *et al*., 2012). These results imply that energy was channelled selectively through the normal pathway (i.e. it is increased and the alternative PEPC pathways is decreased). This does not happen in the DT rhizomes, nor does it suggest that switching to the alternative pathway happens in DT

plants (since the abundance of phosphoenolpyruvate carboxylate 4 did not decline in those plants). If taken together, this might suggest that DT and DS rhizomes regulate energy metabolism slightly differently. More hypotheses can be generated by using holistic systems biology approaches to get a broader picture of the molecular processes underlying desiccation-tolerance in this plant.

Furthermore, the abundance of isocitrate dehydrogenase [NAD] regulatory subunit 1, which is part of the TCA clycle, declined in the DS rhizomes, further complicating the understanding of how the energy balance is regulated in the DS rhizomes. In the DT rhizomes, while the abundance of glycolysis proteins were increased, the abundance of 6-phosphogluconolactonase 1, which is involved in the pentose phosphate pathway was decreased. Therefore, these few example show that merely focusing on expression profiles of individual proteins makes it difficult to tell what the net energy status of the cell is after desiccation stress. Again, this provides a strong case to employ alternative approaches e.g. bioinformatics tools to understand the ultimate molecular status of rhizomes in response to desiccation.

3.3.4.2 Proteins showing contrasting expression patterns

iTRAQ analysis data show that the abundance of the protein chalcone synthase was increased in DT while it declined in the DS rhizomes in response to desiccation. Chalcone synthase is believed to act as a central hub for enzymes involved in the flavonoid pathway (Crosby *et al*., 2011). Flavonoids are important secondary metabolites with various functions including anti-oxidant activity, pigmentation, protection against UV, signal transduction during stress, reproductive functions (flower development, fruit and seeds) (Cain *et al*., 1997). Also, several genes encoding components of flavonoid biosynthesis have been reported to be induced under stress conditions. For example, considerable increases in flavonoid level have been demonstrated in several plants in response to abiotic stresses including drought (Winkel-Shirley, 2002). It is proposed here that increased flavonoid content in DT rhizomes might also play a role in the signalling to areal portions (fronds) during dehydration. Since flavonoids are also polyphenols and considering the fact that there were more polyphenols in the DT rhizome cortical tissues compared to the DS

forms (Chapter 2), this observation implies that it is possible that the polyphenols may have acted as mechanical stabilizers with redox buffering potential, as described for *M. flabellifolia* (Moore *et al.*, 2006).

The contrasting expression patterns observed for this protein can be explained in relation to the environmental conditions in which the plant grows. During summer, there are harsher conditions such as high temperatures, high radiation and therefore the increase in the abundance of chalcone in the DT rhizomes is most likely to help cope with the environmental conditions. In the case of the DS form, the plants grow in winter where the conditions are relatively conducive for growth and reproduction. The decreased abundance of chalcone synthase in response to desiccation may be an indication that the functions it is recruited to perform in summer are not necessary for survival in winter and thus the energy used in the execution of such pathways might be reduced in favour of other processes.

Another protein showing a contrasting expression pattern is heat shock protein 70-2 (also known as heat shock protein, chloroplastic). Since the rhizome is not photosynthetic, it is likely that this is a plastid associated protein. The abundance of this protein was increased in the DT rhizomes but decreased in the DS rhizomes. In the DT rhizomes, the abundance of another HSP family member, HSP-91 was also decreased suggesting the importance of the HSPs in the protection of the DT rhizomes against water-deficit stress. Since drying occurred under higher temperatures in the summer dried plants; it is possible that the increased abundance of these proteins is simply a consequence of that. That is, if dried under elevated temperatures, the HSPs might be important for plastid protection as starch within is degraded for energy requirements associated with dehydration. However, it is worth noting that while the abundance of HSP 70-2 declined in the DS rhizomes, the abundance of heat shock cognate protein 70-1 was increased in the same desiccated rhizomes. Why the abundance of HSP 70-2 was specifically decreased in the DS rhizomes can only be speculated upon as possibly as a need for economization of pathways not absolutely required to facilitate tolerance to dehydration under cooler temperatures. The best approach to obtain an overall picture of potential protection by the HSP family members in the rhizome and to resolve the apparent contradictions is to employ systems based bioinformatics analysis. Thus the subject of the expression patterns exhibited by the HSPs will be discussed further in Chapter 4.

3.3.5 Western blot analysis

SOD-[Cu/Zn] and heat shock protein 70 (HSP70), were selected for western blotting analyses to check the accuracy of protein identification and changes in relative quantity as predicted from the above iTRAQ analysis results. These were selected not only because iTRAQ quantification showed quantitative differences in expression on dehydration of DT *vs* DS rhizomes, but also because they have been cited in previous studies to be associated with DT in resurrection plants (Ingram and Bartels 1996; Farrant *et al*., 2007) including fern allies (Wang *et al*., 2010) and other DT tissues such as seeds (reviewed in Leprince and Buitink, 2010; Mtwisha *et al*., 2006) (see also Chapter 1). Fig 3.7 below present the western blot results for SOD-[Cu-Zn].

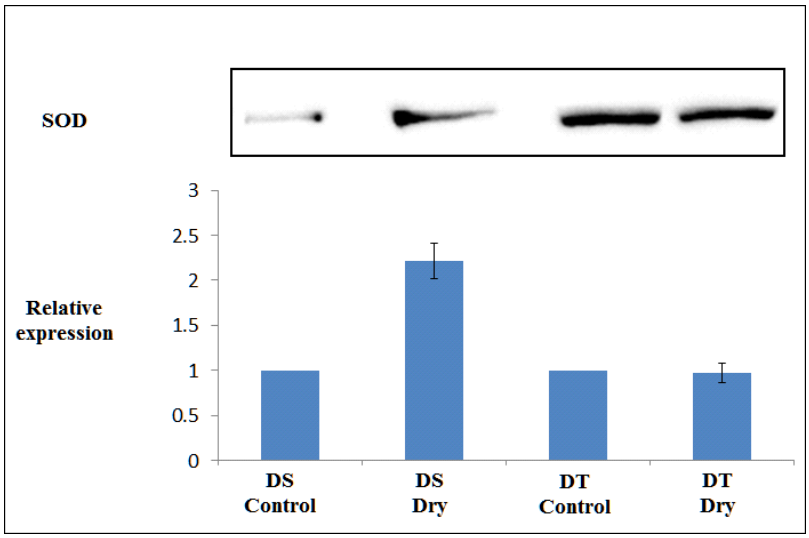


Fig 3.7: Western Blot validation of SOD expression profiles for the rhizomes collected while fronds were DT and those collected while the fronds were DS. The relative quantification of band intensities was performed using ImageLab (BioRad) software and analysed for statistical significance (Student's t-test) using Prism 5 software. The fold changes (relative expression) were calculated by comparing the band intensity values of the dry rhizomes relative to the control (fully hydrated). Three biological replicates were carried out. Error bars indicate the standard error.

Statistical analysis of the western blot results confirmed that the expression levels of SOD-[Cu-Zn] was significantly increased in the DS rhizomes in response to desiccation while the same protein was determined not to be significantly differentially expressed in the DT rhizomes. These differential expression patterns agree with the results of the iTRAQ quantification. Based on the western blot results alone, it is interestingly that the levels of

this protein in the respective hydrated controls show that the DT rhizomes have significantly higher levels than the DS rhizomes. It is suggested here that the combined effects of higher temperatures and desiccation in summer has the potential of causing high levels of oxidative damage to cells. Therefore, even in the hydrated state, this house-keeping anti-oxidant may be important in protecting against the ROS, suggesting that SOD-[Cu-Zn] is constitutively expressed in the DT rhizomes. On the other hand, in the cooler temperatures of the winter season in which the DS plants were growing, high levels of house-keeping anti-oxidants are probably not essential for survival. In winter, drying events are not usually experienced. Therefore, the significant increase of SOD-[Cu-Zn] on desiccation in the DS rhizomes is likely a reaction to the unexpected drying event.

Fig 3.8 below present the western blot results for HSP-70-2.

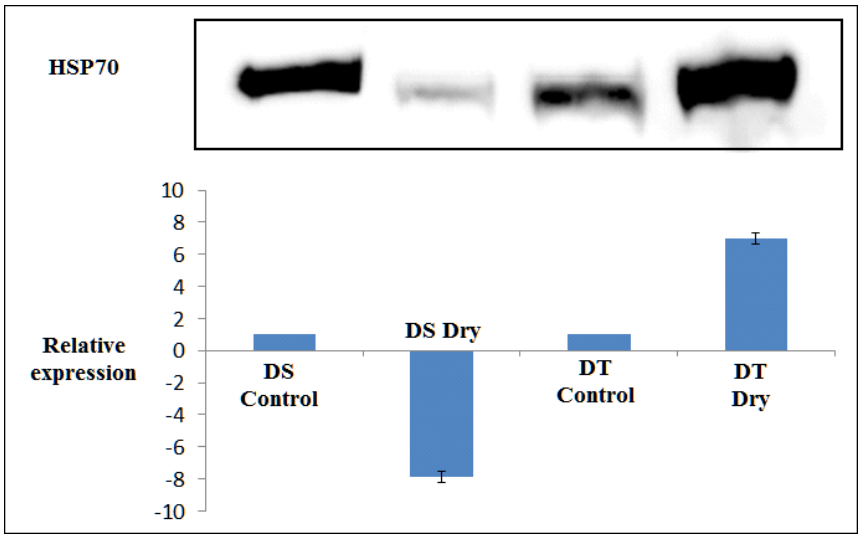


Fig 3.8: Western Blot validation of HSP70-2 expression profiles for the rhizomes collected while fronds were DT and those collected while the fronds were DS. The relative quantification of band intensities was performed using ImageLab (BioRad) software and analysed for statistical significance (Student's t-test) using Prism 5 software. The fold changes (relative expression) were calculated by comparing the band intensity values of the dry rhizomes relative to the control (fully hydrated). Three biological replicates were carried out. Error bars indicate the standard error.

As Fig 3.8 shows, western blot quantification indicates that the levels of HSP70-2 was increased in the DT and decreased in the DS rhizomes. This expression trend agrees with the iTRAQ quantification results. The possible interpretation of these expression trends has been given in section 3.3.4.2 above.

Put together, the agreement between the western blot analysis and iTRAQ on the expression patterns for both HSP70-2 and SOD-[Cu-Zn] shows that despite the multiple cross-species identification strategy employed (*M. caffrorum* to *E. hymale* and then to *A. thaliana*), data generated from this study can be relied on to provide insights into the processes that define desiccation-tolerance or sensitivity in *M. caffrorum*. However, the two techniques (iTRAQ and western blotting) gave different expression values (fold changes). A possible explanation for the observed differences is that the two techniques have different levels of sensitivity. For example, it is suggested that iTRAQ is generally able to achieve more accurate quantification over at least two orders of magnitude of concentration (Casado-Vela *et al*., 2010). On the other hand western blot quantification based on chemiluminescence is limited by its poor detector response linearity. With a linear dynamic range of just over one (Heinicke *et al*., 1992), it does not have sufficient range to accurately quantify immunoblot pictures. Also, antibodies against a given protein may bind to other closely related proteins and thereby giving misleading western blot results. We are aware that more protein targets should have been selected for validating protein expression levels. However, time and financial constraints limited the possibility to acquire more antibodies for western blot analysis.

3.3.6 Summary

In summary, a proteomic investigation has been successfully implemented to identify rhizome proteins that are differentially expressed in the DT and DS phenotypes of *M. caffrorum*, in response to desiccation. While analysis of proteomics data at an individual protein level is capable of providing useful information on mechanisms of desiccation-tolerance in resurrection plants, a more comprehensive and holistic approach using modern bioinformatics tools may reveal much more information. This is because proteins perform their functions in a complex network with other proteins, and not in isolation. As a result, the next chapter (Chapter 4) takes the proteomic investigation described in this chapter further by carrying out network based bioinformatics analysis to obtain deeper insights into molecular processes leading to the acquisition of desiccation-tolerance in rhizomes of *M. caffrorum*.

Chapter 4: Bioinformatics prediction of molecular mechanisms of desiccation-tolerance in *M. caffrorum* rhizomes

4.1 Introduction

In Chapter 3, a high-throughput proteomics approach (using iTRAQ) was presented that produced information on the identity of 236 *M. caffrorum* rhizome proteins, some of which were differentially expressed in response to desiccation stress. However, high-throughput experiments *per se* do not necessarily produce sufficient information to provide a biological understanding of the molecular processes underlying physiological or phenotypic observations. This is because proteins do not work individually, but in an intricate network of interactions. Indeed, Chapter 3 showed clearly that an attempt to understand molecular mechanisms of desiccation-tolerance in the *M. caffrorum* rhizomes based on individual protein expression profiles alone could not suffice because potentially contradictory results were obtained. Since desiccation-tolerance involves, *inter alia,* the integrated responses of many proteins, a systems-level understanding of experimentally derived data is needed to gain in-depth and contextual insights into the protection mechanisms employed by resurrection plants against desiccation.

One of the foundational themes of systems biology is the study of networks of inter-dependent and interacting components that produce the unique properties of the system (Chawla *et al*., 2011). The major strength of network analysis is the fact that it enables the identification of proteins that might have remained undetected in proteomic profiling experiments. These are subsequently added to the initial (query) protein lists and the combined lists used to derive molecular features characteristic of the system. Further, network analysis provides intuitive ways for the visualization of 'omics' data by reducing the intrinsic complexity associated with such data. Different algorithms and methods have been developed to mine biological information from existing datasets of biological networks, including gene-metabolite, protein-protein interaction, transcriptional regulatory, gene regulatory, and co-expression networks (Yuan *et al*., 2008; Konika Chawla *et al*., 2011). However, it is important to note that bioinformatics tools involved in network analysis give only theoretical interpretation of data based on known and predicted

associations stored in the various databases involved in the analysis. Also, we note that our use of TAIR identifiers (Chapters 3 and 4) may impose bias to our analysis given the relationships between *M. caffrorum* proteins *vs* their *A. thaliana* orthologs. Specifically, protein networks and protein-protein interactions observed in *A. thaliana* work may not be a true reflection of what happens in the fern.

This chapter reports the first network based systems-level analysis of *M. caffrorum* rhizome proteome to reveal molecular mechanisms of desiccation-tolerance in the DT and DS phenotypes of the plant. Making use of the publicly available network construction tools, an integrated bioinformatics analysis of the data generated using iTRAQ (Chapter 3) was carried out to derive a molecular interpretation of the data from the perspective of gene ontology (GO) biological process terms, and protein-protein interactions (PPI). Results predict that the DT and DS rhizomes are characterized by two almost contradictory stress-responsive strategies. According to bioinformatics analysis, in the DT rhizomes, pathways related to protein synthesis machinery and the UPS were significantly up-regulated, while the two systems were down-regulated in the dry DS form. The anti-oxidant potential in the desiccated DS rhizomes was predicted to be markedly up-regulated while, in contrast it was predicted to be down-regulated in the DT rhizomes. Furthermore, in the DS rhizomes, pathways related to energy metabolism were predicted to be up-regulated in response to desiccation, while they appeared to be down-regulated in the DT rhizomes. However, there were some similarities between the two phenotypes; rhizomes of the two plant forms activated a similar set of hormones/molecular messengers, namely ABA, JA, indole acetic acid (IAA), auxin (AUX) and sucrose signalling. The exception to this was the putative up-regulation of salicylic acid (SA) and ethylene (ET) mediated signalling in the DS rhizomes only. Based on the bioinformatics tools used, the noteworthy commonly down-regulated biological process was fatty acid β-oxidation. Interestingly, in the DS rhizomes, biological processes normally associated with pathogen defences were hypothetically up-regulated, suggesting a possible cross-talk against both water-deficit stress and pathogen infection. In the DT rhizomes, bioinformatics prediction results suggest an apparent cross-talk and cross-tolerance against water-deficit and heat stresses.

Since the rhizomes are continuously DT (Chapter 2) and given these observations, it is posited that tolerance of extreme water loss has some common features in rhizomes of *M. caffrorum* but that it can, apparently, be achieved by different processes/pathways, depending on the environmental conditions under which the plant is desiccated. It is also proposed that the pathways variously hypothesised to be up- and down-regulated in winter rhizomes might preclude attainment of DT in fronds produced during that season, and conversely, pathways involved in DT in summer rhizomes facilitate tolerance in fronds. In this regard, the current work provides some theoretical indication of pathways involved in the attainment of desiccation-tolerance in *M. caffrorum* rhizomes, and the role that environmental factors (and potential cross-talks among them) might play in attainment thereof. This chapter will also attempt to provide insight as to the potential role of the rhizomes in regulating frond behaviour seasonally. Farrant *et al*., (2009) have shown that the fronds produced towards the end of the winter season produce sporangia for the production of DT spores that are dispersed in spring and early summer. The analyses presented below take these observations into consideration.

4.2 Methods

The following open-source programs were employed to carry out bioinformatics analysis of differentially expressed proteins identified in Chapter 3:

1) ClueGO (Bindea *et al*., 2009; 2013) for enrichment of gene ontology (GO) biological process terms (Ashburner *et al*., 2000);

2) Reduce and Visualize Gene Ontology (REViGO) (Supek *et al*., 2011) for summarization and visualization of enriched GO biological process terms and;

3) Gene Multiple Association Network Integration Algorithm (GeneMANIA) (Warde-Farley *et al*., 2010) for protein-protein interaction and functional module predictions.

4.2.1 GO biological process functional enrichment analysis

The Cytoscape v3.1.1 (Shannon *et al.*, 2003) plug-in ClueGO v2.1.2 was used for enrichment of GO biological process terms. ClueGO is a Java package that extracts the non-redundant biological information for groups of genes/proteins using GO terms and can perform single cluster analysis or cluster – cluster comparisons. In the present study, for input, TAIR identifiers were used for protein cluster lists and ontology terms were derived from *A. thaliana.* Enrichment analysis was performed by comparing frequencies of the annotation terms in the input protein sets (differentially expressed protein sets identified in Chapter 3) with those in the reference list (all the 236 proteins identified in the iTRAQ experiment, see Chapter 3). Two-sided (enrichment/depletion) tests were employed based on the hypergeometric distribution with a statistical significance threshold of $p \leq 0.05$. To correct the p-value for multiple testing, the Bonferroni step-down correction was used to reduce the chances of type 1 error (false positive) rate (Ge *et al*., 2003). P-value doubling was employed to address discreetness and conservatism issues (Rivals *et al*., 2007).

ClueGO networks are drawn using kappa statistics that reflect the relationships between terms based on the similarity of their associated genes. Term-gene matrices containing

terms and their associated genes are created and used to derive term-term similarity matrices calculated using kappa statistics to determine the strength of association between terms (kappa score). A higher kappa score permits connections between closely related terms with very similar associated genes (high stringency). On the other hand, a low score permits connections between less related terms (low stringency). In this work, on a scale of 0 to 1, GO term connectivity (kappa score) was fixed at 0.4. To reduce redundancy, GO term grouping (functional grouping) was used. The minimum number of common parents allowed was set at 3 and the maximum number of different parents was set at 4. No restrictions were imposed on the number of genes to be associated with a term for that term to be considered valid. The ClueGO 'cluster comparison' option was employed to identify biological processes that were common between DT *vs* DS tissues or specific to either of the two, for both the categories of proteins that were increased and those that were decreased in abundance. Since high level terms (closer to the root) in a GO tree can be too general while lower level terms are more specific, enrichment analysis was carried out at the 6^{th} level of depth and deeper in order to achieve an effective comparative analysis.

4.2.2 GO term summarization and visualization

GO biological process terms were summarized using the REViGO tool to further minimize redundancy and simplify the analysis. Node data from statistically overrepresented (enriched) terms in the ClueGO constructed networks were exported and analysed using the REViGO tool. REViGO reduces functional redundancies by summarizing extensive lists of GO terms to show only a representative subset of the terms using a semantic similarity measure clustering algorithm. Cluster representative GO terms were visualized in two-dimensional scatter-plots. REViGO analyses were carried out with the following settings; allowed similarity - small (0.5) [or medium (0.7) for non-specific terms]; GO terms database - *Arabidopsis thaliana*, and semantic similarity measure - SemRel.

4.2.3 Protein-protein integration

The online tool GeneMANIA (http://www.genemania.org), also available as a Cytoscape plug-in, was used to derive the interactome of empirically determined and predicted PPIs

of differentially expressed proteins identified in Chapter 3. GeneMANIA finds association data to form complementary network connections (edges) by searching for related genes in many large, publicly available biological datasets, including protein-protein, protein-DNA and genetic interactions, gene and protein expression data, pathways, protein domains reactions, and phenotypic screening profiles (Warde-Farley *et al*., 2010). The main datasets that are part of the search includes Pfam, BioGRID, Pathway Commons as well as datasets from recent publications. TAIR identifiers of the respective gene sets were used as query genes, and the program was executed with default settings. GeneMANIA can; 1) predict if query genes are functionally connected, 2) find new members of a pathway or complex, 3) predict additional genes that might have been missed and, 4) augment the network with new genes with a specific function.

4.3 Results and preliminary discussion

4.3.1 GO biological process term enrichment

Complete ClueGO generated analyses charts for GO term enrichment analysis results can be found in Figs S1– S6 in the Supplementary Materials. As shown in the Figs S1 – S6, in some cases GO biological processes enrichment analysis produced tens of terms for each dataset. GO biological processes reviewed below are REViGO generated summaries of overrepresented terms. Using REViGO allowed clustering of GO terms into groups of related biological processes and identification of the most unique or less dispensable terms. Thus, in the scatter-plots presented in Figs 4.1 – 4.6 below, only one representative term *per* cluster is shown.

4.3.1.1 Biological processes commonly up-regulated in both DT and DS

Considering the fact that the DT and DS plants were dried under different environmental conditions (summer and winter respectively), the hypothetically commonly up-regulated biological processes are important as they can be considered to be part of the critical molecular network that regulate desiccation-tolerance in *M. caffrorum* since they were up-regulated irrespective of other environmental conditions. Representative GO biological process terms predicted to be up-regulated in both DT and DS rhizomes are presented in Fig 4.1 below.

As shown in Fig. 4.1, the DT and DS rhizomes were both predicted to up-regulated a common set of biological processes relating to several hormones, which suggests that the hormones and other signalling molecules play a key role in the acquisition of desiccation-tolerance in *M. caffrorum*. However, despite being predicted to be commonly up-regulated in the two forms of this plant, it is possible that these hormones and signalling molecules do not necessarily regulate the same pathways in the same way in the two rhizome types. Different hormone expression levels and combinations are likely to lead to different outcomes in the two rhizome types.

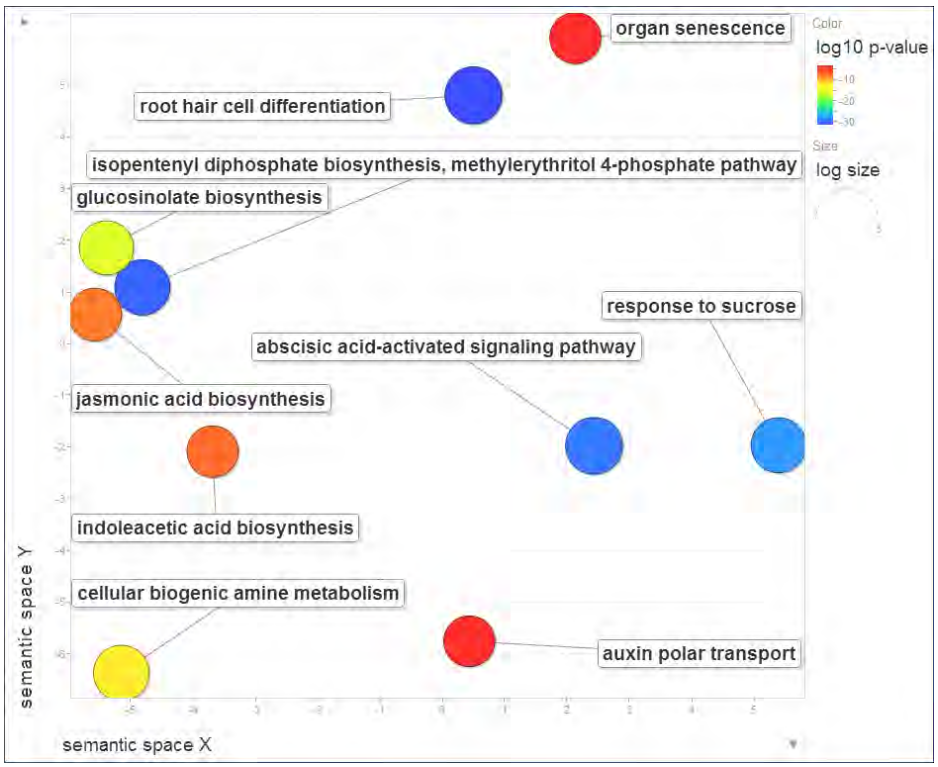


Fig 4.1: The scatter-plot shows the GO biological process cluster representatives of terms up-regulated in both DT and DS rhizomes. The bubble colour indicates the P-values obtained from the ClueGO enrichment analysis (colour code is shown on the upper right corner). Bubble size indicates the GO term frequency in the underlying GOA (Camon *et al*., 2004) database. More general terms are larger, and more specific terms are represented by smaller bubbles. The GO terms are clustered together in the semantic space according to functional similarity and the units on the scatter-plots have no intrinsic significance.

The hypothetical up-regulation of biological processes involving ABA-mediated signalling pathway, and JA biosynthesis shows the importance of these hormones in the rhizomes of this plant. The proposed roles for JA were discussed in Chapter 3, particularly its potential role as a signalling molecule in the regulation of glutathione and ascorbate metabolism (Shan and Liang, 2010). ABA-mediated signalling has been suggested to be involved in the regulation of genes encoding protectants such as heat shock factors and LEA proteins, aldehyde dehydrogenase, galactinol and raffinose family oligosaccharide (Deng *et al*., 2006; Wang *et al*., 2009; Kirch *et al*., 2001; Wu *et al*., 2011). In the leaves and roots of *H. rhodopensis*, JA and ABA served as signals triggering the response to desiccation (Djilianov *et al*., 2013). Therefore it is likely that these pathways played similar roles in the *M. caffrorum* rhizomes.

The biological processes 'indolacetic acid biosynthesis' and 'auxin polar transport' are associated with root related processes (Marchant *et al*., 1999). The hypothetical up-regulation of these biological processes is likely to be linked to the predicted up-regulation of 'root hair cell diferentiation'. Root hair development has been demonstrated to be an adaptive response to low water potentials, and the process is mediated by ABA accumulation, AUX transport in the root tips and ROS (Schnall and Quatrano, 1992; Foreman *et al*., 2003; Yamaguchi and Sharp, 2010; Xu *et al*., 2013), which were apparently up-regulated in this work. Root hair cell differentiation refers to a situation where a relatively un-specialised cell acquires the features of a root hair cell. The desiccation-tolerance of root-hairs was not tested in this work, but it is possible that if these structures lose viability on drying, most likely shortly after rehydration, it is necessary to produce new root-hairs for continued water and nutrient uptake in the metabolically active plant.

While the role of sucrose signalling in resurrection plants has not been extensively studied; in other plants e.g. *Brassica oleracea* and *A. thaliana,* it appears sucrose has a role in the induction of responses associated with ROS scavenging (Nishikawa *et al*., 2005; Couée *et al*., 2006; Ramel *et al*., 2009). In addition, many JA, ABA and other stress-inducible genes are co-regulated by sugars (Reinbothe *et al*., 1994; Sadka *et al*., 1994). Given the fact that sucrose tends to accumulate during drying in ferns and other DT angiosperms examined to date (Ghasempour *et al*., 1998; Cooper and Farrant, 2002; Whittaker *et al*., 2007; Phillips *et al*., 2008; Farrant *et al*., 2009; Toldi *et al*., 2009; Oliver *et al*., 2011), it is highly probable that sucrose signalling may have a role in the acquisition of desiccation-tolerance in resurrection plants. Therefore, the predicted up-regulation of the 'response to sucrose signalling', in both the DT and DS rhizomes suggests that shifts in sucrose metabolism, and possible resultant sucrose signalling plays a key role in the acquisition of desiccation-tolerance in *M. caffrorum* rhizomes.

Glucosinolates have not been reported to be present in ferns, and are found almost exclusively in the order Brassicales, and to a lesser extent in the genus Drypetes in the order Euphorbiales (Halkier and Gershenzon, 2006). It is possible that this species does indeed have glucosinolates in their rhizomes. Alternatively, the identification of biological

processes related to glucosinolates in *M. caffrorum* rhizomes, could well be an example of the disadvantages of using resources developed for the model plant, *A. thaliana*, to conduct molecular biology research in evolutionarily distant species. However, useful insights can still be obtained. In terms of roles in abiotic stress, an *A. thaliana* mutant deficient in glucosinolate metabolism was reported to be thermo-sensitive and to be defective in the expression of cytosolic Hsp90 after heat stress (Ludwig-Muller *et al*., 2000), implying a role in the acquisition of tolerance to heat and perhaps to other stresses. The existence of a related pathway in the *M. caffrorum* rhizomes with similar roles cannot be ruled out.

The cellular biogenic amine metabolism also appears to have increased in response to desiccation. One group of the widely reported biogenic amines in resurrection plants are the polyamines. Polyamines are involved in various abiotic stresses including drought, UV, oxidative stress *etc.,* and are thought to act as signalling/messenger molecules to modulate the plant responses to stress (Bhatnagar-Mathur *et al*., 2008; Groppa and Benavides, 2008; Alcázar *et al*., 2010). The accumulation of polyamines tends to correlate with drought tolerance. For example, in *Craterostigma plantagineum* the reported levels of polyamines were higher than those reported for *A. thaliana* after drought treatment (Alcázar *et al*., 2011). It is possible, therefore, that polyamines may act as signalling molecules modulating the response to drying, and perhaps the rehydration process in the *M. caffrorum* rhizomes. Also, polyamines are capable of binding to negatively charged molecules such as proteins, membrane phospholipids, or DNA and thus are able to protect these macromolecules (Dinakar and Bartels, 2013). This characteristic could also be beneficial to the *M. caffrorum* rhizomes during the desiccation process or during rehydration.

The isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway is involved in the production of isoprenoids. Plant isoprenoids are involved in the synthesis of a variety of compounds potentially important in the acquisition of desiccation-tolerance e.g. sterols (essential for membrane architecture), regulatory molecules (ABA, brassinosteroids, strigolactones from roots, *etc.*) (Pulido *et al.*, 2012). Recent work has shown that some intermediates of the isoprenoid biosynthesis pathway act as signal molecules released from the plastid to modulate the expression of stress-related genes

(Xiao *et al*., 2012). Isoprenoids are believed to form part of the defence system against oxidation in all plants and some isoprenoids were reported to enhance drought tolerance in some plants (Demmig-Adams and Adams III, 1996; Davison *et al*., 2002; Munné-Bosch and Alegre 2002; Telfer, 2002; Du *et al*., 2010). In *X. humilis* leaves, isoprenoids were suggested to function in the protection of photosynthetic membranes from desiccation-induced damage (Beckett *et al*., 2012). Therefore, the predicted up-regulation of isoprenoid biosynthesis implies that these compounds have a role in the protection of the *M. caffrorum* rhizomes, possibly of protecting them against desiccation-induced oxidative damage.

Another interesting observation is that, according to the bioinformatics predictive tools, the biological process 'organ senescence' was up-regulated in both the DT and DS rhizomes upon desiccation. Senescence could probably also play roles in regulating controlled turnover of proteins and other molecules in stressed tissues of both the DT and DS rhizomes. For example, it is possible that the rhizomes senesce older or dead rhizome tissues so that the nutrients from those tissues get recycled to other parts of the rhizomes to produce potentially essential protectants.

4.3.1.2 Biological processes commonly down-regulated in both DT and DS

Biological processes predicted to be commonly down-regulated in the DT and DS rhizomes in response to desiccation stress, are shown in Fig. 4.2 on the next page. As shown in Fig 4.2, based on the bioinformatics analysis applied here, few processes were predicted to be down-regulated in common in DT *vs* DS rhizomes in response to desiccation stress. This is an expected result since the iTRAQ results (Chapter 3) showed that there were no proteins whose protein expression levels were commonly declined between the two rhizome types in response to desiccation. These commonly down-regulated GO biological process terms can be viewed as representing processes that are not necessary in the cells during the desiccation process, or in the fully desiccated rhizomes and therefore have to be down-regulated to save energy and resources, redirecting these to

cell protection. Also, down-regulation of some processes may be designed to ensure that the reactions that are likely to cause the accumulation of toxic products are avoided.

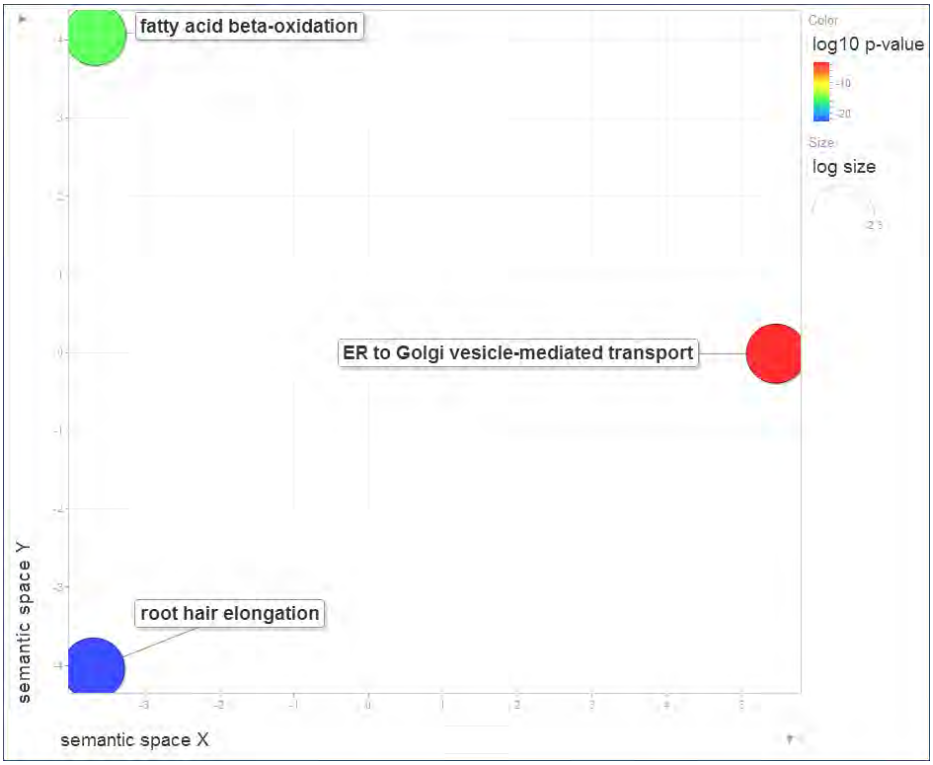


Fig 4.2: The scatter-plot shows the GO biological process cluster representatives of terms down-regulated in both DT and DS rhizomes. The bubble colour indicates the P-values obtained from the ClueGO enrichment analysis (colour code is shown on the upper right corner). Bubble size indicates the GO term frequency in the underlying GOA (Camon *et al*., 2004) database. More general terms are larger, and more specific terms are represented by smaller bubbles. The GO terms are clustered together in the semantic space according to functional similarity and the units on the scatter-plots have no intrinsic significance.

One of the major functions of fatty acid β-oxidation is the catabolism of fatty acids from lipid storage reserves for gluconeogenesis (Poirier *et al*., 2006). In Chapter 2, it was shown that lipid reserves in the rhizome cortical cells of both the DT and DS phenotypes of this plant were reduced in response to desiccation in both the DT and DS plants. It is possible that β-oxidation was involved in the degradation of lipids in the rhizome cortical cells (and, perhaps other tissues) for energy production during drying. However, at the end of desiccation when the lipid reserves were decreased, high levels of fatty acid β-oxidation were probably no longer essential. One of the major consequences of fatty

acid β-oxidation is the generation of H_2O_2 (del Río *et al*., 2002). Therefore, the predicted down-regulation of biological processes associated with fatty acid β-oxidation can be interpreted to be a way of reducing the accumulation of H_2O_2 and therefore protect cells from oxidative damage.

The GO biological process 'ER to Golgi vesicle-mediated transport' refers to the directed movement of substances from the endoplasmic reticulum (ER) to the Golgi apparatus, mediated by COP II vesicles. Molecules transported by the COP II trafficking system include proteins and other soluble cargo (Lee and Miller, 2007). The predicted down-regulation of the COP II trafficking system may be a reflection of the decrease in the production of metabolism associated with active secretion to the plasmalemma, cell wall and/or vacuoles during the last stages of desiccation.

The predicted down-regulation of the GO biological process 'root hair elongation' appears to be an apparent contradiction since a related biological process, 'root hair cell differentiation' was predicted to be up-regulated in both the DT and DS rhizomes. Since this study only analysed protein expression at two points only (fully hydrated *vs* fully dehydrated), it is difficult to predict what transpired during the course of drying. Generally, growth is sensitive to water-deficit. For example, under controlled conditions, *A. thaliana* seedlings subjected to water-deficit terminate shoot and root growth at water potentials of only 21 and 20.6 MPa, respectively (van der Weele *et al*., 2000). Therefore, during the drying process, the elongation of existing root-hairs was probably inhibited during drying. It was proposed above that root hair cell formation may be important during desiccation. It is most likely that the apparent down-regulation of the pathways responsible root hair elongation observed here suggests that the transcripts for this process were stored in the rhizomes but were prevented from being translated, as suggested by Dace *et al*., (1998).

4.3.1.3 Up-regulated biological processes specific to DT rhizomes

Bearing in mind the fact that the DT plants were collected and dried under summer conditions, the biological processes predicted to be up-regulated in the DT rhizomes may also bear the influence of the specific conditions under which the plants were grown, e.g.

higher temperature and high levels of radiation compared to winter. Fig 4.3 below shows some of the biological processes predicted to be up-regulated specifically in the DT rhizomes.

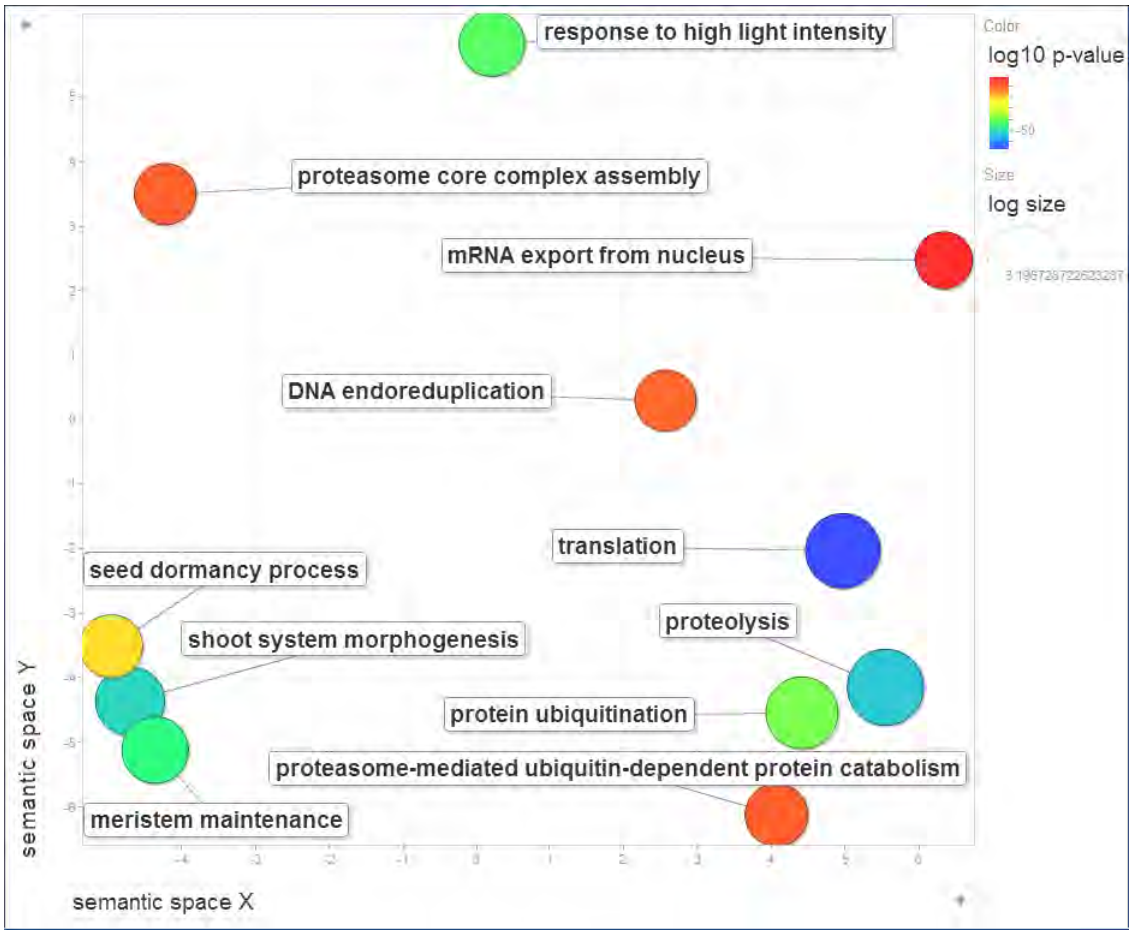


Fig 4.3: The scatter-plot shows the GO biological process cluster representatives of terms up-regulated in the DT rhizomes. The bubble colour indicates the P-values obtained from the ClueGO enrichment analysis (colour code is shown on the upper right corner). Bubble size indicates the GO term frequency in the underlying GOA (Camon *et al*., 2004) database. More general terms are larger, and more specific terms are represented by smaller bubbles. The GO terms are clustered together in the semantic space according to functional similarity and the units on the scatter-plots have no intrinsic significance.

As shown in Fig 4.3, the DT rhizomes seemingly induced dormancy processes in response to desiccation as shown by the hypothetical up-regulation of the GO biological process term ‘seed dormancy processes’. Seed dormancy is a well-documented phenomenon that is

induced, often along with desiccation-tolerance in many (non-recalcitrant) seeds. These pathways are proposed to maintain the seed in a relatively quiescent non-germinative mode until environmental conditions conducive to germination and subsequent plant growth for that species occurs. If indeed summer dormancy pathways in the DT rhizomes were up-regulated, this could ensure better survival in the dry season. It is suggested here that the rhizomes of *M. caffrorum* do not go into dormancy but rather they become quiescent. As mentioned in Chapter 1, dormancy in resurrection plants is unlikely. Rather, vegetative tissues in resurrection plants normally become quiescent i.e. if water becomes available, they revive full metabolic activities.

The hypothetical up-regulation of the GO biological process terms 'proteasomal-mediated ubiquitin-dependent protein catabolism', 'protein ubiquitation', 'proteolysis' and, 'proteasome core complex assembly' suggests that selective protein degradation likely mainly *via* the UPS has an important role in the desiccation stress response in the DT rhizomes. The importance of the UPS in ensuring the survival of plant tissues against water-deficit stress was reviewed in Chapter 1. The apparent up-regulation of pathways involved in the UPS in the DT rhizomes may be due to the possible high rate of protein denaturation as a result of not only water-deficit, but also high temperature stress associated with the summer. Protein recycling *via* the UPS may also ensure the synthesis of potential protectants such as the HSP proteins among others, while reducing the number of proteins, which are not required or are counter-productive to the attainment of desiccation-tolerance.

The biological processes 'mRNA export from nucleus' and 'translation' are associated with the protein synthesis machinery. Preservation of the protein synthesis machinery by the DT rhizomes is in keeping with observations made in other resurrection plant species. Studies have shown that in leaves of DT species, protein synthesis continues until the leaves reach an almost air-dry state (Bartels *et al*., 1990; Gaff *et al*., 1997). This adaptation is important in resurrection plants because it is vital that key protectants required for desiccation-tolerance (such as chaperones and LEAs) and enzymes required for the production of other protectants (such as anti-oxidants) are available during the desiccation stress period or in the early stages of rehydration. In addition to stored proteins, it is also

likely that transcripts are stored (prevented from translation) until rehydration as proposed by Dace *et al*., (1998).

Cell multiplication in plants occurs mainly from meristems which comprise undifferentiated actively dividing stem cells (Sablowski, 2011). Meristem maintenance in roots is regulated by the interaction of hormones, including ABA, AUX and JA (Lee *et al*., 2012), which were also predicted to be up-regulated in the present study. It is highly likely that maintenance of integrity of these tissues is essential for the continued growth or re-growth of rhizome tissues such as root-hairs.

The predicted up-regulation of the GO biological processes 'shoot system morphogenesis' in the rhizomes implies that the rhizomes have a role in controlling the frond's capacity to recover from desiccation on rehydration. The accumulation of proteins responsible for shoot system morphogenesis in the desiccated rhizomes may be a strategy to ensure that when the desiccated plants are watered, they produce new fronds, and to ensure the rapid resumption of normal physiological functions. It is notable that this biological process is not predicted to be up-regulated in DS rhizomes where dehydration of fronds results in their death.

The predicted up-regulation of the GO biological process 'response to high light intensity' may be a reflection of the influence of the environmental conditions in summer where plants were likely exposed to high light intensity compared to the DS plants. The exposure to higher light in summer may result in fronds absorbing more energy than can be used for carbon dioxide fixation (Asada, 1999). The accumulation of excess excitation energy in the fronds may in turn cause an increase in ROS (Karpinski *et al*., 1997; Pfannschmidt *et al*., 1999). In *A. thaliana*, it was shown that the high light response involves ABA signalling between cells (Galvez-Valdivieso *et al*., 2009). The up-regulation of processes involved in the response to high light in the rhizomes suggests a possible communication between the fronds and rhizomes *via* ROS and ABA signalling.

The GO biological processes 'RNA methylation' refers to the RNA mediated methylation of DNA molecules. RNA methylation of DNA may be involved in regulating gene expression by switching 'on' or 'off' of certain genes as appropriate (Fu *et al*., 2014). The

predicted up-regulation of this process suggests the active involvement of genetic control of the desiccation stress response in *M. caffrorum* rhizomes.

The biological significance of endoreplication is under intense debate with several theories and hypotheses being put forward. Here, two of the theories are outlined to help speculate on the possible role of endoreplication in the DT rhizome tissues in the moisture deficit stress response. One theory suggests that endoreplication acts as a buffer against DNA damage and mutations by providing extra copies of important genes (Edgar and Orr-Weaver, 2001). If the idea holds, then it is possible that the DT rhizomes underwent DNA endoreplication to provide extra copies of genes essential for the production of protectants needed for protection against water-deficit, or other environmental stresses such as heat which accompany drying in the summer. It is probable that the chances of extensive DNA damage are higher in summer than in winter given the severity of the environmental conditions characteristic of summer.

Another suggestion is that endoreplication may have a role in modulating stress responses. For example, it was demonstrated that increased endoreplication reduced *A. thaliana* leaf sensitivity to desiccation (Cookson *et al*., 2006). It is possible; therefore, that endoreplication in the DT rhizomes may have a key role in the modulation of some processes key to the acquisition of desiccation-tolerance or in the response against other abiotic stresses such as heat or radiation.

Interestingly, preliminary unpublished flow cytometry data on the hexaploid (6C) resurrection plant *X. humilis* show that roots, but not leaves, of this species are octoploid (8C) in the desiccated condition, suggesting endoreplication in those organs during drying (Farrant, pers. com.). Insufficient research has been done on tissues of underground organs of resurrection plants to conclude whether this is a common phenomenon.

4.3.1.4 Up-regulated biological processes specific to DS

Biological processes hypothetically up-regulated specifically in DS rhizomes in response to desiccation are summarized in Fig 4.4. These processes are most likely not merely related to desiccation alone, but also may have been significantly influenced by other stresses in the environment in which the plants were grown.

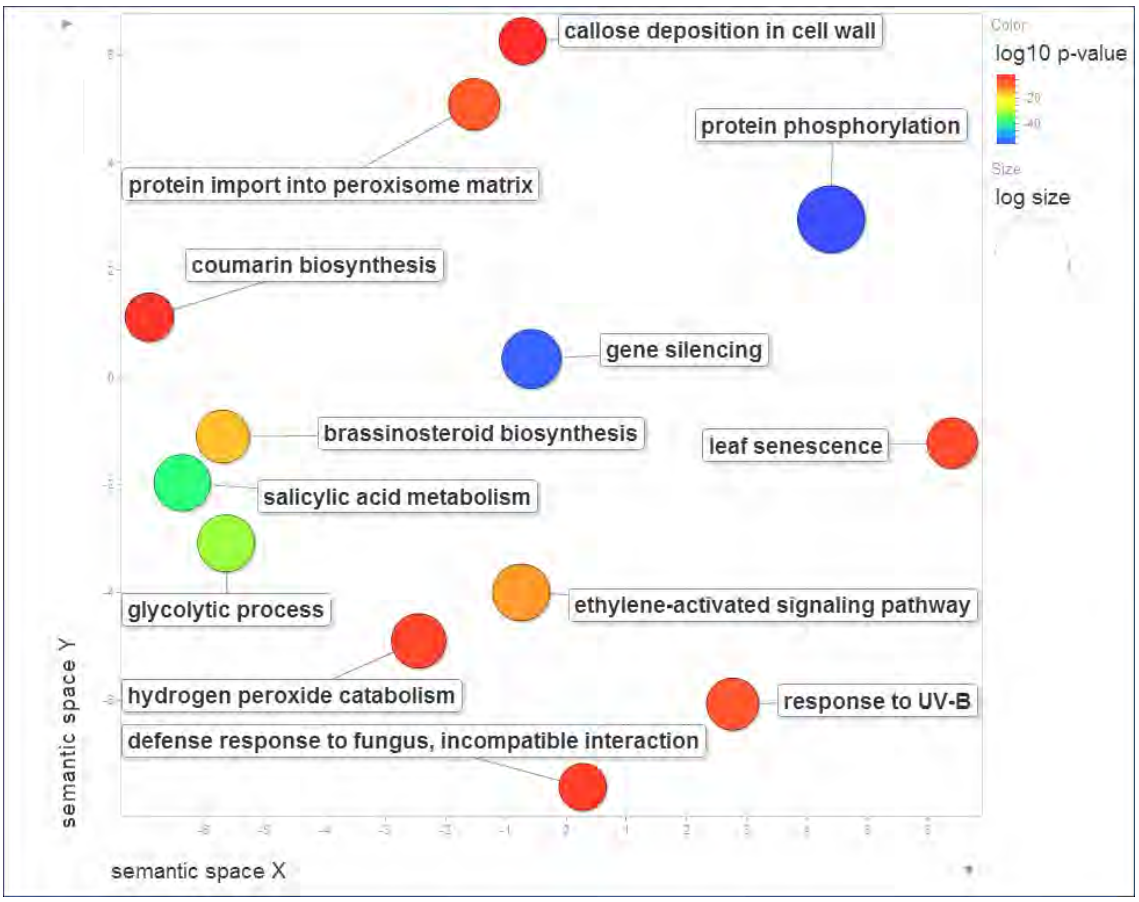


Fig 4.4: The scatter-plot shows the GO biological process cluster representatives of terms up-regulated in the DS rhizomes. The bubble colour indicates the P-values obtained from the ClueGO enrichment analysis (colour code is shown on the upper right corner). Bubble size indicates the GO term frequency in the underlying GOA (Camon *et al*., 2004) database. More general terms are larger, and more specific terms are represented by smaller bubbles. The GO terms are clustered together in the semantic space according to functional similarity and the units on the scatter-plots have no intrinsic significance.

Interestingly, as shown in Fig 4.4, bioinformatics analysis suggests that there appears to be a link in the responses of the DS rhizomes to desiccation stress and biotic stresses, as shown by the predicted up-regulation of biological processes associated with 'defence response to fungus, incompatible interactions'. The up-regulation of biological processes linked to responses against pathogens as a result of desiccation in resurrection plants has also been reported by other researchers. In *C. plantagineum* and *H. rhodopensis*, some transcripts commonly associated with response to pathogen defence increased in abundance during desiccation (Rodriguez *et al*., 2010; Gechev *et al*., 2013). Also, several of the transcripts expressed during rehydration in *C. plantagineum* encode pathogen

responsive proteins (Rodriguez *et al*., 2010). A possible cross-tolerance in the response against both water-deficit stress and pathogen defence will be analysed in the discussion section of this chapter.

Generally, among its several functions, callose is deposited in cell walls in response to both abiotic and biotic stresses, and this response is believed to be mediated by ABA (Nowicki *et al*., 2013; Oide *et al*., 2013). Thus, the predicted up-regulation of the biological process 'callose deposition in cell wall' is consistent with responses to either the desiccation stress treatment employed in this work, or in microbial infections, or, both. In addition, and interestingly, callose can be deposited at the plasmodesmata where it controls the cell-to-cell trafficking of molecules by regulating the size exclusion limit (Iglesias and Meins, 2000; De Storme and Geelen, 2014). As reported in Chapter 2, the rhizome cortical cell walls of both the DT and DS plants were prominently punctuated by plasmodemata and these areas appeared to increase in size during drying. It is possible therefore, that as one of its functions, callose was involved in regulating transport between cells. The fact that this biological process was not predicted to be significantly up-regulated does not preclude the possibility that the callose could still have played the same regulatory role. Why this should be more evident in DS compared to DT rhizomes is not clear. SA has been implicated in defensive roles against many different abiotic and biotic stresses (Janda *et al*., 2007). SA also appears to have roles in water-deficit stress response. In the leaves of the resurrection plant *H. rhodopensis*, SA was maintained at a steady high level during the whole process of desiccation (Djilianov *et al*., 2013), suggesting that, together with other hormones, SA may have a key role in the plant response to desiccation. Thus the hypothetical up-regulation of the GO biological process 'salicylic acid metabolism' suggests that this process may have important roles in the DS rhizomes, and the difference in expression between DS and DT rhizomes could be related to different mechanisms whereby these two phenotypes survive water-deficit in the face of different environmental conditions. Since SA is also widely known for its pathogen response, it is possible that during the winter months this species is faced with different or more virulent pathogens than are present in summer. As this study did not investigate the role of pathogens during desiccation, no further comment can be made here.

The hypothetical up-regulation of the GO biological process 'coumarin biosynthesis' can also be linked to responses against pathogens since coumarin derivatives have been shown to have anti-fungal properties against soil-borne plant pathogenic fungi (Brooker *et al*., 2007).

Water-deficit has been shown to enhance or weaken the responses of plants to UV-B radiation (Zlatev *et al*., 2012). UV-B can cause DNA damage, protein denaturation, and membrane changes (Zlatev *et al*., 2012). Therefore, the predicted up-regulation of the GO biological process 'response to UV-B' in the DS rhizomes may be linked to the invocation of protective mechanisms in response to desiccation. The putative up-regulation of proteins linked to this response in the rhizomes also suggests a communication between fronds and the rhizomes.

The hypothetical up-regulation of the biological process 'hydrogen peroxide catabolism' is consistent with other findings in resurrection plants where proteins involved in anti-oxidant enzymes were up-regulated against desiccation-induced ROS (Sherwin and Farrant, 1998; Farrant, 2000; Kranner *et al*., 2002). The importance of anti-oxidants in the protection against desiccation-induced ROS accumulation was reviewed in Chapter 1. Briefly, ROS generated during dehydration stress can potentially damage proteins, nucleic acids, and lipids. The putative up-regulation of the biological process 'hydrogen peroxide catabolism' in the DS rhizomes suggests that production of this ROS generating chemical may be more prevalent during drying of DS forms and confirms the observation that while tolerance is achieved in both DS and DT rhizomes, it may be differently achieved due to other factors faced by the plant during achievement of tolerance.

Among its several roles, the plant peroxisome is important in the metabolism of ROS, the biosynthesis of JA (which was hypothetically up-regulated in both the DT and DS rhizomes) and the osmoprotectant glycine betaine, *etc.* (Collings *et al*., 2002). Therefore, the predicted up-regulation of 'protein import into peroxisome matrix' suggests that this process may have played an important role in protection of the DS rhizomes during desiccation, possibly by being involved in ROS metabolism.

Phosphorylation is reportedly an important post-translational modification of proteins during many abiotic stresses, including water-deficit stress. It is well-known that many key regulatory proteins in the central amino acid and carbon metabolism exist in altered phosphorylation states depending on environmental conditions (Oliveira *et al*., 2012). For example, significant changes were observed in the phosphoproteomes of leaves of maize and *C. plantagineum* in response to water-deficit (Röhrig *et al*., 2008; Bonhomme *et al*., 2012). In *H. rhodopensis*, one of the most drought/desiccation associated up-regulated genes encoded a protein phosphatase (Gechev *et al*., 2013). In *Zea mays* L, protein phosphorylation enhanced anti-oxidant enzyme activity in water stressed plants (Xu *et al*., 2009). Thus, the putative up-regulation of 'protein phosphorylation' implies that phosphorylation events may be an essential signalling event leading to the acquisition of desiccation-tolerance in the DS rhizomes. While it is likely that phosphorylation is required for tolerance in the DT rhizomes, the comparatively higher induction in DS rhizomes again might suggest alternative pathways of achieving DT in the two forms of the plant.

Another important biological process that was hypothetically up-regulated in the DS rhizomes is 'glycolytic processes'. Two proteins involved in glycolysis in the DS rhizomes; putative phosphoglucomutase and pyruvate kinase, significantly increased in abundance through iTRAQ quantification (Chapter 3). The predicted up-regulation of proteins involved in glycolysis in the desiccated DS rhizome suggests that glycolysis may be essential for the production of critical downstream products, and probably to prepare the rhizomes for rehydration. In *S. pidophylla*, many glycolysis/gluconeogenesis intermediates accumulated during dehydration (Yobi *et al*., 2013), suggesting that the glycolysis/gluconeogenesis pathway is important in the acquisition of desiccation-tolerance in some resurrection plants. The predicted overrepresentation of the GO biological process terms relating to glycolysis in the DS rhizomes has theoretically resolved the complications noted in Chapter 3, where the levels of some proteins involved in glycolysis were increased while others declined.

In some plants, application of brassinosteroids to drought-stressed plants seems to improve the plant response to water-deficit (Krishna, 2003; Jager *et al*., 2008). In the resurrection

plant *Sporobolus stapfianus*, a proteomics study implicated brassinosteroid biosynthesis in the response of leaves to dehydration (Oliver *et al*., 2011), where the authors proposed that brassinosteroids were involved in the regulation of the cellular protection in response to desiccation. Therefore, the predicted up-regulation of the biological process 'brassinosteroid biosynthesis' implies that brassicanoids play an important role in the desiccation stress response in the DS rhizomes, possibly in the regulation of cellular protection during the drying process.

Notably, in the DS rhizomes, the biological process 'leaf senescence' was predicted to be up-regulated in response to desiccation. Leaf senescence may be important in the re-allocation of nitrogen, phosphorus, and metals to other parts of the plant (Jaradat *et al*., 2013). Since the fronds from the winter growing *M. caffrorum* do not survive desiccation, it is possible that the predicted up-regulation of this metabolism in rhizomes is related to induction of senescence in existing fronds.

In truly DT leaves of angiosperm resurrection plants, senescence is prevented (Griffiths *et al*., 2014). Results from the present study suggests communication between rhizomes and fronds with the rhizomes directing senescence in fronds and the potential re-absorption of cellular components from the DS fronds for their use in other organs e.g. rhizomes. It is believed that ROS and ABA are involved in the regulation of water-deficit-triggered senescence (Munné-Bosch and Alegre, 2004). Other regulatory molecules that promote senescence are brassinosteroids and ethylene (He *et al*., 2001). Thus, the predicted concomitant up-regulation of 'ethylene mediated signalling pathway', 'brassinosteroid biosynthesis' and 'ABA-mediated signalling pathway', suggests that these processes were probably responsible for sending the signal to fronds to promote senescence. The lack of up-regulation of these concomitant processes in DT rhizomes might suggest that senescence is prevented in the drying rhizome in that form of the fern. It is hereby suggested that in winter, water-deficit is unlikely to be the main stress threatening this plant and so other mechanisms are also up-regulated to maintain survival under those conditions. Unusual drying events are likely to be brief and thus loss of fronds (and reabsorbion of nutrients for later frond production) is probably a sustainable option. In

summer, drought is a major threat and therefore pathways to specifically prevent senescence in leaves under such conditions might be more important for survival.

The putative up-regulation of 'gene silencing' is an indication of the importance of regulating gene expression in the desiccation stress response in the rhizomes. It could represent the switching 'off' of genes that are not essential during the stress response and also avoid gene products that can be toxic to the plant.

4.3.1.5 Down-regulated biological processes specific to DT

The GO biological processes that were hypothetically down-regulated in the DT rhizomes in response to desiccation are presented in Fig 4.5 below.

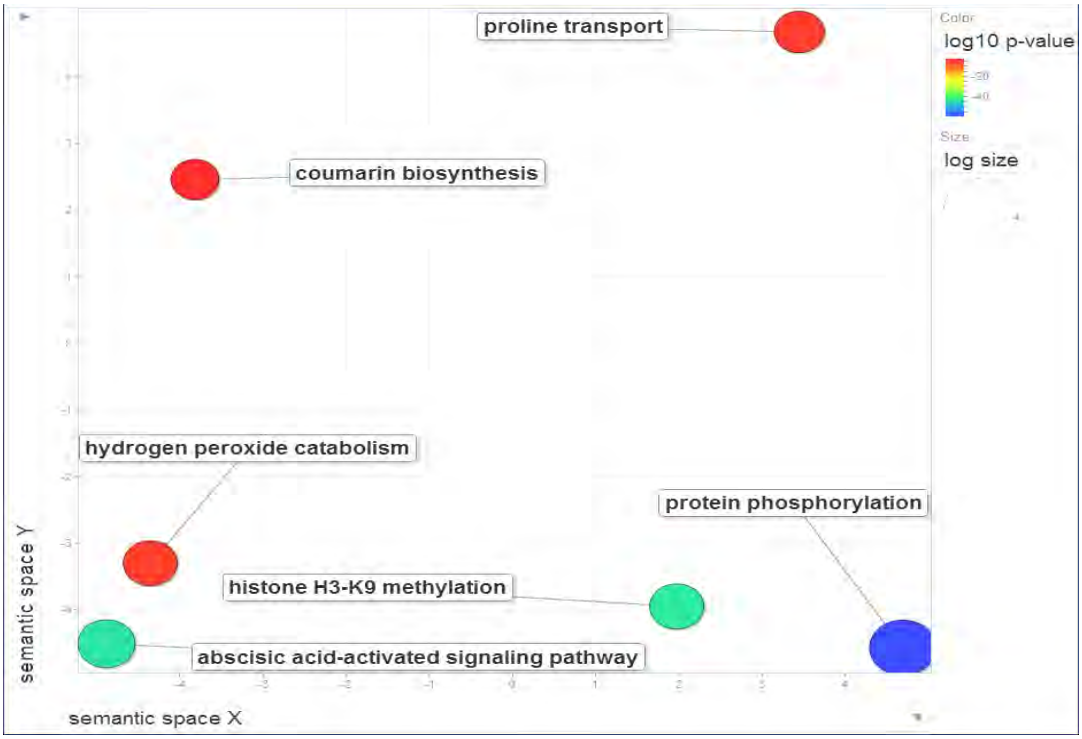


Figure 4.5: The scatter-plot shows the GO biological process cluster representatives of terms down-regulated in the DT rhizomes. The bubble colour indicates the P-values obtained from the ClueGO enrichment analysis (colour code is shown on the upper right corner). Bubble size indicates the GO term frequency in the underlying GOA (Camon *et al*., 2004) database. More general terms are larger, and more specific terms are represented by smaller bubbles. The GO terms are clustered together in the semantic space according to functional similarity and the units on the scatter-plots have no intrinsic significance.

As shown on Fig 4.5, the biological process 'hydrogen peroxide catabolism' which was predicted to be up-regulated in the DS rhizomes was predicted to be down-regulated in response to desiccation. The down-regulation of proteins associated with anti-oxidant activities was observed by the reduced levels of the proteins; L-ascorbate peroxidase and peroxiredoxin-2E (Chapter 3). These and other anti-oxidants were probably significantly reduced during the course of dehydration. Polyphenols (which increased in the DT rhizomes on desiccation – Chapter 2) and other protective strategies may have been employed in the final stages of drying.

The predicted down-regulation of the ABA-mediated signalling pathway presents an apparent contradiction, since the same pathway was predicted to be up-regulated in both DT and DS. Analysis of *A. thaliana* mutants showing altered responses to ABA indicates that the molecular mechanisms of ABA action have genetic redundancy, defined by a signalling pathway with multiple inputs and outputs (see reviews by Bonetta and McCourt, 1998; Leung and Giraudat, 1998). Therefore, results from the present study may be indicative that the ABA-mediated signalling pathway in *M. caffrorum* is also as complex. It is possible that the ABA pathway that was down-regulated operates independent from those that were up-regulated and whose potential roles have already been described. In the context of the results presented thus far, it can be speculated that the putative down-regulation of ABA-mediated signalling in the DT rhizome in response to desiccation was connected to the inhibition of senescence. ABA seems to have dual functions in the drought stress response; cellular protection and promotion of senescence (Griffiths *et al*., 2014).

The putative down-regulation of biological processes associated with coumarin biosynthesis may be explained by the fact that the threat of pathogens in the summer is probably not high compared to the winter season and therefore coumarin biosynthesis was no longer a priority. Besides having a potential as an anti-microbial agent, coumarin is also responsible for the odour of plants and may have some appetite suppressing properties against animals thus protecting the plants from browsers (Link, 1959). These functions may have become non-essential in the desiccated state hence the predicted down-

regulation of coumarin biosynthesis may have helped redirecting resources to critical protective processes.

Protein phosphorylation, which was putatively up-regulated in the DS rhizomes, was predicted to be down-regulated in the DT rhizomes. The benefits of phosphorylation have already been outlined (see section 4.3.1.4 above). The putative down-regulation of protein phosphorylation can be explained by the fact that stress-induced phosphorylation events are largely reversible (Hoyos and Zhang, 2000). Thus, it is possible that phosphorylation occurred at the earlier and mid stages of drying, and was possibly reversed at the end of the drying process as the plant was preparing for quiescence.

The hypothetical down-regulation of the GO biological process 'histone H3-K9 methylation' is yet another example of genetic control of gene expression in response to stress. Methylation of histones on the H3-K9 site is associated with repression of gene expression (Granot *et al*., 2009). Modifications of histones in response to desiccation stress have been reported for some plants. In *Zygophyllum dumosum* Boiss, a drought tolerance desert plant, histone H3-K9 monomethylation appeared to be prominent in the wet season but was undetectable in the dry season (Granot *et al*., 2009). Therefore, the putative down-regulation of this biological process in the DT rhizomes suggests that the process may have an adaptive value in the survival of desiccation and heat stress characteristic of summer.

Reproductive organs, meristems and developing tissues, usually import amino acids to support growth and developmental processes (Kavi Kishor *et al*., 2005). Therefore, it can be speculated that under water-deficit, the DT rhizomes opted to accumulate rather than transport proline to fronds or make more in the rhizome. It has been proposed that accumulation of osmolytes such as proline in the vacuoles during drying may offer advantages including preservation of a normal osmotic pressure in the cells and prevention of mechanical stress, scavenging for ROS and inhibition of lipid peroxidation (Kavi Kishor *et al*., 1995; Hong *et al*., 2000; Ramanjulu and Bartels, 2002; Ashraf and Foolad, 2007).

4.3.1.6 Down-regulated biological processes specific to DS

Biological processes hypothetically down-regulated in the DS rhizomes in response to desiccation are shown in Fig 4.6 below.

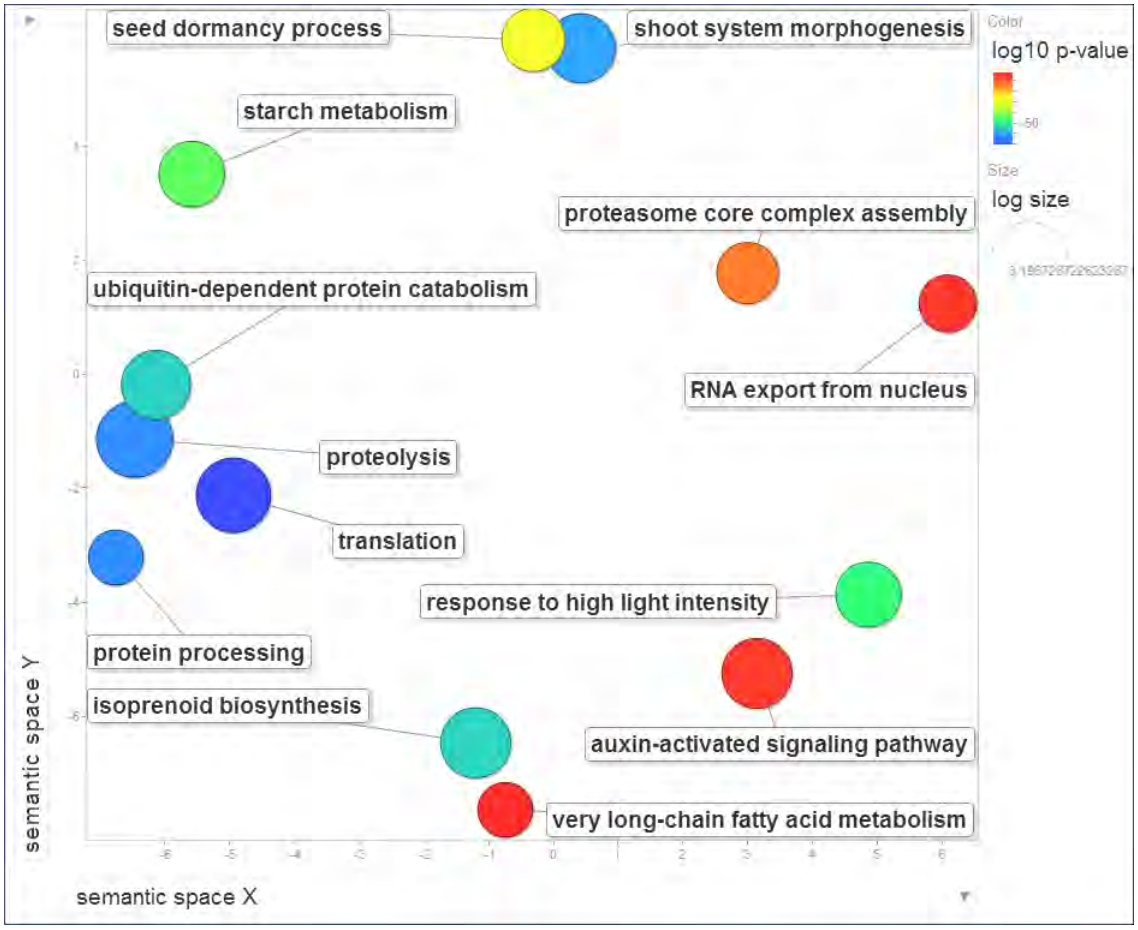


Fig 4.6: The scatter-plot shows the GO biological process cluster representatives of terms down-regulated in the DS rhizomes. The bubble colour indicates the P-values obtained from the ClueGO enrichment analysis (colour code is shown on the upper right corner). Bubble size indicates the GO term frequency in the underlying GOA (Camon *et al*., 2004) database. More general terms are larger, and more specific terms are represented by smaller bubbles. The GO terms are clustered together in the semantic space according to functional similarity and the units on the scatter-plots have no intrinsic significance.

As Fig 4.6 shows, the GO biological processes involved in protein synthesis; 'translation', 'protein processing' and 'RNA export from nucleus' were hypothetically down-regulated in response to desiccation in the DS rhizomes. This is the opposite of the DT rhizomes where the abundance of proteins associated with protein synthesis was significantly

reduced. This could be because high rates of protein synthesis were probably not necessary in the desiccated rhizomes since the DS plants grew in a relatively favourable environment compared to the DT plants. Also, it can be that cells stored in the desiccated rhizomes appropriate transcripts required for protectants upon rehydration. These transcripts could be prevented from translation until moisture becomes available.

Also, the GO biological processes 'ubiquitin-dependent protein catabolic process', 'proteasome core complex assembly' and 'proteolysis', which are associated with protein degradation, were predicted to down-regulate in the DS rhizomes. This is the opposite of the DT rhizomes where biological processes relating to protein degradation were hypothetically up-regulated. It could be assumed that since the DS plants grew in winter when environmental conditions were generally not harsh and there was no need for high levels of protein degradation hence the inhibition of biological processes involved in protein catabolism.

Considering that the DS rhizomes appeared to have up-regulated biological processes associated with pathogen defence (see section 4.3.1.4), it is conceivable that the putative down-regulation of the UPS is a pathogen defence strategy. The UPS has been associated with host immune responses triggered by pathogen attack (Dudler, 2013).

The putative down-regulation of biological processes involved in 'starch metabolism' is consistent with the ultra-structural analysis results of the rhizome cortical cells, where it was shown that the number of starch granules in the rhizome cortical cells were significantly reduced in response to desiccation stress (Chapter 2). The depletion of starch was likely accompanied with the down-regulation of starch metabolism.

In contrast to the DT rhizomes, the 'seed dormancy process' was predicted to be down-regulated on desiccation stress in the DS rhizomes. It was proposed that the up-regulation of dormancy related proteins in the DT may be related to quiescence. In the case of the DS rhizomes, the putative down-regulation of this process may be related to the environmental conditions that prevail in winter. While it is likely that dormancy associated quiescence genes may be more activated in the summer season where moisture is naturally scarce and

the temperature is high, the DS plants growing in winter are probably not genetically programmed to use the same quiescence scheme. It is probable that a different genetic program suitable for the natural winter season was deployed to achieve quiescence.
Long-chain fatty acids are believed to have several functions in plants including promotion of cell elongation, and the promotion of lateral root growth (Qin *et al*., 2007; Roudier *et al*., 2010). As mentioned before, the proteomic investigation reported in this work was carried out at the fully hydrated *vs* extreme dried states, making it difficult to predict the metabolic profiles in the mid stages of drying. It is possible that these growth related processes (cell elongation and lateral root growth) may have been important in the early stages of drying but not essential in the final stages of drying. The continued production of these fatty acids may have ceased to be essential to the plant in the later stages of drying.

The predicted down-regulation of the biological process 'auxin mediated signalling' may represent another apparent contradictory result associated with the hormonal pathways in the desiccation stress response since auxin polar transport was apparently up-regulated in both the DT and DS rhizomes. Antagonism against other up-regulated hormones, to achieve a balance for an appropriate response to a variety of abiotic and biotic factors is possible. Hormone balances regulate many plant responses to stresses, including in water-deficit (Wilkinson *et al*., 2012). Also, it is believed that abscisic acid (ABA), AUX, , SA, JA, ethylene (ET), and cytokinins (SKs) constitute part of a complex hormonal cross-talk which exerts a critical influence on the core plant immunity pathways (Robert-Seilaniantz *et al*., 2011). Since auxin (AUX) is also involved in the growth and development in plant organs and roots in particular (Marchant *et al*., 1999; Friml, 2003; Xu *et al*., 2013), the putative down-regulation of AUX mediated signalling may suggest the cessation of some growth and developmental processes in the rhizomes during dehydration. It is likely that cessation of this metabolism enables energy to be used in protective processes. The predicted down-regulation of AUX mediated signalling pathway is not necessarily in conflict with the fact that the GO biological process 'auxin polar transport' was hypothetically up-regulated in both the DT and DS rhizomes. This can be explained by the fact that auxin polar transport is characterized by its directionality (polarity). Thus, auxin transport was probably actively promoted gravimetrically towards the rhizome root tips

and yet reduced towards the apical parts. In that way, auxin signalling was probably regulated at the hormone distribution level and not at the biosynthesis level.

Unlike in the DT rhizomes, the GO biological process 'shoot system morphogenesis' was predicted to be down-regulated in the DS rhizomes in response to desiccation. This can be explained by the fact that the fronds of the DS plants are killed during extreme desiccation (Farrant *et al*., 2009). Thus, the DS rhizomes are not programmed to produce fronds upon drying. This observation provides another line of evidence that the rhizomes are the regulatory organs responsible for determining whether fronds produced on rehydration are DT or DS.

The 'Isopentenyl diphosphate biosynthetic process, methylerythritol 4-phosphate pathway', which was apparently up-regulated in both the DT and DS rhizomes presents a contradiction as it appears that the same process was down-regulated in DS. The potential roles of isoprenoids in the acquisition of desiccation-tolerance have already been described (see 4.3.1.1). Thus the putative down-regulation observed here can be explained by the fact that plant isoprenoids are an extremely diverse family of compounds with varied functions (Chappell, 1995; Pulido *et al*., 2012). It is probable that those isoprenoids that were down-regulated were those dealing with growth processes or secondary metabolic processes not essential for plant defence against desiccation.

4.3.2 PPI analysis

The GeneMANIA analysis results are presented in Figs 4.7 - 4.10 and Tables 4.1 – 4.4. Bold black nodes represent the significant proteins identified in the iTRAQ experiment (Chapter 3), and the less bold grey nodes represent proteins predicted to be functionally associated with the significant proteins. The circle size represents functional relevance.

4.3.2.1 Predicted PPI network for up-regulated DT rhizome proteins

The predicted PPI networks for the DT up-regulated proteins are shown in Fig 4.7 and the derived functional annotations are shown in Table 4.1.

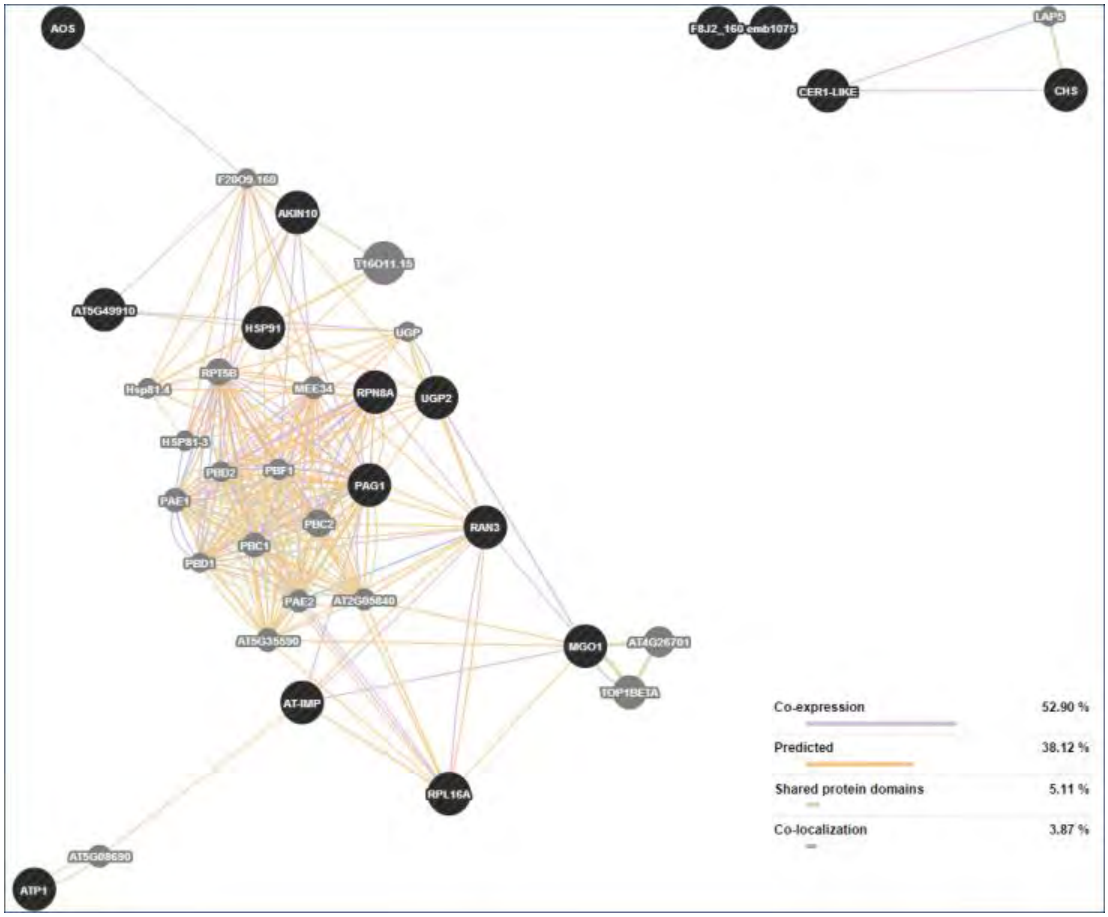


Fig 4.7: Interaction network of DT up-regulated proteins created by GeneMANIA showing the relationships of proteins from the gene list (nodes) connected (with edges) according to the functional association networks from the databases. GeneMANIA retrieved known and predicted interactions between these genes and added extra genes (small grey circles) that are strongly connected to query genes (large circles). Gene names are: F8J2_160 - Pyruvate kinase family protein, ATP1 - ATP synthase subunit 1, AT5G49910 - chloroplast heat shock protein 70-2, emb1075 - Pyridoxal phosphate (PLP)-dependent transferases superfamily protein, CER1-LIKE - Fatty acid hydroxylase superfamily, AKIN10 - SNF1 kinase homolog 10, CHS - Chalcone and stilbene synthase family protein, AOS allene oxide synthase, HSP91 - heat shock protein 91, UGP2 - UDP-glucose pyrophosphorylase 2, MGO1, DNA topoisomerase I alpha, RPN8A - RP non-ATPase subunit 8A, PAG1 - 20S proteasome alpha subunit G1, AT-IMP - importin alpha isoform 1, RAN3 - RAN GTPase 3, RPL16A - ribosomal protein large subunit 16A.

Table 4.1: Predicted functional classes up-regulated in the DT rhizomes.

Feature	FDR	Genes in network
proteasome complex	8.17E-18	11
proteasome core complex	1.16E-14	8
proteolysis involved in cellular protein catabolic process	3.42E-11	11
cellular protein catabolic process	3.92E-11	11
protein catabolic process	4.70E-11	11
cellular macromolecule catabolic process	9.26E-11	11
macromolecule catabolic process	2.78E-10	11
ubiquitin-dependent protein catabolic process	4.22E-10	10
modification-dependent protein catabolic process	4.35E-10	10
modification-dependent macromolecule catabolic process	4.35E-10	10
response to cadmium ion	3.36E-04	7
protein folding	3.62E-04	6
cellular macromolecule localization	3.71E-02	5
apoplast	7.09E-02	5

As shown in Fig 4.7, the most dominant functional classes of the putatively up-regulated DT rhizomes proteins are involved in protein catabolism. These include 'ubiquitin-dependent protein catabolic process', 'modification-dependent protein catabolic process' and, 'modification-dependent macromolecule catabolic processes'. These results agree with those obtained from GO biological process enrichment analysis using ClueGO, and they serve to reaffirms the significance of the UPS in the desiccation stress response in the DT rhizomes.

The hypothetically up-regulated 'protein folding' is associated with proteins of the HSP chaperone system. As Table 1 shows, GeneMania predicted that the genes identified from the iTRAQ experiment reported in Chapter 3 (HSP system; AT5G49910 - chloroplast heat shock protein 70-2, HSP91 - heat shock protein 91), were functionally associated with other genes relating to the HSP chaperone system, which includes; HSP 81.3 – Heat shock protein 81.3, F2009 – DNAJ heat shock family protein, T16011.15 – DNAJ heat shock family protein, HSP-4 –Heat shock protein 18.4 (Table 4.1). The hypothetical up-regulation of these proteins suggests a cross-tolerance against both desiccation and heat

stress. As mentioned in Chapter 1, the HSP system is responsible for correctly folding proteins damaged by stress. Those proteins that are not correctly folded are forwarded to the UPS system (Chapter 1). The predicted up-regulation of protein folding suggests that many proteins required to be folded in specific ways to be functional in response to desiccation stress/high temperature stress associated with the summer. The accumulation of proteins responsible for protein folding may be important either during the desiccation period or in the early stages of rehydration.

The predicted up-regulation of the 'apoplast' can be interpreted as the up-regulation of proteins in the apoplastic space. Among its functions, the apoplast has been proposed to be a means of disposing products of programmed cell death (PCD) in plants (Lam, 2004). In the context of this work, some DT rhizome tissues are more likely to undergo PCD due to the combined effects of desiccation-induced oxidative stress, or high temperatures characteristic of the summer season. It is possible that the predicted up-regulation of the proteins in the apoplast may be linked to the predicted up-regulated 'organ senescence' identified in section 4.3.1.1.

Apparently, proteins associated with the 'response to cadmium ion' were up-regulated in response to desiccation in the DT rhizomes. The hypothetical up-regulation of these proteins may suggest that the Cd concentration increasing in the cells as a consequence of water loss (i.e. localized increase in concentration). Cadmium (Cd) toxicity can cause oxidative stress and the generation of ROS, which can lead to cellular damage just as water-deficit stress (Oono *et al*., 2014). Therefore, it is probable that some of the mechanisms that protect plants against water-deficit can also protect against Cd toxicity in the *M. caffrorum* rhizomes. In rice, it was demonstrated that Cd exposure triggered the up-regulation of drought stress-responsive genes such as LEA3, HSP70, bZIP23 and ABI5 (bZIP23 and ABI5 are thought to be components of ABA-dependent transcriptional networks in rice), and genes encoding several ROS scavenging enzymes (Oono *et al*., 2014). The predicted up-regulation of the 'response to cadmium ion' points to a possible cross-talk or cross-tolerance in which the same protection mechanisms and signalling pathways function to protect the rhizomes against both Cd exposure and water-deficit stress.

4.3.2.2 Predicted PPI network for up-regulated DS rhizome proteins

Protein-protein interaction networks for the DS up-regulated proteins are shown in Fig 4.8 and the derived functional annotations are shown in Table 4.2.

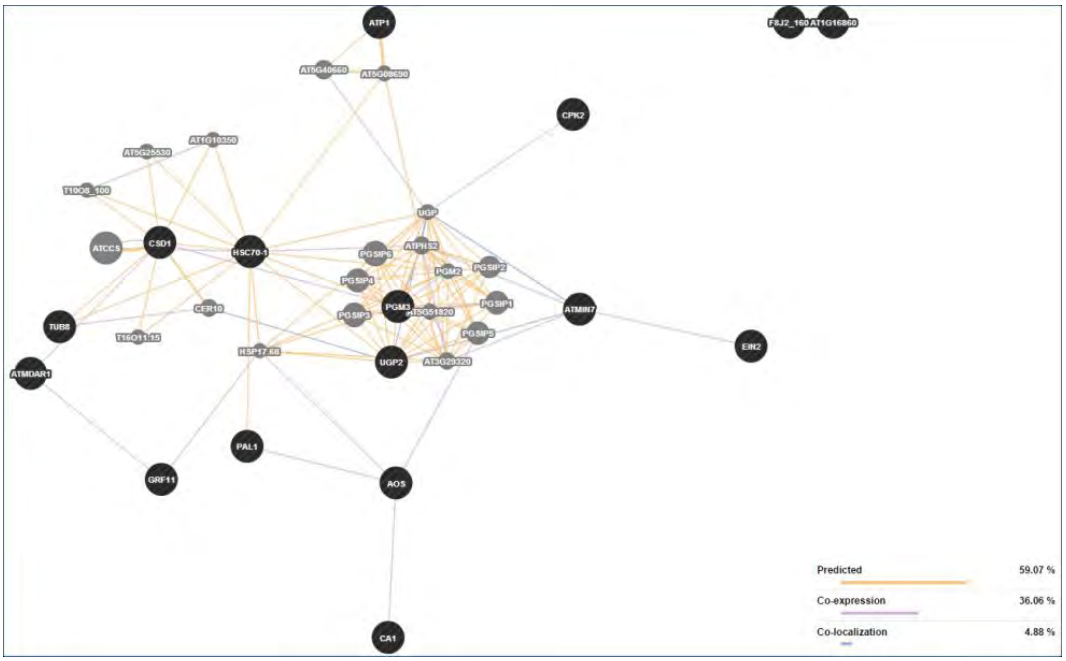


Fig 4.8: Interaction network of DS up-regulated proteins created by GeneMANIA showing the relationships of proteins from the gene list (nodes) connected (with edges) according to the functional association networks from the databases. GeneMANIA retrieved known and predicted interactions between these genes and added extra genes (small grey circles) that are strongly connected to query genes (large circles). Gene names are: F8J2_160 - Pyruvate kinase family protein, AT1G16860 - Ubiquitin-specific protease family C19-related protein, ATMIN7 - HOPM interactor 7, EIN2 - NRAMP metal ion transporter family protein, ATMDAR1 - monodehydroascorbate reductase 1, GRF11- general regulatory factor 11, AOS - allene oxide synthase, ATP1 - ATP synthase subunit 1, CA1 - carbonic anhydrase 1, PAL1 - PHE ammonia lyase 1, UGP2 - UDP-glucose pyrophosphorylase 2, CPK2 - calmodulin-domain protein kinase cdpk isoform 2, PGM3 - Phosphoglucomutase/phosphomannomutase family protein, CSD1 - copper/zinc superoxide dismutase 1, TUB8 - tubulin beta 8, HSC70-1 - heat shock cognate protein 70-1.

Table 4.2: Predicted functional classes up-regulated in the DS rhizomes.

Feature	**FDR**	**Genes in network**
glucuronosyltransferase activity	5.52E-05	4
cellular polysaccharide biosynthetic process	9.39E-04	5
response to cadmium ion	1.00E-03	7
polysaccharide biosynthetic process	1.38E-03	5
cellular carbohydrate metabolic process	1.38E-03	6
cellular carbohydrate biosynthetic process	1.44E-03	5
carbohydrate biosynthetic process	1.44E-03	6
cellular polysaccharide metabolic process	1.46E-03	5
cell wall organization or biogenesis	1.46E-03	6
xylan biosynthetic process	2.77E-03	3
polysaccharide metabolic process	3.44E-03	5
response to oxidative stress	3.44E-03	6
cell wall thickening	3.44E-03	3
callose deposition in cell wall	3.44E-03	3
transferase activity, transferring hexosyl groups	3.44E-03	6
xylan metabolic process	4.31E-03	3
callose localization	4.73E-03	3
polysaccharide localization	5.17E-03	3
protein folding	5.65E-03	5
cell wall polysaccharide biosynthetic process	6.30E-03	3
cell wall macromolecule biosynthetic process	6.30E-03	3
cellular component macromolecule biosynthetic process	6.30E-03	3
stromule	1.42E-02	3
hemicellulose metabolic process	1.49E-02	3
defence response to bacterium	1.82E-02	5
cellular response to ROS	1.93E-02	3
starch metabolic process	2.34E-02	3
secondary cell wall biogenesis	2.70E-02	3
disaccharide metabolic process	2.70E-02	3
cell wall polysaccharide metabolic process	3.19E-02	3
hexose catabolic process	3.19E-02	3
monosaccharide catabolic process	3.19E-02	3
cellular response to oxidative stress	3.19E-02	3
gametophyte development	3.19E-02	5
reactive oxygen species metabolic process	3.93E-02	3
cell wall macromolecule metabolic process	4.75E-02	3
oligosaccharide metabolic process	4.87E-02	3
single-organism carbohydrate catabolic process	4.99E-02	3

Table 4.2 continued

response to superoxide	5.40E-02	2
cellular metal ion homeostasis	5.50E-02	3
cellular cation homeostasis	6.18E-02	3
copper ion binding	6.85E-02	4
glucuronoxylan biosynthetic process	6.85E-02	2
superoxide metabolic process	6.85E-02	2
glucuronoxylan metabolic process	6.85E-02	2
stomatal movement	7.01E-02	3
hexose metabolic process	7.01E-02	3
UDP-glycosyltransferase activity	7.41E-02	4
starch biosynthetic process	7.43E-02	2
cellular ion homeostasis	7.46E-02	3
cell wall modification	8.55E-02	3
carbohydrate catabolic process	8.70E-02	3
cellular chemical homeostasis	9.54E-02	3

As shown in Fig 4.10, the major molecular functions that were predicted to be up-regulated in the DS rhizomes in response to desiccation were mainly those involved in cell wall remodelling, carbohydrate metabolism, polysaccharide metabolism, defence against microbial interaction and response to oxidative stress. The results predict that proteins associated with 'callose deposition in cell wall' and, 'callose localization' were up-regulated; which agrees with ClueGO analysis.

Cell wall remodelling appears to be an important response to desiccation in the DS rhizomes of this plant as shown by the hypothetical up-regulation of such functions as 'cell wall modification', 'cell wall macromolecule metabolic process', 'cell wall polysaccharide biosynthetic process', 'cell wall organization or biogenesis', 'xylan biosynthetic process' *etc*. Changes in cell wall structures in response to desiccation stress are important to stabilize them against damage (Farrant *et al*., 2007). Discussions on modifications in cell wall properties to resist the desiccation stress-induced mechanical damage during dehydration in some resurrection plants can be found in *inter alia* Vicré *et al*., (2004) and Moore *et al*., (2006; 2008; 2013).

Just as in the DT rhizomes, the process of protein folding was also putatively up-regulated in response to desiccation (Table 4.2). This indicates the importance of the HSP system in

the desiccation stress response in the rhizomes of this plant. In addition to the gene, HSC70-1 - heat shock cognate protein 70-1 which was identified in the iTRAQ experiment (Chapter 3), other proteins of the HSP system added by GeneMANIA include; genes of the DNAJ heat shock family proteins (AT5G25530, T10O8_100, T16O11.15, AT5G25530), and HSP17.6II – 17.6 kDa class II heat shock protein. The predated up-regulation of protein folding and the associated HSPs has theoretically helped resolve a potentially confusing situation where iTRAQ identified the level of one protein (heat shock cognate protein 70-1) as increasing and another one (heat shock protein 70-2) as decreasing, making it difficult to understand the overall situation.

Just as was the case for the DT rhizomes, the 'response to cadmium ion' was also hypothetically up-regulated in the DT rhizomes. As already proposed, this could be a desiccation-induced increase in the concentration of cellular Cd.

Another function that was predicted to be up-regulated in the DS rhizomes was the 'stromule'. Stromules (stroma-filled tubules) are highly dynamic structures extending from the surface of all plastids, including proplastids, leucoplasts and amyloplasts which are present in roots and rhizomes although the precise role of stromules has not yet been fully elucidated (Waters *et al*., 2004). It has been proposed that these microfilimanent containing structures increase the surface area associated with plastids which has the potential of increasing transport to and from the cytosol. It is possible; therefore, that stromules might link plastids to mitochondria for energy purposes during desiccation. Also, they could be involved in the movement of organelles to strategic places for protection against desiccation-induced damage. Drought, ABA, salt stress, 1-aminocyclopropane-1-carboxylic acid (the first committed ethylene precursor) and JA have been reported to increase the formation of stromules in the root-hairs of wheat seedlings and the epidermal cells of tobacco hypocotyls (Gray *et al*., 2012), suggesting that the stromule may have defence roles in the response to abiotic stresses and perhaps in biotic stresses too. Also, stromule formation was induced by a DNA-virus in *Nicotiana benthamiana* epidermal leaf tissues (Krenz *et al*., 2012). Thus, the probable up-regulation of 'stromule' in the DS rhizomes is a response to either desiccation stress or pathogen interaction or both.

One notable feature of DS rhizomes is the predicted up-regulation of pathways linked to energy metabolism as shown by the apparent up-regulation of 'hexose catabolic process', 'monosaccharide catabolic process', 'carbohydrate catabolic process' *etc.* As mentioned in Chapter 1, the up-regulation of energy related metabolic processes is important during the dehydration. These results also agree with ClueGO results where biological processes associated with the glycolytic process were predated to be up-regulated. The accumulation of proteins involved in energy production in the desiccated rhizomes is probably also important for rehydration, as energy is needed to revive the plant's metabolic processes.

The hypothetical up-regulation of 'starch biosynthetic process' and 'starch metabolic process' presents a potential contradiction since ClueGO analysis predicted that 'starch metabolism' was down-regulated (4.3.1.5). It can be reasoned that starch metabolism is a broad term. Probably, proteins involved in starch catabolism were down-regulated (as proposed in 4.3.1.5), as the starch reserves declined e.g. in the cortical cells (Chapter 2), in response to desiccation. On the other hand, pathways responsible for starch biosynthesis were likely up-regulated, in preparation for rehydration. The accumulation of proteins responsible for starch synthesis in the dehydrated rhizomes may prepare cells for resumption of active metabolism when the rhizomes are rehydrated.

The predicted up-regulation of proteins associated with 'stomatal movement', and 'gametophyte development' suggests that the rhizomes are involved in the regulation of some processes that take place in the fronds. It is well-known that when roots detect a decrease in soil moisture, plants tend to reduce water loss from transpiration by stomatal closure, and the process is regulated by ABA (Davies *et al.*, 2005; Tardieu, 2005; Tardieu *et al*., 2010). The accumulation of proteins responsible for stomatal movement pathways in the rhizomes shows that the rhizomes are involved in the coordination of the transpiration stream. As mentioned before, Farrant *et al*., (2009) proposed a life cycle for *M. caffrorum* in which fronds produced towards the end of the winter season, produce sporangia for the production of DT spores that are dispersed in spring and early summer. In the present work it is possible, therefore, that the slow desiccation treatment of the DS rhizomes resulted in the accumulation proteins responsible for signalling the fronds to start producing the spores.

4.3.2.3 Predicted PPI network for down-regulated DT rhizome proteins

Predicted protein-protein interaction networks for the DT down-regulated proteins are shown in Fig 4.9 and the derived functional annotations are shown in Table 4.3.

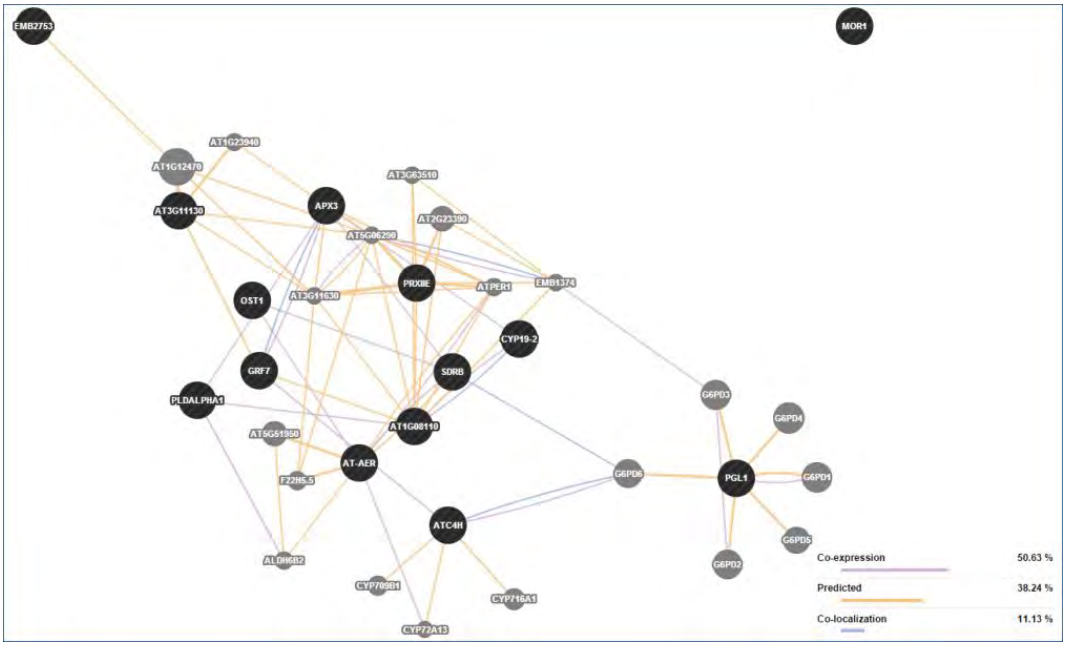


Fig 4.9: Interaction network of DT down-regulated proteins created by GeneMANIA showing the relationships of proteins from the gene list (nodes) connected (with edges) according to the functional association networks from the databases. GeneMANIA retrieved known and predicted interactions between these genes and added extra genes (small grey circles) that are strongly connected to query genes (large circles). Gene names are: AT3G11130 - Clathrin, heavy chain, PLDALPHA1 - phospholipase D alpha 1, CYP19-2 - Cyclophilin-like peptidyl-prolyl cis-trans isomerase family protein, SDRB - short-chain dehydrogenase-reductase B, GRF7 - general regulatory factor 7, MOR1 - ARM repeat superfamily protein, PGL1 - 6-phosphogluconolactonase 1, ATC4H - cinnamate-4-hydroxylase, OST1 - Protein kinase superfamily protein, APX3 - ascorbate peroxidase 3, AT-AER - alkenal reductase, PRXIIE - Thioredoxin superfamily protein, EMB2753 - tetratricopeptide repeat (TPR)-containing protein, AT1G08110 - lactoylglutathione lyase family protein / glyoxalase I family.

Table 4.3: Predicted functional classes down-regulated in the DT rhizomes.

Feature	FDR	Genes in network
NADPH regeneration	2.06E-08	5
NADP metabolic process	5.23E-08	5
pentose metabolic process	5.42E-08	5
nicotinamide nucleotide metabolic process	1.73E-07	5
glucose metabolic process	2.53E-07	6
pyridine nucleotide metabolic process	3.54E-07	5
oxidoreduction coenzyme metabolic process	7.55E-07	5
pyridine-containing compound metabolic process	9.85E-07	5
hexose metabolic process	1.41E-06	6
monosaccharide metabolic process	7.05E-06	6
glucose catabolic process	8.76E-06	5
monosaccharide catabolic process	9.62E-06	5
oxidoreductase activity, acting on the CH-OH group of donors, NAD or NADP as acceptor	9.62E-06	6
hexose catabolic process	9.62E-06	5
oxidoreductase activity, acting on CH-OH group of donors	1.95E-05	6
single-organism carbohydrate catabolic process	2.44E-05	5
carbohydrate catabolic process	1.01E-04	5
coenzyme metabolic process	2.30E-04	5
nucleotide metabolic process	2.69E-03	5
nucleoside phosphate metabolic process	2.99E-03	5
cofactor metabolic process	3.61E-03	5
oxidation-reduction process	6.43E-03	5
nucleobase-containing small molecule metabolic process	6.46E-03	5
organophosphate metabolic process	2.60E-02	5

As shown in Table 4.3, most of the hypothetically down-regulated functions in the desiccated DT rhizomes are mainly those involving redox and energy metabolisms. The predicted down-regulation of proteins in anti-oxidant metabolism agrees with observations made in the ClueGO analysis results.

The hypothetical down-regulation of ‘glucose catabolic process’, ‘monosaccharide catabolic process’, and ‘pentose metabolic process’ *etc.*, suggests the repression of genes responsible for energy generation. This is in contrast to the DS rhizomes where proteins involved in energy generation were predicted to be up-regulated. The hypothetical down-

regulation of proteins linked to energy metabolism is probably associated with the rhizomes going into quiescence, which is characterized by reduced metabolism. Low sugar levels are important at the end of the drying period for quiescence to develop. Also, it can be reasoned that energy is used to make protectants which may include the formation of sucrose and other oligosacharides for glass formation (Chapter 1). Having too much sucrose can be undesirable for oxidative purposes e.g. it may cause the induction of MAILLARD reactions *inter alia*. During rehydration, protectants, including sucrose, are recycled for early energy requirements until the plant is photosynthetically active again.

According to the iTRAQ experiment results (Chapter 3), pyruvate kinase and UTP--glucose-1-phosphate uridylyltransferase 1, which are both involved in glycolysis, were both increased in abundance, and 6-phosphogluconolactonase 1 which is involved in the pentose phosphate pathway, was reduced in abundance. The fact that the levels of two proteins involved in glycolysis were increased and yet the overall effect, according to protein-protein interaction analysis, is not significant shows the value of using network enrichment analysis tools to study molecular processes rather than limiting research to individual proteins.

4.3.2.4 Predicted PPI network for down-regulated DS rhizome proteins

Predicted protein-protein interaction networks for the DS down-regulated proteins are shown in Fig 4.10 and the derived functional annotations are shown in Table 4.4.

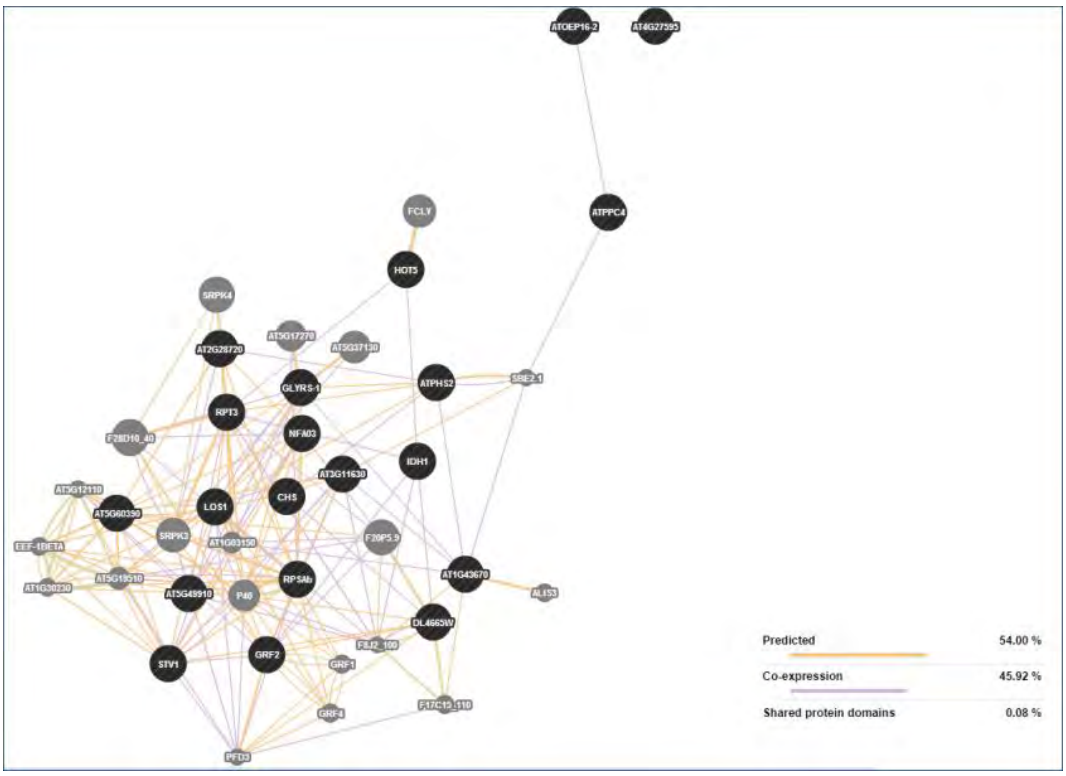


Fig 4.10: Interaction network of DS down-regulated proteins created by GeneMANIA showing the relationships of proteins from the gene list (nodes) connected (with edges) according to the functional association networks from the databases. GeneMANIA retrieved known and predicted interactions between these genes and added extra genes (small grey circles) that are strongly connected to query genes (large circles). Gene names are: AT4G27595 - Plant protein of unknown function, ATPPC4 - phosphoenolpyruvate carboxylase 4, ATOEP16-2 - Mitochondrial import inner membrane translocase subunit Tim17/Tim22/Tim23 family protein, AT5G49910 -chloroplast heat shock protein 70-2, CHS - Chalcone and stilbene synthase family protein, IDH1 - isocitrate dehydrogenase 1, HOT5, GroES-like zinc-binding dehydrogenase family protein, AT1G43670 - Inositol monophosphatase family protein, AT5G60390 - GTP binding Elongation factor Tu family protein, DL4665W - Lactate/malate dehydrogenase family protein, GLYRS-1 - glycyl-t-RNA synthetase / glycine--tRNA ligase, AT3G11630 - Thioredoxin superfamily protein, ATPHS2 - alpha-glucan phosphorylase 2, AT2G28720 - Histone superfamily protein, STV1 - Ribosomal protein L24e family protein, NFA03 - nucleosome assembly protein 1;3, GRF2 - general regulatory factor 2, RPSAb - 40s ribosomal protein SA B, LOS1 - Ribosomal protein S5/Elongation factor G/III/V family protein, RPT3 - regulatory particle triple-A ATPase 3.

Table 4.4: Predicted functional classes down-regulated in the DS rhizomes.

Feature	FDR	Genes in network
translation elongation factor activity	1.01E-05	5
translation factor activity, nucleic acid binding	1.00E-02	5
apoplast	3.43E-02	6
translational elongation	1.23E-01	3
protein phosphorylated amino acid binding	3.40E-01	2
organelle assembly	3.40E-01	2
RNA localization	3.40E-01	2
RNA export from nucleus	3.40E-01	2
phosphoprotein binding	3.40E-01	2
RNA transport	3.40E-01	2

As shown in Table 4.4, the desiccated DS rhizomes appeared to have down-regulated functions relating to protein synthesis such as 'RNA export', 'translation elongation', 'translation elongation factor activity', and 'translation factor activity'. This agrees with ClueGO analysis. Also the predicted down-regulation of 'organelle assembly' agrees with GO biological enrichment analysis results suggesting that the UPS was down-regulated.

In contrast to the situation in the DT rhizomes, the 'apoplast' was predicted to be down-regulated in the DS rhizomes. As proposed before, the apoplast may have been recruited in the DT rhizomes to discharge products of PCD of damaged proteins. Given the relatively less harsh environmental conditions typical of winter, there may have been lower levels of PCD and therefore high levels of proteins involved in the apoplast were not necessary.

4.4 Discussion

4.4.1 Preamble

Desiccation-tolerance is a complex phenomenon which requires an integrated systems biology approach to understand. In this work an integrated approach using network analysis tools enabled the identification of similarities and differences in molecular mechanisms employed by the DT and DS rhizomes of *M. caffrorum* against desiccation stress. GO biological process enrichment analysis revealed the involvement of several phytohormones in the responses of both the DT and DS rhizomes against desiccation, including ABA, JA, AUX and IAA suggesting their importance in the acquisition of desiccation-tolerance in *M. caffrorum* rhizome. In addition to the hormones other signalling molecules such as sucrose signalling were hypothetically up-regulated in response to desiccation. However, these hormones possibly induced slightly different pathways in the two rhizome types. Besides hormones and other signalling pathways, the results indicate that the rhizomes of the two phenotypes regulate their metabolisms differently, presumably in keeping with the specific environments in which the plants grow. Briefly, a summary of the predicted regulation patterns of major molecular pathways by the two rhizomes can be summarized thus;

1) Energy metabolism – up-regulated in DS and down-regulated in DT.
2) Anti-oxidant potential – up-regulated in DS and down-regulated in DT.
3) Pathogen defence – up-regulated in DS.
4) Cell wall remodelling – up-regulated in DS.
5) Protein synthesis – up-regulated in DT and down-regulated in DS.
6) Protein catabolism – up-regulated in DT and down-regulated in DS.
7) HSP chaperone system – up-regulated in both the DT and DS.

This summary shows the complexity of the mechanisms of desiccation-tolerance in the rhizomes of this plant. Although the results obtained from the assessment of just two hydration states (fully hydrated *vs* dry) precludes drawing firm inferences about how the whole plant system functions in different seasons, several hypotheses can still be proposed

for testing in future research. A brief discussion is given below to try and reveal how the plant survives stresses in the respective seasons in which they grow. Also, an attempt is made to consolidate lines of evidence that suggest that the rhizomes appear to regulate the fronds response to desiccation.

4.4.2 Cross-talk in the summer

DT plants used in this study were collected in summer and dried under summer conditions. In summer, temperature can rise to over 30^{o}C with little rain occurring over prolonged periods and relatively higher levels of radiation. The data presented in the results section, point to a possible cross-talk and cross-tolerance against the combined stresses of desiccation and heat. The two stresses all lead to protein denaturation, PCD and elevated levels of ABA (Barua and Heckathorn, 2006; Wituszynska and Karpinski, 2013). Since these stressors were experienced simultaneously by the plants, it is possible that the defences against them shared the same protection mechanisms ('cross-tolerance') or regulatory and signalling pathways ('cross-talk') (Sinclair *et al*., 2013). This potentially evolutionarily adaptive feature may allow an efficient utilisation of resources since the cellular damages caused by the co-occurrence of the stresses are simultaneously countered by the same regulatory system or mechanisms.

As outlined in the results section, there was a hypothetically higher rate of proteins involved in protein turnover which may have been employed to deal with proteins denatured by the combined stresses. Several proteins identified in the iTRAQ analysis (Chapter 3), and predicted by GeneMANIA, revealed that the HSP chaperone system was up-regulated in both the DT and DS rhizomes. As outlined in the results section, predicted the up-regulation of the HSPs in the DT rhizomes in summer in response to desiccation suggests a cross-tolerance to both heat and water-deficit.

Together with other proteins predicted to be up-regulated in the DT rhizomes, the rhizomes seem to employ an efficient pipeline that ensures survival in the relatively harsher season. Proteins that cannot be repaired or correctly folded by the HSP system are probably passed on to the UPS, which was predicted to be up-regulated specifically in the DT rhizomes.

Also, proteins that are not beneficial during the stressful in the stressful periods are also degraded by the UPS. The importance of such a recycling system during the stressful conditions could potentially lead to some pathways being down-regulated to ensure that those proteins get destroyed and amino acids from them used to make new proteins necessary for protection. Protein denaturation and cell death appear to be high in the summer as suggested by the putative up-regulation of pathways responsible for the PCD in the DT rhizomes only. The UPS drives the protein machinery to the synthesis of potentially protectant system (Chapter 1). Indeed proteins involved in protein synthesis accumulated specifically in the DT rhizomes. The three systems; protein folding/chaperone (HSP), protein catabolism (UPS) and the protein synthesis machinery are the key to the survival of the rhizomes in summer. Such an apparently efficiently controlled response is likely coordinated by ABA signalling (among other signalling pathways), and potentially active involvement of gene expression controls e.g. the switching off of the histone H3-K9 methylation pathways, as proposed in the results section. It would be interesting in future studies to investigate whether DNA endoreplication (which was predicted to be up-regulated in the DT rhizomes only) contributes to these proposed key pathways.

Notably, according to bioinformatics predictions both the protein synthesis machinery and protein catabolism were down-regulated in the DS rhizomes. This is likely a means of saving energy resources and directing those to mechanisms appropriate in winter. These results suggest the existence of a finely controlled seasonally relevant protection system which is possibly regulated by the observed variously hypothetically up- and down-regulated hormones driven by other environmental cues.

4.4.3 Cross-talk in the winter

The DS rhizomes seemingly also up-regulated proteins of the HSP family, in response to desiccation stress. Given the relatively cooler conditions in which the plant was dried, it is unlikely that the predicted up-regulation of these proteins is in concomitant defence against both heat and stress. It is suggested that the HSPs are produced merely because desiccation causes protein denaturation just like heat stress and therefore cause the same consequence

of cellular stress. As a result, the up-regulation is probably an adaptive response in which the same protein folding machinery is employed.

Rhizome desiccation under winter (cooler) conditions appeared to induce mechanisms also relating to pathogen stress, which was not observed during drying of the DT rhizomes. It has been reported that moisture deficit increases plant susceptibility to diseases (Colhoun, 1973) and given the observations resulting from the current study, it is possible that a cross-talk between water-deficit and pathogen stresses exists. However, this could also be true of the DT form. It is possible that the pathogens are different in winter and summer and the winter forms require activation of more well-known pathogen pathways that could be identified by the databases associated with the bioinformatics pipeline employed in this study. It is also possible that the summer form of the rhizomes use dehydration to eliminate some pathogens, i.e. pathogens cannot survive desiccation, so they die during the late stages of drying, but the plant tissues survives this stress. These are all theories – and you can postulate them as such. At this stage these are only theories.

Abiotic stress responses are largely regulated by ABA, while the defence response against different biotic stresses is specified by the antagonism between SA and JA/ethylene signalling pathways (Atkinson and Urwin, 2012). As already reported, biological processes relating to JA, SA and ethylene-activated signalling were all identified in the present study. Besides having a key role in the mediation of abiotic stress responses, ABA can also have a positive effect on pathogen defence systems (Asselbergh *et al*., 2008; Ton *et al*., 2009). For example, ABA is required for *b*-aminobutyric acid (BABA) - induced callose deposition during defence against fungal pathogens (Ton and Mauch-Mani, 2004). In addition to identifying most of the hormones believed to be involved in the response to pathogens, this study identified that ‘defence response to fungus, incompatible interactions’, ‘callose deposition in cell wall’ among other pathways were hypothetically up-regulated in the desiccated DS rhizomes. The possible dual roles for ABA, SA, and JA and ethylene indicate that a hormone mediated cross-talk against both pathogens and water-deficit exists in the winter.

4.4.4 The rhizomes as central regulator organs?

Results obtained in the present study give several clues that the *M. caffrorum* rhizomes are responsible for the regulation of the behaviour of the aerial tissues with regards to determining whether the fronds are DT or DS. Drying the rhizomes in summer led to the accumulation of proteins that are likely to have facilitated rapid resumption of normal physiological processes in both the fronds and the rhizomes upon rehydration. Examples include the accumulation of proteins involved in 'shoot system morphogenesis'. Notably, according to bioinformatics predictions the pathways responsible for shoot system morphogenesis were down-regulated in winter rhizomes, showing that the program for new frond formation after desiccation is activated only when plants are dried under summer conditions.

A further suggestion that the rhizomes might influence the physiological status of fronds is provided by the predicted up-regulation of senescence associated proteins in winter dried rhizomes. Since the rhizomes themselves do not appear to senesce on drying, it is proposed that these proteins might induce frond senescence during dehydration. If so, this could be a means of regaining nutrient from senescing fronds during dehydration and as such could be viewed as a 'sacrifice' of the fronds for survival of the rhizome. Rhizomes also appeared to regulate the reproductive stages in the life cycle. This can be seen by the hypothetical up-regulation of the proteins associated with gametophyte development during desiccation of the winter rhizomes. These observations fit in with the life cycle proposed by Farrant *et al*., (2009).

4.4.5 Summary

In summary, the present study employed the strengths of existing network/pathway tools and databases to gain insights into possible processes related to the protection mechanisms employed by the *M. caffrorum* DT and DS rhizomes against desiccation stress. It is worth noting that results produced from any network enrichment analysis, are merely probabilistic inferences based on known and predicted interactions between query proteins/genes and their presumed associates using information stored in various databases.

The information does not always take into account the dynamic and unique nature of every biological system. Thus, the data produced here is aimed at influencing future research by providing pointers for empirically testing some of the findings made in this work. Being the first thorough investigation on molecular mechanisms employed by the resurrection fern rhizomes to protect themselves against desiccation, this work provides an important basis for further investigations of rhizomes of other DT pteridophytes.

Chapter 5: Concluding remarks

The first aim of this research was to establish whether the *M. caffrorum* rhizome was perennially DT or not. This question arose from the observation that the fronds of this plant are DT in the dry season and DS in the wet season (Farrant *et al*., 2009). It was therefore necessary to establish whether this same pattern exists in the rhizomes. The second aim was to identify molecular mechanisms that protect the rhizomes from desiccation stress. Based on ultra-structure and viability investigations conducted, it was established that the rhizome tissues of both winter and summer forms can withstand drying and are perennially DT. However, apparently, the DT state is achieved differently in summer, when temperatures are high and water-deficit stress is a constant threat, than in winter when metabolism is geared to a lower temperature and other stresses (from the data obtained, this is likely to include increased pathogen stresses) might be more of a constant threat.

When the plants were dried under simulated summer or winter conditions, the rhizomes were capable of maintaining cellular integrity and recruiting mechanisms characteristic of most resurrection angiosperms. Using TEM enabled the characterization of organelle organization within the cortical cells. Results showed that cell walls and cell membranes were well preserved during the desiccation stress treatment in plants collected from both seasons. This was also corroborated by the viability studies *via* EL, which showed that the rhizomes did not indicate excessive leakage of metabolites suggesting that membranes had remained intact during desiccation. Fully hydrated cells contained large amounts of starch granules and lipids as shown in the electron micrographs in Chapter 2 and these are hypothesized to contribute to structural stabilization, and acting as nutrient stores. The starch and lipids were almost completely depleted after desiccation, showing that they may have contributed to the cell's energy needs during desiccation stress. On drying, cells from the rhizomes of summer season accumulated large amounts of electron-dense osmophillic substances, presumably mostly polyphenols, compared to the winter collected rhizomes. Polyphenols may have played a role in increasing the anti-oxidant potential of the cells as proposed by, Sgherri *et al*., (2004); Veljovic-Jovanovic *et al*., (2006); Veljovic-Jovanovic *et al*., (2008). TEM also showed that in both the summer and winter rhizomes, desiccation resulted in increasing the visualized size of plasmodesmata in cell walls. These

plasmodesmata may have functioned in cell-to cell communication and, based on bioinformatics analysis, this signalling may have been regulated by callose.

Using proteomics and bioinformatics approaches gave compelling evidence that the rhizomes meets the features of desiccation-tolerance reported for angiosperm resurrection plant tissues in summer (Figure 1.2; reviewed by Vertucci and Farrant, 1995; Oliver and Bewley, 1997; Walters *et al*., 2002; Berjak *et al*., 2007; Farrant *et al*., 2012) but alters metabolism differently in winter to withstand this water loss. This in turn might be related to physiological states of areal parts. In summer, fronds are DT and signals from the rhizomes, one would intuit, contribute towards this process. In winter, the rhizome possibly protects only itself. However, since rhizomes give rise to reproductive fronds during this period, this strategy is not counter intuitive. Survival of the rhizome would be essential to this reproductive strategy. Signals sent to the frond might be different - enabling the recycling of nutrients from those tissues back to the rhizome store reserves for production of new fronds, among them reproductive ones, upon rehydration.

Network enrichment analysis provided an interesting insight into the role of the rhizomes in the regulation of desiccation-tolerance at the rhizome level and possibly also of the whole plant. In addition to apparent signalling to the leaves, rhizomes seem also to regulate some root processes. The indications are that the rhizomes of this plant have a genome plasticity that allows the plant to survive throughout the year. Potential cross-talks and cross-tolerances against desiccation and heat stress were identified in summer, and in winter an apparent cross-talk against desiccation and pathogen stresses was also identified. This work has therefore shown that the mechanisms of DT in resurrection plants cannot be meaningfully studied without considering the role of other biotic/abiotic factors as co-occurrence of multiple stresses appear to modify the molecular mechanisms of DT. The mechanisms of attaining the DT state, at least in *M. caffrorum*, appear to be through a gene plasticity that allows adaptive tolerances to other environment stress.

The present study provides some evidence that the rhizomes function as master regulator organs that control desiccation/sensitivity in the fronds. This role was inferred from the rhizome's up-/down-regulation of pathways that relate to physiological and reproductive functions of the fronds, in response to desiccation. It appears that the rhizome regulate such

functions as leaf senescence, shoot system morphogenesis, stomatal opening and gametophyte development among others. This being the first scientific investigation into the molecular mechanisms underlying DT in the rhizomes of this plant, the work has managed to provide useful insights into the question of how the *M. caffrorum* fern cope with desiccation stress.

The present work used a unique approach in carrying out a proteomic investigation in a resurrection plant. Specifically, a fern rhizome-specific database was used to identify 236 proteins. The database was constructed using RNA sequences from *E. hymale* sequences resources available in the public domain. Thereafter, the bioinformatics approach used took advantage of the well-developed molecular resources for *A. thaliana*, and a systems level investigation that took advantage of publicly available network construction tools. This approach allowed the identification of not only proteins, but also predicted molecular processes (biological processes and molecular pathways, *etc.*). This approach has also shown that despite the lack of well-established molecular resources for resurrection plants, meaningful research can still be conducted leading to testable inferences. Other researchers in the field are strongly recommended to use such approaches.

5.5.1 Suggestions for further work

Several bold hypotheses have been made in this research that need to be followed up in order to obtain a complete picture of how the *M. caffrorum* rhizome acts as a master regulator for the whole of the *M. caffrorum* plant. Thus, it is recommended that further physiology and proteomics or metabolomics work to analyse possible differences in the summer and winter rhizomes be conducted. Since the present work focused only on the fully hydrated *vs* fully dehydrated stages, it may be important to observe changes during desiccation to obtain bigger picture of the mechanism of attaining DT in this plant. It appears that the ability of the rhizomes to cope with desiccation stress appear to be tightly regulated by cross-talks between several hormones that fine-tunes the plant's adaptation to other environmental conditions such as heat and pathogens. It is therefore recommended that empirical investigations be conducted to characterize the hormonal profiles of the rhizome during desiccation.

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Supplementary Materials

Table S1: Proteins identified from the custom database that did not show any significant change in both the DS and DT in response to desiccation stress.

D/Base entry	TAIR	#Unique	Protein description
EhRi_060518	AT1G26480	23	14-3-3-like protein GF14 iota
EhRi_033274	AT5G38480	8	14-3-3-like protein GF14 psi
EhRi_061751	AT1G09780	6	2,3-bisphosphoglycerate-independent phosphoglycerate mutase 1
EhRi_040450	AT1G16470	2	20S proteasome subunit PAB1
EhRi_058322	AT5G43010	17	26S proteasome AAA-ATPase subunit RPT4a
EhRi_018880	AT5G23540	4	26S proteasome non-ATPase regulatory subunit 14
EhRi_048240	AT4G38630	6	26S proteasome non-ATPase regulatory subunit 4
EhRi_045694	AT2G32730	19	26S proteasome regulatory complex, non-ATPase subcomplex, Rpn2/Psmd1 subunit
EhRi_038274	AT5G09900	7	26S proteasome regulatory particle non-ATPase subunit 5A
EhRi_020236	AT2G20580	37	26S proteasome regulatory subunit S2 1A
EhRi_058417	AT2G33150	12	3-ketoacyl-CoA thiolase 2
EhRi_037065	AT3G52580	5	40S ribosomal protein S14-3
EhRi_041109	AT1G07770	9	40S ribosomal protein S15a-1
EhRi_039752	AT5G04800	5	40S ribosomal protein S17-4
EhRi_052714	AT5G35530	43	40S ribosomal protein S3-3
EhRi_041284	AT4G34670	3	40S ribosomal protein S3a-2
EhRi_058584	AT5G58420	15	40S ribosomal protein S4-3
EhRi_052938	AT1G48830	7	40S ribosomal protein S7-1
EhRi_058299	AT2G40010	9	60S acidic ribosomal protein P0-1
EhRi_059411	AT2G27530	2	60S ribosomal protein L10a-2
EhRi_059103	AT3G02360	51	6-phosphogluconate dehydrogenase, decarboxylating 3
EhRi_033447	AT4G02480	4	AAA-type ATPase family protein
EhRi_042322	AT1G36160	15	acetyl-CoA carboxylase 1
EhRi_036245	AT4G35830	13	aconitate hydratase 1
EhRi_032650	AT2G05710	4	aconitate hydratase 3
EhRi_061670	AT5G04740	7	ACT domain-containing protein
EhRi_070660	AT5G09810	6	actin 7
EhRi_044363	AT2G37250	2	adenosine kinase
EhRi_047963	AT4G13940	29	adenosylhomocysteinase
EhRi_061239	AT1G80300	17	ADP,ATP carrier protein 1
EhRi_035492	AT1G15500	4	ADP,ATP carrier protein 2
EhRi_058258	AT4G28390	13	ADP/ATP carrier 3 protein

Table S1 *continued*

EhRi_062710	AT4G39660	3	alanine:glyoxylate aminotransferase 2
EhRi_058372	AT1G20220	6	Alba DNA/RNA-binding protein
EhRi_058378	AT1G23800	11	aldehyde dehydrogenase 2B7
EhRi_058886	AT3G29320	8	alpha-glucan phosphorylase 1
EhRi_061238	AT4G33090	2	aminopeptidase M1
EhRi_018129	AT5G66055	2	Ankyrin repeat domain-containing protein, chloroplastic precursor
EhRi_060239	AT4G24830	4	argininosuccinate synthase
EhRi_048974	AT1G54385	2	ARM repeat superfamily protein
EhRi_055054	AT5G53480	19	armadillo/beta-catenin-like repeat-containing protein
EhRi_032837	AT4G31990	6	aspartate aminotransferase
EhRi_043912	AT4G31180	4	Asx tRNA synthetase (AspRS/AsnRS) class II core domain-contating protein
EhRi_042167	AT5G08680	33	ATP synthase subunit beta-3
EhRi_043056	AT3G06650	30	ATP-citrate lyase B-1
EhRi_043255	AT5G50920	10	ATP-dependent Clp protease ATP-binding subunit ClpC
EhRi_054731	AT1G59453	7	B-block binding subunit of TFIIIC
EhRi_061946	AT5G63980	8	bifunctional 3'(2'),5'-bisphosphate nucleotidase and inositol polyphosphate 1-phosphatase
EhRi_070521	AT2G36530	35	bifunctional enolase 2/transcriptional activator
EhRi_070495	AT5G35360	16	biotin carboxylase subunit CAC2
EhRi_058311	AT1G09210	3	calreticulin 1b
EhRi_040235	AT4G12400	15	carboxylate clamp-tetratricopeptide repeat protein
EhRi_050287	AT5G15450	20	casein lytic proteinase B3
EhRi_046955	AT4G35090	4	catalase 2

Table S1 *continued*

EhRi_058242	AT5G03340	2	cell division control protein 48C
EhRi_038020	AT2G22125	23	cellulose synthase-interactive protein 1
EhRi_053942	AT3G44110	3	chaperone protein dnaJ 3
EhRi_059629	AT2G28000	16	chaperonin-60 alpha
EhRi_059071	AT2G44350	7	citrate synthase 4
EhRi_035239	AT1G52360	10	coatomer subunit beta'-2
EhRi_044217	AT4G31490	9	coatomer subunit beta-2
EhRi_053598	AT4G36910	2	cystathionine beta-synthase domain-containing protein
EhRi_034524	AT3G53110	5	DEAD-box ATP-dependent RNA helicase 38
EhRi_045647	AT1G20920	3	DEAD-box ATP-dependent RNA helicase 42
EhRi_070609	AT5G11200	14	DEAD-box ATP-dependent RNA helicase 56
EhRi_063967	AT1G48430	2	Dihydroxyacetone kinase
EhRi_046234	AT1G59610	6	dynamin-2B
EhRi_050667	AT3G60190	14	dynamin-related protein 1E
EhRi_037640	AT5G25350	5	EIN3-binding F-box protein 2
EhRi_058739	AT2G05990	4	enoyl-[acyl-carrier-protein] reductase [NADH]
EhRi_058826	AT1G11480	12	eukaryotic translation initiation factor-like protein
EhRi_038756	AT3G24200	3	FAD/NAD(P)-binding oxidoreductase family protein
EhRi_070489	AT4G26530	19	fructose-bisphosphate aldolase 5
EhRi_057548	AT3G25530	6	gamma-hydroxybutyrate dehydrogenase
EhRi_031523	AT5G40760	16	glucose-6-phosphate dehydrogenase 6
EhRi_061335	AT5G42740	4	glucose-6-phosphate isomerase
EhRi_040110	AT5G17330	12	glutamate decarboxylase 1

Table S1 *continued*

EhRi_035858	AT4G23100	2	glutamate-cysteine ligase
EhRi_041557	AT5G37600	3	glutamine synthetase 1;1
EhRi_070575	AT4G19880	2	Glutathione S-transferase family protein
EhRi_049816	AT2G30870	3	glutathione S-transferase PHI 10
EhRi_056627	AT1G78380	7	glutathione S-transferase TAU 19
EhRi_044578	AT3G24170	5	glutathione-disulfide reductase
EhRi_049064	AT1G16300	29	glyceraldehyde 3-phosphate dehydrogenase
EhRi_031875	AT1G30580	15	GTP-binding protein
EhRi_043654	AT4G02080	4	GTP-binding protein SAR1A
EhRi_052561	AT2G44100	42	guanosine nucleotide diphosphate dissociation inhibitor 1
EhRi_047262	AT5G62670	105	H(+)-ATPase 11
EhRi_070616	AT1G74310	20	heat shock protein 101
EhRi_043914	AT3G23990	12	heat shock protein 60
EhRi_055733	AT3G12580	42	heat shock protein 70-4
EhRi_031982	AT5G52640	44	heat shock protein 90.1
EhRi_070566	AT5G56000	31	Heat shock protein 90-4
EhRi_05886	AT4G40030	5	Histone H3.3
EhRi_061147	AT2G28740	15	histone H4
EhRi_053790	AT1G53540	10	HSP20-like chaperone
EhRi_059132	AT4G02450	2	HSP20-like chaperone
EhRi_057983	AT4G24190	107	HSP90-like protein GRP94
EhRi_070529	AT1G65930	17	isocitrate dehydrogenase
EhRi_052799	AT3G58610	15	ketol-acid reductoisomerase ketol-acid reductoisomerase

Table S1 *continued*

EhRi_057350	AT4G30920	12	leucine aminopeptidase 2
EhRi_066989	AT1G17420	3	lipoxygenase 3
EhRi_058302	AT3G22400	22	lipoxygenase 5
EhRi_058643	AT5G28540	91	Luminal-binding protein 1
EhRi_035605	AT5G01750	3	lurp1 protein
EhRi_052090	AT5G63190	7	MA3 domain-containing protein
EhRi_038864	AT3G47520	15	malate dehydrogenase
EhRi_037701	AT1G53240	12	malate dehydrogenase 1
EhRi_033839	AT1G50890	10	microtubule-associated protein SPIRAL2-like
EhRi_070843	AT5G09590	3	mitochondrial HSO70 2
EhRi_057069	AT1G63940	4	Monodehydroascorbate reductase
EhRi_040871	AT2G24270	8	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase
EhRi_063374	AT5G25880	52	NADP-dependent malic enzyme 3
EhRi_059367	AT4G09320	3	nucleoside diphosphate kinase 1
EhRi_031329	AT4G23900	8	nucleoside diphosphate kinase IV
EhRi_038750	AT2G43750	12	O-acetylserine (thiol) lyase (OAS-TL) isoform oasB
EhRi_036282	AT4G35360	8	pantothenate kinase 2
EhRi_060620	AT4G35850	5	pentatricopeptide repeat-containing protein
EhRi_050133	AT3G14420	3	peroxisomal (S)-2-hydroxy-acid oxidase GLO1
EhRi_070970	AT3G10340	29	phenylalanine ammonia-lyase 4
EhRi_050267	AT5G14040	3	phosphate transporter 3;1
EhRi_044648	AT4G37870	12	phosphoenolpyruvate carboxykinase [ATP]

Table S1 *continued*

EhRi_046334	AT4G24620	16	phosphoglucose isomerase 1
EhRi_040819	AT1G56190	14	phosphoglycerate kinase
EhRi_044908	AT1G60680	21	probable aldo-keto reductase 2
EhRi_052976	AT1G60710	11	probable aldo-keto reductase 4
EhRi_048958	AT3G27280	5	prohibitin 4
EhRi_050117	AT5G42790	2	proteasome alpha subunit F1
EhRi_050757	AT5G19820	7	protein EMBRYO DEFECTIVE 2734
EhRi_061441	AT1G67230	13	protein little nuclei1
EhRi_058985	AT1G51980	9	putative mitochondrial-processing peptidase subunit alpha-1
EhRi_039190	AT3G51800	5	putative nuclear DNA-binding protein G2p
EhRi_058496	AT2G29560	12	putative phosphoenolpyruvate enolase
EhRi_062143	AT1G20960	7	putative U5 small nuclear ribonucleoprotein helicase
EhRi_059244	AT5G15400	8	putative ubiquitin conjugation factor E4
EhRi_036779	AT1G15690	2	Pyrophosphate-energized vacuolar membrane proton pump 1
EhRi_066612	AT5G50850	4	pyruvate dehydrogenase E1 beta
EhRi_042020	AT5G60860	22	RAB GTPase homolog A1F
EhRi_041661	AT4G17170	11	RAB GTPase homolog B1C
EhRi_059049	AT3G16100	11	RAB GTPase-like protein G3C
EhRi_049419	AT3G55020	4	RabGAP/TBC domain-containing protein
EhRi_048650	AT1G43890	9	Ras-related protein Rab-18
EhRi_058535	AT1G48630	13	receptor for activated C kinase 1B

Table S1 *continued*

EhRi_034140	AT1G19440	3	3-ketoacyl-CoA synthase 4
EhRi_061170	AT3G04790	2	ribose 5-phosphate isomerase A
EhRi_042954	ATCG00490	12	ribulose-1,5-bisphosphate carboxylase/oxygenase large subunit
EhRi_053000	AT5G15270	3	RNA-binding KH domain-containing protein
EhRi_048868	AT4G19210	4	RNAse l inhibitor protein 2
EhRi_051477	AT3G25230	8	rotamase FKBP 1
EhRi_070532	AT3G05500	8	Rubber elongation factor protein
EhRi_058824	AT5G27470	8	seryl-tRNA synthetase / serine--tRNA ligase
EhRi_043632	AT2G03510	13	SPFH/Band 7/PHB domain-containing membrane-associated protein
EhRi_033314	AT3G54670	5	structural maintenance of chromosomes 1
EhRi_031692	AT1G79440	88	succinate-semialdehyde dehydrogenase
EhRi_058753	AT3G20050	14	T-complex protein 1 subunit alpha
EhRi_039426	AT1G24510	3	T-complex protein 1 subunit epsilon
EhRi_048519	AT5G20890	18	TCP-1/cpn60 chaperonin family protein
EhRi_035691	AT1G53300	9	TPR repeat-containing thioredoxin TTL1
EhRi_036370	AT3G18860	3	transducin family protein / WD-40 repeat family protein
EhRi_043135	AT3G60750	15	transketolase
EhRi_041129	AT1G13950	4	translation initiation factor eIF-5A
EhRi_060938	AT3G13920	36	translational initiation factor 4A-1
EhRi_052549	AT3G16640	5	translationally-controlled tumor protein-like protein
EhRi_061700	AT1G06950	27	translocon at the inner envelope membrane of chloroplasts 110
EhRi_056133	AT2G21170	43	triosephosphate isomerase
EhRi_070921	AT5G19770	5	tubulin alpha-3
EhRi_058714	AT5G62690	2	tubulin beta-2/beta-3 chain
EhRi_043579	AT4G05050	16	ubiquitin 11
EhRi_041749	AT3G08900	3	UDP-arabinopyranose mutase 3
EhRi_059330	AT4G10960	4	UDP-D-glucose/UDP-D-galactose 4-epimerase 5
EhRi_049019	AT5G59290	7	UDP-glucuronic acid decarboxylase 3
EhRi_062029	AT2G19830	3	vacuolar protein sorting-associated protein 32-1
EhRi_065167	AT2G41740	4	villin 2
EhRi_038406	AT3G01280	3	voltage dependent anion channel 1
EhRi_056872	AT1G78900	9	V-type proton ATPase catalytic subunit A
EhRi_047548	AT3G18060	5	WD40 domain-containing protein

Table S2: Proteins identified in the Swiss/Prot database.

Accession	Coverage (%)	#Unique	Description
Q9T0K1|GBIS2_ARATH	6	2	(Z)-gamma-bisabolene synthase 2
P46266|1433_PEA	29	4	14-3-3-like protein
O81822|HS177_ARATH	14	3	17.7 kDa class II heat shock protein
P05478|HSP16_SOYBN	13	3	18.5 kDa class I heat shock protein
O23894|PRS6A_BRACM	7	2	26S protease regulatory subunit 6A homolog
Q9SEI4|PRS6B_ARATH	11	3	26S protease regulatory subunit 6B homolog
O48844|PSD1A_ARATH	5	6	26S proteasome non-ATPase regulatory subunit 1 homolog A
Q9SZD4|PRS4A_ARATH	6	2	26S proteasome regulatory subunit 4 homolog A
Q96468|BAS1_HORVU	6	2	2-Cys peroxiredoxin BAS1_ chloroplastic (Fragment)
Q2QWT4|DPNP_ORYSJ	6	2	3'(2')_5'-bisphosphate nucleotidase
P42798|R15A1_ARATH	12	2	40S ribosomal protein S15a-1
Q9M339|RS32_ARATH	22	5	40S ribosomal protein S3-2
O80377|RSSA_DAUCA	9	2	40S ribosomal protein SA
O98454|RK22_SPIMX	14	2	50S ribosomal protein L22_ chloroplastic
Q42699|METE_CATRO	10	3	5-methyltetrahydropteroyltriglutamate--homocysteine methyltransferase
P57691|RLA03_ARATH	14	5	60S acidic ribosomal protein P0-3
Q0DK10|RL11_ORYSJ	18	4	60S ribosomal protein L11
Q9SH69|6PGD1_ARATH	13	3	6-phosphogluconate dehydrogenase_ decarboxylating 1_ chloroplastic
Q2R480|6PGD2_ORYSJ	13	4	6-phosphogluconate dehydrogenase_ decarboxylating 2_ chloroplastic

Table S2 *continued*

Q9FWA3|6GPD3_ARATH	16	3	6-phosphogluconate dehydrogenase_ decarboxylating 3
Q9C8H1|AB11C_ARATH	3	2	ABC transporter C family member 11
Q9SIB9|ACO2M_ARATH	6	2	Aconitate hydratase 2_ mitochondrial
P53498|ACT_CHLRE	34	2	Actin
Q96481|ACT4_SOLLC	28	2	Actin-105 (Fragment)
P23344|ACT2_DAUCA	26	2	Actin-2
P0C542|ACT7_ORYSI	32	3	Actin-7
P30169|ACT7_SOLTU	34	4	Actin-75
P31167|ADT1_ARATH	22	3	ADP_ATP carrier protein 1_ mitochondrial
P40941|ADT2_ARATH	21	4	ADP_ATP carrier protein 2_ mitochondrial
P49076|ARF_MAIZE	41	5	ADP-ribosylation factor
Q9SU63|AL2B4_ARATH	10	4	Aldehyde dehydrogenase family 2 member B4_ mitochondrial
Q8S528|AL2B7_ARATH	15	8	Aldehyde dehydrogenase family 2 member B7_ mitochondrial
Q56YU0|AL2C4_ARATH	7	2	Aldehyde dehydrogenase family 2 member C4
P80607|UPTG_MAIZE	11	2	Alpha-1_4-glucan-protein synthase [UDP-forming
Q9LKJ3|PHSH_WHEAT	10	2	Alpha-glucan phosphorylase_ H isozyme
P26854|ATPAM_MARPO	30	5	ATP synthase subunit alpha_ mitochondrial
Q9TJR9|ATPB_PROWI	16	2	ATP synthase subunit beta_ plastid
P83484|ATPBN_ARATH	26	3	ATP synthase subunit beta-2_ mitochondrial

Table S2 *continued*

Q9LV11|PMA11_ARATH	11	3	ATPase 11_ plasma membrane-type
Q9SH76|PMA6_ARATH	11	2	ATPase 6_ plasma membrane-type
Q42556|PMA9_ARATH	9	2	ATPase 9_ plasma membrane-type
Q9FRX6|AS1_ANTMA	2	2	Aureusidin synthase
P25696|ENO2_ARATH	20	2	Bifunctional enolase 2/transcriptional activator
O04983|ACCC_ARATH	6	2	Biotin carboxylase_ chloroplastic
Q9LEV3|CBSX3_ARATH	16	3	CBS domain-containing protein CBSX3_ mitochondrial
P54774|CDC48_SOYBN	6	3	Cell division cycle protein 48 homolog
P42730|CLPB1_ARATH	29	18	Chaperone protein ClpB1
Q75GT3|CLPB2_ORYSJ	9	4	Chaperone protein ClpB2_ chloroplastic
Q8VYJ7|CLPB4_ARATH	8	3	Chaperone protein ClpB4_ mitochondrial
P29185|CH61_MAIZE	18	4	Chaperonin CPN60-1_ mitochondrial
P29357|HSP7E_SPIOL	40	3	Chloroplast envelope membrane 70 kDa heat shock-related protein
P49298|CISY_CITMA	7	3	Citrate synthase_ mitochondrial
Q2RBN7|CLH1_ORYSJ	2	3	Clathrin heavy chain 1
Q0DJ99|COPD2_ORYSJ	4	2	Coatomer subunit delta-2
Q0DH40|CCB15_ORYSJ	6	2	Cyclin-B1-5
P32260|CYSKP_SPIOL	6	2	Cysteine synthase_ chloroplastic/chromoplastic
Q9SRZ6|ICDHC_ARATH	16	2	Cytosolic isocitrate dehydrogenase [NADP]
O04130|SERA_ARATH	4	2	D-3-phosphoglycerate dehydrogenase_ chloroplastic
Q0JM17|RH56_ORYSJ	6	2	DEAD-box ATP-dependent RNA helicase 56

Table S2 *continued*

Q9SMH3|DYH1A_CHLRE	1	2	Dynein-1-alpha heavy chain_ flagellar inner arm I1 complex
Q8H0T4|UPL2_ARATH	1	3	E3 ubiquitin-protein ligase UPL2
P25698|EF1A_SOYBN	14	2	Elongation factor 1-alpha
O23755|EF2_BETVU	9	6	Elongation factor 2
P35016|ENPL_CATRO	16	4	Endoplasmin homolog
P26301|ENO1_MAIZE	27	4	Enolase 1
P80030|FABI_BRANA	4	2	Enoyl-[acyl-carrier-protein] reductase [NADH]_ chloroplastic
Q40471|IF4A9_TOBAC	24	3	Eukaryotic initiation factor 4A-9
Q6ZCX3|FH6_ORYSJ	2	2	Formin-like protein 6
P46256|ALF1_PEA	12	3	Fructose-bisphosphate aldolase_ cytoplasmic isozyme 1
P46257|ALF2_PEA	16	4	Fructose-bisphosphate aldolase_ cytoplasmic isozyme 2
P29356|ALF_SPIOL	17	5	Fructose-bisphosphate aldolase_ cytoplasmic isozyme
Q9FZ42|GRDH1_ARATH	12	4	Glucose and ribitol dehydrogenase homolog 1
Q9LK23|G6PD5_ARATH	16	7	Glucose-6-phosphate 1-dehydrogenase_ cytoplasmic isoform 1
Q8H103|G6PIP_ARATH	8	4	Glucose-6-phosphate isomerase 1_ chloroplastic
Q0DG35|GLT2_ORYSJ	2	2	Glutamate synthase 2 [NADH]_ chloroplastic
P38562|GLNA4_MAIZE	16	2	Glutamine synthetase root isozyme 4
Q8L7C9|GSTUK_ARATH	4	2	Glutathione S-transferase U20
Q9SAJ6|G3PP1_ARATH	23	2	Glyceraldehyde 3-phosphate dehydrogenase GAPCP1 chloroplastic

Table S2 *continued*

P08735|G3PC1_MAIZE	12	2	Glyceraldehyde-3-phosphate dehydrogenase 1_ cytosolic
P34921|G3PC_DIACA	8	2	Glyceraldehyde-3-phosphate dehydrogenase_ cytosolic
Q9M8L4|GLPK_ARATH	5	3	Glycerol kinase
Q42968|SSG1_ORYGL	5	2	Granule-bound starch synthase 1_ chloroplastic/amyloplastic
P52885|SAR1_TOBAC	25	2	GTP-binding protein SAR1
P31584|YPTV1_VOLCA	19	2	GTP-binding protein yptV1
P93340|GBLP_NICPL	10	3	Guanine nucleotide-binding protein subunit beta-like protein
Q9C7X7|HSP7N_ARATH	39	8	Heat shock 70 kDa protein 18
O65719|HSP7C_ARATH	39	2	Heat shock 70 kDa protein 3
Q9S9N1|HSP7E_ARATH	38	8	Heat shock 70 kDa protein 5
Q9LTX9|HSP7G_ARATH	13	2	Heat shock 70 kDa protein 7_ chloroplastic
P26791|HSP70_DAUCA	19	2	Heat shock 70 kDa protein
P24629|HSP71_SOLLC	42	5	Heat shock cognate 70 kDa protein 1
P09189|HSP7C_PETHY	43	3	Heat shock cognate 70 kDa protein
P36181|HSP80_SOLLC	22	2	Heat shock cognate protein 80
A2YWQ1|HSP81_ORYSI	26	3	Heat shock protein 81-1
Q69QQ6|HSP82_ORYSJ	24	2	Heat shock protein 81-2
Q08277|HSP82_MAIZE	27	5	Heat shock protein 82
P51819|HSP83_IPONI	31	2	Heat shock protein 83
P27323|HS901_ARATH	28	5	Heat shock protein 90-1
Q43468|STIP_SOYBN	6	4	Heat shock protein STI
Q9SQ76|HXK2_SOLTU	6	2	Hexokinase-2
Q43217|H2B3_WHEAT	16	3	Histone H2B.3
P69248|H32_PETCR	17	3	Histone H3.2
Q6WZ83|H4_EUCGL	44	5	Histone H4
O04294|IMA2_ARATH	4	2	Importin subunit alpha-2

Table S2 *continued*

Q8LPJ5|ICDHP_ARATH	9	2	Isocitrate dehydrogenase [NADP]_ chloroplastic/mitochondrial
P28297|ACEA_ARATH	5	2	Isocitrate lyase
Q15GI3|IGS1_PETHY	8	2	Isoeugenol synthase 1
Q01292|ILV5_SPIOL	7	2	Ketol-acid reductoisomerase_ chloroplastic
Q8S950|NACK1_TOBAC	2	2	Kinesin-like protein NACK1
Q05431|APX1_ARATH	16	3	L-ascorbate peroxidase 1_ cytosolic
Q1PER6|APX2_ARATH	11	2	L-ascorbate peroxidase 2_ cytosolic
Q42876|AMPL2_SOLLC	8	2	Leucine aminopeptidase 2_ chloroplastic
Q8GSM2|LOX23_HORVU	5	3	Lipoxygenase 2.3_ chloroplastic
Q9FNX8|LOX4_ARATH	3	2	Lipoxygenase 4_ chloroplastic
P29038|LDH_MAIZE	11	4	L-lactate dehydrogenase
O24581|BIP3_MAIZE	30	5	Luminal-binding protein 3
Q42434|BIP_SPIOL	31	4	Luminal-binding protein
P83373|MDHM_FRAAN	9	2	Malate dehydrogenase_ mitochondrial
Q39043|MD37F_ARATH	30	6	Mediator of RNA polymerase II transcription subunit 37f
Q9C6D2|MTK_ARATH	7	2	Methylthioribose kinase
P37225|MAON_SOLTU	6	2	NAD-dependent malic enzyme 59 kDa isoform_ mitochondrial
Q35322|NDUS3_ORYSJ	13	2	NADH dehydrogenase [ubiquinone] iron-sulfur protein 3
Q9XGZ0|MAOP3_ARATH	12	3	NADP-dependent malic enzyme 3
P47923|NDK2_PEA	9	2	Nucleoside diphosphate kinase 2_ chloroplastic

Table S2 continued

O49203|NDK3_ARATH	18	4	Nucleoside diphosphate kinase III_ chloroplastic/mitochondrial
Q9SKQ0|CP19B_ARATH	36	8	Peptidyl-prolyl cis-trans isomerase CYP19-2
Q38931|FKB62_ARATH	4	2	Peptidyl-prolyl cis-trans isomerase FKBP62
Q3L181|PERR_RAUSE	10	2	Perakine reductase
O64963|PAL1_PRUAV	6	2	Phenylalanine ammonia-lyase 1
P26600|PAL5_SOLLC	7	2	Phenylalanine ammonia-lyase
Q9T074|PCKA_ARATH	9	4	Phosphoenolpyruvate carboxykinase [ATP]
Q9LD57|PGKH1_ARATH	19	3	Phosphoglycerate kinase 1_ chloroplastic
Q9SBN4|PGKH_VOLCA	16	3	Phosphoglycerate kinase_ chloroplastic
Q42962|PGKY_TOBAC	25	2	Phosphoglycerate kinase_ cytosolic
P93844|PLDA2_ORYSJ	6	2	Phospholipase D alpha 2
P52574|REHY_TORRU	7	2	Probable 1-Cys peroxiredoxin
P55844|RL14_PEA	14	2	Probable 60S ribosomal protein L14
C6TBN2|AKR1_SOYBN	18	4	Probable aldo-keto reductase 1
Q93ZN2|ALKR4_ARATH	23	2	Probable aldo-keto reductase 4
Q7XKI5|TIP32_ORYSJ	7	2	Probable aquaporin TIP3-2
P0C0L1|APX6_ORYSJ	13	3	Probable L-ascorbate peroxidase 6_ chloroplastic
Q8H016|LOX6_ORYSJ	2	2	Probable lipoxygenase 6
Q8H1B3|MD37B_ARATH	12	4	Probable mediator of RNA polymerase II transcription subunit 37b

Table S2 continued

Q9LHA8|MD37C_ARATH	49	6	Probable mediator of RNA polymerase II transcription subunit 37c
P22953|MD37E_ARATH	38	2	Probable mediator of RNA polymerase II transcription subunit 37e
Q93WJ8|MDAR4_ARATH	6	2	Probable monodehydroascorbate reductase_ cytoplasmic isoform 4
O49299|PGMC1_ARATH	14	2	Probable phosphoglucomutase_ cytoplasmic 1
O04331|PHB3_ARATH	9	2	Prohibitin-3_ mitochondrial
Q9LK25|PHB4_ARATH	8	2	Prohibitin-4_ mitochondrial
Q9LSU0|PSA3_ORYSJ	16	4	Proteasome subunit alpha type-3
Q9LSU3|PSA6_ORYSJ	6	2	Proteasome subunit alpha type-6
Q9LST6|PSB2_ORYSJ	14	3	Proteasome subunit beta type-2
Q6YZX6|ACOC_ORYSJ	8	2	Putative aconitate hydratase_ cytoplasmic
Q9LRZ2|FB147_ARATH	5	2	Putative F-box protein At3g16820
Q2QSB9|AMPL1_ORYSJ	7	2	Putative leucine aminopeptidase 1
Q06572|AVP_HORVU	6	5	Pyrophosphate-energized vacuolar membrane proton pump
Q38799|ODPB1_ARATH	5	2	Pyruvate dehydrogenase E1 component subunit beta-1_ mitochondrial
P22200|KPYC_SOLTU	6	2	Pyruvate kinase_ cytosolic isozyme
P49103|RAB2A_MAIZE	24	5	Ras-related protein Rab-2-A
O24466|RAE1A_ARATH	19	2	Ras-related protein RABE1a
P0C5D6|SAPK3_ORYSJ	16	2	Serine/threonine-protein kinase SAPK3

Table S2 continued

P43291|SRK2A_ARATH	14	2	Serine/threonine-protein kinase SRK2A
Q02028|HSP7S_PEA	18	4	Stromal 70 kDa heat shock-related protein_ chloroplastic
Q9SAY1|SUT11_ARATH	2	2	Sulfate transporter 1.1
P24704|SODC1_ARATH	18	2	Superoxide dismutase [Cu-Zn] 1
O65768|SODC_CARPA	23	2	Superoxide dismutase [Cu-Zn]
P28769|TCPA_ARATH	6	3	T-complex protein 1 subunit alpha
Q9M4S8|TPIC_FRAAN	15	3	Triosephosphate isomerase_ chloroplastic
Q43772|UGPA_HORVU	12	2	UTP--glucose-1-phosphate uridylyltransferase
P49087|VATA_MAIZE	6	4	V-type proton ATPase catalytic subunit A (Fragment)
Q9SZN1|VATB2_ARATH	12	4	V-type proton ATPase subunit B2

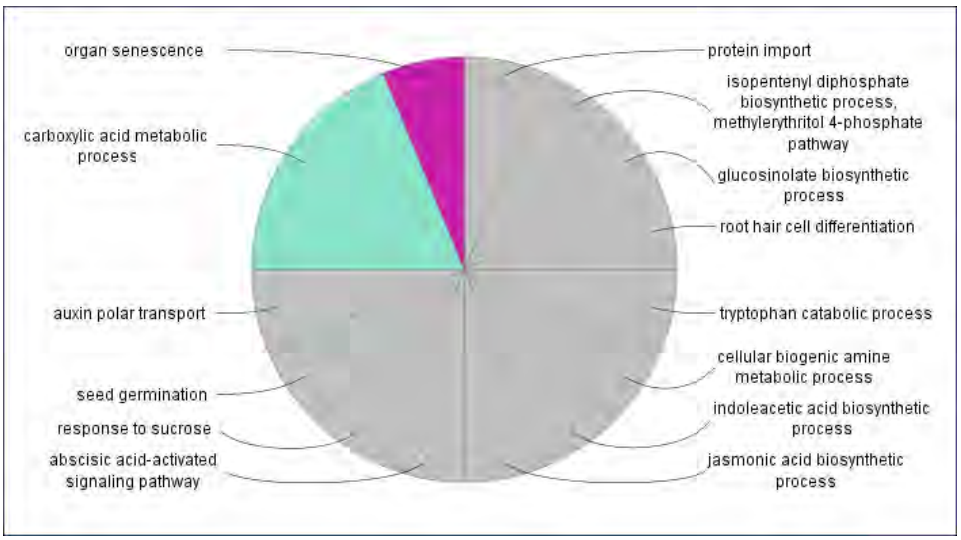

Fig S1: GO biological process terms commonly up-regulated in both DT and DS rhizomes in response to desiccation-tolerance. The colours indicate the statistical significance of each biological process. In this case, carboxylic acid metabolic process is the most significant term followed by organ senescence and then the rest of the biological process terms. The size of the pie chart 'slice' is proportional to the number of genes associated with a particular biological pocess.

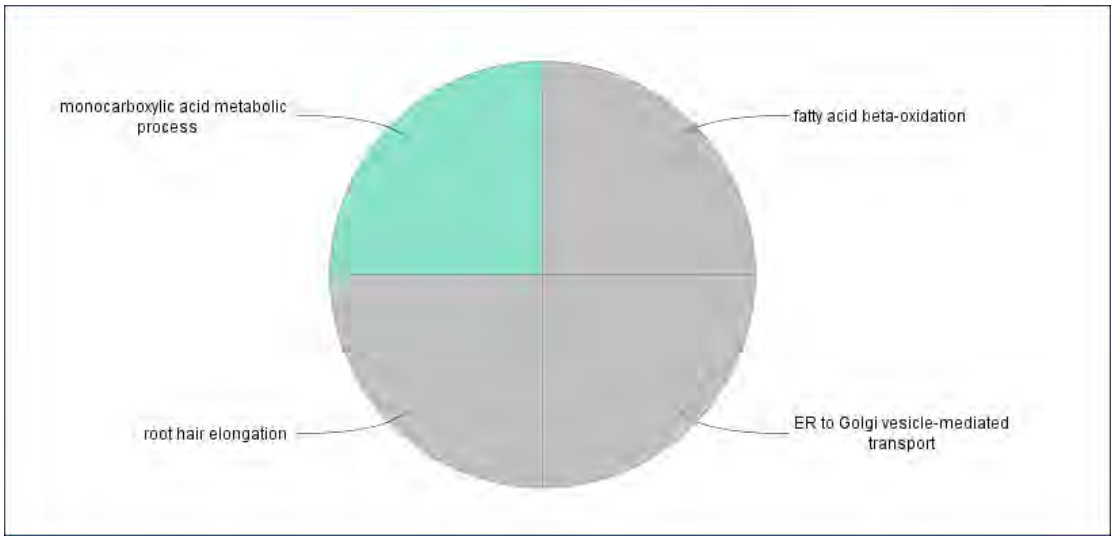

Fig S2: GO biological process terms commonly down-regulated in both DT and DS rhizomes in response to desiccation-tolerance. The colours indicate the statistical significance of each biological process. In this case, monocarboxylic acid metabolic process is the most significant process term. The size of the pie chart 'slice' is proportional to the number of genes associated with a particular biological pocess.

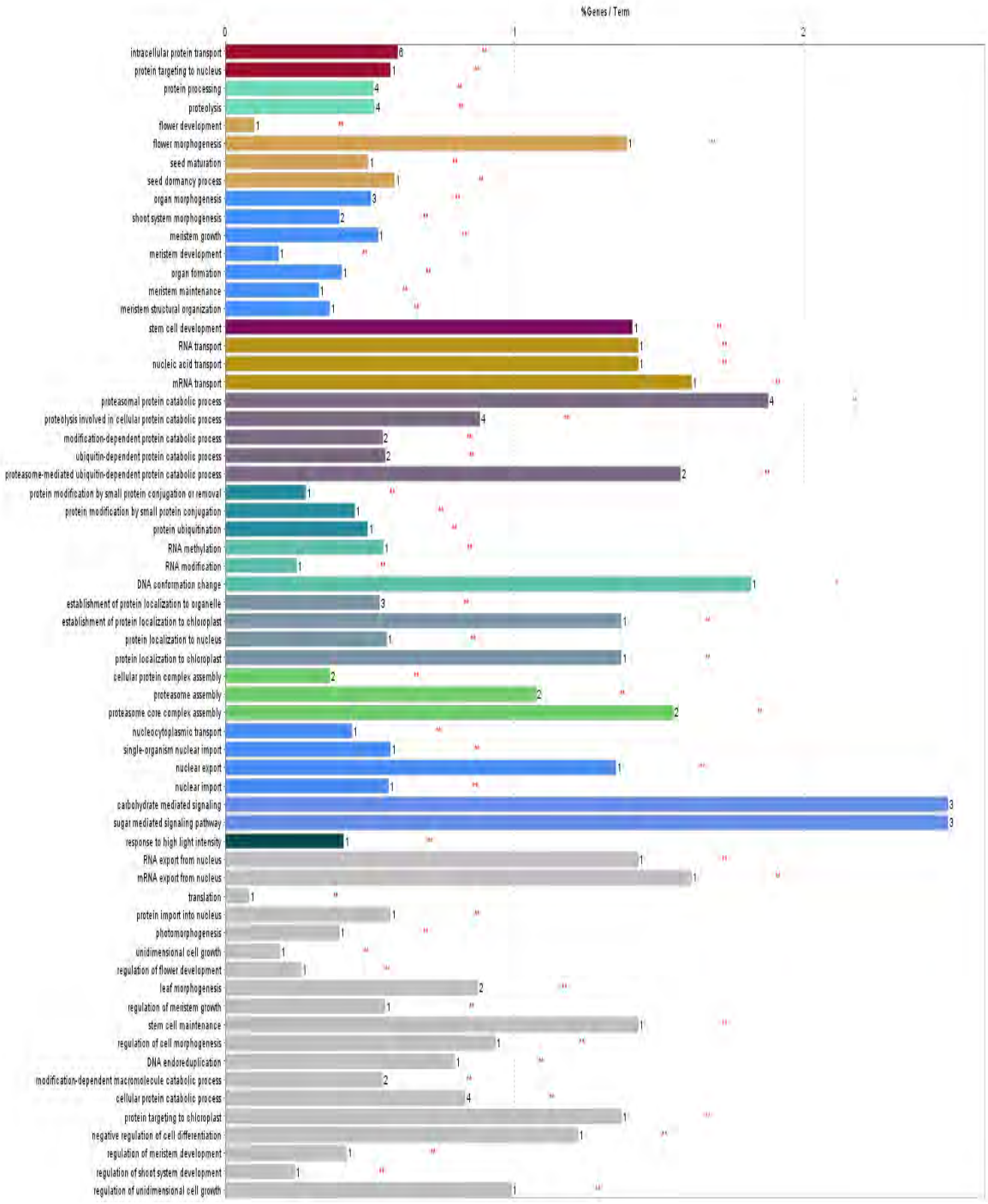

Fig S3: GO biological process terms specific for up-regulated DT rhizome proteins. The bars represent the number of genes associated with the terms and the percentage of genes per term is shown as bar label. The colour gradient of the bars indicates statistical significance with the most statistically significant term on top. The asterisk indicates that all reported GO terms are significant at the corrected P-value of 0.05 and lower.

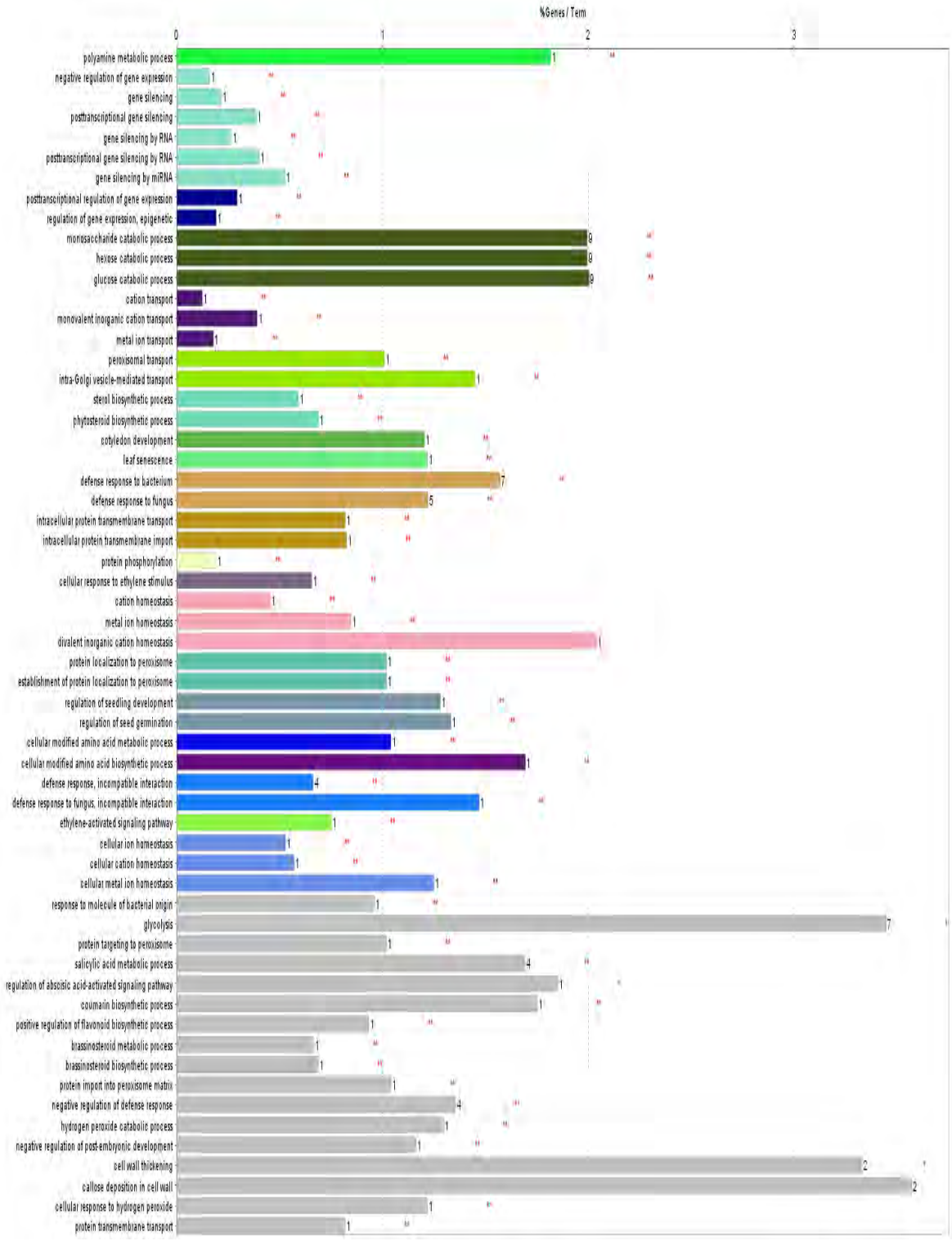

Fig S4: GO biological process terms specific for up-regulated DS rhizome proteins. The bars represent the number of genes associated with the terms and the percentage of genes per term is shown as bar label. The colour gradient of the bars indicates statistical significance with the most statistically significant term on top. The asterisk indicates that all reported GO terms are significant at the corrected P-value of 0.05 and lower.

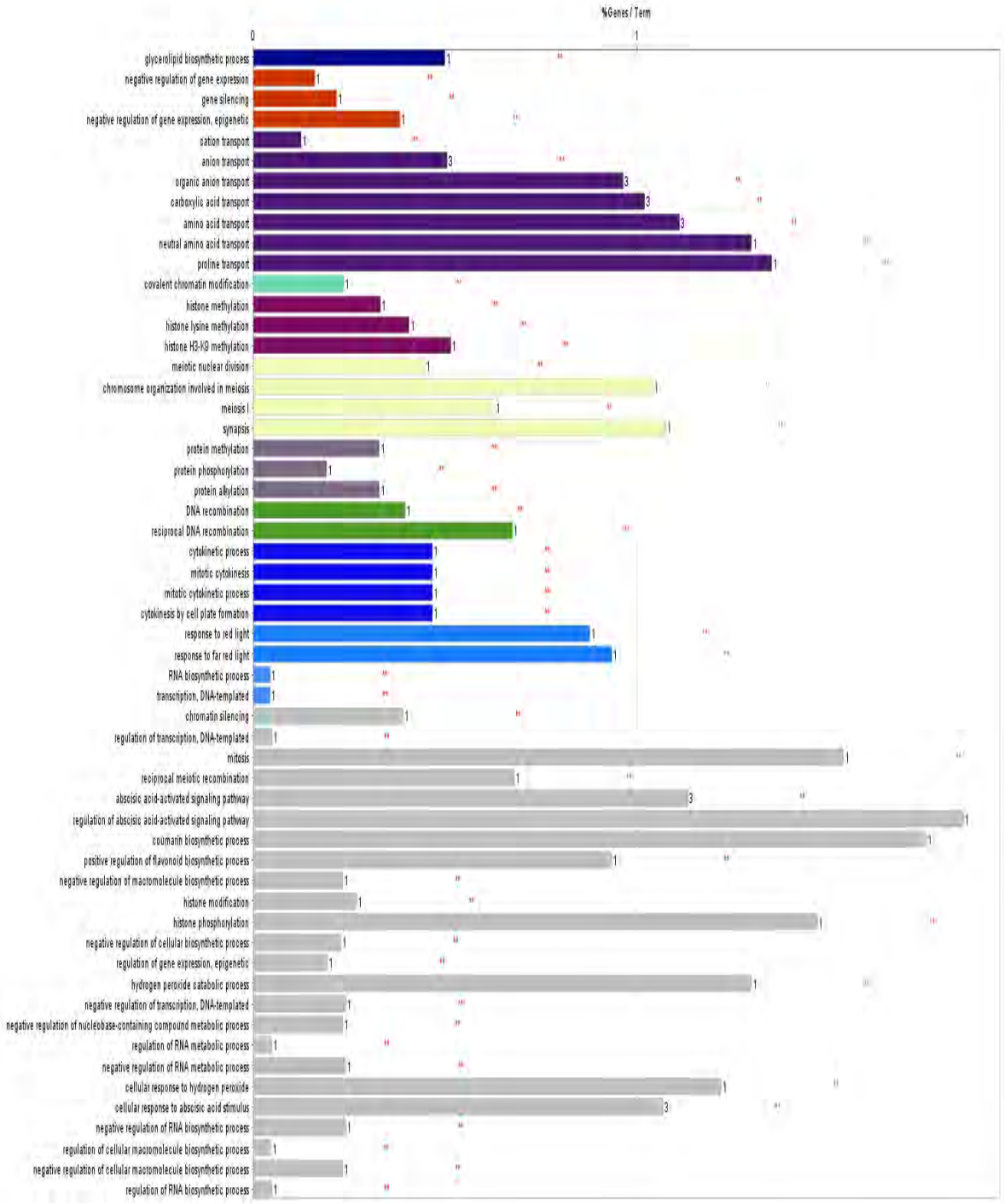

Fig S5: GO biological process terms specific for down-regulated DT rhizome proteins. The bars represent the number of genes associated with the terms and the percentage of genes per term is shown as bar label. The colour gradient of the bars indicates statistical significance with the most statistically significant term on top. The asterisk indicates that all reported GO terms are significant at the corrected P-value of 0.05 and lower.

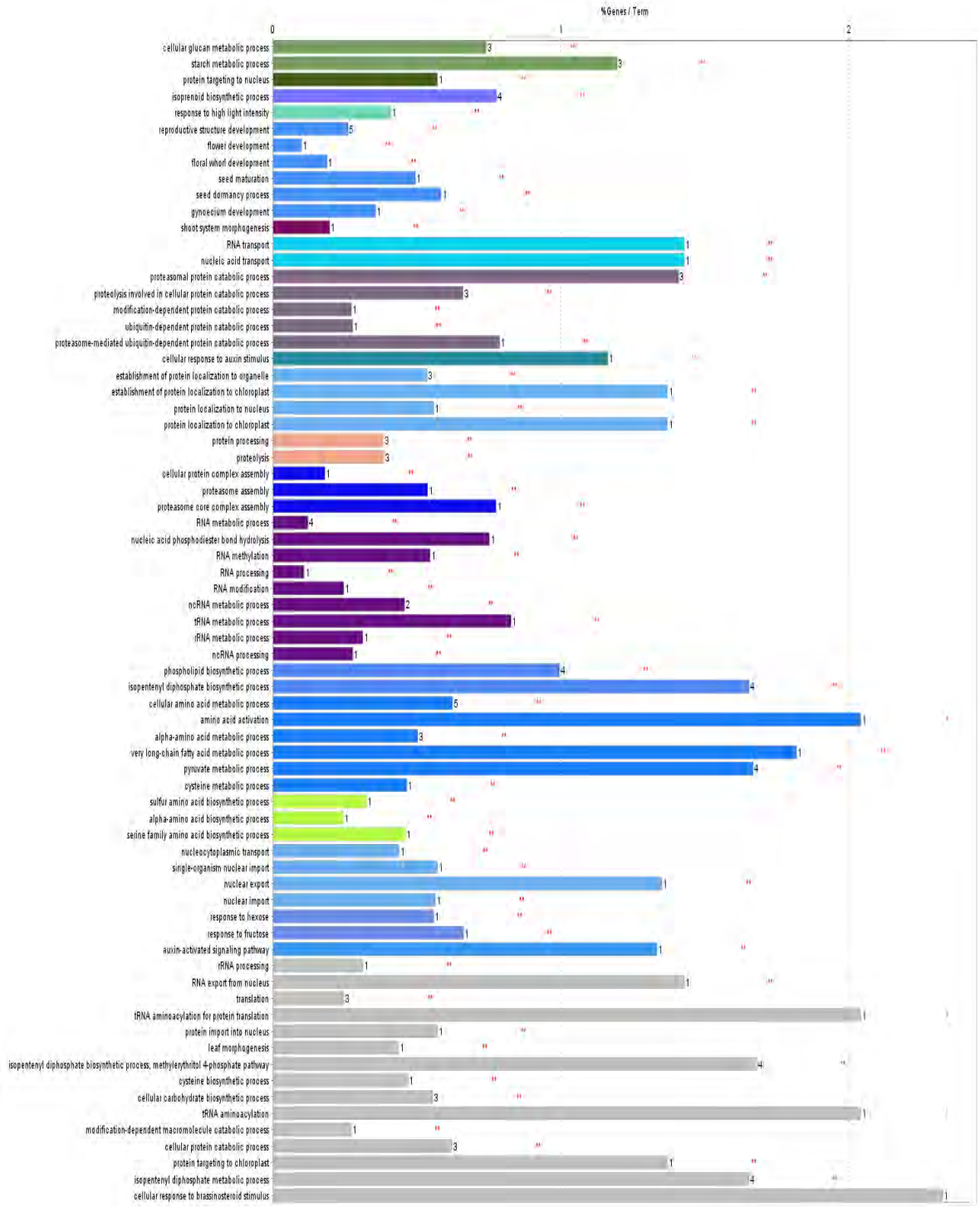

Fig S6: GO biological process terms specific for down-regulated DS rhizome proteins. The bars represent the number of genes associated with the terms and the percentage of genes per term is shown as bar label. The colour gradient of the bars indicates statistical significance with the most statistically significant term on top. The asterisk indicates that all reported GO terms are significant at the corrected P-value of 0.05 and lower.