Analysis of the nuclear proteome of the resurrection plant *Xerophyta viscosa* (Baker) and its response to dehydration stress

Kamal Omer Mohamed Ali Abdalla

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Contents

DECLARATION

I, Kamal Omer Mohamed Ali Abdalla hereby declare that this thesis, submitted in fulfillment of the requirement for the award of Doctor of Philosophy in the Department of Molecular and Cell Biology, University of Cape Town, is wholly my own unaided work apart from the normal guidance of my supervisor, Dr. MS Rafudeen. Neither the substance nor any part of the thesis has been submitted before for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged as complete references.

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Kamal Omer Mohamed Ali Abdalla,

September 2009

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List of figures

List of tables

List of abbreviations

1-DE	one- dimensional electrophoresis
2D-PAGE	two-dimensional polyacrylamide gel electrophoresis
ICAT	isotope-coded affinity tags
cICAT	cleavable ICAT
iTRAQ	isobaric tags for relative and absolute quantification
LC	liquid chromatography
MALDI-TOF MS	matrix-assisted laser desorption ionization mass spectrometry
ESI-MS/MS	electrospray ionization tandem mass spectrometry
2-DIGE	two-dimensional differential in-gel electrophoresis
RP HPLC	reverse phase high-performance liquid chromatography
PSD	post-source decay
CID	collision-induced dissociation
2DLC	two-dimensional liquid chromatography
PMF	peptide mass fingerprint
EST	expressed sequence tag
RWC	relative water content
NIB	nuclei isolation buffer
HBS	homogenized NIB supernatant
NIBA	NIB buffer containing protease inhibitor
MLS	membrane lysate supernatant
DAPI	4',6'-diamidino-2-phenylindole hydrochloride
CHAPS	3-((3-cholamido propyl)-dimethylammonio)-1-propane sulfonate
CBB	coomassie brilliant blue
PCNA	proliferating cell nuclear antigen
XVPx2	*X. viscosa* preoxiredoxin type II
NP	nuclear protein
DNP	nuclear proteins from dehydrated leaf samples
IPG	immobilized pH gradient
DDT	dithiothreitol
IEF	isoelectric focusing
PTM	post-translational modification
TEAB	triethylammonium bicarbonate
SCX	strong cation exchange chromatography

Abstract

Analysis of the nuclear proteome of the resurrection plant *Xerophyta viscosa* (Baker) and its response to dehydration stress

Kamal Omer Mohamed Ali Abdalla, September 2009.

Xerophyta viscosa Baker (family Velloziaceae) can survive extremes of dehydration (desiccation), down to 5% relative water content (RWC) and resumes full physiological activity within 80 h of rehydration. A thorough understanding of this phenomenon may provide further insight into possible mechanisms for improving drought tolerance in other plants. In this respect a comprehensive analysis of the nuclear proteome of this plant and its response to dehydration stress at 35% RWC was carried out. The RWC at 35% represents a distinct phase of the dehydration process where induction of late protection mechanisms is initiated and is a characteristic of desiccation tolerant species.

We optimized nuclei isolation and nuclear protein extraction protocols and successfully employed these protocols to isolate highly purified nuclei and subsequently nuclear proteins from fully hydrated and dehydrated *X. viscosa* leaf samples. The integrity of the purified nuclei was confirmed with light and fluorescent microscopy. The nuclei were uniform spheres, approximately 5 μm in size. The purity and enrichment of the nuclear proteins were confirmed by chlorophyll assay and Western blot analysis. The nuclear proteins were investigated using two-dimensional (2D) and isobaric tags for relative and absolute quantitation (iTRAQ) technologies. Using the 2DE approach, a total of 438 proteins spots were reproducibly detected and analysed of which 18 protein spots were shown to be up-regulated in response to dehydration. These proteins contained both regulatory and functional proteins. The largest category comprised five novel protein factors and two proteins with unassigned functions. The second category comprised proteins involved in gene regulation and signal transduction. The third category comprised stress responsive proteins with chaperone type activities. Other categories include proteins involved in energy metabolism, protein degradation and translation. These results demonstrate that dehydration was controlled by multiple genes within the plant nucleus and *X. viscosa* may possess its own specific nuclear proteins that are involved in desiccation stress. In addition we comprehensively analyzed the nuclear proteome of *X. viscosa* using iTRAQ with two-dimensional liquid chromatography and tandem mass spectrometry to complement the data obtained from the 2DE approach. Using iTRAQ, we reproducibly

identified 128 proteins with confidence ≥ 95% ($\rho < 0.05$). Sixty six percent of the identified proteins showed consistent expression levels. The remaining 34% proteins showed significant changes in expressions. Of the latter, 23% were shown to be up regulated in response to dehydration stress. The remaining 11% were shown to be down regulated. The nuclear proteins of *X. viscosa* up-regulated in response to dehydration stress showed a coordinated response involving both regulatory and functional proteins and were implicated in diverse cellular functions. The characteristic feature of the *X. viscosa* nuclear proteins is the high level of stress molecules among the dehydration responsive proteins with evident functions in defense mechanisms compared to down regulated proteins and proteins showing consistent expression levels. These results demonstrate that enhanced defense capacity is crucial to desiccation tolerance and strongly support the notion that late dehydration responsive proteins are involved in protection of the cellular structures during dehydration. Proteins showing consistent expression levels during dehydration most likely maintain the minimum viability in cells under all conditions or may be indirectly associated with desiccation tolerance. Down-regulated proteins are likely important for plant survival under normal growth conditions. The proteins up-regulated in response to dehydration stress were assumed to be associated directly with the acquisition of desiccation tolerance. The up-regulated proteins were further categorized into nine functional groups to gain more insight into their roles in desiccation tolerance. The largest group was shown to be involved in gene regulation and signal transduction (36%), which reflects the role of the nucleus in gene expression and regulation. The second group included stress responsive molecules such as antioxidants, molecular chaperones and compatible solutes (33%). This reflects the importance of strong defense systems in preventing lipid peroxidation, protein aggregation, membrane leakage and maintaining the integrity of cellular structures during dehydration and in the dried state. The third group contained proteins involved in nucleocytoplasmic transport (10%). This might reflect the capacity of this plant to control the movement of molecules to and from the nucleus during dehydration and the importance of this process in adaptation to dehydration stress. The fourth group contained proteins involved in protein translation (7%). Proteins categorized to other functions, include proteins with miscellaneous and unknown functions. Proteins with unknown functions were considered to be *X. viscosa* nuclear-specific proteins. There was good correlation between the up-regulated proteins identified by 2-DE and iTRAQ approaches. In conclusion, this study revealed that *X. viscosa* nuclear proteome was responsive to dehydration stress and desiccation tolerance is

genetically encoded. Secondly, *X. viscosa* relies on readily inducible protection to combat desiccation and desiccation tolerance is controlled by multiple genes within the plant nucleus. Thirdly, the protective mechanisms of desiccation tolerance utilized by *X. viscosa* appear to involve signal perception genes and modulating gene expression of appropriate genes encoding protective molecules including antioxidants, molecular chaperones, compatible solutes, proteins of translation and degradation machinery, proteins with miscellaneous functions and novel protein factors. Lastly, proteins are crucial to desiccation tolerance allowing *X. viscosa* to possess a unique stress tolerance with versatile and coordinated actions to provide protection for its cellular structures during desiccation and in the dried state. To our best knowledge this is the first study to provide insight into the nuclear (organellar) proteome of a desiccation tolerant plant.

CHAPTER 1

LITERATURE REVIEW

1.1. INTRODUCTION

Water is essential for life of all living organisms for its roles as solvent, transport medium, electron donor in the hill reaction, and as evaporative coolant (Bohnert *et al.,* 1995). In plants and other photoautotrophs, water plays the additional role of providing energy necessary to drive photosynthesis (Mundree et al., 2002). One of the major consequences of dehydration stress is the loss of protoplasmic water which leads to the concentration of ions and results in the inhibition of cell metabolic processes and formation of a glassy state (Mundree et al., 2002). In this state the cell viscosity becomes very high and the chances of molecular interactions increase which cause essential macromolecules to aggregate and the disintegration of organelles (Hoekstra et al., 2001; 2001; Mundree et al., 2002; Alpert, 2006). In non-desiccation tolerant plants this process is often accompanied by membrane damage, resulting in the leakage of solutes and eventually plant death (Blum, 1988). It has been proposed that the critical feature of tolerance to dehydration depends on the abilities of an organism to limit membrane damage during dehydration and to regain membrane integrity and membrane bound activities quickly upon rehydration (Turner, 1986; Tripathy et al., 2000). Water deficiency is one of the main factors that restricts the crop productivity and has a negative impact on national and global economies worldwide (McKersie et al., 1994; Vincent et al., 2005). The understanding of plant adaptation to environmental stress is important for developing strategies to deal with the mentioned challenges.

1.2. WATER DEFICIT AND DESICCATION TOLERANCE IN PLANTS

Water deficit conditions, low temperature (chilling and freezing), heat stress, salinity and oxygen deficiency are some of the important environmental stress factors that affect plant growth. Plants acclimatize and adapt to these stress conditions by undergoing various biochemical and physiological changes (Lawlor and Cornic, 2002; Reddy et al., 2003; Khurana et al., 2008; Jaleel et al., 2008c). Understandably, approximately half to two-thirds of the yield potential of major crops is typically lost due to adverse environmental conditions (Khurana et al., 2008). Plants cope with water deficit by reducing water loss and/or by increasing water uptake (Bray et al., 2000; Khurana et al., 2008). They have evolved two major mechanisms to

deal with water deficit: stress avoidance and tolerance. Drought avoidance refers to adaptations that help the plant in escaping the stressful situations; for instance, short life cycles that cease before drought occurs (e.g. desert ephemerals). Drought tolerance refers to adaptations that allow the plant to withstand stress, with or without a decline in performance (Khurana et al., 2008). Understanding of the basic physiological, biochemical and molecular mechanisms for drought stress perception, transduction and tolerance is still a major challenge in biology. Plant modification for enhanced drought tolerance is mostly based on the manipulation of either transcription and/or signaling factors or genes that directly protect plant cells against water deficit (Khurana et al., 2008).

Acclimation to water deficit results from a series of integrated events occurring at physiological and biochemical levels that helps in the retention and/or acquisition of water, protection of chloroplast functions, and maintenance of ion homeostasis (McKersie et al., 1993; Bohnert and Jensen, 1996; McKersie et al., 1996). These are coordinated array of molecular events starting from perception of stimuli, transduction of stress signals, then regulation of gene expression which leads to alterations of the cell metabolic pathways (Bohnert et al., 1995; Lawlor and Cornic, 2002; Taiz and Zeiger, 2002; Zhu, 2002; Mohesnzadeh et al., 2006).

As sessile organisms, plants have developed adaptive strategies to cope with environmental stress. Understanding the responses of plants to their external environmental is important for basic research and is a attractive target for improving stress tolerance (Ramanjulu and Bartels, 2002). Plant cells have evolved mechanisms to perceive different signals from their surroundings, to integrate them and then to respond by modeling the appropriate gene expression (Ramanjulu and Bartels, 2002).

Water stress in general is a mild form of water deficit, which is a condition where the water status of plants undergoes relatively small changes (Bray, 1997). Desiccation is a severe form of water loss, which characterized by the loss of most of the protoplasmic ‘free or bulk’ water and survival with only the ‘bound water’, water, associated with the cell matrix (Ramanjulu and Bartels, 2002). The majority of higher plants produce desiccation-tolerant seeds and pollen, but it is a rare phenomenon in vegetative tissues (Bewley and oliver, 1992; Vertucci and Farrant, 1995; Ingle et al., 2007). However, a small group of taxonomically diverse plants known as resurrection plants can survive the loss of 80-95% of their cell water for indefinite periods, yet revive and resume normal metabolic processes upon rewatering (Phillips et al., 2002; Tuba et al., 2008). For a plant to be desiccation tolerant it has to possess the ability to

minimize mechanical damage associated with turgor loss, maintain the integrity of macromolecules and membranes by the accumulation of stress-associated proteins and compatible solutes, and minimize toxin accumulation and free radical damage generated as a result of impaired metabolism (Sherwin and Farrant, 1996). Upon rehydration, such plant tissues must also be able to repair desiccation-induced damage (Sherwin and Farrant, 1996).

1.3. RESURRECTION PLANTS

Desiccation tolerant plants were taxonomically classified into two broad groups, fully desiccation tolerant plants as tolerance is unaffected by the rate of drying, and modified desiccation tolerant plants as a certain amount of time is required for the induction of tolerance (Gaff, 1971; Oliver et al., 2000 and 1998). All lower order plants to date are classified under the group of fully desiccation tolerant plants such as *Bryophyta* or true *mosses* which desiccate rapidly (Oliver et al., 2000; 1998). These groups of plants survive the extremely rapid loss of protoplasmic water and rely mainly on rehydration-induced repair mechanisms coupled to constitutive cellular protection (O'Mohany and Oliver, 2001). While high order plants are classified under the group of modified desiccation tolerant plants, these are largely pteridophyte and angiosperms that possess rigid mechanisms to cope with desiccation and rely more on dehydration-induced protection of cellular integrity, with repair mechanisms playing a supplemental role (O'Mohany and Oliver, 2001). They developed complex morphological and biochemical pathways to retard initial water loss and institute cell protective features during dehydration and rehydration (Oliver et al., 1998; Farrant et al., 1999; Dace et al., 1998). Repair mechanisms are likely to include not only regeneration of damaged membranes but also turnover of proteins and RNA damaged or rendered useless because of water depletion or as part of a reorganization strategy to redirect the metabolic emphasis (O'Mohany and Oliver, 2001). Resurrection plants carry out a controlled shutdown of photosynthesis early on during drying (at RWC > 50%) to avoid the generation of ROS, and also employ range of defense systems to limit damage (Farrant, 2000; Ingle et al., 2007).

Desiccation tolerant angiosperms are represented by two groups, those that retain chlorophyll on drying (homiochlorophyllous) and those that lose chlorophyll (poikilochlorophyllus) (Sherwin and Farrant, 1996; Farrant and Sherwin, 1998; Georgieva et al., 2007). The homiochlorophyllous desiccation tolerant plants such as *Craterostigma* retain the majority of their chlorophyll and rely on mechanisms such as anthocyanin production to

neutralize ROS and leaf folding to prevent chlorophyll-light interactions (Sherwin and Farrant, 1998; Ingle et al., 2007). These plants recover rapidly after desiccation and restore photosynthetic activities within 24 hours of rehydration (Bartels, 2005; Bernacchia et al., 1996). Piokilochlorophyllous such as *X. viscosa* dismantle thylakoid membranes and degrade chlorophyll, which must be resynthesized upon rehydration (Tuba et al., 1994; Sherwin & Farrant, 1996; Tuba, 2008). The poikilochlorophyllus desiccation tolerant strategy is anatomically complex, as the dismantling of chloroplast structure is achieved by a deconstruction order during dehydration while the resynthesis of the chloroplast is achieved by a reconstruction order upon rehydration (Tuba et al., 1997; Tuba, 2008). This process is thought to be superimposed on an already existing cellular protection mechanism of vegetative desiccation tolerance (Tuba, 2008). The selective advantage of this strategy is minimum photo-oxidative damage and low energy cost of maintaining the chloroplast structure upon rehydration than to maintain it during the drying period (Oliver et al., 2000; Tuba, 2008). These plants need longer time (about 80 hours) of rehydration to recover their photosynthetic activities (Walford *et al.,* 2004*).*

The poikilochlorophyllus desiccation tolerant plants are currently known in 8 genera of four families (Cyperaceae, Liliaceae, Anthercaeae, Poaceae and Vellozaiceae), and they seem to be restricted to the monocots, occupy soil-less rocky outcrops known as inselbergs, in severe seasonal subtropical climates (Gaff, 1989; Tuba, 2008). Desiccation-induced breakdown of photosynthetic apparatus in poikilochlorophyllus desiccation tolerant (PDT) plants is different from leaf senescence (Tuba, 2008). The dismantling of the photosynthetic apparatus can be seen as a strictly organized protective mechanism, rather than damage to be repaired after rehydration (Tuba, 2008). *Xerophyta scarbida* preserves most of its chlorophyll when it is desiccated in the dark, so most loss seems to be due to photooxidation under natural conditions (Tuba et al., 1997). Dismantling of the thylakoid membranes leads to the formation of nearly isodiametric desiccoplasts, which contain granular stroma, a couple of translucent plastogobuli, which are able to regreen and photosynthesize upon rehydration (Tuba et al., 1993a; Tuba 2008). In *X. villosa* all chloroplast membranes and most mitochondrial cristae disappear on dehydration and the remaining ones appear to decompose within 30 minutes after rewatering (Tuba, 2008). The poikilochlorophyllus desiccation tolerant strategy has evolved in habitats where the plants are exposed to desiccation stress for 5-10 month period (Tuba, 2008). Under these conditions it is more advantageous to dismantle the whole photosynthetic apparatus and

reconstitute it again when water becomes available rather than to maintain it (Tuba, 2008). Resurrection plants are therefore excellent model systems for the study of desiccation tolerance mechanisms and the identification of genes that could potentially enhance drought tolerance of crops by biotechnological methods.

In resurrection plants mechanical stress associated with desiccation such as shrinkage of the plasma membrane away from the cell wall followed by cytorhesis is prevented by changes in cell wall composition to allow folding, packaging of cytoplasm with vacuoles in which water is replaced with compatible solutes and changes in membrane lipids to increase fluidity (Farrant 2000; Willigen et al., 2004; Vicre et al., 2004b; Moore et al., 2005b; 2006). To prevent the damage to macromolecules such as DNA and proteins, resurrection plants also synthesize compatible solutes such as sucrose and trehalose (Muller et al., 1995; Gaff, 1997; Ingram et al 1997; Whittaker et al 2001), and the expression of heat-stable, hydrophilic proteins that are associated with seed development the small heat shock proteins and the late embryogenic accumulating/abundant proteins (LEAs) (Bernacchia and Furini 2004).

1.4. *XEROPHYTA VISCOSA*

Xerophyta viscosa Baker (family Velloziaceae) is a monocotyledonous poikilorophyllous desiccation tolerant plant capable to survive drying of its leave tissues to 5% relative water content (RWC) for prolonged periods and resume full physiological activity within 80 h of rewatering (Illing et al., 2005; Ingle et al., 2007) (Fig. 1.1). The family of Velloziaceae desiccation tolerant plants contains about 200 members in 8 genera and is considered the largest resurrection plant family (Tuba, 2008). *X. viscosa* has been investigated to understand the molecular mechanisms of desiccation tolerance (Farrant, 2000; Mundree *et at.,* 2000; Mundree *et al.,* 2002; Garwe *et al.,* 2003; Marais *et al.,* 2004; Walford *et al.,* 2004; Mundree *et al.,* 2006; Iyer *et al.,* 2007, Ingle *et al.,* 2007). This plant is endemic to southern Africa, Madagascar and southern America (Mundree et al., 2006) and serves as a good model system to study drought tolerance and for the discovery of novel genes that may be used for the genetic enhancement of crop varieties. *X. viscosa* has the unique ability to resurrect after desiccation, thus it must posses specific genes that may confer the plant the ability to cope with the desiccation. Furthermore, it is monocotyledonous, which is a common characteristic shared with many crops, is easily subjected to stress treatments and a tissue culture system has been developed to simplify the laboratory manipulation (Marais et al., 2004).

A number of interesting genes have been identified in *X. viscosa* as up-regulated in response to various abiotic stresses, these genes include *XvPrx1*, *XvPrx2*, *XvGols*, *XvIno1*, *XvGrp94*, *XvALDR4*, *XvSAP1*, *XvVHA-c"1* and *XvCaM* (Mundree et al., 2006; Govender, 2006). In response to desiccation stress, *X. viscosa* undergoes numerous changes to neutralize the harmful effects of elevated reactive oxygen species. It breaks down its chlorophyll and thylakoid membranes during the drying process (Mundree et al., 2006). It also produces several antioxidant enzymes such as peroxiredoxins (Mundree et al., 2006; Ekmekci et al., 2005) which function in antioxidant defense in photosynthesis, respiration, stress response and redox signaling. Resurrection plants accumulate an assortment of carbohydrates, amino acids and polyols that are assumed to be involved in protecting cell integrity upon water loss (Ramanjulu and Bartels, 2002; Mundree et al., 2006). Two genes (*XvGols* and *XvIno1*) involved in the synthesis of osmoprotectants. The *XvGols* was shown to be up-regulated in leaves of *X. viscosa* during dehydration stress (Peters, 2005), and it encodes galactinol synthase and it thought to be an important component in compatible solute biosynthesis. The *XvIno1* is a myo-inositol-1-phosphate synthase gene, which has been shown to be up-regulated during various stresses (Mundree et al., 2006). Myo-inositol is a precursor for a number of important metabolites, which include membrane components, storage molecules, phytohormones and a variety of osmoprotectants (Mundree et al., 2006). The *XvGrp94* gene codes for a glucose related protein and is also homologous to HsP90, it has been shown to be up-regulated during desiccation and heat stress (Walford et al., 2004).

The *XvALDR4* is an aldose reductase gene, which has been shown to be up-regulated during dehydration stress (Mundree et al., 2000; Mundree et al., 2006). Aldose reductase catalyses the reduction of sugars to their corresponding alcohols, it is also involved in detoxification of cytotoxic aldehydes; products of ROS-induced lipid peroxidation (Obserchall et al., 2000; Mundree et al., 2006).

Maintaining cell membrane integrity during osmotic stress is critical to stress-tolerance mechanisms (Mundree et al., 2006). The XvSAP1 is a highly hydrophobic protein and has two membrane lipoprotein lipid attachment sites. It was shown to be up-regulated in leaves of *X. viscosa* during dehydration stress (Garwe et al., 2003). Transgenic *A. thaliana* and *N. tobacum* plants constitutively expressing XvSAP1 displayed increased tolerance to osmotic, salt, heat and dehydration stress (Garwe et al., 2006; Mundree et al., 2006). It is postulated that it could play an important role in maintaining cell membrane integrity.

Salinity and drought can cause hyperosmotic stress, which results in declined cell turgor. This disrupts ionic equilibration in the cell by a cytotoxic buildup of sodium and chloride ions (Mundree et al., 2006). The *XvVHA-c"1* is a V-ATPase gene that has been shown to be up-regulated during dehydration stress in *X. viscosa* leaves. The vacular-ATPase generates a proton motive force across the vacuole membrane, and may be of major importance in the removal of ions from the cytoplasm into the vacuole as the cell shrinks during drying, an important process in damage control (Oliver et al., 1998; Mundree et al., 2006). It is also postulated that XvVHA-c"1 plays a role in creating a proton translocating pore and assists in adaptation to osmotic pressure fluctuation as well as having a housekeeping role to maintain luminal acidification (Mundree et al., 2006).

The stress perception and signal transduction to switch on adaptive responses are critical steps in determining the survival of plants exposed to adverse environments. Plants have multiple stress perception and signal transduction pathways, which may cross-talk at various steps (Knight et al., 2001; Pastori et al., 2002). Signaling pathways use secondary messengers, such as calcium, pH, lipids, inositol triphosphate, cyclic guanosine monophosphate and activated oxygen species (Mundree et al., 2006) Calcium responds to more stimuli than any other messenger and XvCaM, a calmodulin-like protein gene, was shown to be upregulated in leaves of *X. viscosa* during dehydration stress (Mundree et al., 2006). Calmodulin (CaM) is highly conserved and ubiquitous as a calcium receptor in plants.

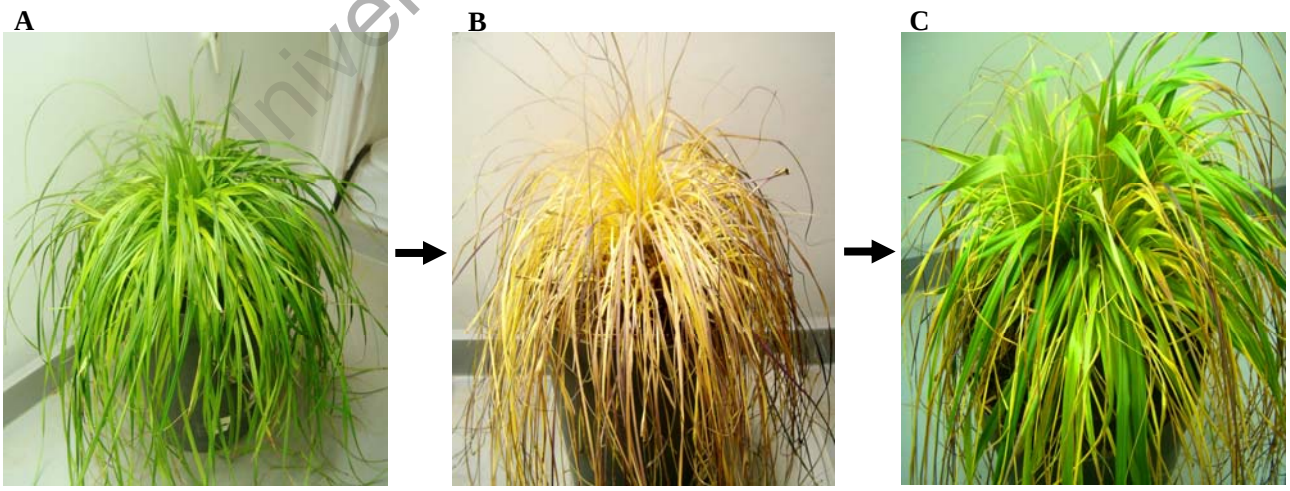


Figure 1.1. (A) Potted fully hydrated (100% RWC) *X. viscosa* plant in a controlled environmental room, (B) the same plant dehydrated at 5% RWC and (C) the same plant after 80 h of rewatering.

1.5. PROTEOMICS AS A TOOL TO STUDY GENE FUNCTIONS

Proteomics is the high-throughput identification and analysis of proteins, usually by biochemical methods (Pandey and Mann, 2000). Traditionally had been associated with displaying a large number of protein spots from a given cell line or organism on two-dimensional polyacrylamide gels (2D-PAGE (O'Farrel, 1975). Proteomics describes proteins on a proteome-wide scale, thereby creating a new way of doing cell research which results in the determination of three-dimensional protein structures, description of protein networks and signaling pathways (Burley et al., 1999; Hunter 2000; Sweetlove, 2006).

Though the complete genome sequences are available for *A. thaliana*, *Oryza sativa* and other plant species, it has become clear that this is not sufficient to elucidate biological functions (Barbier-Brygoo and Joyard, 2004). The existence of an open reading frame (ORF) in genomic data does not necessarily imply the existence of a functional gene (Pandey and Mann, 2000). Despite the advances in bioinformatics, it is still difficult to predict genes accurately from genomic data (Pandey and Mann, 2000). This is particularly true in the case of small genes or genes with little or no homology in databases (Pandey and Mann, 2000). Therefore, verification of a gene product by proteomic methods is an important first step in annotating the genome. Modifications of the proteins that are not apparent from the DNA sequence can only be determined by proteomic approaches. Furthermore, direct determination of protein expression level can be achieved only through proteomic approaches as mRNA levels do not always correlate with the protein level (Gygi et al., 1999). The localization of gene products can be determined experimentally, whilst it is often difficult to predict from the sequence. Several cellular processes such as regulation of protein functions in cell compartments affect gene products rather than genes, the protein-protein interactions and the molecular composition of cellular structures such as organelles can be determined only at the protein level (Pandey and Mann, 2000). Finally, protein studies are necessary in signal transduction because signaling pathways are composed of proteins. Many proteins exist in protein complexes whose function may be elucidated only through a study of the intact complex (Graves and Haystead, 2003). Thus a better approach for the basic description of cell phenotypes would be an exhaustive, quantitative analysis of the proteome (Haberkorn et al., 2002).

In order to analyze global patterns of gene expression at the protein level, proteomics performs proteins analysis of cells or cell fractions by gel-based (such as 2DE, and 2DIGE) or

gel-free approaches (liquid chromatography-based proteomics using isotopic labeling (such as ICAT and iTRAQ), followed by identification of proteins by mass spectrometry (Fig. 1.2.). In gel-based approaches, relative protein abundance is determined by visualization within the gel, and the mass spectrometer is used only for protein identification (Moresco et al., 2008). In gel-free approaches protein identification and relative protein abundance are determined after protein identification using mass spectrometer in combination with software (Moresco et al., 2008). Although protein abundances are not direct measurements of cellular functions, methods for broader biochemical characterization and systematic measurements are not currently available. Such analyses on a large scale probably will be feasible soon relies on the rapid development of mass spectroscopy to generate large quantities of peptide sequences from proteins that are available only in femto-molars (Wilm et al., 1996; Haberkorn et al., 2002).

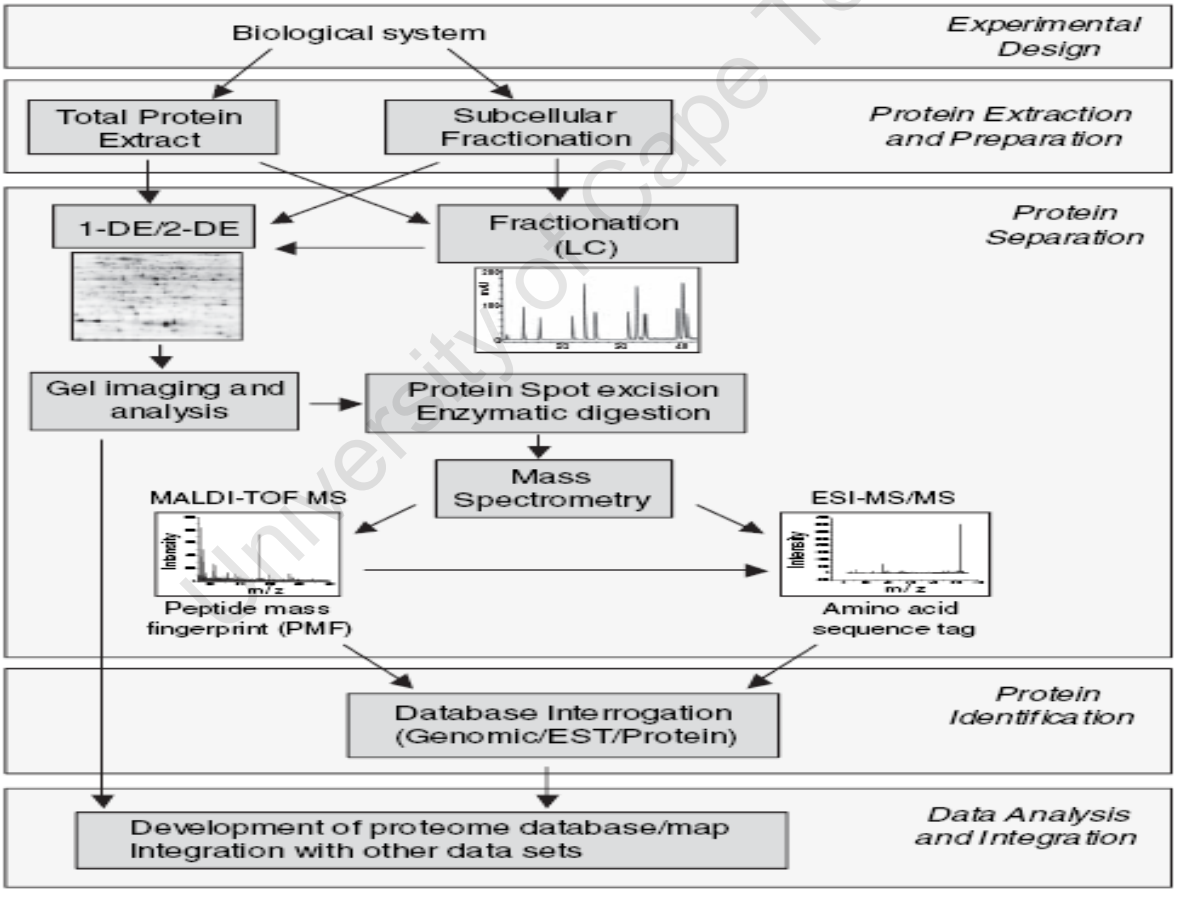


Figure 1.2. Overview of common steps involved in proteomic analysis. These typically include protein separation by one- or two-dimensional electrophoresis (1-DE or 2-DE), respectively or

liquid chromatography (LC), followed by protein identification using spectra generated by MALDI-TOF MS or ESI-MS/MS (Rose et al., 2004).

1.6. OVERVEIW OF PROTEOMIC TECHNOLOGIES

Functional genomics, a shift in the scientific paradigm can be observed in the pre-proteomics era, functional assignments were derived from experiments designed to understand specific cellular processes (Haberkorn et al., 2002). Proteomics is independent of the testing of specific models of cellular behavior, i.e. it represents a generally applicable approach. Using proteomic methods, variations in the abundance and properties of proteins will allow the determination of probable protein function.. These descriptions may then be used for the design of hypotheses in the traditional sense. Technical advances for protein assays include affinity purification using specific antibodies, metals, lectins or other reagents which allow enrichment for modified proteins for detection by mass spectrometry (Haberkorn et al., 2002).

1.6.1. Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE)

Gel-based approaches were once considered the workhorse of proteomics (Moresco et al., 2008). The first approach to characterization of complex protein mixtures was separation by two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), stain, gel analysis and excision of protein spots of interest. This is followed by in-gel digestion of visible protein spots and subsequent offline analysis of individual digest by matrix-assisted laser desorption ionization mass spectrometry (MALDI-MS) (Henzel et al., 1993). The fundamental principle is that proteins are separated first according to their pI using immobilized pH gradient strip, then proteins are separated on the second dimension according to their molecular weights by gel electrophoresis. After digestion (usually with trypsin), individual protein spots (converted to peptides) are removed from the gel matrix and analyzed by MALDI-MS. Protein identification is based on the principle of peptide mapping, where observed peptide peak patterns in individual spectra are compared with predicted digest fragments of proteins in databases (Yates et al., 1993; Ferguson & Smith, 2003). Although 2DE-based proteomics has proven powerful for global analysis of proteins, it still suffers several drawbacks. It is costly, extremely laborious and time-consuming to set-up as it requires too many steps of sample preparation, a typical 2DE experiment can take 2-3 days, and only a single sample can be analysed per gel (Gygi et al., 2000; Righetti et al., 2004, Park, 2004). Other limitations include limited *pI* range, proteins >

150 kD are not seen in 2D gels, bias against membrane proteins (> 30% of all proteins), and detects only high abundance proteins (top 30% typically). 2DE also is difficult to automate (Rose et al., 2004), and as a consequence, running and analyzing 2D gels requires competent technical skills, optimization and good analysis software.

1.6.2. Two-dimensional differential in-gel electrophoresis (2-DIGE)

Given the drawbacks of the classical 2-DE method have given an added momentum to other alternatives, the first publication came from Unlu et al., (1997) with new technique termed 2-DIGE, two-dimensional differential in-gel electrophoresis (Figure 1.3.). In fluorescence 2-D DIGE, two different samples are derivatized (ε-amino group of lysine) with two different spectrally resolvable fluorescent cyanine dyes, CyDye flours (e.g., Cy2, Cy3, and Cy5), similar to those used for differential cDNA microarry analysis, but optimized for 2-DE (Rose et al., 2004). The two labeled protein samples are then combined and separated on a single 2D gel (Patton et al., 2002; Wu et al., 2006) to allow better spot matching and to minimize gel-to-gel variations. The fluorescent cyanine dyes exhibit distinct excitation and emission spectra and so it is possible to rapidly quantify and distinguish between proteins that were present in either of the original two extracts. As the two protein samples are separated on the same gel, any protein that exists in both populations will migrate to the same location on the 2-D gel, dramatically facilitating comparisons of protein expression in the two original samples. Therefore, in principle, a single gel would suffice for full quantitative analysis (Hamdan and Rigehetti, 2002; Wu et al., 2006). Proteins are detected using a dual laser scanning device or xenon arc-based instrument equipped with different excitation /emission filters in order to generate two separate images. The images are then matched by a computer-assisted overlay method, signals are normalized, and spots are quantified. Differences in protein expression are identified by evaluation of a pseudo-colored image and data spreadsheet. DIGE technology can maximally evaluate three different samples using Cy2-, Cy3- and Cy5-based chemistries (Tonge et al., 2001; Zhou et al., 2002; Patton, 2002; Gharbi et al., 2002).

One of the advantages of the 2-D DIGE is that control and treated samples are run in the same gel, consequently no separate standard maps for the control and the treated samples must be created (which requires optimization and many separate gels for each condition and the creation of reference maps) (Hamdan and Righetti, 2002). In the DIGE, though matching is automatic, this technique suffers a number of drawbacks. First, the labeling must be done under

very stringent conditions, as pre-labeling in any focusing technique could generate an incredible number of positional isomers, partially reacted species, and so on. In order to avoid this problem, labeling must be minimal, which results in a decrease in sensitivity and it was reported that the DIGE sensitivity was less than that of silver staining (Hamdan and Righetti, 2002). Secondly, the current DIGE protocol does not call for alkylation during the isoelectric focusing (IEF) step. As a result, spurious multimeric spots, due to scrambled disulfide bonds could appear in the gels, this is also associated with protein comigration and partial comigration (Wu et al., 2006), which makes protein quantification challenging. Cutting of protein spots from a DIGE gel is difficult due to the fact that the real centroid of the spot will not be aligned with the fluorescent spot. This makes it difficult to predict where the covalent fluorescent label will be attached, and the majority of the spots will be present in too low an amount for MS analysis, which will complicate the MS peptide identification. Finally, DIGE is not cost effective for proteomic experiments due to the CyDye labeling cost, the gel must be visualized under special scanner for florescence and the use of specialized software.

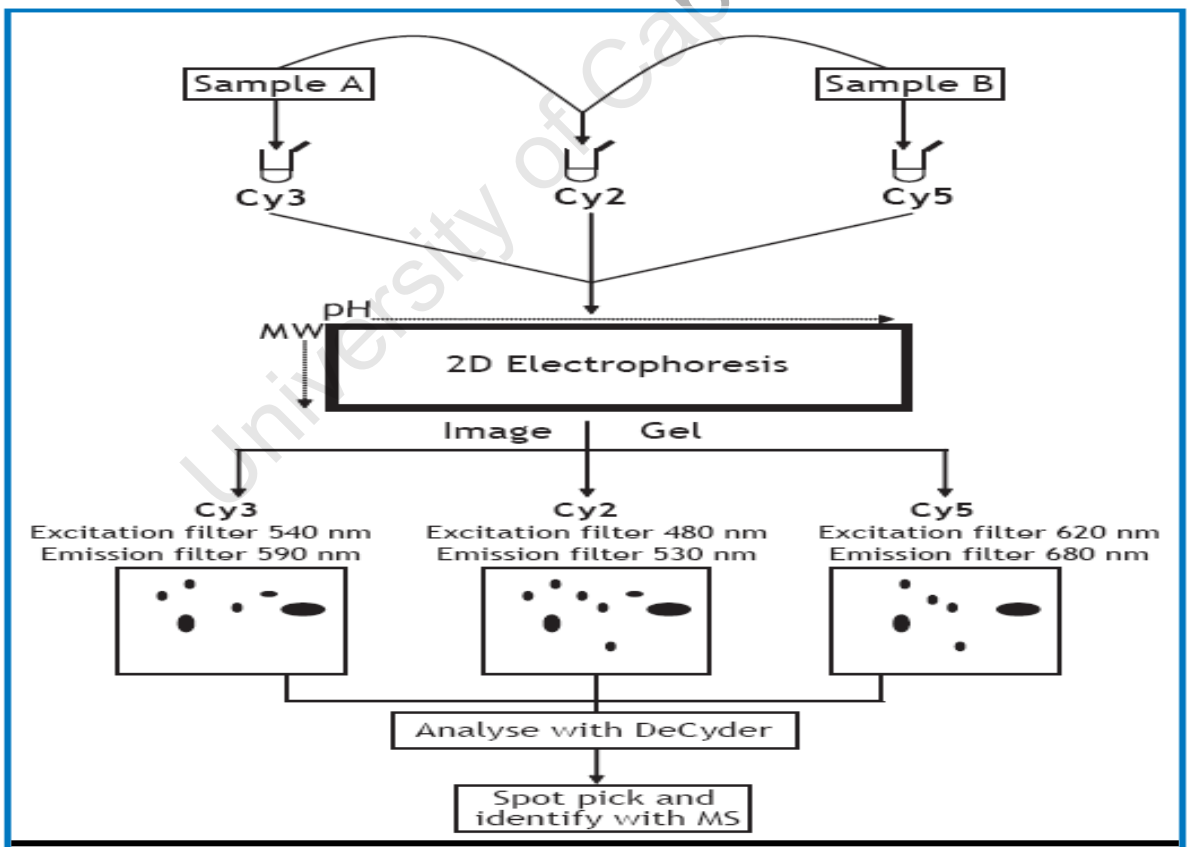


Figure 1.3. Schematic illustration of the difference gel electrophoresis (DIGE) technology platform (Attard et al., 2004).

In the past few years, the labor intensive gel-based technologies have been challenged and complemented by several liquid chromatography-based methods, particularly in the area of high-throughput proteomic research. Among the different LC-based techniques for quantitative proteomics, ICAT is the most commonly practiced technique and iTRAQ is the newest and is an alternative to the ICAT technique.

1.6.3. Isotope-coded affinity tags (ICAT) technology

The ICAT technique was first used by the group of Gygi et al., (1999). Proteins from two states to be compared are labeled at cysteine residues with light and heavy, respectively, ICAT reagents (tags) carrying a biotin moiety. The labeled proteins are then mixed and digested. The peptides mixtures are purified by cation exchange chromatography to remove excess reagents, and then purified by affinity chromatography using immobilized avidin (Wu et al., 2006). Peaks corresponding to the same peptide are identified as doublets in mass spectra due to the mass difference between light and heavy isotopes. The peak intensities of the peptides correlate directly with the relative abundance of the proteins in the two states. Within the same analysis, tandem mass spectrometry can be used to obtained amino acid sequence information on the eluting peptides; that can be used for protein identification (Hamdan and Righetti, 2002). The novelty of this approach lies in the use of the ICAT reagents. A number of limitations to the prototypical ICAT techniques has been reported in the literature (Hamdan and Righetti, 2002; Wu et al., 2006), which include missed identification of proteins with few or no cysteine residue, lost information for post-translational modifications, differential reversed-phase elution of identical peptides labeled with the hydrogen/deuterium isotope pairs, and complicated interpretation of tandem mass spectrometry (MS/MS) spectra due to the addition of the biotin group (Leitner and lindner, 2004). Many, but not all of these limitations have been solved by the new cleavable ICAT (cICAT) reagent that employs carbon 13 isotopes and acid-cleavable biotin group (Wu et al., 2006).

1.6.4. Isobaric tags for relative and absolute quantitation (iTRAQ)

This technique is one of the newest techniques used for the quantitative study of gene expression at the proteome level. It retains post-translational modification (PTM) information and allows multiplex analysis of up to four samples in a single run, thus it is potentially more cost-effective than other labeling approaches. The iTRAQ technique has been developed by

Ross et al., (2004) to overcome the limitations of the cICAT. The iTRAQ labeling technique can analyze up to four sets of proteins at one time (Figure 1.4.; 1.5.). The first step in ITRAQ is preparation of protein samples for iTRAQ labeling. Secondly each protein is reduced, alkylated and digested separately. Thirdly, the iTRAQ is a set of four isobaric reagents, all with a mass around 145 Daltons. Each reagent contains three components; reporter, balance, and reactive groups. The reporter groups of the iTRAQ reagents have molecular weight of 114, 115, 116, and 117 Da, respectively. The balance groups ensure that an iTRAQ-labeled peptide has the same mass irrespective of which one of the four reagents it was labeled with (Lin et al., 2005). The reactive groups are attached to the N-terminal and the lysine residues of protein samples. All samples are then pooled together, separated into up to 20 fractions by strong cation exchange chromatography to reduce sample complexity and each fraction is analyzed by online reverse phase (RP) HPLC-MS/MS. The samples are co-eluted on the RP HPLC and have the same m/z values (isobaric all are 145 Daltons) in the MS. The isobaric 4-plex peptides were fragmented simultaneously and represented in the second MS. The first MS (performing a survey scanning) detects the ions as they elute from the reverse-phase column and it records the m/z and the relative intensity of all of the ions eluting. In the first MS there is individual selection of m/z values from the survey MS scan for further analysis (Moresco et al., 2008). Peptide ions selected from the survey MS are fragmented and the label is cleaved owing to post-source decay (PSD) or collision-induced dissociation (CID) between the reporter and the balancer group. The reporter groups of the iTRAQ reagents will split from the peptide and form small fragments with mass/charges (m/z) of 114, 115, 116, and 117, respectively. The intensity of each of these peaks represents the quantity of a peptide sample. Other peaks in the spectra graph are used to identify peptide sequences and post-transitional modifications. The second MS scans have less noise than the survey scans, which allows for accurate measurements of less abundant peptides.

The iTRAQ technology in proteomic research in combination with mass spectrometry is currently the most powerful technique for relative and absolute qauntitation of proteins, due to its ability to globally label proteins and to detect low abundance, very acidic, very basic, high and low molecular weight proteins (Ross et al., 2004; Lin et al., 2005; Shadforth et al., 2005; Chong et al., 2006; Gan, 2007). The principal advantages of 2DLC-MS/MS methods using iTRAQ labeling include the ability to conduct multiplex experiments, whereby up to 8 samples can be analyzed concurrently under different physiological conditions (Pierce et al., 2007). This

results in reduced systematic error and increased electrospray ionization efficiency leading to higher sensitivity; efficiency and a comprehensive proteome analysis. In addition, because protein identification and quantification is based on MS/MS evidence there is increased selectivity and confirmation of identity (Garbis et al., 2008).

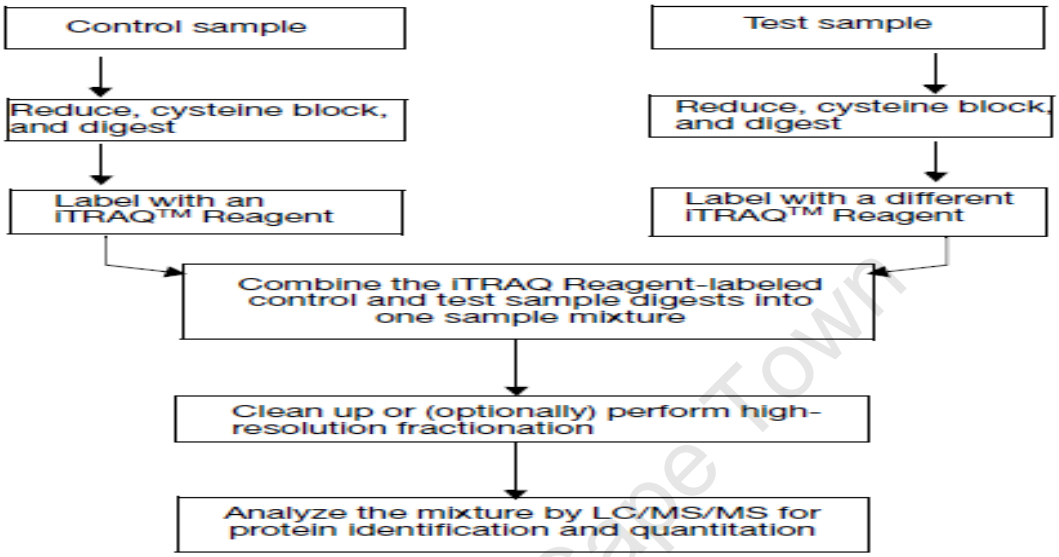


Figure 1.4. Overview of iTRAQ Reagents methodology (Applied Biosystems ITRAQ reagents chemistry reference guide, copyright 2004).

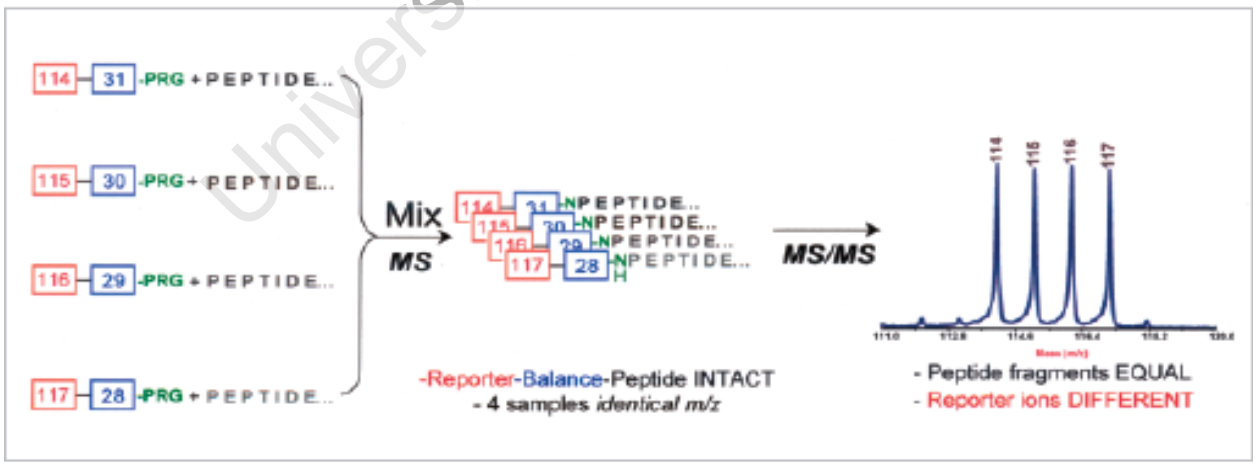


Figure 1.5. The general concept of iTRAQ chemistry (e.g., of a 4-plex experiment). Each trypsin digested peptide sample is labeled with one of the four iTRAQ reagents and then pooled prior to MS analysis (Applied Biosystems product bulletin iTRAQ reagents, www.appliedbiosystems.com).

1.7. MASS SPECTROMETRY IN PROTEOMIC RESAERCH

Mass spectrometric identification of proteins has revolutionized the area of proteomic research, which extends analysis far beyond the mere display of proteins (Shevchenko et al., 1997; Shevchenko et al., 2001; Liska and Shevchenko, 2003; Liska et al., 2005). Over the past decade, mass spectrometers have become increasingly attractive analytical instruments for biologists, due in part to new ionization methods and major improvements in mass accuracy, resolution, sensitivity and ease of use, which have extended the applicability of MS to characterize large intact macromolecules such as proteins (Loboda et al., 2000; Mann and Jensen, 2003; Rose et al., 2004). Identification of proteins and characterization of post-translational modifications can be carried out using MS at the femtomolar level (Wilm et al., 1996; Rose et al., 2004; Graves and Haystead, 2003). It relies on digestion of proteins into peptides by a sequence-specific protease such as trypsin to simplify sample elution for mass spectrometry analysis and to appropriate molecular weights for database identification (Pandey and Mann, 2000). In functional proteomics, considerable effort is frequently expended to obtain a relatively small number of proteins for analysis. In this case, the most important feature of a mass spectrometer is high sensitivity and ability to identify a protein with the highest level of confidence (Shevchenko et al., 1997; Graves and Haystead, 2003). Critically, newer spectrometers are packaged with software that facilitate protein identification and structural analysis and provide a bridge between mass spectra and public sequence databases (loboda et al., 2000; Shevchenko et al., 2001; Aebersold and Mann, 2003; Ferguson and Smith, 2003; Lin et al., 2003; Mann et al., 2001; Rose et al., 2004; Liska et al., 2005).

For MS analysis, samples must be converted to gas-phase ions. Two of the most common methods to accomplish this are matrix-assisted laser desorption ionization (MALDI) and electrospray ionization (ESI) (Shevchenko et al., 1997; 2000; Graves and Haystead, 2003). In MALDI, a laser is used to promote the ionization of analyte molecules embedded in a crystalline matrix (Loboda et al., 2000; Karas and Hillenkamp, 1988). The masses of individual peptides derived from a protein are measured in what is known as a peptide mass “fingerprint” (Rose et al., 2004). Several software packages are then available that can compare the peptide mass list with a predicted ‘theoretical’ list of tryptic peptide fragments for every protein in the public databases, together with equivalent translated genomic and EST databases. In this case the protein is identified based on the in silico match of the experimentally determined versus

predicted peptide masses, together in some cases with the apparent and predicted pI and molecular mass from the 2-D gels, rather than actual amino acid sequence (Rose et al., 2004). In general, peptide mass fingerprint analysis is not as reliable for protein identification as peptide amino acid sequence obtained by MS/MS (Loboda et al., 2000; Graves and Haystead, 2003). Because of this deficiency, a new breed of MALDI mass spectrometers have emerged that can, in addition to peptide mass fingerprinting, also obtain peptide amino acid sequence (Graves and Haystead, 2003). Examples of these are MALDI-Q-TOF (Loboda et al., 2000; Shevchenko et al., 2000) and MALDI-TOF-TOF (Medzihradszky et al., 2000). Although each PMF is usually a viable means of assigning identity to a specific protein, due to the variability in amino acid sequences and the relative distribution of protease cleavage sites between proteins (Godovac-Zimmermann and Brown, 2001) members of protein families with a high degree of sequence similarity can also result in indistinguishable PMFs. This problem is exacerbated by the fact that it is unusual for the full complement of peptides for any given protein to be ionized and detected experimentally by MALDI-TOF (Rose et al., 2004). A common alternative to the PMF approach is de novo sequencing by electrospray ionization tandem mass spectrometry (ESI) MS/MS, which yields amino acid sequences of selected tryptic peptides (Rose et al., 2004). In ESI, a sample is directly introduced into the mass spectrometer as a fine mist of charged droplets (Graves and Haystead, 2003). In an adaptation of electrospray known as nanospray, samples are introduced through microcapillary tubes at very low flow rates (Wilm and Mann, 1996). The first step of tandem MS involves ionization of a sample and separation based upon the mass-to-charge ratio (m/z) of the primary ions. An ion with a specific m/z value is then selected, fragmented, and the fragment ions detected after passing through the second mass spectrometer. This process produces a series of fragment ions that can differ by single amino acids, allowing a portion of the peptide sequence, termed an ‘amino acid sequence tag’, to be determined and used for database searching. When a peptide has been identified in the database, the theoretical fragmentation pattern can be predicted and compared with the observed MS/MS spectrum for assignment of other peaks that can validate the identification. This procedure can be repeated for every fragmented peptide in the sample, leading to additional verification or identification of other proteins in the sample. The spectra are analyzed with software packages (e.g., Mascot, Paragon and ProGroup Algorithms, Matrix Science) (Rose et al., 2004; www.appliedbiosystems.com) to identify the protein (Fig. 1.6). The MALDI-TOF/PMF approach is very rapid, requires relatively little user expertise, can be automated and is tolerant

of contaminants. The single biggest advantage of MALDI analysis is speed. In MALDI, the entire process of sample preparation and analysis in the mass spectrometer can be automated. As a result, MALDI is the one of the preferred methods for high-throughput proteomics operations. However, the data are generally more ambiguous and essentially rely on the availability of a genomic sequence, or at least a substantial expressed sequence tag (EST) collection, for the species under study (Rose et al., 2004). In contrast, MS/MS analysis is technically more challenging, requires specialist training, has a slower throughput and is far more expensive (Rose et al., 2004).

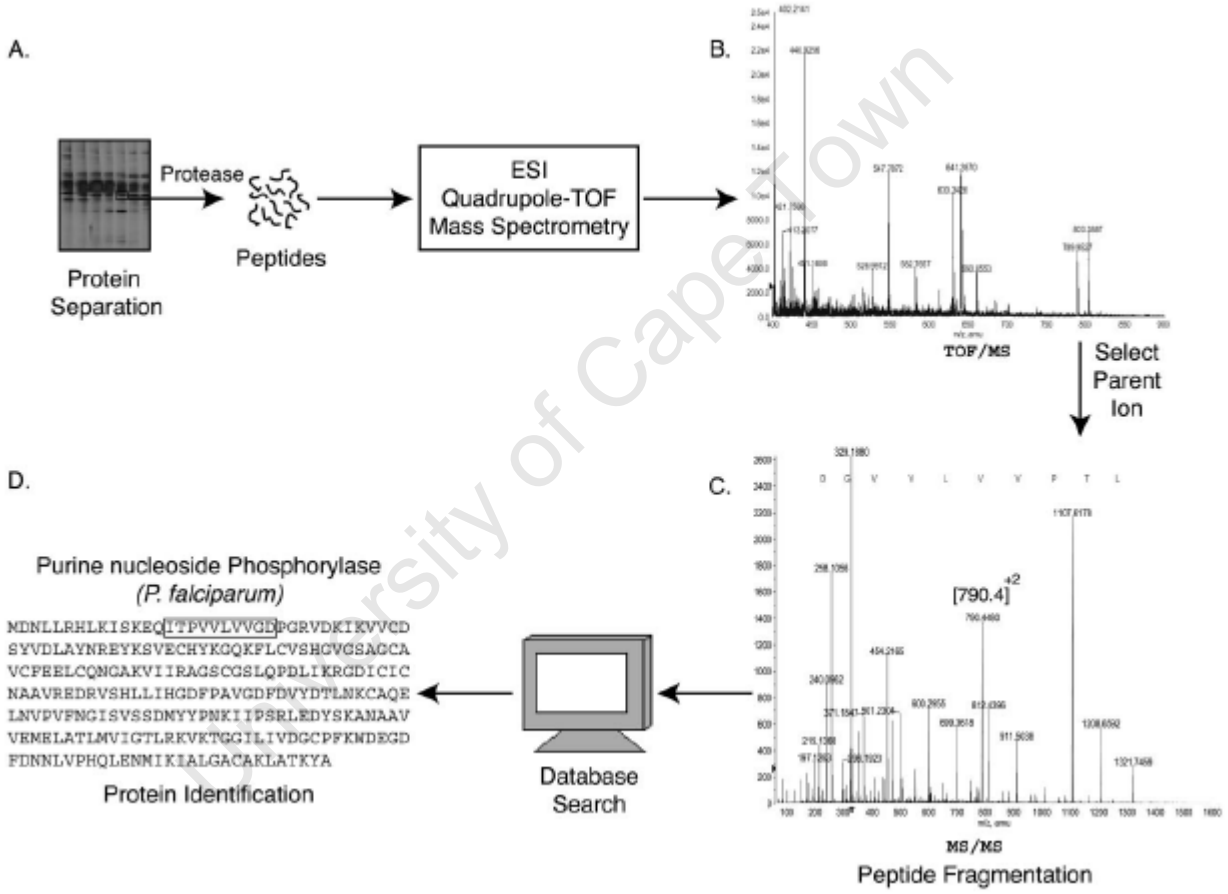


Figure 1.6. Protein identification by tandem MS/MS. (A) A protein sample is resolved by electrophoresis, excised, and in-gel digested with trypsin. The resulting peptides are ionized by ES and analyzed by quadrupole TOF/MS. (B) Selection of a parent ion from the TOF spectrum. (C) MS/MS and amino acid sequencing of the parent ion. (D) Database searching and protein identification by peptide mass tag searching (Graves and Haystead, 2003).

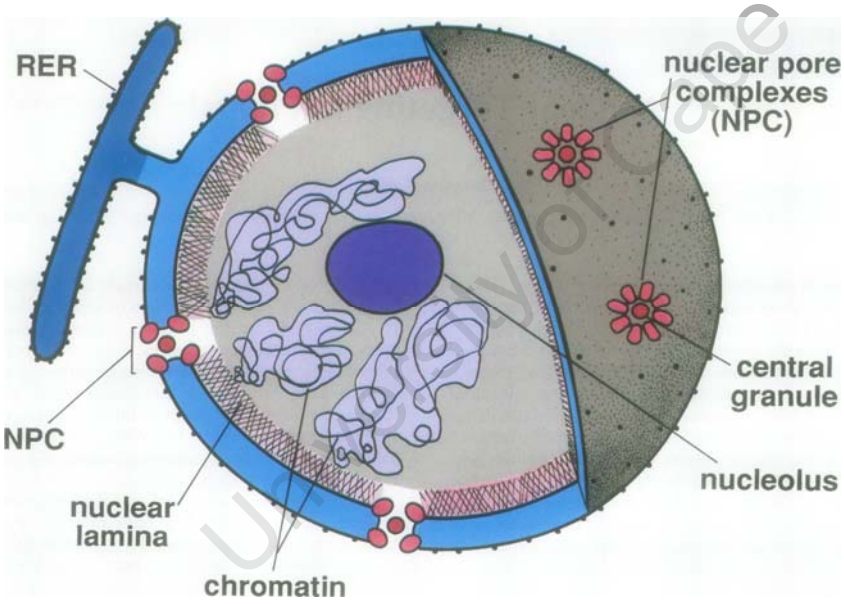
Despite this, ESI MS/MS has a number of advantages over MALDI-TOS/PMF in functional proteomics applications. First, nano-electrospray MS can be used to obtain peptide amino acid sequence in a procedure known as MS/MS (Shevchenko et al., 1997; 2000; Graves & Haystead, 2003). This is the most specific method for protein identification and greater confidence can be achieved in protein assignment. Second, nanospray provides a higher sensitivity than MALDI, allowing for the identification of low-copy proteins (Shevchenko et al., 1997; 2000; Graves and Haystead, 2003). Finally, and perhaps most importantly, MS/MS is less affected by protein mixtures than peptide mass fingerprinting (Graves and Haystead, 2003). This is critically important because frequently, protein "bands" isolated from polyacrylamide gels contain more than one protein (Graves and Haystead, 2003).

1.8. OVERVIEW OF THE NUCLEUS STRUCTURE

The nucleus (Fig. 1.7.) was the first organelle discovered and was originally described by Franz Bauer in 1802 and later popularized by Robert Brown (Dundr and Misteli, 2001; Lamond and Sleeman, 2003). Though the organelle was studied since then it is the least understood subcellular structure (Lamond and Earnshaw, 1998). The organelle attracted the attention of many Scientists because of its fascinating, complex and aesthetically pleasing behavior during cell division and its evident roles in essential processes, such as fertilization and inheritance (Dundr and Misteli, 2001). The nucleus stores and protects the hereditary material, DNA, and is the site for many key processes in the cell (Dundr and Misteli, 2001; Jackson, 2003). These include the orchestration of the switching on and off in time and space of many thousands of genes, the faithful replication of the genome during cell division and the elaborate molecular mechanisms that are responsible for repairing damaged DNA (Dundr and Misteli, 2001; Jackson, 2003). These processes require the cooperation of large numbers of proteins, and also numerous non-protein-coding RNA molecules with specific recognition sites on the genome. Transcription, RNA processing, DNA repair and replication are functionally tightly coupled. The nucleus also coordinates the cell's activities, which include intermediary metabolism, growth, protein synthesis, and reproduction (Dundr and Misteli, 2001; Lamond and Sleeman, 2003). The nucleus is characterized by an extremely dynamic structure, where both euchromatin and heterochromatin move continuously through the fibrillar network of the nuclear matrix, depending on the transcriptional status of the cell (Cremer et al., 2000; Dundr

and Misteli, 2001; Lamond and Sleeman, 2003). Furthermore, there is highly controlled traffic of proteins and RNAs through the nuclear pores complex (Erkmann and Kutay, 2004).

The nucleus contains distinct subcompartments (Dundr and Misteli, 2001). These subcompartments are characterized by the absence of delineating membranes, and some of these compartments can be biochemically isolated in an enriched form (Dundr and Misteli, 2001 Knowles et al., 2006). The absence of defining membranes dramatically distinguishes nuclear compartments from other cellular counterparts. The best-studied nuclear compartments are the nucleolus the splicing-factor compartments (SFCs), the Cajal body (CB) or coiled bodies, the promyelocytic leukaemia oncoprotein (PML) body in human cell, and a rapidly growing family of small dot-like nuclear speckles or SFCs (Dundr and Misteli, 2001).



1.7. Schematic representation of plant nucleus (Raikhel N. 1992).

Most of the nuclear material consists of chromosomes, where DNA is combined with proteins and organized into a precise, compact structure, the chromatin (Dundr and Misteli, 2001). Chromosomes are distinct entities within the nucleus, but can obviously not be considered nuclear compartments (Cremer et al., 2000). Each DNA strand wraps around groups of small basic protein molecules called histones, forming a series of compact bead-like

structures, called nucleosomes, connected by the DNA strand. There are two types of chromatin. Euchromatin is the genetically active portion and is involved in transcribing RNA to produce proteins used in cell function and growth (Cremer et al., 2000; Dundr and Misteli, 2001). Heterochromatin contains inactive DNA and is the portion of chromatin that is the most condensed (Davidson, 2009). Also inside the nucleus is the nucleolus, the most prominent nuclear substructure. It is assembled around the ribosomal DNA (rDNA) repeats, which cluster at chromosomal loci called nucleolar organizers. This is the site where 28S, 18*S*, and 5.8*S* ribosomal RNAs (rRNAs) are transcribed, processed, and assembled into ribosome subunits (Lamond and Earnshaw, 1998). Nucleolus formation is both transcription- and cell cycle–dependent. In most eukaryotic cells, the entire structure breaks down during cell division and reforms during each mitotic cycle (Olson and Dundr, 2005). Thus, the nucleolus is a dynamic structure that forms in response to the requirement for new ribosome synthesis. Within the nucleolar factory, the rRNA is extensively modified during ribosome biogenesis in a process involving a series of specific nucleolytic cleavages as well as base modifications (Olson and Dundr, 2005). In addition to its major role in ribosome production, the nucleolus may also be involved in messenger RNA (mRNA) export or degradation (Lamond and Earnshaw, 1998; Olson and Dundr, 2005). A nucleus may contain up to four nucleoli, but within each species the number of nucleoli is fixed (Davidson, 2009). After a cell divides, a nucleolus is formed when chromosomes are brought together into nucleolar organizing regions.

Many nuclear factors localize either partly or completely in distinct "bodies" or subnuclear compartments. Interest in these subnuclear bodies has been rekindled by recent discoveries that some of them contain factors involved in the processing and transcription of RNA (Lamond and Earnshaw, 1998; Dundr and Misteli, 2001). Speckled pattern results from the association of snRNPs and other protein splicing factors (interchromatin granules), found in the spaces between more densely staining regions of chromatin; interchromatin granule–associated zones that flank interchromatin granules and contain U1, but not the other splicing snRNPs; perichromatin fibrils that are closely associated with active chromatin and contain newly transcribed mRNA precursors and processed mRNA ; and coiled bodies that are fibrillar structures (Dundr and Misteli, 2001; Lamond and Earnshaw, 1998). Interchromatin granule speckles are genuine nuclear structures that can be visualized directly in the electron microscope. Speckles disperse when cells enter mitosis, but snRNPs and protein splicing factors reform into speckle-like structures during telophase, before their reimport into daughter nuclei

(Dundr and Misteli, 2001). Thus, snRNP speckles can occur in the absence of DNA and transcription. Speckles function either as depots supplying splicing factors to active gene loci, or way stations accumulating snRNPs bound either to partially spliced pre-mRNA or to excised introns after release of mRNA from the spliceosome (Lamond and Earnshaw, 1998).

The nuclear envelope is a double-layered membrane that separates contents of the nucleus from the cellular cytoplasm (Merkle, 2004; Davidson, 2009). The outer membrane is a simple continuation of the endoplasmic reticulum in its protein composition. In contrast, the inner membrane has a distinct protein composition and specialized functions. The space between the layers is called the perinuclear space and appears to connect with the rough endoplasmic reticulum. The nuclear envelope separates the nuclear compartment containing the chromosomes from the cytoplasm where protein synthesis occurs. Nucleo-cytoplasmic trafficking of macromolecules is therefore an essential process in eukaryotic cells. All nuclear proteins are synthesised on cytoplasmic ribosomes and must be imported, whereas mRNAs, tRNAs and ribosomal subunits must be exported to the cytoplasm (Dundr and Misteli, 2001). The need for separating transcription and translation and thus for nucleo-cytoplasmic trafficking may arise from the fact that most eukaryotic genes contain introns. In order to prevent the production of proteins from aberrant mRNAs, most of the translation factors are actively excluded from the nucleus to prevent nuclear translation (Bohnsack et al. 2002). The inner surface has a protein lining called the nuclear lamina, which binds to chromatin and other nuclear components. During mitosis, or cell division, the nuclear envelope disintegrates, but reforms as the two cells complete their formation and the chromatin begins to unravel and disperse (Davidson, 2009). The nuclear envelope has several important functions such as separation of the biochemical environment of the nucleus from that of the cytoplasm, and mediates and regulates the selective exchange of molecules between the nucleus and cytoplasm (nucleocytoplasmic transport) (Allen et al., 2000). The nuclear envelope also acts as an anchoring surface for heterochromatin, and in higher organisms, plays a still-enigmatic role in the highly complex dissociation and re-formation of the nucleus during cell division (Georgatos and Theodoropoulos 1999, Ellenberg et al., 1999). Although nuclei are typically depicted as spheres, the shape of the nuclear envelope can diverge greatly from this image. Significant grooves and invaginations, both static and dynamic in nature, have been found in both animal and plant nuclei (Collings et al., 2000). These structural features increase the interaction surface between the nucleus and cytoplasm, and suggest that nuclear and cytoplasmic activities may be

more structurally linked than was previously anticipated (Merkle, 2004). The nuclear envelope is perforated with holes called nuclear pores (Kiseleva et al., 2000). Nuclear pore complexes (NPCs), occupy pores where the inner and outer membranes are fused together (Fig. 1.7). NPCs are large protein conglomerates responsible for the selective import and export of macromolecules traversing the envelope (Ryan and Wente, 2000; Kiseleva et al., 2000; Meier, 2001). Transport between the nucleus and the cytoplasm occurs exclusively through the nuclear pore complexes (NPCs) that span the nuclear envelope and allow the passage of molecules between the two compartments (Ryan and Wente 2000). Small molecules may pass the NPCs by passive diffusion. This process is dramatically slowed down if the molecular mass of macromolecules exceeds 40 kDa, which is referred to as the diffusion barrier of the NPC. However, larger proteins may still enter the nucleus, although this process might take hours (Bohnsack et al. 2002). This may explain why some proteins are actively excluded from the nuclear compartment, even if they do not contain a signal for nuclear targeting and their molecular masses exceed the diffusion limit. The other mode, by which macromolecules can pass the NPC, is called facilitated (Kiseleva et al., 2000). In addition to the nuclear structures mentioned above, the nucleus contains a large number of less well characterized, mostly small structures (Dundr and Misteli, 2001).

1.9. SIGNIFICANCE OF THIS STUDY

Dehydration stress is a major abiotic stress that hampers sustainability of food production and causes food shortages worldwide (McKersie et al., 1994; Vincent et al., 2005). *Xerophyta vsicosa* has been investigated as a model desiccation tolerant plant to understand the molecular basis of abiotic stress tolerance and as a source of novel genes for transgenic crops (Mundree et al., 2002; 2006).

The nucleus contains nearly all the genetic information required for the regulated expression of cellular proteins. It helps in shuttling of regulatory factors and gene product via the nuclear pore, aids in the production of mRNAs and ribosomes and organizes the uncoiling of DNA to replicate key genes. It coordinates innumerable pathways to achieve growth, division and differentiation of the cell (Fink et al., 2008). The dynamic nuclear organization, orchestrated by a complex network of nuclear proteins, is fundamental to an understanding of cellular development and physiology (Moriguchi et al., 2005).

The organization of the nuclear proteins is linked to cell and tissue phenotypes and plays a central role in directing nuclear functions (Knowels et al., 2006). Increasing evidence suggests that nearly one-fourth of total cellular proteins are localized in the eukaryotic nucleus, implying a variety of functions (Moriguchi et al., 2005; Pandey et al., 2006). This indicates that an unexpectedly large number of proteins function in the nucleus. Nuclear proteins have been shown to be implicated in broad and different cellular functions such as cell signaling, gene regulation, structure, translation, proteolysis, physiological responsiveness and a variety of RNA-associated functions (Bae et al., 2003; Khan and komatsu, 2004; Moriguchi et al., 2005; Pandey et al., 2008). Although a recent proteome analysis for the *Arabidopsis* nuclear matrix identified several nuclear matrix proteins with similarity to animal nuclear proteins, most of them were nucleolar or ribosomal components found in mammals (Calikowski et al., 2003). Furthermore, no homolog of the component of intermediate filament-like nuclear lamins has been found in the *Arabidopsis* and *Oryza* genomes, despite their essential role as fundamental nuclear components in animals (Calikowski et al., 2003). These results demonstrate that proteins are organelle and organism specific and pproteomics characterization of individual plant subcellular components provides focussed characterization of protein location in relation to function as it reduces protein complexity and increases technical resolution (Jung et al., 2000, Dreger 2003; Xiao-Sheng Jiang et al., 2005). Therefore, identification of all of the proteins that localize to the nucleus is of particular importance.

The characterization of the complex regulatory mechanisms within the nucleus requires global approaches, such as proteomics that simultaneously identify a wide range of proteins without any prior knowledge. Targeted investigations of the nuclear proteome are therefore needed to decipher the mechanisms governing the regulation of adaptation to dehydration stress. Most studies on dehydration stress have focused on the changes in gene expression, while there is far less information available on their functional products. The changes in gene expression are regulated by a number of different and potentially overlapping signal transduction pathways (Shinozaki and yamaguchi-Shinozaki, 1997). However, the level of mRNA does not always correlate well with the level of protein, the key player in the cell (Gygi et al., 1999). It is thus insufficient to predict protein expression level from quantitative mRNA data. Proteomic approaches allow for direct determination of protein expression level and represent a better alternative for the basic description of cell phenotypes.

Recently, whole proteomes of two resurrection plants under dehydration stress conditions were reported namely, the *Boea hygrometrica* (Jiang *et al.,* 2007) and *X. viscosa* (Ingle *et al.,* 2007). These studies initiate a new era of functional genomics in resurrection plants with the aim of obtaining a greater understanding of the molecular basis of desiccation tolerance. Proteins and genes that were up-regulated in response to dehydration stress in resurrection plants were shown to be potentially useful in conferring stress tolerance to transgenic plants and maintaining its physiological adaptation (Mundree et al., 2002; 2006). Ingle et al. (2007) in their investigation of the whole proteome of the resurrection plant *X. viscosa* at 35% RWC have shown that 37% of the differentially expressed proteins in response to dehydration stress were up-regulated and implicated in different protective cellular pathways including ROS scavenging, glycolytic, and signaling pathways. Proteins responsive to dehydration stress in *X. viscosa* and other resurrection plants at low dehydration points such as 35% RWC were described as late-dehydration responsive and were thought to be associated directly with acquisition of desiccation tolerance (Bartles and Salamini 2001; Jiang et al., 2007; Ingle et al., 2007). Such changes in expression at this low water deficit were shown to be unique to resurrection plants (Bartles & Salamini 2001; Mundree and Farrant, 2000; Illing et al., 2005; Ingle et al., 2007). Non-desiccation tolerant species such as *Arabidopsis* can not survive water deficit to less than 85-95% (v/v) relative humidity during their vegetative growth period, although desiccation is an integral part of the normal development program of higher plants in the context of seed formation (Ramanjulu & Bartels, 2000; Bartles & Salamini 2001). Hence, late-dehydration responsive proteins at 35% RWC are of greatest interest to uncover the molecular basis of desiccation tolerance.

Whereas organellar proteome research is quite advanced in animal, yeast, and *E. coli*, there is relatively little information on plant organellar proteomes, and most of the nuclear proteins studies are restricted to yeast and mammals (Dreger et al., 2001; Anderson et al., Jung et al., 2000; Park, 2004; Pandey et al., 2006). Other plant organelles that have been investigated at the proteome level include chloroplast (Peltier et al., 2000) and mitochondrion (Millar et al., 2001). Only a few plant nuclear matrix proteins have been characterized, and they have no obvious homology with known nuclear matrix proteins in yeast and mammals (Gindullis and Meier, 1999; Gindullis *et al.,* 1999; Calikowski et al., 2003). Unlike resurrection plants, nuclear proteome analyses for small number of non-resurrection plants under normal and abiotic stress conditions have been reported and their molecular functions have been highlighted these include

Arabidopsis (Bae *et al.,* 2003), rice (Khan and Komatsu, 2004), chickpea (Pandey *et al.,* 2006; 2008), and *Medicago* (Repetto et al., 2008). These studies demonstrate that, the nuclear proteins were implicated in diverse protective and signaling pathways with the protein function linked to the investigated stress conditions. However, no nuclear or other organellar proteome research has been carried out in resurrection plants.

Therefore, comprehensive analysis of the nuclear proteome of the resurrection plant *X. viscosa* and its response to dehydration stress at 35% RWC is important. This nuclear proteome is of particular interest as it will provide an insight into the functional role of the organelle in the acquisition of desiccation tolerance. It will also provide a foundation for future investigation of the mechanisms involved in the acquisition of desiccation tolerance at the metabolic and molecular levels. The following objectives were pursued to achieve this goal:

1. Optimization of nuclei isolation protocol to isolate purified nuclear preparations from fully hydrated and dehydrated leaf samples at 35% RWC from *X. viscosa* plants.
2. Confirmation of nuclei integrity.
3. Assessment of possible chloroplast contamination of the purified nuclear preparations.
4. Optimization of protein extraction protocol to extract proteins from the purified nuclei.
5. Confirmation of the enrichment of nuclear protein fractions using Western blot analyses.
6. Development of high resolution 2-D nuclear proteome maps of *X. viscosa* for both control and dehydrated nuclear protein samples at 35% RWC for general image analysis and identification and analysis of up-regulated proteins.
7. Identification and analysis of the nuclear proteome of *X. viscosa* and its response to dehydration stress at 35% RWC using iTRAQ technology.

CHAPTER 2

Nuclei isolation and nuclear protein extraction

2.1. INTRODUCTION

Proteome research aims to unravel the biological complexity encoded by the genome. Single-step proteome characterization of eukaryotic cells is likely to be difficult to achieve due to the number of proteins expressed by a single cell and the complex physiochemical properties of proteins (Jung et al., 2000). Subcellular or organellar proteomics combines biochemical fractionation techniques with proteomic technologies to reduce artefacts and protein complexity while increasing the resolution and representation of endogenous proteins (Jung et al., 2000; Dreger, 2003).

Plant cellular functions such as the storage of genetic material, photosynthesis, respiration, protein degradation, intra- and extra- cellular signalling are often localized to subcellular compartments within the cell. The isolation of these plant organelles in contrast to tissues provides for the characterization of protein expression and possible function in relation to cellular location under specific developmental or environmental conditions. Furthermore, the proteomes of organelles comprise a focused set of proteins that fulfils discrete but varied cellular functions. The analysis of cell organelle proteomes provide additional important information about localization, pathway compartmentalization, and detailed monitoring of protein expression under specific conditions (Dreger 2003; Pandey et al., 2006).

Nuclear proteins have been shown to be implicated in different cellular functions including, signaling, gene regulation, defense, structure, translation, proteolysis and a variety of RNA-associated functions (Bae et al., 2003; Khan and Komatsu, 2004; Moriguchi et al., 2005; Repetto et al., 2008). Increasing evidence shows that nearly one-fourth of total cellular proteins are localized in the nucleus, implying a variety of protein functions in this organelle (Moriguchi et al., 2005; Pandey et al., 2006).

The separation of enriched nuclei from plant tissues is difficult due to the tight association between the nuclear membrane and the ER, and the similar density of nuclei and chloroplasts (Peterson et al., 2000; Khan and Komatsu 2004; Pandey et al., 2006; Rossingol et al., 2006). Although a number of plant organelle proteomes have been characterized (Peltier et al., 2000; Prime et al., 2000; Kruft et al., 2001; Millar et al., 2001; Bae et al., 2003) the various

isolation protocols need modification, optimization and refinement due to the biochemical and morphological diversity of plant tissues. The reliability of a plant organelle proteome is dependent on the purity of the organelle preparation. On the other hand protein extraction and sample preparation are crucial in any proteomics study. Due to the diversity of polypeptide molecular size, charge, hydrophobicity, post-translational modification, complexation and cellular distribution, no single protein extraction protocol or solvent system can capture an entire proteome. Consequently, a range of different extraction protocols, involving many permutations of physical treatments, solvents and buffers, have been reported in the literature (Carpentier et al., 2005; Vincent et al., 2006). Methodological improvements in sample handling, organelle fractionation and protein extraction are therefore crucial to plant subcellular proteomics (Pandey and Mann, 2000). Protein extraction from enriched subcellular organelle is an effective means to decrease protein complexity, enhance the detection of low abundance proteins and increase the overall detectable proportion of the proteome (Jung et al., 2000).

In this chapter, isolation of pure and enriched nuclear preparations and subsequent protein extraction from leaf samples of fully hydrated and dehydrated *X. viscosa* experimental plants are described.

2.2. MATERIALS AND METHODS

2.2.1. Plant material and growth conditions

X. viscosa (Baker) plants were germinated from seeds harvested from plants collected from the Cathedral Peak Nature Reserve located in the Drakensberg mountain range in KwaZulu-Natal, South Africa. The plants were grown in pots until maturation under green house conditions as described by Sherwin & Farrant (1996). The plants were watered once daily using a shower system to simulate rainfall. Prior to this study, 6 experimental plants from the same parental line were transferred from the green house to a controlled environment room with a light intensity of 350 με, 25^0 C, 50% relative humidity, and 16 h day light. The plants were kept in this room for approximately 2 months to acclimate before leaf samples were collected or the plants were dehydrated.

2.2.2. Estimation of water content and dehydration stress

After completion of the acclimation period, relative water content (RWC) was measured for all the plants before collecting leaf samples. Relative water content was determined for leaf

samples and was calculated using the method described by Henson et al., 1981; Jin et al., 2000 and Jiang et al., 2007.

RWC (%) = [(FW-DW)/ (TW-DW)] X 100

Where, FW stands for fresh weight, DW for dry weight, and TW for turgid weight. The fresh weight was determined prior to the incubation of leaf segments in distilled water. The weight of full turgid leaves was determined after incubation of the leaf segments in distilled water in sealed vials for 24 h at room temperature, and the dry weight was determined by drying the turgid leaf segments in an oven at 70^0 C for 48 h. Three independent determinations of the RWC were performed for each of the experimental plants, and the average RWC was calculated. Leaf samples were collected at noon time. Leaves were detached from the plants, dissected using sterile scissors into small segments, weighed out by 5 gm, immediately wrapped in aluminium foil, flash-frozen in liquid nitrogen, and stored at -80^0 C .

For the experiments the fully hydrated leaf samples from six plants (equal amount per plant) were pooled together to normalize growth and developmental variations (Ingle et al., 2005; Michele et al 2006, Pandey et al., 2006, 2008). The six plants described above were left for four weeks until completely restored, and then dehydrated by withholding water until the RWC had reached ca 35%. The RWC of the dehydrated leaves was measured as described above. The dehydrated leaf samples from the 6 plants (equal amount per plant) were pooled together and stored as described above.

2.2.3 Nuclei isolation

2.2.3.1. Isolation of nuclei using the method of Folta and Kaufman 2000

2.2.3.1.1. Tissue treatment and homogenization

The nuclei were isolated from 5 gm of frozen leaf samples of *X. viscosa* using the method of Folta and Kaufman (2000) with some modifications. The leaf samples were ground in liquid nitrogen to a fine powder using a pre-chilled mortar and pestle. The liquid nitrogen was left to evaporate completely, and the powder was transferred from the cold mortar to a new container to prevent freezing. The fine powder was resuspended in 2 vol of extraction buffer (1.0 M hexylene glycol (2-methyl-2, 4-pentandiol), 0.5 M PIPES-KOH (pH 7.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol).

All the subsequent steps were performed on ice or at 4^0 C. The homogenate was passed through a double layer of cheesecloth which had been soaked in extraction buffer. Triton X-100 (25%) was added dropwise to the resulting liquid fraction with constant stirring to a final concentration of 1% to lyse the organelle membranes. The resulting homogenate was then subjected to centrifugation either without or with the use of percoll density gradients.

2.2.3.1.2. Preparation of nuclei without percoll gradients

The lysate (40 ml in extraction buffer containing 1% Triton X-100) was centrifuged at 2000 g for 30 min. The pellet consisted of layers of plant material (starch, cellular debris/non-homogenized tissue) and the nuclei were situated at the top of the pellet as a powdery film of non-clumping material. The pellet was covered in up to 5 ml ice-cold gradient buffer (0.5 M hexylene glycol, 0.5 M PIPES-KOH (pH 7.0), 10 mM $MgCl_2$, 5 mM 2-mercaptoethanol, 1% Triton X-100). The buffer was carefully swirled in the tube until the nuclei were resuspended in the buffer. The nuclei in the 40 ml ice-cold gradient buffer were centrifuged for 5 min at 2000 g to remove residual non-nuclear materials. The nuclear layer was resuspended in 5 ml gradient buffer and centrifuged for 5 min, and the pellet was resuspended in 200 µl (or 3 volumes) of nuclear storage buffer (50 mM Tris-HCL (pH 7.8), 10 mM 2-mercaptoethanol, 20% glycerol, 5 mM $MgCl_2$, and 0.44 M sucrose). Nuclei were stored at -80^0 C.

2.2.3.1.3. Preparation of nuclei with percoll gradients

Percoll (Sigma Aldrich, USA) density gradients were prepared by placing a 6-ml layer of 80% (v/v) percoll diluted in 1 x gradient buffer into the bottom of 50 ml centrifuge tube and a 6-ml layer of 35% (v/v) diluted in 1 x gradient buffer was layered on top. Thirty millilitres lysate was layered onto the top of the column and centrifuged at 2000 g for 30 min at 4^0 C. Following centrifugation, the nuclei located as a sticky band at the interface of the 35% and 80% percoll layers were collected, diluted to 35 ml with 1x gradient buffer and layered on top of 6 ml 35% percoll in a new tube and centrifuged at 2000 g for 10 min. The percoll was discarded and the nuclei-enriched pellet was resuspended in 3 volumes of nuclear storage buffer and stored at -80^0 C.

2.2.3.2. Isolation of nuclei by the method of Abdalla et al., 2009.

2.2.3.2.1. Tissue treatment and homogenization

The fine powder (section 2.2.3.1.1.) was resuspended in 15-25 ml (3-5 ml/g tissue) 1x nuclei isolation buffer (NIB) (Sigma, USA), and filtered once through a 100-µm filter mesh, the filter mesh was gently squeezed to collect all the liquid, the resultant liquid fraction was then passed twice through a double 100- µm filter mesh. The filtrate was centrifuged at 1260 *g* for 10 min at 4^0 C. The supernatant (homogenized NIB supernatant, HBS) was collected for later western blot analysis (purity control), and the pellet (organelle fraction) was resuspended in 0.75 ml 1x NIBA buffer (0.75 ml NIBA/nuclei obtained from 5 g tissues) (NIB buffer containing protease inhibitor cocktails, Sigma). The organellar membranes were differentially lysed by the addition of 10% Triton X-100 to a final concentration of 0.3%, vortexed for 2 min, and incubated on ice for 15 min. The Triton X-100 removed the outer of the two nuclear membranes (Watson and Thompson, 1986). All subsequent steps were performed on ice or at 4^0 C. The resulting organelle lysate was then subjected to centrifugation either without a percoll/sucrose density gradient, with only sucrose density gradient, or with percoll/sucrose density gradient.

2.2.3.2.2. Preparation of nuclei without percoll/sucrose cushions

The organelle lysate (see above), was pipetted by 1.8 ml into 2 ml microcentrifuge tubes and centrifuged at 12,000 *g* for 10 min at 4 °C. The supernatant (membrane lysate supernatant, MLS) was collected for western blot analysis (as a second purity control). The nuclei pellet was washed once in 2 ml NIBA buffer with centrifugation at 12,000 *g* for 5 min at 4 °C. The nuclei pellet was covered by nuclei pure storage buffer (Sigma-Aldrich) and stored at -80^0 C.

2.2.3.2.3. Preparation of nuclei with only sucrose cushion

A sucrose cushion was prepared by pipeting 0.8 ml of 1.7 M sucrose (prepared in 1x NIBA) into 2 ml micro-centrifuge tube, 0.8 ml organelle lysate (section 2.2.3.2.) was layered on top of the sucrose cushion. The nuclei were pelleted by centrifugation at 12,000 g for 10 min at 4 °C, the upper phase (MLS) was collected for western blot analysis (a second purity control), the sucrose cushion was discarded. The nuclei pellet was washed twice in 2 ml NIBA buffer with centrifugation at 12,000*g* for 5 min at 4 °C. The nuclei pellet was covered with nuclei pure storage buffer (Sigma) and stored at -80^0.

2.2.3.2.4. Preparation of nuclei with percoll and sucrose cushions

A gradient isolation tube was prepared by placing 3 ml of 2.3 M Sucrose into the bottom of a 14 ml round-bottom centrifuge tube, and 3 ml layer of 50% percoll (v/v) (diluted in 1x NIB) was layered on top of the sucrose layer. The organelle lysate (section 2.2.3.2.) was pipetted onto the top of the percoll layer, and the organelle lysate/percoll/sucrose was centrifuged at 3,200 g for 30 min at 4 °C. Following the centrifugation, most of the nuclei were banded at the interface between the sucrose and the percoll layers. The upper supernatant phase (MLS) was collected for western blot analysis (as a second purity control). The nuclear suspension was collected into a new tube and double volume NIBA was added to remove the percoll and sucrose contaminations, and centrifuged at 3,200 g for 5 min at 4 °C. The nuclei pellet was washed twice in 2 ml NIBA with centrifugation at 12,000 g for 5 min at 4 °C. The nuclei pellet was covered with nuclei pure storage buffer (Sigma-Aldrich, USA) and stored at -80^0 C.

2.2.4. Confirmation of nuclei integrity and evaluation of chloroplast contamination

The integrity of the purified nuclei was confirmed with microscopic analysis without and with DAPI staining. Six microlitres of nuclei suspension at 120 µg nuclei (wet weight) per 150 µl nuclei storage buffer (Sigma) was mixed with 6 µl of 1 µg/ml of DAPI (4',6'-diamidino-2-phenylindole hydrochloride in 0.1 M potassium phosphate buffer, pH 7.4). Three smears were prepared from the nuclei/DAPI suspension and from the nuclei suspension without DAPI as well. The smears were left to dry at RT in the dark for 5 – 10 minutes. The unstained and DAPI stained nuclei were analysed under oil immersion lens without and with fluorescence microscopy using Nikon-inverted microscope supported with Xiocamera, Xiovision software version 4.4, Japan, and Olympus microscope using Hp photosmart digital camera, USA.

Possible chloroplast contamination in the nuclear fractions was examined by spectrophotometric analysis of chlorophyll in the fractions obtained during the nuclei purification (sections 2.2.3.2.2; and 2.2.3.2.3) according to the method of Pandey et al., (2006).

2.2.5. Protein extraction

2.2.5.1. Protocol 1 (Phenol extraction, methanol/ammonium/acetone precipitation)

This protocol was based on the work of Ingle and co-workers (2005) with some modifications. Protein was extracted from 200 mg (wet weight) nuclei pellet (section 2.2.3.2.4.),

and from 0.5 ml HBS and MLS fractions (see above). Each fraction was resuspended in 1.5 ml of ice-cold extraction buffer (0.5 M Tris-Cl pH 7.5, 10 mM EDTA, 1% triton X-100, and 0.2 M β-mercatoethanol), and the fractions were homogenized by vortexing for 10 min at RT, followed by incubation for 5 min to settle non-protein material. The samples were centrifuged at 14000 g for 5 min at 4^0 C. The supernatant containing the proteins were transferred into new microcentrifuge tubes. Equal volume of ice-cold Tris (0.5 M, pH 8.0)-saturated phenol was added to the supernatant to precipitate the protein. The samples were mixed by vortexing for 1 min at RT, and centrifuged for 1 min at 14000 g, two phase separation were appeared. Eighty percent of the top aqueous phase was gently removed & discarded, and the protein was re-extracted by the addition of equivalent volume of extraction buffer, mixed by vortexing for 3 min, and centrifuged at 14000g for 1 min. Again, eighty percent of the top aqueous phase was removed and 5 volumes of 0.1 M ammonium acetate in methanol was added to the remaining bottom layer. The sample was incubated at -20^0 C for 16-24 h to allow for complete protein precipitation. Then the proteins were pelleted by centrifugation at 14000 g for 5 min at 4^0 C, the supernatants were removed and discarded. The protein pellets were washed once with 1.5 ml of 0.1 M ammonium acetate in methanol with centifugation at 12000 g for 5 min at 4^0 C and the supernatants were discarded. The protein pellets were washed twice with 1.5 ml of ice-cold 80% acetone to remove any salt with centrifugation at 14000 g. The protein pellets were air-dried for 5 min on ice to get rid of acetone and then stored at -80^0 C.

2.2.5.2. Protocol 2 (TES extraction, acetone precipitation)

This protocol was based on the work of Bailly and et al., (2004) with some modifications. Nuclear protein was extracted from 0.5 gm (wet weight) nuclei pellet (section 2.2.3.2.4.), and from 0.5 ml HBS and 0.5 ml MLS fractions. Each protein fraction was resuspended in 0.75 ml TES buffer (30 mM Tris-Cl (pH 7.5), 5 mM EDTA, and 1% SDS) containing 20 mM NaCl, 1 mM PMSF and 20% (w/v) polyvinylpolypyrrolidone (PVPP). This was mixed by inversion for 2 min, homogenized by vortexing for 2 min at RT, and centrifuged at 14000 g for 15 min at 4^0 C to pellet non protein compounds. Eighty percent of the upper aqueous phase containing the proteins was pipetted into a new tube and the protein was quantified using Bradford Reagent (Biorad, Germany). The protein was completely precipitated by the addition of 4 volumes of 100% acetone containing 10 mM 2-mercapoethanol with

incubation at – 20^0 C for 16-24 h, and the sample mixture was cleared by centrifugaton at 14000 g for 30 min at 4^0 C, and stored at -80^0 C.

2.2.5.3. Protocol 3 (High salt extraction, acetone precipitation)

This protocol was adapted from Sigma the CelLytic ^{TM}PN plant nuclei isolation/extraction kit (Sigma, USA). Protein was extracted from 0.6 gm nuclei pellet (wet weight) (section 2.2.3.2.4.). The nuclei pellet was homogenized by vigorous agitation in 0.5 ml sigma extraction buffer containing 5 mM DTT and 1:100 protease inhibitor cocktails (Sigma, USA) for 30 min at 4^0 C, and centrifuged at 12000 x g for 10 min at 4^0 C to pellet non- protein compounds. The supernatant containing the proteins was transferred into new microcentrifuge, 2 ml 80% ice-cold acetone was mixed with the supernatant, and the sample was cleared with centrifugation at 14000 g for 5 min at 4^0 C. The protein pellet was washed twice in 2 ml of ice-cold 80% acetone with centrifugation at 14000 g for 5 min at 4^0 C. The protein pellet was stored at -80^0 C.

2.2.5.4. Protocol 4 (Phenol extraction, isopropanol methanol/ammonium acetate precipitation)

This protocol was based on the Trizol LS reagent Invitrogen procedures (Invitrogen Life Technologies, USA) with some modifications. Proteins were extracted from 100 mg nuclei pellet (wet weight) (section 2.2.3.2.4.) and from 0.5 ml HBS and MLS. Each protein fraction was homogenized in 1 ml Trizol by vortexing for 5 min at RT, followed by incubation for 5 min. Two hundred microlitres of chloroform was added to each sample, and the samples were vortexed for 10 seconds, and incubated for 3 min at RT. The samples were centrifuged at 12000 g for 15 min at 4^0 C. The top aqueous phase containing the RNA was removed and discarded. Three hundred microlitres of 100% ethanol was added to the organic phase containing the proteins to precipitate the DNA, samples were mixed by inversion for 10 times, incubated for 2 min at RT, and centrifuged at 2000 g for 5 min at 4^0 C. The supernatant containing the protein was transferred into a new Eppendorf tube using a pipette. The protein was precipitated by the addition of 1.5 ml of isopropanol, and incubation for 10 min at RT, followed by centrifugation at 12000 g for 10 min at 4^0 C. The top phenol-containing phase was removed and discarded, and the protein pellet was washed thrice with 2 ml of ice-cold 0.1 M ammonium acetate in methanol for further phenol removal with centrifugation at 12000 g for 5 min at 4^0 C. The

protein pellets were washed twice with 2 ml of ice-cold 100% acetone and centrifuged at 12000 g for 5 min at 4^0 C. The protein samples were air-dried in a fume hood for 10 minutes and stored at -80^0 C.

2.2.5.5. Protocol 5 (Urea Lysis Buffer extraction)

The nuclear protein was extracted from 100 mg nuclei pellet (wet weight) (section 2.2.3.2.4.) by resuspension in 500 µl of urea lysis buffer (7 M urea, 2 M thiourea and 2% (w/v) CHAPS), with the addition of a pinch of sand. The sample was homogenized by grinding for 5 min using a mini pestle. The homogenate was centrifuged at 12000 g for 5 min at 4° C. Ninety percent of the top-phase containing the nuclear proteins was pipetted into a new 2-ml centrifuge tube and stored at –80° C.

2.2.5.6. Protocol 6 (Phenol extraction isopropanol/ ethanol precipitation)

Proteins were extracted from 120 mg (wet weight) nuclei pellets (sections 2.2.3.2.2., and 2.2.3.2.3.), and from 0.5 ml HBS and MLS. Each fraction was resuspended in 1 ml of Trizol (phenol/guanidine isothiocyanate solution) (Invitrogen Life Technologies, USA). The nuclear pellet was homogenized by fragmentation and pipetting using 1 ml sterile pipette until a homogenized solution was obtained, and additionally homogenized by a vigorous vortex for 15 min at RT to fully lyse the nuclei and dissolve the proteins. The control fractions were homogenized by vigorous agitation. The samples were incubated for 5 min at RT to settle the debris, followed by the addition of 0.2 ml chloroform and vortexed for 15 seconds. The samples were incubated for 5 min to allow phase separation, then centrifuged at 12,000 *g* for 15 min at 4 °C. The addition of chloroform allows the RNA to enter the upper aqueous phase, the DNA to enter the interphase, and the protein to enter the lower phenol phase. The upper phase containing RNA was removed and discarded; the DNA in the lower phenol protein containing layer was precipitated by the addition of 0.3 ml absolute ethanol.

The phenol/ ethanol supernatant containing the nuclear proteins was mixed carefully by inversion for 15 s and incubated for 3 min at RT then centrifuged at 2000 *g* for 5 min at 4 °C to pellet remaining nucleic acids. The proteins in the phenol/ ethanol supernatant were precipitated by the addition of 1.5 ml 100% isopropanol and incubated for 15 min at RT. The proteins were collected by centrifugation at 15,000 *g* for 15 min at 4 °C. The supernatant was removed and discarded, and the protein pellets was disrupted with the aid of sterile pipette tips in 2 ml of

300 mM guanidine hydrochloride (Sigma) in 95% ethanol to remove residual phenol, the mixture was incubated at RT for 20 minutes and centrifuged at 20.000 x g for 5 minutes at 4^0 C. This step was repeated 6 times to thoroughly remove the phenol from the samples. Finally the protein pellets were washed twice with 2 ml of 100% cold acetone initially kept at -20^0 C to remove any residual salt and centrifuged at 12.000 x g for 5 minutes at 4^0 C. The protein pellets were stored at -80^0 C.

2.2.6. Proteins solubilisation and quantification

The protein pellets were resuspended in minimum volume of urea lysis buffer (7 M urea, 2 M thiourea and 2% (w/v) 3-((3-cholamido propyl)-dimethylammonio)-1-propane sulfonate (CHAPS), and solubilised by vortexing and incubation 4 times for 5 min each at RT.

The protein samples were quantified according to the method of Ndimba et al., 2003. Stock bovine serum albumin (BSA) (Bio Basic, USA) dissolved in the urea lysis buffer was used as standards.

2.2.7. Proteins separation and immunoblotting analysis

2.2.7.1. Proteins separation.

The nuclear proteins and proteins obtained from the HBS and MLS were electrophoresed separately on 12% SDS PAGE and/or on 15% SDS PAGE. Initially the protein samples were electrophoresed at 10 mA per gel for 10 minutes and then the current was increased to 25 mA per gel for 50 minutes at RT. Once the run was ended gels were removed from the plates and stained overnight on a shaker in 200 ml of CBB R-250 staining solution (40% (v/v) methanol, 10% (v/v) acetic acid and 0.01% (w/v) Coomassie Brilliant Blue R-250) and destained in 200 ml destain (40% (v/v) methanol, and 10% (v/v) acetic acid) until protein bands were clearly visible, then the gels were placed in 7% (v/v) acetic acid until gel purple-blue colour background was faded away completely. Finally the gels were incubated in ddH2O for 30 minutes to wash out the acetic acid.

2.2.7.2. Immunoblotting analysis

The enrichment of the nuclear fractions was evaluated only for the method of Abdalla *et al.*, 2009. Two specific nuclear antibodies, anti-H1 antibody, and anti-proliferating cell nuclear

antigen antibody (anti-H1 and pCNA antibodies) and anti-cytosolic/chloroplastic antibody, anti-*X. viscosa* preoxiredoxin type II antibody (anti-XvPrx2 antibodies) were used.

The nuclear proteins, HBS, and MLS proteins (10-50 μg) each were separated on 12% SDS gels. Following the SDS electrophoresis the gels were equilibrated for 10 min in transfer buffer (25 mM Tris-base; 150 mM glycine and 20% methanol added just before use), and filter papers, nitrocellulose membranes and packing pads were soaked in the transfer buffer.

The Hoefer miniVE vertical electrophoresis system (Amersham Biosciences, USA) was used for the blotting procedure. For each antibody the transfer stack was assembled according to the manufacturer's instructions. Electrophoretic transfer was conducted for 2 hrs at 350 mA on ice. Upon completion of the transfer, the membrane was removed from the blotting apparatus and stained in Ponceau S stain to verify transfer efficiency and equal loading of proteins as Ponceau S reversibly stains proteins red. Stained protein bands were scanned and immediately washed 2-3 times for 10 min each time with TBS buffer (10 mM Tris-Cl, pH 7.5 containing 150 mM NaCl) with shaking at RT to remove the stain. The membrane was incubated for 1 h in blocking buffer: 8% (w/v) fat-free milk powder in TBS buffer at RT with shaking.

Membranes were washed twice for 10 min each time in TBS-Tween/Triton buffer (TBSTT) (20 mM tris-Cl, pH 7.5 containing 500 mM NaCl, 0.05% (v/v) tween 20, and 0.2% (v/v) Triton x-100) at RT with shaking and thereafter washed for 10 min with TBS buffer.

The blocking buffer, Tris-buffered saline (10 mM Tris-Cl, pH 7.5, 150 mM sodium chloride containing 8% w/v fat-free milk powder), was used to dilute the primary and secondary antibodies. The primary anti-mouse histone 1 (H1) antibody (GeneTex, USA) was diluted 1:50 from a 0.25 mg/ml stock. The primary anti-rabbit proliferating cell nuclear antigen (PCNA) antibody (kindly provided by Dr. Jorge Vazquez, UNAM, Mexico) was diluted 1:200 from a 1 mg/ml stock. The primary anti-rabbit *X. viscosa* peroxiredoxin II (XvPrx2) antibody (kindly provided by Dr. Govender, UCT) was diluted 1:2000 from a 3 mg/ml stock.

The respective membranes were incubated with anti-PCNA and anti-XvPrx2 antibodies for 90 min at RT while with anti-H1 antibody it was incubated overnight at 4^{o}C with shaking. This was followed by 2 x 10 min washes in TBSTT and 1 x 10 min in TBS buffer at RT and thereafter the membranes were incubated in secondary antibodies for 90 min. The anti-rabbit IgG peroxidase conjugate (Pierce, USA) at a 1:5000 dilution was used as the secondary antibody for the anti-PCNA and anti-XvPrx2 western blots. The anti-mouse IgG peroxidase conjugate (KPL, USA) at 1:5000 dilution was used as the secondary antibody for the anti-H1

western blot and thereafter the respective blots were washed 4 times for 10 min each in TBSTT buffer at RT with shaking. Detection was carried out using the SuperSignal West Pico Chemiluminescent Substarte detection System (Pierce, USA) according to the manufacturer's instructions. Fluorescence was capture on CL-XPosure Film (Pierce, USA). Protein bands were sized by comparison to a prestained protein ladder. Western blot analyses were performed in triplicate for each antibody on nuclei and nuclear proteins obtained from independent nuclei isolation and nuclear protein extraction procedures.

2.3. RESULTS

2.3.1. Plant material and growth conditions

Potted *X. viscosa* plants growing under green house conditions and in the controlled environment room were observed to flourish with green healthy leaves and shoots, and to survive severe dehydration stress as in their natural environment (Fig. 2.1.), which indicate that growth conditions in the greenhouse and the controlled environment room are well suited to their normal growth and development. Prior to this study, six healthy mature plants from same parental line were transferred from the green house to a controlled environmental room. The plants were kept in the room until acclimation and then fully hydrated leaf samples were collected for experiments. The plants were left until the excised leaves were completely restored. Then the plans were dehydrated until ca. 35% RWC was reached, and dehydrated leaf samples were collected for experiments.

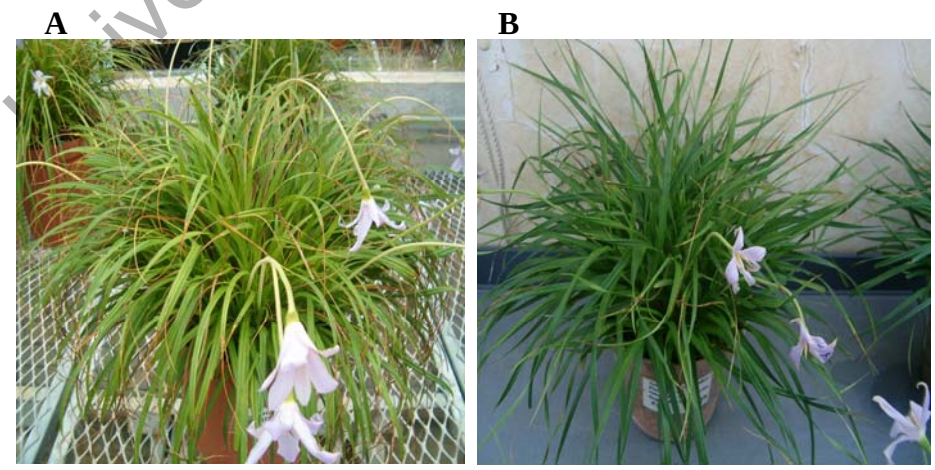


Figure 2.1. Potted *X. viscosa* plants in the greenhouse (A) and in the controlled environment room (B) respectively. Both plants are in bloom with green healthy leaves and shoots.

2.3.2. Estimation of water content and dehydration stress

The RWC measured for the leaf samples of the fully hydrated plants (control samples) remained fairly constant in the average range of 85 to 92% (Fig. 2.2.). Upon dehydration of the plants, plants with RWC between 80% and 90% reached the 35% RWC after 12 days of drying, while plants with RWC over 90% reached this point after 15 days of drying (Fig. 2.2.).

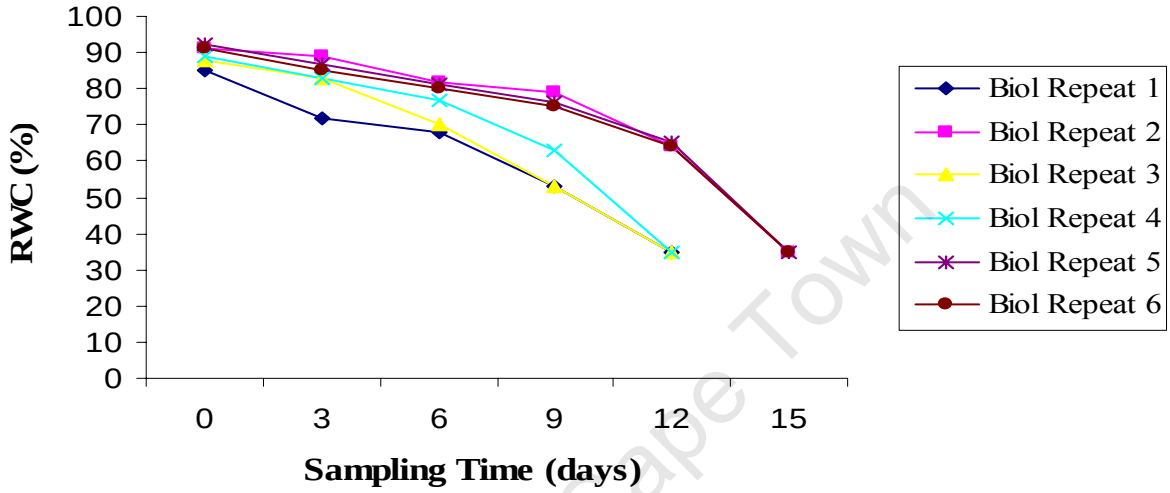


Figure 2.2. *X. viscosa* relative water content data for dehydration treatments. The dehydration treatments were performed in triplicate on six plants (Biol. Repeat 1-6). The error bars (± standard error) are wholly contained within the symbol.

2.3.3. Nuclei isolation and the assessment of nuclei integrity

To isolate the X. *viscosa* nuclei for a comprehensive comparative study of changes in nuclear protein expression in response to dehydration stress at 35% RWC, the method of Folta and Kaufman (2000) was modified and tested. Attempts to use this method resulted in a low yield of nuclei contaminated with debris. The nuclei yield was not optimal for proteomic studies as proteomic analysis requires appreciable amounts of nuclei and consequently nuclear proteins; hence this protocol was not pursued further. The low yield of nuclei obtained from this method could be attributed to the inefficiency of the nuclei extraction buffers and loss of nuclei in the discarded cell debris.

This led us to modify and optimize methods based on Sigma CelLytic PN extraction kit, (Sigma-Aldrich, USA). This kit allows preparation of nuclei with or without the use of percoll and sucrose gradients. The nuclei isolation buffer (NIB) contained in the CelLytic PN kit is

based on a nuclei isolation buffer developed by Conley and et al., (1994) but it does not contain β-mercaptoethanol, phenylmethylsulfonyl fluoride, and antipain (personal communication, Sigma–Aldrich USA, Technical Service).

Through the optimization of the Sigma CelLytic PN extraction kit protocols, pure and enriched nuclei fractions were successfully isolated from the leaf samples of fully hydrated and dehydrated X. *viscosa* experimental plants. Applying of percoll/sucrose gradient for the isolation of *X. viscosa* nuclei resulted in low yield (1 mg nuclei (wet weight) per gm leaves) of non-aggregated intact nuclei (5 μm in size) (Fig. 2.3.). This yield was not sufficient to conduct proteomic studies and hence this approach was not pursued further.

Nuclei of reproducible quality and sufficient quantity were obtained from both fully hydrated and dehydrated leaf samples when using the preparation of nuclei without percoll and sucrose gradients and preparation of nuclei with only the sucrose gradient. The yield of nuclei from these protocols were 12 mg (wet weight), and 9 mg nuclei (wet weight) respectively per gm of fully hydrated leaf samples. The nuclei yield from the dehydrated leaf samples was 49 mg nuclei (wet weight) per gm of leaf samples without using the percoll and sucrose gradient. The integrity and purity of the nuclei was confirmed using microscopic analysis with and without DAPI staining (Fig. 2.3.-2.5.). The purified X. *viscosa* nuclei were intact uniform spheres and approximately 5 μm in size. These results indicate that the isolated nuclei were highly purified.

The difference in yield among the three optimized protocols is mostly likely due to the use of percoll/sucrose gradients or sucrose gradient and loss of nuclei during the wash and recovery steps. It was observed under the microscope that the nuclei density per field obtained without the use of percoll/sucrose was the highest, followed by the nuclei obtained with use of sucrose gradient and lastly the nuclei obtained with the use of percoll/sucrose gradient. The high yield of nuclei from the dried leaves compared to the fully hydrated was expected, and could be attributed to the high biomass of the dried samples compared to the fully hydrated.

A chlorophyll assay was carried out in triplicate to evaluate possible contamination of the nuclear fractions (Figure 2.6.). The homogenized NIB supernatant, which contains cytoplasmic and chloroplast proteins had the highest chlorophyll content, the chlorophyll content in the crude and semi-pure nuclei preparations was less than 0.9% and 0.38% respectively when compared to the homogenized NIB supernatant.

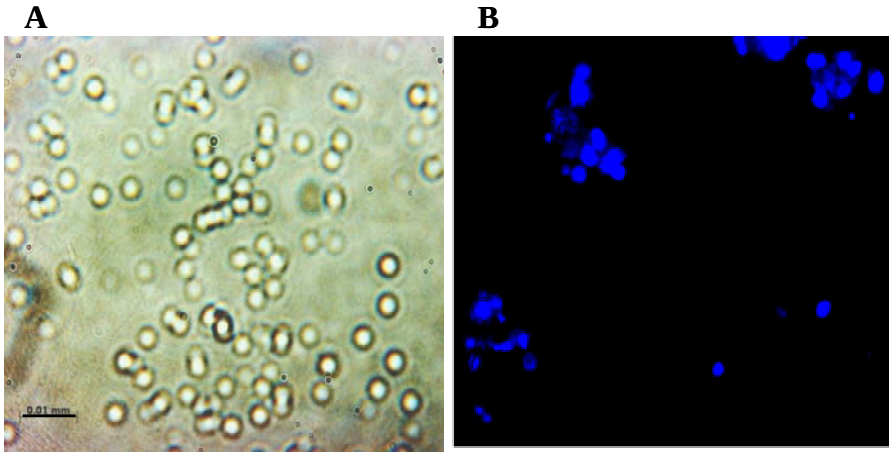


Figure 2.3. Representative micrographs of *X. viscosa* nuclei isolated with the use of percoll/sucrose gradient. (A) Represents unstained nuclei and (B) represents DAPI-stained nuclei. The bar is 10 µm.

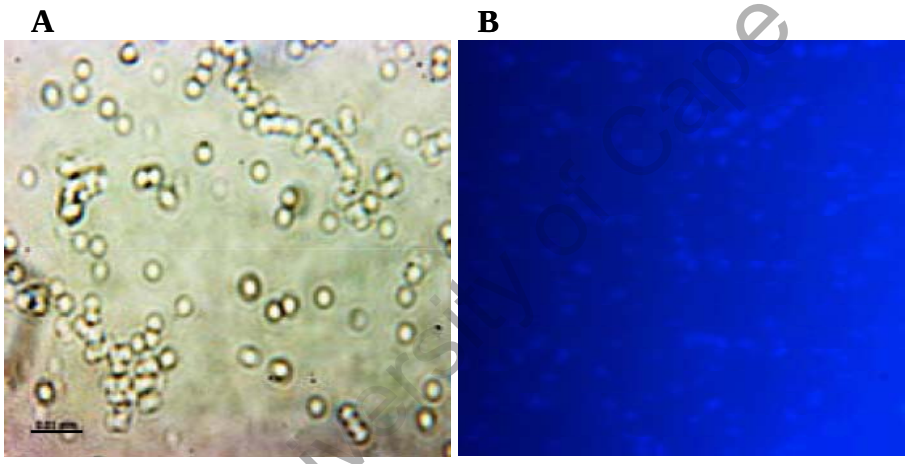


Figure 2.4. Representative micrographs of *X. viscosa* nuclei isolated with the use of sucrose gradient only. (A) represents unstained nuclei and (B) represents DAPI-stained nuclei. The bar is 10 µm.

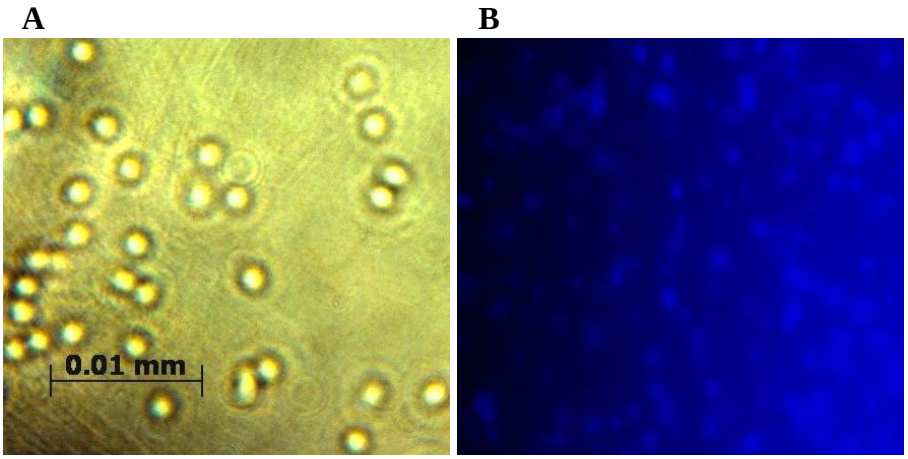


Figure 2.5. Representative micrographs of *X. viscosa* nuclei isolated without the use of gradient. (A) Represents unstained nuclei and (B) represents DAPI-stained nuclei. The bar is 10 µm.

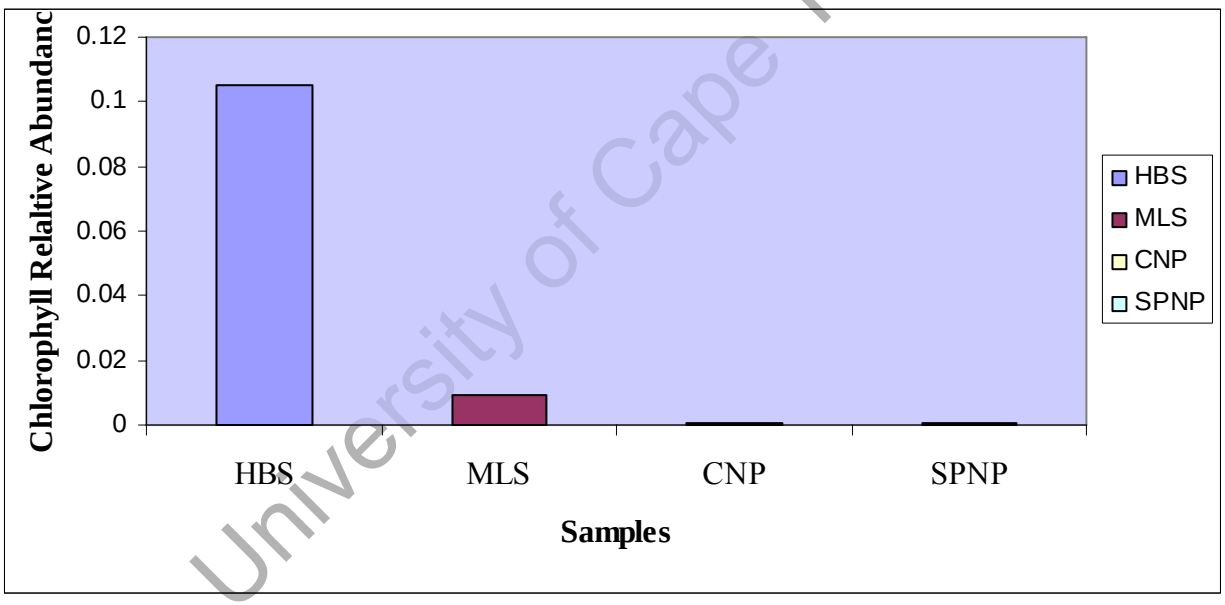


Figure 2.6. Determination of chlorophyll content at different stages during the purification of *X. viscosa* nuclei. (HSB) homogenized NIB supernatant, (MLS) membrane lysate supernatant, (CNP) crude nuclear preparation and (SPNP) semi-pure nuclear preparation.

2.3.4. Nuclear protein extraction and the assessment of the purity of the nuclear fractions

Six different methods of protein extraction were employed in this study to extract *X. viscosa* nuclear proteins, and proteins from the control fractions (HBS and MLS) with only protocol 6 providing best results in terms of reproducibility, yield and quality (an average of 2.5-3.5 mg nuclear protein per gm nuclei). The profiles of the protein fractions obtained from the other protein extraction protocols (Fig. 2.7-2.10) were different to protocol 6 which gave distinct bands in the respective one-dimensional PAGE (Fig. 2.11.). The other five protocols gave non-reproducible low yield of proteins (average 0.07-0.40 mg nuclear proteins per gm nuclei) and smeared lanes in 1D PAGE (Fig. 2.7.-2.10.). We observed that protein lanes with non distinct bands in 1DE correlated with protein impurity; and such proteins gave high false protein concentration not corresponding to their real amounts in one-dimensional PAGE. Using protocol 5 (Urea Lysis Buffer extraction) resulted in least yield of proteins as it seems the urea lysis buffer could not dissolve the nuclear protein efficiently and one-dimensional PAGE could not be done on the sample using this methodology.

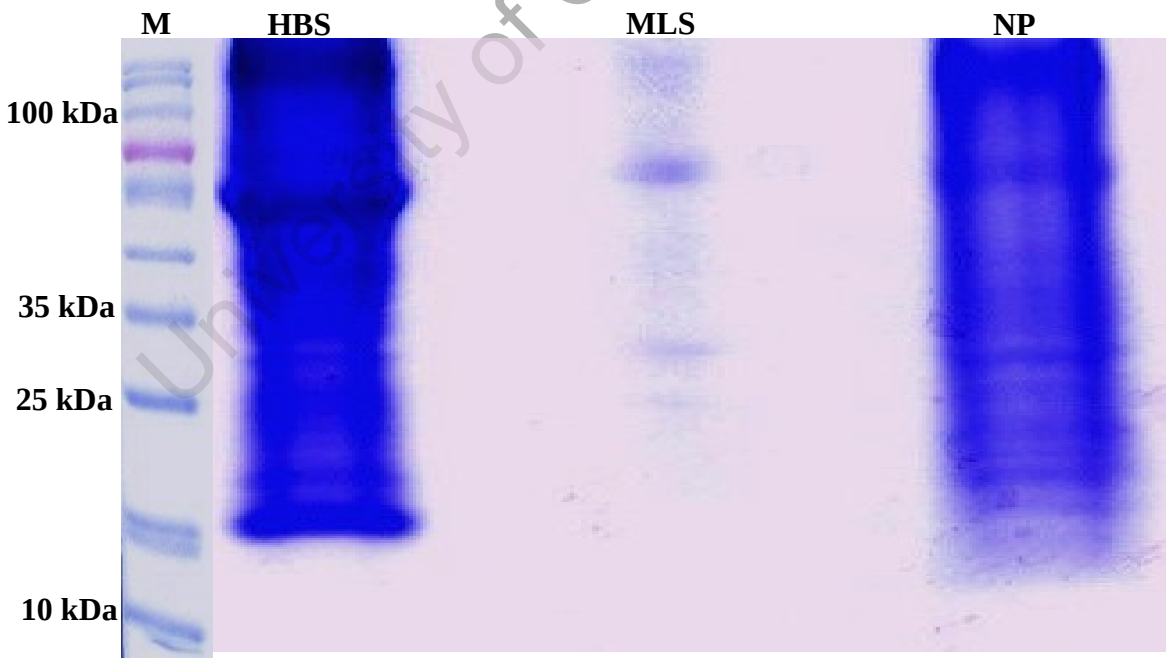


Figure 2.7. 12% SDS-PAGE of *X. viscosa* proteins from nuclei and fractions obtained during the nuclei isolation. Proteins were extracted using protocol 1. Lanes: (M) Biorad marker; (HBS) homogenized NIB supernatant membrane proteins; (MLS) lysate supernatant proteins and (NP) nuclear proteins.

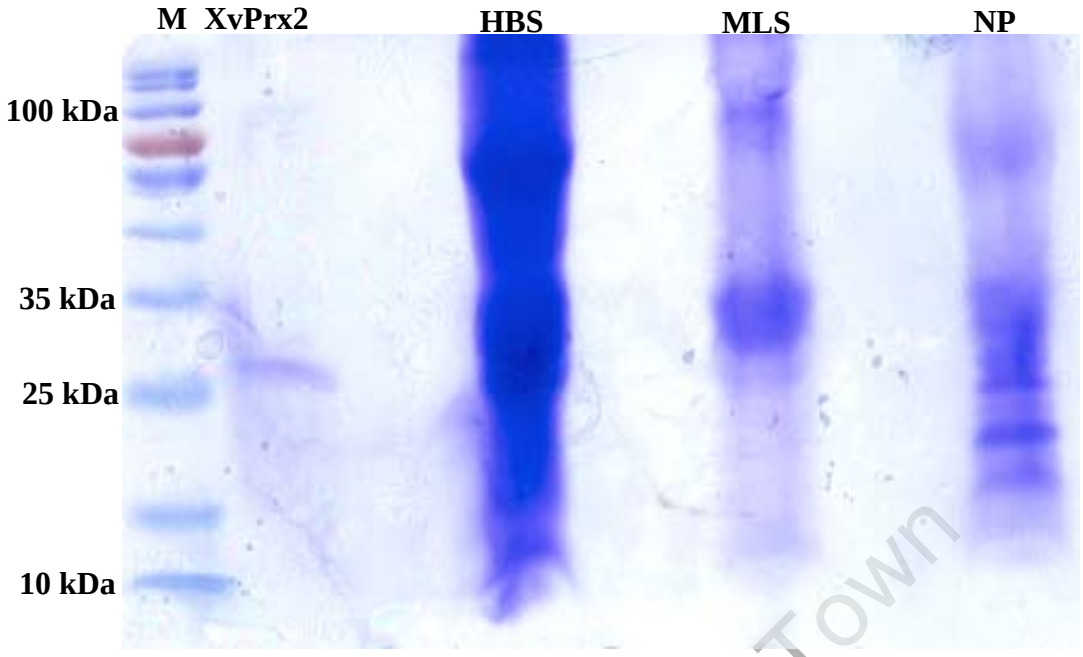


Figure 2.8. 12% SDS PAGE of *X. viscosa* proteins from nuclei and fractions obtained during the nuclei isolation. Proteins were extracted using protocol 2, except XvPrx2. Lanes: (M) Biorad marker; (XvPrx2) recombinant *X. visccosa* peroxiredoxin type II with His-tag (used as control, MW 22- kDa); (HBS) homogenate NIB supernatant proteins; (MLS) membrane lysate supernatant proteins and (NP) nuclear proteins.

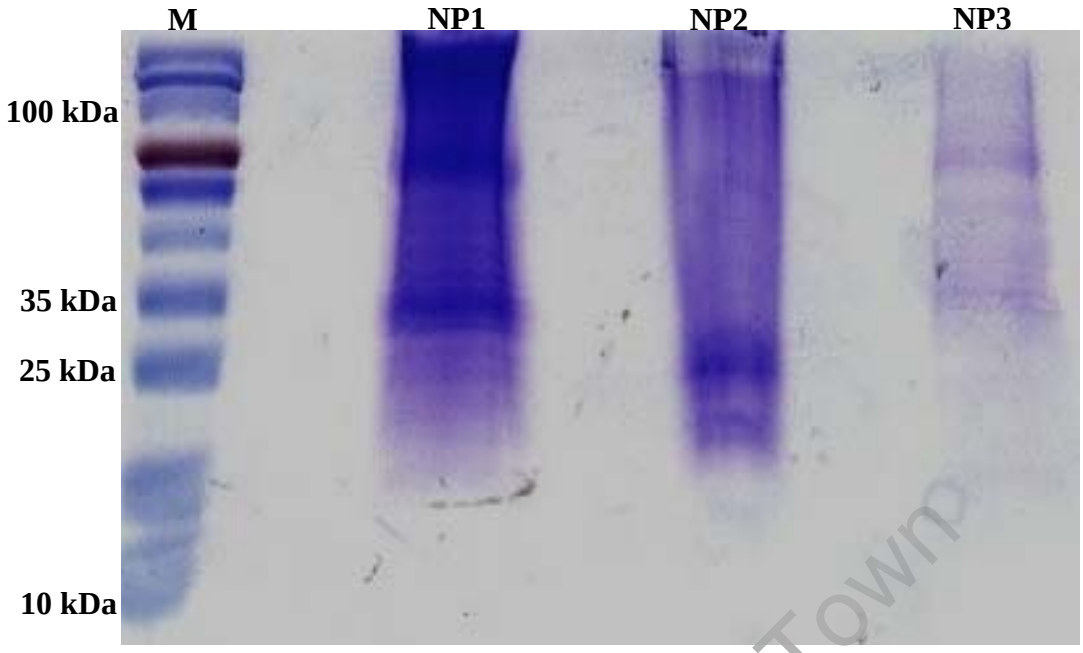


Figure 2.9. 12% SDS-PAGE of three *X. viscosa* nuclear protein profiles. Lanes: (M) Biorad marker; (NP1), (NP2) and (NP3) nuclear proteins extracted using protocol1-3 respectively.

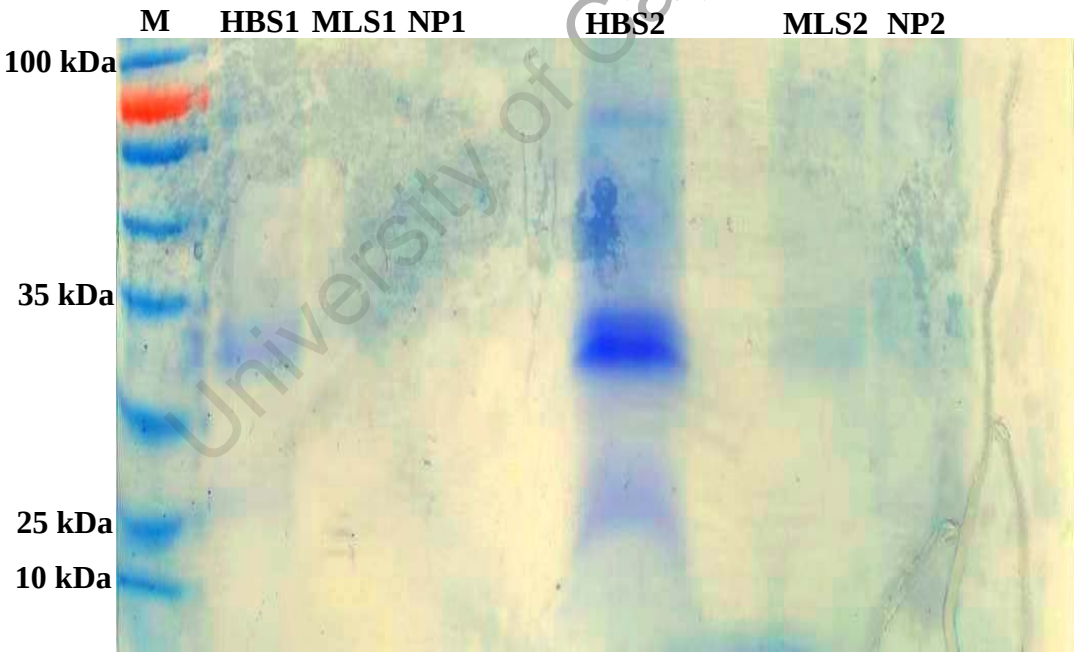


Figure 2.10. 12% SDS-PAGE of *X. viscosa* proteins from nuclei and fractions obtained during the nuclei isolation. Proteins were extracted using protocol 4. Lanes: (M) Biorad marker; (HBS1) and (HBS2) homogenate NIB supernatant proteins (20 and 40 µg respectively); (MLS1) and (MLS2) membrane lysate supernatant proteins (20 and 40 µg respectively); (NP1) and (NP2) nuclear proteins (20 and 40 µg respectively).

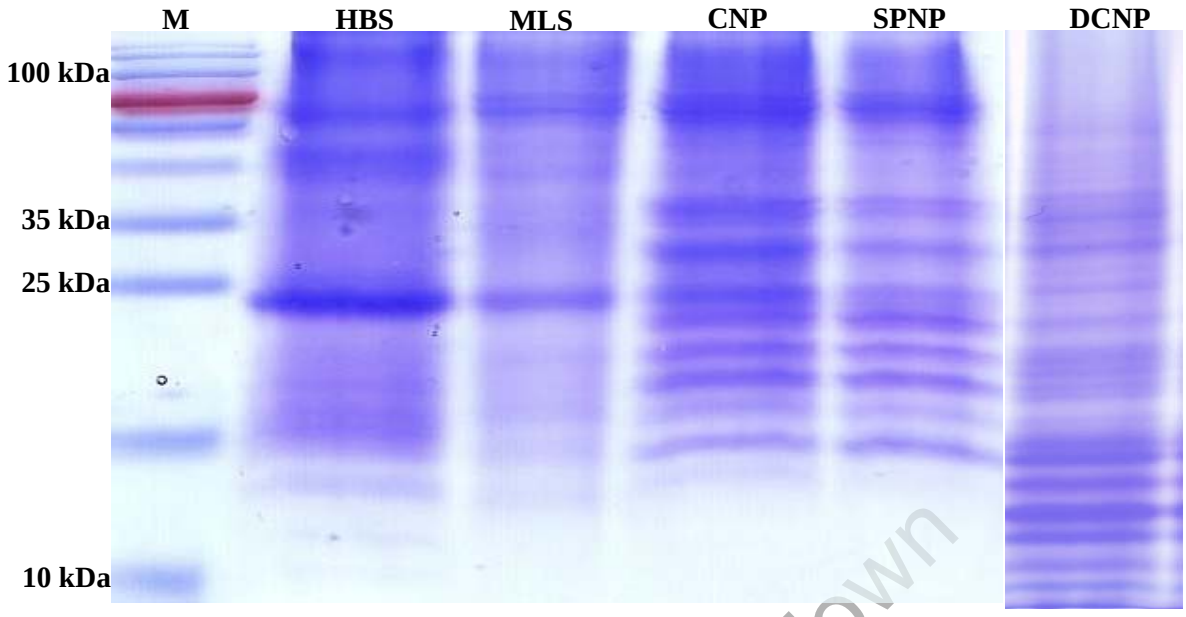


Figure 2.11. 15% SDS-PAGE of *X. viscosa* proteins from nuclei and fractions obtained during the nuclei isolation. Proteins were extracted using protocol 6. Lanes: (M) Biorad marker; (HBS) homogenate NIB supernatant proteins; (MLS) membrane lysate supernatant proteins; (CNP) fully hydrated crude nuclear proteins (extracted form nuclei obtained without the use of percoll and sucrose gradient) and (SPNP) fully hydrated semipure nuclear proteins (extracted from nuclei with the use of sucrose only) and (DCNP) dehydrated crude nuclear proteins.

During the initial selection and optimization for appropriate protocols for nuclei isolation and nuclei protein extraction from *X. viscosa* leaves, protocol 2 of Invitrogen and protocol 4 of Bailly et al., 2004 were validated by western blot analysis using the anti-XvPrx2 antibodies isolated from *X. viscosa* as it was shown to localize in both the cytoplasm and chloroplast of *X. viscosa* (Govender, 2006). As these two methods in particularly gave better protein yields ~ 0.20-0.40 mg per gram wet weight nuclei compared to protocols 1,3 and 5 mentioned previously.

Recombinant XvPrx2 with His-Tag (22 kDa) was loaded as a control alongside with the other protein fractions obtained by the method of Bailly et al., 2004 (protocol 2) to validate the nuclei purity. The cytoplasmic-chloroplastic protein XvPrx2 was clearly detected in the HBS protein fractions of protocol 4 (Fig. 2.12.) and was not detected in either the MLS or the nuclear protein fractions, which indicated that the nuclear fraction is free of cytoplasmic and chloroplastic contaminants. Using the method of Bailly et al. 2004 (protocol 2), the XvPrx2 was

detected in the HSB fraction but not in the MLS or in the nuclear fractions (Fig 2.13.). These results also confirm the purity of the nuclear fraction.

As protocol 6 of the protein extraction gave the best yield and resolution to develop *X. viscosa* nuclear maps (Chapter 3, and 4), it was selected for validation with western blot analysis to assess the enrichment of the nuclear fractions obtained without the use of percoll/sucrose gradients (crude) and with the use of sucrose gradient only (semi-pure).

The enrichment of the nuclear fractions was evaluated by western blot analyses using specific antibodies against two nuclear proteins, the histone H1 and proliferating cell nuclear antigen (pCNA). Histone H1 plays a role in the higher structure of chromatin, regulation of gene expression and determinant of mitotic chromosome structure (Razafimahatratra et al., 1991; Hansen, 2002), while the PCNA is required for processive DNA synthesis (Sanchez et al., 2005). To evaluate possible contamination of the purified nuclei with cytoplasmic and chloroplastic proteins, a specific antibody against cytoplasmic and chloroplastic proteins was used namely, XvPrx2 (MW 17.5 kDa). This is a type II peroxiredoxin isolated from *X. viscosa* and was shown to localize in both the cytoplasm and chloroplast of *X. viscosa* and function as an antioxidant *(*Govender, 2006). For all protein fractions loadings were observed to be at similar levels indicating that transfers onto the membranes were optimal (Fig. 2.14.-2.19.). Histone H1 and PCNA were detected in both the nuclear fractions, while the XvPrxII protein was not detected in the nuclear fractions (Fig. 2.14.-2.19.). Furthermore, the H1 and PCNA were not detected in the cytoplasmic and chloroplastic fractions while the XvPrxII was detected (Fig. 2.14-2.19.). Together these results confirmed the enrichment of the *X. viscosa* purified nuclear fractions.

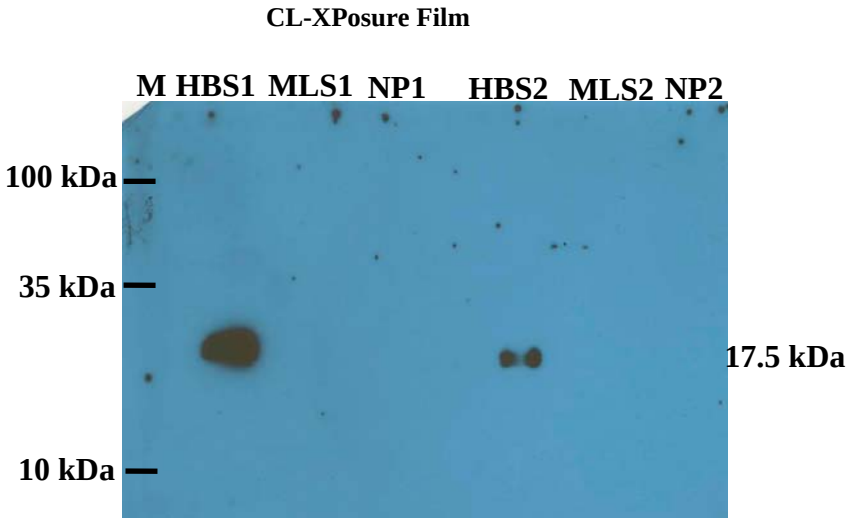


Figure 2.12. Western blot analysis of *X. viscosa* protein fractions probed with XvPrx2 antibodies (17.5-kDa). Proteins were extracted using protocol 4. Lanes: (M) Biorad marker; (HBS1) and (HBS2) homogenate NIB supernatant proteins (20 and 40 µg respectively); (MLS1) and (MLS2) membrane lysate supernatant proteins (20 and 40 µg respectively); (NP1) and (NP2) nuclear proteins (20 and 40 µg respectively).

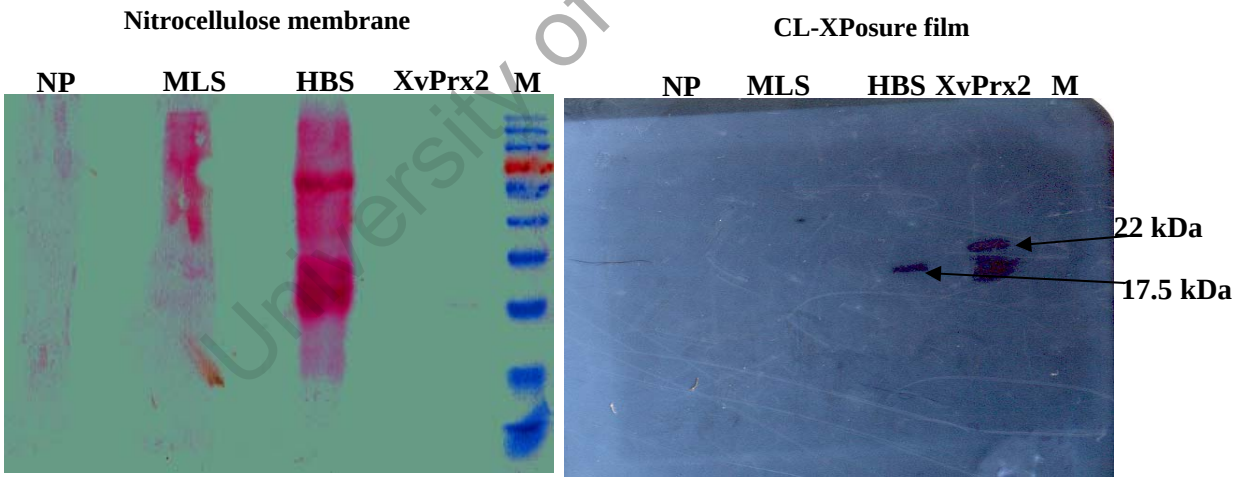


Figure 2.13. Western blot analysis of *X. viscosa* protein fractions probed with XvPrx2 antibodies (17.5-kDa). Proteins were extracted using protocol 2. Lanes: (M) Biorad marker; (XvPrx2) recombinant XvPrx2 (MW 22-kDa); (HBS) homogenate NIB supernatant proteins; (MLS) membrane lysate supernatant proteins and (NP) nuclear proteins.

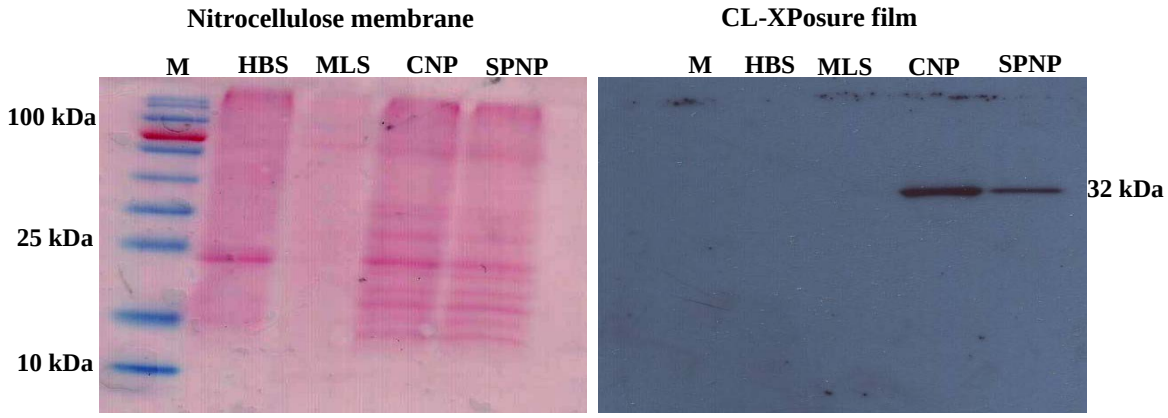


Figure 2.14. Western blot analysis of *X. viscosa* protein fractions probed with histone H1 antibodies (32-kDa). Proteins were extracted using protocol 6. Lanes: (M) Biorad marker; (HBS) homogenate NIB supernatant proteins; (MLS) membrane lysate supernatant proteins; (CNP) crude nuclear protein fraction and (SPNP) semipure nuclear protein fraction.

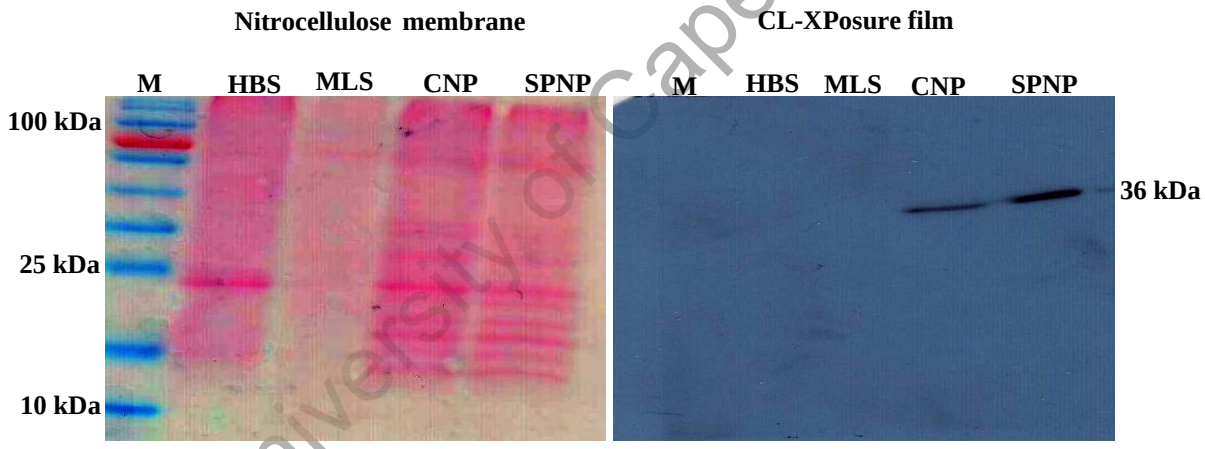


Figure 2.15. Western blot analysis of *X. viscosa* protein fractions probed with PCNA antibodies (36-kDa). Proteins were extracted using protocol 6. Lanes: (M) Biorad marker; (HBS) homogenate NIB supernatant proteins; (MLS) membrane lysate supernatant proteins; (CNP) crude nuclear proteins and (SPNP) semipure nuclear proteins.

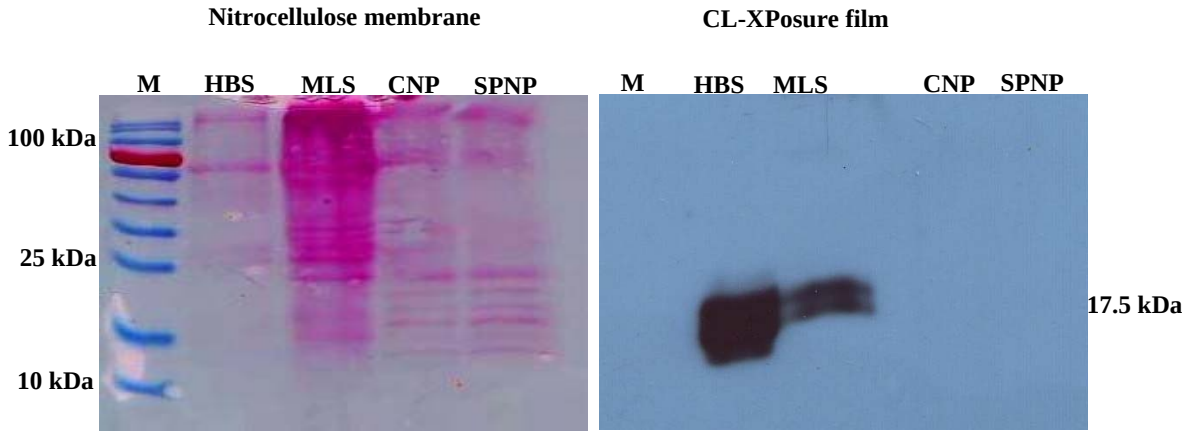


Figure 2.16. Western blot analysis of *X. viscosa* protein fractions probed with XvPrx2 antibodies (17.5-kDa). Proteins were extracted using protocol 6. Lanes: (M) Biorad marker; (HBS) homogenate NIB supernatant proteins; (MLS) membrane lysate supernatant proteins; (CNP) crude nuclear proteins and (SPNP) semipure nuclear proteins.

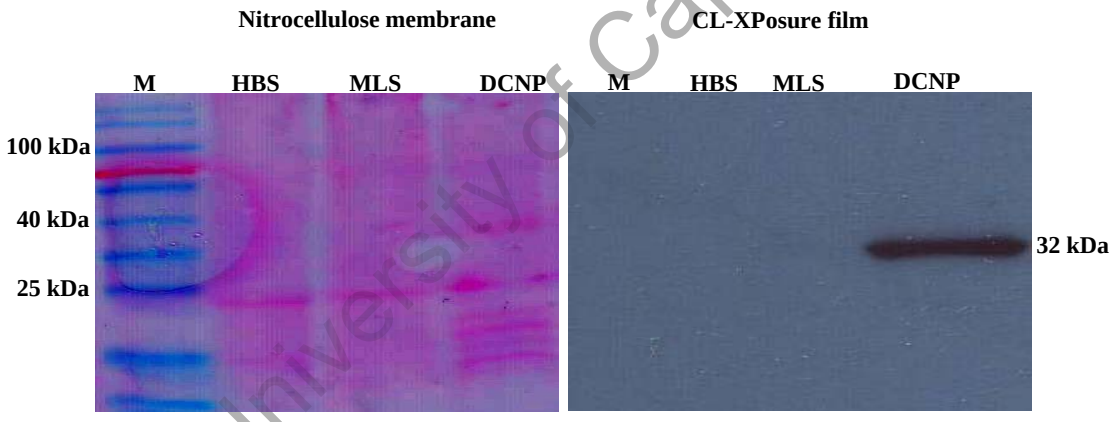


Figure 2.17. Western blot analysis of *X. viscosa* dehydrated protein fractions probed with H1 antibodies (32-kDa). Proteins were extracted using protocol 6. Lanes: (M) Biorad marker; (HBS) homogenate NIB supernatant proteins; (MLS) membrane lysate supernatant proteins and (DCNP) dehydrated crude nuclear protein fraction.

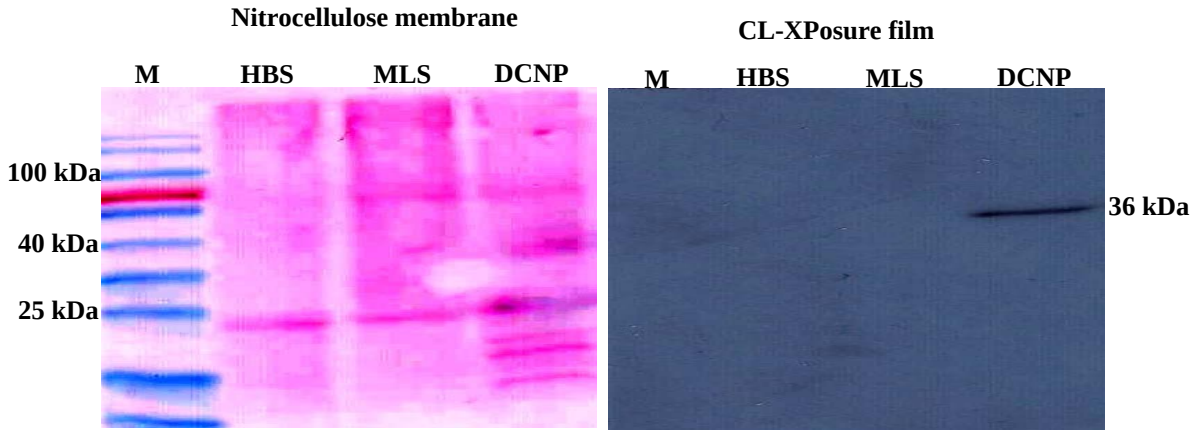


Figure 2.18. Western blot analysis of *X. viscosa* dehydrated protein fractions probed with PCNA antibodies (36-kDa). Proteins were extracted using protocol 6. Lanes: (M) Biorad marker; (HBS) homogenate NIB supernatant proteins; (MLS) membrane lysate supernatant proteins and (DCNP) dehydrated crude nuclear protein fraction.

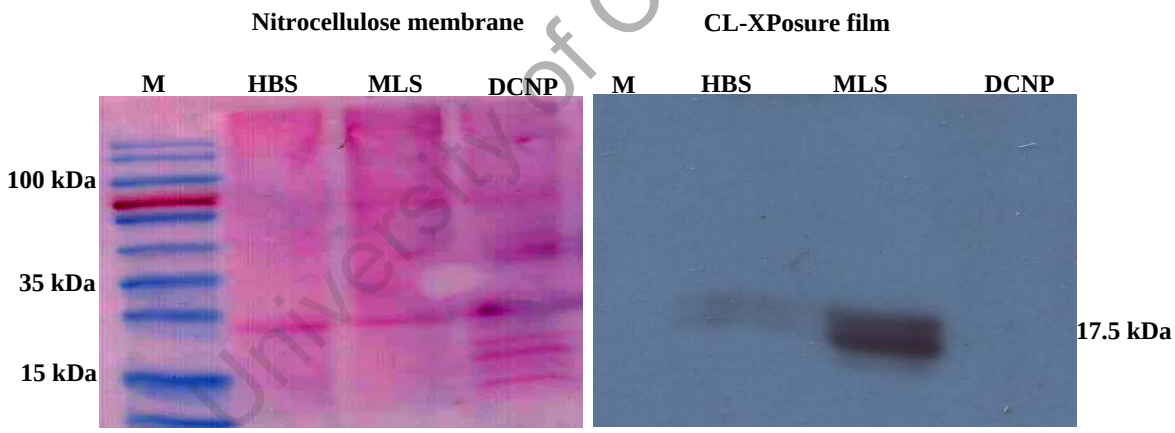


Figure 2.19. Western blot analysis of *X. viscosa* dehydrated protein fractions probed with XvPrx2 antibodies (17.5-kDa). Proteins were extracted using protocol 6. Lanes: (M) Biorad marker; (HBS) homogenate NIB supernatant proteins; (MLS) membrane lysate supernatant proteins and (DCNP) dehydrated crude nuclear protein fraction.

2.4. DISCUSSION

Performing optimal nuclei isolation from plant tissues for proteomic studies is a difficult task as other cellular contaminants might compromise the data obtained. Indeed, the integrity of the nuclei is dependent on the purification procedures. Furthermore, extracting high quality proteins from nuclei and of sufficient quantity is complex due to the large content of DNA and RNA in the nucleus and interference of non-protein compounds with the nuclear protein.

Watson and Thompson (1986) developed a nuclei isolation protocol for pea based on the composition of the homogenization buffer and the use of percoll gradients. In this protocol, plant tissue is homogenized in extraction buffer designed to stabilize the membranes and the homogenate is filtered to eliminate whole cells and large debris. Differential lysis is achieved by the addition of Triton X-100 to the filtrate in the presence of divalent cations where the detergent removes only the outer of the two nuclear membranes. Nuclei are then purified from the cellular debris by percoll density gradient centrifugation. The above protocol was later optimized by Folta and Kaufman (2000) to obtain transcriptionally active nuclei from etiolated seedlings of *Arabidopsis* for nuclear run-on assays as the former protocol suffered from the drawback of requiring large quantities of starting material. The latter protocol follows the original protocol with regard to cell permeation, cell lysis, nuclei stabilization and organelle lysis but the main modification is the ability to isolate nuclei by centrifugation with or without percoll density gradients. The ability to eliminate the percoll gradient step allows a considerable saving in time though there is a 10-fold drop in the amount of nuclei obtained when compared to implementation of the gradient in the protocol. The modified protocol has been used successfully to investigate the nuclear proteome of *Arabidopsis* and rice respectively (Bae et al, 2003; Khan and Komatsu 2004). Pandey et al., (2006) used the modified method of Zhang et al., (1995) with a large quantity of starting material to isolate nuclei from chickpea for nuclear proteomic studies. This protocol contains no percoll density gradient step and differs from the Folta and Kaufman (2000) protocol with respect to tissue treatment and homogenization buffers.

Attempts to use the Folta and Kaufman protocol (2000) to isolate nuclei from fully hydrated and dehydrated leaf samples *X. viscosa* for nuclear proteomic studies resulted in a low yield of nuclei and the preparation was contaminated with cellular debris (data not shown), and this protocol was not pursued further. This prompted the development of a nuclei isolation protocol based on the CelLytic PN Extraction kit from Sigma (Sigma-Aldrich, USA). The

CelLytic PN protocol allows the isolation of nuclei with percoll/percoll gradient, with only a sucrose gradient, and without any gradients. This kit was used to isolate nuclei from the model plant *Arabidopsis* for gel shift assays (Del viso et al., 2007; Gonzalez et al., 2007), rape seeds for ChIP experiments (De Block et bal., 2005), and maize for immunological detection of nuclear proteins (Hernandez et al., 2007). Recently this kit was used to isolate nuclei from seeds of *Medicago truncatula* using sucrose gradients for proteomic study under normal conditions and the nuclear proteins were investigated using 1DE and MS analysis (Repetto et al., 2008).

The nuclear isolation buffer (NIB) contained in the CelLytic PN kit is based on that of Conley and et al., (1994), but has been modified in that it does not contain 2-mercaptoethanol, phenylmethylsulfonyl fluoride and antipain (personal communication, Sigma-Aldrich Technical Service). The most important considerations in *X. viscosa* nuclei isolation was the amount of starting leaf sample, the pore size of the filter mesh, the final concentration of Triton-X 100, the amount of NIBA solution per gram tissue, and the percentage of sucrose and percoll gradients. We observed that the amount of plant tissues should not exceed 5 grams per isolation as more starting material could cause the filter mesh to be blocked by debris and large insoluble material resulting in low yield of nuclei and nuclear proteins. The modification of passing the homogenate through a single and then a double 100 µm filter mesh increased nuclei yield and purity as more soluble cellular materials were passed through, and more insoluble materials were captured. Empirically, we found that 0.3% (v/v) Triton X-100 was the optimal final concentration for optimal differential organellar lysis of *X. viscosa*, and 0.75 ml NIBA was the optimal amount required to resuspend the organelle lysate obtained from 5 gm of leaf tissues. Furthermore, we determined that 1.7 M sucrose was the ideal concentration for the sucrose cushion when using sucrose gradient to isolate the nuclei, and 50% percoll (v/v) was the optimal concentration when using percoll/sucrose to isolate the nuclei form *X. viscosa* leaf tissues. The yield of nuclei using the percoll/sucrose gradient was very low, and it was difficult to get rid of percoll contamination, hence this protocol was not pursued further. The yield of nuclei without the use of percoll and sucrose gradients, and with the use of only sucrose was good and reproducible with an average of 12 mg, and 9 mg (wet weight) nuclei per gm of fully hydrated leaf tissues respectively. While the yield of the nuclei from the dehydrated leaves was 49 mg nuclei (wet wet) per gm leaves, this high yield of nuclei from dehydrated samples was attributed to the high biomass of dry samples compared to fully hydrated leaves.

The integrity of the nuclei isolated without the use of percoll and sucrose gradients (crude), with the use of only sucrose gradients (semi pure) and with the use of percoll/sucrose gradients (high pure) was confirmed by microscopic analysis with and without DAPI staining. These protocols gave intact *X. viscosa* nuclei, which were spherical and approximately 5 µm diameter (Fig. 2.3.-2.5). Pea nuclei were also reported to be uniform spheres with an average diameter of about 20-30 µm, but the *Arabidopsis* nuclei were smaller in diameter 5-10 µm and non-spherical (Folta and Kaufman, 2000). In comparison, the chickpea nuclei were observed to be uniform spheres with an average diameter of approximately 20 µm. These results indicate that the isolated nuclei were highly purified, and the optimized protocols were appropriate for the isolation of good quality nuclei for proteomic studies. A chlorophyll assay was carried out to detect possible chloroplast contamination of the nuclear fractions. The chlorophyll content in the crude and semi-pure nuclei preparations was less than 0.9% and 0.38% respectively when compared to the homogenized NIB supernatant (2.6.). This result demonstrates that the purity of the nuclei obtained from *X. viscosa* leaf samples using the optimized methodology were higher compared to the protocol used to isolate the nuclei from chickpea seedlings (Pandey et al., 2006). These protocols of nuclei isolation from leaf samples *X. viscosa* now provide a basis for functional studies of nuclear genes and gene products using molecular and proteomic approaches which is fundamental for the functional analyses of the *X. viscosa* nuclear proteins.

Numerous protocols for protein extraction from plant tissues have been developed and has been reviewed in detail by Canovas et al., (2004), and the common extraction techniques have been reported by many authors (Carpentier et al., 2005; Vincent et al., 2006; Issacson et al., 2006). Six different methods of protein extraction were employed in this study to extract *X. viscosa* nuclear proteins with only protocol 6 providing the best results in terms of reproducibility, yield and quality. This method was adapted from the method of Kirkland at al., (2006), which was designed to extract total proteins from *Haloferax volcanii* D570 bacterium.

Protocol 1 was adapted from the method of Ingle et al., (2005) and was based on phenol extraction and methanol/ammonium/acetone precipitation. The extraction buffer of this method contains EDTA to inhibit metalloproteases and (poly)-phenol oxidase activities by chelating metal ions, Triton X-100 to dissolve non-protein compounds, and 2-mecaptoethanol as a reducing agent. This method also gave a low yield of proteins with indistinct bands, probably due to incomplete nuclei pellet homogenization, ineffective removal of the nuclear DNA and RNA by the extraction buffer and insufficient protein precipitation and purification. Protocol 2

was adapted from the method of Bailly et al., 2004 and based on TES extraction buffer and acetone precipitation. The buffer contained EDTA and PMSF to inhibit metallproteases and (poly)-phenol oxidase activities, SDS as a strong anionic detergent (removed prior to IEF), NaCl to improve protein extraction (desalting in effect), and PVPP which is a strong H-receptor, making it effective in adsorbing (poly)-phenols (Carpentier et al., 2005). This protocol gave a low protein yield, smeared protein lanes and indistinct bands. The low yield of this protocol could be attributed to the same reasons as mentioned for protocol 2. Protocol 3 was adapted from the Sigma CelLytic PN Extraction kit procedure and involved using buffers with high salt concentration to facilitate the extraction of proteins, although DTT was added to reduce possible (poly)-phenols which covalently bind to proteins, and protease inhibitor cocktail as also added to inhibit proteases that are released upon nuclei rupture. The yield from this protocol was very low. The protein bands were indistinct, probably due the poor extraction capacity of the buffer. The acetone precipitation and pellet washes in desalting the protein sample were also inefficient. Protocol 4 was adapted from the Trizol Invitrogen procedure, which is based on phenol extraction and methanol/ammonium acetate precipitation. Phenol is known for its high clean-up capacity and selectivity as a strong solvent on proteins as it acts as a dissociating agent to decrease molecular interactions between proteins and other materials (Carpentier et al., 2005). However, this protocol gave a low yield of nuclear proteins with indistinct bands, most likely attributed to incomplete homogenization of the nuclei pellet. The nuclei pellet had to be fragmented by pippetting and vigorous vortexing in order to homogenize the sample. Moreover, ammonium acetate in methanol was inefficient in removing interfering substances, which resulted in indistinct protein bands in the 1DE-PAGE (Fig. 2.10.). Protocol 5 was based on urea lysis buffer and applied no precipitation but fractionation to extract the nuclear proteins. This protocol relied completely on the dissolving capacity of the urea buffer to extract the proteins. It gave the poorest results, probably due to the inefficient dissolving capacity of the urea lysis buffer to extract the nuclear proteins, and subsequent proteolytic breakdown of the nuclear proteins.

With the exception of protocol 6, using the mentioned protein extraction protocols we were unable to obtain *X. viscosa* nuclear proteins of sufficient yield and quality to analyse *X. viscosa* nuclear proteome. Non-protein impurities, such as nuclei acids, phenolic compounds, salt and carbohydrates interfere with 2D separation, produce streaking and heterogeneity and subsequent hamper detection of protein spots on 2D maps (Vandahl et al., 2005; Capentier et

al., 2005). Salts in protein samples can also induce protein modification and quench migration of proteins to the pI value (Hamdan and Righetti, 2005). The IEF procedure is tolerant of salt concentrations up to 40-50 mM (Cho et al., 2003; Canas et al., 2007), and this includes contributions by carrier ampholytes (Kirkland et al., 2006).

In order to desalt the nuclei sample and enrich the nuclear fractions we modified and optimized the protocol of Kirkland and et al., (2006) to extract the nuclear proteins from the crude and semi-pure nuclear preparations. Kirkland and co-workers developed this protocol to extract total bacterial halophilic proteins. Kirkland and co-workers used heating to homogenize the bacterial samples. For the *X. viscosa* nuclear proteins this step was replaced by fragmentation, pipetting, and mixing the nuclei pellet in the Trizol until a homogenized solution was obtained, followed by vigorous vortexing to release more nuclear proteins as heating would change the nuclear protein profile. The phenol chlorophorm extraction and ethanol precipitation steps thoroughly remove the nucleic DNA and RNA. The isopropanol precipitation followed by multiple guanidine HCL and acetone washes removed the phenol and salt from the nuclear proteins making them fully compatible with IEF. One dimensional SDS-PAGE analysis of the extracted proteins from the different fractions gave distinct protein bands (Fig.2.11). The reason that the optimized protocol (6) protein yield and quality was better as compared with the other methodologies employed can be attributed to the efficient nuclei homogenization and the ability of phenol/chloroform extraction and ethanol precipitation to thoroughly remove RNA and DNA. Moreover phenol-based methods were reported to be the most efficient in removing interfering substances, enriching nuclear proteins (Vincent et al., 2006) and minimizing proteolysis during extraction (Kirkland et al., 2006), which resulted in improved protein yield and resolution of extracted proteins The isopropanol precipitation followed by 300 mM guanidine HCl and acetone washes with high-speed centrifugation removed the salt from protein samples, thereby improving the yield. The other methods use ammonium sulphate in methanol to precipitate the proteins and acetone washes to remove salt from the protein samples, which are less effective in desalting the proteins. Using the employed methodology a large proportion of soluble protein is retained (Kirkland et al., 2006) as less fractionation and multiple tube transfer steps are required. The cleaner protein samples that result from this methodology of preparation may facilitate the detection of low-abundance proteins in complex samples through any number of mass spectrometric techniques, and facilitate separation in various liquid chromatography techniques (Chapter 3, 4).

The enrichment of the *X. viscosa* nuclear proteins was evaluated by Western blot analyses using specific antibodies for two nuclear proteins, histone H1 and proliferating cell nuclear antigen (PCNA). Histone H1 is a determinant of mitotic chromosome structure (Hansen 2002) and PCNA is required for processive DNA synthesis (Sanchez et al., 2005). To detect possible cytoplasmic and chloroplastic contaminations in the nuclear fractions, an antibody specific to the *X. viscosa* peroxiredoxin II enzyme (XvPrxII) was used as it was shown to localize to both the cytoplasm and chloroplast of *X. viscosa* (Govender, 2006). Both nuclear proteins were detected in the nuclear preparations while the XvPrxII protein was not detected. Furthermore, the nuclear proteins were not detected in the fractions containing cytoplasmic and chloroplast proteins while XvPrxII was detected (Fig. 2.14.-2.19). Together these results indicate that both the nuclear preparations (crude and semi-pure nuclei) had no appreciable level of cellular contamination, and any of the preparations can be used to conduct the proteomic analysis for the X. *viscosa* nuclear proteins. Similar findings were published on characterization of nuclei proteins of *Medicago truncatula* (Repetto *et al.*, 2008) using the Sigma kit. Also, good quality crude transcriptionally active nuclei were isolated from *Arabidopsis* etiolated seedlings by Folta and Kaufman (2000) without the application of percoll and sucrose gradients, and were recommended for nuclear run-on assays and for proteomic studies as well.

Western blot analyses revealed that histone H1 was more abundant in *X. viscosa* crude nuclear preparations compared to the semi-pure nuclear preparations with the yield of the former higher compared to the latter preparations. Moreover, the crude procedures are simpler and quicker than the semi-pure procedures, as it involves no sucrose cushion, and therefore results in minimum loss of nuclei as less wash steps were needed. The sucrose molecules employed in the semi-pure isolation procedures readily enters the nuclei, and could trigger a nucleus response that might interfere with accurate description of nuclear phenomenon. This is an especially important consideration when investigating the nuclei under dehydration stress conditions. Taking these into account, crude nuclei preparations from leaf samples of fully hydrated and dehydration stress *X. viscosa* plants were selected for the analysis of *X. viscosa* nuclear proteome (Chapter 3, and 4).

In conclusion, the protocols, presented in this study resulted in a significant enrichment of *X. viscosa* nuclear proteins and allow proteomic analysis of the nucleus under desiccation stress condition. This will provide an insight into the complex processes in the nucleus and its role in the adaptation of *X. viscosa* to desiccation stress.

CHAPTER 3

Proteomic analysis of up-regulated nuclear proteins during dehydration using 2D-PAGE with MALDI-TOF/TOF MS

3.1. INTRODUCTION

Functional proteomics is defined as a method to identify specific proteins in a cell, tissue, or organism that undergo changes in abundance, localization, or modification in response to a specific biological condition (Graves & Haystead, 2003). In functional proteomics, the aim is to provide information about proteins that are directly relevant to the biological question being studied (Graves & Haystead, 2003). The comparison of control versus treated samples, and the detection of differences in protein expression can provide unique markers of biological activity (Patton, 2002).

Dehydration responsive proteins of *X. viscosa* and other resurrection plants at low dehydration points such as 35% RWC were classified as late-dehydration responsive (Ingle et al., 2007). The changes in expression at this dehydration point were reported to be unique to resurrection plants (Bartles & Salamini 2001; Mundree and Farrant, 2000; Illing et al., 2005; Ingle et al., 2007), and were thought to be associated directly with the acquisition of desiccation tolerance (Jiang et al., 2007; Ingle et al., 2007). Thus, the investigation of up-regulated proteins at 35% RWC is important.

Proteomic analysis under dehydration stress of various tissues and organelles from *X. viscosa* plant will provide more detailed information about the proteome of this plant and its response to dehydration stress than analysis of its whole proteome. It was proposed that nearly one-fourth of total cellular proteins are localized in the nucleus, implying a variety of proteins functions in this organelle (Moriguchi et al., 2005). Targeted investigations of the nuclear proteome are therefore needed to decipher the mechanisms governing the regulation of adaptation to environmental stresses.

In this chapter we describe the development of 2-D nuclear proteome maps of *X. viscosa,* which includes displaying the nuclear proteins of control and dehydrated samples for general image analysis and identification of up-regulated nuclear proteins using MALDI-TOF/TOF MS. Furthermore we also describe the matched functions of these proteins to gain information about their roles in acquisition of desiccation tolerance in *X. viscosa*.

3.2. MATERIALS AND METHODS

3.2.1. Nuclei isolation and nuclear protein extraction

For 2D SDS-PAGE, nuclei were isolated from 5 gm of fully hydrated and dehydrated leaf samples at 35% RWC as described previously (sections 2.2.3.2.1 and 2.2.3.2.2). The integrity of the isolated nuclei were confirmed by microscopic analysis, with and without 4',6-diamidino-2-phenylindole (DAPI) staining as described previously (section 2.2.4.). Possible chloroplast contamination in the nuclear fractions was examined by spectrophotometric analysis of chlorophyll in the fractions obtained during the nuclei purification as described previously (2.2.4.). Nuclear proteins were extracted from 120 mg nuclei as described previously (section 2.2.5.6). Nuclear proteins were solubilised and quantified as described previously (section 2.2.6.). The enrichment of the nuclear fractions was confirmed using two specific anti-nuclear antibodies, the anti-H1, and anti-PCNA antibodies, and an anti-cytosolic/chloroplastic antibody, the anti-XvPrx2 antibody as described previously (section 2.2.7.2.).

3.2.2. Two-dimensional polyacrylamide gel electrophoresis (2DE)

To screen for high gel resolution and reproducibility 2-DE was initially performed using 7-cm immobilized pH gradient (IPG) strips (Bio-Rad, USA) with *pH* range of 3-10; 4-7 and 5-8 respectively. For final imaging and data analysis, isoelectric focusing (IEF) was carried out for the nuclear proteins of the control and dehydrated samples using 11-cm immobilized *pH* gradient (IPG) strips (Bio-Rad, USA) with a pH range of 5-8. Nuclear proteins dissolved in urea lysis buffer as described previously (section 2.2.6.) were additionally solubilized by soft agitation for 40 minutes at 20^0 C, and just before loading the strips the following were added to the protein samples to final concentrations: 1% (w/v) DTT in urea lysis buffer containing 0.001% bromophenol blue, 1.2% carrier ampholyte (pH 3-10) (Bio-Rad, USA), 0.4% ASB14 (Sigma-Aldrich, USA) and urea lysis buffer (7 M urea, 2 M thiourea and 2% CHAPS) was added to final volume of 125 µl and 185 µl for each 7 cm and 11 cm strip respectively. The 7cm and 11 cm strips were loaded with 250 µg and 400 µg protein respectively in rehydration/equilibration tray (Biorad, UK) and left at 20^0 C for about 1 h before covered with 2 ml of mineral oil (Biorad, UK) and rehydrated for 24 h at 20^0 C. Electro-focussing of the proteins was performed using the Biorad Protean IEF Cell (Biorad, USA) as described by the manufacturer. The proteins in the 7 cm IPG strips were focused at a maximum of 4000 V for

20,000 volt-hours (V-h) at 20^0 C with a maximum current of 50 µA/strip, while proteins in the 11 cm IPG strip were focussed at a maximum of 8000 V for 35,000 volt-hours (V-h) at 20^oC with a maximum current of 50 µA per strip. Once the one dimension electrophoresis was completed, the strips were subject to reduction for 15 min with shaking in 2 ml of DTT equilibration buffer (6 M urea, 30% [v/v] glycerol, 2% [w/v] SDS, 50 mM Tris-HCl [pH 8.8], and 1% [w/v] dithiothreitol [DDT]) followed by alkylation in 2 ml Iodoacetamide equilibration buffer (6 M urea, 30% [v/v] glycerol, 2% [w/v] SDS, 50 mM Tris-HCl [pH 8.8], and 4.8% [w/v] iodoacetamide). The equilibrated 7 cm IPG strips were placed in the upper well of 12% polyacryamide gel and set in place with 0.6% [w/v] agarose overlay (Bioscience, USA) prepared in 1x SDS PAGE tank buffer containing trace amount of bromophenol blue. The second dimension was run initially at 10 mA per gel for 20 minutes and then the current was increased to 25 mA per gel for 50 minutes at RT. Once the gel run was completed, gels were removed from the plates and stained overnight or until spots were clearly visible in 200 ml of CBB R-250 stain (40% [v/v] methanol, 10% [v/v] acetic acid and 0.1% [w/v] CBB R-250) with shaking, then transferred to 7% [v/v] acetic acid destain with shaking until gel purple-blue color background was faded away completely. Finally the gels were incubated in ddH2O for 30 minutes to wash out the acetic acid.

The equilibrated 11 cm IPG strip was rinsed briefly in 1x MES tank buffer (Biorad, USA) before being placed in the upper well of 11-cm Criterion precast Bis-Tris 4-12% polyacryamide gel (Biorad, USA) and set in place with 0.6% [w/v] agarose overlay (prepared in 1x Criterion MES tank buffer containing 0.002% bromophenol blue). The second dimension was run at 185 mA/gel for 25 min at room temperature. Upon completion of the run the gels were removed from the cassette and stained overnight on a shaker with colloidal commasie stain (Biorad, USA) and then destained overnight with deionised distilled water until protein spots were clearly visible. To maximize spot reproducibility for each of the control and dehydrated protein samples, five IEF and subsequent Bis-Tris SDS-PAGE gels were run simultaneously. The two dimensional gels were digitized with a flatbed colour scanner Epson perfection V750 (Epson, Germany). Acquired images were analyzed with PDQuest (version 8.0.1) software (Bio-Rad, USA).

3.2.4. Statistical analysis of 2D SDS-PAGE gels

Gel images were acquired by flatbed colour scanner Epson perfection V750 (Germany). Pixel depth was 16 bit, resolution was 300 dpi; brightness and contrast were set to default.

Cropped gel images were exported as TIFF files from the scanner. Acquired images were analyzed with PDQuest software (Bio-Rad, USA) to assemble first and second level match sets. A first level match set (mater gel) represents a "standard image" of five replicate two-dimensional gels for each of the fully hydrated and dehydrated samples. From the 5 replicas master maps were created for each of the fully hydrated and the dehydrated images as well, which contain all spots found in the individual gels. Spots in all images were quantified and matched across gels. Default settings were used during automated spot detection and matching, prior to manual inspection and any necessary editing of spot matching. There was no significant difference in the number of spots detected per gel at the full turgor and 35% RWC. After obtaining the first level match sets a second level match set was performed that allowed a comparison between the two master maps. Spot intensities were normalized in each gel and the second level match set was performed by paired *t*-test ($P < 0.05$) to find proteins up-regulated in expression from two to ten-fold. Only spots present on all five replicate gels of the fully hydrated and dehydrated samples were included in this analysis. A qualitative analysis was also performed to detect 'de novo' proteins (proteins that expressed only during dehydration at 35% RWC). The statistical analyses were repeated five times.

3.2.5. Mass spectrometry identification of proteins

All reagents are analytical grade or equivalent. Gel slices were cut up into 1 mm X 1 mm X 2 mm and destained in 1.5 ml Eppendorf tubes with water:acetonitrile:acetic acid 50:45:5 (Romill; Riedel de Haen) until clear. Samples were dehydrated and desiccated before reduction with 20 mM dithiothreitol (DTT; Sigma) in 100 mM NH_4HCO_3 (Sigma) for 30 minutes at 56 °C. Excess DTT were removed and the gel pieces again dehydrated. Cysteine residues were alkylated with 100 mM iodoacetamide (Sigma) in 100 mM NH_4HCO_3 for 30 minutes at room temperature in the dark. After carbamidomethylation the gel pieces were dehydrated and washed with 50 mM NH_4HCO_3 followed by another dehydration step. Proteins were digested by rehydrating the gel pieces in trypsin (Promega) solution (20 ng/uL) and incubating at 37 °C overnight. Peptides were extracted from the gel pieces once with 50 mM NH_4HCO_3, once with 50 mM NH_4HCO_3/acetonitrile 50/50 and once with 50% acetonitrile water. The combined extracts were acidified by adding trifluoroacetic acid (TFA) (Sigma) to a final concentration of 0.1% v/v. Peptide solutions were dried and reconstituted in 20 µl 0.1% TFA/water and desalted/concentrated using C18 ZipTips (MilliPore). Peptides were eluted from the ZipTip

with 50% acetonitrile/water, 0.1% TFA. The eluted peptides were spotted using the dried droplet technique with two times 0.5 µl overlay. The matrix was 5 mg/ml α-cyano-4-hydroxycinmamic acid (Fluka) with 10 mM $NH_4H_2PO_4$ (Fluka) in 50% acetonitrile.

Mass spectrometry was performed with a 4800 MALDI TOF/TOF (Applied Biosystems, USA). All MS spectra were recorded in positive reflector mode. Spectra were generated with 600 laser shots/spectrum at laser intensity of 3600 (arbitrary units) with a grid voltage of 16 kV. All peptide containing spots were internally calibrated using trypsin autolytic fragments

3.2.6. Proteomic database analysis

Database interrogation was performed with the Mascot algorithm using the MSDB database on a GPS workstation. Search parameters were as follows: Species – Viridiplantae, all entries; Enzyme – trypsin; Maximum number of missed cleavages -1; Fixed modifications – carbamidomethyl (C); Variable modifications oxidation (M); Precursor tolerance - 50 ppm.

3.3. RESULTS

3.3.1. Nuclei isolation and nuclear proteins extraction

To construct 2DE gel electrophoresis maps of the *X. viscosa* nuclear proteome, the nuclei were isolated from organellar lysate of *X. viscosa* leaf samples without the use of sucrose and percoll density gradient. The integrity of the isolated nuclei was analysed by microscopy without and with DAPI staining of nuclei (Fig. 2.5., chapter 2). A chlorophyll assay was performed to assess possible chloroplast contamination of the nuclear fraction (Fig. 2.6., chapter 2). Proteins were extracted from the nuclei using protocol 6 (section 2.2.5.6.) which included phenol extraction, isopropanol precipitation and ethanol/guanidine hydrochloride and acetone wash. The isolation of nuclei acid-free proteins is critical, as nuclei acids severely interfere with isoelectric focusing (IEF) (Capentier et al., 2005). The protein profile of the nuclear fractions appeared distinct from that of the control fractions in a one-dimensional (1D) gel (Fig. 2.11., chapter 2).

Enrichment of the nuclear proteins was analysed using specific antibodies for two nuclear proteins, histone H1, and a proliferating cell nuclear antigen (pCNA). Contamination with non-nuclear proteins was analysed using an antibody to a chloroplast/cytoplasmic protein, *X. viscosa* peroxiredoxin type II (XvPrx2). The nuclear proteins histone H1 and pCNA were found in the nuclear fraction, but not in the chloroplastic and cytoplasmic fractions (discussed in

Chapter 2, Fig. 2.14-2.19). In contrast, the chloroplast/cytoplasmic protein XvPrx2, which was absent from the nuclei was not detected in the purified nuclear fraction. The data suggested that this preparation was enriched in nuclear proteins.

3.3.2. Construction of two-dimensional gel electrophoresis map of nuclear proteins

To develop 2-D nuclear proteome maps for *X. viscosa*, the nuclear proteins were initially separated across a broad-range pH 3.0-10.0 (7 cm) IPG strips (Fig. 3.1A), then subsequently across mid-range pH 4.0-7.0 and 5.0-8.0 IPG strips (Fig. 3.1B and 3.1C respectively). The pH 5.0-8.0 range gave significantly better separation than the pH 3.0.-10 and pH 4.0-7.0 and was selected to establish 2D reference maps for the control and the dehydrated sample and for the up-regulated nuclear proteins in the sample at 35% RWC (Fig. 3.2., 3.3., and 3.4.).

3.3.3. 2-DE analysis of the nuclear proteins

Flatbed gel scanning and image analysis software enabled 459±21 protein spots (average of 438 spots) to be reproducibly detected in all gel images. In order to gain information about proteins whose expression is up-regulated in response to dehydration stress, a difference map was generated which showed proteins that up-regulated from two to ten-fold. The probability of the differences being statistically significant was calculated using student's *t* test; changes in expression were considered significant if the calculated *p* values were < 0.05. A total of 18 nuclear protein spots were found to be up-regulated in response to dehydration stress at 35% RWC (Fig. 3.3.). In addition, a qualitative analysis was performed to detect spots present only during dehydration in all 5 replicate gels at 35% RW ('*de novo*' proteins). The qualitative analysis results showed that no de novo proteins could be detected in the dehydrated sample compared to fully hydrated. These results cannot rule out the presence of de novo proteins in the dehydrated samples as the de novo proteins might be present in undetectable levels. On the other hand post-translational modifications and deletions can alter proteins MW and *pI* leading to a false detection of pre-existing proteins that are modified when dehydration signal is first perceived as de novo proteins. Once this differential analysis has been performed, the up-regulated spots were manually excised from the gel and subsequently characterized by MALDI-TOF/TOF mass spectrometry analysis through their peptide mass fingerprint (PMF) data.

3.3.1. Identification of up-regulated dehydration stress responsive nuclear proteins

Ten of the 18 samples analyzed were identified with high confidence (protein score > 63; $p < 0.05$) using the MSDB database and limiting the search to green plants. A further three samples were identified with low confidence ("borderline hits"), these were spot 3 (Q93Y69 GAG-pol, score 59, confidence 86.40%), spot 8 (A96767 unknown protein, score 61, confidence 84.40%); and spot 14 (Q9BAA0 intron maturase, score 60, confidence 88.95%). The remainder five samples (spots 2, 5, 6, 7, and 15) could not be assigned to any protein in the specific database.

Samples 10, 11 and 18 returned the same protein identity with high confidence. This was investigated by searching the data against all entries in the MSDB database to rule out contaminants such as keratin. None of the samples analyzed indicated the presence of keratin. Analysis of the ions observed in the three samples that matched the database pattern indicates that 23 ions were common to all three samples and a total of ten are unique peptides distributed over the three samples. Seven ions were selected for fragmentation and of which two were common to sample 10 and sample 11. All the sequences assigned were of high confidence (in the ion score range of 64 – 148). If carry over was responsible for this result one would expect the sample containing the carry over to have lower signal intensities and lower confidence identifications. This was not the case with samples 10 and 11. Sample 11 identified with a score of 412 as opposed to 202 for sample 10. Similarly carry over cannot explain identification of sample 18. We hypothesize that the reason why the same protein identity was obtained for samples 10, 11 and 18 could be due to protein degradation, protein isoforms, heterodimer formation or they belong to an extensive protein family.

These results demonstrate that identification of proteins from an organism with un-sequenced genome by comparing peptides on the proteins of interest to orthologous proteins of species that are well characterized could be successfully achieved. (Shevchenko et al., 2001; Liska and Shevchenko, 2003).

Results of MS protein identifications are shown in table 3.1., while the representative 2DE gel indicating the 18 up-regulated protein spots at 35% RWC is shown in Fig. 3.3.A. The intensities of the up-regulated protein spots in the dehydrated samples compared to their counterparts in the fully hydrated samples are shown in Fig. 3.5.

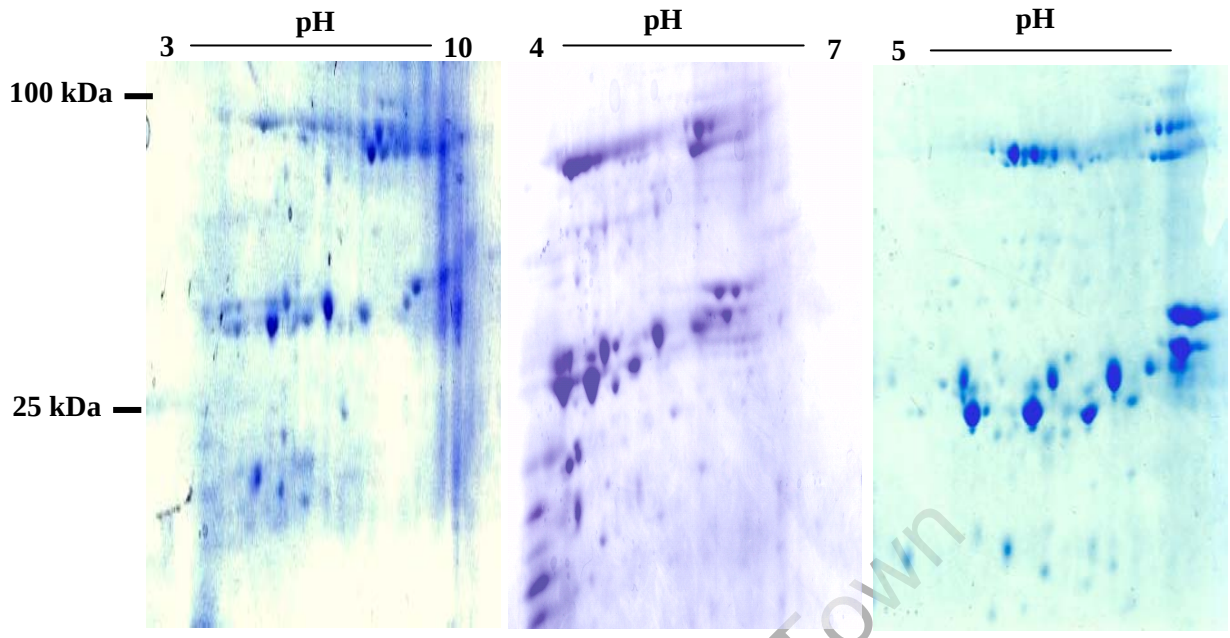


Figure 3.1. Two-dimensional PAGE of *X. viscosa* nuclear proteins of the fully hydrated samples separated across pH ranges: 3-10, 4-7 and 5-8 respectively using 7 cm IPG strips.

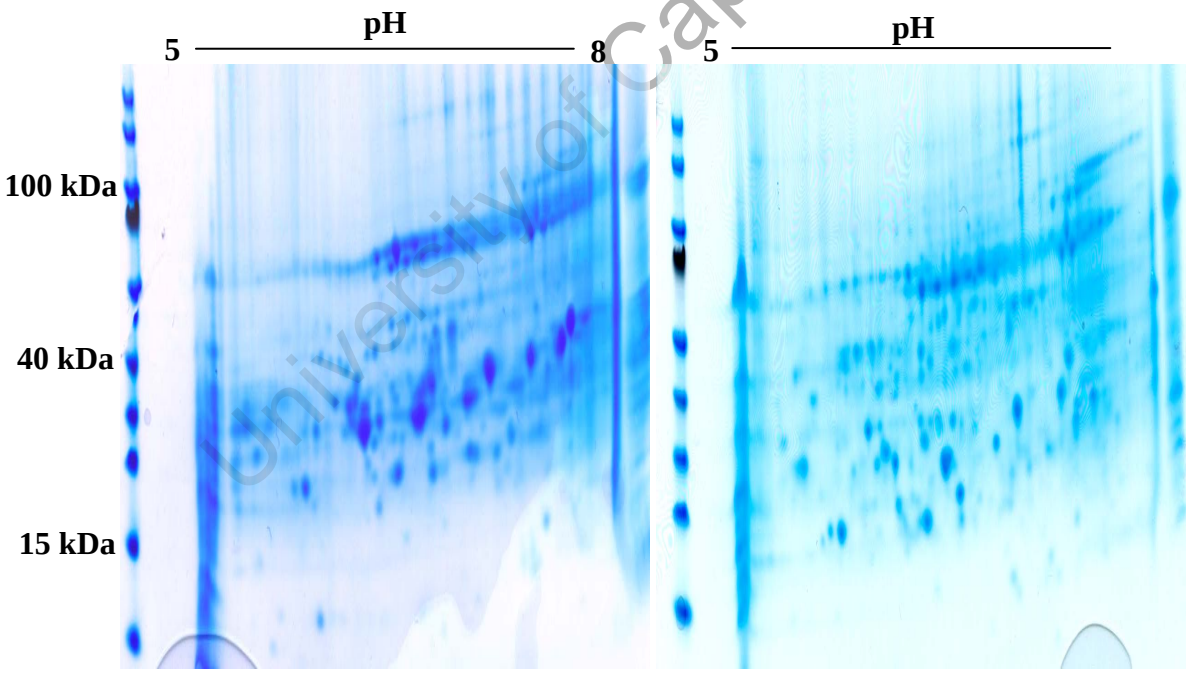


Figure 3.2. Two-dimensional PAGE of *X. viscosa* nuclear proteins of the (A) fully hydrated and (B) dehydrated samples at 35% RWC separated across pH 5-8 using 11 cm IPG strips on 4-12% Criterion gradient gels (Bio-Rad, USA).

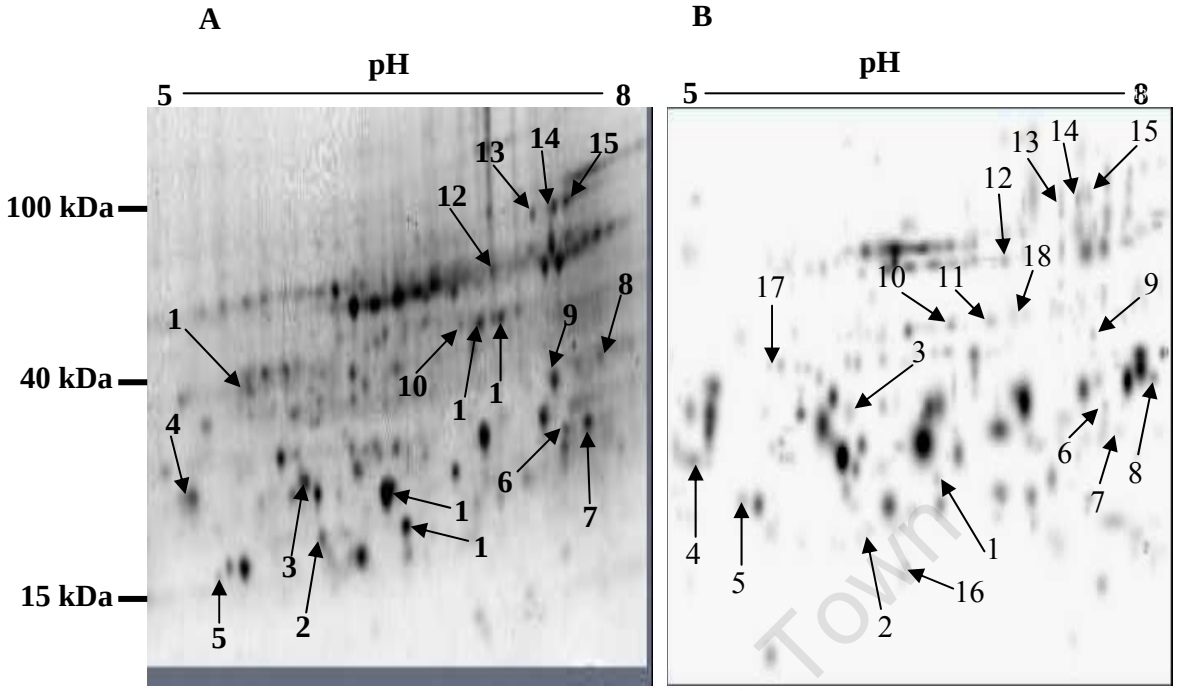


Figure 3.3. Two-dimensional PAGE reference map of the *X. viscosa* up-regulated nuclear proteins at 35% RWC (A) compared to the (B) fully hydrated sample separated across pH 5-8 using11 cm IPG strips on 4-12% Criterion gradient gels (Bio-Rad, USA).

Figure 3.4. PDQuest display of 2-D reference gel of the up-regulated nuclear proteins of *X. viscosa* during dehydration.

Table 3.1. Mass spectrometry identification of up-regulated proteins in the nucleus of *X. viscosa* during dehydration.

Spot No	Accession No.	Protein name	Score	Confidence	Fold-change
1.	Q9LGM1	Non-LTR retro element reverse transcriptase	66	96.98	8.8
2.	Not available	Novel	-	-	5.7
3.	Q93Y69	GAG-pol	59	86.40	6.7
4.	Q9ASH2	Orf100f protein	69	98.67	6.8
5.	Not available	Novel	-	-	4.4
6.	Not available	Novel	-	-	6.8
7.	Not available	Novel	-	-	7.0
8.	A96767	Unknown protein F2P9.19	61	86.40	6.9
9.	Q943P8	Chaperonin	69	99.22	6.9
10.	S21567	EF-Tu precursor	202	100	6.0
11.	S21567	EF-Tu precursor	412	100	7.8
12.	E86388	Chaperonin	77	99.78	7.5
13.	ATP_ARATH	ATP sythase α chain	106	100	7.7
14.	Q9BAA0	Intron maturase	60	88.95	7.4
15.	Not available	Novel	-	-	6.9
16.	Q8LHZ4	Zinc-finger helicase	65	100	7.1
17.	D84580	Ribosomal protein L28	74	100	6.5
18.	S21567	EF-Tu precursor	318	100	6.2

Probability Based Mowse Score Score is -10*Log (P), where P is the probability that the observed match is a random event. Protein scores greater than 63 are significant ($p < 0.05$).

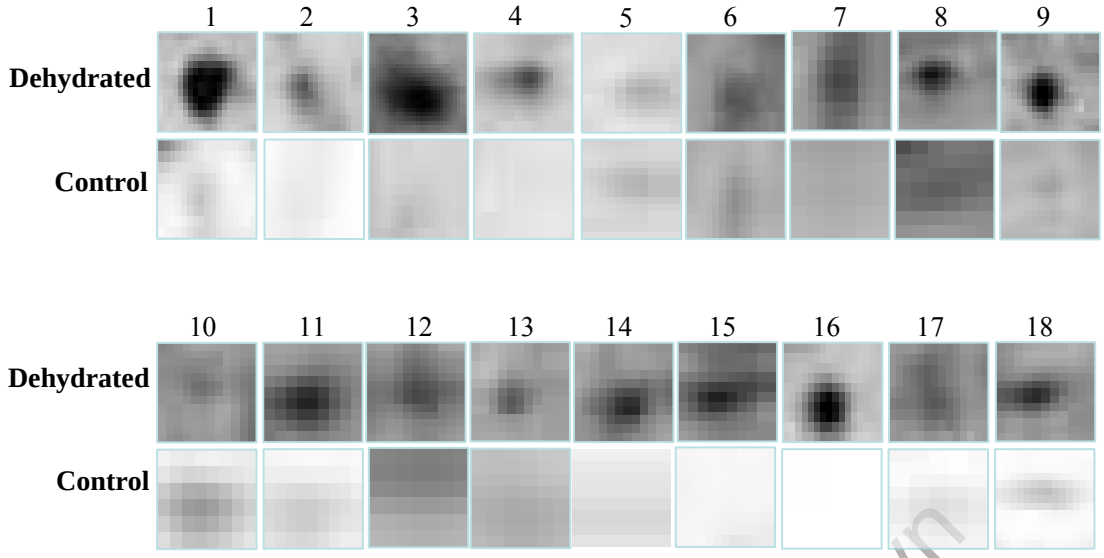


Figure 3.5. Enlarged view of the up-regulated nuclear protein spots of *X. viscosa* during dehydration compared to protein spots of the fully hydrated samples.

3.4. DISCUSSION

Proteins up-regulated during dehydration stress, especially during the late late-responsive phase were thought to be responsible for stabilization of the cellular environment and maintaining the structural and physiological integrity in desiccated cells and are therefore important in keeping the viability in cells under dehydration stress conditions (Bartles & Salamini 2001; Jiang et al., 2007). These proteins are likely to be associated directly with the acquisition of desiccation tolerance (Bartles & Salamini 2001; Jiang et al., 2007). Late dehydration-responsive proteins are of greatest interest in deciphering the molecular mechanisms enabling the resurrection plants to survive desiccation as such changes in expression are unique to resurrection plants: non-desiccation tolerant species such as *Arabidopsis* cannot survive at 35% RWC (Ingle et al., 2007). This was therefore the rationale for investigation of late dehydration responsive proteins at 35% in order to uncover these molecular mechanisms.

In this study, nuclear proteins were extracted from nuclei isolated from leaf samples of fully hydrated (100% RWC) and dehydrated (35% RWC) *X. viscosa* plants, and analyzed by 2DE. The RWC at 35% represents a distinct phase of dehydration process where induction of late protection mechanisms is initiated (Mundree et al., 2000; Illing et al., 2005; Ingle et al., 2007). At this dehydration point *Xerophyta species* cease photosynthetic process by breaking down its chlorophyll and the thylakoid membranes (Mundree et al., 2006; Ingle et al., 2007). Of

the 438 protein spots detected on the gels, quantitative analysis using PDQuest identified 18 that were significantly increased in abundance (from two to ten-fold) (paired *t-test, P* < 0.05) during drying. In addition, qualitative analysis was performed to identify protein spots that were present only in the dehydrated sample at 35% RWC, this analysis showed that no protein spots could be detected as de novo. However, this result cannot rule out the expression of de novo proteins as they may present at non-detectable levels during drying.

The 18 up-regulated protein spots were late-dehydration responsive, and we assume that they play an important role in the latter stages of drying, a characteristic to resurrection plants.

These 18 protein spots were excised from the gels and identified by MALDI-TOF/TOF mass spectrometry. According to the function of matched proteins, these proteins might play a variety of roles during dehydration and can be classified into five categories including:

(i) four proteins were identified as involved in gene regulation, these were spots (1) Q9LGM1 non-LTR retro element reverse transcriptase; (3) Q93Y69 GAG-pol; (14) Q9BAA0 intron maturase; and (16) Q8LHZ4 zinc-finger helicase.

(ii) five proteins were identified as involved in molecular chaperone type activities, these were two chaperonins and three eukaryotic translation elongation factors similar to the bacterial EF-Tu with dual functions of translation and chaperone type activities. The protein spots were (9) Q943P8 Chaperonin, (12) S21567 Chaperonin, (10) S21567 EF-Tu precursor, (11) S21567 EF-Tu precursor and (18) S21567 EF-Tu precursor

(iii) four proteins were identified as proteins of translation machinery, these were spots (10) S21567 EF-Tu precursor, (11) S21567 EF-Tu precursor, (18) S21567 EF-Tu precursor and (17) D84580 Ribosomal protein L28.

(iv) one protein was identified as involved in energy metabolism, this was spot (13) ATP_ARATH ATP sythase α chain.

(v) seven proteins were identified with no-assigned functions, five of these were novel with no homologues in databases, these were spots (2), (5), (6), (7) and (15). There were two proteins with unknown functions, spot (8) with homologue to *Arapidopsis* unknown protein, and spot (4) was a homologue to sugar beet protein.

Proteins involved in gene regulation

Non-LTR retro element reverse transcriptase: retrotransposons are ubiquitous mobile genetic elements that transpose through an RNA intermediate and are found in the genome of most eukaryotes (Capy et al., 1998; Cui et al., 2004; Belfort et al., 2002; Bringaud, et al., 2005). The rice genome draft sequences revealed that retrotransposons account for 15% of its genome (Goff et al., 2002; Yu et al., 2002; Komatsu et al., 2003). In contrast to DNA-type transposable elements, retrotransposons encode reverse transcriptase (RT) activity and move by a replicative mechanism that involves RNA intermediate (Komatsu et al., 2003). Thus, retrotransposons contributed greatly to the expansion and the evolution of the genome. Despite the fact that retrotransposons exist in high copy numbers in the genomes of most eukaryotes, the great majority of them are inactive or defective, and only a small portion of them retain the ability to retrotranspose (Komatsu et al., 2003). They can be divided into two lineages that utilize completely different mechanisms of integration. Those elements with long terminal repeats (LTR), called LTR-retrotransposons, are similar both in structure and retrotransposition mechanism to retroviruses (Whitcomb and Hughes 1992; Bringaud, et al., 2006). Those elements that lack LTR, called non-LTR retrotransposons or retroposons, use a simpler mechanism of transposition. Transcription is the first step of the retrotransposition process and seems to be a major controlling step for plant retrotransposons (Casacuberta and Santiago, 2003). Transcription, and subsequent transposition, is only inducible under certain conditions that in all cases can be considered as stress conditions (Casacuberta and Santiago, 2003). Retrotransposons have been shown to be specifically activated by environmental stress signaling (Grandbastien, 1998; Pandey et al., 2008). It has been shown that sharp microclimate changes can modify the copy number of the BARE-1 retrotransposon in wild barley (Casacuberta and Santiago, 2003). The non-LTR retrotransposons of the protozoan parasite *Trypansoma cruzi* were thought to be randomly distributed in the nuclear genome (Bringaud, et al., 2006). Most of the nonsite-specific non–LTR retrotransposons contain an APE-like domain, which is thought to determine the site of retroelement insertion. Olivares et al., (2003) showed that the *T. cruzi* L1Tc APE-like domain contains an APE activity (Olivares et al., 1997). Furthermore, overexpression of the L1Tc APE-like domain protects *T. cruzi* against DNA damaging stresses (Olivares et al. 2003; Bringaud, et al., 2006), indicating that the L1Tc-encoded APE-like domain is active *in vivo* and may have a protective role (Bringaud, et al., 2006). Consequently, it has been proposed that this APE-repair activity could act as a signal for

new retrotransposition events. It has been shown that mobilization of the human L1 elements can be mediated by endonuclease-independent retrotransposition to repair double-strand break DNA (Morrish et al. 2002; Bringaud, et al., 2006).

Zinc-finger helicase: transcription is a highly regulated process, ensuring the production of appropriate levels of gene products to direct cellular processes. Regulation occurs at every step, from the initiation and elongation of transcription and splicing of pre-mRNAs to the export and translation of the mature transcripts (Worringer and Panning, 2007). Many RNA helicases play critical roles during conformational changes of RNAs and ribonucleoprotein (RNP) complexes, as well as during timing and proofreading events (Jankowsky and Fairman, 2007). The stress perception and signal transduction to switch on adaptive responses are critical steps in determining the survival of plants exposed to adverse environments. Plants have multiple stress perception and signal transduction pathways, which may cross-talk at various steps (Knight et al., 2001, Pastori et al., 2002). Thus, many molecules have tomove in and out of the nucleus at any given time. Proteins involved in signal transduction were shown to play a crucial role in nucleocytoplasmic transport as they provide the identity of these two compartments and ensure the directionality of transport (Pandey et al., 2008). The regulated nuclear accumulation of zinc finger protein 1 (ZPR1) in mammals suggests that this protein may function as a signalling molecule (Galcheva-Gargova et al., 1998). ZPR1 could participate in many nuclear processes. For example, ZPR1 could be a regulated transcription factor (Worringer and Panning, 2007). Disruption of the ZPR1 gene causes defects in the biochemical properties of the nucleolus and ZPR1 protein may therefore contribute to the normal function of the nucleolus (Galcheva-Gargova et al., 1998). The major functions of the nucleolus are thought to be the transcription of rRNA genes, the processing of the pre-rRNA precursor to form mature rRNA, and the assembly of rRNA into preribosomal particles (Woolford and Warner, 1991; Shaw and Jordan, 1995). The nucleic acid composition of the nucleolus differs from other regions of the nucleus because of the abundance of rRNA genes and rRNA transcripts. This distinctive nucleic acid composition (both DNA and RNA) may contribute to the accumulation of ZPR1 in the nucleolus. Interestingly, the nuclear redistribution of ZPR1 observed in mammalian cells is also observed in yeast (Galcheva-Gargova et al., 1998). Because ZPR1 is a nucleolar protein that is essential for cell viability, a plausible role for ZPR1 is to contribute to one step in the process of rRNA expression (Galcheva-Gargova et al., 1998).

Maturases: mobile groups I and II introns, which are present in both prokaryotic and eukaryotic genomes are widely believed to be the ancestors of nuclear pre-mRNA introns (Michel and Ferat, 1995; Mohr and Lambowitz, 2003). These mobile introns encode reverse transcriptases (RTs) that function in intron mobility and also as maturases to promote RNA splicing by helping the intron RNA fold into the catalytically active structure (Mohr et al., 1993; Lambowitz et al., 1999; Belfort et al., 2002; Mohr and Lambowitz, 2003). The mobile yeast mitochondrial DNA aI1 and aI2 and *Lactococcus lactis* Ll.LtrB group II introns, encode proteins with several conserved domains associated with different activities (Michel et al., 1995; Mohr and Lambowitz, 2003). These are: an N-terminal RT domain, with an upstream region Z and conserved sequence motifs (I-VII) characteristic of the fingers and palm domains of retroviral RTs; domain X, a putative RNA-binding domain associated with maturase activity; a C-terminal DNA-binding region and DNA endonuclease domain, which function in intron mobility (Mohr and Lambowitz, 2003). The mobility of these introns occurs by a novel target DNA primed reverse transcription mechanism in which the intron RNA reverse splices directly into one strand of a DNA duplex, while the intron-encoded protein (IEP) uses the C-terminal DNA endonuclease domain to cleave the opposite strand and then uses the 3' end of the cleaved strand as a primer for reverse transcription of the inserted intron RNA (Mohr and Lambowitz, 2003). The reverse transcriptase/maturase proteins encoded by the yeast mitochondrial DNA and *L. lactis* Ll.LtrB introns are intron-specifc splicing factors (Michel et al., 1995; Mohr and Lambowitz, 2003). Recent genome sequencing projects have revealed putative proteins having similarity to group II intron maturases encoded by nuclear genes in *Arabidopsis* (Mohr and Lambowitz, 2003) and rice *(Oryza sativa)* (Goff et al., 2002; Yu et al., 2002; Mohr and Lambowitz, 2003). Some or all of the nuclear maturase proteins may lack mobility functions, but could retain the RNA splicing function. The high degree of conservation of the nuclear maturase proteins in *Arabidopsis* and rice suggests that they have an essential function (Mohr and Lambowitz, 2003). Further, cDNA clones have been obtained for *Arabidopsis* nuclear maturase-1b and maturase-2b, indicating that these genes are expressed (Mohr and Lambowitz, 2003). It has been shown that the ORFs have mitochondrial localization signals and their maturase domains appear conserved. The most likely hypothesis is that the putative maturase proteins are transported into organelles to function in the splicing of group II introns (Mohr and Lambowitz, 2003; Cui et al., 2004). Also, the proteins could have evolved to perform other

cellular functions utilizing their putative RNA-binding activity (e.g. nuclear pre-mRNA splicing) and it remains possible that nMat2 proteins retain RT activity (Mohr and Lambowitz, 2003). Thus, the nuclear-encoded maturases could potentially function as part of a common splicing apparatus for multiple organelle group II introns (Mohr and Lambowitz, 2003). The transfer of group II intron maturases to the nucleus has the potential advantage of facilitating the regulation of organelle gene expression by linking the splicing of one or more organellar introns to global signals that regulate gene expression in response to cellular energy state or environmental stimuli (Mohr and Lambowitz, 2003). In addition, transfer to the nucleus and the adaptation of maturases to function in splicing of multiple groups II introns could reflect steps in the evolution of a common spliceosomal splicing apparatus for nuclear pre-mRNA introns (Mohr and Lambowitz, 2003).

Molecular chaperones and proteases

GAG-pol and chaperonins: all living organisms employ dedicated chaperones and proteases to monitor and control the state of cellular proteins (Krojer, et al., 2008). Proteases are synthesized as part of the gag or gag-pol polyproteins (Lillehoj et al., 1988). Under dehydration stress appreciable amounts of denatured or partially unfolded proteins may arise in cells. In such environments, increased production of proteases appears to help prevent the accumulation of damaged cellular proteins. Proteases catalyze the rate-limiting steps in the degradation of highly abnormal proteins (Goldberg, 1992). This response should lead to an enhanced capacity of the cell to degrade abnormal proteins. It has become reported that in the eukaryotic cytosol and nucleus, large multimeric proteolytic complexes exist which require ATP hydrolysis for their function (Goldberg, 1992). Proteases were reported to be involved in the stability of nuclear inner membrane proteins (Goldberg, 1992).

Chaperonins: different chaperones have been reported to be up-regulated in response to stresses (Berlett et al., 1996; Narberhaus, 2000; Rahman, 2003; Pandey et al., 2008), and play complementary roles in protection of proteins against misfoldings. According to Sørensen et. al. (2003) HSP family and other molecular chaperones play significant roles in relation to stress resistance. Proteins that fail to fold correctly by HSP are generally degraded by proteases. Chaperones perform functions in various intra-cellular processes and play an important role in protein-protein interactions, folding, assembly, intracellular localization, secretion and prevention of unwanted protein aggregation or degradation and reactivation of damaged

proteins (Vierling, 1991; Parsell and Lindquist, 1993; Demirevska et al., 2008). Chaperonins mediate the prevention of protein aggregation under stress conditions and eventually control the degradation of nuclear proteins (Demirevska et al., 2008). Failure of this quality control can lead to protein aggregation, a malfunction that is correlated with the cell death. Chaperone proteins were identified in *Arabidopsis* nuclear matrix (Calikowski et al., 2003) and in yeast nuclear lamins (Willsie and Clegg, 2002). Similar results were also obtained in other experimental animal and human systems (Pouchelet et al., 1983; Gerner et al., 1999; Gerner et al., 2002).

Proteins of translation machinery

Ribosomal protein L28 and protein elongation translation factor ET-Tu: proteins of the translation machinery such as 60S ribosomal protein L28 and translation factors are common in the nuclear proteome (Pandey et al., 2006). It was proposed that protein translation may represent another way of combating dehydration stress by increasing protein levels for translation factors (Pandey et al., 2008). EF-1a is an evolutionarily conserved GTPase that catalyzes the efficient delivery of charged tRNA to the ribosome during protein elongation and is critically involved in translation fidelity. It has multiple and divergent roles in cell physiology affecting the cytoskeleton, peptide synthesis and protein degradation (Talapatra, et al.,2002). Ehanced EF-1a expression would allow for the subsequent expression of cellular stress response proteins under apoptotic conditions where rates of protein production significantly and progressively decline (Talapatra et al., 2002). The role of EF-1a in survival may not require its function in peptide elongation. EF-1a has an established role in maintaining and enhancing translational fidelity (Talapatra et al., 2002). One important effect of reduced translation fidelity is an increase in the frequency of proteins that are no longer able to adopt their native conformation due to amino acid misincorporation. Such changes in protein content and folding could be recognized in the nucleus, and trigger chaperones for the proper modification and folding of nascent polypeptides. If increasing EF-1a levels increases translation accuracy, the overexpression of EF-1a may lead to a reduction of unfolded proteins (Talapatra, et al., 2002).

Eukaryotic elongation factor (eEF-1-α) plays a pivotal role in protein biosynthesis, though present mainly in the cytoplasm, but many eEF-Tα molecules has been identified in the nucleus where it forms a complex with a zinc finger protein (Gangwani et al., 1998). In the *Escherichia coli*, EF-Tu in addition to its function in protein translation elongation it was found

to act as a molecular chaperone (Caldas et al., 2005), interacted with unfolded and denatured involved in protein folding and protein renaturation induced by dehydration stress. In a concentration 20-fold lower than its cellular concentration Caldas et al., (2005) demonstrated that EF-Tu prevented the aggregation of other proteins under heat stress. In mammalian cells EF-1a shares homology with the bacterial translation protein EF-Tu, and both proteins are essential for protein translation (Talapatra et al., 2002). Manipulation of EF-1a protein levels has been reported to alter total cellular protein levels through mechanisms involving both protein synthesis and degradation (Gonen et al., 1996). Many translation factors and ribosomal proteins were identified in both human and plant nuclear and nucleolar proteomes, human nucleolus (Scherl et al., 2002) and in the human nuclear matrix (Holzmann et al., 2000; Anderson et al., 2002), and nuclear proteomes of *Arabidopsis* in response to cold stress (Bae et al., 2003), rice (Khan and Komatsu, 2004), chickpea in response to dehydration stress (Pandey et al., 2008), *Medicago* under normal conditions (Repetto et al., 2008), as well as in *Arabidopsis* nuclear matrix preparations (Calikowski et al., 2003) supporting the concept about the presence of a nuclear translation process, which has been proposed to be a checking mechanism before mRNA exits to the cytoplasm (Repetto et al., 2008; Shaw and Doonan, 2005). The identification of different elements of translation (translation factors, ribosomal subunits) in the nuclear matrix gains relevance from the recent demonstration of nuclear translation (Iborra et al., 2001; Calikowski et al., 2003) and the association of ribosome components with the sites of transcription and nascent RNP complexes (Calikowski et al., 2003).

Energy metabolism

ATP synthase: is a ubiquitous membrane enzyme that plays a key role in biological energy metabolism. The membrane bound F1F0-type ATP synthase of mitochondria catalyses the terminal step in oxidative respiration generating ATP from the electrochemical gradient for cellular biosynthesis (Zhang et al., 2008). The structure of the enzyme is highly conserved in both prokaryotic and eukaryotic organisms (Walker et al., 1984). Orthologs for mitochondrial ATP synthase has been detected in *Arabidopsis* nucleus and other organelles (Heazlewood et al., 2003a; Zhang et al., 2008). Zhang et al., (2008) isolated and characterized both the gene and protein (annotated as AtMtATP6 and Atg46430 respectively) of the A. thaliana mitochondrial F1F0ATPase. Using northern blot analyses they showed that the expression of this gene in *Arabidopsis* suspension-cultured cells was induced by several abiotic stresses such as salt,

drought, and cold. Moreover, over-expression of this gene greatly improved the tolerance of transgenic *Arabidopsis* plants and yeast under salt, drought, oxidative and cold stresses (Zhang et al., 2008). Accordingly, Zhang and co-workers proposed that induction of the F1F0- ATPase plays a role in stress tolerance. Previously, Zhang et al. (2006) reported that this gene was induced by salt and osmotic stresses and the transgenic *tobacco* overexpressing this gene showed enhanced tolerance to salt stresses. According to Zhang et al., (2008), up regulation of the expression of this gene under various stresses is an attempt to maintain the activity of F1F0-ATPase under environmental stress. ATPases were also identified up-regulated in response to dehydration stress at less than 10% RWC in the nuclear proteome of the resurrection plant *Boea hydrometrica* (Jiang et al., 2007), as well during rehydration. This indicates that this protein may play an important role in providing energy for protective and repair reactions during dehydration and rehydration stresses (Jiang et al., 2007).

Unknown proteins

Five protein samples could not be matched to any protein using taxonomy limited and unlimited database search settings (spots 2, 5, 6, 7, and 15). Two other proteins were identified in *Arabidopsis* and sugar beet but with no homology to known functions in databases. These proteins together comprised the largest group (about 39%) in the up-regulated proteins in nucleus of *X. viscosa* in response to dehydration stress. Characterization of the functions of these proteins under dehydration stress could broaden our understanding of desiccation tolerance in plants.

In summary, this study has presented primary investigations of the protein profiles in the nucleus of the resurrection plant *X. viscosa* in response to dehydration stress. From a total of 438 proteins spots that were reproducibly detected and analysed, MS analysis of 18 dehydration-induced nuclear protein spots resulted in the identification of proteins associated with gene transcription and regulation, cell signaling, molecular chaperones and proteolytic type activities, protein translation, energy metabolism, and novel protein factors. Identification of known stress responsive proteins validates and confirms the reliability of approaches used in this study. This study revealed that *X. viscosa* nuclear proteome was sensitive to dehydration stress, and dehydration stress is controlled by multiple genes within the plant nucleus. Furthermore, this study showed that *X. viscosa* relies on readily inducible protection to combat desiccation. The protective mechanisms of desiccation tolerance utilized by *X. viscosa* appear to

involve signal perception genes, and modulating of appropriate gene expression encoding protective molecules. Finally, as non-desiccation tolerant species can not survive severe water loss as it appears that their defense systems are impaired by the over-generation ROS, misfold-proteins and osmotic disorders under dehydration stress, Therefore, we propose that stress tolerance is genetically encoded and *X. viscosa* has evolved a unique (enhanced) defense capacity with versatile and coordinated actions enough to provide protection for its cellular structures during dehydration and in the dried state.

CHAPTER 4

Proteomic analysis of nuclear proteins during dehydration using iTRAQ with 2DLC and Tandem mass spectrometry

4.1. INTRODUCTION

The analysis of dynamic proteome changes in cellular organelles is necessary to elucidate the molecular basis of desiccation tolerance in plants. Although 2DE-based proteomics has proven powerful for whole and subcellular proteomic analysis, it still suffers technical problems that need to be solved (Gygi et al., 2000; Park, 2004). It is costly, extremely laborious and time-consuming to set-up as it requires too many steps of samples preparation (Righetti et al., 2004; Park, 2004). In addition, 2DE very often does not resolve very large (> 100 kDa), and small proteins (< 10 kDa), basic proteins (pI >10), hydrophobic proteins, many insoluble and low-copy proteins are not detected (Rampitsch and Srinivasan, 2006; Celis and Gromov, 1999; Gorg et al., 2004; Rose et al., 2004).

Given the limitations of the 2DE method outlined above for performing comprehensive nuclear proteome analysis for *X. viscosa*, we additionally used isobaric tags for relative and absolute quantitation (iTRAQ) technology with two-dimensional liquid chromatography (2DLC) and nano-electrospray ionization tandem mass spectrometry (ESI-MS/MS) to complement the findings obtained using 2DE (chapter 3) so as to gain insight into the nuclear proteome of *X. viscosa*. iTRAQ is a recent powerful technique for the quantitative study of gene expression at the proteome level, and the principal advantages of 2DLC-MS/MS methods using iTRAQ labeling include the ability to retain post-translational modification (PTM) information and to conduct multiplex experiments in a single run under the same or different physiological conditions (Lin et al., 2005; Pierce et al., 2007). Moreover, it globally labels proteins and detects low abundant, acidic, basic, high and low molecular weight proteins (Shadforth et al., 2005; Ross et al., 2004; Chong et al., 2006; Gan et al., 2007). In this chapter a more thorough identification and analysis of the nuclear proteome of *X. viscosa* and its response to dehydration stress at 35% RWC is described using iTRAQ with 2DLC and nano ESI-MS/MS.

4.2. MATERIALS AND METHODS

4.2.1. Nuclei isolation, nuclear protein extraction and assessment of nuclei purity

Leaf samples were collected from *X. viscosa* experimental plants (section 2.2.1) at full turgor (100% RWC) and during drying at 35% RWC, and nuclei were isolated from 5 gm leaf samples as described previously (sections 2.2.3.2.1 and 2.2.3.2.2). The integrity of the isolated nuclei was confirmed by microscopic analysis, without and with the use of 4', 6-diamidino-2-phenylindole (DAPI) staining as described previously (section 2.2.4.). A chlorophyll assay was performed to assess possible chloroplast contamination of the nuclear fraction as described previously (section 2.2.4). Nuclear proteins were extracted from 120 mg nuclei as described previously (section 2.2.5.6). The enrichment of the nuclear fraction was confirmed using two specific anti-nuclear antibodies, the anti-H1, and anti-PCNA antibodies, and an anti-cytosolic/chloroplastic antibody, the anti-XvPrx2 antibody as described previously (section 2.2.7.2.).

4.2.2. iTRAQ labeling and mass spectrometry identification of the nuclear proteins

All reagents are analytical grade or equivalent. All iTRAQ reagents and buffers were of Applied Biosystems (Foster City, CA).

4.2.2.1. Labeling with multiplex iTRAQ reagents

A 2-plex iTRAQ labeling was conducted for each of the fully hydrated and dehydrated nuclear protein samples respectively using 45 µg for each sample (fully hydrated sample 45 µg X 2 same population; and dehydrated sample 45 µg X 2 same population). Individual protein samples were first reduced, alkylated and then labeled by iTRAQ reagents as follows: each protein pellet of both samples was first dissolved in 20 µl of 8 M urea, 300 mM triethylammonium bicarbonate (TEAB) followed by reduction with the addition of 2 µl of 45 mM DTT and incubation at 37^0 C for 20 minutes, alkylation with 4 ml of 100 mM IAN and incubation at ambient temperature for 20 minutes. The reduced and alkylated samples were trypsin digested. First the digest was preceded by the addition of deionized distilled water to reduce the urea to 2 M, and then 10 µl of 1 mg/ml Lysyl endopeptidase was added to each sample, incubated at 37^0 C for 5 hours, followed by the addition of 10 µl of 1mg/ml trypsin and

incubation overnight at 37^0 C. The 45 µg (in duplicate) digests of the controls were labeled with iTRAQ tags 114.1 and 115.1 respectively, and the 45 µg (in duplicate) digests of the dehydrated samples were labeled with iTRAQ tags 116.1 and 117.1 respectively (prior to this the 4-plex tags were prior dissolved individually in 70 µl ethanol). All tagged samples of control and dehydrated were allowed to react at ambient temperature for 2 hours, and then the four tagged samples were pooled together. The samples workflow and iTRAQ labeling scheme used for this study are illustrated in Figure 4.3. Subsequent sample processing, such as solution phase digestion, iTRAQ labeling, peptide fractionation, and desalting, was conducted in accordance to the manufacturer's specifications and guidelines (Applied Biosystems).

4.2.2.2. Cation exchange chromatography separation

The pooled samples mentioned above were acidified by the addition of 2 µl 1M phosphoric acid and peptides were eluted in multiple steps (~15 steps) through strong cationic exchange chromatography (SCX) using an Applied Biosystems CEX Vision Workstation. This system used PolySulfoethyl A column (2.1 X 200 mm, 5 µm, 300 A^0) (PolyLC Inc.). Samples was dissolved in 4 ml of SCX loading buffer (A: 25% v/v acetonitrile, 10 mM KH_2PO_4, pH 3.0, with phosphoric acid) loaded, and washed isocratically for 20 min at 0.5 ml/min to remove excess reagent. Peptides were eluted with a linear gradient of buffer (B: 25% acetonitrile, 10 mM KH_2PO_4, pH 3.0, plus 1M potassium chloride) over 118 min and twenty cation-exchange fractions were collected.

4.2.2.3. RP HPLC-MS/MS identification

The twenty fractions mentioned above were lyophilized to remove acetonitrile, and resuspended in 5 µl 70% formic acid, diluted to 15 µl with 0.1% TFA, and each of the processed SCX fractions was run via Reverse Phase LC ESI-MS/MS on an Applied Biosystems QSTAR Elite Mass Spectrometer. This system was interfaced with a RPLC Packing Ultimate workstation with a setup to pre-concentrate/desalt the samples on a 5 mm C18 LC Packings PepMap trap column and the RP-HPLC carry out at flow rate of 400 nl/min on an in-line 100 µm X 15 cm Waters Atlantics C-18 column. The HPLC conditions were as follows: 95% buffer A and 5% buffer B with the following linear gradient: 3 min 5% B; 43 min 37% B; 75 min 75% B; and 85 min 95% B. Buffer A consisted of 98% water, 2% acetonitrile, 0.1% acetic acid, and 0.003% TFA. Buffer B contained 80% acetonitrile, 20% water, 0.09% acetic acid, and 0.003%

TFA. Samples were co-eluted on the RP HPLC and have the same m/z values (isobaric) in the MS. The isobaric 4-plex peptides were fragmented simultaneously and represented in the second MS. The first MS (performing a survey scanning) detected the ions as they eluted from the reverse-phase column, it recorded the m/z and the relative intensity of all of the ions eluting at the moment. Then the MS performed individual selection of m/z values from the survey MS scan for further analysis. Peptide ions selected from the survey MS were fragmented in a collision cell using helium. The second MS spectrum contains the relative amount of each tag used for quantification and the peptide fragmentation pattern used to determine the amino acid sequence and post-transitional modification. The second MS scans have less noise than did the survey scans, which allows for accurate measurements of less abundant peptides.

4.2.2.4. Proteomic data interrogation

Data dependent acquisition was performed so that the mass spectrometer switched automatically from MS to MS/MS modes. Protein identification and quantitation on the resulting LC/MS and MS/MS spectra were performed first using the Paragon Algorithm (Applied Biosystems, USA), and the raw peptide identification results from paragon algorithm searches were post-processed by pro-group algorithm (Applied Biosystems, USA) for confidence threshold > 99% (P (less than) < 0.01) using the mass spectrometry data base (MSDB) on a GPS workstation. To exclude any contaminations, the data was also searched against all entries in the AGlallAA_contaminant database. Pro Group Algorithm software separately integrated the reporter ion peak areas and combined the search results in a table with both the identifications and ratios (fold-change). The following database analysis parameters were used: sample type: iTRAQ 4-plex (Peptide Labeled); Cys Alkylation: Iodaacetamide; Digestion: Trypsin; Instrument: QSTAR ESI; Special factors: Phosphorylation emphasis urea denaturation; Species: None selected; Quantitate table: checked; ID Focus: Biological modifications – search for over 170 potential modifications (with the fixed modification i.e., phosphorylations, amidations, pyro-glu, etc), Database: AGlallAA_contaminant_tdr.fasta; Search Effort: Thorough; Minimum Detected Protein Threshold [Unused ProtScore (Conf)]: 2 (99%). Other parameters such as tryptic cleavage, specificity, precursor ion mass accuracy and fragment ion mass accuracy are ESI built-in functions of Protein Pilot software.

The Pro-Group Algorithm software calculates a confidence percentage (the unused score) which reflects the probability that the hit is a false positive, meaning greater than 99%

confidence level, there is a false positive identification chance of less than 1%. While this software automatically accepts all peptides having an identification confidence level > 1%, only proteins having at least one peptide equal or above 99% confidence were initially recorded (Applied Biosystems AB/MDS Sciex. 2007). The low confidence peptides cannot give positive protein identification by themselves, but may support the presence of a protein identified using other peptides with higher confidence (Applied Biosystems AB/MDS Sciex. 2007).

4.3. RESULTS

4.3.1. Nuclei isolation, nuclear protein extraction and the assessment of nuclei purity

Nuclei were isolated from fully hydrated and dehydrated leaf samples at 35% RWC, this low dehydration point represents a distinct dehydration process where induction of late protection mechanisms is initiated (Mundree et al., 2000; Illing et al., 2005, Ingle et al., 2007). At 35% RWC *Xerophyta* species cease photosynthesis and degrade its chlorophyll (Ingle et al., 2007). An important criterion for compartment-specific proteome, such as the nucleus is the purity of the isolated compartment away from other cellular contaminants. The isolation of high-purity nuclei from plant is a difficult task as it might compromise the yield (Khan and Komatsu, 2004; Pandey et al., 2006). In this study, the nuclei were isolated from *X. viscosa* leaf samples without the use of sucrose and percoll density gradient. The integrity of the isolated nuclei was analysed by microscopic analysis without and with the use of DAPI staining. Photomicrograph of nuclei-enriched fraction is presented in Fig. 4.1. These results indicate that the isolated nuclei were highly purified. A chlorophyll assay was performed to assess possible chloroplast contamination of the nuclear fraction. The homogenized NIB supernatant, which contains cytoplasmic and chloroplast proteins retained most of the chlorophyll content, the chlorophyll content in the nuclear fraction was less than 0.9% when compared to the homogenized NIB supernatant (Fig. 2.6, chap. 2.). These results showed that the methodology used to isolate *X. viscosa* nuclei gave purer nuclei compared to the methodology used to isolate nuclei from chickpea seedlings (Pandey et al., 2006).

The nuclear proteins were extracted from the purified nuclei using phenol extraction, isopropanol precipitation and ethanol/guanidine hydrochloride, acetone wash as described previously (Fig. 2.11. chap. 2). Enrichment of the nuclear proteins and possible contamination with non-nuclear proteins were analysed using specific nuclear antibodies, anti-H1 antibody, and anti-proliferating cell nuclear antigen antibody (anti-H1 and pCNA antibodies respectively)

and anti-cytosolic/chloroplastic antibody, anti-*X. viscosa* preoxiredoxin type II antibody (anti-XvPrx2 antibodies). The nuclear proteins histone H1 and PCNA were found in the nuclear fraction, but not in the chloroplastic and cytosolic fractions. In contrast, the cytosolic/chloroplastic protein XvPrx2, which is not a nuclear protein, was not detected in the purified nuclear fraction (Fig. 2.17-2.19, chap. 2). These results, together, suggest that the nuclear preparation is enriched in nuclear proteins.

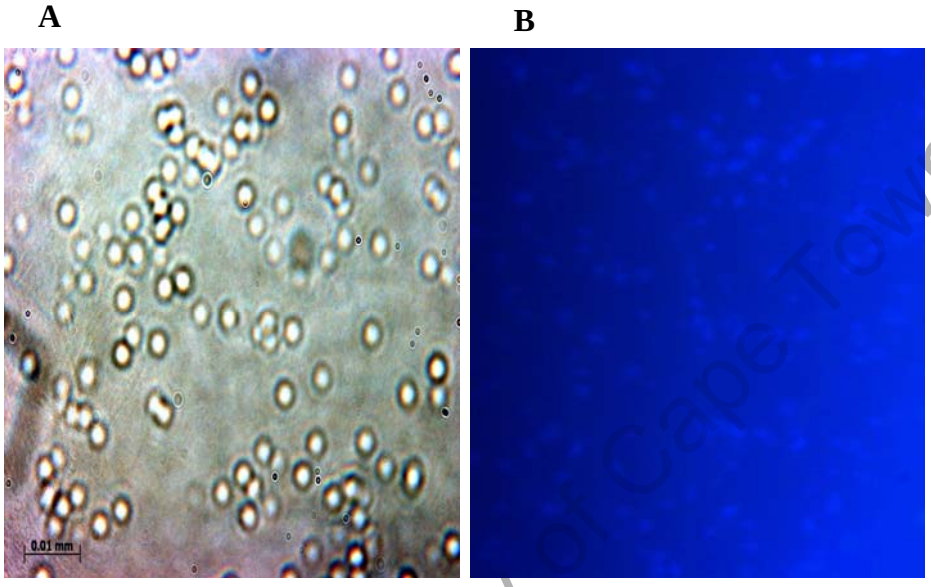


Figure 4.1. Representative micrographs of *X. viscosa* nuclei. (A) Represents unstained nuclei and (B) represents DAPI-stained nuclei. The bar is 10 µm.

4.3.2. Nuclear proteins identification using iTRAQ LC-MS/MS

The overall experimental design workflow of iTRAQ labeling and MS identification used in this study is presented in Fig. 4.2. The Pro Group Algorithm software used in this study identifies protein based only on unique evidences (peptide spectra) displayed by a sequence characteristic to that protein (Applied Biosystems AB/MDS Sciex. 2007). The core philosophy and unique feature of Pro Group Algorithm is that each new protein reported in a list must be a protein detected based on evidence not already explained by better proteins (Applied Biosystems AB/MDS Sciex. 2007). Evidence used to prove the detection of one protein is not used again to prove the detection of a second protein, the score is used only once (Applied Biosystems AB/MDS Sciex. 2007). This avoids using the same data multiple times to justify the detection of multiple proteins and prevents falsely reporting redundant proteins, but still allows

tracking of competitors (Applied Biosystems AB/MDS Sciex. 2007). This in contrast to other software such as Mascot algorithm, which uses total protein score (all peptide evidence for any proteins is counted again and again) to identify other proteins. The ProGroup algorithm software calculates a confidence percentage (the unused score) which reflects the probability that the hit is a false positive, meaning that at 99% confidence level, there is a false positive identification chance of about 1% (Applied Biosystems AB/MDS Sciex. 2007). While this software automatically accepts all peptides having an identification confidence level > 1%, only proteins having at least one peptide above 99% confidence were initially recorded. The low confidence peptides cannot give positive protein identification by themselves, but may support the presence of a protein identified using other peptides with higher confidence (Applied Biosystems AB/MDS Sciex. 2007).

Applying the above mentioned criteria, our proteomic approach was able to confidently and reproducibly (20 times) identify 128 proteins with confidence ≥ 95% ($\rho < 0.05$) from 985 (Tables, 4.1. and 4.3.). Hundred and twelve proteins were identified by at least 2 significant peptides (with confidence ≥ 99% ($\rho < 0.01$), and the remaining 16 proteins were identified with 1 significant tryptic peptide (peptide score 2.0). These gave a sequence confidence of 95%. In all the amino acid sequences were verified to be accurate based on *de novo* sequencing interpretation (Table 4.2.).

The relative quantification of the *X. viscosa* identified nuclear proteins was based on the ratio of the reporter ions corresponding to the dehydrated tryptic peptides (116.1 and 117.1) over the ratio of the reporter ions corresponding to the fully hydrated tryptic peptides (115.1 and 114.1). Proteins giving tryptic peptides with an average reporter ion ratio greater than 2 were classified as up-regulated and those with an average reporter ion less than 0.5 were classified as down regulated (confidence ≥ 99%, protein unused score equal or greater than 2.0 ($\rho < 0.01$). Analogous reporter ion ratio cut-off for differentially expressed proteins was used in other iTRAQ MDLC-MS/MS approaches (De Souza et al., 2005, De Souza et al., 2007, Garbis et al., 2008).

Eighty four proteins (66%) of the 128 identified protein were shown to have consistent expression levels (no significant change in expression), with the mean dehydrated/fully hydrated reporter ion ratios corresponding to the tryptic peptides in the range between 1.99±0.15 to 0.55±0.01 (Table 4.1). The tryptic peptides of the remaining 44 proteins (34%) were showed significant change in expression with the mean dehydrated/fully hydrated reporter

ion ratios corresponding to the tryptic peptides of either greater than 2 or less than 0.5. Of the proteins showing significant change in expression, thirty proteins (23%) were shown to be up regulated in response to dehydration stress with the mean dehydrated /fully hydrated reporter ion ratios corresponding to the tryptic peptides of greater than 2, the remaining 14 proteins (11%) were shown to be down regulated with the mean dehydrated /fully hydrated reporter ion ratios corresponding to the tryptic peptides less than 0.50 (table 4.1.).

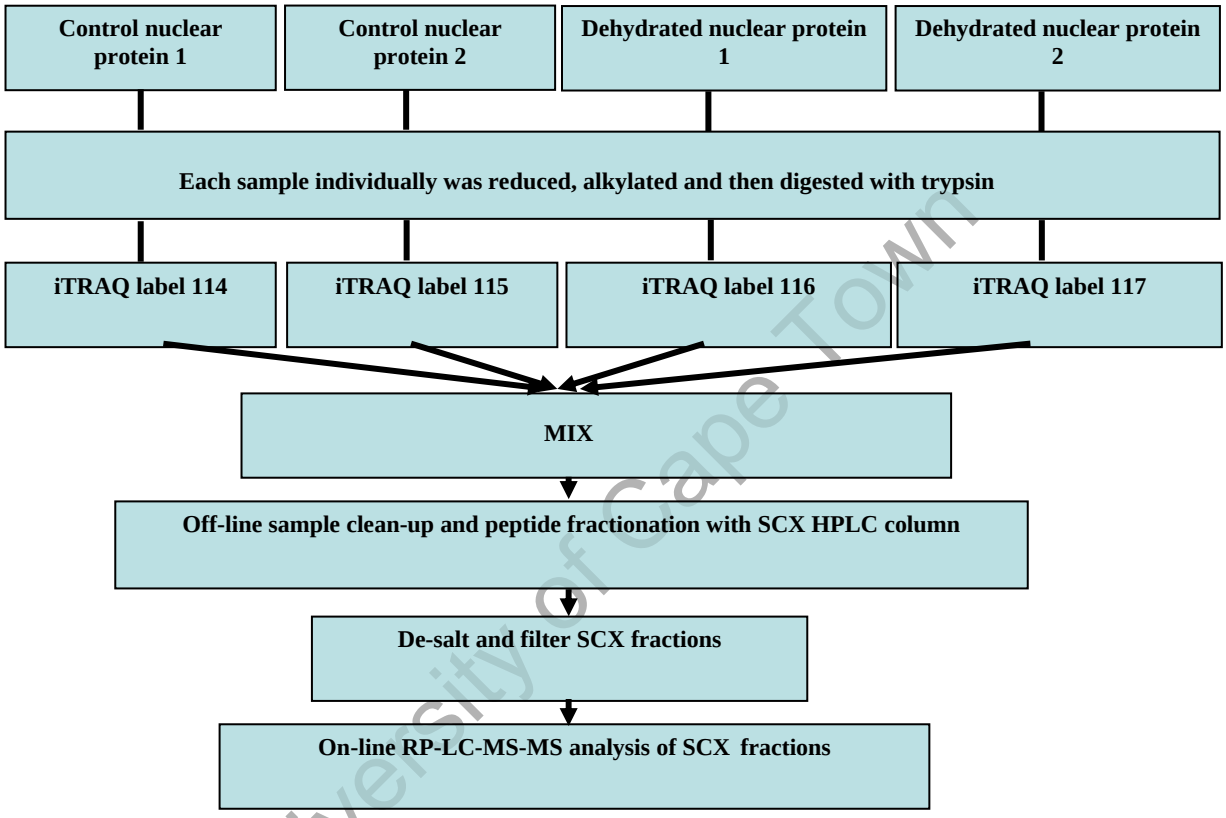


Figure 4.2. Depiction of the experimental design and workflow used for the 4-plex comparative analysis of the *X. viscosa* nuclear proteins of the fully hydrated and dehydrated samples.

4.3.3. Overall functional classification of the identified nuclear proteins

To understand the nuclear proteome of *X. viscosa* and its role in acquisition of desiccation tolerance, the functions of the identified proteins are discussed in detail below and the proteins were sorted into eleven categories according to their matched functions as shown in Fig. 4.3. Of the 128 proteins identified following iTRAQ labelling and mass spectrometric analysis, the most abundant category was classified as being involved in (i) gene regulation, 22%; (ii) protein translation, 18%; (iii) miscellaneous, 15%; (iv) cell signaling, 10%; (v) unknown, 9%; (vi) ROS pathway related, 9%; (vii) protein degradation, 8%; (viii)

nucleocytoplasmic transport, 3%; (ix) molecular chaperones, 3%; (x) energy metabolism, 2%; and (xi) compatible solutes, 1%. Proteins with no assigned function occupied the fifth place (9%), as the function of these proteins are not yet identified or clearly known and are therefore considered to be *X. viscosa* nuclear-specific proteins. Thus, using iTRAQ and de novo sequencing tandem mass spectrometry, followed by database searching, nuclear proteins were comprehensively identified, and it was observed that the proteins were involved in a variety of functions. These results demonstrate that identification of proteins from an organism with un-sequenced genome such as *X. viscosa* can be efficiently achieved by comparing peptides of interest to orthologous proteins of species with well characterized genomes (Shevchenko et al., 2001; Liska and Shevchenko, 2003).

It has been reported that late dehydration responsive proteins such at 35% RWC and specifically up-regulated, were unique to desiccation tolerant species, and thought to be associated directly with acquisition of desiccation tolerance in plants (Ramanjulu and Bartels, 2002; Jiang et al., 2007; Ingle et al., 2007). Therefore, we additionally categorized the up-regulated proteins into nine groups according to their matched functions so as to get more insight into their roles in acquisition of desiccation tolerance:

(i) twenty three percent of the up-regulated proteins were shown to be involved in gene regulation, this comprises the largest category, followed by (ii) twenty percent proteins involved in ROS pathways, (iii) ten percent were proteins involved in molecular chaperone type activities, (iv) thirteen percent were shown to be involved in cell signaling, (iv) ten percent were proteins involved in nucleocytoplasmic transport, (v) seven percent were proteins involved in protein translation. (vi) seven percent were proteins with miscellaneous functions, (viii) seven percent were proteins with unknown functions, and (ix) three percent were proteins involved in synthesis of compatible solutes.

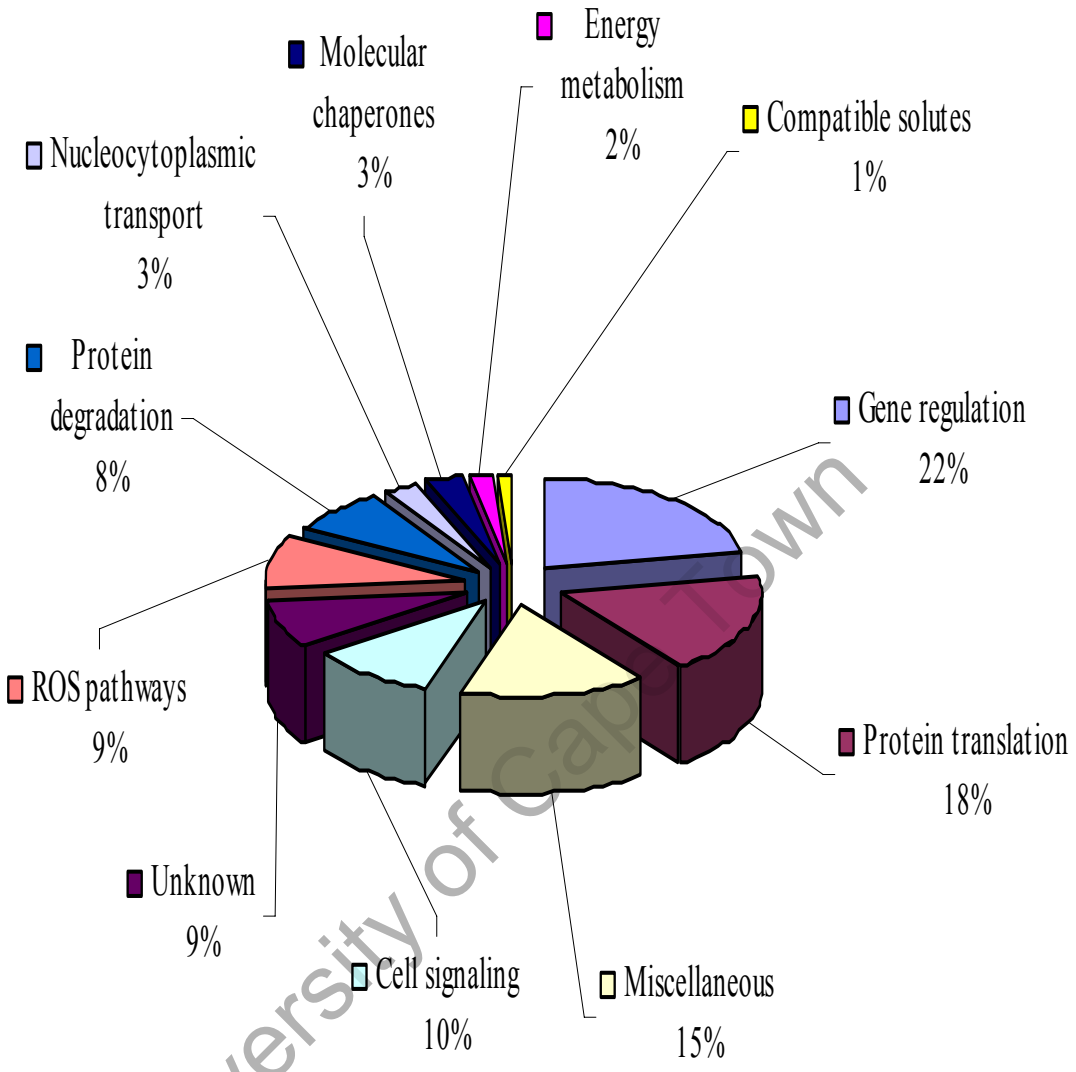


Figure 4.3. Functional category distribution of the identified nuclear proteins of the resurrection plant *X. viscosa* in response to dehydration stress.

Table 4.1. iTRAQ with LC ESI MS-MS/MS identification of *X. viscosa* nuclear proteins during dehydration and functional classification.

Functional Category	Protein Name	Unused Protein Score	Primary Protein ID	Percent Coverage	Number of unique peptides	Mean ratio (±SD)*
	I. Up Regulated Proteins					
ROS pathways	1. NADPH:proto-chlorophyllide oxido reductase B	2.10	gi|968977	46.13	3	17.07±2.0
Nuclearcytoplasmic Transport	2. GTP-binding protein GB2	2.01	gi|7270538	26.54	2	8.99±0.17
Nuclearcytoplasmic Transport	3. Dynamin-related protein 1D	2.11	gi|68566307	13.07	4	7.20±2.5
Cell signaling	4. Casein kinase α1	4.19	gi|79332762	22.07	3	5.43±0.06
Compatible solute	5. UDP glucose 4-epimerase	2.01	gi|8698725	15.1	2	3.83±0.19
ROS pathways	6. NADPH:protochloro-phyllide O. Reducase A	4.32	gi|968975	40.49	7	3.68±0.29
Gene regulation	7. Oligosaccharyl transferase like	2.03	gi|21553942	18.76	3	3.61±0.08
ROS pathways	8. F21D18.15, 1-Cys peroxiredoxin PER1	2.00	gi|8778528	19.9	1	3.32±0.18
Cell signaling	9. Calcineurin B-like protein 2	2.00	gi|9758619	22.1	1	3.21±0.54
Miscellaneous	10. ATP binding	2.71	gi|145334803	27.02	4	2.74±0.03
Cell signaling	11. Putative disease resistance protein	3.26	RRRRRgi|29	15.1	5	2.74±0.17
Molecular chaperone	12. 17.6 kDa class 1 Heat shock protein	2.99	gi|8671873	34.24	3	2.69±0.22
ROS pathways	13. Putative ferredoxin NADP+ reductase	4.15	gi|20465661	28.89	4	2.64±0.05
ROS pathways	14. Gdp-Mannose-3" , 5"-Epimerase	2.05	gi|83754659	17.41	2	2.54±0.04
Nucleocytoplasmic transport	15. GTP-binding protein	4.74	gi|3249104	50.26	4	2.50±0.33

Molecular chaperone	16. Heat shock protein 81-2	11.53	gi|9758623	20.46	7	2.42±0.09
Molecular chaperone	17. Heat shock protein 70	4.16	gi|6746592	20.89	3	2.33±0.17
Protein translation	18. Translation e-factor eEF-Tu	7.52	gi|7268831	40.13	7	2.29±0.10
ROS pathways	19. Malate dehydrogenase (NAD)	2.06	gi|145332399	25.32	3	2.29±0.29
Protein translation	20.At1g07930/T6D22_3= eukaryotic EF-1-α	32.22	gi|15081765	61.92	25	2.23±0.11
Gene regulation	21. Alcohol dehydrogenase (EC 1.1.1.1) class III	2.00	gi|9758553	5.8	1	2.23±0.08
Gene regulation	22. Actin Putative	2.04	gi|30688915	10.33	2	2.21±0.00
Gene regulation	23. Histone H4	20.78	gi|9758835	68.93	13	2.21±0.03
Gene regulation	24. Actin 1	9.35	gi|79324605	37.93	7	2.13±0.02
Gene regulation	25. Histone H3.2	10.68	gi|145334271	48.17	11	2.10±0.05
Gene regulation	26. Putative Histone H2A.5	6.24	gi|75306451	53.33	4	2.10±0.03
Unknown function	27. Putative protein	2.58	RRRRRgi|67	9.35	3	2.10±0.02
Unknown function	28. Unnamed protein product	2.00	gi|9293904	33.7	1	2.06±0.11
Nucleocytoplasmic transport	29. F28C11.12= ADP-ribosylation factor	2.92	gi|8778579	20	2	2.02±0.10
Cell signaling	30. Patatin-like protein	2.00	gi|7270656	17.6	1	2.01±0.08
	II. Proteins showing consistent expres. levels					
Unknown function	31. Putative protein	2.01	gi|7019676	20.04	2	1.99±0.15
Protein degradation	32. 26S proteasome AAA-ATPase subunit RPT3	2.01	gi|8777330	27.21	2	1.94±0.02
Gene regulation	33. Pattern formation protein, putative	2.17	gi|15241777	9.7	2	1.87±0.03

Gene regulation	34. DNA-binding protein	4.00	gi|601843	13.16	2	1.77±0.12
Gene regulation	35.Glyceraldehyde-3-P-DH C subunit	6.59	gi|6721173	43.2	5	1.76±0.04
Gene regulation	36. DNA gyrase subunit B-like	2.27	gi|9955568	20.53	4	1.74±0.03
Protein degradation	37. Ubiquitin-Like protein SMTB	2.02	gi|9758113	24.27	2	1.71±0.16
Gene regulation	38. Peptidylprolyl isomerase ROCI	2.60	gi|7270856	22.67	2	1.67±0.07
Cell signaling	39. Putative phosphor-glycerate kinase	10.35	gi|23198084	51.46	10	1.66±0.05
Unknown function	40. Unknown protein	2.17	gi|5306242	9.26	2	1.63±0.04
Gene regulation	41. Probable histone 2AXa (HTA5)	3.13	gi|75276926	26.76	3	1.64±0.02
Cell signaling	42. Calnexin homolog 1 precursor	4.06	gi|231683	17.92	4	1.63±0.03
Gene regulation	43. Fructose bisphosphate Aldolase like -protein	2.00	gi|7529717	32.1	1	1.59±0.07
Gene regulation	44. Small nuclear ribonucleoprotein	2.14	gi|8778615	34.3	5	1.56±0.03
Cell signaling	45. Magnesium chelatase subunit of Reductase	2.02	gi|9758940	34.52	2	1.56±0.04
ROS pathways	46. Peroxiredoxin Q-like protein	4.29	gi|9279611	43.98	3	1.53±0.01
Cell signaling	47. 14-3-3-like protein GF14 upsilon	4.27	gi|9755651	19.03	5	1.51±0.09
Cell signaling	48. 14-3-3 protein GF14=NCT Ran	2.01	gi|9759623	27.64	2	1.48±0.09
Gene regulation	49. Histone H2A	4.48	gi|75313476	41.18	4	1.46±0.08
Protein degradation	50. AtCIpC Caseino-lytic protease C	10.55	gi|7576225	49.89	9	1.46±0.08
Gene regulation	51. At5g59910= H2B11	5.68	gi|98960893	36	6	1.42±0.13

Unknown function	52. Uncharacterized protein At2g34160	2.00	gi|73921087	22.3	1	1.39±0.08
Protein degradation	53. (20S proteosome) peptidase complex	2.02	gi|79314743	32.1	2	1.33±0.88
Miscellaneous	54. V-ATPase subunit D	2.57	gi|7630068	12.26	2	1.32±0.04
Unknown function	55. Unknown protein	3.74	gi|6714442	22.75	7	1.31±0.08
Protein translation	56. 40S ribosomal protein S15A	4.15	gi|9757906	39.23	3	1.31±0.01
Miscellaneous	57. F22C12. Probable v-type H^+ ATPase	2.11	gi|6692094	27.62	3	1.31±0.02
Gene regulation	58. DEAD-box ATP-dependant RNA helicase 52	2.30	gi|75335836	39.94	8	1.29±0.01
Gene regulation	59. At5g42270/ K5J14_7 =DNA binding	7.35	gi|22022513	37.07	7	1.26±0.02
Miscellaneous	60. Germin-like protein (GLP10)	2.00	gi|6899900	23.2	1	1.25±0.05
Gene regulation	61. Retroelement Pol Polyprotein-like	2.14	gi|8978263	14.86	3	1.23±0.04
Miscellaneous	62. T4012.24= V-type ATPase	2.06	gi|6721109	24.42	4	1.23±0.04
ROS pathways	63. Manganese superoxide dismutase	2.0	gi|79313181	18.7	1	1.19±0.02
Gene regulation	64. Arginine/serine -rich-splicing factor RSP31	2.23	gi|26454661	60.98	4	1.19±0.02
Protein translation	65. Protein synthesis initiation factor-like	2.23	gi|7576200	27.52	8	1.17±0.01
Miscellaneous	66. Glutamine synthetase	2.34	gi|9757970	41.29	2	1.13±0.09
Cell signaling	67. Phospholipase D	2.21	RRRRRgi|223	8.03	2	1.12±0.11
ROS pathways	68. Copper zinc superoxide dismutase	2.08	gi|125662843	24.34	2	1.12±0.22

Gene regulation	69. RNA helicase	4.25	gi|3776021	34.24	4	1.12±0.03
Miscellaneous	70. Carbonic anhydrase 2	2.00	gi|9755742	7.6	1	1.10±0.11
Unknown function	71. At3g49560= Unknown protein	2.5	gi|892133231	22.22	3	1.10±0.01
Gene regulation	72. GAPDH	6.66	gi|8698736	36.59	8	1.09±0.06
Gene regulation	73. RNA helicase	2.08	gi|3776005	34.15	3	1.08±0.02
Protein degradation	74. Putative Polyubiquitin	4.74	gi|28436479	58.15	7	1.05±0.00
Protein translation	75. 40S ribosomal protein S6	2.59	gi|7671467	26.91	3	1.04±0.05
Miscellaneous	76. Ribulose bisphosphate carboxylase	8.11	gi|4204274	60.56	8	1.04±0.02
Miscellaneous	77. Anion-transporting ATPase	2.05	gi|30681260	30.66	4	1.02±0.02
Gene regulation	78. F1E22.8= Small nuclear Ribonucleoprotein	2.14	gi|6686407	32.42	5	1.01±0.26
Energy metabolism	79. Mitochondrial F1 ATP synthase β subunit	8.67	gi|17939849	48.73	12	1.00±0.09
Protein degradation	80. Putative prohibitin	5.58	gi|4586035	86.01	5	0.99±0.00
Protein translation	81. 60S Ribosomal protein L18A	3.94	gi|9294382	16.85	4	0.97±0.03
Molecular chaperone	82. HSP 70 kDa	2.72	gi|9294373	24	4	0.97±0.02
Protein translation	83. 60S ribosomal protein L13	2.1	gi|21537149	20.39	2	0.95±0.16
Protein degradation	84. DegP protease precursor	2.93	gi2565436	36.38	4	0.93±0.01
Unknown function	85. Unknown protein	4.59	gi|24030464	50.18	3	0.92±0.03
Miscellaneous	86. Cytochrome f	3.62	gi|7525046	13.75	3	0.91±0.08

Unknown function	87. Unnamed protein product	2.22	gi|9758504	18.1	2	0.90±0.03
Cell signaling	88. 14-3-3-like protein GF14 phi	2.00	gi|2232146	19.9	1	0.86±0.03
Protein degradation	89. ATP-dependent Clp protease	2.09	gi|9758239	40.04	4	0.82±0.03
Protein translation	90. Ribosomal protein	3.84	gi|806279	24.87	3	0.78±0.05
Nucleocytopalsmic transport	91. Dynamin-2A	3.39	gi|68566309	26.26	6	0.78±0.08
ROS pathways	92. Putative Glycosyltransferase 4	2.43	RRRRRgi|465	8.58	3	0.77±0.05
Miscellaneous	93. Citrate synthase	4.68	gi|145339693	26.72	4	0.76±0.08
Miscellaneous	94. ATP binding / H^+ ion Transporting ATP synthase	6.00	gi|79321468	34.03	3	0.75±0.02
Energy metabolism	95. ATP synthase CF1 α subunit	13.59	gi|7525018	54.44	13	0.75±0.06
Unknown function	96. Unnamed protein product	4.25	gi|2102657	28.86	4	0.74±0.00
Protein translation	97. 40S ribosomal protein	5.23	gi|9759565	41.44	4	0.73±0.02
Cell signaling	98. Putative protein kinase	2.06	RRRRRgi|73	7.15	3	0.71±0.08
Cell signaling	99. At4g09000/ Probable 14-3-3-like protein	2.00	gi|87116612	20.2	1	0.71±0.19
Gene regulation	100. Small nuclear riboprotein Sm-D1	2.04	gi|7269769	37.93	2	0.69±0.04
Miscellaneous	101. NADH-ubiquinone oxidoreductase	2.09	gi|41019517	59.6	2	0.65±0.00
Protein translation	102. 60S Ribosomal protein L2	10.57	gi|4406816	11.05	10	0.64±0.02
Protein translation	103. 50S ribosomal protein L27	2.00	gi|21553582	34.2	1	0.64±0.01
Protein translation	104. 40S Ribosomal protein	3.70	gi|27808637	59	4	0.64±0.14

Protein translation	105. At3g09680= 40S ribosomal protein S23	2.08	gi|98960873	25.35	2	0.62±0.01
Protein translation	106. 40S Ribosomal protein S10-3	2.26	gi|84028256	45.25	3	0.62±0.05
Cell signaling	107. Putative spliceosome associated protein	2.24	gi|4218014	14.88	2	0.59±0.02
Protein translation	108. At5g39850= 40S ribosomal protein S9	3.25	gi|90568002	53.3	3	0.58±0.07
Protein degradation	109. 20S Proteasome α subunit A2	2.15	gi|79322198	30	3	0.57±0.00
Protein translation	110. 60S ribosomal protein L10A	2.00	gi|30680605	25.5	1	0.57±0.00
Cell signaling	111. Nacent polypeptide-associated complex alph-like protein 3	2.03	gi|71151986	45.91	2	0.57±0.00
Protein translation	112. Ribosomal protein CL9	2.01	gi|9798392	24.37	2	0.56±0.02
Protein translation	113. 40S ribosomal protein S17	2.30	gi|9758455	26.95	2	0.55±0.04
Miscellaneous	114. Rubisco large subunit	26.22	gi|7525041	43.01	20	0.55±0.01
	III. Down Regulated Proteins					
Protein translation	115. Putative S-phase-specific ribosomal protein	5.4	gi|7270417	23.66	3	0.49±0.04
Protein translation	116. 30S Ribosomal protein S5	2.05	gi|75101015	29.37	2	0.48±0.01
Protein translation	117. Ribosomal protein 5B	2.26	gi|79324564	45.41	3	0.48±0.02
Miscellaneous	118. T1N15.8; Probable Gutamine sythetase	2.00	gi|8778687	14.7	1	0.47±0.04
Miscellaneous	119. Putative glutamine synthetase	5.68	gi|28393681	41.81	7	0.45±0.03
ROS pathways	120. Catalase	11.26	gi|7270460	47.36	11	0.43±0.01
Unknown function	121. Hypothetical protein	7.37	gi|20198280	36.68	6	0.40±0.02

Protein translation	122. 40S Ribosomal protein	4	gi|464720	81.25	2	0.37±0.02
Protein translation	123. 40S ribosomal protein S19-like	2.00	gi|21555157	14.3	1	0.33±0.01
Unknown function	124. Hypothetical protein	2.01	gi|7269239	25.32	2	0.30±0.02
Miscellaneous	125. NADP-specific isocitrate Dehydrogenase	2.82	gi|6227018	14.15	3	0.29±0.11
Miscellaneous	126. Glycolate oxidase	6.23	gi|62320779	31.06	7	0.28±0.01
Miscellaneous	127. Glycolate oxidase	6.04	gi|62320779	31.1	2	0.26±0.03
Miscellaneous	128. Cytochrome b559	2.01	gi|114152861	48.19	2	0.19±0.02

*****Mean ratio corresponds to the protein reporter ion intensity originating from dehydrated nuclear protein sample (116.1 and 117.1) relative to fully hydrated nuclear protein sample (114.1 and 115.1) with inter assay significance $P < 0.01$. The ± SD was determined from $N = 4$ measurements (2 data pairs corresponding to dehydrated/fully hydrated reporter ion ratio (116:114; 116/115; 117/114; 117/115) for both dehydrated and fully hydrated samples. The number and identity of the unique peptides observed for each protein was reproducibly observed in both duplicate in the 4-plex experiments of both dehydrated and fully hydrated nuclear protein samples. Tryptic peptides observed for a subset of the 4-plex experiments were excluded. Unused protein score 2.0 (confidence ≥ 99%, $\rho < 0.01$).

Table 4.2. Tryptic peptides identified by iTRAQ 2DLC ESI MS/MS of *X. viscosa* nuclear proteins in response to dehydration stress of the proteins listed in table 4.1.

Protein name	Fragment evidence and ion score*	Protein Score*
1. NADPH: proto chlorophyllide oxidoreductase B	{WNVIMAC[CAM]RDFLK[IT4] (2.0); VWEISEK[IT4] (0.05); FGASITDQIK[IT4](0.05)}	2.10
2. GTP-binding protein GB2	{GAAGALLVYDITRR (2.0); AVS[Pho]K[IT4]EEGQ[Dea]Q[Dea]FAK[IT4] (0.01)}	2.01
3. Dynamin-related protein 1D	{SSVLESIVGRDFLPR (2.0); EYFETSPDYGHLAT[Pho]R (0.07); LDLMDK[IT4]GTNALDVINGR (0.02); TF[Oxi]GVLT[Pho]K[IT4]LDLMDK[IT4]GTNAL (0.02)}	2.11
4. Casein kinase α1	{LIDWGLAEFYHPGK[IT4] (2.0); YQ[1Me]L[1Me]ELDPQLEALVGR (2.0); LLLLC[CAM]AVLALR[CRM] (0.19)}	4.19
5. UDP glucose 4-epimerase	{FDAVIHFAGLK[IT4] (2.0);LFLEEIAR[CRM]D[Pho]IQ[Dea]K[IT4](0.01)}	2.01
6. NADPH: protochlorophyllide oxidoreducase A	{SGVYWSWNK[IT4](2.0); R[Dea]LAQ[Dea]VVADPSLTK[IT4]1.22); DSY[Oxi]TVM[CRM]HLDLASLD[1Me]SVR (0.77); R[Oxi]LIIVGS[Pho]TGN[Oxi]TNTLAGN[Dea]VP (0.15); LAQ[1Me]VVADPSLTK[IT4] (0.15); MALQAASLVS[Pho]SAFSVRK[IT4] (0.02); MALQAASLVSSAFSVRK(0.010}	4.32
7. Oligosaccharyl transferase like	{TK[IT4]IEAPVLFR (2.0); QLTGSSISLVSVMQAR (0.02); VVISGSVQLFSDR[Oxi]LIR[Oxi]SGVQK[IT4] (0.01)}	2.03
8. F21D18.15, 1-Cys peroxiredoxin PER1	{LLGLSC[CAM]DDVQ[Dea]SHK[IT4](2.0)}	2.00
9. Calcineurin B-like protein 2	{ESLFADRVFDLFDTK[IT4](2.0)}	2.00
10. ATP binding	{ILVLAATNRPFDLDEAIIR (2.0); NASAASDISSIS[Pho]S[Pho]R[2Ox]S[Pho]SSSVSASRK[IT4] (0.62); LN[Oxi]K[IT4]LSGP[Oxi]VLILGSR (0.08); LFLQSLY[Pho]K[IT4] (0.01)}	2.71
11. Putative disease resistance protein	{LNRM[Oxi]K[IT4]IFTSR[Dea]DLP[Oxi]LK[IT4] (2.0); GLWLFR(1.22); RLSS[Pho]LGN[Dea]IGR[CRM]R[Oxi]LEPLDEVR(0.02); R[Dea]IC[CAM]TGLENK[IT4] (0.01); TK[IT4]NP[Oxi]SQM[Oxi]LK[IT4](0.01)}	3.26

12. 17.6 kDa class 1 Heat shock protein	{FRLPENAK[IT4](2.0); ETPEAHVFK[IT4] (0.96); SN[Oxi]ENEEK[IT4]N[Dea]DK[IT4]WHR (0.03)}	2.99
13. Putative ferredoxin NADP+ reductase	{LRLYSIASSAIGDFGDSK[IT4](2.0);RLVYTND[1Me]GGE[CAM]IVK[IT4]2.0); ES[Pho]K[IT4]K[IT4]Q[Dea]EEGIVVN[Dea]K[IT4] (0.14); EM[DTM]LMPK[IT4] (0.01)}	4.15
14. Gdp-Mannose-3" , 5"- Epimerase	{ISITGAGGFIASHIAR (2.0); VVGT[Pho]QAPVQLGS[Dhy]LRAADG (0.05)}	2.05
15. GTP-binding protein	{AFDLGGHQIAR (2.0); LVQHQPTQH[AAS]PTSEELSIGK[IT4](2.0); WLSQYIK[IT4]0.72); TTLLHMLK[IT4](0.02)}	4.74
16. Heat shock P81-2	{EIFLR[Dea]E[Dhy]LISN[Dea]SSDALDK[IT4](2.0); IRFESLTDK[IT4](2.0); LDGQ[1Me]PELFIHIIPDK[IT4](2.0); RAPFDLFDTK[IT4](2.0); SK[IT4]LDGQ[1Me]PELFIHIIPDK[IT4](2.0); LGIHEDSQNR(1.52); ELISNS[Pho]SDALDK[IT4]IRFESL (0.01)}	11.53
17. H shock protein 70 kDa	{FEELC[CAM]SDLLDR[2Me]VR[Dea]T(2.0); QFAAEEISAQVLRK[IT4](2.0); FIGRRMNEVAEESK[IT4](0.16)]	4.16
18. Translation e-factor eEF-Tu	{K[IT4]YDEIDAAPEER(2.0); VGETVDLVGLRETR (2.0); M[Oxi]VMPGDRVK[IT4] (1.40); EHILLAK[IT4] (1.22); IMNDK[IT4]DEESK[IT4] (0.82); DGAILVVS[Pho]GADGPMPQ[Dea]TK[IT4](0.07); NM[Oxi]ITGAAQMDGAILVVSGADGPMPQ[Dea]TK[IT4](0.01)}	7.52
19. Malate dehydrogenase (NAD)	{RTQDGGTEVVEAK[IT4] (2.0); QGLLRR[Dea]GFASES[Pho]VPDRK[IT4] (0.05); SGGSARS[Pho]RATLR (0.01)}	2.06
20. At1g07930/T6D22_3= eukaryotic EF-1-α	{DM[Oxi]RQTVAVGVIK[IT4](2.0); EHALLAFTLGVK[IT4](2.0); GITIDIALWKIET[Pho]TK[IT4](2.0); GPTLLEALDQIN[HNc]EPK[IT4]R[CRM]PL(2.0); IGGIGTVPVGRVET[Dhy]GMIK[IT4](2.0); MT[1Me]PTK[IT4]PM[Oxi]VVETFSEYPPLGR(2.0); P[2Ox]K[IT4]FLK[IT4]N[Dea]GDAGM[Oxi]VK[IT4](2.0); PLRLPLQDVYK[IT4](2.0); STNLDWYK[IT4](2.0); STTTGHLIYK[IT4](2.0); SVEMH[Oxi]H[Oxi]ESLLE[KXX]ALPGDNVGFNVK[IT4](2.0); VGYNP[Oxi]DK[IT4]IPFVPISGFEGDNM[Oxi]IER(2.0); YY[Oxi]C[CAM]T[Pho]GIDAPGHRDFIK[IT4](2.0); EPK[IT4]F[Oxi]LK[IT4]N[1Me]GDAGMVK[IT4](1.7); YC[CAM]T[IT4]GIDAPGHRDFIK[IT4](1.4); EVSSYLK[IT4](0.85); K[IT4]VGYNPDK[IT4]IPFVPISGFEGDN[Dea]M[DTM]IER(0.72); YC[CAM]T[IT4]GIDAPGHR(0.60); VETGM[CRM]IK[IT4]PGMVVT[Dhy]F[Oxi]APTGLTTEVK[IT4](	32.22

	0.57); RGYVAS[Dhy]NSK[IT4]DDPAK[IT4](0.17); GPTLLEALDQIN[Dea]EPK[IT4]R[CRM]PLD(0.10); VAS[Pho]NS[GCn]K[IT4]DDPAK[IT4](0.04); SFK[IT4]YAWVLDK[IT4](0.04); GY[Oxi]VAS[Pho]N[Dea]SK[IT4]DDPAK[IT4](0.01); VIER[CRM]FQ[Dea]K[IT4]EAAEM[Oxi]NK[IT4](0.01)}	
21. Alcohol dehydrogenase (EC 1.1.1.1) class III	{K[IT4]FGVN[Dea]EFVNPK[IT4](2.0)}	2.00
22. Actin Putative	{VAPEEHPVLLTEAPLNPK[IT4](2.0); IMM[DTM]ERGYTS[Pho]AEREVVR (0.04)}	2.04
23. Histone H4	{DAVTYTEHAR (2.0); DNIQGITK[IT4]PAIR (2.0); IFLENVIRDAVTYTEHAR (2.0); ISGLIYEETRGVLK[IT4](2.0); RISGLIYEETR (2.0); RISGLIYEETRGVLK[IT4] (2.0); TVTAMDVVY[IT4]ALK[IT4] (2.0); TVTAMDVVYALK[IT4]R (2.0); VVLRDNIQGITK[IT4] (2.0); VLRDNIQGITK[IT4]PAIR (2.0); VLRDNIQGITK[IT4]PAIRR (0.55); DNIQGITK[IT4]PAIRR(0.22); IS[Pho]GLIYEET[IT4]R(0.01)}	20.78
24. Actin 1	{AVFPSIVGRPR (2.0); GYSFTTTAEREIVR (2.0); LDLAGRDLTDALMK[IT4] (2.0); VAPEEHPILLTEAPLNPK[IT4](2.0); IWHHTFYNELR(0.77); GYSFTTTAEREIVRDIK[IT4] (0.55); DLAGRDLT[Dhy]DALM[Oxi]K[IT4]ILT[Pho]ER[Orn](0.03)}	9.35
25. Histone H3.2	{EIAQDFK[IT4]TDLR(2.0); LVREIAQDFK[IT4](2.0); STELLIRK[IT4](2.0); YRPGTVALREIR(1.4); YRPGTVALR (1.3); RVTIMPK[IT4] (0.92); DIQLARR (0.85); YRPGTVALREIRK[IT4](0.14); GTVALREIR[Orn]K[IT4](0.04); LPFQR(0.02); R[Dea]TK[IT4]QT[Pho]ARK[IT4]STGGK[IT4](0.01)}	10.68
26. Putative Histone H2A.5	{GVTIAS[HNc]GGVLPNINPVLLPK[IT4](2.0); LH[Oxi]GVT[Pho]IASGGVLPN[Dea]INPVLLPK[IT4] (2.0); LLHGVT[Dhy]IAS[dHx]GGVLPNINPVLLPK[IT4](2.0); LH[Oxi]GVT[Pho]IASGGVLPN[Dea]INPVLLPK[IT4]K[IT4](0.24)}	6.24
27. Putative protein	{GAVIYINTT[Pho]SPFGMAK[IT4] (2.0); LRQIS[Pho]PPPSNN[Dea]ALR(0.44); RR[Oxi]TTTT[Pho]PAAPLQSSSSPN[Dea]VPVGV (0.14)}	2.58
28. Unnamed protein product	{VAEIQNEGLGEHR(2.0)}	2.00
29. F28C11.12= ADP-ribosylation factor	{MLNEDELRDAVLLVFANK[IT4] (2.0); LGLHSLR(0.92)}	2.92
30. Patatin-like protein	{LHQTLTNIVIPTFDIK[IT4](2.0)}	2.00
31. Putative protein	{LFNDELTLDNISRPR (2.0); SLSSVK[IT4](0.01)}	2.01

32. 26S proteasome -- RPT3 AAA-ATPase subunit	{FDAQTGADREVQR (2.0); LVFQVC[CAM]TSK[IT4](0.01)}	2.01
33. Pattern formation protein, putative	{FLQAESLQQLVNSLIR[Dea] (2.0); T[Pho]GWRTIISLLSIT[IT4]ARH[IT4](0.17)}	2.17
34. DNA-binding protein	{AAVEAQLRK[IT4] (2.0); AWEESEK[IT4] (2.0)}	4.00
35. Glyceraldehyde-3-P-DH C subunit	{FGIVEGLM[Oxi]TTVHSITATQK[IT4](2.0); VIN[Dea]D[NaX]R[Orn]FGIVEGLMTTVHSITATQK[IT4](2.0); K[IT4]VVISAPSK[IT4](1.52); SSIFDAK[IT4](1.05); AAS[Pho]FN[Dea]IIPSSTGAAK[IT4](0.02)}	6.59
36. DNA gyrase subunit B-like	{SSLETVLTVLHAGGK[IT4](2.0); HSS[Pho]T[Pho]LSSPPFSSPS[Pho]PR(0.24); GYS[Pho]S[IT4]EQIQVLEGLD[Oxi]PVR(0.02); ELIK[IT4]N[Dea]AAT[Pho]R[Orn]IN[Dea]LQRL(0.01)}	2.27
37. Ubiquitin-Like protein SMTB	{QSVDFNSIAFLFDGRR (2.0); IK[IT4]R[Oxi]STQLK[IT4]K[IT4](0.02)}	2.02
38. Peptidylprolyl isomerase ROCI	{HVVFGQVVEGLDVVK(2.0);VYFDMTIDGQPAGR(0.60)}	2.60
39. Putative phosphor-glycerate kinase	{ADLNVPLDDNQNITDDTRIR(2.0); K[IT4]LASLADLYVNDAFGTAHR (2.0); LASLADLYVNDAFGTAHR(2.0); LVAS[IT4]LPEGGVLLLENVR (2.0); RPFAAIVGGSK[IT4](2.0); FYK[IT4]EEEK[IT4] (0.14); K[IT4]S[Pho]VGD[1Me]LNSVDLK[IT4] (0.11); T[Pho]MAK[IT4]K[IT4]SVGDLNSVDLK[IT4]GK[IT4]K[IT4]-VFVR[Dea]ADLNVPLDDNQN[Dea]ITDD[Oxi]TRIR(0.06); ELDYLVGAVSN[Dea]PK[IT4]R[Dea]PFAAIVGGSK[IT4](0.02); ST[Pho]GGAAVTRSSR[CRM]ASFGHIPSTSVS[IT4]AR (0.02); F[Oxi]S[IT4]LAPLVPR (0.01)}	10.35
40. Unknown protein	{TIC[CAM]NH[Oxi]D[Oxi]ENT[Pho]SAAFFGLLMVFLGK[IT4](2.0); LGLAAK[IT4] (0.17)}	2.17
41. Probable Histone 2AXa (HTA5)	{HIQLAVRN[Dea]DEELS[1Me]K[IT4](2.0); HIQLAVR(1.10); STGAGS[Pho]GTTK[IT4]GGR(0.03)}	3.13
42. Calnexin homolog 1 precursor	{LS[1Me]HVYTAILK[IT4]PDNEVR(2.0); SEGHEDYGLLVSEK[IT4](2.0); FQEGLEC[CAM]GGAYLK[IT4](0.04); LIFGGK[IT4](0.02)}	4.06
43. Fructose bisphosphate Aldolase like -protein	{K[IT4]PWSLSFSFGR(2.0)}	2.00

44. Small nuclear-ribonucleoprotein	{GFDQATNIILDESHER(2.0); ER[Orn]GNVGGGGGGS[Pho]R (0.07); SGS[Pho]RERGNVGGGGGGSR (0.03); ERSGSR[Dea]ER[Dea]GN[Dea]VGGGGGGSR[Dea](0.01); T[Pho]ES[IT4]DK[IT4]DSPVSSP[Oxi]VAAEK[IT4] (0.01); SGSRERGNVGGGGGGS[Pho]R[Oxi]R (0.01); S[Pho]K[IT4]S[IT4]PARAGN[Dea]NVPSLSRSNSRK[IT4] (0.01)}	2.14
45. Magnesium chelatase subunit of Reductase	{FILIGSGNPEEGELRPQLLDR (2.0); ST[Pho]TVRSLVDLLPEITVVSGD[Dhy]PYNS[1Me]DPR (0.02)}	2.02
46. Peroxiredoxin Q-like protein	{YK[IT4]LPYT[Dhy]LLSDEGNK[IT4](2.0); QAC[CAM]AFRDSYEK[IT4] (1.40); NGVVQLI[1Me]Y[IT4]N[Dea]NQFQPEK[IT4](0.89)}	4.29
47. 14-3-3-like protein GF14 upsilon	{SAQDIALADLAPTHPIR (2.0); MSS[Dhy]D[Dhy]SSREENVYLAK[IT4](1.70); SSDSS[Pal]R[Dea]EENVYLAK[IT4](0.47); YLAEFK[IT4]YLAEFK(0.09); NLLSVAYK[IT4] (0.01)}	4.27
48. 14-3-3 protein GF14=NCT Ran	{LLDSHLIPSATASESK[IT4] (2.0); IVS[Pho]S[Pho]IEQK[IT4]EESRK[IT4](0.01)}	2.01
49. Histone H2A	{GTIAGGGVIPHIHK[IT4] (2.0); HLQLAIRGDEELDTLIK[IT4] (2.0); HLQLAIR (0.31); GLLAAK[IT4] (0.17)}	4.48
50. AtCIpC Caseino-lytic protease C	{LAEEGK[IT4]LDPVVGR (2.0); NPNRPIASFIFSGPTGVGK[IT4] (2.0); RVIGQDEAVK[IT4] (2.0); RVLELSLEEAR (2.0); AIDLIDEAGSRVR (1.7); TRVIGQDEAVK[IT4] (0.74); YRGEFEER (0.08); NTLLIMT[Pho]SN[Oxi]VGSSVIEK[IT4]GGR (0.02); AIRRAR[Oxi]VGLK[IT4](0.01); LDPVVGRQPQIER (0.01); DR[Dea]EIELK[IT4]AEIAN[Oxi]VLSR(0.01); RAPS[Pho]ALDYLGR[Dea]P(0.01); SSVIEK[IT4](0.01);LDPVVGRQ[Dea]PQI(0.01)}	10.55
51. At5g59910= H2B11	{QVHPDIGISSK[IT4] (2.0); AMGIM[CRM]N[Oxi]SFIN[Dea]DIFEK[IT4] (1.70); LAQEASK[IT4] (0.92); K[IT4]PTITSR (0.48); SVETYK[IT4] (0.46); IYIFK[IT4] (0.12)]}	5.68
52. Uncharacterized protein At2g34160	{K[IT4]PLFFYVNLAK[IT4](2.0)}	2.00
53. 20S proteosome; peptidase complex	{AITVFSPDGHLFQVEYALEAVRK[IT4] (2.0); ADAR[Dea]VLIN[Dea]K[IT4] (0.02)}	2.02
54. V-ATPase subunit D	{GELDELEREDFFR (2.0); GELDELEREDFFRLK[IT4] (0.57)}	2.57
55. Unknown protein	{TPLQALLSDRDK[IT4] (2.0); AK[IT4]GENSSVDIPTQNSIQR (1.52); EP[Oxi]SISS[Pho]DRK[IT4]FVEQADEAK[IT4] (0.16); LQALLSDRDK[IT4] (0.02);	3.74

	GQNPSTIVVGEAVVQVK[IT4]NPT[Pho]PAR (0.02); ER[Dea]GFLP[Oxi]LSAALLSM[Oxi]PELHSK[IT4] (0.01); ELLAS[Pho]LSAK[IT4] (0.01)}	
56. 40S ribosomal protein S15A	{HGYIGEFEYVDDHR(2.0); IVVELNGRLNK(2.0); ISVLNDALK(0.15)}	4.15
57. F22C12. Probable v-type H^+ ATPase	{FIRQEAEEK[IT4] (2.0); IRQEYEK[IT4] (0.10); K[IT4]K[IT4]IDY[Oxi]SMQ[Dea]LNAS[Pho]RIK[IT4](0.01)}	2.11
58. DEAD-box ATP-dependant RNA helicase 52	{GLDIPHVAHVVNFDLPNDIDDYVHR(2.0); LNDLLERGRVSLQ[Dea]MVR (0.09); YVK[IT4]PTPVQR (0.07); VGSS[Pho]T[Pho]DLIVQR[Oxi] (0.05); R[Oxi]SHLMDLLHAQR[Orn]ENGN[Dea]Q[Dea]GK[IT4] (0.03); S[Pho]PGNN[Dea]DRGGYGGAN[Dea]SGYGGRGQGYGGR (0.03); SGGR[Dea]FGGR[CRM]DFRRESF[Oxi]SR (0.02); SGGRFGGRDFRRESFSR(0.01)}	2.30
59. At5g42270/ K5J14_7 =DNA binding	{EISK[IT4]DEISDALER (2.0); GQAGGLTFFAPSEERLESGLYSR (2.0); RTPGFTGADLQNLMNEAAILAAR(2.0); QVTVDRPDVAGR(0.77); DEISDALERIIAGP[Oxi]EK[IT4] (0.51); FR[Orn]GGQ[Dea]GGAGGPGGLGGPMDFGRS[Pho]K[IT4] (0.06); IGQ[1Me]VAVGGAGGN[Dea]PFLGQSMSS[Pho]QK[IT4] (0.01)}	7.35
60. Germin-like protein (GLP10)	{ID[Pho]Y[3Ox]APGGLNPPHTHPR(2.0)}	2.00
61. Retroelement Pol Polyprotein-like	{S[Dhy]PRLSLAAIS[Pho]NP[Oxi]SSPR (2.0); AMRLVIS[Pho]S[Pho]LGSADR (0.12); LAFLS[Pho]GGSN[Oxi]PSTPRSTSSPSLR[Dea] (0.02)}	2.14
62. T4012.24= V-type ATPase	{RGQVLEVDGEK[IT4] (2.0); GY[Oxi]PGY[IT4]MYTDLATIYER (0.03); QIY[Oxi]P[Oxi]PIN[Dea]VLPSLSR (0.02); ELLHRIPAK[IT4] (0.01)}	2.06
63. Manganese superoxide dismutase	{FN[Dea]GGGHVNHSIFWK[IT4](2.0)}	2.00
64. Arginine/serine -rich-splicing factor RSP31	{TLFVINFDPIRTK[IT4] (2.0); RPSPGQGR[1Me]RPS[Pho]PD[Oxi]Y[Oxi]GR (0.1); RPS[Pho]PDYGRRPSPGQ[Dea]GR[Dea] (0.07); SPGYDR[Oxi]Y[Pho]RSR[Dea]S[Pho]PVPR (0.06)}	2.23
65. Protein synthesis initiation factor-like	{AVGIWGC[CAM]K[IT4] (2.0); RPAVASNT[Pho]YQSDATQAGGGDS[Dhy]R (0.13); QT[Pho]Q[Dea]ANRLS[Pho]R[Oxi]GPSMNSSGR (0.04); QT[Pho]QANRLSR[Oxi]GPSMN[Dea]SSGRR[Oxi]0.02); DAAQERQ[Dea]TQ[Dea]ANR[Oxi]LSR[Oxi] (0.02); ADIQVPH[Iod]IAP[Oxi]PSQTQK[IT4] (0.01); AGGAYTMNTAS[Pho]AVTVR[Dea]STIR[Oxi] (0.01); S[Pho]S[Pho]GEENEEGTRYGASIRK[IT4] (0.01)}	2.23

66. Glutamine - synthetase	{VFSN[Dea]PD[Cox]VAAEVPW[Oxi]YGIEQEYTLLQK[IT4] (2.0); LW[Oxi]GVANRGASIR (0.34)}	2.34
67. Phospholipase D	{NK[IT4]VTDDGSLGS[IT4]I[1Me]FF (2.0); EHIEEIFAR(0.21)}	2.21
68. Copper zinc superoxide dismutase	{AVVVHADPDDLGK[IT4] (2.0); GGHELSLATGN[Dea]AGGR[Oxi] (0.08)}	2.08
69. RNA helicase	{ RDELTLEGIK[IT4] (2.0); VQVGVFSATMPPEALEITRK[IT4] (2.0); VLQ[Dea]S[Pho]GVH[Pho]VVVGTPGRVFDLLR[Dea] (0.24); RSGSS[Pho]RVLIT[Pho]T[Pho]DLLAR[Orn] (0.01)}	4.25
70. Carbonic anhydrase 2	{YAGVGAAIEYAVLHLK[IT4](2.0)}	2.00
71. At3g49560= Unknown protein	{GLLT[dHx]DPTLPLLTDSALK[IT4](2.00);NFAAITGVNAGIAS VMK[IT4](0.49); N[Dea]DQQNPIQ[Dea]QFQ[Dea]VK[IT4](0.01)}	2.50
72. GAPDH	{GTMTTTHSYTGDQR(2.0); TFAEEVNAAFRDAAEK[IT4] (2.0); LLDASHR(1.05); QASHLLK[IT4] (0.92); AVALVLPNLK[IT4] (0.48); YDSTLGIFDADVK[IT4]PSGDS[1Ac]ALSVDGK[IT4] (0.15); AS[Pho]AT[IT4]FSVAK[IT4]PSLQGFSEFSGLR[Oxi] (0.05); LLDASHRDLR(0.01)}	6.66
73. RNA helicase	{RILVATDLVGR(2.0); LSEMEK[IT4] (0.07); LSLK[IT4]NVR (0.01)}	2.08
74. Putative polyubiquitin	{ESTLHLVLR(2.0); QLEDGRTLADYNIQK[IT4](2.0); ES[Pho]TLHLVLRLRGGMQ[Dea]IFLK[IT4] (0.26); LIFAGK[IT4] (0.23); DGR[Oxi]T[IT4]LAD[Oxi]YN[Dea]IQK[IT4] (0.20); MQIFVK[IT4] (0.04); GGMQIFLK[IT4] (0.01)}	4.74
75. 40S ribosomal protein S6	{K[IT4]LEIDDDQK[IT4](2.0); LVTPLTLQRK[IT4] (0.49); GYVFK[IT4] (0.10)}	2.59
76. Ribulose bisphosphate carboxylase	{GYYDGRYWTMWK[IT4] (2.0); QVQC[CAM]ISFIAY[1Cl]K[IT4]PP[Oxi]S[Pho]F (2.0); SPGYYDGRYWTMWK[IT4] (2.0); QVQC[CAM]ISFIAY[Amn]K[IT4]PPS[1Me]FT (0.82); K[IT4]FETLSYLP[Oxi]DLT[Pho]DS[Pho]E[NaX]LAK[IT4] (0.72); YWTMWK[IT4] (0.51); IIGFDNT[Pho]R[2Ox]QVQ[Dea]C[CAM]ISFIAYK[IT4]P[Oxi]PSF T (0.03); EHGNS[Pho]PGYYDGR (0.03)}	8.11
77. Anion-transporting ATPase	{IVFDTAPTGHTLR(2.0); GML[1Me]VEQLGELK[IT4] (0.02); EEFRSASQMNGGTGVK[IT4] (0.02); FTR[Oxi]IVFDT[Pho]APTGHTLR[Dea] (0.01)}	2.05
78. F1E22.8= Small nuclear Ribonucleoprotein	{VSQLEHVFIRR(2.0); VSQLEHVFIRGSK[IT4] (0.07); LLHEAS[Dhy]GHIVTVELK[IT4](0.05); IYLDR[Dea]F (0.01); GK[IT4]SS[Pho]SLGVGR[Dea]GRGAMR(0.01)}	2.14

79. Mitochondrial F1-ATP synthase β subunit	{AHGGFSVFAGVGER(2.0); VGLTGLTVAEYF[Oxi]R[Orn] (2.0); MLS[dHx]PHILGEEHYNTAR (1.4); TREGNDLYREM[Oxi]IESGVIK[IT4](1.3); VVDLLAPY[IT4]QR (1.15); RVLSS[IT4]LLR[CRM]SS[Pho]SGR[Dea]SAAK[IT4] (0.29); VLQNYK[IT4] (0.26); K[IT4]T[Pho]YDYGGK[IT4](0.20); SGIPGSTHAS[Pho]VAS[Pho]RRVLSSLLR (0.03); ASRRVL[1Me]S[Pho]S[Pho]LLRSSS[Dhy]GR (0.02); GRK[IT4]VLN[Dea]TGAPIT[Pho]VPVGR (0.01); SS[Pho]SGRSAAK[IT4]LGNR[AGA]NPR[Pho] (0.01)}	8.67
80. Putative prohibitin	{VLPSIIHETLK[IT4](2.0); VYPEGTHLM[Oxi]IPWFERPIIYDVR (1.70); PY[IT4]LVESTSGSR (1.05); RS[Pho]AVIR[CRM]AEGEAK[IT4]SAQ[Dea]L (0.82); S[Pho]AQLIGQAIANNQ[Dea]AFLTLR(0.01)}	5.58
81. 60S Ribosomal protein L18A	{GGFRFHQYQVVGR(2.0); FHQYQVVGR(0.92); VRFPC[CAM]IQIIK[IT4](0.82); NYGIWLR(0.20)}	3.94
82. Heat shock P 70 kDa	{ATAGDTHLGGEDFDNR (2.0); ARFEELNMDLFRK[IT4] (0.64); PAGGS[Pho]GGAGPK[IT4] (0.07); LDAADK[IT4](0.01)}	2.72
83. 60S ribosomal protein L13	{LAPTIGIAVDHR(2.0); R[Oxi]K[IT4]N[Dea]R[CRM]S[Pho]LEGLQTNVQRLK[IT4] (0.1)}	2.10
84. DegP protease precursor	{VFAIGNPFGLDHTLTTGVISGLRR(2.0); VTRPILGIK[IT4](0.89); VGDEVTVQVLR(0.03); GASDLRVT[Dhy]LADQTT[Pho]FDAK[IT4] (0.01)}	2.93
85. Unknown protein	{TAIQAK[IT4]PDSVYFVVS[1Me]R (2.0); TIEVEVDK[IT4]PLGLTLGQK[IT4] (2.0); IGGLN[Dea]GLR(0.59)}	4.59
86. Cytochrome f	{IGNLSFQNYR[AGA]P[Oxi]NK[IT4]K[IT4] (2.0); GRGQIYPDGSK[IT4] (1.52); DVHFLK[IT4] (0.1)}	3.62
87. Unnamed protein product	{AVLSGIRET[Pho]VSYQAAR (2.0); K[IT4]EGY[Oxi]T[Pho]FD[Dhy]GFANC[CAM]AVVSALIK[IT4] (0.22)}	2.22
88. 14-3-3-like protein GF14 phi	{AAQ[Dea]D[Dhy]IAN[Oxi]AELAP[Oxi]THPIR(2.0)}	2.00
89. ATP-dependent Clp protease	{IAS[IT4]GDVPETIEGK[IT4]K[IT4] (2.0); R[Orn]II[1Me]GQDEAVK[IT4](0.06); Q[Dea]LT[Pho]K[IT4]LE[Cox]VK[IT4]EIADILLK[IT4](0.02); VVDEGYN[Dea]PSYGAR[Oxi](0.01)}	2.09
90. Ribosomal protein	{DITPMGGFPHY[Oxi]GIVK[IT4](2.0); HGSLGFLPR(1.7); RVVTLR(0.14)}	3.84
91. Dynamin-2A	{AFVPPQHFIR(2.0); LR[CRM]LP[Oxi]S[Dhy]VLSGLQGK[IT4](1.22);	3.39

	LVQ[Dea]RR[CRM]MER[Oxi]QR (0.12); IALVDT[Pho]LAS[Pho]QIR (0.02); YS[Pho]DPAQNGDAASPGSGSN[Dea]RR (0.02); VDLPGLDQR90.01)}	
92. Putative Glycosyl transferase 4	{WGGN[Dea]SVATTSLGRGSGSS[Pho]R (2.0); FS[IT4]QNVS[Pho]DANSSGLNGVR (0.32); IFSQ[1Me]NVS[Pho]D[Oxi]AN[Dea]SSGLNGVR (0.11)}	2.43
93. Citrate synthase	{HLPDD[1Me]PLFQLVSK[IT4] (2.0); VVPGYGHGVLR(2.0); ALGLPLERPK[IT4] (0.66); EQLK[IT4]DYVWK[IT4]TLN[Oxi]S[Pho]GK[IT4](0.02)}	4.68
94. ATP binding / H^+ ion Transporting ATP synthase	{AREVLQREDDLNEIVQLVGK[IT4] (2.0); ESEYGYVRK[IT4] (2.0); RTTLVANTSNM[Oxi]PVAAR (2.0)}	6.00
95. ATP synthase CF1 α subunit	{ELIIGDRQTGK(2.0); IAQIPVSEAYLGR(2.0); LIESPAPGIISRR(2.0); QHTLIIYDDLSK(2.0); RSVYEPLQTGLIAIDSMIPIGR(2.0); VTIRADEISNIIRER(1.52); ADEISNIIRER(1.40); RPPGREAYPGDVFYLHSR(0.25); SVYEPLQTGLIAIDSMIPIGR(0.20); LRELLK(0.08); ERIEQYNR(0.07); VTIRADEISNIIR(0.05); GIRPAINVGISVSR(0.02)}	13.59
96. Unnamed protein product	{HTPGTFTNQMQT[Dhy]SFSEPR (2.0); VIVAIENPQDIIVQSARPYGQR (2.0); FAQYTGAN[Dea]AIAGR (0.21); MVLQ[Dea]M[Oxi]RGTIAAGQ[Dea]K[IT4] (0.04)}	4.25
97. 40S ribosomal protein	{GSFETIHIQ[1Me]D[Oxi]S[Pho]TGHEFATR (2.0); TIRYPDPLIK[IT4] (2.0); GTK[IT4]PWVSLPK[IT4] (0.89); PWVSLPK[IT4](0.34)}	5.23
98. Putative protein kinase	{LLITNSS[Pho]TISFPLH (2.0); GFYLK[IT4] (0.05); AN[Dea]LK[IT4]EDLLINSSK[IT4] (0.01)}	2.06
99. At4g09000/ Probable 14-3-3-like protein	{AAQDIAN[Dea]S[Dhy]ELAP[Oxi]THPIR(2.0)}	2.00
100. Small nuclear riboprotein Sm-D1	{NPVTLDHLSVR (2.0); AGRGRGR[Dea]GR[Dea] (0.04)}	2.04
101. NADH-ubiquinone oxidoreductase	{ILTDYGFEGHPLRK[IT4] (2.0); DNQF[Oxi]IFK[IT4] (0.09)}	2.09
102. 60S Ribosomal protein L2	SLDFGERN[Dea]GYLK[IT4] (2.0); SIPEGAVVC[CAM]NVEHHVGDR (2.0); AM[Oxi]IGQVAGGGRTEK[IT4] (2.0); ASGDYAIVIAH[Oxi]NPDSD[Oxi]TT[Dhy]R[Orn] (2.0); GAGSVFK[IT4] (1.05); VVTEIIHDPGR (1.0); GVVTEIIHDPGR (0.28); LRGQAAAS[1Me]AAK[IT4] (0.18); SIPEGAVVC[CAM]N[Dea]VEHHVGDRGVLAR[Dea] (0.05); AIVIAH[Oxi]NPDS[Pho]DTTR (0.01)}	10.57
103. 50S ribosomal	{DHTIFSLIDGLVK[IT4](2.0)}	2.00

protein L27		
104. 40S Ribosomal-protein	{AHGLAPEIPEDLYHLIK[IT4] (2.0); FRLILVESR (1.7)}	3.70
105. At3g09680= 40S ribosomal protein S23	{GHAVGDIPGVR (2.0); QLR[Dea]ITQR (0.08)}	2.08
106. 40S Ribosomal protein S10-3	{RVNQAIF[Oxi]LLTTGAR (2.0); RQAVDISPLRR (0.19); QAVDISPLRR (0.07)}	2.26
107. Putative spliceosome associated protein	{IAPGVGANLLGQHSAER (2.0); T[Pho]T[Dhy]RIAPGVGAN[Dea]LLGQ[Dea]HSAER (0.24)}	2.24
108. At5g39850= 40S ribosomal protein S9	{RIFEGEALLR (2.0); ERLDAELK[IT4](1.22); RIFEGEALLRR (0.03)}	3.25
109. 20S Proteasome α subunit A2	{HITIFSPEGR (2.0); YLGLLAT[Pho]GMTADSRSLVQQAR (0.10); ATSAGM[CRM]K[IT4]EQEAVN[Dea]FLEK[IT4] (0.05)}	2.15
110. 60S ribosomal protein L10A	{FPTLVSHQESLESK[IT4](2.0)}	2.0
111. Nacent polypeptide-associated complex alph-like protein 3	{NILFVISK[IT4]PDVFK[IT4] (2.0); LGMK[IT4]PITGVSR[Dea]VT[Pho]VK[IT4] (0.03)}	2.03
112. Ribosomal protein CL9	{RLVSLPEIRETGEYIAELK[IT4](2.0); ELK[IT4]M[Oxi]EDERIEAEK[IT4](0.01)}	2.01
113. 40S ribosomal protein S17	{RM[Oxi]DFVPDESAIK[IT4] (2.0); MTLDFHTNK[IT4](0.30)}	2.30
114. Rubisco large subunit	{AMHAVIDRQK[IT4] (2.0); AMHAVIDRQK(2.0); DN[Dea]GLLLHIHR (2.0); EGDR[CRM]ESTLGFVDLLRDDY[Oxi]VE[Dhy]K[IT4] (2.0); E[Cox]STLGFVDLLRDD[Dhy]Y[Oxi]VEK[IT4] (2.0); GGLDFTK[IT4]DDENVNSQPFM[Oxi]R (2.0); GHYLNATAGTC[CAM]EEMIK[IT4] (2.0); LS[Hex]GGDHIH[Oxi]AGTVVGK[IT4] (2.0); TFQGPPHGIQVERDK[IT4] (2.0); TFQGPPHGIQVER (2.0); YGRPLLGC[CAM]TIK[IT4] (2.0); YGRPLLGCTIKPK(2.0); LEDLRIPP[Oxi]AYT[1Me]K[IT4] (0.92); ASVGFK(0.52); LGLSAK[IT4]0.48); ALRLEDLR(0.15); RAVFAR(0.07); S[Pho]GGD[Dhy]HIHAGTVVGK[IT4] (0.05); LEGDR[Dea]E[CAM]STLGFVDLLRDDYVEK[IT4] (0.02); GDHIH[Iod]AGT[Pho]VVGK[IT4] (0.01)} {AILPLSM[Oxi]YFPPSEYVK[IT4](2.0)} {ADRDESSPYAAMLAAQDVAQR(2.0); IGRIEDVTPIPTDSTR(2.0); TK[IT4]EP[Oxi]K[IT4]VENVTLGPAVR(2.0); ELGITAIH[1Me]VK[IT4] (1.40)}	26.22
115. Putative S-phase-specific ribosomal	{VFEVSLADLQNDED[Dhy]NAYR[Oxi]K[IT4] (2.0); RTC[CAM]YAQSSQIR (1.70); LRAEDVQGR[Dea]N[Dea] (1.70)}	5.40

protein		
116. 30S Ribosomal protein S5	{VM[Oxi]LRPASPGTGVIAGGAVR (2.0); AS[Dhy]ALSSLSS[Pho]LSLH[Oxi]TR (0.05)}	2.05
117. Ribosomal protein 5B	{RVNQAIF[Oxi]LLTTGAR (2.0); RQAVDISPLRR (0.19); QAVDISPLRR (0.07)}	2.26
118. T1N15.8; Probable Gutamine sythetase	{TLPGPVTDPSK[IT4]LPK[IT4] (2.0)}	2.00
119. Putative glutamine synthetase	{TLPGPVTDPSK[IT4]LPK[IT4(2.0); EHIAAYGEGNER (2.0); DIVDAHYK[IT4] (0.92); EHIAAYGEGNERR (0.70); SK[IT4]ART[Pho]LPGPVTDP[Oxi]SK[IT4]LP[Oxi]K[IT4](0.02);S WGVANR (0.02); WGVANR (0.02)}	5.68
120. Catalase	{FPDMVHALKPNPK(2.0); FSTVIHER (2.0); GFFE[Dhy]VT[Pho]HDISNLTC[CAM]ADFLR (2.0); GPILLEDYHLVEK[IT4] (2.0); LFIQIIDPADED[Pho]K[IT4]FDFDPLDVTK[IT4] (1.7); FIQ[Dea]IIDP[Oxi]ADEDK[IT4]F[Oxi]D[Oxi]FDPLDVTK[IT4] (1.3); PASS[Pho]YNSPF[Oxi] (0.12); GFAVK[IT4] (0.07); GSPETLRDPR (0.04); LANFDRERIPER (0.02); SHIQENWR(0.01)}	11.26
121. Hypothetical protein	{EAFPGDVFYLHSR (2.0); RPPGREAFPGDVFYLHSR (2.0); SVHEPM[Oxi]QTGLK[IT4] (2.0); GIRPAINVGLSVSR (1.22); EAFPGDVFYLH[Oxi]S[Pho]RLLER (0.11); GGLTN[Dea]ERK[IT4]M[Oxi]EP[Oxi]DAFLK[IT4]ER(0.04)}	7.37
122. 40S Ribosomal protein	{GPVREGDVLTLLESER(2.0); GPVREGDVLTLLESEREAR(2.0	4.00
123. 40S ribosomal protein S19-like	{QFVIDVLHPGR(2.0)}	2.00
124. Hypothetical protein	{TFIAIK[IT4]PDGVQR (2.0); C[CAM]R[Oxi]S[Dhy]ASR[Dea]AARS[Pho]LLSSAK[IT4]NAR (0.01)}	2.01
125. NADP-specific isocitrate Dehydrogenase	{TIEAEAAHGTVTR(2.0); QMWRS[Pho]PNGTIRNILN[Dea]GTVFR (0.80); SPN[Dea]GTIRNILNGTVF[Oxi]R[CRM] (0.02)}	2.82
126. Glycolate oxidase	{AIALTVDTPRLGR[AGA]R (2.0); RIAIQ[Dea]AGAAGIIVS[Pho]NHGAR(2.0); VPVFLDGGVRR (2.0); ILFRPR(0.10); RGTDVFK[IT4] (0.08); MAHPD[1Me]GEYATAR (0.04); AGFK[IT4]AIALTVDT[Pho]PRLGR[Pho]R (0.01)}	6.23
127. Cytochrome b559	{QGIPLITGRFDSLEQLDEFSR(2.0); FDSLEQ[Dea]LDEFSRS[Pho] (0.01)}	2.01
128. Hypothetical protein	{TK[IT4]EDVWLVS[Pho]NGVIARSTR(2.0)}	2.00

*The number and amino acid sequences of the unique peptides for the *X. viscosa* nuclear proteins identified by iTRAQ LC ESIMS-MS/MS are shown. Unique ion scores (unused score) and protein score which are derived from the ion scores are provided. Ion and protein scores ≥ 2.0 indicate identity at $\rho < 0.01$.

Modifications on amino acid residues abbreviated as: **CAM:** carbamidomethyl, **IT4:** iTRAQ 4-plex, **Oxi:** oxidation, **Me:** methyl group, **Pho:** phospho group, **Dea:** deamidated, **Me:** methyl group, **CRM:** carbamy group, **Dhy:** dehydrated , **DTM:** dethiomethyl, **AAS:** Delta:H(2)(2), **HNc:** HexNAC, **2Ox:** dioxidation, **KXX:** Cation:K, **GCn:** glucuronyl, **Orn:** Arg_> Orn, **dHx:** dHex, **Amn:** Amino group, **AGA:** Arg_ > Glu SA, **Cox:** carboxyl group, **3Ox:** trioxidation, **Pal:** Palmitoyl.

Table 4.3. Summary of ID statistics for the detected proteins in the nucleus of *X. viscosa* in response to dehydration stress.

Unused protein score (confidence)	Proteins detected	Protein before grouping	Distinct peptides	Spectra identified	% Total spectra
> 2.0 (99%)	128	2089	2397	4712	33.4
> 1.3 (95%)	226	2707	3005	5603	39.7
> 0.47 (66%)	424	3455	3960	7043	49.9
Cutoff applied >0.10 (20%)	**985**	**5766**	5528	9188	65.1

4.4. DISCUSSION

Analysis of the up-regulated nuclear proteins of *X. viscosa* in response to dehydration stress using 2DE and MALDI mass spectrometry (chapter 3) showed that dehydration stress was controlled by coordinated actions of different stress molecules reflected in multigenic responses within the plant nucleus. Hence the need for comprehensive understanding of the physiological response of plants under dehydration stress is of critical importance. Unlike non-resurrection plants, which maintain water potentials above that of the surrounding environment and attempt to continue to function during dehydration periods (Mundree et al., 2006), *X. viscosa* and other resurrection plants use a very different strategy, they desiccate and then rehydrate once water becomes available again (Mundree et al., 2002). A thorough understanding of this phenomenon may provide further insight into possible mechanisms for improving drought tolerance in crop plants.

Our study revealed that 34% of the identified proteins in the nucleus of *X. viscosa* in response to dehydration stress were significant differentially expressed (23% up-regulated with change-fold greater than 2, and 11% down-regulated with change-fold less than 0.5). The

majority of the identified proteins (66%) were shown to have consistent expression levels (no significant change in expression) (change-fold between 2 to greater than 0.50).

Overview of functions of the up-regulated nuclear proteins

X. viscosa and other resurrection plants posses a number of protection mechanisms, instated during dehydration to neutralize the stresses associated with severe cellular water deficit (Mundree et al., 2002). One of these mechanisms is the induction of dehydration responsive-proteins. Proteins induced during dehydration with different kinetic and consequently represent proteins that functions at specific stages during the program that leads to dehydration tolerance (Jiang et al., 2007). Protein up-regulated may contribute to stabilize the cellular environment for maintenance of basic structural and physiological integrity in desiccated cells (Jiang et al., 2007). Many studies suggest that late dehydration responsive proteins are responsible for the acquisition of desiccation tolerance in vegetative tissues, and are thought to be of great interest to uncover the molecular mechanisms behind this phenomenon (Ramanjulu and Bartels, 2002; Jiang et al., 2007; Ingle et al., 2007). Therefore, detailed investigation of the up-regulated proteins in the nucleus of *X. viscosa* in response to late dehydration point at 35% is of particular interest.

To gain more insight into the roles of the up-regulated proteins in acquisition of desiccation tolerance, we discussed their functions in detail below and categorized them into nine groups according to their matched functions:

(i) twenty three percent were shown to be involved in gene regulation, this comprise the largest category, followed by (ii) twenty percent proteins involved in ROS pathways, (iii) thirteen percent were shown to be involved in cell signaling, (iv) ten percent were proteins involved in molecular chaperone type activities, (iv) ten percent were proteins involved in nucleocytoplasmic transport, (v) seven percent were proteins involved in protein translation. (vi) seven percent were proteins with miscellaneous functions, (viii) seven percent were proteins with unknown functions, and (ix) three percent were proteins involved in synthesis of compatible solutes.

Up-regulated proteins involved in gene regulation

Alcohol dehydrogenase (ADH) (EC 1.1.1.1) class III (2.23±0.08 fold-change). This is a glycolytic enzyme increased in abundance by more than twofold in the nucleus of *X. viscosa* in

response to dehydration. ADH catalyses the conversion of ethyl aldehyde to ethyl alcohol in anaerobic glycolysis, and the ADH gene in *Arabidopsis* is up-regulated in abscisic acid (ABA)-dependent manner in response to dehydration (de Bruxelles et al.., 1996; Ingle et al., 2007). However, carbohydrate metabolism is modulated in *X. viscosa* and other resurrection plants during dehydration, particularly towards the synthesis of sucrose (Bianchi et al., 1991; Whittaker et al., 2001; Ingle et al., 2007), and possibly towards the synthesis of compatible solutes such as sorbitol (Mundree et al., 2000). The accumulation of sucrose is independent of photosynthetic activity because it occurs predominately at lower RWC such as at 35% after the cessation of photosynthesis (Cooper & Farrant 2002). The function of sucrose accumulation remains unclear, but it has been suggested that it may act as a water replacement molecule to stabilize membrane and protein structure or in vitrification of the cytoplasm in desiccated cells, and as an energy source during rehydration (Hoekstra et al., 2001; Buitink & Leprince 2004; Vicré et al. 2004a; Ingle et al., 2007). On the other hand, previous studies have also reported the presence of many dehydrogenases in the nucleus involved in intermediary metabolism in the nuclei (Yamamoto et al., 1997; Markova et al., 2006; Pandey et al., 2008; Repetto et al., 2008). These dehydrogenases may be involve in monitoring metabolic status in response to various stimuli and transmit the changes to the transcriptional apparatus (Repetto et al., 2008), where they interact with DNA-bound proteins to modulate their regulatory activity (Repetto et al., 2008). Thus, ADH may also be involved in regulation of gene transcription and regulation during dehydration stress through nuclear targeting of metabolism.

Actins 1 and actin putative were up-regulated with fold-change (2.13±0.02 and 2.22±0.00 respectively). Actin is not only a major cytoskeletal component in all eukaryotic cells but also a nuclear protein that plays a role in gene transcription (Percipalle and Visa, 2006). Recent studies performed *in vitro* have suggested that actin, in direct contact with the transcription apparatus, is required in an early step of transcription that is common to all three eukaryotic RNA polymerases. In addition, there is evidence from *in vivo* studies that actin is involved in the transcription elongation of class II genes. In this case, actin is bound to a specific subset of premessenger RNA binding proteins, and the actin–messenger RNP complex may constitute a molecular platform for recruitment of histone-modifying enzymes (Percipalle and Visa, 1999). It is well documented that actin plays a central role in the coordination of signal transduction pathways in the cytoplasm, and it was believed that actin-based mechanisms

of transcription regulation may sense extracellular signals via cytoplasmic changes in the actin pools. It was speculated that actin can modulate the overall transcriptional activity of the cell in response to extracellular signals (Percipalle and Visa, 2006). In this way actin is involved in enhancing plant adaptation to dehydration stress by its role in cell signaling and in gene transcription and regulation.

Three histones were up-regulated, histone H4, histone H3.2, and putative histone H2A.5 with fold-change (2.21±0.03, 2.10±0.05, and 2.10± 0.03 respectively). Histones are the basic proteins of the nucleus and their variants functionally differentiate individual nucleosomes and, hence, act as key regulators of chromatin structure and function (Yi et al., 2006). The basic repeating unit of the genome packing structure in eukaryotes is the nucleosome, itself composed of an octamer of the four core histones H3, H4, H2A and H2B (Shia et al., 2006; Yi et al., 2006). A fifth histone, the linker histone or H1, which involved in connecting the nucleosomes together (Yi et al., 2006). In addition to their structural role, histones might also serves as indicators, signaling the nature of the packaged DNA via their post-translational modifications (Shia et al., 2006; Yi et al., 2006). In particular Histone variants 2 and 3 are responsible for the functional diversity of nucleosomes, and histone synthesis is connected with DNA replication (Shia et al., 2006; Yi et al., 2006). The regulatory circuits of histone gene expression, in addition to the primary sequence of the encoding genes, are both of critical importance to achieve a specific chromatin organization at the right time and place (Govin et al., 2005). Access to certain genes can be granted or denied, depending on which posttranslational modifications (PTMs) are present on particular histones (Yi et al., 2006). For example, trimethylation of lysine 4 on histone H3 is associated with gene activation. In addition, combinations of PTMs (known as histone codes) may work together to modulate the degree of activation or repression (Shia et al., 2006; Yi et al., 2006). Thus, histones play important roles in gene transcription and regulation, cell-cycle progression and development.

Oligosaccharyl transferase (OT) like (3.61±0.08 fold-change), is a membrane-associated enzyme complex that catalyzes N-glycosylation, an essential and highly conserved protein modification reaction that occurs in some prokaryotic and all eukaryotic organisms (Yan et al., 2003; Zubkov et al., 2004). This co- and posttranslational modification reaction affects a large number of both secretory and membrane proteins (Zubkov et al., 2004). Protein

posttranslational modifications are also an important mechanism in the regulation of gene expression in eukaryotes **(**Isshiki et al., 2006). OT catalyzes the transfer of a preassembled high mannose oligosaccharide (Glc3Man9GlcNAc2) from a lipid-linked dolichol pyrophosphate onto an asparagine residue in an Asn-X-Ser_Thr consensus sequence on nascent polypeptides as they are translocated into the lumen of the rough endoplasmic reticulum (Zubkov et al., 2004). Recent mutagenesis studies have shown that OT activity is essential for normal cell viability (Lennarz WJ. 2007). These mutations were shown to cause the disruption of the interactions between the enzyme subunits Ost4p, Ost3p, and Stt3p (Lennarz WJ. 2007). OT might involved in signal transduction pathways and regulatory functions including intracellular transport, as under normal conditions it might reside in the ER while during dehydration stress it enters the nucleus.

Up-regulated proteins involved in ROS pathways

Dehydration stress impairs plant photosynthetic apparatus and hence the plant cannot utilize light energy during dehydration stress, this situation leads to an increased level of ROS (Smirnoff, 1993; Jiang and Zhang, 2001; Bian et al., 2009). ROS generated under stress conditions triggers protein degradation (Jiang et al., 2007) and damages nucleic acids, as H_2O_2 can readily permeate cell membranes and put DNA at major risk by generating highly reactive hydroxyl radicals through the interaction of H_2O_2 with transitional metal ions such as Fe^{2+} in Fenton-type reactions (Qureshi et al., 2007). Our study revealed that six proteins were up-regulated in the nuclear proteome of *X. viscosa* in response to dehydration stress with a potential role in protection against oxidative stress, these proteins are:

NADPH:protochlorophyllide (Pchlide) oxidoreductase B (POR B) (17.07±2.03) and NADPH: protochlorophyllide (Pchlide) oxidoreducase A (POR A) (3.68±0.29) were up-regulated in the *X. viscosa* nuclear proteome in response to dehydration stress. The POR family is highly conserved proteins in plants (Buhr et al., 2008). The PORA and PORB enzymes were nuclear gene products that were synthesized as larger precursors in the cytosol and imported into the plastid post-translationally (Reinbothe et al., 2000). PORA and PORB displayed different substrate specificities with regard to Pchlide A and Pchlide B (Reinbothe et al., 2003a; 2003b), but interacted together to form larger light-harvesting POR (known as LHPP) (Reinbothe et al., 1999; Reinbothe et al., 2006). The LHPP were found to be involved in light

trapping and the dissipation of excess light during the transition of dark grown seedlings to the greening stage (Reinbothe et al., 2006). Plants exposed to low or high light intensity in natural environment can suffer oxidative damage due to generation of reactive oxygen species (ROS) (Qureshi et al., 2007). Although ROS is necessary for signaling and stress-induced gene expression, excess ROS can cause damage to proteins, DNA and lipids in the cell. Thus removal of excess free radicals from the cell is necessary through specific detoxification mechanisms. POR A was found to play a photoprotective role in non-resurrection etiolated plants during greening, and its expression conferred adaptation on etiolated seedlings (Buhr et al., 2008). Recently, Buhr and co-workers (2008) have provided evidence that LHPP functions in antioxidant defense in stress response, and is implicated in protection of plant cell and organelles from excess reactive oxygen species through a mechanism involving energy transfer. As light is absorbed by PORA-bound Pchilide b is transferred to PORB-bound Pchlide a, this induces the conversion of Pchlide a to Chlorophyllide (chlide) a. This energy transfer eliminates the possibility of Pchlide b photo-reduction and prevents the excited triplet states of either Pchlides a or b accumulation and provoke singlet oxygen production. If ROS is not removed immediately it can cause protein modifications and damage to the cellular and molecular machinery (Zolla and Rinalducci, 2002). Genes encoding antioxidant enzymes were up-regulated during dehydration stress, supporting their important role in enhancing drought tolerance in plants (Holmstrom et al., 1996; Shulaev and Oliver, 2006). The nucleus was found to have its own ROS antioxidant systems to protect its DNA and other molecules, the nuclear anti-oxidant machinery includes enzymes that were previously considered to be localized in other subcellular compartments (Pandey et al., 2008). As POR B and POR A are synthesized in the cytosol and have plastid localization signals and their POR domains appear conserved, the most likely hypothesis is that the putative POR proteins are transported into the nucleus during dehydration to function as ROS scavengers. During stress the imbalance between photosynthetic light capture and NADPH utilization in carbon fixation may alter the redox state and lead to photo-inhibition (Qureshi et al., 2007). POR B and POR A activation during dehydration stress could provide a mechanism whereby the regeneration of $NADP^+$ could provide the observed protective effect. The transfer of POR group to the nucleus during dehydration stress has the potential advantage of facilitating the regulation of organelle gene expression by linking the nuclear signal pathway to global signals that regulate gene expression in response to dehydration stress. This response should lead to an enhanced capacity of the

nucleus to scavenge ROS and prevent membrane damage by controlling lipid peroxidation. It was noted that different species have different antioxidant activity responses in the face of water deficit (Jiang and Zhang, 2001). The increase in abundance of the PORB and PORA in the nuclear proteome of *X. viscosa* in response to dehydration suggests their involvement in detoxification process of ROS. These increased levels of PORA and PORB may indicate a major role of these enzymes in the nucleus during dehydration period, and seem to be unique to the resurrection plant *X. viscosa* nucleus as it was not detected in the whole proteome of this species (Ingle et al., 2007) or in the nuclear proteomes of *Arabidopsis* and chickpea under cold and stress dehydration stress (Bae et al., 2003; Pandey et al., 2008) respectively when 2DE approach was used. Furthermore, the observed increased level of PORs may be able to increase the ratio of $NADP^+$/NADPH, thereby resulting in the availability of $NADP^+$ as an electrons acceptor, this will reduce the generation of superoxide radicals. We hypothesize that this could be an important mechanism of desiccation tolerance in *X. viscosa* which might enable the cell to survive severe water loss.

1-Cys peroxiredoxin PER1 enzyme (3.32±0.18 fold-change), was the first Prx type identified in plants and was characterized as a dormancy related protein in both seed and leaf tissues and it was shwon to enhance plant ability to cope with ROS exacerbated by abiotic stresses (Mowla et al., 2002; Haslekas et al., 1998; lee et al., 2000). A 1-Cys Prx termed XvPer 1 (*X. viscosa* peroxiredoxin type I) was isolated from *X. viscosa* leaf samples, and was shown to be up-regulated in response to dehydration stress and ABA treatment (Mowla et al., 2002; Mowla, 2005). The 1-Cys Prx was shown to localize in the nucleus and in the cytosol implicating in the protection of macromolecules from ROS (Govender, 2006; Stacy et al., 1996; 1999). *Tobacco* leaves in which the rice 1-Cys Prx was over-expressed were found to be less susceptible to H_2O_2-mediated oxidative damage (Apel and Hirt, 2004). This supports the hypothesis that 1-Cys Prx protects the embryo and the aleurone layer from oxidative damage during desiccation of the seed. It was also found in vitro that 1-Cys Prx protected DNA from nicking (Dietz, 2003). Since the protein is nuclear localized it was postulated that it might protect DNA and the nuclear components of translation (Stacy et al., 1999). The over-expression of *Arabidopsis* 1-Cys Prx was found to be under the control of antioxidant and ABA responsive elements (Govender 2006; Haslekas et al., 2003a). There is evidence that the protein could be involved in cell signaling as it was found to possess a nuclear bipartite signal targeting

the protein to the nucleus of barley embryos and aleurone cells (Stacy et al., 1999). Large numbers of antioxidants defense genes in *X. humilis* were found to be up-regulated in response to dehydration (Collet et al., 2004). Ingram and Bartels (1996) found that genes encoding antioxidant enzymes were up-regulated during dehydration, and removal of ROS resulting from oxidative stress was important for survival drought stress conditions. An inhibitor to lipooxygenase was found to be up-regulated in desiccated leaves of the resurrection plant *Craterostigma plantagineum* that prevents lipid hydroperoxidation (Bianchi et al., 1991). In the resurrection plants *X. viscosa* and *C. wilmsii* ascorbate peroxidase was found to be up-regulated in dehydration stress plants (Sherwin and Farrant, 1998) supporting the importance of antioxidants in desiccation tolerance. The 1-Cys Prx enhances the plant ability to cope with dehydration stress, either by functioning as direct scavenger of ROS or by being involved in signal transduction pathways related to the adaptation of environmental stresses.

Gdp-mannose-3′,5′-epimerase (2.54±0.04 change-fold), this enzyme catalyses the conversion of GDP-mannose to GDP-l-galactose or GDP-l-gulose, and represents the first step in the de novo synthesis of ascorbate (Wolucka et al., 2001; Ingle et al., 2007). It has been demonstrated that application of the signaling molecule methyl jasmonate leads to transcriptional up-regulation of the gene encoding Gdp-mannose- 3′,5′-epimerase and increased ascorbate biosynthesis in tobacco cell cultures (Wolucka et al., 2005). Furthermore, Wolucka et al., (2005) reported that ascorbate was up-regulated in leaves of non-resurrection plants in response to abiotic and biotic stresses. Exogenous ascorbic acid (vitamin C) was shown to increase resistance to salt stress and reduce lipid peroxidation in plants (Shadforth and Neumann, 2005). In *Arabidopsis,* Gdp-mannose-3′,5′-epimerase was shown to co-purify with HSP70 protein, which indicates that HSP70 might involved in regulation of this enzyme (Wolucka and Montagu, 2003). Interestingly, HSP70 protein (2.328 change-folds) was also shown to be up-regulated in the nucleus *X. viscosa* in response to dehydration stress.

Malate dehydrogenase (NAD) (2.29±0.29 fold-change). Dehydration responsive functional network in the nucleus whether ABA acts upstream of ROS or vice-versa is controversial as gene expression via ROS independent ABA signaling is also common in plants (Pandey 2008). Despite the deleterious effect of the ROS, recent studies indicate that at low concentration they act as signaling molecules (Apel and Hirt, 2004). NADPH enzymes such as

oxidase, a so-called "respiratory burst oxidase homologue" mediating ABA induced ROS (Pandey et al., 2008), are known nuclear membrane proteins that may produce O_2^- in the vicinity of membrane ion channels (Ushio-Fukai, 2006). The resulting oxidative species, in turn, act as secondary messengers to control a variety of physiological responses. It is clear that this respiratory burst must have an adequate supply of NADPH for ROS generation, which suggests an increase in NADP(H) is a requirement for this signaling pathway (Apel and Hirt, 2004). The required NADPH for this reaction might be generated by malate dehydrogenase making it an important member of the ROS pathway (Apel and Hirt, 2004). The capacity to scavenge ROS and to reduce their damaging effects is important for stress-tolerance (Apel and Hirt, 2004). Under normal conditions all plants are able to cope with the levels of ROS, but dehydration stress exacerbates the levels of ROS and when plants antioxidant defense systems fail to eliminate excess ROS then injury occurs (Apel and Hirt, 200). This indicates that up-regulation of any of antioxidant element in response to stresses may reduce or prevent oxidative damage of ROS and improve desiccation tolerance in plants. Malate dehydrogenase was also reported in the nuclear proteomes of *Arabidopsis* in response to cold stress and chickpea in response to dehydration stress (Bae et al., 2003; Pandey et al., 2008).

Ferredoxin $NADP^+$ reductase (2.64±0.05 change-fold): functions in eliminating excess reducing power and preventing uncontrolled over reduced states that occur in normal physiological and stress conditions (Eckardt, 2006). It has been reported that purified cyanobacterial flavodoxins (Fld) are able to mediate plant ferredoxin (Fd)-dependent reactions *in vitro*, including the $NADP^+$ and thioredoxin reduction (Eckardt, 2006). The expression of cyanobacterial Fld in tobacco compensated for the decline in Fd under adverse environmental conditions and resulted in transgenic lines exhibiting increased tolerance to oxidative stress, including redox-cycling herbicides, extreme temperatures, high irradiation, water deficit, and UV radiation (Eckardt, 2006). This data indicates that the ferredoxin-thioredoxin reductase system plays a key role in plant tolerance to oxidative stress.

Up-regulated proteins involved in cell signaling:

Casein kinase α1 (5.43±0.06 fold change), belongs to the group of protein kinases designated as serine/threonine-selective enzymes that function as regulator of signal transduction pathways in most eukaryotic cells, these signal transduction pathways are related

to ROS scavenging machinery and other cell signals (Davidson et al., 2005). The name of this enzyme is connected to its substrate for historical reasons (Burnett and Kennedy, 1954). Casein kinase was reported in the chickpea nuclear proteome and was shown to be up-regulated in response to dehydration stress (Pandey et al., 2008). Changes in protein phosphorylation were observed in plants under dehydration stress (Ramanjulu and Bartels, 2002; Rohring et al., 2006). In mammalian, yeast and plant cells several protein kinases were shown to be involved in the main signal transduction pathways responsible for the transduction of signals from the cell membrane to the nucleus and were identified in response to dehydration, suggesting that protein kinases were involved in stress signaling in plants (Morano and Thiele, 1999; Ramanjulu and Bartels, 2002). However, the details of intracellular signaling pathways in plants leading to adaptive changes are still scarce (Ramanjulu and Bartels, 2002). Many kinases were reported also in the analyzed nuclear proteomes of *Arabidopsis* (Bae at al., 2003), rice (Khan and Komatsu, 2004); chickpea (Pandey et al., 2006; 2008) and *M. truncatula* (Repetto et al., 2008).

Calcineurin B-like protein 2 (3.20±0.18 fold-change), is a calcium-binding protein involved in signal transduction in response to diverse abiotic stresses include drought, wounding and cold (Tong et al., 1995; Luan et al., 2002; Ramanjulu and Bartels, 2002). The role of calcium in signaling in plants during abiotic stress has been demonstrated unequivocally (Ramanjulu and Bartels, 2002). Calcium channels have been detected in the nuclear membranes of plant cells (Quareshi et al., 2007). Calcium-dependent protein kinases were shown to be up-regulated in plants under drought and salt stress conditions showing the importance of calcium signaling in gene expression (Ramanjulu and Bartels, 2002). Calinuerin B-like proteins are implicated in variety of signaling pathways in animals (Tong et al., 1995) and in adaptation to salt stress in plants and yeasts (Nakamura et al., 1993; Pardo et al., 1998; Liu and Zhu, 1998). Calcium binding proteins were also reported in the nuclear proteomes of *Arabidopsis* (Bae at al., 2003), rice (Khan and Komatsu, 2004); chickpea (Pandey et al., 2006; 2008) and *M. truncatula* (Repetto et al., 2008).

Disease resistance protein (2.73±0.17 fold-change). Plants respond to biotic (pathogens) and abiotic stresses in a similar manner, and many genes induced by pathogens were found to be induced by abiotic stresses (Qureshi et al., 2007; Wolucka et al., 2005). Plant disease

resistance is regulated by two classes of genes: the major resistance (R) genes and the defence-responsive or defence-related genes. Most of the R genes that have been characterized are race-specific (Hammond-Kosack and Parker, 2003), whereas most defence-responsive genes, which encode the components of the signal transduction pathways leading to defense responses of the host after the recognition event triggered by an R gene product, are not be race-specific (Maleck et al., 2000; Zhou et al., 2002; Wen et al., 2003). PR protein, which is the general name of a variety of novel proteins, has been known to be essential component of systemic acquired resistance (Wang et al., 2006). The accumulation of PR protein(s) by the induction of defense signalling molecule(s) is a sign of active plant defence. *Oryzae sativa* (rice) OsPR1a, OsPR1b, OsPR5, OsPR8, and OsPR10 genes are inducible to all of the known signalling molecules of defense responses, jasmonic acid and ethylene (Agrawal et al., 2000a,b, 2001a; Rakwal et al., 2001a,b; Park et al., 2004; Wang et al., 2006). The expression of another rice PR gene, JIOsPR10, is also upregulated by jasmonic acid (Wang et al., 2006). Wang et al., (2006) have shown that OsDR8, a disease resistance gene, codes for thiamine in rice and has a dual phenotypic impact on rice for both defence regulation and thiamine synthesis. Methyl jasmonate and its free acid jasmonic acid are important cellular regulators involved in activation of plant defence mechanisms in response to insect-driven wounding, various pathogens and environmental stresses, such as drought, low temperature and salinity (Qureshi et al., 2007). The above indicates that protection or defence mechanisms in plants against abiotic and biotic stresses share common elements which are complex and interconnected.

Patatin-like protein (2.01±08 fold-change) Patatins are a group of plant storage glycoproteins that show lipid acyl hydrolase activity (Andrews et al., 1988; Banerji and Flieger, 2004). The patatin-associated lipolytic activity was thought to be a means of defense against plant parasites and has been shown to function in plant signal transduction as well (Strickland et al., 1995; Holk et al., 2002; Banerji and Flieger, 2004). Lipase/hydrolase was also reported in the nuclear proteome of *Arabidopsis* in response to cold stress and is involved in signal transduction (Bae et al., 2003). As mentioned earlier, plants responses to abiotic and biotic stresses shared common elements, thus patatin could be implicated in signal transduction in the nucleus of *X. viscosa* in response to dehydration stress.

Up-regulated proteins involved in molecular chaperone type activities

Heat shock protein 17.6 kDa class 1 (2.69± 0.22 fold-change), heat shock protein 81-2 (2.42±0.09 fold-change) and HSP 70 kDa (2.33±0.17 fold-changes), were shown to be up-regulated in the nuclear proteome of *X. viscosa* in response to dehydration stress. All cells counter the potentially deleterious effects of environmental stress via the coordinated synthesis of a battery of proteins called heat shock proteins (Hsps), which both protect the cell and facilitate rapid recovery from stress-induced cellular damage (Morano and Thiele**,** 1999). Heat shock proteins function as molecular chaperones and belong to a class of proteins whose function is to interact with and stabilize proteins that are partially or totally unfolded, as is the case when proteins are in the process of being synthesized, translocated across a membrane, or damaged by conditions of cellular stress (Leroux et al., 1997). Many chaperones are expressed at higher levels during biological stresses, and are members of heat shock protein (HSP)1 families. Whereas some chaperones (HSP70, HSP40, and HSP60) are involved in protein folding under normal conditions *in vivo*, others such as HSP104, inducible HSP70s, and small HSPs (Leroux et al., 1997) are known to play important roles in protecting organisms from stress. The large family of 70 kDa heat-shock or stress proteins (HSP) is the most evolutionary conserved and most prominent among all of the stress proteins. They are rapidly synthesized when cells are stressed by heat or by a wide range of adverse stress conditions in order to provide cyto-protection against macromolecular damage. Constitutive members of this family in non-stressed cells have a role as molecular chaperones (Agueli et al., 2001) and have been localized in several cellular compartments, including the centrosomes. Heat shock proteins are induced by dehydration and other abiotic stresses (Craig et al., 1994). Many Hsps are also maintained at high levels in unstressed cells and are required for a number of cellular processes from protein biosynthesis, processing and transport to signal transduction (Morano and Thiele, 1999). Hsps were shown to be localized in the nucleus and in other subcellualr compartments; their multilocation indicates their importance in many stages of protein turnover and diverse cellular processes (Craig et al., 1994). Heat shock proteins were also reported in the nuclear proteomes of *Arabidopsis* (Bae at al., 2003), rice (Khan and Komatsu, 2004); and chickpea (Pandey et al., 2008)

Up-regulated proteins involved in nucleocytoplasmic transport

The stress perception and signal transduction to switch on adaptive responses are critical steps in determining the survival of plants exposed to adverse environments. Plants have multiple stress perception and signal transduction pathways, which may cross-talk at various steps (Knight et al., 2001). Thus, a lot of molecules have to move in and out of the nucleus at any given time. Proteins involved in signal transduction were shown to play a crucial role in nucleocytoplasmic transport as they provide the identity of these two compartments and ensure the directionality of transport (Pandey et al., 2008).

GTP-binding protein GB2 (8.99±0.17 fold-change) and GTP-binding protein (2.50±0.33 fold-change). These proteins are nuclear and are highly up-regulated under dehydration stress (Pandey et al 2008). They well known to be involved in a wide range of regulatory functions in all organisms. Their functions include growth, differentiation, cytoskeletal organization, and intracellular vesicle transport and secretion (Hall, 1990). In this context the GTP-binding proteins are the major regulators of nucleocytoplasmic transport.

Dynamin-related protein ID (7.20± 2.50 fold-change) was up-regulated by the dehydration stress in nuclear proteome of *X. viscosa*. Dynamin-related protein fulfils GTPAse function and plays a crucial role in nucleocytoplasmic transport (Pandey et al., 2008). It is encoded by a nuclear gene—Dynamin, which is a large GTPase protein required for endocytosis in animal cells and assembles into a multimeric spiral at the neck of clathrin-coated pits (Sever, 2002). Numerous studies have revealed that dynamin and dynamin-related proteins (DRPs) are involved in not only endocytosis but also diverse cellular membrane-remodeling events including vesicular transport, division of organelles and cytokinesis (Praefcke and McMahon, 2004). The *Arabidopsis* genome has 16 DRPs grouped into 6 subfamilies (DRP1-6) (Hong et al. 2003a). Members of two of these subfamilies (DRP1 and DRP2) are candidates for plant dynamin that are involved in membrane trafficking. Endocytosis is an essential phenomenon in eukaryotic cells for engulfing external materials, for intermediating cellular signals and for regulating the abundance and distribution of plasma membrane proteins (Mellman, 1996). Compared with what is known about endocytosis related molecules in animal cells, little is known about endocytosis-related molecules in higher plants (Murphy et al., 2005). However, the *Arabidopsis* genome has possible homologues of some of the major molecules in animal

endocytosis, such as dynamin and clathrin (Fujimoto et al., 2007). Dynamin-like GTPase was also reported in the nuclear proteome of *M. truncatula* (Repetto et al., 2008).

Up-regulated proteins of translation machinery

EF-Tu and eEF-1-α with change-folds (2.29±0.10) and (2.23± 0.11) respectively were shown to be up-regulated in the nuclear proteome of *X. viscosa* in response to dehydration stress. The functions of these proteins were discussed in detail previously (chap. 3)

Up-regulated proteins with miscellaneous functions

Our study showed that ADP-ribosylation factor (ARF) (2.02± 0.10 fold-change) was up-regulated in the nuclear proteome of *X. viscosa* in response to dehydration stress. This protein (ARF) is a ubiquitous, highly conserved 21-kDa GTP binding protein (members of the small ras-like GTPase superfamily), originally identified in mammalian cells as a protein required for ADP ribosylation of the R subunit of the heterotrimeric G protein G_S, thereby leading to activation of adenylate cyclase (Stearns et al., 1990; Lee et al., 2002; Zuk et al., 2003). While ARF is largely localized in Golgi stacks, it may also be present in other cellular compartments (Stearns et al., 1990). ARF functions as an activator of the enzyme phospholipase D and is required in coated vesicle assembly of the Golgi apparatus (Stearns et al., 1990; Zuk et al., 2003). ADP ribosylation factors function as specific regulators of vesicle trafficking in animal and plants and may be involved in the regulation of ADP- ribosyl transferase (Zuk et al., 2003). Zuk et al., (2003) in their studies of transgenic potato with ADP ribosylation factor, demonstrated that transgenic potato expressed high levels of sucrose, decreased level of glycol-alkaloids and showed increased antioxidant capacity. Thus, ADP-ribosylation factor may contribute to the adaptation of *X. viscosa* to dehydration stress by facilitating overexpression of sucrose, a compatible solute that acts as a water replacement molecule to stabilize membrane and protein structure and as an energy source during dehydration. On the other hand, activation of phospholipase D generates phospholipid signaling which might regulate nucleocytoplasmic transport with a possibility that it plays a role in adaptation to dehydration stress (Frank et al., 2000).

ATP binding (2.74±0.03 fold-change). Members of ATP binding cassette super family of integral membrane transporters functions in cellular detoxification, cell-to-cell signaling, and

channel regulation (Terasaka et al., 2005). More recently, members of the multidrug resistance P-glycoprotein (with ATP binding function) (MDR/PGP) subfamily of ABC transporters have been shown to function in the transport of the phytohormone auxin in both monocots and dicots (Terasaka et al., 2005). Mutagenic studies on *Arabidopsis* showed that ATP binding protein was essential for normal functioning of roots (Terasaka et al., 2005). Mutants P-glycoprotein (pgp4) exhibited reduced basipetal auxin transport in roots and a small decrease in shoot-to-root transport consistent with a partial loss of the redirective auxin sink in the root compared with the wild type (Terasaka et al., 2005). Seedlings over expressing normal PGP4 exhibited increased shoot-to-root auxin transport. Heterologous expression of PGP4 in mammalian cells resulted in 1-N-naphthylthalamic acid-reversible net uptake of indole-3-acetic acid (Terasaka et al., 2005). These results indicate that PGP4 functions primarily in the uptake of redirected or newly synthesized auxin in epidermal root cells (Terasaka et al., 2005).

Up-regulated proteins with unknown functions

Two proteins were identified with unknown functions, putative protein (2.10±0.02) and unknown protein (2.06±0.01).

Up-regulated proteins involved in synthesis of compatible solute

UDP glucose 4-epimerase (3.83±0.19 fold-change) was up regulated in the nuclear proteome of *X. viscosa* in response to dehydration stress. In higher plants, nucleotide sugars act as biosynthetic substrates and intermediates in the uptake of free sugars released from the breakdown of nutritional or storage carbohydrates and other sources (Liu et al., 2007). Nucleotide sugars can be modified at their glycosyl moieties to generate different sugars and intermediates by nucleotide sugar interconversion enzymes (Liu et al., 2007). UDP-glucose 4-epimerase (UGE) is one of these enzymes which interconverts UDP-d-glucose (UDP-Glc) and UDP-d-galactose (UDP-Gal) (Liu et al., 2007). UGE is essential for *de novo* biosynthesis of UDP-Gal, a precursor for the biosynthesis of numerous different carbohydrates, glycolipids, and glycosides (Zhang et al., 2006). UDP glucose 4-epimerase (EC 5.1.3.2) in *Arabidopsis* has five genes encoding functional UDP-D glucose/ UDP-D-galactose 4-epimerase (named UGE1 to UGE5), while in rice, there are at least four *UGE* genes (Zhang et al., 2006). In transgenic plants there is a correlation between UGE1 expression and resistance to toxic levels of galactose UDP-glucose 4-epimerase (UGE). There are many carbohydrate biosynthetic enzymes using

UDP-Gal as a substrate, one of these is galactinol synthase (GolS (Liu et al., 2007). GolS catalyses the first committed step in the biosynthesis of raffinose and plays a key regulatory role in carbon partitioning between sucrose and raffinose (Liu et al., 2007). In plants, galactinol and raffinose have been shown to be important for improving stress tolerance in plants (Liu et al., 2007). Expression of the rice gene OsUGE-1 was found to be induced by various abiotic stresses, including cold, drought, high salinity and ABA (Liu et al., 2007). Transgenic *Arabidopsis* over-expressing OsUGE-1 was shown to confer tolerance to salt, drought and freezing stress without altering plant morphology (Liu et al., 2007). In addition, comparison of soluble sugars in wild-type and transgenic plants over-expressing OsUGE-1 showed remarkably higher levels of raffinose in transgenic plants than wild-type plants (Liu et al., 2007). Thus, it is very likely that enhanced tolerance to abiotic stress by over-expressing OsUGE-1 may be due to elevated level of raffinose (Liu et al., 2007). Raffinose as a compatible solute may contribute to the desiccation properties of *X. viscosa* by facilitating the maintenance of favorable turgor pressure during water loss and may also serve as a protective agent by stabilizing protein structure (Mundree et al., 2002). Compatible solutes have also been shown to be involved in antioxidant defense mechanisms, protecting DNA from the degradative effects of ROS (Kishor et al., 1995; Mundree et al., 2002).

The overall functions of the up-regulated proteins in the nucleus of *X. viscosa* demonstrate that acquisition of desiccation tolerance is controlled by multiple genes. The first and largest group of these genes was shown to encode for proteins involved in gene regulation and signal transduction, which reflects the role of nucleus in gene expression and regulation. This is followed by genes that encode for stress response molecules such as antioxidants, molecular chaperones and compatible solutes. These results demonstrate the important roles of antioxidant proteins in neutralizing the damaging effects of ROS, which can denature proteins, cause DNA mutation, lipid peroxidation and consequently membrane damage. Strong antioxidant defense systems enable the prevention of lipid peroxidation during dehydration and maintaining of cellular integrity (Ingram and Bartels, 1996). Molecular chaperones prevent the aggregation of misfolded proteins and thus protect cellular integrity during dehydration stress. Compatible solutes have been reported to stabilize cellular membranes and maintain cell turgor (Mundree et al., 2002; 2006), and thereby might enable *X. viscosa* to survive the debilitating effects of water loss to the cell. The available studies suggest direct correlation between stress

tolerance and antioxidant defense capacity (Ingram and Bartels, 1996). The enhancement of defense mechanisms in *X. viscosa* nucleus revealed by this study might therefore be critical in enabling this plant to survive severe water loss. Up-regulation of proteins implicating in nucleocytoplasmic transport indicates the presence of cross-talking between the nucleus and the cytoplasm during dehydration, and emphasizes the importance of this action in adaptation to dehydration stress. Proteins of translation machinery are integral components of adaptation to desiccation stress as the enhancement of the translation machinery would allow for expression of cellular stress response proteins under dehydration where rates of protein production significantly and progressively decline. Other features of the up-regulated proteins in the nucleus of *X. viscosa* in response to dehydration stress include proteins with miscellaneous functions, and unknown proteins or proteins with unassigned functions. The prediction of a possible function for these proteins might improve our knowledge about desiccation tolerance in plants.

Overview of functions of the down-regulated nuclear proteins

Approximately eleven percent of the analyzed proteins were down-regulated in the nuclear proteome of *X. viscosa* in response to dehydration stress.

Down-regulated proteins involved in protein translation

Putative S-phase-specific ribosomal protein (0.49±0.04); 30S ribosomal protein S5 (0.48±0.01); ribosomal protein 5B (0.48±0.02); 40S ribosomal protein (0.37±0.02); and 40S ribosomal protein S19-like (0.33±0.01) were shown to be down-regulated. Ribosomes are responsible for protein synthesis in all cells and thereby link transcriptomes with proteomes and are standard in the case of any nuclear proteome (Pandey et al., 2006). The identification of different elements of translation such as ribosomal subunits in the nuclear matrix gains relevance from the recent demonstration of nuclear translation (Calikowski et al., 2003) and the association of ribosome components with the sites of transcription and nascent RNP complexes (Calikowski et al., 2003). Recent studies showed that some ribosomal proteins, in addition to their functions as stabilizers of rRNA structure in the ribosome to guarantee the efficiency of protein synthesis, may also possess extra-ribosomal functions such as DNA repair, regulation of transcription in nucleus, cell growth and apoptosis (Jing et al., 2006). Ribosomal proteins were reported previously in the nuclear proteomes of *Arabidopsis*, (Bae et al., 2003), rice (Khan and

Komatsu, 2004), chickpea (Pandey et al., 2008), and *Medicago* (Repetto et al., 2008). The decreased levels of ribosomal proteins in the nuclear proteome of *X. viscosa* in response to dehydration stress reflect their important roles in the physiology of the plant under well watered conditions and in the recovery of the plant from the dehydration stress.

Down-regulated proteins involved in miscellaneous cell functions

Two glutamine synthetases (GS) (0.47±0.04; and 0.45±0.03), were revealed by our study to be down regulated in response to dehydration stress. GS functions as the major assimilatory enzyme for ammonia produced from N fixation, and nitrate or ammonia nutrition (Miflin and Habash, 2002). It also reassimilates ammonia released as a result of photorespiration and the breakdown of proteins and nitrogen transport compounds (Miflin and Habash, 2002). The enzyme is the product of multiple genes with complex promoters that ensure the expression of the genes in an organ- and tissue-specific manner and in response to a number of environmental stresses affecting the nutritional status of the cell (Miflin and Habash, 2002). During the growth and development of plants, nitrogen is moved into and out of proteins in the different organs and transported between organs in a limited number of transport compounds, but a significant portion is released as NH_3 and reassimilated via GS (Miflin and Habash, 2002). GS functions in conjunction with a number of other key enzymes to enable the plant to balance its carbon and nitrogen metabolism in different parts of the cell, at different times of the day, in different organs and under a wide range of environment conditions (Miflin and Habash, 2002). Recent studies with transgenic plants suggests that altered or overexpression of GS may accelerate development (Miflin and Habash, 2002). Earlier flower and seed development were observed in transgenic wheat lines containing a *Phaseolus vulgaris* GS1 gene under the control of the rbcS promoter (Miflin and Habash, 2002). Presumably GS is involved in ammonia metabolism pathways that are active in hydrated *X. viscosa* plants.

Two Glycolate oxidases (0.27±0.03; and 0.28±0.01) were shown to be down regulated in response to dehydration stress. The plant glycolate oxidase catalyzes the oxidation of glycolate to glyoxylate as well as the oxidation of glyoxylate to oxalate and plays an essential role in photorespiration pathway in the plant (Jones et al., 2000). Glycolate oxidase was also reported in the *Arabidopsis* and chickpea nuclear proteomes under normal growth conditions (Bae et al., 2003; Pandey et al., 2006).

NADP-specific isocitrate Dehydrogenase (NADP-IDH) (0.29±0.11), is involved in the supply of 2-oxoglutarate for ammonia assimilation and glutamate synthesis in higher plants through the glutamine synthetase/glutamate synthase (GS/GOGAT) cycle (Palomo et al., 1998). The synthesis of 2-oxoglutarate represents a connecting point between carbon and nitrogen metabolism because this keto acid provides the carbon skeleton for the assimilation of inorganic nitrogen into amino acids in higher plants through the GS/GOGAT cycle. The result from this cycle, glutamine and glutamate are the donors for the synthesis of major nitrogen compounds in higher plants: amino acids, chlorophyll, polyamines, and nucleic acids (Palomo et al., 1998). 2-Oxoglutarate is also a co-substrate of a group of dioxygenases involved in different metabolic pathways, including phytohormone, flavonoid, and alkaloid biosynthesis and in the hydroxylation of Pro residues of Hyp-rich glycoproteins (Palomo et al., 1998). In angiosperms NADP-IDH has been studied in pea and several members of the Solanaceae and cucumber families (Palomo et al., 1998). It occurs as several isozymes located in different subcellular compartments, including the cytosol, chloroplast, and mitochondria (Palomo et al., 1998). However, Palomo and co-workers (1998) reported a non-coordinated expression pattern of NADP-IDH and GS/GOGAT in advanced stages of pine cotyledon development and in the hypocotyl, where NADP1-IDH abundance was inversely correlated with the presence of GS, GOGAT, and ribulose-1,5-bisphosphate carboxylase/oxygenase but was associated with the differentiation of the organ. Hence, they diminished the role of the enzyme in nitrogen assimilation and suggested that NADP-IDH may have other, as-yet-unknown, biological functions.

Cytochrome b559 (0.19±0.02), forms part of plant respiratory and antioxidant-defense systems (Buser et al., 1992). Cytochromes were also reported in the nuclear proteome of *Medicago* (Repetto et al., 2008).

Down-regulated proteins involved in ROS pathways

Catalase (0.43±0.01), enzyme catalyzes the change of hydrogen peroxide to water and is an important member of antioxidant defense systems in plants (Scandalios, 1994). The decreased level of catalase in the nuclear proteome of *X. viscosa* in response to dehydration stress at 35% RWC was probably due to the fact that this enzyme is efficient against oxidative

stress during moderate dehydration or when the plant is well-watered. This result suggested that the protective action of catalase does not appear to be an efficient way of scavenging hydrogen peroxide under low water deficit such at 35% RWC; hence the nucleus utilizes other alternative protective antioxidant mechanisms located within the nucleus to dispose of the ROS. These results demonstrate that plant utilize different strategies to deal with ROS. Proteins of antioxidant defense systems were reported in the nuclear proteomes of *Arabidopsis* (Bae et al., 2003), rice (Khan and Komatsu, 2004), chickpea (Pandey et al., 2006; 2008), and *Medicago* (Repetto et al., 2008).

Down-regulated hypothetical proteins

Our study revealed two proteins down regulated (0.40±0.02; and 0.30±0.02) in the nuclear proteome of *X. viscosa* in response to dehydration stress with hypothetical functions.

In summary the reduction in expression level of these proteins may have resulted from repression of gene expression and/or increased protein turnover (Jiang et al., 2007), and support the notion that these proteins are likely to be involved in metabolic pathways that were active in well-watered tissues but inactivated by the decrease in osmotic potential (Jiang et al., 2007). Proteins that are down-regulated in response to dehydration stress may not be directly associated with stress tolerance per sec. However, it cannot be ruled out that the reduction in expression was actively triggered by dehydration for purposes such as resource reallocation thereby activating physiological mechanisms that are necessary for desiccation tolerance (Jiang et al., 2007).

Overview of functions of the proteins showing consistent expression levels

The majority of the identified proteins in the nucleus of *X. viscosa* in response to dehydration was shown to have consistent expression levels (no significant changes in expression) and comprised 66% of the total identified proteins. Proteins showing consistent expression levels during dehydration are likely to be structural proteins or proteins associated with the basic metabolic activities that are important to keep the minimum viability in cells under all kinds of conditions. They may therefore not directly play roles in desiccation tolerance; but are necessary for survival (Jiang et al., 2007).

Proteins showing consistent expression levels involved in gene regulation

Two glyceraldehyde-3-P-DHs (1.76±0.04) and (1.09±0.06), a fructose bisphosphate aldolase like –protein (1.59±0.07), and a phosphor-glycerate kinase (1.66 ±0.05). These enzymes are known to have non-glycolytic function in the nucleus (Pandey et al., 2006; 2008). GAPDH has been proposed to act as a tRNA-binding protein and may participate in RNA export (Pandey et al., 2008). Zheng et al., (2003) discovered GAPDH can itself activate transcription. The OCA-S transcriptional coactivator complex contains GAPDH and lactate DH, two proteins previously only thought to be involved in metabolism. GAPDH moves between the cytosol and the nucleus and may thus link the metabolic state to gene transcription (Zheng et al., 2003). Fructose bisphosphate aldolase was reported to be involved in DNA-binding and thus it might be implicated in regulation of gene expression (Ronai et al., 1992). The phosphorylation of fructose-bisphosphatase might be the signal inducing the movement of the enzyme to the nucleus where it could induce the expression of other genes (Gizak and Dzugaj, 2003). Phosphor-glycerate kinase is known to function as a primer recognition protein involved in DNA synthesis and is known to posses a bipartite nuclear localization signal in the N-terminus (Pandey et al., 2006).

Two DNA-binding proteins (1.77±0.12; and 1.26±0.02), and a DNA gyrase subunit B-like (1.74±0.03) were identified in the nuclear proteome of *X. viscosa* in response to dehydration stress with no significant expression levels. The latter is a member of the topoisomerase superfamily (Corbett, et al., 2004). These enzymes are assembled as oligomeric complexes with distinct domains that coordinate ATP binding and hydrolysis with DNA binding, cleavage, and transport (Corbett, et al., 2004). The topology of cellular DNA is managed by topoisomerases, enzymes that pass DNA strands through each other to relieve excess supercoiling and resolve DNA knots and catenanes (Corbett, et al., 2004). Whereas all organisms contain at least one topoisomerase, the bacterium *E. coli* possess four, each with distinct roles: topoisomerase (topo) I, topo III, topo IV, and DNA gyrase (Corbett, et al., 2004). Topo I and topo III pass single DNA strands through one another to relax negative supercoils or aid RecQ-family helicases in certain DNA repair processes, respectively (Corbett, et al., 2004). DNA gyrase and topo IV use ATP to power the transport of one intact DNA duplex through another, an activity that can alter DNA superhelicity as well as promote chromosome decatenation (Corbett, et al., 2004). DNA gyrase function is important for counteracting

positive supercoiling forces arising from DNA-unwinding events and for maintaining appropriate levels of supercoiling in the chromosome (Corbett, et al., 2004). DNA-binding proteins were also reported in the nuclear proteome of *Arabidopsis* (Bae et al., 2003), and in the nuclear proteome of *Medicago* (Repetto et al., 2008), where it was reported to function as unspecified transcription factors involved in gene transcription and regulation.

Pattern formation protein (1.87±0.03), was reported to be involved in regulatory circuits during development (Schellmann et al., 2007). The current data suggest that this gene cassette comprises a transcriptionally active complex consisting of a R2R3-type Myb-related transcription factor, a bHLH (basic helix–loop–helix) protein and a WD40 protein (Schellmann et al., 2007). bHLH proteins are involved in the patterning process, where in some cases, different homologues act specifically only in one of the patterning systems while in other cases, the family members are involved in all patterning systems although with different impacts (Schellmann et al., 2007).

Peptidyl prolyl isomerase (PPI) ROCI (regulator of chromosome condensation 1) (1.67±0.07), belongs to a ubiquitous class of proteins which are expressed in both prokaryotic and eukaryotic cells alike (Shaw, 2002). In addition to their primary catalytic function to facilitate the *CIS*-trans isomerisation of peptide bond N-terminal to proline within polypeptide chains, several PPI are involved in chaperone-like activities and regulation of gene transcription and regulation. They perform these functions in conjunction with protein kinases, histone H3 and transcription elements (Shaw, 2002).

Histone 2AXa (1.64±0.02), histone H2A (1.46±0.08), and H2B11 (1.42±0.13) expressed with consistent expression levels in the nucleus of *X. viscosa* in response to dehydration stress at 35% RWC. Histones are highly conserved components of eukaryotic chromatin. During the past decade, an increasing number of reports has emphasized the roles of histone modification and histone variants in the regulation of gene expression (Yi et al., 2006). Nemergut et al., (2001) have demonstrated that H2A/H2B was involved in nuclear envelope assembly and nuclear transport through an interaction involving Ran-specific exchange factor, RCC1 (regulator of chromosome condensation 1) with chromatin. The interaction of RCC1 to

H2A/H2B establishes the polarity of the Ran-GTP gradient which was proposed to be responsible for the aforementioned functions (Nemergut et al., 2001).

RNA helicases: DEAD-box ATP-dependant RNA helicase 52 (1.29±0.01), RNA helicase (1.12±0.03) and RNA helicase (1.08±0.02) expressed with consistent expression levels in the nucleus of *X. viscosa* in response to dehydration stress at 35% RWC. These enzymes belong to a large group of enzymes, which also include the known DNA helicases and function in virtually all aspects of RNA metabolism including RNA duplex unwinding, protein displacement from RNA and strand annealing (Jankowsky and Fairman, 2007). Most RNA helicases belong to helicase superfamily 2 (SF2) and are grouped into the DEAD-box, the DEAH and the DExH subfamilies. A few RNA helicases belong to helicase superfamily 1 (SF1). RNA helicases perform their function mostly as parts of large macromolecular assemblies such as the pre-mRNA splicing machinery (Jankowsky and Fairman, 2007). Several DEAD-box proteins have been shown to catalyze strand annealing, in addition to their duplex unwinding activity (Jankowsky and Fairman, 2007). RNA helicase were also reported in the nuclear proteomes of *Arabidopsis* and *M. Truncatula* (Bae et al., 2003; Repetto et al., 2008)

Arginine/serine-rich-splicing factor RSP31 (1.19±0.02) and a Spliceosome associated protein (0.59±0.02) were also revealed by our study to express with consistent levels in the nucleus of *X. viscosa* in response to dehydration stress at 35% RWC. Multi-cellular organisms regulate splicing of pre-mRNAs depending on cell type, developmental state or external stimulus (Reddy, 2004). Many proteins involved in mRNA processing also interact with transcription and mRNA export machinery. This results in truncated or extended proteins with altered (increased, decreased or loss of) activity, cellular localization, regulation, and/or stability (Reddy, 2004). The serine/arginine-rich proteins (SR proteins) are a highly conserved family of structurally and functionally related non-snRNP proteins with multiple roles in pre-mRNA splicing (Reddy, 2004). Ser/Arg-rich (SR) proteins can bind to specific RNA sequences and assemble the spliceosome at weak splice sites in alternative splicing **(**Isshiki et al., 2006). The SR proteins have one or two RNA recognition motifs (RRMs) in the N terminus and an Arg/Ser-rich (RS) domain for protein–protein interaction in the C terminus **(**Reddy, 2004; Isshiki et al., 2006). The activity of SR proteins for alternative splicing depends on specific RNA sequences called exonic splicing enhancers (ESEs) **(**Isshiki et al., 2006). Moreover, SR proteins are

essential splicing factors for constitutive splicing and are highly conserved in metazoans and plants **(**Isshiki et al., 2006). The functions of SR proteins in mRNA metabolism, however are not only in constitutive and alternative splicing but also in nuclear export, mRNA stability, and translation **(**Isshiki et al., 2006). Three human SR proteins, ASF/SF2, SRp20, and 9G8, shuttle between the nucleus and cytoplasm depending on the phosphorylation of the RS domain and could promote the export of intronless mRNAs **(**Reddy, 2004; Isshiki et al., 2006). With the aid of the green fluorescent protein (GFP) fusion, RS was shown to be localized in the nucleus in a speckled pattern **(**Isshiki et al., 2006). Alternative splicing is an important mechanism in the regulation of gene expression in eukaryotes **(**Isshiki et al., 2006). It enables the generation of proteins with different functions and structures through variations in the splicing patterns of pre-mRNA from one gene **(**Isshiki et al., 2006).

Three small nuclear ribonucleoproteins (1.56±0.03; 1.01±0.26; and 0.69±0.04) were also revealed by our study to express with consistent levels in the nucleus of *X. viscosa* in response to dehydration stress at 35% RWC. Alternative splicing is an important mechanism in the regulation of gene expression in eukaryotes **(**Isshiki et al., 2006). The selection of alternative splice sites is determined by the assembly of the spliceosome, a large complex containing five small nuclear ribonucleoproteins (snRNPs) and non-snRNP proteins **(**Isshiki et al., 2006). In the human genome, ~74% of transcripts are alternatively spliced **(**Isshiki et al., 2006). The basic mechanism of splicing in higher plants is similar to the one observed in vertebrates **(**Isshiki et al., 2006). Recently, it was demonstrated that the regulation of alternative splicing in higher plants is important in physiology and development **(**Isshiki et al., 2006). Small nuclear ribonucleoproteins were reported in the nuclear proteomes of *Arabidopsis* and *M. truncatula* (Bae et al., 2003; Repetto et al., 2008).

Retroelement Pol Polyprotein-like (1.23±0.04), belongs to DNA mobile elements, which can rearrange genomes and other individual gene structure and also affect gene regulation through various promoted activities: transposition, insertion, excision, chromosome breakage, and ectopic recombination (Capy et al., 1998; Bringaud et al., 2006). Retroelements were reported in the nuclear proteomes of *Arabidopsis*, *Chickpea* and *Medicago* (Bae et al., 2003; Pandey et al., 2006; 2008; Repetto et al., 2008). The functions of retroelements were highlighted in chapter 3.

Protein showing consistent expression levels involved in protein degradation

Ubiquitin-Like protein SMTB (1.71±0.16), 26S proteasome AAA-ATPase subunit RPT3 (1.94±0.02), AtCIpC Caseino-lytic protease C (1.46±0.08), 20S proteosome peptidase complex (1.33±0.88), putative polyubiquitin (1.05±0.00), DegP protease precursor (0.93±0.01), ATP-dependent Clp protease (0.82±0.03), 20S proteasome α subunit A2 (0.57±0.00) and putative prohibitin (0.99±0.00). Intracellular proteolysis might have an important role in the reorganization of plant metabolism under stress (Feller, 2004; Grudkowska and Zagdanska, 2004). The contribution of cysteine proteases to total proteolytic activity increases drastically in response to water deficit in wheat (Zagdanska and Wisnievski, 1996) and some experimental evidence suggests that drought-sensitive species and varieties have higher proteolytic activity compared to the resistant ones (Roy-Macauley et al., 1992; Hieng et al., 2004). Proteolysis of cellular proteins is a highly complex, temporally controlled and tightly regulated process which plays important roles in the regulation of many basic cellular processes such as cell cycle, growth regulation, signal transduction pathways and the response to stress and extracellular modulators (Ciechanover, 1998). Peptide hydrolysis is exergonic; therefore, it is surprising that up to 80% of protein degradation in *E. coli* requires ATP hydrolysis (Shanklin et al., 1995). However, this coupling of proteolysis to ATP hydrolysis is emerging as a general principle for both prokaryotes and eukaryotes (Maurizi, 1992). The AAA-ATPase enzyme is characterized by conserved 200-250 residues that include Walker A and B motifs (Vij, 2008). All known AAA+ proteins contain either 1 or 2 AAA+ domains, and the family can be divided into 2 groups on this basis. The most common function of AAA+ domain is to catalyze protein folding or unfolding in an ATP-dependent manner (Vij, 2008). Other cellular functions of AAA^+ gene family comprise nuclear envelope reconstruction, cell cycle, post mitotic Golgi reassembly, suppression of apoptosis, DNA damage response and endoplasmic reticulum associated degradation (ERAD) (Vij, 2008). In the eukaryotic cell secretory pathway, a significant proportion of unwanted proteins that enter the ER are specifically extracted from the ER and targeted to the cytosol, where they are degraded by the ERAD (Vij, 2008). In ERAD, protein ubiquitylation plays a role in both protein extraction from the ER and proteasome mediated protein degradation (Vij, 2008). This crucial protein modification is mediated by a set of ubiquitin enzymes (Vij, 2008). The proteasome is a multicatalytic proteinase complex that is involved in an ATP/ubiquitin-dependent proteolytic pathway (Vij, 2008). In eukaryotes, there

are two types of complexes with sedimentation coefficients of 20S (20S proteasome) and 26S (26S proteasome) (Oguchi et al., 2001). The 26S proteasome consists of a 19S regulatory complex and the 20S proteasome as the catalytic core (Oguchi et al., 2001). Proteasomes have been shown to be involved in not only degradation of misfolded or truncated proteins, but also degradation of many rate-limiting enzymes (e.g. ornithine decarboxylase), transcriptional regulators and critical regulatory proteins (e.g. cyclins) and involved in diverse biological functions including cell cycle progression (Coux et al., 1996). This protein degradation is necessary for viability (Coux, 1996). The genes that code for proteasomes were reported to be up-regulated in *Arabidopsis* in response to wounding (Genschik et al., 1992). In rice the genes were reported to be expressed in all organs and stages, and were stimulated by wounding (Oguchi et al., 2001). Proteasomes are present in the nucleus and cytosol of all eukaryotic cells and some particles are also found associated with the endoplasmic reticulum and with the cytoskeleton (Coux, 1996). In eukaryotes, the major energy-dependent protease system of the cytosol is the ubiquitin system (Shanklin et al., 1995), which degrades proteins by first coupling a small protein, ubiquitin, to the protein destined for degradation (Shanklin et al., 1995); a multi-subunit protease (also referred to as the proteasome) then degrades the tagged protein ATP hydrolysis is required for both the coupling of ubiquitin to the target protein and the degradation by the proteasome (Shanklin et al., 1995). Energy-dependent Clp proteases are composed of a proteolytic component, ClpP, and a regulatory ATPase, which can be either ClpA, or ClpC, or ClpX, and have homologues in most organelles (Demirevska et al., 2008). The responses of protein degradation machinery should lead to an enhanced capacity of the cell to degrade abnormal proteins. Proteins of degradation pathways were reported also in the nuclear proteomes of *Arabidopsis*, rice, and chickpea (Bae et al., 2003; Khan and Komatsu, 2004; Pandey et al., 2008).

Proteins showing consistent expression levels involved in miscellaneous functions

Cytochrome f (0.91±0.08) with consistent expression level was identified in the nuclear proteome of *X. viscose*. Plants use a diverse array of cytochromes in their biosynthetic and detoxificative pathways in particularly ROS pathways and were reported to be localized in different plant cell compartments (Brumme et al., 1998). Different cytochromes were also reported in the nuclear proteome of *Medicago* (Repetto et al., 2008)

V-ATPase subunit D (1.32±0.04), v-type H^+ ATPase (1.31±0.02), v-type ATPase (1.23±0.04) and anion-transporting ATPase (1.02±0.02). These proteins are a family of ATP dependent proton pumps that are responsible for acidification of intracellular compartments in eukaryotic cells and are present in a variety of intracellular compartments (Arata et al., 2002). Acidification of vacuolar compartments plays an important role in a variety of cellular processes; including receptor mediated endocytosis, intracellular targeting, protein processing and degradation, and coupled transport (Arata et al., 2002). The v-ATPase may be of major importance in preventing cells shrinking during dehydration resulting in cell death thus it enhances cell viability during dehydration stress by maintaining a balanced cell homeostasis. V-ATPaes were also reported in the nuclear proteome of chickpea (Pandey et al., 2006; 2008).

Germin-like protein (GLP10) (1.25±0.05) was reported to be ubiquitously distributed in the plant kingdom, (Kim et al., 2003). In several cereal species the "true" germins possess oxalate oxidase activity; however, none of the germin-like proteins are oxalate oxidates (Kim et al., 2003). Several other enzyme activities or functions have been suggested also for germin-like protein such as superoxide dismutase, ADP-glucose pyrophosphatase/phosphodiesterase, and auxin binding protein (kim et al., 2003). The exact function of these proteins in plants is not well understood. Germin-like proteins were reported also in the nuclear proteome of *Arabidopsis* (Bae et al., 2003).

Glutamine synthetase (1.13±0.09): this enzyme is involved in miscellaneous metabolic pathways; its function was discussed previously.

Carbonic Anhydrase 2 (1.10±0.11): is a zinc-containing metalloenzyme that catalyses the reversible inter-conversion of CO_2 and HCO_3, and is widely distributed in animals, plants, archaebacteria, and eubacteria (Yu et al., 2007). Plants have four types of CAs (a, b, c and d). CA is involved in a variety of biological processes including pH regulation, CO_2 transfer, ion exchanger, respiration, biosynthesis, and photosynthetic CO_2 fixation (Yu et al., 2007). Carbonic anhydrase was shown to localize in different subcellular compartments (Yu et al., 2007). Carbonic anhydrase was also reported in the nuclear proteomes of chickpea and *Medicago* (Pandey et la., 2006; Repetto et al., 2008).

Ribulose 1,5-bisphosphate carboxylase (1.04±0.02), and Rubisco large subunit (0.55± 0.01). This enzyme is a bifunctional enzyme which catalyzes the first reaction of photosynthetic Co_2 fixation and photorespiratory carbon oxidation (Lee et al., 1991; Lawler and Cornic, 2002). It is obviously that this enzyme is involved in basic metabolic functions necessary for *X. viscosa* survival under normal and dehydration stress conditions, as its expression remains constant under both conditions. Dioxygenase and Rubisco were reported in the nuclear proteome of *Arabidopsis*, chickpea and *M. truncatula* (Bae et al., 2003; Pandey et al., 2006, Repetto et al., 2008). Ribulose 1,5-bisphosphate carboxylase might be involved in other functions such as a signaling or stress molecule thus, its function and relation to the nucleus deserves further investigations.

Citrate synthase (0.76±0.08). The nuclear genome plays a dominant role in determining mitochondrial structure and function and in providing most of the products that regulate the expression and maintenance of the mitochondrial genome (Liao et al., 1991). Liao et al., 1991 examined this complex interplay through studying the effects of perturbation of mitochondrial function on expression of two nuclear genes encoding the mitochondrial and peroxisomal forms of citrate synthase in *Saccharomyces cerevisiae*. A mitochondrial form (CS 1) that is analogous to the protein found in mitochondria of higher eucaryotes and a non-mitochondrial form (CS 2) that is sequestered within peroxisomes. The mitochondrial isoform is encoded by CITI, and the peroxisomal isoform is encoded by CIT2 (Liao et al., 1991). Their studies demonstrated an increased expression of CIT2 in cells with altered mitochondrial function, which was thought to be related to an important physiological function and proposed an intercross path of communication from mitochondria to the nucleus in yeast cells (Liao et al., 1991). They also hypothesized that such events could provide signals to the nucleus allowing the cell to monitor mitochondrial DNA content and mitochondrial mass or the general state of mitochondrial activity during cell growth and division.

NADH-ubiquinone oxidoreductase (0.65±0.00). Mitochondria from various organisms, especially plants, fungi and many bacteria contain so-called alternative NADH: ubiquinone oxidoreductases that catalyse the same redox reaction as respiratory chain complex I, but do not contribute to the generation of transmembrane proton gradients (Kerscher, 2000). Plant mitochondria contain complex I and up to four alternative NAD(P)H: ubiquinone

oxidoreductases, which are associated with both faces of the mitochondrial inner membrane and display different induction kinetics and Ca^{2+} requirements (Kerscher, 2000).

Proteins showing consistent expression levels involved in ROS pathways

Peroxiredoxin Q-like protein: (1.53±0.01). Peroxides are reactive compounds that pose an oxidation threat to cells when they accumulate to high concentrations (Gross et al., 1977; Dietz et al., 2006). In the presence of Fe_2+, hydrogen peroxide (H_2O_2) is reduced in the Fenton reaction to yield the hydroxyl radical OH^- within diffusion distance, OH^- reacts with virtually any biomolecule to abstract an electron. Due to its ability to diffuse over significant distances within and between cells, H_2O_2 serves a signalling function in cellular communication (Foyer and Noctor, 2000). This dual role of H_2O_2 as a potentially damaging compound and as a messenger demands a balanced defence system. H_2O_2 concentrations increase in response to various abiotic and biotic stresses and take part in the reactive oxygen regulatory network (Harding et al., 1997; Dietz et al., 2006). Cells express a set of hydrogen peroxide-decomposing enzymes including peroxiredoxins which reduce H_2O_2 and also detoxify alkyl hydroperoxides and peroxinitrite (Dietz et al., 2006). Experimental evidence shows that Prx proteins function in plant cell as antioxidant, modulator of cell signaling pathways, and redox sensor (Dietz et al., 2006). Thus, Prx proteins modulate signaling pathways that involve ROS and reactive nitrogen species (RNS), which regulate developmental processes, adaptation to biotic and abiotic stresses (Dietz et al., 2006).

Manganese superoxide dismutase (1.19±0.02), and copper zinc superoxide dismutase (1.12±0.22). Manganese, copper and zinc are essential micro-elements for normal plant growth and development (Culotta et al., 1995). However elevated levels of these same metals can drastically impair cell growth and function (Culotta et al., 1995). As these ions would catalyze the conversion of hydrogen peroxide (H_2O_2) to the powerful oxidant hydroxyl radical (OHz), which has the capacity to damage cellular components (Culotta et al., 1995). Alternatively, manganese, copper and zinc toxicity may also be raised from the inappropriate binding of the metals to nitrogen, oxygen, and sulfur ligands in biomolecules, thereby inactivating enzymes and disrupting cellular function. To balance the growth inhibitory and stimulatory effect of these ions, all organisms have evolved various metal homeostasis factors that properly control the cellular accumulation, distribution, and detoxification of the metal (Culotta et al., 1995). As

another means of maintaining manganese, copper and zinc ions homeostasis, many eukaryotic organisms contain one or more forms of a metal binding metallothionein that acts to chelate, sequester, and thereby detoxify manganese, copper and zinc ions. Thus, the manganese, copper and zinc dismutases identified in the nuclear proteome of *X. viscosa* in response to dehydration stress might involved in maintaining manganese, copper and zinc ions homeostasis.

Glycosyl transferase 4 (0.77±0.05) belongs to a ubiquitous group of enzymes, which is widely distributed in the plant kingdom, and it was proposed to play an important role in the glycosylation pathways in plants in response to oxidative stress (Lim et al., 2006).

Proteins showing consistent expression levels involved in cell signaling

Protein kinase (0.71±0.08) and phospholipase D (PLD) (1.12±0.11) form part of signaling pathways in plants and were also reported in the nuclear proteome of *chickpea* in response to dehydration stress (Pandey et al., 2008). They were shown to function as a receptor of signals and might be involved in relaying these signals to the heterochromatin region to regulate gene expression (Pandey et al., 2008). On the other hand phospholipase D was reported to be involved in phospholipid signaling pathway which plays an important role in diverse early signaling cascade in animal cells (Frank et al., 2000). In plants, phospholipid signaling (involving phospholipases A2, C and D) was reported to play a role in adaptation to dehydration stress, where dehydration genes were isolated from *Arabidopsis* encoding for a phosphoatidyl inositol-specific phospholipase C, and phosphoatidylinositol-4-phosphate 5-kinase (Frank et al., 2000). Genes encoding PLD were isolated from various plant species and were shown to be triggered by dehydarion and involved in different signaling pathways (Frank et al., 2000).

Calnexin homolog 1 precursor (1.63±0.03) is molecular chaperone which assists in proper protein folding (Qureshi et al., 2007). Also it is a calcium binding protein involved in signal transduction pathways (Repetto et al., 2008). The functions of molecular chaperone-like and calcium binding proteins were highlighted in detail previously and in chapter 3. Calnexin was also reported in the nuclear proteome of *M. truncatula* (Repetto et al., 2008).

14-3-3-like protein GF14 upsilon (1.51±0.09); 4-3-3 protein GF14=NCT Ran (1.48±0.09); 14-3-3-like protein GF14 phi (0.86±0.03); and probable 14-3-3-like protein

(0.71±0.19) are a family of conserved regulatory molecules expressed in all eukaryotic cells (Fu et al., 2000). A striking feature of the 14-3-3 proteins is their ability to bind a multitude of functionally diverse signaling proteins, including kinases, phosphatases, and transmembrane receptors (Fu et al., 2000). This plethora of interacting proteins allows 14-3-3 to play important roles in a wide range of vital regulatory processes, such as mitogenic signal transduction, apoptotic cell death, and cell cycle control (Fu et al., 2000). Emayan et al., (2007) demonstrated that tobacco cells transformed with antisense construct of 14-3-3 protein no longer accumulates ROS suggesting a role as an important component in the ROS pathway. 14-3-3-like proteins were reported in the nuclear proteome of *Arabidopsis* and chickpea (Bae et al., 2003; Pandey et al., 2008) Thus; 14-3-3 proteins might play important and versatile roles in enhancing the ability of *X. viscosa* to survive desiccation stress.

Nacent polypeptide-associated complex α-like protein 3 (0.57±0.00). The nascent-polypeptide-associated complex (NAC) plays a prominent role in regulating the association of signal recognition particle (SRP) with nascent chains and in preventing the binding of ribosomes with non-secretory nascent chains to the endoplasmic reticulum (Wickner, 1995). Both SRP and NAC are ribosomally bound and may compete for signal sequences (Wickner, 1995). Also SRP in association with NAC was reported to function as a molecular chaperone (Wickner, 1995).

Magnesium chelatase (1.56±0.04) which is composed of three subunits, namely CHLD, CHLI and CHLH, catalyzes the insertion of Mg_2+ into protoporphyrin IX (Shen et al., 2006; Kobayashi et al., 2008). This is the first committed step in chlorophyll (Chl) biosynthesis because the enzymes prior to this step are also shared with the heme biosynthetic pathway (Shen et al., 2006). This reaction is a key regulatory step for the tetrapyrrole biosynthetic pathway and plays an important role in plastid-to-nucleus signal transduction (Kobayashi et al., 2008). Shen et al., (2006) demonstrated that Arabidopsis ABAR/CHLH specifically binds ABA, and mediates ABA signalling as a positive regulator in seed germination, post-germination growth and stomatal movement, showing that ABAR/CHLH is an ABA receptor. Arabidopsis has two CHLI isoforms, CHLI1 and CHLI2, it has been suggested that CHLI2 has limited function in Mg-chelatase, and is thus not essential for Chl biosynthesis under normal growth

conditions but it assumed this isoform may be involved in Mg-chelatase activity under particular physiological conditions that are currently not understood (Kobayashi et al., 2008).

Proteins showing consistent expression levels involved in nucleocytoplasmic transport

Dynamin-2A (0.78±0.08). The function of this protein is related to nucleocytoplasmic transport and was discussed previously in detail. Dynamin was also reported in the nuclear proteome chickpea (Pandey et al., 2008).

Proteins showing consistent expression levels involved in protein translation

Sixteen proteins of translation machinery were identified with consistent expression levels in the nuclear proteome of *X. viscosa* in response to dehydration stress: 40S ribosomal protein S15A (1.31±0.01), 60S ribosomal protein L13 (0.95± 0.16), 40S ribosomal protein S6 (1.04 ±0.05), ribosomal protein (0.78±0.05), 40S ribosomal protein (0.73±0.02), ribosomal protein L18A (0.97±0.03), 60S ribosomal protein L2 (0.64±0.02), 50S ribosomal protein L27 (0.64±0.01), 40S ribosomal protein (0.64±0.14), 40S ribosomal protein S23 (0.62±0.01), 40S ribosomal protein S10-3 (0.62±0.05), 40S ribosomal protein S9 (0.59±0.07), 60S ribosomal protein L10A (0.57±0.00), ribosomal protein CL9 (0.56± 0.02), 40S ribosomal protein S17 (0.55± 0.04) and protein synthesis initiation factor-like (1.17±0.01). The function of ribosomal proteins and protein synthesis initiation factors were highlighted in detail previously and in chapter 3 as well. Ribosomal proteins and protein initiation and elongation factors were also reported in the nuclear proteomes of *Arabidopsis* (Bae et al., 2003; Calikowaski et al., 2003), rice (Khan and Komatsu, 2004), chickpea (Pandey et al., 2006; 2008), and *Medicago* (Repetto et al., 2008).

Proteins showing consistent expression levels with molecular chaperone type activities

Heat shock protein 70 kDa (0.97±0.02). Heat shock proteins are present in cells under normal conditions because of their essential role in protein maintenance, they are induced when a cell undergoes various types of environmental stresses like heat, cold and oxygen deprivation (Demirevska et al., 2008) According to Sørensen et. al. (2003) HSP family and other molecular chaperones play significant roles in relation to stress resistance. HSP 70 kDa was reported also in the nuclear proteome of *Arabidopsis* (Bae et al., 2003). The function of this protein was discussed in detail previously.

Proteins showing consistent expression levels involved in energy metabolism

Three enzymes involved in energy metabolism: mitochondrial F1 ATP synthase β subunit (1.00±0.09), ATP binding/H^+ ion Transporting ATP synthase (0.75±0.02), and ATP synthase CF1 α subunit (0.75±0.06). These enzymes are involved in generating energy for the cell in the from of ATP, their functions was described in detail in chapter 3.

Proteins showing consistent expression levels involved in unknown functions

Among the proteins showing consistent level before and after dehydration, eight proteins matched proteins with unknown functions, putative protein (1.99±0.15), unknown protein (1.63±0.04), uncharacterized protein (1.39±0.08), unknown protein (1.31±0.08), unknown protein (1.10±0.01), unknown protein (0.92±0.03), unnamed protein product (0.90±0.03) and unnamed protein product (0.74±0.00). Investigation of functions of these proteins under dehydration stress could broaden our understanding of desiccation tolerance in plants.

To simplify the roles of the *X. viscosa* nuclear proteome in acquisition of desiccation tolerance, the identified proteins in response to dehydration stress can be broadly divided into four groups: (i) proteins involved in gene expression and regulation including cell signaling and nucleocytoplasmic transport; (ii) stress responsive proteins; (iii) miscellaneous and (iv) novel or proteins with unassigned functions. Proteins implicated in gene expression and regulation and cell signaling dominated other proteins in the nucleus of *X. viscosa* in response to dehydration stress (Fig. 4.4). These results are in complete agreement with the previous findings in the investigated nuclear proteomes of *Arabidopsis* under cold stress (Bae et al., 2003), and chickpea under dehydration stress (Pandey et al., 2008). However, our results are in contrast with other organellar proteomes, such as mitochondrial and chloroplastic, where proteins involved in ATP generation or in electron transport were predominant (Millar et al., 2001; Peltier et al., 2000). Our results demonstrate that inducible gene regulation is fundamental properties of adaptive mechanisms and reflect the important role of the nucleus in gene expression and regulation.

It has been proposed that the critical feature of tolerance to dehydration depends on the abilities of an organism to limit membrane damage during dehydration and to regain membrane

integrity and membrane bound activities quickly upon rehydration (Tripathy et al., 2000). Dehydration in non-desiccation tolerant plants is often accompanied by membrane damage, resulting in the leakage of solutes (Blum, 1988). Hence, the ability to increase membrane stability in response to severe water loss is characteristic of desiccation tolerant species (Turner, 1986). It was not surprising that proteins implicated in defense mechanisms against dehydration stress in the nucleus of *X. viscosa* were shown to be the second largest group after proteins involved in gene expression and regulation. The identification of known stress-related proteins confirms the reliability of approaches used in this study. Antioxidants, molecular chaperones and compatible solutes are essential stress-related elements that prevent and protect membrane damage that is caused by ROS and water loss (Mundree et al., 2002; 2006). Proteins implicated in cell signaling are integral part of desiccation tolerance as expression of genes in response to abiotic stresses results from a signal transduction cascade that commences with perception of the stimulus (Ramanjulu and Bartels, 2002). Proteins involved in nucleocytoplasmic transport, protein translation, miscellaneous and unknown proteins are other features of the up-regulated proteins identified in this study, and reflect the important roles of these proteins in adaptation to dehydration stress. These results demonstrate that, *X. viscosa* relies heavily on dehydration-inducible protection during dehydration as about 23% of the total identified proteins were up-regulated in response to dehydration stress at 35% RWC implicated in diverse protective functions. The protective mechanisms of dehydration tolerance utilized by *X. viscosa* appear to involve signal perception inducible genes, and modulating of appropriate gene expression encoding protective molecules such as antioxidants, molecular chaperones, proteins of translation machinery, compatible solutes, proteins with miscellaneous functions and novel protein factors such as ones reported here. Our results suggest that *X. viscosa* evolved unique strong defense capacity with versatile and coordinated actions enough to provide protection of cellular structures during dehydration and in the dried state. Previous nuclear proteome analyses of non-desiccation tolerant species of *Arabidopsis* and chickpea under cold and dehydration stress respectively (Bae et al., 2003; Pandey et al., 2008) showed that these angiosperms possess defense systems, as they were shown to express stress-inducible proteins including antioxidants, molecular chaperones and proteases, however they cannot survive at late dehydration points such as 35% RWC (Sherwin and farrant, 1996; Bartels and Salamini, 2001; Ingle et al., 2007). Because it seems that the defense systems of non-desiccation tolerant plants are impaired by over-generation of misfold-proteins, ROS and osmotic disorders, we propose that stress

tolerance is genetically encoded. The increased activities of stress-molecules during dehydration may serve as good indicators for dehydration tolerance in plants.

In conclusion, the comprehensive proteome analysis of *X. viscosa* nucleus and its response to dehydration stress presented here provides an insight into the functional role of the organelle. Furthermore, it will provide a foundation for future investigation of the mechanisms involved in the acquisition of desiccation tolerance at the metabolic and molecular levels. It will also initiate further organellar proteome research in resurrection plants. To our best knowledge this the first study to provide an insight into a plant organellar proteome using iTRAQ labeling technique in combination with two-dimensional liquid chromatography and nano-electrospray ionization tandem mass spectrometry.

CHAPTER 5

Conclusions and future prospects

This thesis has investigated the nuclear proteome of the resurrection plant *Xerophyta viscosa* (Baker) and its response to dehydration stress at 35% RWC with the aim to get detailed insights of the nuclear proteins and their roles in acquisition of desiccation tolerance. This should help in better understanding some of the adaptive mechanisms developed by resurrection plants and identify proteins involved in stress tolerance. This thesis has revealed that the *X. viscosa* nuclear proteome is sensitive to dehydration stress, and *X. viscosa* relies on readily inducible protection to combat desiccation. The protective mechanisms of desiccation tolerance utilized by *X. viscosa* appear to involve signal perception genes, and modulating of appropriate gene expression encoding protective molecules including antioxidants, molecular chaperones, compatible solutes, proteins of translation and degradation machinery, proteins with miscellaneous functions and novel protein factors. It also revealed that desiccation tolerance is controlled by multiple genes within the plant nucleus. This thesis also emphasizes the crucial roles of proteins in desiccation tolerance. Under normal conditions all plants are able to grow and develop normally, but under dehydration stresses such as at 35% RWC, non-desiccation-tolerant species can not survive, because it appears that their defense systems are impaired at this low dehydration point by osmotic disorders, over-generation of ROS and misfold-proteins. This failure would result in cell membrane injury, electrolyte leakage and eventually plant death. Thus, we propose that stress tolerance is genetically encoded, and *X. viscosa* has evolved unique strong defense capacity with versatile and coordinated actions enough to provide protection for its cellular structures during desiccation stress and in the desiccated state.

The first part (chapter 2) of this thesis involved modifications, optimizations and refinements of nuclei isolation and nuclear protein extraction protocols that enabled isolation of purified intact nuclei and enriched nuclear protein fractions for a detailed analysis of the nuclear proteome. These results indicate that the isolated *X. viscosa* nuclei were highly purified. Hence, proteins from the purified nuclei were extracted and used for further proteome analysis.

The second part (chapter 3) of this thesis involved the development of a suitable 2-D electrophoresis system to separate and display the nuclear proteins for the control and dehydrated samples for general image analysis and further identification and analysis of up-regulated nuclear proteins to gain information about the dynamics of the proteome in response

to dehydration stress. From an average total of 438 proteins spots that were reproducibly detected and analysed, a total of 18 protein spots were found to be up-regulated in response to dehydration. Selected analysis of dehydration-induced nuclear protein spots showed that dehydration was controlled by a coordinated response of different stress molecules within the X. *viscosa* nucleus implicating both regulatory and functional proteins. These included proteins involved in gene regulation and signal transduction, molecular chaperones, protein degradation and translation, energy metabolism, and novel protein factors. In addition, to their detailed discussed functions, the identified nuclear proteins were sorted into different categories (chapter 3). The largest category comprised five novel proteins (28%) which could not be matched to any protein using taxonomy limited and unlimited database search settings. These results showed that *X. viscosa* has its own specific stress nuclear proteins that it used to combat desiccation stress. In addition two proteins were identified in *Arabidopsis* and sugar beet but with no homologies to known functions in databases. The prediction of a possible function for the novel and the proteins with unknown function might give more insights into the role of the nucleus in acquisition of desiccation tolerance. The second largest group was five proteins involved in molecular chaperone type activities. A consequence of any adverse environmental stress is the generation of appreciable amounts of denatured or partially unfolded proteins in cells. Molecular chaperones are key elements in preventing protein aggregation and malfunction under dehydration stress and in controlling the state of cellular proteins (Sørensen et. al. 2003; Vierling, 1991; Parsell and Lindquist, 1993; Leroux et al., 1999; Demirevska et al., 2008). This response should enhance cell viability under dehydration stress. Interestingly, among proteins with chaperone type activities, three eukaryotic translation elongation factors similar to the bacterial EF-Tu were identified up-regulated in the nuclear proteome of *X. viscosa* in response to dehydration stress. Among these was eEF-1-α which was shown to be involved in protein-protein interactions with a zinc-finger protein (Gangwani et al., 1998). Investigations of the oligomeric state of the nucleus would be of interest. Other categories include proteins involved in translation. It proposed that enhanced protein expression would allow for the expression of cellular stress response proteins under stress conditions and may lead to cell protection during dehydration stress where rates of protein production significantly and progressively decline (Talapatra t al., 2000). The final category included proteins involved in energy metabolism, which demonstrates that enhanced energy generation during dehydration stress is important for protective reactions and might play an important role in adaptation to dehydration stress.

The final part of this thesis (chapter 4) involved the employing the gel-free iTRAQ MS/MS system to greatly increase the number of proteins that can be analyzed, with the additional benefit that hydrophobic proteins lost during isoelectric focusing (IEF) can be studied (Suzuki et al., 2006). Employing the iTRAQ labeling technique in combination with 2DLC and nano-ESI MS-MS we were able to confidently and reproducibly identify 128 proteins with confidence ≥ 95% ($\rho < 0.05$). Sixty six percent of the identified proteins were shown to have consistent expression levels (between 2 to 0.5 fold). The remaining 34% proteins showed significant change in expression (greater than 2 or less than 0.5 fold). Of the latter 23%, were shown to be up regulated in response to dehydration stress with expression levels greater than 2 fold. The remaining 11% were shown to be down regulated with expression levels less than 0.5 fold. The nuclear proteins of *X. viscosa* in response to dehydration stress showed a coordinated response implicating both the regulatory and functional proteins, and were involved in diverse cellular functions. These included gene regulation and signal transduction, which dominated others in the nucleus and comprised 32%; followed by proteins involved in translation 18%; miscellaneous 15%; unknown 9%; antioxidants 9%; protein degradation 8%; nucleocytoplasmic transport 3%; molecular chaperones 3%; energy metabolism 2%; and compatible solutes 1%.

The characteristic feature of the identified proteins in the nucleus of *X. viscosa* in response to the low dehydration point is the high level of stress molecules among the dehydration responsive molecules with evident functions in defense mechanisms compared to down regulated or proteins showing consistent expression levels. Six proteins of the 30 up-regulated proteins were shown to be involved in ROS scavenging pathways compared to one among the 14 down-regulated and 4 among the 84 proteins showed consistent expression levels. Other stress molecules include molecular chaperones where 3 proteins among the up-regulated were involved in molecular chaperone type activities compared to 1 protein among proteins showing consistent expression levels and none were detected among the down-regulated proteins. A protein involved in the synthesis of compatible solute was detected only in the up-regulated proteins. Three proteins involved in nucleocytoplasmic transport were among the up-regulated proteins compared to one among the proteins showing consistent expression levels, with none identified among the down-regulated proteins. The characteristic features of proteins that showed consistent expression levels are the high number of proteins involved in other functions such as gene regulation (21 proteins), protein translation (16 proteins), proteins with miscellaneous functions (12 proteins), protein degradation (9 proteins), and energy metabolism

(3 proteins). The characteristic features of the down-regulated proteins are proteins involved in miscellaneous functions (6 proteins), and proteins of translation machinery (5 proteins). These results together demonstrate that enhancement of defense capacity is crucial to desiccation tolerance and strongly supports the notion that late dehydration responsive proteins are involved in protection of the cellular structures during dehydration, and hence are responsible of acquisition of desiccation tolerance in vegetative tissues (Jiang et al., 2007; Ingle et al., 2007). Thus up-regulated proteins are of greatest interest to uncover the molecular basis of desiccation tolerance in vegetative tissues. Our results support the notion that down-regulated proteins are involved in metabolic pathways that were active in well-watered tissues but inactivated by the decrease in osmotic potential (Jiang et al., 2007). Proteins showing consistent expression levels during dehydration identified in this study support the notion that these proteins are likely associated with the basic metabolic activities that are important to keep the minimum viability in cells under all kinds of conditions. These proteins may not directly play roles in desiccation tolerance; but, they appear necessary for survival (Jiang et al., 2007). Interestingly, our study showed good correspondence between the two groups of up-regulated proteins identified by the 2-DE and iTRAQ approaches as the same proteins were detected by both methodologies such as chaperones, ET-Tu, ATP synthase, retro elements, proteases, ribosomal proteins and RNA helicases.

Previous whole proteomic analyses of *X. viscosa* leaf tissue detected 430 protein spots which represented proteins from all the sub-cellular organelles but an over-representation of chloroplast proteins (Ingle et al., 2007). Thus our adapted protocols of nuclei isolation and nuclear protein extraction resulted in a significant enrichment of *X. viscosa* nuclear proteins which helped in identification and subsequent correlation of the protein function in relation to its nuclear location. This study demonstrates that the fractionation of the proteome into subcellular fractions allows the enrichment of the nuclear proteins and subcellular fractionation and proteomics are an ideal combination to uncover proteins function. Subcellular fractionation allows access to intracellular organelles and multiprotein complexes, low abundant proteins and signaling complexes can be enriched, and at the same time the complexity of the sample can be reduced. Furthermore analyzing subcellular fractions and organelles allows tracking proteins that shuttle between different compartments in the cell. Importantly, subcellular fractionation is a flexible and adjustable approach that may be efficiently combined not only with 2D gel electrophoresis but also with gel-free techniques such as iTRAQ. However, the main hurdle

with subcellular proteomics is optimization of organelle isolation and organellar protein extraction methodologies to obtain enriched organelle, and sufficient protein of good quality for the proteomic study.

Gene expression profiling and understanding gene function can be approached through RNA based system biology approaches, such as transcriptomics. These techniques are powerful and highly automated, allowing massive screening of hundreds of genes simultaneously. However, the success of those approaches depends greatly on the genomic progress. Other techniques like cDNA microarrays, cDNA amplified fragment length polymorphism (AFLP) and serial analysis of gene expression (SAGE) are restricted to model organisms or species with characterized genomes (Carpentier et al., 2008). Due to the lack of genomic information, these powerful transcript-based techniques are inapplicable to non-model species such as *X. viscosa*. Gene sequences are rarely identical from one species to another and orthologous genes are usually riddled with nucleotide substitutions (Carpentier et al., 2008). An alternative to these techniques is the proteomics approaches, which allow the study of gene expression through its end products, the proteins. Protein sequences are more conserved and proteins from an organism with an un-sequenced genome are compared to orthologous proteins of species that are well characterized (Shevchenko et al., 2001; Liska and Shevchenko, 2003). Proteomic analysis has several advantages over transcriptomics in that it provides a view of the end-point of gene expression (Ingle et al., 2007), i.e. the actively translated component of the mRNA pool, and allows the investigation of post-transcriptional and posttranslational regulatory events (Ingle et al., 2007).

Proteins identified in the nuclear proteome of the resurrection plant *X. viscosa* in response to dehydration stress supports existing knowledge and uncovers novel proteins, neither detected before in the whole proteome of this plant, nor in the nuclear proteomes of previously investigated non-desiccation tolerant plants under normal or abiotic stress conditions. More than 85% of the identified proteins are verified as nuclear proteins as evident by the literature citations. Our results also show evidence that there is cross-talking between different cellular compartments in response to dehydration stress. These results, in part, are in agreement with the previous reports on plant nuclear proteomes. Nevertheless, until a more complete survey of the proteomes of nucleus in several desiccation-tolerant plants is conducted using similar protein approaches and identification technology, it will be difficult to determine the presence or absence of specific proteins between plant species.

In conclusion, the comprehensive proteome analysis of *X. viscosa* nucleus presented here provides an insight into the functional role of the organelle, and will provide a foundation for future investigation of the mechanisms involved in the acquisition of desiccation tolerance at the metabolic and molecular levels. To our best knowledge this is the first study to provide an insight into the nuclear or organellar proteome of a desiccation tolerant plant.

For a complete understanding of cellular functions and the roles of different proteins in acquisition of desiccation tolerance further studies are necessary:

1. Investigations on the physiological, molecular and biochemical roles of the up-regulated nuclear proteins including the novel proteins identified here to determine their precise roles in desiccation tolerance. This could lead to a better understanding of the role of nucleus in the mechanisms enabling the resurrection plants to withstand severe water loss.
2. Further proteomic studies on other tissues and organelles from *X. viscosa*. This will enrich the database on novel stress-responsive proteins and help in understanding the biochemical and molecular mechanisms of desiccation tolerance at a cellular level. Also, it will help in identifying proteins that shuttle between cell organelles during dehydration stress.
3. The use of transgenic technology to express desiccation inducible nuclear proteins into model plants to establish the role of the protein and whether drought tolerance is improved.
4. Finally, a systems biology approach combining proteomics methodologies, metabolomics, transcriptomics and computational approaches that could offer a comprehensive description of regulatory networks used by resurrection plants to tolerate desiccation.

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