MOLECULAR AND PHYSIOLOGICAL STUDY OF WATER-DEFICIT STRESS ON SELECTED *ERAGROSTIS* SPECIES

Zekarias Gebremedhin Ginbot

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Faculty of Science
University of Cape Town
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Abstract

Eragrostis nindensis and *Eragrostis tef* are wild and domestic grasses respectively that belong to the subfamily Eragrostideae. *E. nindensis* is desiccation tolerant while *E. tef* is desiccation sensitive. The responses of these plants to water-deficit stress were studied using molecular and physiological approaches.

A cDNA library of *E. nindensis* was screened to identify differentially expressed genes during dehydration. Physiological studies included monitoring changes in photosynthesis, respiration, ultrastructure and membrane integrity of plants during dehydration and rehydration.

The differential screening of the cDNA library, using a radio-labelled cDNA from hydrated and dehydrated leaves respectively, revealed two genes, referred as Nin-19 and Nin-44, that were differentially expressed in dehydrated leaves of *E. nindensis*. These genes were sequenced and partially characterized. Nin-19 did not show considerable identity with any known genes and was not studied any further. Nin-44 was identified as a dehydrin-like gene with approximately 99 % identity to seven water-deficit stress responsive genes on a section of about 60 bp near the 3' end. As its sequence was found to represent a partial insert size, two forward and reverse primers were designed to find the full length through RT-PCR. Despite repeated attempts, no products that could be used in subsequent procedures were achieved using this technique. Hence, further characterization of this gene also could not be performed and different approaches were suggested.

The physiological studies showed that *E. nindensis* is desiccation tolerant but *E. tef* is not, the latter dying below RWC of about 33 %. Difference among plants in physiological responses became evident after 6 days of dehydration treatment, which resulted in a decline of RWC to 65%, 39% and 33% in *E. nindensis*, *E.tef* (R) (red-seeded) and *E. tef* (W) (white-seeded) plants respectively. A significant decrease in photosynthesis, transpiration, stomatal conductance, and an increase in electrolyte leakage occurred in all species after 6 days of dehydration, but leaves of *E. tef* (W) did not recover from this

level of dehydration when watered. Instead, new leaves were observed to re-grow from the stem nodes. The leaves of red-seeded variety of *E. tef* did recover fully from RWC of 39 %. After a further 3 days dehydration both varieties of *E. tef* died. On the other hand, *E. nindensis* was found to survive extreme water-deficit (~10 % RWC tested here) and recovered full physiological activity when watered. The electrolyte leakage study on these plants indicated major injury on *E. tef* (W), being intermediate in *E. tef* (R) and very low in *E. nindensis*, which coincided with the trend of declining in RWC and other metabolic activities measured. The ultrastructural study on *E. tef* varieties also showed evidence of the damage caused by dehydration, but the difference among these species was not significant enough to indicate the level of susceptibility of the plants to dehydration damage.

The study demonstrated that *E. tef* varieties are not drought tolerant and showed a considerable difference in their responses to water-deficit stress with each other and with respect to *E. nindensis*. However, *E. tef* (R) seems to have a better control over transpiration and some form of repair mechanism operational at least until dehydration to 39 % RWC. This is proposed to be a better performing cereal in conditions of water stress. On the other hand *E. nindnsis* did not suffer major injury from the dehydration treatment and confirmed to be desiccation tolerant.

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Abbreviations

ABA	abscisic acid
BSA	bovine serum albumin
CDNA	copy DNA
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNAse	deoxyribonuclease
E. nindensis	*Eragrostis nindensis*
E. tef (W)	white-seeded *Eragrostis tef*
E. tef (R)	red-seeded *Eragrostis tef*
EDTA	ethylenediaminetetra-acetic acid
g	grams
HCL	hydrochloric acid
IPTG	isopropyl-β-D-thiogalactopyranoside
Kb	kilobase(s)
kDa	kilodalton(s)
λ	Lambda
l	litre(s)
LA	Luria-Bertani agar
LB	Luria-Bertani broth
M	molar concentration
mg	milligrams
MgSO4	magnesium sulphate
min	minutes
ml	millilitre(s)
mM	millimolar
mRNA	messenger RNA
µg	microgram(s)
µl	microlitre(s)
µmol	micromole

μS	microsimon
OD	optical density
ORF	open reading frame
pfu	plaque forming units
PVP	polyvinylpyrrolidone
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RWC	relative water content
SDS	sodium dodecyl sulphate
SM	suspension media
spp.	species
TBE	tris borate EDTA
TE	tris-EDTA
Tris	tris(hydroxymethyl)aminomethane
X-GAL	5-bromo-4-chloro-3-indolyl-β-D-galactoside

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CHAPTER ONE

Literature Review

1.1 Introduction

Plants often experience unfavorable conditions such as water-deficit, salinity, chilling, freezing, high temperature, flooding, high or low light in their natural environments, which are collectively known as abiotic stresses. Under these stresses, growth, development and productivity of a plant can be reduced, and in extreme cases, they result in the death of the plant (Chen and Murata, 2002).

Tollenaar (2002) defined stress, in the context of modern stress tolerant cultivars and hybrids, as any factor that results in reduction of yield. Stress tolerant plants can be defined as having genotypes that are less affected by mild stresses that occur under relatively good growing conditions. Any factor in the plants' environment, be it physical, chemical or biological, if it is deficient or in excess, is known to affect plant growth and performance and hence it is considered as stress to the plant. Stress reduces the rate of CO_2 uptake by individual leaves and whole plant, hence decreasing photosynthetic efficiency which in turn results in decreased growth of plant parts and productivity (Amthor and McCree, 1990).

As plants are unable to move away from a stress, they have to develop mechanisms to survive if endemic to environments, which periodically experience extreme conditions. To date, a number of plants have been identified which employ such mechanisms that enable them to survive extreme stress. Currently, intensive research is underway to understand the molecular and physiological basis of this survival in order to form a basis for the production of better quality crops by conventional and/or bioengineering means in currently marginal areas (Smallwood *et al.*, 1999). In particular, the molecular and ecophysiological adaptation of plants to water-deficit stress (desiccation) has been targeted for research. This thesis will deal exclusively with this stress.

1.2 Plant water-deficit stress

Plants suffer from water-deficit stress when subjected to drought, high or low temperature and salinity. The term "drought" denotes a period without appreciable precipitation, during which the water content of the soil is reduced to such an extent that plants suffer from lack of water. Decrease in soil water content is often coupled with strong evaporation caused by dryness of the air and high levels of radiation (Larcher, 1995). According to Turner (1997), the ability of a crop (plant) to perform satisfactorily in areas subjected to water-deficits has been termed as its drought resistance.

Plant water-deficit can also be caused by high and low temperatures. During high temperature plants experience high evapotranspiration rates where the plant loses more water than it absorbs from the soil (Young and Britton, 1990). Under inadequate water supply, this results in water-deficit. Low freezing temperatures also affect availability of usable water. During freezing, plants become short of liquid water that could be used in metabolic reactions and in translocation of organic and inorganic compounds to all parts of the plant. Stomatal exchange of gasses and absorption of materials through the roots also decreases.

Salinity is also another factor that may lead to the development of water-deficit. Soil salinity is defined as a measure of the total amount of soluble salt in soil. As salinity levels increase, plants extract water less easily from soil, aggravating water stress conditions. High soil salinity can cause nutrient imbalances resulting in the accumulation of elements toxic to plants, and reduce water infiltration into plant tissues (Kotuby-Amacher, 1997).

Water-deficit, which might be caused by any of the above factors, affects nearly all the plant's growth processes. But the response of plants depends upon the duration of exposure and the stage of growth (Siddique *et al.*, 1999). It is one of the environmental factors determining the productivity and distribution of plants. It affects the rate and out put of physiological processes of the plant and induces various biochemical and physiological responses.

1.3 Water-deficit associated stresses

Water is essential to plant life. Even plants that live in arid deserts need moisture and have developed sophisticated techniques to retain moisture within the plant body. Plants require water for structural maintenances and as an essential ingredient of metabolic processes. When water is limiting, plants suffer from structural and metabolic strains. The most observable effect of water-deficit stress on plants is its effect on the relative growth rate, which is the result of increase in cell number and volume (Earnshaw, 1993). Plant cell division is affected by water-deficit stress directly or indirectly, as it depends on the supply and normal functioning of other metabolic processes, and hence plant growth can be halted. Under extreme water-deficit, cells can die as a result of desiccation and other associated stresses. Here only a few of these stresses are mentioned.

1.3.1 Desiccation stress

Desiccation stress refers to the condition of stress associated with dehydration of a plant to air-dry state (Alpert and Oliver, 2002; Vander Willigen *et al.*, 2001). Bartels and Nelson (1994) also described cellular desiccation (dehydration) as a consequence of drought, salt and cold stresses that lead to depletion of cellular water. Similarly, when the rate of transpiration exceeds water uptake, plants are said to be under water-deficit that could lead to desiccation (Bray, 1997).

Water provides support for the cytoplasmic components of a cell. Under water-deficit conditions, plant cells lose water to the environment resulting in loss of turgidity and disruption of cellular compartmentation, which result in delocalization of organelles and lytic enzymes, which can damage the cell (Vander Willigen *et al.*, 2001). Extreme desiccation also promotes crystallisation of proteins and solutes in the cytoplasm that would severely injure the cell (Leopold, 1990).

Cell membranes have been regarded for a long time as the site of desiccation injury, mainly because the earliest symptom of injury is enhanced leakage of cytoplasmic solutes during rehydration (Simon, 1974). The disorganization of membranes and their apparently enhanced permeability may be simply a consequence of physical rupture of

the membrane due to tearing during cellular collapse, or alternatively, it may involve more subtle changes in the physical organization of lipid or protein components in an otherwise intact membrane (Walters *et al.,* 2002). Ultrastructural studies of dry tissue confirmed that membrane disorganization is a common phenomenon under lethal desiccation stress; cells that are dried at a tolerant stage remain organized (Crevecoeur *et al.*, 1976; Dasgupta *et al.*, 1982).

Desiccation is considered by some to be a necessary prerequisite for the completion of the life cycle in species producing seeds that are "orthodox" with regard to their ability to withstand storage at low moisture. Other species do not exhibit maturation drying of their seeds, and are called "recalcitrant". This latter group includes many tropical trees, aquatic grasses and a few temperate species, such as oak and sycamore (Farrant *et al.*, 1993). Surviving under desiccation stress may arise from either tolerance or a mechanism that permits avoidance of the situation that are discussed in section 1.5.

1.3.2 Oxidative stress

In plants several abiotic and biotic stresses are known to induce formation of oxygen radicals, activated oxygen species (AOS), which have an important role in the activation of defence responses, and also cause damage to tissues under stress. Freezing anoxia and desiccation stresses have been linked with oxidative stress.

Water-deficit provokes the formation of AOS due to an excess of excitation energy in relation to reduced CO_2 assimilation (Lawlor and Cornic, 2002). Under moderate water stress conditions, photosynthetic CO_2 assimilation is reduced due to an increased resistance to CO_2 diffusion consecutive to stomatal closure. As limitation of CO_2 fixation continues inactivation of electron transfer reactions, an excess of reducing power is generated in chloroplasts of water stressed plants. This excess results in the formation of active oxygen species causing cellular damage such as lipid peroxidation or protein modification (Lawlor and Cornic, 2002; Smirnoff, 1993).

Another way that contributes towards the formation of ROS is change and restructuring of cellular membranes caused by water-deficit. Upon water loss, cytoplasmic amphiphillic compounds increase in concentration and partition into membranes (Hoekstra *et al.*, 2001). Such partitioning-induced membrane restructuring is reported to cause impairment of the electron transport chain, which is thought to lead to increased formation of reactive oxygen species. Such reactive oxygen species, regardless of how they are formed, cause an extensive peroxidation and de-esterification of membrane lipids, from which plants suffer at the intermediate ranges of water loss (Hoekstra *et al.*, 2001).

1.3.3 Light Stress

Light is the ultimate source of energy and the most important ecological factor affecting plant growth. A variation in quality, intensity and duration of light affects plant's overall performance (Manske, 2001). All shade plants are reported to be sensitive to light and may be damaged even by brief exposure to moderate irradiation. However, light itself is not stress to plants that grow on open ground unless electron transfer to the Calvin cycle is prevented by other stresses such as water-deficit stress (Larcher, 1995).

High-intensity visible light, under limiting terminal acceptors of photosynthesis, can generate superoxide radicals (O_2^-) and singlet oxygen (O_2^{-1}), that are natural stress factors that plants have to cope within their environments (Smith *et al.,* 1990). In water-stressed cotton plants, excess light is reported to cause the ratio of oxygenation to carboxylation to increase while the absolute rates of both decreases (Demmig-Adams and Adams III, 1992; Lawlor and Cornic, 2002). Exposing *Phaseolus vulgaris* L. to excess light has been reported to cause photoinhibition, decrease in the rate of photosynthesis, which results when plants are exposed to photon flux densities much higher than those prevailing during normal growth conditions (Greer *et al.*, 1986).

1.4 Coping with water-deficit stress

According to Turner (1986) the mechanisms of plant adaptation to water-deficits are divided into morphological and physiological adaptations. In relation to drought resistance, this author categorised adaptive mechanisms as *drought escape*, the ability of a plant to complete its life cycle before serious soil and plant water-deficit develops; *drought tolerance with high tissue potentials,* the ability of a plant to endure periods with out significant water while maintaining a high tissue water potential, and *drought tolerance with low tissue water potentials*, the ability of a plant to endure periods without significant water and to endure low tissue water potential. According this author, both the later ones are considered to be tolerances to describe all mechanisms that enable plants to perform better in stressful environment.

In other reviews, adaptation is explained with respect to the contribution of a character to the fitness of an individual organism to survive in its present environment, or with respect to evolutionary origin of a character where it involves a heritable modification in structure or function (Jones and Jones 1989).

Quantification of adaptation is found to be difficult, because it is unlikely that any plant is in a state of perfect adaptation to its environment. A plant is made up of a collection of ancestral characteristics and the process of adaptation is occurring continually (Jones and Jones 1989). So it can be said that adaptation to an environment depends on the possession of an optimum combination of these characters that minimise deleterious effects and maximise advantageous effects.

Plants subjected to water-deficit and associated stresses strive to survive by employing different mechanisms at different levels of stress such as macromolecular stabilization, membrane modification, osmolyte biosynthesis, transcription control, control of metabolism, ionic concentration and others as detailed in the Figure 1.1 (Bohnert *et al*, 1995).

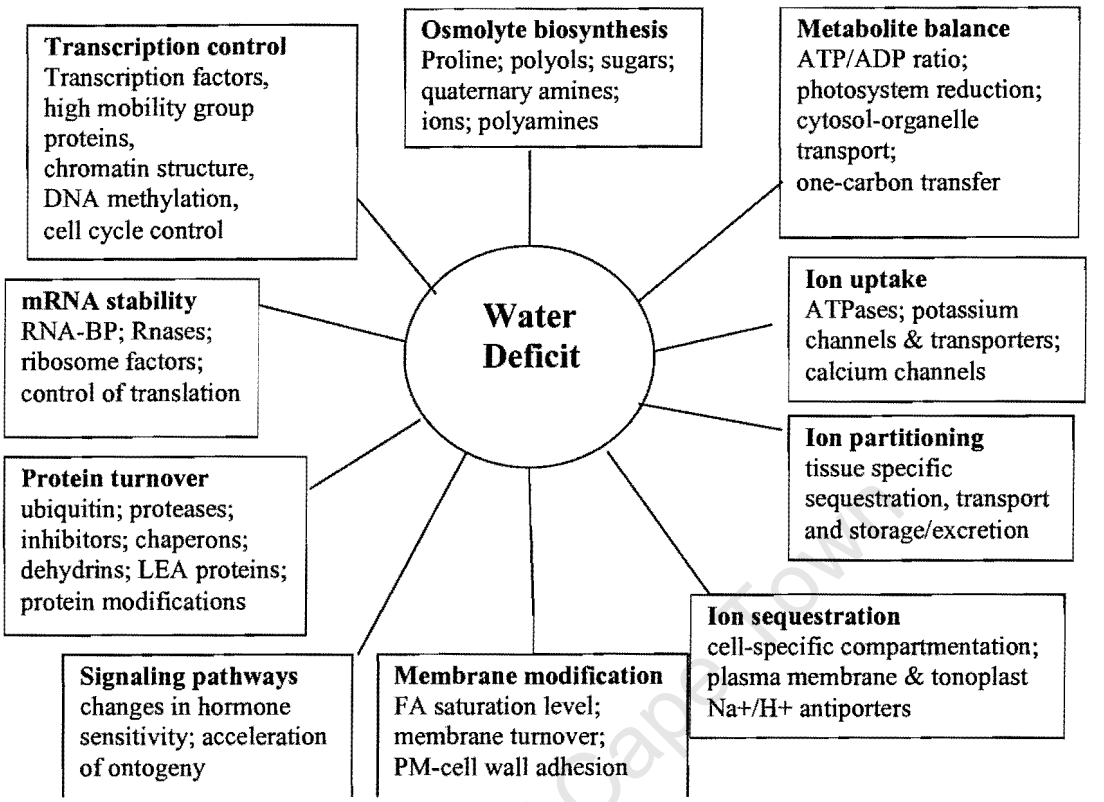


Fig. 1.1 Changes in cellular processes in response to water-deficit. These changes allow the plant to maintain and restore conditions that allow for continued growth under stress. Taken from Bohnert *et al*, 1995, The Plant Cell.

With regard to water-deficit-associated oxidative stress, plants have evolved protective mechanisms such as dissipation of excess heat energy and anti-oxidant scavenging systems (Lawlor and Cornic, 2002; Vander Willigen *et al.*, 2002). In vegetative tissues, genes encoding enzymatic antioxidants such as ascorbate peroxidase, superoxide dismutase, glutathione reductase and amphiphilic molecules such as tocopherol, quinones, flavonoids and phenolics are upregulated during drying (Rey *et al.*, 1999; Hoekstra *et al.*, 2001). These enzymes participate in dehydration tolerance and damage reduction. Compatible solutes have also been reported to play a role in scavenging ROS when they are produced as a result of oxidative stress (Chen and Murata, 2002).

Gluthathione, gluthathione reductase and flavonoid pigments also involve in protecting plants from excessive light damage when water is limiting (Ho and Sachs, 1989). Photoinhibition and photooxidation damages are avoided through combination of high rates of photosynthetic electron transport and high rates of thermal energy dissipation, changing leaf angle with the direction of light, developing greater surface reflectance and producing screening pigments other than chlorophyll (Demmig-Adams and Adams III, 1992; Lawlor and Cornic, 2002).

However, only plants that employ the full spectrum of necessary adjustments can survive extreme water-deficits. Such plants are known as desiccation tolerant plants. According to Leprince (1993), desiccation tolerance is defined as the ability of an organism to survive a rapid desiccation, suspend its metabolism and revive on subsequent rehydration of its cellular structure. It can be achieved by mechanisms that incorporate either cellular protection or cellular recovery (repair) strategies, but usually both. Organisms or organs that cannot survive from dry state, die or fail to ensure normal growth on subsequent rehydration are called as desiccation intolerant (Leprince, 1993; Vertucci and Farrant, 1995).

Desiccation tolerance occurs in a wide range of organisms from animal and plant kingdoms including nematodes, arthropods, crustaceans, algae, fungi, mosses, ferns, seeds and pollen, as well as the vegetative tissue of a few angiosperm species (Leprince, 1993; Farrant and Kruger, 2001).

There are many species of plants from ferns and angiosperms, which are known as resurrection plants that can resume normal physiological functions after air-drying (Stewart, 1989, Gaff, 1971, 1989). More than two thirds of the desiccation tolerant angiosperms are monocots and nearly half of these monocots are from the family Poaceae (Vander Willigen *et al.*, 2001). This remarkable desiccation tolerance property is ecologically important as an adaptive strategy for colonising dry habitats (Leprince, 1993).

In resurrection plants, desiccation tolerance is induced at moderate to severe water-deficits with decline of photosynthesis and respiration, hydrolysis of starch and water-soluble proteins and degradation of chlorophyll (Gaff, 1989).

Based on the survival times, desiccation tolerant plants are grouped as poikilochlorophyllous and homoiochlorophyllous. Poikilochlorophyllous plants are those that dismantle their photosynthetic apparatus when desiccated and reconstitute it after rehydration (Benko *et al.,* 2002). These plants show the ultimate desiccation tolerance of being able to survive full equilibration to air of 0 % relative humidity for longer times. On the other hand, homoiochlorophyllous plants keep their photosynthetic apparatus intact and a greater range of desiccation tolerant values and a spectrum of organelle loss is observed during drying (Gaff, 1989; Benko *et al.,* 2002).

Some desiccation tolerant plants repair all the physically and chemically damaged parts and normalize their metabolic processes (McKesie, 1996; Vander Willigen *et al.* 2001), and some e.g. angiosperms protect against damage (Cooper, 2001). Based on studies on the desiccation tolerant moss *Tortula ruralis* and others, Bewley (1995) proposed the dependence of desiccation tolerance on the ability of the plant to limit cellular damage during the desiccation phase, retain physiological and cellular integrity in the dry state and the presence of repair mechanism upon rehydration of the cellular damage occurred during drying.

Efforts to get better performing plants through breeding drought resistant varieties had always been difficult as researchers have no way to determine genetic influences on the basic mechanisms of drought injury and tolerance (National Research Council, 1996). In contrast to the breeding approach, the introduction of small genes by genetic engineering seems to be a more attractive and rapid approach to improving stress tolerance (Cushman and Bohnert, 2000).

Genes that are induced in plants subjected to dehydration stress have been identified. Ndima *et al.*, (2001), characterized a stress responsive gene from resurrection plant

Xerohpyta viscosa Baker whose transcripts increase under heat and low temperature stress. The products of such gene are thought to function in protecting cells by producing important metabolic proteins and cellular protectants, and in regulating genes that are involved in transducing the stress response signals (Shinozaki and Yamaguchi-Shinozaki, 2000). The mechanism of expression of such genes in response to water-deficit has been obtained from the investigations of DNA elements and sequence specific DNA binding proteins (Bray, 1997).

Bray (1997) stated that cellular events leading to water-deficit induced gene expression include perception of the stress, signal transduction, identification of potential members of signal transduction pathways by DNA sequence homology and perception and signal transduction of abscisic acid (ABA). These studies are very relevant for agricultural development in such areas as understanding drought resistance, preservation of seed, pollen, germplasm or somatic embryos that are encapsulated in artificial seeds (Stewart, 1989; Vertucci and Farrant 1995; Leprince, 1993).

A net loss of protein is reported to occur in desiccation tolerant plants during air-drying, whereas 40 to 60% fall in protein content was recorded in desiccation sensitive plants as hydrolysis of proteins predominates over protein synthesis (Gaff, 1989). But some specific proteins and mRNA species increase in amount in dehydrating plants (Hughes *et al.*, 1989). This accumulation of proteins and mRNA is believed to indicate gene induction due to the stress and additional regulatory mechanisms such as translational regulation and post-translational modification may be required for a fully functional gene product (Bray, 1997). The ability to respond to the stress and integrate this complex response system determines the degree of desiccation tolerance of a plant. In the following section some of the compounds known to have a role in water-deficit stress tolerance are discussed.

1.4.1 Compatible Solutes

During dehydration, cellular volume decrease causes crowding of cytoplasmic components and increased viscosity of the cell contents, increasing the chances for molecular interactions that can cause protein denaturation and membrane fusion (Hoekstra *et al.,* 2001). A broad range of compounds have been identified for model membranes and protein systems that can prevent such adverse molecular interactions. Among these compounds are proline, glutamine, glycine-betaine, carnitine, manitol, sorbitol, fructans, polyols, trehalose, sucrose and oligosaccharides. These compounds are preferentially excluded from the surface of proteins, thus keeping proteins preferentially hydrated (Hoekstra *et al.,* 2001, Shinozaki and Yamaguchi-Shinozaki, 2000).

Irrespective of whether the dehydration is caused by drought, freezing or osmotic shock, many plants and microorganisms are reported to accumulate compatible solutes in response to cellular dehydration (Hoekstra *et al.,* 2001) and this is believed to be a mechanism by which plants cope with water-deficit (Chen and Murata, 2002, Kleines *et al.,* 1999). These compatible solutes help in keeping the cytoplasm osmotically balanced and can accumulate to high concentrations without impairing normal physiological functions (Bartels and Nelson, 1994).

For example, sucrose is found to increase during dehydration in several resurrection plants studied to date (Mundree and Farrant, 2000; Whittacker *et al.,* 2001). High concentration of C8-sugars (2-octulose) was found to accumulate in fully hydrated leaf tissues of the desiccation tolerant resurrection plant *Craterostigma plantagineum*. Upon dehydration this octalose level decreases and the sucrose concentration increases to 90% of the total sugar content (Kleines *et al.,* 1999; Whittaker *et al.,* 2001). During rehydration, this process is reported to be reversed which is a clear indication of the role of sucrose in desiccation tolerance.

Current genetic transformation techniques have allowed the introduction of new pathways for the biosynthesis of various compatible solutes in to plants resulting in the

production of transgenic plants with improved tolerance to stress (Chen and Murata, 2002).

1.4.2 Heat-Shock Proteins (HSPs)

HSPs were originally discovered in an experiment involving heat stressing of *Drosophilia* larva (Wischmeyer, 2002). They are identified by their molecular weights as HSP 12, HSP 25, HSP 27, HSP 72, and HSP 90. HSP induction has been studied in higher plants including soybean, pea, tobacco, tomato and maize. However, they are not restricted to heat stress only. HSPs are induced by chemicals, drugs, hydrogen peroxide, transition series metals, anoxia, prolonged ischemia and desiccation; and the present accepted common pathway for the induction of SHP appears to be the presence of denatured proteins within the cell caused by the above stresses (Wischmeyer, 2002; Hoekstra *et al.*, 2001).

The study of HSPs is relevant to plant water-deficit studies as water-deficit causes protein denaturation. HSPs are members of an intricate set of machinery known as molecular chaperons that involve in the proper folding of proteins in a cell (Wischmeyer, (2002). They also prevent aggregation of unfolded proteins and allow renaturation of aggregated proteins. Recently HSP12 protein was found associated with membranes of desiccated yeast cells (Hoekstra *et al.*, 2001). Hence, in water-deficit stress HSPs protect membranes from the damaging effects of desiccation, but the exact mechanism remains to be determined (Alpert and Oliver, 2002).

In plants, HSPs are encoded in the cell nucleus, synthesized in the cytosol and transferred to the chloroplast and mitochondria. Their expression need a sub lethal exposure to the stress and protect against a subsequent stress that otherwise would be lethal (Wischmeyer, 2002).

1.4.3 Late embryogenesis abundant (LEA) proteins

In plant cells, a number of proteins are induced during stresses involving dehydration such as drought, salt and cold. LEA proteins are a subset of such stress-induced proteins, initially discovered as non-storage proteins, which accumulate abundantly during late stage of cotton embryo development, hence the name LEA for late embryogenesis abundant (Zhang *et al.,* 2000).

Genetic expression studies revealed that LEA proteins are generally associated with plant cells under water-deficit such as desiccation of seeds, dehydration of vegetative tissues, low temperature, increased salt solutions or application of the plant growth regulator ABA (Bartels, 1999). LEA proteins disappear from the plant tissue during the first hours of germination or in response to stress relief which is an indication that their expression is either developmentally or environmentally regulated (Bartels, 1999).

LEA proteins are extremely hydrophilic and are boiling-soluble, indicating that the proteins are hydrated and non-globular. These are the characteristics that led to the suggestion that LEA proteins are involved in the protection of plant cells from dehydration, and their accumulation also coincides with desiccation and freezing tolerance of plants (Zhang *et al.,* 2000).

According to Zhang *et al.,* (2000) LEA proteins are classified into at least seven groups based on the amino acid sequence homology and specific motifs each of which is suggested to have a different function during water-deficit. It has been hypothesized that LEA proteins may play a protective role in plant cells under various stress conditions which is essential for the survival of the plant under extreme stress conditions (Hoekstra *et al.*, 2001).

A number of genes that encode for LEA proteins have been isolated and characterized (Hsing, 1996) and extensive studies are underway at the levels of DNA, RNA and protein, hoping to figure out the biological functions and structure of these proteins.

1.4.4 Abscisic Acid (ABA)

ABA is a plant hormone (phytohormone) that plays an important role in several events such as formation of a mature seed, imposition of dormancy during embryogenesis, and signaling water-related stresses (Iglesias and Babiano, 1999). It is thought to initiate its effects on cells by binding to a receptor protein, which is not yet known. During water deficiency, the concentration of ABA in leaves increases and promotes stomatal closure and hence decreased transpiration (Larcher, 1995).

Studies show that under deteriorating soil water relations, leaf turgor alone does not control the stomatal conductance or leaf expansion in crop species, but the soil water conditions can override leaf water relations in determining the physiological activity of the shoot (Turner, 1997). So, soil water relations can provide a signal to the shoot that conditions are deteriorating and reductions in growth and transpiration are required to maintain hydration. Under these conditions, ABA is found to be necessary to act as the signal for deteriorating soil water conditions (Turner, 1997).

ABA is also found to signal the switch on of the desiccation tolerance program in developing seeds (Hoekstra *et al.*, 2001). The evidence for this came from exogenous application of ABA where it was found to induce desiccation tolerance in leaves of *Borya nitida* in the absence of any concomitant water stress (Gaff, 1989), which is the proof for its role in desiccation tolerance.

ABA is also known to be involved in cold acclimation and can induce freezing tolerance in a number of plant species when applied exogenously (Xing and Rajashekar, 2001). The endogenous ABA level was found to increase in plants under drought and salinity stress (Shinozaki and Yamaguchi-Shinozaki 2000).

ABA accumulation was observed along with the expression of numerous stress related genes which are believed to provide plants with a tolerance to drought, high salinity and cold (Jang *et al.,* 2002). In the same paper, application of exogenous ABA was found to result in the expression of some genes that are typically induced in response to water

stress and cold (Xing and Rajashekar, 2001). Shinozaki and Yamaguchi-Shinozaki (2000) also reported that ABA plays an important role in slow and adaptive responses involving dehydration-induced gene expression. However, the recent finding that ABA mutants expressed responsive genes to dehydration and cold treatment raised questions on how it involves in gene expression and its role in signaling.

1.5 Plants used in this study: *Eragrostis nindensis* and *Eragrostis tef*

The genus *Eragrostis* is a member of the tribe Eragrosteae, sub-family Eragrostoidae, of the Poaceae (Graamineae) consisting of approximately 300 species of both annuals and perennials (Stallknecht *et al.*, 1993). *Eragrostis nindensis*, *Eragrostis paradoxa*, *Eragrostis curvla Eragrostis hispida*, *Eragrostis pilosa*, *E. invalida* and *E. tef* (Zucc.) Trotter are some of the different species of the genus *Eragrostis*. 50 % of *Eragrostis* species are reported to be native to Africa with a common somatic chromosome number of 2n = 40 (Tef has 2n = 40 chromosomes) (Constanza *et al.*, 1979). From these only *Eragrostis tef* (Zucc.) Trotter is known as important cereal (National Research Council, 1989; Lester and Bekele, 1981).

Eragrostis tef (Zucc.) Trotter, an ancient cultigen (a plant that only grows under cultivation), is indigenous to Ethiopia and Eritrea[1] and yet is seldom grown elsewhere. This plant is reported to be drought tolerant species and used as fodder and as a nurse crop in South Africa (Lester and Bekele, 1981), Kenya, Uganda and Pakistan. It is not desiccation tolerant. On the other hand, *Eragrostis nindensis* is one of the 11 resurrection grasses from the subfamily Eragrostideae widely distributed in Namibia and other arid areas of South Africa (National Research Council, 1989; Vander Willigen *et al.*, 2001). It is reported to be a poikilochlorophyllous perennial grass that grows in shallow soils (Vander Willigen *et al.*, 2001).

1 Since Eritrea has been one of the provinces of Ethiopia for some years, it is not mentioned in the literature.

Tef[2] is a highly nutritious cereal grain that survives a wide range of seasonal fluctuations in temperature and moisture, in differing altitudes and soil types. At least 1000 mm of annual rain fall is needed for optimum production (Taddese, 1969). In more arid areas of Ethiopia and Eritrea, where crops such as maize and sorghum fail, tef is often grown as an insurance crop in view of its more limited water requirements (Shiferaw and Baker, 1996). It provides over 2/3 of the human nutrition in Ethiopia and produces both grain for human food and fodder for animals in a short growing season. Publications of late 20th century described tef grain as being marketed as a healthy food product and used as a late planted emergency forage for livestock (Stallknecht *et al.*, 1993).

Scientists used seed colour, e.g. white and red seed colour, as one of the characters to distinguish between varieties. Mengesha *et al.* (1965) reported higher aluminium, potassium and copper content in red-seeded tef than white-seeds tef. But, traditionally, the white-seeded tef is preferred as food crop in Ethiopia and Eritrea; red and brown seeded types are second choice as food (Constanza *et al.*, 1979).

Although it is not consistently proven, tef is believed to contain high Fe, which is supported by the absence of Fe-deficiency among the Ethiopian population (Mamo and Pearsons, 1987). Table 1. shows results of mineral content from two varieties of tef obtained by Mamo and Parsons, (1987). The protein level of tef is reported to be between 10 to 12 % and it contains very high calcium, phosphorous, iron, copper, aluminium, barium and thiamine (Stallknecht *et al.*, 1993).

One of the main agricultural problems with the cereal tef is the long, slender shoot, which is prone to lodging, particularly during panicle development (Gorham and Hardy, 1990). Tef is a fine-stemmed annual grass with many tillers and open panicle inflorescence. It produces small seeds and has shallow roots with varying height depending on the cultivar and the growing environment (Stallknecht *et al.*, 1993). Due to the presence of Kranz-

[2] Tef and *E. tef* are used to refer the cereal tef.

type of anatomy and high chlorophyll a/b ratio, tef is classified as a C4 grass (Kebede *et al.,* 1989).

Table 1.1 Mineral content of two varieties of tef (*Eragrostis tef*) seeds.

Variety	mg/g seed						µg/g seed				
	% H_2O	N	P	K	Ca	Mg	Na	Fe	Mn	Cu	Zn
White seeded	9.7	20.2	4.53	5.56	1.11	1.41	118.1	51.4	33.6	10.4	36.5
Brown seeded*	8.9	20.5	4.66	5.63	1.04	1.38	131.0	49.0	40.5	9.11	38.2
Mean	9.3	20.35	4.59	5.59	1.08	1.39	124.55	50.2	37.05	9.76	37.35

Mamo and Parsons, 1987 Trop. Agric.

* Represents the same plant mentioned here as red seeded.

Ethiopia and Eritrea are known for the type of bread, thin and round-flat, made from the pinhead-sized tef seeds ground into flour and baked on a flat traditional stove that is made from local clay soil. This type of bread, the main daily ration, is eaten with "tsebhi", a type of sauce known locally.

Apart from observations and preliminary local studies, there is no report on the tef water relations or any adaptive mechanisms of this cereal plant to drought stress and no clear methodologies or modern screening techniques have been developed to identify more drought tolerant varieties. However, the use of *E. nindensis,* desiccation tolerant, in crop improvement through somatic hybridisation (forced marriage) has been proposed (Gaff, 1989). In his study, the author fused leaf cells of different *Eragrostis* species, including *E. nindensis,* with tef and found out that *E. nindensis* is one of the eligible partners for tef hybridisation (Gaff, 1989). In the literature reviewed, no study has been done on the gene expression associated with the desiccation tolerance of *E. nindensis*, and the possibility of using this species as a source of genes to transform economically important food crops including tef.

1.6 Aims and concluding remarks

In recent years extensive studies have been conducted investigating mechanisms and strategies that desiccation tolerant plants employ to survive in stressful environments (Potts, 2001). Gene expression and physiological studies have been carried out in growth chambers and in the field. Changes in physiological parameters and gene expression are reported when plants are exposed to water-deficit stress (Koziel *et al.*, 1993). Plant transformation techniques have been developed and performed on some plants. Stress sensitive plants were transformed with genes from stress tolerant plants and tested for the expression of these genes (Shibata and Liu, 2000; Hansen and Wright, 1999). A number of genes and proteins have been identified and are being tried to transform economically important plants (Wischmeyer, 2002, May *et al.*, 1995). However, the success was limited as the stress responses are complex. It is believed that discovery of novel genes and determination of their expression patterns in response to stress, and an improved understanding of their roles would enable scientists to come up with greater stress tolerant plants.

It is known that most of the cultivated plant species have wild relatives that exhibit excellent tolerance to abiotic stresses (Bohnert *et al.,* 1995). *E. nindensis* is such a wild desiccation tolerant relative of *E. tef*. So, the objective of the current study is two-fold: (1) **to identify and characterize genes that are differentially expressed in the desiccation tolerant *E. nindensis* during water stress and (2) to compare aspects of physiology of *E. nindensis* with *E. tef* varieties** during water stress. The differential gene expression study and the methods used will be presented in Chapter 2, and the physiological comparison between species and an ultrastructural study on *E. tef* leaf meristematic tissues are dealt with in Chapter 3.

Identification of stress responsive genes and examining the physiological responses of these relative plants upon dehydration stress is hoped to provide knowledge for ultimate improvement of tef with respect to drought tolerance.

CHAPTER TWO

Gene expression study on *E. nindensis* during water stress

2.1 Introduction

The molecular mechanisms that are fundamental to the development of water deficit tolerance are pivotal to the understanding of desiccation tolerance. In desiccation tolerant plants, the desiccation tolerance property is believed to be switched on by dehydration and the plant hormone abscisic acid. In the green vegetative tissue of mosses, ferns and angiosperm resurrection plants, a slight dehydration triggers gene expression associated with desiccation tolerance (Hoekestra *et al.*, 2001). This author also explained that in anhydrobiotic (orthodox) seeds, this expression occurs during development as part of the normal maturation program. As a result, seed embryos acquire desiccation tolerance partly before maturation drying. Once the stress is perceived, plants respond in various ways. Genes responsible for ameliorating the stress condition are transcribed and levels of the mRNA from these genes increase in relevant plant tissues. Such stress also induces various biochemical and physiological responses. Identification of the most relevant mechanisms of these responses is under intensive investigation.

The strategy of molecular genetics in understanding and improving stress tolerance of plants include finding of differential gene expression by comparing stressed and non-stressed situations in a suitable model system, followed by the isolation of induced genes (Bartels and Nelson, 1994). The role of such genes can then be tested in transgenic plants under different stress levels. Recently, a number of techniques have been developed to identify differentially expressed genes. These involve the creation of cDNA or genomic libraries, which are subsequently screened by a variety of techniques (Sambrook *et al.*, 1989). Since cDNA library is constructed from a population of mRNA transcripts via reverse transcription, it represents genes expressed in a particular tissue at a particular time. Differential hybridisation, subtractive hybridisation and differential display screening techniques have been utilised to identifying differentially expressed genes from cDNA libraries (Bauer *et al.*, 1993).

Differential hybridisation probes DNA fixed to replica membranes with labelled cDNA made from mRNA from stressed or control samples. Positive clones are then recovered from the original source. Differential hybridisation was the first method routinely used to identify differentially expressed genes, and has advantage over subtractive hybridisation in that it is able to show the presence of either repressed or induced genes (Sambrook *et al.*, 1989).

In subtractive hybridisation, cDNA from experimental cells is hybridised to a molar excess of mRNA from control cells. The mRNAs that are common to the two populations are separated from the differentially expressed sequences and the single stranded subtracted cDNA is recovered for further analysis. Subtractive hybridisation technique increases the chance of cloning or finding scarce differentially expressed genes (CLONTECHniques Archives, 1997).

Differential display is a PCR-based technique for identifying changes in the population of transcripts. It involves reverse transcription of mRNA from both stressed and control cells and systematic amplification of the 3' termini using a set of four anchored oligo(dT) primers and an arbitrary decamer (Bauer *et al.*, 1993). The region between these primers is amplified by PCR in the presence of radioactive nucleotide. Subsequently, the PCR products are resolved on a gel and the differences are determined (Bauer *et al.*, 1993; Cushman and Bohnert, 2000).

Although *E. nindesnsis* is reported to be desiccation tolerant (Gaff and Ellis, 1974; Vander Willigen *et al.*, 2002), the molecular mechanisms by which this resurrection grass achieves this have not been characterised. One of the approaches of studying desiccation tolerance is studying the gene expression patterns of the plant under water-deficit stress.

The objective of this study was to identify and characterize genes differentially expressed in dehydrated *E. nindensis*. Differential hybridisation was utilised to screen a cDNA library constructed from mixed mRNA population from hydrated and dehydrated leaves of *E. nindensis*.

2.2 Materials and Methods

2.2.1 cDNA library plating and titering

A cDNA library, constructed from a mixed population of mRNAs from hydrated and dehydrated *E. nindensis* leaves, in lambda ZAP XR vector (Stratogene), was donated by Clare Vander Willigen, Dept. of Molecular and Cellular Biology, University of Cape Town, South Africa. Plating and titering of the library was performed following the protocol described in the Stratagene manual (1999). XL1-blue MRF' *E. coli* host cells were made competent by inoculating a single colony into 50 ml of LB broth supplemented with 0.2 % maltose and 10 mM $MgSO_4$. After incubation for 16 h at 30 ^{0}C, cells were recovered by centrifugation and diluted to an OD_{600} = 0.5.

1 µl from a tenfold serial dilution (10^{-2} to 10^{-5}) of phage containing the library in suspension media (SM) was added to 200 µl diluted XL1-blue host cells. The infected cells were plated onto NZY agar plates before being incubated at 37 ^{0}C overnight. Plaques from the 10^{-4} dilution were counted to determine the titer of the library.

2.2.2 cDNA Library excision

Library "in vivo" excision was performed as described in the Stratagene Manual (1999). An aliquot of the cDNA library with a titer of 6.4 x 10^9 pfu/ml was added to XL-1-Blue MRF' cells and ExAssist helper phage (Stratagene) in a multiplicity of infection (MOI) ratio of 1:10:100 (library to host cells to helper phage). After incubation at 37 ^{0}C for 16 h, the excised Bluescript phagemid packaged as filamentous phage particles was recovered by centrifugation. These excised phagemids were used to infect SOLR (an *E. coli* mutant strain) cells. Infected SOLR cells were plated on agar plates containing 50 µg/ml ampicillin supplemented with IPTG (isopropyl-1-thio-β-D-galactopyranoside) in DMF and X-gal (5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside) to a final concentration of 100 µg/ml and 1.5 mg/ml respectively.

2.2.3 PCR test and restriction analysis

Plasmid DNA purified from 12 randomly selected white colonies (Vernone *et al.,* 2002) from step 2.2.3, was used as the template in a PCR using T3 and T7 primers. Reaction conditions were as follows: 94 °C for 40 sec, 55 °C for 60 sec, 72 °C for 1 min for 30 cycles with a final extension step at 72 °C for 5 min. Molecular weights of the PCR products were determined from agarose electrophoresis by comparison with the migration of a *PstI* digest of bacteriophage λ DNA. *EcoRI* and *XhoI* restriction digestion was performed on selected PCR products and selected plasmid DNA (Current Protocols, 1990), and the products electrophoresed as above.

2.2.4 Slot blotting of the Library

Plasmid DNA was slot blotted onto replica nylon membranes for differential hybridization (Meinkoth and Wahl, 1984). 0.2 µg DNA from randomly selected samples was applied to each well. After denaturation with 1.5 M NaCl 0.5 M NaOH, samples were neutralised with 1.5 M NaCl 0.5 M Tris-HCl pH 8. Each well was rinsed with 0.2 M Tris-HCl pH 7.5 and 2 x SSC (standard saline citrate) buffer. Membranes were surface dried by blotting using Whatman 3 MM paper then baked at 80 ^{0}C for 2 h before being stored at 4 ^{0}C.

2.2.5 RNA extraction, reverse transcription and cDNA labelling

RNA was isolated from hydrated and dehydrated leaves of *E. nindensis* using the guanidium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi, 1987), and reverse transcribed with MMLV reverse transcriptase using an oligo dT primer in the presence of [α-^{32}P]dCTP (3000 mCi/µl specific activity) (Sigma Genosys Biotechnologies Inc.). Labelled cDNA was purified from unincorporated nucleotides by Sephadex G 50 chromatography using STE buffer (TE buffer containing 0.1 M NaCl) (Maniatis *et al.* 1982). The percentage incorporation of [α-^{32}P]dCTP and quantity of cDNA synthesised was calculated from the radioactivity incorporated.

2.2.6 Membrane hybridisation

Membranes were separately probed with labeled cDNA from hydrated and dehydrated leaves of *E. nindensis* (LabVelocity Inc.). Pre-hybridization and hybridizion reactions were performed for 3 h and 16 h respectively at 68 ^{0}C. 10^7 cpm of the denatured probe was used in 10 ml of hybridization solution. Membranes were washed sequentially at 20 ^{0}C with wash solution (2 x SSC 0.1 % SDS) and low stringency solution (0.2 x SSC 0.1 % SDS), and then at 42 ^{0}C with moderate stringency solution (0.2 x SSC 0.1 % SDS) and finally at 68 ^{0}C with high stringency solution (0.1 x SSC 0.1 % SDS). After rinsing with 2 x SSC, the membranes were wrapped with Saran plastic film, autoradiographed and genes differentially expressed identified.

2.2.7 Sequencing and sequence analysis of differentially expressed genes

To confirm the presence of inserts in positive clones, a PCR was performed on seven clones (step 2.2.6). Two differentially expressed clones in dehydrated leaves of *E. nindensis* were sub-cultured and the plasmid DNA sequenced. Sequences were analysed using DNAman software.

2.3 Results

The presence of clones containing inserts of significant length in the cDNA library was initially determined. The titer of the library was found to be 6.4 x 10^6 pfu/µl. However, approximately 80 % of the SOLR colonies appeared as blue non-recombinant colonies on the IPTG-X-gal supplemented plates. Furthermore, only 25 % of plasmid preparations from randomly selected white colonies showed inserts when restriction digested with *EcoRI* and *XhoI*. The sizes of the inserts found were in the range 0.7 kb to 1.3 kb (Fig. 2.1 (a)). To confirm these results, PCR was performed on selected plasmids using T3 and T7 primers which flank the multiple cloning site (MCS). PCR confirmed that the size of the inserts was as determined by restriction digestion (Fig. 2.1 (b)).

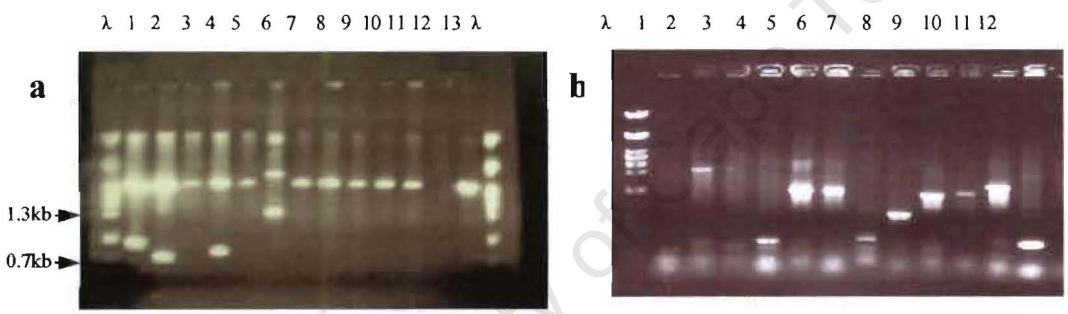


Fig. 2.1. *EcoRI*/*XhoI* restriction digestion (a) and PCR amplification (b) of insert DNA from randomly selected white colonies. (b). The molecular weight marker (λ) is a *PstI* digest of bacteriophage λ DNA. Numbers above the figures represent DNA bands from randomly selected clones of the excised library.

As the first step in the preparation of a radio-labelled cDNA probe, RNA was extracted from hydrated and dehydrated leaves from *E. nindensis*. The quality of the RNA was investigated using denaturing agarose gel electrophoresis. The RNA was judged to be of good quality as sharp bands with few digestion fragments were observed (Fig. 2.2). Radio-labelled cDNA was prepared from this RNA. Approximately 45 % of the [α-^{32}P]dCTP was incorporated, yielding a specific activity of approximately 10^7 cpm/µg.

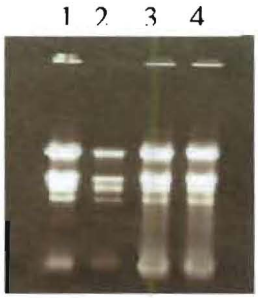


Fig. 2.2. Agarose gel electrophoresis in the presence of formaldehyde of total RNA extracted from dehydrated (lanes 1 and 2) and hydrated (lanes 3 and 4) leaves of *E. nindensis*.

Plasmid DNA from white colonies bound to replica nylon membranes was probed using radio-labelled cDNA from both hydrated and dehydrated leaves. Out of 94 samples, 15 hydridized to both cDNA probes (Fig. 2.3). Of these 15 clones, 7 appeared to be expressed at higher levels in dehydrated leaves, of which 2 clones were more significantly expressed and were designated Nin-19 and Nin-44. 3 clones appeared to be expressed at higher levels in hydrated leaves (clone 4A of membrane 1 and clones 7D and 11B of membrane 2). A further 5 clones appeared to be equally expressed in both hydrated and dehydrated leaves (e.g. clone 8A of membrane 1 and clone 7A of membrane 2). These presumably represented genes required for general cell metabolism, the so-called "house-keeping" genes. PCR was performed to confirm the presence of inserts in 7 differentially expressed genes. All 7 were indeed found to contain inserts of different .

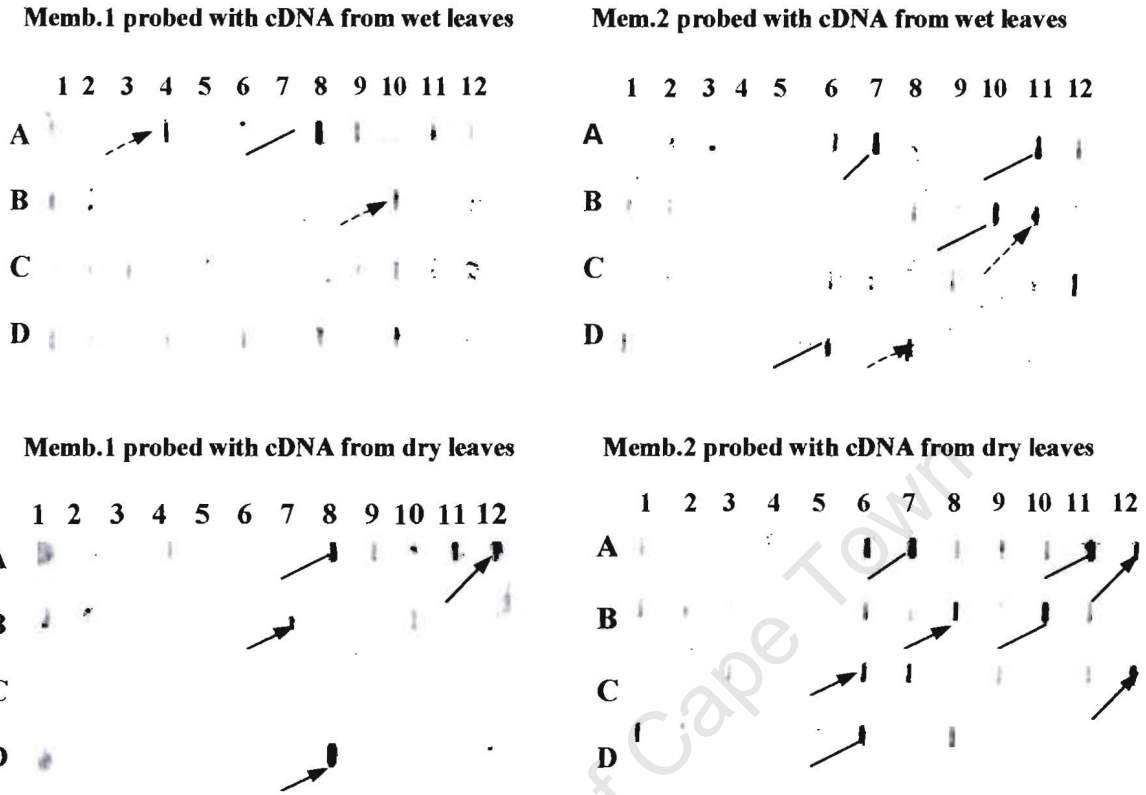


Fig. 2.3. Differential screening of the *E. nindensis* cDNA library on replica membranes using α ^{32}P-labelled cDNA from hydrated (top) and dehydrated (bottom) leaves. Selected genes are indicated as follows:- → : genes expressed more significantly in dehydrated leaves, ⇢: genes expressed more significantly in hydrated leaves, — : genes expressed equally in both hydrated and dehydrated leaves).

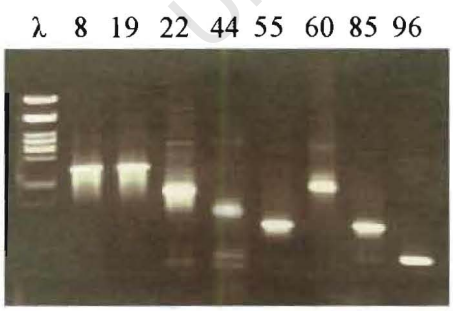


Fig. 2.4. PCR amplification of differentially expressed clones. Numbers on top represent the clones used as template DNA. Clone 96 is a control from a non-recombinant blue colony.

Table 2.1. Size of some differentially expressed clones

Clones	Size (kb)
8	1.3
19	1.3
22	1.0
44	0.7
55	0.6
60	1.1
85	0.6

To identify and characterize the genes of *E. nindensis* expressed during dehydration stress which were present in clones Nin-19 & Nin-44 (Fig. 2.4), these clones were sub-cultured and sequenced using T3 and T7 primers. Despite several attempts, only partial sequences for both samples were obtained with the T3 primer. No sequence data was obtained using the T7 primer. The length of the single strand sequences obtained for Nin-19 and Nin-44 clones were 571 and 458 bases long respectively. These represent approximately 40 % of the size of the Nin-19 insert and 65 % of the size of Nin-44 insert.

The sequence obtained for the Nin-19 clone had a base percent composition of 52 % GC and 48 % AT. No polyA sequence representing the 3' end of the message, nor the 5' end of the gene represented by an ATG codon could be located (Fig. 2.5). Since the reverse sequencing using the T7 primer was unsuccessful, the full length of the gene was not determined.

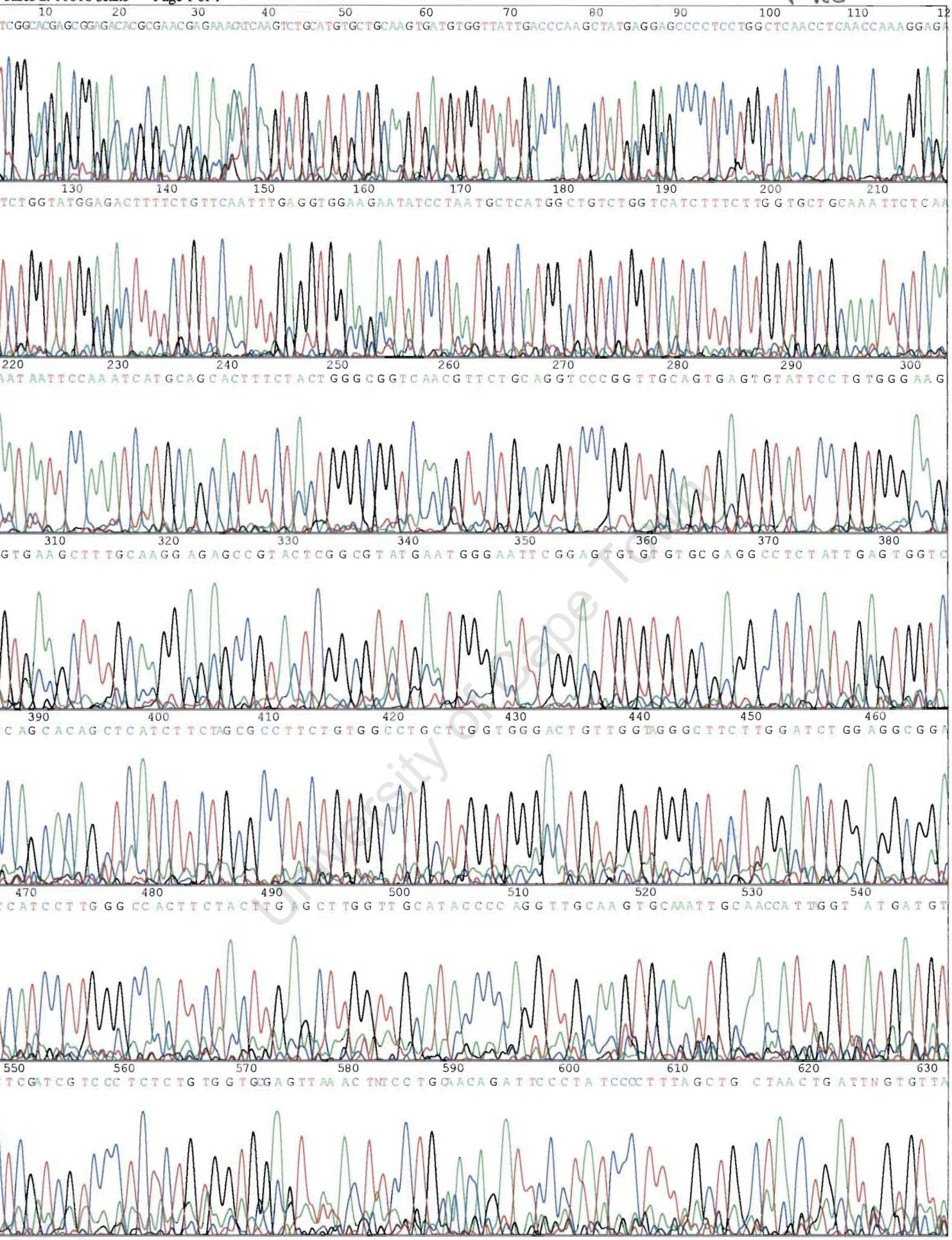


Fig. 2.5. Sequence chromatogram of Nin-19 DNA insert.

The forward sequence obtained was translated and showed a maximum open reading frame of 403 bp that encoded 134 amino acids with a molecular weight of 15.1 kDa (Fig. 2.6). The other two reading frames resulted in maximum lengths of 102 and 103 amino acids respectively.

Nin-19

```
1         ATTCGGCACGAGCGGAGACACGCGAACGAGAAACATCAAGTCTGCATGTGCTGCAAGTGA
1           F  G  T  S  G  D  T  R  T  R  N  I  K  S  A  C  A  A  S  D
61        TGTGGTTATTGACCCAAGCTATGAGGAGCCCCTCCTGGCTCAACCTCAACCAAAGGAGAA
21          V  V  I  D  P  S  Y  E  E  P  L  L  A  Q  P  Q  P  K  E  K
121       GTCTGGTATGGAGACTTTTCTGTTCAATTTGAGGTGGAAGAATATCCTAATGCTCATGGC
41          S  G  M  E  T  F  L  F  N  L  R  W  K  N  I  L  M  L  M  A
181       TGTCTGGTCATCTTTCTTGGTGCTGCAAATTCTCAAGAATAATTCCAAATCATGCAGCAC
61          V  W  S  S  F  L  V  L  Q  I  L  K  N  N  S  K  S  C  S  T
241       TTTCTACTGGGCGGTCAACGTTCTGCAGGTCCCGGTTGCAGTGAGTGTATTCCTGTGGGA
81          F  Y  W  A  V  N  V  L  Q  V  P  V  A  V  S  V  F  L  W  E
301       AGCTGTGAAGCTTTGCAAGGAGAGCCGTACTCGGCGTATGAATGGGAATTCGGAGTGTGT
101         A  V  K  L  C  K  E  S  R  T  R  R  M  N  G  N  S  E  C  V
361       GTGCGAGGCCTCTATTGAGTGGTCACCAGCACAGCTCATCTTCTAGCGCCTTCTGTGGCC
121         C  E  A  S  I  E  W  S  P  A  Q  L  I  F  *  R  L  L  W  P
421       TGCTTGGTGGGACTGTTGGTAGGGCTTCTTGGATCTGGAGGCGGATTCATCCTTGGGCCA
141         A  W  W  D  C  W  *  G  F  L  D  L  E  A  D  S  S  L  G  H
481       CTTCTACTTGAGCTTGGTTGCATACCCCAGGTTGCAAGTGCAAATTGCAACCATTAGGTA
161         F  Y  L  S  L  V  A  Y  P  R  L  Q  V  Q  I  A  T  I  R  Y
541       TGATGTTCTCGATCGTCCCTCTCTGTGGTG
181         D  V  L  D  R  P  S  L  W
```

Fig. 2.6. Nucleotide and derived amino acid sequence of the Nin-19 insert. Asterisks indicate the stop codons.

Comparison of the Nin-19 sequence with known gene sequences in the NCBI data base revealed no significant homology to any other known DNA sequences. Further investigation of this clone was not performed.

The sequence obtained from Nin-44 had a putative polyA-tail sequence representing the 3' end of the message and two GT-rich microsattelite sequences towards the 3' end of the gene, one 100 bp and the second 170 bp upstream of the polyA sequence (Fig. 2.7). The 5' end of this gene was not located as the ATG codon furthest from the stop codon was only 111 bp. The percent base composition was 44 % GC and 56 % AT. Confirmation of this sequence using the T7 primer was also unsuccessful.

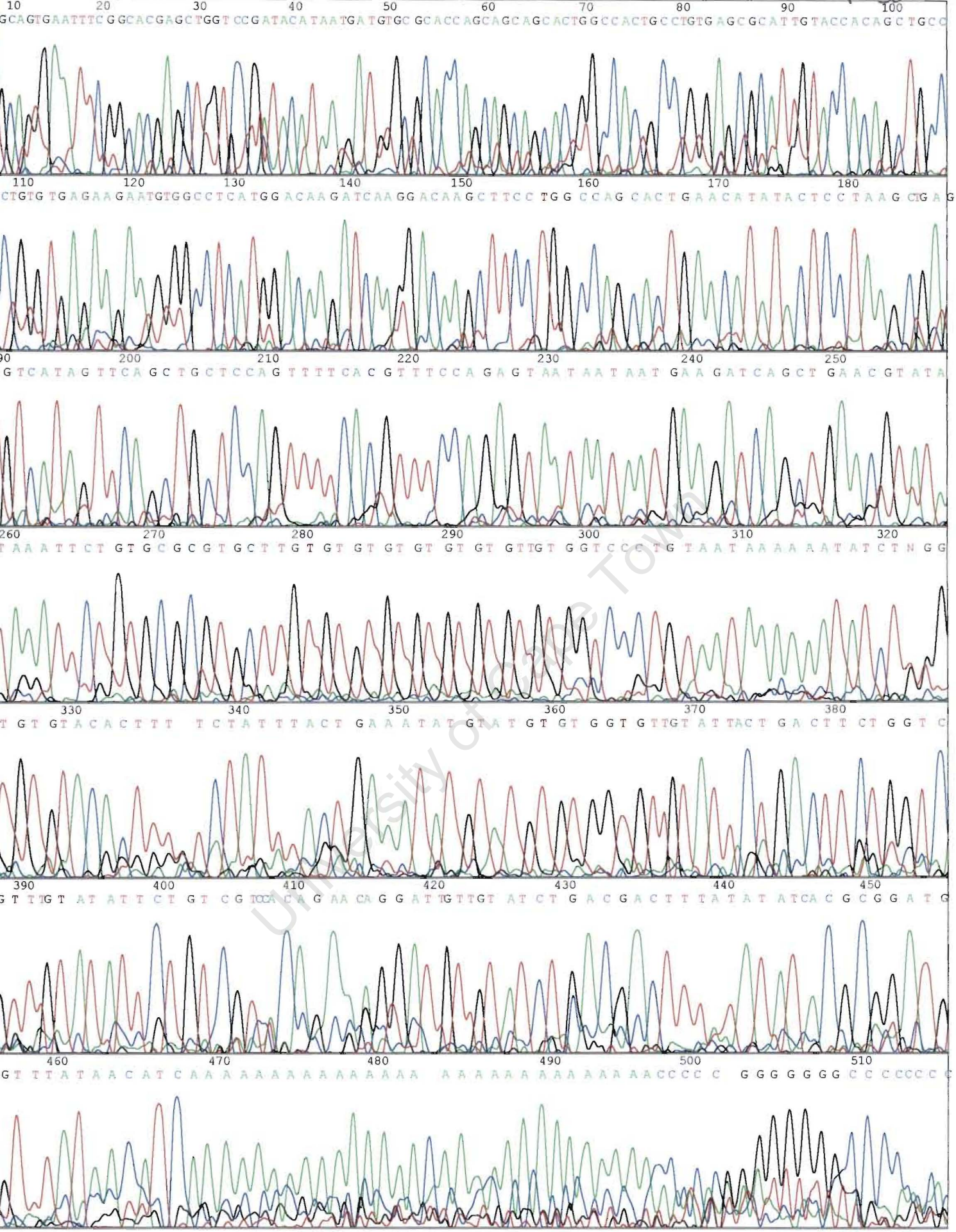


Fig. 2.6. Sequence chromatogram of Nin-44 DNA insert.

Translation of the Nin-44 sequence showed a maximum open reading frame of 129 bp that encoded 43 amino acids with a molecular weight of 4.8 kDa excluding the GT-rich portion (Fig. 2.8). The other two reading frames showed 37 and 32 amino acid long proteins respectively.

Nin-44

```
1          CTGGTCCGATACATAATGATGTGGCACCAGCAGCAGCACGGCCACGCCGGAGCGCATTGT
1           L  V  R  Y  I  M  M  W  H  Q  Q  Q  H  G  H  A  G  A  H  C
61         ACCACAGCTGCCACCGGTGAGAAGAAGGGCCTCATGGACAAGATCAAGGACAAGCTTCCT
21          T  T  A  A  T  G  E  K  K  G  L  M  D  K  I  K  D  K  L  P
121        GGCCAGCACTGAACATATACTCCTAAGCGAGTGTCATAGTTCAGCTGCTCCAGTTTTCAC
41          G  Q  H  *  T  Y  T  P  K  R  V  S  *  F  S  C  S  S  F  H
181        GTTTCCAGAGTAATAATAATGAAGATCAGCTGAACGTATAATAAATTCTGTGCGCGTGCT
61          V  S  R  V  I  I  M  K  I  S  *  T  Y  N  K  F  C  A  R  A
241        TGTGTGTGTGTGTGTTGTGGTCCCTGTAATAAAAAATATCTTGGGTGTGTACACTTTT
81          C  V  C  V  C  V  V  V  P  V  I  K  N  I  L  G  V  Y  T  F
301        TCTATTTACTGAAATATGTATGTGTGGTGTTGTATTCTGACTTCTGGTCAGTTTGTATAT
101         S  I  Y  *  N  M  Y  V  W  C  C  I  L  T  S  G  Q  F  V  Y
361        TCTGTCGTCACAGAACAGGATTGTTGTATCTGACGACTTTATATATACGCGGATGTGTTT
121         S  V  V  T  E  Q  D  C  C  I  *  R  L  Y  I  Y  A  D  V  F
421        ATAACATCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
141         I  T  S  K  K  K  K  K  K  K  K  K
```

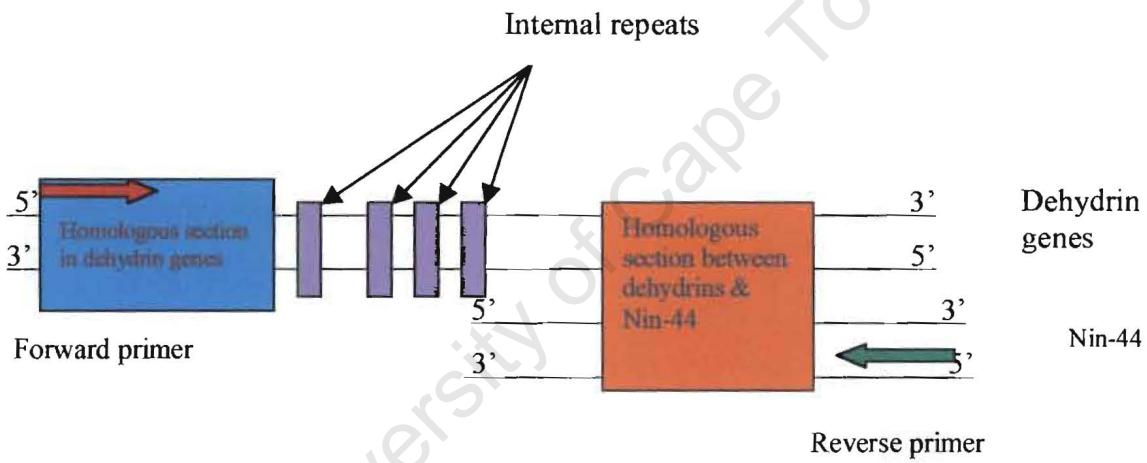
Fig. 2.8. Nucleotide and derived amino acid sequence of the Nin-44 insert. Underlined sections represent the GT rich sequences and asterisks indicate the stop codons.

Nin-44 was found to contain a 60 bp sequence near the 5' end with 99 % identity at the amino acid level to the 3' end of 7 different water-deficit stress inducible genes (dehydrins) from monocot plant species (Fig. 2.9) which might indicate a common origin and co-evolution of these grasses. The amino acid sequence of this region is homologous to a 17 amino acid internal repeat (TGEKKGVMENIKDKLPG) present in multiple copies in these genes. This repeat motif is highly polar with an isoelectric point (pI) of 9.4. The 5' end of the first repeat (from the 5' end of these genes) contains 10 different amino acid residues that are not identical amongst all dehydrins. Dehydrins from durum wheat, barley (Dhn8 and Dhn4) and rice (Rab21) showed the same amino acid sequence [5' *SEDDGMGGRR(K)* KKGIKDKIKEKLPGG 3'] whereas LEA-G2 from barley and WCS66 and WCS120 from wheat shared a similar sequence [5' *ME(D)H(N)QAHG(I)AGE* KKGIMEKIKEKLPGG 3'] (Fig. 2.9). This extended region of 10 residues did not appear in the Nin-44 sequence also suggesting that the Nin-44 sequence obtained represented the 3' end of a dehydrin-like gene.

Fig. 2.9. Comparison of the derived amino acid sequence of Nin-44 with different water stress inducible genes from barley, rice and wheat. Common section is represented by red color. The homologous sections of the other genes near the 5' end is represented by blue color and the internal repeating sequence is represented by violet color. The section from which the forward primer was designed is underlined.

To try and obtain sequence 5' to that obtained from sequencing the Nin-44 clone, a 21 bp forward primer representing the repeating motif and a 22 bp reverse primer derived from the 3' end of the Nin-44 DNA sequence were designed for RT-PCR amplification (Fig. 2.10). The former primer was made degenerate (degeneracy of 24) to include all possible codon usages based on dehydrin protein sequence. Reverse transcription was initially performed using mRNA from dehydrated *E. nindensis* leaves with the reverse primer. The first strand synthesised was then used as template DNA in a PCR with both primers and the products electrophoresed on a 1 % agarose gel (Fig. 2.11).

Primer design



Forward Primer 5' **GA(CTG) AAG ATC AA(AG) GA(CG) AAG CT(CT)** 3'

Reverse Primer 5' **CTG GAG CAG CTG AAC TAT GAC** 3'

Fig. 2.10. RT-PCR primer design. Sections are represented by different colours (c.f. Fig. 2.9). Degenerate base are put in brackets.

Electrophoresis of the RT-PCR products resulted in two major bands with sizes 0.7 & 0.8 kb (Fig. 2.11 A). These bands were excised, purified and used as the template in a second round PCR. However, instead of the defined products expected, a smear was obtained (Fig. 2.11 B and 2.11 C). Although this reaction was performed numerous times with variation of the PCR conditions, no improvement was observed in the quality of the product. As a result, further characterization of the clone was not performed.

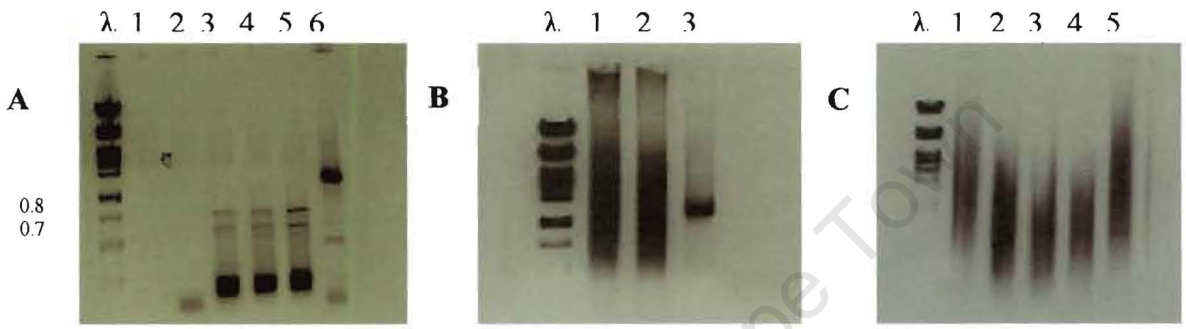


Fig. 2.11. RT-PCR amplification of mRNA extracted from dehydrated leaves of *E. nindensis*. A) First round RT-PCR using Mg^{++} concentrations of 1.5, 2.0, 2.5 and 3.0 mM (lanes 1 – 5). Lane 6 was a positive control to ensure that the PCR was functioning. The 0.7 and 0.8 kb bands were excised and used for second round PCR (B). B) Second round PCR using a Mg^{++} concentration of 3.0 mM with the excised 0.8 kb band (lane 1) or the excised 0.7 kb band (lane 2) as the DNA template. Lane 3 was a positive control. C) Another second round PCR using Mg^{++} concentrations of 0.0, 0.5 and 4.0 mM with the excised 0.8 kb band as DNA template (lanes 1, 2 and 5); or using a Mg^{++} concentration of 3.5 mM with the excised 0.7 kb band (lanes 3 an 4). The molecular weight marker (λ) is a *PstI* digest of bacteriophage λ DNA.

2.4 Discussion

Screening of 94 clones of the cDNA library of *E. nindensis* led to a partial characterization of a dehydrin-like gene, Nin-44. The majority of the clones did not show inserts either on restriction digestion or by PCR amplification. This was confirmed by blue/white colour selection on IPTG-X-gal plates, where over 80 % of the clones were blue non-recombinant colonies. One of the reasons for this is the possibility of self-recircularisation of vectors without the incorporation of inserts on construction of the library.

EcoRI/XhoI digestion or PCR amplification of white colonies showed that approximately 75 % of such colonies were false positives (or their insert DNA to be of less than 200 bp) as no products were visualized on an agarose gel. Small sized inserts may have resulted from poor control of reverse transcription in making the library due to incomplete first strand synthesis, the use of degraded mRNA or inappropriate restriction digestion. False positives can also be produced either by damage to the host β-galactosidase C-terminal portion or interruption of the N- terminal of the β-galactosidase fragment produced upstream of the multiple cloning site (MCS) on the plasmid (Borrebaeck, 1998). A host cell with such damage does not cleave 5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside and so remains a white colony.

Despite all efforts, the sequencing of the selected clones, Nin-19 and Nin-44, was not entirely successful. Only single strand sequences of both samples were obtained. It is known that sequencing fails due to a number of reasons such as poor template quality, incorrect amount of template, contamination, primer-dimer formation and presence of multiple primer binding sites (Hartwell center for Bioinformatics and Biotechnology). It has also been reported that the α-complementing β-galactosidase sequences of ExAssist interference-resistant helper phage used in the excision process interferers with sequencing as the oligonucleotide primers hybridise to β-galactosidase sequences (Stratagene Manual, 1999). However, since sequencing with the T3 primer was at least partially successful, it is unlikely that all the above factors were applicable. Failure to

sequence using the T7 primer might be due to the presence of multiple primer binding sites in the template DNA.

Good homology between the sequence of the Nin-44 insert with dehydrin genes over a short segment near the 3' end was observed. The presence of a putative polyA-tail sequence at the 3'end of the single strand sequence obtained suggested that this sequence represented the 3' end of the gene. RT-PCR performed to determine sequence 5' to that obtained by sequencing the Nin-44 insert resulted in a number of bands being present on the gel. The production of more than one band might have been due to primer binding to more than one homologous region of the Nin-44 gene. It is not clear why the second round PCR resulted in a smear since the primers used in both first and second PCRs were identical. Optimisation of the PCR conditions, such as changing the annealing temperature, Mg^{++} concentration and amount of template DNA had no effect on the quality of the product.

Further investigation of the Nin-44 sequence should be performed using a different approach. One approach could be to use the Nin-44 sequence to probe restriction digested genomic DNA. Alternatively, the method presented here could be applied to a new cDNA library constructed from mRNA of *E. nindensis*.

CHAPTER THREE

Physiological response Of *Eragrostis nindensis* and *Eragrostis tef* plants to water-deficit stress

3.1 Introduction

Water stress in plants is manifested in different ways including physiological disorders, protein damage and misfolded protein structure, leading to cellular injury and death (Wischmeyer, 2002). Loss of cellular water can be recognized primarily by loss of turgor, change in cell volume, change in solute content, alteration in cell wall-plasma membrane connections (Bray, 1997), and general changes in the metabolism. The sum total of these events is referred to as "physiological" changes or adaptations to the stress.

Visual symptoms of stress-induced injury are difficult to quantify when plant species or genotypes are screened for differential susceptibility to a specific stress. Alternatively, a number of physiological parameters and structural damage indicators have been used to study the effects of water-deficit stress (Earnshaw, 1993). In this chapter, comparisons of plant water status, membrane integrity, ultrastructural changes and changes in photosynthetic and respiratory values of the *Eragrostis* species were made at different water-deficit levels during dehydration and rehydration treatments. The following paragraphs provide a framework of understanding on plant water relations and how water-deficit stress develops.

The water status of a plant is usually defined in terms of its water content, water potential, or the components of water potential (Turner, 1986). The use of relative water content, in which the water content is expressed on the basis of the fully hydrated water content, is a useful measure, since it allows comparison among different plant species and tissue types (Turner, 1986). This measure will be used in the current study.

Water-deficit in plants develops as a consequence of water loss from the leaf as the

stomata opens to allow the up take of carbon dioxide from the atmosphere for photosynthesis. The water lost by transpiration from the leaf mesophyll cells is replaced by water drawn from the soil through the root, stem, and leaf via xylem. During transpiration, water vapor (i.e., water in the gaseous state) diffuses from the inside of the leaf out into the atmosphere when stomates are open. Since stomates must be open in order for photosynthesis to occur, most terrestrial green plants are forced into a trade-off such that water must be lost through transpiration in order for the plant to undergo photosynthesis (i.e. the photosynthesis-transpiration compromise) (Passioura, 1982).

During water stress leaves close their stomata as a control mechanism to reduce the loss of water. This protects the inward passage of CO_2, and results in reduction of CO_2 assimilation per unit leaf area. The evaporation of water from leaf surfaces also allows cooling of leaves that otherwise would become too warm in the direct sunlight to which they are exposed.

When soil water potential decreases plants respond by decreasing their transpiration rates. Leaf area and conductance regulate how much water can be potentially transpired by a plant (Schulze *et al.*, 1994). Plants with high leaf area and high conductance will use water at higher rates. As water in the soil depletes, plants cannot sustain the same rate of use. In a short time scale water use is reduced when leaf conductance decreases, but as dehydration persists other mechanisms such as leaf rolling and leaf shedding become an important mechanism to reduce water loss in many plants (Passioura 1982).

Beyond certain water potential thresholds, plants loose turgor and wilt (Larcher, 1995). Usually wilting is observed during the day and plants are observed to recover during night when the atmospheric water demand is low and the plant reaches equilibrium with soil water potential. If there is no water input to the soil, plants will reach a point, termed the "permanent wilting point" where they cannot recover during the night. This is controlled by the particular capacity of the plant to use water at low water potentials effectively, which varies across species (Orians and Solbrig 1977).

Another major impact of water-deficit tress is cell membrane modification (Tourdot-Marechal *et al.*, 2000). Water-deficit causes membrane damage, which is estimated by measuring cellular leakage from affected leaf tissues into an aqueous medium using conductivity meter. This measure is an indicative of the degree of the membrane damage of cells (Farrant and Kruger, 2001; Vander Willigen *et al.*, 2001) and can be used as a screen for stress tolerance. Ultrastructural studies on tissues exposed to water-deficit stress also indicate the extent of tissue damage. This technique has been used successfully in studies of desiccation tolerance (Sargent *et al.,* 1981; Schwab and Heber, 1984; Bohnert *et al.,* 1995; Farrant and Sherwin, 1999; Farrant and Kruger, 2001; Vander Willigen *et al.*, 2001) to investigate the subcellular organization of stressed tissues.

The understanding of physiological responses, by comparing tolerant and sensitive plants to water-deficit stress, is important to develop or improve drought tolerance in economically important plants. Although there have been many studies on the effect of water-deficit on many crop plants, there has been no comparative study reported on the cereal tef (*E. tef*) with its desiccation tolerant relatives. So the objective of this part of the project was to study and compare physiological responses and cellular damage of two varieties of desiccation sensitive *E. tef* with that of the desiccation tolerant *E. nindensis* upon dehydration and rehydration.

The physiological processes that were studied were rates of transpiration, net photosynthesis and respiration, and stomatal conductance. The damage to cellular membranes was also investigated by measuring electrolyte leakage using a conductivity meter. Furthermore, the effect of water-deficit on cellular ultrastructure of *E. tef* varieties was studied using transmission electron microscopy.

3.2 Materials and methods

3.2.1 Plant material

E. nindensis was collected from Windhoek, Namibia, and planted in a mixture of sand and potting soil and maintained in a glass house, at the University of Cape Town, prior to experimentation. Plants were kept hydrated by regular watering of the soil.

Seeds of two varieties of *E. tef*[3] (white and red-seeded) were collected from the central high land province of Eritrea (altitude of about 2000 m above sea level). These seeds were sown directly in to sand and potting soil in replicate pots and maintained in a glass house as above.

One week before the start of the dehydration treatments, all plants species were transferred to a low light growth chamber for acclimation. The day light temperature was set to 25 ^{0}C during the 15 hr photo-period, and 17 ^{0}C, during a 9 hr dark period. These conditions simulate the natural growth temperature conditions of the major tef growing areas of Ethiopia and Eritrea. Relative humidity was 50 %. Light was supplied by warm white fluorescent bulbs at a photon flux density of 300 μmol $m^{-2}s^{-1}$.

3.2.2 Dehydration/rehydration treatments

Dehydration treatment (withholding of water) was started four weeks after germination of *E. tef* seeds. Since the RWC below which *E. tef* plants cannot recover was not known, treatment plants were divided to four groups for rehydration after different times of dehydration. The first group of the samples were watered after 6 days, the second after 9 days and the third after 12 days of dehydration treatment. The last group was allowed to dry for 15 days before rehydration. Similar sets of samples were prepared from *E. nindensis*. A separate set of controls from each group was watered every day until the treatment was ended. All parameters were measured every 3 days during dehydration,

[3] For shorthand representation purposes, the two *E. tef* varieties will be named as *E. tef* (R) for red-seeded and *E. tef* (W) for white-seeded varieties.

and at 0, 1, 2, 3, 6, and 9 days during the rehydration. The following measurements were recorded for all species during dehydration and rehydration.

3.2.3 Leaf relative water content (RWC)

RWC was determined as described by Sherwin and Farrant (1996). Essentially, leaf samples (three different leaves from each plant) from the different treatments were cut into 3 cm lengths and weighed. These leaves were fully submerged on distilled water for 4 h, and then gently blotted on paper towel and their turgid weight was measured. The dry weight was determined after oven drying for 24 h at 103 ^{0}C measured and the percent relative water content was calculated using the method of Weatherly (1950) as: %RWC = [(fresh weight – dry weight)/(turgid weight – dry weight)] x 100. Difference of RWC among species and varieties was tested statistically using T-test at 95 % confidence interval.

3.2.4 Gas Exchange

Measurements of gas exchange were performed using LCA-3 (Analytical Development Company Ltd., Hoddesdon, UK) infrared gas analyser (IRGA) operated in differential mode at an ambient CO_2 concentration of 350 ppm. Data on net photosynthetic rate (A), transpiration (E), and stomatal conductance (Gs) measurements were taken from 10h00-11h00 during the day, and respiration measurements were taken at night from 19h00-20h00. As the small individual leaves of *Eragrostis* plants did not cover the area of the leaf chamber, leaf area used was determined as described by Gollan *et al.*, (1985) and used for calculation. Leaves were placed on millimetre graph paper and the area was determined as: [Area = Nº of full squares covered by the leaf + ½ (Nº of half squares traced on the margins of the leaf.)]. Three readings were taken from three leaves of each plant, standardized to the leaf area and used for comparison among treatments.

3.2.5 Electrolyte leakage

Membrane integrity of leaf tissues was determined by measuring electrolyte leakage using a CM100 Multiple Cell conductivity meter (Reid and Associates, Durban, South Africa). Three replicates of leaf samples from each group of treatments and controls were used. Leaves were placed in 3 ml ultra pure water (milli-Q) and conductivity was read every min for 60 minutes. Rate of leakage was calculated as the slope of line generated from the time course of leakage and was corrected by leaf dry weight ($\mu S.gdwt^{-1}\ min^{-1}$).

3.2.6 Ultrastructural study

Leaf bases which contained meristems from *E. tef* (W) and *E. tef* (R) dehydrated for 6 and 9 days respectively, and rehydrated for a subsequent 3 days, were used for the ultrastructural study. Samples were fixed according to the method described by Sherwin and Farrant (1996) for resurrection plants. Samples were cut into small segments using sharp blades and fixed over-night in 2.5 % glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) and 0.5 % caffeine, then washed 3 times by 0.1 M phosphate buffer and fixed for another 1 h in one part 2 % osmium tetraoxide (OsO_4) and one part 0.2 M phosphate buffer. After fixation, tissues were dehydrated using an ethanol gradient, infiltrated with epoxy resin (Spurr, 1969) over two days and polymerised for 16 h at 60 ^{0}C. Sections (95 nm thick) were cut using a Reichart Ultracut-S (Leica, Vienna, Austria); mounted on copper grids and stained with uranyl acetate and lead citrate (Reynolds, 1963) for 10 min each. Sections were viewed with transmission electron microscopy (Zeiss109 TEM).

3.3 Results

For convenience of comparison, results of dehydration and rehydration treatments are combined in one section. Figure 3.1 (a) shows the % RWC of the three species during dehydration and rehydration treatments. For the first three days of dehydration, there was no significant water loss from any of the three plants. After 6 days, RWC of *E. tef* species had dropped sharply by approximately 60 % (to 33 %) and by 55 % (to 39 %) in *E. tef* (W) and *E. tef* (R) respectively. This difference was significant at 95 % confidence interval [T-value = -3.286, $T_{(4, 0.025)} = 2.776$]. But 6 days of dehydration of *E. nindensis* resulted in only 25 % decrease of RWC (to 63 %) and the decline was gradual. However, after 9 days of dehydration, the RWC of all plants had dropped to about 10 % with no differences between them thereafter.

When the plants were watered after 6 days of dehydration, the RWC of *E. nindensis* and *E. tef* (R) recovered to the control levels within 24 h (Fig. 3.1 (b)) and the existing leaves survived. Dehydrated leaves of *E. tef* (W) did not rehydrate and these existing leaves died. However, new leaves emerged from stem nodes after 4 days of rehydration (Fig. 3.2). When the plants were watered after 9 days of dehydration, where the % RWC of all samples had dropped to about 10 %, only *E. nindensis* was able to recover (Fig. 3.1 (c)).

The rate of electrolyte leakage obtained from the 3 species during the time course of dehydration and rehydration is shown in Figure 3.3 (a). Leakage rates from the two *E. tef* varieties increased three fold in 6 days of dehydration, attaining maximum values after 12 days. After 6 days of dehydration, a maximum leakage (12.7 $\mu S\ gDw^{-1}\ min^{-1}$) was recorded for *E. tef* (W). In comparison to *E. tef* species, leakage from *E. nindensis* was consistently low throughout the dehydration treatment. Control plants showed no change in electrolyte leakage.

On rehydration following 6 days of dehydration, electrolyte leakage from *E. tef* (W) leaves remained elevated (Fig. 3.4 (b)) indicating membrane damage that was not repaired on rehydration. In comparison, leakage from *E. tef* (R) dropped to the control

levels after 24 h, indicating an ability to repair membrane damage. After 9 days drying, both *E. tef* varieties had elevated leakage rates. *E. nindensis* maintained low leakage on rehydration and fully recovered when watered (Fig. 3.4 (c)).

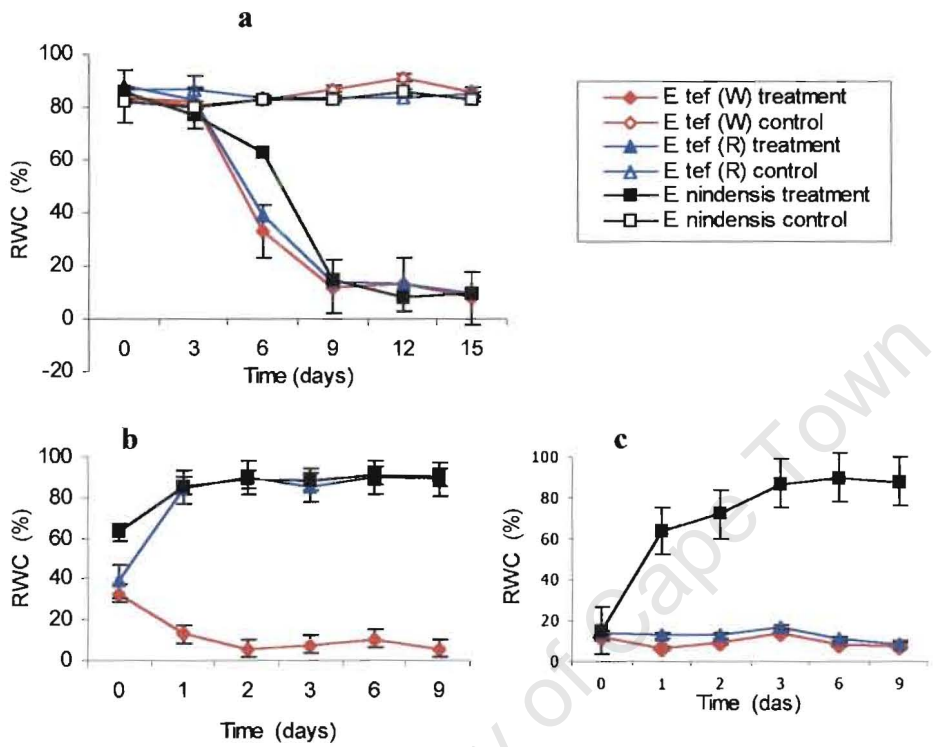


Fig. 3.1 Leaf relative water content (% RWC) of plants measured during dehydration (a), and during two step rehydration: (b) after 6 days dehydration and (c) after 9 days of dehydration (♦ = *E. tef* (W) treatment, ◊ = *E. tef* (W) control, ▲ = *E .tef* (R) treatment, Δ = *E. tef* (R) control, ■ = *E. nindensis* treatment, □ = *E. nindensis* control). Error bars represent the average deviation.

Fig. 3.2 *E. tef* (W) showing new leaves growing from the stem nodes upon rehydration (Arrows indicate the new leaves).

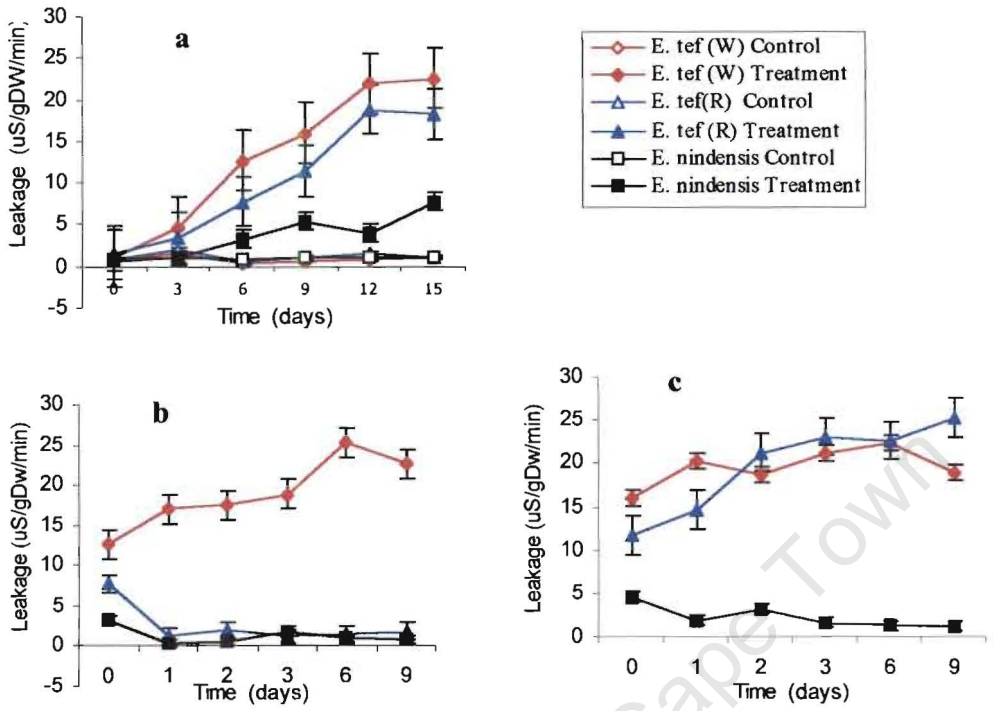


Fig. 3.3 Rate of Electrolyte leakage (μS. $gDw^{-1}min^{-1}$) from plants measured during dehydration (a), and during two step rehydration: (b) after 6 days dehydration and (c) after 9 days of dehydration (♦ = *E. tef* (W) treatment, ◊ = *E. tef* (W) control, ▲ = *E. tef* (R) treatment, Δ = *E. tef* (R) control, ■ = *E. nindensis* treatment, □ = *E. nindensis* control). Error bars represent the average deviation.

Gas exchange measurements showed a similar trend during dehydration of the plant species studied. However, the initial value of stomatal conductance (Gs) of *E. nindensis* leaves (260 mmol $m^{-2}.s^{-1}$) was 1.7 times higher than those of *E. tef* plants (Fig. 3.4 (a)). After 6 days of dehydration treatment, Gs values have dropped by 75 %, 70 % and 40 % in *E. nindensis*, *E. tef* (R) and *E. tef* (W) plants respectively, and lower Gs values were recorded from all species after 9 days of dehydration treatment. Control plants did not show any change of Gs values.

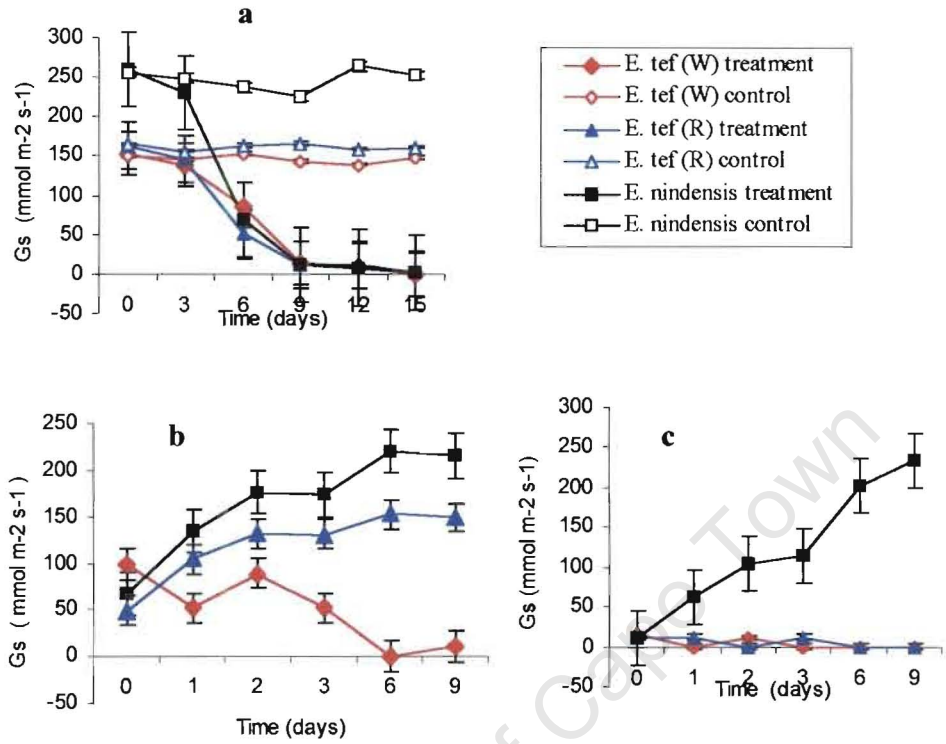


Fig. 3.4 Stomatal conductance (mmol.m^{-2}.s^{-1}) of plants measured during dehydration (a), and during two step rehydration: (b) after 6 days dehydration and (c) after 9 days of dehydration (♦ = *E. tef* (W) treatment, ◊ = *E. tef* (W) control, ▲ = *E. tef* (R) treatment, Δ = *E. tef* (R) control, ■ = *E. nindensis* treatment, □ = *E. nindensis* control). Error bars represent the average deviation.

Rehydration of the plants after 6 days of dehydration, resulted in the recovery of stomatal conductance in *E. nindensis* and *E. tef* (R), and the trend of recovery in these plants was similar. Gs results for *E. tef* (W) showed slight recovery after 48 h, but could not recover fully (Fig. 3.4 (b)). When the plants were rehydrated after 9 days of dehydration, Gs values from neither of the *E. tef* varieties recovered, but *E. nindensis* showed full recovery within 6 days rehydration period (Fig. 3.4 (c)).

Transpiration rates (E) ahowed the same trend as found for stomatal conductance except that all species had similar initial values (Fig. 3.5 (a)). The initial value of transpiration rate from *E. nindensis* leaves dropped sharply to minimum value (90 % decrease

compared to the control values) in 6 days of dehydration treatment. There was a 55 % and 35 % decrease in *E. tef* (R) and *E. tef* (W) respectively, over the same period. The decline in transpiration in *E. tef* plants was not as sharp as that of *E. nindensis*. After 9 days of dehydration, readings did not show significant difference among the species. Control plants maintained high transpiration rates (Fig. 3.5 (a)).

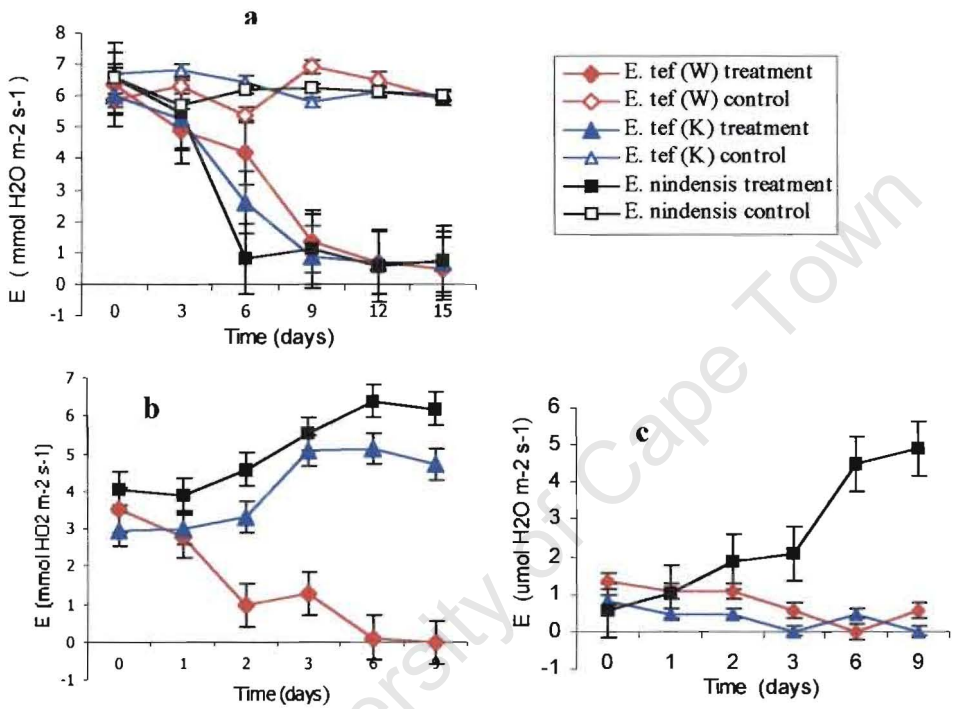


Fig. 3.5 Transpiration rate (mmol.m^{-2}.s^{-1}) of plants measured during dehydration (a), and during two step rehydration: (b) after 6 days dehydration and (c) after 9 days of dehydration (♦ = *E. tef* (W) treatment, ◊ = *E. tef* (W) control, ▲ = *E .tef* (R) treatment, Δ = *E. tef* (R) control, ■ = *E. nindensis* treatment, □ = *E. nidensis* control). Error bars represent the average deviation.

When plants were rehydrated after 6 days of dehydration, transpiration rates of *E. nindensis* and *E. tef* (R) were fully recovered, but showed different patterns of recovery. *E. nindensis* recovered fully to the level of controls within 6 days of rehydration, but the recovered *E. tef* (R) showed slightly lower maximum values of transpiration relative to the controls (Fig. 3.5 (b)). However, after 9 days of dehydration treatment, neither of the *E. tef* species showed recovery of transpiration upon rehydration (Fig. 3.5 (c)).

Fig. 3.6 shows CO_2 assimilation (A) during the dehydration and rehydration treatments. Initial net photosynthetic rates of 12.1 μmol $m^{-2}.s^{-1}$, 11.9 μmol $m^{-2}.s^{-1}$ and 11.0 μmol $m^{-2}.s^{-1}$ were recorded from *E. tef* (W), *E. tef* (R) and *E. nindensis* respectively (Fig. 3.6 (a)). In the first three days of dehydration, there was no significant difference in net photosynthesis in any of the species, but another three days subsequent dehydration showed a 70 % decrease in the *E. tef* (W) and about 40 % decrease in *E. tef* (R) and *E. nindensis* compared to controls. After 9 days of dehydration, net photosynthetic rates of *E. tef* (R) and *E. nindensis* had dropped by about 90 % of the control values. The decline was more gradual in *E. nindensis* and *E. tef* (R) than in *E. tef* (W).

Upon rehydration (after 6 days of dehydration) values of net photosynthetic rates for *E. tef* (R) continued to decline for 2 days, but reversed afterwards reaching an optimal value after 6 days (Fig. 3.6 (b)). *E. nindensis*, on the other hand, recovered immediately reaching maximal rate after 6 days of rehydration. *E. tef* (W) did not recover photosynthesis at all. After 9 days of dehydration neither of the *E. tef* species recovered assimilation (Fig. 3.5 (c)) but *E. nindensis* did.

Changes in respiration as the result of the stress were similar to those of net photosynthetic rates except the fact that the decline was more gradual in all the plants (Fig. 3.7 (a)). By 12th day, recordable respiration had virtually ceased in all three species. Respiration rates in *E. tef* (R) and *E. nindensis* showed rapid recovery within 24 h of rehydration from a dehydration of 6 days (Fig. 3.7 (b)), but only *E. nindensis* showed full recovery when rehydrated after 9 days of dehydration. As for all other measures, *E. tef* (W) did not recover respiration after 6 days of drying (Fig. 3.7 (c)).

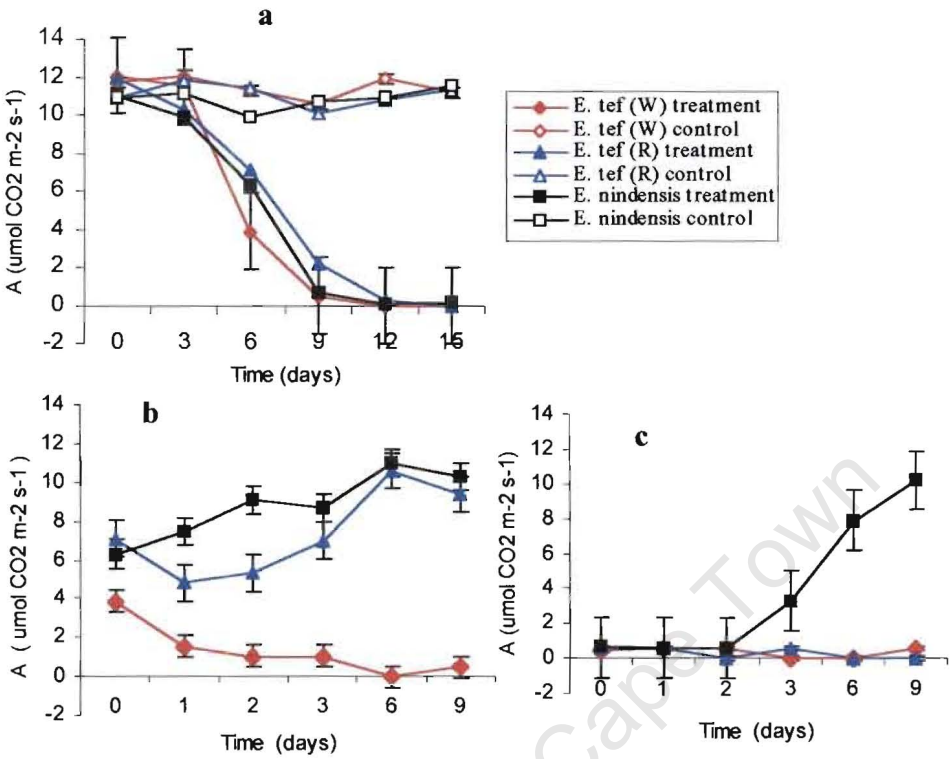


Fig. 3.6 Net photosynthetic rate (μmol $m^{-2}.s^{-1}$) of plants measured during dehydration (a), and during two step rehydration: (b) after 6 days dehydration and (c) after 9 days of dehydration (♦ = *E. tef* (W) treatment, ◊ = *E. tef* (W) control, ▲ = *E.tef* (R) treatment, Δ = *E.tef* (R) control, ■ = *E. nindensis* treatment, □ = *E. nindensis* control). Error bars represent the average deviation.

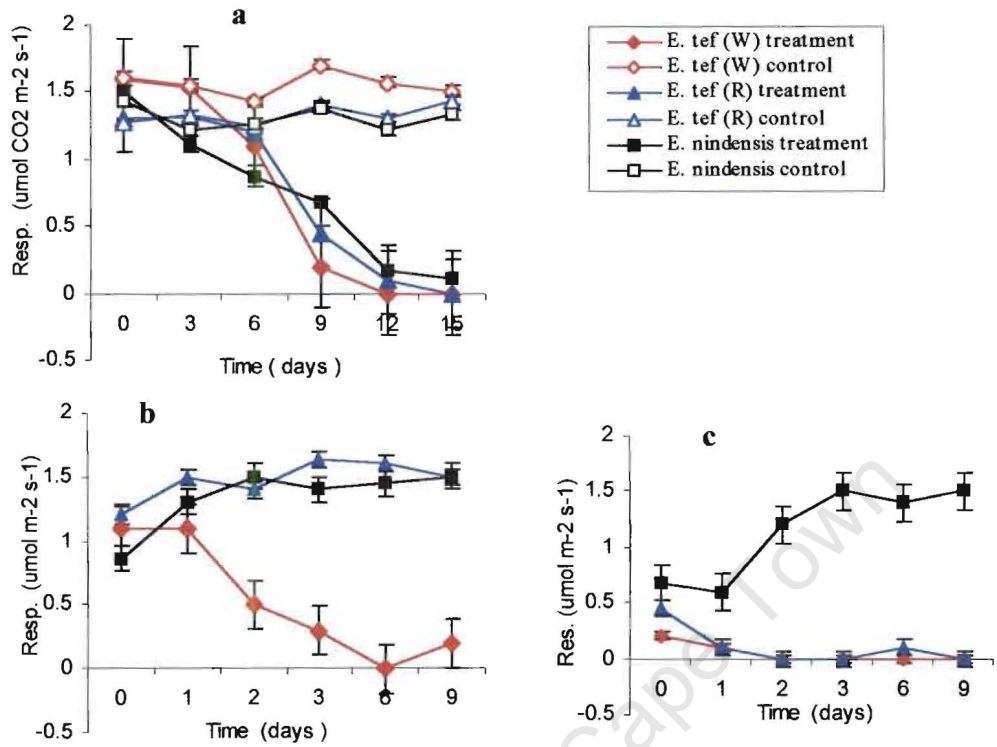


Fig. 3.7 Respiration rate (μmol $m^{-2}.s^{-1}$) of plants measured during dehydration (a), and during two step rehydration: (b) after 6 days dehydration and (c) after 9 days of dehydration (♦ = *E. tef* (W) treatment, ◊ = *E. tef* (W) control, ▲ = *E .tef* (R) treatment, Δ = *E. tef* (R) control, ■ = *E. nindensis* treatment, □ = *E. nindensis* control). Error bars represent the average deviation.

An ultrastructural study was performed on leaf bases of *E. tef* (W) and *E. tef* (R) to characterise the difference in the extent of tissue damage upon dehydration and rehydration. The effect of water-deficit stress on the ultrastructure of *E. nindensis* has been investigated by other researchers (Vander Willigen, *et al.* 2001), and formation of numerous vacuoles with maintained membrane integrity was reported, which enabled the plant to recover upon rehydration.

In the transmission electron micrographs of hydrated tissues (controls) of both *E. tef* varieties, the mesophyll cells contained large vacuoles and the cytoplasm and organelles were confined to the cell periphery (Fig. 3.8 & Fig. 3.9). There was no difference between cellular structures of hydrated tissues of these plants. The stacking of

chloroplasts was normal and starch grains were evident. Mitochondria were present and had the appearance indicative of active tissue.

In the tissues dehydrated for 6 days, the effect of the stress was evident in both plants from the extent of subcellular disorganization (Fig. 3.8, C & D and Fig. 3.9, C & D). Detachment of plasma membrane from the cell wall was observed in some cells (arrowed in Fig. 3.8, C). As reported for *E. nindensis* (Vander Willigen *et al.*, 2001), some small vacuoles were visible at this stage of dehydration in cells of both plant species instead of the one large vacuole. The fine structure of the chloroplast was affected in both plants (Fig. 3.8, C & D and Fig. 3.9, C & D). The thylakoid membranes had become separated in some chloroplasts (Fig. 3.8, C and Fig. 3.9, D) but in others were still intact and the granal stacking was evident (Fig. 3.8, D). In general, *E. tef* (W) showed more damage than *E. tef* (R). In the latter several cells appeared undamaged after 6 days of dehydration (Fig. 3.9, C). In neither variety did 6 days of dehydration cause any cell wall fractures.

Rehydration of the 6 days dehydrated tissues of *E. tef* (W) and *E. tef* (R), depicted in Fig. 3.8, E & F and Fig. 3.9, E & F respectively, showed recovery of general subcellular detail. The plasma membrane became closely associated with the cell wall again and chloroplasts regained thylakoid stacking and starch grains. In the case of *E. tef* (W) some of the cells did not show recovery and had disintegrated chloroplasts (Fig. 3.8, E).

After 9 days of dehydration on the other hand, the subcellular organization of tissues in both *E. tef* species was severely compromised and there was no difference between the plants. Rehydration of the samples at this stage did not allow recovery of subcellular organization in either species. The extent of the damage caused by the 9 days dehydration and rehydration in both plants is shown in Fig. 3.8, G and Fig. 3.9, G).

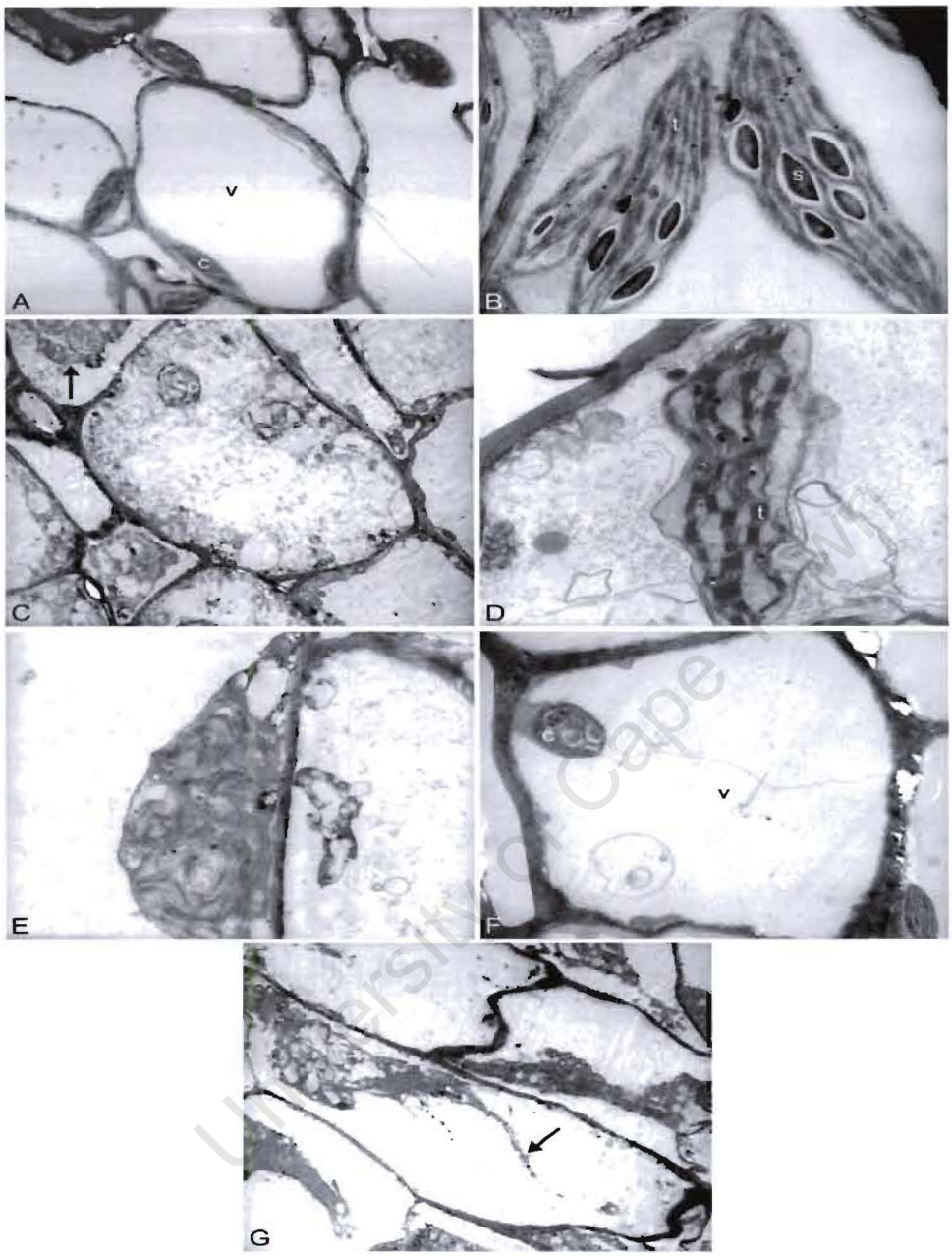


Figure 3.8. Mesophyll cells of hydrated (A & B), dehydrated for 6 days (C& D) and rehydrated after 6 days (E & F) and after 9 days (G) of *Eragrostis tef* (w). Note the changes in stacking of thylakoid membranes (t) and chloroplast (c) integrity, changes in vacuole (v) and plasma membrane detachment from cells walls (indicated by arrows) on drying and rehydration. Starch grains (s).

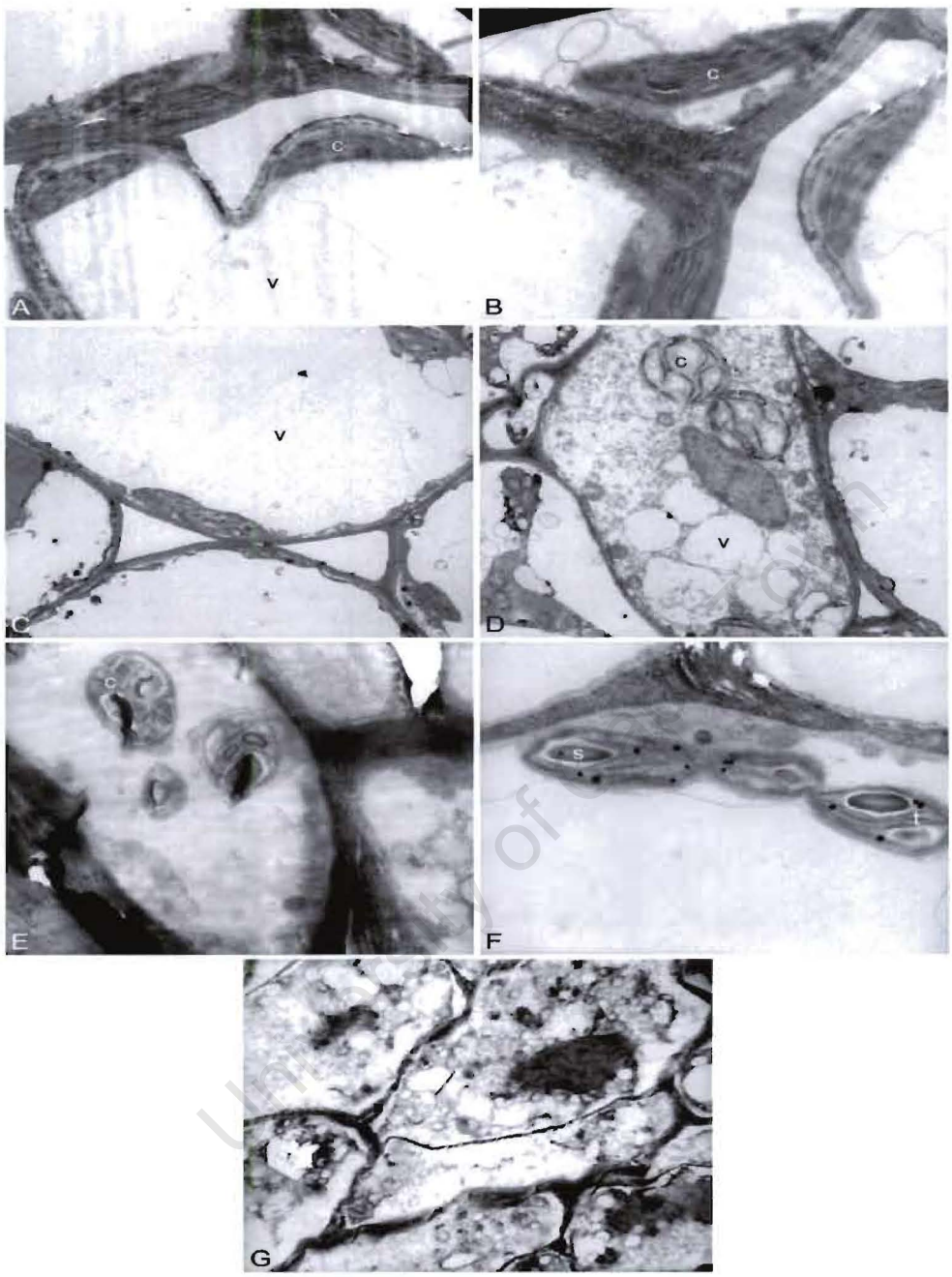


Figure 3.9. Mesophyll cells of hydrated (A & B), dehydrated for 6 days (C& D) and rehydrated after 6 days (E & F) and after 9 days (G) of *Eragrostis tef* (R). Note the changes in stacking of thylakoid membranes (t), chloroplast (c) integrity and changes in vacuole (v) on drying and rehydration. Starch grains (s).

3.4 Discussion

The data presented here for *E. nindensis* support the observation that this plant is desiccation tolerant, and thus a resurrection species (Gaff, 1977; Vander Willigen *et al.*, 2001). It survived drying to 10 % RWC, and full metabolic recovery occurred thereafter (Fig. 3.1, 3.3, 3.4, 3.5, 3.6 and 3.7), thus in this section more emphasis will be made on the results of *E. tef* varieties and a comparison with that of *E. nindensis* will be made when appropriate.

Prior to 6 days of dehydration the *E. tef* varieties showed little differences in all physiological responses and recovered when watered. However, after 6 days of drying *E. tef* (R) recovered whereas existing leaves of *E. tef* (W) died upon rehydration. *E. tef* (R) had a significantly higher RWC than *E. tef* (W) after 6 days (39 % and 33 %; T-value = -3.286, $T_{(4,\ 0.025)} = 2.776$) and this could have been the reason for the survival of the former. This difference in RWC was likely to be due to the difference in control of stomatal conductance (Gs) and transpiration (E).

Reducing stomatal conductance helps plants to reduce transpiration, and hence maintain higher RWC (Lawlor and Cornic, 2002). Transpiration rate in *E. nindensis* had dropped to its minimum level in 6 days indicating the presence of a controlling mechanism against transpirational water loss, whereas in *E. tef* (W) it was decreased by only 30 % (Fig. 3.5, a) leading to a major decline in RWC indicating poor control over transpiration which had resulted in subsequent leaf death. The intermediate E and Gs values from *E. tef* (R) indicated a better performance of this variety over *E. tef* (W) in the short term.

It has been reported that RWC of between 30 - 35 % are lethal for many species (Vertucci and Farrant, 1995; Walters *et al.*, 2002) and *E. tef* (R), which had higher RWC than this critical value, showed survival at 6 days of dehydration, whereas *E. tef* (W) which had dried to about this critical RWC did not. The ultrastructural study also supported this observation. *E. tef* (R) showed some subcellular damage, but a large proportion of cells remained undamaged (Fig. 3.9 C). *E. tef* (W) on the other hand showed considerably larger proportion of damaged cells at 6 days of drying. Some repair

of damage is also obviously possible in *E. tef* (R), but not in *E. tef* (W) since the former showed reduction in electrolyte leakage on rehydration after 6 days of drying but the latter did not.

Although existing leaves of *E. tef* (W) died after 6 days, the whole plant was still able to recover by producing new leaves from meristem at the bases of older leaves when watered (Fig. 3.2). After drying to below 30 % RWC (after 9 days of dehydration), neither of the *E. tef* varieties recover, nor can they produce new leaves. This happens because as drying continues, more and more damage accumulates until presumably a critical level, which is not repairable is reached and hence the plant dies. At this critical level there may be repair, but cannot keep up when critical number of cells damaged (Vertucci and Farrant, 1995; Walters *et al.*, 2002).

The pattern of decrease in net photosynthetic rates observed from *E. tef* varieties and *E. nindensis* was typical for many plants suffering water-deficit stress (reviewed by Lawlor and Cornic, 2002). Under water-deficit stress plants reduce stomatal conductance to maintain their RWC, but this causes limitations to mesophyll photosynthesis as the CO_2 supply is reduced (Gollan *et al.*, 1985; Grieu *et al.*, 1988, Lawlor and Cornic, 2002). This was confirmed by the results obtained with respect to CO_2 assimilation (Fig. 3.6), suggesting that assimilation was limited by closure of stomata, which in turn was limited by water availability. But resurrection plants can override this by actively shutting down photosynthesis at higher water contents than would normally result in shutting down stomata (Farrant, 2000; Vander Willigen *et al.*, 2001).

Separation of thylakoid membrane was reported to be one of the strategies employed by resurrection plants to switch off photosynthesis in order to minimize free radical formation that damages the photosynthetic apparatus (Farrant, 2000; Vander Willigen *et al.*, 2001; Benko *et al.*, 2002). When the thylakoid membrane is disrupted, the electron transport system required for photosynthesis cannot function. The separation of thylakoid observed in *E. tef* varieties upon dehydration (Fig. 3.8, E and Fig. 3.9, D) and its reconstitution in some cells of *E. tef* varieties upon rehydration (Fig. 3.8, C and Fig .3.9,

F) might be considered as an indication of avoiding damage of the photosynthetic apparatus. However, recovery of thylakoids did not occur when *E. tef* varieties were dried to lower water contents and more likely that separation of thylakoids might have reflected damage rather than a protection mechanism.

In agreement with previous studies on plant gas exchange parameters, respiration was the last to be affected by drying and the first to recover from water-deficit stress (Farrant and Kruger, 2001; Vander Willigen *et al.*, 2001; Lawlor and Cornic, 2002). After 6 days of dehydration respiration values for *E. nindensis* and the *E. tef* varieties were still higher (Fig. 3.7, a) and dropped to lower levels only after 12 days of dehydration from which only *E. nindensis* had recovered. This might be due to the presence of some mechanisms (present in most plants, even desiccation tolerant ones) that protect the components of respiratory system for longer periods of drying. However, once damaged, as Farrant and Kruger (2001) have noted, other physiological processes, such as photosynthesis cannot recover as respiration supplies energy required for the repair and recovery process. Those authors suggest that enzymes of resurrection plants required for respiration are particularly well protected in the dry state.

It has been shown that plants under extreme water deficit conditions, once other metabolism such as photosynthesis stops, respiration continues for some time using stored carbohydrates (Lawlor and Cornic, 2002). In the current study after 9 days of dehydration, the damage was so extensive to compromise respiration in *E. tef* varieties (Fig. 3.7, a). This observation is supported by ultrastructural data (Fig. 3.8 and 3.9) showing mitochondrial damage and subcellular deterioration.

In some literature, *E. tef* is reported as a drought tolerant cereal (Kebede *et al.*, 1989; Shiferaw and Baker, 1989). However, our results showed that both *E. tef* varieties had lost about 60 % of the RWC within only 6 days of dehydration period. Based on these results, even though the growth conditions in this study were not the same as in the field it is very hard to consider *E. tef* as drought tolerant or even resistant as it could not maintain its RWC. Comparing the two varieties of the cereal tef, *E. tef* (R) was found to

perform better under water-deficit stress and is slightly more drought resistant than *E tef* (W).

In conclusion, to date, where food shortage has become a major problem in Africa, and farming practices are still traditional and thus are inadequate to feed the population, an understanding of plant water relations is very relevant and would contribute in the selection of a better performing cereals for current environmental conditions. Therefore, further investigation of the physiology of *E. tef*, one of the crops with low nutrient requirement, could aid selection of good performing varieties for the traditional tef growing countries. Based on the results of this study, compared to *E. tef* (W), growing of *E. tef* (R) would be recommended in low rainfall regions.

CHAPTER FOUR

Summary and concluding remarks

The aim of this study was to investigate gene expression in the resurrection plant *E. nindensis* and to compare some physiological responses to drying and rehydration in *E. nindensis* with two varieties of the cereal tef, *E. tef* (W) and *E. tef* (R). From the few studies available, *E. nindensis* is described as desiccation tolerant (Gaff, 1977; Gorham and Hardy, 1989; Vander Willigen *et al.,* 2001) while *E. tef* is not, which was also confirmed by this study.

In chapter 2, gene expression in *E. nindensis* under water-deficit stress was investigated. Differential hybridization was used to screen 94 randomly selected clones of the cDNA library previously constructed from mRNA of *E. nindensis*. Two genes that were found to be expressed in dehydrated leaves were partially characterized. The nucleotide and protein sequence of one of the clones (Nin-44) showed high levels of identity with dehydrins, and hence was identified as a dehydrin-like gene. However, further characterization of both the clones was not performed, partly because the inserts did not represent complete gene sequences, and partly because the RT-PCR did not produce specific bands of DNA that could have been purified and further analyzed.

The physiological responses and changes in cellular ultrastructure caused by dehydration and rehydration treatments were dealt with in Chapter 3. A 6 days of dehydration treatment caused a 60 % loss of RWC in the *E. tef* plants, whereas only 30 % decrease of RWC was observed in *E. nindensis*. The physiological and ultrastructural data showed little difference among the *E. tef* plants in response to drying and yet *E. tef* (R) recovered upon rehydration whereas *E. tef* (W) did not. Presence of some form of repair mechanism upon rehydration was speculated in *E. tef* (R) that enabled it to recover. The data showed that *E. tef* plants are not drought tolerant. On the other hand, these results confirmed that *E. nindensis* is indeed a desiccation tolerant grass.

In conclusion, learning about the biochemical mechanisms by which plants tolerate environmental stresses, such as water-deficit stress, is necessary for genetic engineering approaches to improving crop performance under stress. The complexity of molecular and physiological responses of plants under water deficit stress indicates the need for further and broader investigation in biochemical pathways that are responsible for deriving such responses. Studying plants under stress would help to understand the plasticity of these metabolic pathways and the limits to their functioning, which would be considered as a great achievement in the development of agriculture.

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Appendix

Media and solutions

Solution D (for 10 ml)

- 4 M Guanidium thiocyanaate	4.7 g
- 25 mM sodium citrate	250 µl of 1 M stock
- 0.5 % Sarkosyl	500 µl of 10 % stock
- 2-mercaptoethanol	70 µl
- DEPC water added to	10 ml

NB. Filter sterilise before adding 2-mercaptoethanol if the solution is not fresh.

Hybridization/Prehybridization solution:-

- 25 ml 20 x SSC
- 4 ml 100 x Denhardt's solution
- 10 ml 10 % SDS
- 1 ml sheared salmon sperm DNA (10 mg/µg)
- Bring to 100 ml with H_2O

100 x Denhardt's solution:-

- 5 g Ficoll
- 5 g Polyvinylpyrrolidone (PVP)
- 5 g BSA (bovine serum albumin)
- Bring to 250 ml with ddH_20. Filter sterilise.

20 x SSC :-

- 175.3 g NaCl
- 88.2 g sodium citrate
- Bring to 1 litre with H_2O

LB Agar (per liter)

- 10 g NaCl
- 10 g Tryptone
- 5 g Yeast extract
- 20 g Agar
- Add deionised water to a final volume of one liter, adjust pH to 7 with 5 N NaOH, autoclave and pour into petri dishes (25 ml/100 mm plate)

LB Tetracycline Agar (per liter)

- Prepare 1 litre of LB agar
- Autoclave
- Cool to 55 ^{0}C
- Add 1.5 ml of 10 mg/ml Tetracycline (filter sterilised)
- Pour into petri dishes (~25 ml/100 mm plate)
- Store in a dark, cool place or cover plates with foil if left at room temperature for extended period of time as tetracycline is sensitive to light.

LB Broth (per liter)

- 10 g NaCl
- 10 g Tryptone
- 5 g Yeast extract
- Add deionised water to a final volume of one liter, adjust pH to 7 with 5 N NaOH and autoclave.

LB Tetracycline Broth (per liter)

- Prepare 1 liter of LB broth
- Autoclave
- Cool to 55 ^{0}C
- Add 1.5 ml of 10 mg/ml Tetracycline (filter sterilised)
- Store in a dark, cool place as tetracycline is sensitive to light)

NZY Agar (per liter)

- 5 g NaCl
- 2 g $MgSO_4.7\ H_2O$
- 5 g yeast extract
- 10 g NZ amine (casein hydrolysate)
- 15 g Agar
- Add deionised water to a final volume of one liter, adjust pH to 7.5 with NaOH, autoclave and pour into petri dishes.

NZY Broth (per liter)

- 5 g NaCl
- 2 g $MgSO_4.7\ H_2O$
- 5 g yeast extract
- 10 g NZ amine (casein hydrolysate)
- Add deionised water to a final volume of one liter, adjust pH to 7.5 with NaOH and autoclave.

LB Top Agar (per liter)

- Prepare 1 liter of LB broth
- Add 0.7 % (w/v) agarose
- Autoclave

NZY Top Agar (per liter)

- Prapare 1 liter of NZY broth
- Add 0.7 % (w/v) agarose
- Autoclave

SM Buffer (per liter)

- 5.8 g NaCl
- 2.0 g $MgSO_4.7\ H_2O$
- 50 ml 1 M Tris-HCl (pH 7.5)

- 5.0 ml 2 % (w/v) gelatin
- Add deionised water to a final volume of one liter and autoclave.

Plasmid prep solution I (10x)

Component	stock conc	final conc.	/100 ml
Tris-HCl, pH 8.0	1 M	25 mM	2.5 ml
EDTA, pH 8.0,	0.5 M	50 mM	10 ml
Glucose	20% (w/v)	1% (w/v)	5 ml
Water	-	-	82.5 ml

Note: do not autoclave this solution once the components have been mixed as the glucose caramelizes.

Plasmid prep solution II (1x)

Component	stock conc	final conc./100 ml	/10 ml
NaHO 10 M	0.2 M	2.0 ml	0.2 ml
SDS 25 %(w/v)	1 % (w/v)	4 ml	0.4 ml
water	-	94 ml	9.4 ml

Plasmid prep solution III (1x)

Component	stock conc	final conc.	/100 ml	/10 ml
Potassium acetate	5 M	3 M	60 ml	6 ml
Glacial acetic acid	-	-	11.5 ml	1.15 ml
Water	-	-	28.5 ml	2.85 ml

Salt-saturated isopropanol

Component	stock conc	final conc.	/ 400 ml
Isopropanol	-	-	300 ml
NaCl	5 M	-	100 ml

A white precipitate forms and two phases appear. Use the top phase.

0.5 M EDTA pH 8.0 (2 liters)

- Add 336.2 g Na_2EDTA to about 1400 ml deionized water.
- Add 45 g NaOH (pH should move to 8.0 as ingredients dissolve)
- Adjust volume to 2000 ml with deionized water.

2 % gelatin (200 ml)

- Mix 4 g gelatin with 200 ml deionized water.
- Autoclave to sterilise.

40% glucose (1 liter)

- Heat 600 ml deionized water in 1 liter beaker on hot plate with stirring.
- Gradually add 400 g glucose.
- When glucose has completely dissolved pour into graduated cylinder and fill to 1000 ml with deionized water.
- Mix well and pour about 100 ml into each of several bottles.
- Autoclave to sterilise.

Lambda diluent (100 ml)

Mix: - 1 ml sterile 1 M Tris-HCl, pH 8
- 0.2 ml sterile 1 M $MgCl_2$
- 98.8 ml sterile H_2O

Lambda diluent + gelatin(100 ml)

Mix: - 1.0 ml sterile 1 M Tris-HCl pH 8
- 0.2 ml sterile 1 M $MgCl_2$
- 0.5 ml 2 % gelatin
- 98.3 ml sterile H_2O

LB (1 mM $MgCl_2$ 1 liter)

Mix: - 10 g Difco Bacto tryptone
- 5 g Difco Bacto yeast extract

- 5 g NaCl
- 1 ml 1 M $MgCl_2$
- Adjust volume to 1 liter with dH_2O
- Add 1.1 ml 1 N NaOH. (This should bring pH to 7.2.)
- Autoclave to sterilise.

2 X LB (1 mM $MgCl_2$ 1 liter)

Mix: - 20 g Difco Bacto tryptone
- 10 g Difco Bacto yeast extract
- 10 g NaCl
- 2 ml 1M $MgCl_2$
- Adjust volume to 1 liter with dH_2O.
- Add 2.2 ml 1 N NaOH. (This should bring pH to 7.2.)
- Prepare 5- 500 ml bottles with 200 ml LB each.
- Autoclave to sterilise.

LB plates

- Include 15 g agar with ingredients for 1 liter LB

OR:

- Thoroughly mix 200 ml sterile 2 X LBM with 200 ml sterile melted 3 % agar.
- Label and date the bottom of each plate to be poured.
- Pour approximately 30 ml LBM per plate (in a stack)
- Place a weight on the top plate to minimize to condensation and let sit overnight to solidify.
- Store plates in a plastic sleeve in the cold room.

LB top agar (400 ml)

- 200 ml LB
- 200 ml 1.6 % agar
- 400 µl 1 M $MgCl_2$
- Mix well and autoclave to sterilise (if ingredients used are not sterile)

LB+ Agar plates

Mix together the following pre-sterilised solutions:

- 200 ml 2 X LB
- 200 ml 3 % agar (melt agar in microwave first then add the other ingredients)
- 3 ml 40 % glucose
- 0.4 ml 75 mM calcium chloride
- 0.4 ml 4 mM ferric chloride
- Pour as described for LB plates.

LB+ top agar (1 liter)

- Prepare as LB+ agar plates except use 250 ml 1.6 % agar and 250 ml sterile deionized water.

1 M magnesium sulphate (1 liter)

- Mix 246.5 g $MgSO_4.7\ H_2O$ with deionized water
- Adjust volume to 1 liter with deionized water. Autoclave to sterilise.

1 M $MgCl_2$ (1liter)

- Dissolve 203.31 g $MgCl_2.6\ H_2O$ in deionized water.
- Adjust the volume to 1 liter.
- Autoclave to sterilise.

3 M potassium acetate

- Dissolve 29.4 g of potassium acetate in 100 ml of H_2O. Autoclave and store at room temperature.

5 M potassium acetate (200 ml)

- Mix 98.15 g potassium acetate with 50 ml deionized water.
- Adjust volume to 200 ml with deionized water

RNase stock solution

- Prepare a 10 mg/ml solution in 10 mM Tris-pH 7.5 15 mM NaCl.
- Heat to 100 ^{0}C for 15 minutes. Allow to cool slowly to room temperature.
- Add an equal volume of sterile 80 % glycerol.
- Store at –20 ^{0}C.

10 % SDS (100 ml)

- Add 10 g BDH brand "specially pure" sodium dodecyl sulphate to 50 ml deionized water
- Bring volume to 100 ml with deionized water.

2.5 X SDS-EDTA dye mix (10 ml)

Mix: - 1.25 g Ficoll 400
- 2.5 ml 10 % SDS
- 2.0 ml 0.5 M EDTA
- 0.5 ml 1 % bromophenol blue
- 0.5 ml 1 % xylene cyanol FF
- Adjust volume to 10 ml with dH_2O.
- Store at room temperature (SDS will precipitate at 4 ^{0}C)

10 X SMO (1 liter)

Mix: - 500 ml 1 M Tris H_2O pH 8.0
- 58.4 g sodium chloride
- 20.0 g $MgSO_4.7\ H_2O$
- Bring to 1 liter with deionized water.
- Autoclave to sterilise.

SM (1 liter)

- Mix 100 ml sterile 10 X SMO with 900 ml sterile deionized water.

SM+ (1 liter)

Mix 100 ml sterile 10 X SMO, 5 ml 2 % sterile gelatin and 900 ml sterile deionized water.

3 M sodium acetate (100 ml)

Mix 40.8 g sodium acetate with 50 ml deionized water. Bring volume to 100 ml with deionized water.

TE (1 liter)

Mix: - 500 ml deionized water

- 1.21 g Trizma base (final concentration of 10 mM)
- 0.34 g Na_2EDTA (final concentration of 1 mM).
- Bring to 1 liter with deionized water and pH to 7.5 by addition of 15-20 drops of concentrated HCl. Autoclave to sterilise

X-Gal plates

- Melt 200 ml 3 % agar in a 500 ml size bottle
- Pour 200 ml 2 X LB into the 3 % agar and mix well
- Add 0.8 ml dimethylformamide containing 16 mg X-Gal mix well
- Pour plates immediately; makes 12-16 plates