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**A BIOLOGICAL STUDY OF THE CELLULAR RESPONSE TO
HEAT STRESS IN THE SOUTH AFRICAN ALGA
*GRACILARIA GRACILIS***

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Thesis submitted in fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Molecular and Cell Biology, Faculty of Science,

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DECLARATION

This thesis was prepared under the supervision of Associate Professor Vernon Coyne and co-supervision of Doctor Suhail Rafudeen at the Department of Molecular and Cell Biology, University of Cape Town, South Africa. I hereby declare that this thesis, submitted in fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Molecular and Cell Biology at the University of Cape Town, is the result of my own investigations, apart from the referenced work of others.

Taryn Boom

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June 2012

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ABSTRACT

Gracilaria gracilis is a commercially important alga, previously harvested from the wild South African population in Saldanha Bay as a feed for marine organisms and as a source of commercially important agar. Since 1974 however, a number of sporadic population collapses has lead to the destruction of this once flourishing resource. After numerous failed attempts at re-establishing this industry, the need to develop an alternative farming strategy became evident. In order to devise such a solution, a better understanding of the tolerances and responses of this alga to the environmental parameters responsible for the downfall of the population is required. Although the exact reasons remain unclear, Jaffray *et al.*, 1997 have reported that increased water temperature in Saldanha Bay may be a contributing factor as the population collapses have repeatedly occurred during summer months. Thus the effect of heat stress on *G. gracilis* has been selected for this study.

As very little is known about the tolerance and response of *G. gracilis* to heat stress, this study aimed to create an initial platform from which future studies could be performed to gain a greater insight into the cellular response of *G. gracilis* to heat stress. The first objective of this study was thus to identify conditions required to induce a cellular response to heat stress in *G. gracilis*. Studies monitoring the growth and viability of this alga indicated that the upper limit of thermal tolerance of cultivated *G. gracilis* was 30 °C; and that a 24 hour time-course experiment, exposing the alga to 35 °C, was sufficient to induce a heat shock response. Thus, thallus segments heat shocked for up to 8 hours remained viable and grew demonstrating thermal tolerance; whereas after 18 hours of heat shock, the absence of growth and viability marked the onset of cell death.

Since the production and reduction of reactive oxygen species (ROS) is an early indicator of heat stress in plants and marine algae, the concentration of intracellular ROS, and in particular the removal of hydrogen peroxide, was investigated to validate the use of the devised experiment to induce heat stress in *G. gracilis*. The increase in intracellular ROS within the first 30 minutes of heat stress followed by a reduction in ROS concentrations after two hours validated the induction of heat stress. The decrease in the concentration of ROS in response to heat stress was in part attributed to an increase in antioxidant enzyme activity and the release of hydrogen peroxide from the thallus segments into the surrounding medium.

The second objective of the study was to gain an insight into the molecular pathways involved in maintaining thermal tolerance and the onset of cell death. Thus, a comparative proteomic approach was used to identify differentially expressed proteins in *G. gracilis* in response to heat stress. Two dimensional polyacrylamide gel electrophoresis was employed to compare the total soluble protein profiles of thallus segments heat shocked at 35 °C for various durations up to and including 24 hours. Of the 555 protein spots reproducibly detected on each of these 2D SDS-polyacrylamide gels, 76 proteins were up-

III

regulated and 38 proteins were down-regulated in response to heat stress. The ten most statistically significant differentially expressed proteins were selected for identification using tandem mass spectrometry. Seven of these proteins were successfully identified, their biological functions determined and used in conjunction with the observed physiological response of the alga to heat stress to construct a preliminary model of *G. gracilis*' heat stress response.

One of the seven identified proteins was indicated to be a 70 kDa heat shock protein. Thus, Hsp70 was selected as a potential molecular indicator of heat stress in *G. gracilis*, the third objective of this study. An endoplasmic reticular *Hsp70* was sequenced and the transcriptional up-regulation of the gene in response to heat stress was verified using real-time PCR. The results indicated that the ER Hsp70 is a potential molecular indicator of heat stress in *G. gracilis*, and could potentially be used to validate heat stress in this alga in future experimental studies.

ABBREVIATIONS

1D	one dimensional
2D	two dimensional
3'	three prime
5'	five prime
β	beta
Δ	delta
μ	specific growth rate
μl	microliter (s)
μmol	micromolar (s)
&	and
~	approximately
°C	degrees Celsius
%	percentage
®	trademark
A	
A	adenine
aa	amino acid (s)
Amp ^r	ampicillin resistance
AMPS	Ammonium persulphate
ANOVA	analysis of variance
ASC	sodium ascorbate
ASW	artificial seawater
ATP	adenosine triphosphate
B	
BLAST	basic local alignment search tool
bp	base pair (s)
BSA	bovine serum albumin
C	
C	cytosine
cDNA	complementary deoxyribonucleic acid
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
Co.	Company
Ct	threshold cycle
D	
DEPC	diethylpyrocarbonate
DFC	2', 7'-dichlorofluorescein
V	

dF/dt	rate of change of fluorescence over time
dH ₂ O	distilled water
DNA	deoxyribonucleic acid
DOI	digital object identifier
dpi	dots per inch
DTT	DL-Dithiothreitol
E	
E	efficiency
EDTA	ethylenediaminetetraacetic acid
ER	endoplasmic reticulum
<i>et al.</i>	et alia
EtBr	ethidium bromide
F	
FAO	Food and Agriculture Organization
fg	femtogram
FNR	ferredoxin-NADP ⁺ reductase
G	
g	gram (s)
G	guanine
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
gDNA	genomic deoxyribonucleic acid
GR	growth rate
GroEL	chaperonin GroEL
H	
h	hour (s)
HCl	hydrochloric acid
H ₂ DCF-DA	2', 7'-dichlorofluorescein diacetate
H ₂ O	water
H ₂ O ₂	hydrogen peroxide
Hsc70	70 kDa heat shock cognate
Hscs	heat shock cognates
Hsp	heat shock protein
Hsp70	70 kDa heat shock protein
HSP70	70 kDa heat shock protein multigene family
HSPs	heat shock protein multigene families
I	
I.D.	identification
IEF	isoelectric focusing
IgG	immunoglobulin G

Inc.	incorporated
IPG	immobilized pH gradient
IPTG	Isopropyl β -D-1-thiogalactopyranoside
ISBN	international standard serial number
ISSN	international standard book number
K	
kDa	kilodalton (s)
KOH	potassium hydroxide
L	
l	liter (s)
LA	Luria Bertani agar
LB	Luria Bertani broth
LC-MS/MS	tandem mass spectrometry
LPS	lipopolysaccharide
Ltd	limited
M	
M	molar (s)
m ⁻²	per square meter
mg	milligram (s)
min	minute (s)
ml	milliliters (s)
mM	millimolar (s)
MOPS	3-(N-morpholino) propanesulfonic
MOWSE	molecular weight search
MS BLAST	mass spectrometry-driven basic local alignment search tool
Mw	molecular weight
N	
NADP ⁺	β -Nicotinamide adenine dinucleotide phosphate sodium salt
NADPH	β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt
NCBI	National Centre for Biotechnology Information
ng	nanogram (s)
nm	nanometer (s)
No.	number
O	
ORF	open reading frame
OSPAR	Oil Spill Prevention, Administration and Response Fund

P

p.	page
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction (s)
pH	potential of hydrogen
pI	isoelectric point
PMSF	Phenylmethanesulfonyl fluoride
pp.	pages
PPP	pentose phosphate pathway

Q

qPCR	real-time PCR
------	---------------

R

RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RNase	ribonuclease
ROS	reactive oxygen species
rpm	revolutions per minute
RT	reverse transcriptase

S

s ⁻¹	per second
SDS	sodium dodecyl sulfate
SGR	specific growth rate

T

T	thymine
TAE	tris-acetate-EDTA buffer
TBS	tris-buffered saline
TBST	tris-buffered saline with Tween 20
TE	tris-EDTA buffer
TEMED	N, N, N', N'-Tetramethylethylenediamine
™	trademark
Tris	tris(hydroxymethyl)aminomethane
TSP	total soluble protein
TTC	2, 3, 5-triphenyltetrazolium chloride

U

U	unit (s)
ULB	urea lysis buffer
US\$	American dollar (s)
UV	ultraviolet

V
v volume
V volt (s)
Vh volt hour (s)
Vis visible

W
w weight

X
X-gal 5-bromo-4-chloro-3-indolyl β -D-galactopyranoside

University of Cape Town

CHAPTER 1
LITERATURE REVIEW
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1.1 Introduction

Aquaculture, defined as the cultivation of either marine or freshwater organisms, dates back to the 17th century (Beveridge and Little, 2002; Choppin and Sawhney, 2009; OSPAR, 2009). Over the last half a century, significant growth has occurred in this sector, increasing the cultivation from below 1 million tonnes in the 1950s to 66.7 million tonnes in 2006, which had an estimated value of US\$ 85.6 billion (FAO, 2009b). The Food and Agriculture Organization of the United Nations has suggested that aquaculture is possibly the fastest growing food production sector in the world and has further projected that given the growth in the global population, an additional 40 million tonnes of aquatic food will be required annually by 2030 (FAO, 2009a; OSPAR, 2009).

At present, nearly half of global aquaculture is obtained from mariculture, a specialised branch of aquaculture involving the cultivation of marine organisms for food and other products (FAO, 2009a; OSPAR, 2009). In this sector, seaweed aquaculture contributed 45.9 % of the total biomass. Although seaweed mariculture is comprised of approximately 220 cultivated species, alga from the six genera: *Laminaria* (kombu), *Undaria* (wakame), *Porphyra* (nori), *Eucheuma*, *Kappaphycus* and *Gracilaria* (this study) together contributed 94.8 % of the total algal biomass harvested for commercial use (Choppin and Sawhney, 2009; FAO, 2009b).

Gracilaria species are well known for their rapid growth rates and tolerance to a wide range of environmental conditions, and are thus well suited for commercial cultivation (Santelices and Doty, 1989; Armisen, 1995; Kain and Destombe, 1995; Polifrone *et al.*, 2006; Troell *et al.*, 2006). Although farmed for a number of commercial applications, *Gracilaria* is most importantly harvested for the production of agar, the most valuable

phycocolloid on the market (McHugh, 1991; Oliveira *et al.*, 2000; Polifrone *et al.*, 2006). Phycocolloids, algal gelatinous substances, are of great economic value as they serve as stabilising and gelling agents in a number of commercial products. The high quality agar isolated from *Gracilaria* species is predominantly used for the following purposes: as a gelling agent in foods such as sweets, jellies, mayonnaises and cream cheeses; as the principle component of bacteriological culture bases; and in the pharmaceutical industry as inert drug carriers to allow for the slow release of drugs into the body (Chapman and Chapman, 1980; Lee, 1999).

1.2 *Gracilaria gracilis*

Gracilaria gracilis is a member of the commercially valuable *Gracilaria* genus, which has been harvested in South Africa since the 1950s (Schroeder, 2001). This red alga consists of long, thin, branched, reddish-brown thalli (Figure 1.1) and is of economic interest to South Africa for use as fodder for marine organisms such as abalone and for the production of the phycocolloid agar (Woelkerling, 1990; Butterfield, 2000; Rothman *et al.*, 2009). Together with *Gracilaria chilensis*, *Gracilaria gracilis* is considered to be the most economically important species for agar production, based on the yield and quantity of the agar (Oliveira *et al.*, 2000).

Owing to developments in molecular technologies, the species previously identified as *G. confervoides* (Stackhouse) Greville in Italy and southern Africa and *G. verrucosa* (Hudson) Papenfuss in China, Japan, Argentina and Namibia (Bird *et al.*, 1994; Bird and Kain, 1995) has been reclassified by Steentoft *et al.* (1995) as the same organism and renamed *Gracilaria gracilis* (Stackhouse) M. Steentoft, L. M. Irvine & W. F. Farnham. Thus the current taxometric classification of this seaweed is as follows:

KINGDOM: Plantae
PHYLUM: Rhodophyta
CLASS: Florideophyceae
ORDER: Gracilariales
FAMILY: Gracilariaceae
GENUS: *Gracilaria*
SPECIES: *gracilis*



Figure 1.1 *Gracilaria gracilis* thalli.

1.3 Cultivation of *G gracilis*

Although *Gracilaria* species were traditionally harvested directly from natural populations, the increase in the demand for agar has resulted in the rapid depletion of natural stocks and the development of medium to large scale cultivation in a number of countries (Santelices and Doty, 1989; FAO, 2003; Polifrone *et al.*, 2006; Ye *et al.*, 2006; Choppin and Sawhney, 2009). Farming strategies using both spores and vegetative propagation in sheltered bays, ponds or tanks have been very successful in countries such as Chile, China, Indonesia and Vietnam, Taiwan, Norway and Namibia to name but a few (Dawes, 1995; Zemke-White and Ohno, 1999; FAO, 2003; Ye *et al.*, 2006).

In South Africa however, despite economic interest from local companies and research into suspended cultivation, the establishment of a successful farming strategy has been unsuccessful, resulting in the reliance on the natural stocks occurring in Saldanha Bay and Langebaan Lagoon (Figure 1.2; Anderson *et al.*, 1989; Jaffray and Coyne, 1996; Anderson *et*

al., 1998; Anderson *et al.*, 2001; Rothman *et al.*, 2009), the only area along the coastline which is capable of supporting the agar industry (Anderson *et al.*, 1989). At first, the natural population in these areas met the demand of the industry. In 1951, the first 20 tonnes of dry weight beach-cast seaweed was harvested and exported for the extraction of agar (Figure 1.3 A; Rothman *et al.*, 2009). The industry flourished for the following 22 years, with an average yield of between 500 and 1000 tonnes of dry weight per year facilitated by the development of infrastructure in the 1960s (Figure 1.3 B; Rothman *et al.*, 2009).



Figure 1.2 Images illustrating the position of the natural populations of *Gracilaria gracilis* harvested for commercial exploitation in South Africa. (A) South Africa (B) The positioning of Saldanha Bay and Langebaan 100 km north of Cape Town (C) Protective structure of the Saldanha Bay and Langebaan Lagoon area which harbours the natural population of *G. gracilis* (www.maps.google.co.za). The block in panel A and B, indicate the position of enlarged image depicted in the subsequent panel.

In 1974 however, the South African *G. gracilis* population collapsed after the construction of an ore-jetty and breakwater, which changed the layout of Saldanha Bay (Anderson *et al.*, 1989; Rotmann, 1990). Since then, a number of collapses have occurred, resulting in the contraction and eventual breakdown of this economy (Anderson *et al.*, 1996; Schroeder, 2001). As a result, South Africa no longer contributes to this sector (Schroeder, 2001), and until a suitable cultivation strategy for this alga has been developed, this industry will continue to be non-viable in South Africa (Anderson *et al.*, 1989; Rotmann, 1990).

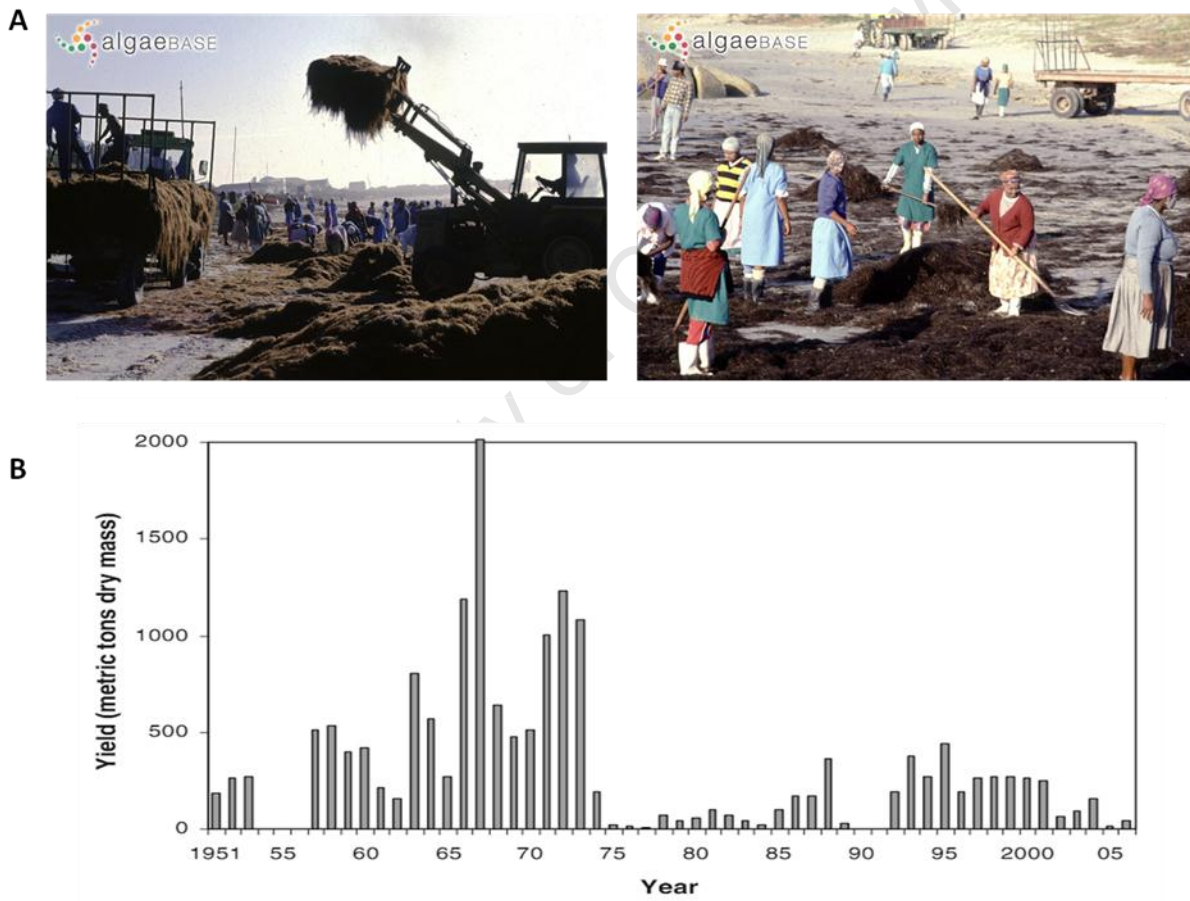


Figure 1.3 (A) Images of harvesting beach-cast *G. gracilis* along the Saldanha Bay coastline for use in the agar industry (Robert Anderson; www.algaebase.org). (B) The commercial yields of *Gracilaria* collected from Saldanha Bay (Rothman *et al.*, 2009).

In order to establish a farm-based cultivation strategy, it is essential to understand the tolerances and responses of *G. gracilis* to environmental parameters, especially those deemed responsible for the collapses (Raikar *et al.*, 2001; Ealand, 2011). Even though the exact constituents remain unclear (Anderson *et al.*, 1990; Anderson *et al.*, 1993; Anderson *et al.*, 1996), Jaffray *et al.* (1997) have suggested that an increase in water temperature may be one of the contributing factors. Therefore, this study was devised to obtain a better understanding of the effects of elevated temperature on *G. gracilis* by beginning to unravel the complex mechanisms involved in the maintenance of thermal tolerance and the pathways implicated in the onset of cell death.

1.4 Thermal tolerance of *G. gracilis*

The wild South African *G. gracilis* population inhabits the cool waters of Saldanha Bay and the Langebaan Lagoon along the west coastline of the country. These areas are characterised by cool temperatures, with reported mean monthly waters of 13 to 18 °C, and an annual mean temperature of 13 to 14 °C (Bolton, 1986; Engledow and Bolton, 1992; Bolton *et al.*, 2003). Previous research by Engledow and Bolton (1992) reported that this alga has a thermal tolerance for a wide range of temperatures ranging from between 5 to 25 °C, with the latter being optimal for growth of this species; whereas temperatures greater or equal to 30 °C exceeded the thermal tolerance of the species and resulted in cell death. Therefore the thermal tolerance of the wild population was determined to be 25 °C, above which the alga did not survive. The wide thermal tolerance range of this species is not unusual as *Gracilaria* species have been reported to be adaptable to a wide range of growth temperatures (Santelices and Doty, 1989; FAO, 2003).

1.5 Heat stress in macroalgae

Macroalgae are exposed to a wide variety of environmental stressors from short hourly bursts to long gradual seasonal variations (Burritt *et al.*, 2002). Seaweed in the intertidal zones, such as *G. gracilis*, must be able to withstand some of the harshest abiotic stressors, including heat (Todd and Lewis 1984; Rautenberger and Bischof, 2006), salinity (O'Neil and Prince, 1988), UV irradiation (Nepple and Bachofen, 1997; Hanelt *et al.*, 1997) and pollutants (Gledhill *et al.*, 1997) to name but a few.

Heat stress, defined as a stress resulting from an increase in environmental temperature, is one of the major abiotic stressors influencing the growth and productivity of plants. Failure of plants to effectively deal with heat stress has resulted in many incidences of severe developmental retardation, stunted growth, reduced quality and loss of crops worldwide (Suzuki and Mittler, 2006; Huang and Xu, 2008; Neilson *et al.*, 2010). This stress does not only affect terrestrial plants, but has also been reported to be one of the most important factors limiting the cultivation of marine algae (Lüning, 1990).

Unlike the ability of terrestrial plants to become physiologically inactive during periods of severe stress, and microalgal species to migrate to a more optimal environment in response to heat stress (Kübler and Davison, 1993; Sinha *et al.*, 2000), macroalgae have evolved to tolerate rapid fluctuations in temperature by developing a number of physiological adaptation and cellular responses (Knight and Knight, 2001; Ramanjulu and Bartels, 2002; Wang *et al.*, 2003; Fujita *et al.*, 2006; Qureshi *et al.*, 2007; Gupta *et al.*, 2010). Although very little is known about algal physiological adaptations to heat stress, the general cellular response of an organism to heat stress is highly conserved in all taxa (Jolly and Morimoto, 2000).

1.6 Cellular response to heat stress

The general cellular response to heat stress consists of a number of interconnected pathways that carefully balance the maintenance of thermal tolerance and the onset of cell death (Lindquist, 1986; Barry *et al.*, 1990; Jolly and Morimoto, 2000). Therefore although severe heat stress leads to irreversible cellular damage and cell death, sub lethal doses of heat stress induces a number of cellular responses which protects the cells against irreversible damage and facilitates the continuation of normal cellular and physiological activities, maintaining thermal tolerance (Jolly and Morimoto, 2000).

The general cellular response to heat stress, depicted schematically in Figure 1.4, is a conserved taxonomic response (Lindquist, 1986; Barry *et al.*, 1990; Jolly and Morimoto, 2000). In this model, exposure to elevated temperatures is hypothesised to cause a disruption in cellular components. Cellular damage to heat-labile components, such as the photosystem II in the mitochondria, causes a rapid accumulation of reactive oxygen species within the cell (ROS; Timperio *et al.*, 2008). If unsuccessfully managed by the antioxidant response, which consists of enzymatic (primary oxidative response) or non-enzymatic antioxidants (secondary antioxidant response; Mittler, 2002; Lesser, 2006), oxidative stress facilitates the onset of cell death by either inducing ROS-mediated apoptosis via the up-regulation of caspases (Gill and Tuteja, 2010) or causing extensive damage to the macromolecules of the cells by oxidising lipids, nucleic acids, polysaccharides and proteins, which in turn triggers cell death (Larkindale and Knight, 2002; Suzuki and Mittler, 2006; Gupta *et al.*, 2010).

However, the heat shock response is rapidly induced in response to proteotoxicity caused by the accumulation of misfolded proteins within the cells (Cadenas, 1989; Contreras *et al.*, 2005; Lesser, 2006). This pathway is characterised by the discontinued synthesis of most

cellular proteins and the up-regulation of a distinct set of heat shock proteins (HSPs; Gupta *et al.*, 2010). These proteins are hypothesised to function as a last resort to re-establish

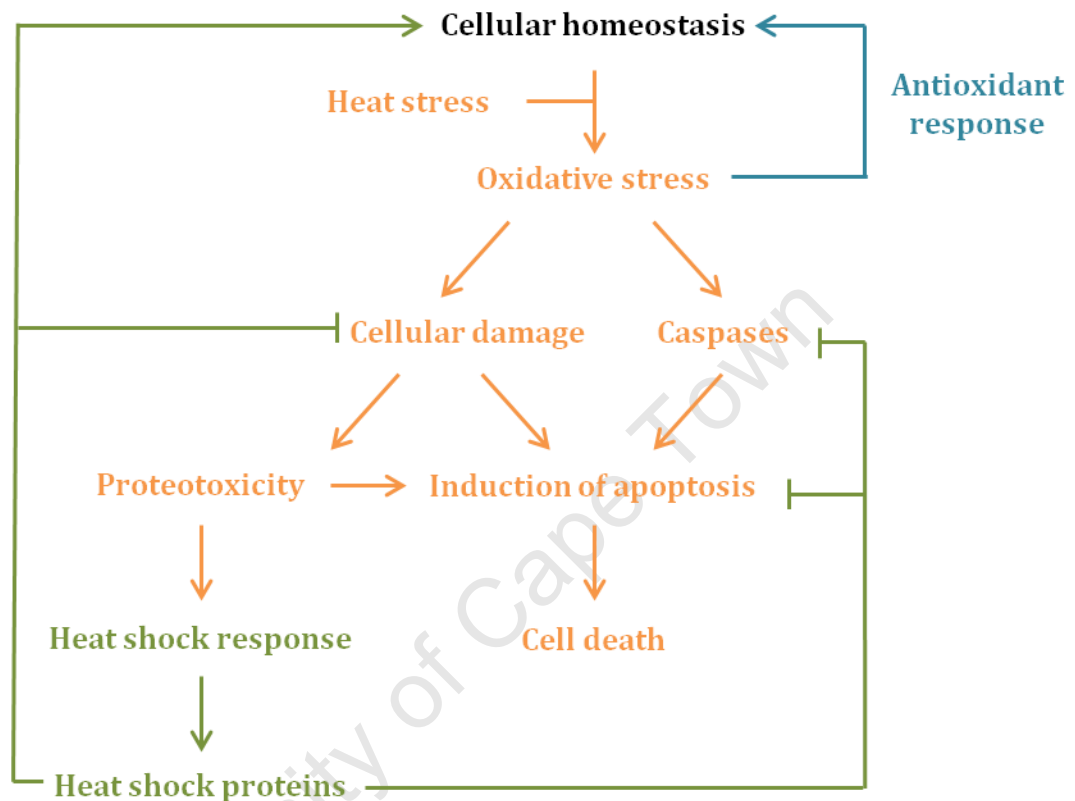


Figure 1.4 Schematic of the conserved cellular responses to heat stress, representing the pathways involved in the two possible outcomes of heat stress: the maintenance of thermal tolerance or the induction of apoptosis. If unsuccessfully managed, heat stress causes oxidative stress, which eventually results in cell death by either directly activating ROS-mediated apoptosis or inducing cellular damage which can in turn trigger apoptosis (orange). In response to heat stress, however, a number of general cellular responses are up-regulated to maintain the thermal tolerance of the cell for as long as possible. The antioxidant response (blue) decreases the oxidative stress of the cell and restores cellular homeostasis, whereas the heat shock response (green) reduces the proteotoxicity of the cell, which in turn decreases cellular damage, which helps to restore cellular homeostasis and inhibits the induction of apoptosis. The arrows and blunted lines represent induction and repression of the various pathways, respectively.

cellular homeostasis and delay the onset of cell death by: assisting in the correct folding of misfolded or denatured proteins to directly reduce cellular proteotoxicity, selectively degrading misfolded or denatured proteins which cannot be correctly refolded, and blocking apoptotic pathways at several levels to maintain thermal tolerance for as long as possible (Jolly and Morimoto, 2000; Timperio *et al.*, 2008; Gupta *et al.*, 2010). Therefore, if the stress intensifies or persists for a period of time greater than that which can be endured by the HSPs, the balance between thermal tolerance and cell death shifts, resulting in the induction of apoptosis and marks the onset of cell death.

1.7 Heat stress proteins

As discussed in Section 1.6, the synthesis of heat stress proteins (HSPs) are an essential part of the cellular response to heat stress in that they function to maintain cellular homeostasis once proteotoxicity has occurred (Lee and Schöffl, 1996; Jolly and Morimoto, 2000). This diverse set of proteins was originally observed by Ritossa (1962) who reported an increase in the expression of a number of proteins in the common fruit fly *Drosophila melanogaster* after exposure to heat stress. Although these proteins are still known as heat shock proteins, an increase in the expression of these proteins has been reported after exposure to a number of non-heat related stresses (Cooper and Ho, 1983; Wang *et al.*, 2004).

A number of different types of heat shock proteins have been identified, and are classified based on their approximate molecular weight, cellular localisation and expression patterns. The first method of classification separates the proteins into five heat shock protein families (denoted using capital letters; HSP) based on the molecular weight of the proteins, namely: HSP100s, HSP90s, HSP70s, HSP60s and small HSPs (15 to 42 kDa; Vierling, 1991;

Wang *et al.*, 2004; Qureshi *et al.*, 2007; Al-Whaibi, 2010). Each of these protein families has a slightly different function, but they all work together to re-establish cellular homeostasis, by assisting in the stabilisation, folding, translocation and degradation of cellular proteins (Wang *et al.*, 2004). Within each family, the individual proteins are classified according to their expression patterns and cellular localisation. Proteins that are produced rapidly in response to heat stress are known as heat shock protein (Hsp) whereas proteins whose expression patterns remain relatively constant in response to this stress are known as heat shock cognates (Hscs; Reddy *et al.*, 2010). Although the Hscs are not rapidly synthesised during heat stress, these proteins contain high sequence similarity to the Hsps and belong to the same heat shock family, however they function as molecular chaperones under non-stressed conditions. The localisation of these proteins can be determined using computational tools which identifies the probable subcellular location from which the proteins function. Heat shock proteins have been reported to reside within a number of cellular compartments, including the cytoplasm, endoplasmic reticulum, chloroplast and mitochondria (Miernyk, 1999).

1.8 Heat shock proteins as potential molecular indicators of heat stress

Molecular indicators, sometimes referred to as bio-indicators or biomarkers, are defined as a biological property, biochemical feature, or facet that can be used to measure the presence or progression of a stress or the effects of a disease treatment (Xuefeng and Sylvester, 2011). Thus, based on the direct correlation between the level of heat stress and the amount of HSPs synthesised, a central idea has emerged that heat shock protein levels may be an effective indicator of heat stress in various organisms (Lee and Schöffl, 1996; Schöffl *et al.*, 1998; Timperio *et al.*, 2008; Gupta *et al.*, 2010). Although a number of HSPs have already been reported as potential molecular indicators of various stressors (Gupta *et*

al., 2010), the 70 kDa heat shock proteins (Hsp70s) have been selected by convention as the molecular indicators of heat stress (Mirkes *et al.*, 1994; Ryan and Hightower, 1996). This has been attributed to the conserved nature of Hsp70s, the rapid induction of these proteins in response to heat stress and the correlation of Hsp70 expression with the maintenance of thermal tolerance in an organism in response to heat stress (Gupta *et al.*, 2010).

1.9 Proteomics

In the past, genomic and transcriptomic studies were performed to investigate the stress response of an organism. The static nature of the genome and the unreliable correlation between transcript abundance and protein levels has led to the development of proteomics, which investigates the entire protein complement of the cell (Gypi *et al.*, 1999; Jung *et al.*, 2000; Cho, 2007). Since the protein complement of a cell is constantly changing in response to stimuli, proteomics has become a powerful tool for identifying key proteins and molecular pathways involved in the stress response of an organism (Anderson and Anderson, 1996; Shepard *et al.*, 2000; Haberkorn *et al.*, 2002; Brumbarova *et al.*, 2008; Huang and Xu, 2008). By identifying the proteins and pathways involved, a hypothesis can thus be constructed to interpret the mechanisms resulting in the tolerance or intolerance of the organism to a stress (Abdalla, 2009).

At present, a number of proteomic techniques are available that can be used to investigate the identity, expression, structure, complexes, pathways and networks of proteins involved in a stress response (Cho, 2007). To gain a better understanding of an organism's phenotype in response to a particular stressor, two dimensional gel electrophoresis

coupled with tandem mass spectrometry and bioinformatics is routinely employed (Figure 1.5; Haberkorn *et al.*, 2002; Cho, 2007; Moresco *et al.*, 2008).

Two dimensional gel electrophoresis is a gel-based technique which enables scientists to separate out complex mixtures of proteins on a polyacrylamide gel in two dimensions according to their isoelectric points and molecular weights (O'Farrel, 1975). These proteins can be visualised using staining techniques and the relative abundance of each protein spot determined using 2D analysis software (Moresco *et al.*, 2008). After analysis, the differentially expressed protein spots can be digested with trypsin, removed from the gel and analysed using tandem mass spectrometry.

Tandem mass spectrometry is an effective method for identifying proteins with a high level of confidence. The technique uses two mass spectrometers, connected in series to determine the amino acid sequence of tryptic digested peptide fragments (de Hoffmann, 1996; Shevchenko *et al.*, 1997; Graves and Haystead, 2003). Electrospray ionisation tandem mass spectrometry directly introduces fragmented peptides into the first spectrophotometer as a fine mist of charged droplets (Graves and Haystead, 2003). After which, the peptides are ionised and separated based on the mass-to-charge ratio of the primary ions. These ions are then further selected, fragmented and detected after passing through the second mass spectrometer. The peptide sequences obtained are identified using database searching, and used to identify the fragmented protein differentially expressed on the 2D polyacrylamide gel. The identified proteins can be examined using bioinformatic techniques to determine their biological function and proposed roles in the stress response (Rose *et al.*, 2004).

Although highly effective as a power tool for protein analysis, there are a number of disadvantages associated with utilising 2D gel electrophoresis coupled with tandem mass

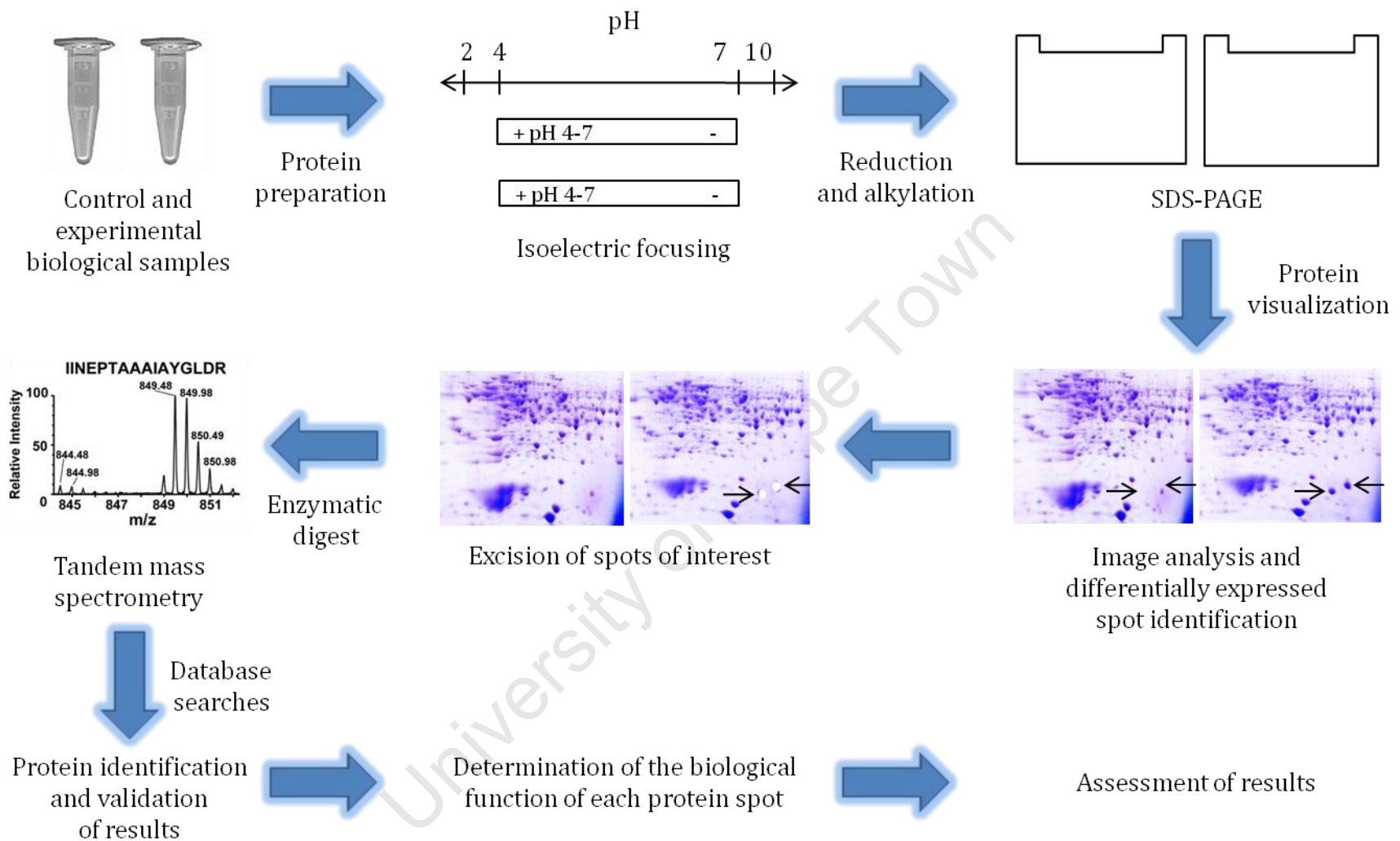


Figure 1.5 Schematic of a proteomic approach to identify differentially expressed proteins in response to a stressor with the use of two dimensional gel electrophoresis coupled with tandem mass spectrometry and database searching.

spectrometry that should be taken into account. This is an expensive, laborious and technically challenging technique (Gygi *et al.*, 1999). Only proteins that can be solubilised and separated on a 2D polyacrylamide gel can be accurately examined (Rose *et al.*, 2004). Although proteins from poorly studied organisms can still be identified if there is sufficient sequence homology between the protein of interest and a homologue from another species, it should be noted that this technique is less optimal for non-model organisms for which there is little sequence information (Jorrín-Novo *et al.*, 2009).

1.10 Concluding remarks and aim of study

Gracilaria gracilis is a commercially important alga harvested from wild South African populations in Saldanha Bay for the production of the economically valuable phycocolloid agar and for use as feed for marine organisms (Woelkerling, 1990; Butterfield, 2000; Rothman *et al.*, 2009). Since 1974, sporadic collapses of this alga have led to the destruction of this once flourishing resource. After numerous failed attempts to restore the feasibility of harvesting this crop from wild populations, a need for the development of an alternative cultivation strategy was identified (Schroeder, 2001). In order to devise a suitable solution, further knowledge is required to understand the tolerances and responses of *G. gracilis* to the environmental parameters responsible for its demise (Raikar *et al.*, 2001; Ealand, 2011). Since the population collapses have repeatedly occurred during the summer months, it was reported that the elevation of water temperature in Saldanha Bay may have played a role in the subsidence (Jaffray *et al.*, 1997). Therefore, the tolerance and response of *G. gracilis* to elevated temperature was selected for this study.

As very little is known about the cellular response to heat stress in this algal species, the aim of this study was to create an initial platform from which further investigations could

be performed to better understand the effects of heat stress on *G. gracilis* and the mechanisms involved in thermal tolerance and the induction of cell death as a response to heat stress. In doing so, the three main objectives of this study were to (i) determine the conditions required to induce a heat shock response in *G. gracilis* (ii) gain insight into the protein pathways involved in the process of maintaining thermal tolerance and the induction of cell death in *G. gracilis* using a 2D SDS-PAGE proteomic approach and (iii) identify a potential molecular indicator of heat stress in *G. gracilis*.

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CHAPTER 2

INDUCTION OF A HEAT SHOCK RESPONSE IN THE CULTIVATED SOUTH AFRICAN ALGA *GRACILARIA GRACILIS*

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2.1 Summary

In this chapter, the conditions required to induce a cellular response to heat stress in cultivated *Gracilaria gracilis* (Stackhouse) Steentoft, Irvine *et* Farnham were investigated. Studies monitoring the growth and viability of this alga indicated that the upper limit of thermal tolerance of cultivated *G. gracilis* was 30 °C; and that a 24 hour time-course experiment, exposing the alga to 35 °C, was sufficient to induce a heat shock response. Thus, thallus segments heat shocked for up to 8 hours remained viable and grew implying thermal tolerance; whereas after 18 hours of heat shock, the absence of growth and viability marked the onset of cell death.

Since the production and reduction of reactive oxygen species (ROS) is an early indicator of heat stress in plants and marine algae (Schrek and Baeuerle, 1991; Rijstenbil *et al.*, 1994; Mittler, 2002; Nedelcu *et al.*, 2004; Weinberger *et al.*, 2005; Lesser, 2006; Zuppini *et al.*, 2007), the concentration of intracellular ROS and in particular the removal of hydrogen peroxide was investigated to validate that 35 °C induced heat stress in cultivated *G. gracilis*. The concentration of intracellular ROS increased within the first 30 minutes, remained elevated for the first hour, and returned to basal levels after 2 hours. The decrease in intracellular H₂O₂ was determined by monitoring the change in three antioxidant enzymes (catalase, ascorbate peroxidase and glutathione peroxidase) that break down intracellular H₂O₂ into water, and changes in extracellular ROS released from thalli during heat stress (Lamb and Dixon, 1997; Weinberger *et al.*, 1999; Lesser, 2006). Increased antioxidant enzyme activity after half an hour of heat stress, and the accumulation of H₂O₂ in the surrounding medium from 6 hours after the start of the experiment, validated the induction of the heat stress response in this alga.

2.2 Introduction

Gracilaria gracilis is a commercially valuable alga, previously harvested in South Africa from wild populations in Saldanha Bay. Since 1974, sporadic collapses in these populations, occurring during summer, has led to the destruction of this once flourishing resource and emphasised the need for the development of a suitable cultivation strategy for this alga (Anderson *et al.*, 1989; Rotmann, 1990). In order to do so, it is essential to understand the tolerances and responses of *G. gracilis* to environmental parameters, especially with regard to factors responsible for the collapses (Raikar *et al.*, 2001; Ealand, 2011). Even though these exact constituents remain unclear (Anderson *et al.*, 1990; Anderson *et al.*, 1993; Anderson *et al.*, 1996), Jaffray *et al.* (1997) have suggested that an increase in water temperature may be one of the contributing factors. Thus the investigation of this alga's tolerance and response to elevated temperature was selected for this study.

Engledow and Bolton (1992) laid the foundation for studies in this research area by investigating the thermal tolerance of the wild *G. gracilis* population. However, the wild *G. gracilis* populations were deemed an unsuitable source of *G. gracilis* for this study due to the discovery of a phenotypically indistinguishable algal species, *Gracilariopsis longissima*, which recently invaded the habitat and is only distinguishable from *G. gracilis* using molecular techniques (Ealand, 2011). Therefore, *G. gracilis* was obtained from Irvin & Johnson Abalone Culture Division, South Africa for this study. This source of alga has been separated from the wild population for at least ten years (personal communication, Irvin & Johnson Abalone Culture Division), during which it has been acclimatised to a mean temperature of 2.5 °C higher than that recorded for the wild population (Engledow and Bolton, 1992). Thus, the first objective of this study was to investigate the upper limit of thermal tolerance of this cultivated *G. gracilis* and to compare these findings to that of the wild population (Engledow and Bolton, 1992).

After determining the thermal tolerance of cultivated *G. gracilis*, the second objective was to investigate the conditions required to induce a heat shock response in this species, with the intention of distinguishing between thermal tolerance and the induction of cell death. As intertidal algae have been reported to tolerate limited exposure to temperatures greater than that of their thermal limit without physiological damage (Kübler and Davison, 1993; Burritt *et al.*, 2002), 35 °C was selected for this study, which is higher than the thermal tolerance of cultivated *G. gracilis*. Thus this objective investigated the duration of heat stress at 35 °C required to observe the stress response of the species so as to include both the initial period of thermal tolerance followed by the onset of cell death.

The third and final objective of this chapter was to validate that exposure to 35 °C induced a heat shock response in the alga. Since the production and reduction of reactive oxygen species (ROS) is an early indicator of heat stress in plants and marine algae (Schrek and Baeuerle, 1991; Rijstenbil *et al.*, 1994; Mittler, 2002; Nedelcu *et al.*, 2004; Weinberger *et al.*, 2005; Lesser, 2006; Zuppini *et al.*, 2007), the concentration of ROS and the reduction of hydrogen peroxide by three antioxidant enzymes or the removal of H₂O₂ out into the surrounding media (Lamb and Dixon, 1997; Weinberger *et al.*, 1999; Lesser, 2006), was investigated to validate that 35 °C induced heat stress in cultivated *G. gracilis*.

2.3 Materials and methods

All media and solutions used in this study are listed in Appendix A.

Experimental data was obtained from three biological repeats (each consisting of three technical repeats) unless otherwise stated. In the context of this study, technical and biological repeats were defined as data obtained from algal samples exposed to the same environmental conditions but acquired from either the same or triplicate experiments, respectively.

2.3.1 Preparation of cultivated *G. gracilis*

2.3.1.1 Sample acquisition and maintenance conditions

Gracilaria gracilis was obtained every fortnight from the Irvin & Johnson Abalone Culture Division, Danger Point, South Africa. Healthy thalli were selected and briefly rinsed in distilled water to remove visible epiphytes. Cleaned thallus segments were maintained at 15 °C for a minimum of 2 weeks prior to acclimatisation (Section 2.3.1.2) in 45 l tanks fitted with a flow-through system filtering seawater at a rate of 1 l/min. Water movement was achieved by pumping compressed air through plastic airlines and the alga was illuminated by cool white fluorescent tubes at $\sim 45 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 16-hour light: 8-hour dark cycling photoperiod. During this maintenance period, both the alga and the tanks were cleaned twice a week with filtered seawater to remove any accumulated sedimentation.

2.3.1.2 Acclimatisation conditions

Post maintenance (Section 2.3.1.1), *G. gracilis* was acclimatised at 15 °C for 10 days in an Adaptis A350 Multi-Application Growth Chamber (Convion®). Thus, similarly

structured thalli (included an equivalent number of apical segments) were incubated in conical flasks at a ratio of 1 g thalli: 200 ml precooled artificial seawater (ASW; Appendix A.1.1) supplemented with PES-enriched seawater medium to achieve a ½ strength growth medium (Appendix A.2.2.1; enriched ASW). Sufficient water circulation was obtained by pumping compressed air through plastic tubing and the seaweed was illuminated at $\sim 45 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ by cool white fluorescent tubes with a 16-hour light: 8-hour dark cycling photoperiod. Every 2-3 days, the alga was rinsed briefly in enriched ASW and transferred to flasks containing freshly prepared media.

2.3.2 Heat shock experiments

Heat shock experiments were performed in temperature controlled horizontal orbital shaker incubators (Dynamic & Evolution International Corporation). When starting an experiment, *G. gracilis* was transferred directly from the acclimatisation conditions at 15 °C (Section 2.3.1.2) to enriched ASW at the appropriate temperature at a ratio of 1 g thalli: 200 ml enriched ASW. Flasks were shaken at 75 rpm to ensure adequate water movement and illumination was provided at $\sim 45 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ by cool white light with a cycling 16-hour light: 8-hour dark photoperiod. Post heat shock, thalli were removed from the flasks, and processed immediately as metabolically active tissue or frozen in liquid nitrogen and stored at -80 °C until required.

2.3.3 Growth experiments

In this study, two individual growth experiments were conducted. First, to investigate the effect of a range of incubation temperatures on the growth rate of cultivated *G. gracilis*, the fresh weight of the thallus segments was monitored over a 21 day incubation period at 15 (control), 20, 25, 30, 35 and 40 °C. After verifying 35 °C as a suitable temperature to induce a heat shock response in *G. gracilis*, a second experiment was devised to monitor the effect of the duration of heat stress on the growth rate of the alga. Thus *G. gracilis* was heat shocked for 0 (omission of heat stress, control), 0.5, 1, 2,

4, 6, 8, 18 and 24 hours (various durations up to and including 24 hours) at 35 °C (Section 2.3.2) prior to monitoring the growth of the alga at 15 °C for 21 days.

Both of these growth experiments were performed in Adaptis A350 Multi-Application Growth Chambers (Convicon®) at the appropriate temperature and for the required time-course. In each experiment, 0.2 ±0.01 g inocula of *G. gracilis* were incubated in 800 ml enriched ASW under identical incubation conditions as described in Section 2.3.1.2, unless otherwise stated. Every 2-3 days *G. gracilis* was rinsed briefly in enriched ASW, blotted dry and intact thallus fragments weighed prior to being transferred to freshly prepared flasks.

The specific growth rate (μ), indicating the average change in the fresh weight of cultivated *G. gracilis* per day, over the 21 day growth period, was calculated using the following equation:

$$\mu = [100 (\ln (N_t/N_o))]/t$$

Where N_t = fresh weight of thalli at end of the growth trial

N_o = fresh weight of thalli at the start of the growth trial

t = number of days of the growth experiment

A decrease in the fresh weight of the tissue over the 21 day growth period, observed in response to cell death, was recorded as a zero specific growth rate.

2.3.4 Viability assays

After the growth experiments (Section 2.3.3), the viability of the thallus segments was assessed to (i) determine the effect of various temperatures on the viability of *G. gracilis* (first growth experiment) and to (ii) investigate the effect of the duration of heat shock at 35 °C on *G. gracilis* viability (second growth experiment). This assay was adapted from Nam *et al.* (1998) and performed as described in Appendix B1. Where thallus segments weighed less than 1.0 ± 0.01 g required for the assay, tissue from the technical repeats was pooled together to make up the necessary weight. Thus, for these time points, only a single technical repeat was used for statistical analysis.

2.3.5 Quantitation of intracellular reactive oxygen species

The concentration of intracellular ROS was monitored for thallus incubated at 15 (control) and 35 °C (experiment) for various durations up to and including 24 hours (Section 2.3.2). Directly after each predetermined incubation period, 1.0 ± 0.01 g thallus segments were sampled at each temperature, blotted dry and stored at -80 °C for a maximum of 48 hours prior to processing. The concentration of intracellular ROS was investigated for each sample as described in Appendix B.2 [adapted from Contreras *et al.* (2005)].

2.3.6 Quantitation of extracellular hydrogen peroxide

The accumulation of extracellular hydrogen peroxide (H_2O_2) released in response to heat stress was determined by quantifying the concentration of H_2O_2 in the surrounding medium. Thus 1.0 ± 0.01 g thallus segments were either incubated in 200 ml enriched ASW (Appendix A.2.2.1) at 15 or 35 °C for a 24 hour time-course (Sections 2.3.2). Directly after 0, 0.5, 1, 2, 4, 6, 8, 18 and 24 hours of heat shock, 1 ml of medium was

removed from each flask and immediately analysed for the accumulation of H₂O₂ using the method adapted from Zuppini *et al.* (2007) and described in Appendix B.3.

2.3.7 Investigation of antioxidant enzyme activities

The activities of three antioxidant enzymes (catalase, ascorbate peroxidase and glutathione) were monitored from the same thallus segments. Thus 2.0 ±0.01 g of *G. gracilis* thallus segments were incubated at 15 or 35 °C for various durations up to and including 24 hours (Sections 2.3.2). After the completion of each incubation period, a thallus sample was removed from each culture. The samples were separated into 0.5 ±0.01 g aliquots and stored at -80 °C until required. Within 96 hours of freezing, all three of the antioxidant enzyme activities were analysed as described below:

2.3.7.1 Determination of catalase activity

Catalase activity was analysed according to the protocol described in Appendix B4, which was adapted from Aebi (1984).

2.3.7.2 Examination of ascorbate peroxidase activity

Ascorbate peroxidase activity was examined as described in Appendix B5, a method modified from Chen and Asada (1989).

2.3.7.3 Assessment of glutathione peroxidase activity

Glutathione peroxidase activity was assessed in accordance with Appendix B6, adjusted from Ursini *et al.* (1985).

2.3.8 Statistical analyses

Descriptive statistics and statistical analyses were performed using SigmaStat for Windows (version 3.10; Systat Software Inc.). Where a single control sample was compared to multiple experimental samples, a one-way analysis of variance (ANOVA) was deemed appropriate to examine the significance of each experimental sample relative to the control sample. However, the student's *t*-test was selected to investigate the significance of the experimental sample relative to the corresponding control sample where a control sample was included for every experimental sample. Data sets that were not normally distributed or that displayed unequal variances were transformed to meet the requirements for parametric statistical testing. Where data could not be adequately transformed, a non-parametric statistical test was conducted instead.

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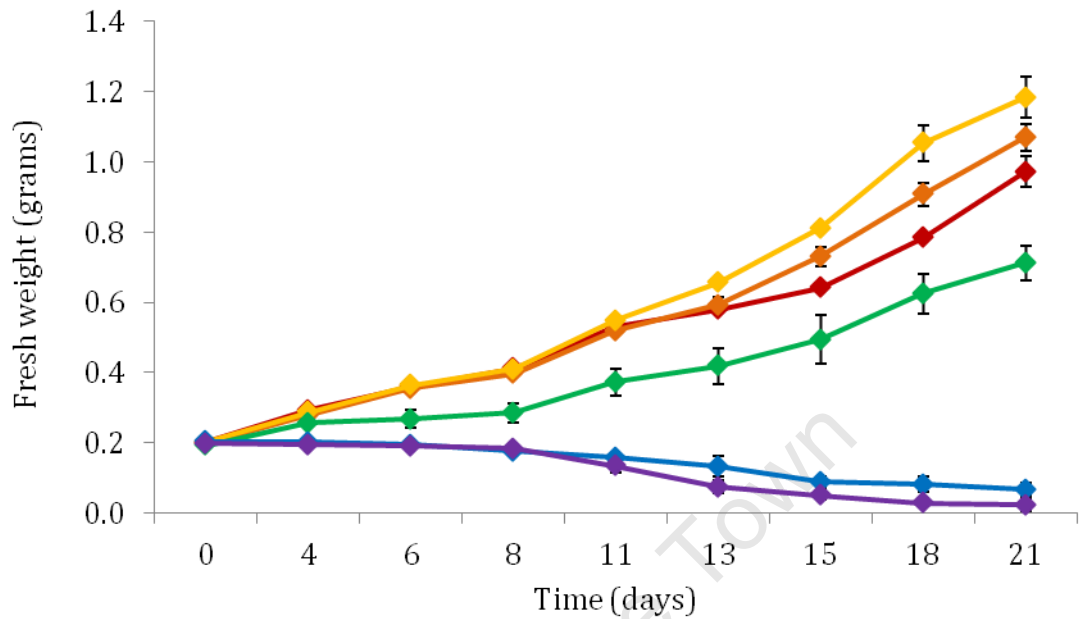
2.4 Results

2.4.1 Determination of the upper limit of thermal tolerance in cultivated *G. gracilis*

The upper limit of thermal tolerance in cultivated *G. gracilis* was investigated by determining the highest temperature at which both growth and viability of the alga were not adversely affected. Thus, *G. gracilis* was incubated at a range of temperatures between 15 and 40 °C for 21 days. Throughout this period, the fresh weight of the thallus segments (Figure 2.1 A) was monitored, and the specific growth rate (SGR; Figure 2.1 B) and viability (Figure 2.2) of the alga was determined at the close of the experiment.

G. gracilis incubated between 15 and 30 °C remained viable and increased in fresh weight over the 21 day incubation period. When compared to the control (15 °C; specific growth rate of 7.4), thallus segments incubated at 20 and 25 °C maintained increased SGRs of 8.0 and 8.4, respectively, whereas thalli incubated at 30 °C maintained a statistically significant lower SGR of 6.1. Although the SGR varied in *G. gracilis* cultured at different temperatures, the observation that *G. gracilis* remained viable and continued to grow at these temperatures indicated that the alga tolerates temperatures between 15 and 30 °C. Alga incubated at 35 and 40 °C did not survive the 21 day incubation period, gradually reducing in biomass throughout this growth study. These results implied that cultivated *G. gracilis* could not tolerate temperatures ≥ 35 °C, and further deduced that the upper limit of thermal tolerance tested for this alga was 30 °C.

A



B

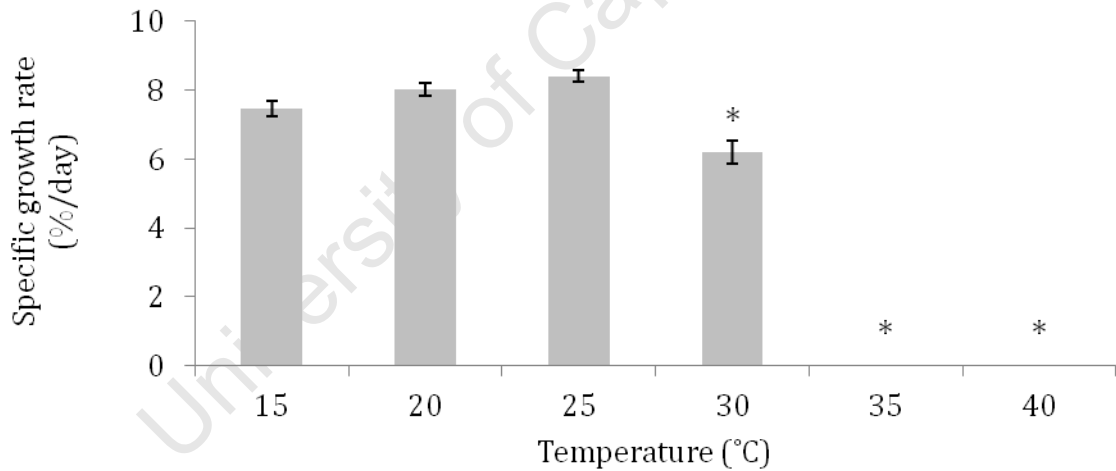


Figure 2.1 (A) The fresh weight and (B) specific growth rate of cultivated *G. gracilis* incubated at 15 (—◆—), 20 (—◆—), 25 (—◆—), 30 (—◆—), 35 (—◆—) and 40 °C (—◆—) for 21 days. Non-parametric Student-Newman-Keuls test detected statistically significant differences in the specific growth rate of *G. gracilis* between the control (15 °C) and experimental samples ($P < 0.05$, denoted by asterisks '*'). Data represents the mean \pm standard error from three biological repeats. Error bars not visible are overlapped by the data points.

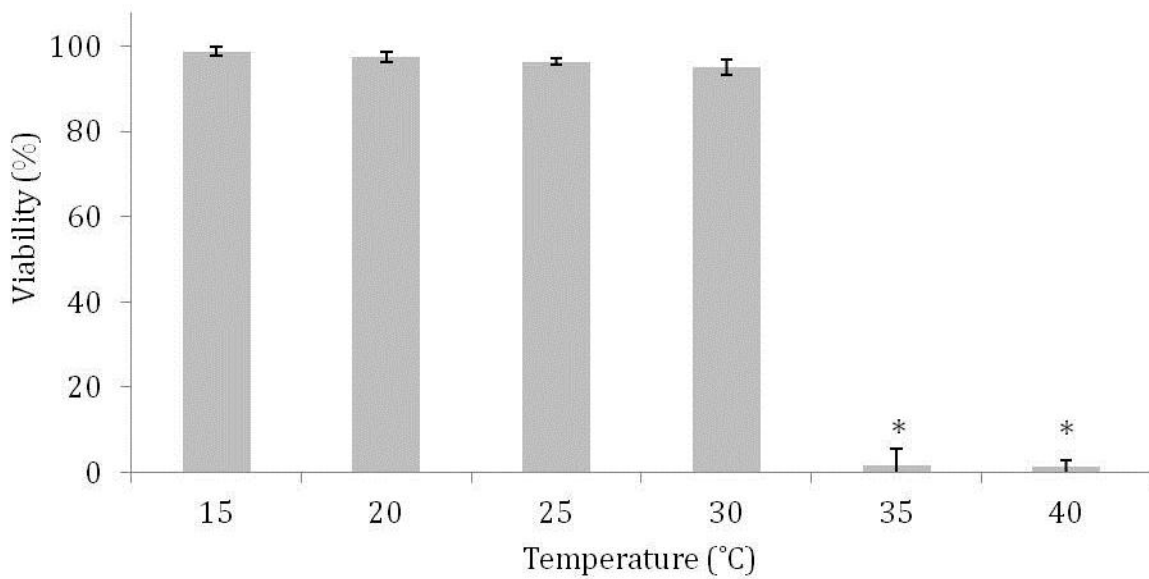


Figure 2.2 Viability of cultivated *G. gracilis* exposed to a range of temperatures for 21 days. Parametric one-way ANOVA detected statistically significant differences in the viability of *G. gracilis* between the control (15 °C) and experimental samples ($P < 0.001$, denoted by asterisks '*'). Data represents the mean \pm standard error from three biological repeats.

2.4.2 Investigation of the conditions required to induce a heat shock response in cultivated *G. gracilis*

Intertidal algae have been reported to tolerate temperatures higher than that of the thermal limit of a species for limited periods without physiologically harming the algae (Kübler and Davison, 1993; Burritt *et al.*, 2002). Therefore in this study, an incubation temperature of 35 °C, which is higher than that of the thermal tolerance of cultivated *G. gracilis*, was selected to induce a heat shock response in the agarophyte (Section 2.4.1). To determine a suitable time-course, which spans a period of thermal tolerance and the onset of cell death in this alga, thallus segments were heat shocked for various durations up to and including 24 hours (Section 2.3.2), before monitoring the effect of the heat shock on the thalli for 21 days at 15 °C (Section 2.3.3). During this recovery period, the fresh weight of the thallus segments (Figure 2.3 A) was monitored; and at

the close of the experiment the specific growth rate (SGR; Figure 2.3 B) and viability (Figure 2.4) of the alga was examined.

Cultivated *G. gracilis* heat shocked for ≤ 8 hours tolerated the heat shock in that the alga remained viable and grew over the 21 day incubation period. When compared to control samples [*G. gracilis* omitting heat shock (0 hours); SGR of 7.8], thallus segments heat shocked for between 0.5 to 4 hours maintained significantly faster SGRs of between 8.6 and 9.3, whereas thalli heat shocked for 6 and 8 hours had slower SGRs of 7.3 and 5.3. Even though the SGRs of the thallus segments varied in response to the duration of heat shock, the continued viability and growth of these thalli indicated that cultivated *G. gracilis* could tolerate heat shock at 35 °C for up to 8 hours. In contrast, thalli heat shocked for ≥ 18 hours did not survive, slowly reducing in biomass throughout the recovery period. These results inferred that a 24 hour heat shock experiment at 35 °C would sufficiently induce periods of thermal tolerance and the onset of cell death in cultivated *G. gracilis*.

2.4.3 Validation of the heat shock experiment

The production of an oxidative burst and the reduction of intracellular hydrogen peroxide (H_2O_2) in cultivated *G. gracilis* were investigated to validate that this alga was heat shocked when exposed to 35 °C for 24 hours. A rapid and transient oxidative burst was induced in thallus segments exposed to 35 °C (Figure 2.5). The concentration of ROS increased after half an hour of exposure, remained elevated for 30 minutes, and then decreased back towards basal levels demonstrating the induction of heat stress. Further reduction in the concentration of ROS after 18 hours of heat stress was hypothesised to infer a loss of membrane stability, facilitating the leaching of cellular content, including ROS, out into the surrounding media.

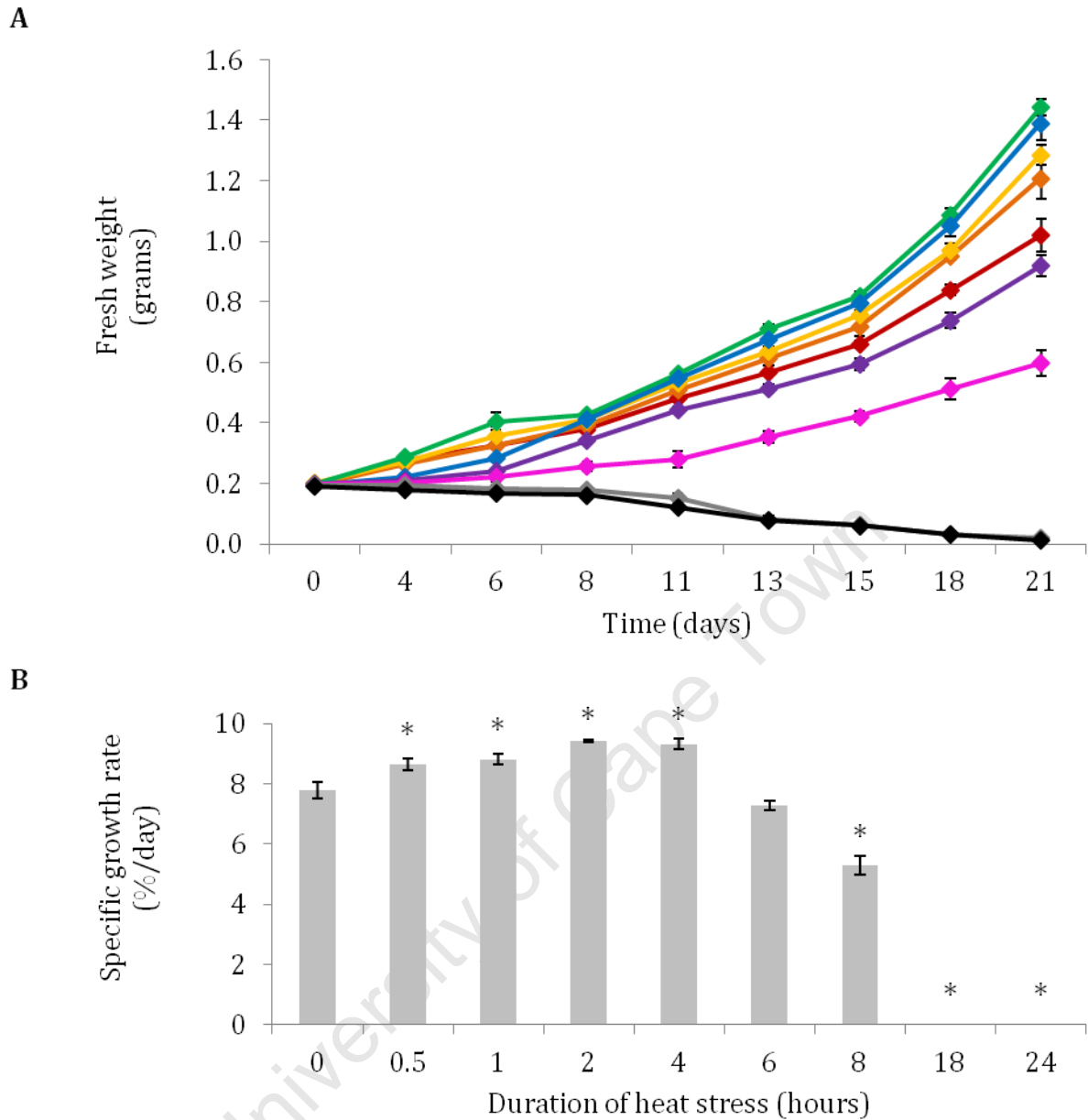


Figure 2.3 (A) The fresh weight and (B) specific growth rate of cultivated *G. gracilis* heat stressed at 35 °C for 0(—), 0.5(—), 1(—), 2(—), 4(—), 6(—), 8(—), 18(—) and 24(—) hours. Parametric one-way ANOVA detected statistically significant differences in the specific growth rate of *G. gracilis* between the control (0 hours) and experimental samples (denoted by asterisks '*'). At 0.5 hours the statistical difference was at $P < 0.05$, and between 1-4 and 8-24 hours the statistical difference was at $P < 0.001$. Data represents the mean \pm standard error from three biological repeats.

The reduction of intracellular H₂O₂ was further assessed using two indirect methods; by initially monitoring the concentration of H₂O₂ released from thallus segments out into the surrounding media (Weinberger *et al.*, 1999; Lesser, 2006), and by subsequently investigating the rate at which three preselected antioxidant enzymes (catalase, ascorbate peroxidase and glutathione peroxidase) reduce intracellular H₂O₂ to water (Lamb and Dixon, 1997; Weinberger *et al.*, 1999).

The concentration of H₂O₂ began to accumulate in the medium surrounding cultivated *G. gracilis* after 6 hours of exposure to 35 °C (Figure 2.6), whereas the trends in the activities of the antioxidant enzymes increased within the first half an hour of exposure (Figure 2.7). Catalase activity remained elevated for the first hour of heat stress, before

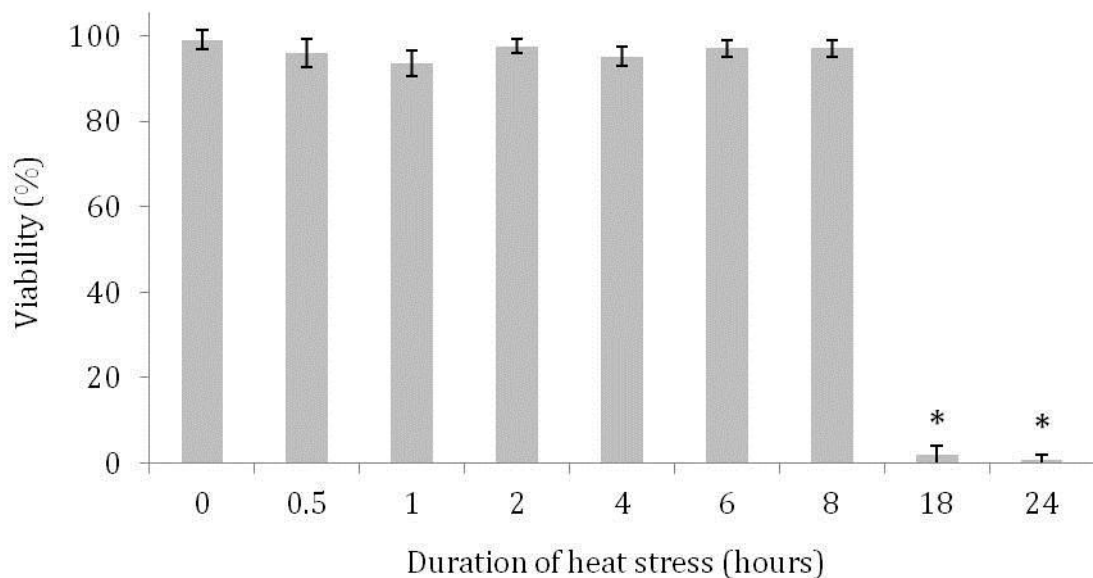


Figure 2.4 Viability of cultivated *G. gracilis* exposed to varying durations of heat stress at 35 °C (after a 21 day recovery period at 15 °C). Parametric one-way ANOVA detected statistically significant differences in the viability of *G. gracilis* between the control (0 hours) and experimental samples ($P < 0.001$, denoted by asterisks “*”). Data represents the mean \pm standard error from three biological repeats.

reducing back towards basal levels after 2 hours (Figure 2.7 A). A further decrease in catalase activity occurred from 18 hours of exposure. *T*-tests comparing the mean catalase activity in thallus segments incubated at 15 and 35 °C for 1, 18 and 24 hours failed to detect statistically significant differences in this enzyme's activity. However, the power of these performed tests (0.359, 0.146 and 0.225 respectively) was well below that of the desired 0.800 indicating that a type II error (false negative) may have been committed.

The activity of ascorbate peroxidase and glutathione peroxidase increased after half an hour of exposure to 35 °C and remained elevated for the 24 hours (Figures 2.7 A and B).

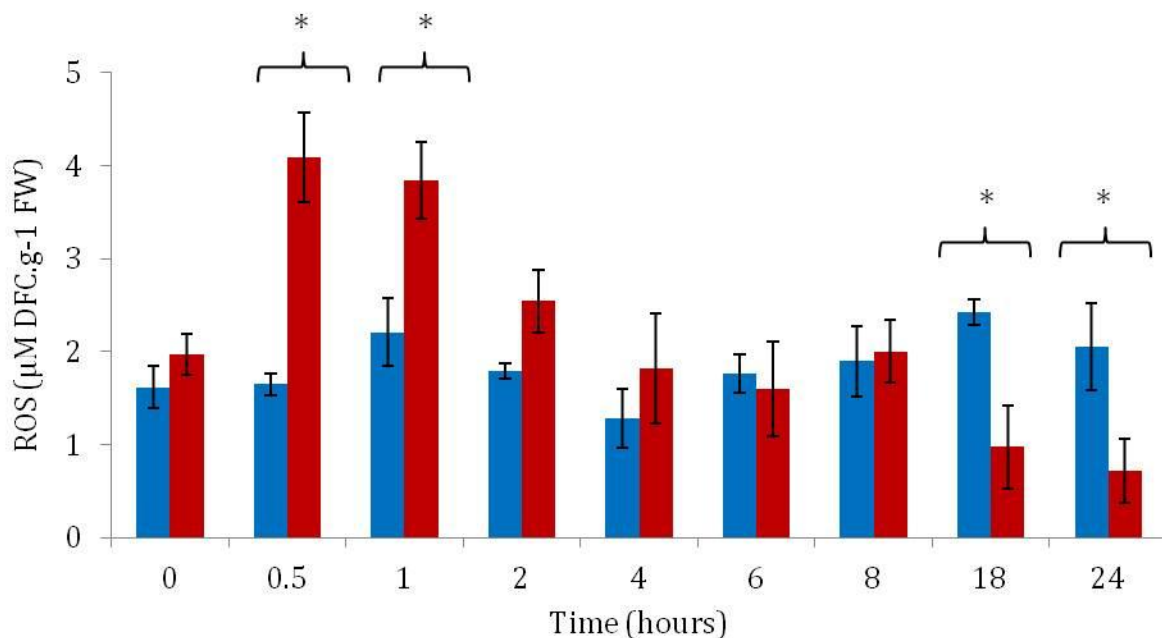


Figure 2.5 The accumulation of intracellular reactive oxygen species (ROS) in cultivated *G. gracilis* in response to varying durations of heat stress. The blue and red bars represent the data obtained from thallus segments incubated at 15 (control) and 35 °C (experimental samples) respectively. Parametric *t*-tests detected statistically significant differences in the concentration of ROS between the control and experimental samples at 0.5, 1, 18 and 24 hours ($P < 0.05$, denoted by asterisks '*'). Data represents the mean \pm standard error from three biological repeats. Data from the sample set that was exposed to 0.5 hours of heat shock was \log_{10} transformed to meet the requirements of parametric statistical testing.

Statistically significant differences in the activity of ascorbate peroxidase between the control and experimental samples were observed after 0.5, 1, 8 and 18 hours of exposure to 35 °C. Although an increase in the mean activity of ascorbate peroxidase in thalli exposed to 35 °C compared to those exposed to 15 °C was detected after 2, 4, 6 and 8 hours, the powers of the *T*-tests were 0.196, 0.05, 0.079 and 0.416 respectively, again demonstrating that a type II error could have been committed. Similarly, the activity of glutathione peroxidase in thallus segments exposed to 35 °C remained elevated for the 24 hour duration, starting after half an hour of exposure, when compared to the activity in thalli incubated at 15 °C. However, statistical differences were not observed between means of the control and experimental samples at any given time point. This

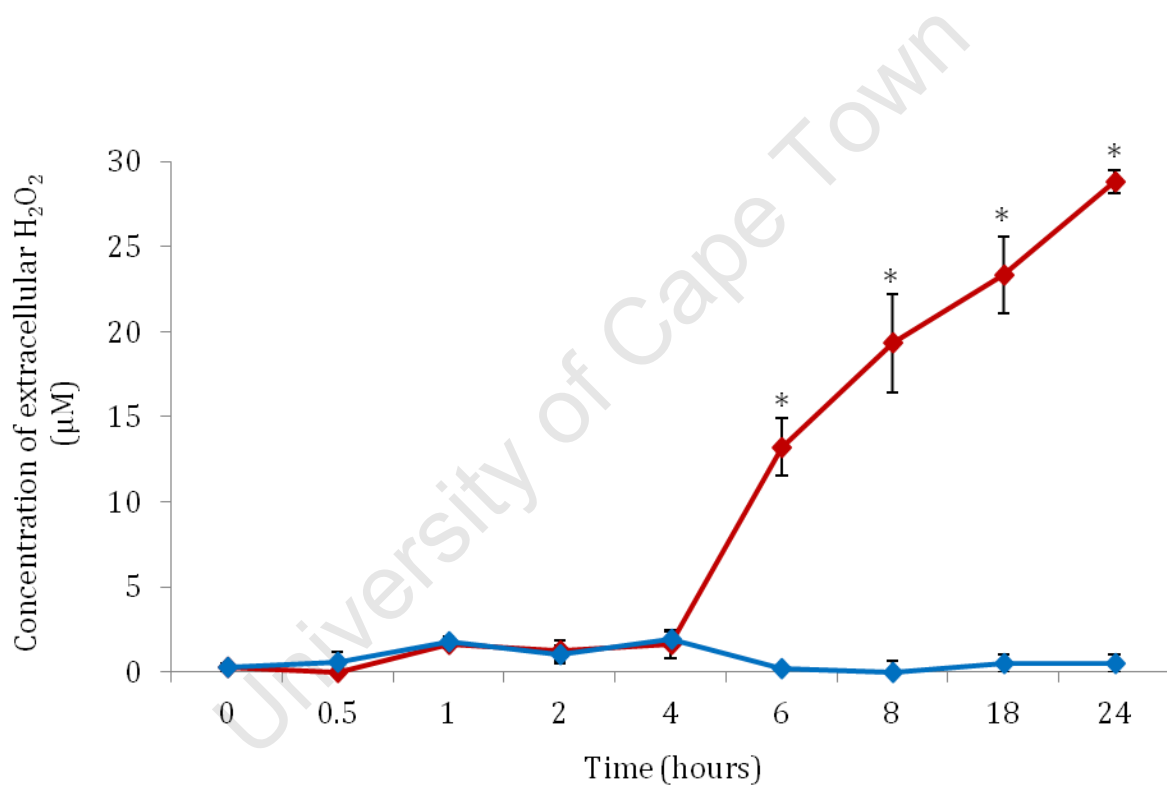


Figure 2.6 Quantitation of extracellular hydrogen peroxide (H_2O_2) released by cultivated *G. gracilis* in response to varying durations of heat stress. The blue and red lines represent data obtained from flasks incubated at 15 (control) and 35 °C (experimental samples) respectively. Parametric *t*-tests detected statistically significant differences in the concentration of H_2O_2 in the surrounding medium between the control and experimental samples after 6 hours of heat shock ($P \leq 0.001$, denoted by asterisks '*'). Data represents the mean \pm standard error from three biological repeats.

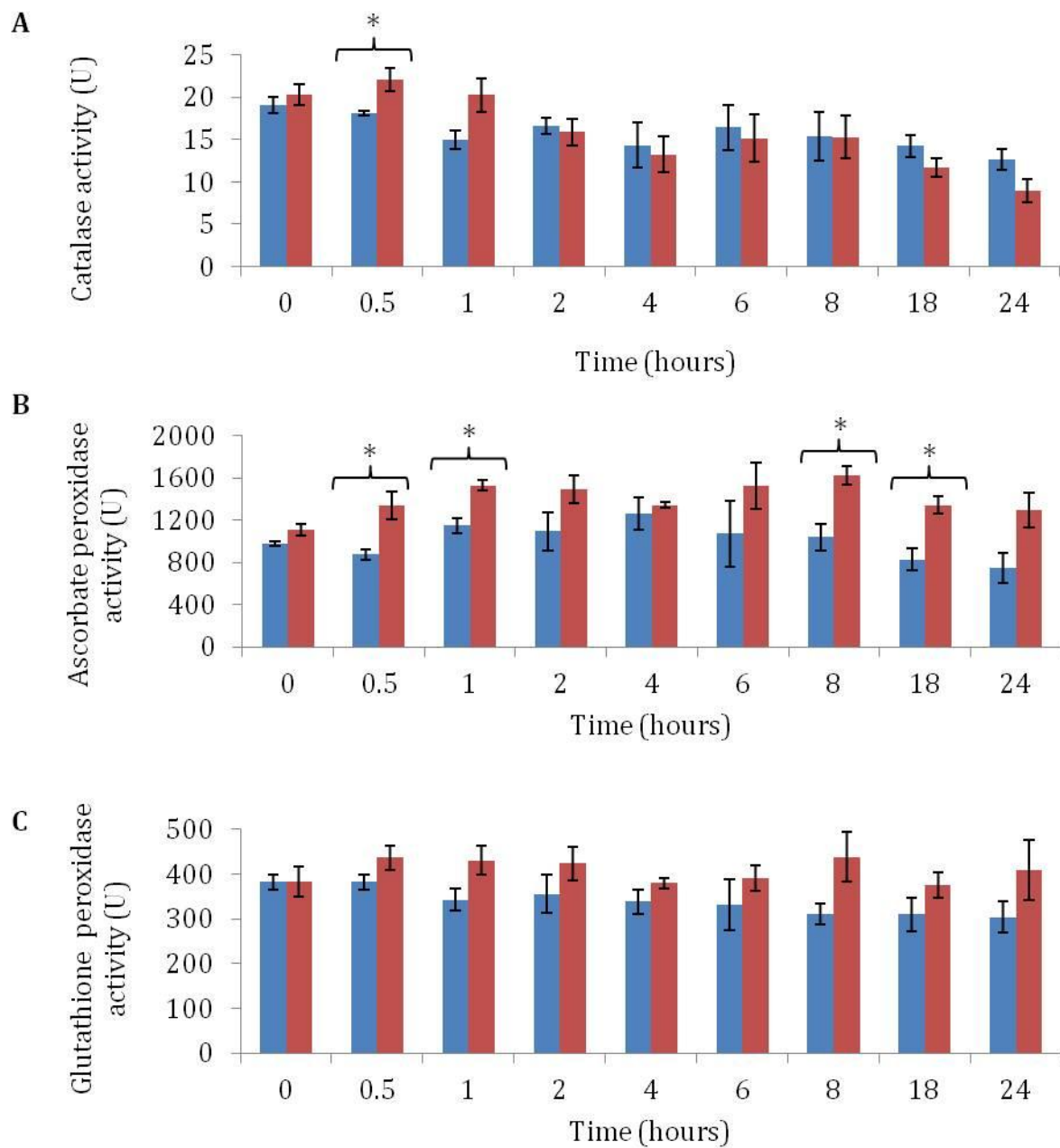


Figure 2.7 Quantitation of (A) catalase, (B) ascorbate peroxidase and (C) glutathione peroxidase activity in cultivated *G. gracilis* in response to varying durations of heat stress. The blue and red bars represent the data obtained from thallus segments incubated at 15 (control) and 35 °C (experimental samples) respectively. Parametric *t*-tests detected statistically significant differences in the enzyme activity between the control and experimental samples at a specific time point ($P < 0.05$, denoted by asterisks '*'). Data represents the mean \pm standard error from three biological repeats.

may, once again, be attributed to the power of the *T*-tests being well below that of the desired 0.800, again demonstrating that a type II error may have been committed. Although an increase in the number of biological repeats in these assays could have increased the power of the statistical tests, potentially to reveal additional significance (Williams *et al.*, 1997; Cumming *et al.*, 2007), what can be concluded is that the increased trends in the activity of these antioxidant enzyme activities confirmed that cultivated *G. gracilis* was heat shocked when exposed to 35 °C for 24 hours.

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2.4 Discussion

To devise a suitable heat shock experiment to induce a heat shock response in cultivated *G. gracilis*, knowledge of the thermal tolerance of this species is required. In previous studies, the upper limit of the thermal tolerance of *Gracilaria* species had been determined by investigating the highest temperature at which both growth and viability of the alga was not adversely affected (McLachlan and Bird, 1984; Engledow and Bolton, 1992; Levy and Friedlander, 1994; Steentoft and Farnham, 1997). Engledow and Bolton (1992) reported that the wild South African population of *G. verrucosa* (later reclassified as *G. gracilis*) inhabiting the temperate regions of Saldanha Bay and the Langebaan Lagoon, retained growth and viability between 5 to 25 °C; whereas temperatures ≥ 30 °C exceeded the thermal tolerance of the alga.

In this study, the effect of elevated temperature on *G. gracilis* cultivated at the Irvin & Johnson Abalone Culture Division, South Africa was investigated by similarly monitoring the growth and viability of this alga in response to a range of temperatures for 21 days. As reported for the wild population, the optimal growth temperature of the cultivated *G. gracilis* was 25 °C (Engledow and Bolton, 1992). A positive correlation between the specific growth rate (SGR) of *G. gracilis* and increased incubation temperatures towards that of the optimal growth temperature was detected as previously reported by Rueness and Tananger (1984), Engledow and Bolton (1992) and Macchiavello *et al.* (1998). The increase in the specific growth rate from 7.4 to 8.4 at 15 and 25 °C respectively was within the expected range (between 5.0 and 10.0) reported for *Gracilaria* species (McLachlan and Bird, 1984; Rueness *et al.*, 1987; Molloy and Bolton, 1996). However, in contrast to the wild population, the cultivated species did not only survive at 30 °C for the duration of the growth experiment, but grew at a SGR of 6.1. It did not however survive temperatures equal to or greater than 35 °C. These results inferred that the thermal tolerance of cultivated *G. gracilis* was higher than that of the wild population and that 35 °C is beyond the thermal limit of the species.

Although previous studies reported that there was little to no evidence of *Gracilaria* ecotypes and instead suggested that thermal tolerance is a conserved taxonomic trait of *Gracilaria* species (Bolton and Lüning, 1982; Lüning, 1984; McLachlan and Bird, 1984; Rietema and van den Hoek, 1984; Lüning and Freshwater, 1988; Engledow and Bolton, 1992; Levy and Friedlander, 1994), this study suggests otherwise. The cultivated *G. gracilis* population used in this study has been separated from the wild population for more than 10 years, where the mean monthly water temperature has increased from 13 °C to 18 °C in the wild to 14 °C to 21 °C in the on-shore cultivation raceways (Bolton, 1986; Engledow and Bolton, 1992; Irvin & Johnson Abalone Culture Division, personal communication). It is hypothesised that the increased mean incubation temperature to which the seaweed has been exposed for many years (omitted from the previously reported studies) is responsible for the difference in the upper limit of thermal tolerance between the wild and cultivated *G. gracilis*.

Thus, an experiment was designed to induce a heat shock response in cultivated *G. gracilis* based on the upper limit of thermal tolerance of the alga. As intertidal algae have been reported to survive limited exposure to temperatures above that of the thermal limit of the species without physiological damage to the organism (Kübler and Davison, 1993; Burritt *et al.*, 2002), *G. gracilis* was exposed to 35 °C, a temperature greater than its thermal tolerance, to induce heat stress in this cultivated alga.

A 24 hour time-course experiment was conducted at this temperature to incorporate both an initial period of thermal tolerance and a subsequent period in which cell death was induced. Cultivated *G. gracilis* exposed to ≤ 8 hours of heat stress was not adversely affected. In fact, *G. gracilis* incubated for between 0.5 and 4 hours did not only tolerate short periods of exposure to heat stress but maintained a statistically significant faster SGR over the recovery period when compared to a control sample. The uncoupling of thermal tolerance and growth of intertidal algae is not uncommon as a number of these species have been reported to continue functioning for limited periods above the thermal limit of the organism (Davison, 1987; Zimmermann *et al.*, 1989; Kuebler *et al.*,

1991). Kübler and Davison (1993) reported that the Rhodophyte *Chondrus crispus* not only continued to photosynthesise and respire at a temperature above that of its thermal limit for up to 9 hours, but continued to exhibit elevated photosynthetic rates for a number of hours after it had been returned to tolerable growth conditions. Although further investigation would be required to determine the relationship between the photosynthetic and respiration rates of the cultivated *G. gracilis* and the duration of heat stress at 35 °C, the possibility that elevated photosynthetic rates, coupled with the ability of some algae to modify their growth rates within minutes of an environmental change (Strömgren, 1982; Lüning, 1992), may be responsible for the increased SGR of cultivated *G. gracilis* following exposure to short durations of heat stress at 35 °C.

Thallus segments exposed to 35 °C for ≥18 hours did not survive, reducing in biomass over the incubation period. After heat shock, thalli began to discolour within the first 24 hours and despite having been returned to an unstressed incubation environment, thalli started to fragment within the first 7 days of the recovery period. This tissue degradation is characteristic of the third and final phase of programmed cell death in which the classic phases are: (i) cell death signals are induced (induction phase), (ii) the cells are committed to die (effector phase) and (iii) tissue begins to degrade (degradation phase; Stein and Hansen, 1999).

Since it has been reported that ROS production and reduction is an early indicator of heat stress in plants and marine algae (Schrek and Baeuerle, 1991; Rijstenbil *et al.*, 1994; Mittler, 2002; Nedelcu *et al.*, 2004; Weinberger *et al.*, 2005; Lesser, 2006; Zuppini *et al.*, 2007), changes in the concentration of ROS and more specifically intracellular hydrogen peroxide (H₂O₂) was investigated in cultivated *G. gracilis* to validate that exposure to 35 °C induced a heat shock response in the alga.

In this study, a rapid and transient accumulation of ROS (alternatively known as an oxidative burst; Lesser, 2006) was detected in cultivated *G. gracilis* after 0.5 and 1 hour of exposure to 35 °C prior to returning towards that of basal levels after 2 hours of exposure. This temporary accumulation of ROS is inferred to validate the induction of heat stress in this alga. After 18 hours of exposure to 35 °C however, the concentration of intracellular ROS decreased below that of basal levels. Although additional research would be required to determine the mechanisms involved in the reduction of internally located ROS after the onset of cell death (Section 2.4.3), the potential loss in membrane stability through lipid oxidation, previously reported as a physiological symptom of cell death in plant and algal species (Cadenas, 1989; Nedelcu *et al.*, 2004; Contreras *et al.*, 2005; Lesser, 2006; Ross *et al.*, 2006; Darehshouri *et al.*, 2008), may be responsible for the leaching of cellular contents out into the surrounding medium.

The reduction of intracellular H₂O₂ by enzymatic and non-enzymatic processes has been reported in several algal species (Collén *et al.*, 1995; Mtolera *et al.*, 1995; Contreras *et al.*, 2005; Xue *et al.*, 2005; Ross *et al.*, 2006). Although the exact mechanism of H₂O₂ reduction in *G. gracilis* thallus segments remains unclear, the reduction of intracellular H₂O₂ was assessed in this study by monitoring the concentration of H₂O₂ released from thallus segments out into the surrounding medium and investigating the rate at which three preselected antioxidant enzymes (catalase, ascorbate peroxidase and glutathione peroxidase) reduce intracellular H₂O₂ into water (Lamb and Dixon, 1997; Weinberger *et al.*, 1999; Lesser, 2006).

Weinberger *et al.* (1999) reported that the release of H₂O₂ into the surrounding medium occurred within minutes of the addition of the disease elicitor neoagarohesane. This process was hypothesised to be a potential inducer of the disease response in *Gracilaria conferta*. However, the release of H₂O₂ into the surrounding medium was thought to coordinate a series of cellular responses that result in cell death in other species of cyanobacteria and algae (Korsmeyer, 1995; Levine *et al.*, 1996; Vardi *et al.*, 1999; Apel and Hirt, 2004; Ross *et al.*, 2006; Zuppini *et al.*, 2007). The accumulation of H₂O₂ in the

medium surrounding cultivated *G. gracilis* after 6 hours of heat stress indicates that the accumulation of H₂O₂ in this alga is more likely to be attributed to the onset of cell death rather than the induction of thermal tolerance.

The rapid induction of the three antioxidant enzymes assayed in this study occurred within the first 30 minutes of heat shock, indicating that the enzymes are involved in the induction of thermal tolerance in this species. Catalase activity increased in conjunction with the accumulation of intracellular ROS, increasing in activity between 0.5 and 1 hours of heat shock prior to returning to basal levels after 2 hours and subsequently, dropping to levels lower than that of the control sample after 18 hours. In contrast, the activity of the peroxidases increased within the first 0.5 hour of heat shock and remained elevated for the duration of the heat shock. These two different profiles are hypothesised to reflect the putative functions of these enzymes in *G. gracilis* based on previous studies where catalase was reported to counteract ROS accumulation by rapidly scavenging high concentrations of intracellular H₂O₂, while peroxidases were reported to function in a more general stress response (Lesser, 2006; Cheeseman, 2007; Gill and Tuteja, 2010).

Thus in this chapter, the conditions required to induce heat stress in cultivated *G. gracilis* were determined. Collectively these results validated that the 24 hour time-course experiment exposing cultivated *G. gracilis* to 35 °C was sufficient to induce heat stress in this alga: incorporating an initial period of thermal tolerance (≤ 8 hour) in which thallus retained growth and viability and a second period (≥ 18 hours) in which cell death was induced. On further analysis, the decrease in the growth rate of the thallus segments and the accumulation of extracellular H₂O₂ in the surrounding medium after 6 hours of heat shock, suggests that although thermal tolerance is maintained for up to 8 hours of heat stress, the activation of cellular mechanisms involved in initiating the first phase of cell death may occur after as little as 6 hours of exposure to 35 °C.

CHAPTER 3

ANALYSIS OF DIFERENTIALLY EXPRESSED PROTEINS IN *GRACILARIA GRACILIS* IN RESPONSE TO HEAT STRESS

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3.1 Summary

In this chapter, a comparative proteomic approach was used to identify differentially expressed proteins in *G. gracilis* in response to heat stress. Thus, two dimensional polyacrylamide gel electrophoresis was employed to compare the total soluble protein profiles of thalli heat shocked at 35 °C for various durations up to and including 24 hours. Of the 555 protein spots reproducibly detected on each of these 2D SDS-polyacrylamide gels, 76 proteins were up-regulated and 38 proteins down-regulated in response to heat stress.

The ten most statistically significant differentially expressed proteins were selected for identification using tandem mass spectrometry. The putative identity and function of seven protein spots was successfully determined. Four of these proteins were directly associated with stress responses and three functioned within metabolic processes. One and two dimensional western blot analyses validated the differential expression and identification of three of the four stress response proteins: ferredoxin NADP⁺ reductase, chaperonin GroEL and heat shock protein 70.

3.2 Introduction

Proteins are the functional components of cells, constantly changing in response to stimuli to facilitate the adaptation of an organism to its surroundings (Anderson and Anderson, 1996; Shepard *et al.*, 2000; Brumbarova *et al.*, 2008). It is this dynamism of protein networks which makes functional proteomics, which monitors and analyses the spatial and temporal properties of protein networks (Hemby and Bahn, 2006), such a powerful tool for understanding the protein networks responsible for the adaptations of organisms to various stressors (Monti *et al.*, 2005; Brumbarova *et al.*, 2008; Contreras *et al.*, 2008; Jorrín-Novo *et al.*, 2009).

Thus, a proteomic approach was employed to gain further insight into the protein pathways involved in the process of maintaining thermal tolerance and the induction of cell death in *G. gracilis*. The first objective was to identify proteins that were differentially expressed in response to the heat stress, using a comparative proteomic approach, and subsequently, to examine the expression profile of the differentially expressed protein spots in relation to the physiological state of the seaweed over the course of the heat shock (as determined in Chapter 2).

Although identification of the majority of the differentially expressed proteins would have been preferable in order to obtain a comprehensive overview of the pathways involved in thermal tolerance and the onset of cell death in *G. gracilis*, financial constraints limited the number of proteins that could be selected for identification by tandem mass spectrometry. Thus, the second objective of this study was to identify the ten most statistically significant differentially expressed proteins and to further determine their putative functions using Blast2GO. This would provide the first step to elucidating the pathways involved in the induction of thermal tolerance and the onset of cell death in this alga.

As with all high-throughput proteomic approaches, the results obtained from the above investigation should be validated by the use of complementary biochemical or cellular biological techniques (Jorrín *et al.*, 2007; Jorrín-Novo *et al.*, 2009). Thus the third and final objective of this study was to verify the putative identity of the proteins that had been identified using tandem mass spectrometry by comparing the observed and theoretical isoelectric points and molecular weights of these proteins, and to use 2D and 1D western blot analyses to further validate the identities and expression profiles of three of the proteins.

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3.3 Materials and Methods

All media and solutions used in this study are listed in Appendix A.

Experimental data was obtained from three biological repeats (each consisting of three technical repeats) unless otherwise stated. In the context of this study, technical and biological repeats were defined as data obtained from algal samples exposed to the same environmental conditions but acquired from either the same or triplicate experiments, respectively.

3.3.1 Preparation of *G. gracilis*

3.3.1.1 Sample collection and maintenance conditions

Gracilaria gracilis was obtained every fortnight from the Irvin & Johnson Abalone Culture Division, Danger Point, South Africa. Healthy thalli were selected and briefly rinsed in distilled water to remove visible epiphytes. Cleaned thallus segments were maintained at 15 °C for a minimum of 2 weeks prior to acclimatisation (Section 3.3.1.2) in 45 l tanks fitted with a flow-through system, filtering seawater at a rate of 1 l/min. Water movement was facilitated by pumping compressed air through plastic airlines and the alga was illuminated by cool white fluorescent tubes at $\sim 45 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 16-hour light: 8-hour dark cycling photoperiod. During this maintenance period, both the alga and the tanks were cleaned twice a week with filtered seawater to remove any accumulated sedimentation.

3.3.1.2 Acclimatisation conditions

After maintenance (Section 3.3.1.1), *G. gracilis* was acclimatised at 15 °C for 10 days in an Adaptis A350 Multi-Application Growth Chamber (Convion®). Thus, similarly

structured thallus segments were incubated in conical flasks at a ratio of 1 g thalli: 200 ml precooled artificial seawater (ASW; Appendix A.1.1) supplemented with PES-enriched seawater medium to achieve a ½ strength growth medium (Appendix A.2.2.1; enriched ASW). Sufficient water circulation was obtained by pumping compressed air through plastic tubing and the seaweed was again illuminated at $\sim 45 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ by cool white fluorescent tubes under a 16: 8 light: dark cycling photoperiod. Every 2-3 days, *G. gracilis* was rinsed briefly in enriched ASW and transferred to flasks containing freshly prepared media.

3.3.2 Heat shock experiments

In this study, two individual heat shock experiments were conducted. First, to gain an initial understanding of protein expression changes in *G. gracilis* in response to heat stress, a preliminary heat shock experiment (Experiment 1) was performed. Thus thallus segments were heat shocked at 35 °C for 0 (omission of heat stress, control), 0.5, 1, 2, 4, 6, 8, 18 and 24 hours. As the objective of this study was to obtain an overview of the differentially expressed proteins, this experiment was only performed using two biological repeats and omitted technical repeats to minimise costs. After analysis, a second heat shock experiment (Experiment 2) was performed. In this experiment, thallus segments were heat shocked at 35 °C for 0, 6 and 24 hours, representing omission of heat stress (control), thermal tolerance and the onset of cell death, respectively (Chapter 2). As Experiment 2 was executed to validate the results obtained from Experiment 1, this experiment was performed in triplicate, where each biological repeat consisted of three technical repeats.

Both of these experiments were performed in temperature controlled horizontal orbital shaker incubators (Dynamic & Evolution International Corporation) for the required duration. When starting each heat shock experiment, *G. gracilis* was transferred directly from acclimatisation conditions of 15 °C (Section 3.3.1.2) into enriched ASW at the appropriate temperature, at a ratio of 1 g thalli: 200 ml of enriched ASW. Flasks were

shaken at 75 rpm to ensure adequate water movement, and illuminated at $\sim 45 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ using a 16-hour light: 8-hour dark cycling photoperiod for the duration of the experiment. Post heat shock, thallus segments were removed from the flasks, frozen in liquid nitrogen and stored at $-80 \text{ }^\circ\text{C}$ until required.

3.3.3 Preparation of total soluble protein

3.3.3.1 Extraction and quantification of total soluble protein

Post heat shock (Section 3.3.2), total soluble protein (TSP) was extracted from frozen *G. gracilis* tissue samples (Appendix B.7) and stored at $-80 \text{ }^\circ\text{C}$ until required. Prior to use, TSP was defrosted on ice, vortexed for 1 hour at room temperature and quantitated using a modified Bio-Rad protein assay (Bio-Rad; Appendix B.8).

3.3.3.2 Determination of the integrity of the total soluble protein

After TSP quantification (Section 3.3.3.1), the integrity of the frozen total soluble protein was immediately determined using one dimensional (1D) gel electrophoresis (Appendix B.9). Thus 25 μg of each TSP sample was prepared and loaded onto a 12 % SDS-polyacrylamide gel. The protein was electrophoresed using the Mini-PROTEAN Tetra Cell electrophoresis system as per the manufacturer's instructions. After electrophoresis, the gel was removed from the gel assemblage and the TSP visualised using a coomassie staining technique (Appendix B.11). An image of the stained protein gel was captured using a Bio-Rad GelDoc EQ-system™ (Bio-Rad) fitted with a white light conversion screen. The image was analysed for signs of protease degradation. TSP exhibiting a good structural integrity was immediately used for either 2D gel electrophoresis or western blot analysis (Sections 3.3.4 and 3.3.10 respectively).

3.3.4 Two dimensional gel electrophoresis

After preparation of TSP (Section 3.3.3), 250 µg TSP isolated from each thallus sample was separated on a 2D SDS-polyacrylamide gel (Appendix B.10). Thus, TSP was loaded by rehydration onto immobilised pH gradient (IPG) strips (pH 4-7; Bio-Rad) and separated in the first dimension according to the protein's net charge via isoelectrofocusing. After the initial separation, TSP was subjected to chemical reduction and alkylation prior to loading the IPG strip onto a premade 12 % SDS-polyacrylamide gel where the proteins were further separated in the second dimension according to the protein's molecular weight via electrophoresis. To minimise variability between gels, TSP samples from a single biological repeat were electrophoresed concurrently using the Criterion Dodeca Cell electrophoresis system (Bio-Rad; Appendix B.10.4) according to the manual.

3.3.5 Detection of differentially expressed proteins using 2D analysis software

Two dimensional SDS-polyacrylamide gels containing the resolved TSP (Section 3.3.4) were removed from the gel assemblage and visualised by coomassie staining (Appendix B.11). The stained protein gels were scanned at a resolution of 300 dots per inch (dpi) using a flat-bed Epson perfection V750 colour scanner (Epson) and images were captured using the SilverFast® 6 software package (LaserSoft Imaging®). Black and white TIFF images were saved for the detection of differentially expressed proteins using the 2D gel analysis program *Melanie 7.0* (Swiss Institute of Bioinformatics), while colour JPEG file formats were saved for visual representation of the gel images. Once the TIFF images were uploaded to *Melanie 7.0*, the differentially expressed proteins were identified according to the software manual (Appendix B.12).

3.3.6 Hierarchical cluster analysis

Hierarchical clustering analysis was performed to group the expression profiles of the ten preselected proteins identified from the abovementioned 2D analysis (Section 3.3.5), using Minitab 16 Statistical Software (Minitab Inc.) as described by the software's help file. A cluster variable procedure was used to classify variables into groups. A single linkage method of analysis with a 95 % similarity level was selected for analysis of the data.

3.3.7 Preparation and identification of proteins using tandem mass spectrometry

Protein spots selected for identification by tandem mass spectrometry were excised from three coomassie stained 2D SDS-polyacrylamide gels (Sections 3.3.4) using an automated EXQuest Spot Cutter (Bio-Rad). Replicate gel plugs were placed in a single sterile centrifuge tube and dried for 30 minutes using a Savant Speedvac® plus SC210A (Thermo Quest) on the 'low' temperature setting. Dehydrated protein plugs were sent to the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, United States of America) for protein analysis using tandem mass spectrometry (LC-MS/MS; Appendix B.13). Data from the LC-MS/MS analysis, for each protein spot, was searched against the non-redundant NCBI database using the Mascot software. From this investigation, a list of the possible protein identifications was received via the W. M. Keck Foundation Biotechnology Resource Laboratory on-line repository, which ranked the possible protein identifications from the highest to lowest probability-based molecular weight search (MOWSE) scores. The highest ranked identification for each of these protein spots was selected as its putative identity. For this study, a minimum requirement for identification of a protein required the presence of least two peptides matching a homologue in the NCBI database and a MOWSE score of more than 56 ($P < 0.05$; Wong *et al.*, 2006).

3.3.8 Verification of protein identities

Verification of the protein identities obtained from tandem mass spectrometry was accomplished by comparing the observed molecular mass and isoelectric point of each selected protein spot with the theoretical molecular mass and isoelectric point of the homologue obtained from the NCBI database (www.ncbi.nlm.nih.gov). The observed molecular mass and isoelectric point of each protein spot was calculated from a 2D SDS-polyacrylamide gel image which depicted TSP isolated from *G. gracilis* and concurrently resolved with 2D SDS-PAGE standards (Bio-Rad; Section 3.3.4). The theoretical values were deduced from the full length amino acid sequences of the homologue proteins using the pI/Mw tool on the ExPASy Bioinformatics Resource Portal (web.expasy.org/compute_pi).

3.3.9 Determination of the molecular functions of the selected proteins

The molecular function of each of the putatively identified proteins was determined using the universal gene ontology annotation, visualisation and analysis tool for functional genomics research, Blast2GO (www.blast2go.org). This tool was used as directed by the website.

3.3.10 Validation of protein identities

Of the proteins identified via mass spectrometry (Section 3.3.7), suitable commercially available antibodies were available for three of the four stress response proteins (ferredoxin NADP⁺ reductase, chaperonin GroEL and heat shock protein 70). Thus validation of these three proteins was accomplished as described below:

3.3.10.1 Validation of protein identities using 2D western blot analysis

Validation of the identities of the proteins was assessed using 2D SDS-polyacrylamide gel western blot analysis. Thus, 250 µg of TSP was isolated from an acclimatised thallus sample (Section 3.3.1.2) and electrophoresed on a 2D SDS-polyacrylamide gel (Section 3.3.4). After electrophoresis, TSP was transferred onto a nitrocellulose membrane (Appendix B.14) and stained with Ponceau S staining solution (Appendix B.15). Images of the spots on the membrane were obtained with a flat-bed Epson perfection V750 colour scanner (Epson) at a resolution of 300 dpi and captured using the SilverFast® 6 software package (LaserSoft Imaging®). The exact location of the relevant protein spot on the membrane was determined by comparing this 2D protein profile with that of the coomassie stained 2D-SDS-polyacrylamide gel prior to excision (Section 3.3.5). Post imaging, the membrane was rinsed in 1X TBS (Appendix A.2.1.6) for 15-30 min with agitation to remove the Ponceau S stain, after which western blot hybridisation was performed using the appropriate antibody (Appendix B.16). Images of the protein spot/s visualised with the appropriate antibody were obtained at a resolution of 300 dpi using a flat-bed Epson perfection V750 colour scanner (Epson) and captured using the SilverFast® 6 software package (LaserSoft Imaging®). In order to validate the identity of the protein, the position of the protein spot on the Ponceau S stained membrane image was compared to the position of the protein spot visualised via western blot analysis. Agreement in the positioning of the spots was taken to validate the identity of the protein.

3.3.10.2 Validation of the differential expression of selected proteins using 1D western blot analysis

Validation of the differential expression of the proteins was performed using 1D SDS-polyacrylamide western blot analysis. Thus for the assessment of each protein, 25 µg of TSP extracted from thalli exposed to 35 °C for 0, 6 and 24 hours (Section 3.3.2), was electrophoresed on an 8 % polyacrylamide gel. The gel was loaded such that each

protein sample was loaded onto each half of the gel. Post electrophoresis, the gel was cut in half. One half of the gel was stained using a coomassie staining technique (Appendix B.11) and images of the visualised proteins were captured on a light box (Bio-Rad GelDoc EQ-system™; Bio-Rad) using the Quantity One® software (version 1.7; Bio-Rad). TSP on the other half of the gel was transferred to a nitrocellulose membrane (Appendix B.14) and stained with Ponceau S staining solution (Appendix B.15). Post staining, the membrane was scanned at a resolution of 300 dpi using a flat-bed Epson perfection V750 colour scanner (Epson) and an image was captured using the SilverFast® 6 software package (LaserSoft Imaging®). After imaging, the membrane was rinsed in 1X TBS (Appendix A.2.1.6) for 15-30 min with agitation to remove the Ponceau S stain, after which western blot hybridisation was performed using the appropriate antibody (Appendix B.16). Each membrane was again scanned and an image of the visualised protein band(s) captured as described above. To quantify expression of the relevant protein in thalli exposed to 35 °C for 6 and 24 hours in relation to the control (omitted heat shock), an additional image of the visualised protein band(s) was captured on a light box (Bio-Rad GelDoc EQ-system™; Bio-Rad) using the Quantity One® software (version 1.7; Bio-Rad). Densitometric analysis of the protein band(s) was performed using the above mentioned software and statistically significant differences in the abundance of each protein were determined using SigmaStat for Windows (version 3.10; Systat Software Inc.; Section 3.3.11). Differential expression of each of the proteins was compared to the abundance of the appropriate protein as determined by 2D polyacrylamide gel electrophoresis and associated quantitation techniques (Sections 3.3.4 and 3.3.5). Agreement in the abundance of these proteins as determined by the two methods was taken to validate differential expression of the proteins.

3.3.11 Statistical analysis

Descriptive statistics and statistical analyses were performed using SigmaStat for Windows (version 3.10; Systat Software, Inc.) unless otherwise stated. Where a control sample was compared to multiple experimental samples, a one-way analysis of variance (ANOVA) was deemed appropriate to examine the significance of each experimental

sample relative to the control sample. Data sets that were not normally distributed or displayed unequal variances were transformed to meet the requirements for parametric statistical testing. Where data could not be adequately transformed, a non-parametric statistical test was conducted instead.

3.4 Results

3.4.1 Overview of the proteomic experiments

Two proteomic experiments were performed to identify differentially expressed proteins in *G. gracilis* in response to heat stress (Figure 3.1). A preliminary study (Experiment 1; Figure 3.1 Ai) was performed to examine the differential expression of the proteins separated on 2D SDS-polyacrylamide gels, in duplicate, for all nine time points investigated in Chapter 2 (Figure 3.1 Bi). Thus, total soluble protein was isolated from each sample and electrophoresed on 2D SDS-polyacrylamide gels (Appendix C.1). Post electrophoresis, the protein spots were visualised and differential expression assessed using the 2D analysis software *Melanie 7.0*. Owing to financial constraints, only the ten most statistically significant differentially expressed protein spots were selected for further investigation. Hierarchical clustering was used to examine the expression profiles of these 10 protein spots, which lead to the selection of three time points that were used to validate the differential expression of these proteins in a second proteomic experiment (Experiment 2, Figure 3.1 Aii).

In Experiment 2, tissue was harvested from three biological repeats from thallus segments exposed to 35 °C for 0, 6 or 24 hours (Figure 3.1 Bii). From each of these thalli, total soluble protein was harvested in triplicate and electrophoresed on 2D SDS-polyacrylamide gels. The abundance of the ten most statistically significant, differentially expressed protein spots was compared to the expression values obtained for these protein spots in Experiment 1. Agreement between the relative expression values of a specific protein spot confirmed that the protein was differentially expressed in heat stressed *G. gracilis*. Once validated, all ten protein spots were sent to the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale University, United States of America) for identification using tandem mass spectrometry and database searching. The identities of the successfully identified protein spots were verified by comparing the observed and theoretical molecular weight and isoelectric point of each protein.

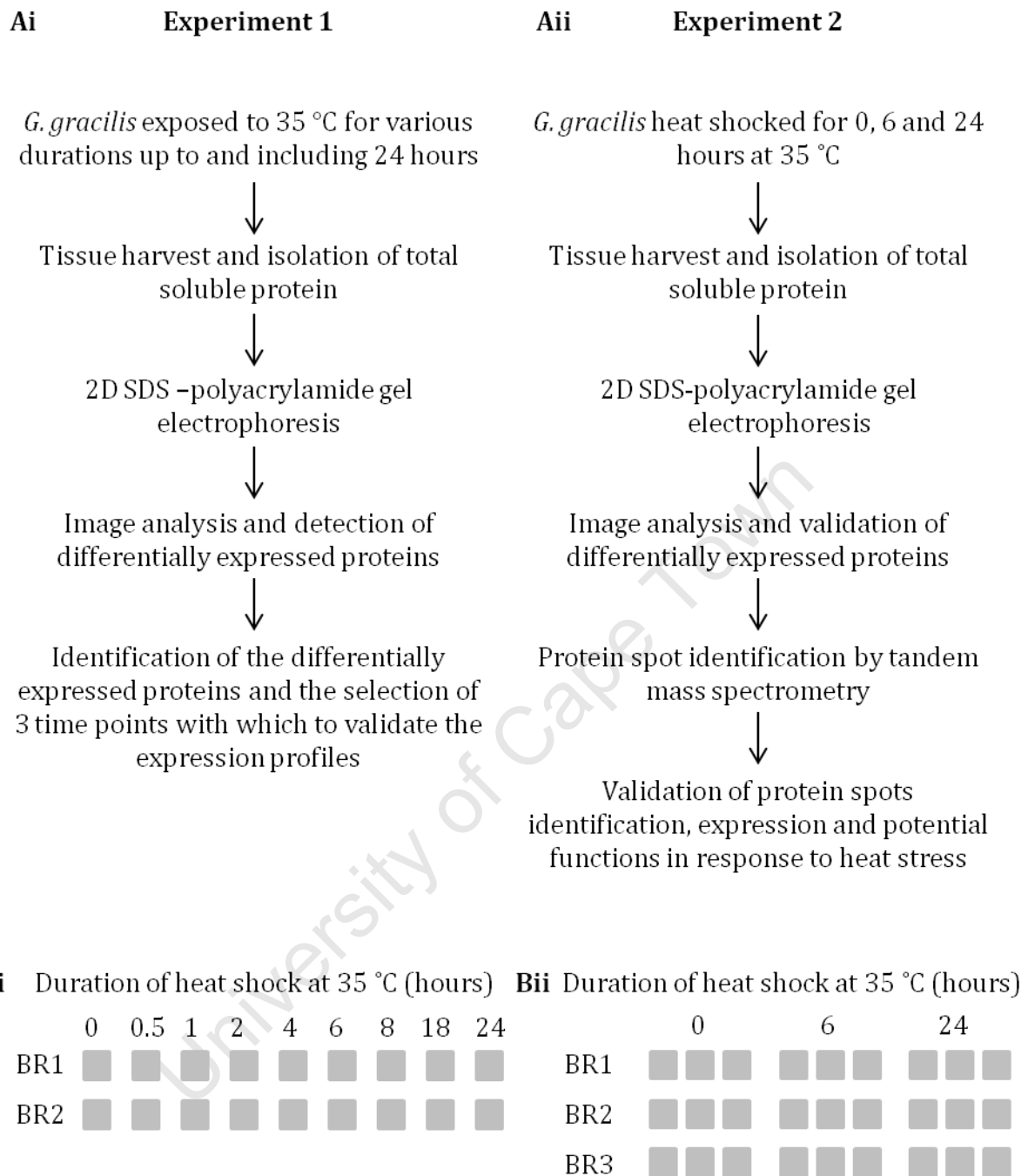


Figure 3.1 Overview of the experimental design for identification of differentially expressed proteins in *G. gracilis* in response to heat stress. (A) Flow schematic of the proteomic approach employed for (i) Experiment 1 and (ii) Experiment 2. (B) The experimental plan indicating biological repeats (BR1, BR2 and BR3) and technical repetitions (depicted as grey squares) for both (i) Experiment 1 and (ii) Experiment 2.

After which, the putative function of each of the proteins was determined using Blast2GO. Since commercially available antibodies were available for three of the four proteins thought to play a direct role in stress response, 1D and 2D western blot analysis was used to validate the identity and expression profile of the three putative stress response proteins from *G. gracilis*.

3.4.2 Identification of differentially expressed proteins in *G. gracilis* in response to heat stress

Two dimensional gel electrophoresis, coupled with the 2D gel analysis software *Melanie 7.0*, was used to identify *G. gracilis* proteins that are differentially expressed in response to heat stress. In both Experiment 1 and 2, an average of 555 protein spots were reproducibly visualised on each of the 2D SDS-polyacrylamide gels over the pI range of 4 to 7. In the first experiment (Section 3.4.1), 114 of these proteins were found to be differentially expressed in response to heat stress (*Melanie 7.0*; ANOVA; $P < 0.05$). Of these proteins, 76 were recorded to be up-regulated (49 during thermal tolerance and 27 during the onset of cell death) and 38 were down-regulated (9 during thermal tolerance and 29 during the onset of cell death). Only 2 proteins were identified as de novo proteins (only translated in response to heat stress). Although spot number 1 (Figure 3.2) was initially indicated to be up-regulated in response to heat stress, further separation of the TSP on an 18 % 2D SDS-polyacrylamide gel (Appendices C.2) demonstrated that this protein was actually newly synthesised and occurred in close proximity to another stably expressed protein. These two spots are indistinguishable when visualised on a 12 % polyacrylamide gel.

Owing to financial constraints, only the ten most statistically significant differentially expressed protein spots were selected for further analysis (Figures 3.2). The relative abundance of each of these protein spots after various periods of heat shock (Figure 3.3), compared to the abundance of each protein spot prior to heat shock, was used to elucidate expression patterns using hierarchical clustering. Three distinct expression

profiles were evident (Figures 3.4 and 3.5). In cluster 1, the relative abundance of de novo protein spots (1 and 5) rapidly increased after one hour, peaked between 4 and 6 hours before decreasing steadily after 18 hours of heat stress. In cluster 2, protein spots 7 and 10 increased from already present basal levels, and peaked between 4 and 6 hours of heat stress before decreasing towards basal levels. The relative intensities of the protein spots in cluster 3 remained constant during periods in which the alga exhibited thermal tolerance (Chapter 2), only increasing after 18 and 24 hours during the induction of cell death. Taking these profiles into account, three sampling points: 0 (control), 6 (thermal tolerance) and 24 (onset of cell death) hours were selected for use

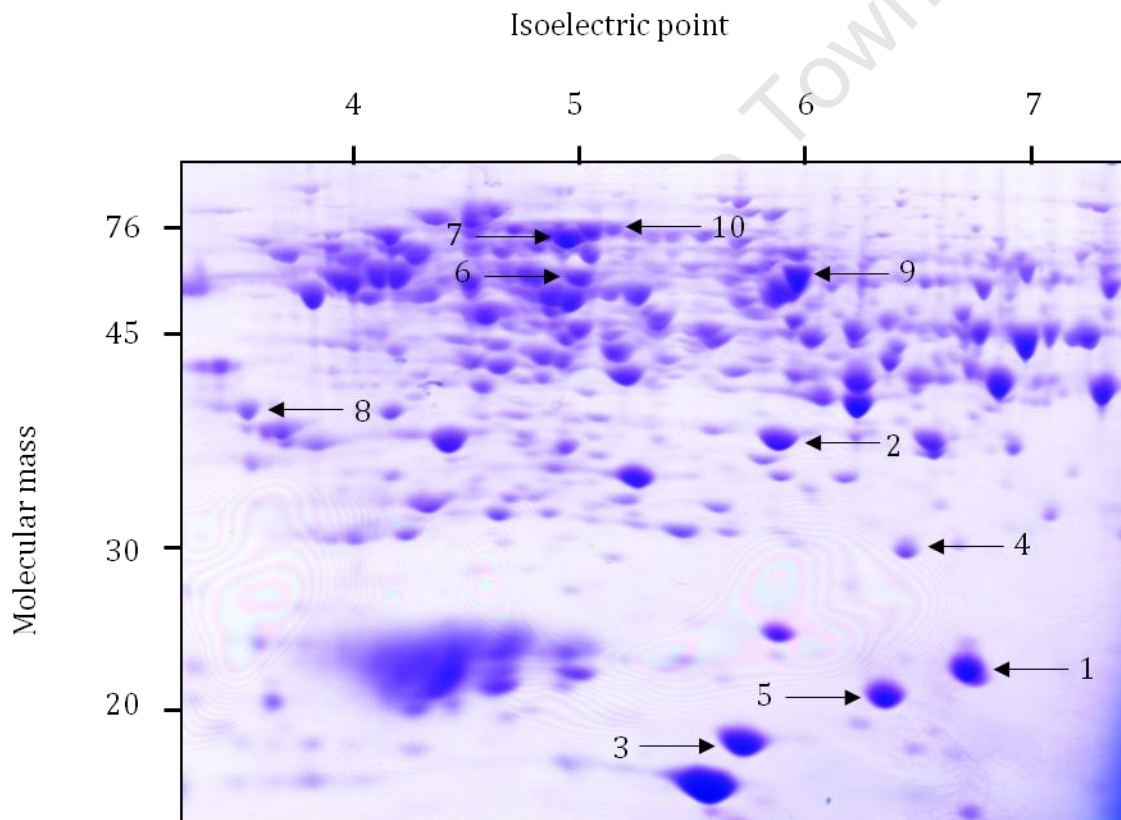


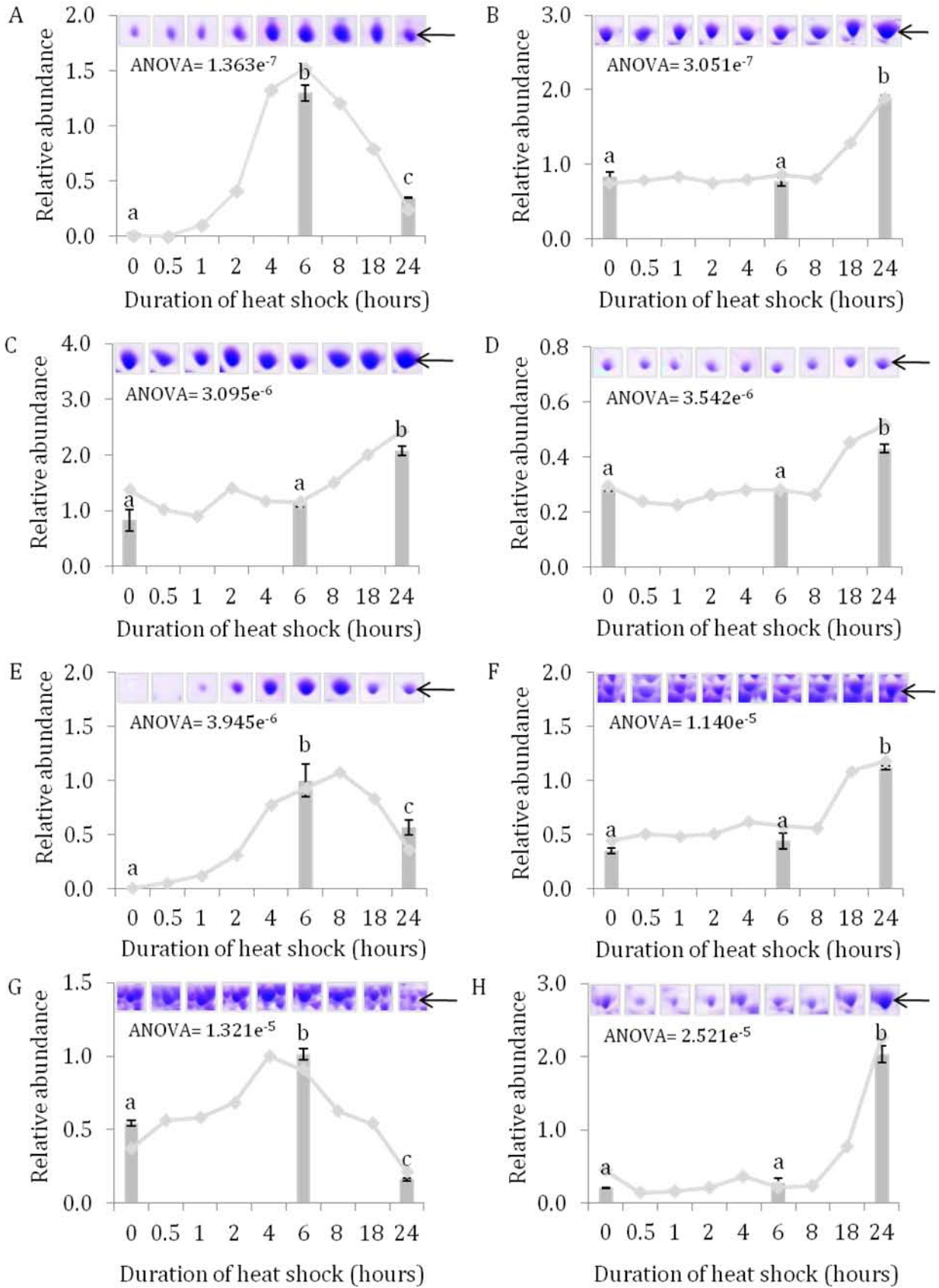
Figure 3.2 A representative image of a coomassie stained total soluble protein profile of *G. gracilis* separated on a 12 % 2D SDS-polyacrylamide gel. Total soluble protein was isolated from a thallus segment heat shocked for 6 hours at 35 °C. The arrows indicate the position of the ten most statistically significant differentially expressed proteins spots indentified over the 24 hour heat shock experiment using the 2D analysis program *Melanie 7.0*.

in Experiment 2 to validate the expression profiles of the preselected protein spots. Agreement between the relative abundance values of each protein spot in Experiments 1 and 2 was taken as validation of differential expression of the protein spots in response to heat stress in *G. gracilis* and confirmed the selection of these protein spots for identification via tandem mass spectrometry.

3.4.3 Putative identification of differentially expressed proteins using tandem mass spectrometry

Although identification of all the protein spots that were differentially expressed in *G. gracilis* in response to heat stress (Section 3.4.2) would have provided the best insight into the mechanisms involved in the induction of thermal tolerance and the onset of cell death in this alga, only the ten most statistically significant differentially expressed protein spots were selected for putative identification using tandem mass spectrometry due to financial constraints. Since the *G. gracilis* genome has not been sequenced, a mass spectrometry-driven BLAST (MS BLAST) search was performed for this study.

Of the 10 protein spots analysed, seven putative identities were obtained: ferredoxin-NADP⁺ reductase, phycocyanin alpha subunit, glyceraldehyde-3-phosphate dehydrogenase, chaperonin GroEL, heat shock protein 70, patatin and transketolase. All seven of the putatively identified protein spots had at least two peptides matching to a homologue in the NCBI database, a protein score of >63 with a significance of $P < 0.05$, and high homology to proteins from plant or algal species (Table 3.1). The unique tryptic peptides obtained for each of the successfully identified protein are reported in Table 3.2 and the alignments of these peptides to their appropriate homologues are presented in Appendix C.3. The three unsuccessfully identified proteins were designated as novel proteins since they had no homology to proteins present in the database.



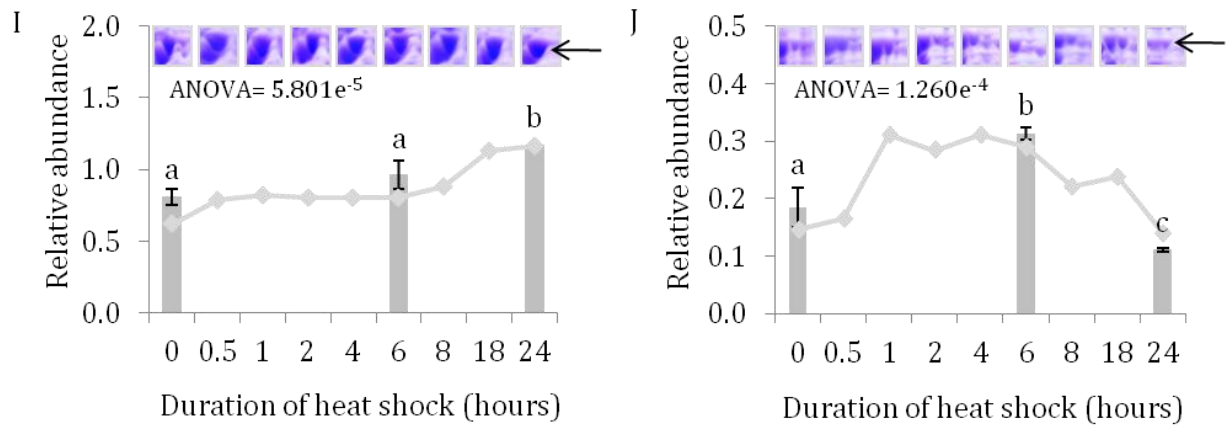


Figure 3.3 Relative abundance of the ten most statistically significant protein spots that were differentially expressed in *G. gracilis* after exposure to heat stress at 35 °C. Protein spots labeled as 1-10 in Figure 3.2 are depicted in panels A-J respectively. The images are representative coomassie stained 2D SDS-polyacrylamide gel images taken from Experiment 1 after the various durations of heat stress. The arrows have been included to indicate the exact spot being assessed. In each panel, the line and bar graph represents the relative abundance of the protein spot from Experiments 1 and 2 respectively. All data represents the mean \pm standard error from three biological repeats where appropriate. The lack of an error bar in some cases is due to overlapping data points. The ANOVA values depict the significance of the differential expression of the protein spot over the time course in Experiment 1 (*Melanie 7.0*), whereas the letters indicate statistical differences in the relative abundance of the protein spots in Experiment 2. In this case, either a parametric one-way ANOVA or a non-parametric Student-Newman-Keuls Test, where data could not be adequately transformed, was performed ($P < 0.05$).

3.4.4 Verification of the putative identifies of the selected proteins

The putative identities of the selected protein spots were verified by comparing the observed molecular weight and isoelectric point of each protein spot on a 12 % 2D SDS-polyacrylamide gel with that of the theoretical molecular weight and isoelectric point of the closest protein homologue obtained from tandem mass spectrometry and database searching. In this study all seven of the putative protein identities were verified using this method of analysis (Table 3.3).

3.4.5 Validation of the putative identifies and expression profiles of the selected proteins

The identities and expression profiles of protein spots obtained from a proteomics investigation should be validated using complementary approaches such as cell biology, physiology and/or biochemical techniques (Jorrín-Novo *et al.*, 2009). Thus, in order to select a number of these protein spots for validation, the hypothetical functions of the proteins were determined using Blast2GO. Of the seven proteins successfully identified using tandem mass spectrometry, four were involved in a stress response and three functioned in metabolic processes (Table 3.3). Thus, the four stress response proteins

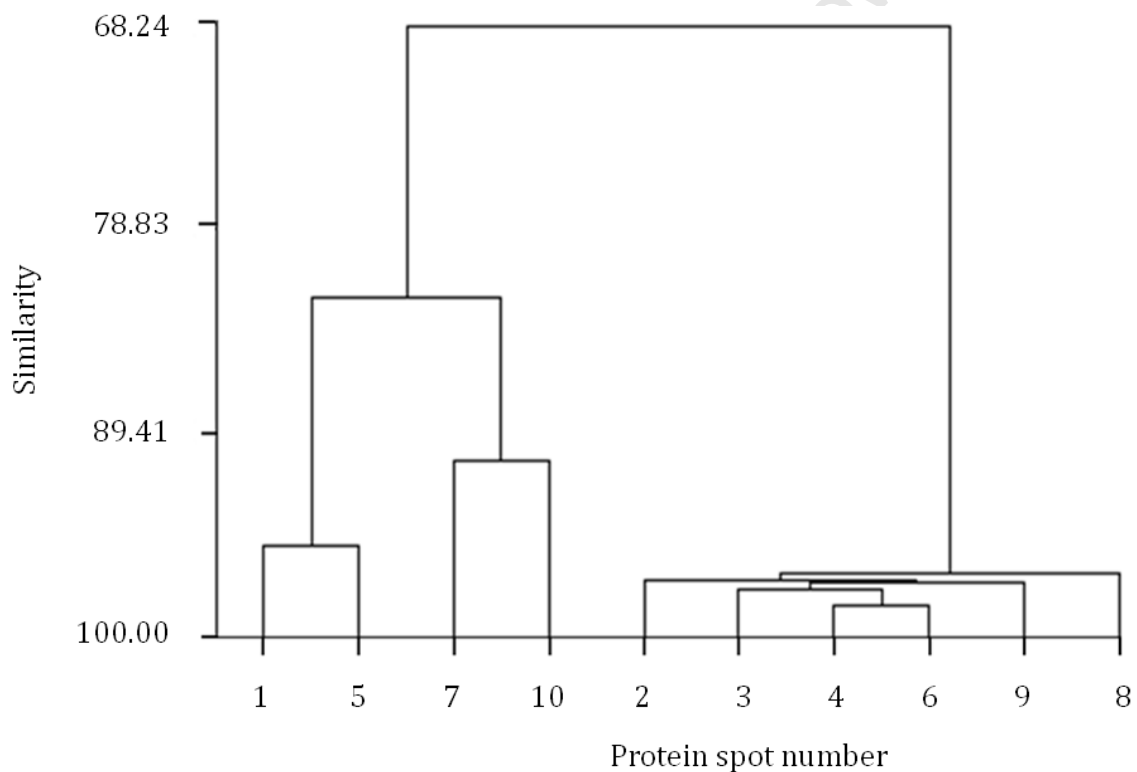


Figure 3.4 Dendrogram demonstrating hierarchical clustering of the expression profiles of the 10 most differentially expressed proteins in *G. gracilis* in response to the 24 hour heat shock experiments (Figure 3.2). Cluster 1 (protein spot numbers 1 and 5) represents a 91.14 similarity level, cluster 2 (protein spot numbers 7 and 10) represents a 90.84 similarity level and cluster 3 (protein spot numbers 2, 3, 4, 6, 8 and 9) represents a 95.23 similarity level.

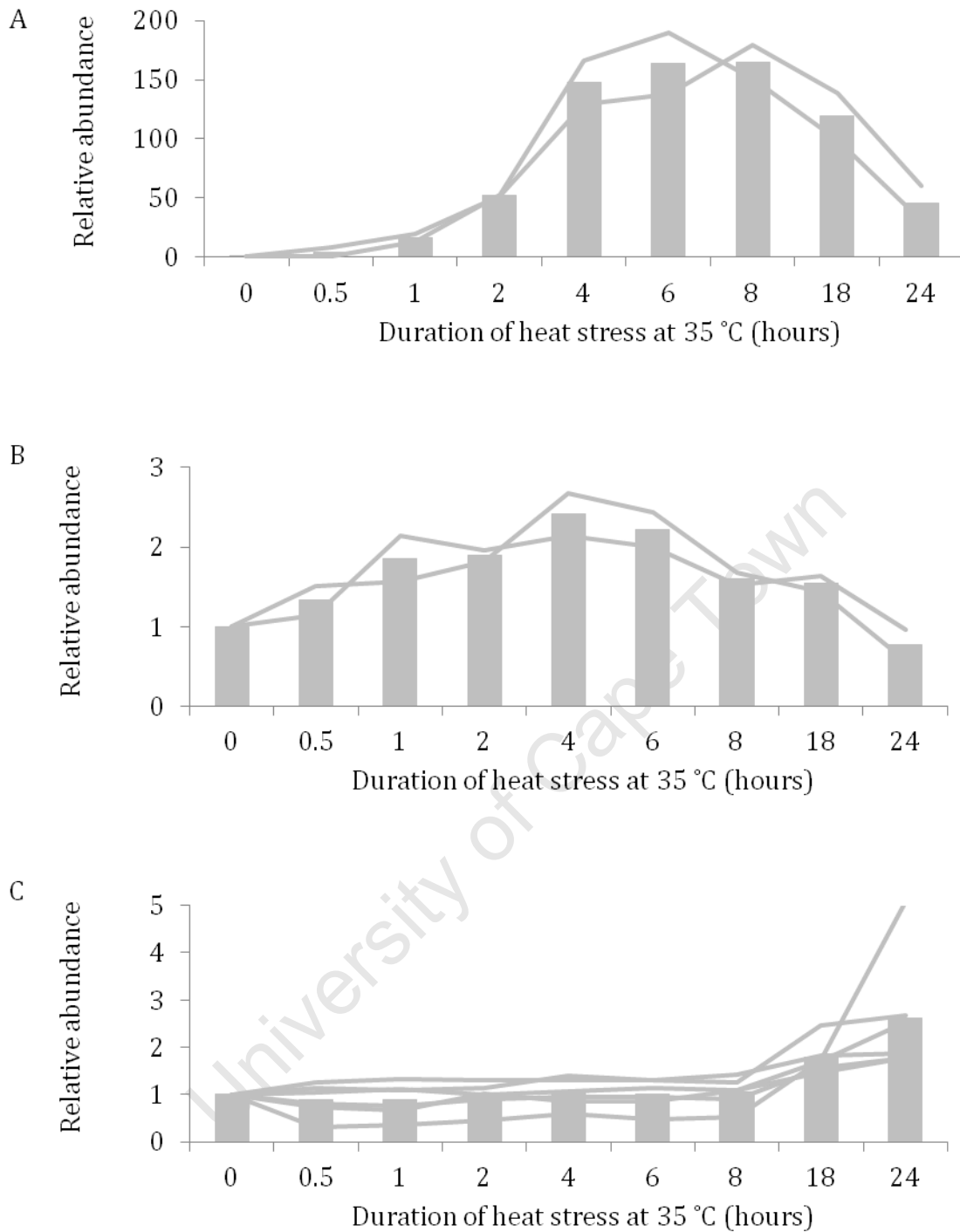


Figure 3.5 Relative abundance of the 10 preselected protein spots following heat shock of *G. gracilis* at 35 °C for various time intervals. Panels A, B and C represent the data obtained from clusters 1, 2 and 3, respectively (Figure 3.4). Each line graph represents the relative abundance of a protein spot from the cluster, whereas the bar graph represents the average relative abundance of all the protein spots in the particular cluster.

Table 3.1 Putative identification of the ten most statistically significant differentially expressed proteins in *G. gracilis* in response to 24 hours of heat shock.

Spot no. ^a	Protein identification	Accession number	Score	Expectation value	No. of unique peptides ^b	Sequence coverage (%) ^c
1	Novel ^d	-	-	-	-	-
2	Ferredoxin NADP ⁺ reductase (<i>Cyanophora paradoxa</i>)	Q00598	174	4.4 ⁻¹¹	4	7.7
3	Phycocyanin alpha subunit (<i>Gracilaria tenuistipitata</i> var. liui)	YP_063694	288	1.7 ⁻²²	3	24.7
4	Glyceraldehyde-3-phosphate dehydrogenase (<i>Gracilaria gracilis</i>)	P30724	558	1.7 ⁻⁴⁹	11	42.8
5	Novel ^d	-	-	-	-	-
6	Chaperonin GroEL (<i>Gracilaria tenuistipitata</i> var. liui)	YP_063549	348	1.6 ⁻²⁸	5	14.8
7	Heat shock protein 70 (<i>Giffithsia japonica</i>)	AAM94003	513	5.1 ⁻⁴⁵	5	30.3 ^e
8	Novel ^d	-	-	-	-	-
9	Patatin (<i>Solanum tuberosum</i>)	P15477	518	1.6 ⁻⁴⁵	7	23.1
10	Transketolase (<i>Nostoc punctiforme</i> PCC 73102)	YP_001867861	155	3.5 ⁻⁹	2	3.1

^a The protein spot identification numbers are equivalent to that in Figure 3.2.

^b The number of unique peptides matching the protein sequence.

^c The percentage of the protein sequence covered by the matching peptides.

^d Novel proteins had no sequence similarity to any of the identified proteins (10 May 2012).

^e Percentage of sequence coverage determined from a partial protein sequence present in the NCBI database.

Table 3.2 Sequence information obtained for the putatively identified protein spots using tandem mass spectrometry and database searching.

Spot no ^a	Protein identification	Homologue	Tryptic peptides identified ^b
2	Ferredoxin NADP ⁺ reductase	Q00598	R.LYSIASTR.H; R.LDYAISR.E; K.MYIQNR.I; K.TVSLSVK.R
3	Phycocyanin alpha subunit	YP_063694	K.TPITEAIASADSQGR.F; R.FLSNGELQSINGR.Y; R.LITGAAQSVYTK.F
4	Glyceraldehyde-3-phosphate dehydrogenase	P30724	K.YDSILGTFDSDVVAGEDSITVDGK.T; R.NPLELPWKEMEIDIVVEATGVFVDAVGAGK.H; R.GTDESTIIDSSLTMVMGDDMLK.V; K.GEGVGTFFVGVNDHLYSHDK.F; K.EEVNGALLK.A; R.GMMTTTHSYTGDQR.L; K.VLDDEFGVVR.G; K.KVLITAPGK.G; R.SAALNIVPTTTGAAK.A; K.AVALVPTLAGK.L; R.VVDLGEVMASQWK
6	Chaperonin GroEL	YP_063549	K.GMDTLVEAVAITLGPK.G; K.TGRPLLI AEDIEKEALATIIVNK.L; K.VGAATETEMR.D; K.ITLIQQELLPILEKVTK.T; K.LRLEDAINATK.A
7	Heat shock protein 70	AAM94003	R.IINEPTAAAIAYGLDR.K; R.TTPSYVAFTDNER.L; K.MKDVAESYLK.E; K.DAGTIAGLNVSRI; R.VEIIANDQGNR.T
9	Patatin	P15477	K.QMLLLSLGTGTNSEFDK.T; K.DSPETYEEALKR.F; R.LAQEDPAFSSIK.S; K.YLLQVLQEK.L; K.TYTAEEAAK.W; R.HSQNNYLR.V; K.GIIPAILEFLEGQLQEVDDNNK.D
10	Transketolase	YP_001867861	K.VTTIIGYGPSPNK.A; R.FLAIDAVEK.A

^a The protein spot identification numbers are equivalent to that in Figure 3.2.

^b Tryptic peptides identified for each of the unique peptide stated in Table 3.1.

Table 3.3 Verification of the putative identification and determination of the theoretical function of the ten most statistically significant differentially expressed proteins in *G. gracilis* in response to 24 hours of heat shock.

Spot no. ^a	Protein identification (Homologue)	Observed M _r (kDa) ^b	Observed isoelectric point (pI) ^b	Theoretical M _r (kDa) ^c	Theoretical isoelectric point (pI) ^c	Biological process ^d
1	Novel ^e	20	7.0	-	-	-
2	Ferredoxin NADP ⁺ reductase	40	6.5	40.491	8.36	Stress response
3	Phycocyanin alpha subunit	15	6.0	17.587	6.57	Metabolic process
4	Glyceraldehyde-3-phosphate dehydrogenase	30	7.0	44.337	7.0	Metabolic process
5	Novel ^e	20	7.0	-	-	-
6	Chaperonin GroEL	55	5.0	56.848	5.80	Stress response
7	Heat shock protein 70	75	5.0	68.081	4.96	Stress response
8	Novel ^e	40	4.0	-	-	-
9	Patatin	50	6.0	42.613	5.47	Stress response
10	Transketolase	75	5.0	72.624	5.77	Metabolic process

^a The protein spot identification numbers are equivalent to that in Figure 3.2.

^b Observed molecular weight and pI was approximated and rounded to the nearest 5 kDa or 0.5 pI respectively. These figures were determined by comparing the position of the protein spot with that of the position of the 2D SDS-PAGE standards on a 12 % 2D SDS-polyacrylamide gel (Bio-Rad; Appendix C.4).

^c Estimated molecular weight and theoretical pI of the protein homologue (web.expasy.org/compute_pi).

^d Functional classification of the proteins as determined using Blast2GO (www.blast2go.org)

^e Novel proteins lacked sequence similarity to proteins currently in the NCBI database and thus theoretical molecule weights and isoelectric points could not be determined.

were selected for validation using 1D and 2D western blot analysis. However, suitable commercially available antibodies were only available for three of these proteins, thus limiting the validation process to three of the four stress response proteins: ferredoxin NADP⁺ reductase (FNR), chaperonin GroEL (GroEL) and heat shock protein 70 (Hsp70).

Therefore, two dimensional western blot analysis was used to validate the identification of the three previously mentioned stress response proteins by successfully matching their position on a coomassie stained 2D SDS-polyacrylamide gel (Figure 3.2) with their position on 2D western blots (Figure 3.6). After confirmation of the identification of these protein spots, 1D western blot analyses were used to further confirm the differential expression of each of these proteins (Figures 3.7, 3.8 and 3.9). In Figure 3.7, an increase in the relative abundance of 40 kDa FNR protein band, after 24 hours of exposure to 35 °C, validated that this protein increased in abundance after the onset of cell death. In Figure 3.8, an increase in the relative abundance of the 60 kDa GroEL, after 24 hours of heat stress, validated that this protein also increased in abundance after the onset of cell death. Whereas, in Figure 3.9, an increase in the relative abundance of the 70 kDa Hsp70 protein band after 6 hours of heat stress, followed by a decrease in the relative abundance of this protein band after 24 hours of heat stress, validated that this protein increased in abundance during thermal tolerance but decreased in abundance after the onset of cell death. These results thus affirmed the differential expression of these three proteins as indicated by two dimensional gel electrophoresis coupled with spot density analyses.

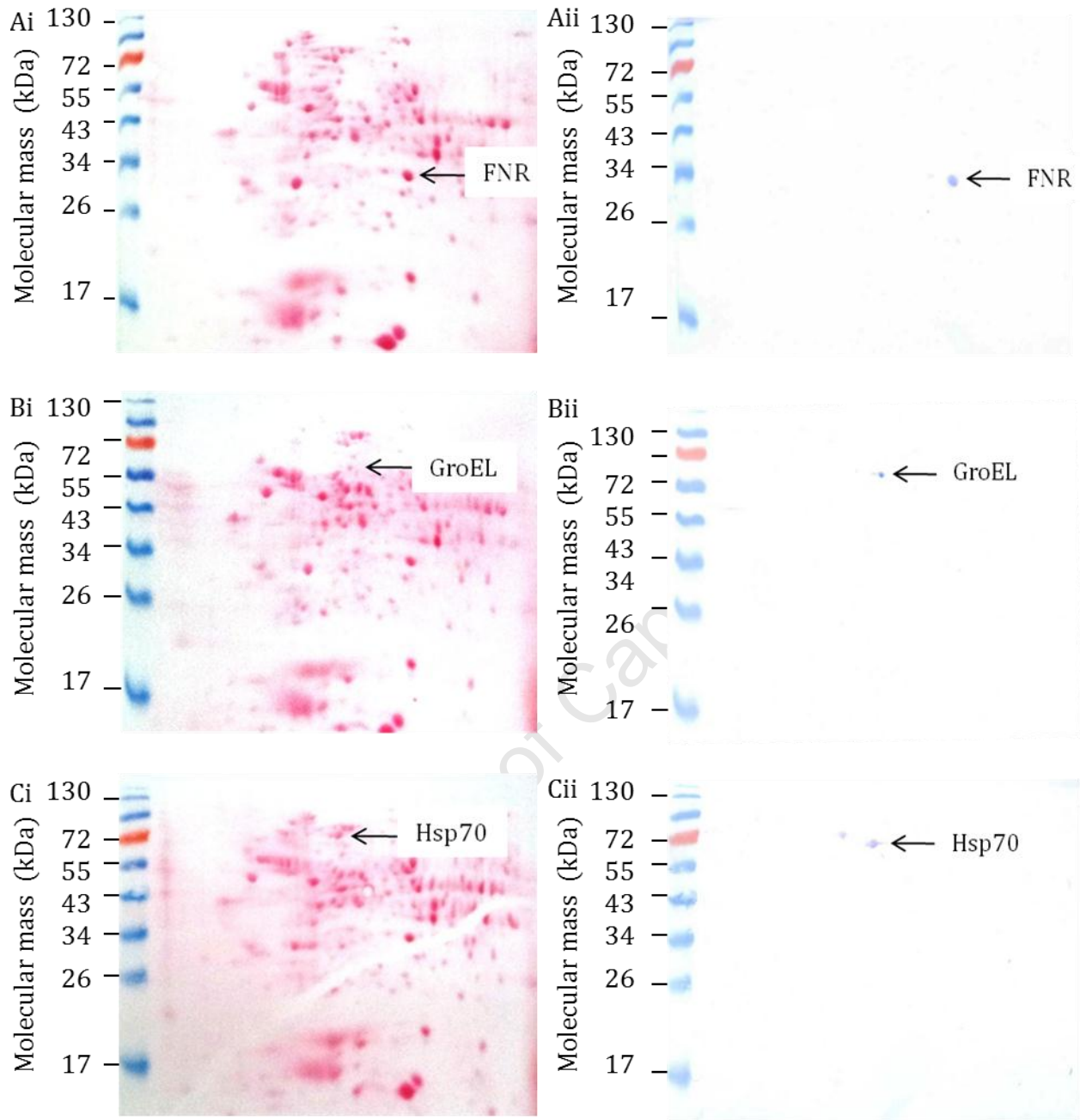


Figure 3.6 Validation of the positioning of (A) ferredoxin NADP⁺ reductase (FNR; spot 2), (B) chaperonin GroEL (GroEL; spot 6) and (C) heat shock protein 70 (Hsp70; spot 7) on a 12 % 2D SDS-polyacrylamide gel using 2D western blot analysis. (i) Ponceau S stains of the membranes prior to western blot analysis, showing the position of the protein spots putatively identified by tandem mass spectrometry. (ii) Position of the protein spot following western blot analysis. The spot numbers in brackets refer to the original identification of these proteins as indicated in Figure 3.2.

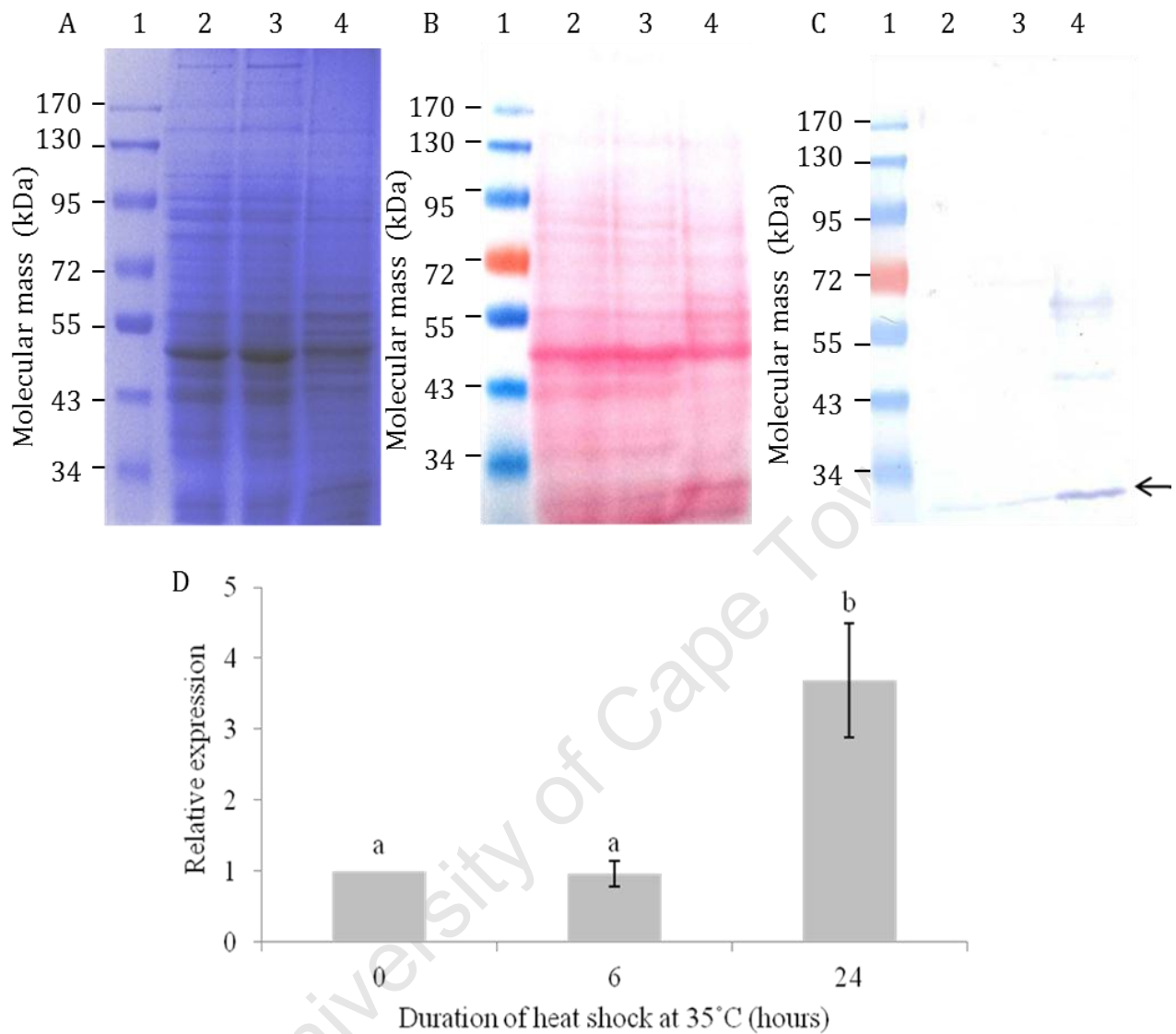


Figure 3.7 Validation of the relative expression of ferredoxin NADP⁺ reductase (FNR) in response to 6 and 24 hours of heat shock at 35 °C. (A) Coomassie stained 1D SDS-polyacrylamide gel, (B) Ponceau S stained nitrocellulose membrane, (C) western blot indicating the FNR band (arrow) and (D) densitometric analysis of the protein's expression. Lane 1: PageRuler™ prestained protein ladder (Fermentas). Lanes 2-4: Total soluble protein isolated from *G. gracilis* thalli heat stressed for 0 (control), 6 and 24 hours, respectively. In D, a parametric one-way ANOVA detected statistically significant differences in the relative expression of FNR between the control (0 hours of heat stress) and experimental samples ($P < 0.005$; denoted by lettering). Data represents the mean \pm standard error from three biological repeats.

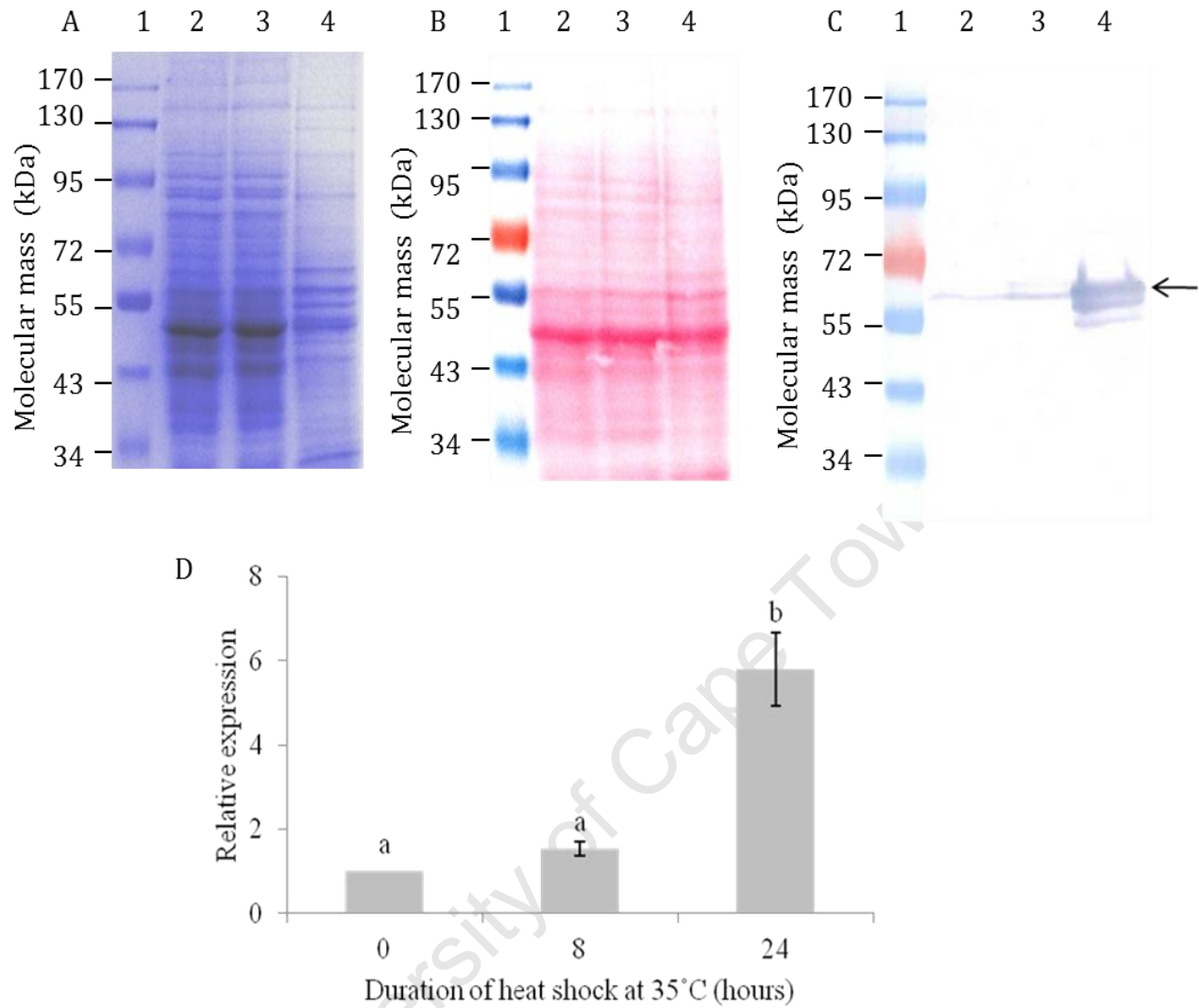


Figure 3.8 Validation of the relative expression of chaperonin GroEL (GroEL) in response to 6 and 24 hours of heat shock at 35 °C. (A) Coomassie stained 1D SDS-polyacrylamide gel, (B) Ponceau S stained nitrocellulose membrane, (C) western blot indicating the GroEL band (arrow) and (D) densitometric analysis of the protein's expression. Lane 1: PageRuler™ prestained protein ladder (Fermentas). Lanes 2-4: Total soluble protein isolated from *G. gracilis* thalli heat stressed for 0 (control), 6 and 24 hours, respectively. In D, a parametric one-way ANOVA detected statistically significant differences in the relative expression of GroEL between the control (0 hours of heat stress) and experimental samples ($P < 0.005$; denoted by lettering). Data represents the mean \pm standard error from three biological repeats. Data was \log_{10} transformed prior to analysis to meet the requirements of parametric statistical testing.

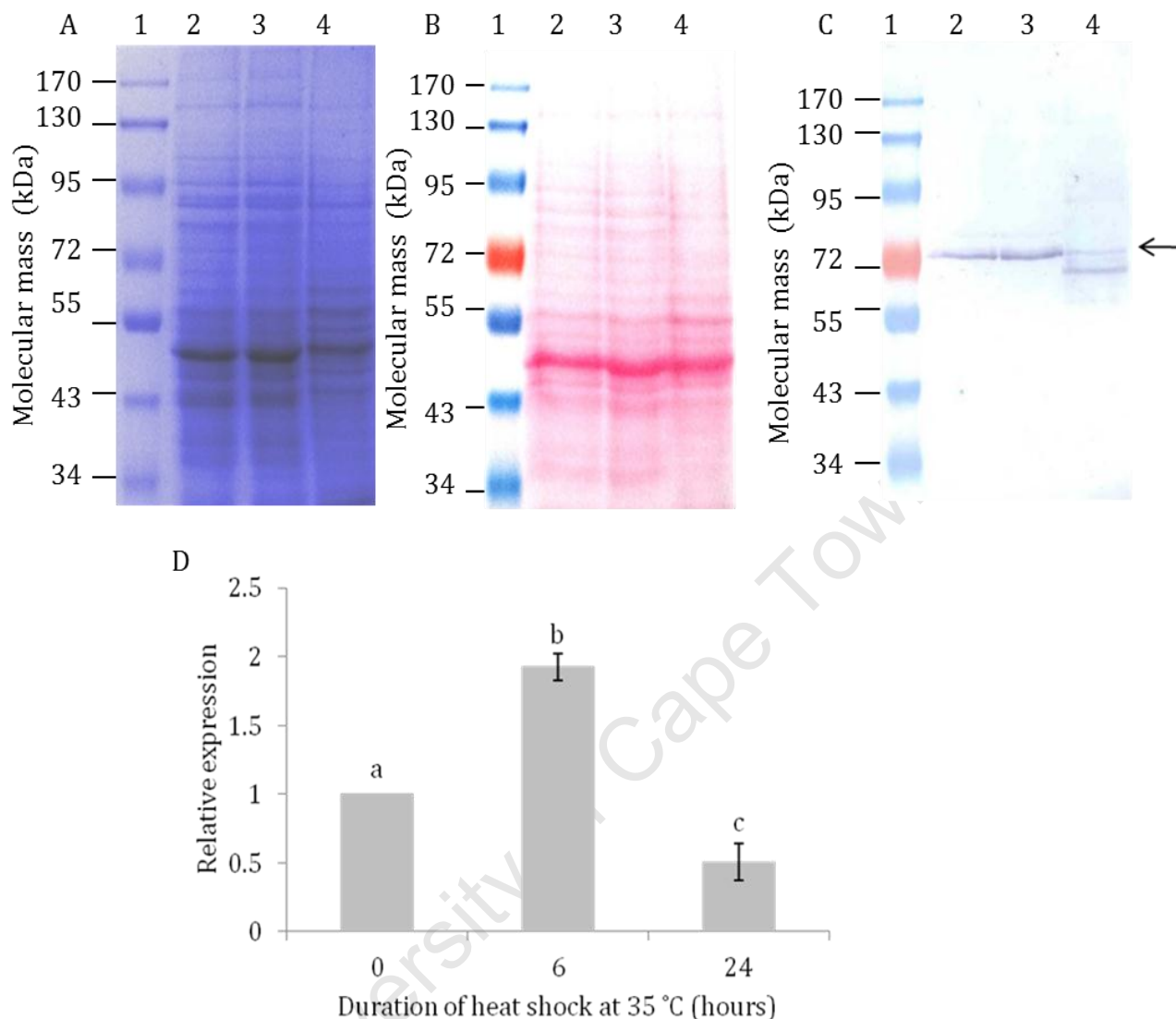


Figure 3.9

Validation of the relative expression of Hsp70 in response to 6 and 24 hours of heat shock at 35 °C. (A) Coomassie stained 1D SDS-polyacrylamide gel, (B) Ponceau S stained nitrocellulose membrane, (C) western blot indicating the Hsp70 band (arrow) and (D) densitometric analysis of the protein's expression. Lane 1: PageRuler™ prestained protein ladder (Fermentas). Lanes 2-4: Total soluble protein isolated from *G. gracilis* thalli heat stressed for 0 (control), 6 and 24 hours, respectively. In D, a parametric one-way ANOVA detected statistically significant differences in the relative expression of Hsp70 between the control (0 hours of heat stress) and experimental samples ($P < 0.005$; denoted by lettering). Data represents the mean \pm standard error from three biological repeats. Data was \log_{10} transformed prior to analysis to meet the requirements of parametric statistical testing.

3.5 Discussion

Proteins are the functional components of cells, constantly changing in response to stimuli and facilitating the adaptation of an organism to an ever changing environment (Anderson and Anderson, 1996; Shepard *et al.*, 2000; Brumbarova *et al.*, 2008). Thus proteomics, the study of the entire protein complement expressed by the genome (Jung *et al.*, 2000), has become a powerful tool for analysing and interpreting changes in the proteome which can further elucidate the biological state and cellular response of an organism to a particular stimulus (Monti *et al.*, 2005; Brumbarova *et al.*, 2008; Contreras *et al.*, 2008; Jorrín-Novo *et al.*, 2009).

In this study, a comparative proteomic approach employing 2D gel electrophoresis coupled with mass spectrometry was used to separate out a complex mixture of total soluble proteins and help identify those that were differentially expressed in *G. gracilis* in response to heat stress (López *et al.*, 2001; Neilson *et al.*, 2010). Although highly effective, this technique is not always practical as it is expensive to perform large experiments owing to the three biological and three technical repeats recommended for the interpretation of these results (Jorrín-Novo *et al.*, 2009). This was the case in this study as the devised heat shock experiment consisted of nine time points (Chapter 2), requiring a total of 81 2D SDS-polyacrylamide gels. Thus, in order to minimise the cost of performing a single large experiment, an alternative strategy employing two smaller experiments was devised that only required a total of 45 2D SDS-polyacrylamide gels (Figure 3.1).

Using this dual experimental approach a number of differentially expressed proteins were identified. Of the 555 protein spots reproducibly detected on each 2D SDS-polyacrylamide gel, 114 were differentially expressed, with 76 up-regulated and 38 down-regulated in response to heat stress. Further investigation demonstrated that these proteins were not

constantly up or down-regulated throughout the entire time course of the heat shock, but were more specifically correlated to a specific biological state of the alga. Thus, 49 of these proteins were up-regulated and 9 down-regulated during thermal tolerance, while a different subset of proteins, comprising an additional 27 up-regulated and 29 down-regulated proteins, were differentially expressed during the onset of cell death. This said, some overlap did occur, as a number of the proteins which were hypothesised to help maintain thermal tolerance in *G. gracilis* in response to heat stress, were still abundant during the onset of cell death when compared to the levels present in control thalli. Although the levels of these proteins were decreasing rapidly during cell death, the abundance of these proteins was still higher than that present in control thalli. This was hypothesised to be due to the time required to degrade proteins after their synthesis has been halted (Stein and Hansen, 1999).

Even though it would have been preferable to identify the majority of the differentially expressed protein spots and to determine their biological function in heat stressed *G. gracilis*, financial constraints limited this investigation to ten proteins. Thus the ten most statistically significant, differentially expressed proteins were selected for further investigation to gain an initial insight into the pathways involved in eliciting thermal tolerance and the onset of cell death in *G. gracilis*.

Of the 10 proteins selected for sequencing using tandem mass spectrometry and database searching, 3 proteins were designated to be novel due to their lack of sequence homology to any characterised proteins in the database. Re-analysis of the regulation of these proteins did however indicate that two of them were de novo only present in thalli exposed to heat stress, suggesting that these proteins function in eliciting thermal tolerance in *G. gracilis*. The third unidentified protein rapidly increased in abundance after thallus segments were exposed to 35 °C for 18 hours. This protein was thus hypothesised to function in the onset of cell death rather than maintaining thermal tolerance in this species.

The remaining seven proteins were putatively identified using tandem mass spectrometry. After which, the putative identities of the proteins were validated by comparing the observed and theoretical molecular weights and isoelectric points of each protein, and the biological processes in which they are involved as determined using Blast2GO.

Four proteins, namely ferredoxin NADP⁺ reductase, chaperonin GroEL, heat shock protein 70 and patatin, were identified to be involved in the stress response, while three proteins spots, putatively identified as phycocyanin alpha subunit, glyceraldehyde-3-phosphate dehydrogenase and transketolase, functioned in metabolic processes. The putative role of each of these proteins in heat stressed *G. gracilis* is discussed below:

3.5.1 Proteins directly involved in the stress response

Proteins involved in stress responses have traditionally been reported to be involved in stabilisation of the cellular environment by maintaining the basic structure and physiological integrity of the cell (Jiang *et al.*, 2007). These proteins are not only involved in stress tolerance but may also influence the balance between survival and the onset of cell death, two pathways which are closely linked by continuous regulatory cross-talk (Jolly and Morimoto, 2000).

Both heat shock protein 70 (Hsp70) and chaperonin GroEL (GroEL) are functionally classified as molecular chaperones, a group of unrelated proteins which assist in the stabilisation, assembly and disassembly of proteins (Ellis, 1993; Morano and Thiele, 1999; Jolly and Morioto, 2000; Hall, 2002; Periago *et al.*, 2002). Even though tandem mass spectrometry did not distinguish between the constitutive and inducible isoforms of these proteins, their increased expression in response to heat stress suggests that these proteins are both the inducible isoforms. Previous studies have reported that inducible Hsp70s have

been associated with the short-term survival of an organism in response to heat stress by restoring proteomic damage (Lee and Schöffl, 1996; Miernyk, 1999; Sarto *et al.*, 2000; Periago *et al.*, 2002) and impeding the apoptotic pathway at several levels (Jolly and Morioto, 2000). Thus the accumulation of Hsp70 in *G. gracilis* during thermal tolerance promotes cell survival whereas the degradation of this protein accelerates cell death. Unlike the expression of Hsp70 however, the expression of GroEL remained stable throughout the period of thermal tolerance and then increased rapidly after 18 hours of heat shock, after the onset of cell death. Therefore it is hypothesised that rapid accumulation of GroEL in *G. gracilis* does not aid in thermal tolerance but rather promotes the onset of cell death. Indeed, Hsp60 (GroEL homologue) has been reported to increase the rate of apoptosis by accelerating caspase activation in humans (Bukau and Horwich, 1998; Samali *et al.*, 1999; Jolly and Morimoto, 2000), thus substantiating the possible role of GroEL in inducing cell death in *G. gracilis*.

Expression of ferredoxin NADP⁺ reductase (FNR) and patatin remained stable during the period of thermal tolerance and increased rapidly after the onset of cell death. FNR is a flavoenzyme that catalyses the reversible electron transfer between NADP(H) and ferredoxin (Girardini *et al.*, 2002; Palatnil *et al.*, 2003; Medina and Gómez-Moreno, 2004). This enzyme has been implicated in cellular defense against oxidative damage by maintaining NADP⁺/NADPH homeostasis (Carrillo and Vallejos, 1987; Krapp *et al.*, 1997). During cell death however, Girardini *et al.* (2002) and Lui and Chen (2002) have reported that an up-regulation of FNR was hypothesised to induce apoptosis in response to DNA damage. Patatins, on the other hand, are a group of storage proteins that demonstrate nonspecific lipid acyl hydrolase activity which cleaves fatty acids from membranes (Sahsah *et al.*, 1998). This protein has been hypothesised to not only produce signaling molecules during stress (Strickland *et al.*, 1995; Holk *et al.*, 2002; Banerji and Flieger, 2004), but has also been reported to facilitate the destabilisation of cellular membranes during cell death (Shewry, 2003). Thus it is highly likely that these proteins play a role in cell death in *G. gracilis*.

As this study is focused on working towards a better understanding of the biological response of *G. gracilis* to heat stress, three of the four stress responsive proteins to which commercially available antibodies were available were investigated further in order to validate their putative identities and expression profiles. Two dimensional western blot analysis was successfully used to confirm the identification of the protein spots, while 1D western blot hybridisation confirmed the expression profiles of the proteins in response to heat stress. However, a number of additional bands were visible on each of the 1D western blot. Western blot analysis using anti-FNR antibodies resulted in the presence of a single band after 0 and 6 hours of heat shock, whereas two high molecular weight bands were observed after 24 hours. It has been well documented that FNR aggregates into dimers and larger oligomers which appear as high molecular weight bands on SDS-PAGE gels (Matthijs *et al.*, 1987). A study by Lazazzera *et al.* (1996) reported the presence of a single FNR protein band during periods without stress, whereas the presence of oligometric forms was observed during severe cell stress in *E.coli*. The authors hypothesised that high molecular weight oligometric forms of FNR may activate the binding of FNR to DNA, thus functioning as a transcription factor and facilitating the synthesis of additional proteins.

GroEL was visualised as a triplet after 24 hours of heat stress, during the onset of cell death. Similar GroEL doublets/triplets (between 53 to 64 kDa) have been observed on immunoblots in a number of studies (Allan *et al.*, 1988; Hoffman *et al.*, 1989; Michel, 1993). Wu *et al.* (1993) and Lin *et al.* (2007) hypothesised that the presence of multiple GroEL bands on an SDS-PAGE may either represent posttranslational modifications or the various formations of the two characteristic heptameric rings of this protein in association with the small accessory protein GroES (Schumann and Ferreira, 2004; Horst *et al.*, 2005).

Similarly, low molecular weight Hsp70 bands were detected after 24 hours of heat stress. An additional ~20 kDa degradation product could also be observed after 24 hours if the protein was separated on an 8 % polyacrylamide gel (Appendix C.5). The appearance of

similar sized Hsp70 degradation products have been previously reported and are not unexpected during the onset of cell death (Ireland *et al.*, 2004; Ruchalski *et al.*, 2006; Wang *et al.*, 2010).

3.5.2 Proteins involved in metabolic processes

Metabolic processes are those pathways involved in the growth, reproduction and maintenance of cellular structures (Garrett and Grisham, 2005; Seager and Slabaugh, 2008; Engel, 2009). Expression of proteins involved in these processes is therefore constantly changing in response to stimuli. In this study, three *G. gracilis* proteins differentially expressed in response to heat stress function in metabolic processes. The abundance of transketolase increased during thermal tolerance, while glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and phycocyanin increased during the onset of cell death after 18 hours of heat shock.

Transketolase is a thiamine dependent enzyme involved in the non-oxidative branch of the pentose phosphate pathway (PPP). It is responsible for generating reducing equivalents which are essential for the production of ribose required for nucleic acid synthesis (Alexander-Kaufman and Harper, 2009). During cell stress however, this enzyme links the PPP to glycolysis, increasing the production of cytosolic NADPH (Slekar *et al.*, 1996; Alexander-Kaufman and Harper, 2009). This reducing agent is required for the elimination of harmful reactive oxygen species by detoxification enzymes during oxidative stress (Tunc-Ozdemir *et al.*, 2009). Thus increased abundance of transketolase during thermal tolerance may facilitate detoxification of harmful reactive oxygen species in *G. gracilis*.

The expression of GAPDH and phycocyanin remained constant during thermal tolerance and was up-regulated after the onset of cell death. GAPDH has several functions (Nicholls *et*

al., 2011; Tristan *et al.*, 2011). In addition to catalysing the sixth step of glycolysis which aids in the breakdown of glucose into ATP and pyruvate, GAPDH has been reported to induce pro-apoptotic mitochondrial membrane permeabilisation (Tarze *et al.*, 2007). Hara *et al.* (2005) and Tristan *et al.* (2011) further hypothesised that S-nitrosylation of GAPDH during oxidative stress allows binding to an E3 ubiquitin ligase which mediates translocation of this stable complex into the nucleus where it facilitates nuclear protein degradation and the induction of apoptosis. Phycocyanin is a light harvesting biliprotein which captures light energy which is transferred to chlorophyll during photosynthesis. It is reported to have antioxidant and radical scavenging properties (Reddy *et al.*, 2003). Studies by Reddy *et al.* (2003) and Subhashini *et al.* (2004) suggest that elevated levels of phycocyanin from the blue-green alga *Spirulina platensis* induces apoptosis in both the mouse macrophage cell line RAW 264.7 stimulated with LPS, and the human chronic myeloid leukemia cell line-K562 via the cytochrome C pathway which activates caspases. Thus increased expression of these proteins may facilitate the onset of cell death in heat shocked *G. gracilis*.

This study indicated that, from a total of 555 reproducible protein spots detected on each 2D SDS-polyacrylamide gel, 114 were differentially expressed in response to heat stress, resulting in the maintenance of thermal tolerance of this species at 35 °C for the first 8 hours of heat stress, and was followed by the onset of cell death after 18 hours. Further analysis identified seven of the differentially expressed proteins and indicated how these proteins facilitate the biological state of the organism during heat stress. Although further research is required to decipher the intricate molecular mechanisms that enable *G. gracilis* to survive heat stress via thermal tolerance and induce cell death, this study has examined some of these proteins and their molecular functions. Consequently, this research has identified a number of stress response proteins which may prove useful as possible molecular indicators of heat stress in *G. gracilis* in future studies.

CHAPTER 4

IDENTIFICATION OF A POTENTIAL MOLECULAR INDICATOR OF HEAT STRESS IN *GRACILARIA GRACILIS*

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4.1 Summary

In this chapter, a potential molecular indicator of heat stress was identified in cultivated *G. gracilis*. Thus, a heat shock protein 70 (Hsp70) was sequenced using traditional PCR and the rapid amplification of cDNA ends techniques. A full length 2177 bp cDNA sequence was obtained, consisting of a single open reading frame of 1986 bp, flanked by a 98 bp 5'- and a 93 bp 3'-terminal untranslated region. The open reading frame encoded for a 662 amino acid residue protein with a hypothetical molecular mass of 72.577 kDa. Blastx analysis inferred that the protein shared high similarity to a number of plant and algal Hsp70s localised to the endoplasmic reticulum (ER). Further sequence and phylogenetic analyses validated the identification of this protein as a putative ER Hsp70 from *G. gracilis*.

Transcriptional regulation of the ER *Hsp70* was examined after the alga was exposed to 35 °C for various durations, using real-time PCR. Thus, the abundance of ER *Hsp70* transcript increased rapidly within the first hour of heat stress, after which expression of the gene decreased to basal levels over the next five hours. This result indicated that the ER *Hsp70* gene could potentially be used as a molecular indicator of heat stress in *G. gracilis* in future experimental studies.

4.2 Introduction

In Chapter 3, a comparative proteomic approach was used to identify proteins that are differentially expressed in *G. gracilis* in response to heat stress. Of the seven proteins successfully identified, one was classified as a member of the HSP70 multigene family. This highly conserved family has been extensively researched and reported to help maintain the homeostasis of a cell under both stressed and non-stressed conditions (Song *et al.*, 2006). The vital roles of these proteins as molecular chaperones which include folding nascent polypeptides, assembling of protein complexes, translocation of proteins across membranes and facilitating in the degradation of damaged proteins (Harlt, 1996; Song *et al.*, 2006; Reddy *et al.*, 2010) are essential for survival during exposure to stressors. Thus this multigene family was selected for investigation as a potential molecular indicator of heat stress in *G. gracilis*.

The HSP70 family consists of a number of proteins (Ingolia *et al.*, 1980), classified according to (i) cellular localisation and (ii) expression patterns (Reddy *et al.*, 2010). Although a specific cellular localisation is not essential for defining a potential molecular indicator, it is important that an inducible heat shock protein and not a heat shock cognate protein is selected. Thus the first objective of this study was to sequence and characterise an *Hsp70* from *G. gracilis*.

After classification, the second objective of this study was to validate the induction of the *Hsp70*'s expression, in response to heat stress, using real-time PCR. As *Hsp70*s are rapidly induced, whereas *Hsc70* expression remain relatively constant in response to heat stress (Reddy *et al.*, 2010), an increase in abundance of the gene transcript would not only validate the classification of this protein as a heat shock protein, but also identify it as a

potential molecular indicator of heat stress in *G. gracilis* for use in future experimental studies.

4.3 Materials and Methods

All media and solutions used in this study are listed in Appendix A.

Experimental data was obtained from three biological repeats (each consisting of three technical repeats) unless otherwise stated. In the context of this study, biological and technical repeats were defined as data obtained from algal samples exposed to the same environmental conditions but acquired from either triplicate or the same experiment respectively.

4.3.1 Preparation of *G. gracilis* for experiments

4.3.1.1 Sample acquisition and maintenance conditions

Gracilaria gracilis was obtained every fortnight from the Irvin & Johnson Abalone Culture Division, Danger Point, South Africa. Healthy thalli were selected and briefly rinsed in distilled water to remove visible epiphytes. Cleaned thallus segments were maintained at 15 °C for a minimum of 2 weeks prior to acclimatisation (Section 4.3.1.2) in 45 l tanks fitted with a flow-through system filtering seawater at a rate of 1 l/min. Water movement was achieved by pumping compressed air through plastic airlines and the alga was illuminated by cool white fluorescent tubes at $\sim 45 \mu\text{mol photons m}^{-2} \text{s}^{-1}$ with a 16-hour light: 8-hour dark cycling photoperiod. During this maintenance period, both the alga and the tanks were cleaned twice a week with filtered seawater to remove any accumulated sedimentation.

4.3.1.2 Acclimatisation conditions

After maintenance (Section 4.3.1.1), *G. gracilis* was acclimatised at 15 °C for 10 days in an Adaptis A350 Multi-Application Growth Chamber (Convion®). Similarly structured thallus segments were incubated in conical flasks at a ratio of 1 g thalli: 200 ml precooled artificial seawater (ASW; Appendix A.1.1) supplemented with PES-enriched seawater medium to achieve a ½ strength growth medium (Appendix A.2.2.1; enriched ASW). Sufficient water circulation was obtained by pumping compressed air through plastic tubing and the seaweed was illuminated at ~45 µmol photons m⁻² s⁻¹ by cool white fluorescent tubes with a 16-hour light: 8-hour dark cycling photoperiod. Every 2-3 days, *G. gracilis* was rinsed briefly in enriched ASW and transferred to flasks containing freshly prepared media.

4.3.2 Heat shock experiment

A single heat shock experiment was conducted in this chapter to determine the effects of various durations of heat shock at 35 °C on *G. gracilis*. When starting an experiment, *G. gracilis* was transferred directly from the acclimatisation conditions of 15 °C (Section 4.3.1.2) into prewarmed enriched ASW in a temperature controlled horizontal orbital shaker incubator (Dynamic & Evolution International Corporation). Alga was added to flasks containing the media at a ratio of 1 g thalli: 200 ml of enriched ASW. Flasks were shaken at 75 rpm to ensure adequate water movement and illuminated at ~45 µmol photons m⁻² s⁻¹ using a 16-hour light: 8-hour dark cycling photoperiod for the duration of each experiment. Thus, thallus segments were heat shocked for either 0 (omission of heat stress; control), 0.5, 1, 2, 4, 6, 8, 18 and 24 hours. After heat shock, thalli were removed from the flasks, frozen in liquid nitrogen and stored at -80 °C until required.

4.3.3 Sequencing of a putative ER *Hsp70* gene from *G. gracilis*

4.3.3.1 Preparation of template DNA

Genomic DNA (gDNA) and RACE-Ready complementary DNA (cDNA) were isolated in this study for use as template for the amplification of *Hsp70* gene fragments from *G. gracilis*.

4.3.3.1.1 Genomic DNA

Genomic DNA was isolated from an acclimatised *G. gracilis* thallus segment (Section 4.3.1.2; Appendix B.17) and quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop 1000 software; version 3.7.1; Thermo Scientific). Absorbance readings were determined at 260 nm where one unit of absorption at 260 nm represents 50 µg ml⁻¹ of DNA. The ratio of sample absorbencies at 260: 280nm and 260: 230 nm were used to assess the purity of the DNA samples. Values of ~2.0 indicated the isolation of pure DNA without the co-precipitation of contaminants. Post quantification, 1 µg of gDNA was electrophoresed on a 1.5 % TAE-agarose gel (Appendix B.18) together with a *Pst* I-digested lambda DNA molecular weight marker (Appendix B.19) and visualised using a short wavelength UV light box (Bio-Rad GelDoc EQ-system™; Bio-Rad). An image was captured using the Quantity One® Software (version 1.7; Bio-Rad) and assessed. Genomic DNA which possessed good structural integrity and displayed no signs of co-precipitated contaminants was aliquoted and stored at 4 °C until required.

4.3.3.1.2 5'- and 3'-RACE-Ready complementary DNA prepared from total RNA

5'- and 3'-RACE-Ready complementary DNA (cDNA) was prepared using the SMART™ RACE cDNA Amplification Kit (Clontech laboratories) as described in the manual. As this kit

required freshly isolated RNA from tissue hypothesised to obtain high concentrations of the transcript to be amplified, total RNA was isolated from 100 mg tissue obtained from a single thallus segment which had been heat shocked at 35 °C for 2 hours, using the peqGOLD Plant RNA Kit (peqGOLD; Appendix B.20). After isolation, total RNA was treated with DNase I (Appendix B.21) and quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop 1000 software; version 3.7.1; Thermo Scientific). Absorbance readings were taken at 260 nm where one unit of absorption at 260 nm represents 40 µg ml⁻¹ of total RNA. The ratio sample absorbencies at 260: 280nm and 260: 230 nm were used to assess the purity of the RNA samples. Values of ~2.0 demonstrated the isolation of pure RNA without the co-precipitation of contaminants. After quantification, 1 µg of total RNA was electrophoresed on a 1.2 % formaldehyde-agarose gel (Appendix B.22) and visualised using a short wavelength UV light box (Bio-Rad GelDoc EQ-system™; Bio-Rad). An image of the total RNA was captured using the Quantity One® Software (version 1.7; Bio-Rad) and analysed for signs of degradation. Total RNA with a good structural integrity was immediately used for the production of 5'- and 3'-RACE-Ready cDNA.

4.3.3.2 Amplification of a putative *Hsp70* gene from *G. gracilis*

Traditional polymerase chain reaction (PCR) and the rapid amplification of cDNA ends (RACE) were used to amplify the full length coding region of a putative *Hsp70* gene from *G. gracilis*.

4.3.3.2.1 Primer design

Nine primers were used for the amplification of an *Hsp70* gene from *G. gracilis* (Appendix B.23.1). Degenerate primers F1 and R1 were designed by David Rautenbach for his study in

which he amplified a partial *Hsp70* sequence from *Haliotis midae* (unpublished; available from the Marine Biotechnology Unit, University of Cape Town). The additional 7 primers were designed in this study using the FastPCR software package (Kalendar *et al.*, 2009). Two types of primers were designed; the degenerate primer R2 was designed from a multiple sequence alignment of *Hsp70* protein sequences from the rhodophyta species *Cyanidioschyzon merolae* and four chlorophyta species *Cherffelia dubia*, *Chlamydomonas reinhardtii*, *Ostreococcus lucimarinus*, and *Ostreococcus tauri* using ClustalX (version 1.81; Thompson *et al.*, 1997; Appendix C.6). The 6 sequence specific primers F2–F5 and R3–R4 were designed from nucleic acid sequence information obtained in this study from *G. gracilis*. Thus, sequence specific primers F2 and F3 were designed from the sequenced PCR product amplified using F1 and R1. The sequence specific primers F4, F5, R3 and R4 were designed from the consensus DNA sequence produced in DNAMAN (version 4.13; Lynnon Biosoft) from a multiple sequence alignment of the three PCR products amplified with the primer sets F1 and R1, F2 and R2, and F3 and R2.

4.3.3.2.2 Amplification Reactions

Seven PCR products were amplified to obtain the full length sequence of a putative *Hsp70* gene from *G. gracilis* (Figure 4.1). Two amplification protocols were used. To amplify the three overlapping, internally located regions of the *Hsp70* gene, traditional PCR was employed using the primer set combinations F1 and R1, F2 and R2 and F3 and R2 (Appendices B.23.1 and B.24.1). To amplify the 5'- and 3'-ends of the gene the amplification of cDNA ends was performed using the primers F4, F5, R3 and R4 (Appendix B.23.1 and B.24.2).

4.3.3.3 Purification of amplified *Hsp70* gene fragments

After amplification, the *Hsp70* gene fragments were electrophoresed on a 1.5 % TAE-agarose gel (Appendix B.18) and visualised using a long wavelength UV light box (Bio-Rad GelDoc EQ-system™; Bio-Rad). For each reaction, the PCR product band, of the expected size, was excised from the gel and extracted using the Biospin Gel Extraction Kit (Bioer Technology Co.). Each purified DNA product was quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop 1000 software; version 3.7.1; Thermo Scientific) and stored at 4 °C until use.

4.3.3.4 Ligation of the purified *Hsp70* gene products into cloning vectors

The purified *Hsp70* gene products (Section 4.3.3.3) were ligated into the pGEM®-T Easy or pTZ57R/T vectors using the pGEM®-T Easy Vector System (Promega) or Fermentas InsTAclone™ PCR Cloning Kit (Fermentas) as described in the relevant manuals. The choice of vector was completely arbitrary, and depended on the availability of the kits. The specific vector used in each ligation reaction is indicated in Table 4.1.

4.3.3.5 Transformation of competent cells with recombinant plasmids

In preparation for the transformation reactions, *Escherichia coli* JM109 cells were made competent using the CaCl₂ shock treatment by Dagert and Ehrlich (1979). Once competent, the cells were transformed with the appropriate recombinant cloning vectors by following the standard heat shock protocol outlined by Sambrook *et al.* (1989).

Table 4.1 Bacterial strains and plasmids

Strain/plasmid	Genotype/relevant features	Reference
<i>E.coli</i> JM109	<i>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB) F'(traD36 proAB' lacI^q lacZΔM15)</i>	Sambrook <i>et al.</i> (1989)
pGEM®-T Easy	Amp ^r , β-galactosidase	pGEM®-T and pGEM®-T Easy Vector Systems (Promega Corporation)
pTZ57R/T	Amp ^r , β-galactosidase	InstAclone™ PCR Cloning Kit (Fermentas)
pHsp70_1	Derivative of pGEM®-T Easy containing 417 bp <i>G. gracilis Hsp70</i> gene fragment amplified using the F1 and R1 primer set.	This study
pHsp70_2	Derivative of pTZ57R/T containing 1316 bp <i>G. gracilis Hsp70</i> gene fragment amplified using the F2 and R2 primer set.	This study
pHsp70_3	Derivative of pTZ57R/T containing 1003 bp <i>G. gracilis Hsp70</i> gene fragment amplified using the F3 and R2 primer set.	This study
pHsp70_4	Derivative of pTZ57R/T containing 647 bp <i>G. gracilis Hsp70</i> gene fragment amplified using the primer R3.	This study
pHsp70_5	Derivative of pTZ57R/T containing 1826 bp <i>G. gracilis Hsp70</i> gene fragment amplified using the primer F4.	This study
pHsp70_6	Derivative of pTZ57R/T containing 380 bp <i>G. gracilis Hsp70</i> gene fragment amplified using the primer R4.	This study
pHsp70_7	Derivative of pTZ57R/T containing 672 bp <i>G. gracilis Hsp70</i> gene fragment amplified using the primer F5.	This study
p245	Derivative of pDNR-Lib containing 120 bp <i>G. gracilis</i> reference gene 245 gene fragment	Ealand (2011)

4.3.3.6 Selection and confirmation of successfully transformed *E. coli* cells

After transformation (Section 4.3.3.5), 100 µl of the transformation culture was plated onto a LA plate (Appendix A.1.2) containing a final concentration of 100 µg/ml ampicillin (Appendix A.2.20.1), 40 µg/ml X-Gal (Appendix A.2.20.2) and 50 µM IPTG (Appendix A.2.20.3). The plates were incubated at 37 °C for 16 hours, after which successfully transformed *E. coli* cells were identified as white colonies. These colonies were restreaked onto LA plates supplemented with 100 µg/ml ampicillin to validate the success of the transformation. A single colony from each culture was used to inoculate a 5 ml Luria-Bertani broth (LB; Appendix A.1.3) containing 100 µg/ml ampicillin and incubated at 37 °C for an additional 16 hours. After incubation, plasmid DNA was isolated from each of the cultures (Ish-Horowitz and Burke, 1981) and quantified using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop 1000 software; version 3.7.1; Thermo Scientific) at an absorbancy of 260 nm where one unit of absorption represented 50 µg ml⁻¹ of DNA. The ratio of sample absorbencies at 260: 280nm and 260: 230 nm were further used to assess the purity of the DNA samples, where values of ~2.0 indicated the isolation of pure DNA without the co-precipitation of contaminants.

To confirm the success of the transformation procedure, traditional PCR was employed to amplify the insert DNA fragment from each recombinant plasmid using the M13F and M13R primer set (Appendices B.23.2 and B.24.1). Amplification products were electrophoresed on a 1.5 % TAE-agarose gel (Appendix B.18) and visualised using a short wavelength UV light box (Bio-Rad GelDoc EQ-system™; Bio-Rad). An image was captured using the Quantity One® Software (version 1.7; Bio-Rad) and the fragment size of each PCR product harboured within the various plasmids validated based on the expected fragment sizes (Figure 4.1).

4.3.3.7 Sequencing of the ER *Hsp70* gene from *G. gracilis*

The cloned *G. gracilis* DNA was sequenced by Macrogen Ltd., Korea using both the M13F and M13R primers (Appendix B.23.2). For each clone, the sequence data from both the forward and reverse sequencing reaction was manually edited using the Chromas software (version 2.1; Technelysium PTY, Ltd.), and aligned using DNAMAN (version 4.13; Lynnon Biosoft) to obtain a consensus sequence for each fragment. To attain the full length sequence of the *Hsp70* gene, the DNA sequences from all 7 clones were assembled and assessed using DNAMAN. From this analysis, an open reading frame was identified and the gene sequence determined.

4.3.4 Characterisation of the putative Hsp70

4.3.4.1 Homology search

A homology search was performed using the blastx algorithm (Altschul *et al.*, 1997) provided by the National Centre for Biotechnology Information (NCBI; blast.ncbi.nlm.nih.gov/Blast.cgi).

4.3.4.2 Conserved domains, family signatures and functional sites

Conserved domains, family signatures and functional sites were identified using the conserved domain finder on the NCBI website (www.ncbi.nlm.nih.gov) and PROSITE (www.expasy.org/prosite).

4.3.4.3 Prediction of a signal peptide and cleavage site

A signal peptide was predicted by examining the hydrophobicity plot obtained for the gene sequence using DNAMAN (version 4.13; Lynnon Biosoft). The identification of the signal peptide and position of the cleavage site in the protein sequence, using the SignalP 4.0 Server (Sigrist *et al.*, 2010) of the Center for Biological Sequencing Analysis (www.cbs.dtu.dk/services/SignalP), further suggested that a signal peptide was present.

4.3.4.4 Phylogenetic tree construction

Two phylogenetic trees were constructed in this study. The first tree was constructed to validate the targeted cellular location of the putative ER *Hsp70* gene from *G. gracilis*. Cytoplasmic, endoplasmic reticular, mitochondrial and chloroplastic Hsp70 proteins from a land plant *A. thaliana*; a diatom *T. pseudonana*; Chlorophyta: *C. dubia*, *C. reinhardtii*, *G. theta*, *O. lucimarinus* and *O. tauri*; and the rhodophyta: *C. merolae*, *G. tenuistipitata* and *G. gracilis* were selected for this study (Appendix C.6). The second tree was constructed to validate the phylogenetic placement of this protein amongst ER Hsp70 protein sequences from a diverse range of phyla. Thus the ER Hsp70s included in the first phylogenetic study were supplemented with additional ER Hsp70 sequences from the land plants *C. sativus* and *Z. mays*; rhodophyta: *P. haitanensis*, *P. purpurea* and *P. yezoensis*; animals: *G. gallus*, *H. sapiens* and *P. abelii*, *R. norvegicus* and *X. laevis* and protozoa: *P. falicparum* and *P. yoelli* (Appendix C.6).

Prior to phylogenetic analysis, the variable N- and C-terminal regions, which included the signal peptide and retention signal, were removed from each sequence. After which, multiple sequence alignments were constructed using ClustalX (version 1.81; Thompson *et al.*, 1997) and phylogenetic trees generated using MEGA (version 3.1; Tamura *et al.*, 2007).

The phylogenetic relationships were analysed using the Neighbor Joining construction method. One thousand bootstrap replicates were created. Bootstrap values greater or equal to 30 were reported for each branch to reflect the percentage of the 1000 trees that contained that branch.

4.3.5 Transcriptional regulation of the *G. gracilis* ER *Hsp70*

4.3.5.1 Primer design for Real-time PCR

Two primer sets were designed to investigate transcriptional regulation of the ER *Hsp70* from *G. gracilis* (Appendix B.23.3). Primers QHSP70F (1511 nt – 1531nt) and QHSP70R (1623 nt – 1646 nt), employed to amplify a 135 bp fragment of the ER *Hsp70* gene from *G. gracilis* (Section 4.3.3), were designed using FastPCR software (Kalendar *et al.* 2009). Primers 245F and 245R, used to amplify a 120 bp fragment of the reference gene 245 (*RG 245*), were designed by Ealand (2011).

4.3.5.2 Preparation of total RNA

Total RNA was isolated from *G. gracilis* thalli heat shocked at 35 °C for varying intervals of up to and including 24 hours (Section 4.3.2), using the peqGOLD Plant RNA Kit (peqGOLD; Appendix B.20). Although the peqGOLD Plant RNA Kit's (peqGOLD) PerfectBind RNA resin and spin-column technology has been reported to remove most of the genomic DNA (gDNA) contamination from the isolated RNA, the manual suggests the inclusion of an additional gDNA removal treatment for sensitive RNA applications. Hence, an additional DNase I treatment was performed on freshly isolated RNA samples (Appendix B.21).

The efficiency of the DNase I treatment was evaluated by testing for the presence of genomic DNA in treated RNA samples using traditional PCR. Thus, for each biological repeat and primer set (Section 4.3.5.1), a number of PCR reactions were prepared to amplify the ER *Hsp70* or *RG 245* fragment from different templates (Appendix B.24.1; Section 4.3.5.1). For each RNA extraction, 2 µl of total RNA was added as template; for the two positive controls 50 ng gDNA (Appendix B.17) or 1 ng of the plasmid DNA harbouring the fragments of interest (pHsp70_3 or p245; Table 4.1) was used; and for the negative control, sterile water was added in place of template. After amplification, the PCR reaction mixes were electrophoresed on a 1.5 % TAE-agarose gel (Appendix B.18) and visualised using a short wavelength UV light box (Bio-Rad GelDoc EQ-system™; Bio-Rad). Images were captured using the Quantity One® Software (version 1.7; Bio-Rad) and the presence of amplicons identified. The omission of amplicons inferred that the DNase I treatment was effective at removing residual gDNA from total RNA.

4.3.5.3 Conversion of total RNA to complementary DNA

Total RNA, with a high structural integrity and free of genomic DNA was used to synthesise complementary DNA (cDNA) using the ImProm-II™ Reverse Transcription System (Promega; Appendix B.25). In addition, a reaction excluding reverse transcriptase (No RT control) was performed for each biological repeat. After completion of this procedure, the synthesised cDNA was immediately used as template in the real-time PCR reactions (Section 4.3.5.4).

4.3.5.4 Real-time PCR

Real-time PCR (qPCR) was performed to determine the abundance of the ER *Hsp70* transcript in *G. gracilis* thalli heat shocked for various durations up to and including 24

hours at 35 °C. Thus for each biological repeat and primer set, a number of qPCR reactions were prepared in triplicate, to amplify a fragment of the ER *Hsp70* and *RG 245* from different templates (Appendix B.26; Section 4.3.5.1). For each cDNA conversion, 1 µl of undiluted cDNA was added as template; for the negative control, sterile water was added in place of template; and for the positive control 1 ng of plasmid DNA harbouring the fragments of interest (pHsp70_3 or p245; Table 4.1) was used. These samples were run concurrently in a Rotor-Gene™ 6000 (version 1.7; Corbett Research; Rotor-Gene™ Series Software) together with those required for the construction of the standard curves. The standard curves were constructed using a ten-fold serial dilution of pHsp70_3 and p245 (Table 4.1) which harboured a DNA fragment of the ER *Hsp70* or *RG 245* respectively. The plasmid DNA concentrations used for the construction of the standard curves ranged from 1 ng to 1 fg and 100 ng to 100 fg for pHsp70_3 and p245 respectively.

After completion of the run, the dissociation curves, reaction efficiencies, correlation efficiencies and Ct values were determined using the Rotor-Gene™ Series Software (version 1.7; Corbett Research). As the correlation efficiencies for the amplification of the ER *Hsp70* and *RG 245* were slightly different, the Pfaffl mathematical model was used to calculate the relative quantification of the target gene in relation to the reference gene and calibrated to the control thallus sample (omitted heat shock; Pfaffl, 2001).

Relative abundance of the ER *Hsp70* in *G. gracilis* was thus calculated according to the following equation and expressed, at each time point, as the *n*-fold change relative to the control sample.

$$\text{Fold Change Expression} = \frac{(E_{\text{target}})^{\Delta\text{Ct}_{\text{target}}(\text{control-sample})}}{(E_{\text{ref}})^{\Delta\text{Ct}_{\text{ref}}(\text{control-sample})}}$$

Where E_{target} = PCR amplification efficiency of the target gene (*Hsp70*)
 E_{ref} = PCR amplification efficiency of the reference gene (*RG 245*)
 $\Delta\text{Ct}_{\text{target}}$ = Change in the Ct values of target gene (*Hsp70*)
 $\Delta\text{Ct}_{\text{ref}}$ = Change in the Ct values of the reference gene (*RG 245*)
Control = Sample omitting heat shock
Sample = Heat shocked sample

4.3.6 Statistical analysis

Descriptive statistics and statistical analysis was performed using SigmaStat for Windows (version 3.10; Systat Software, Inc.). Where a control sample was compared to multiple experimental samples, a one-way analysis of variance (ANOVA) was deemed appropriate to examine the significance of each experimental sample relative to the control sample. Data sets that were not normally distributed or displayed unequal variances were transformed to meet the requirements for parametric statistical testing.

4.4 Results

4.4.1 Identification of an ER *Hsp70* from *G. gracilis*

Amplification of an ER *Hsp70* gene from *G. gracilis* was performed using traditional polymerase chain reactions and the rapid amplification of cDNA ends. Thus seven overlapping gene fragments were successfully cloned, sequenced and assembled to produce a 2177 bp sequence (Figure 4.1). After translation of the nucleotide sequence, a single open reading frame (ORF) of 1986 nucleotides was identified in the plus three frame. This ORF was flanked by a 98 bp 5'- and 93 bp 3'-untranslated region, indicated by the presence of an initiation (ATG) and termination (TAG) codon respectively. The gene encoded for a 662 amino acid (aa) residue protein with a hypothetical molecular mass of 72.577 kDa (Figure 4.2).

A blastx search of the GENBANK database revealed that the *G. gracilis* sequence has strong homology to a number of Hsp70 protein sequences from plant and algal species (Table 4.2). The top sequence similarities of 70-71 % were to two Hsp70 sequences from *C. merolae* and *H. vulgare* and two luminal binding proteins, otherwise known as an ER luminal Hsp70 (Kabani *et al.*, 2003), from *P. menziesii* and *G. hirsutum*. Thus the newly sequenced gene isolated from *G. gracilis* was identified as a putative *Hsp70* that codes for an Hsp70 protein that is localised to the endoplasmic reticulum (ER).

The classification of the putative *G. gracilis* ER Hsp70 protein was validated using a number of software applications. Identification of all three of the classical Hsp70 family signature motifs: IDLGTTYYS (aa 36-43), VFDLGGGTFDVTLL (aa 222-235) and VVLVGGSTRIPKVQQ (aa 359-373) confirmed that this protein is a Hsp70, while the presence of a signal peptide

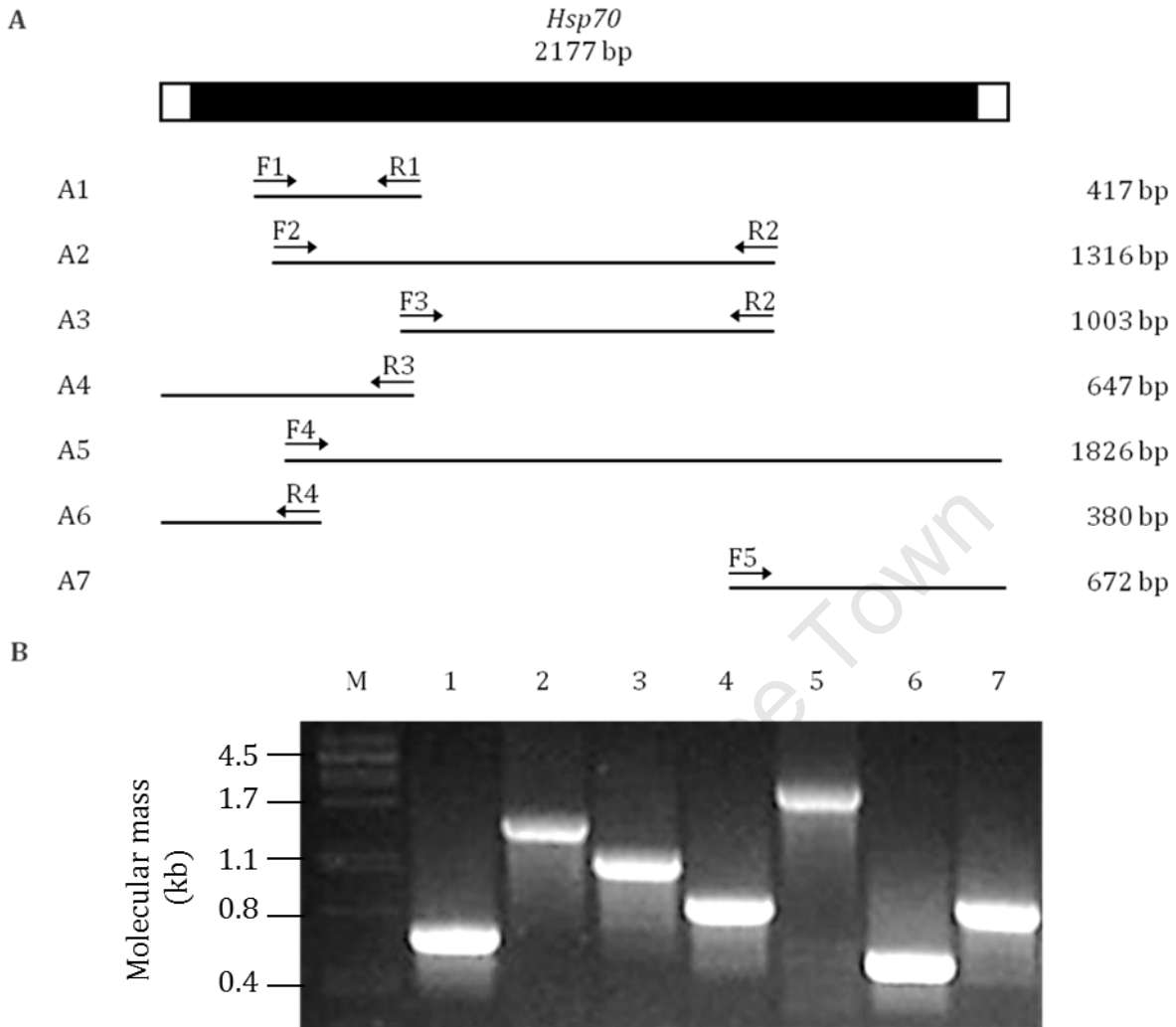


Figure 4.1 (A) Diagram demonstrating the strategy employed to amplify the putative ER *Hsp70* gene from *G. gracilis* using the 9 primers. F1 to F5 represents the forward primers and R1 to R4 represent the reverse primers. The shaded and unshaded areas of the schematic represent the coding and the untranslated regions of the gene respectively. A1-A7 represents the amplified amplicons. The sizes depict the length of the individual amplicons. (B) PCR amplification of the depicted amplicons on a 1.5 % TAE agarose gel. Amplicons were amplified from the recombinant plasmids pHsp70_1-pHsp70_7 harbouring the depicted PCR fragments (Table 4.1), using M13F and M13R primers. Lane M: Lambda DNA digested with *Pst* I. Lanes 1-7: Amplified products incorporating A1 to A7 respectively. The difference between the fragments sizes in (A) and (B) results from the incorporation of 263 bp of the pGEM[®]-T Easy Vector, for A1, and 152 bp of pTZ57R/T Vector, for A2-A7, in between the cloned *Hsp70* fragment and the M13 primer sites on the respective plasmids.

1 FCCCGTCTTCCATTCTAATTGATTTTCGGTCACTTAACCTCTTTAACTGTCCCGCCTCGTTCCCCGATTTGG
71 TTGGGAGCTTCTTACTTCTGTTACCACCATGTCCGTCTCTTCTTACAAACGCCAATCGGCCTTCTTTTTG
1 M S V S S Y K R Q S A F F L
141 TTATTTCGCGTCCCCTCATCGTCGCCTGCACCGCTAGTACGGAGAATGTGGGCACCGTTATTGGAATTGATC
15 L F A S L I V A C T A S T E N V G T V I G I D
211 TCGGAACCACTTACTCTTGTGTGGAGTTATGCAAACGGCAAGGTTGAGATAATCGCCAATGATCAGGG
38 L G T T Y S C V G V M Q N G K V E I I A N D Q G
281 AAACCGAATCACTCCTTCATACGTGGCCTTCACTTCTGAAGAGCGACTGATTGGAGATGCCGCTAAGAAT
62 N R I T P S Y V A F T S E E R L I G D A A K N
351 CAAGCCGCCATGAACCCCGTCAACACGGTGTTCGATGTGAAGCGTCTCATCGGAAGAAAGTACACTGATG
85 Q A A M N P V N T V F D V K R L I G R K Y T D
421 CCACTGTTTCCAGCGCGATAAGAAGCTCTTGGCCCTTCAAGATCGTCAGCCAGGGTGATAAGCCGATGATTT
108 A T V Q R D K K L L P F K I V S Q G D K P M I S
491 CATTGACGTCAAGGGTGAGAAGAAGACATTCTCTCCGGAACAGATTTCCGCCATGGTTCTCGGAAAGATG
132 I D V K G E K K T F S P E Q I S A M V L G K M
561 AAGAAGACTGCTGAGGAGTATCTTGGTAGAGAAGTTAAGAACGCTGTGGTTACCGTCCCGGCCTACTTCA
155 K K T A E E Y L G R E V K N A V V T V P A Y F
631 ATGACGCCCAGAGACAGGCTACGAAGGATGCTGGTACTATTGCCGGCCTGAATGTGCATGCGTATTATCAA
178 N D A Q R Q A T K D A G T I A G L N V M R I I N
701 CGAACCTACTGCGGCTGCCATTGCGTACGGCCTCAACAAGAAGGGCGAGAAGAACATTCTTGTCTTTGAC
202 E P T A A A I A Y G L N K K G E K N I L V F D
771 CTCGGTGGTGGTACTTTTTGATGTCACCTCTTCTTACTATCGACGATGGAGTATTCGAAGTCTTGCCACCA
225 L G G G T F D V T L L T I D D G V F E V L A T
841 ACGGAGATACACATCTTGGTGGTGAAGACTTTGACCAGAGAGTCATGGATTACATGATGAAGCTGTTCAA
248 N G D T H L G G E D F D Q R V M D Y M M K L F K
911 ACGCAAGTACGGCAAAGACCTTTCCAAGGATAAGCGTGTCTTGGCAAGCTTCGCCGAGAATGTGAGAAG
272 R K Y G K D L S K D K R A L A K L R R E C E K
981 GCCAAGCGCGCTCTTTCTTCTCAGACTCAGGTGCGAATTGAAATCGAGTCTCTGTATGACGGTCAGGACT
295 A K R A L S S Q T Q V R I E I E S L Y D G Q D
1051 TCTCTGAGACTCTCACTCGTGCAGGTTTTGAGGAACTCAACTCTGATCTCTTCCAGACGTACTCTGAAGCC
318 F S E T L T R A R F E E L N S D L F R R T L K P
1121 GGTGGAGAAGGTGCTCAAGGATGCTGACATGACTAAAGGTGAGGTTTCATGAAGTTGTGCTTGTGGTGGGA
342 V E K V L K D A D M T K G Q V H E V V L V G G
1191 TCTACTCGTATCCCGAAGGTGCAGCAGCTTGTGAGCAACTTCTTTAACGGAAAGGCCCCAGCAAGGGTA
365 S T R I P K V Q Q L V S N F F N G K A P S K G
1261 TCAACCCAGATGAGGCTGTTGCATATGGAGCTGCTGTTTCCAGCCGGTATTCTGTCTGGTGTGGTGGTGA

388 I N P D E A V A Y G A A V Q A G I L S G D G G E
 1331 GACTACGGACAACATTGTGTTGGTTGACGCTGCGCCTCTTTCACCTGGTATTGAGACAGTGGGTGGTGTG
 412 T T D N I V L V D A A P L S L G I E T V G G V
 1401 ATGAGCAAGATCATCCCCGCTAACTCCAAGGTCCCGGCCAGTAAGAGTCAGACTTTCACCACTTACCAGG
 435 M S K I I P R N S K V P A S K S Q T F T T Y Q
 1471 ATAACCAGGAAGTTGTAAGTATCCAGGTGTACGAGGGTGAG**CGCACGATGACCAAGGACTG**CCACATGCT
 482 D N Q E V V S I Q V Y E G E R T M T K D C H M L
 1541 TGGCAAGTTCGATCTGAGTGGCATCCGAAAGGCGCGACGAGGTGATCCGCAAATTCCTGTGACTTTTGC
 505 G K F D L S G I R K A R R G D P Q I L V T F D
 1611 ATTGATGCTAACGGTATCCTGAGTGTGTCTGCTGAGGAGAAGGGTGCATCCAACAAGGAAAGTATTACTA
 482 I D A N G I L S V S A E E K G A S N K E S I T
 1681 TCACTAACGATAAGGGTAGACTTTCTGAGGAAGAAATCGAGCGAATGGTTCGTGAAGCAGAGGAGATGAA
 505 I T N D K G R L S E E E I E R M V R E A E E M K
 1751 GGATGAGGATGAGAAGATCAAGAAGAAGGTTGAGGCGAAGAACAACCTAGAAAACCTCATCTTCACTGTG
 528 D E D E K I K K K V E A K N N L E N F I F T V
 1821 AAGGGCACCTTGAAGAGAAGGACAAGTTAGAAGGAAAGCTGGACGAGGAAGACATCGAAGCGCTGGAAT
 552 K G T L E E K D K L E G K L D E E D I E A L E
 1891 CTGCGGTGAAGGAAGCGCAGGAATGGATGGACACGGAGGCTGAGGACGCTGACGCTGAGGACATCGAGTC
 575 S A V K E A Q E W M D T E A E D A D A E D I E S
 1961 GCGGTTGAACGACATGAAGGAGGTGGTTGAACCAATAATGAAGAAGGTTGGTGGTGGTCCCGGCGCGCT
 698 R L N D M K E V V E P I M K K V G G G P G G A
 2031 GGTGGTGGCCCTGAGGAAGAGGACGACGACGATGATGACGCCACGAAGAATTGTAGACAAACACTCTTT
 622 G G A P E E E D D D D D D A H E E L
 2101 AGCTTTTAACTCTCGCTCCATGTTGTCCTAACGCGGTTTCGATGTACATGTAAAAATTTCCGCACTCGCG
 2171 TTAACGT

Figure 4.2 The nucleotide and derived protein sequence (in bold) of the ER *Hsp70* gene from *G. gracilis*. The initiation (ATG) and termination (TAG) codons are underlined. The highly conserved Hsp70 family signatures are indicated in red, while the putative signal peptide and endoplasmic reticulum retention signal is recorded in blue and green, respectively.

and a putative signal peptidase cleavage site between the 26th and 27th amino acids (Figure 4.3) suggested that the protein is not cytoplasmic. Identification of a 3'-terminal ER

retention signal HEEL (aa 659-662; Figure 4.2), indicated that this Hsp70 is more specifically localised to the ER.

Phylogenetic analyses further confirmed the identification of the protein as an ER Hsp70 (Figures 4.4 and 4.5). The relationships displayed in these trees are consistent with the traditional taxonomy of plant and algal species. Phylogenetic analysis clearly showed that the putative ER Hsp70 from *G. gracilis* clustered with Hsp70s localised to the ER, and more specifically, clustered together with the rhodophyta ER Hsp70 sequence from *C. merolae*. The strong association between these two sequences suggested that the protein is an ER Hsp70 protein from *G. gracilis*.

Table 4.2 Sequences that displayed the highest sequence homology to the putative ER Hsp70 from *G. gracilis* as identified by a blastx search (26 April 2012).

Accession Number	Description	Sequence Identity (%)	E-value
BAC67670	Heat shock 70 kDa protein [<i>Cyanidioschyzon merolae</i> strain 10D, Rhodophyta]	71	0
AAA62325	HSP70 [<i>Hordeum vulgare</i> subsp. Vulgare, domesticated barley]	71	0
CAA89834	Luminal binding protein [<i>Pseudotsuga menziesii</i> _Douglas-fir]	71	0
CBI34546	unnamed protein product, partial [<i>Vitis vinifera</i> , grape vine]	70	0
ACJ11746	luminal binding protein [<i>Gossypium hirsutum</i> _upland cotton]	70	0

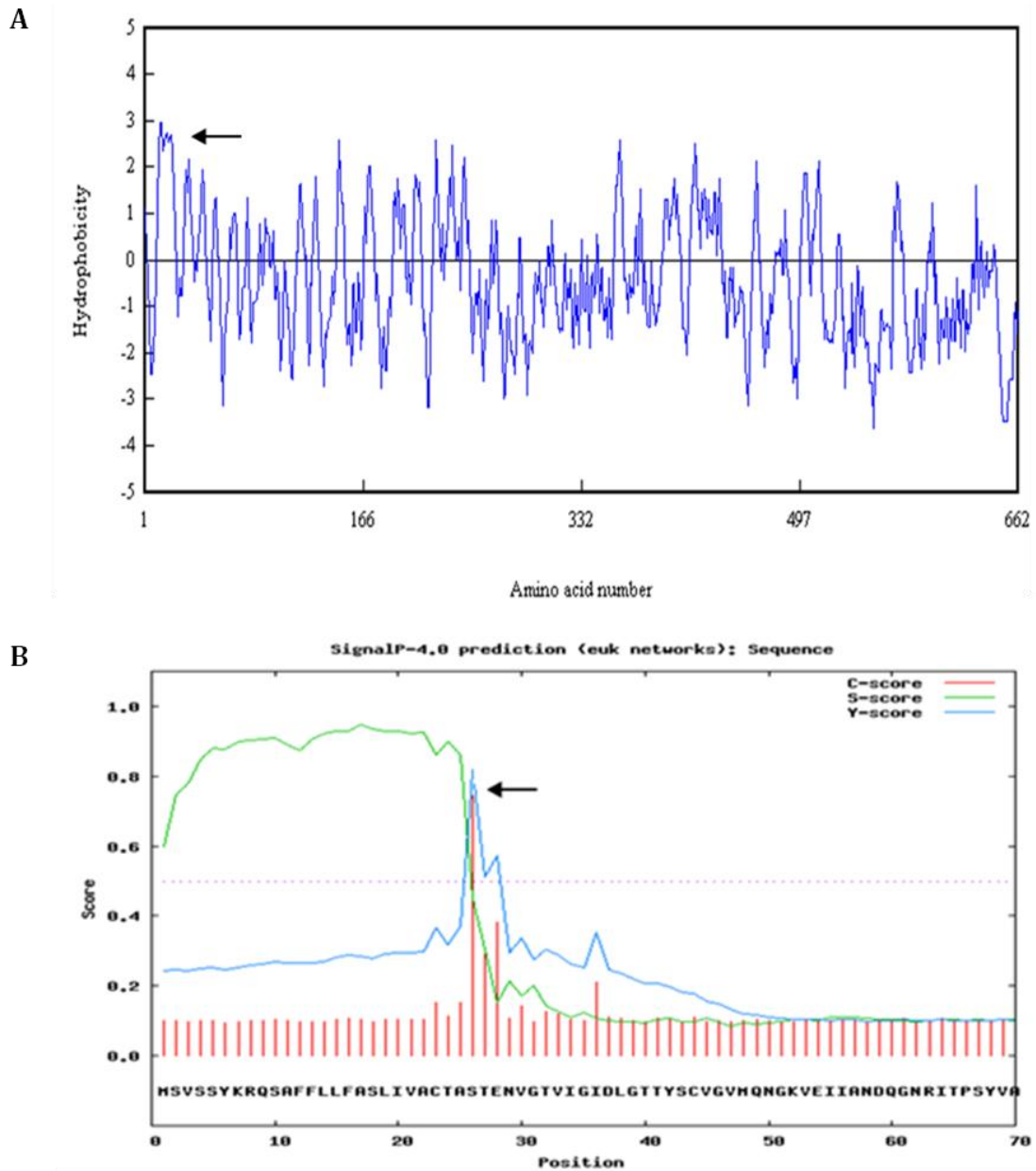


Figure 4.3 Position of the proposed signal peptide and signal peptidase I cleavage site. (A) Hydrophobicity plot of the deduced 662 aa sequence of the ER Hsp70 from *G. gracilis*. The arrow represents the proposed signal peptide. (B) Graphical output of the first 70 deduced amino acids of the ER Hsp70 from *G. gracilis*, from which the presence and location of a signal peptide cleavage site the protein, was predicted. The C-score, S-score and Y-score represent the raw cleavage site score, the signal peptide score and the combined cleavage site score respectively. The arrow represents the position of the proposed signal peptidase I cleavage site between the 26th and 27th amino acid.

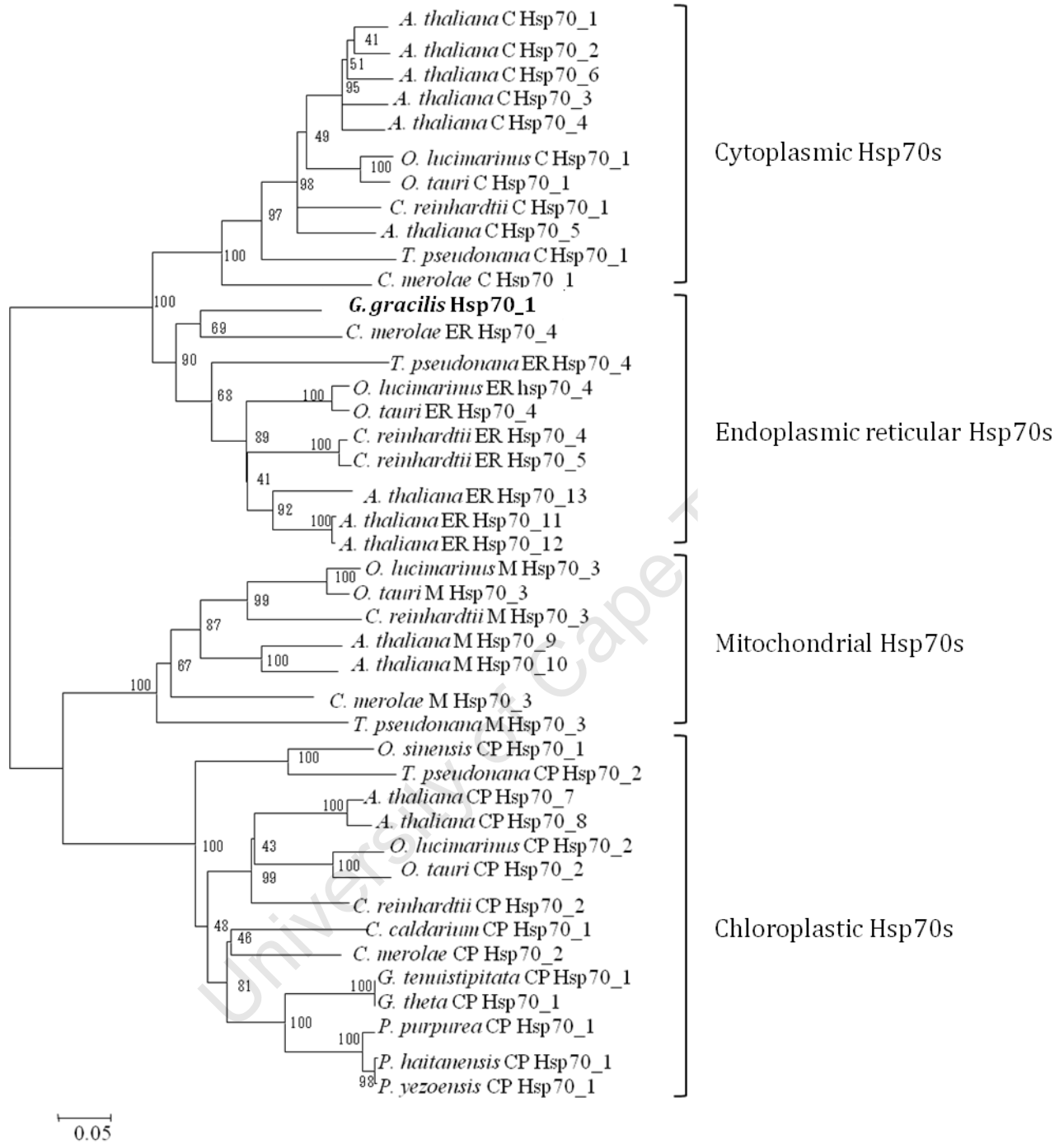


Figure 4.4 A Neighbor Joining phylogenetic tree indicating the targeted cellular compartmentalisation of the putative ER Hsp70 protein from *G. gracilis* (indicated in bold). The phylogenetic tree was constructed in MEGA (version 3.1; Tamura *et al.*, 2007). Bootstrap values greater or equal to 30 are recorded at the nodes. The bar depicts 5 base substitutions per 100 nucleotide sites.

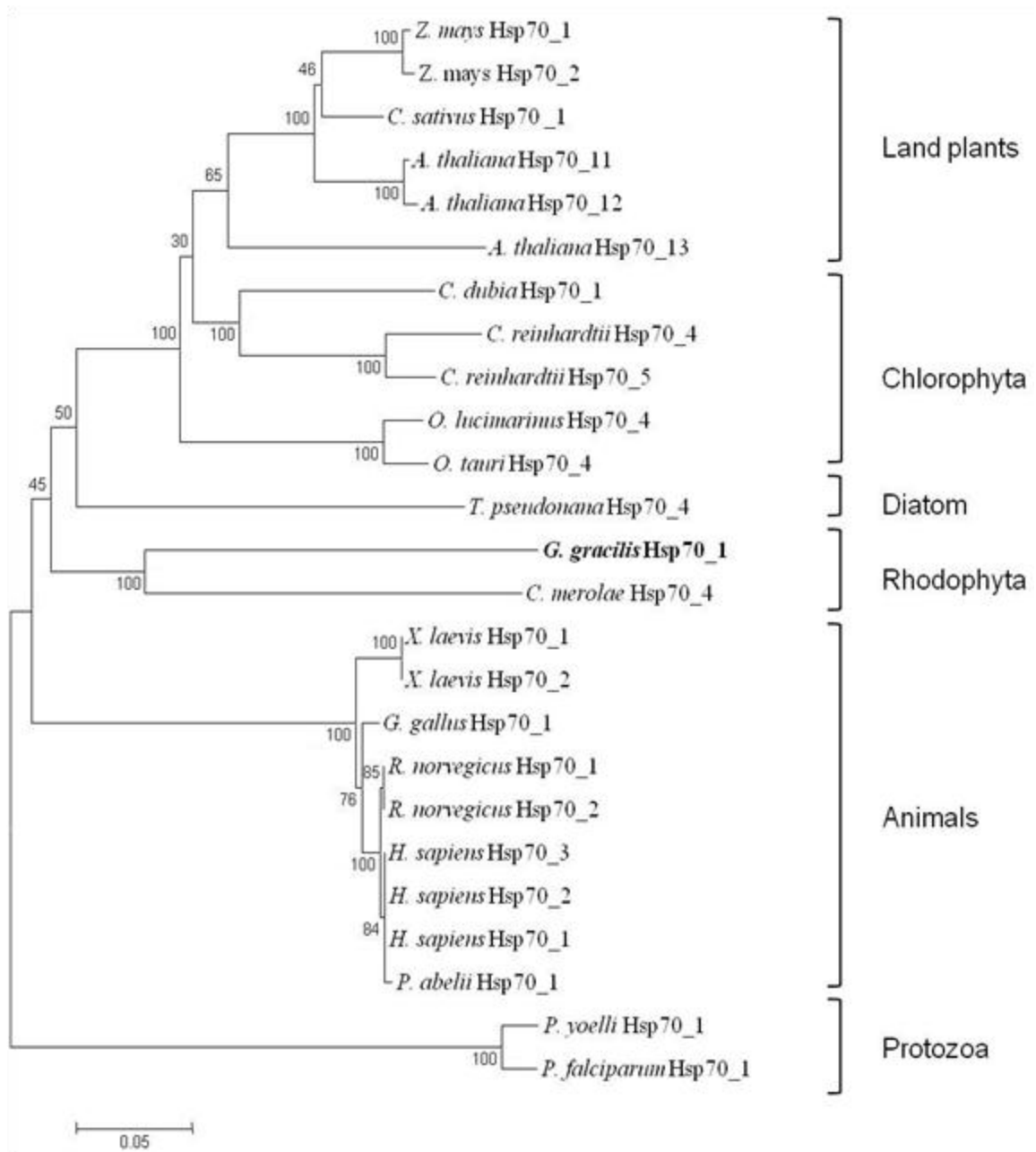


Figure 4.5 A Neighbor Joining phylogenetic tree depicting the relationship of the ER Hsp70 protein *G. gracilis* (indicated in bold) to a number of ER Hsp70 proteins from a diverse range of phyla. The phylogenetic tree was constructed in MEGA (version 3.1; Tamura *et al.*, 2007). Bootstrap values greater or equal to 30 are recorded at the nodes. The bar depicts 5 base substitutions per 100 nucleotide sites.

4.4.2 Investigation of the relative abundance of the ER *Hsp70* gene using real-time PCR

4.4.2.1 Assessment of total RNA for cDNA synthesis

Extraction of good quality total RNA is essential for accurate evaluation of gene expression using real-time PCR (Fleige and Pfaffl, 2006). Thus, a number of investigations were made to assess the purity, structural integrity and efficacy of DNase I treatment prior to the synthesis of cDNA. The purity of total RNA was determined using a NanoDrop® ND-1000 Spectrophotometer (Thermo Scientific). Absorbance values of between 2.02-2.43 and 1.76-2.19 at 260: 230 and 260: 280 respectively, indicated that the total RNA samples were of a high purity. The integrity of the RNA was assessed by evaluating 1 µg of total RNA from each extraction on a 1.2 % formaldehyde-agarose gel (Figure 4.6). The absence of

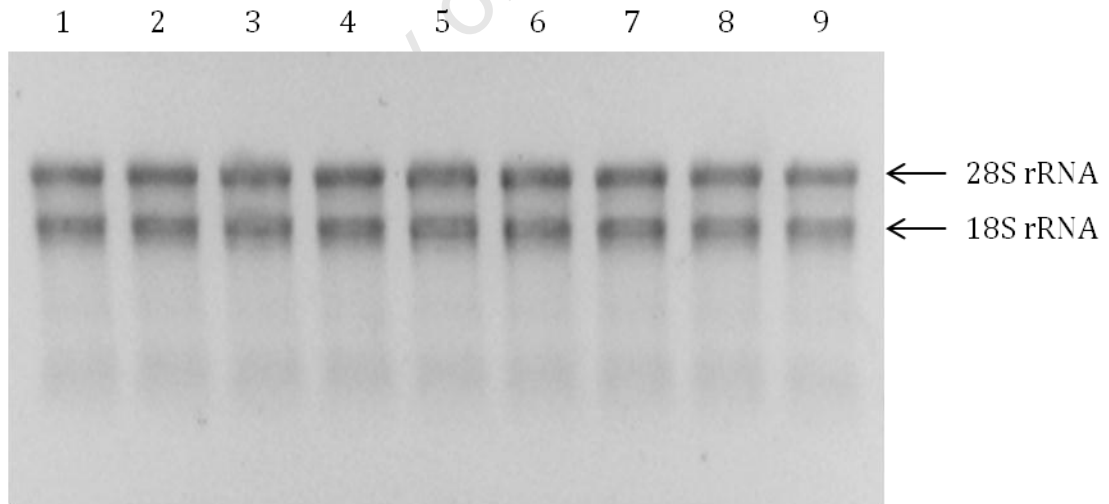


Figure 4.6 An assessment of RNA integrity and quality by electrophoresis of 1 µg total RNA on a 1.2 % formaldehyde-agarose gel. Lanes 1-10: Total RNA isolated from *G. gracilis* thallus samples heat shocked at 35 °C for 0, 0.5, 1, 2, 4, 6, 8, 18 and 24 hours respectively, using the peqGOLD Plant RNA Kit (peqGOLD).

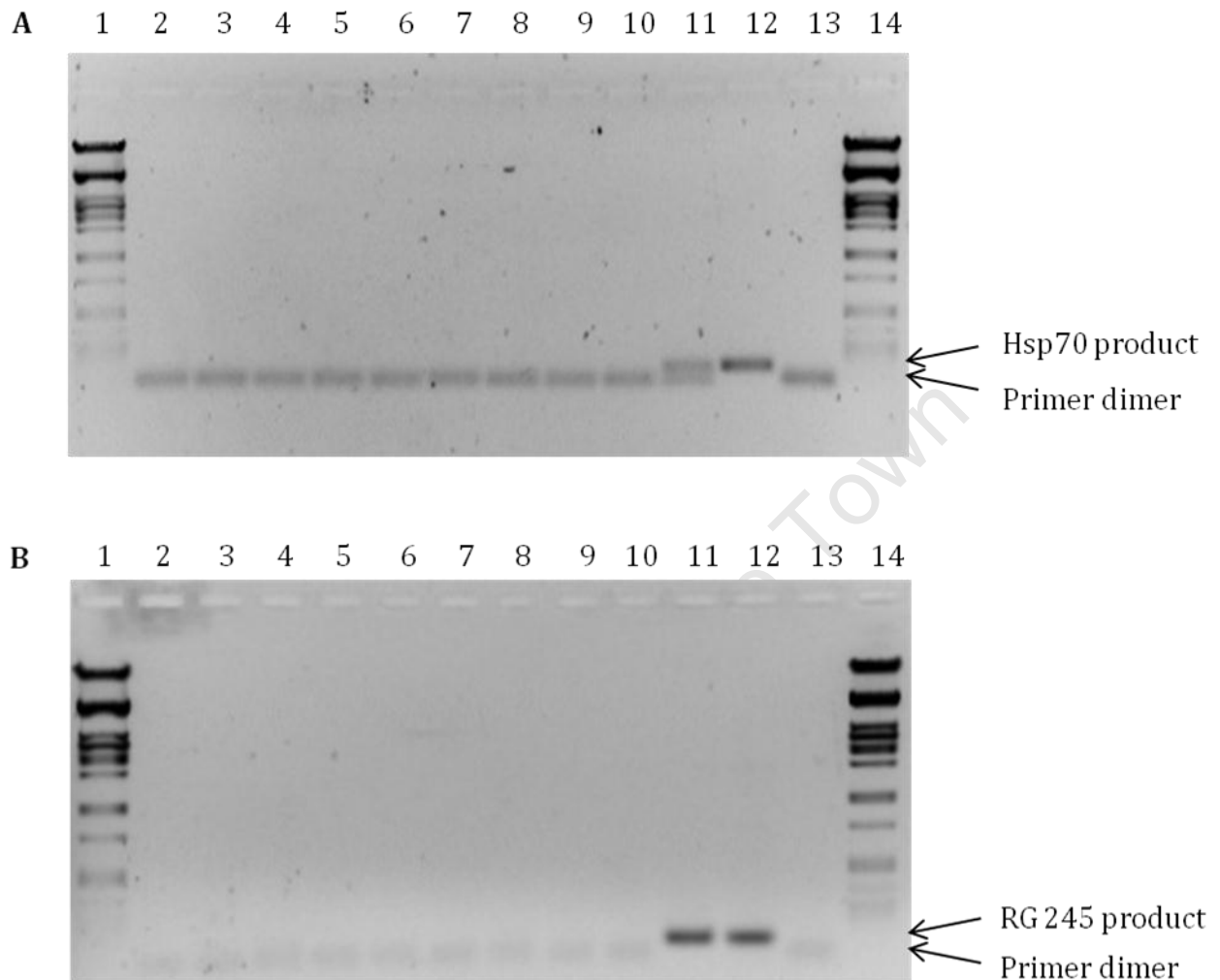


Figure 4.7 An assessment of the presence of genomic DNA in treated RNA samples using PCR. Panels A and B depict the PCR amplicons electrophoresed on a 1.5 % TAE-agarose gel using (A) ER Hsp70 specific primers or (B) *RG 245* specific primers (Section 4.3.5.1). Lanes 1 and 14: Lambda DNA digested with *Pst* I. Lanes 2-13: PCR reactions containing different templates. Lanes 2-10: 500 ng total RNA isolated from *G. gracilis* samples heat shocked at 35 °C for 0, 0.5, 1, 2, 4, 6, 8, 18 and 24 hours respectively. Lane 11: 100 ng genomic DNA isolated from an acclimatised *G. gracilis* sample. Lane 12: 1 ng of the plasmid harbouring the fragments of interest (pHsp70_3 or p245) and lane 13: sterile water.

ribonuclease degradation indicated that total RNA maintained a high structural integrity. The efficacy of the DNase I treatment was evaluated by testing for the presence of genomic DNA in treated RNA samples using PCR (Figure 4.7). The absence of amplicons indicated the successful removal of gDNA. Thus, these results indicated that the extracted total RNA was of a good quality and could be used for the synthesis of cDNA.

4.4.2.2 Assessment of a suitable reference gene for real-time PCR

Since a suitable reference gene for evaluating the relative abundance of transcripts, expressed in response to heat stress, had not yet been established in *G. gracilis*, a suitable reference gene needed to be identified for this study. Reference gene *RG 245*, currently available in the laboratory and previously reported to be stably expressed in *G. gracilis* in response to disease elicitors (Ealand, 2011), was selected and assessed for its potential use as a reference gene for heat stress. The change in Ct values of *RG 245* in *G. gracilis* was therefore monitored for the duration of the heat shock experiment (Figure 4.8). The variation in Ct values, over the 24 hours of heat stress, for each biological repeat was recorded as less than or equal to 1.9. This result indicated that *RG 245* was stably expressed and thus could be used as a suitable reference gene in this study.

4.4.2.3 Relative abundance of ER *Hsp70* using Real-time PCR

Real-time PCR was employed to quantitate the relative abundance of ER *Hsp70* in *G. gracilis* in response to heat stress. Melt curves of the ER *Hsp70* and *RG 245* amplicons (Figure 4.9) were reproducible for all biological repeats. In each case, a single peak was observed, indicating that only the target gene was amplified in the reaction. Standard curves constructed for each biological repeat and data set were used to calculate the amplification and correlation efficiencies for both ER *Hsp70* and *RG 245*. Although the correlation

efficiencies were similar at 1.02 and 0.998 for ER *Hsp70* and *RG 245*, the reaction efficiencies differed slightly at 0.87 and 0.999 respectively. Thus, the Pfaffl method of analysis, which takes the differences between the reaction efficiencies of the gene of interest and the reference gene into account, was employed to determine the relative

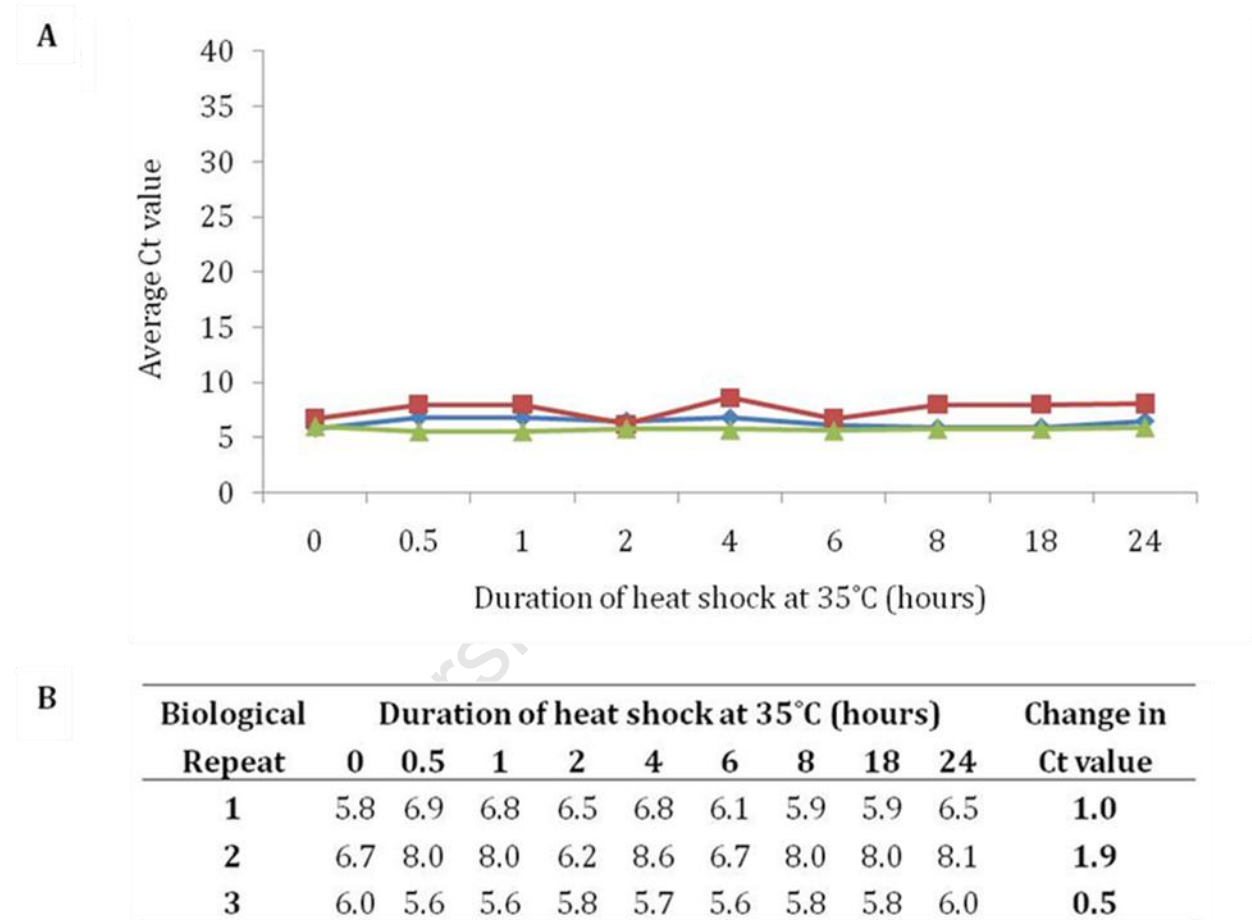


Figure 4.8 Comparison of the cycle threshold (Ct) values of *RG 245* in *G. gracilis* after exposure to 35 °C for various durations up to and including 24 hours. (A) Graphical representation of the average Ct value of *RG 245* after exposure to heat stress. The blue, red and green lines represent the average Ct value for biological repeats 1, 2 and 3 respectively. (B) Tabulated results of the variation in the Ct values of *RG 245* for each biological repeat.

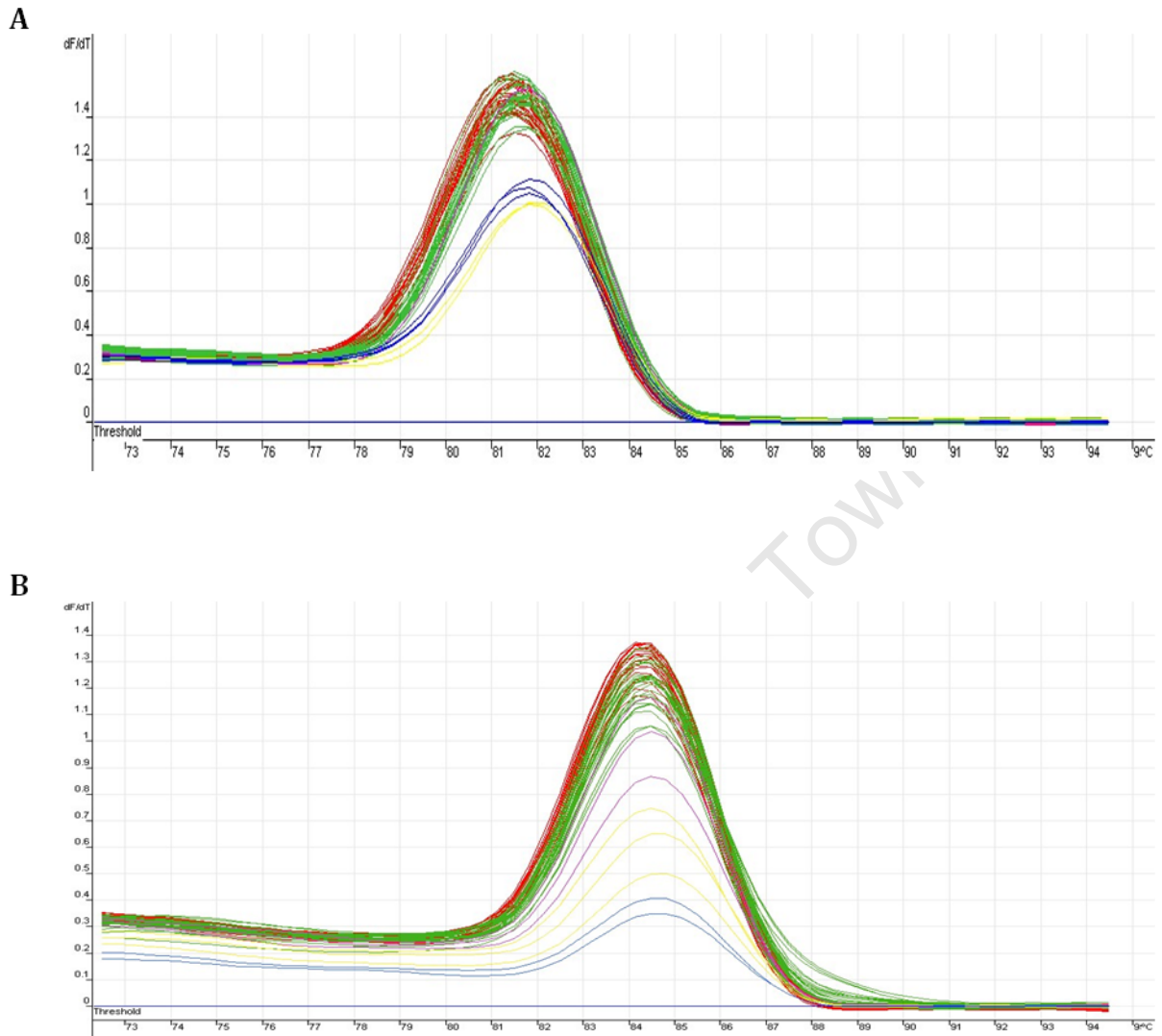


Figure 4.9 Melt curve analyses of the ER *Hsp70* and *RG 245* amplicons, amplified from transcripts present in *G. gracilis* after exposure to heat stress at 35 °C for up to and including 24 hours. The x-axis represents the range of melting temperature from 72 to 95°C applied to the qPCR amplicons. The y-axis represents the derivative of the raw data after smoothing (dF/dT) as calculated by Rotor-Gene 6000 software. Panels A and B represent the melt curves for the reference gene *RG 245* and the target gene ER *Hsp70* respectively. The red, green, purple, yellow and blue curves represent the standard curve, experimental, positive (pHsp70_3 and p245) and negative controls (No RT and sterile water) respectively.

abundance of ER *Hsp70* transcripts in *G. gracilis* in response to heat stress (Fleige and Pfaffl, 2006; Figure 4.10).

A rapid increase in transcript levels was recorded within the first 30 minutes of heat shock, peaking after 1 hour at a 4.95 fold change. For the next 5 hours, the transcript levels decreased to basal level; dropping to the lowest recorded fold change of 0.13 after 24 hours. Although a trend in the ER *Hsp70* transcript levels was apparent, no statistically significant increases were identified in the abundance of this transcript in response to heat

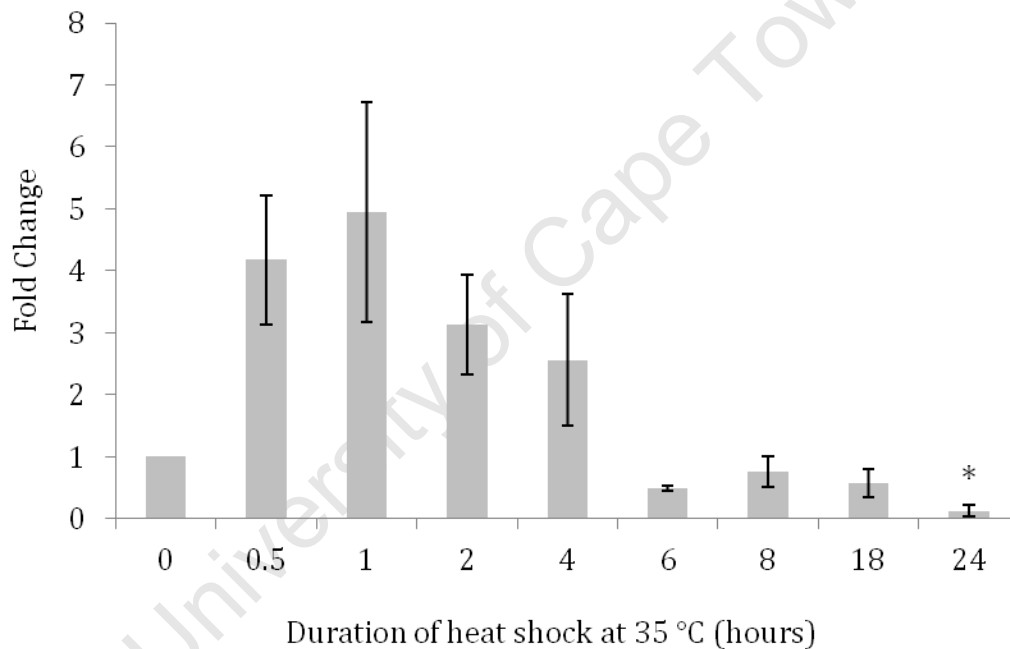


Figure 4.10 Relative abundance of ER *Hsp70* transcripts in *G. gracilis* after exposure to heat stress at 35 °C. A parametric one-way ANOVA detected statistically significant differences in the relative abundance of ER *Hsp70* transcripts between the control and experimental samples ($P \leq 0.001$; denoted by an asterisk '*'). Data represents the mean \pm standard error from three biological repeats. Data was transformed prior to analysis to meet the requirements of parametric statistical testing.

stress. This result may however be attributed to type II errors (false negatives) as the power of the individual *T*-tests which assessed the difference in the level of ER *Hsp70* transcript between the control and experimental samples heat shocked for 0.5, 1, 2 and 4 hours (data not shown) were well below that of the desired 0.800.

4.5 Discussion

At present, very little is known about the complex mechanisms involved in the maintenance of thermal tolerance and the onset of cell death in *G. gracilis* in response to heat stress. Thus in order to gain a better understanding of the intricate molecular pathways involved in this stress response, a number of additional studies will need to be performed. The development of a molecular indicator of heat stress, which could be used to infer the presence or progression of the stress (Xuefeng and Sylvester, 2011), would therefore be a useful tool in advancing research in this field, by indicating the physiological state of the alga without requiring tedious traditional indices such as growth studies.

In Chapter 3, a number of differentially expressed proteins were reported to be up-regulated in response to heat stress. Of the seven proteins successfully identified, one was classified as a member of the HSP70 multigene family. HSP70s have been extensively researched and reported to help maintain the homeostasis of a cell under stressed conditions (Song *et al.*, 2006). These proteins play a vital role as molecular chaperones, in facilitating the folding of nascent polypeptides, transporting proteins across membranes, preventing the accumulation of protein aggregates and averting the onset of cell death (Hartl, 1996; Song *et al.*, 2006; Reddy *et al.*, 2010).

Although the exact identity of the Hsp70 could not be determined using tandem mass spectrometry, the rapid increase in abundance of this protein during thermal tolerance followed by the decrease in abundance during the onset of cell death (Appendix C.7) implies that this protein could be used to monitor the progression of heat stress in *G. gracilis*. Thus, correlation of Hsp70 expression with the maintenance of thermal tolerance in *G. gracilis* in response to heat stress, together with the abundance of literature reporting the importance of this protein family in maintaining cellular homeostasis (Lee

and Schöffl, 1996; Wang *et al.*, 2004; Hendrick and Hartl, 1995; Qureshi *et al.*, 2007) and the convention of selecting Hsp70s as a potential molecular indicator for heat stress (Mirkes *et al.*, 1994; Ryan and Hightower, 1996), indicates that the HSP70 multigene family probably contains a molecular indicator of heat stress for *G. gracilis*.

The primary objective of this study was to identify a specific potential molecular indicator of heat stress in *G. gracilis* by sequencing an Hsp70 and monitoring the relative abundance of the transcript in response to heat stress. Thus, an Hsp70 was sequenced using traditional PCR and the rapid amplification of cDNA ends techniques. A full length 2177 bp cDNA sequence was obtained, consisting of a single open reading frame of 1986 bp, flanked by a 98 bp 5'- and a 93 bp 3'-terminal untranslated region. This gene encoded for a 662 amino acid protein, with a hypothetical molecular mass of 72.577 kDa. Although no introns were observed between nucleotides 147 and 1522, the presence of introns in the flanking regions could not be determined from the attained sequence information. This is owing to the use of genomic DNA, which contain introns, as template for amplifying the internal region of this gene whereas, cDNA, which does not contain introns, was employed to amplify the flanking regions of this gene. The absence of introns in the central 458 amino acid region of this gene, inferred that this protein may be an inducible isoform as introns are generally found in the constitutive expressed HSP70s and not in the inducible isoforms of this protein (Yost and Lindquist, 1986). The lack of introns in inducible isoforms of HSP70s allows rapid transcription and accumulation of this protein after exposure to heat stress (Huang *et al.*, 1999). However further research will be required to assess the presence or absence of introns in the flanking regions of this gene.

A blastx search of the coding region of the gene revealed that this protein shared strong homology, of between 70 and 71 %, to a number of plant and algal Hsp70 protein sequences. The top five sequence similarities were to two Hsp70s from *C. merolae* and

H. vulgare, two luminal binding proteins, otherwise known as an ER luminal Hsp70 (Kabani *et al.*, 2003), from *P. menziesii* and *G. hirsutum*, and an unnamed protein product from *V. vinifera*. Even though this protein was not identified in the database, a conserved domain blast search of this sequence indicated that it contained conserved multi-domains of the Hsp70 family (pfam00012; 26 April 2012). Thus the newly sequenced protein was hypothesised to also be an Hsp70 protein. Further analyses of the five homologue sequences indicated that all of these proteins contained a putative 5'-terminal signal peptide. Four of these proteins also contained a 3'-terminal endoplasmic reticulum retention signal (Appendix C.8). The fifth protein sequence that did not contain an ER specific retention signal was however assessed to be truncated on the 3' end and was thus omitted from further analysis owing to insufficient sequencing information. Thus, in agreement with the remaining four proteins it was hypothesised that the newly sequenced Hsp70 from *G. gracilis* was more specifically an endoplasmic reticular Hsp70 (ER Hsp70).

The only gene sequence to not contain an ER specific retention signal was the sequence from *H. vulgare*. However, after further analysis, the aforementioned gene sequence was revealed to lack a retention signal, suggesting that this was a partial gene sequence. Therefore, in agreement with the additional four gene sequences selected for this analysis, the Hsp70 from *G. gracilis* was hypothesised to be an

Classification of this protein as an ER Hsp70 from *G. gracilis* was validated using a number of software applications. A conserved domain and family signature search revealed the presence of a 604 amino acid Hsp70 multi-domain which contained all three conserved Hsp70 family signatures and validated that this protein is an Hsp70. Identification of a 5'-terminal signal peptide and a 3'-terminal ER retention signal confirmed that this protein was more specifically an Hsp70 located in the endoplasmic reticulum. Classification of this protein as an ER Hsp70 was supported by the two phylogenetic trees which revealed that

the ER Hsp70 from *G. gracilis* clustered with the ER Hsp70 from the rhodophyte species *C. merolae*.

Transcriptional regulation of ER *Hsp70* was examined in *G. gracilis* in response to heat stress using real-time PCR. The rapid increase in abundance of ER *Hsp70* transcripts occurred within the first thirty minutes of heat shock, correlating to a number of reported studies in which *Hsp70* transcripts in plant, mold, fungus and alga were reported to rapidly increase in abundance within minutes of exposure to elevated temperatures (Cooper and Ho; 1983; Rezaie *et al.*, 2000; Černila *et al.*, 2003; Cheng *et al.*, 2006). Although an increase in the abundance of the ER *Hsp70* transcript was apparent, the increase was not statistically significant. Cumming *et al.* (2007) and Ealand (2011) highlighted the error in over-interpreting a lack of statistical significance obtained from real-time results as indicating no biological significance, especially in cases where the change in gene expression was low or an insufficient number of biological repeats were employed to counteract the biological variation of such a technique. Thus, although real-time PCR should be repeated to obtain statistically relevant real-time data, a rapid increase was observed in the fold change of the ER *Hsp70* transcript in *G. gracilis* in response to heat stress. Thus, it is reasonable to conclude that the *G. gracilis* ER Hsp70 could be used as a molecular indicator of heat stress in future studies.

CHAPTER 5

GENERAL DISCUSSION

Since the 1950s, industry has successfully harvested *G. gracilis* from the wild populations in Saldanha Bay for the production of agar (Rothman *et al.*, 2009). Subsequently, a number of sporadic collapses have led to the dissolution of this industry and emphasised the need for the development of an alternative farming strategy if this sector is ever to be re-established in South Africa (Anderson *et al.*, 1996; Schroeder, 2001). Of the various farming strategies currently available, land based cultivation systems are particularly attractive as it would create a controlled environment in which *G. gracilis* could potentially flourish. In order to establish such a farming system however, a better understanding of the tolerances and responses of *G. gracilis* to the environmental parameters hypothesised to be responsible for the wild population collapses is essential. Research in this field has many advantages. In particular, understanding the tolerance of this alga to various growth conditions may be useful for optimising cultivation conditions to increase algal yield. In addition, understanding the cellular responses of *G. gracilis* to environmental parameters could potentially facilitate the creation of genetically engineered strains or alternatively assist in the development of diagnostic tools for monitoring the health of the alga in cultivation systems.

Although the exact reasons for the collapses in the wild *G. gracilis* population remains unclear, a number of potential stressors have been identified (Anderson *et al.*, 1990; Anderson *et al.*, 1993; Anderson *et al.*, 1996). It has been hypothesised that elevated water temperatures may have contributed to the collapses, which have consistently occurred during the summer months (Jaffray *et al.*, 1997). Therefore in order to better understand the tolerance and response of *G. gracilis* to heat stress, this study identified and successfully

addressed the following three objectives: (i) to determine conditions required to induce a cellular response to heat stress in cultivated *G. gracilis*, (ii) to gain insight into the molecular pathways involved in the maintenance of thermal tolerance and the onset of cell death and (iii) to identify a potential molecular indicator which could be employed as a useful tool in future studies.

Thus in order to determine conditions required to induce a cellular response to heat stress in cultivated *G. gracilis*, the upper limit of thermal tolerance of this alga was investigated. Studies monitoring the growth and viability of this alga indicated that the highest temperature at which *G. gracilis* remained viable and grew was 30 °C (Section 2.4.1). As intertidal algae have been reported to tolerate limited exposure to temperatures greater than that of their thermal limit without physiological damage (Kübler and Davison, 1993; Burritt *et al.*, 2002), 35 °C, a temperature higher than the upper limit of thermal tolerance, was selected to induce heat stress in *G. gracilis*. Exposure to 35 °C for 24 hours was found to be an adequate duration for the heat shock experiment as it included both an initial period of thermal tolerance in which alga could recover (≤ 6 hours) and a latter period (≥ 8 hours) in which the onset of cell death committed *G. gracilis* to die (Section 2.4.2; Lindquist, 1986; Barry *et al.*, 1990; Jolly and Morimoto, 2000). Studies monitoring the growth and viability of *G. gracilis* indicated that thallus segments remained viable and grew for the first 8 hours of exposure, demonstrating thermal tolerance; whereas the absence of thallus growth and viability after 18 hours of heat shock marked the onset of cell death (Section 2.4.2). These results indicated that the abovementioned experimental conditions could be used, not only to study the complex mechanisms involved in either the maintenance of thermal tolerance or the onset of cell death, but also to research the fine balance between these two phases.

Thus with the conditions required to induce heat stress defined above, the second objective was fulfilled by gaining insight into the molecular pathways involved in the maintenance of

thermal tolerance and the onset of cell death. This was achieved by investigating both the production and reduction of reactive oxygen species (ROS) in *G. gracilis* in response to heat stress (Section 2.4.3) and by employing a comparative proteomic approach to gain a better understanding of the pathways involved in the maintenance of thermal tolerance and the onset of cell death in *G. gracilis* (Chapter 3). The comparative proteomic approach, which utilised two dimensional gel electrophoresis, identified 114 protein spots which were differentially expressed during thermal tolerance or at the onset of cell death (Section 3.4.2). This result highlights the molecular difference between the two cellular responses to heat stress, and implies that specific molecular pathways facilitate each of these phases. Although 114 differentially expressed protein spots were identified, financial constraints limited the extent of the information obtained from this technique as only ten protein spots were selected for further identification using tandem mass spectrometry. None-the-less, successful identification of seven of these proteins, followed by the determination of their putative functions, provides an initial insight into the complex mechanisms involved in both the maintenance of thermal tolerance and the onset of cell death (Section 3.4.3). A better understanding of these pathways could however be obtained from a more comprehensive analysis of these results in the future, as less than 10 % of the protein spots representing differentially expressed proteins were analysed using tandem mass spectrometry in this study, and furthermore, only 70 % of the proteins selected for this analysis were successfully identified by sequence identity to homologues already present in the NCBI database. To obtain a better understanding of the molecular response of *G. gracilis* to heat stress, future research aimed at sequencing and annotating the genome of this species, coupled with identification and investigation of the functions of the remaining 107 differentially expressed proteins, could provide a great deal of information regarding the pathways that are involved in these two possible outcomes of heat stress in *G. gracilis*.

Although further research will be required to gain a more extensive and complete overview of the cellular response of *G. gracilis* to heat stress, the information obtained from this study, presented in Figure 5.1, was used to gain an initial insight into the hypothesised

	Duration of heat shock (hours)									
	0	0.5	1	2	4	6	8	18	24	
Viability	No difference									
Growth rate	No difference	Increase				No difference	Decrease			
Intracellular ROS	No difference	Increase		No difference			Decrease			
Extracellular H ₂ O ₂	No difference					Increase				
Catalase	No difference	Increase		No difference			Decrease			
Ascorbate and Glutathione peroxidase	No difference	Increase			No difference	Increase				
Hsp70	No difference	Increase						Decrease		
Transketolase	No difference	Increase							No difference	
GroEL	No difference			Increase					Increase	
FNR	No difference			Increase					Increase	
Patatin	No difference			Increase					Increase	
GAPDH	No difference			Increase					Increase	
Phycocyanin	No difference			Increase					Increase	

Figure 5.1 Schematic indicating the physiological, enzymatic and proteomic changes in *G. gracilis* in response to heat stress. Gray shading (from darkest to lightest), indicates an increase, decrease or no difference in values when compared to the control sample (exposed to 0 hours of heat stress). The cells that have been left blank indicate that no growth or inability was recorded for the samples. ROS, reactive oxygen species; H₂O₂, hydrogen peroxide; Hsp70, 70 kDa heat shock protein; GroEL, chaperonin GroEL; FNR, ferredoxin-NADP⁺ reductase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

cellular response that resulted in (i) thermal tolerance between 0.5 and 8 hours of heat shock where growth and viability persisted (Section 2.4.2) and (ii) cell death between 18 and 24 hours of exposure to 35 °C, where neither growth nor viability was recorded (Section 2.4.2). For each of the possible outcomes of heat stress, a schematic was constructed to depict the pathways involved in thermal tolerance and cell death in *G. gracilis* (Figures 5.2 and 5.3). These schematics are consistent with the conserved cellular response of organisms to heat stress as described in the literature review (Section 1.6). In addition to the pathways presented in this conserved model, additional information obtained from this study has been included in the schematics constructed for *G. gracilis*.

5.1 Hypothesised cellular response resulting in the maintenance of thermal tolerance

During the first 8 hours of heat stress, a number of cellular changes were recorded and hypothesised to function in the maintenance of thermal tolerance in *G. gracilis* (Chapters 2 and 3; Figure 5.2). A rapid and transient oxidative burst was recorded in *G. gracilis* in which intracellular ROS was detected after 0.5 and 1 hour of heat stress, prior to returning towards that of basal levels after two hours of exposure (Section 2.4.3). This temporary accumulation of ROS is hypothesised to indicate that the cellular response to heat stress in *G. gracilis* is rapidly induced. This hypothesis is supported by a number of studies which suggest that the rapid production and reduction of ROS in plants and marine algae is an early indicator of heat stress and further suggests that a heat shock response has been induced (Schrek and Baeuerle, 1991; Rijstenbil *et al.*, 1994; Mittler, 2002; Nedelcu *et al.*, 2004; Weinberger *et al.*, 2005; Lesser, 2006; Zuppini *et al.*, 2007)

Intracellular ROS has been reported to be rapidly reduced in several algal species by enzymatic and non-enzymatic processes (Collén *et al.*, 1995; Mtolera *et al.*, 1995; Contreras *et al.*, 2005; Xue *et al.*, 2005; Ross *et al.*, 2006). Although the exact mechanisms for reducing

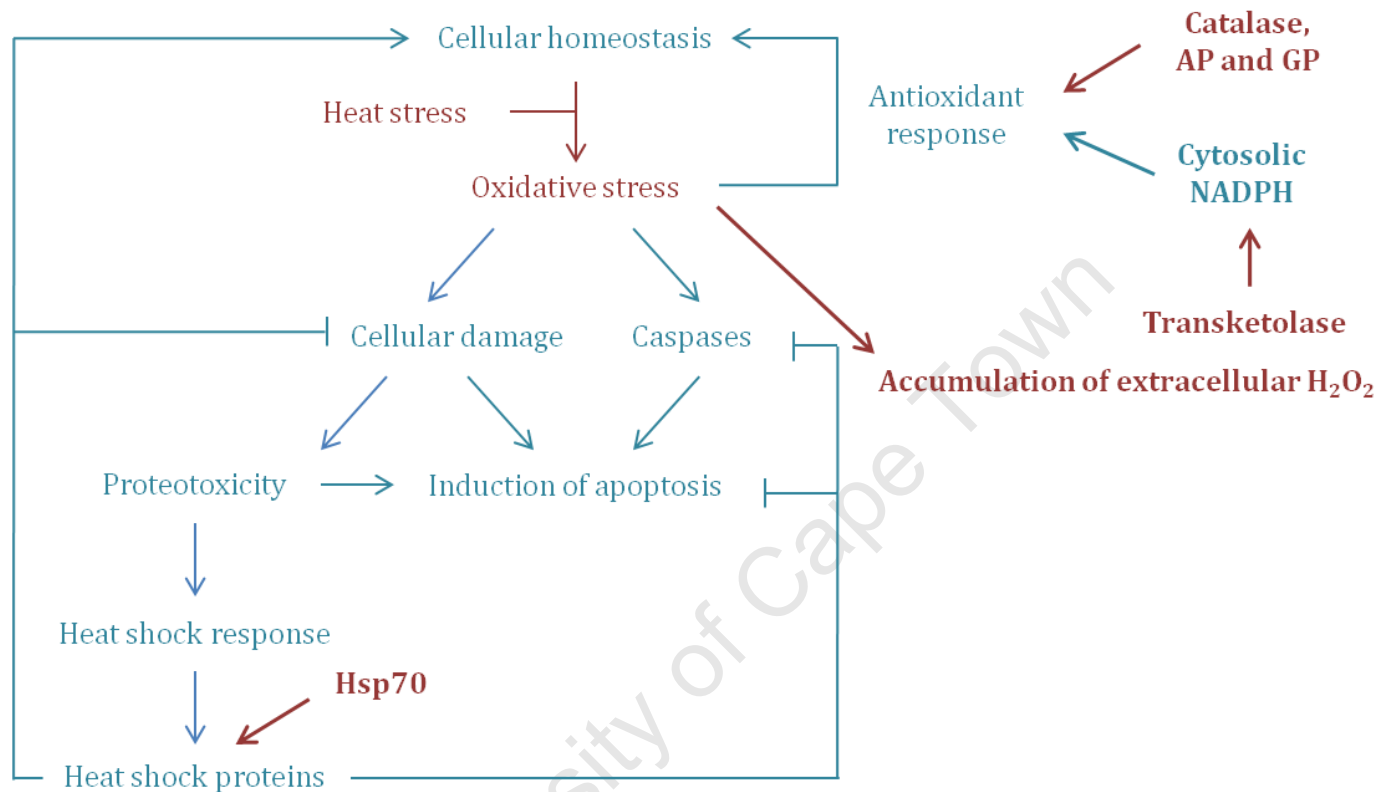


Figure 5.2 Schematic of the hypothesised cellular response to heat stress in *G. gracilis* during the maintenance of thermal tolerance between 0.5 and 8 hours of exposure to 35 °C. Unbolded text, components of the conserved cellular response to heat stress as presented in the literature review (Section 1.6); bolded text, additional components of the cellular response to heat stress in *G. gracilis* not originally presented in the conserved model (Section 1.6); red, components investigated in this study via experimental research; blue, components examined from literature. AP, ascorbate peroxidase; GP, glutathione peroxidase; NADPH, β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt; H₂O₂, hydrogen peroxidase; Hsp70, 70 kDa heat shock protein. The solid arrows and blunted lines represent the induction or repression of cellular pathways during thermal tolerance, respectively.

intracellular ROS in *G. gracilis* remain unclear, the activity of three well characterised antioxidant enzymes (catalase, glutathione peroxidase and ascorbate peroxidase), which reduce hydrogen peroxide to water (Lamb and Dixon, 1997; Weinberger *et al.*, 1999; Lesser, 2006) were monitored in *G. gracilis*. The rapid increase in catalase activity between 0.5 and 1 hour of heat shock, which occurred in conjunction with the accumulation of intracellular ROS, suggests that this enzyme reduces the oxidative burst, whereas glutathione peroxidase and ascorbate peroxidase, which increased in activity after 30 minutes of heat stress and remained elevated throughout exposure to heat stress, play a more general role in reducing intracellular ROS (Section 2.4.3).

In support of the increase of antioxidant enzyme activities in response to heat stress, the increase in the relative abundance of transketolase during thermal tolerance (Section 3.4.3) is hypothesised to facilitate detoxification of harmful reactive oxygen species by increasing the levels of cytosolic NADPH required by a number of antioxidant enzymes. This hypothesis is supported in studies by Slekar *et al.* (1996) and Alexander-Kaufman and Harper (2009) who suggested that during thermal tolerance, transketolase can link the pentose phosphate pathway to glycolysis thus increasing the production of cytosolic NADPH. This reducing agent is indirectly required for the elimination of harmful ROS by a number of detoxification enzymes, including glutathione peroxidase and ascorbate peroxidase (Tunc-Ozdemir *et al.*, 2009). In these examples, NADPH is required for the conversion of oxidised glutathione to reduced glutathione and dehydroascorbate to ascorbate, which are directly required for the conversion of hydrogen peroxide to water by glutathione peroxidase and ascorbate peroxidase respectively (Dalton *et al.*, 1986).

Although ROS accumulation is reduced by the antioxidant response, cellular damage can nevertheless rapidly manifest within the cell, resulting in oxidation of lipids, nucleic acids, polysaccharides and proteins, which can either induce ROS-mediated apoptosis via up-regulation of caspases (Gill and Tuteja, 2010) or directly induce cell death as the damage to

macromolecules accumulates (Larkindale and Knight, 2002; Suzuki and Mittler, 2006; Gupta *et al.*, 2010). The cell is thought to up-regulate damage repair pathways in response to accumulated cellular damage. One such pathway is the heat shock response. This pathway, widely accepted to be up-regulated in response to proteotoxicity, assists in maintaining thermal tolerance and inhibiting the onset of cell death (Hartl, 1996; Jolly and Morimoto, 2000; Song *et al.*, 2006; Reddy *et al.*, 2010). This pathway is hypothesised to be rapidly up-regulated in *G. gracilis* in response to heat stress. As one of the central proteins in the pathway, Hsp70 was found to be up-regulated within the first 30 minutes of exposure to heat stress (Section 3.4.3). This protein is hypothesised to help maintain thermal tolerance and inhibit the onset of cell death by restoring proteomic damage within the cell and blocking apoptotic pathways. The function of this protein is widely supported in the literature, and has been reported to specifically restore proteomic damage by re-folding nascent polypeptides, assembling protein complexes, translocating proteins across membranes, degrading damaged proteins and actively assembling protein complexes (Hartl, 1996; Song *et al.*, 2006; Reddy *et al.*, 2010). Hsp70s have also been reported to repress cell death by inhibiting caspase activation by cytochrome c, blocking metabolic events downstream of caspase activation, or inhibiting SAPKs/JNKs activation (Jolly and Morimoto, 2000).

Although *G. gracilis* maintained thermal tolerance for the first 8 hours of heat stress, it should be highlighted that the decreased thallus growth rates (Section 2.4.2) and accumulation of extracellular hydrogen peroxide observed after 6 hours of heat stress (Section 2.4.3) may be the first indication that a shift between thermal tolerance and cell death is about to occur. This hypothesis is supported by studies which indicated that the release of extracellular hydrogen peroxide co-ordinates a series of cellular responses that result in cell death in species of cyanobacteria and algae (Korsmeyer, 1995; Levine *et al.*, 1996; Vardi *et al.*, 1999; Apel and Hirt, 2004; Ross *et al.*, 2006; Zuppini *et al.*, 2007).

5.2 Hypothesised cellular response resulting in cell death

Growth and viability were not detectable in *G. gracilis* exposed to 35 °C for 18 hours, implying that a shift between thermal tolerance and cell death had taken place (Section 2.4.2). Furthermore, a number of changes in the cellular response provided an initial insight into the mechanisms involved in cell death in *G. gracilis* (Chapters 2 and 3; Figure 5.3). Extracellular hydrogen peroxide accumulated rapidly during cell death (Section 2.4.3), verifying the hypothesis that the production of extracellular hydrogen peroxide may trigger a series of cellular responses that result in the onset of cell death in *G. gracilis*.

In addition, a number of differentially expressed proteins were identified and their potential roles in facilitating the onset of cell death determined. The postulated functions of these proteins are based on previously reported information regarding the roles of these proteins in response to heat stress in various organisms. The decreased abundance of Hsp70 is hypothesised to remove the positive effect that Hsp70 has in maintaining thermal tolerance; specifically, by no longer reducing the concentration of impaired proteins nor blocking apoptotic pathways. Therefore, reduced Hsp70 levels are hypothesised to lead to a rapid increase in impaired proteins and consequently, cell death.

In contrast to Hsp70 degradation, a number of proteins increased in abundance after exposure to 18 hours of heat stress (Section 3.4.3). Of the 27 proteins determined to be up-regulated after 18 hours, five proteins were identified and hypothesised to facilitate in the onset of cell death (Section 3.4.4). These five proteins, namely chaperonin GroEL (GroEL), phycocyanin, patatin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and ferredoxin NADP⁺ reductase (FNR), will be discussed in more detail below.

The increased abundance of GroEL and phycocyanin in *G. gracilis* (Section 3.4.3) is hypothesised to participate in the induction of apoptosis. The hypothesised function of GroEL is substantiated by research which showed that up-regulated Hsp60 expression in humans (a GroEL homologue found in mammals) increased the rate of apoptosis by accelerating caspase activation (Bukau and Horwich, 1998; Samali *et al.*, 1999; Jolly and Morimoto, 2000). The hypothesised function of phycocyanin is supported by research that suggested that elevated levels of phycocyanin from the blue-green alga *Spirulina platensis* induced apoptosis in both the mouse macrophage cell line RAW 264.7 stimulated with LPS, and the human chronic myeloid leukemia cell line-K562 via the cytochrome C pathway which in turn activated caspases and induced the onset of cell death (Reddy *et al.*, 2003) and Subhashini *et al.*, 2004).

Up-regulation of patatin (Section 3.4.3) is hypothesised to facilitate destabilisation of cellular membranes during cell death. This hypothesis is supported by a study that reported that this group of storage proteins, which demonstrate nonspecific lipid acyl hydrolase activity, facilitate destabilisation of cellular membranes in tubers during cell death (Shewry, 2003). The suggested function of this protein in *G. gracilis* is further supported by the observed reduction in intracellular ROS to levels below that of the control sample after 18 hours of exposure to heat stress (Section 2.4.3), which most likely occurred as a consequence of leakage into the culture media through unstable cellular membranes. Indeed, a number of studies have reported that a potential loss in membrane stability through lipid oxidation is a physiological symptom of cell death in plant and algal species (Cadenas, 1989; Nedelcu *et al.*, 2004; Contreras *et al.*, 2005; Lesser, 2006; Ross *et al.*, 2006; Darehshouri *et al.*, 2008), and thus the hypothesised function of patatin in *G. gracilis* would be consistent with that reported for other plant and algal species.

The increased abundance of GAPDH (Section 3.4.3) is suspected to induce pro-apoptotic mitochondrial membrane permeabilisation and facilitate nuclear protein degradation. A

study by Tarze *et al.* (2007) supports the concept that GAPDH may induce pro-apoptotic mitochondrial membrane permeabilisation in humans during the onset of cell death. Hara *et al.* (2005) and Tristan *et al.* (2011) further hypothesised that S-nitrosylation of GAPDH during oxidative stress allows binding to an E3 ubiquitin ligase, thus allowing this complex to translocate into the nucleus where it facilitates nuclear protein degradation. The up-regulation of FNR (Section 3.4.3) has been correlated with DNA damage and may indicate that DNA damage has already taken place. Research by Girardini *et al.* (2002) and Lui and Chen (2002) reported that up-regulation of FNR is hypothesised to induce apoptosis in response to DNA damage. Therefore, it can be concluded that after the onset of cell death, a number of cellular pathways are up-regulated to induce cellular imbalances that facilitate cell death.

To address the final objective of this study, a potential molecular indicator of heat stress for this alga was identified. An *Hsp70* gene which encoded for a 70 kDa heat shock protein localised to the endoplasmic reticulum was successfully cloned, sequenced and characterised (Section 4.4.1). Transcriptional regulation of the ER *Hsp70* was examined by real-time PCR which indicated that the transcript rapidly increased in abundance within the first 30 minutes of heat stress (Section 4.4.2). Although it was reasonable to conclude that this study identified the ER *Hsp70* as a potential molecular indicator of heat stress in *G. gracilis*, future research will need to be performed to validate this hypothesis. In doing so, a repetition of the real-time results could be performed to obtain statistically relevant real-time data which could be used to confirm the transcriptional response of the *G. gracilis* ER *Hsp70* to heat stress. Cloning and expression of the full length ER *Hsp70* gene would be useful as the expressed protein could be used to raise ER *Hsp70* antibodies from which translational analysis could be performed to validate the use of this protein as a molecular indicator of heat stress in *G. gracilis*.

Thus, overall this study successfully fulfilled the three objectives of this study by (i) identifying the conditions required to induce a cellular response to heat stress in cultivated *G. gracilis*, (ii) by gaining an initial insight into the molecular pathways involved in the maintenance of thermal tolerance and the onset of cell death and (iii) by identifying a potential molecular indicator which could be employed as a useful tool in future studies. Therefore, this study did not only incorporate vital initial studies required to lay a platform from which future experimental studies can be performed, but it also provided an initial insight into the convoluted cellular response of *G. gracilis* to heat stress. In doing so, this research has also contributed to the general understanding of an organism's response to heat stress and the poorly studied link between the maintenance of thermal tolerance and the onset of cell death.

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APENDIX A

MEDIA AND SOLUTIONS

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Regarding the preparation of all media, solutions and buffers, glassware was washed in dH₂O and autoclaved at 121 °C for 40 min prior to use. Distilled water (dH₂O) was purified using a Milli-RO Plus (Millipore) water purification system, ultra pure water was obtained by further purifying dH₂O using a Milli-Q Plus (Millipore) water purification system, and DEPC treated water (Appendix A.2.19.1) was used for RNA related applications. To sterilise purified water (sterile dH₂O), dH₂O was autoclaved at 121 °C for 40 min before use.

After preparation, all media, solutions and buffers were autoclaved at 121 °C for 20 min, before being stored at room temperature, unless otherwise stated.

A.1 Media

A.1.1 Artificial Seawater (ASW)

NaCl (Saarchem)	123.5 g
MgCl ₂ .6H ₂ O (Saarchem)	23.5 g
KCl (Saarchem)	3.3 g
CaCl ₂ .2H ₂ O (Saarchem)	9.5 g
MgSO ₄ .7H ₂ O (Saarchem)	31.5 g
NaHCO ₃ (Saarchem)	0.9 g
dH ₂ O to	5 l

A.1.2 Luria Bertani agar (LA)

Tryptone (Biolab)	10 g
Yeast Extract (Biolab)	5 g
NaCl	5 g
Agar (Biolab)	15 g
dH ₂ O to	1 l

A.1.3 Luria Bertani broth (LB)

Tryptone	10 g
Yeast Extract	5 g
NaCl	5 g
dH ₂ O to	1 l

A.2 Solutions

A.2.1 General stock solutions

A.2.1.1 1 M Hydrogen peroxide (H₂O₂)

30 % H ₂ O ₂ (Sigma)	1.022 ml
Sterile dH ₂ O to	10 ml

Do not autoclave. Light sensitive. Store at 4 °C.

A.2.1.2 0.1 M Phosphate buffer (pH 7.0)

0.2 M Potassium phosphate, mono-potassium salt (Appendix A.2.1.2.1)	39 ml
0.2 M Potassium phosphate, di-potassium salt (Appendix A.2.1.2.2)	61 ml
dH ₂ O to	100 ml

Adjust the pH to 7.0 (Beckman Φ 70 pH meter).

A.2.1.2.1 0.2 M Potassium phosphate, mono-potassium salt

KH ₂ PO ₄ (Merck)	27.2 g
dH ₂ O to	1 l

A.2.1.2.2 0.2 M Potassium phosphate, di-potassium salt

K ₂ HPO ₄ (Merck)	34.8 g
dH ₂ O to	1 l

A.2.1.3 0.5 M Ethylenediaminetetraacetic acid (EDTA; pH 8.0)

EDTA (Saarchem)	93.05 g
NaOH (Saarchem)	10 g
dH ₂ O to	500 ml

Dissolve solutes in 400 ml dH₂O and adjust the pH to 8.0 (Beckman Φ 70 pH meter). Make up to a final volume of 500 ml.

A.2.1.4 1 M Tris (hydroxymethyl) aminomethane-hydrochloric acid (Tris-HCl)

Tris (Sigma)	60.55 g
dH ₂ O to	500 ml

Dissolve Tris in 400 ml dH₂O. Adjust to the required pH (Beckman Φ 70 pH meter) using HCl (Saarchem). Make up to a final volume of 500 ml.

A.2.1.5 Urea Lysis Buffer (ULB)

Urea (Bio Basic Inc.)	24 g
Thiourea (Sigma)	7.61 g
3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate (CHAPS; Calbiochem®)	1.0 g
Sterile dH ₂ O to	50 ml

Dissolve urea, thiourea and CHAPS in 25 ml sterile dH₂O at 30 °C, using agitation. Once the solutes are fully dissolved, make up to a final volume of 50 ml with sterile dH₂O. Aliquot out and store at -20 °C. Before use, defrost on ice and vortex to ensure the suspension of solutes. Discard unused ULB. Do not refreeze.

A.2.1.6 1X Tris buffered saline (TBS)

10X TBS (Appendix A.2.1.6.1)	100 ml
Sterile dH ₂ O to	1 l

Do not autoclave.

A.2.1.6.1 10X TBS

Tris	30.25 g
NaCl	43.8 g
dH ₂ O to	500 ml

Dissolve solutes in 400 ml of dH₂O. Adjust the pH to 7.4 (Beckman Φ 70 pH meter) and make up to a final volume of 500 ml.

A.2.1.7 20 % Sodium dodecyl sulfate (SDS)

SDS (Saarchem)	20 g
dH ₂ O to	100 ml

Dissolve SDS in dH₂O at 37 °C, using agitation.

A.2.1.8 70 % Ethanol

Absolute ethanol (Merck)	70 ml
Sterile dH ₂ O to	100 ml

Do not autoclave.

A.2.1.9 Tris-EDTA buffer

1 M Tris (pH 7.6; Appendix A.2.1.4)	1 ml
0.5 M EDTA (pH 8.0; Appendix A.2.1.3)	200 µl
dH ₂ O to	100 ml

A.2.1.10 6X Tracking dye

Bromophenol Blue (Saarchem)	62.5 mg
Sucrose (Saarchem)	10 g
0.5 M EDTA (pH 8.0; Appendix A.2.1.3)	1 ml
dH ₂ O to	25 ml

A.2.2 Solutions for the enrichment of ASW

A.2.2.1 PES-enriched seawater medium

(Provasoli, 1968)

NaNO ₃ (BDH)	350 mg
Na ₂ glycerophosphate.5H ₂ O (Merck)	50 mg
Fe-solution (Appendix A.2.2.1.1)	25 ml
PII metal solution (Appendix A.2.2.1.2)	25 ml
Vitamin B ₁₂ (Sigma)	10 µg
Thiamine (Sigma)	0.5 mg
Biotin (Sigma)	5 µg

Tris	500 mg
dH ₂ O to	100 ml

Dissolve NaNO₃, Na₂ glycerophosphate.5H₂O and Tris in 100 ml dH₂O. Autoclave and cool to room temperature. After cooling, add the remaining constituents and adjust the pH to 7.8 (Beckman Φ 70 pH meter). Light sensitive. Store at 4 °C. To obtain a ½ growth medium (enriched ASW), dilute PES-enriched seawater medium in ASW (Appendix A.1.1) at a ratio of 1: 100 (v/v) prior to use.

A.2.2.1.1 Fe-solution

Fe(NH ₄) ₂ (SO ₄) ₂ .6H ₂ O (BDH)	702 mg
EDTA	600 mg
dH ₂ O to	1 l

Store at 4 °C.

A.2.2.1.2 PII metal solution

EDTA	100 mg
H ₃ BO ₃ (Saarchem)	114 mg
FeCl ₃ .6H ₂ O (Saarchem)	4.9 mg
MnSO ₄ (Sigma)	16.4 mg
ZnSO ₄ .7H ₂ O (Sigma)	2.2 mg
CoSO ₄ .7H ₂ O (Saarchem)	0.48 mg
dH ₂ O to	100 ml

Store at 4 °C.

A.2.3 Solutions for viability assay

A.2.3.1 0.8 % 2, 3, 5-triphenyltetrazolium chloride (TTC) solution

TTC (Sigma)	80 mg
ASW containing 50 mM Tris (pH 7.6; Appendix A.2.3.1.1)	10 ml

Do not autoclave. Light sensitive. Prepare fresh and cool to 15 °C before use.

A.2.3.1.1 ASW containing 50 mM Tris (pH 7.6)

NaCl	4.94 g
MgCl ₂ .6H ₂ O	0.94 g
KCl	0.13 g
CaCl ₂ .2H ₂ O	0.38 g
MgSO ₄ .7H ₂ O	1.26 g
NaHCO ₃	0.04 g
Tris	1.21 g
dH ₂ O to	200 ml

Dissolve solutes in 150 ml dH₂O. Adjust the pH to 7.6 (Beckman Φ 70 pH meter) and make up to a final volume of 200 ml.

A.2.3.2 0.2 M Potassium hydroxide (KOH) in 25 % ethanol

KOH (Saarchem)	1.12 g
Absolute ethanol	25 ml
Sterile dH ₂ O to	100 ml

Do not autoclave.

A.2.4 Solutions for the detection of intercellular reactive oxygen species

A.2.4.1 10 mM 2', 7'-dichlorofluorescein (DFC)

DFC (Sigma)	0.0401 g
40 mM Tris-HCl (pH 7.0; Appendix A.2.4.2) to	10 ml

Do not autoclave. Prepare fresh before use.

A.2.4.2 40 mM Tris-HCl (pH 7.0)

Tris	0.4845g
dH ₂ O to	100 ml

Dissolve Tris in 60 ml dH₂O. Adjust the pH to 7.0 (Beckman Φ 70 pH meter) and make up to a final volume of 100 ml.

A.2.4.3 5 mM 2', 7'-dichlorodihydrofluorescein diacetate (H₂DCF-DA)

H ₂ DCF-DA (Sigma)	0.0244 g
Absolute ethanol to	10 ml

Do not autoclave. Light sensitive. Store at -20 °C.

A.2.5 Solutions for the detection of extracellular released hydrogen peroxide

A.2.5.1 Xylenol orange assay mix

100 mM Ammonium ferrous sulphate (Appendix A.2.5.1.1)	0.5 ml
1 M Sorbitol (Appendix A.2.5.1.2)	20 ml
3 mM Xylenol Orange (Appendix A.2.5.1.3)	6.67 ml
1 M Sulphuric acid (Appendix A.2.5.1.4)	5ml
Sterile dH ₂ O to	100 ml

Do not autoclave. Prepare fresh before use.

A.2.5.1.1 100 mM Ammonium ferrous sulphate

Ammonium ferrous sulphate (Sigma)	0.039g
dH ₂ O to	1 ml

A.2.5.1.2 1 M Sorbitol

D-Sorbitol (Sigma)	9.1 g
dH ₂ O to	50 ml

A.2.5.1.3 3 mM Xylenol orange

Xylenol orange (Sigma)	0.107g
Sterile dH ₂ O to	50 ml

Do not autoclave. Prepare fresh daily.

A.2.5.1.4 1 M Sulphuric acid

H ₂ SO ₄ (Merck)	544 µl
Sterile dH ₂ O to	10 ml

Do not autoclave. Add sulphuric acid slowly to sterile dH₂O.

A.2.6 Solutions for the catalase assay

A.2.6.1 Catalase reaction buffer

1 M Hydrogen peroxidase (Appendix A.2.1.1)	2.222 ml
0.1 M Phosphate buffer (pH 7.0; Appendix A.2.1.2) to	100 ml

Do not autoclave. Light sensitive. Prepare fresh before use.

A.2.7 Solutions for the ascorbate peroxidase assay

A.2.7.1 0.1 M Sodium ascorbate

Sodium L-ascorbate (BDH)	0.0991 g
0.1 M Phosphate buffer (pH 7.0; Appendix A.2.1.2) to	5 ml

Do not autoclave. Light sensitive. Prepare fresh before use.

A.2.7.2 Ascorbate peroxide extraction buffer

0.1 M Sodium ascorbate (Appendix A.2.7.1)	500 µl
0.5 M EDTA (pH 8.0; Appendix A.2.1.3)	20 ml
0.1 M Phosphate buffer (pH 7.0; Appendix A.2.1.2) to	100 ml

Do not autoclave. Light sensitive. Prepare fresh before use.

A.2.7.3 Ascorbate peroxidase reaction buffer

0.1 M Sodium ascorbate (Appendix A.2.7.1)	444 µl
1 M Hydrogen peroxide (Appendix A.2.1.1)	1.6 ml

0.1 M Phosphate buffer (pH 7.0; Appendix A.2.1.2) to 100 ml
Do not autoclave. Light sensitive. Prepare fresh before use.

A.2.8 Solutions for the glutathione pathway assay

A.2.8.1 0.1 M β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH)

NADPH (Sigma) 0.0833 g
0.1 M Phosphate buffer (pH 7.0; Appendix A.2.1.2) to 1 ml

Do not autoclave. Light sensitive. Store at -20 °C.

A.2.8.2 Glutathione peroxidase reaction buffer

L-Glutathione reduced (Sigma) 0.0061 g
1 M Hydrogen peroxide (Appendix A.2.1.1) 800 μ l
0.1 M NADPH (Appendix A.2.8.1) 88.88 μ l
0.1 M Phosphate buffer (pH 7.0; Appendix A.2.1.2) to 100 ml

Do not autoclave. Light sensitive. Prepare fresh before use.

A.2.9 Solutions for the isolation of total soluble protein

A.2.9.1 Protein extraction buffer

1 M Tris-HCl (pH 7.5; Appendix A.2.1.4) 5 ml
0.5 M EDTA (pH 8.0; Appendix A.2.1.3) 200 μ l
Triton® X-100 (Promega) 100 μ l
2-Mercaptoethanol (Merck) 200 μ l
0.2 M PMSF (Appendix A.2.9.1.1) 50 μ l
Sterile dH₂O to 10 ml

Do not autoclave. Prepare immediately before each extraction and store on ice until required.

A.2.9.1.1 0.2 M Phenylmethanesulfonyl fluoride (PMSF)

PMSF (Sigma)	0.0348 g
Absolute ethanol	1 ml

Do not autoclave. Aliquot out and store at -20 °C. Defrost on ice and vortex well before use.

A.2.9.2 0.1 M Ammonium acetate in methanol

Ammonium acetate (Saarchem)	3.85 g
Methanol (Merck) to	500 ml

Do not autoclave. Store at -20 °C.

A.2.9.3 80 % Acetone

Acetone (Merck)	400 ml
Sterile dH ₂ O to	500 ml

Do not autoclave. Store at -20 °C.

A.2.10 Solutions for the quantification of total soluble protein

A.2.10.1 Bovine serum albumin (BSA; 10 mg/ml)

BSA (Roche)	100 mg
Sterile dH ₂ O to	10 ml

Do not autoclave. Store at -20 °C.

A.2.10.2 0.1 M Hydrochloric acid (HCl)

1 M HCl (Appendix A.2.1.1)	10 ml
dH ₂ O to	100 ml

A.2.11 Solutions for the preparation and electrophoresis of SDS-PAGE gels

A.2.11.1 4X Tris-HCl/SDS (pH 8.8)

Tris	91 g
SDS	2 g
Sterile dH ₂ O to	500 ml

Add Tris and SDS to 300 ml sterile dH₂O. Adjust the pH to 8.8 (Beckman Φ 70 pH meter) and make the final volume up to 500 ml. Do not autoclave. Filter sterilise and store at 4 °C.

A.2.11.2 10 % Ammonium persulphate (AMPS)

AMPS (Promega)	1 g
Sterile dH ₂ O to	10 ml

Do not autoclave. Filter sterilise and store at 4 °C.

A.2.11.3 4X Tris-HCl/SDS (pH 6.8)

Tris	6.05 g
SDS	0.4 g
Sterile dH ₂ O to	100 ml

Add Tris and SDS to 40 ml sterile dH₂O. Adjust the pH to 6.8 (Beckman Φ 70 pH meter) and make the final volume up to 100 ml with sterile dH₂O. Do not autoclave. Filter sterilise and store at 4 °C.

A.2.11.4 1X SDS PAGE running buffer

5X SDS PAGE running buffer (Appendix A.2.11.4.1)	200 ml
Sterile dH ₂ O to	1 l

Do not autoclave. Cool to 4 °C prior to use.

A.2.11.4.1 5X SDS PAGE running buffer

Tris	7.55 g
Glycine (Saarchem)	36 g
SDS	2.5 g
Sterile dH ₂ O to	500 ml

Do not autoclave.

A.2.11.5 5X Protein sample application buffer

1 M Tris (pH 6.8; Appendix A.2.1.4)	2.25 ml
Glycerol (Merck)	5 ml
20 % SDS (Appendix A.2.1.7)	2.5 ml
Bromophenol Blue	5 mg
2-Mercaptoethanol	2.25 ml

Do not autoclave. Dilute 1: 5 with protein sample and boil. the mixture for 5 min at 100 °C before loading onto a SDS-PAGE gel.

A.2.12 Solutions for the preparation and loading of proteins onto a 2D SDS-polyacrylamide gel

A.2.12.1 10 % ASB-14

ASB-14 (Sigma)	0.1 g
Sterile dH ₂ O to	1 ml

Do not autoclave. Aliquot out and store at -20 °C. Defrost on ice and vortex well before use.

A.2.12.2 30 % DL-Dithiothreitol (DTT)

DTT (Promega)	0.3 g
Sterile dH ₂ O to	1 ml

Do not autoclave. Aliquot out and store at -20 °C. Defrost on ice and vortex well before use.

A.2.12.3 Equilibration buffer stock solution

Urea	72.1 g
1.5 M Tris-HCl (pH 8.8; Appendix A.2.12.3.1)	50 ml
SDS	4 g
Glycerol	40 ml
Sterile dH ₂ O to	200 ml

Dissolve Urea in Tris-HCl and 25 ml sterile dH₂O at 30 °C. Once fully dissolved, add SDS and glycerol. Make up to a final volume of 200 ml with sterile dH₂O. Do not autoclave. Aliquot buffer into 10 ml volumes and store at -20 °C. Defrost aliquots on ice for 1 hour before use.

A.2.12.3.1 1.5 M Tris-HCl (pH 8.8)

Tris	90.83 g
dH ₂ O to	500 ml

Dissolve Tris in 400 ml dH₂O. Adjust to pH to 8.8 (Beckman Φ 70 pH meter) using HCl (Saarchem) and make up to a final volume of 500 ml.

A.2.12.4 Equilibration buffer 1

DTT	0.1 g
Equilibration buffer stock solution (Appendix A.2.12.3)	10 ml
Bromophenol blue	Trace amounts to colour

Do not autoclave. Prepare immediately before use. Vortex well to ensure that the DTT has fully dissolved prior to use.

A.2.12.5 Equilibration buffer 2

Iodoacetamide (Bio-Rad)	0.48 g
Equilibration buffer stock solution (Appendix A.2.12.3)	10 ml
Bromophenol blue	Trace amounts to colour

Do not autoclave. Prepare immediately before use. Vortex well to ensure that the iodoacetamide has fully dissolved before use.

A.2.12.6 0.5 % Agarose in 1X SDS PAGE running buffer

Agarose (Lonza)	0.5 g
1X SDS PAGE running buffer (Appendix A.2.11.4)	100 ml
Bromophenol blue	Trace amounts to colour

In preparation of this solution, add the agarose to 1X SDS PAGE running buffer and microwave until the agarose has fully dissolved. Prior to use, re-melt the set agarose solution in a microwave and allow cooling to ~40 °C before use.

A.2.13 Solutions for the visualisation of protein on SDS-PAGE gels

A.2.13.1 Coomassie blue staining solution

Coomassie brilliant blue R250 (BDH)	0.5 g
Methanol	225 ml
Glacial acetic Acid (Merck)	50 ml
Sterile dH ₂ O to	500 ml

Do not autoclave. Filter sterilise before use.

A.2.13.2 Coomassie blue destaining solution

Methanol	50 ml
Glacial acetic acid	70 ml
Sterile dH ₂ O to	1 l

Do not autoclave.

A.2.14 Solution for the electrotransfer of proteins onto nitrocellulose membrane

A.2.14.1 Towbin buffer

Tris	3.03 g
Glycine	14.42 g
Methanol	200 ml

Sterile dH₂O to 1 l

Do not autoclave. Store at 4 °C.

A.2.15 Solution for the visualisation of proteins on a nitrocellulose membrane

A.2.15.1 Ponceau S staining solution

Ponceau S (Sigma)	0.1 g
Glacial acetic acid	5 ml
Sterile dH ₂ O to	100 ml

Do not autoclave. Light sensitive. Reusable.

A.2.16 Solutions for western blot analysis

A.2.16.1 Blocking buffer

Skim milk powder (Clover)	5 g
1X TBS (Appendix A.2.1.6)	100 ml

Do not autoclave. Prepare fresh before use.

A.2.16.2 Anti-Hsp70 primary antibody solution

Anti-Hsp70 (rabbit polyclonal serum; SPC-103C/D; StressMarq Biosciences Inc.)	2 µl
Blocking buffer (Appendix A.2.16.1)	10 ml

Do not autoclave. Aliquot and store at -20 °C. Defrost on ice before use. Reusable, but discard when hybridisation signal begins to fade.

A.2.16.3 Anti-GroEL primary antibody solution

Anti-Gro-EL (rabbit immunoglobulin G (IgG) Fraction; G6532; Sigma)	1 µl
Blocking buffer (Appendix A.2.16.1)	10 ml

Do not autoclave. Aliquot and store at -20 °C. Defrost on ice before use.
Reusable. Discard when hybridisation signal begins to fade.

A.2.16.4 Anti-FNR primary antibody solution

Anti-FNR (rabbit polyclonal serum; AS101625; Agrisera)	3.33 µl
Blocking buffer (Appendix A.2.16.1)	10 ml

Do not autoclave. Aliquot and store at -20 °C. Defrost on ice before use.
Reusable. Discard when hybridisation signal begins to fade.

A.2.16.5 Anti-Hsp70 combined with Anti-Rubisco primary antibody

Anti-Hsp70 (rabbit polyclonal serum; SPC-103C/D; StressMarq Biosciences Inc.)	10 µl
Anti-Rbcl (Rubisco large subunit; rabbit polyclonal serum; AS03037; Agrisera)	0.5 µl
Blocking buffer (Appendix A.2.16.1)	50 ml

Do not autoclave. Aliquot and store at -20 °C. Defrost on ice before use.
Reusable, but discard when hybridisation signal begins to fade.

A.2.16.6 1X Tris buffered saline with Tween 20 (TBST)

Tween® 20 (Saarchem)	1 ml
1X TBS (Appendix A.2.1.6)	1 l

Do not autoclave. Light sensitive.

A.2.16.7 Goat anti-rabbit secondary antibody solution

Affinity Purified A Peroxidase Labeled Goat anti-rabbit IgG Liquid Conjugate (KPL)	2 µl
Blocking buffer (Appendix A.2.16.1)	20 ml

Do not autoclave. Aliquot and store at -20 °C. Defrost on ice before use.
Reusable. Discard when hybridisation signal begins to fade.

A.2.17 Solutions for the isolation of genomic DNA

A.2.17.1 DNA extraction buffer

Tris	1.21 g
EDTA	1.86 g
NaCl	2.92 g
dH ₂ O to	100 ml

Dissolve Tris, EDTA and NaCl in 80 ml dH₂O and adjust the pH to 8.0 (Beckman Φ 70 pH meter) using HCl (Saarchem). Make up to a final volume of 100 ml.

A.2.17.2 Proteinase K (20 mg/ml)

Proteinase K (Sigma)	20 mg
Sterile dH ₂ O to	1 ml

Do not autoclave. Aliquot out and store at -20 °C.

A.2.17.3 RNase A (10 mg/ml)

RNase A (Sigma)	0.1 g
1 M Tris-HCl (pH 7.5; Appendix A.2.1.4)	100 μ l
5 M NaCl (Appendix A.2.17.3.1)	3 ml
Sterile dH ₂ O to	10 ml

Heat for 15 min at 100 °C and allow to cool at room temperature. Do not autoclave. Aliquot out and store at -20 °C.

A.2.17.3.1 5 M NaCl

NaCl	29.22 g
dH ₂ O to	100 ml

A.2.18 Solutions for the electrophoresis of DNA on 1.5 % TAE-agarose gel

A.2.18.1 1X TAE (Tris-acetate-EDTA buffer)

50X TAE (Appendix A.2.18.1.1)	20 ml
dH ₂ O to	1 l

A.2.18.1.1 50X TAE

Tris	242 g
Glacial acetic acid (Saarchem)	57.1 ml
0.5 M EDTA (pH 8.0; Appendix A.2.1.3)	100 ml
dH ₂ O to	1 l

A.2.18.2 Ethidium bromide (EtBr; 10 mg/ml)

EtBr (Sigma)	0.1 g
Sterile dH ₂ O to	10 ml

Do not autoclave. Contains a powerful mutagen. Wear gloves at all times and. clean all spills immediately with isopropanol (Merck).

A.2.19 Solutions for the preparation and electrophoresis of total RNA

A.2.19.1 Diethylpyrocarbonate (DEPC) treated water

DEPC (Sigma)	1 ml
Sterile dH ₂ O to	1 l

Add DEPC sterile dH₂O. Loosen the cap and leave to stand in a fume hood for a minimum of 12 hours before use.

A.2.19.2 70 % Ethanol (RNA)

Absolute ethanol	70 ml
DEPC treated water (Appendix A.2.19.1) to	100 ml

Do not autoclave.

A.2.19.3 Phenol: chloroform: isoamyl alcohol (25: 24: 1)

Phenol (pH 4.0; Sigma)	5 ml
Chloroform (Merck)	4.8 ml
Isoamyl alcohol (Merck)	0.2 ml

Light sensitive. Prepare fresh before use.

A.2.19.4 Sodium acetate (pH 5.2)

Sodium acetate (Saarchem)	40.8 g
DEPC treated water (Appendix A.2.19.1) to	100 ml

Dissolve sodium acetate in 80 ml DEPC treated water and adjust the pH to 5.2 (Beckman Φ 70 pH meter) with glacial acetic acid (Merck). Make up to a final volume of 500 ml. Do not autoclave. Filter sterilise.

A.2.19.5 10X MOPS (pH 7.0)

MOPS (Sigma)	10 g
Sodium acetate	0.5 g
0.5 M EDTA (RNA; Appendix A.2.19.5.1)	5 ml
DEPC treated water (Appendix A.2.19.1) to	250 ml

Dissolve solutes in 240 ml DEPC treated water and adjust the pH to 7.0 with NaOH (Saarchem). Make up to a final volume of 250 ml. Do not autoclave. Filter sterilise and store at 4 °C. Light sensitive.

A.2.19.5.1 0.5 M EDTA (RNA)

EDTA	93.05 g
NaOH	10 g
DEPC treated water (Appendix A.2.19.1) to	500 ml

Dissolve EDTA and NaOH in 400 ml DEPC treated water and adjust the pH to 8.0 (Beckman Φ 70 pH meter). Make up to the final volume of 500 ml.

A.2.19.6 1X 3-(N-morpholino) propanesulfonic acid (MOPS) running buffer (pH 7.0)

10X MOPS (pH 7.0; Appendix A.2.19.5)	100 ml
DECP treated water (Appendix A.2.19.1) to	1 l

Light sensitive. Do not autoclave. Store at 4 °C.

A.2.19.7 RNA sample application buffer

10X MOPS (Appendix A.2.19.5)	300 µl
37 % Formaldehyde (Merck)	80 µl
Formamide (Merck)	900 µl
EtBr (RNA; 10 mg/ml; Appendix A.2.19.7.1)	2 µl
DEPC treated water (Appendix A.2.19.1) to	220 µl

Do not autoclave. Light sensitive. Store at 4 °C. Wear gloves at all times and. clean all spills immediately with isopropanol.

A.2.19.7.1 EtBr (RNA; 10 mg/ml)

EtBr	0.1 g
DEPC treated water (Appendix A.2.19.1) to	10 ml

Do not autoclave. Contains a powerful mutagen, therefore wear gloves at all times. Clean all spills immediately with isopropanol.

A.2.20 Solutions for the preparation and selection of successfully transformed *E.coli* cells

A.2.20.1 Ampicillin (100 mg/ml)

Ampicillin (Sigma)	2 g
Sterile dH ₂ O to	20 ml

Do not autoclave. Filter sterilise and store aliquots at 4 °C. Dilute 1: 1 000 in media to obtain a final concentration of 100 µg/ml.

A.2.20.2 Bromo-chloro-indolyl-galactopyranoside (X-gal; 20mg/ml)

X-gal (Sigma)	0.2 g
N, N-Dimethylformamide (Sigma)	10 ml

Do not autoclave. Filter sterilise and store aliquots at -20 °C. Dilute 1: 500 in media to obtain a final concentration of 40 µg/ml.

A.2.20.3 10 mM Isopropyl β-D-1-thiogalactopyranoside (IPTG)

IPTG (Fermentas)	0.2383 g
Sterile dH ₂ O to	10 ml

Do not autoclave. Filter sterilise and store aliquots at 4 °C. Dilute 1: 200 in media to obtain a final concentration of 50 µM.

University of Cape Town

APPENDIX B
STANDARD METHODS
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All procedures were performed at room temperature, unless otherwise stated.

B.1 Viability assay

(Adapted from Nam *et al.*, 1998)

This viability assay uses a 2, 3, 5-triphenyltetrazolium chloride solution to determine the viability of *G. gracilis* thallus segments. Triphenyltetrazolium chloride is a colourless substance that when reduced by respiratory enzymes such as dehydrogenases, is converted into 1, 3, 5-triphenylformazan, an insoluble red compound (Chang *et al.*, 1999). Thus the viability of *G. gracilis* can be determined by measuring the concentration of triphenylformazan in the tissue. Before determining the viability of experimental thallus segments, two control samples were prepared. A positive control was prepared by acclimatising *G. gracilis* at 15 °C for 10 days (viable tissue sample; Section 2.3.1.2) and a negative control was prepared by exposing tissue to 60 °C for 30 min (non-viable tissue sample; Section 2.3.2).

To determine the viability of the experimental thalli, 0.1 g of each thallus, 0.1 g of the positive control and 0.1 g of the negative control was processed concurrently. Each thallus was placed into a separate sterile microfuge tube, immersed in 1.5 ml of 0.8 % 2, 3, 5-triphenyltetrazolium chloride solution (Appendix A.2.3.1) and overlaid with 500 µl mineral oil (Bio-rad). The tubes were tightly sealed and incubated at 15 °C for 3 h in the dark. Post incubation, thalli were rinsed four times in 2 ml ASW (Appendix A.1.1), blotted dry and transferred to sterile microfuge tubes containing 1 ml of 0.2 M potassium hydroxide in 25 % ethanol (Appendix A.2.3.2). The tubes were then incubated at 60 °C for 30 min, after which 1 ml of Hexane (Sigma) was added to each tube. The tubes were vortexed for 30 seconds and centrifuged at 12 000 rpm for 1 min. The top layer from each tube was transferred to a cuvette and the absorbance of each solution was measured at 545 nm using a DU®530 UV/Vis Spectrophotometer (Beckman).

The viability of each experiment thallus segment, indicating the percentage of viable cells, was calculated using the following equation:

$$\text{Percentage viability} = [(S-TC)/(C-TC)] \times 100$$

Where S = absorbance of experimental tissue

TC = absorbance of negative control

C = absorbance of positive control

B.2 Detection of intracellular reactive oxygen species

(Adapted from Contreras *et al.*, 2005)

This assay uses a solution of 2', 7'-dichlorofluorescein diacetate (H₂DCF-DA) to measure the concentration of reactive oxygen species within *G. gracilis* thalli. Dichlorofluorescein diacetate is a non-fluorescent compound that can permeate cells. Once in the cells, H₂DCF-DA is rapidly de-esterified to dihydrodichlorofluorescein (H (2) DCF). In turn, H (2) DCF can be rapidly oxidized by intracellular ROS to fluorescent 2', 7'-dichlorofluorescein (DCF). The fluorescence of DCF is measured in this assay and used as an indirect measure of determine the concentration of intracellular ROS within the cells (Karlsson *et al.*, 2010).

Thus, prior to determining the concentration intracellular reactive oxygen species (ROS), a standard curve was constructed to represent the linear relationship between the known concentrations of 2', 7'-dichlorofluorescein (DFC) and resultant fluorescence at an excitation wavelength at 488 nm and an emission wavelength of 525 nm. In doing so, 10 mM DFC stock solution (Appendix A.2.4.1) was diluted 1: 10 000 with 40 mM Tris-HCl (pH 7.0; Appendix A.2.4.2) to prepare a 1 µM DFC working stock solution. This solution was used to prepare known concentrations of DFC, in sterile microfuge tubes, by diluting the 1 µM DFC working stock solution with 40 mM Tris-HCl (pH 7.0) as described below:

Final DFC concentration (nM)	1 µM DFC (µl)	40 mM Tris-HCl (pH 7.0) (µl)
0	0	1000
200	200	800
400	400	600
600	600	400
800	800	200
1000	1000	0

After preparing the various concentrations of DFC, 150 µl of each solution was dispensed in triplicate, into a 96 well polystyrene black plate (Porvair Sciences). The fluorescence in each well was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm using a Modulus™ Microplate Multimode Reader (Turner BioSystems). The results obtained were used to construct a standard curve, from which the concentration of DFC in any given sample could be deduced from its fluorescence.

The levels of intracellular ROS in the tissue samples were therefore quantified by measuring the DFC fluorescence in the cell lysates. Thus, the experimental samples were assessed concurrently with a negative control. For each experiment sample, 1.0 g of tissue was transferred to a 50 ml sterilin (Sigma) containing 25 ml of ASW (Appendix A.1.1) supplemented with 25 µl 5 mM H₂DCF-DA (Appendix A.2.4.3), whereas the 1.0 g of the negative control tissue (*G. gracilis* acclimatised at 15 °C for 10 days; Section 2.3.1.2) was transferred into a sterilin containing 25 ml of ASW (omitting H₂DCF-DA). All tubes were incubated for 1 h at 15 °C, after which the thallus segments were rinsed in 2 ml ASW, blotted dry and ground up in liquid nitrogen using a pestle and mortar. The fine powder from each thallus was further transferred to a sterile eppendorf containing 2 ml of 40 mM Tris-HCl (pH 7.0). Tubes were briefly vortexed and centrifuged at 12 000 rpm for 15 min, after which 150 µl of the supernatant from each tube and 150 µl of 40 mM Tris-HCl (pH 7.0; to obtain the background fluorescence) was dispensed, in triplicate, into a 96 well polystyrene black plate. The fluorescence of each solution was determined. Samples with oversaturated fluorescence were further diluted 1: 10 with 40 mM Tris-HCl (pH 7.0) and re-examined.

The fluorescence of the DFC in each experimental sample was determined using the following equation:

$$\text{Fluorescence of intercellular DFC} = (\text{ETF}-\text{BF})-(\text{CTF}-\text{BF})$$

Where ETF = fluorescence of experimental tissue

BF = fluorescence of background

CTF = fluorescence of control tissue

Thus the concentration of DFC/g of *G. gracilis* was determined using the previously constructed standard curve, and further used to indirectly measure the concentration of intercellular ROS in the experimental thallus segments.

B.3 Detection of extracellular released hydrogen peroxide

(Adapted from Zuppini *et al.*, 2007)

Extracellular released hydrogen peroxide from *G. gracilis* into the surrounding media was detected using a xylenol orange assay mix. Here, hydrogen peroxide in the media reacts with sorbitol, converting hydrogen peroxide into a peroxy radical, and in turn converts iron (II) to iron (III). As this solution contains sulphuric acid, the iron (III) can then complex with xylenol orange, resulting in a colour change of the solution from orange to purple. This change of absorbency can be used to indirectly measure the amount of hydrogen peroxide in surrounding media (Jiang *et al.*, 1991).

Therefore, before determining the concentration of extracellular hydrogen peroxide (H_2O_2), a standard curve was constructed to represent the relationship between known concentrations of H_2O_2 and the resultant absorbencies of xylenol orange at 560 nm. Thus, in preparation, a 1 M H_2O_2 stock solution (Appendix A.2.1.1) was diluted 1: 10 000 with enriched ASW (Section 2.2.1) to prepare a 100 μM H_2O_2 working stock solution. Using this solution, known concentrations of H_2O_2 were further prepared, as described on the next page:

Final H_2O_2 concentration (μM)	100 μM H_2O_2 (μl)	ASW (μl)
0	0	1000
20	200	800
40	400	600
60	600	400
80	800	200
100	1000	0

After preparation, 500 μl of each solution was aliquot, in triplicate, into sterile microfuge tubes containing 500 μl of the xylenol orange assay mix (Appendix A.2.5.1). Tubes were briefly vortexed, left to stand for 45 min and absorbencies measured at a wavelength of 560 nm using a DU®530 UV/Vis Spectrophotometer (Beckman). Using these results, a standard curve was constructed, from which an unknown concentration of H_2O_2 could be deduced from the solutions absorbancy of xylenol orange.

Thus, to determine the concentration of H_2O_2 in the surrounding media of experimental thallus samples, in triplicate, 500 μl of the media containing unknown concentrations of H_2O_2 were aliquot into sterile microfuge tubes containing 500 μl of the xylenol orange assay mix. Tubes were briefly vortexed and left to stand for 45 min. Post incubation the absorbance of each solution was measured at a wavelength of 560 nm using a DU®530 UV/Vis Spectrophotometer (Beckman). Solutions with oversaturated absorbencies were diluted 1: 10 with enriched ASW and re-examined. The concentration of extracellular H_2O_2 released from each sample could therefore be determined from the previously constructed standard curve.

B.4 Catalase assay

(Adapted from Aebi, 1984)

Catalase is a powerful antioxidant enzyme which rapidly converts hydrogen peroxide into water and oxygen (Aebi, 1984). In this assay, the reduction of hydrogen peroxide is measured spectrophotometrically and used as an indirect measure to determine the activity of the catalase in the cell lysate.

Thus, prior to determining the catalase activity in the experimental tissue sample, a standard curve representing the relationship between known concentrations of hydrogen peroxide (H_2O_2) and its absorbencies at 240 nm was constructed. Known concentrations of H_2O_2 were prepared in sterile microfuge tubes by diluting the 1 M H_2O_2 stock solution (Appendix A.2.1.1) with 0.1 M phosphate buffer (pH 7.0; Appendix A.2.1.2) as described on the following page:

Final H₂O₂ concentration (mM)	1 M H₂O₂ (μl)	0.1 M Phosphate buffer (μl)
0	0	1000
5	5	995
10	10	990
15	15	985
20	20	980
25	25	975

After preparation, the absorbancy of each known H₂O₂ concentrations at 240 nm was determined, in triplicate, using a DU®530 UV/Vis Spectrophotometer (Beckman). Using these results a standard curve was constructed, from which the catalase activity of cellular lysate could be determined.

Thus, to investigate the catalase activity in the experimental thallus samples, 0.5 g of tissue from the each sample was ground in liquid nitrogen using a pestle and mortar. Finely ground tissue was transferred to sterile microfuge tubes containing 1 ml of 0.1 M phosphate buffer (pH 7.0) and briefly vortexed. Tubes were centrifuged at 10 000 rpm for 5 min. The supernatants containing total soluble protein (TSP) were further transferred to a sterile microfuge tubes and placed on ice until use. The concentration of the TSP in each sample was further determined using the modified Bio-rad protein assay (Appendix B.8). Post quantification, 100 μ l TSP was added to 900 μ l of the catalase reaction buffer (Appendix A.2.6.1) constituting a final concentration of 20 mM H₂O₂. The tubes were briefly vortexed and the decrease in the absorbance at 240 nm was monitored every 30 seconds, over a 3 minute incubation period. For each sample, the rate of catalase activity/min/100 μ l of TSP was determined from the constructed standard curve. By further determining the concentration of TSP present in 100 μ l of the protein extract, the units of catalase activity in each sample could be calculated, where:

$$\text{Catalase activity (U)} = 1 \text{ mol H}_2\text{O}_2\text{converted/mg total soluble protein/min}$$

B.5 Ascorbate peroxidase assay

(Adapted from Chen and Asada, 1989)

Ascorbate peroxidase is an antioxidant enzyme which converts hydrogen peroxide and ascorbate into water and dehydroascorbate (Chen and Asada, 1989). In this assay, the reduction of sodium ascorbate is measured spectrophotometrically and used to determine the activity of ascorbate peroxidase in the cell lysate

Therefore, before determining the ascorbate peroxidase activity in a tissue sample, a standard curve of the known concentrations of sodium ascorbate (ASC) versus its absorbencies at 290 nm was constructed. In preparation, 0.1 M ASC stock solution (Appendix A.2.7.1) was diluted 1: 100 in 0.1 M phosphate buffer (Appendix A.2.1.2) to prepare a 1 mM ASC working stock solution. Known concentrations of ASC were prepared in sterile microfuge tubes by diluting the 1 mM ASC working stock solution with 0.1 M phosphate buffer as described below:

Final ASC concentration (μM)	1 mM ASC (μl)	0.1 M Phosphate buffer (μl)
0	0	1000
100	100	900
200	200	800
300	300	700
400	400	600
500	500	500

After preparation, the absorbance of each known ASC concentration sample was determined at 290 nm, in triplicate, using a DU[®]530 UV/Vis Spectrophotometer (Beckman). The standard curve was constructed and the resultant straight line equation calculated.

Thus, to investigate the ascorbate peroxidase activity in the experimental thallus samples, 0.5 g of tissue from the each sample was ground in liquid nitrogen using a pestle and mortar. Finely ground tissue was transferred to sterile microfuge tubes containing 1 ml of ascorbate peroxidase extraction buffer (Appendix A.2.7.2) and briefly vortexed. Tubes were centrifuged at 10 000 rpm for 5 min. The supernatants containing total soluble protein (TSP) were further transferred to sterile microfuge tubes and placed on ice until use. The

concentration of the TSP in each sample was further determined using the modified Bio-rad protein assay (Appendix B.8). Post quantification, 100 μ l of TSP was added to 900 μ l of the ascorbate peroxidase reaction buffer (Appendix A.2.7.3), constituting a final concentration of 400 μ M ASC. The tubes were briefly vortexed and the decrease in the absorbance at 290 nm was monitored every 30 seconds over a 3 minute incubation period. For each sample, the rate of ascorbate peroxidase activity/min/100 μ l of TSP was determined from the previously constructed standard curve. By further determining the concentration of TSP present in 100 μ l of the protein extract, the units of ascorbate peroxidase activity in each sample could be calculated, where:

Ascorbate peroxidase activity (U) = 1 mol ASC converted/mg total soluble protein/min

B.6 Glutathione peroxidase assay

(Adapted from Ursini *et al.*, 1985)

Glutathione peroxidase is an antioxidant enzyme which converts hydrogen peroxide and monomeric glutathione into water and glutathione disulphide. This reaction is coupled to the recycling of glutathione disulphide back into monomeric glutathione utilizing glutathione reductase and β -Nicotinamide adenine dinucleotide 2'-phosphate reduced tetrasodium salt (NADPH, Ursini *et al.*, 1985). Since Glutathione peroxidase is the rate limiting factor of these coupled reactions, the reduction in NADPH can be measured spectrophotometrically and used to determine the activity of glutathione peroxidase in the cell lysate

Thus, prior to the determining the glutathione activity in a tissue sample, a standard curve of the known concentrations of NADPH versus the absorbencies of the various concentrations of NADPH at 340 nm was constructed. The 0.1 M NADPH stock solution (Appendix A.2.8.1) was diluted 1: 1000 with 0.1 M phosphate buffer (Appendix A.2.1.2) to prepare a 100 μ M NADPH working stock solution. Known concentrations of NADPH were prepared in sterile microfuge tubes by diluting the 100 μ M NADPH working stock solution with 0.1 M phosphate buffer as described on the next page.

Final NADPH concentration (μM)	100 μM NADPH (μl)	0.1 M Phosphate buffer (μl)
0	0	1000
20	200	800
40	400	600
60	600	400
80	800	200
100	1000	0

After completion, the absorbance of each known NADPH concentration was determined at 340 nm, in triplicate, using a DU®530 UV/Vis Spectrophotometer (Beckman). From these results, a standard curve was constructed and the resultant equation was calculated.

Thus, to determine the glutathione peroxidase activity in *G. gracilis* samples, 0.5 g of tissue from the each sample was ground in liquid nitrogen using a pestle and mortar. Finely ground tissue was transferred to sterile microfuge tubes containing 1 ml of 0.1 M phosphate buffer (pH 7.0) and briefly vortexed. Tubes were centrifuged at 10 000 rpm for 5 min. Supernatants containing total soluble protein (TSP) was transferred to a sterile microfuge tubes and placed on ice until use. Prior to the commencement of the assay, the concentration of the TSP in each sample was further determined using the modified Bio-rad protein assay (Appendix B.8). Post quantification, 100 μl TSP was added to 900 μl of the glutathione peroxidase reaction buffer (Appendix A.2.8.2) constituting a final concentration of 80 μM NADPH. The tubes were briefly vortexed and the decrease in the absorbance at 340 nm monitored every 30 seconds over a 3 minute incubation period. For each sample, the rate of the glutathione pathway activity/min/100 μl of TSP was determined from the constructed standard curve. By further determining the concentration of TSP present in 100 μl of the protein extract, the units of glutathione peroxidase activity in each sample could be calculated, where:

$$\text{Glutathione peroxidase activity (U)} = \frac{1 \text{ mol NADPH converted}}{\text{mg total soluble protein/min}}$$

B.7 Isolation of total soluble protein

(Adapted from Ingle *et al.*, 2005)

To isolate total soluble protein from a thallus sample, half a gram of frozen tissue was ground in liquid nitrogen using a pestle and mortar. Finely ground tissue was transferred to a sterile microfuge tube containing 1 ml protein extraction buffer (Appendix A.2.9.1), vortexed and centrifuged at 12 000 rpm for 5 min, 4 °C. The resultant supernatant was transferred to a sterile microfuge tube containing an equal volume of phenol (pH 8.0; Sigma), vortexed and centrifuged at 12 000 rpm for 10 min, 4 °C. This supernatant was discarded and 1 ml of protein extracted buffer was added to the microfuge tube. Once again, the tube was vortexed, centrifuged at 12 000 rpm for 10 min, 4 °C and the resultant supernatant discarded. The remaining solution was divided into two sterile microfuge tubes containing 3X volume of precooled 0.1 M ammonium acetate in methanol (Appendix A.2.9.2). The tubes were gently mixed, and incubated at -20 °C for 2 h. Post incubation, one of the two tubes was centrifuged at 12 000 rpm for 5 min, 4 °C. The resultant supernatant was discarded and the content of the second tube was transferred to the first tube containing the pelleted protein. This tube was further centrifuged at 12 000 rpm for 5 min, 4 °C. The resultant supernatant was discarded and the collective pellet containing the precipitated protein from both tubes was washed once with 1 ml of precooled 0.1 M ammonium acetate in methanol and twice with 1ml of precooled 80 % acetone (Appendix A.2.9.3). After each wash step, the supernatant was discarded. The resultant protein pellet was dried for 5 min and resuspended in 200 µl ULB (Appendix A.2.1.5). The tube was vortexed for 1 hr. If the protein had not fully resuspended in this time, an addition of 50 µl of ULB was added to the pellet and the microfuge tube was vortexed for an additional 30 min. After the protein had been successfully resuspended, the concentration of the protein sample was determined using the modified Bio-rad protein assay (Appendix B.8) and further divided into a number of sterile microfuge tubes and stored at -80 °C. These protein samples were defrosted on ice and vortexed for 1 h prior to use.

B.8 Quantification of total soluble protein

(Adapted from Bio-rad protein assay; Bio-Rad)

Before quantitating the total soluble protein isolated from a thallus sample, a standard curve was constructed to represent the relationship between the known concentrations of bovine serum albumin (BSA) and its absorbency at 595 nm after performing a modified Bio-rad protein assay (Bio-Rad). Thus, known concentrations of BSA were prepared in

sterile microfuge tubes by diluting a 10 mg/ml BSA stock solution (Appendix A.2.10.1) with ULB (Appendix A.2.1.5) as described below:

Final BSA concentration (mg/ml)	BSA stock solution (μl)	ULB (μl)
0	0	1000
0.2	20	980
0.4	40	960
0.6	60	940
0.8	80	920
1.0	100	900

After completion, in triplicate, 10 μ l from each known BSA concentration was aliquot into sterile microfuge tubes containing 90 μ l of 0.1 M HCl (Appendix A.2.10.2). The tubes were briefly vortexed, after which 900 μ l of the diluted Bio-rad protein assay dye reagent concentrate (1: 5; Bio-Rad) were added. The tubes were again briefly vortexed and allowed to stand for 10 min prior to determining the absorbance of the solutions at 595 nm using the DU[®]530 UV/Vis Spectrophotometer (Beckman). Using these results, a standard curve was constructed, from which an unknown concentration of total soluble protein in cellular lysate could be determined from its absorbancy at 595 nm after the completion of the assay.

Thus, to determine the concentration of total soluble protein isolated from each thallus sample, 10 μ l of each protein sample was aliquot into a sterile microfuge tube containing 90 μ l of 0.1 M HCl. The tubes were vortexed prior to the adding 900 μ l of the diluted Bio-Rad protein assay dye reagent concentrate (1: 5; Bio-Rad) were added. The tubes were briefly vortexed and allowed to stand for 10 min prior to determining the absorbance at 595 nm (DU[®]530 UV/Vis Spectrophotometer; Beckman). Solutions with oversaturated absorbencies were diluted 1: 10 with ULB and re-examined. The concentration of the total soluble protein in each sample was determined from the standard curve.

B.9 Preparation and electrophoresis of a one dimensional SDS-polyacrylamide gel

B.9.1 Preparation of a one dimensional SDS-polyacrylamide gel

(Ausubel *et al.*, 1989; unit 10.2)

After deciding on the appropriate percentage of polyacrylamide required for the 1D SDS-polyacrylamide gel, the preferred resolving gel was prepared, as described below:

Reagent	Percentage of polyacrylamide in the resolving gel	
	8 %	12 %
	Volume	
40 % Acrylamide (Sigma)	1.0 ml	1.5 ml
4X Tris-HCl/SDS (pH 8.8; Appendix A.2.11.1)	1.25 ml	1.25 ml
10 % AMPS (Appendix A.2.11.2)	25 μ l	25 μ l
N, N, N', N'-Tetramethylethylenediamine, (TEMED; Merck)	5 μ l	5 μ l
dH ₂ O to	5 ml	5 ml

Seven and a half milliliters of the freshly made gel mixture was poured between mini-PROTEAN Tetra Cell (Bio-Rad) plates, preassembled as per the manufacturer's instructions. Isopropanol (Merck) was gently poured across the top of resolving gel and incubated at room temperature for 45 min to ensure polymerisation of gel mixture. After incubation, the isopropanol was removed and the upper surface of the gel was rinsed with dH₂O and blotted dry with 3 mm filter paper (Whatman).

Once ready, 4 milliliters of a 4 % stacking gel mixture was freshly prepared in a clean beaker as described next:

Reagent	Volume
40 % Acrylamide	0.5 ml
4X Tris-HCl/SDS (pH 6.8; Appendix A.2.11.3)	0.4 ml
10 % AMPS (Appendix A.2.11.2)	25 μ l
TEMED	2 μ l
dH ₂ O to	4.0 ml

An appropriate quantity of the stacking gel mixture was then poured over the set resolving gel mixture, and a clean gel comb was inserted. Once again, the gel was incubated at room temperature for 45 min to ensure the complete polymerisation of the gel mixture. The gel was then placed in the mini-PROTEAN Tetra Cell electrophoresis system (Bio-rad) and filled with 1X SDS-PAGE running buffer (Appendix A.2.11.4). Once the comb was removed and the wells were rinsed, the gel was ready to be used (Appendix B.9.2).

B.9.2 Preparation and loading of protein samples onto a one dimensional SDS-polyacrylamide gel

Twenty five micrograms of the total soluble protein (Appendix B.7) was aliquot into individual sterile microfuge tubes. 5X protein sample application buffer (Appendix A.2.11.5) was added to each tube at a ratio of 1: 5 (v/v). The freshly prepared mixtures were vortexed and boiled for 5 min. Post incubation, the tubes were vortexed again and centrifuged at 10 000 rpm for 5 seconds prior to loading onto a 1D polyacrylamide gel. For the determination of the approximate molecular masses of the electrophoresed proteins, 3 µl PageRuler™ Prestained Protein Ladder (Fermentas) was loaded onto the gel. The protein ladder was defrosted on ice, vortexed and immediately loaded onto each gel.

B.9.3 Electrophoresis of a one dimensional SDS-polyacrylamide gel

Proteins loaded onto a 1D SDS-polyacrylamide gel were electrophoresed using the mini-PROTEAN Tetra Cell electrophoresis system (Bio-rad) as described by the manual. A constant voltage of 100 V for 3 h was employed to ensure appropriate separation of the proteins. After electrophoresis, the gel was removed from the gel plates and the proteins visualised using a coomassie staining technique (Appendix B.11) or directly transferred onto nitrocellulose paper for western blot analysis (Appendices B.14 and B.16).

B.10 Preparation and electrophoresis of a two dimensional SDS-polyacrylamide gel

B.10.1 Preparation of a protein sample for loading onto a 2D SDS-polyacrylamide gel

B.10.1.1 Rehydration of an immobilised pH gradient strip

Total soluble protein (Appendix B.7) isolated from the appropriate tissue sample was prepared for the rehydration on an immobilised pH gradient strip, as described below:

Reagent	Volume
10 % ASB-14 (Appendix A.2.12.1)	5.6 μ l
Bio-Lyte Buffer (pH 3-10; Bio-Rad)	7.0 μ l
30 % DTT (Appendix A.2.12.2)	9.3 μ l
Total soluble protein (Appendix B.7)	250 μ g
Bromophenol Blue	Trace amount
ULB (Appendix A.2.1.5)	Up to 140 μ l

The mixture was vortexed, and centrifuged at 10 000 rpm for 5 seconds, before loading onto a 7 cm ReadyStrip™ Immobilised pH Gradient (IPG) strip (pH 4-7; Bio-Rad) in a rehydration tray (Bio-Rad). The strip was overlaid with 2 ml with mineral oil (Bio-Rad) and incubated at room temperature for 14 h.

B.10.1.2 Isoelectrofocusing of total soluble proteins

After the rehydration (Appendix B.10.1.1), the IPG strip was removed from the rehydration tray, blotted dry and placed into a clean focusing tray (Bio-Rad). The IPG strip was overlaid with 1.5 ml mineral oil and bubbles trapped underneath the strip were removed. The focusing tray was placed into the PROTEAN® Isoelectrofocusing (IEF) Cell (Bio-Rad) and the proteins were focused along the immobilised pH gradient using the 4 step profile as indicated next:

Step	Voltage (V)	Ramping Profile	Duration
1	250	Linear	20 min
2	4 000	Linear	2 h
3	4 000	Linear	20 000 Vh
4	500	Linear	Until removed

After the isoelectrofocusing profile was complete, the IPG strip was removed from the focusing tray and placed into a clean rehydration tray, with the gel layer facing upwards. 50 μ l mineral oil was dispersed over the strip prior to storage at -80 °C until required.

B.10.1.3 Equilibration of total soluble protein

Frozen immobilised pH gradient (IPG) strip (Appendix B.10.1.2) containing the focused total soluble protein from the appropriate tissue sample were removed from -80 °C and defrosted on ice for 15 min prior to equilibration. The defrosted strips were blotted dry and placed with the gel layer facing upwards in a clean focusing tray (Bio-Rad). After the correct positioning of the protein strips, 2 ml of equilibration buffer 1 (Appendix A.2.12.4) was added to the strip and incubated at room temperature, with agitation, for 10 min. Post incubation, the buffer was discarded, and 2 ml of equilibration buffer 2 (Appendix A.2.12.5) was added to the strip prior to an additional incubation at room temperature, with agitation, for 10 min. After incubation, the buffer was discarded and the IPG strip was rinsed in 1X SDS-PAGE running buffer (Appendix A.2.11.4) and blotted dry prior to loading directly onto a 2D SDS-polyacrylamide gel (Appendix B.10.2).

B.10.2 Preparation of a two dimensional SDS-polyacrylamide gel

After deciding on the appropriate percentage of polyacrylamide required for the 2D SDS-polyacrylamide gel, the preferred mixture was prepared as described on the next page:

Reagent	Percentage of polyacrylamide in the gel	
	12 %	18 %
	Volume	
40 % Acrylamide (Sigma)	2.4 ml	3.6 ml
4X Tris-HCl/SDS (pH 8.8; Appendix A.2.11.1)	2 ml	2 ml
10 % AMPS (Appendix A.2.11.2)	40 µl	40 µl
N, N, N', N'-Tetramethylethylenediamine, (TEMED; Merck)	8 µl	8 µl
dH ₂ O to	8 ml	8 ml

After completion, an appropriate volume of the freshly made gel mixture was poured between the stacked mini-PROTEAN® 3 System Multi-Caster apparatus (Bio-Rad) plates, preassembled as per the manufacturer's instructions. Isopropanol (Merck) was gently poured across the top of gel and left to stand for 45 min to ensure complete polymerisation of gel mixture. After incubation, the isopropanol was removed and upper surface of the gel and washed three times with dH₂O to remove any residual isopropanol. The gel was stored in a mini-PROTEAN® 3 Dodeca™ Cell (Bio-Rad) cassette with a thin layer of dH₂O on the top of the gel before use.

B.10.3 Loading of the IPG strip onto 2D SDS-polyacrylamide gel

In order to prepare the 2D SDS-polyacrylamide gel (Appendix B.10.2) for the loading of an immobilised pH gradient strip rehydrated with the appropriate protein sample (Appendix B.10.1), a thin layer of dH₂O was removed from the gel and blotted dry with 3 mm filter paper (Whatman). The IEF strip (Appendix B.10.2.3) was loaded onto the gel, ensuring that the strip was placed in the correct electrical orientation. A 4 mm x 4 mm square of filter paper (Whatman) hydrated with 3 µl of PageRuler™ Prestained Protein Ladder (Fermentas) was placed next to the positive end of the gel strip. The strip and filter paper was overlaid with 0.5 % agarose in 1X SDS-PAGE running buffer (Appendix A.2.12.6) and allowed to dry for 5 minutes.

B.10.4 Electrophoresis of two dimensional SDS-polyacrylamide gels

All gels from a single biological repeat were electrophoresed concurrently using the mini-PROTEAN® 3 Dodeca™ Cell (Bio-Rad) as described by the manual. Thus preloaded 2D SDS-polyacrylamide gels already placed within the gel cassettes (Appendix B.10.3) were placed in the mini-PROTEAN® 3 Dodeca™ Cell and the reservoirs filled with 1X SDS-PAGE running buffer (Appendix A.2.11.4). 12 % polyacrylamide gels and 18 % polyacrylamide gels were electrophoresed at 100 V for 3 and 18 h at 4 °C respectively. Post electrophoresis, gels were removed from the glass plates and the protein was visualised by coomassie staining (Appendix B.11) or transferred onto nitrocellulose paper for western blot analysis (Appendices B.14 and B.16).

B.11 Visualisation of proteins on a SDS-polyacrylamide gel

The visualisation of total soluble protein on a 1D or 2D SDS-polyacrylamide gel (Appendices B.9 and B.10) was performed using a coomassie blue staining technique. Thus each gel was rinsed in 100 ml dH₂O for 5 min and stained in 100 ml coomassie blue staining solution (Appendix A.2.13.1) for 1 h at 37 °C without agitation. After incubation, the coomassie blue staining solution was removed and the gel was destained for 18 h in 250 ml coomassie blue destaining solution (Appendix A.13.2) at 25 rpm.

B.12 Detection of differentially expressed protein spots using 2D gel analysis software

Visualised 2D SDS-polyacrylamide gels (Appendix B.11) were scanned at a resolution of 300 dots per inch (DPI) using a flat-bed Epson perfection V750 colour scanner (Epson) and images captured using the SilverFast® 6 software package (LaserSoft Imaging®). Black and white TIFF format files were uploaded into the 2D gel analysis software program *Melanie 7.0* (Swiss Institute of Bioinformatics) omitting filtering to avoid artificial effects on the image and the software was used to identify differentially expressed protein spots. All images were cropped and rotated to ensure equal sizing and positioning of the gels within the program. Spot detection was prepared using the following parameters (smooth = 4; saliency= 0.5; min Area = 100). Six well distributed landmarks were added to reduce error as a result of global deformations of the gels and increase the efficacy during the matching

of the protein spots. Gels were separated into classes owing to the duration of heat shock at 35 °C prior to the isolation of total soluble protein from *G. gracilis*. Matching of protein spots across classes was performed by the 2D gel analysis software program and manually edited to eliminate software errors caused by the incorrect matching of spots or the incorporation of artifacts as spots. After matching, protein spots that were not consistently expressed in all the polyacrylamide gels were further discarded. Two exceptions were made, in the case of the two de novo proteins (protein spots 1 and 5; Appendix C.1), which were clearly up-regulated after 1 hour of heat stress. After editing the protein profiles, the relative intensity of the protein spots were normalised by expressing the intensity of each protein spot in a gel as a proportion of the total protein intensity detected for the entire gel and the change in densities of the protein spots in response to heat stress was determined. One-way ANOVA statistics analysis data was generated for each spot using *Melanie 7.0* (Systat Software, Inc.). A $p < 0.05$ indicate a statistically significant change in the spot density in response to heat stress.

B.13 Putative identification of protein spots using mass spectrometry

The putative identities of the differentially expressed protein spots were identified at the W. M. Keck Foundation Biotechnology Resource Laboratory (Yale School of Medicine; Yale University; United States of America). Thus three dehydrated gel plugs, containing the protein of interest, were washed, rehydrated, digested using either modified trypsin (Promega), endopeptidase Lys-C (Wako), endoproteinase Glu-C (Roche) or chymotrypsin (Roche) and analysed using the electrospray ionization LC-MS/MS (Tandem mass spectrometry) method of protein identification on a LTQ-Orbitrap XL mass spectrometer (Thermo Scientific). The Mascot Distiller software program was used to generate Mascot compatible files followed by the use of the Mascot algorithm (version 2.2.0; Hirosawa *et al.*, 1993) for uninterrupted MS/MS spectra. Data from the LC-MS/MS analysis was searched against the non-redundant NCBI database using the Mascot software with the predicted amino-acid sequences derived from the spectra for each tryptic peptide fragments and a probability-based molecular weight search (MOWSE) score provided. Putative identities of the protein spots were obtained from the W. M. Keck Foundation Biotechnology Resource Laboratory on-line repository. Thus a protein identity was considered successful when 2 or more peptides matched the same protein in the database and the MOWSE score was significant at $p < 0.05$.

B.14 Electrotransfer of protein onto a nitrocellulose membrane

(Towbin *et al.*, 1979)

Electrotransfer of proteins from either a one (Appendix B.9) or two dimensional (Appendix B.10) SDS-polyacrylamide gel onto a nitrocellulose membrane were performed as described below. Prior to each transfer, a single piece of Protran® Pure Nitrocellulose transfer and immobilisation membrane (PerkinElmer™) and two pieces of 3 mm filter paper (Whatman®) were cut to the same size as the gel and soaked in Towbin buffer (Appendix A.2.14.1). After the electrophoresis of the total soluble protein on a SDS-polyacrylamide gel (Appendix B.9.3 or B.10.4), the gel was removed from between the gel plates and incubated in Towbin buffer (Appendix A.2.14.1) for 15 min, 4 °C. Post incubation, an electrotransfer assemblage was prepared. Thus the holder cassettes were opened and layered with a foam pad, followed by a piece of filter paper, the SDS-polyacrylamide gel, the nitrocellulose paper and the last two pieces of filter paper and foam pad. The holder cassette was closed cautiously to prevent the addition of air bubbles, placed into the mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad) and the tank reservoirs filled with Towbin Buffer. The proteins were electrotransferred at a constant voltage of 100 V for 1 h at 4 °C.

B.15 Visualisation of protein on a nitrocellulose membrane

Post electrotransfer, the nitrocellulose membrane was removed from the holder cassettes (Appendix B.14) and incubated in 250 ml Ponceau S stain (Appendix A.2.15.1) for 15 min. To temporarily visualise the proteins on the nitrocellulose membrane, the membrane was rinsed in distilled water for 5 min. Post visualisation the membrane was rinsed in 1X TBS (Appendix A.2.1.6) with agitation to remove the majority of the stain prior to the analysis of the protein of interest by western blot hybridisation (Appendix B.16). During the beginning stages of this procedure, the residual stain is removed prior to the visualisation of the selected protein spots/bands.

B.16 Western blot analysis

After the visualisation of the total soluble protein on the nitrocellulose membrane (Appendix B.15), western blot analysis was performed as described below. All membrane washes and antibody solution incubations were performed at 50 rpm. To begin, the membrane was incubated for 1 h in blocking solution (Appendix A.2.16.1), followed by incubation in 10 ml of the appropriate primary antibody solution (Appendices A.2.16.2 to A.2.16.5) overnight at 4 °C. Post incubation, the membrane was washed four times in 25 ml 1X TBST (Appendix A.2.16.6) for 15 min each, prior to incubation in 10 ml of goat anti-rabbit secondary antibody solution (Appendix A.2.16.7) for 3 h. Post incubation, the membrane was washed 4 times 1X TBST for 15 min each, followed by a single wash in 1X TBS (Appendix A.2.1.6) for 5 min. The colourmetric detection of the protein/s of interest was/were performed using the TMB Membrane Peroxidase Substrate (KPL) as indicated on the instruction sheet. Thus, 1 ml of the substrate was added to the membrane under low light conditions, and the visualisation reaction left develop for up to 10 minutes. Once the protein/s could be detected, the membrane was washed twice in 50 ml dH₂O for 10 min at 50 rpm prior to the placement of the membrane onto filter paper (Whatman) where it was dried overnight in the dark.

B.17 Isolation of genomic DNA

(Adapted from Wattier *et al.*, 2000)

Genomic DNA was isolated from 0.25 g of acclimatised *G. gracilis* thallus segment. Thus the thallus was ground with a pestle and mortar using liquid nitrogen. The resultant fine powder was transferred into a microfuge tube containing a mixture of 1.5 ml DNA extraction buffer (Appendix A.2.17.1), 150 µl SDS (20 %; Appendix A.2.1.7) and 2.75 µl Proteinase K (20 mg/ml; Appendix A.2.17.2). The tube was vortex for 30 seconds prior to its incubation at 37 °C with agitation. Post incubation, the tubes were centrifuges at 13 000 rpm for 15 min and the consequential aqueous phase was transferred into a sterile microfuge tube. After the addition of 2 µl of RNase A (10 mg/ml; Appendix A.2.17.3) the tube was further incubated at 37 °C for 1 hour. Post incubation the tube was placed on ice for 30 min and centrifuged at 13 000 rpm for 15 min at 4 °C. A milliliter of the resultant supernatant was again transferred into a sterile microfuge tube, to which 700 µl of precooled isopropanol (Merck) was added. The tube was mixed by inverting the tubes gently until a homogenous layer could be visualised and stored overnight at -20 °C. Post incubation, the tube was centrifuges at 13 000 rpm for 15 min at 4 °C after which the

supernatant was discarded. The remaining pellet was washed twice with 1 ml of precooled 70 % ethanol (Appendix A.2.1.8) and air dried at room temperature for 5 min. The pellet containing genomic DNA was further resuspended in 50 μ l Tris-EDTA buffer (Appendix A.2.1.9) and stored at 4 °C until required.

B.18 Electrophoresis of DNA on 1.5 % TAE-agarose gels

(Ausubel *et al.*, 1989; unit 2.5)

To prepare a 1.5 % (w/v) TAE-agarose gel, 1.5g of agarose (Lonza) was added to 100 ml 1X TAE (Appendix A.2.18.1) in a sterile bottle. The solution was shaken well and allowed to stand for 5 min. After incubation, the agarose was dissolved by heating. Periodically, the solution was swirled and examined for homogeneity. Once dissolved, 4 μ l of ethidium bromide solution (10 mg/ml; Appendix A.2.18.2) was added to the solution, mixed and allowed to cool to 55 °C. Once cooled, the solution was poured into a cleaned gel-casting trays sealed with masking tape at the open ends. After which, the gel comb was inserted and the gel allowed to cool. Once set, the masking tape was removed from the gel trays and placed into an electrophoresis tank. An appropriate volume of 1X TAE was added to the tank to completely cover the gels. DNA samples were prepared by mixing the DNA: 6X tracking dye at a ratio of 1: 6 (v/v; Appendix A.2.1.10). For the determination of fragment sizes, 8 μ l of the premade lambda DNA digested with *Pst* I molecular weight marker (Appendix B.19) was loaded into one of the empty gel lanes. The electrical leads were attached as to allow for the migration the DNA down the length of the gel. Gels were electrophoresed until the dye front had reached the bottom of the gel as described by Sambrook *et al.* (1989) prior to visualisation.

B.19 Preparation of lambda DNA digested with *Pst* I molecular weight ladder

(Adapted from the *Pst* I product information sheet; Fermentas)

In preparation of lambda DNA digest with *Pst* I, 40 μ l of a 500 ng/ml Lambda DNA (Promega), 20 μ l 10X recommended Orange restriction digest buffer (Fermentas), 2 μ l of 10 units/ μ l *Pst* I (Fermentas) and 140 μ l of sterile water were added into a sterile microfuge tube. The tube was mixed gently by inversion and incubated for 16 h at 37°C. Post incubation, 36 μ l of the 6X tracking dye (Appendix A.2.1.10) was added to the tube, mixed thoroughly and stored at 4 °C, ready for use.

B.20 Isolation of total RNA

(Adapted from the peqGOLD Plant RNA Kit; peqGOLD)

All reagent used in this section, came as part of the kit, unless otherwise stated. 100mg of *G. gracilis* thalli isolated from each time point was ground up in liquid nitrogen using a pestle and mortar. Finely ground tissue was transferred into separate sterile microfuge tube containing 450 µg of RNA Lysis Buffer T. Tubes were briefly vortexed and incubated for 30 min with agitation. Post incubation, tubes were centrifuged at 10 000 rpm for 5 min. The supernatant from each sample was transferred onto a DNA removal column and centrifuged for a further 2 min at 10 000 rpm. Equal volumes of 70 % Ethanol (RNA; Appendix A.2.19.2) was added to the flow-through and mixed thoroughly with a pipette prior to the transfer onto a PerfectBind RNA Column. The columns were centrifuged at 10 000 rpm for 2 min and the filtrate discarded. The columns were washed once with 500 µl of Wash Buffer I and twice with 650 µl of Wash Buffer II. Post washes the columns were dried with an additional centrifugation at 10 000 rpm for 2 min. The columns were removed from the collection tubes and placed into a sterile microfuge tubes, to which 80 µl of RNase-free water preheated to 70 °C was added and incubated for 5 min. Post incubation, total RNA was eluted from the columns via centrifugation at 10 000 rpm for 1 min, and store at -80 °C until required.

B. 21 DNase I treatment of total RNA

(Adapted from DNase I, RNase free certificate of analysis; Fermentas)

Post quantification of total RNA, 5 µg total RNA (Appendix B.20), 10 µl of the 10 X Reaction Buffer with MgCl₂ (Fermentas), 5 µl of the DNase I, RNase-free (Fermentas) and DEPC treated water (Appendix A.2.19.1) up to a final volume of 100 µl was added into a sterile microfuge tube. Tubes were incubated at 37 °C for 45 min. Post incubation, the DNase I was inactivated by the addition of 100 µl phenol: chloroform: isoamyl alcohol (25: 24: 1; Appendix A.2.19.3). The tubes were mixed thoroughly using pipetting and centrifuged for 10 min at 14 000 rpm. Post centrifugation the aqueous layer was transferred into a sterile microfuge tube containing 10 µl 3 M sodium acetate (Appendix A.2.19.4) and 300 µl of absolute ethanol (Merck). Again, the tubes were mixed thoroughly using a pipette and centrifuged for 10 min at 14 000 rpm. The supernatant was discarded and pellet air dried for 5 min. Total RNA was resuspended in 30 µl of DEPC treated water (Appendix A.19.1). RNA was stored on ice prior to electrophoresis and the use in cDNA conversion (Appendix B.25).

B.22 Preparation and electrophoresis of RNA on a 1.2 % formaldehyde-agarose gel

(Ausubel *et al.*, 1989; unit 2.5)

A 1.2 % formaldehyde gel was prepared by melting 0.75 g agarose (Lonza) in 44 ml DEPC-treated water (Appendix A.2.19.1). Once cooled to approximately 60 °C, 6 ml of 10X MOPS (Appendix A.19.5) and 10 ml of 37 % formaldehyde (Merck) was added to the solution and poured into sealed gel-casting platforms. Combs were inserted and the gel was allowed to set. Prior to electrophoresis combs and masking tape were removed from the casting platforms and placed into a 1X MOPS running buffer (Appendix A.2.19.6). Total RNA was prepared for electrophoresis by adding 1 µg total RNA (DNase free; Appendix B.21), 5 µl of RNA sample application buffer (Appendix A.2.19.7) and DEPC treated water up to a final volume of 10 µl. Tubes were heated at 65 °C for 15 min and snap cooled on ice for 5 min prior to loading on the gel. The electrical leads were attached as to allow for the migration the DNA down the length of the gel. Gels were electrophoresed for 1 hour as described by Sambrook *et al.* (1989) prior to visualisation.

B.23 Primers

B.23.1 Primers used to amplify the ER *Hsp70* gene sequence from *G.gracilis*

All DNA oligonucleotide primers were synthesised in the Department of Molecular and Cell Biology, University of Cape Town.

Primer	Primer sequence	Reference
F1	5'-GGNAARGTNGARATHATHGC-3'	(Rautenbatch; unpublished)
F2	5'-TCACTTCTGAAGAGCGACTG-3'	This study
F3	5'-CTACTTCAATGACGCCAGAC-3'	This study
F4	5'-AGCCGCCATGAACCCCGTCAACACG-3'	This study
F5	5'-GGTGAGCGCACGATGACCAAGGACTGCCACAT-3'	This study
R1	5'-NCCNGCRTCYTTNGTNGC-3'	(Rautenbatch; unpublished)
R2	5'-CCRTTNGCRTCDATRRCRAANG-3'	This study
R3	5'-CTCTGGGCGTCATTGAAGTAGGCCGGGAC-3'	This study
R4	5'-ACACCGTGTTGACGGGGTTCATGGCGGCTT-3'	This study

Where: N= A, G, C or T; H= A, C or T; R= A or G; Y= C or T and D= A, G or T.

B.23.2. Primers used for sequencing of clone inserts

Primer	Primer sequence	Reference
M13F	5'-CGCCAGGGTTTTCCAGTCACGAC-3'	(Yanisch-Perron <i>et al.</i> , 1985)
M13R	5'-GAGCGGATAACAATTTTCACACAGG-3'	(Yanisch-Perron <i>et al.</i> , 1985)

B.23.3 Primers used in Real-time PCR

Primer	Primer sequence	Reference
QHSP70F	5'-CGCACGATGACCAAGGACTG-3'	This study
QHSP70R	5'-CTCAGCAGACACACTCAGGATAC-3'	This study
245F	5'-CTGCGAGAGCGGTACCAAATCG-3'	(Ealand, 2011)
245R	5'-GCCTTAGCTGGTGGTCTGG-3'	(Ealand, 2011)

B.24 Amplification protocol

B.24.1 Traditional polymerase chain reaction protocol

(Supertherm Taq polymerase PCR reagents; Southern Cross Biotechnology).

The following reagents were added into a sterile microfuge tube as described below:

Reagents	Volume (μ l)
100 ng/ μ l gDNA	2
10X Taq polymerase buffer	5
25 mM MgCl ₂	6
5 mM dNTPs	2
10 μ M Forward Primer (Appendix B.23)	2.5
10 μ M Reverse Primer (Appendix B.23)	2.5
Sterile water	28
1U / μ l Supertherm Taq polymerase	2
Total Volume	50

Once added, the tube was closed, gently mixed by light tapping and further centrifuged at 5 000 rpm for 5 seconds to position the solution at the bottom of the tube and to remove any residual bubbles. The tube was then added to hot lid XP thermocycler (Bioer) and the PCR profile was performed as follows:

Temperature (°C)	Time (s)	Cycles
95	300	1
95	30	
55	30	35
72	60	
72	300	1

B.24.2 Rapid amplification of cDNA ends protocol

(SMART™ RACE cDNA Amplification Kit; Clontech laboratories, Inc.)

The following reagents were added at the appropriate volumes, into a sterile microfuge tube:

Reagents	Volume (µl)
RACE-Ready cDNA	2.5
10X Advantage 2 PCR Buffer	5
50X Advantage 2 Polymerase Mix	1
10 mM dNTPs	1
10µM Designed Primer	1
10X Universal Primer Mix A	5
PCR-Grade Water	34.5
Total volume	50

The tubes were then closed, gently mixed via light tapping and centrifuges at 5 000 rpm to remove any residual bubbles and to position the solution in the bottom of the tube. The tube was added to a hot lid XP thermocycler (Bioer) and the PCR cycling profile performed as indicated next:

Temperature (°C)	Time (s)	Cycles
94	300	1
94	30	
68	30	30
72	180	
72	300	1

B.25 Conversion of total RNA to complementary cDNA

(Adapted from the ImProm-II™ Reverse Transcription System; Promega)

For each conversion, the following reagents were added into a sterile microfuge tube (where x = 2 µg total RNA):

Reagents	Volume (µl)
Total RNA (DNA free; Appendix B.8.2)	x
Oligo (dT)15 Primes	1
Random Primes	1
RNase free water up to	10

After which, the tubes closed, mixed gently and incubated at 72 °C for 5 min. After incubation the tubes were placed on ice for 5 min before adding the following reagents:

Reagents	Volume (µl)
5X Impro-II™ Reaction Buffer	8
25 mM MgCl ₂	4.8
10 mM dNTPs (Fermentas)	2
Recombinant RNasin® Ribonuclease Inhibitor	1
RNase-free water to	38

Once prepared, the microfuge tubes were left to stand for 5 min and then further incubated at 42 °C for an additional 5 min. Two microliters of reverse transcriptase was added to each tube and returned to 42 °C for a further 16 h. After completion of this incubation period, all the reactions were incubated at 70 °C for 15 min to inactivate the reverse transcriptase.

Newly synthesised cDNA was either used directly in real-time PCR or stored at -80 °C until required.

B.26 Real-time PCR

(Adapted from the SensiMix™ SYBR Kit; Quantace)

A master mix was prepared in a sterile microfuge tube to include the following reagents:

Reagents	Volume (µl)
Sensimix™ SYBR	6.25
Forward Primer (10 µM)	0.25
Reverse Primer (10 µM)	0.25
Sterile dH ₂ O to	11.5

The master mix was then mixed gently and aliquot out in 11.5 µl volumes, into real-time strip tube (Gene Target Solutions). To each tube, the appropriate volume of template/sterile water was added to make up a final volume of 12.5 µl. The tubes were then closed, and gently tapped to ensure sufficient mixing while preventing the addition of bubbles. The tubes were then placed into the Rotor-Gene™ 6000 (Corbett Research) and the following cycling conditions were performed:

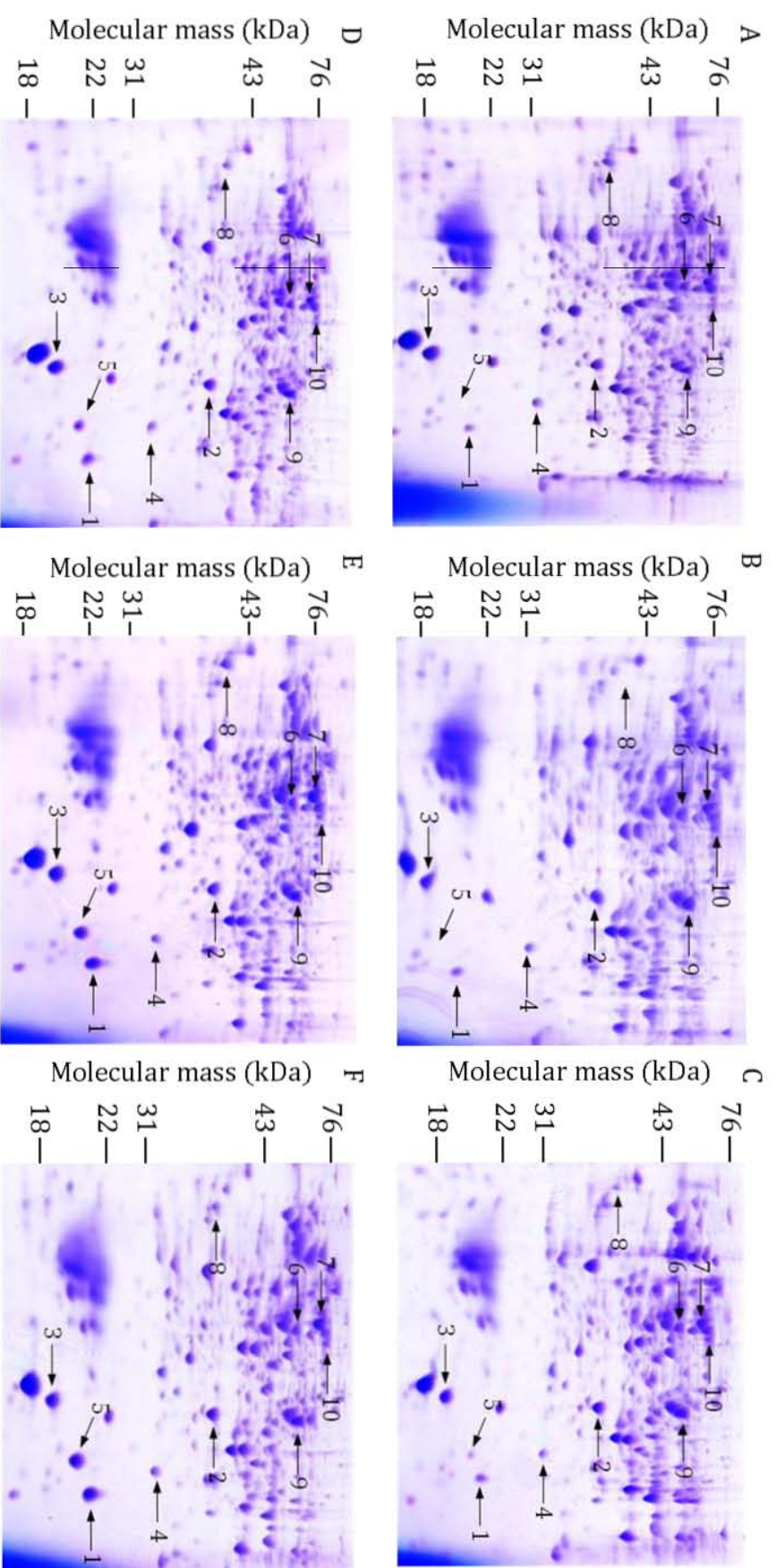
Temperature (°C)	Time (s)	Cycles
95	300	1
95	30	
62	30	35
72	30	
72	300	1
20	Hold	

Post cycling, the data was obtained and assessed using the Rotor-Gene™ Series Software (version 1.7).

APPENDIX C
SUPPLEMENTARY DATA
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C.1 2D electrophoresis analysis of *G. gracilis* total soluble protein subjected to heat stress



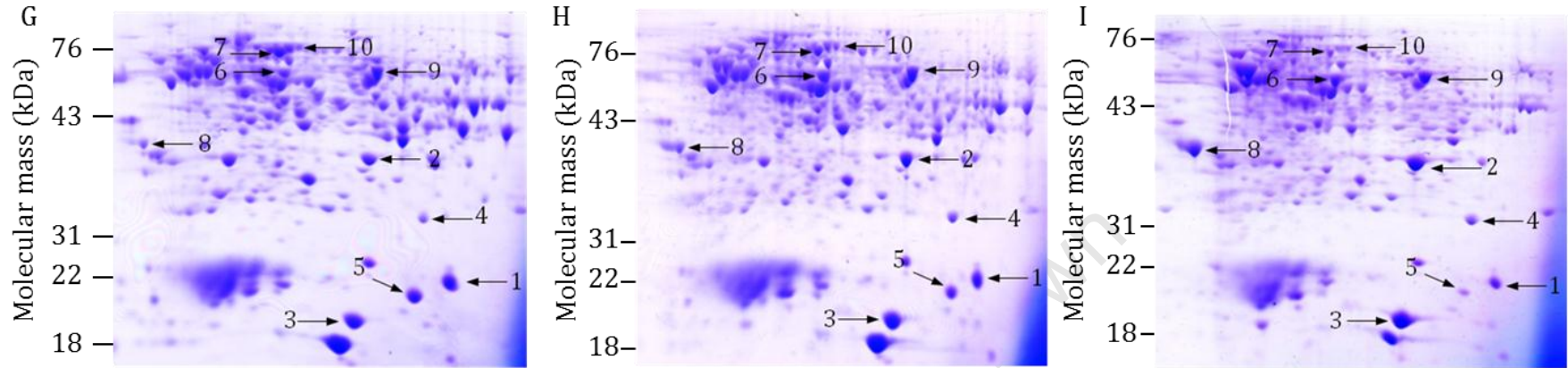


Figure 1 Representative images of the coomassie stained total soluble protein (TSP) profiles of *G. gracilis* separated on 12 % 2D SDS-polyacrylamide gels. Each gel contained TSP isolated from thallus segments heat shocked for various durations at 35 °C. Gels A-I represent total soluble protein, isolated from tissue heat shocked for 0, 0.5, 1, 2, 4, 6, 8, 18 and 24 hours respectively. The arrows indicate the position of the ten most statistically significant differentially expressed protein spots, as previously indicated in Figure 3.2.

C.2 Separation of two overlapping protein spots on an 18 % 2D SDS-polyacrylamide gel

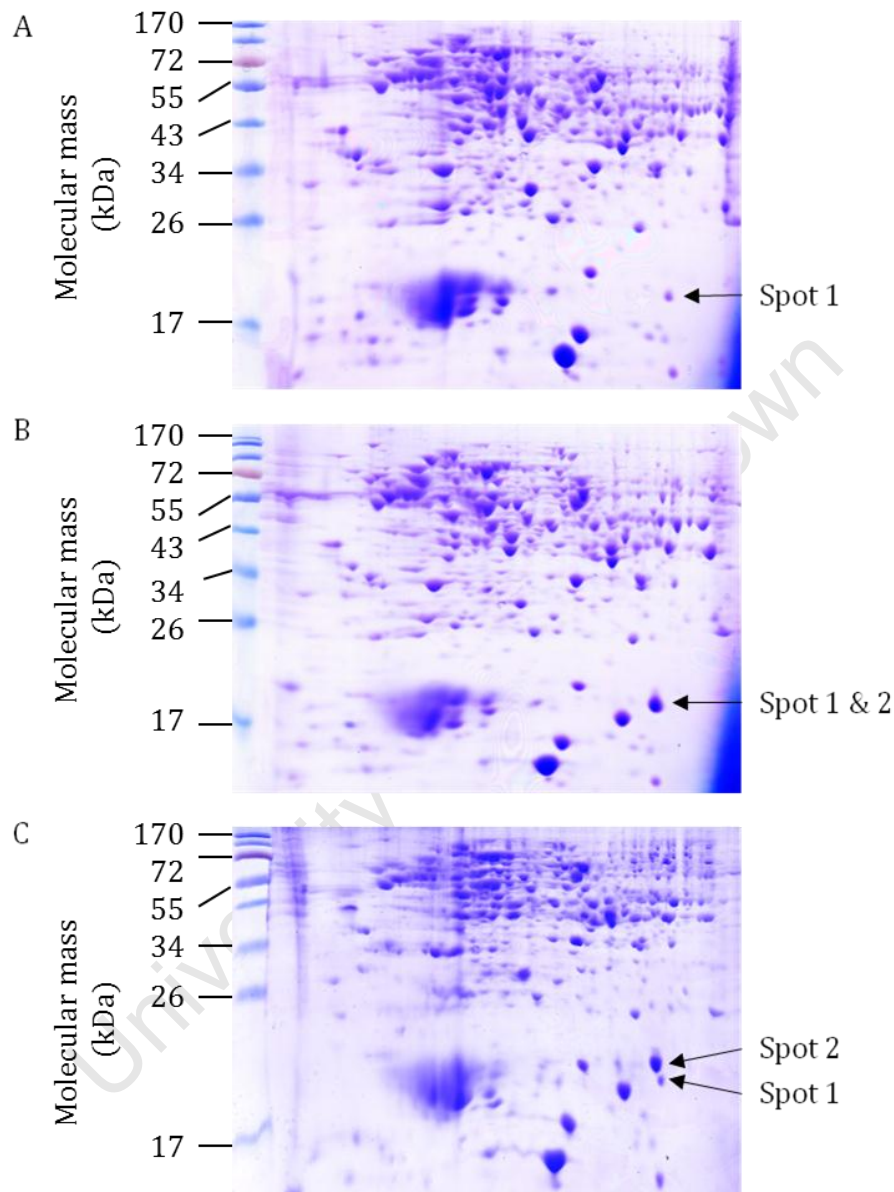


Figure 2 Separation of two overlapping protein spots on an 18 % 2D SDS-polyacrylamide gel. (A) Total soluble protein (TSP) isolated from an acclimatised *G. gracilis* thallus segment and separated out on a 12 % SDS-polyacrylamide gel. (B) and (C) TSP isolated from tissue heat shocked at 35 °C for 6 hours and separated out on a 12 % and 18 % SDS-polyacrylamide gel respectively. In C, the de novo protein spot (Spot 2) was successfully separated away from the stability expressed protein spot (Spot 1).

C.3 Alignments of the unique tryptic peptides to the appropriate protein homologues

A	1	MAFVASVPVF	ANASGLKTEA	KVCQKPALKN	SFFRGEEVTS	RSFFASQAVS
	51	AKPATTFEVD	TTIRAQAVDA	KKKGDIPNL	FRPANPYIGK	CIYNERIVGE
	101	GAPGETKHII	FTHEGKVPYL	EGQSIGIIPP	GTDKDGKPHK	<u>LRLYSIASTR</u>
	151	<u>HGDFGDDKTV</u>	<u>SLSVKR</u> LEYT	DANGNLVKGV	CSNYLCDLKP	GDEVMITGPV
	201	GTTMLMPEDQ	SATIIMLATG	TGIAPFRSFL	RRMFEETHAD	YKFENGLAWLF
	251	LGVPTSSTLL	YREELEKMQK	ANPNNFRLDY	<u>AISREQTDSK</u>	<u>GEKMYIQNRI</u>
	301	AEYANEFWNM	IQKPNTFVYM	CGLRGMEDGI	QQCMEDIAKA	NGTTWDAVVK
	351	GLKKEKRWHV	ETY			
B	1	<u>MKTPITEAIA</u>	<u>SADSQGRFLS</u>	<u>NGELQSINGR</u>	YQRASASLEA	ARSLTSNAQR
	51	<u>LITGAAQSVY</u>	<u>TKFPFTTQMP</u>	GPTYASSAIG	KAKCARDIGY	YLRMTTYCLV
	101	VGATGPMDEY	LIAGLEEINR	SFELSPSWYI	EALQYIKNSH	SLSGQAANEA
	151	NTYLDYAINA	LS			
C	1	MAFVAPVSSV	FSTSSKSAVC	SGRSSFAQFS	GLKKVNNTAR	LQTAEQGSFAF
	51	GGVSDANDAF	FNAVNTMGAP	ARTSNAPSMK	VRVAINGFGR	IGRNFIRCWA
	101	GRTDSNMDVV	CINDTSGVKT	ASHLLKYDSI	<u>LGTFDSDVVA</u>	<u>GEDSITVDGK</u>
	151	<u>TIKVVSNRNP</u>	<u>LELPWKEMEI</u>	<u>DIVVEATGVF</u>	<u>VDAVGAGKHI</u>	<u>QAGAKKVLIT</u>
	201	<u>APGKGEVGT</u>	<u>FVVGVDHLY</u>	<u>SHDKF</u> DIVSN	ASCTTNCMAP	<u>FMKVLDDEFG</u>
	251	<u>VVRGMMTTH</u>	<u>SYTGDQRLD</u>	AGHRDLRRAR	<u>SAALNIVPTT</u>	<u>TGAAKAVALV</u>
	301	<u>VPTLAGK</u> LNQ	IALRVPTPNV	SVCDVVMQVS	<u>KKTFKEEVNG</u>	<u>ALLKAANGSM</u>
	351	KGI IKYSDEP	LVSCDYRGTD	<u>ESTIIDSSLT</u>	<u>MVMGDDMLKV</u>	VAWYDNEWGY
	401	<u>SQRVVDLGEV</u>	<u>MASQWK</u>			
D	1	MAKKILYQDN	ARKALEK <u>GMD</u>	<u>TLVEAVAITL</u>	<u>GPKGRNVVLE</u>	RKFGAPQIIN
	51	DGVTIAKEIE	LQDLAENTGV	ALIRQAASKT	NDVAGDGTTT	ATVLAHAIVK
	101	GLKNVAAGAN	PITLKKGIQK	AVKFVVGKIA	EYSKPICSMQ	DVTHIGSISS
	151	GNDVEVGTM	ANAIQQVGKE	GIISLEEGQS	TSTELDIKEG	MKFDKGFISP
	201	YFVTDTSRME	VIQDNPYILI	TDKK <u>ITLIQQ</u>	<u>ELLP</u> ILEKVT	<u>KTGRPLLI</u> IA
	251	<u>EDIEKEALAT</u>	<u>IIVNKL</u> RGII	NVVAVRAPGF	GDRRKLLED	IAILTSGVVI
	301	TQDMGLSLDS	MSLDQLGSAR	RVQITKDSTT	IVADAQKDLI	QARCDQIRRQ
	351	LEAATNSYAK	DKLHERLAKL	SGGVAVV <u>KVG</u>	<u>AATETEM</u> RDK	<u>KLRL</u> EDAINA
	401	<u>TKAA</u> IEEGIV	PGGGSTFVHL	SEVLRVWAIN	NLVEEELVGA	LIIVDSLHP
	451	LKRIVENAGN	NGSIIIEKIK	NSNFSTGYNA	DIGQIVDMYK	EGIIDPAKVT
	501	RSALQNAASI	ASMILTTECL	IAEQVDN		

E	1	MAFVASVPVF	ANASGLKTEA	KVCQKPALKN	SFFRGEEVTS	RSFFASQAVS
	51	AKPATTFEVD	TTIRAQAVDA	KKKGDIPLNL	FRPANPYIGK	CIYNERIVGE
	101	GAPGETKHII	FTHEGKVPYL	EGQSIGIIPP	GTDKDGKPHK	<u>LRLYSIASTR</u>
	151	HGDFGDDKTV	<u>SLSVKRLEYT</u>	DANGNLVKGV	CSNYLCDLKP	GDEVMITGPV
	201	GTTMLMPEDQ	SATIIMLATG	TGIAPFRSFL	RRMFEETHAD	YKFENGLAWLF
	251	LGVPTSSTLL	YREELEKMQK	ANPNNFRLDY	<u>AISREQTDSK</u>	<u>GEKMYIQNRI</u>
	301	AEYANEFWNM	IQKPNTFVYM	CGLRGMEDGI	QQCMEDIAKA	NGTTWDAVVK
	351	GLKKEKRWHV	ETY			
F	1	MATKSFLLIL	FFMILATTSS	TCAKLEEMVT	VLSIDGGGK	<u>GIIPAILLEF</u>
	51	<u>LEGQLQEVND</u>	<u>NKDARLADYF</u>	DVIGGTSTGG	LLTAMITTPN	ENNRPFAAAK
	101	DIVPFYFEHG	PHIFNYSGSI	LGPMYDGKYL	<u>LQVLQEKLGE</u>	TRVHQALTEV
	151	AISSFDIKTN	KPVIFTKSNL	AKSPELDAKM	YDICYSTAAA	PIYFPPHHFV
	201	THTSNGARYE	FNLVDGAVAT	VGDPALLSLS	VATRLAQEDP	<u>AFSSIKSLDY</u>
	251	<u>KQMLLLSLGT</u>	<u>GTNSEFDKTY</u>	<u>TAEAAKWGP</u>	LRWMLAIQQM	TNAASSYMTD
	301	YYISTVFQAR	<u>HSQNNYLRVQ</u>	ENALNGTTTE	MDDASEANME	LLVQVGETLL
	351	KKPVSKDSPE	<u>TYEEALKRFA</u>	KLLSDRKKLR	ANKASH	
G	1	MTVATQSAKE	LSVEELAINS	<u>IRFLAIDAVE</u>	<u>KAKSGHPGLP</u>	MGAAPMAFVL
	51	WDRFLKFNPK	NPKWFNRDRF	VLSAGHGSM	QYALLYLTGY	DSVTIEDIKQ
	101	FRQWGSKTPG	HPENFETPGV	EVTTGPLGQG	IANGVGLAIA	EAHLAAKFNK
	151	PDTKIVDHYT	YVIVGDGCNM	EGISGEACSF	AGHLGLGKLI	ALYDDNHISI
	201	DGSTDVAFTE	DVSKRFEAYG	WHVQHVEDGN	TDLAAIAKAI	EAAKAVTDKP
	251	SFIKVTTIIG	<u>YGSPNKANTA</u>	GVHGAALGAD	EIALTRKNLG	WEYEPFDVPO
	301	EALNHTRKAV	ERGASYEAEW	NKTFADYKAK	YAQEAAEFER	YLSGKLPDQW
	351	DNVLPPTYTPE	DKGLPTRKHS	ETCLNKLAAY	LPELIGGSAD	LTHSNLTEIK
	401	GKGDFQKGHY	ENPNIHFGVR	EHAMGAICNG	IALHTSGLIP	YGATFLIFTD
	451	YMRAAIRLSA	LSQAGVIWVM	THDSIQGED	GPTHQPIETL	ASLRAIPNLL
	501	VFRPADGNET	SGAYKIAIEK	AKQNAPSLLA	FTRQNVPNLA	GTSVEGVAKG
	551	GYTVVDSEGT	PDIILIGTGS	ELSLAVSAAE	KLKAEGKKVR	VVSLPSWELF
	601	EAQDAAYKES	ILPKAVTKRL	SVEAAASFGW	HKYVGTEGDT	VSIDRFGASA
	651	PGNVCLEKFG	FSVDNVLAKA	KQLLG		

Figure 3 Alignments of the unique tryptic peptides obtained from *G. gracilis* using mass spectrometry to that of the appropriate homologues. (A) Ferredoxin NADP+ reductase (Q00598), spot 2 (B) Phycocyanin alpha subunit (YP_063694), spot 3 (C) Glyceraldehyde-3-phosphate dehydrogenase (P30724), spot 4 (D) Chaperonin GroEL (YP_063549), spot 6 (E) Heat shock protein 70 (AAM94003), spot 7 (F) Patatin (P15477), spot 9 (G) Transketolase (YP_001867861), spot 10. The numbers to the left of the sequence indicate the amino acid positioning of each sequence fragment. The unique peptides are underlined. The spot protein identification numbers are equivalent to that in Figure 3.2.

C.4 Determination of the approximate molecular weight and isoelectric focusing points of the differentially expressed protein spots using 2D SDS-PAGE standards

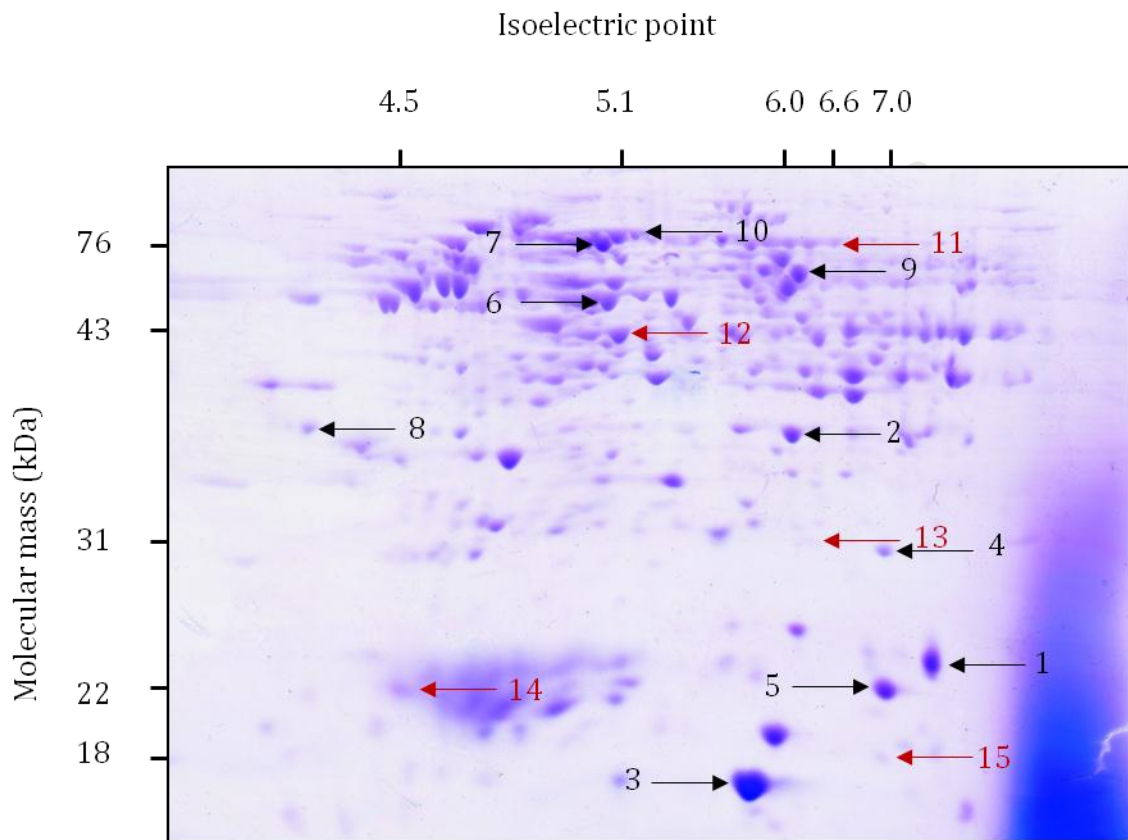


Figure 4 An image of a coomassie stained total soluble protein profile of *G. gracilis* with 2D SDS-PAGE Standards (Bio-rad), separated on a 12 % 2D SDS-polyacrylamide gel. Total soluble protein was isolated from a thallus segment heat shocked for 6 hours at 35 °C, to include the up-regulated de novo proteins. The black arrows indicate the position of the ten most statistically significant, differentially expressed proteins (Section 3.4.2). The red arrows indicate the position of the 2D SDS-PAGE standards where spot 11: hen egg white conalbumin; spot 12: bovine muscle actin, spot 13: bovine carbonic anhydrase, spot 14: soybean trypsin inhibitor and spot 15: equine myoglobin.

C.5 Visualisation of the Hsp70 low molecular weight degradation product

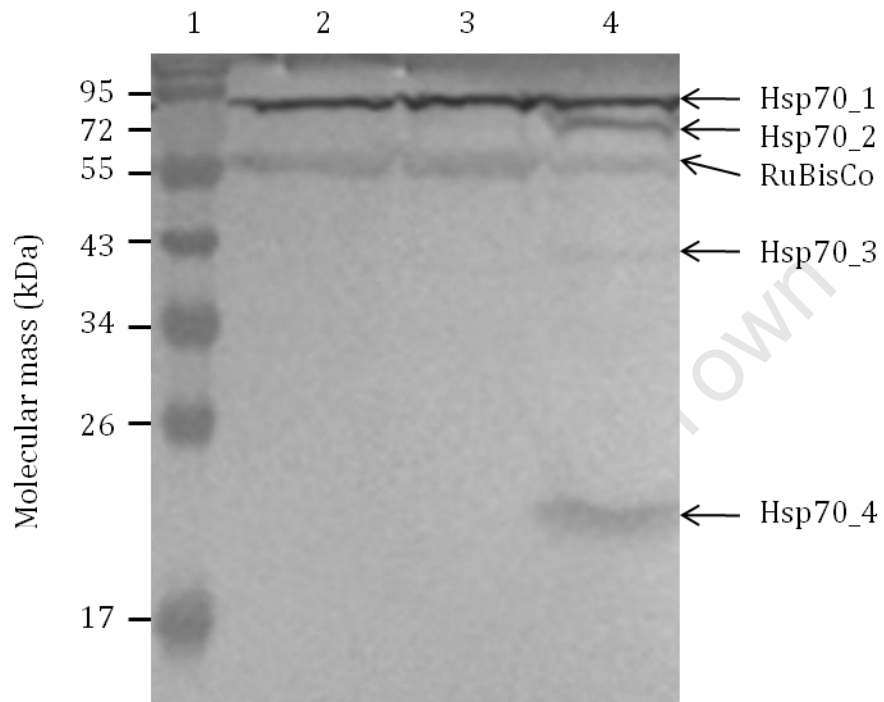


Figure 5 Western blot analysis of Hsp70 in *G. gracilis* in response to heat stress. Lane 1: PageRuler™ prestained protein ladder (Fermentas). Lanes 2-4: Total soluble protein isolated from *G. gracilis* thallus segments heat stressed for 0 (control), 6 and 24 hours, respectively. The various fragments are indicated on the right of the image. RuBisCo: loading control, Hsp70_1: Hsp70 protein band maintaining a high integrity, Hsp70_2 – Hsp70_4: Hsp70 degradation products.

C.6 Additional information regarding the Hsp70 sequences used in this study

Table 2 Additional information regarding the Hsp70 proteins sequenced used in this study.

Hsp70 Identification ^a	Accession number	Reference website
<i>A. thaliana</i> C Hsp70_1	AT5G02500	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> C Hsp70_2	AT5G02490	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> C Hsp70_3	AT3G09440	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> C Hsp70_4	AT3G12580	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> C Hsp70_5	AT1G16030	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> C Hsp70_6	AT1G56410	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> CP Hsp70_7	AT4G24280	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> CP Hsp70_8	AT5G49910	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> M Hsp70_9	AT4G37910	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> M Hsp70_10	AT5G09590	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> ER Hsp70_11	AT5G28540	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> ER Hsp70_12	AT5G42020	www.ncbi.nlm.nih.gov
<i>A. thaliana</i> ER Hsp70_13	AT1G09080	www.ncbi.nlm.nih.gov
<i>C. sativus</i> ER Hsp70_1	CAB72128	www.ncbi.nlm.nih.gov
<i>Z. mays</i> ER Hsp70_1	O24581	www.ncbi.nlm.nih.gov
<i>Z. mays</i> ER Hsp70_2	P24067	www.ncbi.nlm.nih.gov
<i>C. dubia</i> ER Hsp70_1	CAC37635	www.ncbi.nlm.nih.gov
<i>C. reinhardtii</i> C Hsp70_1	126835	genome.jgi-psf.org/Chlre3/Chlre3.home.html
<i>C. reinhardtii</i> CP Hsp70_2	137452	genome.jgi-psf.org/Chlre3/Chlre3.home.html
<i>C. reinhardtii</i> M Hsp70_3	185673	genome.jgi-psf.org/Chlre3/Chlre3.home.html
<i>C. reinhardtii</i> ER Hsp70_4	133650	genome.jgi-psf.org/Chlre3/Chlre3.home.html
<i>C. reinhardtii</i> ER Hsp70_5	133859	genome.jgi-psf.org/Chlre3/Chlre3.home.html

Table 2 Additional information regarding the Hsp70 proteins sequenced used in this study (continued)

Hsp70 Identification ^a	Accession number ^b	Reference
<i>G. theta</i> CP Hsp70_1	YP_063608	www.ncbi.nlm.nih.gov
<i>O. lucimarinus</i> C Hsp70_1	28169	genome.jgi-psf.org/Ost9901_3/Ost9901_3.home.html
<i>O. lucimarinus</i> CP Hsp70_2	48839	genome.jgi-psf.org/Ost9901_3/Ost9901_3.home.html
<i>O. lucimarinus</i> M Hsp70_3	15148	genome.jgi-psf.org/Ost9901_3/Ost9901_3.home.html
<i>O. lucimarinus</i> ER Hsp70_4	12592	genome.jgi-psf.org/Ost9901_3/Ost9901_3.home.html
<i>O. tauri</i> C Hsp70_1	22076	genome.jgi-psf.org/Ostta4/Ostta4.home.html
<i>O. tauri</i> CP Hsp70_2	28374	genome.jgi-psf.org/Ostta4/Ostta4.home.html
<i>O. tauri</i> M Hsp70_3	28024	genome.jgi-psf.org/Ostta4/Ostta4.home.html
<i>O. tauri</i> ER Hsp70_4	15769	genome.jgi-psf.org/Ostta4/Ostta4.home.html
<i>T. pseudonana</i> C Hsp70_1	269120	genome.jgi-psf.org/Thaps3/Thaps3.home.html
<i>T. pseudonana</i> CP Hsp70_2	YP_874583	genome.jgi-psf.org/Thaps3/Thaps3.home.html
<i>T. pseudonana</i> M Hsp70_3	269240	genome.jgi-psf.org/Thaps3/Thaps3.home.html
<i>T. pseudonana</i> ER Hsp70_4	27656	genome.jgi-psf.org/Thaps3/Thaps3.home.html
<i>C. merolae</i> C Hsp70_1	3804	merolae.biol.s.u-tokyo.ac.jp
<i>C. merolae</i> CP Hsp70_2	5604	merolae.biol.s.u-tokyo.ac.jp
<i>C. merolae</i> M Hsp70_3	1601	merolae.biol.s.u-tokyo.ac.jp
<i>C. merolae</i> ER Hsp70_4	914	merolae.biol.s.u-tokyo.ac.jp
<i>G. gracilis</i> ER Hsp70_1	-	-
<i>G. tenuistipitata</i> CP Hsp70_1	AAT79683	www.ncbi.nlm.nih.gov
<i>P. haitanensis</i> CP Hsp70_1	ABF20063	www.ncbi.nlm.nih.gov
<i>P. purpurea</i> CP Hsp70_1	AAC08201	www.ncbi.nlm.nih.gov
<i>P. yezoensis</i> CP Hsp70_1	ABF54971	www.ncbi.nlm.nih.gov

Table 2 Additional information regarding the Hsp70 proteins sequenced used in this study (continued)

Hsp70 Identification ^a	Accession number ^b	Reference
<i>G. gallus</i> ER Hsp70 1	AAA48785	www.ncbi.nlm.nih.gov
<i>H. sapiens</i> ER Hsp70 1	AAF42836	www.ncbi.nlm.nih.gov
<i>H. sapiens</i> ER Hsp70 2	NP_005338	www.ncbi.nlm.nih.gov
<i>H. sapiens</i> ER Hsp70 3	P11021	www.ncbi.nlm.nih.gov
<i>P. abelii</i> ER Hsp70 1	Q5R4P0	www.ncbi.nlm.nih.gov
<i>R. norvegicus</i> ER Hsp70 1	NP_037215	www.ncbi.nlm.nih.gov
<i>R. norvegicus</i> ER Hsp70 2	P06761	www.ncbi.nlm.nih.gov
<i>X. laevis</i> ER Hsp70 1	Q91883	www.ncbi.nlm.nih.gov
<i>P. falicparum</i> ER Hsp70 1	XP_001352050	www.ncbi.nlm.nih.gov
<i>P. yoelli</i> ER Hsp70 1	EAA16958	www.ncbi.nlm.nih.gov

^a Hsp70s were identified using a species name, a cellular localisation code and a number. Hsp70 protein sequences were obtained for a number of different species including: 3 land plants *Arabidopsis thaliana*, *Cucumis sativus* and *Zea mays*; 5 chlorophyta *Cherffelia dubia*, *Chlamydomonas reinhardtii*, *Guillardia theta*, *Ostreococcus lucimarinus* and *Ostreococcus tauri*; a diatom *Thalassiosira pseudonana*; 6 rhodophyta *Cyanidioschyzon merolae*, *Gracilaria gracilis* (this study), *Gracilaria tenuistipitata*, *Porphorya haitanensis*, *Porphorya purpurea* and *Porphorya yezoensis*; 5 animals *Gallus gallus*, *Homo sapiens*, *Pongo abelii*, *Rattus norvegicus* and *Xenopus laevis* and 2 protozoa *Plasmodium falicparum* and *Plasmodium yoelli*. The species name is followed by a cellular localisation code: cytoplasm (C), chloroplast (CP), mitochondria (M) or endoplasmic reticulum (ER). The Hsp70 number was given at random to distinguish between multiple Hsp70s from a single organism.

^b Accession number or protein I.D. as indicated on the referenced websites.

C.7 The relative expression of Hsp70 in *G. gracilis*, in response to various durations of exposure to 35 °C.

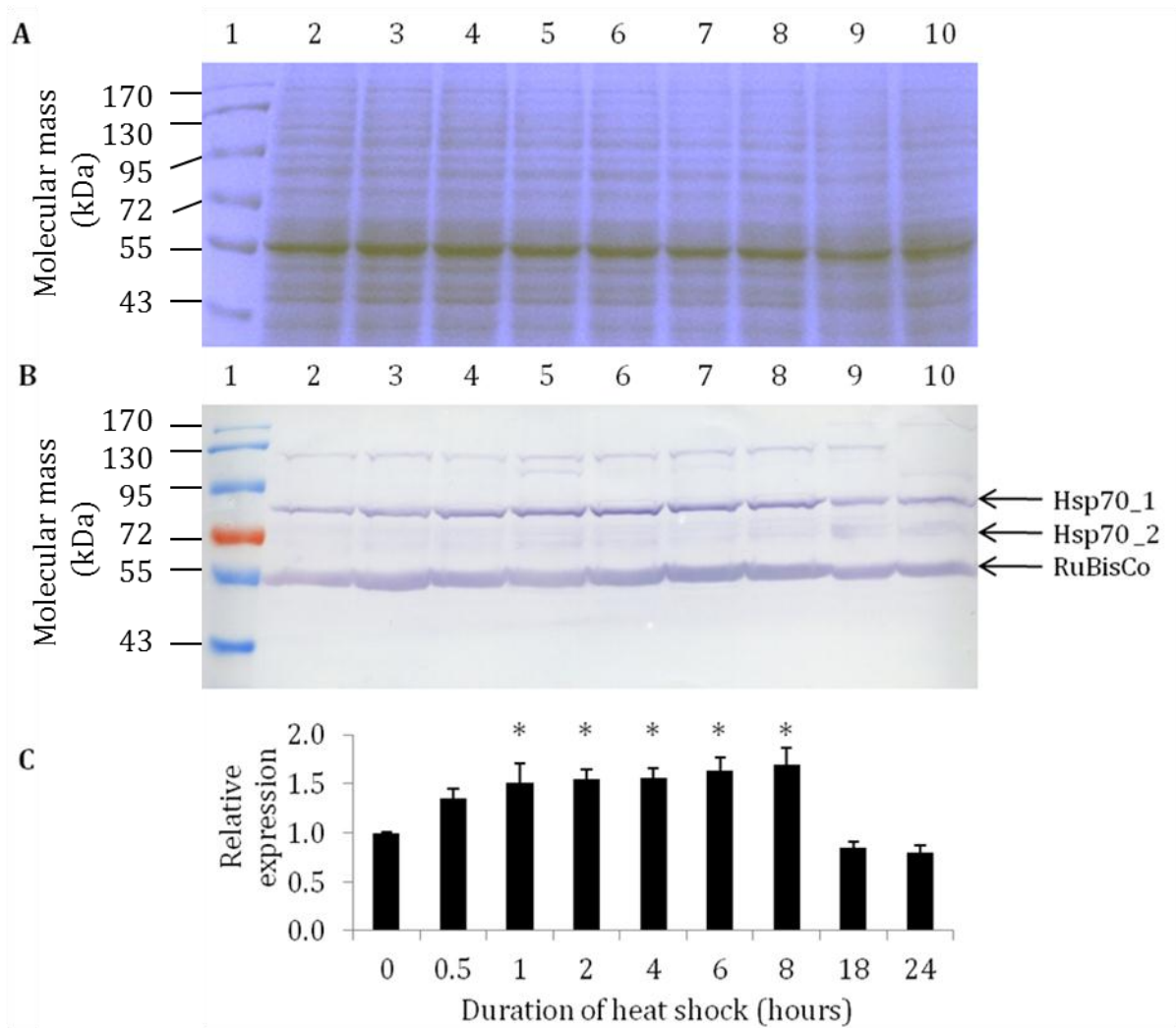


Figure 6 The relative expression of Hsp70 in *G. gracilis*, in response to various durations of exposure to 35 °C. (A) Coomassie stained 1D SDS-polyacrylamide gel, (B) western blot analysis of Hsp70, where Hsp70_1: non-degraded Hsp70, Hsp70_2: a Hsp70 degradation product and RuBisCo: loading control (C) Densitometric analysis of the Hsp70_1 band. Lane 1: PageRuler™ prestained protein ladder (Fermentas). Lanes 2-10: Total soluble protein isolated from *G. gracilis* thallus segments heat stressed for 0 (control), 0.5, 1, 2, 4, 6, 8, 18 and 24 hours respectively. In C, a parametric one-way ANOVA detected statistically significant differences in the relative expression of Hsp70_1 between the control and experimental samples ($P < 0.001$, denoted by asterisks '*'). Data represents the mean \pm standard error from three biological repeats.

C.8 Multiple sequence alignment of the putative ER Hsp70 from *G. gracilis* and its top five sequence similarities.

ER Hsp70					
BAC67670	1	MAHHRCDA AACVFNQHAQEARTHARTHARTLARMHHRRLRKQSE SASLQPLDSIARNGGAANTTTAFGVV			70
AAA62325					
CAA89834					
CBI34546					
ACJ11746					
			← Signaling peptide →		
ER Hsp70	1		MSVSSYKRQSAFFLLFASLIVACTASTENVGTV		33
BAC67670	71	VVVC DVVPRRVKELGQVDMATRRFSYRRVSIWNWALVAVLVHVCCLFGRAVLVGAKDASSGGGKIEGPV			140
AAA62325	1		MDRARG SALLLGVLLAGSLFALCAAKEEAKKLGTV		35
CAA89834	1	MGRKQKCAGFNNAGKDFNGFMFLAAFITAGFLFSSVIAAEEAAKLGTV			48
CBI34546	1		MESSWRRRGS LIVAAIVCFGFLAAISIAKEEATKLGTV		38
ACJ11746	1		MARSWRASGS LVALAIVLSGCFFAISIAKEEAAKLGTV		38
ER Hsp70	34	KYTDATVQRDKLLPFKIVSQGDKPMISIDVK .GEKKTFSPEQISAMVLGKMKKTAE EYLGREVKNAVVT			102
BAC67670	141	R FDEETVQKDIKLLPYKVVNKDGKPYIRVEVRDGEVKTFSPEEISAMILGKMKKIAEDYLGKPVKNAVVT			210
AAA62325	36	KFEDKEVQRDMRLVPYKIVNKEGKPYIQVKIKDGETKVFSPEEVSAMILGKMKETA EAYLGKKINDAVVT			105
CAA89834	49	KYEDKEVQKDIKLLPYKIVNKGKPYIQVKIRDGEIKVFSPEEISAMILKMKETAESYLGRKIKDAVVT			118
CBI34546	39	KFEDKEVQKDMKLVPIVIVNKGKPYIQVKIKDGETKVFSPEEISAMILTKMKETA EAFGLGKKIKDAVVT			108
ACJ11746	39	KFEDKEVQRDMKLVPIVIVNKGKPYIQVKIKDGETKVFSPEEISAMVLTMKKETA EAFGLGKKIKDAVVT			108
			← Hsp70 signature motif 1 →		
ER Hsp70	103	IGIDLGTTYSCVGMQNGKVEI IANDQGNRITPSYVAFTSEERLIGDAAKNQAAMNPVNTVFDVKRLIGR			172
BAC67670	211	IGIDLGTTYSCVGVFKNGKVEI IANEQGNRITPSYVAFTDKERLIGDAAKNQLALNPERTIFDVKRLIGR			280
AAA62325	106	IGIDLGTTYSCVGVYKNGHVEI IANDQGNRITPSWVGFTDGERLIGEA AKNQA AVNPERTVFDVKRLIGR			175
CAA89834	119	IGIDLGTTYSCVGVYKNGHVEI IANDQGNRITPSWVAFTDTERLIGEA AKNQA AMNPERTVFDVKRLIGR			188
CBI34546	109	IGIDLGTTYSCVGVYKNGHVEI IANDQGNRITPSWVAFTDSEERLIGEA AKNQA AVNAERTIFDVKRLIGR			178
ACJ11746	109	IGIDLGTTYSCVGVYKNGHVEI IANDQGNRITPSWVAFTDSEERLIGEA AKNQA AVNAERTIFDVKRLIGR			178

ER Hsp70	451	QTFTTYQDNQEVVSIQVYEGERTMTKDC	HMLGKFDLSGIRKARRGDPQILVTFDIDANGILSVSAEEKGA	520				
BAC67670	561	QIFTTTHVDRQSSVLIQVYEGERVATKDNHLLGKFELTGIPPA	PRGVPQIEVTFEIDANGILQVSAEEKTA	630				
AAA62325	455	QVFTTYQDQQTTVSIQVFEGERSMTKDCRLLGKFDLSGIPAA	PRGTPQIEVTFEVDANGILNVKAEDKGT	524				
CAA89834	468	QVFTTYQDQQTTVSIKVYEGERSLTKDCRELGKFDLSGIPPA	PRGVPQIEVTFEVDANGILNVRAEDKGT	537				
CBI34546	458	QVFTTYQDQQTTVSIQVFEGERSLTKDCRQLGKFDLNGIPPA	PRGTPQIEVTFEVDANGILNVKAEDKGT	527				
ACJ11746	458	QVFTTYQDQQTTVSIQVFEGERSLTKDCRLLGKFDLTGIPPA	PRGTPQIEVTFEVDANGILNVKAEDKGT	527				
ER Hsp70	521	SNKESITITNDKGRLS	EEEEIERMVREAEEMKDEDEKIKKKVEAKNNLENFI	FTVKGTLEEKDKLEGLDE	590			
BAC67670	631	GKREKITIRNDKGRLLKDEEIQRMVREAEYAEVDAKLKRK	VDAKNNFENYIYQVRQMYEDKDKKT.KLST		699			
AAA62325	525	GKSEKITITNEKGRLSQEEIDRMVKEAEFAEEDKKVKE	RIDARNQLETYVYNMKN	TVGDKDKLADKLES	594			
CAA89834	538	KKTEKITITNDKGRLSQEEIERMVKEAEFAEEDKKVKD	KIDARNNLETYVYNMKSTINEKDKLADKIDS		607			
CBI34546	528	GKSEKITITNDKGRLSQEEIDRMVREAEFAEEDKKVKE	KIDEVEAVCNPIITAVYQRS	GAPGAGSDGG	597			
ACJ11746	528	GKSEKITITNDKGRLSQEEIERMVREAEFAEEDKKVKE	RIDARNSETYIYNMKNQINDKDKLADKLES		597			
ER Hsp70	591	EDIEALES	AVKEAQEWM	DTAEADADAEDIESRLNDMKEVVEPI	MKKVGGGPGGAGGAPEEEDDDDDDA..	658		
BAC67670	700	DDIDK	LKDSVESAQDWL	DEHGEASDAAAI	EERMKA	FQDVVQPIILKTYESAKGTGKDSSADSSADDDRDS	769	
AAA62325	595	EEKEK	MEGSWPNNN				608	
CAA89834	608	EDKEK	IETAIKEALEWLD	DNQSAEKEDFE	EKLKEVEAVCSPI	IKQVYEKTGGSSGGDDEDEDS.....	671	
CBI34546	598	EDDDS.....					602	
ACJ11746	598	DEKEK	VETAVKEALEWLD	DNQSAEKEDYE	EKLKEVEAVCNPI	ITAVYQRS	GAPGGSTEEEDDS.....	662
ER retention signal								
ER Hsp70	659	..HEEL					662	
BAC67670	770	EEHDEL					775	
AAA62325								
CAA89834	672	..HEEL					675	
CBI34546	603	..HDEL					606	
ACJ11746	663	..HDEL					606	

Figure 7 Multiple sequence alignment of the putative ER Hsp70 from *G. gracilis* (this study) and its top five sequence similarities, as identified using blastx. The accession numbers of each of the sequence obtained from the GENBANK database are indicated on the far left of each sequence. The numbers to the left and right of the sequences indicate the amino acid positioning of each sequence fragment. The amino acids in red, green and blue represent sequence identities of 100 %, >75 % and >50 % respectively. The position of the highly conserved Hsp70 family signatures 1, 2 and 3, the signaling peptides and the ER retention signals, are indicated with arrows and labels.

APENDIX D

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