

Characterisation of *galactinol synthase II (XvGolSII)* from the resurrection plant *Xerophyta viscosa* (Baker)

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Submitted in fulfillment of the requirements for the degree of Master of Science in the Department of Molecular and Cell Biology, University of Cape Town, South Africa.

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Dedication

I would like to dedicate my thesis to God my Father. Without His unconditional love, none of this would have been possible. This journey has taught me to trust in Him fully and in all things acknowledge Him and He will direct my path.

His love has surrounded me from the start and if I was able to see further it is because I am standing on the shoulders of a giant.

Thank you.

University of Cape Town

'But I rejoiced in the Lord greatly

that now at last your care for me has flourished again;

though you surely did care, but you lacked opportunity.

**Not that I speak in regard to need, for I have learned that in whatever
state I am, to be content;**

I know how to be abased, and I know how to be abound.

**Everywhere and in all things I have learned both to be full and to be
hungry, both to abound and to suffer need.**

I can do all things through Christ who strengthens me.'

Phillipians 4:10-13

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DECLARATION

I hereby declare that this thesis entitled:

Characterisation of *galactinol synthase II (XvGolSII)* from the resurrection plant *Xerophyta viscosa* (Baker)

is my own work and has not previously in its entirety or in part been submitted at any university for another degree, and that all the sources I have used and cited have been stipulated and acknowledged through complete referencing.

I know the meaning of plagiarism and declare that all of the work in the document, save for that which is properly acknowledged, is my own.

Signature

Date

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Last but not least, to a wonderful man, Mario Mufweba. Your love and strength has carried me in this time. You’ve been the wind beneath my wings. Thank you.

List of abbreviations

µg	microgram
µm	micrometre
µl	microlitre
ABA	abscisic acid
Amp	ampicillin
bp	base pair
BSA	bovine serum albumin
CaMV	cauliflower mosaic virus
cDNA	complementary DNA
DTT	dithiothreitol
EtBr	ethidium bromide
EtOH	ethanol
GFP	green fluorescent protein
His	histidine
kb	kilobase
LB	Luria Bertani
mg	milligram
ml	milliliter
ng	nanogram
N =	number of sample size
ROS	reactive oxygen species
RT	room temperature
RWC	relative water content
SDS PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
YFP	yellow fluorescent protein

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ABSTRACT

The monocotyledonous *Xerophyta viscosa* belongs to a unique group of angiosperms known as resurrection plants. These plants possess a number of unique characteristics which allow them to survive and cope for extended periods with extreme abiotic stresses such as dehydration and cold stress. It is therefore of great interest to understand and elucidate the various molecular mechanisms which are specifically regulated in response to abiotic stress by identifying genes and proteins which may contribute to abiotic stress tolerance. These genes could potentially be utilized in the development of crops with improved tolerance to abiotic stresses. The aim of this study was to preliminarily characterize *XvGolSII*, a galactinol synthase, which had been isolated from a *X. viscosa* cold stress cDNA library. In this study, the *XvGolSII* cDNA was sequenced and both the nucleic and amino acid sequence analysed through *in silico* analysis. The *XvGolSII* cDNA sequence was shown to be 1434 bp in length, with an open reading frame (ORF) of 1018 bp. This ORF encodes a 339 amino acid protein with a molecular weight of 38.7 kDa, containing a characteristic hydrophobic carboxy-terminal pentapeptide, APSAA. Recombinant *XvGolSII* protein was successfully expressed in *E. coli* BL21 cells using the pET29b expression vector. The recombinant *XvGolSII* protein showed *in vitro* galactinol synthase activity via an activity assay using HPAEC-PAD, where it produced galactinol from the substrates *myo*-inositol and UDP-galactose. Subcellular localisation examination, using an *XvGolSII*-YFP fusion protein, indicated localisation to the cell membrane of onion epidermal cells. Quantitative real time PCR analysis showed *XvGolSII* to be significantly down-regulated during dehydration stress while mildly up-regulated during the early stages of cold stress, though the latter increase was not significant. Western blot analyses did not detect *XvGolSII* in total protein extracted from *X. viscosa* leaf tissue during dehydration and rehydration treatments, using polyclonal antibodies generated from the *XvGolSII* recombinant protein. This study successfully characterized *XvGolSII* at the molecular level and provides a basis for further investigation of the role of *XvGolSII* in abiotic stress tolerance.

Chapter 1

Literature Review

1.1 INTRODUCTION

Abiotic stresses such as drought, salinity, and extreme temperatures lead to a series of morphological, physiological, biochemical, and molecular changes that adversely affect plant growth and productivity. Worldwide, abiotic stress reduces the average yield of major crop plants by more than 50%. In many regions, drought and salinity are becoming extensive and may result in salinization of more than 50% of all arable land by the year 2050 (Wang *et al.*, 2003). Cold stress, including chilling and freezing may also lead to secondary stresses due to reduced absorption of water and cellular dehydration caused by ice formation (Zhu *et al.*, 1997). These secondary stresses are characterised by excess production of reactive oxygen species (ROS) which damage macromolecules and lipids in addition to disrupting homeostasis and ion distribution within the cell. The plants response to abiotic stress is complex and involves both molecular and physiological changes. At the physiological level, the plant responds to water stress by triggering stomatal closure, reducing transpiration rates and water potential, growth inhibition and decreasing photosynthesis. At the molecular level the initial abiotic stress signals (e.g. osmotic and ionic effects, temperature, membrane fluidity changes) activates the downstream signalling process and transcription controls which in turn triggers stress-responsive mechanisms to restore homeostasis and protect and repair damaged macromolecules (Figure 1.1). The stress response mechanisms comprise various components such as detoxification of ROS, protection of proteins using chaperones, osmoprotection and osmotic adjustment of the cellular milieu via compatible solutes and tight control over water and ion movement.

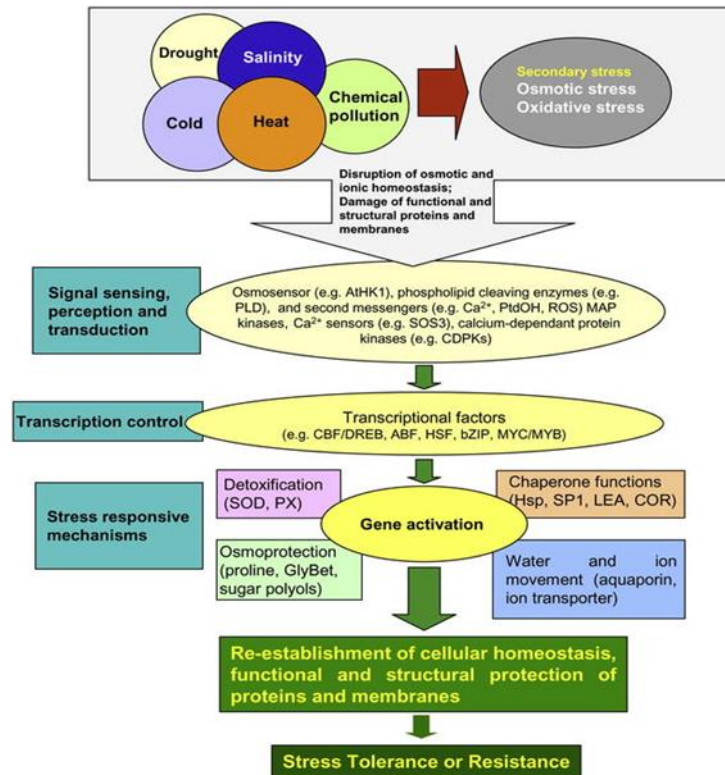


Figure 1.1: Schematic diagram depicting the complex nature of the plant response to abiotic stress. Primary stresses such as drought and salinity and secondary stresses give rise to signal cascades, which in turn result in transcription control and activation of stress responsive mechanisms (Wang *et al.*, 2003).

1.2. Resurrection plants and abiotic stress

A unique group of higher plants, termed resurrection plants, possess an effective mechanism for coping with abiotic stresses, water deficit in particular. Resurrection plants are able to survive the loss of up to 95% of their cellular water content, remain in an air-dry quiescent state for months to years, but upon re-watering regain full metabolic activity in existing tissues within 24 – 72 hours, depending on the species (reviewed in Farrant, 2007; Farrant *et al.*, 2012). This ability provides resurrection plants with a great competitive advantage over other species for certain ecological niches, usually on rocky surfaces where not only is water frequently scarce but extremes of temperature are also experienced (Scott, 2000; Proctor *et al.*, 2007). Resurrection plants are thus studied in attempts to understand the mechanisms of desiccation tolerance, often with the ultimate aim of identifying genes which can be utilised for bioengineering crops for improved tolerance to water stress (Farrant, 2000).

A number of studies, ranging from the physiological to the molecular levels, have been conducted on a range of resurrection plants in order to determine the mechanisms of desiccation tolerance associated with these plants (recently reviewed in Farrant *et al.*, 2012; Gechev *et al.*, 2012). A review of such studies indicates that the mechanisms are complex (briefly summarized in Table 1.1), and while there are some general similarities among species, particularly with respect to mechanisms associated with the more extreme stages of water loss (where some mechanisms are akin to those associated with desiccation tolerance in orthodox seeds (Farrant & Moore, 2011; Farrant *et al.*, 2012)), significant differences also occur among species. Despite increasing reports on putative mechanisms of desiccation tolerance in general (reviewed in Leprince & Buitink, 2010; Farrant *et al.*, 2012), our understanding of the phenomenon of vegetative desiccation tolerance in any one species is far from complete. We have been systematically characterising mechanisms of tolerance in the monocotyledonous resurrection plant *Xerophyta viscosa* Baker (Fig 1.2) and the current study was undertaken to further elucidate the role of osmoprotection (Figure 1.1) in facilitation of tolerance of abiotic stresses faced by this species in its natural environment.

Xerophyta viscosa (Baker) is endemic to southern Africa and is able to dry down to 5% relative water content (RWC), and once water becomes available is able to restore its metabolism to that of a fully turgid plant (Farrant, 2000). It is poikilochlorophyllous in that chlorophyll is degraded and thylakoid membranes dismantled during the early stages of drying (this being complete upon loss of only 20% RWC) in order to minimize the extensive formation of reactive oxygen species (ROS) associated with photosynthesis during water deficit stress. This phenomenon has implications with respect to photosynthetically associated accumulation of protectant osmolytes.



Figure 1.2: A flowering *Xerophyta viscosa* plant found in mountain top habitats like Cathedral Peak in the Drakensberg mountains, which stretch across Lesotho and South Africa.

Table 1.1: A summary of the various responses observed in resurrection plants during osmotic stress (adapted from Bernacchia & Furini, 2003).

Resurrection plants employ	<ul style="list-style-type: none"> • Damage repair mechanism • Cell protection mechanism
Signal transduction pathways can be	<ul style="list-style-type: none"> • ABA-dependent • ABA-independent
Regulatory factors involved	<ul style="list-style-type: none"> • AB13 • CDT1 • HD-ZIPs • Myb factors • Translation factors
Molecular responses	<ul style="list-style-type: none"> • Protective proteins → LEA, structural, hydrophilic • Protective molecules → Sucrose, osmosprotection, glass formation • Metabolic enzymes → Sugar conversions, sucrose synthase, sucrose-P-synthase
Morphological responses	<ul style="list-style-type: none"> • Poikilochlorophyllous → chlorophyll loss • Homoiochlorophyllous → no chlorophyll loss

One of the responses of higher plants, including resurrection plants, to abiotic stress is the synthesis and accumulation of compatible solutes (osmolytes) such as amino acids, quaternary compounds and a number of sugars such as sucrose, trehalose and non-structural carbohydrates termed raffinose family oligosaccharides (RFOs). In a number of studies to date it has been demonstrated that sucrose universally accumulates in the leaves and roots of all angiosperm resurrection plants (Farrant, 2007; Bartels & Salamini, 2001; Peters *et al.*, 2007). It is proposed that the ubiquitous presence of sucrose plays a crucial role in glass formation and the stabilization of the subcellular milieu during maintenance in the dry state (Hoekstra *et al.*, 2001; Berjak *et al.*, 2007). In seed literature it is extensively known that RFOs, particularly raffinose and stachyose, may be vital in stabilization of the subcellular milieu by playing a role in vitrification or acting as a water replacement. Raffinose and stachyose have indeed been shown to be the most commonly accumulated sugars in a number of resurrection plants (Farrant, 2007; Buitink *et al.*, 2000; Sherwin & Farrant, 1998; Peters *et al.*, 2007). Correlations between RFO accumulation and cold, drought and salinity stress have been reported in a number of studies, implying a role for RFOs in stress adaptation (Mundree *et al.*, 2002).

1.3 Raffinose family oligosaccharides (RFOs)

Raffinose family oligosaccharides (RFOs) are synthesized by a set of glycosyltransferases that sequentially add galactose units from galactinol to sucrose (Peterbauer *et al.*, 2001). The most common RFOs are the trisaccharide raffinose, the tetrasaccharide stachyose and the pentasaccharide verbascose. The biosynthesis of raffinose and stachyose is mediated by raffinose synthase and stachyose synthase, respectively. Raffinose synthase is one of the key enzymes that is able to channel sucrose into the RFO biosynthetic pathway (Sui *et al.*, 2012). RFOs have been reported in several studies as the principle carbohydrates in carbon translocation in plants.

These non-structural carbohydrates share a fundamental characteristic with compatible solutes; as non-reducing sugars, they can accumulate to high concentrations within the cell without affecting metabolic processes. The RFO biosynthetic pathway is initiated with the synthesis of the galactosyl donor galactinol, catalysed by the enzyme galactinol synthase (GolS), making use of UPD-galactose and *myo*-inositol as substrates. This is the first committed step in the classical RFO biosynthetic pathway (Peters & Keller, 2009). Figure 1.3 illustrates the role galactinol plays in the formation of raffinose family oligosaccharides.

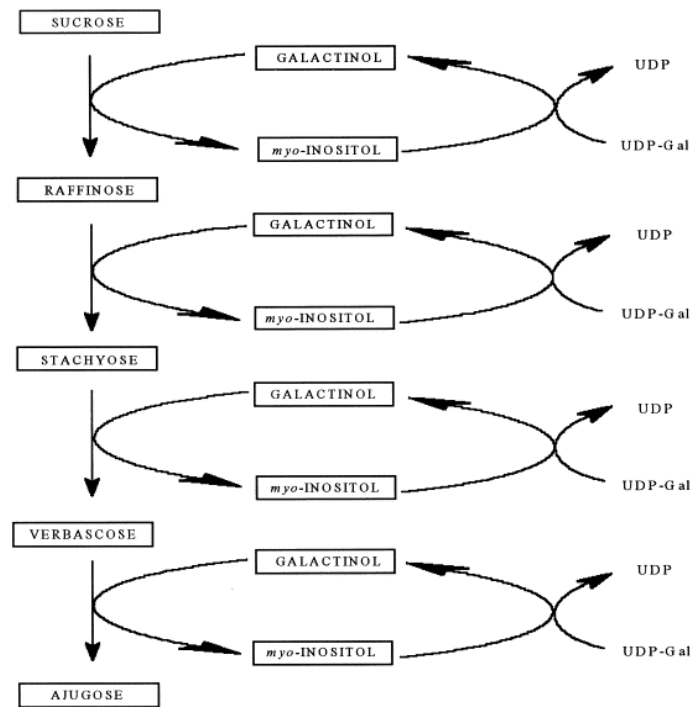


Figure 1.3: Schematic of the role of galactinol in the formation of higher raffinose family oligosaccharides in the galactinol-dependent RFO synthesis pathway (Albini *et al.*, 1999).

A novel galactinol-independent galactosyltransferase was found in cold-acclimated leaves of *Ajuga reptans*. The enzyme was tentatively termed galactan-galactan galactosyltransferase (GGT) and it was shown to produce the next higher and lower degree of polymerization (DP) RFO from two molecules of RFO. Raffinose, stachyose and verbascose were able to serve as substrates for this enzyme, implying that it may be involved in RFO chain elongation (Bachmann *et al.*, 1994). Karner *et al.* (2004) also demonstrated that there was no significant relationship observed between the extractable GalS activity and the amount of RFOs in the seeds from seven pea (*Pisum sativum* L.) genotypes. These findings suggest that RFO accumulation may be controlled not only by the presence of GalS but also the concentration of the initial substrates *myo*-inositol and sucrose (Karner *et al.*, 2004).

1.4 The roles of RFOs

Raffinose family oligosaccharides possess diverse roles within plants such as carbon transport and storage and also serve as compatible solutes for protection against damage caused by abiotic stress (Hanna *et al.*, 2006; Bachmann *et al.*, 1994). Raffinose family oligosaccharides together with galactinol have been proposed to fulfil important functions in

the defence against oxidative stress in plants (Nishizawa *et al.*, 2008) as well as in seeds (Buitink *et al.*, 2000). It has also been proposed that galactinol and raffinose may serve as signals that mediate the stress response (Valluru & Van den Ende, 2011; ElSayed *et al.* In Press).

1.4.1 RFOs as compatible solutes

Compatible solutes allow for the maintenance of favourable turgor during times of water stress and may function as osmoprotectants by stabilizing integral macromolecules such as proteins (Fig 1.4) (Mundree *et al.*, 2002). There is a vast amount of literature on the stabilizing effects of a range of sugars on biomolecules, cells and organisms, which indicates a functional role of sugars in the stress tolerance of a number of organisms. It is also noteworthy that the natural stress tolerance of any organism cannot be attributed to one single compound and that in addition to sugar synthesis there are a number of other physiological adaptations that have to take place to allow an organism to survive extreme abiotic conditions (Hincha *et al.*, 2006).

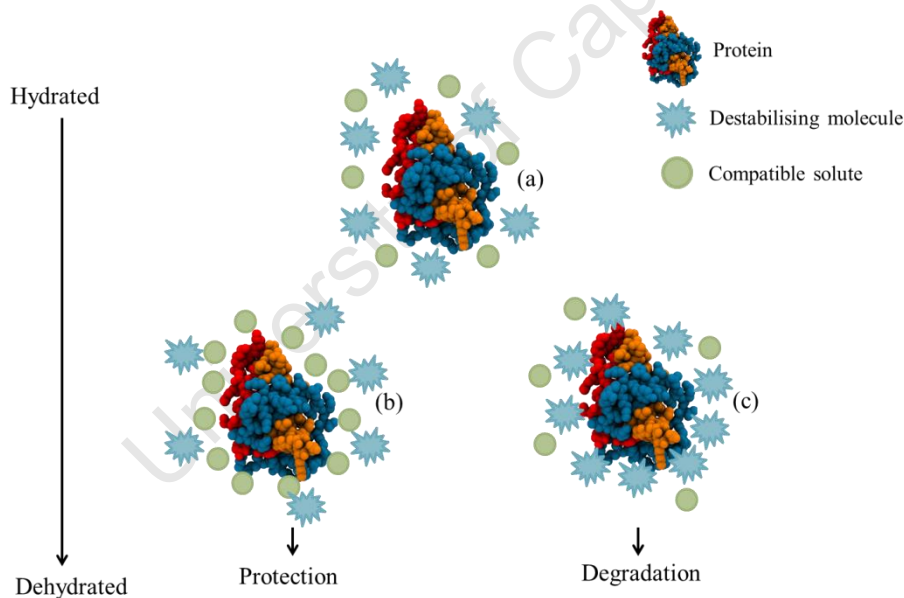


Figure 1.4 Diagram illustrating the function of compatible solutes. a) In the hydrated state, the presence of water reduces the interaction of destabilising molecules. (b) In tolerant cells the synthesis of compatible solutes preferentially excludes the binding of destabilising molecules and stabilises native protein conformation. (c) In sensitive cells the lack of compatible solutes results in the preferential binding of destabilising molecules to the protein surface, leading to degradation (adapted from Mundree *et al.*, 2002).

RFOs have been implicated as protective compounds in the cellular dehydration response, especially within many plant seeds. High chain length RFOs have been shown to afford artificial liposomes with protection against dehydration *in vitro* (Hincha *et al.*, 2003). The accumulation of RFOs in orthodox seeds of different plant species has been implicated in playing a role in development of desiccation tolerance and seed longevity during seed maturation (Black *et al.*, 1996; Koster & Leopold 1988, Blackman *et al.*, 1992).

Raffinose in particular has been associated with greater cold hardiness as it protects biomembranes and proteins acting as a compatible solute. In order for RFOs to be effective as cryoprotectants they need to be mainly located in the cytoplasm (Bachmann *et al.*, 1994). In the frost-hardy evergreen labiate *A. reptans* it was seen that the highest accumulation of RFOs occurred during the winter season, when plants are exposed to the most extreme of low temperatures with the average day temperatures between 0 and -5°C (Peters & Keller, 2009). The onset of low temperature in a number of plants including alfalfa, *Arabidopsis*, cabbage, salt grasses, spruce and the photoautotrophic alga *Chlorella vulgaris* has shown a positive correlation to the increase in mass of its RFOs. These observations strongly infer that RFOs have a functional role in low temperature stress (Peters & Keller, 2009).

1.4.2 The role of RFOs in protection against oxidative stress

An excess in concentration of ROS occurs as a result of a number of environmental factors such as drought, chilling, heat and high-light irradiation. ROS, particularly superoxide and hydroxyl radicals, can cause damage to crucial cellular components such as DNA, proteins and lipids. Plants have therefore evolved complex mechanisms to prevent damage from ROS, ranging from antioxidant enzymes such as superoxide dismutase, catalase and peroxidases to water soluble (eg. ascorbate and glutathione) and lipid soluble (eg. carotenoids and tocopherols) free radical scavengers (Noctor & Foyer, 1998; Mundree *et al.*, 2002; Munne-Bosch & Alegre, 2002; Kranner *et al.*, 2006; Farrant *et al.*, 2007; Nishizawa *et al.*, 2008).

A study was conducted in which the leaves of wild-type *Arabidopsis thaliana* were treated with methyl viologen (MV), a herbicide that inhibits photosynthesis and produces ROS (Nishizawa *et al.*, 2008). It was observed that there was an increase in the transcript levels of a number of galactinol synthase (GolS) and raffinose synthase (RS) isoenzymes, including the total activities of the GolS isoenzymes, and levels of galactinol and raffinose. It was also demonstrated that transgenic plants with high intracellular levels of galactinol and raffinose

were correlated with increased tolerance to MV treatment and salinity or chilling stress (Nishizawa *et al.*, 2008). In this study it was also observed that lipid peroxidation was significantly lower than that of the wild-type plants. The findings of this study suggest a novel function of galactinol and raffinose as having the ability to scavenge hydroxyl radicals and therefore protect plant cells from oxidative damage (MV treatment) (Nishizawa *et al.*, 2008).

1.4.3 RFOs and carbon storage and translocation

Plants possess the inherent ability to store and translocate a portion of its fixed carbon produced during photosynthesis, affording the plants with the flexibility required for growth and survival under a variety of environmental conditions. Plants typically do this by synthesizing starch for storage and translocation of sucrose for metabolic activities and synthesis of integral structural moieties. However, other sources of transportable soluble carbohydrates occur in the plant kingdom, with RFOs being the most prominent, after sucrose (Unda *et al.*, 2012).

In *A. reptans* RFOs function as the main transport, as well as storage carbohydrates. It was demonstrated that *A. reptans* contains two pools of RFOs, a storage pool in the mesophyll and a transport pool in the phloem, with the mesophyll being the primary site of RFO synthesis. RFOs may serve as carbon storage pools in plants which are tolerant to low temperature stress since the enzymes responsible for RFO synthesis are thought to be less sensitive to low temperatures than those which catalyse the formation of starch (Bachmann *et al.*, 1994). In a number of plants, stachyose has been shown to function as a long-distance transporter of carbon skeletons and is symplastically loaded into the phloem (Bachmann & Keller, 1995).

1.4.4 RFOs and OMI metabolism during abiotic stress

Myo-inositol can be seen as a key branching point as it can lead to the galactinol/RFO pathway and/or to *O*-methyl-inositol (OMI). It has been demonstrated that inositols such as pinitol (3-*O*-methyl-*D*-chiroinositol) and ononitol (1*D*-4-*O*-methyl-*myo*-inositol), together with their *O*-methyl ethers, play roles in osmotic regulation in a number of plants (ElSayed *et al.* In Press; Majumder *et al.*, 2010)

In a study conducted on *Coleus blumei* it was demonstrated that these plants accumulate novel carbohydrates, namely high-DP RFOs and OMI, in response to salinity stress (Gilbert

et al., 1997). A reduction in RFO levels was also observed in coleus leaf tissues due to the imposition of drought stress. This reduction in RFO levels may be partially due to the reduction in overall photosynthesis rates in the stressed plants. Reduced enzyme activity of *GolS* was also observed under these stress conditions and this is also expected to lower overall RFO levels. This study also demonstrated the induction of OMI synthesis, which diverts *myo*-inositol away from the RFO biosynthetic pathway, and this may also in part account for the observed reduction in RFO levels under stress (Pattangul & Madore, 1999). It can therefore be said that the conversion of *myo*-inositol to its methyl derivative essentially removes it from the pathway leading to galactinol biosynthesis. This process can be seen in figure 1.5. Therefore, the activation of the *myo*-inositol 6-O-methyl transferase (IMT), together with the observed drought-induced depression of *GolS* activity, would be expected to significantly impact the ability of these leaves to synthesize RFOs by limiting the synthesis of galactinol (Pattangul & Madore, 1999).

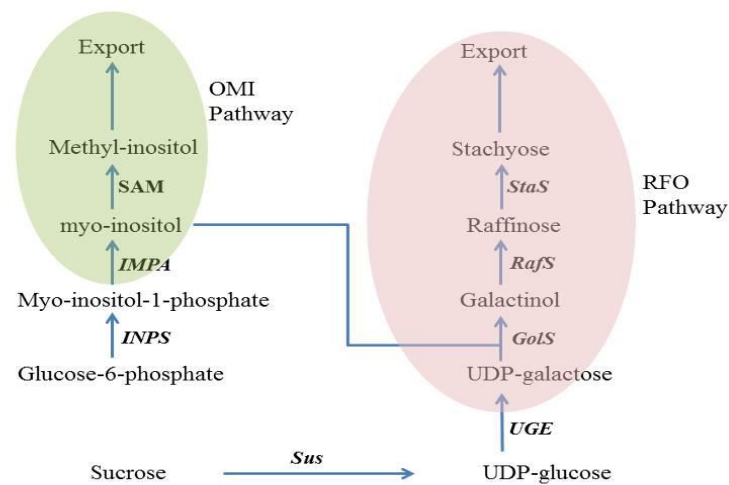


Figure 1.5 Modulation of transcripts encoding enzymes involved in sucrose, methyl-inositol and raffinose metabolism by water stress. Transcripts differentially modulated by water stress are shown in italics. *GolS* galactinol synthase; *IMPA* inositol monophosphatase, *SAM* S-adenosylmethionine, *INPS* *myo*-inositol-1-phosphate synthase; *RafS* raffinose synthase; *Sus* sucrose synthase; *TPP* trehalose-phosphatase; *UGE* UDP-glucose-4-epimerase (adapted from Pattangul & Madore, 1999 and .Unda *et al.*, 2012)

1.4.5 RFOs and the polymer trap model

Phloem loading is the starting point for export of carbohydrates and other nutrients from the leaves of plants. In many plant species phloem loading involves an apoplastic step which is driven by plasma-membrane transporters and energy is supplied by the proton motive force. In other plants phloem loading is a symplastic process, driven by the downhill concentration gradient from the mesophyll to the phloem. In species that load via the symplast, sucrose from the mesophyll diffuses into specialized cells called companion cells (CCs) in the minor veins, termed intermediary cells, and here it is converted to raffinose and stachyose. These RFO molecules are larger than that of sucrose and are therefore unable to diffuse back into the mesophyll through the intermediary cell plasmodesmata. These RFOs thus accumulate in the phloem, in a process known as polymer trapping, to a combine concentration that is similar to that of sucrose in apoplastic loaders. Therefore three strategies for sucrose are recognised: Apoplastic loading; symplastic loading with polymer trapping and symplastic loading without polymer trapping. Species that utilise the polymer trap mechanism are easily identified by the presence of RFOs in leaf extracts and intermediary cells in minor veins (Rennie & Turgeon, 2009; Turgeon *et al.*, 1993).

1.4.6 RFOs and stress-mediated signalling

Abiotic stresses within plants result in major changes in sugar status and in turn affect the expression of a variety of genes by either down- or up-regulation of their expression. Sugars are able to act as primary messengers and regulate signals that in turn regulate the expression of various genes involved in sugar metabolism (Gupta & Kaur, 2005). It was observed in a study conducted on rice (*Oryza sativa* cv Tainan 5) cell cultures that sucrose and glucose exhibit dual functions in gene regulation as illustrated by the up-regulation of growth-related genes and the down-regulation of stress-related genes. It was demonstrated that sugar co-ordinately but differentially activated or repressed gene expression and regulated both the transcription rate and mRNA stability (Ho *et al.*, 2001).

It has also been proposed that *myo*-inositol derived galactinol and associated RFOs are able to act as putative signalling compounds. There is however still debate on the nature and mechanism of action of RFOs in signalling and gene expression (Valluru & Van den Ende, 2011).

1.5 Galactinol synthase (GolS)

As previously mentioned galactinol synthase catalyses the first step in the biosynthesis of RFOs by reversibly synthesizing galactinol from UDP-galactose and *myo*-inositol (Unda *et al.*, 2012, Saravitz *et al.*, 1987).

The *GolS* gene has been identified in a number of different plant species, such as maize (*Zea mays*), tomato (*Solanum lycopersicum*), rice (*Oryza sativus*) and *Arabidopsis thaliana* (Taji *et al.*, 2002; Downie *et al.*, 2003). The full cDNA of a *GolS* gene (*LeGOLS-1*) was isolated from tomato (*Solanum lycopersicum*), together with its genomic sequence. The expressed coding region of the tomato *GolS* possessed the ability to synthesize galactinol from UDP-galactose and *myo*-inositol. Southern hybridization analysis of the *GolS* indicated that a single copy of the gene exists in the genome or may be significantly different from other *GolS* genes found in tomato. It was also found that the tomato *GolS* gene contained 2 introns that were situated in highly conserved regions (Downie *et al.*, 2003). Purification of a GolS protein from mature zucchini squash revealed the enzyme to be 42 kDa with an isoelectric point of 4.1. Analysis of the pH of the enzyme showed that the GolS has a broad pH optima from 7.0 to 8.0 (Smith *et al.*, 1991). In the *Arabidopsis* genome databases at least seven *GolS*-related genes can be found. These were aptly named *AtGolS1*, 2, 3, 4, 5, 6 and 7 (Taji *et al.*, 2002). All reported GolS proteins have a putative serine phosphorylation site at position 263. At the carboxy-terminal end of all known GolS proteins is a characteristic hydrophobic pentapeptide, APSAA.

1.6 GolS and its role in carbon partitioning

Saravitz *et al* studied the galactinol synthase activity and soluble sugars within the developing seeds of four soybean genotypes and reported that the level of GolS activity in the leaf tissue is essential for controlling the partitioning of carbohydrates between sucrose and raffinose saccharides (Saravitz *et al.*, 1987)

The relationship between GolS activity and sugar composition in the leaves and seeds of several crop species was studied (Handley *et al.*, 1983). It was found that there was a positive correlation between GolS activity and the fraction of the total soluble carbohydrate made up of the raffinose saccharides in leaves. A negative correlation was found between the activity of the enzyme and the percentage of sucrose. Galactinol synthase may therefore play a role in the shuttling of carbon to RFOs at the expense of sucrose.

1.7 GolS and the abiotic stress response

Since the only known function of galactinol is in the synthesis of RFOs, galactinol synthesis is likely to be a key regulated step in the biosynthesis of RFOs (Downie *et al.*, 2003). It was therefore speculated that since GolS catalyses this key metabolic step, the gene can be used as an experimental tool for manipulating the level of RFOs in vegetative tissue and to analyse the function of RFOs (Taji *et al.*, 2001).

Taji *et al.* (2002) demonstrated that transgenic *A. thaliana* plants overexpressing a *GolS* galactinol synthase gene, that encodes the first enzyme in the RFO biosynthetic pathway, resulted in the accumulation of raffinose and displayed more desiccation tolerance than the wild type (Taji *et al.*, 2002).

It was further shown that three different *GolS* genes (*AtGolS1*, 2 and 3) within Arabidopsis are differentially induced by drought, low temperature and high salinity. Transgenic Arabidopsis plants overexpressing *AtGolS2* accumulated galactinol and raffinose and also displayed drought-stress tolerance. This study suggested that *AtGolS1* and 2 functions mainly in high-salinity and cold tolerance, whereas *AtGolS3* functions in the tolerance of cold stress (Taji *et al.*, 2002).

It has been demonstrated that GolS mRNA levels increased upon exposure to cold in the vegetative tissues of *A. thaliana*, with transcripts disappearing upon re-exposure to room temperature (Liu *et al.*, 1995). A rice homologue of GolS, belonging to a group of ABA-independent genes, was shown to be induced during short term desiccation stress and was related to acquired chill resistance. In addition to these reports in Arabidopsis, the activity of GolS within kidney bean seeds has also been shown to increase on exposure to cold (Taji *et al.*, 2002; Liu *et al.*, 1995). It can thus be deduced from these observations that GolS is a key metabolic enzyme in directing the biosynthesis of RFOs, RFOs in turn function in water deficit and cold stress (Liu *et al.*, 1995).

A galactinol synthase gene (*XvGolS*) was previously isolated from *Xerophyta viscosa* and functionally expressed. The *XvGolS* transcript levels were shown to increase under water deficit stress, this correlating with an increase in raffinose levels (Peters *et al.*, 2007). These

authors have postulated that this sugar, in conjunction with sucrose, serves both as a storage carbon and stress protectant.

1.8 Transcriptional regulation of *GolS*

Although *GolS* genes are induced by a number of stresses in both stress-sensitive and stress-tolerant plant species the mechanism of transcriptional regulation is not fully established. Transcription factors belonging to different families have been shown to be involved in the desiccation-induced gene regulation leading to the induction of protective mechanisms, inhibition of photosynthesis, and in many cases, growth retardation (Gechev *et al.*, 2012)

A *GolS* gene (*BhGolS1*) was characterized in the resurrection plant *Boea hygrometrica* (Wang *et al.*, 2009). The *BhGolS1* was found to be dehydration and ABA-inducible and conferred dehydration tolerance in a transgenic tobacco system. WRKY genes encode a large group of transcription factors and are defined by a 60-amino acid domain containing the amino acid sequence WRKY (tryptophan-arginine-lysine-tyrosine) at its amino-terminal end. It was demonstrated in this study that a WRKY transcription factor is able to interact via W-boxes, with the *BhGolS1* promoter and activate *BhGolS1* expression.

Heat shock factors (HSFs) are transcriptional regulators of the heat shock response. The major targets of the HSFs are the genes encoding heat shock proteins (HSPs) which are known for their protective functions in counteracting cytotoxic effects. Panikulangara *et al.*, (2004) demonstrated that a novel heat-inducible *GolS* gene (*GolS1*) was HSF-dependently expressed in *Arabidopsis* and the authors concluded that *GolS1* is a true target of HSF regulation confirmed by total transcriptome analysis of HSF knockout mutants. It was also suggested that HSFs are involved in drought- and salinity-induced *GolS1/GolS2* expressions in *Arabidopsis*.

Taji *et al* (2002) demonstrated that a cold-inducible *GolS* gene in *Arabidopsis* (*AtGolS3*) is controlled by the dehydration responsive-element binding factor and the C-repeat binding factor (*DREB1A/CBF*), which are genes that encode key transcription factors involved in cold-responsive gene expression. The *AtGolS3* promoter contains four DRE related motifs and one ABA-responsive element (ABRE). It may be concluded that combinations of these *cis*-acting elements may be involved in cold-specific expression of *AtGolS3* (Taji *et al.*, 2002).

1.9 Aims of this study

The aim of this study was to characterise a second galactinol synthase (*XvGolSII*) from *X.viscosa*. Previous studies (discussed above) have shown that different GolS isoforms occur in plant species and these isoforms respond differently to the same abiotic stress. Given the importance of GolS in the RFO biosynthetic pathway and the role of RFOs in protection against oxidative and osmotic stress, it was necessary that *XvGolSII* be characterised at the molecular level and a possible link to abiotic stress be investigated. To this end, characterisation involved *in silico* analyses, production and purification of recombinant *XvGolSII* protein, GolS enzyme activity assays, subcellular localization studies and the quantification of protein and mRNA expression in response to dehydration and cold stress, respectively.

Chapter 2

Bioinformatics and phylogenetic analysis of *XvGolSII*

2.1 INTRODUCTION

Before proceeding, *in silico* analysis is necessary in order to determine whether *XvGolSII* has characteristic or unique sequences and physiochemical properties at the DNA and protein level, respectively, so as to better inform our experimental approach. Phylogenetic analyses of the sequence information can be used to infer the evolutionary and structural similarity of *XvGolSII* to other *GolS* sequences. Similarly, the use of functional genomic tools to analyse the expression pattern of *GolS* genes from large-data sets obtained from model plants such as *Arabidopsis* may provide useful information with respect to *XvGolSII*. The putative amino acid sequence will also allow determination of whether the *XvGolSII* protein undergoes any post-translational modifications as these may imply regulatory roles.

The full length cDNA sequence of *XvGolSII* was isolated from an *X. viscosa* cold-stress (4°C) cDNA library (Iyer, pers. comm.). All genes in this cDNA library were cloned into the pDNR-Lib vector (Appendix C, Fig. C1).

2.2 MATERIALS AND METHODS

2.2.1 Sequencing analysis of *XvGolSII*

2.2.1.1 Restriction digests of *XvGolSII*

The sequencing of pDNR-Lib::*XvGolSII* was performed via restriction enzyme digestion using *HindIII* and *Sall* restriction enzymes. These enzymes would yield three fragments of *XvGolSII*, free of pDNR-Lib vector sequence.

2.2.1.2 Cloning of *XvGolSII* fragments into pSK (Bluescript)

A double restriction digest of the pSK vector (Appendix C, Fig. C2) was carried out with the *HindIII* and *Sall* enzymes and cleaned up using the Invisorb MSB Spin PCRapace kit (Germany), according to the manufacturer's instructions.

The three *XvGolsII* fragments obtained from the *HindIII* / *Sall* restriction digest of pDNR-Lib::*XvGolsII* were purified from an agarose gel using the Biospin Gel Extraction Kit (Biospin, Japan), according to the manufacturer's instructions. These three fragments were then respectively ligated to the previously prepared pSK vector. These constructs were then transformed into *E. coli* (DH5- α) cells using a standard transformation protocol (Appendix A3).

2.2.1.3 Sequencing analysis

The pSK plasmids containing the respective three *XvGolsII* fragments were sequenced (three clones for each fragment) via the Sequencing Unit (Department of Molecular and Cell Biology, UCT, South Africa). Nucleotide sequence of each fragment of *XvGolsII* was determined using the DYEnamic ET DYE Terminator Cycle sequencing kit from MegaBACE (Molecular Dynamics, USA), according to the manufacturer's instructions. Sequencing reaction products were separated using the MegaBACE 500 Sequence system (Amersham Pharmacia Biotech). Sequences were edited *in silico* using the Chromas v2.13 software package. Assembly of sequences to obtain the full-length *XvGolsII* sequence was carried out using the DNAMAN v5.2.10 (Lynnon BioSoft, 1994-2001).

2.2.2 BLAST and phylogenetic analysis of *XvGolsII*

Amino acid homology searches were carried out using the BLAST algorithms and National Centre for Biotechnology Information (NCBI) databases (Atschul *et al.*, 1997). Fifteen amino acid sequences, including *XvGolsII*, were used to carry out a multiple sequence alignment (Appendix B, Fig. B1) and generate a phylogenetic tree. Both these analyses were conducted in MEGA5 in which ClustalW was used for the multiple sequence alignment (Temura *et al.*, 2011). The evolutionary history was inferred using the Maximum Likelihood method, based on the JTT matrix-based model (Jones *et al.*, 1992). The tree with the highest log likelihood (-2852.9423) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ (neighbor-joining) method with MCL (modular cladistic approach) distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of

substitutions per site (next to the branches). All positions containing gaps and missing data were eliminated. There were a total of 245 positions in the final dataset.

2.2.3 Bioinformatic analysis of *XvGolSII* amino acid sequence

The deduced amino acid sequence of *XvGolSII* was obtained by translation of the cDNA sequence using DNAMAN software v5.2.10 (Lynnon BioSoft Copyright©, 1994-2001). The ProtoParam tool (www.expasy.org) was used to predict physicochemical properties of the *XvGolSII* protein such as its molecular weight, pI, estimated half-life, as well the stability properties of the protein. The solubility of the protein was calculated using the Recombinant Protein Solubility Prediction tool (School of Chemical Engineering and Material Science, University of Oklahoma, <http://biotech.ou.edu/>). The NetPhos2.0 Server (Blom *et al.*, 1999) was used to predict possible phosphorylated amino acids within the *XvGolSII* protein. The computational hydropathy plot of *XvGolSII* was carried out through the Genome Consortium for Active Teaching Server at Davidson College (<http://gcat.davidson.edu/rakarnik/KD.html>). The putative subcellular localization of *XvGolSII* was predicted using the Prediction of Protein Sorting and Localization Sites in Amino Acid Sequences (PSORT) WWW Server.

2.2.4 Expression analysis of *Arabidopsis GolS* genes

In order to gain further insight into the gene expression of *XvGolSII* during abiotic stress the *Arabidopsis* eFP Browser online tool (Winter *et al.*, 2007) was used to interpret and visualize the gene expression of three *Arabidopsis GolS* genes under various abiotic stresses. The eFP Browser online tool uses data from large-scale data sets to generate pictographic representations of the experimental samples used to generate the data sets. This tool has been used to present *Arabidopsis* gene expression data from the AtGenExpress consortium (Kilian *et al.*, 2007).

2.3 RESULTS AND DISCUSSION

2.3.1 Cloning of *XvGolSII* into pSK and sequencing analysis

Sequencing of the full-length *XvGolSII* gene in the pDNR-Lib vector and of the amplified *XvGolSII* PCR product was unsuccessful (data not shown). We therefore performed restriction digestion analyses on pDNR-Lib::*XvGolSII* which indicated that *HindIII* and *Sall* restriction enzymes would yield three *XvGolSII* fragments free of pDNR-Lib vector sequence (data not shown). Each fragment was separately cloned into pSK (Bluescript) and sequenced. The full-length *XvGolSII* sequence was successfully obtained through subsequent sequence assembly using DNAMAN (Figure 2.1).

2.3.2 Bioinformatic analysis of *XvGolSII*

The *XvGolSII* cDNA fragment was shown to be 1434 base pairs (bp) long with the longest open reading frame (ORF) predicted to be 1018 bp, using the DNAMAN software. This ORF encodes a putative protein of 339 amino acids with a molecular weight of 38.7 kDa (Fig. 2.1). The ProtParam tool predicted the *XvGolSII* to be an acidic protein with a pI of 5.03 and therefore carries no electrical charge at this pH. The half-life of the *XvGolSII* protein was predicted to be 10 hours within *E. coli*, *in vivo* and indicates the time it takes for half of the amount of protein in a cell to disappear after its synthesis. The instability index, which estimates the protein stability *in vitro*, was predicted to be 42.43, classifying this protein as unstable. This stability information may be useful in subsequent expression and handling of the protein. The protein was predicted to have a 55.6% chance of solubility when over-expressed in *E. coli*. (Gasteiger *et al.*, 2005)

At the carboxy-terminal end of all known GolS proteins is a characteristic hydrophobic pentapeptide, APSAA (Appendix B, Fig. B1). This can also be seen for *XvGolSII* (Fig. 2.1).

```

1      ATGGCTCCACCAGAGGTGCGCCGGCGCGTTCGACGCAGCCGCCTTCACCGGAAAGCTAGCG
1      M A P P E V A G A V D A A A F T G R L A
61     GTGCCCAACGCCGGTACTCCAAGCGTGCCTACGTGACTTTTCTCGCCGGCAGCGGCGAT
21     V P N A G Y S K R A Y V T F L A G S G D
121    TACTGGAAGGGCGTCTGTTGGGCTAGCCAAGGGGCTGAGGAAGGTAAAGAGCGCGTACCCG
41     Y W K G V V G L A R G L R K V K S A Y P
181    TTGATCGTGGCCATGCTTCCAGATGTTCCCGAGGATCACCGGGAATCCTACGGTCCCAG
61     L I V A M L P D V P E D H R E I L R S Q
241    GGTGTCATCGTCAGGAGATCGAGCCGGTGTACCCGCCGGAGAACCAGATTCAGTTCGCC
81     G C I V R E I E P V Y P P E N Q I Q F A
301    ATGGCTTACTACGTATCAACTACTCCAAGCTTCGCATCTGGAACCTTGAGGAGTACAGC
101    M A Y Y V I N Y S K L R I W N F E E Y S
361    AAGATGATATACCTGGATGCGGATATCCAAGTTTACGACAACATCGACCACCTCTTCGAC
121    K M I Y L D A D I Q V Y D N I D H L F D
421    ATGCCAGACGGGTACTTCTACCGGTGATGGACTGCTTCTGCGAGAAGACATGGAGCCAT
141    M P D G Y F Y A V M D C F C E K T W S H
481    TCGCGCCAGTTTTCCATCGGCTACTGCCAGCAGTGCCTGACAAGGTCAAGTGGCCGGCG
161    S R Q F S I G Y C Q Q C P D K V K W P A
541    GACATGGGCAATCCTCCCGCCTTCTACTTCAATGCAGGGATGTTGTITACGAGCCGAGC
181    D M G N P P A F Y F N A G M F V Y E P S
601    CGCCTCACTTGCATAGCCTCCTTGAGACCCTCCGCGTCAACCACCCACCCCTTCGCG
201    R L T C D S L L E T L R V T P P T P F A
661    GAACAGGATTTCTGAACATGTTTTTTGAGAAGAACTACAAGCCCATCCCTCTTGATTAC
221    E Q D F L N M F F E K N Y K P I P L D Y
721    AATCTTGTTTGGCCATGCTGTGGCGTCATCCCGAGAACGTGACCTTCAAAAAGTCAAAA
241    N L V L A M L W R H P E N V D L Q K V R
781    GTCGTTCACTACTGTGCCGAGGTTCAAAACCATGGAGGTACACAGGAAAAGAAAGCTAAT
261    V V H Y C A A G S K P W R Y T G K E A N
841    ATGGACAGAGAGGACATCAAGATGCTGGTGGCTAAATGGTGGGACGTCTACAACGACGAG
281    M D R E D I K M L V A K W W D V Y N D E
901    TCGCTCGACTTCAAGCCGGAGGATGTAGTCCCGAAGGCGATGCCTTAACGAAGACTCCA
301    S L D F K P E D V V P E G D A L T K T P
961    ATCATGGCAACCTTTCCGGAGCCCGGATTAACCTTTATCCCTGCACCTCTGCAGCCTAG
321    I M A T F P E P A I N F I P A P S A A *

```

Figure 2.1: The deduced XvGolSII amino acid sequence (green) of the longest ORF identified in the cDNA sequence. A conserved hydrophobic pentapeptide (APSAA) can be seen in orange.

Reported GolS proteins display a putative phosphorylation of the serine residue at position 263 (Sprenger and Keller, 2000). According to analysis of putative sites of phosphorylation within the XvGolSII amino acid sequence, using the NetPhos2.0 server, 4 serine (positions 7, 59, 139 and 186), 4 threonine (positions 183, 190, 197 and 255) and 7 tyrosine (positions 6, 11, 84, 99, 104, 213 and 277) residues are potentially phosphorylated during post-translational modification of the protein. These phosphorylation sites are above the threshold value of 0.5 (Fig. 2.2). It is generally accepted that protein phosphorylation and dephosphorylation are involved in the regulation of fundamentally all cellular functions and play roles in signal transduction within the cell (Graves and Krebs, 1999). A number of GolS proteins from various plant species have been shown to have putative serine phosphorylation sites (Taji *et al.*, 2002; Unda *et al.*, 2012; Downie *et al.*, 2003).

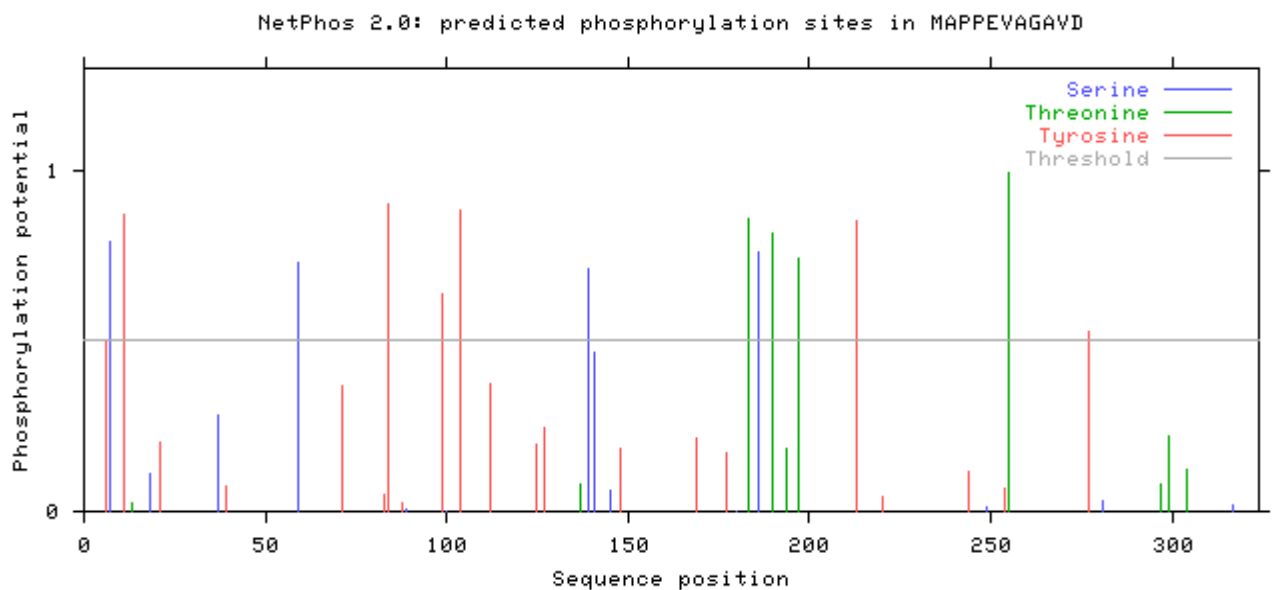


Figure 2.2: NetPhos2.0 depicting possible phosphorylation sites within the XvGolSII amino acid sequence.

A hydropathy plot (Kyte & Doolittle, 1982; window size 9) indicated that XvGolSII is mostly hydrophilic (Fig. 2.3).

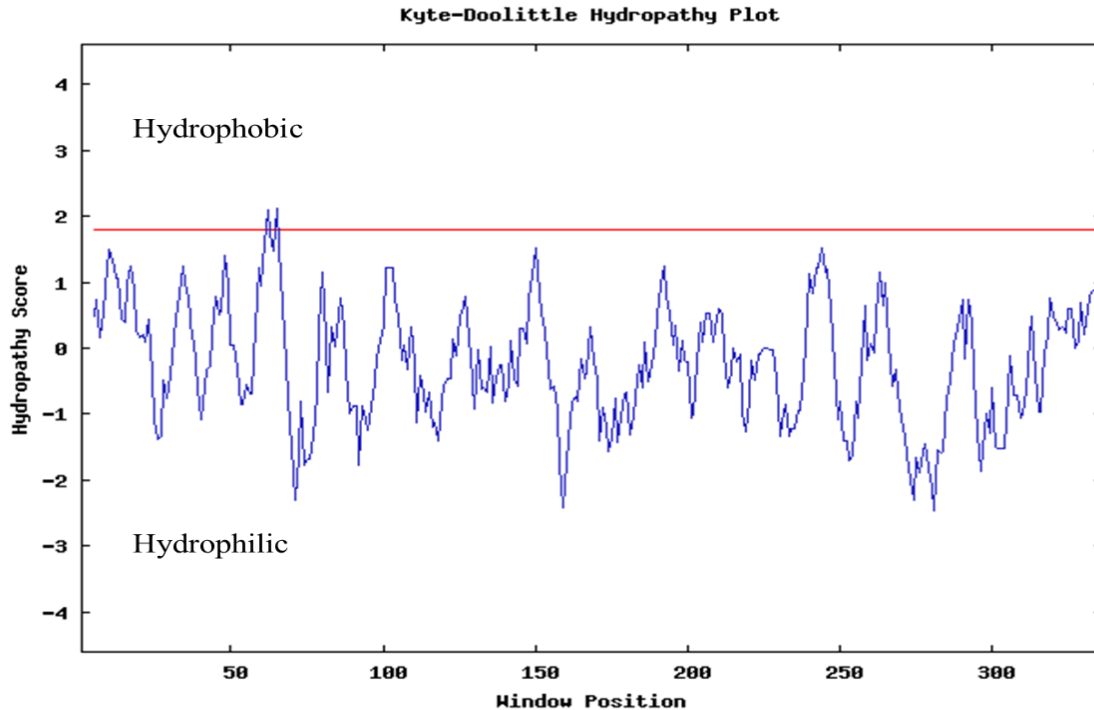


Figure 2.3: A hydrophobicity plot (window size 9) of XvGolSII protein. The red line indicates the threshold value above which peaks are predicted to be transmembrane and below which peaks are predicted to be surface proteins.

Expression of the *Arabidopsis* GolS genes, *AtGolS1*, 2 and 3 under abiotic stress, was analysed using the *Arabidopsis* eFP Browser (Appendix F, Fig. F1, F2 and F3). *AtGolS1* showed slight increases in expression, seen in the leaves under drought stress after 3 hours, increases in expression during cold stress (4°C on crushed ice in cold chamber) was also observed in both the leaves and roots after 24 h. *AtGolS2* showed an increase in expression in response to cold, in both the leaves and roots after 24 h. An increase in expression of *AtGolS2* was seen during osmotic and salt stress, maximally at 3 h for both stresses. *AtGolS3* expression was shown to be primarily up regulated during cold stress, showing maximal up regulation at 24 h of exposure. These results correspond well with those by Taji *et al* (2002) which also investigated the expression of *AtGolS1*, 2 and 3 in response to dehydration and cold stress.

According to the PSORT server it is predicted that XvGolSII is most likely to localise to the cytoplasm (Table 2.1). It was also shown that the protein contains no localisation signals. XvGolSII sub-cellular localization was also compared to galactinol synthase proteins from various plant species as per the PSORT server (Table 2.1). Most GolS proteins, with the

exception of XvGolS and the maize GolS proteins localized to the cytoplasm. Interestingly, the first GolS characterized from *X. viscosa*, XvGolS, localizes to the chloroplast compared to XvGolSII which localizes to the cytoplasm. The localisation of GolS proteins to the cytoplasm has not been experimentally determined; however studies have examined plant tissue specific expression of GolS. Two cold-inducible GolS isoforms, GolS-1 and GolS-2, in *Ajuga reptans* has been shown to be differentially recruited to the mesophyll and phloem cells (Sprenger & Keller, 2000).

Table 2.1: The predicted subcellular localisation of GolS from various plant species, as per the PSORT server. The localisation score is shown in brackets.

Plant species	Gene	Accession number	Subcellular localization
<i>Arabidopsis thaliana</i>	<i>AtGolS1</i>	BAB78530	Cytoplasm (9)
<i>Arabidopsis thaliana</i>	<i>AtGolS2</i>	BAB78531	Cytoplasm (8)
<i>Arabidopsis thaliana</i>	<i>AtGolS3</i>	BAB78532	Cytoplasm (8)
<i>Xerophyta viscosa</i>	<i>XvGolS</i>	ABK27907	Chloroplast (8)
<i>Xerophyta viscosa</i>	<i>XvGolSII</i>	-	Cytoplasm (11)
<i>Solanum lycopersicum</i>	<i>LeGolS1</i>	AAO72744	Cytoplasm (10)
<i>Zea mays</i>	<i>ZmGolS1</i>	AAQ07248	Chloroplast (11)
<i>Zea mays</i>	<i>ZmGolS2</i>	AAQ07249	Chloroplast (14)
<i>Coffea arabica</i>	<i>CaGolS3</i>	ADM92590	Cytoplasm (5)
<i>Brassica napus</i>	<i>BnGolS1</i>	ADG03603	Cytoplasm (9)
<i>Gossypium hirsutum</i>	<i>GhGolS1</i>	AFG26331	Cytoplasm (7)

2.3.3 BLAST and Phylogenetic analysis of *XvGolSII*

From the BLAST search fourteen GolS amino acid sequences were chosen with varying percentage identities to that of XvGolSII. The percentage identity of XvGolSII with galactinol synthases from other plant species can be seen in figure 2.4. These sequences were aligned and can be seen in Appendix B (Fig. B1, 2 and 3).

Table 2.2: GolS protein sequence identities from various plant species as compared to XvGolSII.

Plant species	Accession number	Query coverage	Max. identity	E-value
		(%)	(%)	
<i>Coffea arabica</i>	ADM92588	99	84	0.00
<i>Coptis japonica</i>	BAF99254	99	81	0.00
<i>Populus trichocarpa</i>	AEJ87262	99	83	0.00
<i>Verbascum phoeniceum</i>	ABQ12640	100	80	0.00
<i>Arabidopsis thaliana</i>	BAE99313	97	81	0.00
<i>Capsicum annuum</i>	ABQ44212	98	77	2e-179
<i>Brassica napus</i>	ADG03603	98	76	2e-179
<i>Xerophyta viscosa</i>	ABK27907	97	74	8e-175
<i>Vitis vinifera</i>	CAN79630	99	74	1e-174
<i>Zea mays</i>	ACF88041	98	74	4e-174
<i>Medicago sativa</i>	AAM97493	98	72	3e-164
<i>Solanum tuberosum</i>	ADW78849	98	71	3e-179
<i>Oryza sativa</i>	NP_001049939	97	77	4e-178
<i>Cucumis melo</i>	AAL78686	98	76	2e-176

The protein sequences for the different plant sequences were obtained from a sequence homology search carried out using BLAST algorithms against XvGolSII. Accession numbers, coverage of query sequence, percentage identity and E-value are indicated.

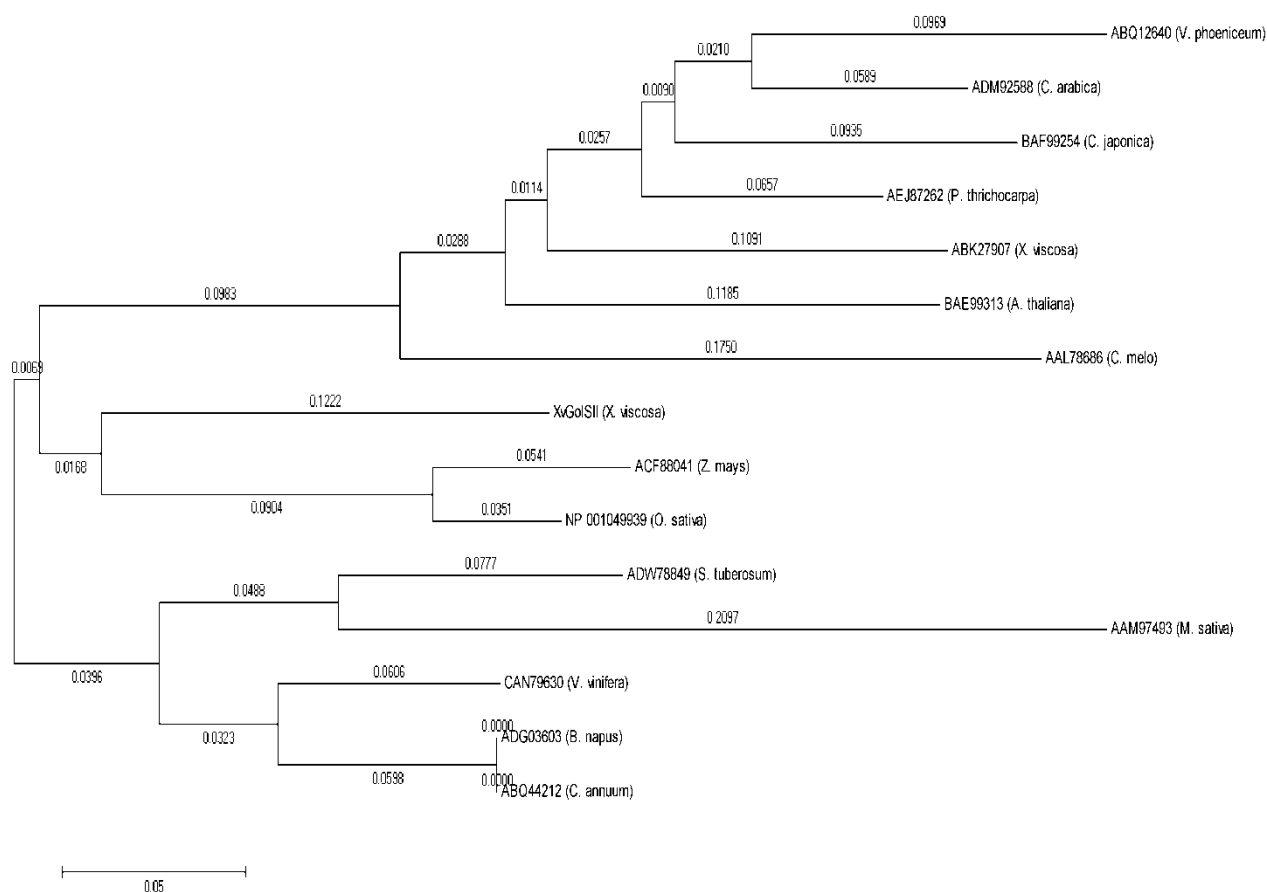


Figure 2.4: The molecular phylogenetic analysis of GolS from various plant species as determined by the Maximum Likelihood method (Tamura *et al.*, 2011; Jones *et al.*, 1992). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 15 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 245 positions in the final dataset.

XvGolSII shows high sequence similarity to a number of different galactinol synthases within various plants including coffee (*Coffea arabica*), mullein (*Coptis japonica*) and other flowering plants such as *Arabidopsis thaliana*. These results also indicate that the *X. viscosa* GolS (*XvGolS*) protein differs significantly from that of *XvGolSII* (Fig 2.1), suggesting homologues of GolS within *X. viscosa*. A phylogenetic tree with no E-value discrimination can be seen in Appendix B, Fig. B4.

2.4 CONCLUSION

In this chapter, the *X. viscosa* galactinol synthase gene, *XvGolSII* was successfully isolated, cloned and sequenced. Subsequent to successful sequencing of *XvGolSII*, *in silico* analyses were carried out. Sequencing analysis revealed that *XvGolSII* is 1434 bp in size with an open reading frame (ORF) of 1018 bp which codes for a protein of 339 amino acids. It was shown that the amino acid composition of *XvGolSII* is similar to a number of GolS proteins found in a number of other plant species such as *A. thaliana*, *O. sativa* and *Z. mays* and contained the characteristic GolS hydrophobic pentapeptide, APSAA. No signal peptides were identified within the *XvGolSII* sequence and the protein was predicted to localize to the cytoplasm in contrast to *XvGolS* which was predicted to localize to the chloroplast. Phylogenetic analysis also revealed that *XvGolSII* is significantly dissimilar from *XvGolS*. *In silico* analysis revealed 15 potential sites of phosphorylation of *XvGolSII* which suggests possible post-translational modification of the enzyme. In the next chapter, we will experimentally determine whether the *XvGolSII* protein is catalytically active and able to catalyze the conversion of *myo*-inositol and UDP-galactose to galactinol.

Chapter 3

Expression of XvGolSII recombinant protein

3.1 INTRODUCTION

For proteins to be used for biochemical analysis, therapeutics or structural studies, three factors regarding their production need to be successful: expression, solubility and purification. These factors need to be optimized to produce sufficient quantities of purified protein. A common problem of high level expression of recombinant proteins in bacteria such as *Escherichia coli* is the formation of insoluble protein aggregates known as inclusion bodies. This aggregation of insoluble protein is in broad terms due to a kinetic competition between protein folding and aggregation (Klein and Dhurjani, 1995).

The choice of protein expression vector is important as specific vectors have affinity tags which are able to enhance the solubility of some of the expressed recombinant proteins to which they are attached. An example of this is the pET29b(+) expression system (Novagen, USA), where the expressed recombinant protein contains a hexahistidine (His₆) fusion tag that can bind to nickel-nitrilotriacetic acid (Ni-NTA) resin matrix, making purification of the desired protein easy. The T7 promoter and the lacI gene facilitate inducible expression of the cloned gene with isopropyl β -D-thiogalactopyranoside (IPTG). The His₆ tag enables the fusion partner to maintain its solubilizing functionality and also double as an affinity tag (Esposito and Chatterjee, 2006; Thomas and Baneyx, 1996).

The aim of this chapter was to obtain soluble purified XvGolSII recombinant protein using the pET29b(+) vector system (Novagen, USA) after expression in *E. coli* BL21(DE3). The purified recombinant XvGolSII protein was used to assess galactinol synthase enzyme activity in converting the substrates UDP-galactose and *myo*-inositol to galactinol.

3.2 MATERIALS AND METHODS

3.2.1 Expression of XvGolSII protein in pET29b

The pET29b(+) expression system (Novagen, USA) was used to express recombinant XvGolSII-protein in *E. coli*-BL21(DE3). The XvGolSII gene was cloned into the multiple cloning site of pET29b(+) at restriction sites *Eco*RI (5' end) and *Sac*I (3' end).

3.2.1.1 Subcloning of *XvGolsII* into pET29b

Plasmid DNA mini-preparations were obtained from *E. coli* cells (DH5- α), that were previously transformed with the construct pDNR-LIB::*XvGolsII* and grown overnight at 37°C. The *XvGolsII* primers (Appendix B, Table B1) were designed with *Eco*RI and *Sac*I restriction sites so as to allow cloning in-frame into the pET29b(+) expression vector. The PCR conditions were as follows: 94°C for 1 min; 2 cycles of (94°C for 30 s, 52°C for 40 s, 72°C for 90 s); 2 cycles of (94°C for 30 s, 56°C for 40 s, 72°C for 90 s); 26 cycles for (30 s, 56°C for 40 s, 72°C for 90 s); and a final extension step at 72°C for 7 min.

Aliquots of both the pET29b(+) vector (2 μ g) and *XvGolsII* fragment (1 μ g) were digested with the restriction endonucleases *Eco*RI and *Sac*I and purified using the EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Canada). A ligation was subsequently performed to obtain the pET29b(+):*XvGolsII* construct. This construct was then transformed into *E. coli* (DH5- α) cells using a standard transformation protocol (Appendix A3). Putative transformants were screened using the aforementioned *XvGolsII* primers. Plasmid DNA preparations were prepared from colonies yielding the desired size product from these PCR reactions. Colony PCR and restriction digests were performed on these constructs to verify successful ligation of *XvGolsII* into the pET29b(+) vector. Further confirmation that the cloning was successful and the *XvGolsII* gene was in the correct reading frame was obtained by sequencing the constructs using the S-tag forward and the T7 terminator reverse primers (Appendix B). The DNA Sequencing Unit (Department of Molecular and Cell Biology, UCT, South Africa) performed nucleotide sequencing using the DYEnamic ET DYE Terminator Cycle sequencing Kit from MegaBACE (Molecular Dynamics, USA) according to manufacturer's instructions. Sequencing reaction products were separated using MegaBACE 500 Sequence system (Amersham Pharmacia Biotech).

3.2.1.2 Expression of recombinant XvGolSII protein

3.2.1.2.1 Expression of recombinant XvGolSII protein using Overnight Express™ Instant TB Medium

E. coli BL21(DE3) cells were transformed with the pET29b(+):*XvGolSII* construct. A single colony was inoculated into 5ml of Overnight Express™ Instant TB Medium supplemented with kanamycin (50µg/ml) and incubated at 30°C overnight, shaking. This was used as an inoculum for 30ml Overnight Express™ Instant TB Medium supplemented with kanamycin (50µg/ml) and incubated at 30°C overnight, shaking. A negative control representing BL21(DE3) cells transformed with only pET29b(+) was treated in the same manner as described above.

3.2.1.2.2 Expression of XvGolSII protein using IPTG induction

E. coli BL21(DE3) cells were transformed with the pET29b(+):*XvGolSII* construct. As a pilot expression, 10ml of LB broth (1% tryptone, 0.5% yeast extract, 0.5% NaCl) supplemented with 50µg/ml kanamycin, was inoculated with 500µl of the overnight transformation and incubated at 37°C, shaking for approximately 2 hours. When the culture had reached an OD₆₀₀ of 0.5, it was split into two 5ml cultures, to one of which IPTG was added to a final concentration of 1mM to induce overexpression of the XvGolSII protein and incubated with vigorous shaking at 30°C. The remaining 5ml LB broth was left uninduced. A 500µl aliquot was removed from the respective cultures (induced and uninduced) and centrifuged in an Eppendorf for 30 seconds at 10 000 x g and the supernatant aspirated. The first aliquots removed were the time zero samples and thereafter time points 1, 2, 3, 4 hours and overnight were removed. Pellets were stored at -20°C until further analysis. The optimum expression time was determined through SDS-PAGE analysis (Appendix A7).

For the large scale induction, 100 ml of an overnight LB-kanamycin culture (pET29b(+):*XvGolSII*) was inoculated into 1L LB-kanamycin broth and incubated with vigorous shaking at 30°C until an OD₆₀₀ of between 0.4 and 0.6 was reached. At this OD₆₀₀ reading the culture was induced by addition of IPTG (1mM final concentration) and incubated for the determined optimal time (overnight) with vigorous shaking at 30°C. The culture was aliquoted into 4 GSA centrifuge tubes and the cells harvested by centrifugation at 3000 x g for 10 minutes at 4°C. Cell pellets were stored at -20°C or at -80°C for longer

periods. Frozen cell pellets were thawed on ice for 30 – 45 minutes and resuspended through gentle pipetting, in an initial amount of 1 – 2 ml lysis buffer (50 mM NaH₂PO₄; 300 mM NaCl; 10 mM imidazole; pH 8.0), ensuring a homogenous solution was obtained. Depending on the size and viscosity of the pellet, 5 – 10 ml of lysis buffer and a small amount of lysozyme (on the tip of a spatula) was added to the solution. Tubes were placed on ice and allowed to shake for 1 hour at 4°C to facilitate cell lysis. Five milliliter aliquots of the cell lysate were sonicated in glass bottles in 5 bursts of 30 seconds each with intermittent 20 second breaks using the Virsonic Ultrasonic Cell Disrupter. The resulting lysate was centrifuged at 10 000 x g for 30 minutes at 4°C. The supernatant was retained and stored at -20°C.

3.2.2 Protein purification

3.2.2.1 Small scale purification of recombinant XvGolSII protein

A small scale purification of the XvGolSII protein was performed according to the protocol outlined in the HIS-Select® Nickel Affinity Gel technical guide (Sigma, USA). Twenty five to 50 µl of the HIS-Select nickel affinity gel (Sigma, USA) was added to an Eppendorf tube which was centrifuged for 30 s at 5000 x g. The supernatant was carefully removed and discarded. Two hundred microlitres of equilibration buffer without imidazole (50 mM sodium phosphate, pH 8.0; 0.3 M NaCl) was added to the gel and mixed well. The gel/buffer mixture was centrifuged for 30 s at 5000 x g and the supernatant removed and discarded. One hundred microlitres of protein lysate (section 3.2.1.2.2) was added to the gel and gently mixed for 1 minute. The mixture was centrifuged for 30 s at 5000 x g and the supernatant saved. The gel was washed twice with 500 µl wash buffer without imidazole (50 mM sodium phosphate, pH 8.0; 0.3 M NaCl) by gently mixing for 10 s and the mix was centrifuged for 30 s at 5000 x g. The wash buffer (supernatant) was retained for analysis. Fifty microlitres of elution buffer (50 mM sodium phosphate, pH 8.0; 0.3 M NaCl; 250 mM imidazole) was added to the gel and mixed well. The mixture was centrifuged for 30 s at 5000 x g to elute the XvGolSII protein. All the fractions were analysed through SDS-PAGE electrophoresis.

3.2.2.2 Large scale purification of XvGolSII protein using the Ni-NTA column

Purification of XvGolSII protein was adapted from the QIAexpressionist™ handbook (Qiagen). Recombinant XvGolSII protein was purified using a borosilicate glass liquid chromatography column of 1 cm in diameter and 20 cm in length (Sigma, USA). One milliliter of nickel-nitrilotriacetic acid (Ni-NTA) resin was added to the column and allowed to settle. The resin was washed with 5 ml of wash buffer 1 (50 mM NaH₂PO₄; 300 mM NaCl; 20 mM imidazole, pH 8.0). The crude protein extract was added to the column, allowing the recombinant XvGolSII to bind to the Ni-NTA resin and the flow-through was collected. The resin was washed with 10 ml wash buffer 1 and buffer 2 (50 mM NaH₂PO₄; 300 mM NaCl; 20 mM imidazole; 5% glycerol, pH 8.0), respectively, and the subsequent flow-through collected. The recombinant protein was eluted with 10 ml elution buffer (50 mM NaH₂PO₄; 300 mM NaCl; 250 mM imidazole; 0.05% Tween-20, pH 8.0) and collected in 1 ml fractions. Recombinant protein was stored at -20°C.

3.2.2.3 Purification of XvGolSII protein using high performance liquid chromatography (HPLC)

Purification of recombinant XvGolSII protein was achieved using a Waters Delta Prep 3000 HPLC machine (Waters Chromatography Division, Milford, MA), making use of a 321 HPLC pump (Gilson, Inc., USA). The HisTrap™ HP 5 ml column (GE Healthcare) was used to purify XvGolS protein on the basis of the hexa-histidine attached to the carboxy-terminus of the recombinant protein. Fifteen milliliters of crude protein extract was passed through a 0.22 µm filter to remove all particulate matter and loaded onto the column. A buffer gradient was created using a low (50 mM sodium phosphate, pH 8.0; 0.5 M NaCl; 20 mM imidazole, pH 8.0) and high imidazole phosphate buffer (50 mM sodium phosphate, pH 8.0; 0.5 M NaCl; 500 mM imidazole, pH 8.0), over a 50 minute time course. Spectrophotometric readings were taken using a Waters 484 Tunable Absorbance Detector. Fractions were collected using an FC 204 Fraction Collector (Gilson, Inc., USA), which were eluted at a flow-rate of 3 ml/min.

3.2.2.4 Concentrating of recombinant XvGolSII protein

Fractions 5 to 16 obtained from HPLC purification was pooled and concentrated using an Amicon® Ultra-15 Centrifugal Filter Device (Millipore Corporation, USA) with a molecular weight cut-off of 30 kDa, according to the manufacturer's instructions. For subsequent antibody generation, the imidazole buffer was replaced with a 1 x PBS buffer (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 1.76 mM KH₂PO₄, pH 7.4). The protein sample was concentrated down to 3 ml of sample. The protein concentration was determined using the Bradford method (Bradford, 1976, Appendix A8).

3.2.3 Western blot analysis of recombinant protein

3.2.3.1 Western blotting

Samples obtained from purification of recombinant XvGolSII protein using the NI-NTA column was run on an SDS-PAGE gel. This gel was then placed in transfer buffer (25 mM Tris; 150 mM glycine; 10% methanol) for 10 minutes. The mini-PROTEAN electrophoresis system (Bio-Rad, USA) was used for the blotting procedure. The proteins were transferred onto nitrocellulose membranes (Osmonics Nitrobind, Krackeler Scientific, Inc., USA). The filter paper, fibre pads and membrane were soaked in transfer buffer. Avoiding air bubbles, the fibre pads were placed on the cathode module of the electrophoresis system, followed by 2 sheets of filter paper, the gel, the membrane, 2 sheets of filter paper, and finally another fibre pad. Transfer of protein on to the membrane was done overnight at 4°C at 40 mA.

3.2.3.2 Chromogenic detection of XvGolSII using anti-His tag antibody

Upon completion of the transfer the membrane was incubated in Ponceau S stain for 2 minutes, with gentle agitation to ensure transfer of the protein and visualize equal loading. The membrane was destained with distilled water until the bands were visible. The membrane was washed with TBS buffer (10 mM Tris-Cl, pH 7.5; 150 mM NaCl) for 10 minutes. The membrane was subsequently incubated in blocking buffer (10% (w/v) skim milk powder in TBS buffer) for 1 hour at RT, with gentle agitation and washed twice for 10 minutes with TBS-Tween buffer (20 mM Tris-Cl, pH 7.5; 500 mM NaCl; 0.05% (v/v) Tween-20). The membrane was once again washed for 10 minutes with TBS buffer at RT. The membrane was

incubated for 2 hours in primary antibody [1:1000 dilution of anti-His tag antibody (Cell Signaling Technology, Inc.)] in blocking buffer at RT, thereafter washed twice for 10 minutes in TBS-Tween buffer at RT, with shaking. The membrane was stained with alkaline phosphatase solution [one tablet of NBT/BCIP ready to use tablets dissolved in 10 ml water (Roche, Germany)] until a visible signal was evident. The reaction was terminated by washing the membrane twice with distilled water. The membrane was photographed immediately.

3.2.4 Galactinol synthase activity assay

The aim of the assays was to determine whether the recombinant XvGolSII protein could catalyze the formation of galactinol from the substrates UDP-galactose and *myo*-inositol.

3.2.4.1 Method A: non-enzymatic hydrolysis

This method was adapted from Peters *et al.* (2007). Aliquots of recombinant XvGolSII (20 μ M) were assayed for activity in a final volume of 40 μ l assay buffer (50 mM Hepes/KOH, pH 7.4; 2 mM MnCl₂; 10 mM DTT) containing final concentrations of 50 mM *myo*-inositol and 5 mM UDP-galactose. All assay reactions were carried out in triplicate (Table 3.1). Samples were incubated at 30°C for 30 min. The reactions were stopped by immersing the tubes in boiling water for 2 min.

Table 3.1 Galactinol synthase activity assay reactions, including test reactions and controls, showing reaction components of each.

Tube	Components
Test reaction	- XvGolSII protein - <i>myo</i> -inositol - UDP-galactose
Control 1	- XvGolSII protein - <i>myo</i> -inositol
Control 2	- XvGolSII protein - UDP-galactose
Control 3	- <i>myo</i> -inositol - UDP-galactose

XvGolSII activity assay reactions were analysed by high-performance anion-exchange chromatography (HPAEC), with pulsed amperometric detection (PAD). Carbohydrate profiling was achieved using a Dionex BioLC ion chromatograph system equipped with an AS50 autosampler, an ED50 electrochemical detector, an LC30 oven and a GS50 pump. The detector cell incorporated a gold working electrode and a pH Ag/AgCl reference electrode. Chromatographic data were recorded on a personal computer equipped with Chromeleon software.

Profiling of galactinol, myo-inositol and UDP-galactose (Sigma-Aldrich, USA) was achieved using a CarboPac MA1 column (250 mm × 4 mm) coupled with a CarboPac MA1 guard column. Isocratic elution was performed with 480 mM NaOH at a flow rate of 0.4ml/min and injection volume of 20ul. Amounts were determined from peak areas calculated from galactinol and myo-inositol standard curves. A test reaction gave an average of 1.86 mg/ml galactinol in original volume (calculated after dilution).

The following standards were run at a concentration of 5 µg/ml: galactinol; *myo*-inositol; UDP-galactose; and UDP. Test samples and controls 1 and 3 were diluted by the addition of 5ml of MilliQ water, and control 2 with 0.5 ml MilliQ water and used as is.

3.2.4.2 Method B: Enzymatic hydrolysis

Fractions containing galactinol, which was synthesized by the recombinant XvGolSII, were collected after separation on a BC-100 column. After separation on the column, NaOH was added to the column. These samples were concentrated in a vacuum concentrator centrifuge and resuspended in 50 µl McIlvaine buffer (48.5 mM citric acid, 103 mM disodium phosphate, pH 5.0). Enzymatic hydrolysis of galactinol was achieved via *Aspergillus niger* α-galactosidase (Megazyme International Ltd, Bray, Wicklow, Ireland) which was added to a final concentration of 2 U per 50 µl reaction volume and this was then incubated for 1.5 hr at 40 °C. Samples were then desalted and analysed by HPLC PAD, using the Benson BC-100 column (Peters *et al.*, 2007).

3.3 RESULTS AND DISCUSSION

3.3.1 Subcloning of *XvGolsII* into pET29b(+)

Analysis through colony PCR (Fig. 3.1) and restriction digests with *SacI* and *EcoRI* (Fig 3.2) confirmed that *XvGolsII* was successfully cloned into pET29b(+). Five distinct bands (lanes 1, 2, 3, 4 and 5) were seen at the expected molecular weight of approximately 1kb (Fig. 3.1 and 3.2, respectively). No band was seen in the no template control (lane 6), as expected. Sequence analysis of the constructs corroborated these results and confirmed that *XvGolsII* was cloned in-frame with no, or silent mutations (data not shown).

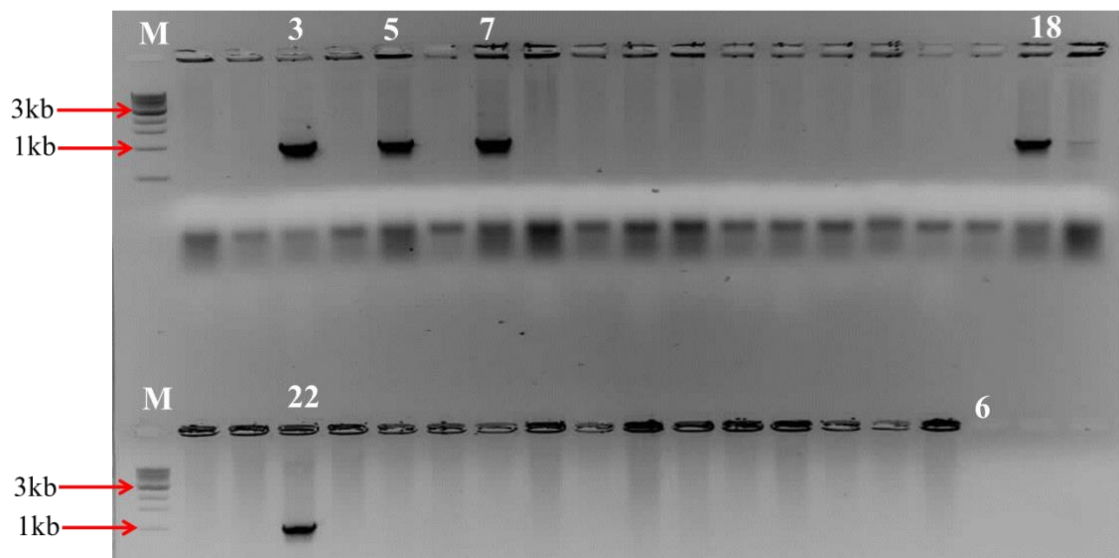


Figure 3.1: 1% TBE agarose/EtBr gel showing colony PCR products of the pET29(+):*XvGolsII* constructs to confirm successful cloning. Lanes: (M) 1kb molecular weight marker; (1, 2, 3, 4, 5) individual colonies that were screened, showing successful cloning of *XvGolsII* into pET29b(+); (6) no template control. The unnumbered lanes indicate no amplification of the product.

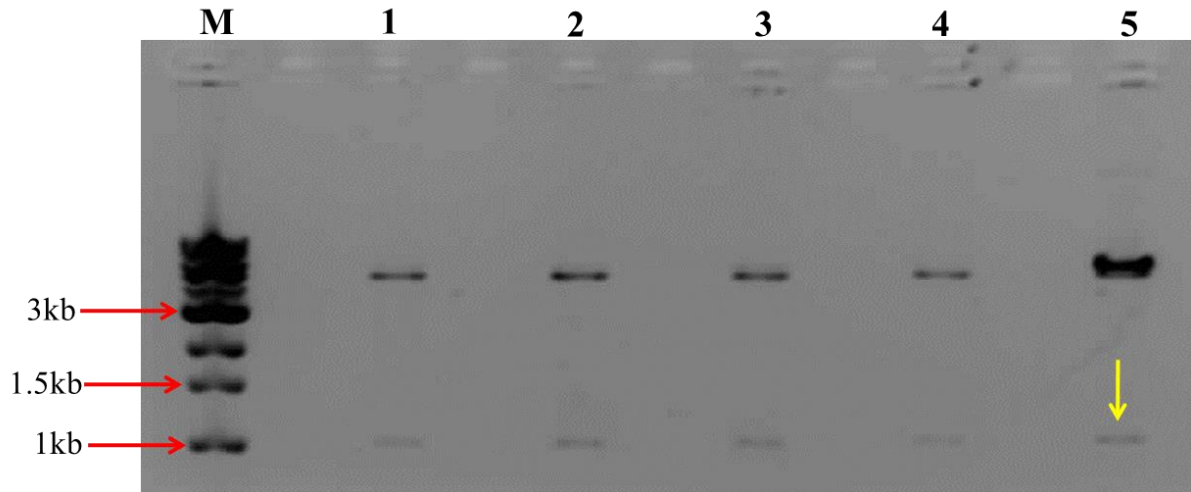


Figure 3.2: 1% TBE agarose/EtBr gel showing restriction digest of pET29b(+):*XvGolSII* isolated from 5 individual colonies. Constructs were digested with *SacI* and *EcoRI*. Lanes: (M) 1kb molecular weight marker; (1, 2, 3, 4, 5) constructs isolated from colonies 3, 5, 7, 18 and 22, respectively. Yellow arrow indicates the 1kb *XvGolSII* released from each construct.

3.3.2 pET29b(+) expression of recombinant XvGolSII protein

3.3.2.1 Expression and small-scale purification of XvGolSII protein

Subsequent to a small scale purification it was evident that a good yield of XvGolSII protein was achieved using the Overnight Express™ technique (Fig. 3.3). *In silico* analysis of XvGolSII predicted a protein with a molecular mass of 38 kDa. This size is similar to GolS that was purified from zucchini leaf and kidney bean respectively (Lui *et al.*, 1995). However, we observed a distinct band at approximately 45 kDa (Fig. 3.3). This additional mass can be attributed to the presence of an s-tag, a hexa-histidine tag and additional translated pET vector sequence. These contribute an approximate mass of 5.72 kDa. This brings the total mass of the recombinant protein to approximately 45 kDa.

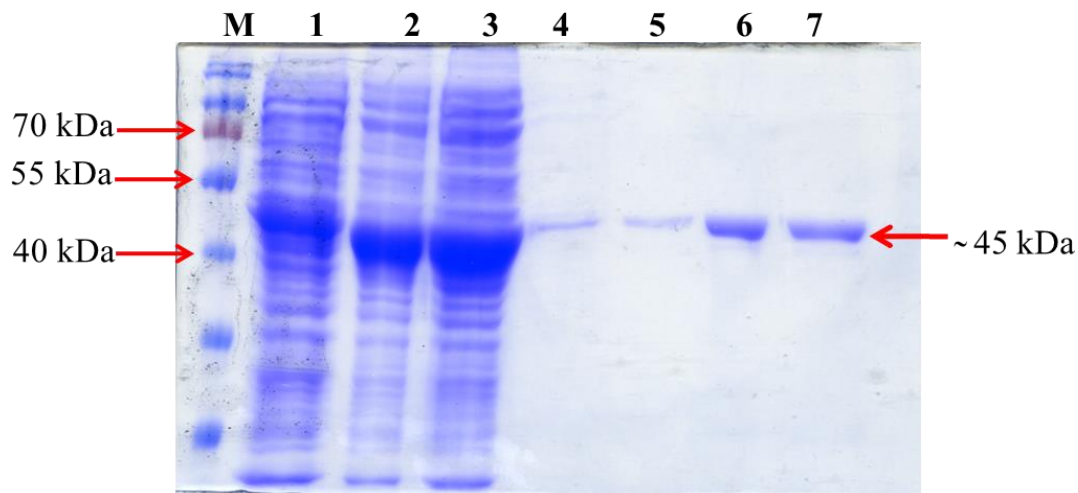


Figure 3.3: 12% SDS-PAGE of protein samples from *E.coli* BL21 (pET29b(+):XvGolSII), colony 3, following small scale purification of the protein synthesized using Overnight Express™. Lanes: (M) Fermentas pre-stained protein ladder; (1) pET29b(+) only (no insert control); (2) crude extract (clear lysate); (3) protein flow-through; (4) wash 1; (5) wash 2; (6) eluent 1; (7) eluent 2. The gel was stained with Coomassie blue.

The XvGolSII protein was concentrated using the Amicon® Ultra-15 centrifugal filter (Millipore Corporation, USA) with a molecular weight cut of (MWCO) of 30 kDa and quantified using the NanoDrop® ND-1000 spectrophotometer (Thermo Scientific, USA).

3.3.2.2 Large scale purification of XvGolSII protein using the Ni-NTA column

Analysis of the XvGolSII protein samples obtained from purification using the Ni-NTA column showed successful purification of the XvGolSII protein, isolated from three separate *E. coli* colonies (colonies 3, 5 and 7). However, after protein quantification using the Bradford method (reaction setup can be seen in Appendix A8), it was seen that the levels of XvGolSII protein obtained was relatively low (data not shown).

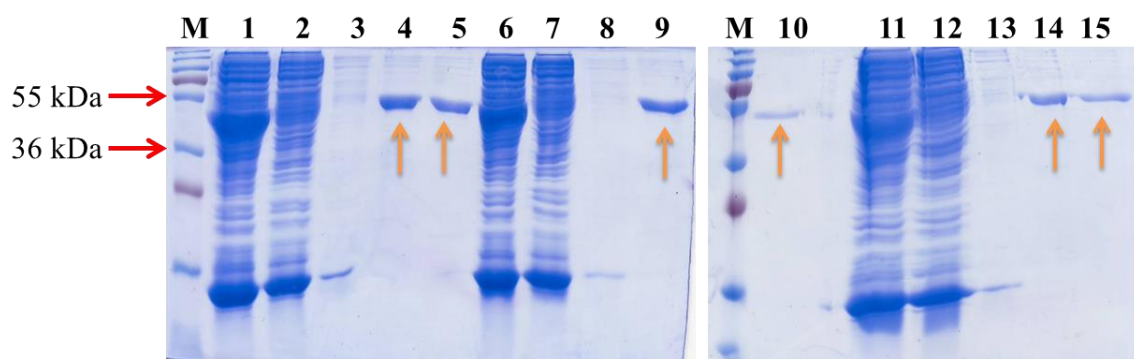


Figure 3.4: 12% SDS-PAGE of protein samples from *E.coli* BL21 (pET29b(+):*XvGolSII*), colonies 3, 5 and 7, following large scale purification of the protein using the Ni-NTA column, synthesized using Overnight Express™. Lanes: (M) Fermentas pre-stained protein ladder; (1) col 3 crude extract; (2) col 3 flow-through; (3) col 3 wash 1; (4) col 3 eluent 1; (5) col 3 eluent 2; (6) col 5 crude extract; (7) col 5 flow-through; (8) col 5 wash 1; (9) col 5 eluent 1; (10) col 5 eluent 2; (11) col 7 crude extract; (12) col 7 flow-through; (13) col 7 wash 1; (14) col 7 eluent 1; (15) col 7 eluent 2. The gel was stained with Coomassie blue.

3.3.2.3 Expression of XvGolSII protein using IPTG induction

Due to the low levels of XvGolSII protein obtained using the Overnight Express™ expression after concentration, IPTG induced expression of the protein was employed. IPTG induction of expression of *XvGolSII* was successful as shown by distinct bands at approximately 45 kDa in Figure 3.5 (A, lanes 4, 6 and 8; B, lanes 2 and 4). This corresponds well with the band seen for the Overnight Express™ expression of the XvGolSII protein (Fig. 3.4). It is evident from Figure 3.5, that the level of expression of the protein increased with increased periods of incubation, with maximal expression observed when cells were incubated overnight.

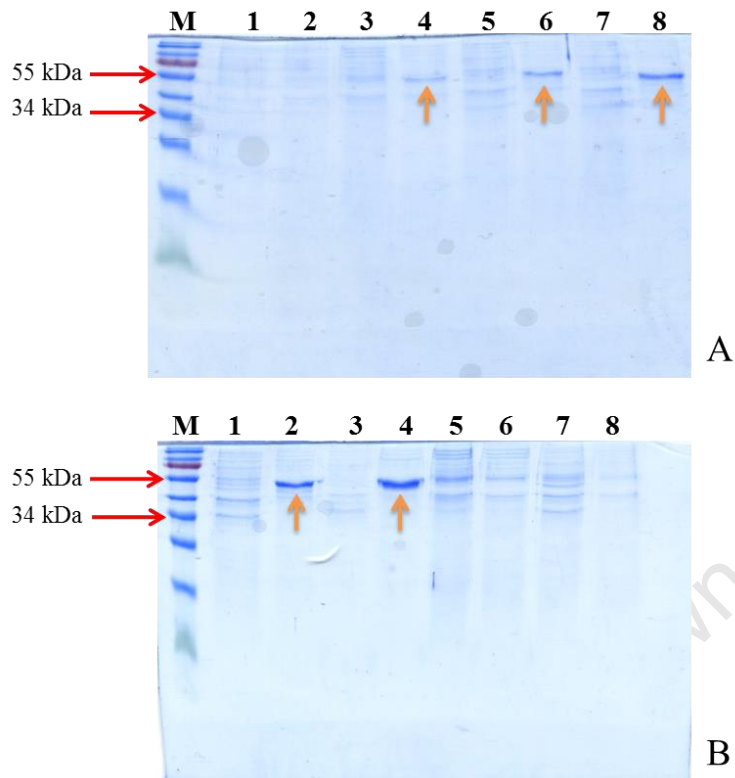


Figure 3.5: 12% SDS-PAGE electrophoresis of protein samples from *E. coli* BL21 (pET29b(+):*XvGolSII*), colony 5, pilot IPTG-induced expression of XvGolSII protein. Lanes: **Gel A:** (M) Prestained protein marker; (1) uninduced T0; (2) induced T0; (3) uninduced T1; (4) induced T1; (5) uninduced T2; (6) induced T2; (7) uninduced T3; (8) induced T3. **Gel B:** (M) Prestained protein marker; (1) uninduced T4; (2) induced T4; (3) uninduced TO/N; (4) induced TO/N; (5) pET29b(+) uninduced T1; (6) pET29b(+) induced T1; (7) pET29b(+) uninduced T2; (8) pET29b(+) induced T2. The orange arrows indicate the XvGolSII recombinant protein. The gel was stained with Coomassie blue.

3.3.2.4 Protein purification using high performance liquid chromatography (HPLC)

To ensure high purity of the XvGolSII recombinant protein that was to be used in the subsequent galactinol synthase activity assay, HPLC purification of the protein was employed. The XvGolSII protein obtained from the IPTG induction (3.3.2.3) was purified using HPLC and maximally eluted at approximately 9 minutes (Fig. 3.6), corresponding to a voltage of approximately 39 mV, which is equivalent to 0.039 OD units of absorbance. From SDS-PAGE electrophoresis it was shown that maximal elution of the protein was in fraction 10 (Fig. 3.7).

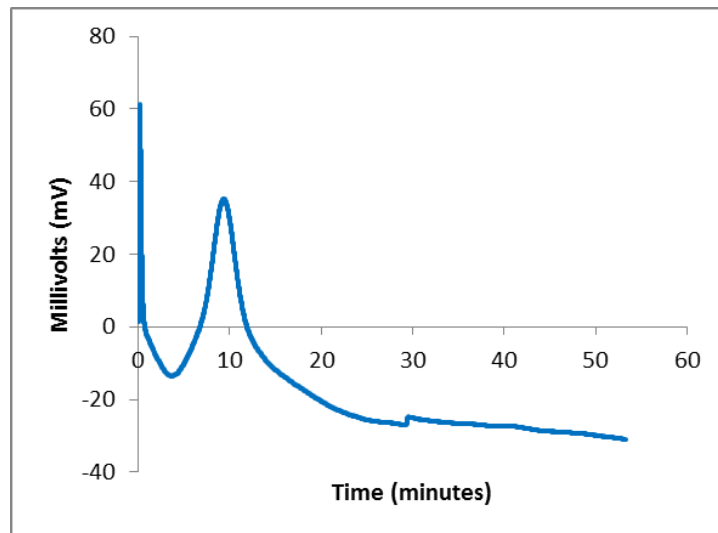


Figure 3.6: HPLC graph of XvGolSII recombinant protein purification showing the voltage (mV) acquired as each fraction eluted with relation to time (minutes). The voltage divided by 1000 is an indication of the absorbance.

The 12 HPLC fractions (fractions 5 – 16) were pooled and concentrated as previously using the Amicon filter. During this process the buffer was exchanged for a 1 x PBS buffer. To determine the concentration of the concentrated protein a Bradford assay was performed. It was determined that the concentration of the recombinant XvGolSII protein was at 4.47 $\mu\text{g}/\mu\text{l}$.

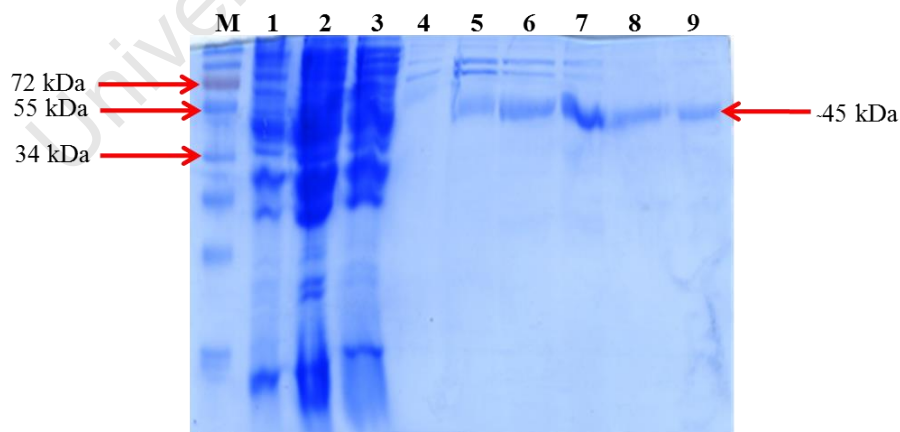


Figure 3.7: SDS-PAGE electrophoresis showing fractions obtained after HPLC purification of XvGolSII protein. Lanes: (M) Fermentas prestained protein ladder; (1) Crude protein extract; (2) unbound protein eluted from column; (3) protein flowthrough; (4) fraction 4; (5) fraction 8; (6) fraction 9; (7) fraction 10; (8) fraction 13; (9) fraction 15

3.3.2.5 Western blot analysis of Recombinant XvGolSII protein

Recombinant XvGolSII protein obtained from *E. coli* BL21 (pET29b(+):XvGolSII), colony 5, was used for western blot analysis. The primary antibody specific for the hexahistidine tag detected a His-tagged protein, as a distinct band is visible at approximately 45 kDa (Fig. 3.6). No band is visible in lane 1 where only the vector was used. There were no non-specific bands seen on the blot which suggests efficient purification of recombinant XvGolSII protein.

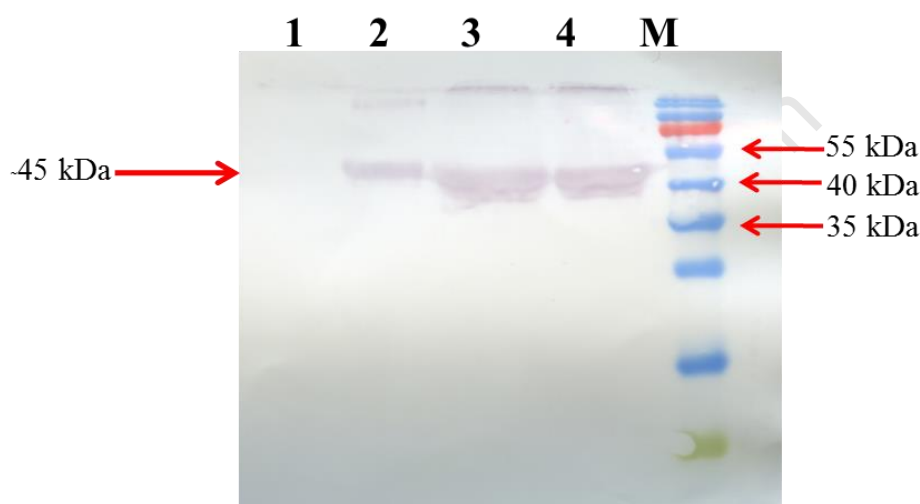


Figure 3.8: Western blot analysis of recombinant XvGolSII protein obtained from *E. coli* BL21(pET29b(+):XvGolSII), colony 5, using chromogenic detection. Lanes: (1) no insert control (pET29b(+) only); (2) eluent; (3) protein flow-through; (4) crude extract; (M) Fermentas pre-stained protein ladder.

3.3.3 Galactinol synthase activity assay

In order to confirm that this protein was in fact a galactinol synthase capable of synthesizing galactinol, an activity assay was conducted (adapted from Peters *et al.*, 2007). Commercially bought standards of myo-inositol, galactinol, UDP and UDP-galactose were run on HPAEC-PAD, as references for the activity assay. In Figure 3.9 distinct peaks were seen for the myo-inositol, galactinol and UDP-galactose standards but due to unknown reasons, UDP was not detected and no galactinol was synthesized from the controls (Fig. 3.10). In the test reaction only a small peak was seen for galactinol at 10.24 min (Fig. 3.11), but this provides conclusive evidence that the protein is acting as a galactinol synthase capable of catalyzing the formation of galactinol from myo-inositol and UDP-galactose.

There may be a number of reasons for the minimal conversion of substrates to galactinol. This may have been due to the period of time that the protein was frozen before the assay was performed, which was approximately one month. What may also have affected the activity of the protein are the freeze-thaw cycles that the protein was subjected to prior to the assay.

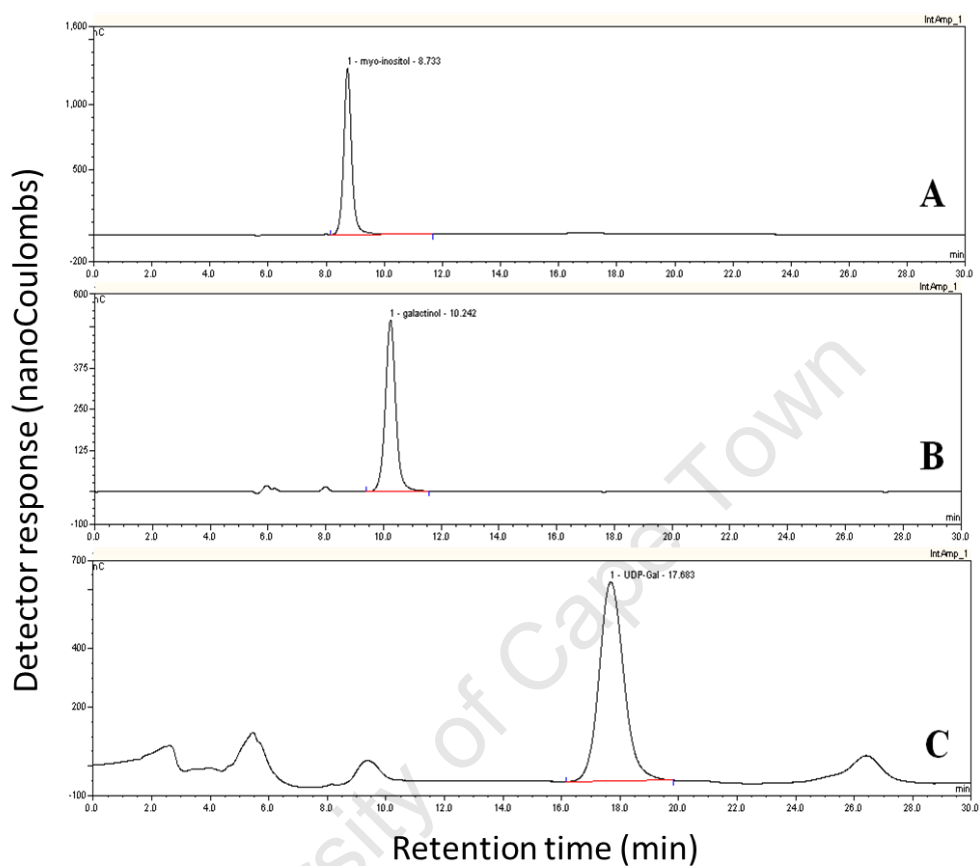


Figure 3.9: HPLC-PAD chromatograms representing commercially bought standards of A) *myo*-inositol, showing a retention time of 8.733 minutes; B) galactinol, showing a retention time of 10.242; and C) UDP-galactose, showing a retention time of 17.683 minutes.

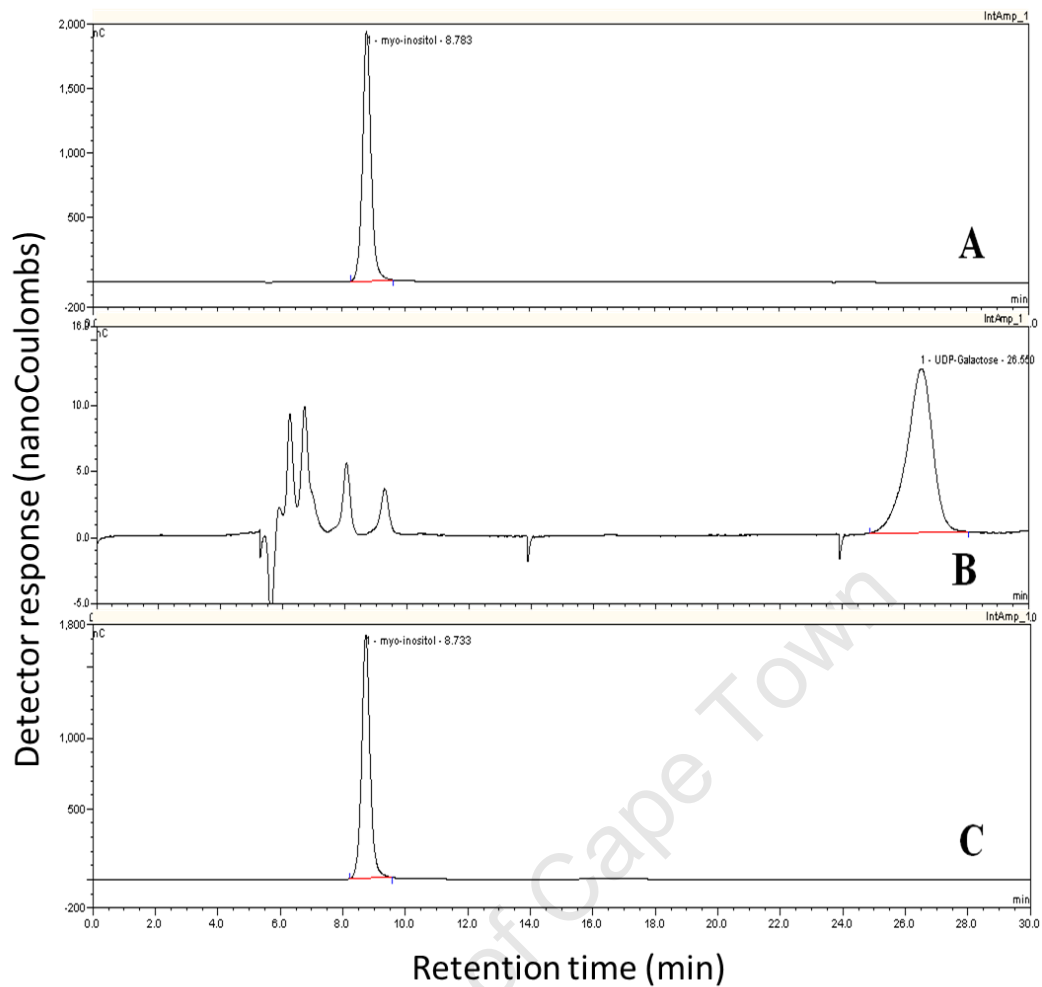


Figure 3.10: HPLC-PAD chromatograms representing galactinol synthase activity assay controls: A) *myo*-inositol + GolS; B) UDP-galactose + GolS; and C) *myo*-inositol + UDP-galactose.

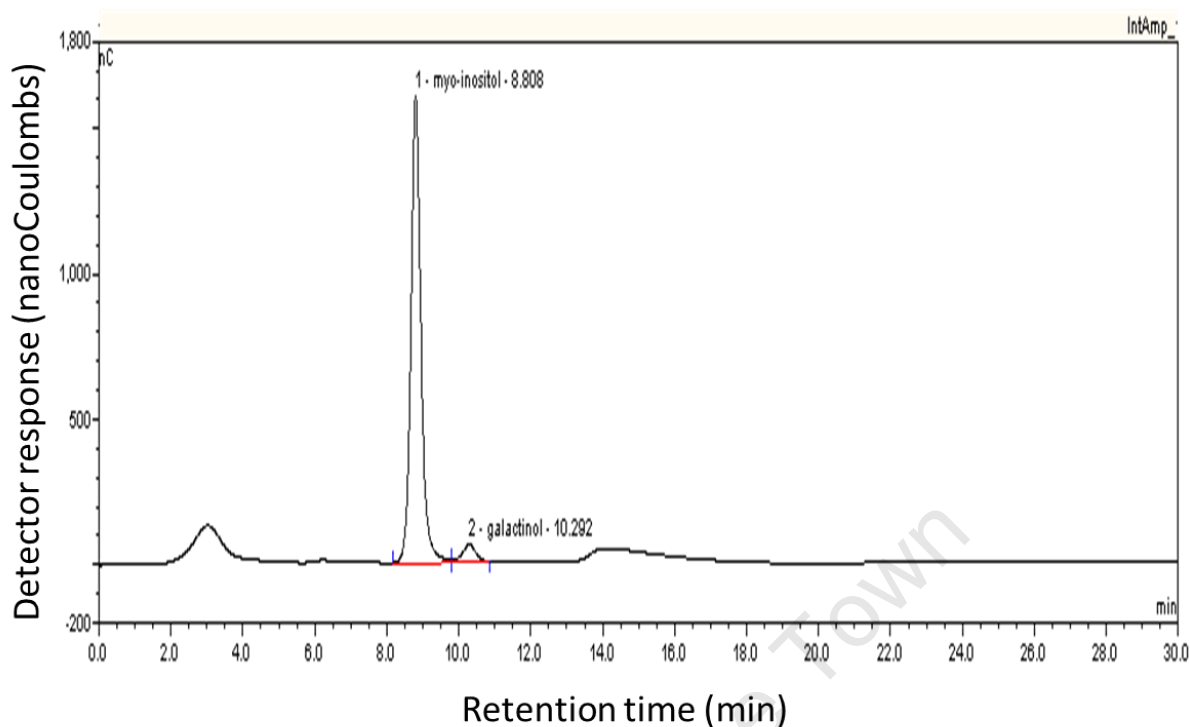


Figure 3.11: HPLC-PAD chromatogram representing an *in vitro* galactinol synthase activity assay which was conducted in the presence of *myo*-inositol and UDP-galactose, using purified recombinant galactinol synthase protein.

In order to further confirm that *XvGolSII* is in fact a galactinol synthase, an additional activity assay was performed by Dr. Shaun Peters (University of Zurich, Institute of Plant Biology, Switzerland). This assay includes an enzymatic hydrolysis of the galactinol synthesized by the recombinant *XvGolSII*. The galactinol produced by the recombinant *XvGolSII* was hydrolysed with the enzyme α -galactosidase which is specific for the α -1,1 galactosidic linkage in galactinol, yielding galactose and *myo*-inositol (Peters *et al.*, 2007). As can be seen from Figure 3.12A, galactinol was synthesized when using crude protein extract obtained from *E.coli* transformed with the pET29b(+):*XvGolSII* construct. The galactinol peak coincides with that of the galactinol standard peak. No galactinol peak was seen from the activity assay run on crude protein extract from *E. coli* that was transformed with the pET29 vector alone.

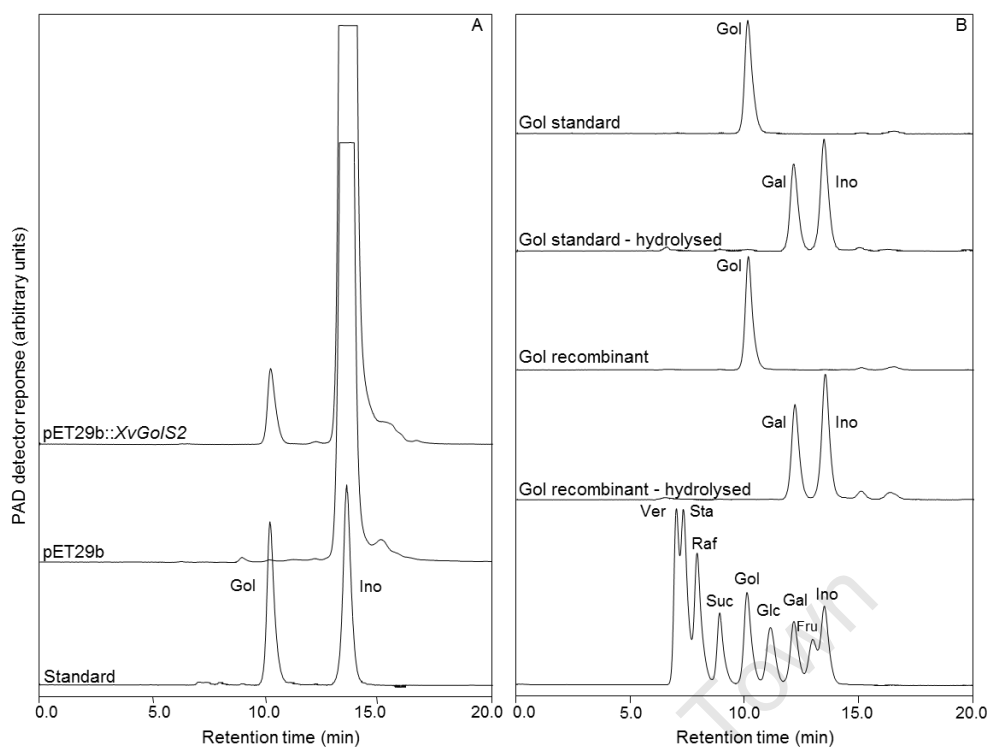


Figure 3.12: (A) *In vitro* GolS activity assays conducted with crude protein extracts obtained from *E. coli* (BL21) cultures transformed with pET29B::XvGolSII and induced with 1 mM IPTG for 3 h. Control crude extracts represent *E. coli* cultures transformed with the pET29b vector. (B) Enzymatic hydrolysis of galactinol (Gol) produced by recombinant XvGolSII. Fractions were collected from the BC-100 HPLC column and digested with an acid α -galactosidase. Gol standards represent 40 mmol of commercially purchased Gol. Standard sugars eluting from 7 min onward are Ver, verbascose; Sta, stachyose; Raf, raffinose; Suc, sucrose; Gol, galactinol; Glc, glucose; Gal, galactose; Fru, fructose; Ino, myo-inositol.

It is noteworthy that fractions of galactinol obtained from the initial activity assay showed a similar retention time (10.24 mins) to that obtained in the above assay for XvGolSII activity.

3.4 CONCLUSION

In this chapter recombinant XvGolSII protein was expressed in *E. coli* BL21 (DE3) cells and it was demonstrated *in vitro* that *XvGolSII* encodes a functional galactinol synthase in that it produced galactinol from the substrates *myo*-inositol and UDP-galactose. Previously it was shown that the first characterised galactinol synthase, XvGolS, also showed *in vitro* activity using the activity assay with enzymatic hydrolysis (Peters *et al.*, 2007). In the following chapter the subcellular localisation of XvGolSII will be investigated in onion (*Allium cepa*) epidermal cells using fluorescent confocal microscopy.

University of Cape Town

Chapter 4

YFP localisation of XvGolSII

4.1 INTRODUCTION

In a eukaryotic cell most of the proteins are encoded in the nuclear genome and synthesized in the cytosol, and may need further organization into various subcellular compartments (Emanuelsson *et al.*, 2000). The subcellular compartment or organelle to which a protein localizes may be directly or indirectly indicative of its function. Its localisation may also be fundamentally important for gaining insight into the regulatory mechanisms by which the protein is controlled (Kumar 2003).

The discovery of the green fluorescent protein (GFP) from the bioluminescent jellyfish *Aequorea victoria* has transformed the study of the localisation of proteins by allowing simple examination of proteins in living cells. Coloured variants of GFP include yellow fluorescent protein (YFP) and cyan fluorescent protein (CFP) (Davis, 2004).

Enhanced green fluorescent protein (EGFP) is a 238 amino acid protein and has an approximate molecular weight of 27 kDa (Scott *et al.*, 1999). GFP localises to the cytoplasm and nucleus (Feilmeier *et al.*, 2000). pEYFP encodes for an enhanced yellow-green variant. The enhanced yellow fluorescent (EYFP) is a coloured variant of EGFP. The EYFP contains four amino acid substitutions: Ser-65 to Gly; Val-68 to Leu; Ser-72 to Ala; and Thr-203 to Tyr, with a molecular weight of 27 kDa that shifts the emission from green to yellowish-green. The fluorescence level of EYFP is similar to that of EGFP. EYFP has an excitation maximum of 513 nm and an emission maximum of 527 nm.

Sanford *et al.* (1987) first developed and described a method of using high-velocity projectiles to deliver exogenous genetic material into plant tissue. This method of particle acceleration was thus called the “biolistic” method (Sanford, 1988), which utilizes instrumentation to accelerate DNA coated projectiles into cells, past the cell wall and membrane (Finer *et al.*, 1999). This study makes use of the PDS-1000/He Biolistic Particle Bombardment Delivery System (BioRad Laboratories, Germany) to introduce EYFP constructs into onion epidermal cells (Appendix F).

In this chapter the subcellular localisation of XvGolSII protein is examined using YFP localisation techniques. For the purposes of a localisation study a vector with a strong promoter and terminator was required. For this reason biolistic tools were used to bombard onion epidermal cells (*Allium cepa*) with the 35S-XvGolSII-YFP-NosT construct. The cauliflower mosaic virus promoter (CaMV 35S) functions as an enhancer that elevates gene expression (Tian *et al.*, 2004; Weigal *et al.*, 2000), while the Nos-terminator (NosT), which is the nopaline synthase terminator, signals the termination of gene expression (Ngaya *et al.*, 2009)

4.2 MATERIALS AND METHODS

The 35s-YFP-NosT vector used in this study was kindly donated by Seidal *et al.* (2005). In brief, the CaMV35S-promoter from the rsGFP-vector (Davies and Vierstra, 1998) was cloned into the 3' multiple cloning site of the EYFP vector (Clontech) which was digested with *Bam*HI and *Hind*III. The Nos-terminator (NosT) was amplified by PCR using a forward primer with a *Not*I restriction site and a reverse primer with the *Eco*RI restriction site which is endogenous to the Nos-terminator. The PCR product was subsequently digested with *Not*I and *Eco*RI and cloned into the 5' multiple cloning site of the EYFP vector, creating the resultant 35S-YFP-NosT vector.

4.2.1 Cloning of XvGolSII into p35S-YFP-NosT

The XvGolSII gene was amplified from pDNR-Lib-XvGolSII using gene specific primers, XvGolSIIBamHI-F (incorporating a *Bam*HI site) and XvGolSIIKpnI-R (incorporating a *Kpn*I site). The standard PCR reaction volumes (Appendix A) was used with the following cycling conditions: 94°C for 1 min; 2 cycles of (94°C for 30 s, 52°C for 40 s, 72°C for 90 s); 2 cycles of (94°C for 30 s, 56°C for 40 s, 72°C for 90 s); 26 cycles for (30 s, 56°C for 40 s, 72°C for 90 s); and a final extension step at 72°C for 7 min. The PCR products were electrophoresed on a 1% agarose/EtBr gel from which the band of interest was excised and purified using the Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's instructions (Appendix A6).

The purified XvGolSII fragment was digested with *Bam*HI and *Kpn*I and incubated at 37°C for 3 hours. The digested fragment was purified using the Bio Basic EZ-10 Spin Column PCR Purification Kit according to the manufacturer's instructions (Appendix A5). Similarly,

the pEYFP vector was digested with *Bam*HI and *Kpn*I and purified using the Bio Basic EZ-10 Spin Column PCR Purification Kit according to the manufacturer's instructions (Appendix A5). The digested *XvGolSII* fragment and p35S-YFP-NosT vector were ligated according to standard ligation conditions (Appendix A). For this ligation a 1:3 vector to insert ratio, was used.

The p35S-*XvGolSII*-YFP-NosT construct was transformed into *E.coli* DH5- α cells under standard transformation conditions (Appendix A2). Colony PCR was performed using the *XvGolSII*BamHI-F and *XvGolSII*KpnI-R primers to screen for positive clones. Three colonies positive for the p35S-*XvGolSII*-YFP-NosT colonies were inoculated into 3 x 5 ml LB broths supplemented with 100 μ g/ml ampicillin. Plasmid DNA was extracted from the respective clones using the standard plasmid extraction method (Appendix A4). The resultant constructs were subsequently digested with *Bam*HI and *Kpn*I to ascertain that the *XvGolSII* fragment successfully ligated into pEYFP. The samples were sequenced to verify that *XvGolSII* was successfully cloned into the p35S-YFP-NosT vector with no errors and was translationally cloned in frame with YFP.

4.2.2 Preparation of gold particles

Sterile techniques were observed and all procedures were performed within a laminar flow hood at all times. Sixty milligrams of gold particles (0.6 μ m) were weighed out in an eppendorf tube and sterilized as described by Sanford *et al.*, (1993). The particles were washed by adding 1 ml of absolute ethanol and vortexing for 1 minute. The gold particles were then pelleted in a microcentrifuge for no longer than 10 seconds. The supernatant was carefully removed and discarded. The ethanol wash steps were carried out four times. Sterile distilled water was added to the tubes and vortexed for 1 minute and particles pelleted by centrifugation for no longer than 10 seconds. The supernatant was carefully removed and discarded. The washing with distilled water step was repeated 6 times. The particles were resuspended in 1 ml of 50% glycerol. Fifty microlitre aliquots of the particles were placed into 1.5 ml eppendorf tubes while gently agitated and stored at room temperature.

4.2.3 Precipitation of gold particles

Two tubes of the prepared gold were used for precipitation of the DNA onto the gold particles. The 2 tubes of gold were for the precipitation of 35S-YFP-NosT only and 35S-

Xv*GolsII*-YFP-NosT. The gold was mixed on a vortex mixer, ensuring that the tube was constantly shaking to prevent coagulation of the gold particles. Three micrograms of each of the respective plasmid DNA was added to the gold particles. While the tubes were shaking in the vortex mixer, 50 µl of CaCl₂ was added to the tubes and mixed for 30 s. Twenty microlitres of 0.1 M spermidine was added to the mix and vortexed at high speed for 3 minutes. The gold particles were pelleted by 3 quick pulses in a microfuge and the supernatant carefully removed and discarded. Two-hundred and fifty microlitres of absolute ethanol was added to the gold particles and mixed for 1 minute and the particles pelleted as previously. The supernatant was removed and 75 µl of absolute ethanol added to the gold. The tubes were kept shaking continuously.

Macrocarrier discs were placed into the macrocarrier holders and the holders were inserted into petri dishes. While the Eppendorfs were shaking, 12.5 µl of the gold was removed from the tube and aliquoted onto the middle of each microcarrier. Once gold was placed on all the macrocarrier discs they were allowed to dry in the laminar hood for 20 minutes. The 1100 psi rupture discs were briefly dipped in isopropanol and allowed to air dry for 30 minutes.

4.2.4 Transformation of onion cells by projectile bombardment

The PDS-1000/He Biolistic Particle Bombardment Delivery System (BioRad Laboratories, Germany) was used to introduce DNA into onion (*Allium cepa*) epidermal cells (Appendix F). The onion epidermal cells were transformed as described by Scott *et al.* (1999). The gold particles were accelerated by pressurized helium, which was released by a rupture disc and a partial vacuum which was able to propel a microcarrier sheet laden with gold microcarriers toward target cell bodies at high velocity.

Segments of the inner layers of an onion were made with a scalpel and placed into Petri dishes. Three onion segments per construct were bombarded. Each segment of onion received 2 shots. The samples were incubated for 48 hr at 25°C in the dark in preparation for subsequent confocal microscopy.

4.2.5 Confocal microscopy of YFP fusion protein

For identification of nuclei and cell walls, cells were stained with 20 µg/ml Hoechst stain / 0.01% aniline blue in 0.07 M K₂HPO₄. Uniform squares were cut out of the epidermal layer of each onion segment, carefully removed and placed in a droplet of sterile distilled

water on a glass slide. One hundred microlitres of the Hoechst / Aniline Blue solution was added on top of the sectioned onion epidermal layer and staining was allowed to take place for 20 min before the cells were viewed. The Hoechst stain was used as a DNA fluorochrome, while the Aniline Blue was for visualisation of the cell membranes. The transformed onion epidermal cells were viewed using the LSM 510 META confocal laser scanning microscope (Carl Zeiss, Germany). To view YFP protein and YFP-GolSII fusion protein localisation, an excitation filter of 488 nm and the emission filter was a GFP absorption bandpass of 500 – 550 nm. The staining of the cell wall and the nuclei were viewed using the blue channel with an excitation filter of 325 – 372 nm and an emission filter with a bandpass of 435 – 485 nm. Brightfield light was used to view onion epidermal monolayer cells. The Zeiss LSM core software (Zeiss, Germany) was used to capture all images and insert the appropriate scale bars. The Axiovision 4.7, Photoshop (Zeiss, Germany) software was used to process all images.

4.3 RESULTS AND DISCUSSION

4.3.1 Cloning of *XvGolSII* into p35s-YFP-NosT

As can be seen from figure 4.1, a 1 kb insert was successfully amplified in 3 separate PCR reactions, from pDNR-Lib-*XvGolSII*, as was expected for the *XvGolSII* fragment.

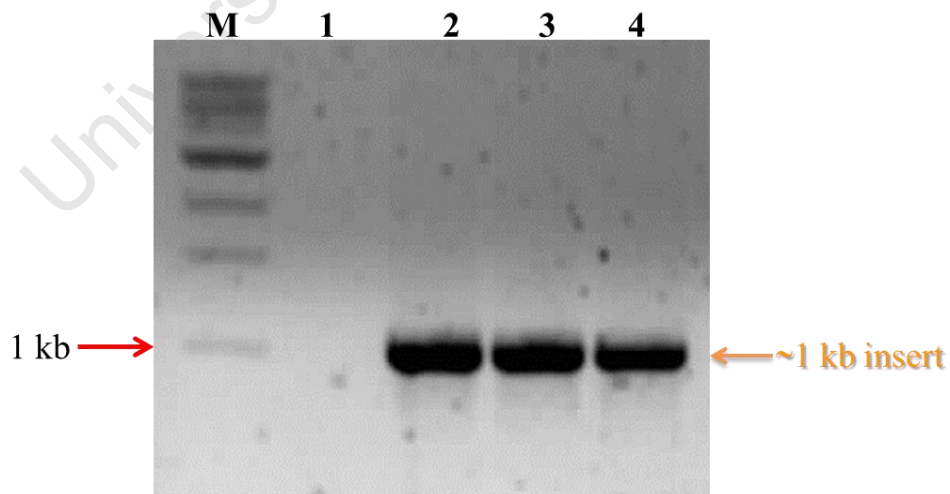


Figure 4.1: 1% (w/v) Agarose/EtBr gel electrophoresis of *XvGolSII* PCR reactions. Lane 1: No template control. Lane 2 – 4: *XvGolSII* fragment amplified from pDNR-Lib-*XvGolSII*, using localization primers. M: 1 kb DNA ladder (NEB). The orange arrow indicates the *XvGolSII* PCR product.

The band of interest was then excised, purified and cloned into the linearized 35S-YFP-NosT vector. Colony PCR confirmed that 10 out of 30 colonies were positive for the *XvGolsII* insert (data not shown).

An enzyme digest of the 35S-*XvGolsII*-YFP-NosT construct isolated from 3 positive clones released the approximate 1 kb (*XvGolsII*) insert. This can be seen in lanes 2, 4 and 6 of figure 4.2. Thus it was confirmed through colony PCR, enzyme digests and sequencing analysis (data not shown) that *XvGolsII* was successfully ligated to 35s-YFP-NosT and the *KpnI* and *BamHI* restriction sites successfully incorporated.

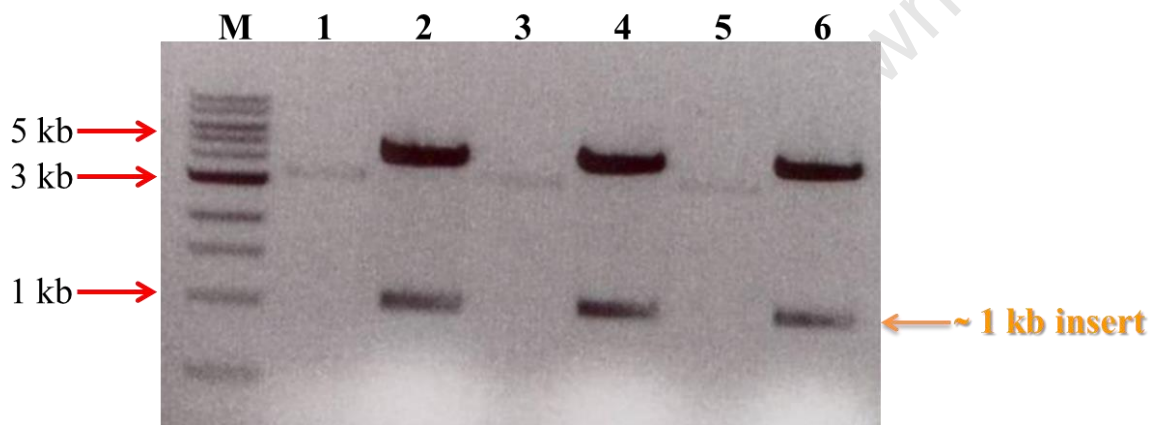


Figure 4.2: 1% (w/v) Agarose/EtBr gel electrophoresis of 3 undigested and digested p35S-*XvGolsII*-EYFP-NosT clones. Lanes 1, 3 and 5: undigested p35S-*XvGolsII*-YFP-NosT clones. Lanes 2, 4 and 6: p35S-*XvGolsII*-YFP-NosT clones digested with *BamHI* and *KpnI*. M: 1 kb DNA ladder (NEB). The yellow arrow indicates the release of the *XvGolsII* insert.

4.3.2 Localisation of *XvGolsII*-YFP in onion epidermal cells

Cells from the epidermal layer of onions (*Allium cepa*) have been useful as a model for light microscopy as the cells are large, comparatively transparent and occur naturally as a monolayer.

It is seen that the p35S-YFP-NosT vector as a control localises to the cytoplasm, as expected (Fig. 4.3). For reasons that are unclear the onion cell walls were not sufficiently stained by the aniline blue. Staining of the nuclei by the Hoechst stain is also prominently seen in figure 4.3 (indicated by arrows).

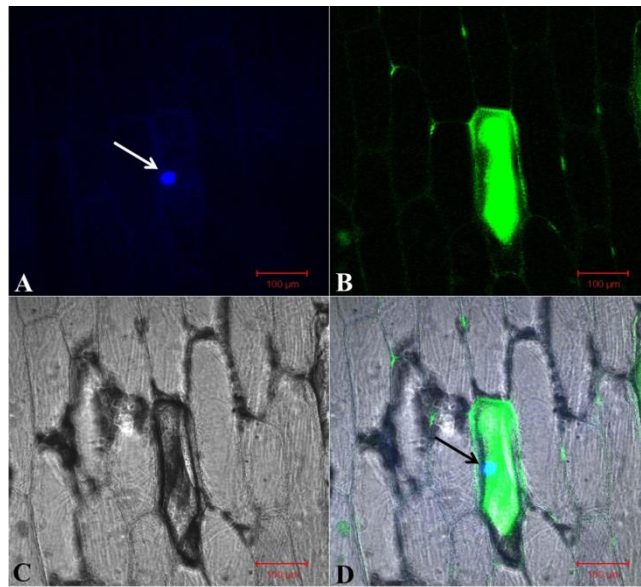


Figure 4.3: Subcellular localisation of the YFP protein in onion epidermal cells, showing Hoechst staining of the nucleus in a cell that shows positive localisation of the YFP protein to the cytoplasm. (A) cells viewed through the blue channel; (B) the green fluorescent channel; (C) cells seen under brightfield light; (D) an overlay of (A), (B) and (C). The stained nucleus is indicated by the arrows.

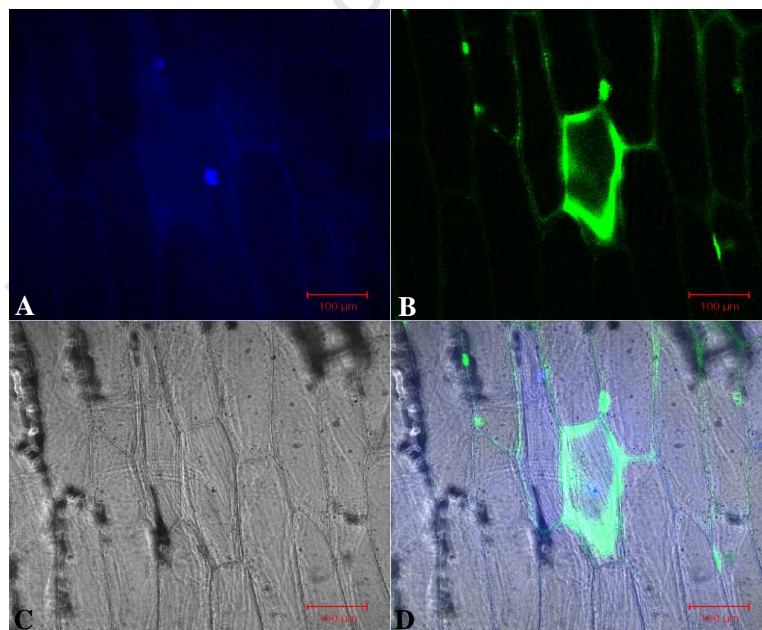


Figure 4.4: Subcellular localisation of the YFP-XvGolSII fusion protein. Localisation of the fusion protein to the cell membrane is seen in a cell with a Hoechst stained nucleus. (A) cells viewed through the blue channel; (B) the green fluorescent channel; (C) cells seen under bright light; (D) an overlay of (A), (B) and (C).

The YFP-XvGolSII fusion protein appeared to be localised to the cell membrane (Fig. 4.4). Zhou *et al* (2012b) demonstrated via GFP localisation that a cotton galactinol synthase (*GhGolsI*) targeted the cell membranes of onion epidermal cells (Zhou *et al.*, 2012b). These results were in contrast to the *in silico* analyses of both XvGolSII and GhGolSI proteins, in which localisation to the cytoplasm was predicted (see section 2.3.2).

These results provide an understanding into the subcellular localisation of XvGolSII, however, it would be beneficial to gain insight into its tissue specific localisation as this may help us to infer potential roles of XvGolSII within *X. viscosa* during abiotic stress.

Sprenger and Keller (2000) report on two cold-inducible GolS isoforms (GolS-1 and GolS-2) found in *Ajuga reptans*, which are differentially recruited for their physiological roles, i.e. GolS-1 for RFO synthesis for storage in the mesophyll and GolS-2 for translocation in the phloem. It was shown that GolS-1 expression was by far more dominant than expression of GolS-2 and therefore it is most likely that GolS-1 is involved in the build-up of the large RFO storage pool in *A. reptans* (Sprenger and Keller, 2000).

Three cDNAs for maize GolS (ZmGolS) were identified from the Pioneer Hi-Bred expressed sequence database (Zhao *et al.*, 2004). Tissue prints of the ZmGolS localised them to the embryo of maize seeds.

It has been reported (Beebe & Turgeon, 1992) that the synthesis of galactinol within the cucurbit (*Cucurbita pepo*) occurs in both the mesophyll and intermediary cells, predominantly the latter. Immunocytochemical and C¹⁴-labelling techniques were used in this study.

4.4 CONCLUSION

Generation of an XvGolSII::YFP fusion protein was successfully achieved and indicated a subcellular localisation of the protein to the cell membrane. These results are in contrast to what was predicted through *in silico* analysis, in which XvGolSII was predicted to localise to the cytoplasm. It would be interesting, in future, to gain insight into the tissue-specific localisation of XvGolSII by means of techniques such as immuno-localisation. In the following chapter, quantitative real-time RT PCR and western blot analyses will be used to determine transcript and protein levels respectively, as it is essential to quantitatively assess

the mRNA and protein expression levels during abiotic stress so as to determine whether XvGolSII transcript and protein expression are induced by specific abiotic stresses.

Chapter 5

Gene expression analyses of *XvGolSII*

5.1 INTRODUCTION

An essential mechanism by which plants are able to cope with abiotic stresses, such as water deficit and cold, is through the regulation of their gene expression. It has been shown previously that in certain resurrection plants, such as *Craterostigma wilmsii*, *Myrothamnus flabellifolius* and *Eragrostis nindensis*, the RFOs accumulate upon water deficit stress (Farrant, 2007). Peters *et al* (2007) showed using Northern blot analyses that *XvGolS* transcript levels increase in *X. viscosa* during dehydration. Though previous studies on resurrection plants have measured GolS activity during various abiotic stresses (Wang *et al.*, 2012; Zhou *et al.*, 2012a; Taji *et al.*, 2002; Bachmann *et al.*, 1994), to my knowledge, there has been no study which measured GolS protein levels specifically.

A number of studies have demonstrated a weak or moderate correlation between mRNA and protein levels (Gygi *et al.*, 1999; Bajic, 2006). Often protein activity is controlled independently of gene expression in response to stress via post translational modification (PTM) such as phosphorylation, glycosylation and ubiquitination amongst others and serve to activate, de-activate, degrade and/or increase the binding affinity and activity of the protein (Miura & Hasegawa, 2010; Arc *et al.*, 2011). In addition, resurrection plants have been shown to store various mRNA transcripts upon dehydration as part of a re-hydration strategy (Ingle *et al.*, 2007; and Dace *et al.*, 1998; Bajic, 2006). This highlights the importance of measuring both transcript and protein levels in response to stress.

This section of the study encompasses the respective dehydration and cold stress treatments of *X. viscosa* plants, followed by RNA extraction and subsequent cDNA synthesis. For dehydration and cold stress treatments, *XvGolSII* mRNA expression levels were assessed using quantitative real-time RT PCR. Due to time constraints, *XvGolSII* protein expression levels were only measured for the dehydration treated plants using western blot analysis.

5.2 MATERIALS AND METHODS

5.2.1 Plant material

The *X. viscosa* plants used in this study was previously collected from the Cathedral Peak Nature Reserve in the Drakensberg Mountains (KwaZulu-Natal, South Africa). These

plants were maintained under greenhouse conditions previously described by Sherwin & Farrant (1996). Four weeks prior to stress treatments; plants were transferred to convirons (Conviron Adaptis A350, Canada) and allowed to acclimatize to controlled environmental conditions. Growth conditions were set at 60% humidity, 22°C temperature, light intensity of 150-200 $\mu\text{mol}/\text{m}^{-2}/\text{s}^{-1}$, and a photoperiod of 16 hour light phase and 8 hour dark phase was maintained for the plants used for the dehydration treatment and an 8 hour light phase and 16 hour dark phase for the cold stress treatment. During this period of acclimatization the dead leaf material at the tips of the leaves was removed allowed to restore for an additional 2 weeks. The plants were watered once a week and precaution was taken to avoid over-watering of the plants. In this study 3 individual plants were chosen per stress treatment i.e. dehydration and cold stress treatments, respectively. Care was taken to choose plants of similar size and phenotype as to minimize biological variation amongst plants during the stress treatments.

5.2.2 Cold and dehydration stress treatment of *X. viscosa*

The dehydration treatment of *X. viscosa* was performed by S. Sattar and can be seen in Appendix A10.

Three biological repeats were used in the cold stress treatment of *X. viscosa*. Subsequent to acclimatization at 22°C the plants were subjected to the same 8 hour day and 16 hour night cycle, with the day temperature being 8°C dropping to a 4°C night temperature. One leaf per biological was sampled at 0, 6, 24, 48 and 72 hours during the course of the cold treatment. All sampling was performed during the light phase of the treatment.

5.2.3 RNA isolation

5.2.3.1 Dehydration-stress treatment

The RNA isolation for the dehydration treated plants was performed by S. Sattar (Appendix A11).

5.2.3.2 Cold-stress treatment

All plastics and pestle and mortars used were autoclaved twice. One leaf per biological, per sampling point, was used for RNA extraction. Each leaf was individually ground to a fine powder in a pestle and mortar in the presence of liquid nitrogen. In order to prevent degradation of RNA the ground tissue was maintained at 4°C. The RNA isolation was performed using the RNeasy[®] Plant Mini Kit (QIAGEN), according to the manufacturer's instructions.

5.2.3.3 DNase treatment and phenol:chloroform:iso-amyl alcohol clean up

Each RNA sample was treated with deoxyribonuclease I (DNase I, Fermentas USA) to remove any genomic contamination. One microgram of RNA was treated with DNase I and the reaction was set up as per the manufacturer's guidelines. The samples were incubated at 37°C for 1 hour. An equal volume of phenol:chloroform:iso-amyl alcohol (ratio of 25:24:1) was then added to the reaction, mixed by inversion and centrifuged at 14 000 x g at 4°C for 10 min. The upper aqueous was then carefully removed and placed in a sterile Eppendorf tube and a 0.1 volume of 3M sodium acetate (pH 5.2) was added to this and gently mixed by inversion. To this a 5 x volume of 100% EtOH was added, mixed by inversion and subsequently centrifuged at 14 000 x g at 4°C for 10 min to precipitate the RNA. The supernatant was carefully discarded and the pellet was allowed to air-dry for 2 min. The pellet was then resuspended in 20 µl of RNase-free water.

5.2.4 cDNA synthesis

5.2.4.1 Dehydration-stress treatment

The purified RNA extracted from dehydration-treated *X. viscosa* leaves was obtained from Shakiera Sattar (Appendix A11). It was this RNA that was used for cDNA synthesis. For each time point for both dehydration and rehydration, the RNA extracted from two leaves was pooled and approximately 325 ng was used for each cDNA synthesis reaction. This was performed for each biological repeat.

The conversion of RNA to cDNA was performed in duplicate thus serving as technical repeats for the reaction. cDNA synthesis was performed using the ImProm-II™ Reverse Transcription System (Promega Corporation, USA). The cDNA synthesis reactions were set up according to the protocol outlined in the manual. Random hexamers and oligo (dT) primers were used in these reactions at a ratio of 10:1. The concentration of RNA used for each conversion was 325 ng in a total volume of 10 µl. The RNA and primer mix was prepared separately from the master mix as per Table 5.1.

Table 5.1: The volumes of oligo-dT and random hexamers and RNA used in the primer/RNA mix.

Reaction component	Volume (µl)
Oligo-dT (0.5 µg/µl)	0.1
Random hexamer primer (0.5 µg/µl)	1
RNA (325 ng)	X
Nuclease-free water	Up to 25µl

The ratio of random hexamers:oligo-dT was 10:1. The X represents the varying volumes from each sample needed for a total of 325ng of RNA.

The reactions were performed in 0.2 ml PCR tubes and thermo-cycling was performed in the GeneAmp PCR System 9700 (Perkin Elmer). Each tube, containing the RNA and primer mix, was then incubated at 70°C for 5 minutes. Tubes were immediately placed on ice for 5 min. Subsequently, the master mix reaction was set up as per Table 5.2.

Table 5.2: The volumes of components used in master mix reaction for cDNA synthesis, for a 20 µl reaction.

	20 µl reaction
Nuclease-free water	6.1
5x reaction buffer	4.0
MgCl ₂ (3mM final)	2.4
dNTP mix(final 0.5mM each)	1
Inhibitor (1U/µl final)	0.5
Reverse Transcriptase	1.0
Total	15

The final cDNA conversion set up consisted of 7.5 µl of the master mix and 2.5 µl of the RNA and primer mix per tube to give a total reaction volume of 10 µl. Once the master mix was added to the RNA and primer mix the tubes were placed at 25°C for 5 minutes to allow

for annealing. For the extension step the tubes were placed at 42°C for 1 hour. The reaction tubes were then incubated at 70°C for 15 minutes to inactivate the reverse transcriptase. For subsequent quantitative real-time analysis 1 µl of cDNA was used.

5.2.4.2 Cold-stress treatment

The cDNA synthesis for the cold treated plants was performed using the M-MLV Reverse Transcription kit (Promega Corporation, USA), according to the manufacturer's instructions. Approximately 500 ng of purified RNA (5.2.3.2) was used for cDNA synthesis. Oligo (dT) primers were used in these reactions. The cDNA conversion was performed in duplicate, this served as technical repeats for the reaction. All components of the reactions were added separately and 1 µl of RNasin® Plus RNase Inhibitor (Promega, USA) was added to each reaction. The reaction setup can be seen in Table 5.3. The reactions were initially placed in a 70°C heating block to in order to melt secondary structures within in the RNA template and immediately cooled on ice for 5 min to prevent secondary structures from reforming. The tubes were then placed in a 40°C heating block for the initial 10 min and then the temperature of the heating block was raised to 55°C for the final 50 min. The reaction was then inactivated by incubating at 70°C for 15 min. The duplicate cDNA reactions for each time point, was subsequently pooled.

Table 5.3: Reaction set up volumes (in µl) for the cDNA synthesis of RNA samples isolated from cold treated plants.

Component	Volume (µl)
RNA (500 ng)	X
Oligo(dT)	1
M-MLV RT 5X Reaction Buffer	5
deoxyadenosine triphosphate (dATP)	1.25
deoxythymidine triphosphate (dTTP)	1.25
deoxycytidine triphosphate (dCTP)	1.25
deoxyguanosine triphosphate (dGTP)	1.25
RNasin® Plus RNase Inhibitor	1
M-MLV RT (H-) Point Mutant	1
Water	Up to 25 µl

The X represents the varying volumes from each sample needed for a total of 500 ng of RNA.

5.2.5 Quantitative real-time reverse transcription PCR

5.2.5.1 Quantitative real-time primer design

5.2.5.1.1 Dehydration-stress treatment

The *XvGolSII* primers used in the dehydration-stress treatment experiment were the forward, GolSRT_Fwd II and reverse, GolSRT_RvII (Appendix B1.3 and B1.4) primer set, designed using Beacon Designer (Premier Biosoft International). Primer amplification specificity and primer dimer formation was analysed by end point PCR (data not shown).

These primers were designed to yield a PCR product of 100 bp and had an annealing temperature of 58°C. The cycling conditions were as follows: an initial denaturation at 98°C for 8 min followed by 30 cycles at 95°C for 10 sec, 58°C for 8 sec and 72°C for 10 sec. The *18S* gene was used as the reference gene for the dehydration-stress experiments. The *18S* RNA primer set (Appendix B1.7 and B1.8) yielded a 131 bp product. The cycling conditions were as follows: an initial denaturation at 94°C for 2 min followed by 40 cycles at 94°C for 30 sec, 58°C for 8 sec and 72°C for 15 sec.

5.2.5.1.2 Cold-stress treatment

An *XvGolSII* specific forward, GolS_RT_F and reverse, GolS_RT_R (Appendix B1.9 and B1.10) primer set were designed to yield a 117 bp PCR product. The cycling conditions for these primers were as follows: an initial denaturation at 95°C for 5 min followed by 40 cycles at 95°C for 10 sec, 55°C for 15 sec and 72°C for 20 sec, with a final extension at 72°C for 5 min. Based on the study of previous literature with respect to cold stress treatments, four potential reference genes were tested (Nicot *et al.*, 2005, Li *et al.*, 2011, Jain *et al.*, 2006 and Schmidt & Delaney 2010). These reference genes were *18S*, *EF1a*, *L2 ribosomal protein* and *actin*, and were subsequently isolated from *X. viscosa* cDNA (data not shown). Real-time PCR melt curve and amplification plot analysis of these potential reference genes in response to cold stress allowed us to choose *actin* as the most suitable reference gene (Fig. D3A and Fig. D4). The *actin* primers were designed to yield a 133bp product. The cycling conditions were as follows: an initial denaturation at 95°C for 10 min followed by 40 cycles at 95°C for 15 sec, 60°C for 15 sec, 72°C for 15 sec, with a final extension at 72°C for 10 min.

5.2.5.2 Construction of real time standard curves

5.2.5.2.1 Dehydration-stress treatment

Standard curves were generated for both the gene of interest (*XvGolsII*) and reference gene (*18S RNA*) to determine the PCR efficiencies. Pooled cDNA was initially used to construct the standard curves, however, the efficiencies were low and it was decided to use purified PCR product for both *XvGolsII* and *18S RNA* to construct the standard curves.

The full length 18S RNA gene was amplified using gene specific primers (Appendix B1.7 and B1.8) and PCR amplified as per section 5.2.5.1.1. The *XvGolsII* was amplified using the same PCR cycling conditions described in section 3.2.1.1 using the pET29b::*XvGolsII* as template (Chapter 3, section 3.2.1.1). The resulting PCR products were purified using the Promega Wizard Gel clean-up kit, according to the manufacturer's instructions (Appendix A6). In constructing the standard curves, 1/5 dilutions were used. These standard curves were subsequently imported into every qPCR run. The gene expression at each sample point was therefore expressed as n-fold change, relative to time point zero (100% RWC). Relative quantification of the expression was achieved using Pfaffl equation (Pfaffl, 2001) (Fig 5.1), with the modification of adding 1 to the efficiency (E) in order to account for the efficiency value generated by the Corbett Rotor Gene 6000 Real-Time PCR instrument (Qiagen, Germany).

$$\text{Ratio} = \frac{(E_{\text{target}})^{\Delta\text{Ct}_{\text{target}}(\text{control} - \text{sample})}}{(E_{\text{reference}})^{\Delta\text{Ct}_{\text{reference}}(\text{control} - \text{sample})}}$$

Figure 5.1 Equation representing the Pfaffl mathematical equation for calculating relative expression ratios in qPCR. The ratio of the gene of interest (target) expressed in a sample versus a calibrator sample (control) in comparison to a reference gene (reference). E_{target} and $E_{\text{reference}}$ represent the amplification efficiency of the target and reference gene, respectively. $\Delta\text{Ct}_{\text{target}}$ and $\Delta\text{Ct}_{\text{reference}}$ is the difference in Ct values of target gene in the control and sample and reference gene in the control and sample, respectively. PCR amplification efficiencies (E) were calculated according to $E = 10^{[-1/\text{slope}]}$ (Pfaffl, 2001). In order to convert Corbett Rotor-gene efficiency to Pfaffl efficiency 1 was added.

5.2.5.2.2 Cold-stress treatment

Standard curves for both *XvGolSII* and *actin* were constructed using pooled cDNA from each time point, from which 1/10 serial dilutions were made. Standard curves were constructed for each biological replicate. The gene expression at each sample point was therefore expressed as n-fold change, relative to time point zero (22°C). Relative quantification of the expression was calculated using the Pfaffl equation, as per section 5.2.5.2.1.

5.2.5.3 Preparation of real-time PCR reactions

5.2.5.3.1 Dehydration-stress treatment

The relative expression of *XvGolSII* under dehydration stress conditions was measured by real-time PCR using the SensiMix dT Kit (Quantace, Australia). The PCR reactions were performed using the Corbett Rotor Gene 6000 Real-Time PCR instrument (Qiagen, Germany). The reactions were prepared as follows: the primer mix for each of the genes (*18S* and *XvGolSII*) were prepared in separate Eppendorf tubes, with equal volumes of gene-specific forward and reverse primers. The reaction mix was then prepared as per Table 5.4.

Table 5.4 Real time PCR reaction set up, shown for a 12.5 µl real time reaction.

Component	Per 12.5 µL Reaction (µl)
Sensimix dT	6.25
Sybr Green	0.25
Nuclease free water	4.75
Primer mix (10 µM) (either 18S fwd and rev, or GolS fwd and rev)	0.25
Template	1

To each real time reaction tube 11.5 µl of mix was added, after which the template was added. A no reverse transcription control (RNA only) and a no template control were carried out for each biological repeat during each real time run. For the no template control (NTC), water was added to the mix instead of template. For the standard curves the undiluted and serial dilutions of the pooled, purified cDNAs (see 5.2.5.2.1) for both *XvGolSII* and *18S* RNA, served as the templates. The thermal cycling conditions were as follows: an initial enzyme activation step at 95°C for 8 min followed by 40 cycles of 95°C for 10 sec, 58°C for

8 sec and elongation at 72°C for 10 sec. All reactions were performed in triplicate. At the end of the elongation step the fluorescence for each reaction was obtained.

5.2.5.3.2 Cold-stress treatment

The relative expression of *XvGolSII* under cold-stress conditions was investigated by real time PCR using the KAPA SYBR® FAST qPCR Master Mix (Kapa Biosystems, USA). The PCR reactions were performed using the Corbett Rotor-Gene 6000 HRM Real-Time PCR instrument (Qiagen, Germany). To each real time reaction tube 18 µl of the reaction mix (Table 5.5) was added, after which 2 µl of template was added. A no reverse transcription control (RNA only) and an NTC were carried out as per section 5.2.5.3.1. The thermal cycling conditions were as follows: an initial enzyme activation step at 95°C for 3 min followed by 40 cycles of 95°C for 5 sec, 60°C (*actin*) / 64°C (*XvGolSII*) for 15 sec and elongation at 72°C for 20 sec. All reactions were performed in triplicate. At the end of the elongation step the fluorescence for each reaction was obtained.

Table 5.5 Real time PCR reaction set up, shown for a 20 µl real time reaction. *Actin* and *XvGolSII* primers were used at a concentration of 10 µM and 5 µM, respectively.

Component	<i>Actin</i>	<i>XvGolSII</i>
KAPA SYBR® FAST qPCR Master Mix	10	10
Forward primer	0.4	0.2
Reverse primer	0.4	0.2
Water	7.2	7.6

5.2.5.4 Statistical analysis

All statistical tests and graphs were performed and generated using GraphPad Prism software version 6 for Windows (GraphPad Software Incorporation, 1992-2007). One-way ANOVA with Dunnett's post-test was performed for both the cold- and dehydration-stress treatment analysis. All dehydration RWCs were compared to time-point zero (100% RWC), while all rehydration RWCs were compared to the last point of dehydration (2% RWC). For the cold stress experiment all time points were compared to time-point zero (22°C).

5.2.6 Western blot analysis of *XvGolSII* protein expression under dehydration stress

The aim of this section was to determine the expression levels of the *XvGolSII* protein under varying relative water contents (RWCs). The *XvGolSII* antibody was used to

detect the XvGolSII protein within total crude protein extract of *X. viscosa*. The crude extract at the respective RWCs was obtained from S. Sattar (Appendix A12).

5.2.6.1 Antiserum production and antibody purification

Recombinant protein that was purified using HPLC (see Chapter 3, section 3.3.2.4) was used to raise polyclonal antibodies in rabbits after ethical clearance was obtained from UCT Animal Ethics Committee. Two New Zealand rabbits were bled for pre-immune sera. The 2 rabbits were then injected with 1 ml antigen emulsion samples at a concentration of 1mg/ml on day 1 and then given 1 ml booster injections at weekly intervals for three weeks based on the protocol by Ed Rybicki (1979) with the following modifications: the rabbits were bled every two weeks commencing at least 4 weeks after the first injection of the antigen.

ELISA analysis of XvGolSII antibody titre was carried out as described in the Appendix (A9).

The antibody was purified as follows: To one volume of serum two volumes of borate-buffered saline (35 mM boric acid; 37 mM NaCl; 17.5 mM NaOH; 0.02% HCl) was added. Crushed polyethylene glycol 6000 (PEG 6000) was added to the diluted serum to a final concentration of 14% (w/v) and gently dissolved by inversion. The mixture was centrifuged at 12 000 x g for 10 min at 4°C and the pellet resuspended in the original serum volume using 1 x PBS buffer. PEG 6000 was added to the mixture to a final concentration of 14% (w/v) and the pellet gently resuspended. The centrifuge step was repeated and the pellet resuspended in half the original serum volume using 1 x PBS containing 60% (v/v) glycerol. The purified antibodies were stored at -20°C in 500 µl aliquots.

5.2.6.2 Testing and optimisation of XvGolSII antibodies

Varying concentrations of purified XvGolSII recombinant protein (4.47 µg [undiluted]; 10^{-1} ; 10^{-2} ; 10^{-3} ; 10^{-4} dilutions) were run on an SDS-PAGE gel. This gel was then placed in transfer buffer (25 mM Tris; 150 mM glycine; 10% methanol) for 10 minutes. The mini-PROTEAN electrophoresis system (Bio-Rad, USA) was used for the blotting procedure. The transfer of the protein onto the nitrocellulose membrane was performed as per section 3.2.3.1.

Subsequent to the transfer the membrane was incubated in staining solution (0.5% [w/v] Ponceau S, 1% [v/v] glacial acetic acid) with gentle agitation for 2 min. The membrane was destained in distilled water until the bands were visible. The membrane was then washed twice for 10 min with TBS buffer at room temperature. The membrane was incubated for 1 hr in blocking buffer (10% skim milk powder in TBS buffer). After incubation with blocking buffer the membrane was washed twice for 10 min each time in TBS-Tween/Triton buffer and washed once for 10 min in TBS buffer at room temperature. The membrane was incubated with XvGolSII antibodies in skim milk/TBS solution at a 1/500 dilution overnight at 4°C, with gentle shaking. The membrane was washed twice for 10 min each time in TBS-Tween/Triton buffer at room temperature and then washed for 10 min in TBS buffer. The membrane was then incubated for 1 hour with the goat anti-rabbit peroxidase conjugate (Sigma-Aldrich, Germany) in skim milk/TBS solution at a 1/20 000 dilution. The membrane was subsequently washed 4 times for 10 min each in TBS-Tween/Triton buffer at room temperature.

The XvGolSII protein was detected using the SuperSignal® West Pico Chemiluminescent Substrate Kit (Pierce, USA) according to the manufacturer's instructions. The western blots were exposed to the BioRad XR ChemiDoc for visualisation of any protein-antibody interactions.

5.2.6.3 Western blot analysis of dehydrated and rehydrated total protein samples

For both the dehydrated and rehydrated protein extracts all three biological repeats were analysed for each of the RWCs. Two micrograms of the recombinant XvGolSII protein was used as a positive control and 40 µg of total protein was used per RWC.

Western blot analysis was carried out as per section 3.2.3.1 with the exception that chemiluminescent detection using x-ray film was employed in addition to the use of the BioRad XR ChemiDoc.

5.3 RESULTS AND DISCUSSION

5.3.1 RNA isolation

RNA quality and integrity was assessed through gel electrophoresis, using a 1.2% (w/v) formaldehyde-agarose gel. Samples were electrophoresed at 80V. The 25S and 18S ribosomal RNA bands were clearly visible on the gel electrophoresis image, indicating good quality RNA (Appendix D2).

5.3.2 cDNA synthesis

5.3.2.1 Dehydration-stress treatment

Approximately 325ng of purified RNA was used for each cDNA synthesis reaction. The integrity of the cDNA was analysed through electrophoresis, using a 1% (w/v) EtBr/agarose gel. A DNA smear was seen from the electrophoresis results and this was an indication of successful cDNA synthesis (data not shown).

5.3.3 Analysis of *XvGolSII* gene expression

5.3.3.1 Dehydration-stress treatment

In order to study the gene expression changes of *XvGolSII* during dehydration, the mRNA levels were monitored using quantitative real-time PCR. To correct for sample-to-sample and PCR variations the expression levels of *18S* was used for normalization. It has been previously reported that *18S* was successfully used as a reference gene in qRT-PCR dehydration stress studies (Bresler 2010; Maredza 2007). The relative expression levels remained reasonably similar across all 3 biological replicates. A down-regulation of *XvGolSII* was observed, particularly at the RWC range of 58 – 2%. The P-values were as follows: 0.0389 (47 - 58% RWC), 0.0065 (21 – 42% RWC), 0.0011(12 – 18% RWC), 0.0021(6 – 9% RWC) and 0.0003 (2% RWC).

The most significant fold expression change is observed at 2% RWC (Figure 5.2). This observation may imply that *XvGolSII* is repressed during dehydration stress. An approximate 1.1 fold decrease in transcript abundance is observed at this RWC. Upon rehydration there appears to be a further down-regulation but statistically this change is not significant as compared to 2% RWC.

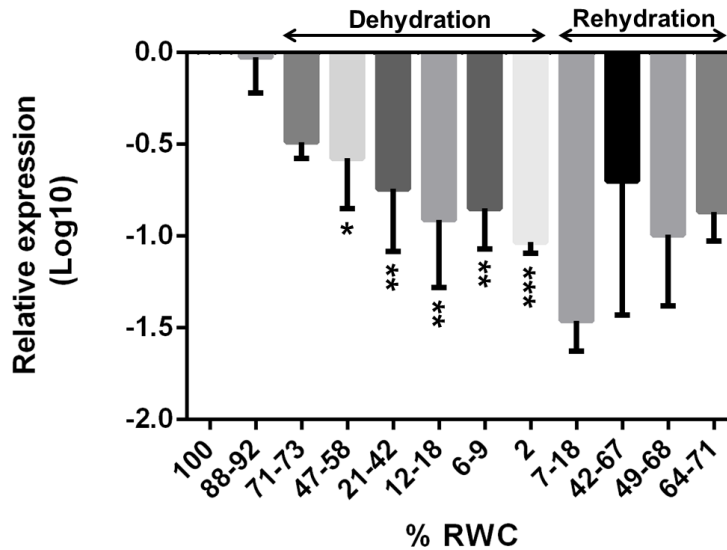


Figure 5.1: A bar graph illustrating the relative gene expression levels of *XvGolsII* during the dehydration and rehydration treatment of *X. viscosa*. Each data point represents the mean and the error bars represent the standard error of the mean (SEM), of relative expression levels of *XvGolsII* (N = 3), compared to *18S* ribosomal RNA, calculated using the Pfaffl method (Pfaffl MW., 2001). The \log_{10} change in expression levels is represented on the Y-axis and the % RWC values are represented on the X-axis. The level of significance in expression change is indicated by asterisks (*). The data represented here is statistically significant at RWC ranges 47 - 58% (*P = 0.0389), 21 - 42% (**P = 0.0065), 12 - 18% (**P = 0.0011), 6 - 9% (**P = 0.0021) and 2% (**P = 0.0003). The graph and SEM values were generated using the GraphPad Prism software (Version 5).

5.3.3.2 Cold stress treatment

The change in the transcription pattern and abundance of *XvGolsII*, under cold stress treatment, was monitored using real-time PCR. The expression levels of *actin*, in the same cDNA samples, were used for normalisation to correct for sample-to-sample and PCR efficiency variation. An up-regulation of less than 1-fold was seen after 6 and 24 hours of the cold treatment. However, after a one-way ANOVA of the data it was shown that this up-regulation was not statistically significant. At 48 and 72 hours of the cold treatment a more than 3- and 4-fold down-regulation of *XvGolsII* is observed, respectively. One-way ANOVA analysis showed this down-regulation to be statistically significant. It is possible that we may have missed a significant increase in transcript levels between 6 and 24 hours of the treatment and in future sampling points at 12 and 18 hours should be included. Translation of the

mRNA may subsequently have occurred and thus a decrease in mRNA observed at time points 24, 48 and 72 hours.

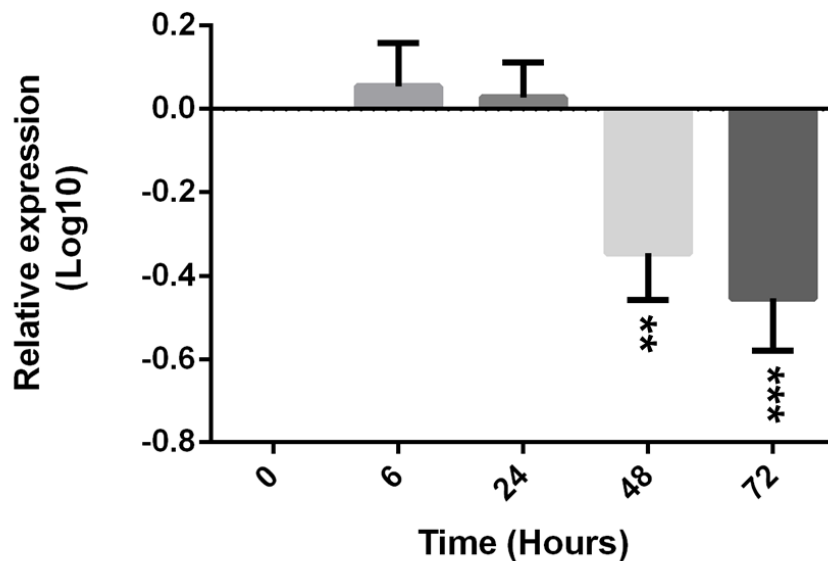


Figure 5.2: A bar graph illustrating the relative gene expression levels of *XvGolSII* during the cold stress treatment of *X. viscosa*. Each data point represents the mean and the error bars represent the standard error of the mean (SEM), of relative expression levels of *XvGolSII* (N = 3), compared to *actin*, calculated using the Pfaffl method (Pfaffl MW., 2001). The \log_{10} change in expression levels is represented on the Y-axis and the sampling times (hours) are represented on the X-axis. The level of significance in expression change is indicated by asterisks (*). The data represented here is statistically significant at 48 hours (**P = 0.0045) and 72 hours (***P = 0.0006). The graph and SEM values were generated using the GraphPad Prism software (Version 5).

It has been shown that the genes encoding GolS isoforms can exhibit differential gene expression with respect to water deficit, cold and salinity stress. The activity of GolS within kidney bean seeds has been shown to increase on exposure of the plants to cold (Liu *et al.*, 1998). When the expression pattern of three Arabidopsis genes encoding GolS enzymes (*AtGolS1*, *AtGolS2* and *AtGolS3*) was analyzed it was found that these three *GolS* genes were differentially expressed in response to various abiotic conditions (Taji *et al.*, 2002). *AtGolS1* and 2 were induced by drought and high-salinity stress but not by cold stress. *AtGolS3*, on the other hand, was induced by cold but not by drought and high-salinity stress.

Two distinct GolS isolated from *Ajuga reptans* sink and source leaves, designated *Gols-1* and *Gols-2*, respectively were both cold-inducible in sink and source leaves whether they were grown under warm or cold conditions (Sprenger & Keller, 2000). Peters *et al* (2007) showed that *XvGolS* was induced by dehydration using northern blot analyses but no study was made to determine if this gene was also induced in response to cold stress. Our results suggest that *XvGolSII* is not or specifically down-regulated in dehydrated and hydrated *X. viscosa* plants unlike *XvGolS*. Initial cold stress induced *XvGolSII* expression but our results are statistically not significant, possibly due to missing earlier time points closer to the onset of the cold stress treatment. Alternatively, it is also possible that the cold stress treatment was not severe enough to induce a significant increase in *XvGolSII* expression. However, as the cold stress treatment proceeded *XvGolSII* expression was down-regulated.

5.3.4 Western blot analysis of dehydrated and rehydrated *X. viscosa* protein samples

Western blot analysis was performed using *XvGolSII* specific polyclonal antibodies, to determine whether *XvGolSII* protein was expressed during the dehydration-rehydration treatment of *X. viscosa*. Before the western blots could be performed the *XvGolSII* antibodies were first purified and tested against recombinant *XvGolSII* protein.

5.3.4.1 Testing of *XvGolSII* antibodies

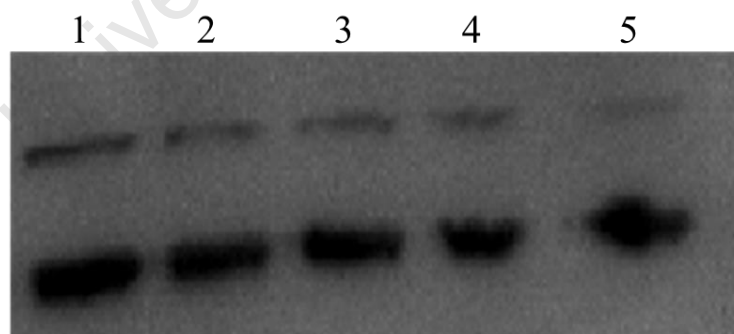


Figure 5.3: Western blot showing the chemiluminescent detection of recombinant *XvGolSII*, at varying amounts of the protein. Lanes: (1) 10 μ g; (2) 8 μ g; (3) 6 μ g; (4) 4 μ g; (5) 2 μ g.

In testing the *XvGolSII* antibody against varying amounts of recombinant *XvGolSII* protein it was found that 2 μ g of recombinant *XvGolSII* was a sufficient amount of protein needed for

positive detection by the XvGolSII antibody (Fig. 5.3). The optimal titre of the XvGolSII antibody was determined to be a 1:500 dilution (data not shown). The secondary antibody (goat anti-rabbit peroxidase conjugate) was optimal at a 1:20 000 dilution (data not shown).

It was thus decided to use 2µg of the recombinant XvGolSII protein as a positive control in subsequent western analysis of the crude protein under dehydration-rehydration treatments.

5.3.4.2 Western blot analysis of dehydrated and rehydrated total protein samples

Total crude protein that was previously extracted (Appendix A12) from *X. viscosa* leaf tissue for each time point from each biological replicate was separated using SDS-PAGE.

Recombinant XvGolSII protein was used as a positive control at the previously determined concentration of 2 µg and can be seen in Figure 5.4 and 5.5, indicated by a red arrow. The recombinant XvGolSII protein ran at ~45 kDa due to the mass of the affinity tags (discussed in Chapter 3, section 3.3.2.1).

Equal loading of the total crude protein for each time point was successfully achieved, as indicated by Ponceau Red staining of the nitrocellulose membranes (Fig. 5.4a and 5.5a). XvGolSII protein was not detected in hydrated nor in the respective dehydrated and rehydrated leaf tissues (Fig. 5.4b and 5.5b). Since recombinant XvGolSII protein was detected, it is inferred that there was no technical problem with the western blot and XvGolSII protein is not present in hydrated leaves, nor is it induced by severe dehydration and rehydration.

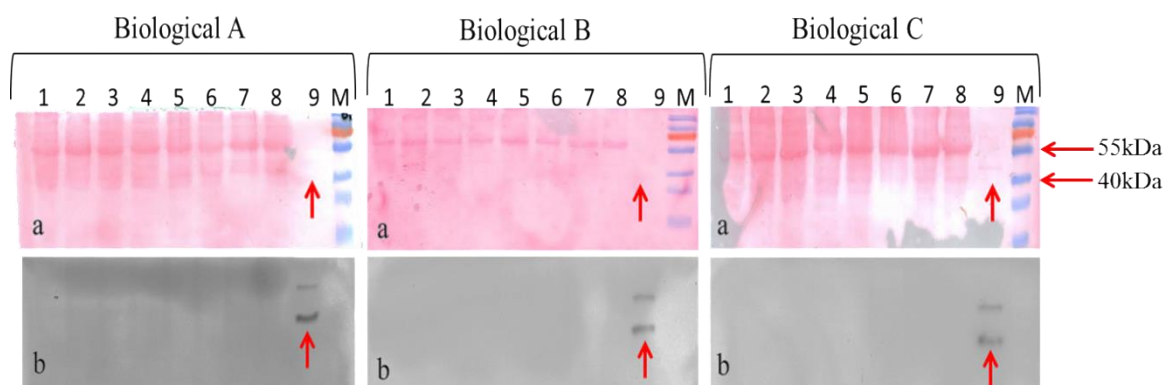


Figure 5.4: Western blot analysis of XvGolSII protein expression during dehydration treatment of *X. viscosa* plants, biological replicates a, b and c, respectively. Ponceau stained nitrocellulose membranes (a) showing equal loading of total crude protein; (b) chemiluminescence image of the corresponding Ponceau stained nitrocellulose membrane, taken using the Chemidoc molecular image, in which XvGolSII polyclonal antibodies were used to probe for XvGolSII protein. Lanes 1-8 contains 40 µg of total crude protein extract for the various RWCs. Lane 1: 100% RWC; lane 2: 88-92% RWC; lane 3: 71-73% RWC; lane 4: 47-58% RWC; lane 5: 21-42% RWC; lane 6: 12-18% RWC; lane 7: 6-9% RWC; lane 8: 2% RWC, lane 9: 2 µg recombinant XvGolSII protein; lane M: 5 µl prestained protein ladder (Fermentas). The red arrows indicate the recombinant XvGolSII protein.

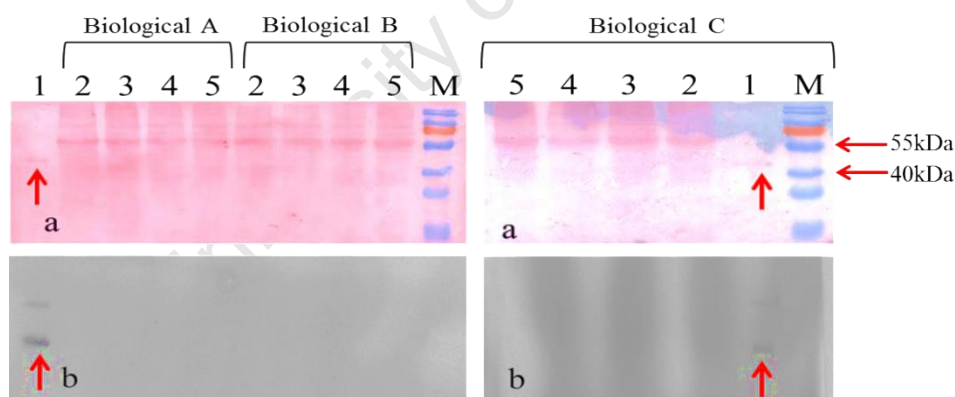


Figure 5.5: Western blot analysis of XvGolSII protein expression during rehydration of *X. viscosa* plants, biological replicates a, b and c, respectively. Ponceau stained nitrocellulose membranes (a) showing equal loading of total crude protein; (b) chemiluminescence image of the corresponding Ponceau stained nitrocellulose membrane, taken using the Chemidoc molecular image, in which XvGolSII polyclonal antibodies were used to probe for XvGolSII protein. Lanes 2-5 contains 40 µg of total crude protein extract for the various RWCs. Lane 1: 2 µg recombinant XvGolSII protein; lane 2: 7-18% RWC; lane 3: 42-67% RWC; lane 4: 49-68% RWC; lane 5: 64-71% RWC; lane M: 5 µl prestained protein ladder (Fermentas). The red arrows indicate the recombinant XvGolSII protein.

This result concurs with the real-time qPCR result which showed that *XvGolSII* mRNA transcript was not induced or present in dehydrated and rehydrated leaf tissue. Due to time constraints we were unable to perform western blot analysis to assess the protein expression pattern during the cold stress treatment. It is therefore important to include this in future work, so as to provide further clarity on the results obtained from the real-time qPCR.

5.4 CONCLUSION

In this chapter, the mRNA transcript expression of *XvGolSII* in response to dehydration and cold stress was investigated through quantitative real time PCR. A significant down-regulation of *XvGolSII* was observed in response to dehydration, particularly at 58 – 2% RWC. In response to cold stress a mild up-regulation of *XvGolSII* was observed at 6 and 24 hours of exposure but this was not statistically significant. A significant down-regulation of the gene was observed at 48 and 72 hours of exposure. Polyclonal *XvGolSII* antibodies were generated to the recombinant *XvGolSII* protein and after optimisation against the recombinant protein the antibody was used in subsequent western blot analyses. The protein expression in response to dehydration and rehydration was similar to that obtained for the real-time qPCR in that no *XvGolSII* protein was detected at the various RWCs. Due to time constraints we were unable to perform western blot analysis of the protein expression pattern during cold stress. This important aspect needs to be investigated in future, as this may provide further clarity into the results obtained from the qRT PCR.

Chapter 6

Conclusion

The aim of this dissertation was to molecularly characterize a putative galactinol synthase (*XvGolSII*) in the resurrection plant *Xerophyta viscosa* and elucidate its potential role in dehydration and cold stress tolerance. Molecular characterization of *XvGolSII* entailed *in silico* analyses, recombinant protein expression, enzyme activity assays, measurement of transcript and protein levels in response to dehydration and cold stress treatments and investigation of the subcellular localization of the *XvGolSII* protein.

Sequencing analysis of *XvGolSII* revealed its cDNA sequence to be 1434 bp long with the longest open reading frame (ORF) predicted to be 1018 bp in length. *In silico* analysis predicted this ORF to encode a 339 amino acid protein with a molecular weight of 38.7 kDa. The carboxy-terminal end of *XvGolSII* was shown to contain the characteristic hydrophobic pentapeptide, APSAA. *In silico* analysis of the *XvGolSII* protein sequence also predicted 15 potential sites of phosphorylation within the sequence and with no signaling peptides. In future work, selected putative phosphorylation sites could be mutated via site-directed PCR mutagenesis in order to determine the role of phosphorylation, if any, in *XvGolSII* functional activity.

Recombinant *XvGolSII* protein was successfully expressed in *E. coli* BL21 (DE3) cells and purified through both His-tag affinity purification and HPLC. Activity assays were performed using the recombinant *XvGolSII* protein and it was shown to be catalytically active, possessing the ability to convert the substrates *myo*-inositol and UDP-galactose to galactinol. The purified protein was subsequently successfully used for antibody generation.

Subcellular localisation of the *XvGolSII*::EYFP fusion protein showed localisation to the cell membrane. It is however important to understand the tissue-specific localisation of the protein so as to determine where this enzyme could be exerting its effect and thereby providing better insight into its potential role in response to stress. Future work should therefore include techniques such as immuno-localisation studies of *XvGolSII* in different

tissues such as roots, stems, phloem or mesophyll tissue, in order to understand tissue-specific roles of the enzyme.

Examination of the transcription (mRNA) levels during both dehydration and cold stress treatment was conducted using quantitative real-time PCR analysis and protein expression levels were examined through western blot analysis using XvGolSII-specific polyclonal antibodies. During the dehydration treatment of *X. viscosa*, quantitative real-time analysis showed a statistically significant down-regulation in *XvGolSII* mRNA transcript expression at RWC ranges 47 - 58% ($P = 0.0389$), 21 - 42% ($P = 0.0065$), 12 - 18% ($P = 0.0011$), 6 - 9% ($P = 0.0021$) and 2% ($P = 0.0003$). This result corresponded with the results obtained from the western blot analysis in which no XvGolSII protein was detected in the both the dehydrated and rehydrated samples across all three biological replicates.

Quantitative real time analysis of the cold treated *X. viscosa* plants showed a mild but not statistically significant up-regulation of *XvGolSII* mRNA transcript expression during the early phase of the treatment (6 - 24 hours). This up-regulation was followed by a statistically significant down-regulation of transcript expression at 48 and 72 hours of the cold treatment with P values of 0.0045 and 0.0006, respectively. It is possible that we may have missed a statistically significant increase in transcript expression between 6 and 24 hours and in future this treatment should include a sampling point at 12 and even 18 hours after exposure to the cold treatment. This gene was initially isolated from a cold stress library where *X. viscosa* plants were subject to 4°C cold stress treatment for 12 h before RNA extraction and subsequent creation of the cDNA library. At these points the *XvGolSII* transcripts may have been expressed and subsequently translated to produce the protein and hence the down-regulation in transcript expression observed at time points 48 and 72 hours. It is also possible that the cold stress treatment was not severe enough for significant up-regulation of *XvGolSII* transcript, though the *X. viscosa* cold stress library and previous studies (Bachmann & Keller, 1995; Liu *et al.*, 1998; Taji *et al.*, 2002) have used similar low temperatures.

Due to experimental design constraints only one leaf per biological per time point could be sampled. We may therefore, in future, require more biological and technical replicates in order to see significant changes in response to cold stress.

Due to time constraints we were unable to extract total proteins from the cold treated plants and perform western blot analysis. This should however be completed in order to understand the expression of the XvGolSII protein during the cold stress treatment and to gain a better understanding into the results obtain from the quantitative real time analysis. It would be interesting to assess the activity of XvGolSII within crude extracts of plants exposed to both dehydration- and cold-stress treatment. If it is confirmed that *XvGolSII* is induced only in response to cold as opposed to dehydration then this would be the first report of differential *GolS* expression in a resurrection plant.

Previous studies have shown that water loss and salinity stress specially induces differential expression of GolS isoforms (Taji *et al.*, 2002). In future other stress treatments on *X. viscosa* such as salinity or oxidative damage can be explored to determine whether these induce XvGolSII expression. It has also been suggested that RFOs play a role in carbon partitioning (Saravitz *et al.*, 1987; Liu *et al.*, 1998) and it may be that XvGolSII has a minor role in abiotic stress but is involved in reallocating carbon. However, given that responses to stress and carbon partitioning are intimately linked, one would have still expected to see XvGolSII induction if this was the specific case.

Another useful strategy that could be employed in the future, with the aim of further understanding the regulation of expression of *XvGolSII*, is the investigation of gene promoter regions which are involved in the activation of gene expression under various abiotic stresses. This would require isolation and sequencing of the promoter region of *XvGolSII* and linking it to reporter genes such as luciferase or GUS. The *XvGolSII* promoter activity and expression profile could then be assessed by measurement of the expression of both the mRNA and protein of the reporter genes, as expression of these gene would be driven by the XvGolSII promoter. This could be assessed in either a quantitative way or through visualization of the activity of the reporter genes in different tissues.

We also suggest that sequence motifs within the *XvGolSII* promoter sequence should be isolated via the splinkerette method and analysed to determine *in silico* which transcription factors could control the promoter activity. It is currently known that various transcription factor regulate GolS promoter activity, these include WRKY transcription factors, heat shock factors, dehydration responsive-element binding factors and C-repeat binding factors (Wang *et al.*, 2009; Panikulangara *et al.*, 2004; Taji *et al.*, 2002).

In conclusion, the aim of gaining a better understanding of XvGolSII on a molecular basis has been achieved. We have successfully characterized XvGolSII through *in silico* analyses, recombinant protein expression, gene expression profiling and subcellular localization.

Appendices

Appendix A

Protocols

A1 Standard PCR reaction

A2 Standard ligation protocol

A3 Standard transformation protocol

A4 Plasmid extraction

A5 PCR product purification

A6 Purification of DNA from agarose gels

A7 Standard SDS-PAGE conditions

A8 Bradford assay

A9 ELISA analysis of XvGolS antibody titre

A10 Dehydration treatment

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A12 Total crude protection extraction

Appendix B

Primer sequences and bioinformatics

Appendix C

Vector maps

Appendix D

Real-time analysis

Appendix E

Biolistic particle delivery system

Appendix F

Arabidopsis eFP Browser abiotic stress expression profiles for *AtGolS1, 2 and 3*

Appendix A

Protocols

A1 Standard PCR reaction

All PCR reactions were performed using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, Singapore). For each amplification, 25 μ l reactions were set up. Unless stated otherwise, NEB Taq polymerase was used for each PCR reaction. The concentration of each of the PCR components is summarized in Table A1.

Component	Final concentration
Primer concentration	0.4 μ M
dNTP mix	200 μ M
NEB Taq polymerase	0.04 U/ μ l
Standard Taq buffer	1X
Template DNA	Varied

Table A1: PCR reagents and final working concentrations in a standard protocol.

A2 Standard ligation protocol

Purified DNA fragments were ligated to the relevant linearized vectors in a reaction comprising 50 ng vector with corresponding amount of insert DNA for a 1:3 ratio of vector:insert. In each ligation reaction 5U of T4 ligase was used. The reaction volume was made up to 20 μ l, mixed well and incubated at 4°C overnight.

A3 Standard transformation protocol

E.coli competent cells were thawed on ice for approximately 30 minutes. Approximately 1-10 ng of DNA was added to the competent cells and gently mixed. The transformation mix was incubated on ice for 10 minutes. The cells were heat shocked at 37°C for 5 minutes and immediately placed on ice for 2 minutes. Nine times more LB broth was added to the transformation mix and incubated at 37°C for 1 hour with vigorous shaking. The transformation mix was plated on LB agar plates, supplemented with the appropriate antibiotic, and incubated at 37°C overnight.

A4 Plasmid extraction

Plasmid DNA was extracted using a method adapted from the QIAGEN® plasmid purification handbook. Bacterial cells (ca. 3 ml) were centrifuged for 15 min at 7000 x g at RT. The supernatant was discarded and pellet was resuspended in 500 µl of P1 buffer. Five hundred microlitres of chilled P2 buffer was added to the mix and vigorously inverted 4 to 6 times and left for 5 minutes at RT. Five hundred microlitres of P3 buffer was added, mixed immediately and incubated on ice for 15 min. The mix was centrifuged for 5 minutes at 7000 x g and the supernatant was retained. Nine hundred microlitres of supernatant was added to 600 µl of isopropanol and left for 2 minutes at RT. This was centrifuged for 9 minutes at RT. The supernatant was carefully decanted and 300µl ethanol added to wash the pellet through centrifugation for 2 minutes at 7000 x g. The ethanol was removed and pellets were left to dry. The dried pellets were resuspended in 40 µl of either water or TE buffer.

A5 PCR product purification

Amplified DNA was purified using the Bio Basic EZ-10 Spin Column PCR Purification Kit (Bio Basic Inc., Canada). Three volumes of binding buffer was added to the PCR mix, mixed well and transferred to a EZ-10 column and was allowed to stand at RT for 2 minutes. The column was centrifuged at 10 000 x rpm for 2 minutes and the flow-through removed. Five hundred microlitres of wash solution was added to the column and centrifuged at 10 000 x rpm for 2 minutes. This wash step was repeated and the column centrifuged for an additional minute to remove any residual wash solution. The column was then transferred to a sterile 1.5 ml microfuge tube and 40 µl of elution buffer added to the column. The column was incubated at 37°C for 5 minutes. To elute the plasmid DNA the column was centrifuged at 10 000 x rpm for 2 minutes.

A6 Purification of DNA from agarose gels

The DNA fragments excised from agarose gels were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, USA). The excised gel slice was placed in a pre-weighed 1.5 ml Eppendorf tube and its mass determined. Membrane binding solution was added to the gel slice in a ratio of 10 µl of solution per 10 mg of agarose gel slice. The mixture was vortexed and incubated at 50 - 65°C for 10 minutes or until the gel was completely dissolved. The tube was vortexed every few minutes to increase the rate of melting of the gel. The dissolved gel mixture was then added to a mini-column and incubated

for 1 minute at RT. The mini-column assembly was centrifuged at 14 000 x rpm for 1 minute and the supernatant discarded. The mini-column was washed by adding 700 µl membrane wash solution and centrifuged at 14 000 x rpm for 1 minute. The supernatant was discarded and the wash step repeated. The column was transferred to a clean 1.5 ml Eppendorf tube and 50 µl of nuclease-free water added directly to the centre of the column and allowed to stand for 1 minute at RT. The column was centrifuged at 14 000 x rpm for 1 minute to elute the DNA.

A7 Standard SDS-PAGE conditions

SDS-PAGE was carried out using 5% stacking gel (40 % acrylamide (w/v), 20 % SDS (w/v), 1 M Tris (pH 6.8), 10% ammonium persulphate (AMPS) (w/v), 20 µl TEMED) and a 12% resolving gel (40 % acrylamide (w/v), 20 % SDS (w/v), 3 M Tris (pH 8.8), 10 % AMPS (w/v), 20 µl TEMED). Each gel was run at 20 mA (constant current). Protein integrity was determined by staining gels with Coomassie blue (Bio Basic Inc., Canada) (0.1-0.5 % Coomassie brilliant blue, 40% methanol, 10% acetic acid, 50% dH₂O) for 30 minutes at 37°C and destained using destain solution (40% methanol, 10% acetic acid, 50% dH₂O) until bands were clearly visible.

A8 Bradford assay

Bradford assays were performed using Bradford reagent (BioRad, USA). All reactions were performed in triplicate with a total volume of 1 ml. Component volumes are summarized in table A9.

Table A9: Component volumes of the Bradford assay for BSA concentrations ranging from 0 to 10 µg/µl

	Blank	1.2 µg/µl	2.5 µg/µl	5.0 µg/µl	7.5 µg/µl	10 µg/µl
BSA (2mg/ml)	0	0.6	1.25	2.5	3.75	5
Buffer (µl)	10	10	10	10	10	10
dH ₂ O (µl)	790	789.4	788.75	787.5	786.25	785
Bradford reagent (µl)	200	200	200	200	200	200
Total volume = 1 ml						

A9 ELISA analysis of XvGolSII antibody titre

A 96-well polysorb ELISA plate was coated with 300 ng of XvGolS protein (100 μ l total volume) in 1 x PBS buffer, covered with clingwrap and incubated at 37°C for 2 hr. After incubation excess protein was tipped out and the wells washed three times with TBS/Tween buffer (100 mM NaCl; 10 mM Tris, pH 7.5; 0.1% Tween-20). The wells were then blocked with 5% skim milk powder in TBS/Tween buffer for 1 hr at room temperature. The wells were filled with 200 μ l of the blocking solution. Excess blocking solution was tipped out and the TBS/Tween wash steps repeated. The first column of the plate was used as a substrate blank, containing all the components except primary antibody. Dilutions (10^{-2} , 10^{-4} , 10^{-6} and 10^{-8}) of the various bleeds (primary antibody) were made using TBS/Tween buffer and placed in the respective wells (Fig. A1) and incubated at room temperature for 1 hr. Excess antibody was tipped out and the TBS/Tween wash steps repeated. Secondary goat anti-rabbit alkaline phosphatase was added to each well at a dilution of 1/5000 in TBS/Tween and incubated at room temperature for 30 min. Excess secondary antibody was tipped out and the TBS/Tween wash steps repeated. The wells were washed once with 10% diethanolamine/0.5 mM MgCl₂, pH9.6. Substrate (1 mg/ml p-nitrophenyl phosphate [PNP] in 10% diethanolamine/0.5 mM MgCl₂, pH9.6) was then added to the wells in a volume of 100 μ l and allowed to stand at room temperature for 30 min. Readings were taken using the Titretrek Multiscan plate reader with a 405 nm filter.

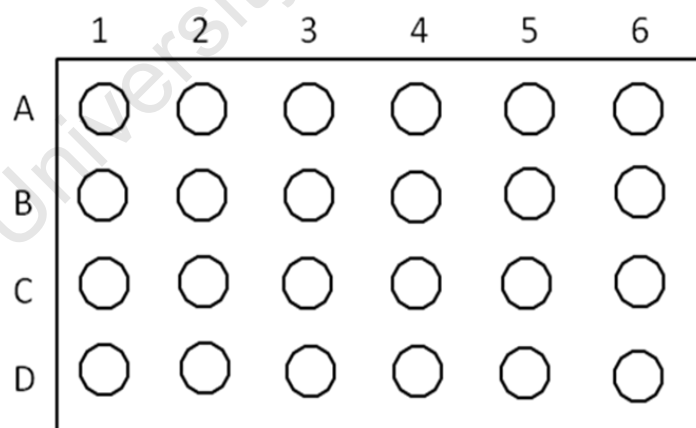


Figure A1: Schematic diagram of the setup of the ELISA plate. Column 1 represents the blank. Columns 2 – 6 represents the pre-bleed; bleed 1; bleed 2; bleed 3 and bleed 4, respectively. Rows A –D represent the dilutions of 10^{-2} ; 10^{-4} ; 10^{-6} and 10^{-8} , respectively.

A10 Dehydration treatment

Three *X.viscosa* plants were dehydrated by withholding water for a 16-day period. The dehydration process was monitored by determining the relative water content (RWC) of the leaf samples. For each time point 4 leaves were excised per plant. Leaf samples were collected just before the start of dehydration and then on days 3, 5, 7, 9, 11, 13 and 15 of the dehydration stress treatment. On the 16th day the plants were watered and the soil kept damp throughout the remainder of the experiment. Leaf samples were then collected 16, 30, 42 and 64 hours after rehydration. The absolute water content (AWC) was determined at the beginning of the treatment. The leaves were torn vertically in half and the portion containing the central vein was used to immediately determine the fresh weight (FW) for each plant and then placed in a 70°C oven for 2 days and subsequently placed in a desiccator for 10 min and weighed to determine the dry weight (DW). The remaining half of the leaf was cut up into pieces, placed in a labeled foil packet and immersed in liquid nitrogen and stored at -80°C. Sampling of the leaves was carried out at 10 am in the morning.

$$AWC = (FW-DW)/DW$$

AWC was calculated for each leaf. For each plant, three AWC values were selected and the average of these values recorded as the whole plant RWC. The full turgor weight (FTW_i) for each plant was determined from only the initial sampling point, prior to the start of the dehydration treatment. The FTW_i value was calculated using the AWC equation and RWCs at each time point (n) were calculated using the following formula:

$$RWC_n = (AWC_n/FTW_i) \times 100$$

A11 RNA extraction

The RNA extraction method was adapted from Kershini Govender PhD Thesis, UCT. All plastic and glassware used in the extraction were double-autoclaved and all solutions were prepared using diethylpyrocarbonate (DEPC) water.

Leaf material that was previously stored at -80°C was ground in liquid nitrogen using pestle and mortars. A small amount (a small spatula full) of autoclaved acid purified calcinated sand and polyvinylpyrrolidone (PVPP) was added to the leave material whilst grinding.

Seven hundred and fifty microlitres of Trizol (Invitrogen Life Technologies, USA) was added to the eppendorfs containing ground plant material and vortexed for 5 min. The mixture was then incubated for 5 min at room temperature. Two hundred microlitres of chloroform was added to the homogenised samples and gently mixed by inversion for approximately 1 min. Tubes were incubated for 3 min at room temperature then centrifuged for 15 min at $12\ 000 \times g$ at 4°C . The aqueous phase was carefully transferred to a fresh eppendorf tube and the organic phase stored at -80°C for protein extraction. Five hundred microlitres of isopropanol was added to the aqueous phase and incubated for 10 min at room temperature to allow for precipitation of the RNA. The RNA was pelleted by centrifugation for 10 min at $12\ 000 \times g$ at 4°C and the supernatant discarded. The RNA pellet was washed in 1 ml of cold 75% ethanol (EtOH) by centrifugation at $6\ 000 \times g$ at 4°C and the EtOH discarded. The samples were briefly centrifuged again and the residual EtOH carefully removed with a pipette. The RNA pellet was air-dried for approximately 10 min (longer if needed). Fifty microlitres of DEPC water was added to the RNA pellet and incubated at 55°C to aid in resuspension of the pellet.

A12 Total crude protein extraction from plant material

Protein extraction was performed individually from each leaf sampled per plant for western blot analysis. The total protein was extracted from organic phase retained during RNA extraction (Appendix A11). Three hundred microlitres of cold absolute EtOH was added to this, inverted approximately 25 times and incubated at RT for 5 min. The protein extract was centrifuged at $2\ 500 \times g$ for 10 min at RT and the supernatant containing soluble protein was transferred to a fresh 2 ml microcentrifuge tube containing 1.5 ml isopropanol. The soluble protein extract was incubated at RT for 10 min to aid precipitation of protein and subsequently centrifuged at $10\ 250 \times g$ for 10 min at 4°C . The protein pellet was washed thrice with 2 ml of 0.1M ammonium acetate (Appendix B8), washed once with 2 ml cold acetone and thereafter air dried for ca. 10 min. The pellet was resuspended in a total volume of 100 μl of urea lysis buffer (Appendix B9) supplemented with 125 mM of DTT. The protein suspension was vortexed at RT for 30 min to aid resuspension. The total protein extraction was then quantified using the Bradford Reagent (Biorad, Germany) method

(Appendix A8) and based on these concentrations, aliquots of extracted protein was electrophoresed according on a 12% polyacrylamide gel (standard SDS-PAGE conditions; Appendix A7) to check protein integrity as well as attempt equal loading.

Appendix B

Primer sequences and bioinformatics

Table B1: The list of primers used in this study. Restriction enzyme sites are seen in bold and underlined.

Number	Primer name	Primer sequence	Amplicon length (bp)
B1.1	pETB19EcoR1	5'- <u>AGAATTC</u> GATGGCCCCACCTGAGGTC-3'	
B1.2	XvB19pETRv	5'- <u>AGAGCTCA</u> AAGGCTGCAGATGGTGCTGG-3'	1000bp
B1.3	Gol19YFPFw	5'-AA <u>GATCCA</u> AATGGCACCACCAGAGGTC-3'	
B1.4	Gol19YFPRev	5'-AA <u>GGTACC</u> GCGGCTGCAGATGGTGC-3'	1000bp
B1.5	GolSRT_Fwd II	5'-GCCAGCAGTGCCCTGACAAG-3'	
B1.6	GolSRT Rev II	5'-GGCGGCTCGGCTCGTAAAC-3'	100bp
B1.7	Xv18S RT-F	5'-GCAAATTACCCAATCCTGACACG-3'	
B1.8	Xv18S RT-R	5'-TGGCACCAGACTTGCCCTCC-3'	131bp
B1.9	GolSII_qRT_F	5'-CGCCGGCTACTCCAAGCG-3'	
B1.10	GolSII_qRT_R	5'-GATCAACGGGTACGCGCTC-3'	117bp
B1.11	Actin RT_F	5'-CGACCACGCCACACCGGAGTTAT-3'	
B1.12	Actin RT_R	5'-CATCCCAATTGCTTACAATCCCGTGCTCTA-3'	133bp

Oryza	MAPPQLAGKMTAK---AAAAVKPAT-----RAYVTFLAGGDYWKGVVGLAKGLRKKVGS	52
Zea	-MSPELTGKMAAKAAAAAAVKPAT-----RAYVTFLAGGDYWKGVVGLAKGLRKKVGS	54
Brassica	-MAPELTQTTTVKSAVTITKPSPPVHG--DRAYVTFLAGNGDYVKGVVGLAKGLRKKVKS	57
Capsicum	-MAPELTQTTTVKSAVTITKPSPPVHG--DRAYVTFLAGNGDYVKGVVGLAKGLRKKVKS	57
Vitis	-MAPEIISAS-GKPSRFLKPAASLPB-----RAYVAFLAGNGDYVKGVVGLAKGLRKKVKS	53
Solanum	-MAPNVFGLA-TKATGLAKAKSLAS-----RAYVTFLAGNGDYWKGVVGLVKGLRKA	53
Xerophyta	-MAPEIVSKR--AANYAGKQVAAPR-----NAYVTFLAGAGDYVKGVVGLAKGLRKKVGS	52
Medicago	-MSPDIITAA---TNITNTQSKATK-----RAFVTFLAGNGDYVKGVVGLAKGLRKKVKT	51
Coptis	-MAP-AVT--VDGFTSTGKVATLN-TGYSKRAYVTFLAGSGDYVKGVVGLAKGLRKKVKS	55
Populus	-MAP-GVP--MDVISCTGKVSTAS-TGYSKRAFVTFLAGNGDYVKGVVGLAKGLRKKVKS	55
Coffea	-MAPQVEVP--AAEFTAAGKVSTLSNTGYSKRAYVTFLAGNGDYVKGVVGLAKGLRKN	57
Verbascum	-MAP-----IIGKIPALG---SRKAYVTFLAGTGDYKGVVGLAKGLRKKVKS	44
XvGolSII	-MAPPEVAGAVDAAAFTEGKLAVPN-AGYSKRAYVTFLAGSGDYWKGVVGLAKGLRKKVKS	58
Arabidopsis	-MAP-EIS--VNPMLSEKAHQAP---PRRAYVTFLAGNGDYVKGVVGLAKGLRKKVKS	52
Cucumis	-MAL-----ELSENVLTTG--ASTERAYVTFLAGNGDYVKGVVGLAKGLRKKVATA	47
	. *:*.***** ** * ** . * . * . * . * . *	
Oryza	YPLVVAVLPDVPESHRRILISQGCIVREIEPVYPPENQTFAMAYVINYSKLRIEWE	112
Zea	YPLVVALLPDPVPESHRRILVSQGCILREIEPVYPPENQTFAMAYVINYSKLRIEWE	114
Brassica	YPLVVAILPDPVPEHRRVLVEQGCIVREIEPVYPPENQTFAMAYVINYSKLRIEWE	117
Capsicum	YPLVVAILPDPVPEHRRVLVEQGCIVREIEPVYPPENQTFAMAYVINYSKLRIEWE	117
Vitis	YPLVVAVLPDPVPEHRRLEESQGCIVREIEPVYPPENQTFAMAYVINYSKLRIEWE	113
Solanum	YPLVVACLDPVPEHRRILINQGCIVREIEPVYPPENQTFAMAYVINYSKLRIEWE	113
Xerophyta	YPLVVAVLPDPVPEHRRKLVSQGCIVREIEPVYPPENQTFAMAYVINYSKLRIEWE	112
Medicago	YPLVVAVLPDVPQEHRRNILTSQGCIVREIVPVYPPENQTFAMAYVINYSKLRIEWE	111
Coptis	YPLVVAMLPDPVPEHRRKILRSQGCILKEIEPIYPPENQIFAMAYVINYSKLRIEWE	115
Populus	YPLVVAMLPDPVPEHRRDILRSQGCIVREIEPIYPPENQIFAMAYVINYSKLRIEWE	115
Coffea	YPLVVAILPDPVPEHRRILRSQGCIVREIEPIYPPENQIFAMAYVINYSKLRIEWE	117
Verbascum	YPLVVAILPDPVPEHRRILKRSQGCIVKEIEPIYPPENQIFAMAYVINYSKLRIEWE	104
XvGolSII	YPLIVAMLPDVPEDHRRILRSQGCIVREIEPVYPPENQIFAMAYVINYSKLRIEWE	118
Arabidopsis	YPLVVAMLPDPVPEHRRILRSQGCIVREIEPVYPPENQIFAMAYVINYSKLRIEWE	112
Cucumis	YPLVVAVLPDVPPIEHRKILKASQGCIVREIEPIYPPENQIFAMAYVINYSKLRIEWE	107
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Oryza	YERMVYLDADIQVFDNIDELFELPKGHFYAVMDCFCEKTSHTPQYQIGYCQQCPDKV	172
Zea	YERMVYLDADIQVFENIDELFELEKGYFFAVMDCFCEKTSHTPQYRIGYCQQCPDKV	174
Brassica	YSKMIYLDGDIQVYENIDHLFDLDPGYFFAVMDCFCEKTSHTPQYKIGYCQQCPDKV	177
Capsicum	YSKMIYLDGDIQVYENIDHLFDLDPGYFFAVMDCFCEKTSHTPQYKIGYCQQCPDKV	177
Vitis	YSKMIYLDGDIQVYDNIDHLFELPDGHFYAVMDCFCEKTSHTPQYKIGYCQQCPDKV	173
Solanum	YSKMIYLDGDIQVFDNIDHLFDLDPGYFFAVMDCFCEKTSHTPQYKIGYCQQCPDKV	173
Xerophyta	YSKMIYLDADIQVYDNIDHLFDLPGGRFYAVMDCFCEKTSHTPQYKIGYCQQCPDKV	172
Medicago	YDKMIYLDGDIQVFENIDHLFDLNNYFFAVMDCFCEASWRHTKQYIEIGYCQQCPDKV	171
Coptis	YSKMIYLDADIQVFENIDHLFDTPNGYFFAVMDCFCEKTSHTSPQYVSVGYCQQCPDKV	175
Populus	YSKMIYLDADIQVFENIDHLFDTPNGYFFAVMDCFCEKTSHTSPQHSIGYCQQCPDKV	175
Coffea	YSKMIYLDADIQVYDNIDHLFDADGYFFAVMDCFCEKTSNSPQYSIGYCQQCPDKV	177
Verbascum	YSKMIYLDADIQVYENIDHLLDTPNGYFFAVMDCFCEATWAHSRQFSIGYCQQCPDKV	164
XvGolSII	YSKMIYLDADIQVYDNIDHLFDMPDGYFFAVMDCFCEKTSHTSRQFSIGYCQQCPDKV	178
Arabidopsis	YSKMIYLDADIQVFDNIDHLFDLSDAYFFAVMDCFCEKTSHTSLQYSIGYCQQCPDKV	172
Cucumis	YKMIYLDGDIQVYDNIDHLFEEDGHFYAVMDCFCEKTSHTSPQYKIGYCQQCPDKV	167
	* . * : * * * . * * * : : * * . * : * . * : * * * * * : * * : * . * : * * * * * : * * *	
Oryza	PTAELGPPPA-LYFNAGMFVHEPSMATAKSLDRLVTTPTFAEQDFLNMFFREQYKPI	231
Zea	PTTELGPPPP-LYFNAGMFAHEPSMATAKALLDRLVTPPTFAEQDFLNMFFRDQYRPI	233
Brassica	PKEELGPPPS-LYFNAGMFVFEPLDTEYEDLLRRLKITPPTFAEQDFLNMFFEKIYKPI	236
Capsicum	PKEELGPPPS-LYFNAGMFVFEPLDTEYEDLLRRLKITPPTFAEQDFLNMFFEKIYKPI	236
Vitis	P-AELGQPPS-LYFNAGMFVFEPLSTYEDLLETLRITPATFAEQDFLNMFFXDVYKPI	231
Solanum	T-QDLGPKPS-LYFNAGMFVFEPLSIYDILLKTVKVTPTPTFAEQDFLNMFFRDYKPI	231
Xerophyta	P-AELGQPPA-LYFNAGMFVHEPSLATAEKLLATLKVAPTPTFAEQDFLNMFFKDIYRPI	230
Medicago	P-TNFGPKPP-LYFNAGMFVYEPNMATYHDLQKLVTEPTFAEQDFLNMFFNDKYKPI	229
Coptis	P-AEMGSPPP-LYFNAGMFVFEPSRLTFESLIENLRITAPTFAEQDFLNMFFNHVYKPI	233
Populus	P-AEMGSPPP-LYFNAGMFVFEPSRLTYESLLETQITPPTFAEQDFLNMFFQTYKPI	233
Coffea	P-ADMGSPPP-LYFNAGMFVFEPSRLTYENLLETQITPPTFAEQDFLNMFFQTYKPI	235
Verbascum	P-TEMGSPPP-LYFNAGMFVFEPNQTTYENLLETLCITPPTFAEQDFLNMFFEKTYKPI	222
XvGolSII	P-ADMGNPPA-FYFNAGMFVFEPSRLTCDLLETLRVTPPTFAEQDFLNMFFFEKTYKPI	236
Arabidopsis	P-EDMESPPPLLYFNAGMFVFEPSRLTYESLLETQITPPTFAEQDFLNMFFKTYKPI	231
Cucumis	P-PASGSPPP-LYFNAGMFVFEPSRETYKSLETLRVTPPTFAEQDFLNMFFKTYKPI	225
	. * . : * * * * * . * * . * : : : . : * * * * * : * * : * * * *	
Oryza	PLIYNLVLAMLWRHPENV-QLEKVKVVHYCAAGSKPWRYTGKEANMDREDIKMLVKKW	290
Zea	PNVYNLVLAMLWRHPENV-QLEKVKVVHYCAAGSKPWRYTGKEANMDREDIKMLVKKW	292
Brassica	PLVYNLVLAMLWRHPENV-ELDKVKVVHYCAAGSKPWRYTGKEANMEREDIKMLVKKW	295
Capsicum	PLVYNLVLAMLWRHPENV-ELDKVKVVHYCAAGSKPWRYTGKEANMEREDIKMLVKKW	295
Vitis	PLVYNLVLAMLWRHPENV-ELDKVKVVHYCAAGSKPWRYTGKEDNMQREDIKMLVKKW	290
Solanum	PNNYNLVLAMLWRHPENV-DLDKVKVVHYCAAGSKPWRYTGKEANMDREDIKMLVKKW	290
Xerophyta	PLTYNLVLAMLWRHPENV-ELDKVMVVHYCAAGSKPWRYTGKEANMDREDIKMLVKKW	289



Figure B1: Multiple sequence alignment between XvGolSII and other GolS protein sequences from various plant species using the ClustalW2 server. Fifteen GolS amino acid (aa) sequences in total are aligned: *O. sativa*, *Z. mays*, *B. napus*, *C. annuum*, *V. vinifera*, *S. tuberosum*, *X. viscosa* (GolSI and II), *M. sativa*, *C. japonica*, *P. trichocarpa*, *C. arabica*, *V. phoeniceum*, *A. thaliana* and *C. melo*. An asterisk (*) represents positions which have a single, fully conserved aa across all species. A period (.) indicates conservation between groups of weakly similar properties. A colon (:) indicates conservation between groups of strongly similar properties.

XvGolSII	ATGGCTCCACCAGAGGTGCGGGGCGCGTGGCGCGCCGCTTACCGGAPAGCTAGGGGTGCCAAGCGCGGGCTACTCCAAAGCGTGCCTACGTTACT	99
XvGolSATGGCGCGGAGTCTGTGAGCAAGAGGGCTCCGACTACGCCGGGAAGCGTGGCGACTCCGCCAAAGCGCTACCTACGTTACT	81
Consensus	gggcccg ga g c g a cta g g ggc ctcc g gctactgt act	
XvGolSII	TTTCTGCGCGGCGAGCGCGATTACTGGCAAGGGCGTGGTGGCTGGCAAGGGGGTGGCAAGGGTAAGAGCGCGTACCCTTGGTCTGCTGCCATGCTTC	199
XvGolS	TTTCTGCGCGGAGCTGGCGACTAGCTTAAGGGCGTGGTGGCTGGCAAGGGGGTGGCAAGGGTAAGAGCTGCTTACCCTTACTGCTTGGTGGCTGCTCC	181
Consensus	tt ct gc gg gg ga tac aagggcgctggtg ct gc aaggggctg g aa gta gc taccct tt t gt gc tgct c	
XvGolSII	CGGATGTTCCCGAGGATCACCGGCAATCTCCAGCTCCAGGGTGCATCGTCAGGAGCATCGAGCGGTGTACCCTCCGGAGAACCAGATTCACTTCGC	299
XvGolS	CGGACGTTCTCCGAGGATCACCGGCAAGCTCTGGTGTCCAGGGTGCATCGTTCAGGCAATCGAGCCCGTGTACCCTCCGGAGAACCAGATCCCAATTCGC	281
Consensus	c ga gttcc ga ga caccgg a t ct gtcccaggg tgcactgt agg a atcgagcc ggtgacct cc gagaaccaga ca ttccg	
XvGolSII	CATGGCTTACTACGTTATCAACTACTCCAAGCTTCGCATCTGGCACTTTCAGGAGTACAGCAAGATGATATACCTGGATGCCGATATCCAAGTTACGAC	399
XvGolS	CATGGCTTACTACGTTATCAACTACTCCAAGCTTCGCATCTGGCACTTTCAGGAGTACAGCAAGATGATATATTTGGAGCGGATATCCAAGTTACGAT	381
Consensus	catggc ta tacgt atcaacta tc aagct cgcact tgg a tttg gtagta agcaagatgata tggg gc ga atccaagt tacga	
XvGolSII	AACATGACCACCTCTTGACATGCCGCGGGTAGTTCTACGGGTTATGGACTGCTTCTGCGAGAAGACATGGAGCCATTCCGGCCAGTTTCCATCG	499
XvGolS	AACATGACCACCTCTTGACATGCCGCGGGTAGTTCTACGGGTTATGGACTGCTTCTGCGAGAAGACATGGAGCCATTCCGGCCAGTACAAPATTG	481
Consensus	aacat gaccacct tt gac tgcc g cggg ttctacgc gt atggactgcttctgcgagaagacatggagcca cgc cagt at g	
XvGolSII	GCTACTGCCAGAGTGCCTGACAAGGTCATGCGCCGGCGGACTGGCCATCTCCCGGCTTCTACTTCAATGCAGGATGTTGTTTACGAGCCGAG	599
XvGolS	GCTACTGCCAGAGTGCCTGACAAGGTCATGCGCCGGCGGACTGGCCATCTCCCGGCTTCTACTTCAATGCAGGATGTTGTTTACGAGCCGAG	581
Consensus	gctactgccagagtg cc acaaggt a tggccggcggg tggg a cc cc gc t tacttcaa gcagg atggt gtt acgagc ag	
XvGolSII	CCGCTTACTTCGATGCTCTCTTTCAGACCTCCGCGTCACTCCCACTCCACCCCTTCGCGGACGCTTCTCTGAACATGTTTTCAGGATGATCTAC	699
XvGolS	CCGCTTACTTCGATGCTCTCTTTCAGACCTCCGCGTCACTCCCACTCCACCCCTTCGCGGACGCTTCTCTGAACATGTTTTCAGGATGATCTAC	681
Consensus	cc c ac cga a ctccctg ac ct gt c cca c ac cccttcgc ga ca ga t cttgaacatggt tt ag a a ctac	
XvGolSII	AGGCCATCCCTCTGATTACAACTTGTGTTGGCATGCTGGCCCTCACTCCGAGAACGTCGCTTCCAAAGTCAAGTCTGTTCACTACTGTGCG	799
XvGolS	AGGCCATCCCTCTGATTACAACTTGTGTTGGCATGCTGGCCCTCACTCCGAGAACGTCGCTTCCAAAGTCAAGTCTGTTCACTACTGTGCG	781
Consensus	a gccat cc ctt tta aa ctgt t gc atgt tggcg ca cc gagaa gtcga ct a aa gtca gtcgt ca tactgtgc g	
XvGolSII	CGGCTTCCAAACCATGGAGGTACACGGCAAGAAGCTAATATGGACAGAGAGCATCAAGATCTGGTGGCTAAATGGTGGGACCTTACAACGACGA	899
XvGolS	CGGCTTCCAAACCATGGAGGTACTCTGTAAGAAGCAATATGGACAGAGAGCATCAAGATCTGGTGGCTAAATGGTGGGACCTTACAACGACGA	881
Consensus	c gg tc aa ccatggaggt a ac gg aaagaag aa atggacagaga gacatcaagatg tggg aa tggggggac tctacaacgacga	
XvGolSII	GTGCTGCACTCAAGCCGGAGGATGTAGTCCCGGAGGCGATCCCTTAACGAAGACTCCAATCATGCGCACTTTCGGGAGCCCGCAATTAATTTATC	999
XvGolS	GTGCTGCACTCAAGCCGGAGGATGTAGTCCCGGAGGCGATCCCTTAACGAAGACTCCAATCATGCGCACTTTCGGGAGCCCGCAATTAATTTATC	981
Consensus	tcgct ga t caag gg g a ga ga c c t a g a t cgg gc cg c	
XvGolSII	CCTGCACCATCTGACGCTAG.....	1020
XvGolS	GTGGCGTATCTAAGACCTTCAGCAGCTTA	1013
Consensus	ct agc	

Figure B2: Nucleotide sequence alignment of XvGolS and XvGolSII using DNAMAN (Version 7).

XvGolSII	MAPPVYAGAVDAAFTGKRLAVENAGYSKRAYVTFLAGGDDYKGVVGLAK	50
XvGolS	MAPEIYSRRAANYKQVAAPFN.....AYVTFLAGGDDYKGVVGLAK	44
Consensus	map v a a n ayvtfilag gdy kgvvglak	
XvGolSII	GLRKVRSAYPLIVAMLPDVPEDHREILRSQGCIVREIEPVYPFENCIQFA	100
XvGolS	GLRKVRSAYPLIVAVLPDVPEDHREILRSQGCIVREIEPVYPFENCIQFA	94
Consensus	glrkv saypl va lpdvp hr l sqgcivr iepvypfenq qfa	
XvGolSII	MAYYVINYSKLRIRWFVEYSKMIYLDADIQVYDNIHDFMPTGYYFAYVM	150
XvGolS	MAYYVINYSKLRIRWFVEYSKMIYLDADIQVYDNIHDFMPTGYYFAYVM	144
Consensus	mayyviny sklrir w f eyskmiyldadiqv ydnidhfd p g fyavm	
XvGolSII	DCFCEKTSWHSRQFSIGYCQCQPKVWPADMGNPPAFYFNAGMFVVEPS	200
XvGolS	DCFCEKTSWHSRQFSIGYCQCQENKVTWPDELGPPAFYFNAGMFVVEPS	194
Consensus	dcfcktswh s q igycqcq kv wpa g ppa yfnagmfv eps	
XvGolSII	RLTCSLIEILRVTPPTPFAEQDFLNMFFEKNYRPIPLIYNLVLAMLWRH	250
XvGolS	LATAERLIITLKVRETTTPFAEQDFLNMFFEKDIYRPIPLIYNLVLAMLWRH	244
Consensus	t ll tl v p tpfaeqd lnmff y pip l ynlvlamlwrh	
XvGolSII	PENVLIDKVVVVHYCAAGSKPWRYTGKEANMDREDIKMLVKKWVDYNDE	300
XvGolS	PENVLIDKVVVVHYCAAGSKPWRYTGKEANMDREDIKMLVKKWVDYNDD	294
Consensus	penv l kv vvhycaagskpwrytgke nmdredikmlv kwd ynd	
XvGolSII	SIDFRPE....DVVEGDALITKPIIMTFPEPAINFIFAPSA	338
XvGolS	SLIYKGPAAIMDLPEAPGKQLKLPALSEAGEVRYTKAPSA	336
Consensus	sid k d pe l a apsa	

Figure B3: Amino acid sequence alignment of XvGolS and XvGolSII using DNAMAN (Version 7).

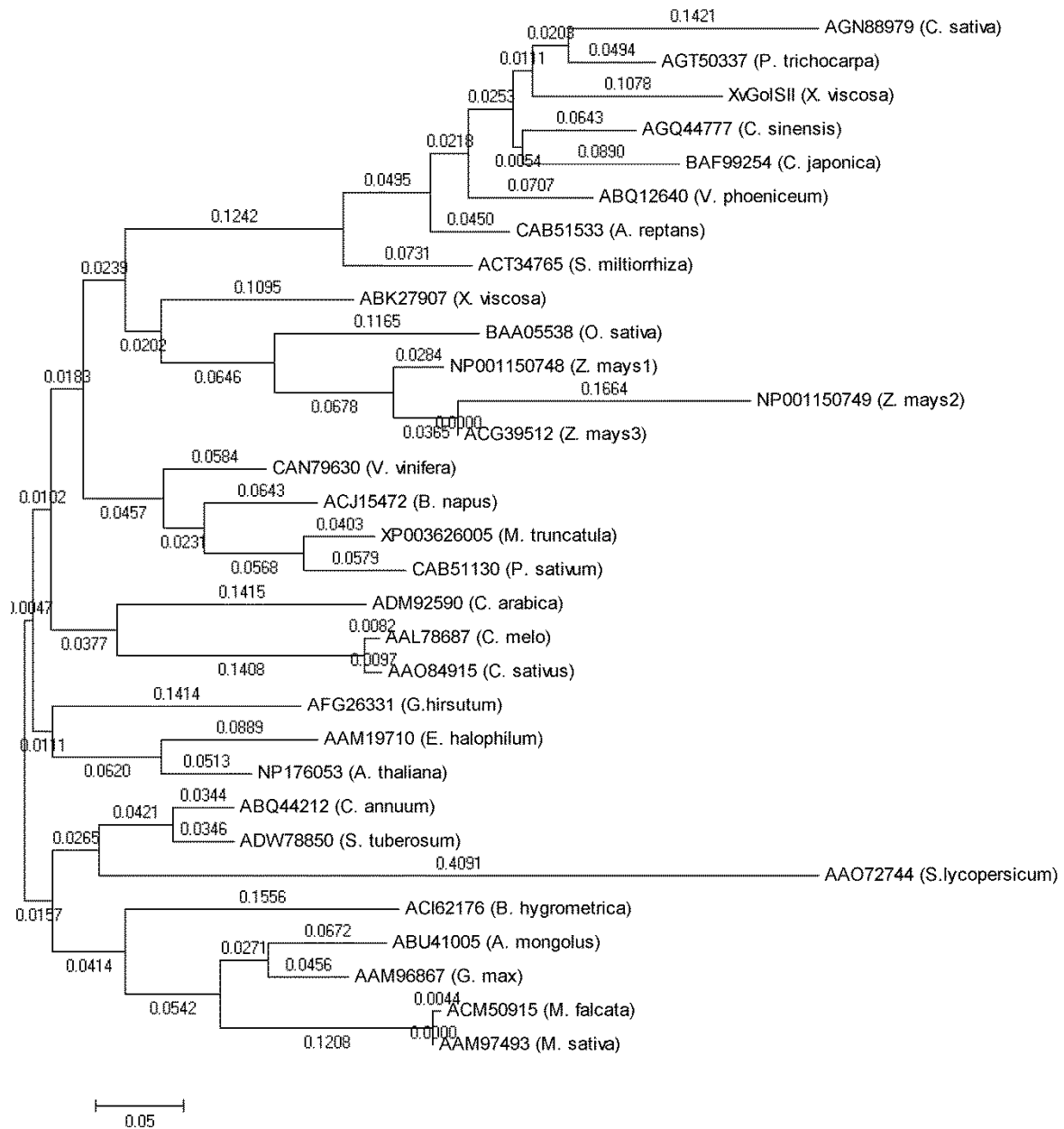


Figure B4: The molecular phylogenetic analysis of GolS from various species inferred by using the Maximum Likelihood method based on the JTT matrix-based model. The tree with the highest log likelihood (-4868.8031) is shown. Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was < 100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 31 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 235 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura *et al.*, 2011; Jones *et al.*, 1992).

Appendix C

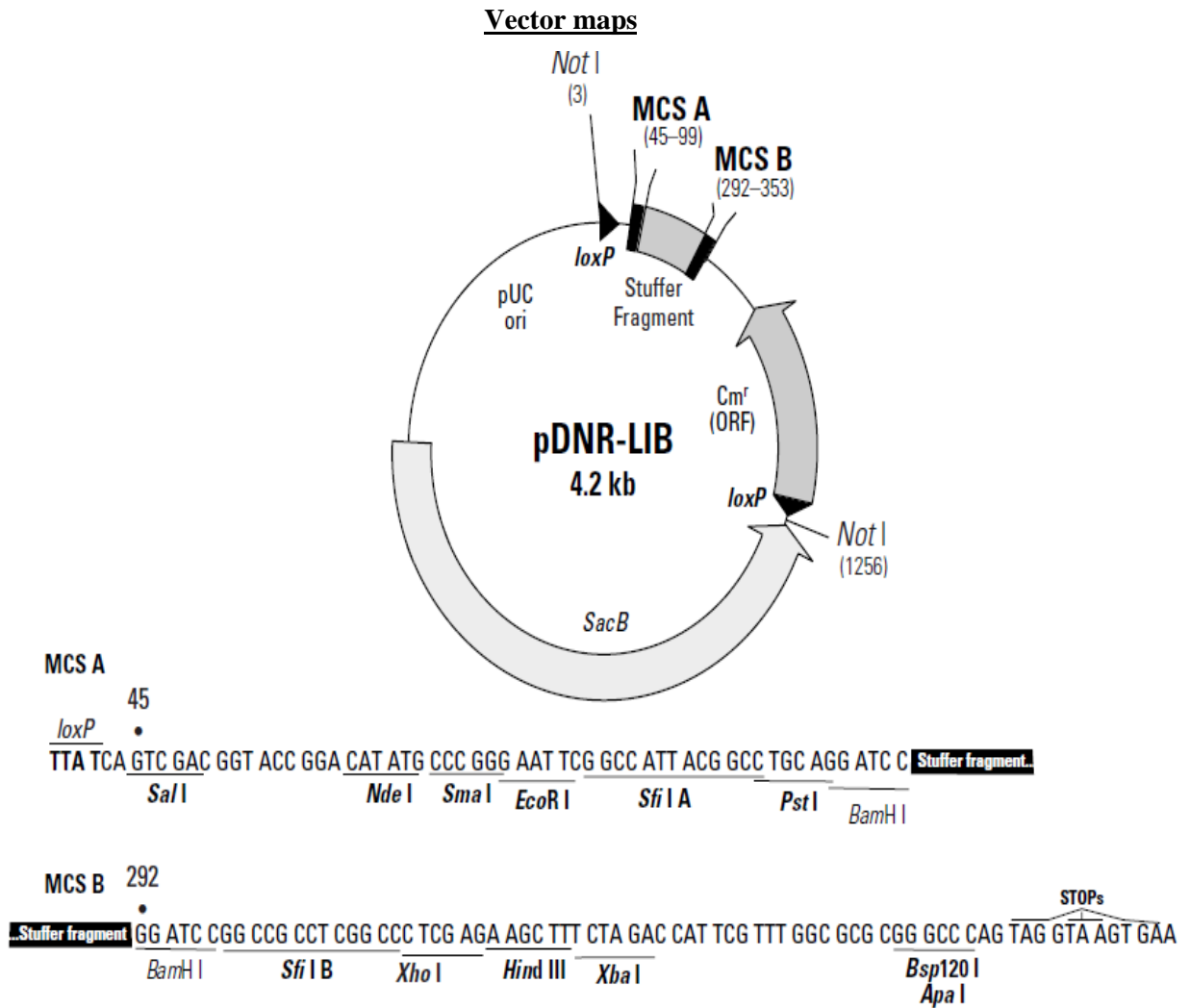


Figure C1: Multiple cloning site and restriction map of pDNR-Lib vector.

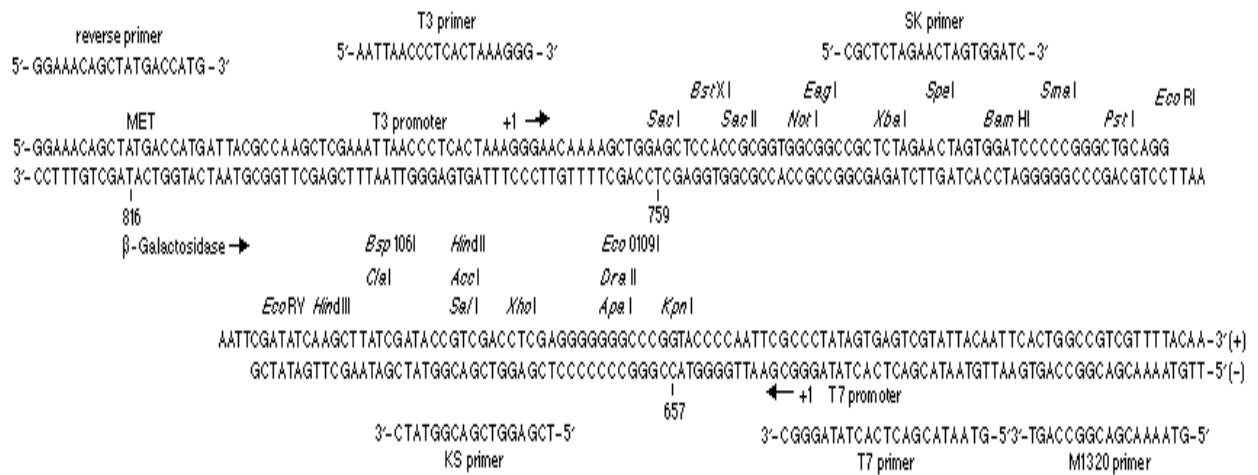
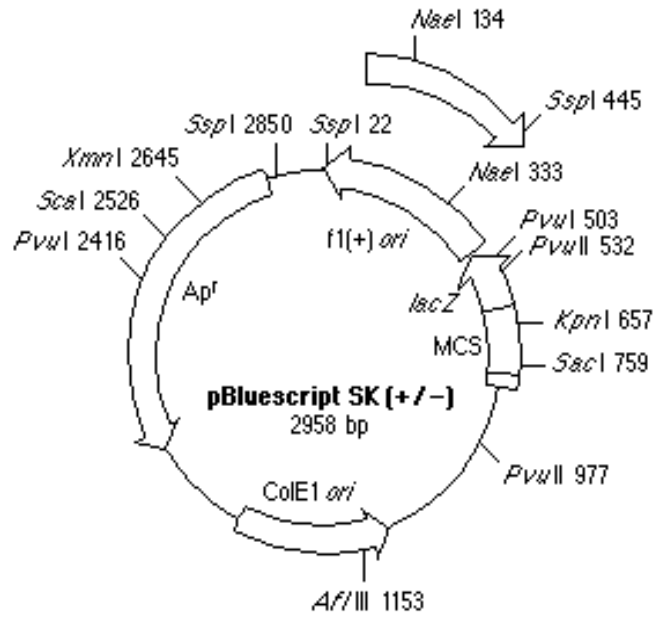


Figure C2: Features of pBluescript SK, including multiple cloning site and restriction map

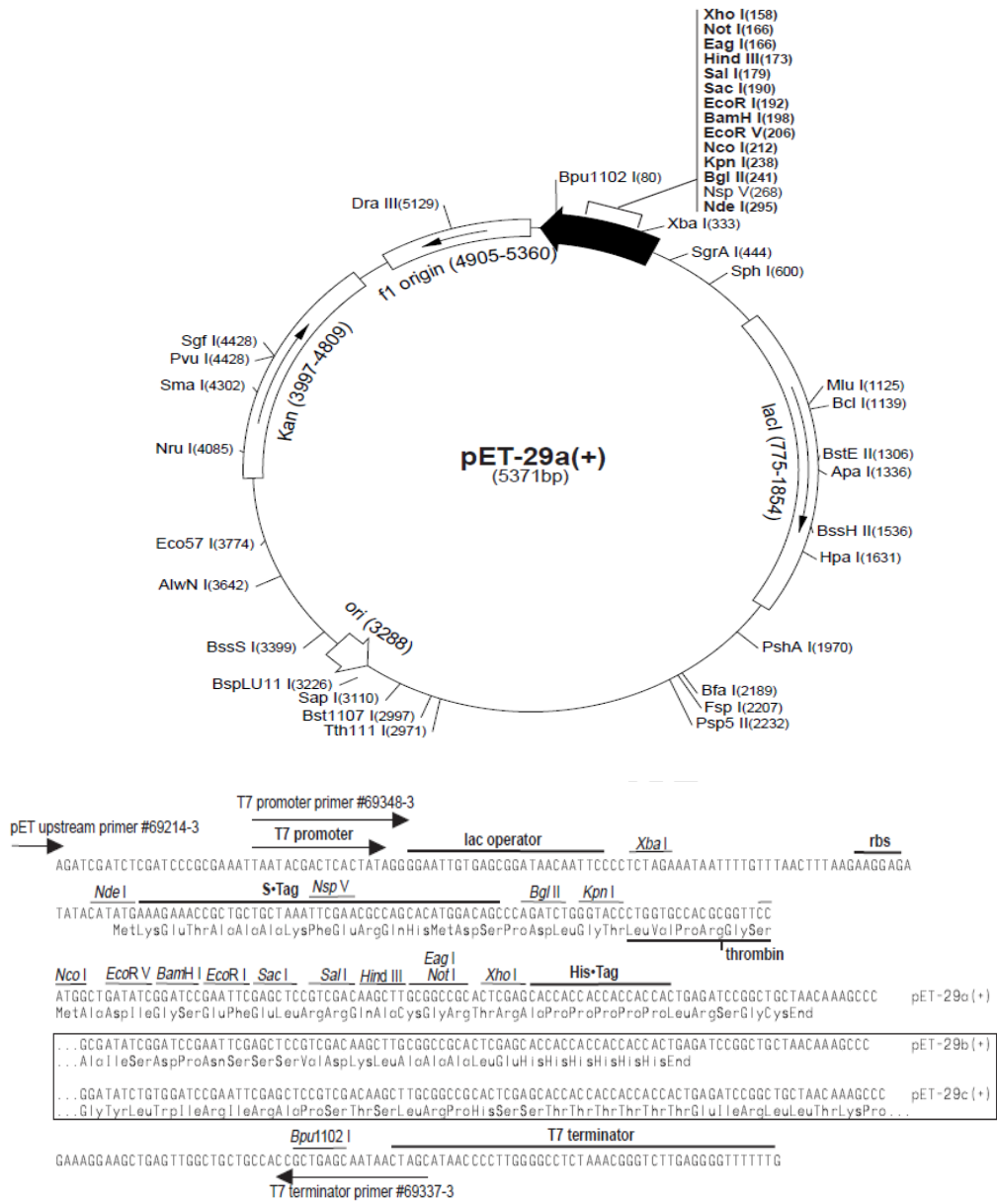


Figure C3: Features of pET29, including multiple cloning site and restriction map.

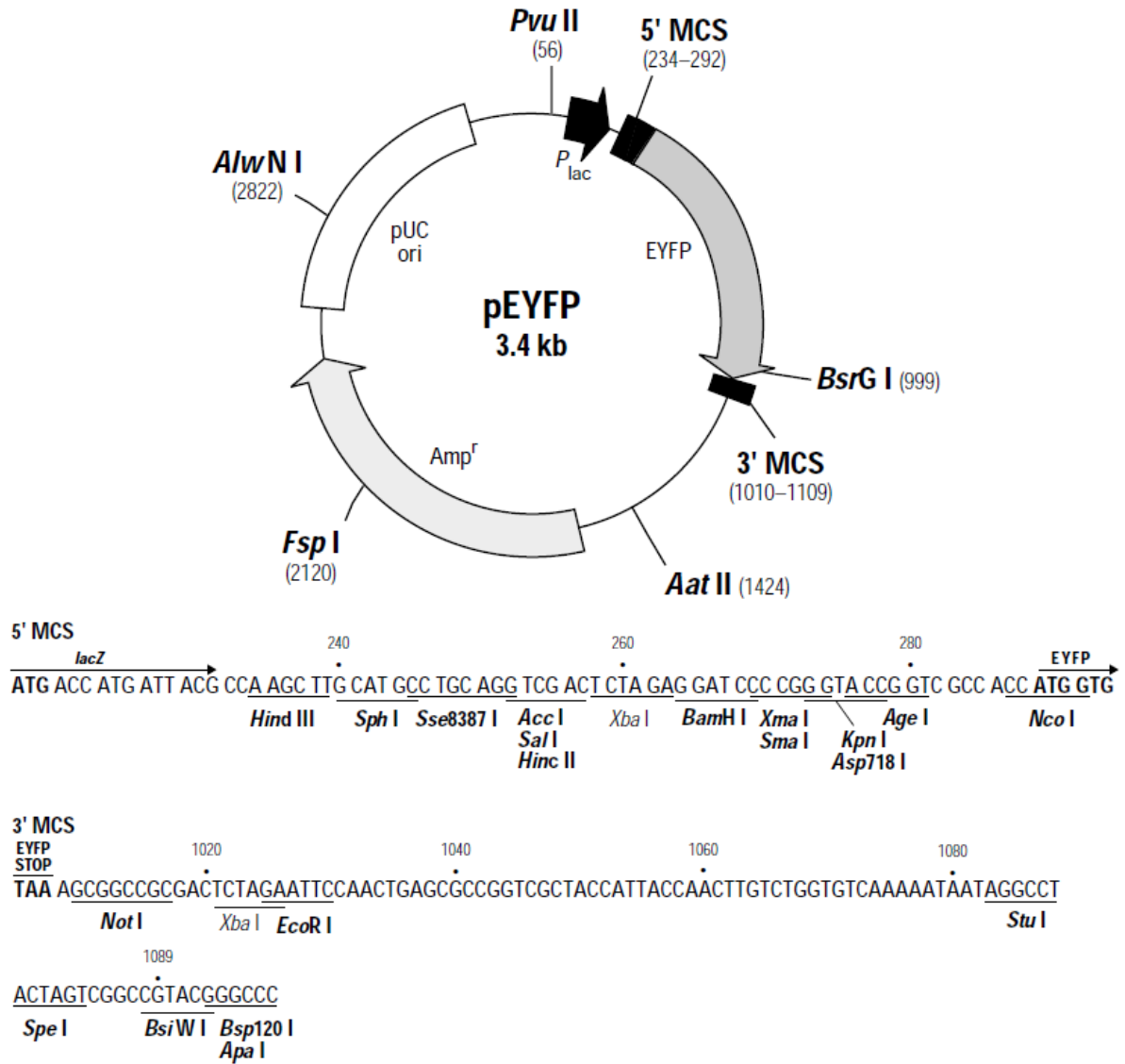


Figure C4: Multiple cloning site and restriction map of the pEYFP vector.

Appendix D

Real-time analysis

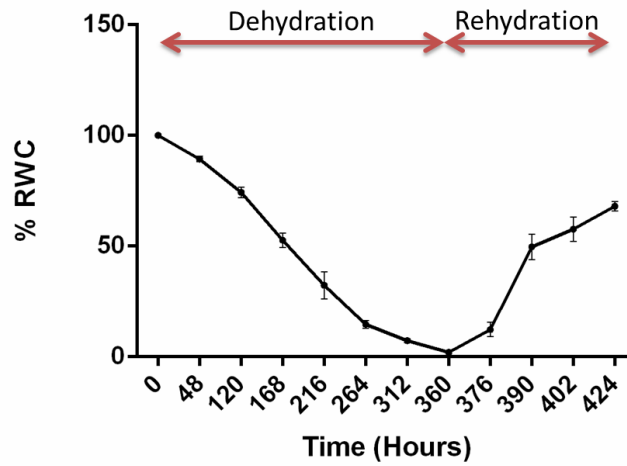


Figure D1: Graph illustrating the %RWC of the three *X. viscosa* plants, biologicals a, b and c, during the dehydration-rehydration treatment. Each point represents the average of the %RWC calculated for each sampling time point. Error bars represent the SEM across all three biological. The graph and SEM values were generated using the GraphPad Prism software (Version 6).

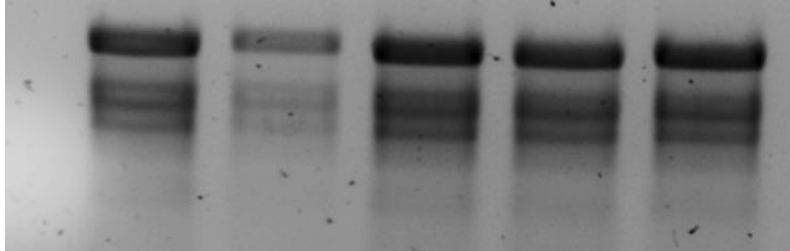


Figure D2: Assessment of RNA quality and integrity by electrophoresis of 1 μ g total RNA through a 1.2 % formaldehyde-agarose gel. This is representative of all time points across all biological replicates.

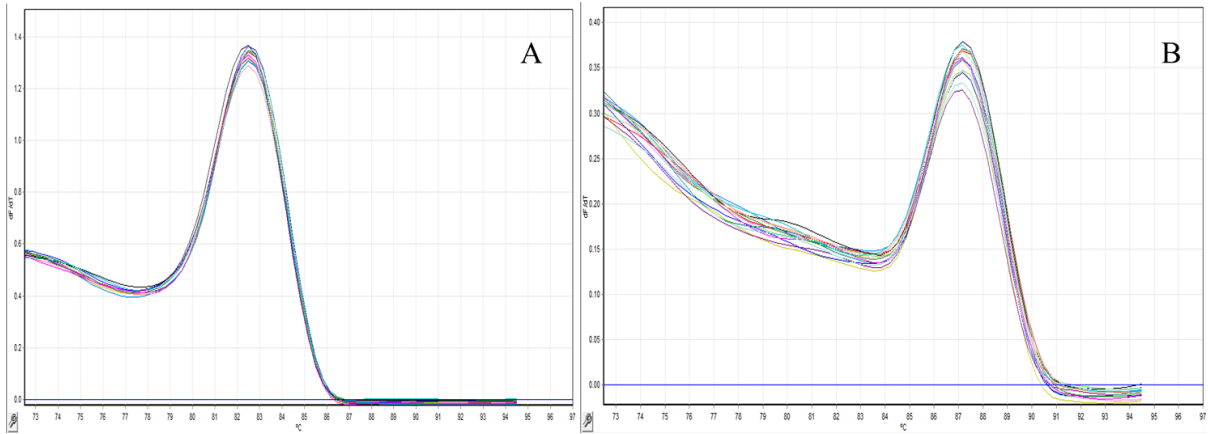


Figure D3: Melt curve analysis of *actin* and *XvGolsII* used in the cold stress treatment. The x-axis represents the range of melting temperatures (73 - 95°C) applied to the qPCR amplicons. The y-axis represents the derivative of the raw data after smoothing (dF/dT) as calculated by Rotor-Gene 6000 software. (A) represents the melt curve for *actin* and (B) for *XvGolsII*.

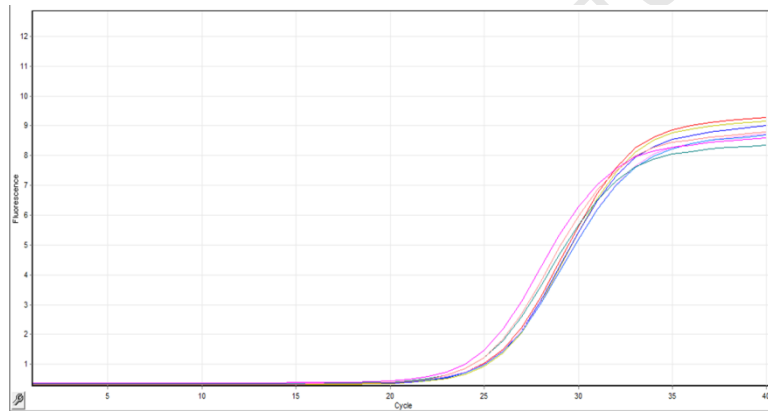


Figure D4: Amplification plot of *actin* used in the cold stress treatment. The x-axis represents the Ct values and the y-axis represents the fluorescence values.

Table D1: A table representing the one-way ANOVA calculations, indicating the significance of *XvGolsII* relative expression across different RWCs. The calculations were performed using the GraphPad Prism software.

Number of families	1								
Number of comparisons per family	7								
Alpha	0.05								
Dunnett's multiple comparisons test									
	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value				
100 vs. 88-92	0.0243	-0.5267 to 0.5753	No	ns	0.9998				
100 vs. 71-73	0.4876	-0.06334 to 1.039	No	ns	0.0944				
100 vs. 47-58	0.5753	0.02432 to 1.126	Yes	*	0.0389				
100 vs. 21-42	0.7429	0.1920 to 1.294	Yes	**	0.0065				
100 vs. 12-18	0.9109	0.3599 to 1.462	Yes	**	0.0011				
100 vs. 6-9	0.8497	0.2988 to 1.401	Yes	**	0.0021				
100 vs. 2	1.033	0.4818 to 1.584	Yes	***	0.0003				
Test details									
	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF	
100 vs. 88-92	0	-0.0243	0.0243	0.1884	3	3	0.1289	16	
100 vs. 71-73	0	-0.4876	0.4876	0.1884	3	3	2.588	16	
100 vs. 47-58	0	-0.5753	0.5753	0.1884	3	3	3.053	16	
100 vs. 21-42	0	-0.7429	0.7429	0.1884	3	3	3.943	16	
100 vs. 12-18	0	-0.9109	0.9109	0.1884	3	3	4.834	16	
100 vs. 6-9	0	-0.8497	0.8497	0.1884	3	3	4.509	16	
100 vs. 2	0	-1.033	1.033	0.1884	3	3	5.481	16	
Number of families									
	1								
Number of comparisons per family									
	4								
Alpha									
	0.05								
Dunnett's multiple comparisons test									
	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value				
2 vs. 7-18	0.4286	-0.4818 to 1.339	No	ns	0.4927				
2 vs. 42-67	-0.3335	-1.244 to 0.5768	No	ns	0.6812				
2 vs. 49-68	-0.04085	-0.9512 to 0.8695	No	ns	0.9998				
2 vs. 64-71	-0.1655	-1.076 to 0.7448	No	ns	0.9545				
Test details									
	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF	
2 vs. 7-18	-1.033	-1.461	0.4286	0.3149	3	3	1.361	10	
2 vs. 42-67	-1.033	-0.6993	-0.3335	0.3149	3	3	1.059	10	
2 vs. 49-68	-1.033	-0.992	-0.04085	0.3149	3	3	0.1297	10	
2 vs. 64-71	-1.033	-0.8673	-0.1655	0.3149	3	3	0.5255	10	

Table D2: A table representing the one-way ANOVA calculations, indicating the significance of *XvGolsII* relative expression across different time points within the cold stress. The calculations were performed using the GraphPad Prism software.

Number of families	1								
Number of comparisons per family	4								
Alpha	0.05								
Dunnett's multiple comparisons test	Mean Diff.	95% CI of diff.	Significant?	Summary	Adjusted P Value				
0 vs. 6	-0.05496	-0.2821 to 0.1722	No	ns	0.8888				
0 vs. 24	-0.02812	-0.2553 to 0.1990	No	ns	0.9881				
0 vs. 48	0.3461	0.1189 to 0.5732	Yes	**	0.0045				
0 vs. 72	0.4542	0.2271 to 0.6814	Yes	***	0.0006				
Test details	Mean 1	Mean 2	Mean Diff.	SE of diff.	n1	n2	q	DF	
0 vs. 6	0	0.05496	-0.05496	0.07858	3	3	0.6994	10	
0 vs. 24	0	0.02812	-0.02812	0.07858	3	3	0.3578	10	
0 vs. 48	0	-0.3461	0.3461	0.07858	3	3	4.404	10	
0 vs. 72	0	-0.4542	0.4542	0.07858	3	3	5.78	10	

University of Cape Town

Appendix E
Biolistic particle delivery system

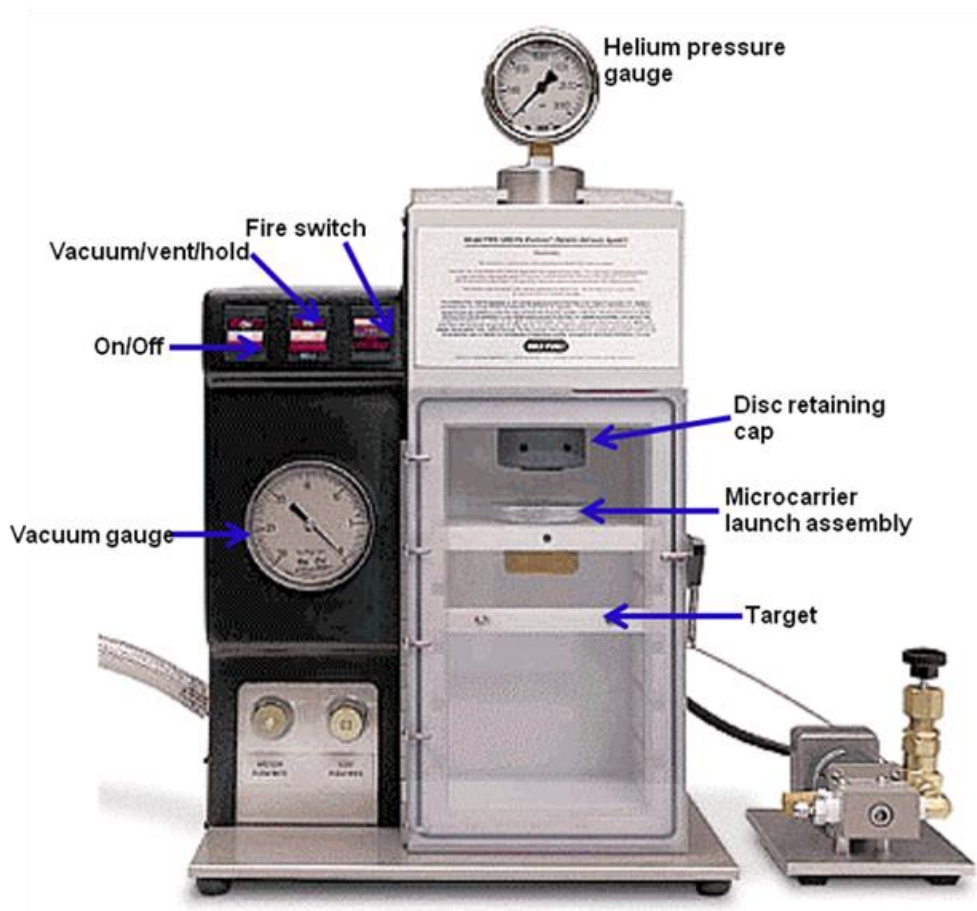


Figure F: A representation of a Biolistic® -PDS-1000 Particle Delivery System (BioRad, USA).

Appendix F

Arabidopsis eFP Browser abiotic stress expression profiles for *AtGolS1*, 2 and 3

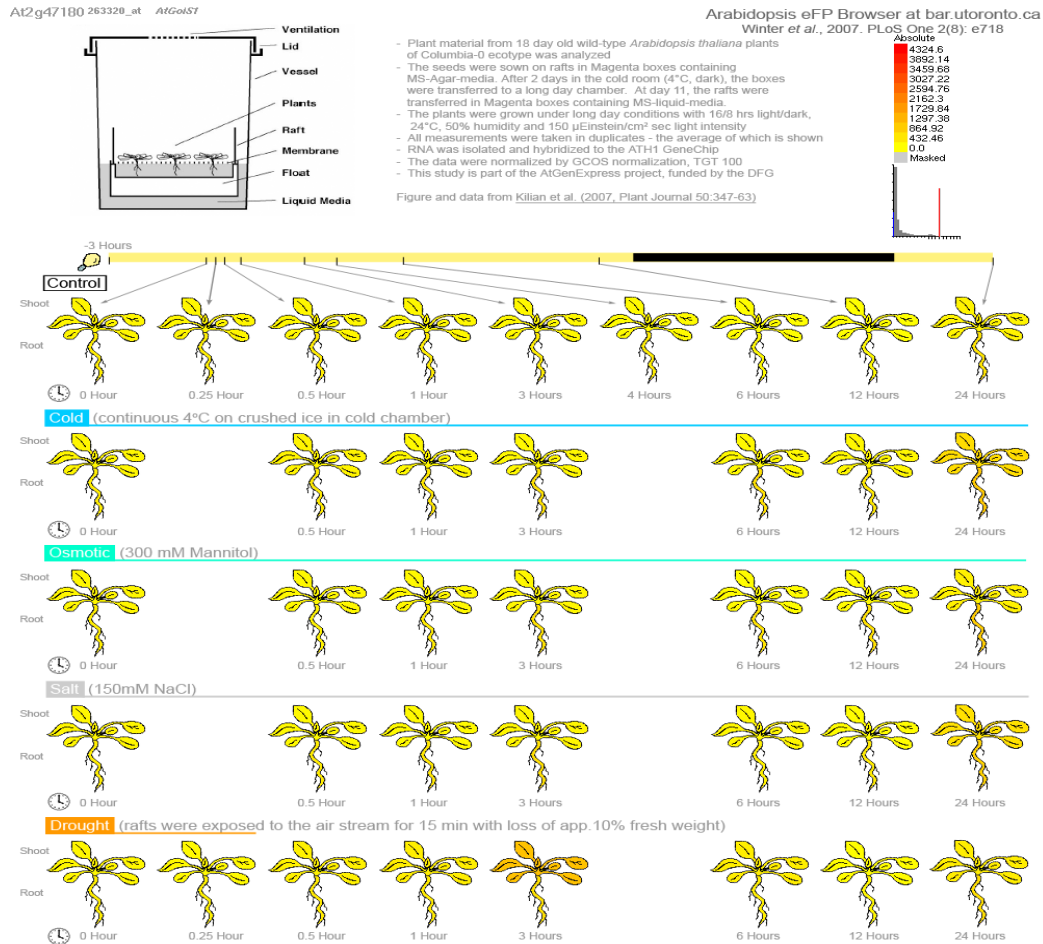


Figure F1: Expression profiles of *AtGolS1* under abiotic stress, as analysed through the Arabidopsis eFP browser.

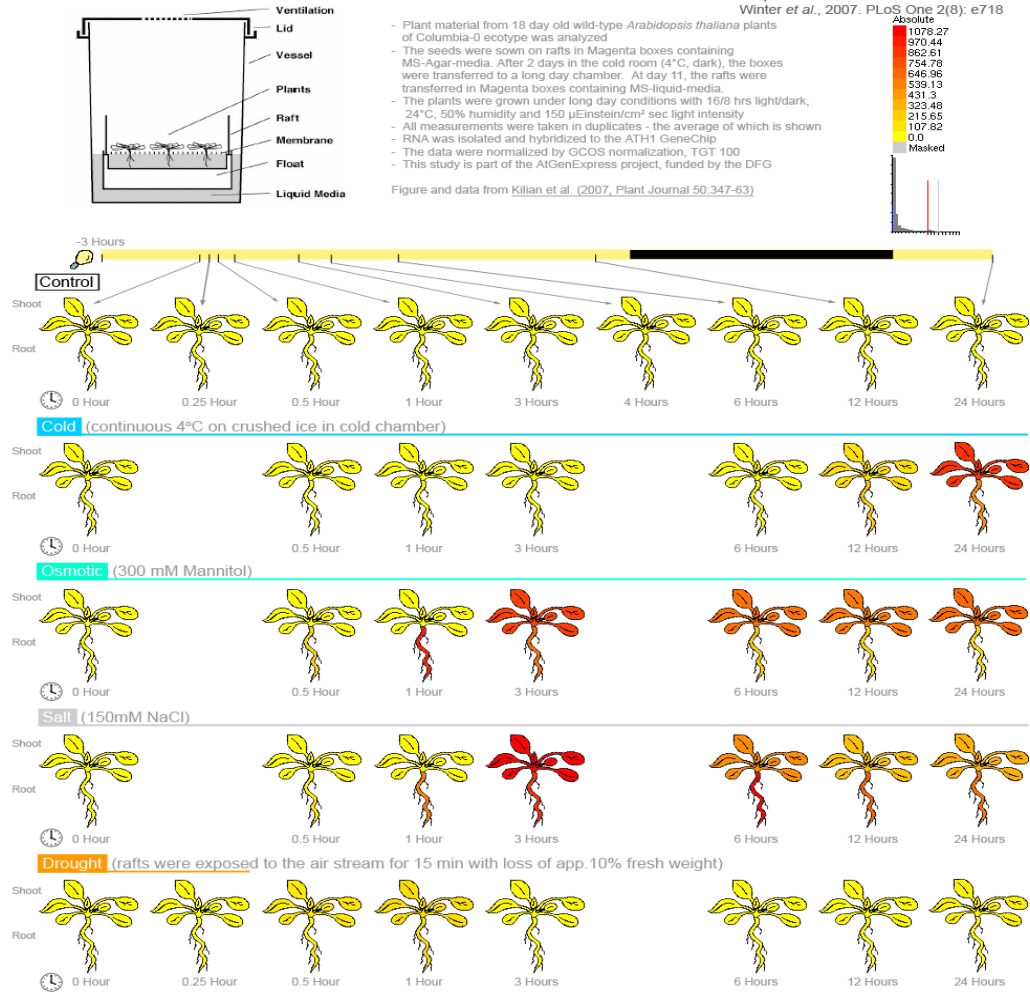
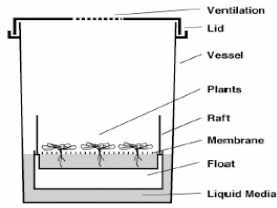


Figure F2: Expression profiles of *AtGols2* under abiotic stress, as analysed through the Arabidopsis eFP browser.



- Plant material from 18 day old wild-type *Arabidopsis thaliana* plants of Columbia-0 ecotype was analyzed
 - The seeds were sown on rafts in Magenta boxes containing MS-Agar-media. After 2 days in the cold room (4°C, dark), the boxes were transferred to a long day chamber. At day 11, the rafts were transferred in Magenta boxes containing MS-liquid-media.
 - The plants were grown under long day conditions with 16/8 hrs light/dark, 24°C, 50% humidity and 150 µEinsteincm² sec light intensity
 - All measurements were taken in duplicates - the average of which is shown
 - RNA was isolated and hybridized to the ATH1 GeneChip
 - The data were normalized by GCOS normalization, TGT 100
 - This study is part of the AtGenExpress project, funded by the DFG
- Figure and data from Kilian et al. (2007, Plant Journal 50:347-63)

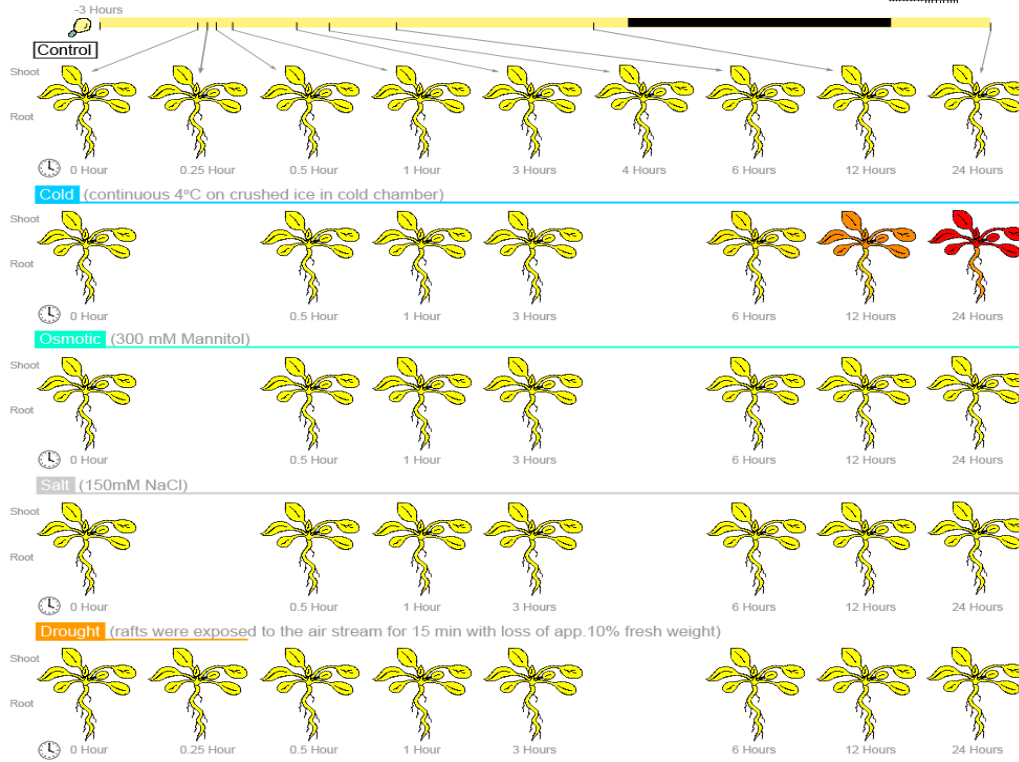
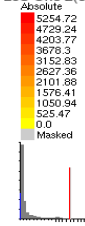


Figure F3: Expression profiles of *AtGolS3* under abiotic stress, as analysed through the Arabidopsis eFP browser.

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