XvGolS, a galactinol synthase is transcriptionally upregulated under water deficit: The role of raffinosaccharides in abiotic stress tolerance in 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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DECLARATION

I hereby declare that this thesis entitled:

XvGolS, a galactinol synthase is transcriptionally upregulated under water deficit: The role of raffinosaccharides in abiotic stress tolerance in 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.

Signature Date

'I would not exchange the laughter of my heart for the fortunes of the multitudes; nor would I be content in converting my tears, invited by my agonized self, into calm. It is my fervent hope that my whole life on this earth will ever be tears and laughter'

- Khalil Gibran

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List of abbreviated carbohydrates

suc	sucrose
glu	glucose
gol	galactinol
raf	raffinose
sta	stachyose
ver	verbascose
gal	galactose
UDP-gal	uridine 5'-diphosphogalactose
fru	fructose
ino	myo-inositol

Abstract

As part of an ongoing project to genetically manipulate maize for increased abiotic stress tolerance, we have isolated and identified a number of genes from the resurrection plant *Xerophyta viscosa* (Baker) that are differentially expressed during water deficit. A cDNA was isolated from a library constructed from the leaves of water stressed *X. viscosa* plants, which showed high identity to galactinol synthase (GolS) enzymes from a variety of plant species. GolS is fundamental to the biosynthesis of raffinose family oligosachharides (RFOs), sucrosyl carbohydrates that are unique to plants and implicated in carbon translocation and abiotic stress tolerance. We subsequently designated this cDNA *XvGolS*.

Molecular in planta characterization of this GolS indicated that transcript levels increased in the leaves in response to water deficit and a low temperature shock of - 20°C. The GolS protein was evident in the leaves at full turgor, in the absence of any stress despite no transcripts being detected at the corresponding time points. Exogenous application of ABA did not result in transcript increases being observed, implying that *XvGolS* expression was ABA independent. XvGolS was heterologously expressed in *E.coli* and functionally identified as a bona fide GolS by the galactinol forming ability of the recombinant protein.

High Pressure Liquid Chromatography (HPLC) analysis of carbohydrate extracts from the leaves of plants subjected to water deficit or low temperature acclimation (1°C) stress regimes revealed that the concentrations of the raffinosaccharides raffinose and stachyose increased in response to water deficit with low levels of verbascose also detected in the analysis. Due to shortages of live plants we could only conduct the low temperature experiment with a single replicate. We provide preliminary evidence to suggest that a plant exposed to a stress regime of 1°C induces raffinosaccharide pathways as evidenced by putative increases in raffinose during an 8 d exposure period whilst a plant acclimated to 1°C did not respond in the same manner. Collectively, we consider this data to provide evidence that RFOs are involved in the stress tolerance response in the vegetative (leaves) tissue of *X.viscosa*.

Chapter 1

Introduction and Literature Review

Introduction

Plants face a multitude of abiotic stress factors such as salinity, water deficit, low temperature, heat and pathogen attack. For research purposes it is tempting to compartmentalise such stress factors into modular events but the reality in a natural setting is very different. Such stresses often occur as a complex combination of events on a daily basis. Understanding this concept allows for the realisation that environmental stress responses that have evolved over eons are in fact extremely complex, displaying multiple interconnected signalling and regulatory elements to ensure the optimal cellular response for any given combination of stress.

Comprised wholly of sessile organisms, the plant kingdom has evolved a clear gradation of abilities to deal with stress with all plants displaying the ability to tolerate abiotic stress to varying degrees (Bohnert et al., 1995). Some plants are able to survive moderate stress, whilst others are very sensitive to minute fluctuations in environmental conditions. Others display the remarkable ability to withstand extremes of environmental stress by employing numerous protection and repair mechanisms that facilitate the recovery of cellular metabolism once the stress has been relieved. Such a group of plants belonging to the family Vellociacea is the genus *Xerophyta*. Members of the genus display the ability to withstand severe water deficit by being desiccation tolerant.

Water is a fundamentally important component of the metabolism of all living organisms, facilitating many vital biological reactions by being a solvent, a transport medium and evaporative coolant (Bohnert et al., 1995). In plants and other photoautotrophs, water plays the additional role of providing the electrons necessary to drive photosynthesis. Water molecules are split, in a process termed autolysis, to yield the electrons that are used to drive the energy yielding Photosystem II reaction centre (Salisbury and Ross, 1992).

1.1 Leaf after death

A small group of angiosperms belong to a class of plants displaying "resurrection" capability. Such plants are typically characterised by their ability to tolerate and survive extremes of vegetative desiccation, subsequently resuming normal cellular metabolism within a short period after water becomes available again (for review see Scott, 2000). Some resurrection plants dismantle their photosynthetic machinery and lose chlorophyll during water deficit and

are termed poikilochlorophyllous, whilst others preserve photosynthetic structures and chlorophyll and are termed homoiochlorophyllous (Tuba et al., 1998). The ability to withstand extremes of desiccation is not exclusive to angiosperm plants, with some lower plants such as mosses also displaying similar tolerances (Oliver and Wood, 1997). Experimental data suggest that resurrection plants follow two strategies with respect to the mechanisms that allow for tolerance, with the angiosperms protecting cellular integrity during drying whilst the lower plants such as mosses repair cellular damage induced by desiccation, upon rehydration (Bewley and Oliver, 1992).

Resurrection species are widely distributed throughout the world with representatives on all continents, except Antarctica. However, many of the angiosperm resurrection species are concentrated in arid climates such as southern Africa, South America and western Australia (Gaff, 1987). A few species have also been recorded on continental Europe, in the Balkan mountain ranges (Stefanov et al., 1992). Despite where resurrection plants occur, the angiosperms are nearly always found in similar habitats, occurring in depressions in shallow bedrock on mountain ranges or rocky outcrops (Porembski and Barthlott, 2000). These environments tend to be microclimatically extreme, showing lower relative humidity and higher temperatures than that of the immediate surrounding environment, which often may be subtropical or tropical. Thus, resurrection plants have, over evolutionary time, come to have a competitive advantage over other species in these unstable environments. Due to their ability to withstand desiccation, they can remain in metabolic stasis until water becomes available and subsequently resume growth and reproduction long before desiccation sensitive species.

Xerophyta viscosa is a resurrection plant, endemic to southern Africa, that is able to withstand water deficit stress to as low as 5% relative water content (RWC), restoring metabolism to a level comparable to that of the fully turgid state within 72 h after water becomes available (Farrant, 2000). Mechanisms of desiccation tolerance have been extensively studied in *Xerophyta* species (Dace et al., 1998; Farrant, 2000; Mundree et al., 2000; Sherwin and Farrant et al., 1998,) as well as other angiosperms such as *Craterostigma plantinegum* (Ingram and Bartels, 1996; Norwood et al, 2000), *Craterostigma wilmsii* (Cooper and Farrant, 2002; Vicré et al., 1999), *Myrothamnus flabelifolius* (Farrant and Kruger 2001; Farrant 2000; Farrant et al., 1999, 2003; Koonjul et al., 2000; Moore et al., 2004) *Sporobulus stapfianus* (Neale et al., 2000; Whittaker et al., 2002, 2004) and the moss *Tortula ruralis* (Oliver and Bewley,

1997, Wood et al., 2000). What has emerged from such work is that responses to desiccation stress involve the co-ordinated expression of a number of genetic pathways, as well as multilayered regulation of these pathways (Table 1.1) that will not be covered in detail by this work. One of the most marked responses to desiccation stress is the accumulation of compounds collectively termed osmoprotectants which are thought to facilitate drying by minimising intracellular damage and it is this aspect which will be further reviewed.

Table 1.1 A summation of responses observed in resurrection plants in response to water deficit (modified from Bernacchia and Furini, 2003)

Resurrection plants utilise	• cellular protection • damage repair mechanisms • cellular repair mechanisms
↓	
Signal transduction pathways can be	• ABA dependent • ABA independent
↓	
Regulatory factors involved	• *ABI3* • *CDT 1* • HD-ZIPs • Myb transcription factors • Translation factors
↓	
Molecular responses	• **Protective proteins** – late embryogenesis abundant (LEA); structural, hydrophilic • **Protective molecules** –sucrose; osmoprotection, glass formation. Anthocynains; chlorophyll masking pigments • **Metabolic enzymes** – sugar conversions; sucrose synthase, sucrose-P-synthase
↓	
Physiological responses	• poikilochlorophylly • homoiochlorophylly • leaf folding

1.2 Compatible solutes: contributors to stress tolerance

A major consequence of drought stress is the exudation of protoplasmic water from the cell via osmosis. This water loss leads to the concentration of ions such as Cl^- and NO_3^-, which effectively inhibit metabolic functions at high concentrations (Hartung et al., 1998). Additionally, the concentration of protoplasmic constituents and the loss of water from the cell lead to the formation of what is termed a glassy state. In this state whatever liquid is left in the cell has a very high viscosity, increasing the chances of molecular interactions that can cause protein denaturation and membrane fusion (Hartung et al., 1998; Hoekstra et al., 2001).

In order to counteract the loss of cell turgor when water exits the cell, amounts of compatible solutes are increased and maintained in the cytosol and vacuolar compartments (Cushman, 2001). However, protection may also occur at concentrations too low to justify osmotic effects (Goufi et al., 1999; Zhifang and Loescher, 2003). These low concentration effects expand the functional roles of compatible solutes to include free radical scavenging (Shen et al., 1997), and stabilisation of membrane structures (Hincha et al., 2003; Galinski, 1993). Compatible solutes are represented in all kingdoms ranging from the Archaebacteria to the higher mammals (McNeil et al., 1999). By definition solutes are synthesized in response to osmotic stress, occurring at high intracellular concentrations without hindering normal cellular metabolism (Ramanjulu and Bartels, 2002) and are collectively represented by certain polyols, sugars, amino acids, betaines and related compounds (Bohnert and Jensen 1996; Hayashi and Murata, 1998; McNeil et al., 1999; Ramanjulu and Bartels, 2002). The properties of compatible solutes facilitate the maintenance of favourable turgor pressure during water stress and in addition may serve as protective agents by stabilizing proteins (Carpenter et al., 1990; Santoro et al., 1992; Papageorgiou and Murata 1995).

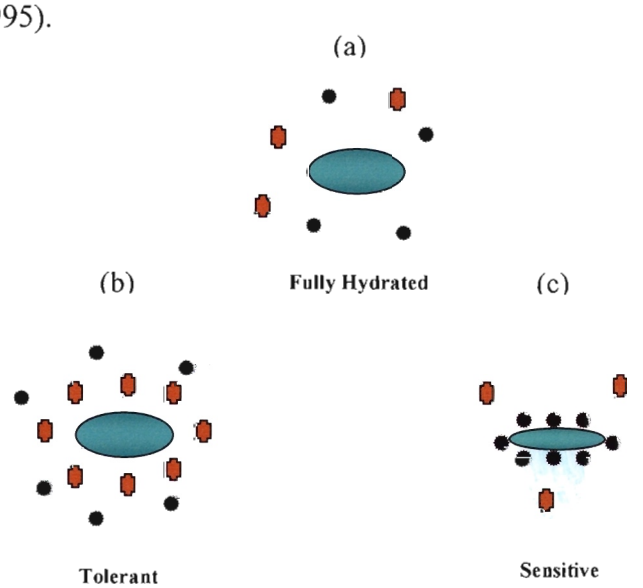

Figure 1.1 Diagram illustrating the function of compatible solutes. (a) In the hydrated state, the presence of water reduces the interaction of destabilising molecules (black circles). (b) In tolerant cells the synthesis of compatible solutes (red bars) preferentially excludes the binding of destabising molecules. (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 Hoekstra et al., 2001.

1.3 Photosynthesis: The origins of plant carbohydrates

Plants, as well as other photoautrophs, possess the ability to synthesise organic compounds by harnessing light radiation. This is made possible by the presence of chlorophyll which is able to absorb incident light radiation and utilise the inherent energy associated with this to drive biosynthetic reaction centers that effectively transform light energy into carbon based molecules used to power cellular metabolism. Given that carbohydrates, in whatever form, owe their origins to the photosynthetic fixation of carbon, attention will be given to providing a brief overview of the process as described in Salisbury and Ross (1992) and Campbell (1995). The complete process of photosynthesis occurs in two phases. Firstly, water is split to produce oxygen in a process called autolysis. This process relies on the light dependent reactions of the photosynthetic process. Secondly CO_2 is fixed in a series of metabolic reactions to yield carbohydrates. This process, although occurring in the absence of light, still depends indirectly on light via the products of the light dependent reactions that precede. Chloroplasts, specialised photosynthetic structures, occur ubiquitously in all photoautotrophic organisms. Chloroplasts contain molecules termed chlorophyll that initiate the primary events in photosynthesis. These molecules are able to harness light energy by increasing their energy states (the excited form). This energy is then subsequently used in the light reactions.

The light reactions of photosynthesis are dependent on two components which occur within the chloroplast. The first of these is photosystem II (PSII), which consists of a complex of six polypeptides noncovalently linked to each other. In addition, a reaction center termed P680 is coupled to the complex. PSII is primarily involved in the reactions which split water (Figure 1.2 a). The second essential component is photosystem I (PSI), consisting of 11 different polypeptides which function primarily in the reduction of $NADP^+$ to NADPH. PSI and PSII are linked via the Cytochromeb6-Cytochrome f Complex, which serves to shuttle electrons generated during autolysis to PSI, thereby generating energy in the form of ATP.

The nett products of the elaborate electron transport chain of PSI and PSII are ATP and NADPH. During the dark reactions, plants are able to absorb CO_2 through their stomatal apertures. This CO_2 is subsequently fed in the Calvin cycle (Figure 1.2 b), a metabolic process by which the carbon is fixed in a series of redox reactions that utilise the NADPH and ATP generated during the light reactions. The most important intermediate in this cycle is the

molecule glyceraldehyde-3-phosphate which is utilised in the synthesis of the carbohydrate glucose (glu).

Although simplified greatly, this overview of photosynthesis provides the basis of understanding how photoautotrophs like plants are able to harness incident light radiation from the sun and ultimately use it to synthesise carbon based metabolites. The fate of these metabolites is many fold. Excess carbohydrates can be translocated to non-photosynthetic storage organs as will be described later, whilst glu can be used to synthesise the disaccharide sucrose (suc), which serves as the carbon skeleton for the biosynthesis of specialised sucrosyl carbohydrates (raffinosaccharides) that will be described in detail.

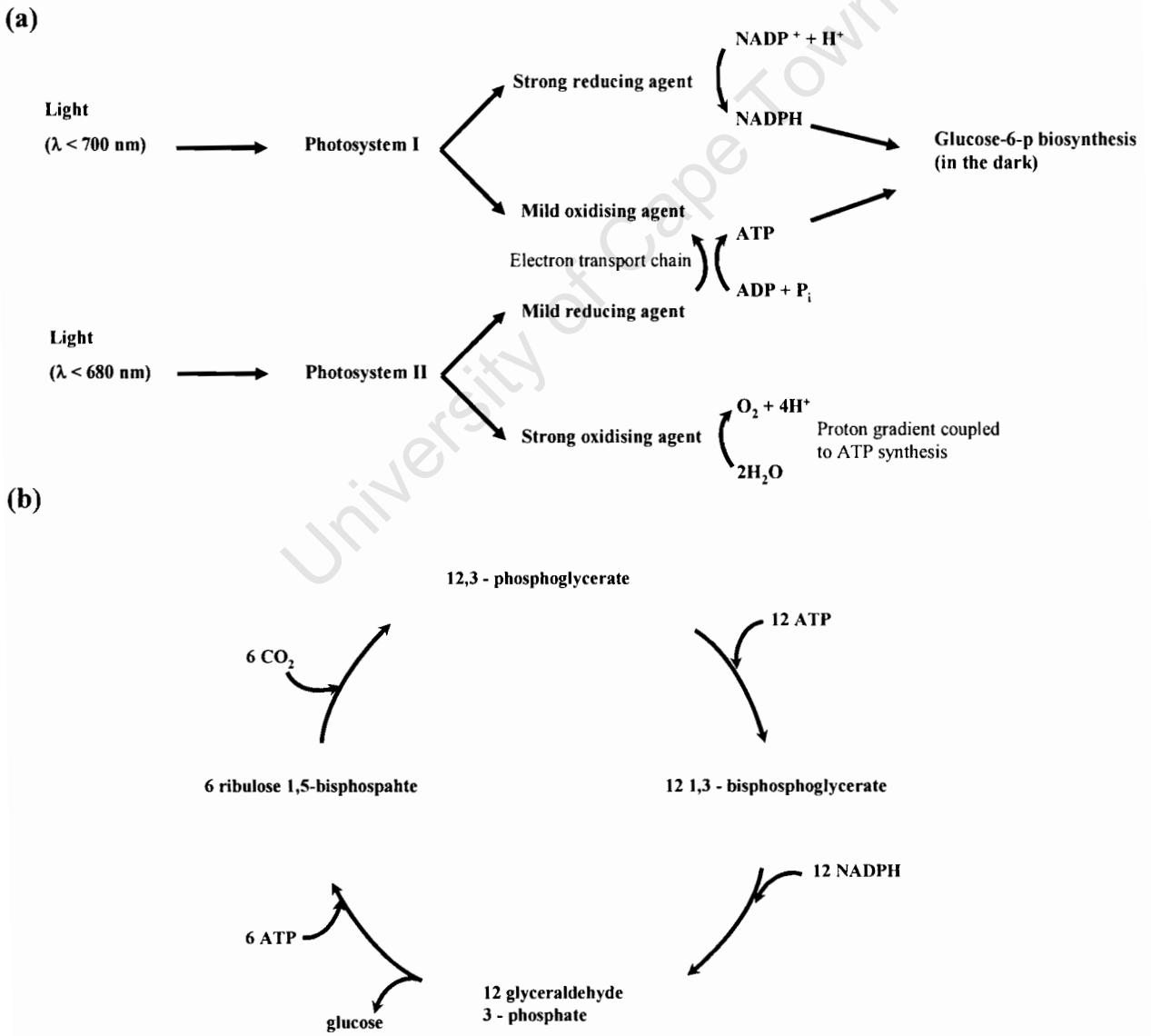

Figure 1.2 schematic representations of the basic process of photosynthesis (a) and the Calvin cycle (b), where glucose biosynthesis occurs (After Campbell, 1995)

1.4 The raffinose family of oligosaccharides (RFOs) are important sucrosyl carbohydrates

RFOs are the most widely distributed non-structural carbohydrates in the plant kingdom, being synthesised in leaves and stored in the roots and tubers of a wide variety of species (Peterbauer et al., 2002). Structurally, RFOs may be considered as extensions of sucrose to which galactosyl residues are attached via α1→6 glycosidic linkages. The biosynthesis of RFOs begins with the synthesis of galactinol (gol), catalysed by the enzyme gol synthase. Gol (**O-α-D**-galactopyranosyl-(1→1)-L-*myo*-inositol) is an unusual carbohydrate-cylitol hybrid (Figure 1.3) found exclusively in plants and serves solely as the galactosyl donor for subsequent RFO biosynthetic reactions (Karner et al., 2004). The first member of the RFOs, raffinose (raf), is synthesised by the α-galactosyltransferase raffinose synthase, which transfers a galactosyl residue from gol to a sucrose molecule. Subsequent stepwise addition of galactosyl monomers by stachyose and verbascose synthase result in the RFO members stachyose (sta) and verbascose (ver) respectively.

RFOs with degrees of polymerisation (DP) of up to 15 have been reported in the alpine specialist plant *Ajuga reptans* (Bachmann et al., 1994; Bachmann and Keller, 1995). These studies also identified a novel chain elongation enzyme, galactan-galactan transferase (GGT), in the RFO biosynthesis pathway of *A. reptans*. It was shown that GGT functioned independently of gol and rather, utilised RFOs themselves as galactosyl donors in the biosynthesis of higher DP RFOs (Bachmann et al., 1994). However, to date little has been elucidated about the functional importance of high DP RFOs. What is clear in the case of *A. reptans*, however, is that RFOs are ultilised as a translocatable and storable carbon source.

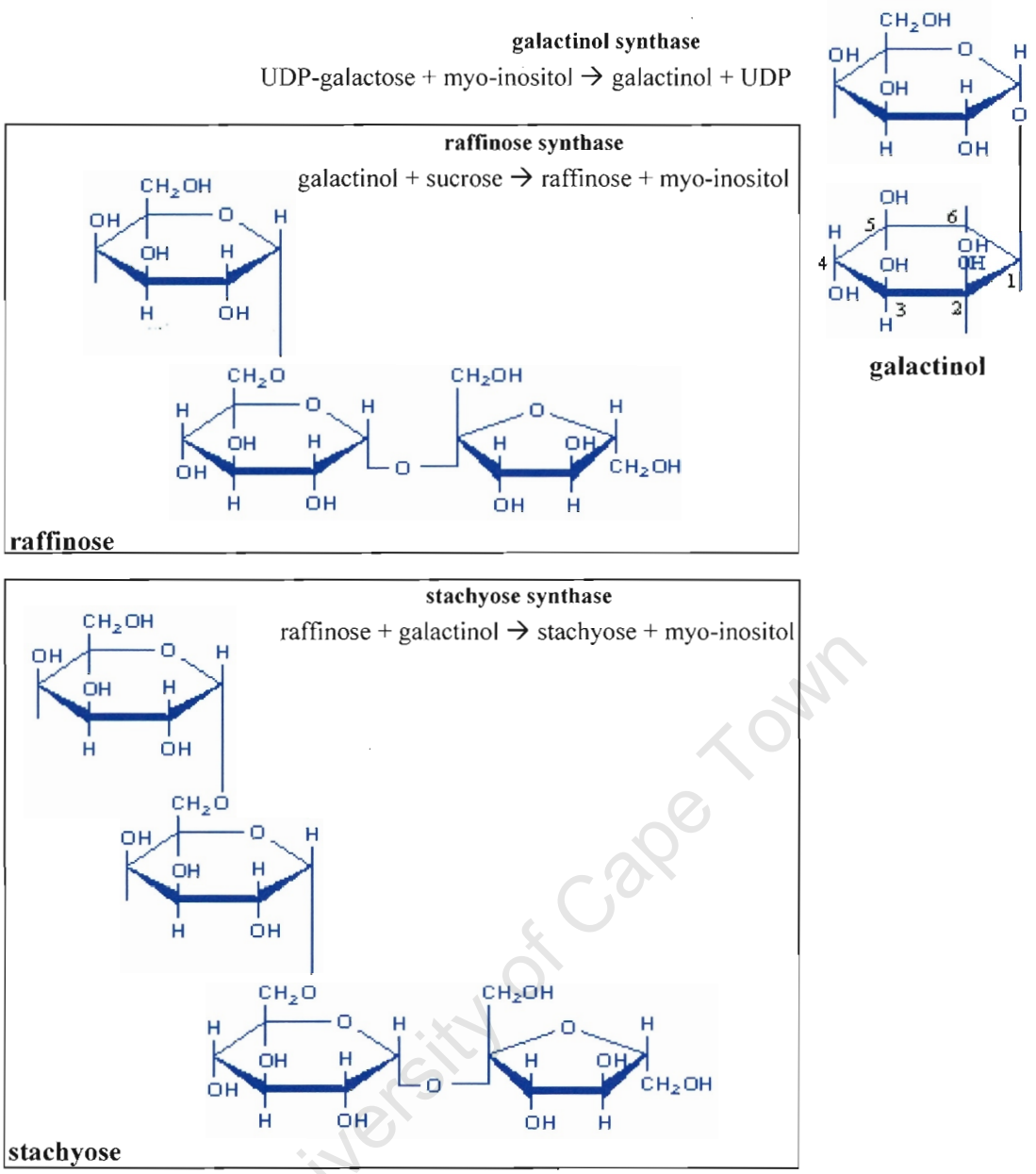

Figure 1.3 Illustration of the biosynthetic pathways of the lower RFOs raffinose and stachyose. Biosynthetic enzymes are indicated by bold lettering (adapted from http://biologi.uio.no/plfys/haa/gif/form251.gif)

1.4.1 RFOs have been identified in the phloem translocation stream

Carbon translocation in plants, in the form of sugars, from photosynthetically active tissue (source) to storage organs or other non-photsynthetically active tissue (sink) has been extensively studied for decades. It was known since 1968 that sucrose serves as the major translocate for a number of plants (cited in Turgeon, 1995). However, there are definitive requirements that must be met in order for a sugar to be considered as a good translocate. Firstly, these sugars need to be non-reducing so that no metabolic interactions along the transport pathway cause any changes. Secondly, the candidate compound should be able to

occur at relatively high concentrations if it is to serve a storage function (Bachmann et al., 1995). Drew (1984) cites a concentration of 10 mg/g dw as a reasonable starting point. Most importantly though, the compound should be metabolically flexible, being easily deposited at storage sites when photosynthates are in excess and remobilized when metabolism demands (Bachmann et al., 1995).

This stated, it is evident that other sugars besides sucrose may fulfill the function of a translocate. Sucrose is by far the principle carbohydrate formed during photosynthesis, but subsequent modifications result in the presence of an array of other sucrosyl sugars. In addition, plants do not exclusively transport one type of sugar. Phloem exudates often contain a number of different sugars in varying proportions (Table 1.1), including polyols and RFOs.

Table 1.2 Phloem translocates observed in selected families of dicotyledonous plants[a]

Family	Transport compounds					
	S	R	St	M	So	D
Fabaceae	+++	--	--	--	--	--
Bignoniaceae	++	++	++	--	--	--
Juglandaceae	++++	+++	+++	--	--	--
Salicaceae	++++	+	+	--	--	--
Magnoliaceae	+++	tr	tr	--	--	--
Oleaceae	++	++	+++	++++	--	--
Rosaceae	+++	tr	tr	--	++++	--
Celastraceae	++	+	+++	--	--	+++

[a] Relative amounts of transport compounds in the sieve sap are given according to Zimmermann and Ziegler (1975). S=sucrose, R=raffinose, St=stachyose, M=mannitol, So=sorbitol, D=dulcitol, tr = trace amounts (adapted from Turgeon, 2000)

1.4.2 The "Polymer Trap" relies on RFO biosynthesis

Two models of phloem loading have come to be accepted in contemporary phloem loading theory (Figure 1.4). Historically, apoplastic phloem loading was first proposed by Geiger et al. (1974). In this model phloem loading occurs when sucrose, synthesised during photosynthesis, finds its way into the apoplast either actively, via a transporter, or passively. From the apoplast, sucrose transporters shuttle sucrose into companion cells from where it enters the phloem bundles (sieve elements). However, subsequent observations showed that the abundance of plasmodesmata at the sieve element, companion cell complex (SE-CCC)

interface varied significantly between different species. Turgeon (1996) cites that species with abundant plasmodesmata are termed type 1 (open) and those with few plasmodesmata are termed type 2 (closed).

These observations led to the speculation that other types of phloem loading existed. Subsequent tests on the validity of the apoplastic model revealed that treatments with *p*-chloromecuri-benzene sulfonic acid (PCMBS), an inhibitor of sucrose-proton co-transport in *Coleus blumei* (Turgeon and McGowan, 1990) and *Olea europaea* L. (Flora and Madore, 1993) did not affect the accumulation of photosynthates in companion cells, leading to the proposal of an alternative model for phloem loading in RFO translocating species (Turgeon, 1991). In the symplastic phloem loading model (Fig 1.4) sucrose is synthesized in mesophyll cells during photosynthesis. It then diffuses via extensive plasmodesmal connections to the bundle sheath and into minor vein companion cells. Sucrose is then utilized in the synthesis of RFOs, which are structurally too large to diffuse back into the bundle sheath and mesophyll and enter the phloem, possibly by diffusion.

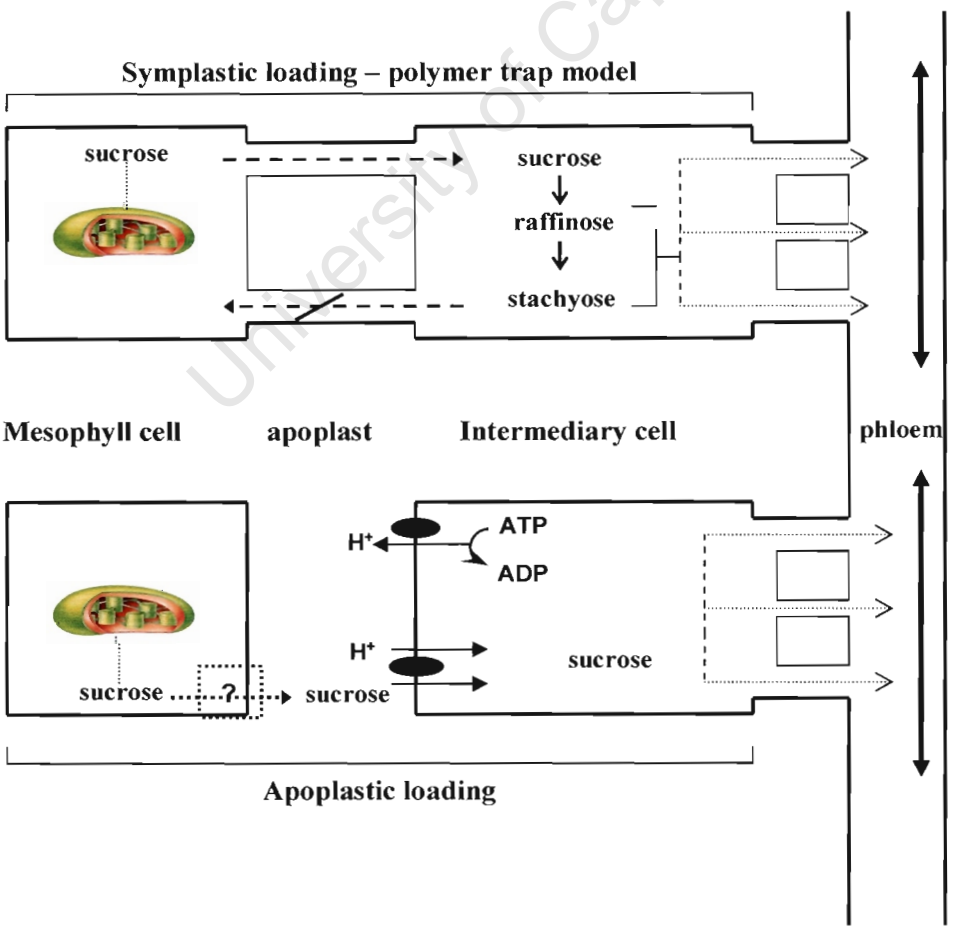

Figure 1.4 Schematic representation of the two accepted mechanisms of phloem loading in plants. Refer to text for details. Adapted from Turgeon (2000)

1.4.3 Gol is not a component of the translocation stream

As described earlier, gol is an unusual molecule occurring solely in plants which synthesise RFOs and its sole function elucidated to date is to serve as the galactosyl donor during RFO biosynthesis. In the cucurbit species, gol present in leaf tissue is made in the intermediary cells (Turgeon, 2000). Although gol is not strictly speaking a sugar, it does fulfil the requirements of a compound that is phloem mobile. Haritatos et al. (1996) determined that in *Cucumis melo* L., gol occurs in the intermediary cells at concentrations that are two thirds that of raffinose. Consequently, if gol is in its free form and not bound by any enzymes then it to should enter the sieve elements and be transported.

Surprisingly, little or no gol has been detected in phloem exudates from a number of RFO translocating species examined to date (Mitchell et al., 1992; Turgeon et al., 1993). Turgeon (2000) suggests a simple explanation for these observations. As previously described, gol is comprised of gal and *myo*-inositol monomers. Given that *myo*-inositol is a very ubiquitous cyclitol essential to a number of metabolic pathways (Loewus and Murthy, 2000), if gol were lost in large quantities to sink tissues then *myo*-inositol would have to be constantly re-synthesised to replenish the pool. The mechanisms that enable this phenomenon to occur are poorly understood. However it has been suggested that either a selective transport system into the phloem stream prevents gol from entering or that there is a reclamation system in the sieve tubes that retrieves essential molecules from the phloem sap, akin to the human renal system (Turgeon, 2000).

1.4.4 GolS is not the master regulator of RFO biosynthesis

As aforementioned, RFO biosynthesis begins with GolS and the synthesis of gol. Traditionally, the belief has been held that GolS activity is the key regulatory factor in RFO biosynthesis (Handley et al., 1983). Although GGT was identified in *A. reptans* (Bachmann et al., 1994), similar RFO biosynthetic enzymes that function independently of gol as a galactosyl donor have not been identified in other plant species to date. It is thus logical to assume that expression of GolS is essential to RFO biosynthesis, in plants lacking gol independent RFO biosynthetic enzymes, as gol is the only known galactosyl donor in the synthesis pathway (Karner et al., 2004). Recent studies into the role of RFOs in stress tolerance (Cunningham et al., 2003; Downie et al., 2003; Zhao et al., 2003) failed to correlate GolS activity and RFO accumulation in all cases. Downie et al. (2003) described a poor

correlation of GolS mRNA levels with the accumulation of raffinose in tomato seeds. Additionally studies conducted on pea cultivars with naturally contrasting raffinose content found no significant difference in GolS activity in the developing seeds of these cultivars (Peterbauer et al., 2001). Although much of the work has been conducted on developing and germinating seeds, evidence exists to postulate other regulatory factors in RFO biosynthesis.

Indeed, work on mutant soybean seeds has shown that mutants with reduced *myo*-inositol levels resulted in drastically reduced gol and RFO contents (Hitz et al., 2002). Similar results have also been reported for transgenic potato tubers (Keller et al., 1998). Karner et al. (2004) showed that pea seeds fed exogenously with *myo*-inositol had increased gol levels, suggesting that sufficient GolS activity was present to utilize the excess *myo*-inositol. In addition it was also shown that barley mutants with defective phytic acid pathways showed elevated levels of *myo*-inositol and gol (*myo*-inositol is utlised in phytic acid biosynthesis – see Loewus and Murphy, 2001). It is thus evident that RFO biosynthesis is not regulated by a single enzyme and that *myo*-inositol may play a key role in this regard. However, given that sucrose is also essential to RFO biosynthesis downstream of gol synthesis it is wholly possible that regulation of RFO biosynthesis pathways is very much more complex, with a number of enzymes and/or metabolites functioning in concert to facilitate optimal concentrations of RFOs.

1.4.5 A role for RFOs in stress tolerance

RFOs are found abundantly in desiccation tolerant orthodox seeds and are often absent or detectable in trace quantities in recalcitrant seeds that are desiccation sensitive (Lin and Huang, 1994; Sun et al., 1994). In orthodox seeds representing many different species, RFO accumulation has been shown to coincide with the onset of desiccation tolerance during the maturation stage of seed development (Koster and Leopold, 1988; Leprince et al., 1993; Horbowicz and Obendorf, 1994; Black et al., 1996). In addition, RFO content has been positively correlated with seed longevity in storage (Horbowicz and Obendorf, 1994; Lin and Huang, 1994; Bernal-Lugo and Leopold, 1995). However, there are conflicting reports that indicate that large amounts of RFOs are not a prerequisite for desiccation tolerance or longevity in seeds (Hoekstra et al., 1994). Other reports promulgate a function for RFO accumulation in seeds as readily metabolisable carbon storage sources which are utilised during the germination process (Downie and Bewley, 2000).

Recent in vitro studies have implicated RFOs in protecting membranes during dehydration. RFOs were found to directly interact with the phospholipid headgroups of synthetic liposome membranes in a manner that suggested direct molecular interactions via hydrogen bonding. In addition, the higher DP RFOs were shown to protect membranes during desiccation better than lower DP RFOs (Hincha et al., 2003). It has also been shown that overexpression of a single gol synthase cDNA in Arabidopsis led to transgenic plants accumulating higher levels of gol and raffinose (200-500 μg/g fwt) than wild type plants and improved the drought tolerance of those transgenic plants (Taji et al., 2002). RFOs have been reported to increase significantly in response to low temperature in *Ajuga reptans*. Although these reports clearly indicated that RFOs were utilised in phloem translocation and subsequently stored in the roots, there is also evidence indicating seasonally related fluctuations in RFO concentration and profile, with the highest concentrations (110 mg/g fwt) occurring over winter months when temperatures are below 0°C (Bachmann et al., 1994). Thus it is evident that in planta RFO accumulation correlates with low temperature stress tolerance in *A. reptans*. Given that this plant is able to survive in sub-zero temperatures in its natural environment without leaf senescence, it is likely that RFOs may serve a dual function as storage carbohydrates and stress protectants. Studies have also reported RFO accumulation in roots in response to salinity stress (Gilbert et al., 1997) and low temperature (Bachmann et al., 1995; Madore and Lucas, 1995).

1.4.6 Are combinations of sugars necessary to facilitate stress tolerance?

An intriguing physiological possibility is that absolute concentrations of sugars are not necessarily important in providing protection under conditions of abiotic stress but rather a combination of certain sugars may serve to provide the optimal protective effect. To date, research has not absolutely investigated this possibility however; evidence does exist to implicate sugars acting in combination during abiotic stress. Santarius and Milde (1977) observed increases of sucrose and raffinose in chloroplasts of frosthardy leaves. Taji et al. (2002) also observed increases of sucrose in response to drought stress in Arabidopsis plants overexpressing a GolS enzyme. Although raffinose levels also increased, these were considered to be a result of the overexpression of GolS and the possible combined sucrose-raffinose increases were not given attention. Hincha et al. (2003) demonstrated the protective effects of RFOs on liposomes during desiccation however; no attention was given to evaluating the effects of combinations of RFOs or combinations with other sugars known to increase in response to desiccation and low temperature stress. Lin et al. (1999) observed that

high amounts of sucrose alone did not prevent desiccation induced damage during germination of the seeds of five crop species. It was however observed that amongst the nett sugar changes that occurred during germination only the RFO quantity and the RFO/sucrose ratio showed significant positive correlations in four out of the five species examined. Zhao et al. (2004) also alludes to the possibility that RFO accumulation may occur in assisting sucrose to protect plant cells against the effects of osmotic stress.

Campana Cue et al. (2001) provide intriguing evidence that lends credence to the possibility that some sugars may directly interact with each other in a situation that is prevalent during desiccation stress. The study showed that during the crystallization of sucrose, the galactose ring of raffinose successfully stablised two hydrogen bonds with the fructose ring of sucrose. In addition it was concluded, on the basis of equilibrium energy states, that the process of the raffinose adsorption to the sucrose crystal is more favourable than the processes of the adsorption of the sucrose molecule. It is known that vitrification of the cell contents occurs during desiccation stress (Hartung et al., 1998). Under these conditions cellular metabolites such as sucrose may indeed undergo full or partial crystallization, facilitating spontaneous raffinose adsorption. Although an interesting hypothesis, to the author's knowledge no research into whether this phenomenon is possible in planta has been conducted. Intriguing research questions related to resurrection plants arise if one entertains the possibility of raffinose-sucrose interactions during water deficit stress. Firstly, is the interaction in planta possible? Secondly, does the hybrid molecule confer better stress protection at lower concentrations than the individual sugars? And thirdly, is there a reversion of the molecule during rehydration?

1.5 Sugars emerge as important role players in signal transduction

The process of photosynthesis affords plants and other phototrophic organisms the unique position of being able to meet their metabolic requirements by autonomously producing metabolic substrates. Ultimately, the sole purpose of photosynthesis is to generate metabolites in the form of carbohydrates. If ATP is considered to be the energy currency of the plant cell, then carbohydrate synthesis, translocation and storage is surely what most of this currency is expended on during plant metabolism.

The role of sugars in gene expression is not a novel concept. In bacteria for example, the effect of glucose on the lactose (*lac*) operon has been well characterised. The *lac* operon encodes β-galactosidase which cleaves β-galactosides to glucose, which is then fed into the glycolytic pathway. In the absence of glucose, cyclic AMP levels are high. A receptor protein called the cAMP receptor protein (CRP) then facilitates the formation of a cAMP-CRP complex which binds promoter elements in the *lac* operon, initiating the transcription of the lactose utilizing genes (Maloy et al., 1994). Similar pathways have been elucidated in yeast, with entire signalling pathways linked to glucose being characterised (for review see Smeekens and Rook, 1997; Koch et al., 2000). It stands to reason then, that if both bacteria and yeast have signal transduction pathways orchestrated by sugars, then plants which have more diverse carbohydrates profiles and usages should also display sugar dependent signalling pathways. This is indeed the case with much attention recently given to elucidating the role of sugars in plant gene regulation (Koch et al., 2000; Loretti et al., 2001; Quintero et al., 2001; Rook and Bevan, 2003).

Unlike bacteria and yeast, plants are multicellular organisms showing complex tissue differentiation and organization. Consequently, the mechanisms employed in sensing and responding to external stimuli should also involve a greater level of complexity. Loretti et al. (2001) define an efficient sugar sensing machine as having the following components: (1) The presence of a sensor able to perceive the concentration of sugars in the apoplast; (2) a sensor able to respond to intracellular carbohydrate flux; and (3) a means of sensing carbohydrates in the cytosol. In addition the network should also be able to monitor the presence and changes of sucrose and other disaccharides which are normally present in the cytosol or being moved around to different compartments. It is thus evident that with such a complex requirement for sugar signalling, the research question has proved an intriguing challenge for contemporary researchers. What has been elucidated thus far will be outlined in the following paragraphs.

A growing body of research evidence implicate hexokinases (HXKs) as pivotal sugar sensors in plants. It has been shown that glucose analogues which were substrates for HXKs were able to modulate sugar-related genes when fed to cucumber plants (Graham et al., 1994). Similarly, phosphorylated sugars such as glucose-6-phosphate were unable to elicit the same effect as glucose when infiltrated into *Zea mays* protoplasts via electroporation and inhibitors of HXK activity were found to eliminate any gene effects brought about by glucose (Umemura et al.,

1998; Pego et al., 1999). At low concentrations mannose and 2-deoxyglucose were observed to repress photosynthetic genes (Jang and Sheen, 1994). These sugars serve as HXK substrates but competitively inhibit glucose-6-phosphate production. In these experiments it would appear that the glucose phosphorylation step represents the point at which signal transduction occurs and not the phosphorylated sugar itself.

Jang et al. (1997) provide further evidence for the HXK\glucose relationship. Transgenic Arabidopsis plants overexpressing the AtHXK1 or AtHXK2 are glucose hypersensitive with respect to sugar responses in seedling development and gene expression. Conversely, plants transformed with anti-sense constructs showed hyposensitivity to glucose. In yeast, Hhk2 has been implicated in the glucose mediated repression of genes involved in the utilisation of alternate carbon sources. Although HXKs appear to play a significant role in glucose mediated gene expression, the complexity of in planta phenomenon cannot preclude many other mechanisms of sugar sensing and signalling. Indeed the recent identification of an Arabidopsis fructokinase (FRK) mutant that is mannitol insensitive (Pego and Smeekens, 2000) implies a role for FRKs in sugar sensing. This is strengthened by observations in tomato that FRK1 and FRK2 transcripts increase in response to exogenous application of glucose, fructose and sucrose. Given that the Arabidopsis genome has yielded a total of six HXK or HXK-like genes, three FRK genes and a number of FRK-like genes (Pego and Smeekens, 2000), it is evident that we are far from understanding how the mechanism operates in plants.

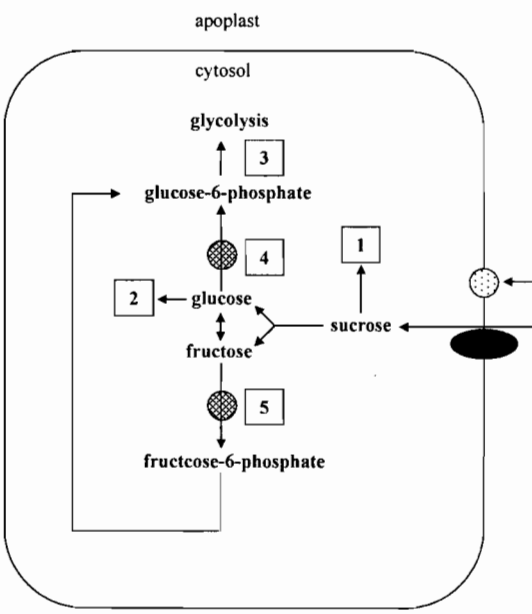

Figure 1.5 Schematic representation of multiple sugar sensing pathways in plants. Apoplastic sucrose is sensed either by dual functional sucrose transporter or a dedicated membrane sensor. Intracellular sucrose is sensed inside the cell (1), or after breakdown into hexose components by a hexose sensor (2) or by a HXK acting as a hexose sensor (4). FRK may be a supplemental hexose sensor, sensing fructose fluxes (5). Independent sensing pathway senses fluxes into metabolism (3) (after Loretti et al., 2001)

1.5.1 The master stress hormone may link stress and sugar signalling

A variety of plant genes have been found to be regulated by abiotic stress, particularly water deficit (Bray, 1993; Zhu et al., 1996; Shinozaki and Yamaguchi-Shinozaki, 1997). In addition, a significant subset of these genes has been found to be also regulated by the plant phytohormone abscisic acid (ABA) (Zhu, 2002). It has also been demonstrated that genes induced by exogenous ABA application are also induced by low temperature and/or water deficit in ABA deficient (*aba*) or ABA insensitive (*abi*) Arabidopsis mutants (Ingram and Bartels, 1996). Such observations have led to the development of a model for stress related signal transduction that sees ABA play a crucial role in the activation of stress related genes which display ABA dependence (for review see Shinozaki and Yamaguchi-Shinozaki, 2000). Recent evidence has begun to emerge that may greatly extend the role of ABA to include signalling events that involve sugars.

The characterisation of Arabidopsis sugar insensitive mutants has provided evidence to link ABA to sugar signal transduction, with several of these mutants displaying the *aba* and *abi* phenotypes. Huijser et al. (2000) described the sugar insensitive Arabidopsis mutant *sun6*, which displays a glucose insensitive (*gin*) phenotype as well as an *abi* phenotype, as being identical to the *abi*4 mutant. Similar studies have also reported mutants with the *gin* phenotypes, isolated in sugar response screens, as being allelic to *abi*4 (Arenas-Huertero et al., 2000; Laby et al., 2000). Other sugar insensitive mutants have been found to be allelic to genes involved in the biosynthesis of ABA (Gibson, 2000). The expression of the ABA induced *Rab16A* gene was found to be repressed by glucose in rice and barley embryos, suggesting that glucose interferes with ABA signal transduction in these plants (Toyofuku, 2000). There is thus a growing body of evidence that convincingly demonstrates some level of interaction between ABA and sugar signalling pathways. There are however, conflicting reports on what the exact role of these interactions are. Arenas-Huertero et al. (2000) suggested that sugar responses are directly mediated by ABA. This model has been refuted in Rook et al. (2001), suggesting that ABA affects sugar response by regulating the responsiveness of specific tissues to sugar signals.

In context, the question that needs to be addressed is firstly, does sugar signalling play a role in abiotic stress? And secondly, is crosstalk between phytohormone and sugar signalling pathways possible? Conclusive evidence to indicate that sugar signalling is part of the stress

response does not exist to date. However, sugars have been shown to regulate the expression of wound inducible genes (Johnson and Ryan, 1990; Sadka et al., 1994) and pathogenesis induced genes (Xiao et al., 2000). It has also been described that in yeast, glucose sensing and signalling pathways play a central role in yeast longevity by modulating oxidative stress resistance (Fabrizio et al., 2001). Similarly, Xiao et al. (2000) described delayed senescence and increased stress tolerance in Arabidopsis plants expressing antisense constructs for hexokinase thereby providing evidence that sugar metabolism and sensing are somehow involved in the regulation of aging and stress tolerance. Another plant phytohormone, ethylene, has been shown to interact with sugar sensing pathways in experiments where overexpressing mutants are glucose insensitive and those with an ethylene insensitive phenotype were glucose hypersensitive (Zhou et al., 1998).

In summary, it is evident that sugar signalling plays an important role in plant metabolism. Although successful inroads have been made in describing some sugar sensing mechanisms, the level of complexity of these systems in plants clearly indicates the need for continued efforts to understand the global sugar sensing and signalling systems as well as their interactions with other more well characterized signal transduction pathways. There is no direct functional evidence of molecular crosstalk between traditional stress response signalling pathways (such as ABA) and sugar response pathways, however given that enzymes such as invertases, which play a central role in maintaining carbohydrate homeostasis, are regulated by both stress stimuli and hormones (Roitsch, 1999) it may yet be shown that such genes serve as central modulators – integrating sugar, stress and hormone signals.

1.6 Targeting carbohydrate and carbohydrate based metabolic pathways for improved stress tolerance

The advent of genetic engineering has proffered an attractive alternative to introducing stress tolerance phenotypes into agronomically important crop lines. As opposed to traditional breeding and marker-assisted selection, directly introducing a small number of genes into a plants genome provides a rapid approach to improving stress tolerance (Cushman and Bohnert, 2000). Contemporary engineering efforts have relied on transferring one or more genes, controlled by a constitutively expressed promoter, that are components of metabolic or signalling pathways. A number of approaches have been reported involving the overexpression of osmoprotectants (Bohnert and Sheveleva, 1998; Apse and Blumwald, 2002;

Chen and Murata, 2002), detoxification enzymes (Bartels, 2001; Apse and Blumwald, 2002), genes involved in ion homeostasis (Yoshida, 2002) and genes encoding proteins induced during stress (Malik et al., 1999; Iba 2002).

1.6.1 A broad variety of carbohydrate species increases during abiotic stress

Compatible solute biosynthesis, as mentioned previously, may be a critical component in the global stress response of a variety of organisms. Importantly, a number of compounds thought to have osmoprotectant effects, that have been shown to increase in response to abiotic stress are carbohydrates or carbohydrate based. This statement is ratified by observations that one class of compatible solutes, namely polyols, has been correlated with salinity and /or water deficit stress in bacteria, yeast, marine algae, higher plants and animals (Bohnert and Jensen, 1995; McNeil et al., 1999). Straight chain polyols such as mannitol or sorbitol, or cyclic polyols such as *myo*-inositol have their origins in the glucose-6-phosphate pool (Bohnert and Jensen, 1995) providing a direct link, in the context of photoautotrophs, between photosynthesis and the modification of primary photosynthates into compounds which may directly protect during stress. In the yeast *Sacchoromyces cerevisiae* it has been reported that the carbohydrate trehalose is effective in maintaining membrane integrity during cycles of rehydration and desiccation (Crowe and Crowe, 1986). In the context of resurrection plant physiology sucrose has been shown invariably to increase during the drying process in a variety of desiccation tolerant plants (Ghasempour et al., 1998; Leprince at al., 1993). In the resurrection plant *Craterostigma plantagineum* an unusual eight carbon sugar 2-octulose is the primary photosynthetic storage sugar. During dehydration, octulose is rapidly converted to sucrose such that sucrose becomes the primary sugar in desiccated leaves (Bianci et al., 1991). Sucrose is also the only sugar utilised by the desiccation tolerant moss *Tortula ruralis*, accounting for 10% of the dry weight of gametophytic cells (Oliver and Bewley, 1997). Interestingly, *C. planatgineum* roots in the fully hydrated plant display detectable levels of the RFO stachyose that do not alter significantly during dehydration (Bernacchia and Furini, 2004). Further information of the complete carbohydrate spectrum of *C. plantgineum* in the dehydrated state is not available hence it is unknown at this stage if desiccation tolerance in the root tissue of this plant is due to the presence of stachyose and/or increases in sucrose levels.

1.6.2 "Sweet" success

The genetic engineering of metabolic pathways involving multiple compounds classified as compatible solutes has been undertaken in a number of higher plants (Table 1.3). Most of these studies have however, focused on model plant systems such as Arabidopsis and tobacco and not on agronomically important crops. One of the most successful recent undertakings in this regard was the engineering of trehalose biosynthesis pathways in rice (Garg et al., 2002). Trehalose is a non-reducing disaccharide of glucose and is thought to be a stress protectant in bacteria, yeast and invertebrates (Crowe et al., 1992). Most plants, with the exception of some resurrection plants, do not accumulate detectable amounts of trehalose (Wingler, 2002) but the discovery that trehalose biosynthetic genes have homologs in Arabidopsis and several crop plants (Goddijn and van Dun, 1999) has led to speculation that the inherent ability to synthesise and utilize trehalose may be widely distributed in the plant kingdom.

Garg et al. (2002) successfully reconstructed a trehalose biosynthesis pathway in *Oryza indica* rice by transforming the plants with a fusion construct containing the trehalose-6-phosphate synthase and the trehalose-6-phosphate phosphatase genes from *E. coli*, under the control of a constitutively active promoter. Transgenic plants were shown to accumulate trehalose (17 μg/g fw) under normal growing conditions and these amounts were significantly elevated during drought stress. In addition, transgenic plants also performed better under high salinity conditions as opposed to non-transformed lines (performance was gauged as root and shoot vigour during the stress). The most important results of this study, however, was that unlike previous attempts to engineer trehalose biosynthesis in tobacco (Pilon-Smits et al., 1998) and potato (Goddijn et al., 1997) there was no evidence of undesirable pleiotropic effects. Transgenic plants were fertile and displayed normal growth phenotypes thereby producing grain yields that were comparable to wild type untransformed plants.

Table 1.3 Examples of osmoprotectant engineering in plants. Maximal accumulation represents measured amounts of product formed in transgenic plants under conditions of abiotic stress. NR = Not reported

	Gene origin	Plant	Maximal accumulation	Tolerance	Reference
Glycine betaine	*Arthrobacter*	Arabidopsis	1.2 μmol g^{-1} fw	salinity	Hayashi et al. (1997)
	Arthrobacter	Rice	5.3 μmol g^{-1} fw	salinity, low temperature	Sakamoto et al. (1998)
	Arthrobacter	Canola	19 mmol g^{-1} dw	salinity, low temperature	Huang et al. (2000)
	E. coli	Tobacco	0.035 μmol g^{-1} fw	salinity, low temperature	Holstroom et al. (2000)
Proline	Mothbean	Tobacco	4 mg g^{-1} fw	salinity	Hong et al. (2000)
Mannitol	*E. coli*	Arabidopsis	10 μmol g^{-1} fw	salinity	Thomas et al. (1995)
		Tobacco	6 μmol g^{-1} fw	salinity	Tarczynski et al. (1992)
Sorbitol	Apple	Persimmon	61.5 μmol g^{-1} fw	salinity	Sheveleva et al. (1998)
D-Ononitol	Ice-plant	Tobacco	35 μmol g^{-1} fw	drought, salinity	Sheveleva et al. (1997)
Trehalose	*E. coli*	Tobacco	NR	drought	Pilon-Smits et al. (1998)
	Yeast	Tobacco	3.2 mg g^{-1} dw	drought	Holmstrom et al. (1996)
Raffinose	Arabidopsis	Arabidopsis	200-500 mg g^{-1} fw	drought	Taji et al. (2000)

1.6.3 Raffinosacharides as targets for metabolic engineering

This review has provided compelling evidence for RFOs being important compounds in conferring stress tolerance in both seeds and vegetative tissues of many plants species. Very little molecular evidence exists in terms of overexpression of RFOs improving the performance of transgenic plants under stress. However, as previously discussed, Taji et al. (2000) successfully showed that overexpression of a GolS enzyme in Arabidopsis led to increased levels of raffinose and a drought tolerance phenotype in transgenic plants. In addition Pennycooke et al. (2003) observed that down-regulation of an α-galactosidase enzyme specific to the hydrolysis of RFOs also led to increased levels of raffinose and a low temperature tolerance phenotype in transgenic plants.

A differential screen of a cDNA library constructed from the leaves of drought stressed *X. viscosa* plants identified 30 transcriptionally up-regulated and 20 transcriptionally down-regulated genes (Ndima et al. 2001). Amongst these was a cDNA clone found to show significant identity to gol synthase proteins from a number of species (this work, chapter 3). The purpose of this research project was to characterise GolS transcript and protein levels in

planta during various simulated abiotic stress treatments. In addition we aimed to ascertain changes in the carbohydrate profile, with particular focus on raffinosaccharides, in the leaves of plants subjected to water deficit and low temperature stress regimes to ultimately determine if GolS played a contributing role in desiccation tolerance in *X. viscosa*

Chapter 2

Materials and Methods

2.1 Plant material

2.1.1 Plant collection

Mature *Xerophyta viscosa* (Baker) plants were collected from Cathedral Peak Nature Reserve, (Kwazulu-Natal Province, South Africa). The plants were maintained under glasshouse conditions previously described by Sherwin and Farrant (1996). Plants used for carbohydrate extractions and enzyme activity assays were maintained under greenhouse conditions, previously described by Bachmann et al. (1994), at the Institute of Plant Biology, University of Zürich, Switzerland.

2.1.2 In vitro propagation

In vitro cultured plants were propagated from a seed stock collected at Cathedral Peak Nature Reserve. All fine chemicals were purchased from Sigma-Aldrich. MS medium (Murashige and Skoog, 1962) was purchased from Highveld Biological (Pty) Ltd., Lyndhurst, South Africa. Working within a laminar flow hood, approximately 1 g of seeds was added to a 1.5 ml Eppendorf tube containing 1 ml of 70% ethanol (v/v) and vortexed for 3 min, after which the ethanol was decanted. A further sterilisation procedure was conducted by resuspending the seeds in sterilisation solution [10% sodium hypochlorite solution (v/v), 0.02% Triton X-100 (v/v)] for 10 min prior to five washes, with agitation, in milli-Q H_2O.

Seeds were then resuspended in 0.1% agar and plated on seed germination medium [MS agar, 1.5% sucrose (w/v)]. Plates were stored in a controlled environment chamber [16 h light (30 $\mu mol.m^{-2}.s^{-1}$) 25°C, 8 h dark, 60 % relative humidity] until the seeds had germinated to plantlets of approximately 1.5 cm in height. The plantlets were then transferred into sterile 200 ml bottles containing rooting medium [0.1% MS salts (w/v), 0.5% MS vitamins (w/v), 0.8% agar (w/v), 0.1 mg napthalene acectic acid (w/v), 100 µg N^6-benzyle adenine (w/v), pH 5.8 (KOH)] and maintained in a controlled environment chamber described above. Plantlets were transferred to pots containing equal parts of sterilised peat, potting soil and vermiculite after approximately 1 yr of growth, when they had reached 5 cm in height. The plants were 'environmentally hardened' in a controlled environment chamber [16 h light (130 $\mu mol.m^{-2}.s^{-1}$) 25°C, 8 h dark, 60 % relative humidity] for six months before being transferred to greenhouse conditions.

2.2 General methods

Unless otherwise stated, all general methods described below were used during experimental procedures to follow.

2.2.1 Endonuclease restrictions and ligations

For endonuclease restrictions, 1 µg of DNA was digested in a total reaction volume of 20 µl (1X restriction buffer, 2U endonuclease). Insert and vector DNA were ligated at a ratio of 4:1 (v/v), in a total reaction volume of 20 µl (1X ligation buffer, 2U T4 DNA ligase).

2.2.2 Polymerase chain reaction (PCR)

PCR was conducted using an initial denaturation temperature of 94°C (2 min), followed by 25 cycles of 94°C (45 s), primer annealing temperature (50 s), 72°C (1 min) and a final elongation step of 72°C (10 min). Annealing temperatures for primer pairs are outlined for specific experiments. Template DNA (100 ng) was amplified in a total reaction volume of 50 µl [1X PCR reaction buffer (v/v), 1.5 mM $MgCl_2$, 0.5 µM primers, 0.15 mM deoxynucleotide triphosphates, 2 U Taq polymerase]. For colony PCR screens, single colonies were substituted as templates.

2.2.3 Transformations, plasmid isolations, PCR product purification and isolation of agarose bound DNA

Vector constructs were transformed into competent *E. coli* cells (Sambrook et al., 1989) by a heat shock transformation protocol described in the pGEM-T Easy (Promega Corporation, USA) user manual. Plasmids were isolated from 5 ml *E. coli* cultures (JM109 or DH5α), grown overnight at 37°C in Luria Broth (LB), with ampicillin (1 ug/ml), using a High Pure Plasmid Isolation Kit (Roche diagnostics GmbH, Mannheim, Germany) according to the manufacturer's instructions. Purification of PCR products and isolation of DNA fragments from agarose gel pieces was conducted by using a High Pure PCR Product Purification Kit (Roche diagnostics GmbH, Mannheim, Germany), according to the manufacturer's instructions.

2.2.4 Visualisation of ethidium bromide stained gels

Agarose gels were viewed under an ultraviolet trans-illuminator (Protea laboratory services, Cape Town, South Africa) at a wavelength of 260 nm (analysis) or 365 nm (cloning).

2.2.5 Sequencing analysis

All plasmid constructs and PCR products were sequenced using a MegaBACE 500 Automated Capillary DNA Sequencing System (Molecular Dynamics, Amersham Biosciences) using a DYEnamic ET Dye terminator Cycle sequencing Kit for MegaBACE, based on traditional dideoxynucleotide chain termination chemistry previously described by Sanger et al. (1977). All reactions were performed according to the manufacturer's instructions and cycle sequenced on a GeneAmp PCR System 9700 (Perkin Elmer, Applied Biosystems). Sequences were edited in-silico using the Chromas v.1.45 software package.

2.2.6 Nucleic acid tranfers:

All nucleic acid transfers were conducted for 2 h with either 0.4 M NaOH (DNA) or 10X SSC (RNA) using downward rapid transfer method previously described by Koetsier et al. (1993), with modifications (Figure 2.2.6.1). Cellulose cleaning sponges were purchased commercially (Scotch, 3M). Transparency film was cut to frame the gel.

Figure 2.1: Schematic representation of apparatus for rapid nucleic acid transfer adapted from Koetsier et al. (1993)

2.2.7 Soluble carbohydrate extraction

Ground, freeze dried *X. viscosa* leaf material (50 mg) was used to extract soluble carbohydrates. Extractions were conducted in 1.5 ml ethanol (twice for each concentration of 80% and 20% (v/v) respectively). During each extraction, samples were heated at 80°C for 10 min, placed on ice for 2 min and subsequently centrifuged at 15000xg in a bench top centrifuge for 5 min. The supernatants of all extraction steps were pooled and volumes adjusted to 6 ml with dH_2O. Aliquots of 50 µl were desalted and analysed by HPLC-PAD (see 2.2.9 and 2.2.10 respectively). For extractions from root material, 50 mg of fresh root tissue was washed in dH_2O and dried thoroughly on tissue. Carbohydrates were extracted in three

extraction steps using 500 µl of 80% ethanol (v/v), 50% ethanol (v/v) and dH_2O respectively. Extractions were conducted as described for leaf tissue except that after pooling supernatants, volumes were adjusted to 1.5 ml.

2.2.8 Enzyme extractions and GolS activity assays

Freshly harvested *X. viscosa* leaf material (200 mg) was ground in 500 µl of chilled extraction buffer [50 mM Hepes/KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM DTT, 0.1 % (v/v) Triton X-100, 1 mM benzamidine, 1 mM PMSF, 50 mM Na-ascorbate, 2 % (w/v) PVP]. Samples were centrifuged at 12000xg (5 min, 4°C). A 150 µl aliquot of supernatant was desalted by gel filtration at 1400xg (2 min, 4°C) through 5 ml Sephadex G-25 columns (fine, final bed volume of 3 ml). Columns were pre-equilibrated with assay buffer (50 mM Hepes/KOH pH 7.5, 2 mM $MnCl_2$, 10 mM DTT). Pre-equilibration was performed twice with 2 ml of assay buffer. Aliquots (20 µl) of desalted extract were assayed for GolS activity in a final volume of 40 µl assay buffer containing final concentrations of 50 mM myo-inositol and 5 mM UDP-galactose. Samples were incubated at 30°C for 15 min and 30 min, respectively with three replicates for each time point. Reactions were stopped by immersing the reaction tubes in boiling water for 2 min.

2.2.9 Desalting for HPLC analysis

Carbohydrate and enzyme assay samples were liberated of phenolic compounds and ions by desalting through pre-rinsed 1 ml Mobicol spin columns (MoBiTec, Göttingen, Germany), fitted with a 10 µm frit and filled with 150 µl of Biorad AG 1-X8 resin (HCO_3^- form, 200-400 mesh), 100 µl of Polyklar AT, and 50 µl of Biorad AG 50W-X8 (H^+ form, 200-400 mesh) respectively. Desalting was performed by centrifugation of the samples through the columns at 3000xg (4 min, 4°C) and centrifuge rinsing of the column with 325 µl (twice) and 100 µl dH_2O, respectively, at 3000xg (4 min, 4°C). Desalted carbohydrate samples were dried in a vacuum concentrator centrifuge (Univapo 100 ECH, UniEquip GmbH, Germany) to remove excess ethanol and resuspended in dH_2O (100 µl/mg dw) for HPLC-PAD analysis.

2.2.10 HPLC-PAD analysis and quantification of carbohydrates

Water-soluble carbohydrates were identified and quantified from plant extracts and enzyme assays by high performance liquid chromotography (HPLC) using a pulsed amperometric detection system (PAD) (Bachmann et al., 1994). A Ca^{2+}-Na^+ moderated ion partitioning carbohydrate column was used to separate carbohydrates [Benson BC-100 column (7.8 x 300

mm; Benson Polymeric, Reno, Nevada, USA) that was operated at 90°C and isocratically eluted with 0.005% (w/v) Ca/Na_2-EDTA at a flow rate of 0.6 ml/min]. To confirm the identities of certain carbohydrates, samples were also analyzed by anion exchange chromatography using a CarboPac MA1 column (4x250 mm; Dionex, Sunnyvale, CA, USA) operated at 30°C and isocratically eluted with 0.6 N NaOH at a flow rate of 0.4 ml/min.

The chromatographic system consisted of a Gynkotek model 480 High Precision Pump, a Gynkotek Gina 50 autosampler, and the Chromeleon v.6.4 chromatography software system (Dionex, Olten, Switzerland). A Jones column temperature controller (Grace Vydac, Hesperia, CA, USA). Carbohydrates were detected using an ESA Coulochem II electrochemical detector (ESA, Massachusetts, USA), operated with an ESA 5040 analytical cell. The MA1 column was operated with a BioLC® chromatographic system (Dionex, Sunnyvale, CA, USA) consisting of a GS 50 gradient pump, an ED50 electrochemical detector, and an AutoSelect™ AS50 autosampler. Soluble carbohydrates on both systems were quantified in silico, using the Chromeleon v.6.4 software package, against a series of 5 nmol of standard sugars. The quantity of standard sugars used corresponds to the linear response range of the BioLC® chromatographic system.

2.3 Cloning and in silico characterisation

2.3.1 Isolation and cloning of *XvGolS* cDNA

Total RNA was extracted from the leaves of dehydrated (5% RWC) *X. viscosa* plants using the TRIZOL LS reagent (Life Technologies, Invitrogen Corporation, Carlsbad, USA), following the manufacturer's instructions. cDNA was generated by reverse transcription PCR, using an Omniscript™ RT Kit (Qiagen, Germany) according to the manufacturer's instructions. Subsequently, PCR was conducted (annealing temperature of 58°C), using the cDNA as template, with primer A 5'AGCTCGAAATATGGCGCCGGAGATC3'and primer B 5'CATCCCTTGTCGAGCCCAGT3'. The products of five PCR reactions were electrophoresed separately in a 0.8% agarose gel (w/v) prestained with ethidium bromide (EtBr). Bands were excised from the gel using a sterile scalpel, purified, and cloned into the pGEM® -T Easy vector (Promega Corporation, Madison, USA) to create the construct pGEMT::*XvGolS*. All constructs were subsequently sequenced.

2.3.2 Bioinformatic analysis of *XvGolS*

The inferred amino acid sequence of *XvGolS* was obtained by translation of the cDNA sequence using the DNAMAN software package (Lynnon Biosoft v.3.0, 1997) and used to search for similarities in protein sequence databases using the BLAST network service (Altschul et al.1990). Amino acid comparisons were conducted using the CLUSTAL program of DNAMAN. A phosphorylation prediction profile was generated in silico using NetPhos v.2.0 software (Blom et al., 1999). Predictions of the subcellular localisation of XvGolS were generated In silico as described by Reinhardt and Hubbard, 1998. Three dimensional structures of conserved domains were obtained by using the full length protein sequence of XvGolS in protein blast (BLASTp) searches using the BLAST service (http://www.ncbi.nih.nlm.gov).

2.4 DNA isolation and Southern blot hybridisation

Genomic DNA was extracted from leaves of fully hydrated *X. viscosa* plants according to the procedure described by Dellaporta et al. (1983). The concentration and purity of the DNA preparations was determined spectrophotometrically (Sambrook et al., 1989). Aliquots representing 15 µg of DNA were restricted with the restriction endonucleases *Bam*HI, *Eco*RV, *Sac*I and *Hind*III (Boehringer-Mannheim, Germany) respectively, and electrophoresed in an agarose gel [0.8% agarose (w/v), 1xTBE (90 mM Tris-borate, 1 mM EDTA)] prestained with EtBr. The DNA was subsequently transferred and UV cross-linked onto nylon filters (Hybond-XL, Amersham Pharmacia Biotech) that were hybridised at 65°C with radiolabelled probe (A radioactive PCR reaction was conducted on *XvGolS* cDNA using primer A 5'CTGCTTACCCCTTAGTGGTTGC3' and primer B 5'AGCTTAAGCTGCTTCAAACCAGG3' with 50µCi α^{32}P-dCTP) in hybridisation buffer [0.5 M NaH2PO4, 0.001 M EDTA, 7% SDS (w/v), 1% BSA (w/v)]. Filters were washed twice for 10 min in wash buffer B [0.1% SDS (w/v), 0.5·SSC] and autoradiographed at –70°C onto high-performance autoradiography film (Hyperfilm™ MP, Amersham Pharmacia Biotech). The protocol was adapted from Sambrook et al. (1989). The radioactive PCR labelling reaction was carried out at an annealing temperature of 59°C, for an extension time of 10 min, and the cycle repeated 15 times.

2.5 Northern blot analysis

Total RNA was extracted from control and stress-treated *X. viscosa* leaves, representing a total of three plants, using the TRIZOL LS reagent (Life Technologies, Invitrogen Corporation,

Carlsbad, USA), following the manufacturer's instructions. For RNA gel blot analysis, approximately 10 µg of RNA was electrophoresed in an agarose gel [1.2% agarose (w/v), 1xTBE (90 mM Tris-borate, 1 mM EDTA)] prestained with EtBr, transferred and UV cross-linked onto nylon filters (Hybond-XL; Amersham Pharmacia Biotech). To estimate whether equal amounts of RNA were loaded, the gel was visualized by EtBr staining. Hybridisation was conducted at 65°C as described for Southern hybridisation. Filters were washed in wash buffer B for 10 min and exposed to high-performance autoradiography film (Hyperfilm™ MP, Amersham Pharmacia Biotech) at –70°C and developed after 72 h exposure. Subsequently, filters were hybridised with a radiolabelled 18s rRNA cDNA probe prepared using a radioactive PCR reaction as described for Southern hybridisation with an annealing temperature 54°C.

2.6 Western blot analysis

2.6.1 Protein Purification and Antiserum Production

E.coli cells were induced for recombinant XvGolS production as described in 2.7.2. After 2 h of induction the cells were collected by ultracentrifugation (Beckmann, 10000xg, 10 min) and resuspended in 20 µl of 5x sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) loading buffer. Samples were boiled at 100°C and subjected to SDS-PAGE using a 5 % stacking gel [10% acrylamide stock (v/v) (30% acrylamide (w/v), 0.8% bis-acrylamide (w/v)), 125 mM Tris-HCl (pH 6.8), 10% SDS] and a 10 % resolving gel [30% acrylamide stock (v/v) (30% acrylamide (w/v), 0.8% bis-acrylamide (w/v)), 375 mM Tris-HCl (pH 8.8), 10% SDS (w/v)]. Single gels electrophoresed in parallel and stained with Coomasie blue [0.05% Coomassie Brilliant Blue R-250 (w/v), 40% ethanol (v/v), 10% glacial acetic acid (v/v), 50% dH_2O (v/v)] were aligned to unstained gels and the protein band corresponding in size to the recombinant XvGolS was excised using a sterile scalpel and ground in liquid nitrogen before being resuspended in Phosphate buffer saline (1xPBS; 50 mM potassium phosphate, 150 mM NaCl, pH 7.2).

The full length purified recombinant protein was used to raise polyclonal antibodies in rabbits as previously described by Rybicki (1979). Antibodies were purified by adding two volumes of borate-buffered saline were added to one volume of anti-serum. Crushed Polyethylene Glycol (PEG 6000) was added to the diluted anti-serum (14%, w/v), gently mixed by inversion and centrifuged at 12 000xg (10 min, 4°C). After decanting the supernatant, the pellet was dissolved in the original serum volume in 1xPBS and PEG 6000 added as described

above. The pellet was redissolved in half the original serum volume in 1xPBS containing 60% glycerol (v/v). Aliquots were stored at -20°C prior to use in western blot analysis.

2.6.2 Protein extraction and western transfer

Total protein was extracted using the TRIZOL LS reagent (Life Technologies, Invitrogen Corporation, Carlsbad, USA), according to the manufacturer's instructions. Following SDS-PAGE as described in 2.6.1, proteins were transferred onto nylon filters (Hybond-P; Amersham Pharmacia Biotech) using a western transfer apparatus (Bio-Rad). Transfer efficacy was determined by staining filters with Ponceau S solution [2% Ponceau S (w/v), 1.1 M sulfosalicylic acid, 1.8 M tri-chloroacetic acid]. Staining was conducted by immersing the filters in Ponceau S for 2 min, with agitation. Subsequently, the filters were rinsed in dH2O until bands became visible. Parallel SDS-PAGE, using volumetrically equal amounts of protein from the same samples used for western transfers, was conducted and the gels stained with Coomasie blue. Rubisco bands were used to visually determine the loading efficacy of SDS-PAGE used for western transfers.

2.6.3 Immunoblotting

Filters were incubated, with agitation, in blocking solution [2.5% (w/v) skimmed milk in 1x phosphate buffered saline (PBS), pH 7.4] for 2 h, prior to an overnight incubation at 4°C with XvGolS antiserum diluted 1:500. Filters were subsequently washed for 10 min (twice) in blocking solution and incubated for 2 h with secondary antibody (anti-rabbit IgG, peroxidase linked whole antibody from goat; Sigma) diluted 1:5000, prior to a final washing step (2X10 min in 1XPBS, pH 7.4). Antibody binding was detected by using an ECL detection system adapted from the ECL detection kit (Amersham Pharmacia Biotech). Filters were exposed to Kodak BioMax ML Film (Sigma).

2.7 Heterologous expression in *E. coli*

2.7.1 Subcloning

Plasmid DNA mini-preparations were prepared from *E. coli* cultures (JM109), grown overnight, that had been previously transformed with the construct pGEMT-Easy::*XvGolS*. Aliquots of DNA (1 ug) were cut with the restriction endonucleases *Sal*I and *Eco*RI, electrophoresed in a 0.8 % agarose gel (w/v) and the appropriate restriction fragments excised from the gel. The plasmid vector pPROEX™ HTb (Life technologies, Invitrogen Corporation, Carlsbad, USA) was cut and purified in the same manner as described above. A

ligation was subsequently performed to create the construct pPROEX™ HTb::*XvGolS*, which was transformed into *E. coli* (DH5α) by means of a conventional heat shock transformation method outlined in the pGEM®-T Easy vector system user manual (Promega Corporation, USA). Putative transformants were screened by colony PCR using an *XvGolS* forward primer 5'CTGCTTACCCCTTAGTGGTTGC3' and a pPROEX™ HTb specific reverse primer 5'AATCTTCTCTCATCCGCC3' with an annealing temperature 58°C. Plasmid DNA mini-preparations from colonies yielding products from these PCR reactions were sequenced to confirm that *XvGolS* had been cloned, in frame, into the vector.

2.7.2 Expression of recombinant XvGolS

A single colony (*E. coli* DH5α containing pPROEX™ HTb::*XvGolS*) was inoculated into 5 ml of Luria Broth (LB) with ampicillin (1 μg/ml), incubated overnight at 37°C and used as an innoculum for 100 ml of prewarmed (37°C) LB with ampicillin (1 μg/ml) incubated at 37°C. Isopropyl – β – D – thiogalactopyranoside (IPTG) was added to a final concentration of 0.8 mM when the culture had reached an OD_{590} of 0.8. Subsequently, after 4 h of growth, the cells were collected by ultracentrifugation (Beckmann, 10000xg, 10 min). Cell lysates were prepared under native conditions as described in the QIA*expressionist*™ user manual (Qiagen). GolS activity was assayed as described in 2.2.8 using 20 μl aliquots of cleared lysate. Negative controls representing *E. coli* (DH5α) that had been transformed with the pPROEX™ HTb vector were treated in the same manner as described above. Samples were desalted and analysed by HPLC-PAD (see 2.2.9 and 2.2.10). Replicate experiments were conducted using individual colonies and three replicates per experiment were used to determine GolS activity.

2.7.3 Enzymatic hydrolysis

Fractions containing gol synthesised by the recombinant XvGolS were collected after separation on a BC-100 column, prior to the addition of post column NaOH. Aliquots were stored for later analysis, whilst the remaining samples were concentrated in a vacuum concentrator centrifuge and resuspended in 50 μl of McIlvaine buffer (48.5 mM citric acid, 103 mM disodium phosphate, pH5.0). α-Galactosidase enzyme from *Aspergillus niger* (Megazyme International Ltd., Bray, Wicklow, Ireland) was added to a final concentration of

2U per 50 µl reaction volume, and incubated at 40°C for 1.5 h. Samples were desalted and analysed by HPLC-PAD.

2.8 Biochemical characterisation of recombinant XvGolS

Cleared cell lysates were prepared and enzyme assays conducted as described in 2.8.2 and 2.2.8 respectively, and samples were desalted and analysed by HPLC-PAD as described in 2.2.9 and 2.2.10 respectively for the following experimental procedures. Experiments were repeated thrice, with three replicates per experiment, on three different *E. coli* colonies.

2.8.1 Determination of time linearity

Incubation times between 0 and 25 min were used, with three replicates for each time point.

2.8.2 Substrate kinetics

Myo-insitol and UDP galactose concentrations were varied between 0 mM and 50 mM in separate experiments.

2.9 Plant stress treatments

All treatments were performed in a controlled environment chamber [16 h light (500 µmol.m^{-2}.s^{-1}) 25°C °C, 8 h dark, 50% relative humidity], unless otherwise stated. Plants were transferred from greenhouse conditions (2.1.1) and maintained in the chamber for one week, prior to the commencement of stress treatments. Two expanded leaves were harvested at each time point, for further analysis, unless otherwise stated. Water deficit, low temperature and salinity stress treatments were conducted on whole plants whilst ABA treatments were conducted on in vitro cultured plantlets approximately 8 months old.

2.9.1 ABA treatment

ABA solution (100 µM) was exogenously applied to in vitro cultured plantlets. Control plants were sprayed with equivalent volumes of dH_2O. Whole plantlets were sampled at times 0, 24, 48 and 72 h, flash frozen in liquid N_2 and stored at -80°C for subsequent RNA and protein extraction.

2.9.2 Water deficit

Water deficit stress was imposed on whole potted plants by withholding irrigation over a period of 25 days, at the end of which the RWC was determined to be 5%. Leaf samples were

excised at regular intervals for RNA, protein and carbohydrate extraction, flash frozen in liquid nitrogen and stored at -80°C. Sampling times were determined by visual appraisal of the plant using leaf decolouration and folding as benchmarks after which relative water contents were determined for the samples.

The relative water content (RWC) was determined at each sampling interval as follows. The initial weight (W_i) of each sample was recorded before immersing it in Milli-Q water for 24 h. The weight at full turgor (W_t) was recorded and leaf samples subsequently dried at 95°C for 24 h and the dry weight (W_d) was recorded. The RWC was calculated using the formula of Barrs and Weatherley (1962): $RWC= [(W_i-W_d)/W_d]/ [(W_t-W_d)/W_d]*100$. Mean values (six replicates) for each sampling point were plotted on separate axes using the Microsoft Excel software package (Microsoft Office XP).

2.9.3 Low temperature

2.9.3.1 Low temperature shock

A single plant was initially exposed to a low temperature shock of -20°C for 3 h. Leaf samples were harvested every 15 min. After exposure to -20°C, the plant was maintained at 4°C for 5 d, before being transferred into the greenhouse. Subsequent low temperature shock treatments were conducted for 2 h.

2.9.3.2 Low temperature acclimation

Low temperature stress was also conducted by comparing the responses of two plants, one under conditions of acclimation and the other under conditions of non-acclimation to a temperature of 1°C. Acclimated plants were transferred from greenhouse conditions to a growth chamber maintained at 10°C, for 72 h. Subsequently, the temperature was lowered to 1°C and a non acclimated plant was transferred from greenhouse conditions into the chamber. Leaf samples were excised at time 0, 72 h later and subsequently at 48, 72, 96 and 200 h after exposure to 1°C for carbohydrate extraction, flash frozen in liquid nitrogen and stored at -80°C.

2.10 Electron microscopy

2.10.1 Tissue fixation

Leaf segments (approximately 5 mm^2) were excised from leaves of plants at full turgor and the process repeated subsequent to the plants being subjected to a low temperature shock of -20°C

for 2 h. The tissue was fixed by immersing in fixative solution [2.5% gluteraldehyde (v/v) in 0.1 M phosphate buffer (pH 7.2), 0.5% (w/v) caffeine]. Fixed tissue was subsequently treated and sectioned as described in Sherwin and Farrant (1996).

2.10.2 Staining and microscopy

Tissue sections were stained with uranyl acetate and lead citrate as previously described by Reynolds (1963) and viewed with a Jeol 1200 CX transmission electron microscope. Observations were conducted on three sections representing separate leaf samples.

2.11 Evaluation of a phloem exudation technique for *X. viscosa*

The carbohydrate profile of phloem sap was determined after phloem exudation using an exudation technique previously described for dicotyledonous plants, by King and Zeevart (1974), with modifications. Mature *X. viscosa* leaves were excised at their base from potted plants and immersed immediately, at the excision point, in exudation buffer (5 mM Na_2EDTA, 5 mM K_2HPO_4, pH 7.0). Leaves were recut under exudation buffer and placed in a 2 ml Eppendorf tube, closed with parafilm, containing 500 µl of exudation buffer, that was exchanged every 2 h over an 8 h period. Leaves were maintained in a chamber with high relative humidity, placed on a laboratory bench, over the duration of the exudation procedure. Exudates were subsequently liberated of particulate matter by centrifugation at 3000xg (4°C, 4 min) through pre-rinsed syringe filters fitted with a 0.45 µm nylon filter and analysed by HPLC-PAD without desalting.

Chapter 3

Results

3.1 In silico analysis of XvGolS

The nucleotide sequence of the cDNA designated *XvGolS* was determined to have an open reading frame (ORF) of 1020 bp, encoding an inferred polypeptide with a predicted MW of 34 kDa (Figure 3.1) and showed high sequence identities to galactinol synthase enzymes from a variety of plants (Figure 3.2). In silico phosphorylation predictions on XvGolS revealed one serine residue (amino acid position 73) and three threonine residues (amino acid positions 211, 269 and 331) with phosphorylation potentials above the threshold value of 0.5 (Figure 3.4). A conserved domain representing the glycosyl transferases family 8 was found for XvGolS as well as all the known full length GolS sequences lodged in GenBank and spanned approximately three quarters of the length of the XvGolS polypeptide (Figure 3.5).


```
                                                        agctcgaaat      - 10
atggcgccggagatcgtgagcaagagggctgccaactacgccgggaagcaggtggcagct          60
  M  A  P  E  I  V  S  K  R  A  A  N  Y  A  G  K  Q  V  A  A          20
ccgcgaaacgcctacgttactttcctggcgggagctggggactacgttaagggcgtcgtt         120
  P  R  N  A  Y  V  T  F  L  A  G  A  G  D  Y  V  K  G  V  V          40
ggactcgctaaggggctgcgaaaagtaggctctgcttaccccttagtggttgcggtgctc         180
  G  L  A  K  G  L  R  K  V  G  S  A  Y  P  L  V  V  A  V  L          60
cccgacgttcctgacgaacaccggaagctactggtgtcccaggggtgcatcgtaaggcaa         240
  P  D  V  P  D  E  H  R  K  L  L  V  S  Q  G  C  I  V  R  Q          80
atcgagcccgtgtaccctcccgagaaccagacccaattcgccatggcgtattacgtcatc         300
  I  E  P  V  Y  P  P  E  N  Q  T  Q  F  A  M  A  Y  Y  V  I         100
aactattctaagctccgcatttgggagtttgtagagtatagcaagatgatatatttggac         360
  N  Y  S  K  L  R  I  W  E  F  V  E  Y  S  K  M  I  Y  L  D         120
gcagacatccaagtctacgataacatagaccacctgtttgacctgcccggcgggcgtttc         420
  A  D  I  Q  V  Y  D  N  I  D  H  L  F  D  L  P  G  G  R  F         140
tacgcagtaatggactgcttctgcgagaagacatggagccacacgccgcagtacaaaatt         480
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ggctactgccagcagtgtcccaacaaggtgacatggccggcggagctggggcaaccgccg         540
  G  Y  C  Q  Q  C  P  N  K  V  T  W  P  A  E  L  G  Q  P  P         180
gcgctatacttcaacgcaggcatgttcgttcacgagcctagcctcgctaccgccgaaaaa         600
  A  L  Y  F  N  A  G  M  F  V  H  E  P  S  L  A  T  A  E  K         200
ctccttgctactctgaaggttgctccaacgactcccttcgctgagcaagactacttgaac         660
  L  L  A  T  L  K  V  A  P  T  T  P  F  A  E  Q  D  Y  L  N         220
atgttcttcaaggacatctacaggcccattccacttacttataaccttgtcctcgcaatg         720
  M  F  F  K  D  I  Y  R  P  I  P  L  T  Y  N  L  V  L  A  M         240
ctctggcgccaccctgagaatgtcgagctcgacaaggtcatggtcgtccattactgtgct         780
  L  W  R  H  P  E  N  V  E  L  D  K  V  M  V  V  H  Y  C  A         260
gcgggatcgaagccatggaggtatactggtaaagaagaaaacatggacagagaagacatc         840
  A  G  S  K  P  W  R  Y  T  G  K  E  E  N  M  D  R  E  D  I         280
aagatgttggttaagaagtggtgggacatctacaacgacgactcgctggattacaaggga         900
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ccggcggcaacgatggatctggagccggaggcccctggtttgaagcagcttaagctggcg         960
  P  A  A  T  M  D  L  E  P  E  A  P  G  L  K  Q  L  K  L  A         320
gcgctgtcggaggccggcgaggtgcgctatactaaagcaccttcagcagcttaa              1020
  A  L  S  E  A  G  E  V  R  Y  T  K  A  P  S  A  A  *               340
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Figure 3.1 Nucelotide sequence of XvGolS (black), indicating the inferred amino acid sequence of the XvGolS polypeptide obtained from an in silico translation using DNAMAN™ software

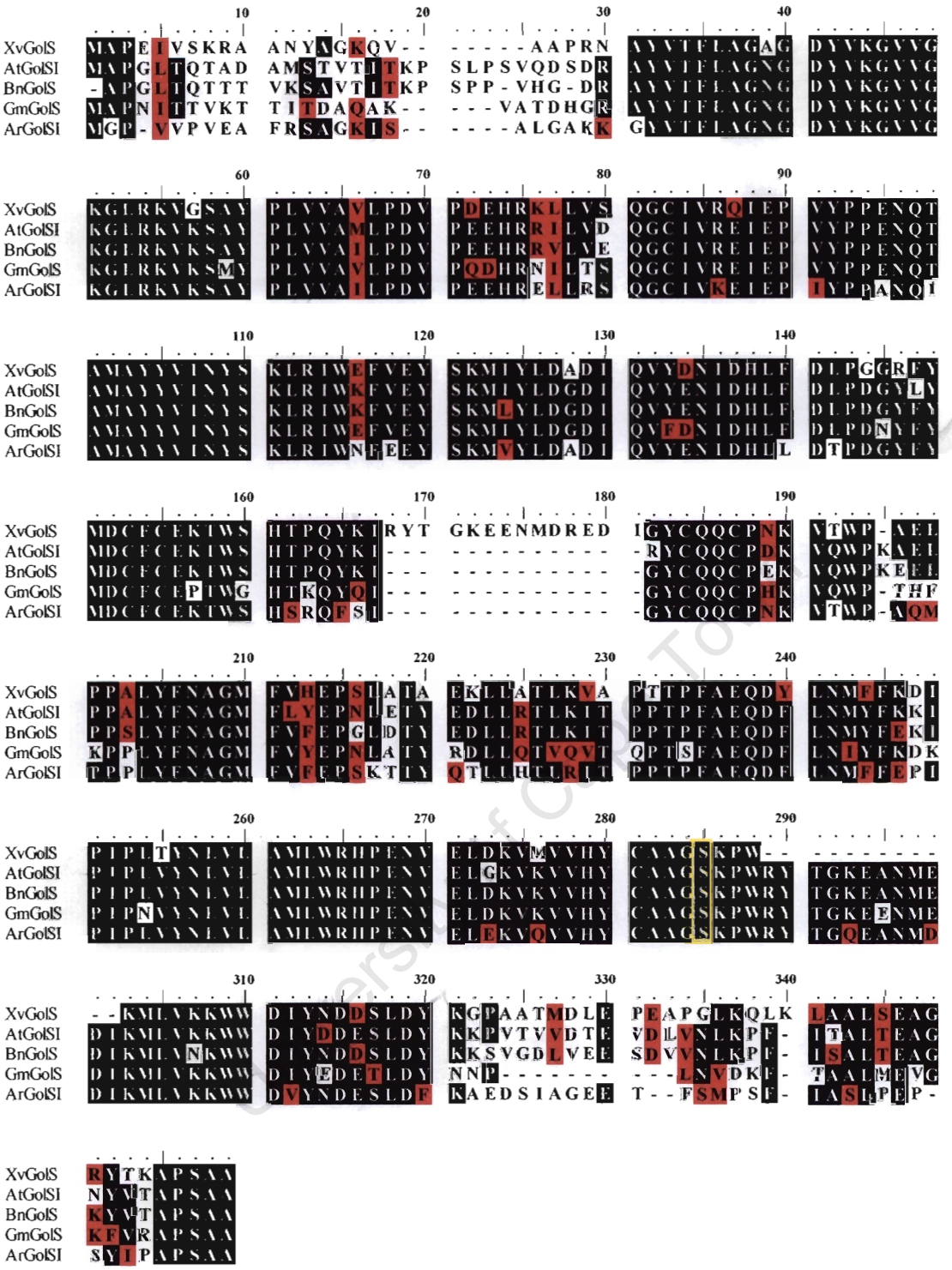


Figure 3.2 ClustalW alignment of GolS amino acid sequences that show highest identity to XvGolS. Identical and similar residues are highlighted in black and red respectively. A conserved serine residue common to most known GolS peptides is bordered in yellow. XvGolS shares identity of 81%, 80%, 78% and 75% to GolS peptides from *A. thaliana* (AtGolSI - AC002337), *Brassica napus* (BnGolS - AF106954), *Glycine max* (GmGolS - AY126715) and *Ajuga reptans* (ArGolSI - AJ237693)

A multiple sequence alignment revealed that the XvGolS polypeptide shared a high degree of identical amino acids with four GolS sequences from *A. thaliana*, *B. napus*, *G. max* and *A. reptans*. A conserved serine residue (ser → 263) known for most full length GolS sequences was present in all the sequences analysed. A distance matrix based phlyogentic tree constructed using neighbour joining parameters indicated that XvGolS formed a clade with GolS enzymes from *Z. mays* and *O. sativa* with the node supporting this clade having a boot strap value of 100%. Sequences from *A. thaliana* were found to cluster into four separate clades that included sequences from *T. halophila*, *A. reptans* and *P. sativum* (Figure 3.3)

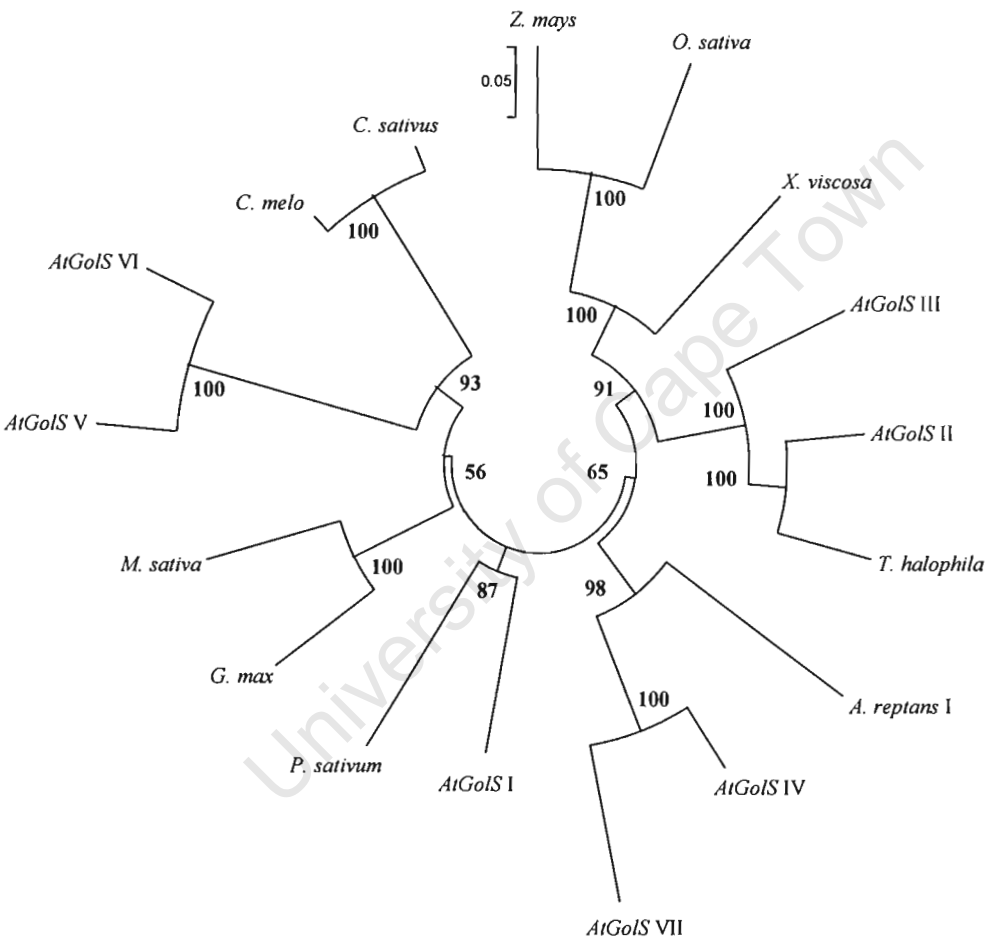


Figure 3.3 Midpoint rooted phylogenetic tree of full length nucleotide GolS coding sequences. Boot strap values are shown as a percentage of 1000 repititions. Sequences represent GolS genes from *Ajuga reptans* (AJ237693), *Arabidopsis thaliana* (AtGolS I-VII - AC002337, AC009323, AC003970, AC002292, AB005244, AL049171, AC004473 respectively), *Brassica napus* (AF106954), *Cucumis sativus* (AY237112), *Glycine max* (AY126715), *Medicago sativa* (AY126615), *Thellungiella halophila* (AF499723), *X. viscosa* (xx) and *Zea mays* (AY192144)

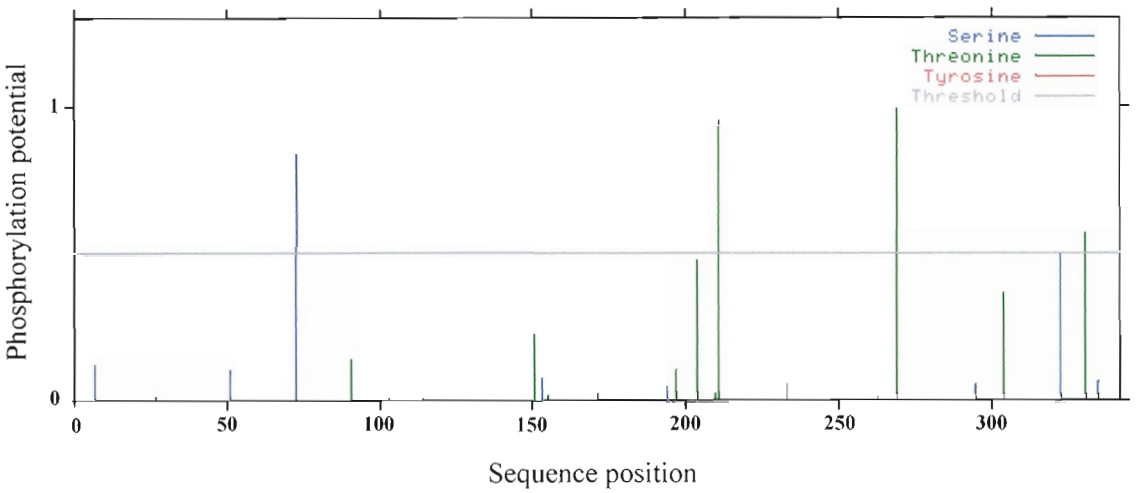


Figure 3.4 Phosphorylation prediction profile of the XvGolS polypetide

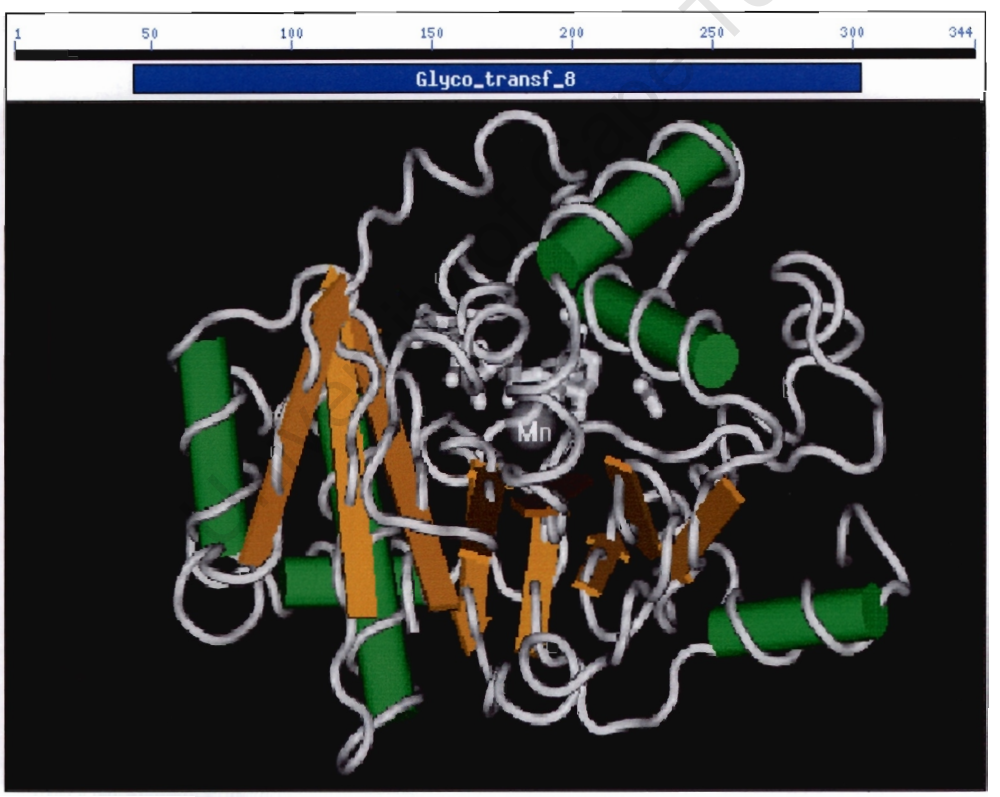


Figure 3.5 Three dimensional structure of the glycosyl-transferases family 8 conserved domain and the amino acid region it spans on the XvGolS polypetide. Orange arrows indicate β-pleated sheets whilst green cylinders represent α helices. The inset denotes the length on the XvGolS peptide over which the domain spans.

3.2 Southern blot analysis

Genomic DNA isolated from *X. viscosa* leaves was digested using a set of restriction endonucleases chosen from a restriction map of the *XvGolS* cDNA (data not shown). From this set SacI was the only endonuclease to have a recognition sequence within the cDNA. The Southern hybridisation revealed the presence of one distinct hybridizing band for genomic DNA digested with BamHI, EcoRV and HindIII (Figure 3.6, lanes 1-3 respectively) and two distinct hybridizing bands for the SacI digest (Figure 3.6, lane 4). Genomic DNA digested with combinations of endonucleases yielded one distinct hybridizing band for BamHI/EcoRV and BamHI/HindIII whilst two distinct bands were evident for BamHI/SacI (Figure 3.6, lanes 5-7 respectively).

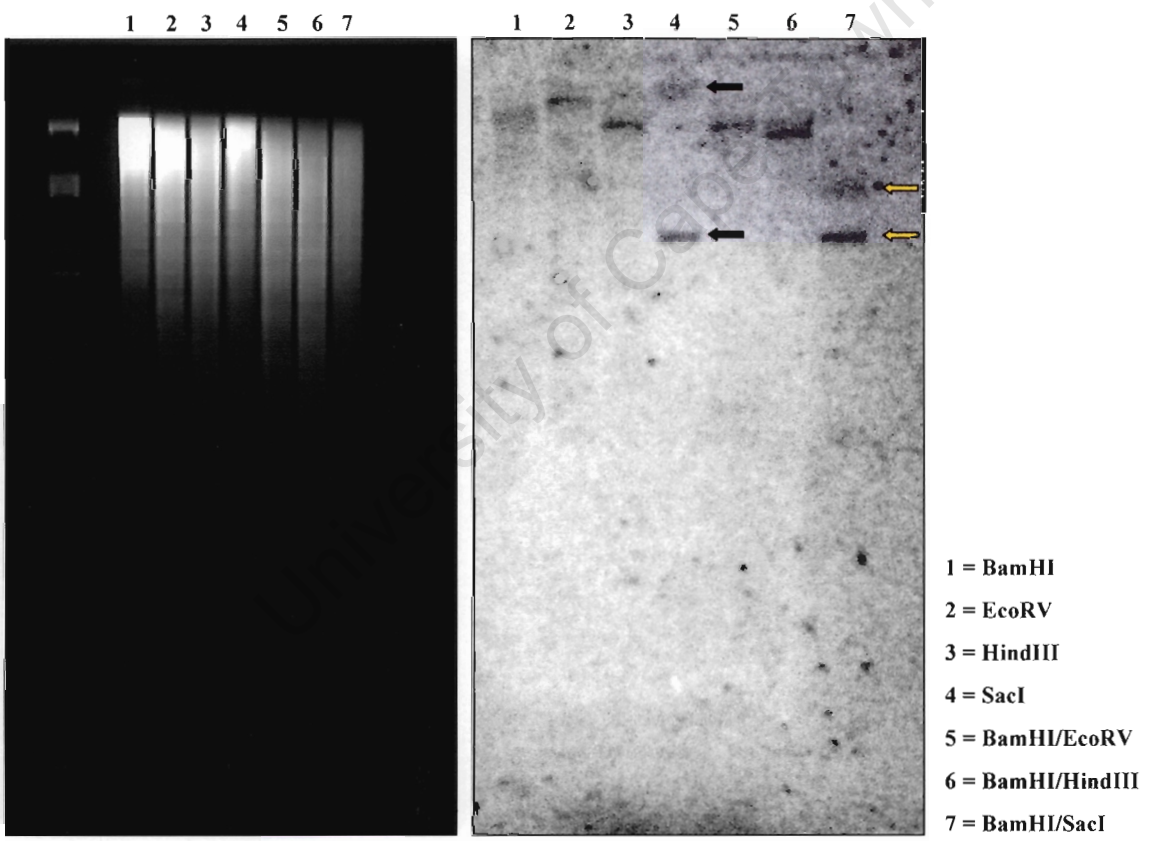


Figure 3.6 Southern hybridization of genomic DNA isolated from *X. viscosa* leaves. Black and yellow arrows indicate hybridizing bands in lanes 4 and 7 respectively.

3.3 Northern and western blot analysis

Total RNA was extracted from the leaves of *X. viscosa* plants subjected to a water deficit stress and a low temperature shock of -20°C for 2 h. Northern blot analyses of these treatments revealed that GolS transcripts were present in leaf tissue representing a RWC of 93%. Relative to 18s rRNA, transcript levels were observed to increase at a RWC of 19%, with similar levels observed at 7% RWC (Figure 3.7 a). When exposed to a low temperature shock, GolS transcripts were observed to increase strongly 1 h after exposure to -20°C. Similar levels of transcript were evident 2 and 3 h post exposure (figure 3.7 b).

Western blots of total protein isolated from the leaves of plants used for the stress treatments indicated that GolS protein increased strongly 15 min after exposure to -20°C (Figure 3.8 a). Protein levels were observed to remain relatively constant thereafter up to 2 h post exposure. Western blots for the NaCl exposure (Figure 3.8 b) indicated that GolS protein was present, despite no transcripts being detected on autoradiographs of the northern blot (data not shown). Protein levels however, showed no increase relative to Rubisco protein up to 6 d of continuous exposure to the salinity conditions (Figure 3.8 b).

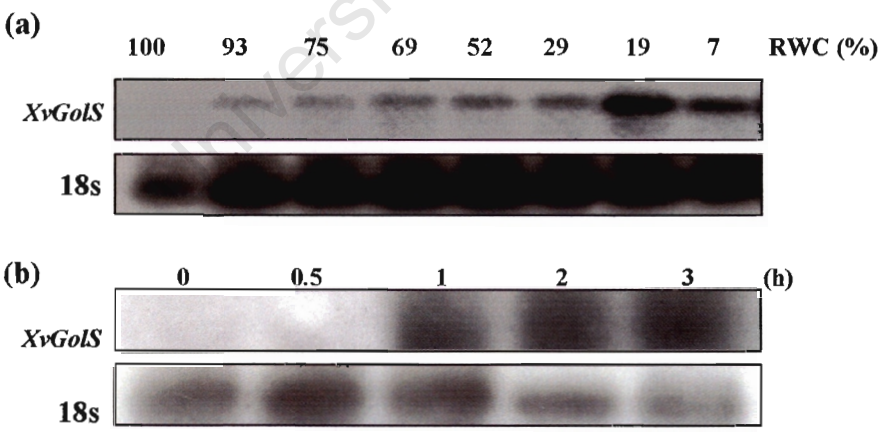


Figure 3.7 Northern blot analysis of total RNA isolated from the leaves of stress treated *X. viscosa* plants (a) water deficit stress (b) low temperature shock (-20°C)

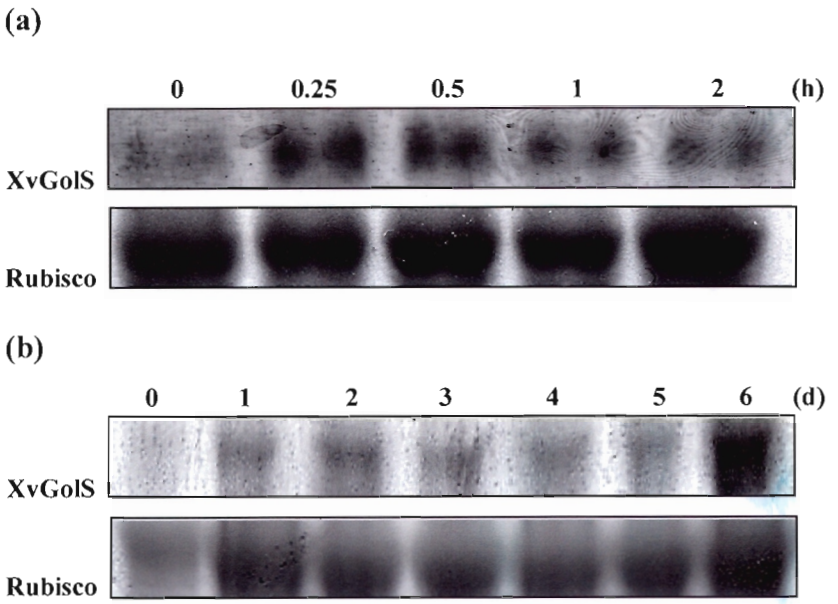


Figure 3.8 Western blots of total protein isolated from the leaves of stress treated *X. viscosa* plants (a) low temperature stress (-20°C), (b) NaCl (150mM)

3.4 Functional expression of *XvGolS*

SDS-PAGE analysis of total protein extracts isolated from *E.coli* colonies transformed with the expression construct pPRO-Ex HTb::*XvGolS*, indicated that a protein with an apparent molecular mass of ~ 40 kD was expressed, increasing progressively, over a 20 h induction period (Fig. 3.9 a). Immunoblots conducted using a His tag specific primary antibody revealed the presence of a His tagged protein with an apparent molecular mass of between 45 and 50 kD in induced samples (to be discussed). The protein was present in detectable quantities in time 0 samples with increased levels observed 1 h after induction of recombinant protein expression but was absent up to 2 h post induction in vector control samples representing *E. coli* colonies transformed with the pPRO-EX HTb vector (Fig. 3.9 b).

High performance liquid chromatography (HPLC) indicated that crude cell lysates isolated from *E. coli* cultures induced for recombinant XvGolS synthesis, displayed in vitro gol forming ability (Figure 3.10). Subsequently, the optimal incubation time for gol synthesis of the recombinant enzyme was determined at 30°C and constant substrate concentrations of UDP-gal and *myo*-inositol of 5 mM and 50 mM respectively. From the analysis the linear range of gol formation occurred at incubation times of between 10 and 20 min (Figure 3.11). All subsequent GolS

activity assays were incubated for 15 min under the temperature and substrate conditions defined above.

Three replicates representing crude cell lysates of single *E. coli* colonies induced for recombinant protein expression displayed maximal GolS activities of between 16157 and 32261nKat/g wet cell mass whilst cell lysates of vector controls consistently showed no detectable GolS activity (Fig 3.12). GolS activity measured from crude cell lysates over the course of a 2 h induction revealed that the gol synthesizing capability of the recombinant enzyme increased progressively over the induction time (Figure 3.13). Low levels of activity (205 ($^{+}$/- 43) nKat/g wet cell mass) were observed in time 0 samples and increased thereafter to a maximum recorded value of 9134 ($^{+}$/- 474) nKat/g wet cell mass 2 h after induction.

Gol synthesized in vitro by recombinant GolS as well as Gol standards were hydrolysed to *myo*-inositol and galactose by an α-galactosidase enzyme (Figure 3.14). The hydrolysis yielded *myo*-inositol and galactose in mole ratios of 1:1 when quantified with the Chromeleon v6.4 software (Dionex, Olten,Switzerland). Substrate kinetic experiments conducted on the recombinant protein yielded a K_m (Ino) of 1.3 mM and V_{max} (Ino) of 3893 nKat and a K_m (UDP-Gal) of 14.1 mM and V_{max} (UDP-Gal) of 11029 nKat.

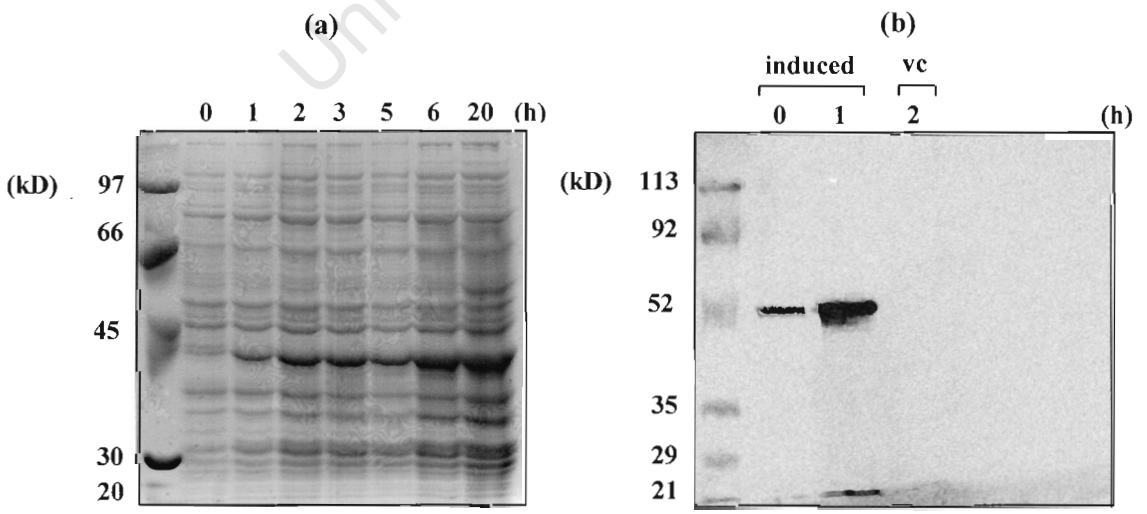


Figure 3.9 SDS-PAGE of total protein isolates from a time course induction of *E. coli* (DH5α) transformed with pPRO-EX Htb::*XvGolS* (a) and, (b) immunoblot of total protein isolates from induced and vector control (vc) samples

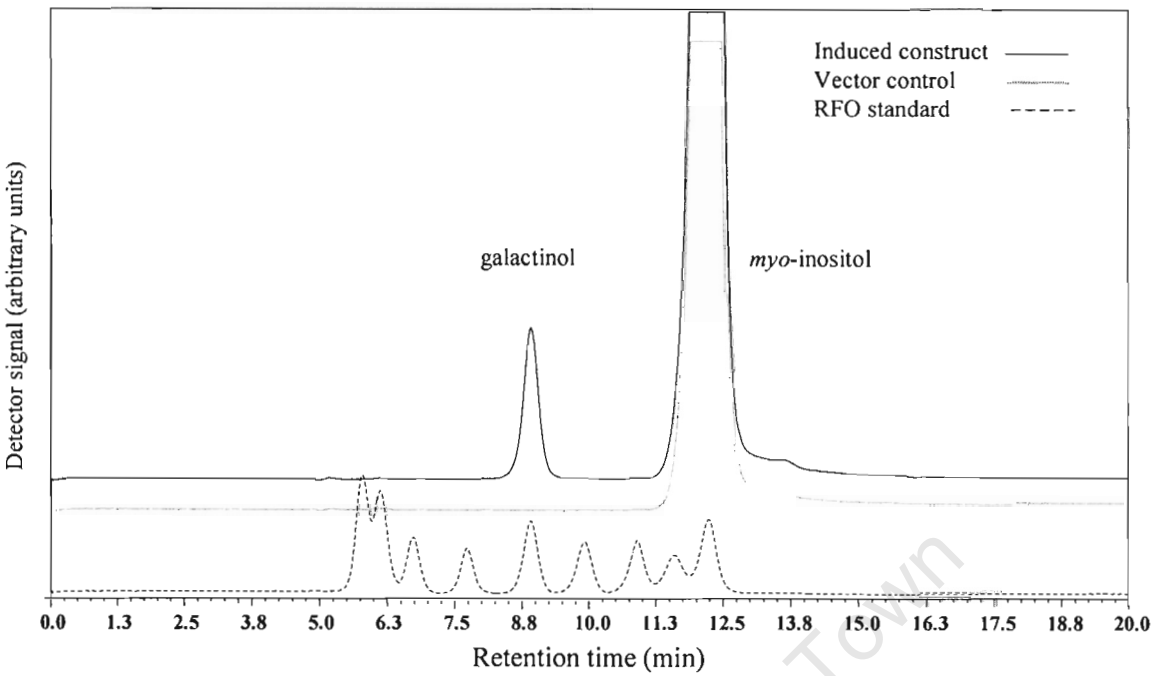


Figure 3.10 HPLC-PAD chromatogram representing an in vitro gol synthesis reaction conducted in the presence of UDP-Gal and *myo*-inositol, using crude *E. coli* cell lysates containing recombinant XvGolS. Standard sugars eluted from 5.88 min represent ver, sta, raf, suc, gol, glu, gal, fru, and *myo*-inositol respectively

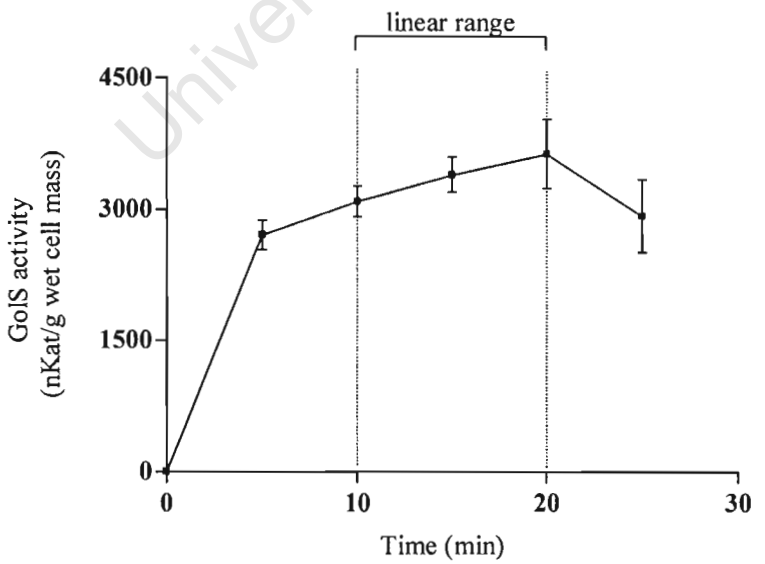


Figure 3.11 Recombinant GolS activity, at varying incubation times, conducted at 30°C with UDP-Gal and *myo*-inositol concentrations of 5mM and 50mM respectively (Error bars indicate the standard error between 9 independent replicates)

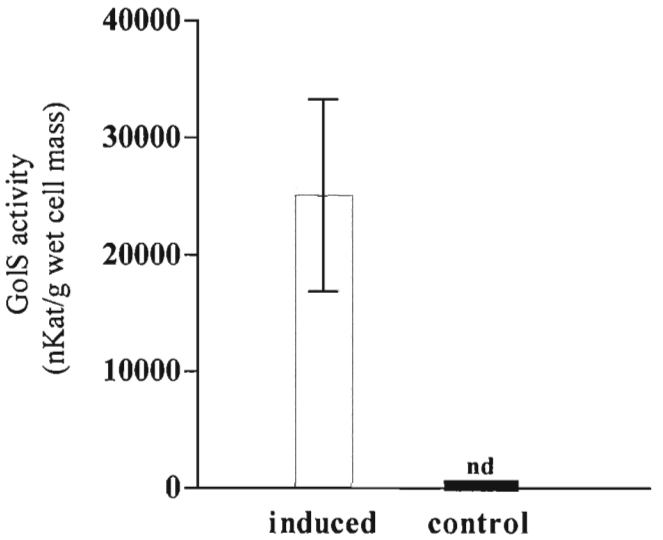


Figure 3.12 GolS enzyme activity in crude cell lysates representing *E. coli* (DH5α) cultures transformed with pPRO-EX Htb::*XvGolS* induced for 2 h. Controls represent cultures that had been transformed with the expression vector only (Error bars indicate the standard error between 9 independent replicates, nd = not detected)

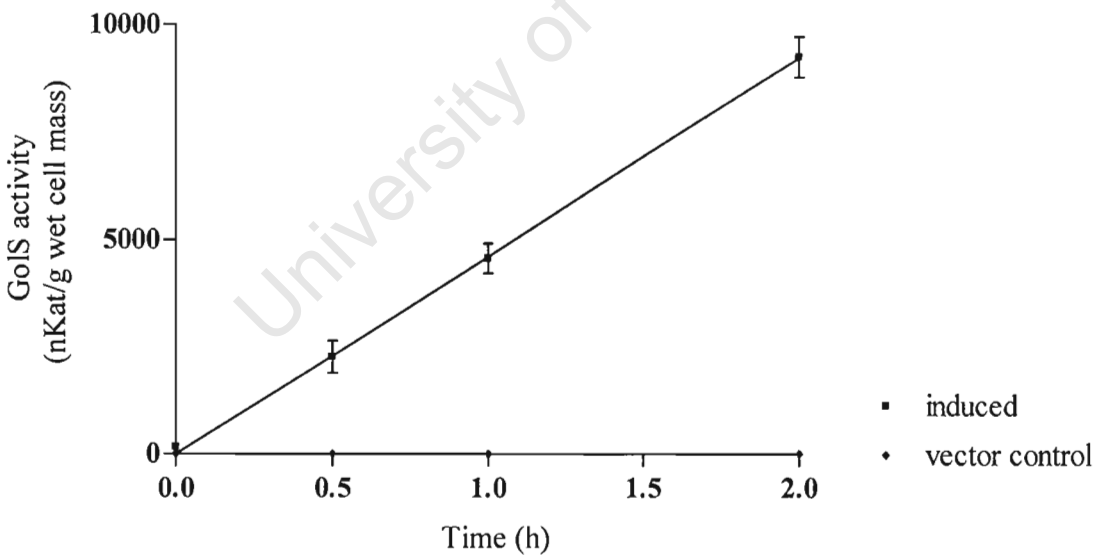


Figure 3.13 GolS enzyme activity in crude cell lysates from *E. coli* (DH5α) cultures transformed with pPRO-EX Htb::*XvGolS*, during a 2 h time course induction (error bars indicate standard error between 9 independent replicates)

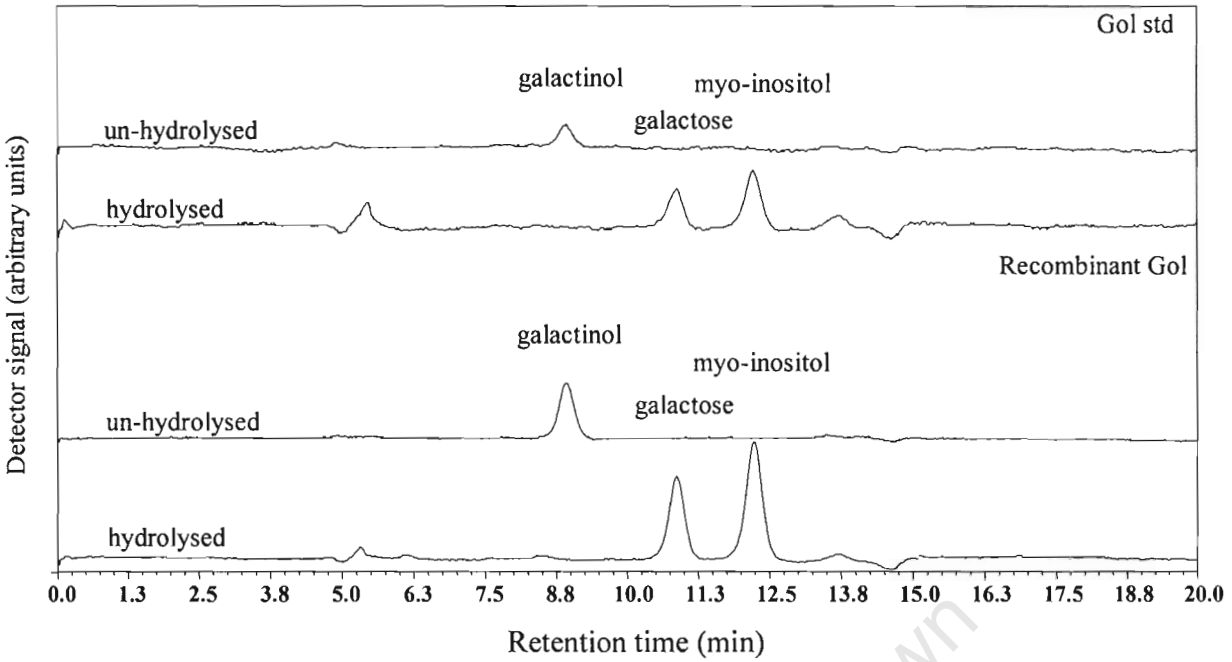


Figure 3.14 HPLC-PAD chromatogram representing the enzymatic hydrolysis of gol synthesised in an in vitro reaction using crude *E. coli* cell lysates containing recombinant XvGolS

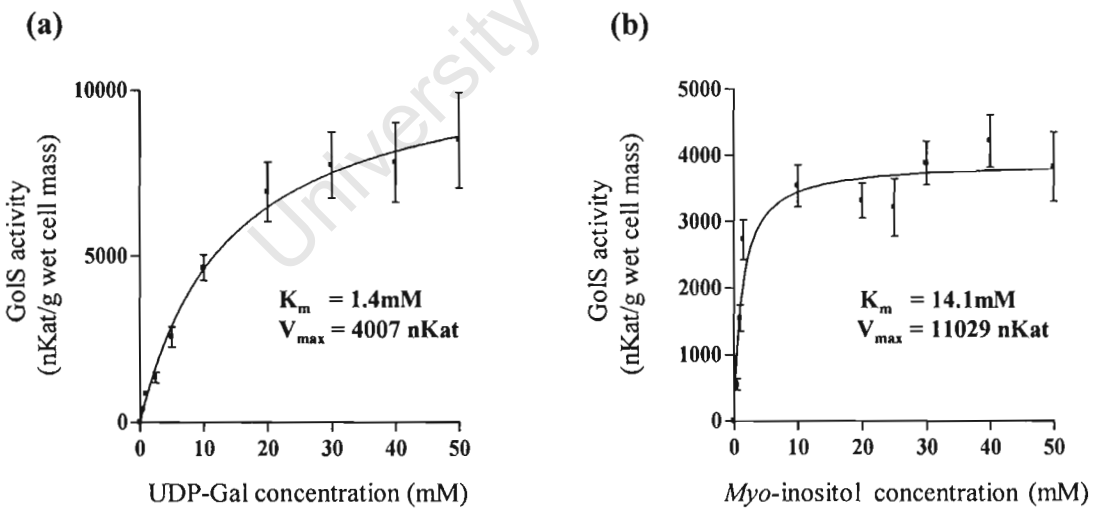


Figure 3.15 Substrate kinetic experiments for recombinant XvGolS using crude extracts from *E. coli* containing recombinant XvGolS. GolS assays were conducted at 30°C with a 15 min incubation period and (a) varying concentrations of UDP-Gal (b) varying concentrations of *myo*-inositol (error bars indicate the standard error between 9 independent replicates)

3.5 Changes in carbohydrate levels during stress treatments

3.5.1 Low temperature acclimation

Individual plants were acclimated to a temperature of 1°C by being exposed to 10°C for 24 h before being transferred into a controlled environment chamber maintained at 1°C. The non-acclimated plant was transferred directly from greenhouse conditions to 1°C. Levels of raf and sta were not found to change considerably in the acclimated plant up to 8 d after exposure to 1°C (Figure 3.16 a). Suc levels in this plant peaked at 31 mg/g dw after 24 h at 10°C but declined steadily thereafter up to 8 d at 1°C. The non-acclimated plant showed a two-fold increase in raf and a 1.6-fold increase in sta after 8 d exposure to 1°C (Figure 3.16 b). Suc levels increased approximately three-fold to a level of 44 mg/g dw after 8 d of exposure to 1°C.

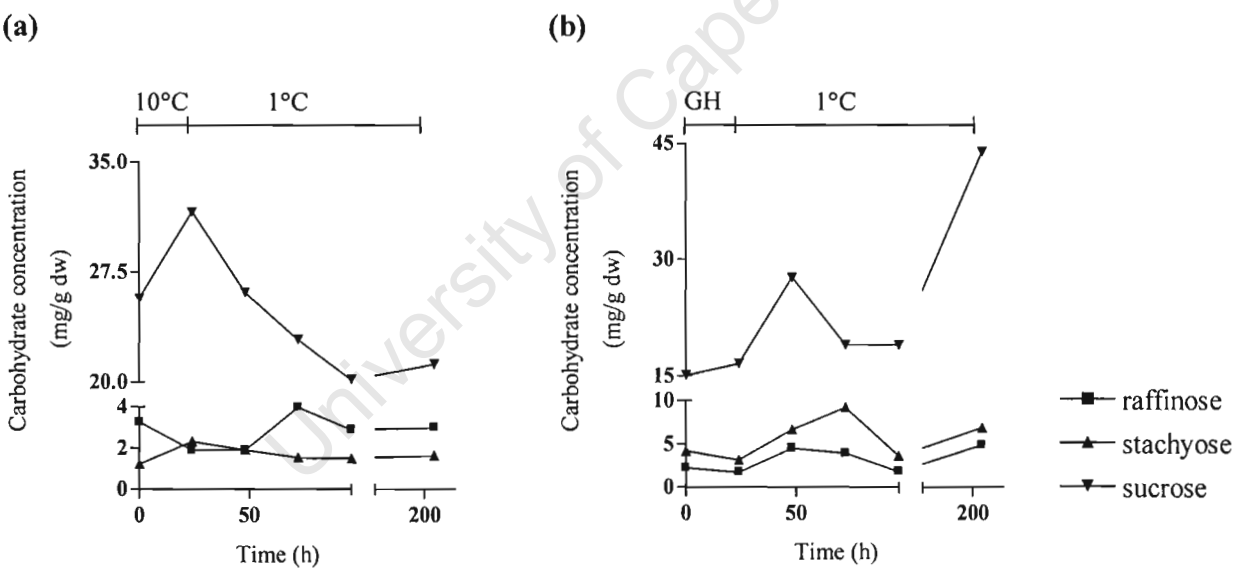


Figure 3.16 Changes in carbohydrates under conditions of acclimation vs. non-acclimation to a temperature of 1°C (a) acclimated plant (b) non-acclimated plant. (GH = greenhouse conditions)

3.5.2 Low temperature shock treatments

A potted *X. viscosa* plant was subjected to a low temperature shock of -20°C for 2h and subsequently transferred into a controlled environment chamber maintained at 4°C. Levels of raf and sta were found to decrease approximately two-fold, 24 h after exposure to -20°C (Figure 3.17 a). Levels of suc were observed to decrease 7.5-fold from 30 mg/ g dw to 4 mg/g dw 96 h after exposure with concomitant 20-fold increases in the hexose sugars glu and fru (1.3 mg/g dw to 28 mg/g dw and 0.9 mg/g dw to 21 mg/g dw respectively, Figure 3.17 b) evident. Leaf pairs were subsequently tested for GolS activity after exposure to varying temperature regimes. Activity was found to marginally higher in leaf pairs exposed to 4°C for 24h when compared to control leaf pairs that had been maintained under greenhouse conditions for 24 h. Activity decreased 2h after exposure to -20°C with no GolS activity measurable 24 h after exposure to -20°C (Figure 3.17 c).

Electron micrographs of leaf tissue at full turgor and after exposure to -20°C for 2 h indicated that in the fully turgid state cellular ultrastructure typically consisted of a large central vacuole surrounded by well defined chloroplasts when viewed at a magnification of 6000x and 15000x (Figure 3.18, a and d respectively). Tissue exposed to -20°C revealed the disappearance of the large central vacuole and the concentration of cellular constituents toward the central part of the cell, indicating that the vacuolar membranehad been disrupted. In addition chloroplasts were not as clearly defined as those in the fully turgid state when viewed at magnifications of 7000x and 15000x (Figure 3.18, b and d respectively).

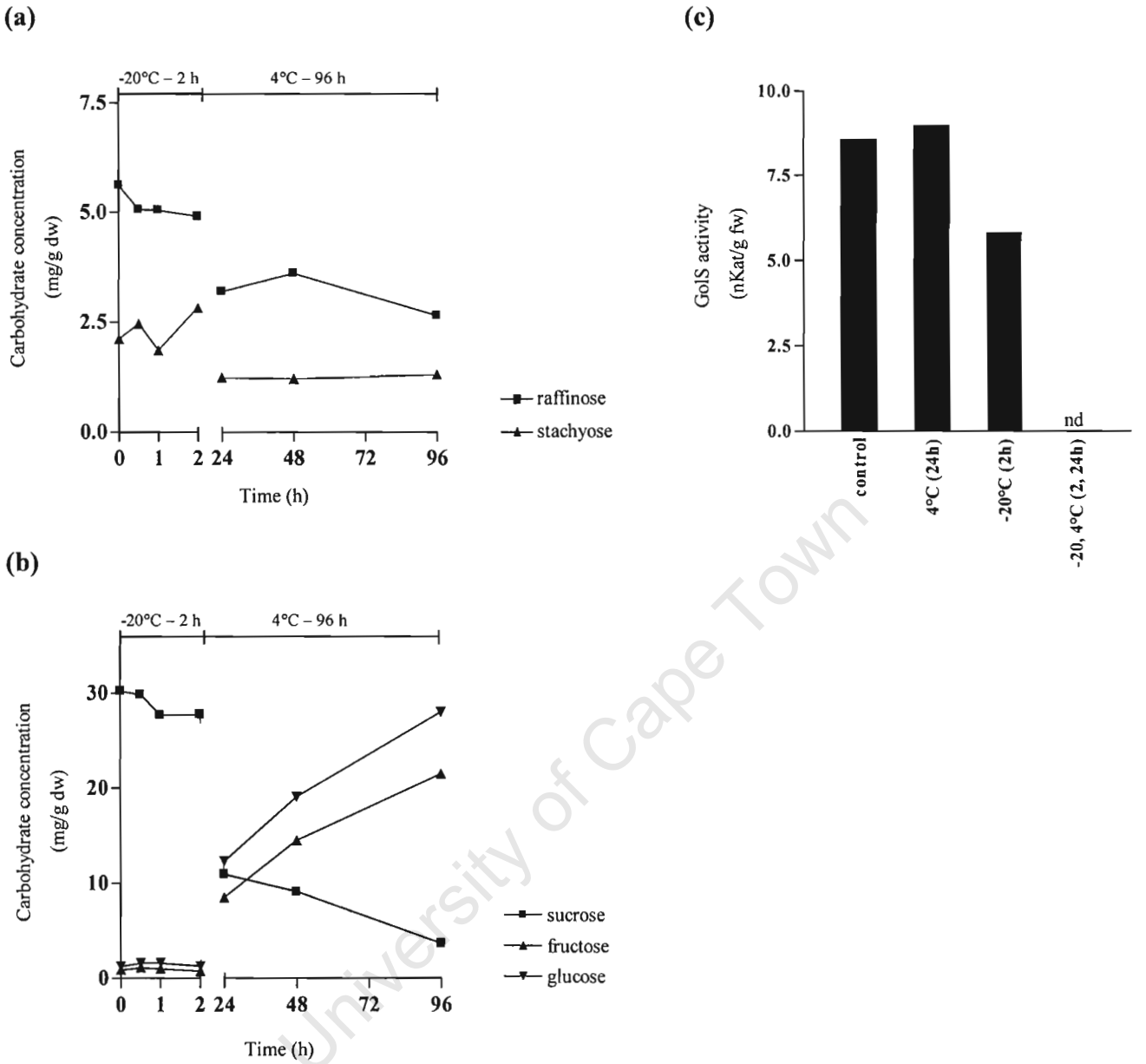


Figure 3.17 Changes in carbohydrate concentrations and GolS activity in the leaves of *X. viscosa* plants exposed to a -20°C low temperature shock and subsequently transferred to 4°C (a) raffinosaccharides, (b) hexose sugars, (c) GolS activity in leaf pairs excised from whole plants and exposed to varying temperature regimes. Control samples were maintained under greenhouse conditions. (nd = not detected)

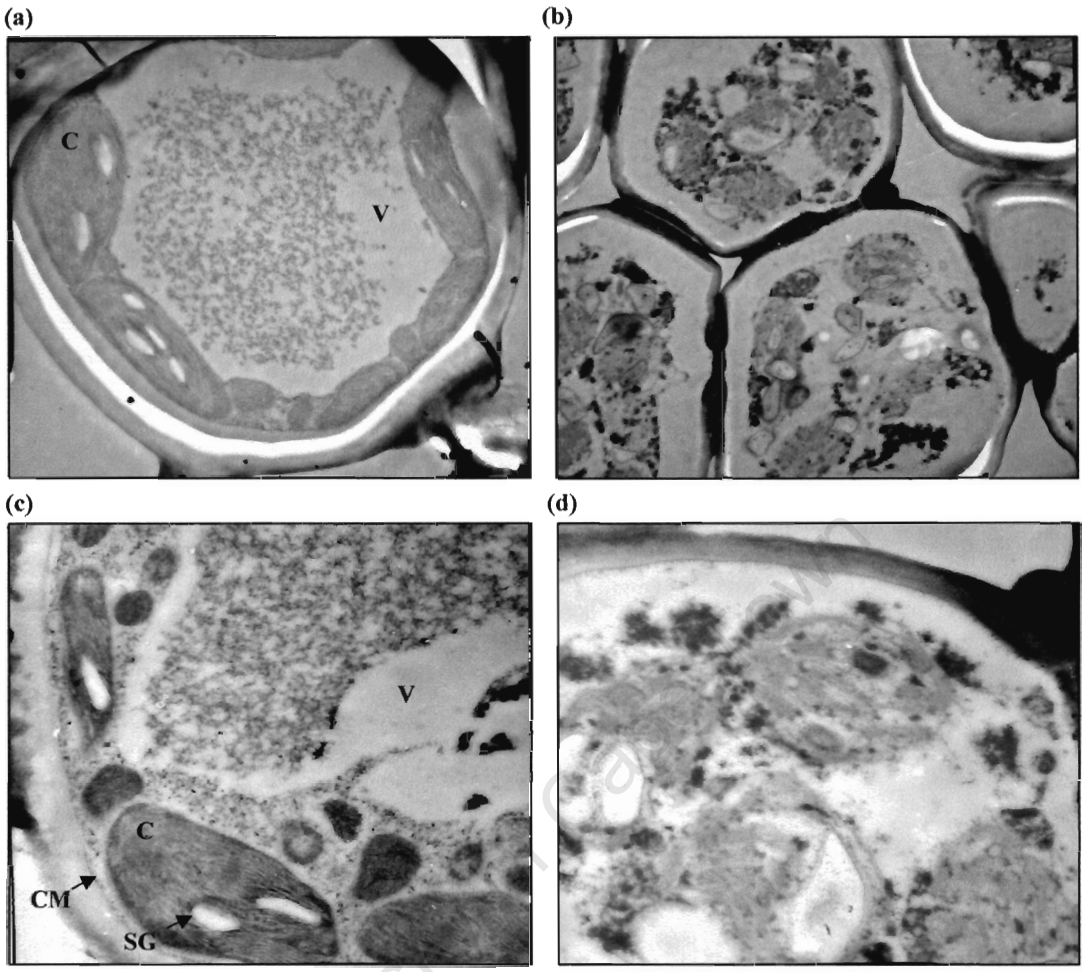


Figure 3.18 Electron micrographs of *X. viscosa* leaf tissue at full turgor (a and c) and after exposure to -20°C for 2 h (b and d). Magnifications of 6000x (a) 7000x (b) and 15000x (c and d) were used for the micrographs C = chloroplast, V = vacuole, CM = cell membrane, SG = starch granule.

3.5.3 Carbohydrate profiles of plants at full turgor and during water deficit are variable

3.5.3.1 Full turgor

Soluble carbohydrates were extracted, during various stress treatments, from the leaves of plants at full turgor. A comparison of these samples from six different plants indicated that noticeable variation occurred in their carbohydrate profiles (Figure 3.19). The most marked difference observed was that an individual plant displayed raf concentrations of 25 mg/g dw, nearly five-fold greater than the five other plants tested. In addition another plant had gol concentrations of 8.2 mg/g dw, more than two-fold greater than the other five plants. This variability was evident for all of the water soluble carbohydrates analysed.

3.5.3.2 Water deficit

Five *X. viscosa* plants were subjected to a water deficit stress as described in materials and methods. Analysis of the carbohydrate profiles in the leaves of these five plants by HPLC (Figure 3.20, a-e) revealed that individual plants displayed varying amounts of raffinosaccharides and suc at full turgor. All plants, with one exception (Figure 3.20 e) displayed increased leaf suc concentrations as well as raf, sta and ver concentrations (Figure 3.20, a-d) in response to water deficit. The maximum amount of raf accumulation in response to water deficit was determined to be 23 mg/g dw in the plants analysed whilst the lowest recorded amount was 12 mg/g dw (Figure 3.20 c and a respectively). Concentrations of suc increased to between 16 – 21 mg/g dw at a RWC of 5%. One plant was found to have raf concentrations of 24 mg/g dw at full turgor (Figure 3.18 e). No ver was detected in this plant and neither raf nor suc levels increased linearly in response to the stress. Rather, a general decline in raf occurred, whilst suc levels fluctuated over the duration of the stress.

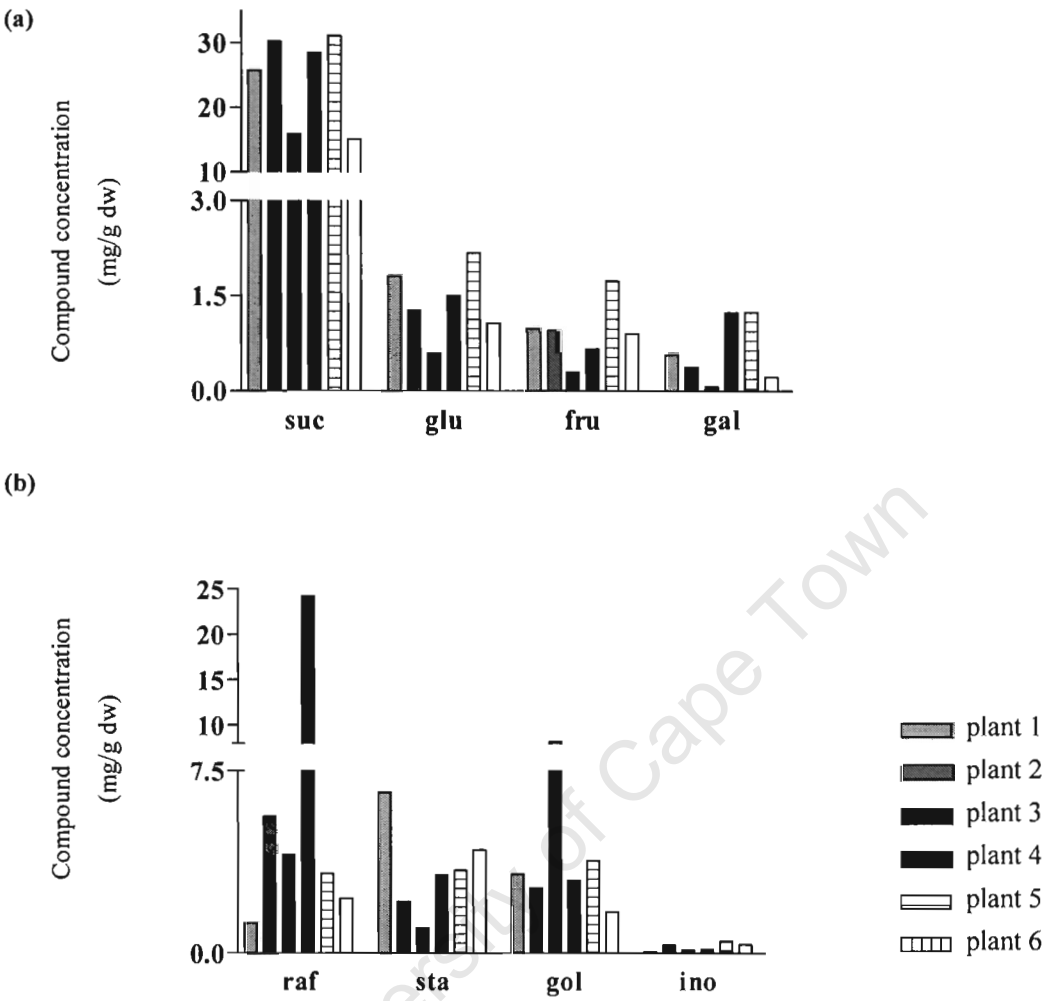


Figure 3.19 Carbohydrate profile in the leaves of individual *X. viscosa* plants at full turgor, (a) non- raffinosaccharides (b) raffinosacharides and biosynthetic intermediates gol and *myo*-inositol

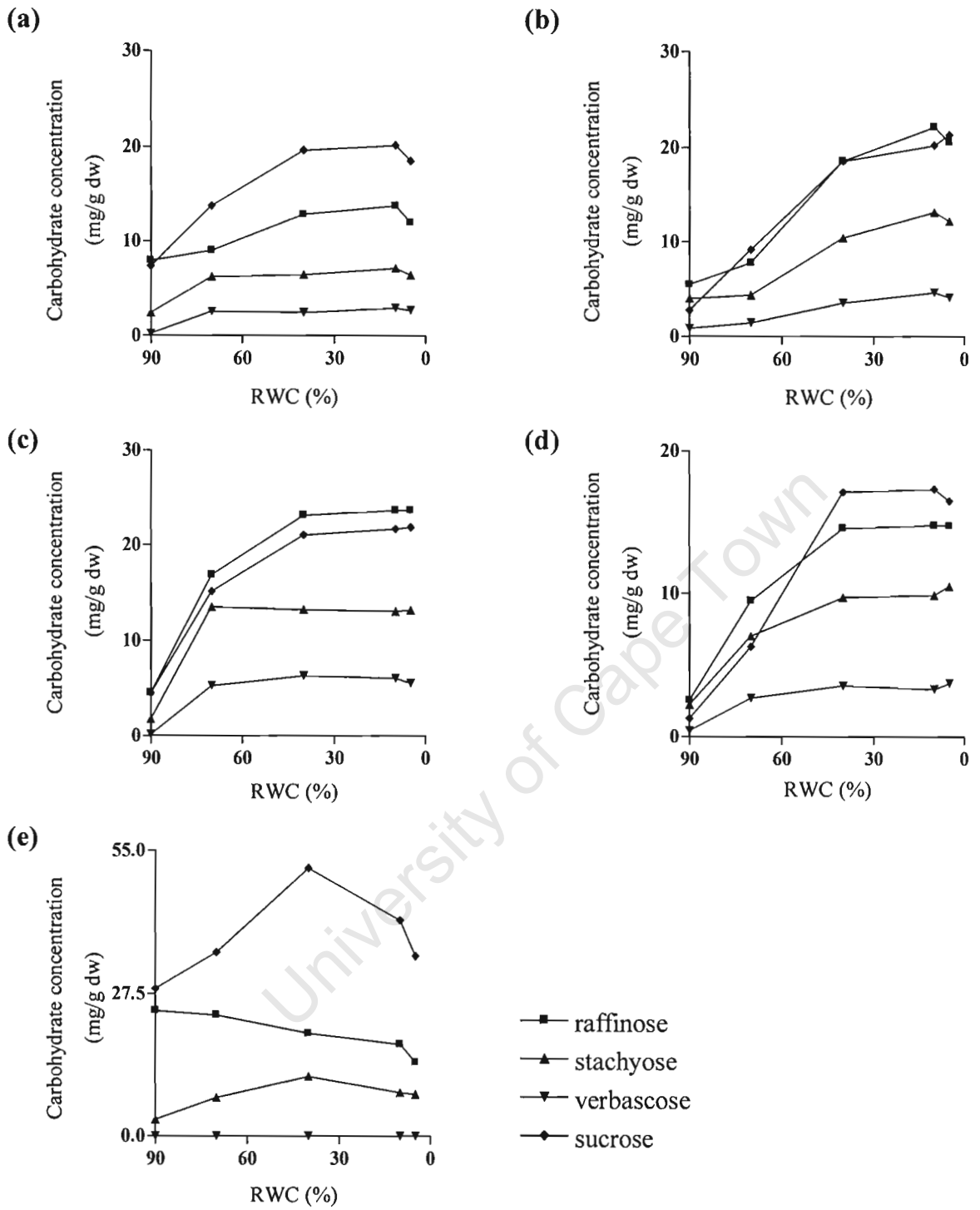


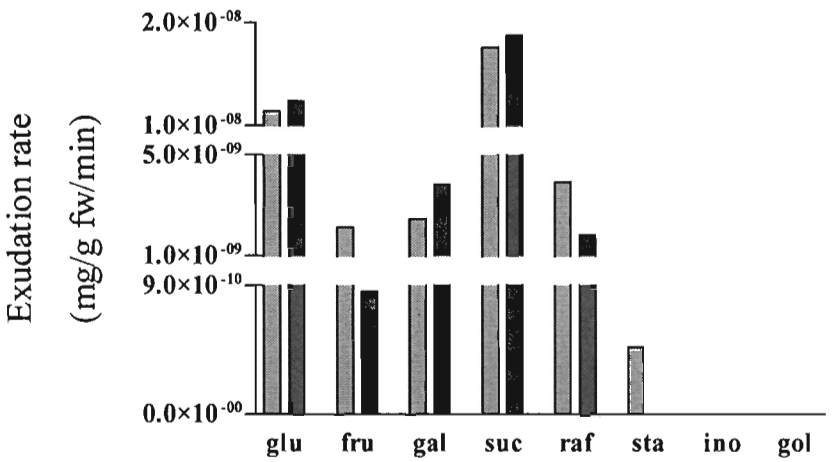
Figure 3.20 Changes in the raffinosaccharide and suc content in the leaves of five individual *X. viscosa* plants subjected to a water deficit stress. Water status is reflected as relative water content (RWC) (a) to (e) represents plants 1 to 5 respectively

3.6 Evaluation of a phloem exudation technique

Phloem exudation experiments conducted on the leaves of two individual *X. viscosa* plants at full turgor revealed that suc, raf and glu occurred predominantly in samples representing 8 h of exudation, with exudation rates of 1.8×10^{-8}, 4×10^{-9} and 1.14×10^{-8} mg/g fw/min (plant 1) and 1.8×10^{-8}, 1.8×10^{-9} and 1.2×10^{-9} mg/g fw/min (plant 2) respectively (Figure 3.21 a). Gal and fru were also identified in the two plants tested. Interestingly sta was detected in plant 1 with a determined exudation rate of 4.7 x 10-10 mg/g fw/min but was conspicuously absent in plant 2. Neither of the plants had detectable levels of ino or gol in phloem exudates (Figure 3.21 a). Water soluble carbohydrate extractions conducted on the leaves used for the experiment procedure revealed the presence of the RFOs raf and sta as well as glu, fru and gal, with suc accounting for 50% and 45% of the total leaf carbohydrate content of each plant respectively. The leaves of both plants also displayed measurable concentrations of ino and gol (Figure 3.21 b).

Detectable levels of the polyol sorbitol were also evident in the phloem exudates of both plants 8 h after exudation was initiated, with exudation rates of 1.7×10^{-9} and 3×10^{-10} mg/g fw/min respectively (Figure 3.22 a). No sorbitol was detected in carbohydrates extracted from the leaves after completion of the exudation procedure (Figure 3.22 b). However, analysis of water soluble carbohydrates in the roots of these plants at full turgor also revealed the presence of sorbitol at low concentrations of 0.16 and 0.25 μg/g fw respectively (Figure 3.22 a). Preliminary HPLC analysis showed the presence of a distinct peak which was determined to be glycerol (data not shown). Subsequently, all phloem exudates representing a series of samples over an 8 h exudation period were found to have glycerol at exudation rates higher than any of the other compounds measured. The glycerol profile during the 8 h exudation period (Figure 3.22 b) was found to occur as an initial burst during the first 2 h of exudation, with amounts decreasing thereafter. It was concluded that glycerol did not represent a typical phloem mobile sugar, but rather occurred as a putative response to wounding when the leaves were excised for the experiment.

(a)



(b)

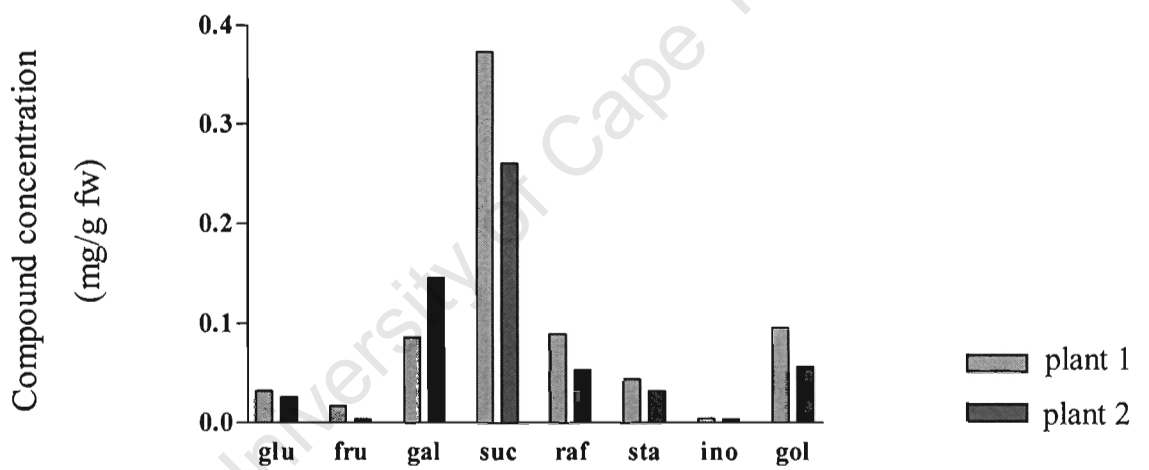


Figure 3.21 (a) Carbohydrate profile of 8h phloem exudates eluted from *X. viscosa* leaves at full turgor, (b) post exudation carbohydrate profile of leaves used for the experimental procedure

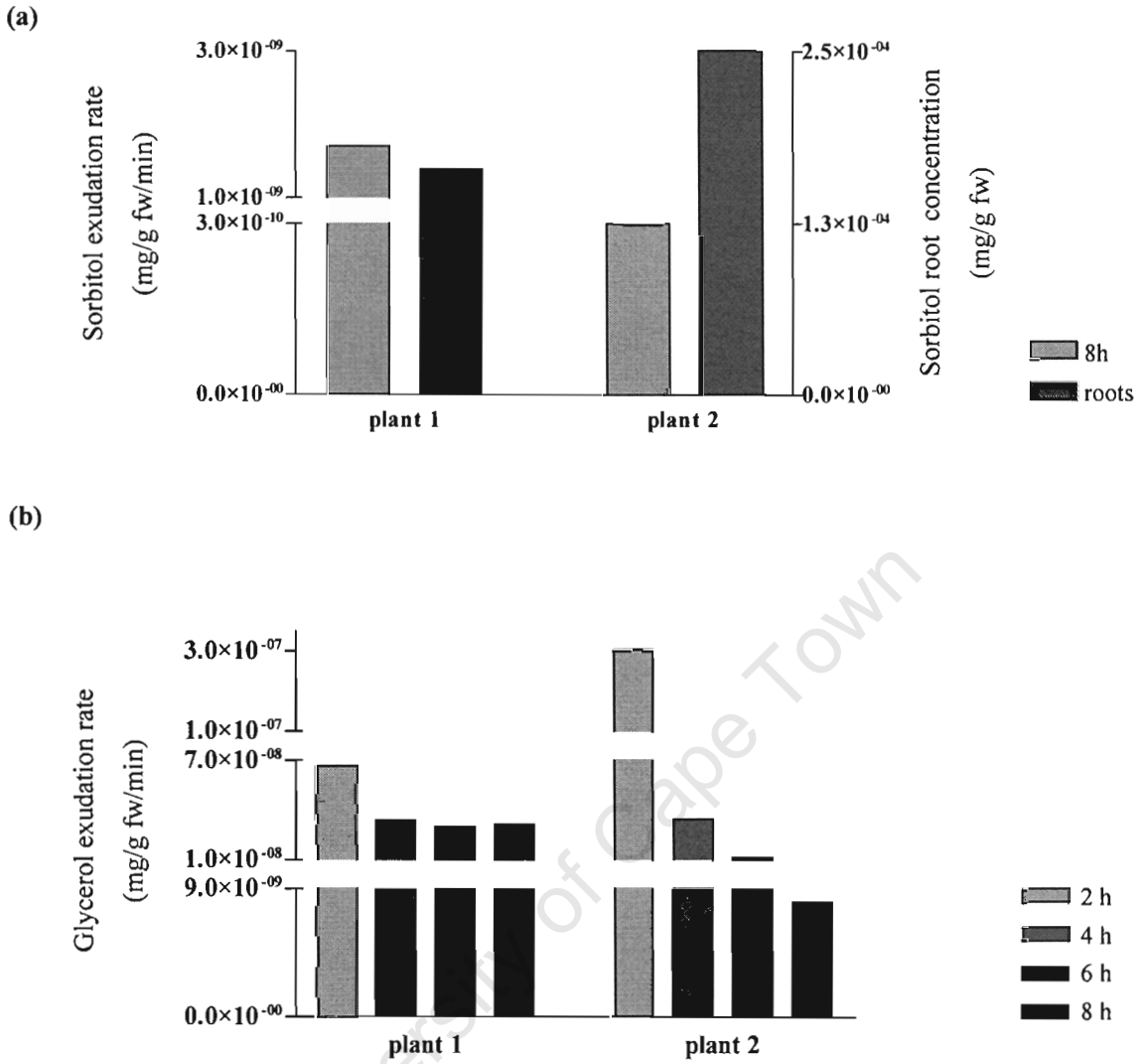


Figure 3.22 Polyols detected during phloem exudation experiments on the leaves of *X. viscosa* plants at full turgor; (a) sorbitol exudation rate and sorbitol root concentrations, (b) glycerol exudation profile over an 8 h exudation period.

Chapter 4

Discussion

4.1 XvGolS shows identity to known GolS enzymes

A translation of the cDNA sequence obtained for *XvGolS* indentified a full length cDNA of 1030p. The cDNA was found to have an open reading frame (ORF) of 1020bp, encoding a polypeptide with a predicted MW of 34 kDa (Figure 3.1). A BLAST search, using the nucleotide cDNA sequence, indicated that *XvGolS* shared homology with gol synthase enzymes from a variety of species. Multiple sequence alignments using the inferred amino acid sequences of a number of these GolS enzymes revealed that XvGolS shared identities greater than 70% to GolS enzymes from *A. thaliana* (81%) *B. Napus* (80%), *G. max* (78%), and *A. reptans* (71%, Figure 3.2).

Sprenger and Keller (2000) have reported a putative serine phosphorylation site (Ser-263) for a deduced subset of GolS proteins, implying a potential regulatory role for this residue. This residue was cited to be conserved in 14 of 17 full-length sequences analysed (Downie et al., 2003). An in-silico phosphorylation prediction profile of XvGolS, based on algorithms described by Blom et al. (1999), indicated a putative serine phosphorylation site at amino acid position 263(Figure 3.4). However, the prediction score of this residue (0.053) was below the threshold limit of 0.5, implying that the residue does not have phosphorylation potential. Interestingly a significant serine phosphorylation residue was located at amino acid position 73, with a prediction score of 0.838 and significant threonine residues were also predicted at positions 211, 269 and 331 with prediction scores of 0.952, 0.992 and 0.571 respectively. Downie et al. (2003) described LeGolS, a tomato GolS, that lacked both the predicted serine and an intron conserved in all known genomic GolS sequences. The cDNA clone still displayed the ability to synthesis gol. Other putative GolS sequences from *Arabidopsis thaliana* (AtGolS V and VI) have also been reported to lack the serine residue (Taji et al., 2000). This in-silico analysis on XvGolS does not confirm that phosphorylation at this specific residue plays a post-translational regulatory role in gol synthesis however, given the evidence that at least one isoform from tomato lacking this residue still retains functionality (Downie et al., 2003) and a number of other potential phosphorylation sites are predicted for XvGolS by the algorithm, a post translational regulatory role for these residues cannot be precluded. To unequivocally address this question we would have to conduct in gel kinase assays on native GolS protein, to determine if phosphorylation of the protein is possible and subsequently to repeat the experiment after site directed mutagenesis to a create point mutation at position 263 on the XvGolS peptide.

A conserved domain search using the BLAST service indicated that all GolS enzymes share a common catalytic domain belonging to the glycosyl transferases family 8 (Figure 3.5). Members of the family are known to be involved exclusively in the transfer of sugar monomers to donor molecules during lipolysaccharide and glycogen biosynthesis (http://www.pfam.wustl.edu). Much of the identity observed in amino acid alignments represents an area on these polypeptides over which this catalytic domain spans. Other stress related genes have been found to display highly conserved motifs across evolutionary diverse species. For instance Late Embryogenesis Abundant (LEA) proteins contain sequence motifs that are conserved in all higher plants (Bartels & Salamini, 2001), whilst signature sequences have been identified in Cold Binding Factor (CBF)-like proteins involved in cold response pathways from *B. napus*, wheat, rye and tomato (Jaglo et al., 2001). The fact that highly conserved motifs can be found in a range of genes implicates these motifs in having functionally significant roles. What is evident in this analysis is that the conserved domain does not represent a signature sequence but rather an entire domain representing multiple tracts of highly conserved amino acids that are of functional importance in mature GolS proteins. It is thus evident that the strictly conserved function in gol biosynthesis of the galactosyl transferase domain has limited the evolution of sequence diversity amongst GolS enzymes from diverse plant species.

A midpoint rooted phlyogenetic tree was constructed using only full-length GolS coding sequences (Figure 3.3). We obtained a distinct clade that included only monocotyledonous GolS peptide sequences from the data set used. Interestingly, a second clade representing AtGolS II, AtGolS III and *T. halophila* GolS grouped with the monocotyledonous clade with the node supporting this monophyletic group displaying a bootstrap value of 89%. This could be representative of the evolutionary speciation event when monocotyledous and dicotyledonous plants diverged. However, we only used sequences that were available in Genbank and therefore the sample pool used for the phylogentic analysis was limited and did not fully represent evolutionary diverse taxa. Additionally, from this pool we selected only full-length GolS protein coding sequences and thus only three monocot GolS sequences were used in the analysis. Interestingly two of the Arabidopsis isoforms, AtGolS II and III, formed a disctinct clade with a GolS from *T. halophila*, a salt tolerant dicot (Wang et al., 2004). Taji et al (2002) identified AtGolS II as being responsive to salinity stress whilst AtGolS III was observed to be cold responsive. Given the high bootstrap values, this clade provides evidence

to suggest that AtGolS II and III are in fact paralogous genes that have recently evolved and function in the amelioration of different abiotic stresses.

An interesting feature of all the known full length GolS sequences is the presence of a pentapeptide, hydrophobic residue (APSAA) at the carboxyl terminus and it is likely that this residue is of functional significance in GolS enzymes. A membrane anchor analyses was conducted on XvGolS (data not shown). Interestingly although no distinct areas with anchor potential were identified, the APSAA residue displayed the highest score. This observation may very well be an artifact of the residue being composed entirely of hydrophobic amino acids and the fact that the algorithms utilize hydrophobicity as a calculation parameter however, the possibility of a membrane associated functional role for this residue should not be precluded.

Southern blot analysis indicated that a radiolabelled XvGolS cDNA probe hybridized with its genomic counterpart yielding single hybridizing bands for restriction digests with restriction endonucleases that did not have any recognition sequences within the cDNA, even at low stringency (Figure 3.6). A restriction digest of *X. viscosa* genomic DNA with *Sac*I and a double digest with *Bam*HI and *Sac*I resulted in two hybridizing bands appearing on autoradiographs. Given that *Sac*I was the only restriction endonuclease with a single recognition sequence within the cDNA, the direct interpretation of the southern blot implies that a single copy of GolS exists in *X. viscosa*. However, it is known that GolS enzymes represent small, highly homologous, gene families in Arabidopsis (Taji et al., 2000), maize (Zhao et al., 2003). Downie et al. (2000) described *LeGolS*, a GolS enzyme from tomato, which also yielded results for a Southern blot that implied the existence of a single GolS. Further analysis revealed that LeGolS was significantly different from 7 GolS enzymes reported for Arabidopsis (Taji et al., 2002) in that the genomic sequence of LeGolS lacked one intron common to these GolS. The authors concluded that the results of the Southern blot implied that either only copy did indeed exist or that LeGolS was significantly different from other GolS enzymes in tomato to prevent a radiolabelled LeGolS probe from hybridizing with those other GolS genes. Since we do not have a genome sequence for *X.viscosa* nor do we have the genomic sequence of XvGolS, we suggest a similar explanation for the interpretation of the results obtained for the Southern blot presented in this work. Although we do not have evidence to suggest the presence of more that one functional GolS in *X. viscosa*, other published reports for various plant species suggest a high likelihood that other GolS genes are

present in *X. viscosa*. Interestingly, additional GolS genes in *X. viscosa* with sequences that are sufficiently variant to prevent hybridization at low stringency conditions would prove an unusual observation given the highly conserved nature of this gene family.

4.2 Transcript and protein levels increase in response to stress

Northern blot analysis of total RNA isolated from the leaves of *X. viscosa* plants subjected to a water deficit stress indicated that *XvGolS* transcript levels increased at a RWC of 19%, with similar levels observed at a RWC of 7% (Figure 3.7a). Transcripts also increased in response to a low temperature shock of -20°C after 1 h of exposure, thereafter remaining relatively constant at 2 and 3 h post exposure (Figure 3.7b). However, it was observed that leaf tissue harvested at 2 h post exposure was frozen. Given that cellular metabolism cannot occur during this state, the transcript levels observed at 2 and 3 h post exposure are considered to be an artifact of transcript levels observed at 1 h post exposure, brought about by the arrest of metabolism during the freezing process. No transcripts were detected on autoradiographs of gel blots representing RNA isolated from the leaves of plants subjected to a sustained NaCl stress of 150 mM, after 7 d exposure (data not shown). In addition no transcripts were observed on autoradiographs up to 72 h exposure to the exogenous application of 100 µM ABA (data not shown). This finding is consistent with published data on GolS from other plant species (Takahashi et al., 1994; Liu et al., 1998) suggesting that GolS regulation is not mediated by ABA- dependent pathways in vegetative tissue.

Western blots of total protein isolated from the stress treatments described above indicated that GolS protein levels increased 15 min after exposure to -20°C (Figure 3.8 a). No changes in protein levels were evident for the western blots conducted for plants subjected to the NaCl stress (Figure 3.8 b), correlating the absence of transcript in Northern blots for this treatment. Western blots for plants subjected to water deficit stress seemed to indicate that GolS protein decreased during the dehydration process. However, due to the fact that degradation of the Rubisco protein profile was observed during the dehydration process (data not shown) we cannot state conclusively that GolS protein levels decrease in response to water deficit. Interestingly, no GolS protein was evident on western blots representing the ABA treatment (data not shown) although GolS protein was observed in time 0 samples of all other treatments. However, ABA treatments were conducted on seed propagated plants maintained by in vitro culture. It is known that RFOs are principally involved in carbon translocation, effectively shunting carbon from source to sink tissue. Given that all of the plants used in this

particular experiment lacked a well developed root system, it is suggested that genes related to the RFO pathways were not being actively expressed in these plants, accounting for the absence of the GolS protein.

An interesting feature of the western blots conducted was the fact that transcript levels and protein levels were not synchronous in response to the stress treatments. We do not consider this an anomalous result but rather it might provide interesting insight into the potential regulation of RFO biosynthesis. The analysis clearly indicates that XvGolS protein is present in detectable quantities at full turgor, in the absence of any stress. Transcript levels however, appear to increase upon exposure to abiotic stress after protein levels began to increase. A likely hypothesis is that in the event of low temperature stress GolS protein stability inceases, leading to an accumulation of GolS protein and the synthesis of RFOs. Subsequently GolS transcripts are produced in response to the stress and de-novo protein synthesis adds to the levels of protein present. Although we do not have definitive results for water deficit stress, we did observe, with SDS-PAGE, that the total protein profile in the leaves of *X. viscosa* decreased markedly in response to water deficit. If this is true, then the hypothesis presented is considered viable under conditions of water deficit when GolS protein turnover increases as the plant loses free water. Increases in GolS transcripts would then counterbalance the increased turnover and ensure that sufficient GolS protein is present for gol synthesis and RFO accumulation in preparation for the dried state. An alternative scenario

4.3 Carbohydrate profiles vary between plants at full turgor

All *X. viscosa* plants used for carbohydrate analyses under low temperature and water deficit stress treatments represent a pool of plants collected from their natural habitat and maintained under glasshouse conditions for not more that two years. An analysis of the carbohydrate profiles in the leaves of individual plants at full turgor revealed distinct variations between different plants. We attributed this either to previous stress treatments conducted on these plants resulting in a stress "memory effect", or considered the variation to be representative of an inherent variation between individuals in their natural environment. Naturally occurring plants in the collection area typically reside in soil pockets in shallow depressions in the granite bedrock. Although soil composition is homogenous across this habitat range, individual plants may be found in varying amounts of soil. Thus, wild plants inhabit a very discernible micro-habitat with respect to soil quantity and may therefore be exposed to abiotic stresses to varying degrees of severity. Ultimately this may result in individual plants being "primed" to varying degrees of stress tolerance. Indeed, as we have shown, one individual

plant used for an analysis of carbohydrate profile during water deficit displayed raf levels of 25 mg/ g dw, five-fold higher than other plants at full turgor. The analysis revealed no increases in RFOs nor was there any increase in suc level in response to water deficit (Figure 3.20 e). One of the known responses to water deficit stress in a number of resurrection plants, such as *Boea hygroscopica* (Albini et al., 1999), *Craterostigma plantagineum* (Norwood et al., 2000; Bartels and Salamini, 2001), *Ramonda myconi* (Muller et al., 1997) and *Sporobulus stapfianus* (Whittaker et al., 2001), is the accumulation of suc.

In order to test this hypothesis we subsequently conducted a water deficit stress treatment on four plants representing individuals gathered from the wild and maintained under greenhouse conditions for not more than two years. Plants that were similar in size were selected for the experiment. An analysis of the raffinosaccharide and suc profiles in the leaves of these plants clearly indicated that raf and suc levels increased progressively as the plant dried (Figure 3.20 a-d). These findings are consistent with other published reports where raf was shown to increase in vegetative tissue in response to water deficit in Arabidopsis (Taji et al., 2002) and kidney bean (*Phaseolus vulgaris*, Liu et al., 1998). In addition sta and ver were detected in relatively low amounts but also showed a progressive increase during the dehydration process. Importantly, we showed that all plants had varying levels of these carbohydrates at full turgor but showed similar responses under a water deficit stress, albeit that carbohydrate levels accumulated to variable amounts in the dry state. Given that the first plant used in the analysis displayed raf levels at full turgor that were higher than the maximum induced levels of any of the four plants analysed subsequently, we consider these findings of importance to our pool of plants. The carbohydrate status of plants at full turgor is obviously important, considering the varied response observed during water deficit.

Similarly, low temperature stress treatments were conducted on two plants, where one individual was acclimated to a temperature of 10°C for 72 h prior to being exposed to a low temperature stress of 1°C for 8 d (Figure 3.16 a). The other individual was maintained under greenhouse conditions during the acclimation period and subsequently transferred into the controlled environment chamber at 1°C for the stated duration (Figure 3.16 b). The acclimated plant showed elevated amounts of suc in the leaves after the 10°C exposure, prior to being subjected to the low temperature stress. Suc levels then gradually declined over the period that the plant was exposed to 1°C. No other carbohydrates showed marked fluctuations during the experiment. In the non acclimated plant raf levels were observed to increase to a level two-

fold higher than time 0 samples after 8d exposure to 1°C. Suc levels were also observed to increase approximately three-fold during this period. Interestingly, gol levels also appeared to increase in a linear manner during the experiment. It is uncertain if the difference in the response of these two plants is attributable to the experimental conditions given that samples representing time 0 had different carbohydrates profiles with the most marked difference evident in a two-fold difference in suc concentration between the two plants. In addition, shortage of live plants prevented us from conducting replicate experiments and we consider these results as preliminary.

4.4 Low temperature shock

Initial low temperature stress treatments were conducted at 4°C. Preliminary northern blot analysis conducted on leaf material collected during these treatments indicated no increase in transcript levels over 7 d (data not shown). Subsequent low temperature treatments were conducted as a low temperature shock of -20°C due to limitations of equipment able to maintain temperatures between 0 and 4°C. Northern blot analysis revealed increases in GolS transcript levels in the leaves 1 h after exposure and increases in protein 15 min after exposure. However, when soluble carbohydrates were analysed during this period no changes were apparent (data not shown). A subsequent experiment where plants were exposed to -20°C and then transferred to a growth chamber maintained at 4°C revealed that after exposure to -20°C and 24 h at 4°C suc levels began to decline rapidly whilst concomitant increases in the levels of hexose sugars glu and fru were observed (Figure 3.17 a-b).

Such marked decreases could represent a controlled breakdown of suc by cellular invertases into hexose sugar components as part of a signalling response pathway that prepares the plant to initiate mechanisms to assist recovery from the -20°C exposure. It is important to note that only plants maintained at 4°C for 7 d after a -20°C shock were able to recover fully. Alternatively, the pattern observed could represent the result of cellular damage occurring at -20°C. In this scenario, leaves are effectively freeze-thawed during the experiment which may lead to damage to cellular structures. If this is the case then cellular invertases could be released in an uncontrolled manner from lysed vacuolar structures or the cell wall where they are normally stored (Foyer et al., 1997), leading to the uncontrolled breakdown of suc. We compared electron micrographs between unstressed leaves and leaves from plants that had been subjected to a -20°C shock for 2 h (Figure 3.18) and consistently found that in tissue from stressed plants, the integrity of cellular ultrastructure was severely compromised with

chloroplastic and cytoplasmic membranes clearly disrupted. Ideally, we would have correlated the electron microscopy with density gradient centrifugation experiments to separate cell fractions. Subsequently the cytoplasmic fraction would be tested, in vitro, for invertase activity. Given that we did not complete this experiment to complement the electron microscopy we cannot conclusively state the sucrose breakdown we observed was due to the release of invertases from ruptured vacuolar membranes and the cell wall however, it is likely that this was the case.

Compelling evidence reflecting the physiological extremity of the experiment was provided by GolS enzyme assays conducted on crude extracts obtained from excised leaves that had been exposed to varying temperature regimes (Figure 3.17 c). A marked decrease in activity was observed after 2 h exposure to -20°C. No measurable GolS activity was present in crude extracts from leaves that had been exposed to -20°C for 2 h and then transferred to 4°C for 24h. Leaves that had been exposed to greenhouse conditions and 4°C for 24 h showed relatively higher activities. The temperature used for the low temperature shock does not represent a typical environmental temperature that *X. viscosa* would be exposed to in its natural habitat. As such it is considered physiologically extreme. Although GolS transcript levels were shown to increase markedly after 1 h of exposure along with GolS protein increases 15 min after exposure, the carbohydrate and enzyme activity analysis would appear to indicate that the experimental design of low temperature stress treatments we conduct needs to encompass valid temperatures that the plants would be exposed to naturally.

4.5 XvGolS is functionally identified as a bonafide GolS

The full length *XvGolS* cDNA was cloned into the prokaryotic expression vector pPRO-EX HTb, downstream of a *lacZ* promoter, and transformed into *E. coli*. Induction of broth cultures transformed with this construct, using IPTG, resulted in a protein of approximately 45 kDa being expressed (Figure 3.9 a). Although the in-silico predicted molecular mass is 38 kDa, the 7 kDa difference is attributed to the presence of hexa histidine tag attached to the amino terminus of the recombinant protein. Western blots, conducted on total protein extracted from *E. coli* cultures induced for recombinant protein expression, using a 1° antibody specific for the hexa-His tag detected a His tagged protein with an apparent molecular mass of between 45 and 50kD (Figure 3.9 b). It is evident from SDS-PAGE and western hybridisation of protein extracts from induced cultures, that an apparent size difference between the recombinant protein is evident. This is attributed to the fact that two

different molecular weight markers were used for each respective gel. In addition, molecular weight markers provide a guideline for the estimation of protein sizes and electrophoresis conditions may result in disparities in the interpretation of protein sizes between different gels.

Importantly, when liquid cultures were induced for recombinant protein expression and crude cell lysates prepared, it was observed that recombinant protein in these lysates had the ability to synthesise gol in an in vitro reaction (Figure 3.10). Given that gol metabolism is unique to plants in terms of the synthetic enzymes and that bacteria invariably possess enzymes that break down RFOs to their component sugars, this enzyme activity is attributed to a functional XvGolS being expressed by the *E. coli* cells upon activation of the *lac*Z promoter of the expression construct. This gol forming ability was reproducible with different colonies albeit at varying levels (Figure 3.12). The very marked variability in GolS activity between different cultures was considered to be due to innate variations related to the growth conditions of individual colonies used to inoculate broth cultures. Vector controls where only the expression vector pPRO-EX HTb had been transformed into *E. coli*, consistently showed no measurable GolS activity after being induced for recombinant protein expression. It was evident that low levels of GolS activity was present in time 0 samples, prior to the addition of IPTG. This is attributed to the fact that the pPRO-EX HT vector system utilises a *lac*Z promoter for recombinant protein expression. Under natural conditions, lactose serves as the inducer of the *lac* operon in bacteria. However IPTG is a lactose analog and can also induce the operon by relieving transcriptional repression, complexing with the *lac* repressor molecule thereby unmasking the *lac*Z promoter. This repression however is not absolute and there is always a low level of transcription of the operon (cited in Maloy et al., 1994). Similarly, vectors utilising the *lac*Z promoter also display a degree of " promoter leakiness".

Fractions of gol, synthesised by recombinant XvGolS, were collected after separation on the HPLC column and digested with a fungal α-galctosidase (Aspergilus niger). Subsequent HPLC analysis of the products of this reaction indicated that galactose and *myo*-inositol had been generated as compared to the digestion of a commercially available gol standard (Figure 3.14). In addition when the mole ratio of these products were quantified we consistently observed a ratio of 1:1, providing conclusive evidence that XvGolS is indeed GolS.

Experiments conducted to determine the substrate kinetic parameters of the recombinant enzyme (Figure3.15 a-b) determined K_m values for *myo*-inositol and UDP-Gal of 1.3 mM and 14.1 mM respectively. The K_m (UDP-Gal) implies that the recombinant enzyme has a low specificity for this particular substrate. Data is available for GolS K_m values from a variety of plant species. Smith et al. (1991) described a K_m (Ino) and K_m (UDP-Gal) of 6.5 mM and 1.8 mM respectively, for GolS isolated and purified from the leaves of *Curcubita pepo*. Similarly *Cucumis sativus* GolS has a reported K_m (Ino) and K_m (UDP-Gal) of 4.0 mM and 0.16 mM respectively (Handley and Pharr, 1982) and *Ajuga reptans* crude enzyme extracts 6.4mM and 0.53 mM respectively (Bachmann, 1994). The large quantities of *X. viscosa* leaf material required prevented such experiments from being conducted. Given that the data presented in this work represents experiments conducted on crude protein extracts from *E. coli* expressing a recombinant XvGolS, it is not possible to make a meaningful comparison between our data and that reported previously for plant extracts. In addition the recombinant protein was functional despite the presence of an N-terminal hexa histidine tag. The presence of these additional amino acids may conceivably have brought about subtle conformational changes in the active protein resulting in the relatively high Km (UDP-Gal).

4.6 Evaluation of a phloem exudation technique for *X. viscosa*

This work initially focused on the response of *XvGolS* in the leaves of *X. viscosa* plants during abiotic stress. However it is known that RFOs are important in carbon translocation. It was unknown at the time of this work if *X. viscosa* represented a plant that specialised in using RFOs as primary phloem translocates or if suc fulfilled this function. We adapted a phloem exudation technique previously described for dicotyledonous plants by King and Zeevart (1974) and based on the use of EDTA in a phosphate buffer, which prevents the formation of callose and specialised structural phloem proteins (P-proteins) that are normally synthesised to plug sieve plates when leaves are wounded, thereby preventing the loss of phloem sap from the wound point (for review see Cronshaw and Sabnis, 1990).

The experimental procedure detected suc and raf as occurring in relatively high proportions in phloem exudates after 8 h of exudation (Figure 3.21 a). However, relatively high proportions of the hexose sugars glu and fru were also evident in these samples. The principal of the extraction technique involves leaf excision at the petiole and elution of phloem exudates into buffer that is changed every two hours. Typically, samples representing 2 h and 4 h show high proportions of hexose sugars due to leakage of these sugars from damaged cells around the

excision point into the exudation buffer. Samples representing 6 h and 8 h of exudation should ideally be free of hexose sugars or they should represent a small proportion of the total sugar profile. We utilised buffer concentrations of 5 mM Na_2EDTA and 5 mM K_2HPO_4 that had been previously optimised for the dicotyledonous plant *A. reptans*. In addition *X. viscosa* leaves, being monocotyledonous, lacked a petiole and leaves were excised at their base for use in the experiment. We did not conduct experiments to vary buffer concentrations in order to determine optimal conditions for exudation from *X. viscosa* leaves. Rather, the experiment was conducted to crudely investigate if RFOs are present in the phloem translocation stream. Importantly, we found no detectable levels of myo-inositol or gol (Figure 3.21 b), consistent with previous findings that gol is not a component of phloem sap (Turgeon et al., 1993). In addition, carbohydrates extracted from leaves after the completion of the experiment established that myo-inositol and gol were present in detectable quantities in leaf tissue.

The polyols sorbitol and glycerol were also detected during the experimental procedure (Figure 3.22). As previously stated, we concluded that glycerol was not phloem mobile but rather occurred in reponse to wounding during the excision of the leaves. Sorbitol was considered to be potentially phloem mobile, being detected in the phloem exudates and roots of both plants. We have previously shown that an aldose reductase gene is upregulated in reponse to water deficit stress (Mundree et al., 2000). Sorbitol was also found to increase in the leaves in response to water deficit, correlating the increase in transcripts and activity of aldose reductase to the synthesis of sorbitol in response to water deficit.

Carbohydrates were extracted from the roots of plants used for the exudation experiments. A comparison of the carbohydrate profiles of roots and leaves at full turgor revealed that roots and leaves had detectable levels of suc, glu, gal, raf and sta. Importantly, sorbitol and ver were present in root tissue but not in leaf tissue whilst ino and gol were detected in leaf tissue but not in root tissue (data not shown). This analysis has provided evidence to suggest that suc and raf are phloem mobile. Similar analyses conducted on the resurrection plant *C. plantagineum* identified the RFOs raf and sta in phloem exudates as well as in roots (Norwood et al., 2000). The authors' concluded that RFOs served a role a carbon translocation and storage in the roots of *C. plantgineum*. In conjunction with the previous reports on aldose reductase in *X. viscosa*, sorbitol may also fulfil a role in both carbon translocation and stress tolerance given that it was detected in low concentrations phloem exudates and root tissue at

full turgor and that we have previously reported increases in aldose reductase enzyme activity in leaf tissues in response to water deficit.

4.7 Concluding remarks

As mentioned previously, the role of RFOs in abiotic stress tolerance is unclear due to a number of conflicting publications with regard to their role in dessication tolerance in seeds. Recent findings have provided evidence to suggest that RFOs may indeed contribute to dessication tolerance (Taji et al., 2000) and low temperature tolerance (Pennycooke et al., 2003). We conducted this work to analyse the role of XvGolS in abiotic stress tolerance in *X. viscosa*. From the findings presented it is evident that increases in transcript levels observed during water deficit lead to increased levels of RFOs in the leaves of stressed plants. Additionally we have presented preliminary evidence to suggest that under low temperature conditions RFOs also accumulate in the leaves. We cannot conclusively attribute this observation to an increase in XvGolS transcripts as we have not excluded the possibility of other functional GolS enzymes acting to ameliorate the effects low temperature in *X. viscosa*.

In the context of the larger ongoing research project we are undertaking, genes involved in the RFO biosynthetic pathway in *X. viscosa* may prove to be important contributors toward the approach of engineering maize plants that harbour multigene vector cassettes that are able to confer multistress resistance. Arguably other genes with potential osmoprotective functions such as sucrose-P synthase and sucrose-P phosphotase or aldose reductase, could also provide the desired effect. However, it is the authors opinion that RFOs provide an ideal focus point as raffinosaccharides have to date only been implicated in carbon translocation and potential stress tolerance. As such these molecules and their intermediates do not appear to be involved in other criticial areas of metabolism as is evidenced by previous transgenic approaches where undesired pleitropic effects were not observed (Taji et al., 2000; Pennycooke et al., 2003).

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