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**Molecular characterization of *XvINO1*, a *myo*-inositol  
1-phosphate synthase gene from *Xerophyta viscosa***

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**"From your parents you learn love and laughter and how to put one foot in front of the other. But when books are opened you discover you have wings." ---Helen Hayes**

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## Abbreviations

ABA	abscisic acid
ABRE	ABA-responsive element
bp	base pair(s)
cDNA	copy DNA
DEPC	diethylpyrocarbonate
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
g	grams
h	hour
IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside
kb	kilobase(s)
kDa	kilodalton(s)
$\lambda$	lambda
l	litre(s)
LB	Luria-Bertani
M	molar concentration
$\mu$ g	microgram(s)
mg	milligram(s)
$\mu$ l	microlitre(s)
ml	millilitre(s)
$\mu$ m	micrometer(s)
mM	millimolar
MI	<i>myo</i> -inositol
min	minutes
mRNA	messenger RNA
Ni-NTA	nickel-nitrilotriacetic acid
nm	nanometre(s)
ORF	open reading frame
PBS	phosphate-buffered saline

PCR	polymerase chain reaction
PEG	polyethylene glycol
RNA	ribonucleic acid
RNase	ribonuclease
rpm	revolutions per minute
RWC	relative water content
sdH <sub>2</sub> O	sterile distilled water
SDS	sodium dodecyl sulphate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SOD	superoxide dismutase
RACE	rapid amplification of cDNA ends
RFO	raffinose family oligosaccharides
TAIL-PCR	thermal asymmetric interlaced PCR
TBE	tris borate EDTA
TE	tris-EDTA
TEMED	N,N,N',N' - tetramethylethylene diamine
Tris	tris(hydroxymethyl)aminomethane
u	unit(s) of enzymatic activity
UTR	untranslated region
w/v	weight per volume
X-Gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside

## Abstract

*Myo*-inositol 1-phosphate synthase (INO1) catalyses the conversion of glucose-6-phosphate to *myo*-inositol 1-phosphate, which is subsequently dephosphorylated to *myo*-inositol. *Myo*-inositol is a precursor for a number of important metabolites that include membrane components, storage molecules, phytohormones and a variety of osmoprotectants. *Xerophyta viscosa* Baker (Family Velloziaceae) is a monocotyledonous angiosperm which has the ability to resume full physiological function after desiccation. The full-length cDNA for *INO1* from *X. viscosa* was isolated using the RACE technique. Analysis of the *XvINO1* sequence revealed it to be similar to other *INO1* sequences. Southern blot analysis suggested that there may be two copies of *XvINO1* in the *X. viscosa* genome. The expression of *XvINO1* under various abiotic stresses was investigated at the mRNA and protein level. Northern blot analyses revealed that *XvINO1* transcript levels increased during abiotic stress in mature plants. The *XvINO1* cDNA was fused to a histidine tag at the N-terminus, cloned into pCR T7/NT overexpression vector and expressed in *E. coli* BL21(DE3)pLysS cells. The resulting recombinant protein tested positive for glucose 6-phosphate cyclase activity and was used to generate antibody for immunoblot analyses. Western blot analyses indicated the accumulation of *XvINO1* under stress. To further explore the expression of *XvINO1*, the 5' UTR of *XvINO1* was isolated using TAIL-PCR and a 1 kb upstream sequence was compared to 5' UTRs of the same length from other plants. *Arabidopsis* knockout mutants in which inositol transporter genes were deleted by T-DNA insertions were analyzed and showed no significant difference to wild type plants when grown under normal conditions. This work provides evidence that *myo*-inositol metabolism contributes to the adaptation of *X. viscosa* to abiotic stress.

**CHAPTER I**  
**INTRODUCTION AND LITERATURE REVIEW**

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## 1.1 Plant Stress

Abiotic stresses such as drought, low temperature and high salinity are environmental factors that dramatically limit plant growth and crop productivity (Sharma and Lavanya, 2002). Water deficit is considered to be the major single abiotic factor likely to affect crop yields globally. Semi-arid tropics are prone to hot summers and erratic rainfall patterns. The productivity of crops in these regions is severely affected by drought besides several other abiotic and biotic constraints that may occur. Reproductive development is usually severely affected by adverse physical conditions. Synthesis, accumulation and storage of proteins are mostly affected by temperature whereas growth is slowed by almost all stresses. The synthesis of polysaccharides is reduced or modified. As a result, the biomass is decreased and the nutritional value of the product is altered.

## 1.2 Resurrection Plants

Resurrection plants are a group of plant species that have the unique ability to revive from an air-dried state (Farrant and Kruger, 2001). The plants can survive the loss of most of their tissue water until they reach a quiescent stage. Upon rehydration, the plants rapidly revive and are restored to their former state (Scott, 2000). Tissue damage through dehydration and rehydration processes is either minimal or non-existent. In resurrection plants, desiccation prevents growth and reproduction over the dehydrated period, unlike other plant response to dehydration stress. Small numbers of resurrection plant species are represented in most taxonomic groups, ranging from pteridophytes to dicotyledons. The unique ability of withstanding near total water loss is shared with specialised structures of higher plants such as seeds and phloem (Ndima *et al.*, 2002; Vertucci and Farrant, 1995). Studies on the mechanisms involved have been derived mostly from the observations of cellular processes occurring during desiccation (Ndima *et al.*, 2001). The accumulation of osmoprotectants has been suggested to play a role in the protection of membranes against desiccation whereas the up-regulation of several antioxidant protectants is thought to prevent the oxidative damage caused by water loss (Vertucci and Farrant, 1995).

*Xerophyta viscosa* Baker (Family Velloziaceae) is a monocotyledonous angiosperm resurrection plant that can tolerate extreme desiccation (Mundree and Farrant, 2000;

Ndima *et al.*, 2001). The plant can be dehydrated to 5% RWC and upon rehydration, full physiological activity is resumed within 80 hours (Sherwin and Farrant, 1998). *X. viscosa* is being used as a model for the development of drought tolerant transgenic crops (Mundree *et al.*, 2002). Molecular and physiological aspects of how the plant responds to desiccation have been extensively studied (Mundree and Farrant, 2000). Differential screening showed that a number of genes are differentially expressed during dehydration and they had significant homology to dehydrins, lea-like proteins, lectins, putative acid phosphatase, rehydrins, calcium binding proteins and galactinol synthase (Mundree and Farrant, 2000). The expression patterns of some these genes have been studied in detail.

Using the strategy of 'complementation by functional sufficiency', several genes were isolated from *X. viscosa* that are important in NaCl stress tolerance (Mundree and Farrant, 2000) were isolated. One of the clones isolated by this approach was identified as a peroxiredoxin (*XvPer1*) (Mowla *et al.*, 2002; Mundree, *et al.* 2002). Peroxiredoxins are known to have seed-specific expression (Stacy *et al.*, 1996). Further studies showed that *XvPer1* was expressed in the vegetative tissues under stress conditions (Mowla *et al.*, 2002; Mundree, *et al.* 2002). This unique characteristic reveals certain 'seed-specific' behaviour of this resurrection plant.

Furthermore, about 30 genes which are upregulated and 20 which are downregulated under stress conditions were isolated using 'differential screening' (Mundree and Farrant, 2000). The genes that have been identified to be upregulated under water-deficit conditions have been identified as a galactinol synthase (*XvGolS*), an aldose reductase (*ALDRXV4*), a subunit c-like protein of the adenosine triphosphate (*VATPIXV*), a transcription factor (*DREB1A*) (Mundree *et al.*, 2002) and a cell membrane binding protein (*XVSAP1*) (Mundree *et al.*, 2002; Garwe *et al.*, 2003).

### **1.3 Dehydration Stress**

Water deficit in plants develops as a result of demand exceeding the supply of water. The demand is set by plant transpiration and soil evaporation. Transpirational cooling is important for the dissipation of the energy resulting from solar radiation (Blum, 1988). Some of this energy received from solar radiation is important for photosynthesis but

most of it is not utilised and must be emitted. Leaf water potential is reduced as water transpires from the leaf. This affects leaf turgor and, as turgor becomes null, the cells collapse and the leaf wilts. Amongst the factors that respond to leaf wilting are stomata which close and thereby reduce transpiration. The closure of the stomata leads to an increase in leaf temperature as it reduces CO<sub>2</sub> fixation and photosynthetic assimilation. The heat may reach a level where it damages the leaf (Blum, 1988).

Under water deficit stress, turgor maintenance and transpiration are crucial for the survival of plants. The maintenance of high leaf potential through the uptake of water from the soil can sustain turgor (Blum, 1995). The total leaf area is a dominant factor which controls whole plant transpiration. Evolution has adapted plants to dry environments by reducing plant size and growth rate, typical of many desert plants. It is also common that when desiccation develops, lower (older) leaves die first so as to reduce total leaf area and water requirements whereas the upper (younger) leaves retain open stomata and carbon assimilation. This behaviour is common in relatively drought-tolerant plants (Blum, 1988).

At the whole plant level, water deficit also has effects on plant phenology, phasic development, growth, carbon assimilation and reproduction processes. In plants such as maize, a short anthesis to silking interval has been shown to be a main feature of drought tolerant varieties (Blum, 1985). This is due to the fact that water deficit causes delayed flowering (which requires water). Abscisic acid is thought to play a role in this respect.

### **1.3.1 Damages Caused by Dehydration Stress**

The effects of drought stress arise from the fact that water is a fundamentally important component of the metabolism of all organisms (Mundree *et al.*, 2002). The physiological roles of water range from being a solvent to being a reactant in many biochemical reactions. Dehydration has several effects, at cellular and subcellular levels, on the plants. These include mechanical stress, oxidative stress and disruption of membrane and molecular integrity (Vertucci and Farrant, 1995).

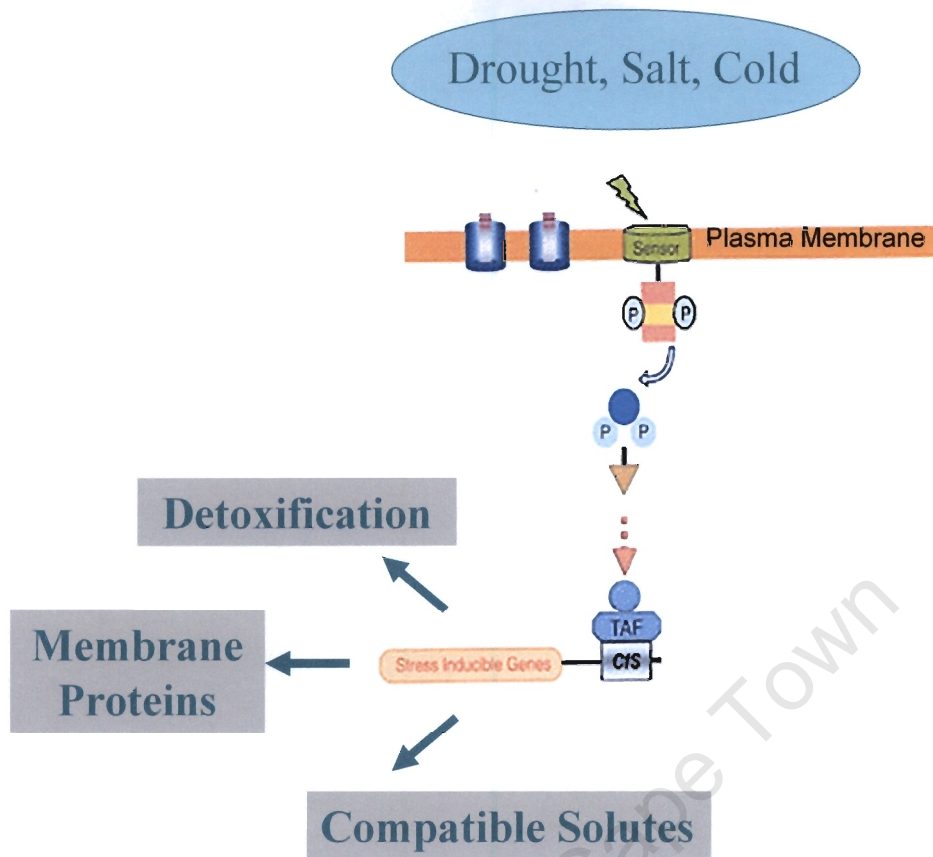
Mechanical stress is a result of considerable reduction in cell volume upon desiccation. Due to water loss, tension is placed on the plasmalemma and this results in the tearing of

the plasmalemma and release of hydrolytic extracellular enzymes into the cytoplasm, leading to cell wall collapse (Vertucci and Farrant, 1995). The contraction of cells during dehydration can lead to loss of membrane material.

The concentration of solutes in the cell increases as water is removed from the cell and this leads to a decline in the fluidity of the aqueous medium (Vertucci and Farrant, 1995). This affects the metabolic status of the cell and, at specific moisture levels, changes in metabolic activities occur. Under limiting water conditions, metabolic processes such as photosynthesis may result in the accumulation of toxic intermediates such as free radicals. During desiccation, the energy harnessed in plants cannot be released via photosynthesis and can lead to the formation of oxygen free radicals (Smirnoff, 1993). Free radicals can also be produced from the partial reduction of oxygen in active mitochondria (Vertucci and Farrant, 1995). If the free radicals are left unquenched they can cause considerable damage to subcellular components such as DNA, proteins and lipids. Membrane integrity of the plant cell is irreversibly disrupted by lipid peroxidation. This results in the leaking out of essential solutes from organelles and from the cell leading to impaired membrane function and metabolic imbalances. DNA damage could mean that proteins that are essential for optimum function of the cell will not be synthesised and the denaturation of important proteins negatively affects the whole plant (Mundree *et al.*, 2002).

### **1.3.2 Protection Mechanisms in Resurrection Plants and Seeds**

Complex protective mechanisms to prevent damage caused by water-deficit stresses have evolved in plants. These mechanisms vary significantly among the different plant species, with some species being highly sensitive to desiccation and others being able to tolerate extreme levels of water loss. Resurrection plants have the unique ability to withstand near total water loss (desiccation) and revive full metabolic activity on rehydration (Farrant and Kruger, 2001; Gaff 1987). The mechanisms of tolerance differ significantly between the lower orders (algae, bryophytes and lichens) and angiosperm resurrection plants and also among the angiosperms (Mundree and Farrant, 2002). The general pathway for the response of plants to abiotic stress is outlined (Figure 1.1).



**Figure 1.1:** Abiotic stress response in plants. At the perception of the stress, the message is relayed via the phosphoinositide signal pathway. The transcription factors (TAF) facilitate the transcription of stress-inducible genes by binding to *cis*-acting elements (Bohnert, pers. comm.).

The raffinose family of oligosaccharides (RFOs) are a group of D-galactose-containing oligosaccharide derivatives of sucrose that are widely distributed in plants. They are characterised by  $\alpha$ -galactosidic linkages between galactose moieties. Raffinose and stachyose are the principal RFOs in the plant kingdom although higher homologues such as verbascose and ajugose are known to occur. The RFOs are almost ubiquitous throughout the plant kingdom and are second only to sucrose in the occurrence of non-structural carbohydrates in the plant kingdom (Obendorf, 1997). They occur in significant quantities in the edible portion of many economically significant crop species which include soybean, sugar beet, cowpea and the common bean. The RFOs are an obstacle to the utilisation of these crops. They are the principal causes of flatulence, that is, the formation of intestinal gas following ingestion by animals and humans (Kerr *et al.*, 1993). RFOs are not digested by monogastrics because of the absence of  $\alpha$ -

galactosidase in the intestinal mucosa. They pass into the large intestine where they are anaerobically fermented by microflora to produce CO<sub>2</sub>, CH<sub>4</sub> and H<sub>2</sub> gases. This results in the acidification of the gut. The resulting flatulence severely limits the use of such plants as legumes in animal, particularly human, diets (Kerr *et al.*, 1993). The unfortunate thing is that these species are otherwise excellent sources of protein and soluble fibre.

There is substantial evidence that RFOs are beneficial to plants (Gorecki *et al.*, 2000). They act as reserve carbohydrates in storage organs such as seeds and tubers (Dey, 1985). These oligosaccharides are mobilised during the early stages of germination. The formation of galactose-containing oligosaccharides may also serve as a detoxifying process as galactose toxicity is well-recognised in plants.

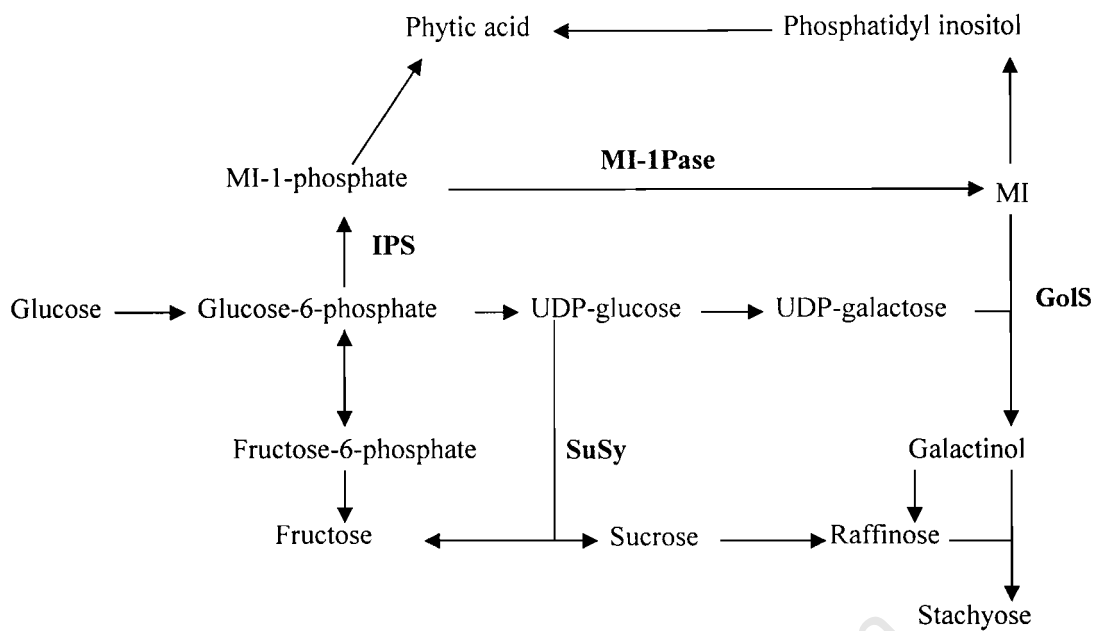
Galactosyl cyclitols and small amounts of free cyclitols also accumulate in seeds of various species in addition to sucrose and RFOs (Gorecki *et al.*, 2000). In lupin seeds, the accumulation of galactosyl cyclitols was found to coincide with an increase in RFO levels and the acquisition of desiccation tolerance whereas only RFOs were accumulated in pea seeds. Galactosyl cyclitols are thought to replace  $\alpha$ -galactosides in some seeds that have very low RFO levels. Buckweed seeds are an example of such seeds and they accumulate fugopyritol (galacto-*chiro*-inositol) as a major soluble carbohydrate in addition to sucrose (Obendorf, 1997).

There are several possibilities of how soluble carbohydrates contribute to desiccation tolerance in plants. The maintenance of membrane integrity appears to be the main role of the compounds in desiccation tolerance (Koster and Leopold, 1988; Bohnert and Jensen, 1996). It is hypothesized that the hydroxyl groups in RFOs (and other soluble sugars) substitute for water and provide the hydrophilic interaction required for the stabilisation of membranes and proteins (Vertucci and Farrant, 1995). High sucrose content may provide hydrogen bonding required for the prevention of lipid phase transitions during drying (Leopold and Vertucci, 1986). However, sucrose alone is not sufficient for desiccation tolerance (Obendorf, 1997). Raffinose has been found to act synergistically with sucrose in desiccation tolerance. It is thought that raffinose prevents the crystallisation of sucrose during drying (Caffrey *et al.*, 1988). It was, therefore,

hypothesized that the presence of RFOs and/ or galactosyl cyclitols in seeds inhibits the crystallization of sucrose during drying and facilitate the formation of a stable glassy state (Vertucci and Farrant, 1995).

Oligosaccharides and galactosyl cyclitols also play an important role in desiccation tolerance by reducing the levels of monosaccharides (e.g. glucose, fructose and galactose) that are used for their synthesis. This results in reduction of available respiratory substrates, thus inhibiting the production of oxygen free radicals prior to drying (Vertucci and Farrant, 1995). Sucrose, RFOs and galactosyl cyclitols may also act as free radical scavengers.

The synthesis of RFOs begins with the galactinol synthase (golS)-catalysed production of galactinol from UDP-galactose and myo-inositol. Galactinol is the galactosyl residue donor in the biosynthesis of RFOs. Raffinose is synthesised by the galactosylation of sucrose by galactinol and myo-inositol is regenerated. This reaction is catalysed by raffinose synthase. Raffinose is the substrate for the synthesis of the next member of the RFO series, stachyose. Addition of a galactosyl moiety from galactinol to the C-6 of the non-reducing  $\alpha$ -D-galactose moiety of raffinose. The reaction is catalysed by stachyose synthase. Stachyose synthase is also able to catalyse the production of galactosylononitol, which could also substitute galactinol in the synthesis of stachyose from raffinose (Peterbauer and Ritcher, 1998). The higher homologues are synthesised by the galactosylation of the previous RFO member by galactinol. The biosynthetic network between *myo*-inositol and RFOs is shown in Figure 1.2. Galactinol is thought to have no other role within the plant except for the synthesis of oligosaccharides (Saravitz *et al.*, 1987).



**Figure 1.2:** The biosynthetic network between RFOs and MI. IPS, Ins(3)P<sub>1</sub> synthase; MI-1Pase, myo-inositol-1-phosphate phosphatase; SuSy, sucrose synthase; GolS, galactinol synthase. (modified from Loewus and Murthy, 2000)

GolS could play a key regulatory role in the carbon partitioning between sucrose and RFOs in the developing seed. The enzyme shows optimal activity between pH 7.5 and 8.0. It requires Mn<sup>2+</sup> for activity and is inhibited by EDTA. Mg<sup>2+</sup> can replace Mn<sup>2+</sup> as a cofactor. Co<sup>2+</sup>, Zn<sup>2+</sup>, Cu<sup>2+</sup> and Ni<sup>2+</sup> have been shown to have an inhibitory effect on enzyme activity (Sprenger and Keller, 2000). There are several known isoforms of golS. Two distinct isoforms were observed in *Ajuga reptans* (Sprenger and Keller, 2000). One of the isoenzymes (GolS-1) is mainly involved in the synthesis of storage RFOs whereas the other one (GolS-2) is for the synthesis of transport RFOs. GolS-1 was found to be primarily expressed in the mesophyll, the site of RFO storage. The GolS-2 isoform is localised in the phloem-associated intermediary cells known for their role in phloem loading (Sprenger and Keller, 2000). The expression patterns of the other GolS isoforms are not well known.

GolS has been found by Liu *et al.* (1998) to be up-regulated under stress conditions as follows: (a) activity in kidney beans increased upon exposure of plants to cold; (b) the GolS mRNA levels also increased significantly upon cold exposure in the vegetative tissues of *Arabidopsis thaliana*, a chill resistant species, with transcripts diminishing

upon re-exposure to room temperature; and (c) a smaller increase was also observed in the siliques with the transcripts likewise disappearing upon re-exposure of the plants to room temperature.

Resurrection angiosperms have two distinct patterns in which they protect themselves from photo-oxidative damage (Mundree *et al.*, 2002). Homoichlorophyllous angiosperms (e.g. *Craterostigma wilmsii*) use leaf folding and anthocyanin accumulation to protect their chlorophyll from the damaging effects of light (Farrant, 2000; Farrant *et al.*, 2003) whereas poichlorophyllous (e.g. *X. viscosa*) dismantle the photosynthetic apparatus (Sherwin and Farrant, 1998; Mundree *et al.*, 2002).

Oxygen free radicals are produced during the drying-induced disruption of photosynthesis and respiration. Free radical formation is prevented and the activity of the species quenched in angiosperm resurrection plants (Farrant, 2000; Mundree and Farrant, 2000). The primary constituents of the protective mechanism include antioxidant enzymes (e.g. superoxide dismutase, catalases and peroxidases) and free radical scavengers (e.g. carotenoids, ascorbate, tocopherols and glutathione) (Sherwin and Farrant, 1998; Mundree *et al.*, 2002). The activity of the enzymes associated with antioxidant metabolism has been studied in the resurrection plant *X. viscosa* (Mundree and Farrant, 2001). The enzymes studied were ascorbate peroxidase (AP), glutathione reductase (GR), and cytoplasmic and chloroplastic superoxide dismutase (SOD). The activities of these enzymes, with the exception of chloroplastic SOD, were found to increase during dehydration. The authors suggested that these enzymes enable antioxidant protection against mitochondrially produced free radicals since respiration was observed to be ongoing to relatively low water content (30% RWC) (Sherwin and Farrant, 1998; Mundree and Farrant, 2001).

A set of hydrophilic and robust proteins accumulate during desiccation in orthodox seeds (which can withstand desiccation) (Vertucci and Farrant, 1995). These proteins are known as late-embryogenesis-accumulated (LEA) proteins and their function is unknown. Their highly conserved nature and timing of accumulation during drying, however, suggests that they have a role to play in desiccation tolerance (Vertucci and Farrant, 1995). LEA proteins are thought to contribute to membrane stability by binding

to macromolecular structures. However, the precise role of LEA proteins has not been defined (Goyal *et al.*, 2005). Some of these proteins do not behave as classical chaperons but exhibit a protective effect in the presence of chemical chaperons such as trehalose.

Another class of stress proteins, the heat shock proteins, are expressed during the natural course of seed development in the absence of heat stress (Vertucci and Farrant, 1995). These proteins are believed to have a role in the stabilisation of protein conformation and, therefore, have a role in desiccation tolerance (Vertucci and Farrant, 1995). The heat shock proteins are thought to preserve macromolecular structure during dehydration or to repair it during rehydration. These proteins are thermostable and are classified into groups according to their amino acid sequences (Bray, 1993).

Vacuole formation and water replacement within vacuoles has been shown to play an important role in desiccation tolerance by some resurrection plants (Willigen *et al.*, 2001). Mechanical damage is avoided in the leaf cells of the resurrection plant *Xerophyta viscosa* by the subdivision of the large central vacuole present in the hydrated leaves into smaller, solute-filled vacuoles in dry leaves (Mundree *et al.*, 2002). Reduction in cell volume is minimised by these vacuoles occupying space in the cytoplasm. The vacuolar contents are thought to be sucrose, sorbitol, anthocyanins and other polyphenolics whose levels have been found to increase during dehydration (Mundree and Farrant, 2001).

#### **1.4 Perception of Stress in Plants**

In order to adapt to the various abiotic stresses, plants use diverse and complex signalling strategies for recognising and responding to these stresses (Blum, 1988). The primary step in the switching on of the molecular responses is the perception of the stress and relaying the information through signal transduction pathways. It is unclear how cells perceive cellular water deficit and how it is transduced to the various responses to the stress. It is thought that plants perceive stress in different ways such as by sensors, receptors, elevated calcium concentrations and changing membrane fluidity. The perception and signalling of the stress is then translocated into biochemical

reactions, metabolic adjustments and altered physiological state (Bray, 1993; Zhu *et al.*, 2002).

#### **1.4.1 Signal Transduction**

There are multiple signal transduction pathways between stress perception and gene expression. Two major pathways are involved in the transcription of the perception of the signal (Bray, 1993; Shen and Ho, 1995). One involves the production of abscisic acid (ABA) and the other is ABA-independent. The ABA-independent pathway is not fully understood. Novel protein synthesis is induced by ABA in the ABA-dependent pathway. This protein synthesis regulates various stress-responsive genes. The regulation of stress responsive genes by ABA may also take place without the synthesis of novel proteins. The genes that are regulated by ABA can either be functional (such as water-channel proteins or enzymes) or they can be regulatory (such as protein kinases) (Zhu *et al.*, 1996). These genes are involved in mediating a number of cellular responses some of which are adaptive.

#### **1.4.2 Changes in Gene Expression**

As with all metabolic processes, gene expression is an important aspect of response to desiccation. The genes that are induced during water deficit stress can be divided into two groups. The first code for proteins that are directly involved in protection whereas the second code for proteins that are involved in signal transduction and gene expression (Mundree *et al.*, 2002). The stress responsive gene expression leads to the synthesis of protection proteins that are important in the adaptation process of plants against diverse stimuli (Sharma and Lavanya, 2002).

According to Ramajulu and Bartels (2002) there is increasing evidence indicating that genes responding to water stress can be categorized in two classes based on the time of their response. Some genes may respond immediately, within seconds or minutes, while others respond later, in hours, days or even weeks. It appears that the early responsive genes may provide initial protection and amplification of signals while the genes that respond later may be involved in adaptation to stress conditions.

Sophisticated patterns exist in the expression of drought-induced genes. Transcription factors acting both in *cis* as well as *trans* have been analysed and a conserved sequence PyACGTGGC has been reported to function as ABA-responsive element (ABRE) (Sharma and Lavanya, 2002). An ABRE that functions as a *cis*-acting DNA element is exemplified by the EmBP-1 protein found in the wheat *em* gene (Mundree *et al.*, 2002). Several other *cis*-acting elements that function in ABA responsive gene expression under different abiotic stress conditions are known.

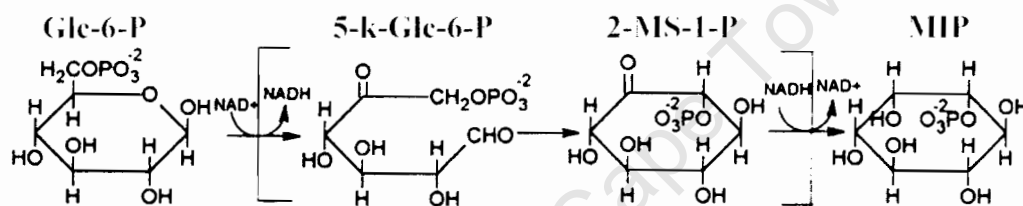
There are *cis*-acting elements that have ABA-independent expression (Shinozaki and Yamaguchi-Shinozaki, 2000). A *cis*-acting element known as a dehydration responsive element (DRE) having the sequence TACCGACAT is involved in ABA-independent gene expression under drought, high salt and low temperature conditions in various dehydration genes such as *rd29A* that is responsible for dehydration and cold-induced expression (Baker *et al.*, 1994; Shinozaki and Yamaguchi-Shinozaki, 1996). cDNAs encoding the DRE-binding proteins, DREB1A and DREB2A, have been isolated and the proteins were shown to specifically bind and activate the transcription of genes containing the DRE sequence (such as *rd29A*) in *Arabidopsis* (Liu *et al.*, 1998).

The result of regulated gene expression during abiotic stress conditions is a net change in the metabolic and physiological activities of the plant so as to counter the deleterious effects of the inducing stress. For example, the upregulation of various antioxidant protectants during drying in the resurrection plants (e.g. *X. viscosa*) enhances the ability of the plants to protect themselves from the damaging effects of oxygen free radicals arising from the respiratory chain (Sherwin and Farrant, 1998).

### **1.5 Myo-Inositol**

*Myo*-inositol (MI) is an essential precursor for a number of cell functions in plants. It is necessary for membrane lipid synthesis, cell signalling, anchoring of proteins, conjugation and transport of hormones, phosphate storage, and in the synthesis of osmoprotectants such as  $\alpha$ -galactosides and galactosyl cyclitols. Despite the fact that MI is part of a complex metabolic pathway, its synthesis occurs via a single defined pathway (Smart and Flores, 1997).

The unique enzyme, myo-inositol-1-phosphate synthase (INO1), common to all eukaryotes, catalyses the synthesis of 1L-myo-inositol-1-phosphate [Ins(3)P<sub>1</sub>] from D-glucose-6-phosphate (Figure 1.3). Ins(3)P<sub>1</sub> is subsequently dephosphorylated to myo-inositol (MI). This biosynthetic conversion of D-glucose-6-phosphate to MI via Ins(3)P<sub>1</sub> constitutes the sole *de novo* pathway for the synthesis of this physiologically important cyclitol in plants (Loewus and Murthy, 2000). Free MI is regarded as a ubiquitous constituent of plant tissues and in some species (e.g. *Actinidia arguta*) it is the major sugar constituent.



**Fig. 1.3;** Enzymatic mechanism of -MI-1-phosphate synthase [a.k.a. Ins(3)P<sub>1</sub> synthase or INO1]. The glucose 6-phosphate (Glc-6-P) is first oxidized to 5-keto- D-glucose-6-phosphate which subsequently undergoes an aldol condensation to 2-myo-inosose-1-phosphate (2-MS-1-P). The last step in the reaction is the reduction of 2-MS-1-P to give *myo*-inositol 1-phosphate (MIP) (Loewus and Murthy, 2000)

The conversion of D-glucose-6-phosphate to Ins(3)P<sub>1</sub> by Ins(3)P<sub>1</sub> synthase involves three substeps; a) NAD<sup>+</sup>-coupled oxidation of C-5 of D-glucose-6-phosphate, b) aldol condensation between C-1 and C-6 of 5-keto- D-glucose-6-phosphate (D-xylulo-5-hexulose-6-phosphate) , and c) NADH-catalysed reduction of 2-*myo*-inosose-1-phosphate (D-2,4,6/3,5-pentahydroxy-cyclohexane-2-phosphate) to yield Ins(3)P<sub>1</sub> (Loewus and Murthy, 2000; Barnet and Corina, 1967).

The structural gene, *INO1*, appears to be highly conserved (Loewus and Murthy, 2000). A transcript homologous to the *INO1* gene was isolated from the dark-weed *Spirodella polyrrhiza* (Smart and Fleming, 1993). This transcript was rapidly and spatially up-

regulated during an ABA- induced morphogenic response. Several possible scenarios were suggested for the role of MI synthesis and they included phytic acid accumulation, lipid synthesis, an ABA signal transducing mechanism involving auxin conjugates, and induced response to stress involving methyl ethers of inositol.

Transgenic *Arabidopsis* plants which overexpressed *INO1* showed no significant difference in whole plant growth habit, germination rate, flowering time, stem thickness and germination/ survival under high salt or low temperature conditions (Smart and Flores, 1997). A four-fold increase in enzymatic activity of Ins(3)P<sub>1</sub> synthase was however observed. It is possible that the extent of expression of *INO1* was not sufficient for stress response.

Recently, a novel salt-tolerant form of *INO1* (*PINO1*) was isolated from the halophytic wild rice *Porteressia coarctata* (Manojee *et al.*, 2004). The enzyme showed distinct differences in a stretch of 37 amino acids between amino acids 174 to 210. Introgression of *PINO1* conferred salinity tolerance in tobacco of up to 300 mM NaCl with retention of photosynthetic competence. Upon deletion of amino acids 174 to 210, the protein lost salt-tolerance.

Cytosolic and chloroplastic forms of *INO1* exist (Loewus and Murthy, 2000). The native cytosolic form is homotrimeric whereas the native chloroplastic form homotetrameric, but they do not differ significantly in their biochemical and kinetic parameters. The two isoforms of the *Oryzae sativa* enzyme have been identified as comprising of 80- and 60-kDa subunits (Haiti *et al.*, 2001). The larger subunit is mostly membrane-bound whereas the smaller subunit accumulates in the stromal fraction. The 80-kDa subunit was found to be proteolytically cleaved to the 60-kDa subunit in a process thought to proteolytic activation (Haiti *et al.*, 2001). It was also found that Ca<sup>2+</sup>-dependent phosphorylation of the 60-kDa subunit in seedlings of salt tolerant *Oryzae* varieties grown under light/dark environment in presence of 100 mM NaCl resulted in increased enzymatic activity (Haiti *et al.*, 2001).

A diurnal fluctuation in the levels of an *INO1*-like transcript from the salt-stressed ice-plant, *Messebryanthemum crystallinum* were observed (Ishitani *et al.*, 1996). An

increase in mRNA during the light period could be coordinated with the gene encoding MI-*O*-methyltransferase. This enzyme catalyzes the methylation of MI to *D*-ononitol which is epimerised to *D*-pinitol. *D*-Pinitol is considered to be the principal osmoregulator in salt-stressed *M. crystallinum*. This effect was, however, not observed in *Arabidopsis* and it was concluded that there is probably no stress-mediated induction of Ins(3)P<sub>1</sub> synthase in *Arabidopsis*.

The synthesis of galactinol, the galactosyl residue donor in the synthesis of RFOs, requires MI. RFOs are involved in phloem transport, seed development, seed desiccation and numerous stress-specific responses of plants (Loewus and Murthy, 2000). *O*-methyl ethers of MI contribute to osmotic regulation in plants that have been exposed to stressful environments. *M. crystallinum* plants that were irrigated with 400 mM NaCl accumulated pinitol to over two-thirds of the soluble carbohydrate fraction and about 10% of the dry weight (Paul and Cockburn, 1989).

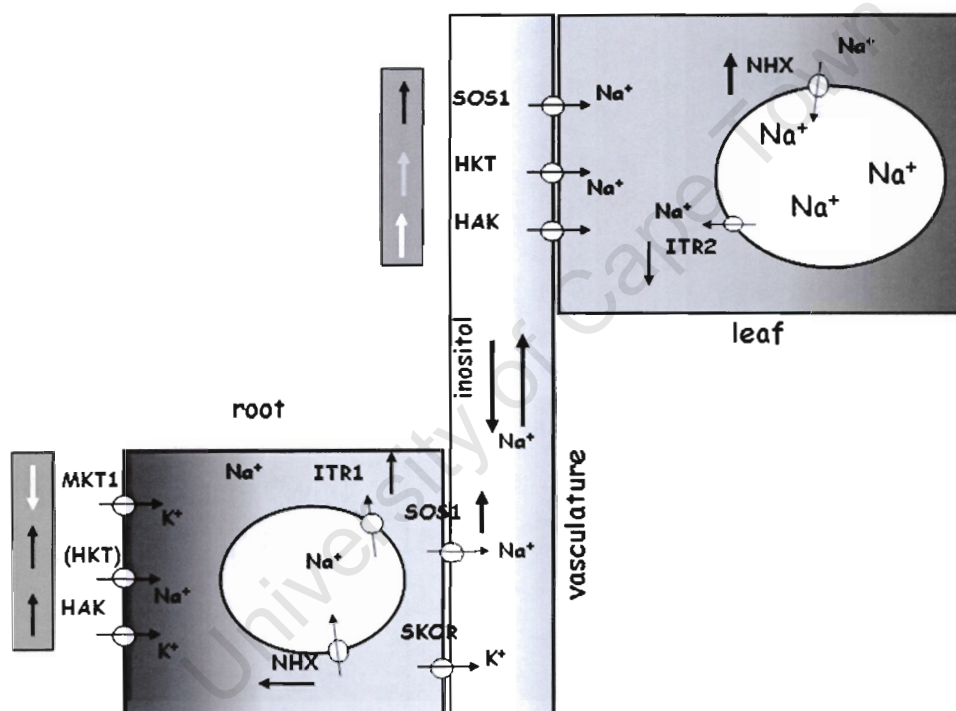
Transgenic tobacco plants containing the *O*-methyltransferase from an ice plant accumulated ononitol and provided better protection under drought and salt-stress conditions than wild type plants (Sheveleva *et al.*, 1997). In the *Arabidopsis* halophytic relative, *Thehungiella halophila*, the *INO1* was found to be one of the significantly upregulated genes under stress conditions (Taji *et al.*, 2004).

### 1.5.1 Myo-Inositol Transportation

The transportation of MI during stress signalling has been studied in a number of systems (Zhang and Majerus, 1998; White *et al.*, 1991). The amount of MI was increased significantly in renal tissues as a result of exposure to NaCl (Nakanishi *et al.*, 1988). Na<sup>+</sup> (H<sup>+</sup>)/ MI symporters (ITRs) have been reported in a number of plants, yeast and animal species (Miyazaki *et al.*, 2004). They are localised in the tonoplasts. The regulation of the ITR genes in plants has been found to vary between the halophytic plants (*M. crystallinum*) and the glycophytes (*A. thaliana*) (Chauhan *et al.*, 2000). It is hypothesised that in *M. crystallinum* ITR1 (MITR1) acts in removing Na<sup>+</sup> from root vacuoles under high salinity. Little is known about the transportation of MI from the roots to the leaves. However, a number of transporters are thought to be involved in this process. These are the salt overly sensitive proteins (SOS) and the high affinity

potassium transporter (HAK). In the leaves MI is compartmentalised into the central vacuole. This compartmentalisation is essential for the maintenance of the osmotic potential of the cell. The proposed mechanism for the transportation and compartmentalization of *myo*-inositol dependant  $\text{Na}^+$  uptake is shown in Figure 1.4.

In *M. crystallinum*, the *mitr1* was found to be expressed strongly in the roots and weakly in leaves whereas it was moderately expressed in the stem (Chauhan *et. al.*, 2000). The transcripts were significantly increased in all organs upon salinity stress. The *mitr2* was only slightly upregulated in leaves under salinity stress.



**Figure 1.4:** Hypothetical model of the uptake of  $\text{Na}^+$  in *M. crystallinum*. The different ion transporters are; SOS: salt-overly sensitive, NHX:  $\text{Na}^+/\text{H}^+$  antiporter, HKT: high affinity  $\text{K}^+$  transporter, HAK:  $\text{K}^+$  transporter family member, MKT:  $\text{K}^+$  channel, SKOR:  $\text{K}^+$  passive transport channel (Bohnert, pers. comm.)

## 1.6 Aims of This Research

The finding that *XvGolS* is one of the genes that is significantly upregulated under stress conditions in *X. viscosa* stimulated interest in looking at the expression of *XvINO1*, which is upstream of *XvGolS*, under similar conditions. The homologues of the gene have been found to be stress-responsive in other plants. This research was aimed at the molecular characterisation of *XvINO1*. The expression patterns of the gene were to be investigated under various abiotic stress conditions such as high salinity, dehydration/rehydration, exogenous ABA and low temperature shock using northern and western blot analyses. The data obtained would provide more information on the mechanisms of desiccation tolerance in the resurrection plant *X. viscosa*. To further understand the metabolism of inositol in plants, Arabidopsis knockout mutants in the inositol transporter genes were investigated.

University of Cape Town

**CHAPTER II**  
**MATERIALS AND METHODS**

University of Cape Town

## 2.1 Plant Material and Growth Conditions

*X. viscosa* plants were collected from Cathedral Peak Nature Reserve in the Drakensburg mountains (Kwazulu-Natal province, South Africa). The plants were grown under green-house conditions as described by Sherwin and Farrant (1996). Prior to imposing of stress treatments, plants were placed in a controlled environment chamber [16 h light ( $350 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )  $25^{\circ}\text{C}$ ; 8 h dark] for two weeks to acclimatize them.

## 2.2 RNA Isolation and RT-PCR

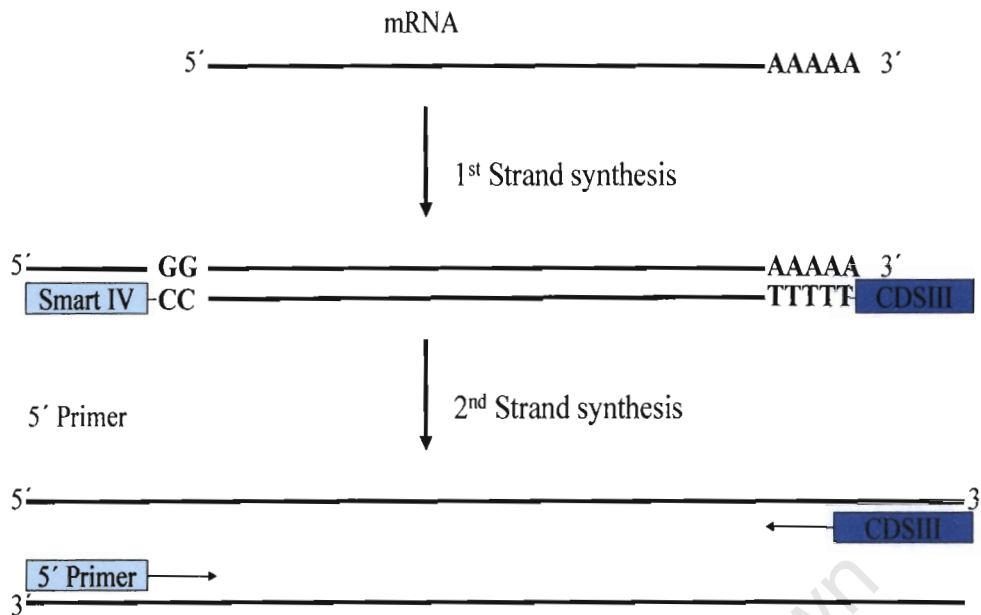
RNA was isolated from *X. viscosa* leaves using the Trizol reagent (Life Technologies, USA). About 0.1 g of plant material was ground in mortar with a pestle in liquid nitrogen. The ground tissue was transferred to a 2 ml eppendorf tube and 0.75 ml of Trizol reagent was added. The mixture was vortexed and placed on ice for 5 min. Two millilitres of chloroform were added, with shaking, followed by centrifugation at  $12\ 000 \times g$  for 15 min at  $4^{\circ}\text{C}$ . The supernatant was transferred to a new tube. Isopropanol (0.5 ml) was added to the supernatant and the mixture left for 10 min at room temperature, followed by centrifugation at  $12000 \times g$  for 10 min at  $4^{\circ}\text{C}$ . The supernatant was discarded and the pellet was washed with 70% ethanol and air-dried. The dried pellet was resuspended in 50  $\mu\text{l}$  DEPC-water and stored at  $-80^{\circ}\text{C}$ .

### 2.2.1 First-strand cDNA Synthesis

The following components were mixed in a 1.5 ml microfuge tube; 1  $\mu\text{l}$  RNA ( $\sim 1 \mu\text{g}$ ), 1  $\mu\text{l}$  smart IV oligonucleotide (Clontech, UK), 1  $\mu\text{l}$  CDS III primer (Clontech, UK) and 2  $\mu\text{l}$   $\text{sdH}_2\text{O}$ . The mixture was spun briefly and incubated at  $72^{\circ}\text{C}$  for 2 min then 2  $\mu\text{l}$  of 5x first strand buffer, 1  $\mu\text{l}$  20 mM DTT, 1  $\mu\text{l}$  10 mM dNTP mix and 1  $\mu\text{l}$  expand RT were added to make a total volume of 10  $\mu\text{l}$ . The tube was incubated at  $42^{\circ}\text{C}$  for 1 hr. One microlitre of the cDNA was used for a 50  $\mu\text{l}$  second-strand synthesis reaction and the rest was stored at  $-20^{\circ}\text{C}$ .

### 2.2.2 Second-strand Synthesis

The second-strand mix containing 40  $\mu\text{l}$   $\text{sdH}_2\text{O}$ , 1  $\mu\text{l}$  first-strand cDNA, 5  $\mu\text{l}$  10x expand buffer, 1  $\mu\text{l}$  50x dNTP mix, 5' primer, 1  $\mu\text{l}$  CDS III primer and 1  $\mu\text{l}$  expand polymerase was made. The following PCR cycle was carried out:



**Figure 2.1:** Schematic presentation of the first and second strand synthesis of cDNA.

### 2.2.3 3' RACE-PCR

Gene-specific amplification of the 3' end of *XvINO1* was carried out using the primers; INO-INTF (GAATCGCGTGCCAACAATGT) and CDSIII. The PCR mix contained 1  $\mu$ l of first strand cDNA, 0.5  $\mu$ M each of forward and reverse primers and 0.8 mM dNTP mix in 50  $\mu$ l of 1x reaction buffer and 0.4  $\mu$ l of high fidelity Taq DNA polymerase. Thirty-six cycles were carried out at 94 $^{\circ}$  for 30 s, 60 $^{\circ}$ C for 30 s, and 72 $^{\circ}$ C for 1 min 30 s. The PCR reaction was nested using the primers CTGCAGAAGCAGCTCAGACC (FINOSINT) and CDSIII. The PCR mix was as before and the same cycle was used.

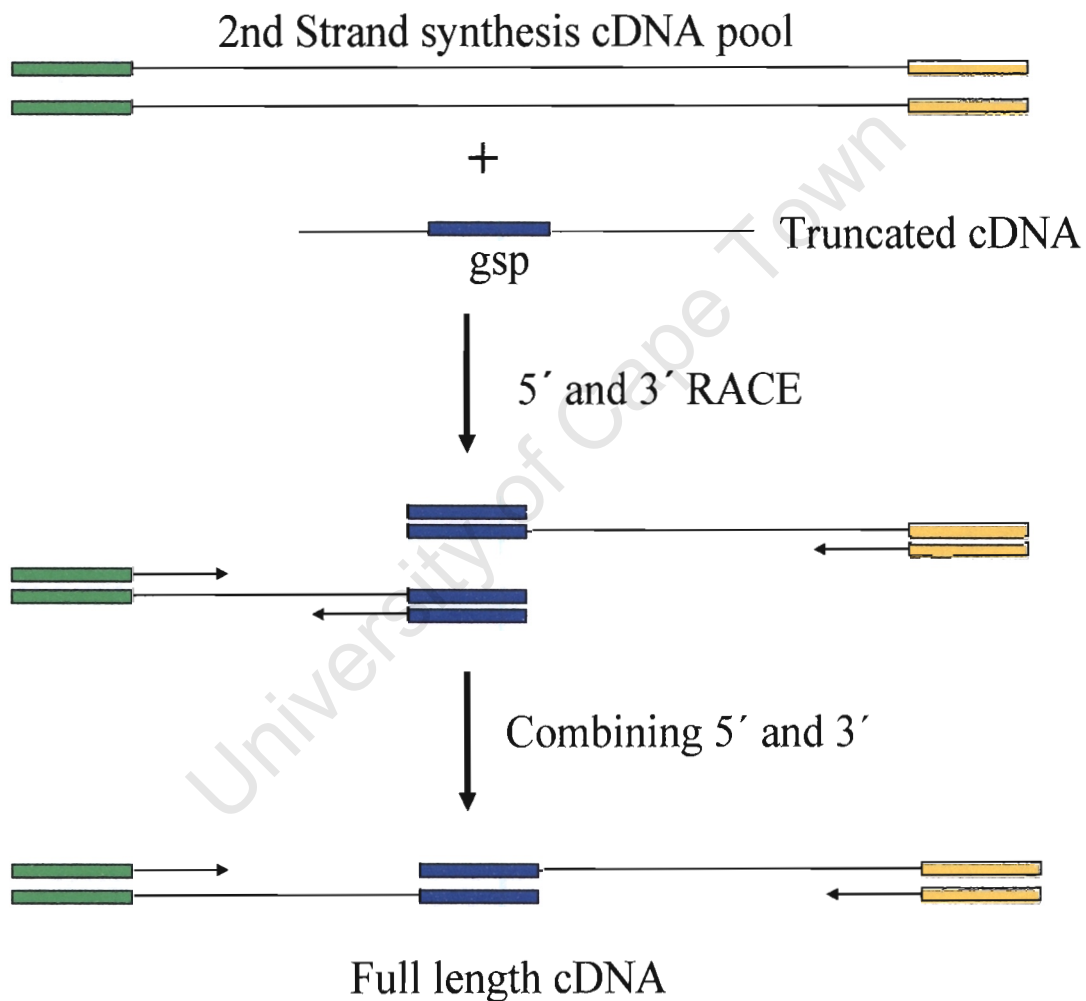
### 2.2.4 5' RACE-PCR

Gene-specific amplification of the 5' end of *XvINO1* was carried out using the primers INOR1 (CGGGACAGCTTCGTCGCGGTTACG) and the 5' Primer. The same reaction was carried out as for the 3' end and nesting was done using GTCATCGACCACATTGCTC (RINOSINT) and 5' Primer.

### 2.2.5 PCR Joining of the 5' and 3' Fragments

The 5' and 3' PCR products were mixed in a 1:1 ratio (5  $\mu$ l each) in a PCR tube. Two microlitres of 10x Expand PCR buffer, 1  $\mu$ l of 10 mM dNTPs and 6  $\mu$ l of sterile distilled

water were added. The mixture was boiled for 5 min and chilled for 2 min on ice. One microlitre of Expand DNA polymerase (10 units) was added followed by incubation at 68°C for 15 min. PCR was carried out using 10 µl, 5 µl, and 1 µl of the products as template. Another reaction was carried out using 1 µl each of the 5' and 3' PCR products. The PCR product was purified using the Roche High Pure PCR Purification Kit. Following the reaction, primers FINOSINT and RINOSINT were used to carry out PCR reaction using the joined 5' and 3' product as template to verify the joining of the two fragments (Figure 2.2).



**Figure 2.2:** Schematic presentation of the gene-specific amplification of the 5' and 3' ends of the target cDNA and subsequent joining of the two ends to obtain the full-length cDNA.

### 2.3 Cloning and Sequence Analysis of *XvINO1*

The full-length *XvINO1* was cloned into the pGEM<sup>®</sup>-T Easy Vector System according to the manufacturer's instruction. The reaction mixture contained 50 ng (2 µl) of the PCR

product, 50 ng (1  $\mu$ l) of the pGEM<sup>®</sup>-Easy vector and 3 units (1  $\mu$ l) of T4 DNA ligase in 1x T4 Rapid Ligation Buffer. The reaction was incubated at 4°C overnight. Two microlitres of the ligation reaction were used to transform *E. coli* DH5 $\alpha$  competent cells. The ligation mixture was added to 50  $\mu$ l of thawed competent cells. The mixture was heat-shocked at 37°C for 5 min then incubated on ice for 2 min. One millilitre of LB broth was added followed by incubation at 37°C for 1 h. One hundred microlitres of the cells were plated on LB agar containing 100  $\mu$ g/ml ampicillin followed by incubation at 37°C overnight. A few colonies were picked and inoculated into LB broth containing 100  $\mu$ g/ml ampicillin. Plasmid miniprep was carried out using the Roche High Pure Plasmid Purification Kit. PCR screening was carried out using the 5' Primer and CDSIII and the purified plasmid as template. Five samples of the plasmid were sequenced using the MegaBACE DNA Sequencing System (Molecular Dynamics, Amersham Biosciences). The BLAST programme of the National Centre for Biotechnology Information (NCBI) (Altschul *et al.*, 1990) was used to search databases for sequence similarities. Nucleotide and amino acid sequence comparisons were done using the DNAMAN programme (Version 4.3, Lynnon BioSoft).

#### **2.4 Southern Blot Analysis**

DNA was isolated using a method based on the plant DNA preparation procedure described by Dellaporta *et al.* (1983). In all cases approximately 1 g of leaf tissue was ground to a fine powder in liquid nitrogen before extraction. DNA was precipitated using isopropanol, resuspended in TE buffer and quantitated spectrophotometrically using a UV spectrophotometer (Beckmann, Germany). Aliquots of 15  $\mu$ g DNA were cut with EcoRI BglII EcoRI/SalI restriction endonucleases at 37°C and electrophoresed on 1% agarose gels at 20V overnight. Electrophoresed DNA was blotted onto nylon membranes (Hybond -N<sup>+</sup>, Amersham) by capillary transfer (Sambrook *et al.*, 1989). DNA was fixed using UV light (300 mJ/s for 3 s).

The *XvINOI* cDNA was labelled with radioactive <sup>32</sup>P using the Megaprime DNA Labeling System (Amersham Biosciences, UK) according to the manufacturer's instructions. One hundred nanograms of *XvINOI* cDNA were mixed with 5  $\mu$ l of the 15 mM primer mix. The mixture was denatured at 95°C for 5 min followed by addition of 10  $\mu$ l of 5x reaction buffer, 5  $\mu$ l (50  $\mu$ Ci) of <sup>32</sup>P, 2  $\mu$ l of 1 U/ $\mu$ l Klenow enzyme and 27

µl of sterile distilled water. The mixture was incubated at 37°C for 1 h. The unincorporated <sup>32</sup>P was removed using Sigma Post-Reaction Clean-Up Columns (Sigma-Aldrich, Germany).

Blots were prehybridised at 65°C for 4 h in prehybridisation solution (1% BSA, 1 mM EDTA, 0.5 M PB, 7% SDS). Hybridisation with the labelled *XvINO1* probe was carried at 65°C for 16 h. The blots were subsequently washed with Wash Buffer A (2 X SSC, 0.5% SDS) at 65°C and more stringently with Wash Buffer B (0.5 X SSC, 0.1% SDS) at 65°C. Following washing, the membranes were autoradiographed at -70°C onto high-performance autoradiography film (Hyperfilm™ MP, Amersham Pharmacia Biotech).

## **2.5 Expression Analysis of *XvINO1* under Abiotic Stress**

### **2.5.1 Plant Material and Growth**

*X. viscosa* plants were grown as described above (2.1).

### **2.5.2 Stress Treatment of Plants**

Whole plants were subjected to various abiotic stress treatments. The experiments were carried out in duplicates (two plants) for each treatment. Leaf samples were collected and the relative water content (RWC) was determined using the formula;

$RWC = [(FW-DW)/DW] \times 100\%$  where FW is the fresh weight and DW is the dry weight

#### **2.5.2.1 Dehydration and Rehydration**

Dehydration of *X.viscosa* was conducted by withholding water from the plants for a period of 15 days in a controlled environment chamber [16 h light (350 µmol.m<sup>-2</sup>.s<sup>-1</sup>) 25°C; 8 h dark]. Leaf samples were taken at full turgor and after 3, 9, 12, 11, 14 and 15 days without water. Upon rehydration, leaf samples were taken at 12, 48 and 72 h after watering.

### **2.5.2.2 Salinity Stress**

To investigate the effect of NaCl on *X. viscosa*, plants were watered with 100 mM NaCl solution in a controlled environment chamber [16 h light ( $350 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) 25°C; 8 h dark]. Leaf samples were collected at 6 h, 12 h, 24 h, 48 h and 72 h.

### **2.5.2.3 Low Temperature**

*X. viscosa* plants were placed at -20°C for 2 h and leaf samples were taken at 0, 30, 60 and 120 min, respectively.

### **2.5.2.4 Exogenous ABA Treatment**

To determine the effect of ABA on *XvINO1* expression, the leaves and soil of plants were sprayed with 150  $\mu\text{M}$  ABA, every 24 h, over a period of 72 h. The plants were equilibrated in a controlled environment chamber [16 h light ( $350 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) 25°C; 8 h dark].

## **2.5.3 RNA Isolation and Northern Blot Hybridisations**

Total RNA was isolated using the Trizol reagent (Gibco-BRL, Germany). *Xerophyta viscosa* leaves (200 mg) were ground in liquid nitrogen and homogenized in 0.75 ml of the reagent. Following incubation for 5 min at room temperature, 0.2 ml chloroform was added and incubated at room temperature for 10 min. Samples were centrifuged at 12 000 x g for 10 min at 4°C and the RNA was precipitated using isopropanol. RNA was quantitated spectrophotometrically, separated on a 1.2% agarose formaldehyde gel and stained with ethidium bromide to verify quantitation.

Fifteen micograms of each sample were run on a 1.2% agarose formaldehyde gel and transferred onto nylon membranes and fixed as described above. Probe-labelling, hybridization, washing, exposure and scanning of the RNA blots were carried as for southern blots.

## **2.6 Heterologous Protein Expression and Purification**

### **2.6.1 pCR T7/ NT Cloning**

*XvINO1* was cloned into an expression vector using the pCR<sup>®</sup>T7 TOPO<sup>®</sup> TA Expression Kit (Life Technologies, Invitrogen, USA). PCR primers flanking the open reading frame of *XvINO1* were designed; ATGTTTCATCGAAAGCTTCAAGG (INOF1) and

CCATGCATAGAATAGCTTACATCGATCGTAC (INOR1). Fifty nanograms of *XvINO1* template were added to the PCR mix containing 0.8 mM dNTPs, 0.5  $\mu$ M each of INOF1 and INOR1, 0.5 units Pfu DNA polymerase (Whitehead Scientific, Cape Town) in 1x reaction buffer. Thirty-six cycles were carried out at 94° for 30 s, 60°C for 30 s, and 72°C for 1 min 30 s. The PCR product was gel-purified as outlined before (2.2.5) and 3' As were added by incubating the following mix at 72°C for 10 min: 30 ng (7  $\mu$ l ) PCR product, 0.1 mM MgCl<sub>2</sub>, 0.5 mM dNTPs, 0.2 Supertherm Taq DNA polymerase (Southern Cross Biotechnology, Cape Town) in 1x reaction buffer, followed by purification (2.3).

Two microlitres of the purified PCR product were mixed with 0.5  $\mu$ l each of the TOPO salt solution and TOPO NT vector. The reaction was left at room temperature for 10 min. The ligation generated the recombinant pCR T7/ NT::*XvINO1* vector. Two microlitres of the ligation reaction were added to a vial of One Shot® TOP10F' competent *E. coli* cells and gently mixed. The mixture was incubated on ice for 10 min. The cells were heat-shocked for 30 s at 42°C and placed on ice. SOC medium (250  $\mu$ l) was added followed by incubation at 37°C for 30 min with shaking (200 rpm). Thirty microlitres of the transformed cells were plated on LB agar plates containing ampicillin (100  $\mu$ g/ml) and incubated at 37°C overnight. Ten colonies were picked and PCR screened. The positive colonies were inoculated into LB broth containing 100  $\mu$ g/ml ampicillin and incubated overnight while shaking (200 rpm). Plasmid purification was carried out as outlined above (2.3) and the product digested with EcoR1 to verify the orientation of the insert. The plasmid was sequenced following the procedure in 2.3.

### **2.6.2 Transformation of One Shot® BL21(DE3)pLysS Cells**

Ten nanograms of pCR T7/ NT::*XvINO1* were added to a vial of One Shot® BL21(DE3)pLysS cells and mixed gently by inverting the tube. The mixture was incubated on ice for 30 min followed by heat-shocking at 42°C for 30 s. The cells were placed on ice and 250  $\mu$ l of SOC medium was added. The tube was incubated at 37°C for 30 min with shaking at 200 rpm. The entire transformation reaction was added to 10 ml of LB broth containing 100  $\mu$ g/ml ampicillin and 34  $\mu$ g/ml chloramphenicol. The cells were grown overnight at 37°C with shaking at 200 rpm.

For the pilot expression, 10 ml of LB containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol were inoculated with 500 µl of the overnight culture. The cells were grown for 2 h at 37°C with shaking. The culture was split into two and IPTG was added to one of the cultures to a final concentration of 1 mM. Five hundred microlitres were pipetted from each culture, put in Eppendorf tubes and centrifuged at maximum speed for 30 s and frozen at -20°C. Samples (500 µl) were taken every hour for 5 h.

### **2.6.3 SDS-PAGE Analysis**

Sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) was set up with the following components: resolving gel (15% (v/v) acrylamide, 250 mM Tris-Cl pH 8.8, 0.1% (w/v) SDS, 0.4% (v/v) APS and 0.04% (v/v) TEMED) and stacking gel (3.3% (w/v) acrylamide, 85 mM Tris-Cl pH 6.8, 0.04% (w/v) SDS, 0.25% (v/v) APS and 0.07% (v/v) TEMED). Each sample from the induction was resuspended in 80 µl of 1x SDS-PAGE sample buffer (0.001% (v/v) bromophenol blue, 40% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, 4% (w/v) SDS and 250 mM Tris). The samples were boiled for 5 min and placed on ice. Five microlitres of each sample were loaded onto the gel and electrophoresed. The acrylamide gel was stained with the staining solution containing 1% (w/v) Coomassie Blue, 45% (v/v) methanol and 10% (v/v) acetic acid at 37°C for 30 min. Destaining of the gel was carried out at room temperature overnight in a solution containing 30% (v/v) methanol and 10% (v/v) acetic acid.

### **2.6.4 Full Scale Induction and Protein Purification**

To scale up the induction of protein expression, 10 ml of LB containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol were inoculated with a BL21(DE3)pLysS transformation reaction. The cells were grown overnight at 37°C with shaking. The following day, 50 ml of LB containing 100 µg/ml ampicillin and 34 µg/ml chloramphenicol were inoculated with 1 ml of the overnight culture. The cells were grown at 37°C with shaking to an OD<sub>600</sub> of approximately 0.5. IPTG was added to a final concentration of 1 mM and the cells were incubated at 37°C with shaking for 4 h. Cells were harvested by centrifugation at 3000 x g for 10 min at 4°C.

The recombinant protein was purified using the Ni-NTA resin (Qiagen, CA). Cells were lysed by resuspending them in 5 ml lysis buffer (200 mM Tris pH 8.0) and freeze-

thawing with brief vortexing at 3 min intervals in a 15 ml centrifuge tube. After the lysis, 1 ml of Ni-NTA resin and 5 ml binding/washing buffer (200 mM Tris pH 8.0, 200 mM NaCl, 10 mM imidazole) were added followed by incubation with gentle shaking at 4°C for 15 min. The mixture was centrifuged at 3000 x g at 4°C for 5 min and the supernatant was aspirated. Ten millilitres of binding/washing buffer were added and the mixture incubated for 10 min at 4°C. The washing process was repeated four times. The protein was eluted using 2 ml of elution buffer (200 mM Tris pH 8.0, 200 mM NaCl, 100 mM imidazole) and centrifuged at 3000 x g at 4°C for 5 min. The supernatant was transferred into an Eppendorf. The elution was repeated with 1 ml elution buffer. The purified protein was desalted using Amicon Ultra-15 columns (Millipore).

After desalting, the protein was quantified using the Bradford (1976) method. Protein standards were prepared by appropriately diluting the BSA protein standard (0, 1, 5, 10, 15, 20 µg/ml). Eight hundred microlitres of each of the standards was mixed with 200 µl of Bradford reagent (Biorad, South Africa). The OD<sub>595</sub> was measured after 30 min incubation at room temperature. A serial dilution of the purified recombinant protein was made and 800 µl of each of the dilutions was mixed with 200 µl of the Bradford reagent and concurrently the OD<sub>595</sub> was measured with the standards. The protein concentration was estimated by extrapolation from the standard curve. The protein was run on SDS-PAGE and stained/destained as described under 2.6.3.

### **2.6.5 Anti-His Western Blotting**

SDS-PAGE was carried out as described in 2.6.3. Proteins were transferred electrophoretically to nitrocellulose membranes (Osmonics, 0.45 µm pore size) using Towbin buffer (25 mM Tris, 192 mM Glycine, 20% methanol) and briefly stained with Ponceau S [0.1% (w/v) Ponceau S in 5% (v/v) acetic acid]. The blot was blocked by incubating in blocking buffer [3% w/v BSA in TBS buffer (10 mM Tris-Cl, 150 mM NaCl)] overnight at room temperature. The membrane was washed twice for 10 min with TBS-Tween-Triton buffer (20 mM Tris-Cl, 500 mM NaCl, 0.05% w/v Tween-20, 0.2% v/v Triton X100), incubated for 3 h with mouse Anti-His antibody in blocking buffer followed by washing with TBS-Tween-Triton buffer. The washed blot was incubated with secondary antibody in blocking buffer for 1 h. The membrane was

washed and stained with NBT/BCPIP (Nitro-Blue Tetrazolium Chloride/ 5-Bromo-4-Chloro-3'-Indolylphosphate p-Toluidine Salt) and rinsed in H<sub>2</sub>O.

### **2.6.6 Antibody Purification**

The purified recombinant protein was used to raise polyclonal antibodies in rabbits. Standard protocols were used according to Rybicki (1979). An enzyme linked immuno-sorbant assay (ELISA) was conducted using anti-serum from rabbits.

Recombinant XvINO1 protein was diluted in a range from 1:10 to 1:1000 in phosphate-buffered saline [PBS (1.8 mM KH<sub>2</sub>PO<sub>4</sub>, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>.7H<sub>2</sub>O, 137 mM NaCl, pH 7.4]. XvINO1 antiserum was diluted in a range from 1:10 to 1:2000 in blocking buffer [1X PBS; 3% (w/v) skim milk powder]. The various recombinant protein dilutions were inoculated into the wells of a Nunc-Immuno™ 96 MicroWell™ Plate, Maxisorp™ surface (Nunc, Denmark) and incubated at 4°C O/N. The bound protein was washed three times with 1X PBS and incubated in blocking buffer for 2 h at room temperature. These samples were washed again thrice with 1X PBS and incubated overnight at 4°C with the various XvINO1 antiserum dilutions. The recombinant protein-antiserum complex was washed thrice with 1X PBS and incubated with anti-rabbit IgG, alkaline phosphatase conjugate (1:10 000 dilution) (Sigma, Germany) for 2.5 h at 37°C. This was washed three times with 1X PBS. The detection was done utilizing ρ-Nitrophenyl phosphate tablets (Sigma Fast™, Germany), according to the manufacturers recommendations. The absorbance at 405 nm was determined using a Titertek Multiskan® Plus MK II (Flow laboratories, UK).

To purify the antibody, two volumes of borate-buffered saline were added to one volume of anti-serum. 14% (w/v) of crushed PEG 6000 was added to the diluted serum and gently mixed by inversion. This was centrifuged at 12 000xg for 10 minutes at 4°C. The pellet was dissolved in the original serum volume using phosphate buffer pH 7.4 (PBS). Polyethylene glycol (PEG) 6000 was added again at a 14% (w/v) concentration and dissolved. It was centrifuged as before and the pellet was dissolved in half the original serum volume using PBS containing 60% (v/v) glycerol. Aliquots were stored at -20°C.

## 2.7 Plant Protein Isolation and Western Blot Hybridization

Total protein was isolated using the TRIZOL LS reagent according to the manufacturer's instructions with the following modifications. Instead of guanidine hydrochloride in 95% ethanol, 0.1M ammonium acetate was used. The protein pellet was washed with 2 ml of cold acetone and thereafter air-dried. Proteins were resuspended in 50  $\mu$ l of Laemmli buffer (625 mM Tris-HCl pH 8.0, 2% SDS, 10% glycerol, 0.02 v/v  $\beta$ -mercaptoethanol) and incubated at 65°C overnight to facilitate resuspension. Proteins were stored at -20°C until needed. Proteins were quantitated using the Bradford reagent (BioRad, USA).

A 12% polyacrylamide separating gel and a 5% stacking gel was used. Protein samples were prepared by adding 5x SDS gel loading buffer to protein sample, boiled for 5 min and cooled on ice. Equal quantities of protein were loaded along with 6  $\mu$ l of pre-stained SDS-PAGE Standards Low Range (Bio-rad, USA) and electrophoresed at a constant current of 40 mA for 2 h. The proteins were transferred onto a nitrocellulose membrane (Osmonics, UK) using the Hoefer<sup>®</sup> Electrotransfer System (Amersham Biosciences, UK) following manufacturer's instructions. Transfer was conducted at 300 mA for 2 h at 4°C. The membrane was stained with Ponceau S (Sambrook *et al.*, 1989) for 10 min to visualize proteins.

Immunoassay was performed according to Sambrook *et al.* (1989). Blocking solution consisted of 3% (w/v) BSA in 1x TBS pH 7.5. Primary antibody was added at a dilution of 1:5000 and incubated overnight at 4°C with agitation. This was followed by 3x 10 min washes using blocking solution. Secondary antibody (anti-rabbit IgG peroxidase-linked whole antibody from goat; Sigma, UK) was added at a dilution of 1:5000 and incubated at room temperature for  $\pm$ 2 hours with agitation. Final washes were performed with TBS (pH 7.5). Antibody cross-reactivity was detected with the SuperSignal<sup>®</sup> West Pico/Femto Chemiluminescent Substrate (Pierce, USA) and exposed to X-ray film (Pierce, USA). Multiple films were used and developed manually after varying exposure times.

## 2.8 Enzyme Activity Assay

The assay was carried out using plant protein extracts as well as with the purified recombinant protein. Three replicates were carried out for each concentration.

### 2.8.1 Enzyme Extraction

The *X. viscosa* plant material was collected from NaCl stressed plants (2.5.2). Two grams of plant leaf material was homogenized in 5 volumes of extraction buffer (50 mM Tris-Cl pH 7.5, 2 mM EDTA). The homogenate was passed through four layers of cheesecloth and centrifuged at 28000 x g for 30 min at 4°C. The supernatant was desalted using the Amicon Ultra-15 columns. The total protein in the extract was quantified using the Bradford method (Bradford, 1976).

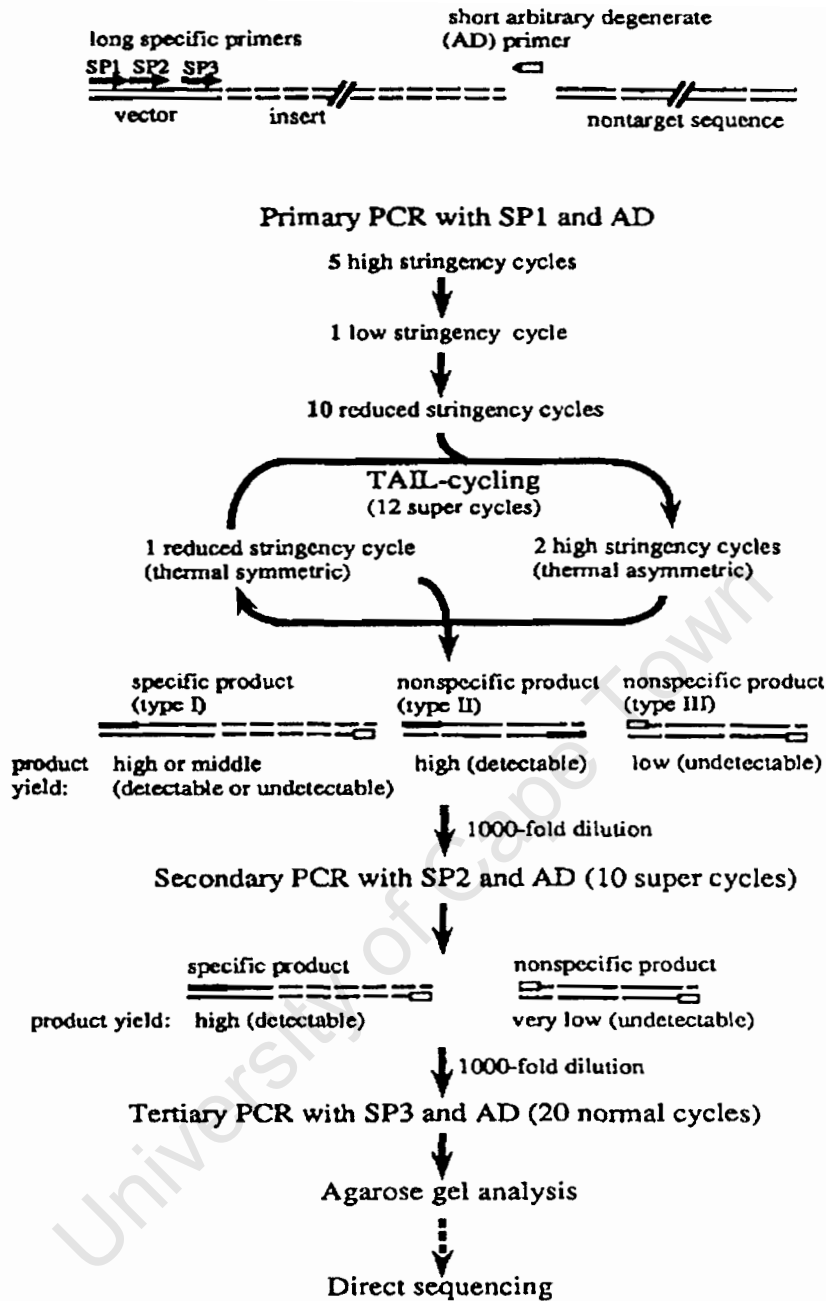
### 2.8.2 Assay of Myo-Inositol-1-Phosphate Synthase

The enzyme was assayed colorimetrically by the periodate oxidation method of Barnett *et. al.* (1970) and further corroborated by the assay described by Adhikari *et. al.* (1987) with some modifications. The assay mixture contained 50 mM Tris-Cl, pH 7.5, 14 mM NH<sub>4</sub>Cl, 0.8 mM NAD<sup>+</sup>, 5 mM β-ME, 5 mM glucose-6-phosphate and enzyme in total volume of 0.5 ml. Suitable blanks without enzyme 0 min control (whereby reaction was stopped at 0 min by addition of 20% TCA) and boiled enzyme were also run. The reactions were incubated at 37°C for 1 h and terminated by adding 0.2 ml of chilled 20% TCA. An equal volume (0.7 ml) of 0.2 mM NaIO<sub>4</sub> was added followed by incubation at 37°C for 1 h. Excess periodate was destroyed by the addition of 1.4 ml of 1 mM Na<sub>2</sub>SO<sub>3</sub>. A non-periodate control in which NaIO<sub>4</sub> and Na<sub>2</sub>SO<sub>3</sub> were replaced by equal volumes of water was also run. Three millilitres of Pi reagent [3.2 ml concentrated H<sub>2</sub>SO<sub>4</sub>, 0.5 g (NH<sub>4</sub>)<sub>2</sub>MoO<sub>4</sub>, 2 g ascorbic acid in 100 ml H<sub>2</sub>O) were added and the reactions were incubated at 37°C for 1 h. The liberated inorganic phosphate was estimated by the method of Chen *et. al.* (1956). Absorbance at 820 nm was measured. The assay was also carried out for the recombinant protein.

## 2.9 5' UTR Isolation

DNA was isolated from *X. viscosa* and *T. halophilla* as described above (2.4). Thermal asymmetric interlaced (TAIL)-PCR was carried out as describe by Liu and Whittier (1994) with modifications, using a set of degenerate primers:

TG(A/T)GNAG(A/T)ANCA(G/C)AGA (AD1), AG(A/T)GNAG(A/T)ANCA(A/T)AGG  
 (AD2), CA(A/T)CGICNGAIA(G/C)GAA (AD3), TC(G/C)TICGNACIT(A/T)GGA  
 (AD4) and gene-specific reverse primers GTTGATCCATTGTTTCCTCCC  
 (THINOR1), GCATCATTCCGAGCTTGGGGACGTGA (XVINOR1)  
 CAAGCATAACCCCTAATTTGGGGACACG (THINOR2),  
 CGGTCTTAAACTCGTAGCGAACCGAC (XVINOR2),  
 CGTATTTGACAGTCTTTGGCTTCAC (THINOR3) and  
 CGGTGGTCTGGTAATCGTAGACCGA (XVINOR3). All the gene-specific primers  
 were within 300 bp of the start codon. The first PCR reaction (TR1) was performed  
 with 2 µl of DNA, 3 µM each of degenerate primers and 0.5 µM of INOR1 and 0.8 mM  
 dNTP mix in 25 µl of 1x reaction buffer and 0.125 µl TaKaRa Ex Taq™ DNA  
 polymerase (TaKaRa, WI). The PCR product was diluted 1:50 and 1 µl of the diluted  
 product was used for the second PCR mix (as above) with INOR2 primer (TR2). The  
 third PCR reaction (TR3) was carried out using a diluted 1:10 TR2 product as template  
 and INOR3. Figure 2.3 shows an outline of the TAIL-PCR strategy. The resulting  
 products were analyzed for *cis*-acting elements using the Plant CARE database  
 (Rombauts *et al.*, 1999).



**Figure 2.3:** Schematic diagram of TAIL-PCR. Boldface segments denote the specific prime (SP), and small rectangles represent arbitrary degenerate (AD) primer (Liu and Whittier, 1994).

## 2.10 Arabidopsis *ITR* Double Knockout Mutants

### 2.10.1 Plant growth

Arabidopsis *ITR* knockout (KO) mutants were obtained from the Arabidopsis Knockout Facility at the University of Wisconsin-Madison. The seeds were surface sterilised and plated on 0.5x MS medium containing 3% (w/v) sucrose and solidified with 8 g/L agar.

Plates were incubated at 4°C overnight and then incubated at 24°C under light (16 h)/dark (8 h) cycles. One week old seedlings were transplanted onto pots containing sterilised soil. The *ITR1* and *ITR4* mutants were crossed with each other to obtain the *ITR1/ITR4* double KO. Phenotype analysis was carried out on *ITR1* and *ITR4* KO mutants as well as the double *ITR1/ITR4* double KO.

### **2.10.2 RNA Isolation and RT-PCR screening**

RNA was extracted from the leaves of two week old seedlings using the RNeasy kit (Qiagen, CA), according to manufacturer's instructions, for RT-PCR analysis. Each 2 µg of total RNA was incubated at 50°C for 1 h with 1.0 µg Oligo-dT primers (Invitrogen, CA) 0.8 mM dNTP, 10 mM DTT and 1 µl of SuperscriptIII™ RNase H<sup>-</sup> reverse transcriptase (Invitrogen, CA) in 20 µl of 1x first strand buffer. The first strand synthesis reaction was stopped by incubating the reaction mixture at 70°C for 15 minutes. PCR reactions were done with gene specific primers, TGAGAGATCAGACAAGAAGAAACAAT and ATACGTATTCCAAATCCGGTGTCATACAT for *ITR1*, ATTTTATGCGTATCGCTGAAATAGGAAGA and GGAACCAATCCCGGTTTACTACTATGT for *ITR3*, TCCGTTTCATTCGACAAACACACT and TGTGCTTTCTCATTGATTATGCTTCCT for *ITR4*.

The reactions were performed with 2 µl of cDNA templates, 0.5 µM each of forward and reverse primers and 0.8 mM dNTP mix in 25 µl of 1x reaction buffer and 0.125 µl TaKaRa Ex Taq™ DNA polymerase (TaKaRa, WI). Thirty cycles at 94° for 30 s, 60°C for 30 s, and 72°C for 1 min 30 s were carried out. The PCR products were resolved on a 1% agarose gel containing ethidium bromide stain and viewed under UV light.

### **2.10.3 DNA Isolation and PCR analysis**

About 100 mg of leaf tissue was ground in an Eppendorf tube with plastic pestle for about 10 seconds. Edwards extraction buffer (200 mM Tris-Cl, 250 mM NaCl, 25 mM EDTA and 0.5% SDS) was added (500 µl). The mixture was ground briefly and vortexed for 5 s then centrifuged at 14 000 rpm for 1 min. The supernatant (300 µl) was

transferred to a new tube and an equal volume of isopropanol was added. The mixture was centrifuged for 5 min at 14 000 rpm. The pellet was air-dried for 15 min and re-suspended in 20  $\mu$ l H<sub>2</sub>O. PCR was carried out as described above (2.9.2) using 2  $\mu$ l of DNA. The JL 270 primer, TTTCTCCATATTGACCATCATATCATTG, was used as a control.

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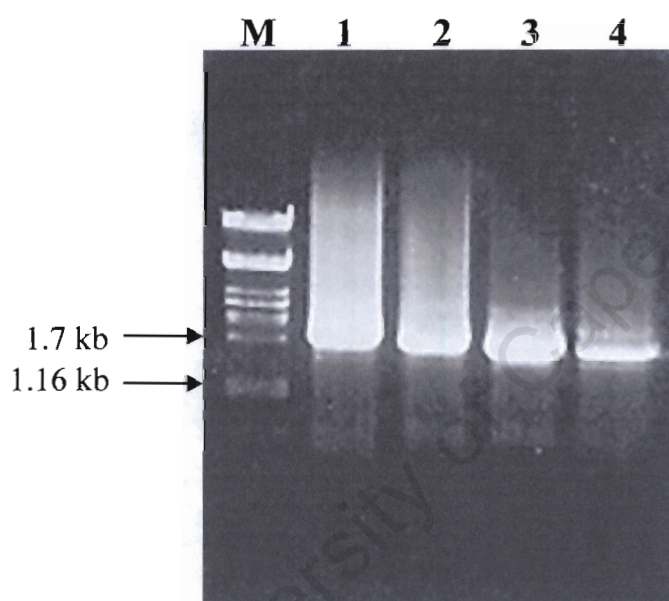
## **CHAPTER III**

### **RESULTS**

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### 3.1 cDNA Isolation and Sequence Analysis

The full-length *XvINO1* cDNA was isolated from salinity stressed *X. viscosa* plants. Amplification of the 3' and 5' ends of *XvINO1* using the modified RACE technique resulted in products of approximately 900 bp each after nesting the reactions. The 3' and 5' PCR products were combined using PCR whereby the products were mixed in a 1:1 ratio, denatured, aliquoted and used as template for the reaction (Figure 3.1). The reaction in which 1  $\mu$ l of the mixture was used as template resulted in the cleanest product which was subsequently purified and sequenced. Analysis of the sequence of the cloned cDNA using the BLAST programme revealed it to be *XvINO1* (Figure 3.2).



**Figure 3.1:** Joining of the 5' and 3' ends of *XvINO1* by PCR. A 1:1 mixture of each product was denatured and incubated at 68°C with Expand DNA polymerase and dNTP mix. Aliquots were used as templates for PCR. Lanes M; PstI- $\lambda$  DNA molecular weight marker; 1, 10 $\mu$ l template; 2, 5  $\mu$ l template; 3, 1  $\mu$ l template; 4, 1  $\mu$ l each of 5' and 3' ends used as template.

The *XvINO1* cDNA sequence consisted of a 1530 bp ORF (Figure 3.2a) encoding 510 amino acids (Figure 3.2b). The 3' UTR of *XvINO1* consisted of one putative polyadenylation signal sequence. The predicted molecular weight of the *XvINO1* is 56.699 kD with a pI of 5.08. The amino acid sequence consists of four sequence motifs that are highly conserved in all eukaryotes.

(a)

```
1      GACTCCCCCT TTCCCCATAT TCTCAAAACC CCCCATTN TTTTCAAAT TTGCTGTCCT
61     TTCCTGCAAA AA
           ATGTTCAT CGAAAGCTTC AAGGTCGAGA GTCCCAACGT CAAGTAT TTC
121    TCGGACCGCA TCGAGTCGGT CTACGATTAC CAGACCACCG AGTTGGTTCA TGAGAACCGC
181    GACGGTTCGT ACCAATGGGT TGTCCGACCC AAGTCGGTTC GCTACGAGTT TAAGACCGAT
241    ACTCACGTCC CCAAGCTCGG AATGATGCTT GTAGGCTGGG GAGGGAACAA TGGCTCGACT
301    CTAACCACTG GAATTATAGC TAATCGAGAG GGGATATCGT GGGTTACGAA GGATAAGGTT
361    CAACAAGCGA ACTACTTCGG TTCACTTACC CAAGCTTCGA CTATTCGGGT TGGTTCGTTT
421    AATGGAGAAG AGATCTACGC TCCATTCAAG AGCTTGCTTC CCATGGTTAA CCCAGATGAT
481    ATGTGTGTTG GAGGATGGGA CATAAGCAAC ATGAACCTTG CAGATTCCAT GGCAAGGGCT
541    AAGGTGCTTG ATATCGACCT CCAGAAACAG CTCAGGCCAT ACATGGAATC AATGGTCCCC
601    TTGCTGGAA TCTACGATCC TGATTTTCATC GCCGCCAATC AGGAATCGCG TGCCAACAAT
661    GTCATCAAAG GCACCAAGAA GGAGCAGTCA GAGCAGATCA TCAAGGACAT TAGGGAATTC
721    AAGGAGAAGA CAAAGGTGGA TAAGGTGGTG GTCCTGTGGA CCGCGAATAC CGAGAGGTAC
781    AGCGATGTGA TCATCGGTCT CAATGATACG ATGGAGAACC TTTTGGGCTC GCTCGAAAAG
841    AACGAGCATG AGATTTCTCC GTCCACTTTA TTTGCGATCG CATGCATCAT GGAGAACGTT
901    CCTTCATCA ATGGCAGCCC TCAAAACACC TTCGTTCCAG GGCTCATCGA TCTTGCTATT
961    AAGAGGAACA CACTCATTGG TGGCGACGAC TTCAAGAGCG GACAGACTAA GATGAAATCT
1021   GTGCTCGTCG ATTTCTTGTG TGGAGCTGGA ATAAAGCCGA CATCCATTGT TAGTACAAT
1081   CACCTCGGCA ACAATGATGG AATGAACCTG TCTGCGCCG AAACCTTTCG TTCCAAGGAG
1141   ATTTCCAAGA GCAATGTGGT CGATGACATG GTCTCCAGCA ATGGCATCCT CTACGAACTT
1201   GGCAGCATC CTGATCATGT AATTGTGATC AAGTATGTGC CATACTCGG GGACAACAAA
1261   AGGGCCATGG ATGAGTACAC ATCGGAGATT TTCATGGGCG GCAAGAGCAC GATTGTATTG
1321   CATAACACTT GCGAGGACTC ACTTCTGGCT GCGCCGATAA TTCTTGACTT GGTTCTTCTC
1381   GCTGAACTTA GCACCCGGAT TCAGCTTAA GCAGAGGGAG AGGATAATTT CCAGTCCTTC
1441   CATCTGTGG CTACAATTCT AAGCTACCTC ACCAAGGCTC CTCTGGTACC TCCGGGCACG
1501   CCGGTGGTGA ATGCCCTGTC GAAGCAGCGC GCAATGCTGG AGAACATCCT CAGAGCTTGC
1561   ATCGGCTTGG CGCCTGAGAA TAACATGATC TTGGAATATA AATGA
                                           AGTAC GATCGATGTA
1621   AGCTATTCTA TGCATGGTTT AAAAAAATGT AAACCTTTTC ATTTAGATCC TAAGTTTGT
1681   TGATGAAATT CT (A) 18
```

(b)

```
1      MFIESFKVES PNVKYFSDRI ESVYDYQTE LVHENRDGSY QWVVRPKSVR YEFKTDTHVP
61     KLGMLLVGWG GNNGSTLTTG IIANREGISW VTKDKVQQAN YFGSLTQAST IRVGSFNNGE
121    IYAPFKSLLP MVNPDDIVFG GWDISNMNLA DSMARAKVLD IDLQKQLRPY MESMVPLPGI
181    YDPDFIAANQ ESRANNVIKG TKKEQVEQII KDIREFKKEK KVDKVVVLWT ANTERYSDVI
241    IGLNDTMENL LGSLEKNEHE ISPSTLFAIA CIMENVFFIN GSPQNTFVPG LIDLAIKRNT
301    LIGGDDFKSG QTKMKSVLVD FLVGAGIKPT SIVSYNHLGN NDGMNLSAPQ TFRSKEISKS
361    NVVDDMVSSN GILYEPGEHP DHVIVIKYVP YVGDNKRAMD EYTSEIFMGG KSTIVLHNTC
421    EDSLAAPII LDLVLLAELS TRIQLKAEGE DNFQSFHPVA TILSYLTKAP LVPPGTPVVN
481    ALSKQRAMLE NILRACIGLA PENNMILEYK
```

**Figure 3.2:** Nucleotide and deduced amino acid sequence of full-length *XvINO1* cDNA. (a) Nucleotide sequence of *XvINO1*. In red are the start (#73) and stop (#103) codons. Underlined is a putative polyadenylation signal. (b) Deduced amino acid sequence of *XvINO1*. In red are the conserved motifs in all plant INO1 sequences.

Comparison of *INO1* sequences is shown in Table 3.1. *XvINO1* shows high sequence identity with other plant *INO1* genes. However, there is lower identity with non-plant species. cDNA sequences compared to *XvINO1* were from *M. crystallinum* (*McINO1*), *Oryza sativa* (*OsINO1*), *Zea mays* (*ZmINO1*), *A. thaliana* (*AtINO1*), *T. halophila* (*ThINO1*), *Homo sapiens* (*HsINO1*) and *Mycobacterium tuberculosis* (*MtINO1*).

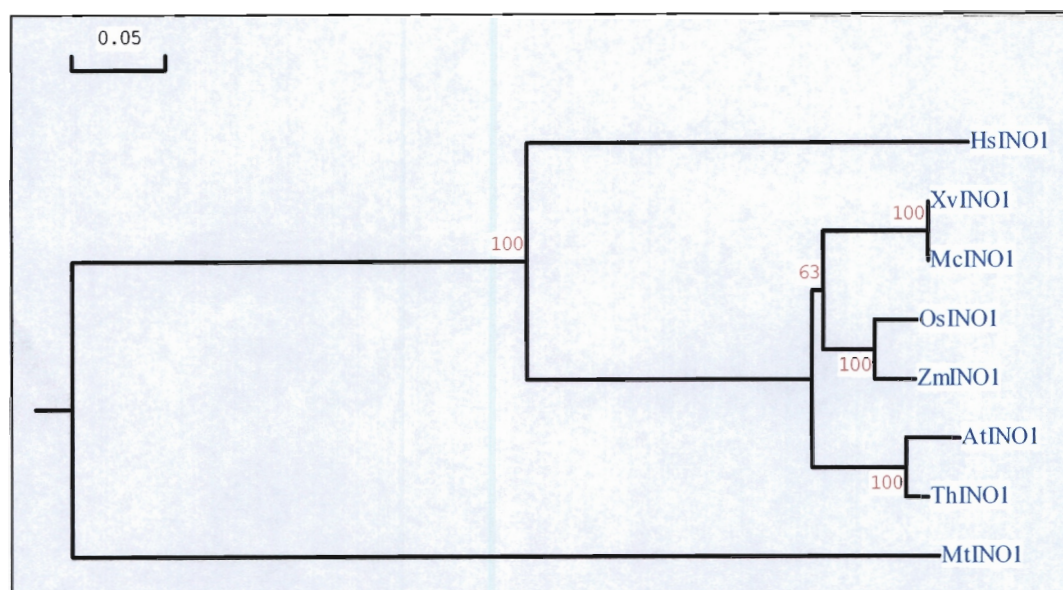
**Table 3.1:** Sequence homology between *INO1* cDNAs from different species.

<b>cDNA</b>	<b>% Identity with <i>XvINO1</i></b>
<i>McINO1</i>	<b>78.60</b>
<i>OsINO1</i>	<b>77.84</b>
<i>ZmINO1</i>	<b>77.14</b>
<i>AtINO1</i>	<b>76.39</b>
<i>ThINO1</i>	<b>75.85</b>
<i>HsINO1</i>	<b>57.40</b>
<i>MtINO1</i>	<b>22.14</b>

The *XvINO1* protein sequence was compared to other *INO1* protein sequences by multiple alignment using the DNAMAN programme (Figure 3.3). The alignment showed that there is high identity between the various *INO1* proteins. At most, the difference in identity is four amino acids in a row. A phylogenetic tree was generated for the plant *INO1* proteins together with the sequences from human *INO1* (*HsINO1*) and *M. tuberculosis* (*MtINO1*) (Figure 3.4). The tree indicates the relationship between the *INO1* protein sequences relative to one another. *MtINO1* occupies a different branch from the other *INO1* proteins, marking the difference between the prokaryotic and eukaryotic sequences clear. The eukaryotic sequences are grouped into animals and plants. *HsINO1* occupies the animals branch. Furthermore, the plant *INO1* proteins can be subdivided into monocots (*XvINO1*, *McINO1*, *OsINO1* and *ZmINO1*) and dicots (*AtINO1* and *ThINO1*). Within the monocots group, *XvINO1* is closely related to *McINO1* whereas *OsINO1* is grouped with *ZmINO1*.

XvINO1	YPIFSIKVESPNMKYFSDRHSVMDYQTELVHEN.RDES YQAVVREPKSVRYDEKDDTHVPPLEVMVLVGGNGNSTLIT	79
AtINO1	YPIFSIKVESPNMKYTENEHSVMDYQTELVVHEKTVNITYQAVVREPKSVRYDEKDDIRVPPLEVMVLVGGNGNSTLIT	80
ThINO1	YPIFSIKVESPNMKYTENEHSVMDYQTELVHEN.VNQTFOAVVREPKSVRYDEKDDIRVPPLEVMVLVGGNGNSTLIT	79
McINO1	YPIFSIKVESPNMKYFSDRHSVMDYQTELVHEN.RDES YQAVVREPKSVRYDEKDDTHVPPLEVMVLVGGNGNSTLIT	79
OsINO1	YPIFSIKVESPNMKYGAAEHSVMDYQTELVHES.HDCASRAVVRPKSVRYDEKDDTHVPPLEVMVLVGGNGNSTLIT	79
ZmINO1	YPIFSIKVESPNMKYFSDRHSVMDYQTELVHEG.KDCASRAVVRPKSVRYDEKDDIRVPPLEVMVLVGGNGNSTLIT	79
XvINO1	GLIANREGISWATKDKVQCANYFGSLTQASTIRVGSFNGEEIYAPFKSLLPMVNPDDIVFGGWDISNMNLADSNARAKVI	159
AtINO1	GLIANREGISWATKDKVQCANYFGSLTQASSIRVGSFNGEEIYAPFKSLLPMVNPDDIVFGGWDISDMNLADANARAKVI	160
ThINO1	GLIANREGISWATKDKVQCANYFGSLTQASSIRVGSFNGEEIYAPFKSLLPMVNPDDIVFGGWDISDMNLADANARAKVI	159
McINO1	GLIANREGISWATKDKVQCANYFGSLTQASTIRVGSFNGEEIYAPFKSLLPMVNPDDIVFGGWDISNMNLADSNARAKVI	159
OsINO1	GLIANREGISWATKDKVQCANYFGSLTQASTIRVGSFNGEEIYAPFKSLLPMVNPDDIVFGGWDISNMNLADAMTRAKVI	159
ZmINO1	GLIANREGISWATKDKVQCANYFGSLTQASTIRVGSFNGEEIYAPFKSLLPMVNPDDIVFGGWDISNMNLADSNARAKVI	159
XvINO1	DIDLQCLRPMESMVPLPGIYDPDFIAANGSRANVVIKGTKEQVEQI IKDIREFEKERKVDKRVVAVLWANTPYSNV	239
AtINO1	DIDLQCLRPMENIVPLPGIYDPDFIAANGSRANVVIKGTKEQVDHI IKDIREFEKERKVDKRVVAVLWANTPYSNV	240
ThINO1	DIDLQCLRPMENIVPLPGIYDPDFIAANGSRANVVIKGTKEQVDRI IKDIREFEKERKVDKRVVAVLWANTPYSNV	239
McINO1	DIDLQCLRPMESMVPLPGIYDPDFIAANGSRANVVIKGTKEQVEQI IKDIREFEKERKVDKRVVAVLWANTPYSNV	239
OsINO1	DIDLQCLRPMESMVPLPGIYDPDFIAANGSRANVVIKGTKEQVEQI IKDIREFEKERKVDKRVVAVLWANTPYSNV	239
ZmINO1	DIDLQCLRPMESMVPLPGIYDPDFIAANGSRANVVIKGTKEQVEQI IKDIREFEKERKVDKRVVAVLWANTPYSNV	239
XvINO1	ITGLNDTMENLLGSLKNEHEIISPSTIYATAQVLEGIPIFINGSPQNTFVPGIIDLAIKRNITGSDIYSGQKRAVSVLV	319
AtINO1	VVGMNDTMENLMSVDRDEAIISPSTIYATAQVLEGIPIFINGSPQNTFVPGIIDLAIKRNITGSDIYSGQKRAVSVLV	320
ThINO1	IVGLNDTMENLMSVDRDEAIISPSTIYATAQVLEGIPIFINGSPQNTFVPGIIDLAIKRNITGSDIYSGQKRAVSVLV	319
McINO1	ITGLNDTMENLLGSLKNEHEIISPSTIYATAQVLEGIPIFINGSPQNTFVPGIIDLAIKRNITGSDIYSGQKRAVSVLV	319
OsINO1	CVGLNDTMENLLASVDKNEAIISPSTIYATAQVLEGIPIFINGSPQNTFVPGIIDLAIKRNITGSDIYSGQKRAVSVLV	319
ZmINO1	CAGLNDTMENLLASVDKNEAIISPSTIYATAQVLEGIPIFINGSPQNTFVPGIIDLAIKRNITGSDIYSGQKRAVSVLV	319
XvINO1	DEIVGAGLRFISVSYNLCNAGGMNSAQCFRSEKLSASWVDDIYSSNAILIYFEGEHPDHVWIKYVPYVGDKRAM	399
AtINO1	DEIVGAGLRFISVSYNLCNAGGMNSAQCFRSEKLSASWVDDIYSSNAILIYFEGEHPDHVWIKYVPYVADSKRAM	400
ThINO1	DEIVGAGLRFISVSYNLCNAGGMNSAQCFRSEKLSASWVDDIYSSNAILIYFEGEHPDHVWIKYVPYVADSKRAM	399
McINO1	DEIVGAGLRFISVSYNLCNAGGMNSAQCFRSEKLSASWVDDIYSSNAILIYFEGEHPDHVWIKYVPYVGDKRAM	399
OsINO1	DEIVGAGLRFISVSYNLCNAGGMNSAQCFRSEKLSASWVDDIYSSNAILIYFEGEHPDHVWIKYVPYVGDKRAM	399
ZmINO1	DEIVGAGLRFISVSYNLCNAGGMNSAQCFRSEKLSASWVDDIYSSNAILIYFEGEHPDHVWIKYVPYVGDKRAM	399
XvINO1	DEYTSIEFMGGKSTIVLHNTCEDSLAAPTILDLVLLAELSTRIGLAEGEEDKHSFHPVATILSYLTKAPLVPPGTPVW	479
AtINO1	DEYTSIEFMGGKSTIVLHNTCEDSLAAPTILDLVLLAELSTRIGLAEGEEDKHSFHPVATILSYLTKAPLVPPGTPVI	480
ThINO1	DEYTSIEFMGGKSTIVLHNTCEDSLAAPTILDLVLLAELSTRIGLAEGEEDKHSFHPVATILSYLTKAPLVPPGTPVW	479
McINO1	DEYTSIEFMGGKSTIVLHNTCEDSLAAPTILDLVLLAELSTRIGLAEGEEDKHSFHPVATILSYLTKAPLVPPGTPVW	479
OsINO1	DEYTSIEFMGGKSTIVLHNTCEDSLAAPTILDLVLLAELSTRIGLAEGEEDKHSFHPVATILSYLTKAPLVPPGTPVW	479
ZmINO1	DEYTSIEFMGGKSTIVLHNTCEDSLAAPTILDLVLLAELSTRIGLAEGEEDKHSFHPVATILSYLTKAPLVPPGTPVW	479
XvINO1	NALSKCRAMLENTIRACVGLAPENNMILEYK	510
AtINO1	NALSKCRAMLENTIRACVGLAPENNMILEYK	511
ThINO1	NALSKCRAMLENTIRACVGLAPENNMILEYK	510
McINO1	NALSKCRAMLENTIRACVGLAPENNMILEYK	510
OsINO1	NALSKCRAMLENTIRACVGLAPENNMILEYK	510
ZmINO1	NALSKCRAMLENTIRACVGLAPENNMILEYK	510

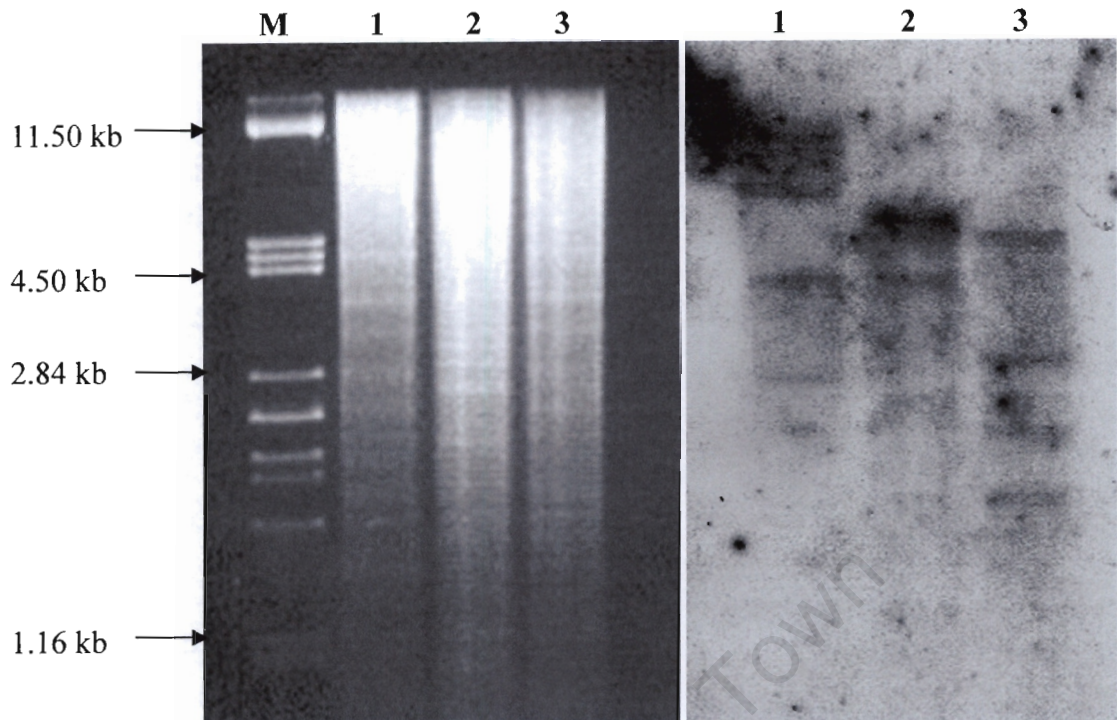
**Figure 3.3:** Multiple sequence alignment of different plant INO1 protein sequences. The sequences are from *X. viscosa* (XvINO1), *A. thaliana* (AtINO1), *T. halophila* (ThINO1), *M. crystallinum* (McINO1), *O. sativa* (OsINO1) and *Z. mays* (ZmINO1).



**Figure 3.4:** Phylogenetic tree of amino acid sequences from plants, human (HsINO1) and *M. tuberculosis* (MtINO1).

### 3.2 Southern Blot Analysis

Southern blot analysis of *X. viscosa* genomic DNA was carried out to determine the copy number of *XvINO1* as well as to confirm the presence of the gene in the *X. viscosa* genome (Figure 3.5). Genomic DNA (15  $\mu$ g) was digested using different restriction endonucleases selected from the restriction map of *XvINO1*. EcoR1 cuts once within the ORF of the cDNA whereas BglII and Sal1 do not cut within the ORF. DNA digested with EcoR1 gave a four bands, that digested with BglII gave two bands. The double-digested DNA (EcoR1/Sal1) resulted in four bands. These results suggest that there could be at least two copies of the gene.



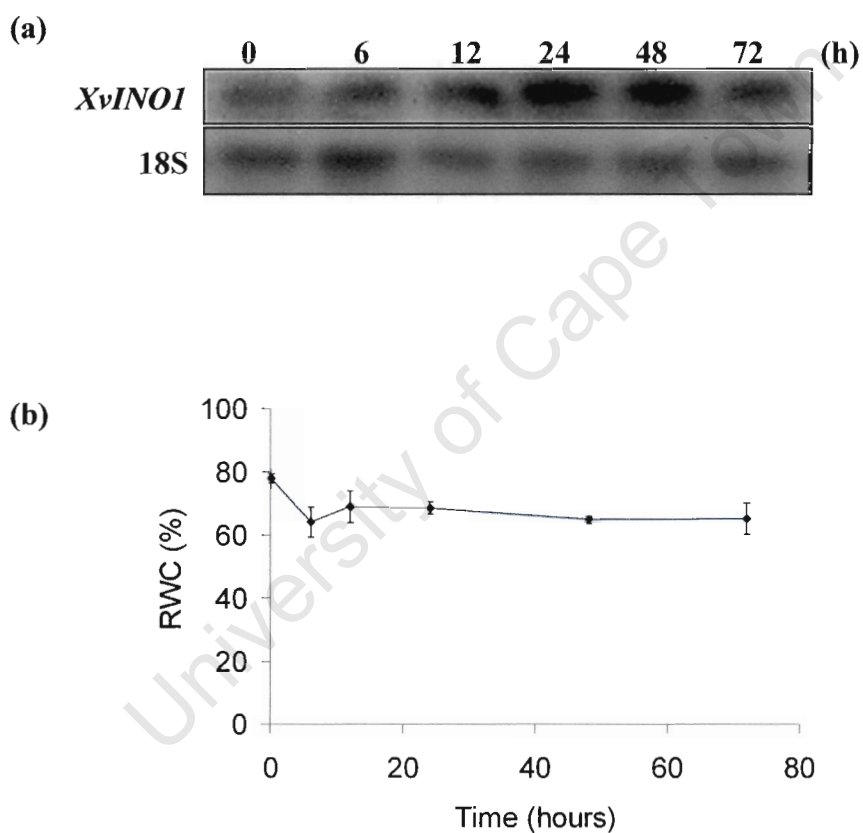
**Figure 3.5:** Southern blot hybridization of genomic DNA isolated from *X. viscosa*. Lanes M, Pst- $\lambda$  DNA molecular weight marker; 1, EcoRI; 2, BglII; 3, EcoRI/ SalI. The panel on the left represents the agarose gel electrophoresis of the restricted DNA whereas the right panel represents the autoradiograph.

### 3.3 Northern Blot Analyses

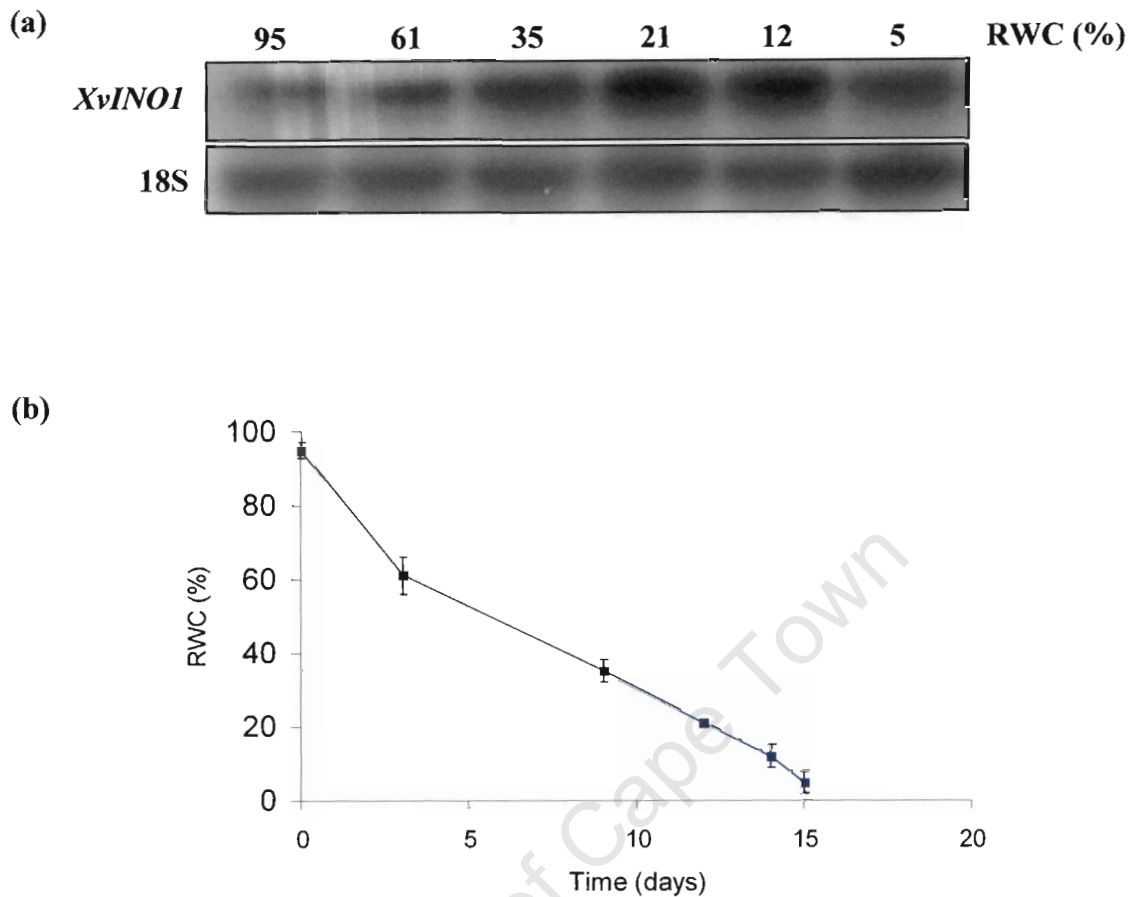
Northern blot analyses were carried out to determine the transcript levels of *XvINO1* when the plant was subjected to various abiotic stresses. For all the treatments, *XvINO1* transcripts could be detected before treatments, indicating that it is constitutively expressed, but the levels changed as the plants were stressed. For salinity stress (Figure 3.6), *XvINO1* transcript levels start to increase after 12 h of the treatment. However, transcript levels decrease at 72 h. When water was withheld from *X. viscosa*, the *XvINO1* transcripts started to increase at 61% RWC and there was a notable decrease at 5% RWC (Figure 3.7). For the rehydration of *X. viscosa* (Figure 3.8), *XvINO1* mRNA levels remained constant and decreased when the plant was fully hydrated (96% RWC). Exogenous ABA treatment resulted in an increase in *XvINO1* transcripts at 6 h of the treatment (Figure 3.9). Low temperature shock ( $-20^{\circ}\text{C}$ ) did not result in a notable

change in the mRNA levels (Figure 3.10), possibly due to lack of metabolic activity when the plant is frozen. The  $-20^{\circ}\text{C}$  low temperature stress was used since it is within the range that *X. viscosa* is subjected to in its natural environment.

The RWC was fairly constant (above 60%) for all stress treatments except for dehydration and rehydration. This ensured that the plants were fully hydrated at during the treatments. For the dehydration, the RWC decreased to below 40% after 9 days and upon rehydration, full turgor was reached 48 h after watering.

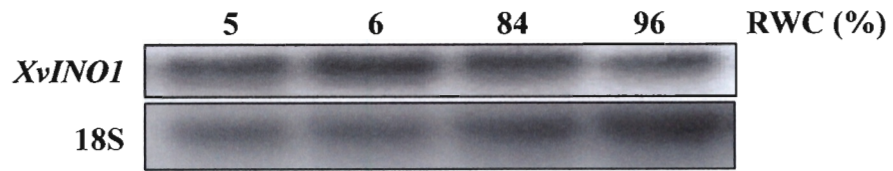


**Figure 3.6:** (a) Northern blot hybridisation of total RNA isolated from salinity stressed *X. viscosa* leaves. Plants were watered with 100 mM NaCl solution in a controlled environment chamber [16 h light ( $350 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ )  $25^{\circ}\text{C}$ ; 8 h dark]. 18S ribosomal RNA was used as a loading control. (b) RWC plot for the salinity treatment.

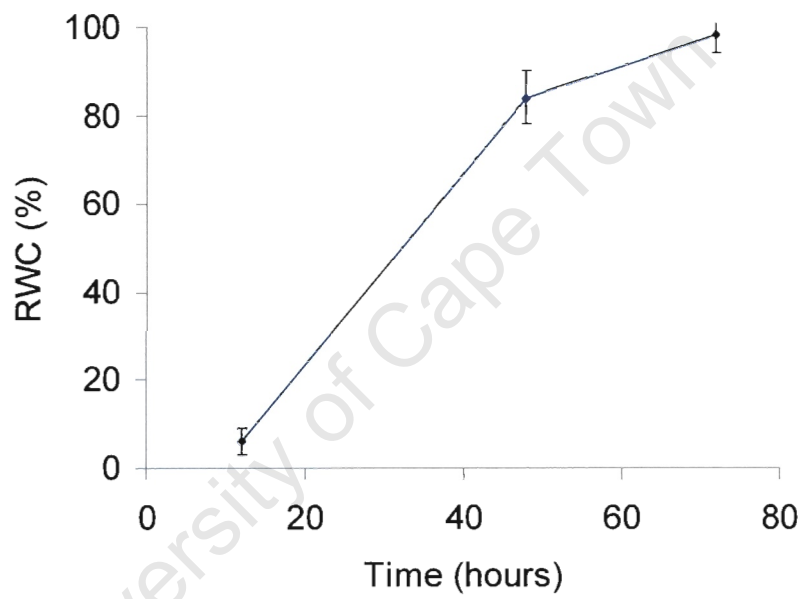


**Figure 3.7:** (a) Northern blot hybridisation of total RNA isolated from water deficit stressed *X.viscosa* leaves. Water was withheld from the plants for a period of 1 month in a controlled environment chamber [16 h light ( $350 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ )  $25^{\circ}\text{C}$ ; 8 h dark]. Leaf samples were taken at full turgor and after 5, 8, 9, 10, 11 and 28 days without water. (b) RWC plot for the treatment.

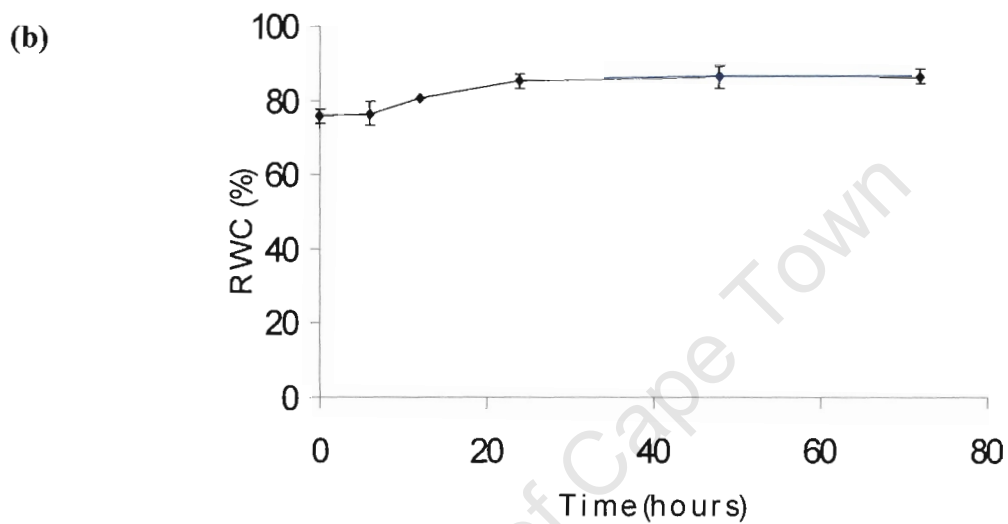
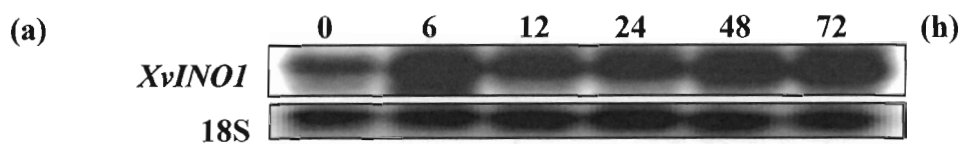
(a)



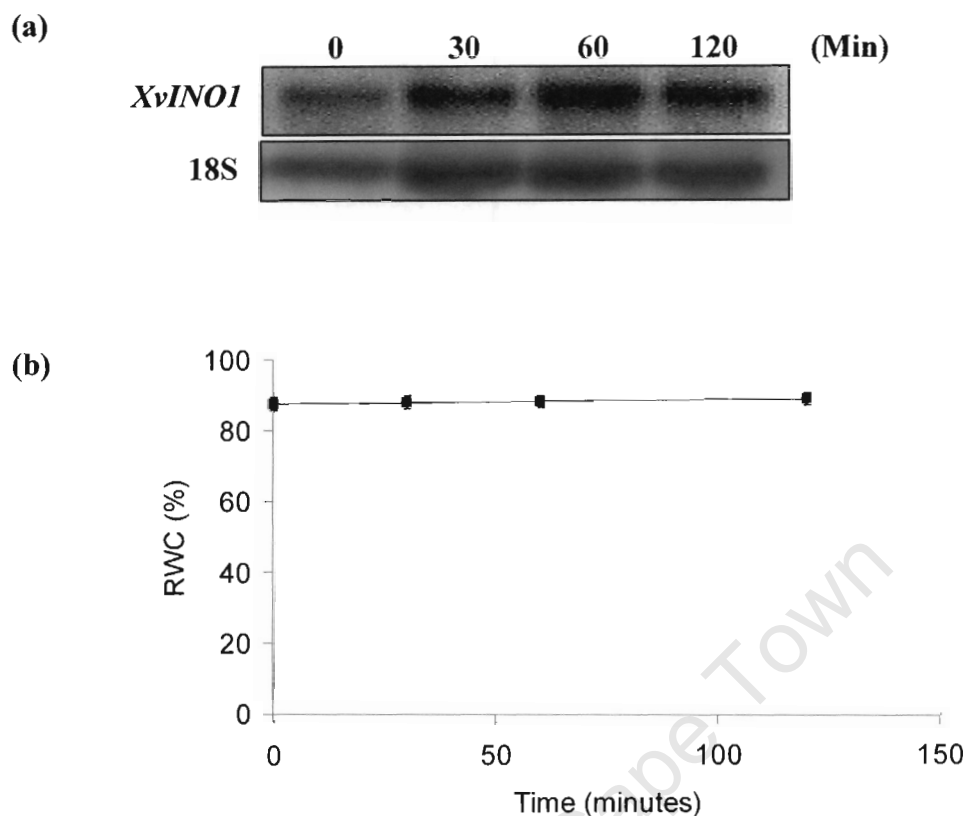
(b)



**Figure 3.8:** (a) Northern blot hybridisation of total RNA isolated from rehydrating *X. viscosa* leaves. The plants were rehydrated by watering them and taking samples at 12, 24 and 72 h after watering. (b) RWC plot.



**Figure 3.9:** (a) Northern blot hybridisation of total RNA isolated from exogenous ABA treated *X. viscosa* leaves. The leaves and soil of plants were sprayed with 150  $\mu\text{M}$  ABA, every 24 h, over a period of 72 h. The plants were equilibrated in a controlled environment chamber [16 h light ( $350 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ ) 25°C; 8 h dark]. (b) RWC plot for the treatment.

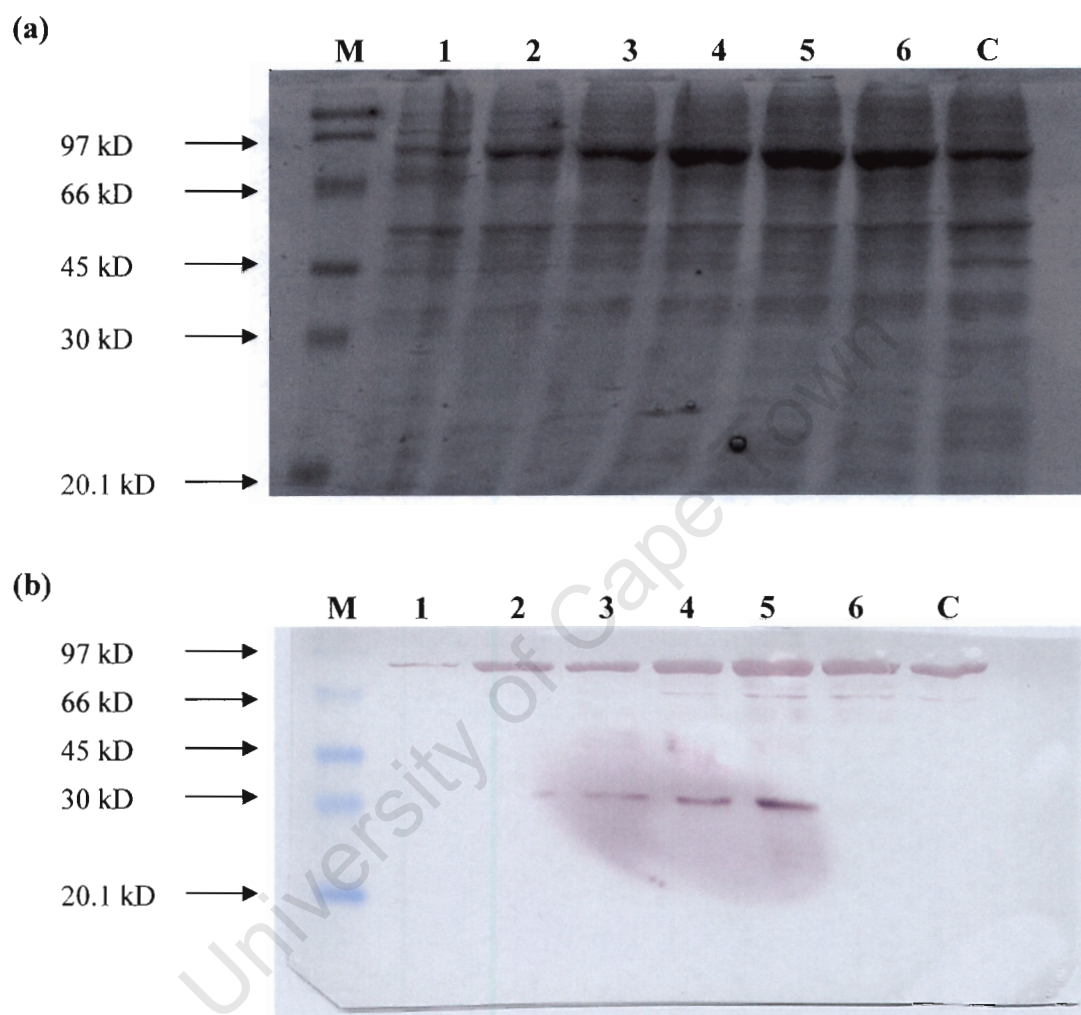


**Figure 3.10:** (a) Northern blot hybridisation of total RNA isolated from low temperature treated *X. viscosa* leaves. *X. viscosa* plants were acclimatised in a controlled environment [16 h light ( $350 \mu\text{mol.m}^{-2}.\text{s}^{-1}$ )  $25^{\circ}\text{C}$ ; 8 h dark] for 2 weeks before placing them at  $-20^{\circ}\text{C}$  for 2 h. (b) RWC plot.

### 3.4 Heterologous Protein Expression and Purification

The *XvINO1* cDNA was expressed in bacterial cells to obtain recombinant protein for use in antibody generating and determination of the enzyme activity. Recombinant protein synthesis was induced at 2 h. A recombinant protein of molecular weight  $\sim 80$  kD was induced in *E. coli* cells transformed with pCR T7/ NT::*XvINO1* (Figure 3.11a). There was an increase in the concentration of the induced protein over a 5 h period. It is noteworthy that there is recombinant protein present before the induction and also in the uninduced sample at 5 h. This could have been due to the strong T7 promoter on the vector.

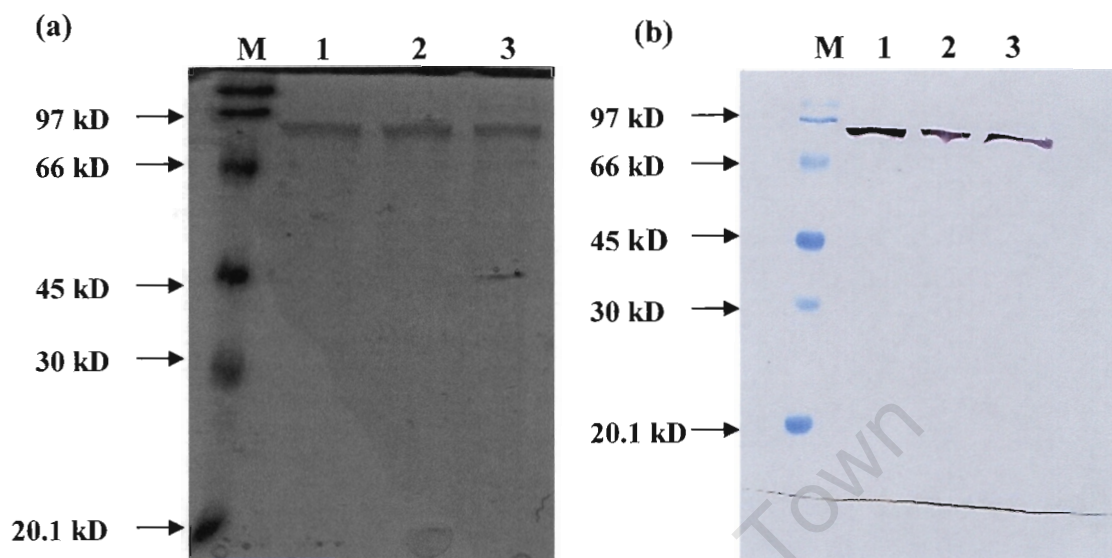
The recombinant protein was detected by the anti-His antibody in an immunoblotting experiment carried out to verify the presence of the His-tag in the expressed protein (Figure 3.11b). The additional bands were due to non-specific binding as a result of experimental error.



**Figure 3.11:** (a) SDS-PAGE of total protein isolated from induced *E. coli* One Shot<sup>®</sup> BL21(DE3)pLysS cells transformed with pCR T7/ NT::*XvINO1* and (b) immunoblot of the total protein isolates. Lanes M, protein molecular weight marker; 1, 0 h; 2, 1h; 3, 2 h; 4, 3 h; 5, 4 h; 6, 5 h; C, uninduced cells at 5 h.

The heterologously expressed recombinant XvINO1 protein was isolated and purified using Ni-NTA column chromatography. Clean protein samples were obtained whose concentrations were ~1 mg/ml (Figure 3.12a). Immunoblot hybridisations of the

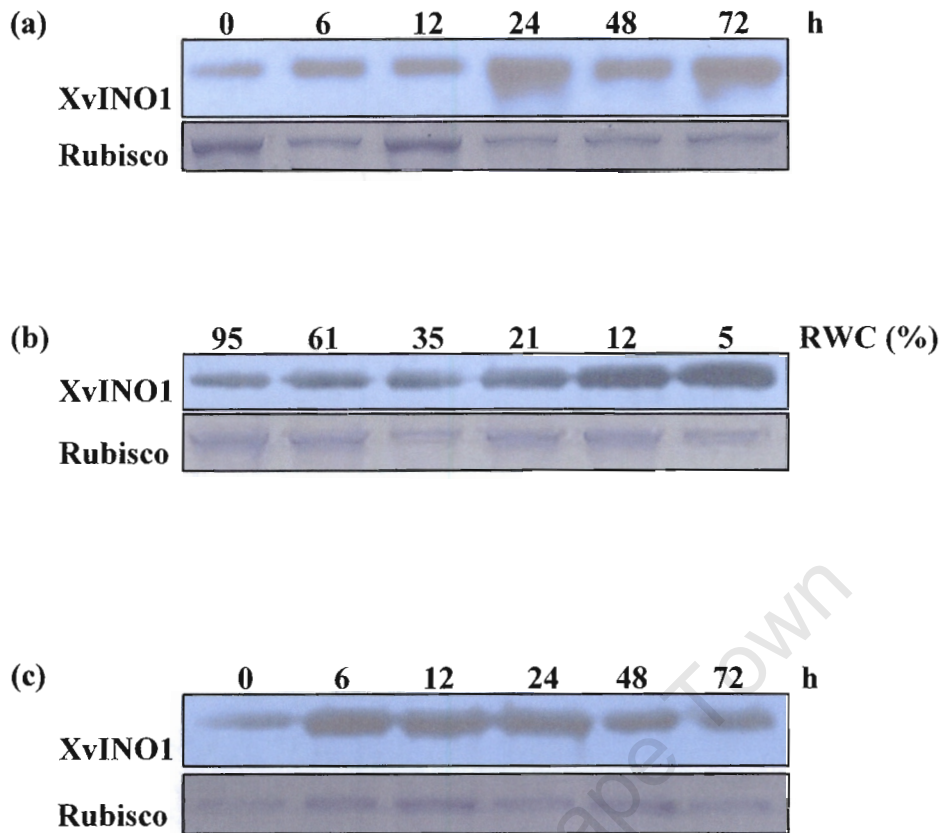
purified protein samples were carried out using anti-His antibodies to confirm presence of the His-tag on the recombinant protein (Figure 3.12b).



**Figure 3.12:** SDS-PAGE analysis of the purified recombinant XvINO1 protein (a) and (b) immunoblot of the purified protein samples. Lanes M, protein molecular weight marker; 1-3, purified recombinant protein.

### 3.5 Western Blot Analyses of Stress Treated Plants

The optimum antibody concentration was determined to be 1:5000 dilution using ELISA. This concentration was used for the immunohybridization of total protein isolated from stress treated plants (Figure 3.13). In all cases, the protein was found to be expressed in untreated (control) leaves before the imposition of stress, consistent with data obtained from northern blot analyses. For NaCl treatment, there is a reasonable accumulation of XvINO1 at 24 and 72 h after treatment (Figure 1.13a). During dehydration, there is a gradual increase with more protein being detected at 12 and 5% RWC (Figure 3.13b). When the plants were treated with exogenous ABA, there was immediate accumulation of XvINO1 with levels increasing as early as 6 h after the treatment (Figure 3.13c), suggesting a strong response to the phytohormone.



**Figure 3.13:** Western blot hybridizations of total protein isolated from leaves of (a) salinity stressed, (b) dehydrated and (c) exogenous ABA treated plants.

### 3.6 Enzyme Activity Assay

The glucose-6-phosphate-*myo*-inositol 1-phosphate cyclase activity was determined using a method modified from the colorimetric method by Barnett *et al.* (1970). The assay is based on the periodate oxidation of the *myo*-inositol 1-phosphate produced from glucose-6-phosphate. The oxidation results in liberation of inorganic phosphate which then colorimetrically determined after it is complexed to phosphomolybdate. The amount of the liberated phosphate is then used to estimate *myo*-inositol 1-phosphate produced in enzymatic reaction. The method is a less tedious way of determining the activity of INO1 activity. Other methods are based on the conversion of radiolabelled glucose 6-phosphate to *myo*-inositol 1-phosphate which is then assayed radiochemically (Barnett *et al.*, 1970; Barnett and Corina, 1967). Determination of the INO1 enzyme activity of the crude extract from the *X. viscosa* plants was not successful (data not shown). The control in which no periodate was used liberated about the same amount of

inorganic phosphate as the one in which the periodate was added. This was most probably due to non-specific phosphatase activity. There are several ways of reducing phosphatase activity. The most common one is to heat the mixture at 60°C for about 2 min and to eliminate Mg<sup>2+</sup> ions (Barnett *et al.*, 1970). In the crude protein extract, it is very difficult to eliminate all the Mg<sup>2+</sup>. Chelating might be an option but the chelating agent might affect the activity of the INO1. Assay with the recombinant protein was successful. The amount of the liberated inorganic phosphate was estimated using a Na<sub>2</sub>HPO<sub>4</sub> standard curve. The activity was determined at two different concentrations of the recombinant enzyme. Specific activities of 2.967 nmol/h/mg and 17.828 nmol/h/mg were obtained for enzyme concentrations of 0.059 mg/ml and 0.118 mg/ml, respectively (table 3.2). These activities do not say much about the kinetics of the enzyme. However, they demonstrate that the recombinant enzyme has ability to convert glucose-6-phosphate to *myo*-inositol 1-phosphate. In order to determine the kinetics of the enzyme, several parameters would have to be investigated. These include the enzyme concentration, substrate concentration, pH, and reaction temperature (Stryer, 1988). The actual kinetics of the recombinant enzyme could also not be determined due to the presence of the His-tag on the protein. The His-tag alters the way the protein folds and hence has an effect on the activity.

**Table 3.2:** Myo-inositol-1-phosphate synthase activities of the recombinant XvINO1 protein. The activity was measured for two different concentrations of the recombinant protein (0.059 and 0.118 mg/ml).

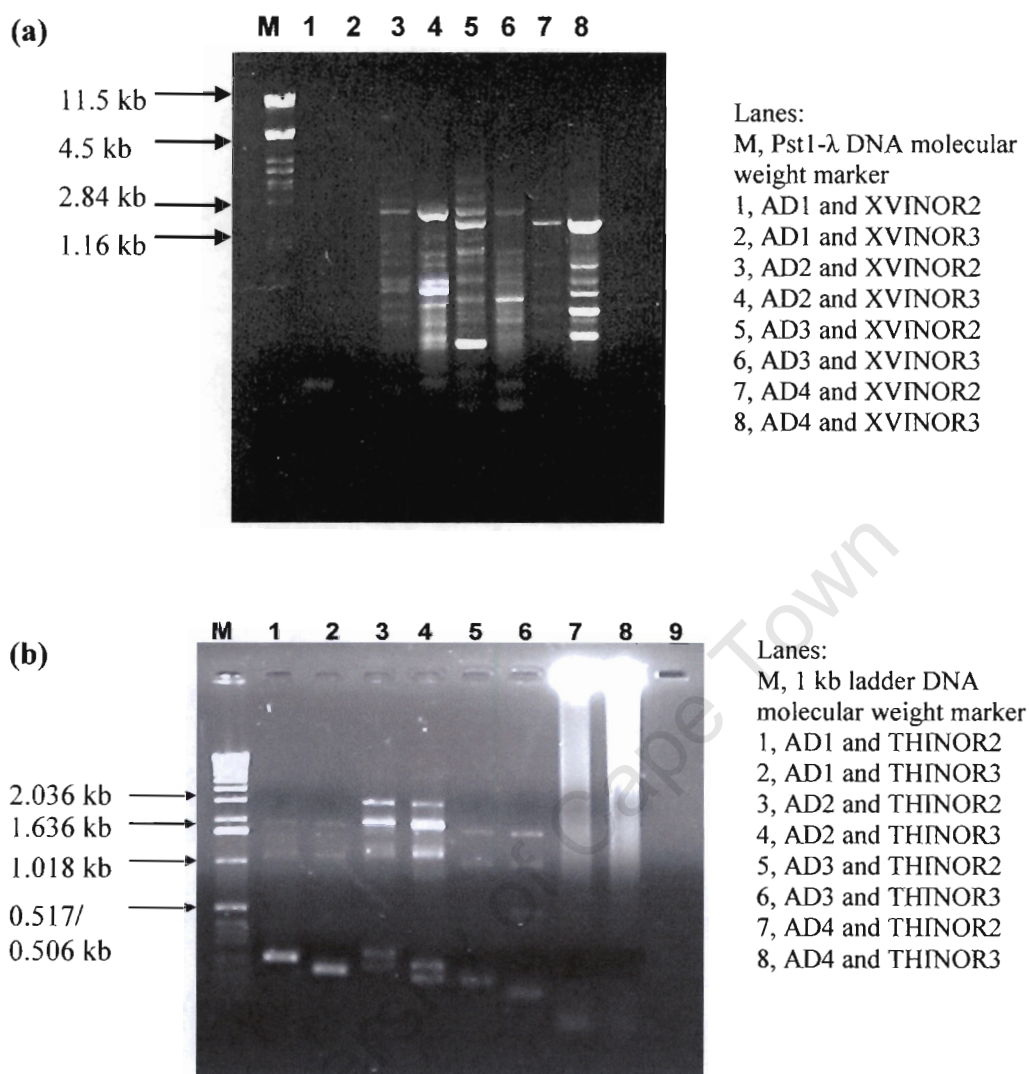
Protein Concentration (mg/ml)	Total Activity (units)	Specific Activity (units/mg)
0.059	2.967	53.949
0.118	17.828	152.915

### 3.7 Promoter Regions Isolation and Sequence Analysis

The *XvINO1* and *ThINO1* 5' UTR sequences were isolated using TAIL-PCR (Liu and Whittier, 1994). A fragment of ~1.2 kb in Figure 3.14a, lane 8 was purified and sequenced. The sequence was verified to be the *XvINO1* 5' UTR. The ~1.6 kb distinct band in Figure 3.14b, lane 4, was purified and sequenced. The fragment was the 5' UTR of *ThINO1* since it contained a 130 bp sequence of the *ThINO1* ORF. Sequences of equal length (1 kb) from *XvINO1* 5'UTR and *ThINO1* 5'UTR were selected for comparison (Figure 3.15). The sequences were compared to the three Arabidopsis *INO1*

5'UTR sequences of similar length. Sequence alignment using the DNAMAN program showed that the promoter regions from the three species differed significantly (Table 3.3). A *cis*-elements scan carried out using the Plant C.A.R.E online tool revealed a number of putative *cis*-acting elements. A few of the elements were selected, based on their relevance to abiotic stress, and are shown in Table 3.4. Of notable significance is the absence of the ethylene-responsive element (ERE) in both the *XvINO1* and *ThINO1* promoter regions whereas the ERE is present in all the *AtINO1* promoter sequences. Also, there are six TCA-elements in the *ThINO1* 5'UTR whereas there is none in the *XvINO1* 5'UTR and two of the Arabidopsis 5'UTR. Only *AtINO1-3* 5'UTR has two TCA-elements.

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**Figure 3.14:** Agarose gel analysis of TAIL-PCR products for amplification of *INO1* sequences from *X. viscosa* (a) and *T. halophila* (b). The product in lane 8 (~1.2 kb) in (a) was purified and sequenced. Similarly, the ~1.6 kb fragment in (b) was purified and sequenced.

**Table 3.3:** Percentage identity of *INO1* 5'UTRs as compared to *XvINO1*. *ThINO1* and the three *AtINO1* isoforms (*AtINO1-1*, *AtINO1-2* and *AtINO1-3*) are shown. Equal sequences (1 kb) of each the 5'UTR regions was used.

Promoter Region	% Identity
<i>XvINO1</i> 5'UTR	100
<i>AtINO1-1</i> 5'UTR	35.92
<i>AtINO1-2</i> 5'UTR	35.43
<i>AtINO1-3</i> 5'UTR	27.38
<i>ThINO1</i> 5'UTR	27.66

**Table 3.4:** Selected *cis*-elements from the *INO1* 5'UTR from the three species. The table shows the number of motif repeats upstream of the start codon for each nucleotide sequence (Rombauts, 1999)

Cis-element	<i>XvINO1</i>	<i>ThINO1</i>	<i>AtINO1-1</i>	<i>AtINO1-2</i>	<i>AtINO1-3</i>	Function
ABRE	4	21	18	7	7	cis-acting element involved in the abscisic acid responsiveness
AuxRR-core	1	2	2	-	-	cis-acting regulatory element involved in auxin responsiveness
ERE	-	-	5	4	3	ethylene-responsive element
HSE	3	6	5	5	6	cis-acting element involved in heat stress responsiveness
RY-element	1	-	1	-	1	cis-acting regulatory element involved in seed-specific regulation
TATA-box	12	22	26	15	7	core promoter element around -30 of transcription start
TCA-element	-	6	-	-	2	cis-acting element involved in salicylic acid responsiveness

### 3.8 Screening and Characterization of KO Mutants

To further explore the metabolism of *myo*-inositol in plants, *Arabidopsis* plants in which two *ITR* genes were inactivated by T-DNA insertion were analysed.

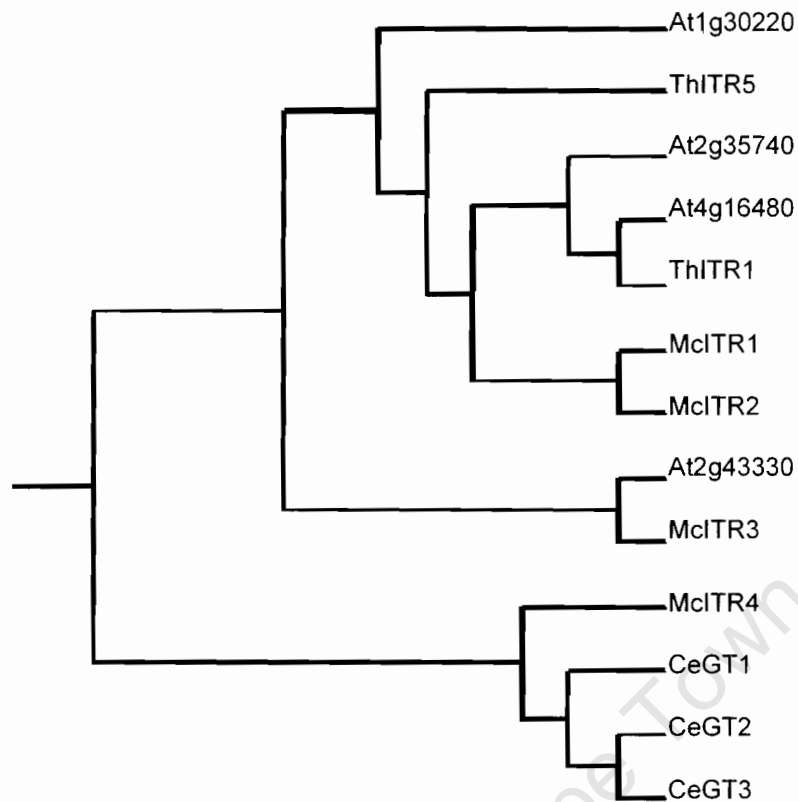
#### 3.8.1 T-DNA Insertions and Homology

The ITR1 and ITR4 KO mutants contained T-DNA insertions at the following sites:

*ITR4* (At1g30220), In exon 5 at AACCGGTGTTTTTTATGAA-insertion- GCGGCTAC  
*ITR1* (At4g16480), In exon 1 at TCCGCCGGAATCGGAGG-insertion-  
TCTTCTCTTCG.

Amino acid sequence alignment of the *A. thaliana* ITR sequences with ITR sequences from other plants as well as other sugar transporters showed that there is high homology between corresponding transporters from various plant species (Figure 3.15). The ITR sequences are separated from the glucose transporters with the exception of McITR4, which is likely to be a glucose transporter. McITR1 and McITR2 are closely related as well as At-ITR1 and its *T. halophilla* homologue (ThITR1).

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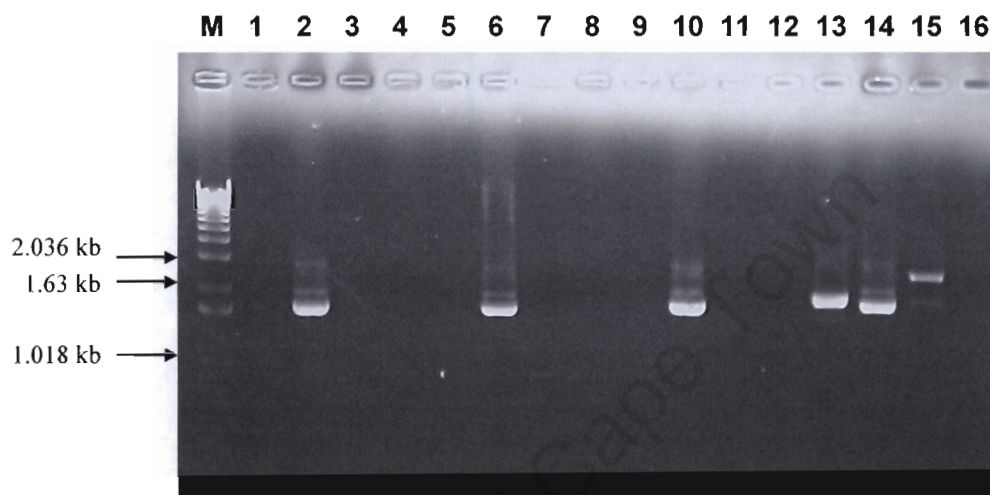


**Figure 3.15:** Alignment of sugar transporters. Sequences from *A. thaliana* four *ITR* homologues (*At-ITR1*: At4g16480; *At-ITR2*: At2g35740; *At-ITR3*: At2g43330; and *At-ITR4*: At1g30220), from *M. crystallinum* four *ITR* sequences (McITR1, McITR2, McITR3 and McITR4), from *T. halophila* two *ITR* sequences (ThITR1 and ThITR5) and from *Caenorhabditis elegans* three glucose transporter sequences (CeGT1, CeGT2 and CeGT3).

### 3.8.2 PCR Screening of KO Mutants

After crossing *ITR1* and *ITR4* KO lines, fifteen plants were randomly selected for PCR screening using gene specific forward and reverse primers. PCR using genomic DNA from the plants revealed that six of the plants were homozygous for *ITR1* KO, seven were wild type and one plant was heterozygous. The same fifteen plants were also selected for *ITR4* KO mutation screening using the primer JL270, which is specific for the T-DNA insertion and the gene specific reverse primer. In this case, only those plants which had the T-DNA insertion would give a PCR product. A total of eight plants contained the T-DNA insertion and three were homozygous for *ITR1/ITR4* double KO mutations (data not shown).

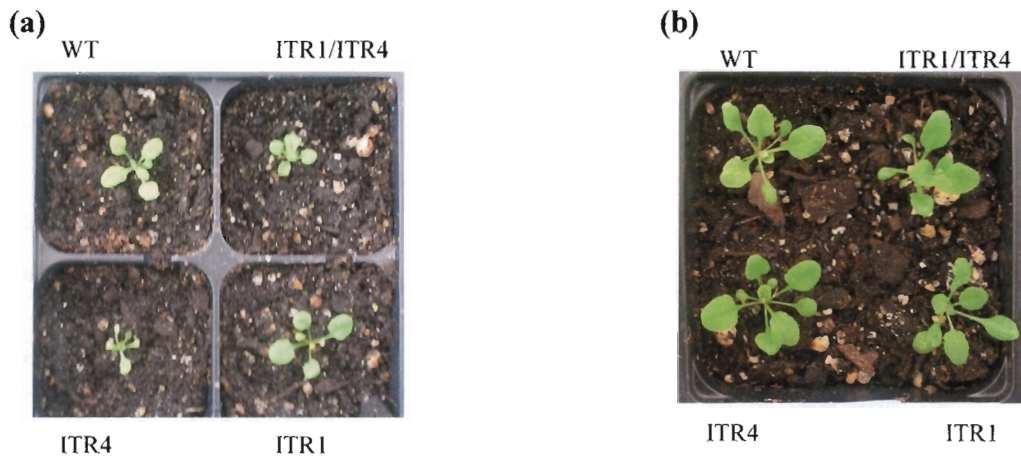
Putative *ITR1/ITR4* double KO plants were germinated on plates and transplanted into pots. Three resulting plants were screened for *ITR1* and *ITR4* expression by RT-PCR (Figure 3.16). All three plants expressed *ITR3* gene but not *ITR1* and *ITR4*. Wild type plants expressed all the three *ITR* genes.



**Figure 3.16:** RT-PCR screening of homozygous double KO mutants for *ITR1*, *ITR3* and *ITR4* expression. Lane M is the 1 kb ladder DNA molecular marker. Lanes 1-4, plant 1 (1, *ITR1*; 2, *ITR3*; 3, *ITR4*; 4, blank), lanes 5-8, plant 2 (5, *ITR1*; 6, *ITR3*; 7, *ITR4*; 8, blank), lanes 9-12, plant 3 (9, *ITR1*; 10, *ITR3*; 11, *ITR4*; 12, blank) and lanes 13-15, wild type (13, *ITR1*; 14, *ITR3*; 15, *ITR4*; 16, blank).

### 3.8.3 Growth of KO Mutants

Under normal growth, there is no significant phenotypic difference between the KOs and the wild type plants (Figure 3.17), suggesting that the plants can survive without the two deleted genes under normal conditions.



**Figure 3.17:** ITR KO mutants under normal growth conditions. (a) Ten days after germination and (b) 3 weeks after germination.

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**CHAPTER IV**  
**DISCUSSION**

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### ***XvINO1* is Highly Homologous to Other *INO1* Genes**

The *XvINO1* full length cDNA was isolated using the modified RACE technique. The 5' and 3' cDNA ends were amplified separately and then joined using a technique developed from PCR. The joining of the two fragments was successful and sequencing confirmed that they were joined at the appropriate position. The technique utilizes the fact that when two DNA fragments with overlapping sequences are denatured, they will be joined to an extent upon re-annealing. The annealed product is then used as a template for a PCR reaction (2.2.5).

The *XvINO1* cDNA obtained using the above technique was 1692 bp with an ORF of 1530 bp, encoding 510 amino acids with a predicted MW of 56.699 kD (Figure 3.2). This polypeptide length is consistent with most known plant *INO1* proteins (Figure 3.4). The *INO1* is conserved at both the nucleotide and amino acid levels, with at least 75% sequence identity with higher plants cDNA sequences. However, there is lower identity between *XvINO1* and non-plant species such as *M. tuberculosis* and *H. sapiens*. Higher identity was observed at the amino acid level where the multiple sequence alignment showed that there were no more than four consecutive amino acids that were different (Figure 3.3). In addition, the predicted amino acid sequence contains four sequence motifs that are highly conserved in *INO1* proteins. These motifs are GWGGNNG, LWTANTERY, NGSPQNTFVPGL and SYNHLGNNDG. One of these conserved sequences (GWGGNNG) is characteristic of the Rossmann fold GXGGXXG motif which is typical of an oxidoreductase (Kleiger and Eisenberg, 2002). This sequence is involved in NAD<sup>+</sup> binding. The sequence SYNHLGNNDG was found to be located in the catalytic region of the *INO1* enzyme (Stein and Geiger, 2002). The other two conserved sequences, LWTANTERY and NGSPQNTFVPGL are within the NAD<sup>+</sup>-binding region (Kleiger and Eisenberg, 2002).

Phylogenetic analysis of the *INO1* amino acid sequences from several plants as well as that from *M. tuberculosis* and *H. sapiens* indicated that these proteins are separated into two main branches representing prokaryotes and eukaryotes. The Mt*INO1* occupies the prokaryotes branch alone. The eukaryotic cluster is further divided into plants and animals. The divergence between the plants is low although there are two groups of plants representing monocots (*XvINO1*, Mc*INO1*, Os*INO1* and Zm*INO1*) and dicots

(AtINO1 and ThINO1). In the monocots group, the XvINO1 is closest to McINO1, reflecting the closeness of the two halophytic monocots. The phylogenetic pattern observed is consistent with the data obtained when the amino acid sequences from INO1 sequences from evolutionarily diverse organisms were analyzed (Majumder *et al.*, 2003), revealing a high degree of evolutionary conservation of INO1.

Southern blot hybridization of restriction endonuclease-digested *X. viscosa* genomic DNA confirmed the presence of *XvINO1* in the *X. viscosa* genome. EcoR1 cuts once within the ORF whereas BglII and Sall are non-cutters. About four bands could be seen in the lane with EcoR1-digested DNA as well as in that with DNA double-digested with EcoR1 and Sall. Two bands were present in the lane with DNA digested with BglII. The results suggest that there could be at least two copies of *XvINO1*. This could mean that the gene belongs to a multigene family. There are three copies of *INO1* in *A. thaliana* and only one in *M. crystallinum* (Ishitani *et al.*, 1996). Soybean contains at least four *INO1* genes whereas *S. indicum* has at least two (Hegeman *et al.*, 2001). The accuracy of the Southern blot technique however depends on a number of factors. Firstly, the extent of digestion of the genomic DNA is important. If the DNA is not properly digested, fewer fragments result. Also, the stringency of washes can determine the outcome of a Southern blotting experiment. It would be more informative to analyze the blot at different stringency washes. Different banding patterns were obtained when the stringency washes were altered (Ishitani *et al.*, 1996). In order to estimate the gene copy number more accurately, information on the genomic sequence is relevant as the gene might contain intronic regions with additional restriction sites. The experiment does, however, confirm the presence of the gene in the genome.

### **Expression of *XvINO1* Under Abiotic Stress**

Changes in *XvINO1* transcript levels in *X. viscosa* leaves were monitored under various abiotic stress conditions. Transcripts and the protein itself could be detected before the stress treatments, suggesting that *XvINO1* is constitutively expressed. This can be explained by the fact that *myo*-inositol is a central metabolite in plant biochemistry, hence the constitutive expression of its gene. Its roles include participating in phytic acid biosynthesis, phosphatidyl inositol (PI) signaling pathway, cell wall biosynthesis, auxin storage and transport and the biosynthesis of various carbohydrate osmoprotectants

(Loewus and Murthy, 2000). These functions make this cyclitol an essential component of normal plant growth and development.

Under salinity stress in whole plants, the *XvINO1* mRNA levels increased markedly after 12 h of the treatment, but decreased at 72 h (Figure 3.6). A similar trend was observed when the plants were dehydrated. The transcripts increased at RWC of 35% and there was a notable decrease at 5% RWC. Upon rehydration, the blots indicated a slight decrease in mRNA levels at full hydration (96% RWC). Immunoblot experiments revealed that *XvINO1* protein levels increase under both salinity and dehydration treatments. The expression of *INO1* under salinity stress has been extensively studied (Ishitani *et al.*, 1996; Nelson *et al.*, 1999; Wang *et al.*, 2003). In the ice plant, *M. crystallinum*, the gene is highly up-regulated at transcript and protein levels in leaves (Ishitani *et al.*, 1996; Nelson *et al.*, 1998). In *Z. mays*, *INO1* was also up-regulated under salinity (Wang *et al.*, 2003). It is hypothesized that under salinity, *myo*-inositol acts as a signal for the uptake of  $\text{Na}^+$  and other ions (Nelson *et al.*, 1999). In addition to it acting as a signal, *myo*-inositol itself could act as a compatible solute during stress. *Myo*-inositol and its sugar derivatives play a role in desiccation tolerance possibly by acting as a replacement for water. The 'Water Replacement Hypothesis' suggests that the hydroxyl groups of the sugars replace water molecules thereby providing the hydrophilic interactions for protein and membrane stability (Clegg, 1986). The decrease in *XvINO1* mRNA at 72 h of salt treatment and 5% RWC during dehydration can be due to the fact that the plant would have accumulated enough protein for the synthesis of the various metabolites downstream of *myo*-inositol required for the adaptation to stress.

For the ABA treatment, the *XvINO1* transcript levels were observed to increase 6 h after the start of the treatment (Figure 3.9). The levels remained elevated for the duration of the treatment suggesting that the expression of *XvINO1* could be via the ABA-dependent pathway or by both the ABA-dependent and ABA-independent pathways. Western blots confirmed the increase in protein during exogenous ABA treatment. The low-temperature shock did not result in any significant changes in the *XvINO1* mRNA levels. This could be because there was no metabolic activity in the plant at  $-20^{\circ}\text{C}$ . The data from the northern blot analyses suggest that *XvINO1* might be regulated at the transcriptional level. However, posttranslational modification of a chloroplastic form of

INO1 from *O. sativa* has been reported (Hait *et al.*, 2001). Under salinity stress, the protein is phosphorylated in a  $\text{Ca}^{2+}$ -dependant manner resulting in increased enzyme activity.

### **Heterologous Protein Expression and Enzyme Activity Assay**

The recombinant XvINO1 was expressed using the pCR T7/ NT system. The recombinant protein obtained after the purification had a MW of ~80 kD, higher than the predicted 56.699 kD. The His-tag on the protein could have contributed to the higher MW. The predicted pI of 5.08 means that the protein is acidic therefore migrates at a slow rate during SDS-PAGE, making it migrate at a higher MW. Using the pCR T7/ NT system, there is induction of protein expression even without the addition of IPTG but to a lesser extent as compared to when IPTG was used for induction. Western blotting using anti-His antibodies confirmed that the induced protein contained the His-tag. The recombinant protein was purified using Ni-NTA column chromatography. A relatively pure protein was obtained and an Anti-His western blot was carried out to verify that the protein was the desired XvINO1. The purified protein was quantified using the Bradford assay and concentrations of ~1 mg/ml were obtained.

The purified recombinant enzyme was used for the *in vitro* enzyme assay. This was done to determine whether the expressed protein had glucose-6-phosphate cyclase activity. The assay with plant extract gave inconclusive results due to problems with non-specific phosphatase activity in the plant extract. Efforts to inhibit phosphatases were not successful.

### **Analysis of XvINO1 5' UTR with respect to other INO1 5' UTR**

TAIL-PCR was used to isolate the 5' UTRs of XvINO1 and ThINO1. This method of isolating fragments upstream of a known DNA sequence is relatively fast. Fragments were selected by comparison of the TR2 and TR3 products. Since the TR3 reaction is a result of nesting TR2, the product is expected to be of slightly lower molecular weight than the TR2 product. The ThINO1 5' UTR was selected for comparison with XvINO1 5' UTR because *T. halophila* is a stress tolerant plant related to the extensively studied *A. thaliana*. This plant can tolerate salinity up to 500 mM NaCl and low temperature down to -15°C (Inan *et al.*, 2004). *T. halophila* has a genome about double the size of

the Arabidopsis genome with a 90-95% sequence identity. *INO1* was found to be one of the genes that were significantly up-regulated under stress in *T. halophila* (Taji *et al.*, 2004). The two upstream regions from stress tolerant plants were compared to the upstream regions from the three *INO1* genes of Arabidopsis.

A number of *cis*-acting elements were revealed by the sequence analysis tool Plant C.A.R.E. (Rombauts *et al.*, 1999) but only a few were selected based on their significance to abiotic stress. Based on the 1 kb upstream region, the *INO1* genes from all three plants are likely to be ABA-responsive but *T. halophila* and Arabidopsis might be more strongly responsive as compared to *X. viscosa*. The *XvINO1* 5' UTR has 4 ABRE whereas that of *ThINO1* has 21, *AtINO1-1* has 18 and *AtINO1-2* and *AtINO1-3* have 7 each. *XvINO1* and *ThINO1* are unlikely to be responsive to ethylene as they both contain no ERE whereas the Arabidopsis *INO1* would be expected to be responsive. The *INO1* genes from *T. halophila* and Arabidopsis would probably be responsive to salicylic acid while *XvINO1* would not be. These findings would need to be consolidated by experimental analyses. *XvINO1* has been found to be responsive to exogenous ABA treatment in this work. The role of *myo*-inositol in both ethylene and salicylic acid response might not be very clear. The limitation of the promoter analysis done here is that the 1 kb regions analyzed could be too short to reveal all the *cis*-acting elements in the upstream regions of the genes. Also, *X. viscosa* might contain multiple copies of *INO1* which might be regulated differently.

### **ITR KO characterization**

*Myo*-inositol metabolism has been implicated in plant abiotic stress tolerance in various plant species (Loewus and Murthy, 2000). This cyclitol plays a central role in the physiology of plant systems and as such it is found ubiquitously in the plant kingdom. Theoretically, impaired *myo*-inositol metabolism is expected to lead to defective plant growth in some way. However, the regulation of inositol metabolism differs from one plant species to another. In the *M. crystallinum*, the major osmoprotectants resulting from *myo*-inositol are ononitol and pinitol (Nelson *et al.*, 1999). The pathway for the biosynthesis of ononitol and pinitol has not been reported in other plants. Under salinity stress, *myo*-inositol is involved in the uptake of Na<sup>+</sup> in *M. crystallinum* (Chauhan *et al.*, 2000). Expression of *INO1* is upregulated in the leaves and downregulated in the roots.

The *myo*-inositol acts both as a signal to the roots and is also directly involved in the compartmentalisation of  $\text{Na}^+$  into the vacuole in the leaves via a  $\text{Na}^+/\textit{myo}$ -inositol symporter. Transportation of *myo*-inositol through the stem occurs through ITRs.

*A. thaliana* knockouts in *ITR1* and *ITR4* were compared to wild type plants under normal conditions. Under normal conditions, there were no significant phenotypic differences between WT, *ITR1*, *ITR4* and *ITR1/ITR4* KO plants. This could be due to the fact that under normal conditions, the plants express *INO1* in all tissues and therefore there is no critical need for the translocation of *myo*-inositol. Also, the other ITR proteins or other hexose transporters could be complimenting the deleted ITR under these conditions. There is, however, need to look at the phenotype under stress conditions.

### **Future Directions**

This work has provided basic evidence that *myo*-inositol could play a role in the survival as well as stress tolerance of *X. viscosa*. Its down-stream metabolism is, however, quite complicated. Of the two main pathways for synthesis of compatible solutes from *myo*-inositol, the RFO pathway has been studied in *X. viscosa* (Peters, unpublished data). The data obtained showed XvGolS to be upregulated under stress conditions at both the transcript and protein levels. Future direction for this work could focus on the down-stream metabolism (e.g. biosynthesis of ononitol and pinitol) as the upregulation of *INO1* in plants with respect to abiotic stress has been reported in plants that are not stress tolerant such as *Z. mays* (Wang *et al.*, 2003). This finding suggests that the accumulation of *myo*-inositol alone is not sufficient for abiotic stress tolerance. However, in the development of stress-tolerant transgenic plants, the use of *INO1* in combination with other genes may be essential as it will provide an increased pool of *myo*-inositol.

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