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Protection mechanisms in the resurrection plant *Xerophyta viscosa*: cloning, expression, characterisation and role of *XvINO1*, a gene coding for a *myo*-inositol 1-phosphate synthase

Arnaud Lehner[A,C], *Denis R. Chopera*[A], *Shaun W. Peters*[B], *Felix Keller*[B], *Sagadevan G. Mundree*[A], *Jennifer A. Thomson*[A] *and Jill M. Farrant*[A,D]

[A]University of Cape Town, Department of Molecular and Cellular Biology, Private Bag, Rondebosch 7701, Cape Town, South Africa.
[B]University of Zürich, Institute of Plant Biology, Molecular Plant Physiology, Zollikerstrasse 107, Zürich 8008, Switzerland.
[C]Present address: Université Paris 7, EA 3514 Electrophysiologie des Membranes, 2, place Jussieu, 75005 Paris, France.
[D]Corresponding author. Email: jill.farrant@uct.ac.za

Abstract. We have used reverse transcription-PCR coupled with 5′- and 3′-RACE to isolate a full length *INO1* cDNA (1692 bp with an ORF of 1530) from the resurrection plant *Xerophyta viscosa* Baker. *XvINO1* encodes 510 amino acids, with a predicted MW of 56.7kD and contains four sequence motifs that are highly conserved in plant *myo*-inositol-1-phosphate synthases (MIPS, EC5.5.1.4), the enzyme that catalyses the first step in the formation of *myo*-inositol (Ino). Northern and western analyses show that the transcript and protein are constitutively present in leaves but their expression increases, temporarily, in response to both accumulative salt stress (~300 mM NaCl) and desiccation (to 5% relative water content). Leaf Ino concentration increases 40-fold during the first 6 h of salt stress, and levels of this and other carbohydrates (galactinol, sucrose, raffinose, stachyose and hexoses) remain elevated relative to control leaves for the duration of salt stress treatment. The timing and pattern of accumulation of these carbohydrates differ under desiccation stress and we propose that they perform different functions in the respective stresses. These are elaborated in discussion of our data.

Additional keywords: carbohydrates, desiccation tolerance.

Introduction

Water deficit and salinity are the main abiotic factors involved in decreased crop productivity (Boyer 1982; Ashraf 1994; Ashraf and Foolad 2007). All aspects of plant growth, but particularly the reproductive phases, are adversely affected by these abiotic stresses (Zhu 2001; Munns 2002; Wang *et al*. 2003; Boyer and Westgate 2004) the estimated loss of yield being $\geq$50% in the major crops (Boyer 1982). It has been estimated that the earth's population will be 9.3 billion by 2050 (Flowers 2004), with most people living in the semiarid tropics where rainfall is erratic and crop productivity is most adversely affected by water deficit and salinity stress. In order to feed this population, it is becoming an imperative to produce crops with enhanced tolerance to these stresses. We use the resurrection plant *Xerophyta viscosa* Baker, a monocotyledonous desiccation tolerant angiosperm able to tolerate loss of 95% of its cellular water, as a model to understand the mechanisms that enable vegetative tissues to withstand abiotic stresses, and as a source of genes to ultimately produce transgenic drought-tolerant plants of agronomic importance to sub-Saharan Africa (Sherwin and Farrant 1996, 1998; Mundree and Farrant 2000; Mundree *et al*. 2000; Garwe *et al*. 2003).

In resurrection plants (Gaff 1971), vegetative tissues are able to dry to equilibrium with the surrounding air (usually to an absolute water content of 0.1 g $H_2O.g^{-1}$ dry mass or a relative water content [RWC] of 5%) for prolonged periods and yet recover full metabolic activity in existing tissues on rehydration (reviewed in Gaff 1989; Bewley and Oliver 1992; Ingram and Bartels 1996; Oliver *et al*. 1998; Farrant 2000; Vicre *et al*. 2003, 2004; Farrant *et al*. 2007). *X. viscosa* is endemic to southern Africa and is a plant in which extensive protection against the damage associated with subcellular desiccation is laid down during drying (as is the case in angiosperm resurrection plants) in order to minimise repair that is needed upon rehydration. These putative protection mechanisms include upregulation of antioxidant activity, synthesis of sucrose (Suc) and oligosaccharides, several classes of stress-associated proteins (Sherwin and Farrant 1996, 1998; Mundree and Farrant 2000; Ndima *et al*. 2001; Mowla *et al*. 2002; Garwe *et al*. 2003; Marais *et al*. 2004; Walford *et al*. 2004; Peters *et al*.

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2007) and many genes, not yet fully characterised, that are likely to code for products involved in subcellular protection against desiccation (Mundree and Farrant 2000; Mundree *et al*. 2002; Iyer *et al*. 2007).

Drought and salinity are known to induce an osmotic stress, resulting in the disruption of homeostasis and ion distribution in the cell (Serrano *et al*. 1999; Zhu 2001; Munns 2002) and the production of reactive oxygen species (ROS), which can lead to lipid peroxidation and the denaturation of proteins (Vertucci and Farrant 1995; Smirnoff 1998). The protectant mechanisms purported to ameliorate the effects of osmotic stresses have been widely reviewed (e.g. Bohnert *et al*. 1995; Cushman 2001; Munns 2002, 2005; Zhu 2002; Wang *et al*. 2003; Flowers 2004; Valliyodan and Nguyen 2006) and include, among other things, the production of stress proteins and the accumulation of compatible organic solutes, especially carbohydrates, organic acids and quaternary ammonium compounds (QACs) such as glycine betaine and amino acids such as proline (Serraj and Sinclair 2002; Ashraf and Foolad 2007). These involve a complex range of adaptations which in turn involve changes in the expression of several genes and transcription factors (Ramanjulu and Bartels 2002; Wang *et al*. 2003; Valliyodan and Nguyen 2006).

Various roles have been assigned to carbohydrates in their protection against osmotic stress and include: (1) acting as water replacement molecules (Crowe *et al*. 1987); (2) facilitating the production of intracellular glasses that put a stasis on metabolism and minimise ROS-associated molecular alterations (Leopold *et al*. 1994; Bernal-Lugo and Leopold 1995; Sun 1997; Berjak 2006; Berjak *et al*. 2007); and (3) amelioration of concentration effects of salt and ions accumulated in vacuoles (Hasegawa *et al*. 2000; Munns 2002). The carbohydrates that have been particularly associated with abiotic stresses include ononitol and pinitol (Keller and Ludlow 1993; Sheveleva *et al*. 1997; Peterbauer *et al*. 1998; Chiera *et al*. 2006), Suc (reviewed in Vertucci and Farrant 1995; Berjak 2006; Berjak *et al*. 2007; Farrant *et al*. 2007), raffinose family oligosaccharides (RFOs) (Keller and Pharr 1996; Avigad and Dey 1997; Tapernoux-Lüthi *et al*. 2004; Peters *et al*. 2007) and *myo*-inositol (Ino) (Vernon and Bohnert 1992; Smart and Fleming 1993; RayChaudhuri *et al*. 1997; Nelson *et al*. 1998; Klages *et al*. 1999; Loewus and Murthy 2000; Chun *et al*. 2003; Majee *et al*. 2004). Indeed, Ino plays a central role in the synthesis of many compounds such as D-glucuronic acid, which is involved in the biogenesis of hemicellulose and other plant cell wall compounds (Loewus and Loewus 1983), auxin esters and glycosides (Loewus and Murthy 2000), galactinol (Gol), the galactosyl donor for RFO synthesis (Keller and Pharr 1996; Sprenger and Keller 2000; Peterbauer and Richter 2001), phytic acid (Cosgrove 1980) and the family of O-methyl inositols, among them pinitol and ononitol (Peterbauer *et al*. 1998; Chiera *et al*. 2006).

The first step in the synthesis of Ino is the conversion of D-Glc-6-P to 1 L-Ino1-P. The enzyme involved in this reaction is the *myo*-inositol-1-phosphate synthase (MIPS, EC 5.5.1.4) (RayChaudhuri *et al*. 1996; Loewus and Murthy 2000; Majee *et al*. 2004). Identified for the first time in *Saccharomyces cerevisiae* Meyen ex Hansen as *INO1* (Donahue and Henry 1981; Majumder *et al*. 1981), the structural gene for the MIPS has been cloned and sequenced from several prokaryotic and eukaryotic sources (Majee *et al*. 2004). A cytosolic and a chloroplastic form of MIPS with the same biochemical properties have been reported and are differentially regulated during salt stress (RayChaudhuri and Majumder 1996).

In this study, we identify and characterise the gene *XvINO1* (GenBank acc. no. EF449773). We attempted to understand the response of gene and MIPS protein expression to desiccation and salt stress using northern and western blot analyses. The changes in leaf concentrations of the polyol Ino and in various sugars (hexoses (Hex), Suc and RFOs) during salt stress were also investigated. The changes in these carbohydrates in response to desiccation were reported earlier in Peters *et al*. (2007) and are referred to in our discussion.

Materials and methods

Plant material

Xerophyta viscosa Baker plants were collected from the Cathedral Peak Nature Reserve (Kwazulu-Natal province, South Africa) and were maintained under greenhouse conditions as described by Sherwin and Farrant (1996). Prior to imposing stress treatments, the plants were acclimated for 1 month to a controlled environment phytotron (16 h light, 350 μmol m^{-2} s^{-1}, 25°C; 8 h dark, 20°C; 50% relative humidity). We are legally limited in the number of plants collected annually (due to their occurrence in a protected reserve) and biological replication was thus limited to 3–5 plants per treatment. However, the desiccation and salt treatments described below were conducted at least twice on different sets of plants.

Experimental treatments

Since carbohydrate metabolism of a plant can vary on a daily rhythm, we first determined changes in gene and protein expression and in carbohydrate levels in leaves on an hourly basis over a 24-h day/night cycle. Water deficit stress was imposed by withholding water from plants for a period of 21 days. Leaf samples were taken at full turgor and after 3, 9, 11, 12, 14, 15 and 21 days without water. Upon rehydration, leaves were sampled after 12, 24, 48 72 h and 7 days after soil watering. In order to study the effect of salinity, fully hydrated acclimated plants were irrigated with 1 L of 100 mM NaCl solution every 24 h over a period of 72 h, this giving a final cumulative salt stress of ~300 mM (assuming minimal evaporation volume, given that there was no water run-off and limited soil surface area exposed for evaporation, Fig. 4). Leaf samples were collected after 6, 12, 24, 48 and 72 h. For all sampling points, 3–5 leaves per plant were flash frozen in liquid nitrogen and stored at –80°C for subsequent extraction and analyses.

Relative water content measurements

RWC measurement was calculated as previously described by Jin *et al*. (2000) by using the following equation: $RWC = 100 \times [(W_f - W_d)/(W_t - W_d)]$ where W_f is the fresh weight before drying in an oven at 70°C for 48 h (W_d). W_t represented the full turgor weight obtained by immersing the leaves during 24 h in water.

Chlorophyll fluorescence

Since chloroplastic isoforms of Ino exist, which might have an ameliorating effect under stress conditions, the effect of salinity stress on the quantum efficiency (F_V/F_M) of photosystem II (PSII) was determined using a portable fluorometer (OS 500, Optiscience, Hudson, NH, USA), as previously reported for the same species under water deficit stress conditions (Sherwin and Farrant 1998). A minimum of five replicates on three separate plants were measured at each sampling point. Leaves were dark-acclimated for 10 min before measurement. A saturating light, with an intensity of ~4 mmol photons m^{-2} s^{-1} with a duration of ~1 s, was utilised to determine initial and maximum fluorescence, F_0 and F_M, respectively. F_V was calculated by subtracting F_0 from F_M, and F_V/F_M was thus determined.

XvINO1 *cDNA isolation*

RNA was isolated from *X. viscosa* leaves using the Trizol LS reagent (Life Technologies, Gaithersburg, MD, USA). RT–PCR was carried out on 1 µg of RNA using the smart IV oligonucleotide and CDS III primers (Clonetech, UK). 3′ and 5′ RACE-PCR was carried out using the primers GAATCGCGTGCCAACAATGT and CDSIII and CGGGACAGCTTCGTCGCGCGTTACG and GAATCGCGTGCCAACAATGT, respectively. In a 20 µL reaction containing 1X Expand PCR buffer, 0.5 mM dNTPs and 10 U of Expand DNA polymerase, the 5′ and 3′ PCR products were mixed in a 1 : 1 ratio (5 µL each). Prior to the addition of the polymerase enzyme, the mixture boiled for 5 min and chilled on ice for 2 min. After addition of the polymerase, the mixture was incubated at 68°C for 15 min. PCR was conducted out using aliquots (10 µL, 5 µL and 1 µL) of the products as template. A separate PCR was conducted using 1 µL each of the 5′ and 3′ PCR products and the products were purified using the Roche High Pure PCR Purification Kit (Roche Applied Science, Indianapolis, IN, USA) following the manufacturer's instructions.

Cloning and sequence analysis of XvINO1

The full length *XvINO1* was ligated into the PGEM-T Easy Vector System (Promega Corporation, Carlsbad, CA, USA) according to the manufacturer's instructions. This construct was subsequently transformed into *Escherichia coli* (DH5α) by conventional heat shock method as outlined in the PGEM-T Easy manual, plated onto Luria Agar (LA) platets supplemented with ampicillin (100 µg mL^{-1}) and incubated overnight at 37°C. Plasmid DNA was isolated from ampicillin resistant colonies, using High Pure Plasmid Purification Kit (Roche) following the manufacturer's instructions and the presence of *XvINO1* verified by PCR using the 5′ Primer and CDSIII. Five positive clones were then sequenced using a MegaBACE DNA Sequencing System (Molecular Dynamics, Amersham, UK). The BLAST program of the National Centre for Biotechnology Information (Altschul *et al*. 1990) was used to search databases for sequences with similarity to *XvINO1*. Nucleotide and amino acid sequence comparisons were done using the DNAMAN program (Lynnon Biosoft, Vaudreuil-Dorion, Quebec, Canada). Prediction of signal peptides was conducted with the Signal Pv3.0 software package (Nielsen *et al*. 1997) using the predicted amino acid sequence of *XvINO1*.

RNA extraction and northern blot analysis

Total RNA was isolated using the Trizol LS reagent (Gibco-BRL, Johannesburg, South Africa). *X. viscosa* leaves (200 mg) were ground in liquid nitrogen and homogenised in 0.75 mL of the reagent. Following incubation for 5 min at room temperature, 0.2 mL chloroform was added followed by a further incubation at room temperature for 10 min. Samples were centrifuged at 12 000*g* for 10 min at 4°C and the RNA was precipitated using isopropanol. RNA was quantified spectrophotometrically, electrophoresed in a 1.2% agarose formaldehyde gel and stained with ethidium bromide to verify quantitation. Fifteen µg of each sample were electrophoresed in a 1.2% agarose formaldehyde gel and transferred onto nylon membrane (Hybond XL, Amersham, UK) using a capillary transfer method (Sambrook *et al*. 1989) and filters crosslinked in a UV cross-linker (Stratalinker 1800, Stratagene, La Jolla, CA, USA). The complete *XvINO1* cDNA was radio-labelled with α–^{32}PdCTP using the Megaprime DNA labelling system (Megaprime, Amersham, UK) according to the manufacturer's instructions. Blots were hybridised with the radio-labelled *XvINO1* probe for 16 h at 65°C and subsequently washed with wash buffer A (2× sodium citrate buffer (SSC), 0.1% SDS) at 65°C and more stringently with wash buffer B (0.5× SSC, 0.1% SDS) at 65°C. The membranes were then autoradiographed, at –70°C, onto high-performance autoradiography film (Hyperfilm MP, Amersham, UK).

Heterologous protein expression and purification

XvINO1 was cloned into an expression vector using the PCR T7 TOPO TA expression Kit (Life Technologies, Invitrogen, Carlsbad, CA, USA) following the manufacturer's recommendations. Primers flanking the open reading frame of *XvINO1* were designed: ATGTTCATCGAAAGCTTCAAGG (INOF1) and CCATGCATAGAATAGCTTACATCGATCGTAC (INOR1) and a standard PCR reaction conducted over 36 cycles at a primer annealing temperature of 60°C. Products were purified using the High Pure PCR Purification kit (Roche), following the manufacturer's instructions. Two µL of the purified PCR product were mixed with 0.5 µL each of the TOPO salt solution and the TOPO NT vector. The reaction was incubated at room temperature for 10 min. The ligation generated the recombinant pCR T7/NT::*XvINO1* vector that was subsequently transformed into *E. coli* using One Shot TOP10F' competent cells (Invitrogen) following the manufacturer's instructions. Positive colonies were inoculated into LB broth supplemented with ampicillin (100 µg mL^{-1}) and incubated overnight at 37°C with agitation. Plasmid DNA was isolated using the High Pure Plasmid Purification Kit (Roche), according to the manufacturer's instructions and the product digested with *Eco*RI to verify the orientation of the insert. Plasmids were sequenced using the MegaBACE DNA Sequencing System (Molecular Dynamics, Amersham, UK). The final transformation was carried out using 10 ng of pCR T7/NT::*XvINO1* in a vial of One Shot BL21(DE3)pLysS cells (Invitrogen) following

the manufacturer's instruction. Recombinant protein expression was conducted with the pCR T7 TOPO TA Expression Kit (Invitrogen) following the manufacturer's instructions. The recombinant protein was purified using the Ni-NTA resin (Qiagen, Valencia, CA, USA) and desalted through Amicon Ultra-15 columns (Millipore, Billerica, MA, USA). Protein content was determined using the Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA) with bovine serum albumin (BSA) as calibration standard (Bradford 1976).

The full length recombinant protein was then used to raise polyclonal antibodies in rabbits as previously described (Mowla *et al*. 2002). Antibodies were purified by adding two volumes of borate-buffered saline to one volume of serum. 14% (w/v) of PEG 6000 was added and the diluted serum was gently mixed by inversion. Following a centrifugation at 12 000*g* for 10 min at 4°C, the pellet was dissolved in the original serum volume by adding 1× PBS. PEG 6000 was added again at a 14% (w/v) concentration and dissolved. The solution was centrifuged as described above and the pellet dissolved in half the original serum volume using PBS containing 60% (v/v) glycerol. Aliquots were stored at –20°C.

Protein extraction and western blot analysis

Proteins were extracted using the TRIZOL LS reagent (Gibco-BRL) following the manufacturer's instructions. Protein contents of the extracts were determined using the Bio-Rad protein assay kit with BSA as the calibration standard (Bradford 1976). Aliquots (15 µg) of protein were separated by SDS–PAGE (12% resolving gel, 5% stacking gel). After separation, proteins were transferred electrophoretically (300 mA, 1 h) onto nitrocellulose membranes (Osmonics, Trevose, PA, USA) using the Hoefer Electrotransfer System (Hoefer, Amersham, UK) following the manufacturer's instructions. The membranes were stained with Ponceau S (Sambrook *et al*. 1989) for 10 min to visualise proteins and incubated in blocking solution [3% (w/v) BSA in TBS] for 1 h at 25°C, followed by incubation at 25°C for 1.5 h in the presence of MIPS antibody diluted (1 : 1000) in blocking solution. After washing thrice (10 min with agitation for each wash) in TBS-Tween buffer [1× TBS, 0.05% (v/v) Tween 20], membranes were incubated for 1.5 h at 25°C with anti-rabbit IgG peroxidase-linked whole antibody from goat (Sigma, St Louis, MO, USA) diluted (1 : 5000) in blocking solution. The secondary antibody was localised using the Supersignal West Pico/femto Chemiluminescent Substrate (Pierce, Rockford, IL, USA) and exposed to X-ray film (Pierce). Films were developed manually after various exposures times.

Extraction, HPLC analysis and quantification of soluble carbohydrates

Soluble carbohydrates were extracted, desalted, analysed and quantified by HPLC as previously described by Peters *et al*. (2007) without any modification. Briefly, soluble carbohydrates were extracted from leaf tissue in 1.5 mL ethanol (twice for each concentration of 80% and 20%, v/v, respectively) and 1.5 mL dH_2O (twice). During each extraction, samples were heated at 80°C for 10 min, placed on ice for 2 min and subsequently centrifuged at 15 000*g* for 5 min. The supernatants of all extraction steps were pooled and volumes adjusted to 6 mL with dH_2O. Aliquots of 50 µL were desalted and analysed by HPLC-PAD. A Ca^{2+}/Na^{+}-moderated ion partitioning carbohydrate column was used to separate carbohydrates (Benson BC-100 column, 7.8 × 300 mm; Benson Polymeric, Reno, NV, USA). To confirm the identities of certain carbohydrates, samples were also analysed by anion exchange chromatography using a CarboPac MA1 column (4 × 250 mm; Dionex, Sunnyvale, CA, USA). Both chromatographic systems were operated as described in Peters *et al*. (2007). Soluble carbohydrates on both systems were quantified *in silico*, using the Chromeleon v 6.4 software package, against a series of 5 nmol of standard sugars. The quantity of standard sugars used corresponds to the linear response range of the both chromatographic systems. We analysed specifically for the presence of Ino, being the product of Ino and MIPS activity. However, we also tested for presence and changes in galactinol (Gol), the sugar downstream from Ino and important in the formation of RFOs, the RFOs themselves, as well as Suc, the major carbohydrate accumulated during desiccation in resurrection plants (Farrant *et al*. 2007) and the Hex, glucose (Glc), fructose (Fru) and galactose (Gal).

Results

Complementary DNA isolation and sequence analysis

The full-length *XvINO1* cDNA was isolated from the leaves of *X. viscosa* plants and following 3′ and 5′ end RACE amplification, purification and sequencing resulted in a cDNA with a 1530 bp ORF (Fig. 1) encoding 510 amino acids (Fig. 2). Use of the BLAST program revealed it to be homologous to other *INO1* genes. The 3′ untranslated region (UTR) of *XvINO1* consisted of one putative polyadenylation signal sequence. The predicted molecular weight of the protein is 56.69 kD with a pI of 5.08. The amino acid sequence consists of four sequence motifs: GWGGNNG, LWTANTERY, NGSPQNTFVPGL, and SYNHLGNNDG that are highly homologous to eukaryotic MIPS (Fig. 2).

The *Xv*MIPS protein sequence was compared with other MIPS protein sequences by multiple alignments using the DNAMAN software (Fig. 2), revealing high identities (92.4%) between the various MIPS proteins. A phylogenetic tree was generated for the plant MIPS together with the sequences from an animal (*Mus musculus* L.), a bacterium (*Mycobacterium tuberculosis*) and a yeast (*Saccharomyces cerevisiae* Meyen ex Hansen). The dicotyledonous plants (*Arabidopsis thaliana* L, *Nicotiana tabacum* L., *Glycine max* Merr., *Mesembryanthemum crystallinum* L.) and monocotyledonous plants (*Avena sativa* L., *Hordeum vulgare* L., *Oryza sativa* L., *Zea mays* L.) for which sequence identity is known, were also used (Fig. 3). The respective sequences grouped into four distinct clades (Fig. 3), with *Xv*MIPS grouping to a clade exclusively containing the sequences from monocotyledonous plants, while sequences from dicotyledonous plants grouped to two distinct clades. Similarly, another clade contained the animal, bacterium and the yeast sequences and showed less similarity to each other and the plant sequences as evidenced by longer branch lengths. An analysis of the *XvINO1* peptide sequence, to identify any sub-cellular targeting signals, revealed no signature sequences for targeting to the chloroplast

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1       GACTCCCCCT TTCCCCATAT TCTCAAAACC CCCCCATTTN TTTTCAAAAT TTGCTGTCCT
61      TTCCTGCAAA AA
                     ATGTTCAT CGAAAGCTTC AAGGTCGAGA GTCCCAACGT CAAGTATTTC
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 TGGTTCGTTC
421     AATGGAGAAG AGATCTACGC TCCATTCAAG AGCTTGCTTC CCATGGTTAA CCCAGATGAT
481     ATTGTGTTTG GAGGATGGGA CATAAGCAAC ATGAACCTTG CAGATTCCAT GGCAAGGGCT
541     AAGGTGCTTG ATATCGACCT CCAGAAACAG CTCAGGCCAT ACATGGAATC AATGGTCCCC
601     TTGCCTGGAA TCTACGATCC TGATTTCATC GCCGCCAATC AGGAATCGCG TGCCAACAAT
661     GTCATCAAAG GCACCAAGAA GGAGCAGGTC 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     CCTTTCATCA ATGGCAGCCC TCAAAACACC TTCGTTCCAG GGCTCATCGA TCTTGCTATT
961     AAGAGGAACA CACTCATTGG TGGCGACGAC TTCAAGAGCG GACAGACTAA GATGAAATCT
1021    GTGCTCGTCG ATTTCCTTGT TGGAGCTGGA ATAAAGCCGA CATCCATTGT TAGCTACAAT
1081    CACCTCGGCA ACAATGATGG AATGAACCTG TCTGCGCCGC AAACCTTTCG TTCCAAGGAG
1141    ATTTCCAAGA GCAATGTGGT CGATGACATG GTCTCCAGCA ATGGCATCCT CTACGAACCT
1201    GGCGAGCATC CTGATCATGT AATTGTGATC AAGTATGTGC CATACGTCGG GGACAACAAA
1261    AGGGCCATGG ATGAGTACAC ATCGGAGATT TTCATGGGCG GCAAGAGCAC GATTGTATTG
1321    CATAACACTT GCGAGGACTC ACTTCTGGCT GCGCCGATAA TTCTTGACTT GGTTCTTCTC
1381    GCTGAACTTA GCACCCGGAT TCAGCTTAAA GCAGAGGGAG AGGATAATTT CCAGTCCTTC
1441    CATCCTGTGG CTACAATTCT AAGCTACCTC ACCAAGGCTC CTCTGGTACC TCCGGGCACG
1501    CCGGTGGTGA ATGCCCTGTC GAAGCAGCGC GCAATGCTGG AGAACATCCT CAGAGCTTGC
1561    ATCGGCTTGG CGCCTGAGAA TAACATGATC TTGGAATATA AATGA
                                                      AGTAC GATCGATGTA
1621    AGCTATTCTA TGCATGGTTT AAAAAAATGT AAACTTTTTC ATTTAGATCC TAAGTTTGTT
1681    TGATGAAATT CT(A)18
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Fig. 1. Nucleotide sequence of the full-length *XvINO1* cDNA from *Xerophyta viscosa*. Putative polyadenylation site in the 3′ UTR is underlined, start (#73) and stop (#1603) codons are double underlined.

or mitochondria, suggesting that *XvINO1* is a cytoplasmic MIPS isoform.

Southern blot analysis of *X. viscosa* genomic DNA was carried out to confirm the presence of the gene in the *X. viscosa* genome (data not shown).

Northern blot analysis

Northern blot analyses were carried out to determine *XvINO1* transcript levels during (1) a day/night cycle and (2) salt and desiccation stress treatments. In all instances, *XvINO1* transcripts were detected at time 0, before the onset of any treatments, suggesting that it is constitutively expressed (Fig. 5). There was no difference in RWC (Fig. 4*A*) or transcript expression levels during a day/night cycle (Fig. 5*A*) indicating that changes in transcript levels upon exposure to abiotic stresses were not due to inherent daily fluctuations in water content. Transcript levels changed considerably in response to both salt and desiccation treatments (Fig. 5*B*, *C*). Although soil irrigation with salt did not result in changes of leaf RWC (Fig. 4*B*), *XvINO1* transcript levels in leaves increased slightly in the first

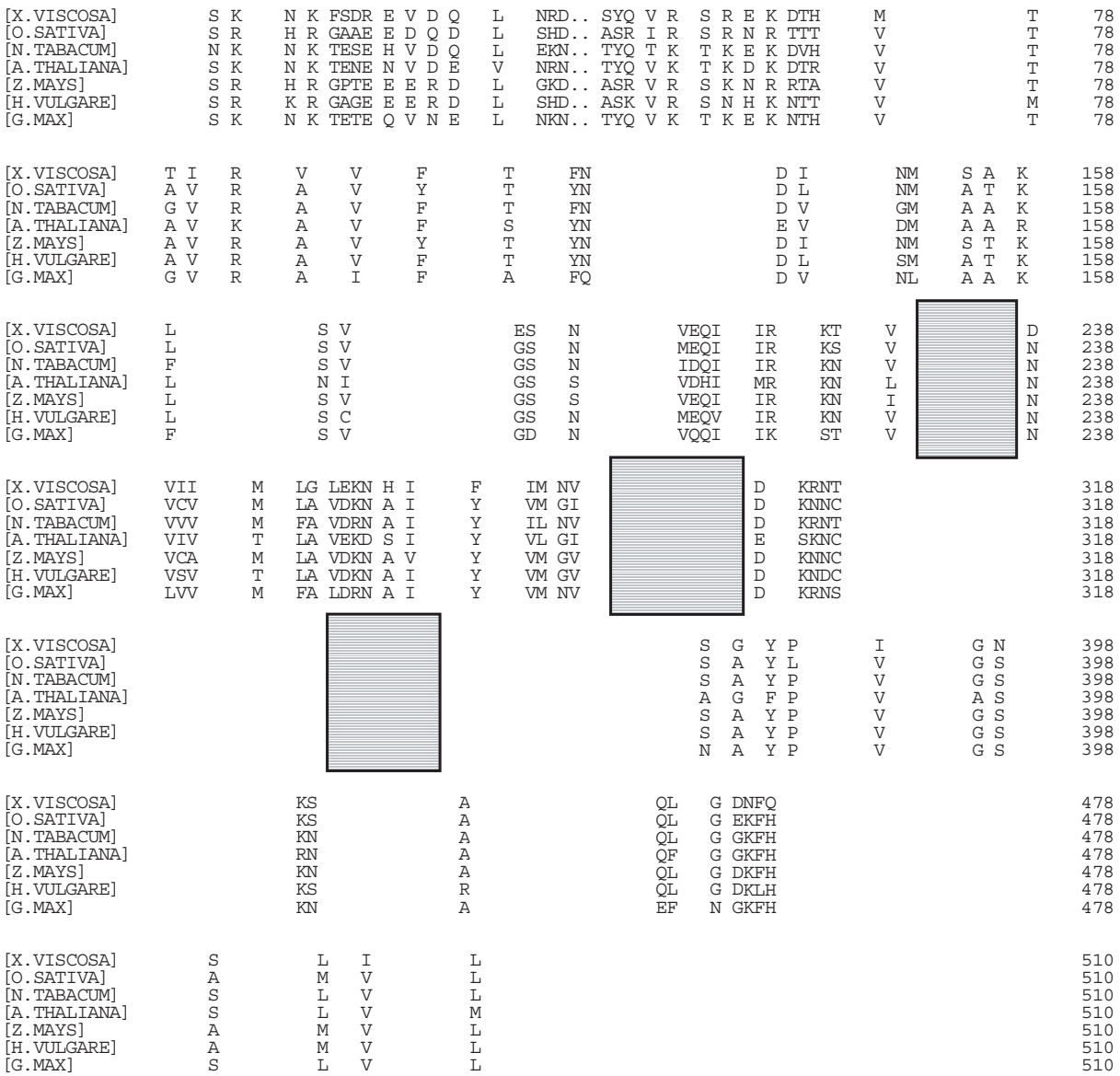

Fig. 2. Comparison of the deduced amino acid sequence of *Xv*MIPS from *Xerophyta viscosa* with MIPS from *Oryza sativa* (accession number O64437), *Nicotiana tabacum* (accession number BAA95788), *Arabidopsis thaliana* (accession number NP_179812), *Zea mays* (accession number AAG40328), *Hordeum vulgare* (accession number ACC17133) and *Glycine max* (accession number ABC55421). Amino acids identical to *Xv*MIPS sequence are boxed in black. Highly conserved domains are boxed in grey.

12 h and there was more substantial transcription thereafter, reaching a maximum after 48 h of cumulative exposure to salinity. Transcript levels declined after 72 h (Fig. 5*B*) and at no time were the plants visibly affected by this salt stress (Fig. 5*D*). When water was withheld from *X. viscosa* plants, there was an initial slow decline in leaf RWC during the first 6 days (to 75%) followed by a rapid decline to the air-dry state (5%) after 10 days (Fig. 4*C*) during which chlorophyll was lost from the leaves which folded along the lamina (Fig. 5*E*) as has been previously reported (Sherwin and Farrant 1998). The rehydration was typically rapid, with leaves reaching full turgor (96% RWC) within 48 h following soil watering (Figs 4*C* and 5*F*). During dehydration, *XvINO1* transcripts started to increase at 61% RWC with levels further increasing to a RWC of 12%. Maintenance in the dry state at 5% resulted in a slight decline in transcript levels, which further declined during early rehydration to levels similar to that of the pre-stress condition when fully hydrated (Fig. 5*C*).

Western blot analysis

Western blot analysis showed that the *Xv*MIPS protein was expressed at relatively constant levels in untreated (control) leaves and throughout a day/night cycle (Fig. 6*A*) suggesting constitutive expression and relatively unchanging concentration of the protein. Application of a salinity treatment resulted in increased accumulation of the protein after 24 h, the levels

Fig. 3. Phylogenetic tree representing the relationship of the *Xerophyta viscosa* MIPS protein sequence to 12 other MIPS homologues. The accession numbers of the sequences used in the comparison are: *Arabidopsis thaliana* (accession number NP_179812); *Nicotiana tabacum* (accession number BAA95788); *Hordeum vulgare* (accession number AAC17133); *Glycine max* (accession number ABC55421); *Avena sativa* (accession number BAB40956.2); *Oryza sativa* (accession number O64437); *Zea mays* (accession number AAG40328); *Mesembryanthemum crystallinum* (accession number AAB03687); *Mus musculus* (accession number NP_076116.1); *Saccharomyces cerevisiae* (accession number AAA66310.1); *Mycobacterium tuberculosis* (accession number NP_214560.1).

remaining at similar elevated levels for up to 72 h of cumulative salinity treatment (Fig. 6*B*). During dehydration, a gradual accumulation in the *Xv*MIPS protein occurred in leaves after RWC had reached 21% (Fig. 6*C*). *Xv*MIPS content remained high during the early stages of rehydration (to 84% RWC), after which it declined to levels comparable to those observed at full turgor (96% RWC; Fig. 6*C*).

Soluble carbohydrate analysis

The soluble carbohydrates found in detectable amounts in leaf extracts were Suc, Hex, Ino, Gol and the RFOs, Raf and Sta (Fig. 7). There were no significant changes in the concentrations of these carbohydrates over a daily period (Fig. 7, left panel). When the plants were subjected to salt treatment, there was a significant and rapid increase in leaf concentrations of all these carbohydrates over the first 6 h, after which concentrations declined, but remained elevated relative to the pre-stress condition (Fig. 7, right panel). The most significant increase was in Ino content, which increased ~42-fold (from 0.35 to 14.78 mg g^{-1} DW) during the first 6 h. During the same time period, there was a 12-, 7.5-, 7-, 4- and 3-fold change in Hex, Gol, Suc, Sta and Raf concentration, respectively, compared with the pre-treatment control levels (Fig. 7, right panel). After this initial increase, the Ino concentration dropped to 9 mg g^{-1} DW (~25-fold higher than the control) for the duration of the salt treatment (Fig. 8 right panel). Hex remained 10-fold higher than control levels and Gol, Suc, Sta and Raf 5-, 5-, 2.5- and 2-fold higher over the remainder of the salt treatment.

By contrast, Peters *et al*. (2007) have reported that during desiccation of *X. viscosa*, there was a decline in leaf concentrations of Ino, Gol and Hex to undetectable levels, although these carbohydrates recovered to original pre-drying control concentrations following rehydration. Suc, Sta and Raf increased as RWC declined below 70% with an ultimate 5-, 4- and 3.5-fold increase, respectively, over the hydrated condition, in dry leaves. While Suc accumulation during desiccation is virtually universal in angiosperm resurrection plants, many, but not all, accumulate Raf and Sta (reviewed in Farrant *et al*. 2007).

Effects of salt on PSII activity

There was no significant change in quantum efficiency (F_V/F_M) of PSII during the first 3 days of salt treatment (Fig. 8), suggesting that electron transfer and presumably the photosynthetic process, was not disrupted by treatment of at least up to 300 mM salt (the cumulative dose after 3 days). This is contrast to the situation during desiccation when F_V/F_M declines from 0.7 in fully hydrated to 0.2 in dry leaves (Sherwin and Farrant 1998); this decline being due to the fact that *X. viscosa* is poikilochlorophyllous and chlorophyll is degraded and thylakoids dismantled to minimise ROS formation during water deficit stress (Farrant 2000; Farrant *et al*. 2007).

Discussion

Cloning and characterisation of XvINO1

Using a reverse transcription-PCR-based cloning strategy employing degenerate primers designed on the basis of

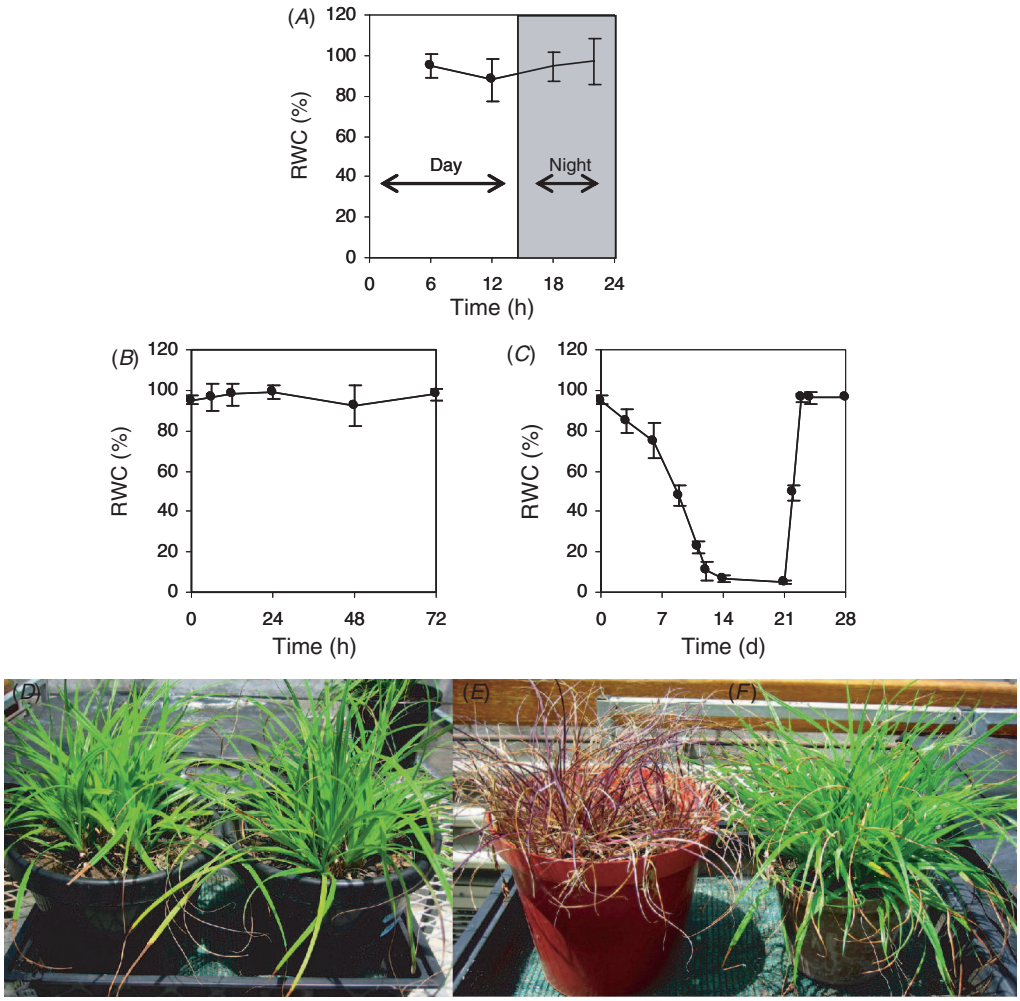

Fig. 4. Relative water content (RWC) of leaves from *Xerophyta viscosa* during the various treatments. (*A*) Day/night cycle; (*B*) salt stress (100 mM); (*C*) dehydration/rehydration. Means of three replicates ± s.d. Where no bars are shown, the spread of the s.d. is less than the size of the symbol. (*D*) Typical appearance of plants after experiencing 3 days of cumulative salt stress, (*E*) upon dehydration to 5% RWC, and (*F*) 72 h following rehydration.

conserved sequences of *INO1*, together with 5′- and 3′-RACE, the complete cDNA encoding the enzyme was isolated and sequenced (Schramm *et al*. 2000). The *XvINO1* cDNA obtained was 1692 bp long with an ORF of 1530 bp, encoding 510 amino acids with a predicted MW of 56.7 kD (Figs 1 and 2). This polypeptide length is consistent with most known plant MIPS proteins. The predicted amino acid sequence contains four sequence motifs that are highly conserved in MIPS proteins. These motifs are GWGGNNG, LWTANTERY, NGSPQNTFVPGL and SYNHLGNNDG. One of these conserved sequences (GWGGNNG) is characteristic of the Rossman fold GXGGXXG motif which is typical of an oxidoreductase (Kleiger and Eisenberg 2002). This sequence is involved in NAD^+ binding. The sequence SYNHLGNNDG was found to be located in the catalytic region of the MIPS enzyme (Stein and Geiger 2002). The other two conserved sequences, LWTANTERY and NGSPQNTFVPGL, are within the NAD^+-binding region (Kleiger and Eisenberg 2002).

Phylogenetic analysis of the *Xv*MIPS amino acid sequence is consistent with the data obtained when the amino acid sequences from MIPS sequences from evolutionarily diverse organisms were analysed (Majumder *et al*. 2003), revealing a high degree of sequence conservation between MIPS of different plant species.

Changes in levels of expression of XvINO1*,* Xv*MIPS and carbohydrate concentrations during salt and desiccation stress*

Both the *XvINO1* transcript and the *Xv*MIPS protein were constitutively expressed in non-stressed leaves of *X. viscosa* and expression did not change notably during daily cycling. Similar data have been reported for glycophytic plant species

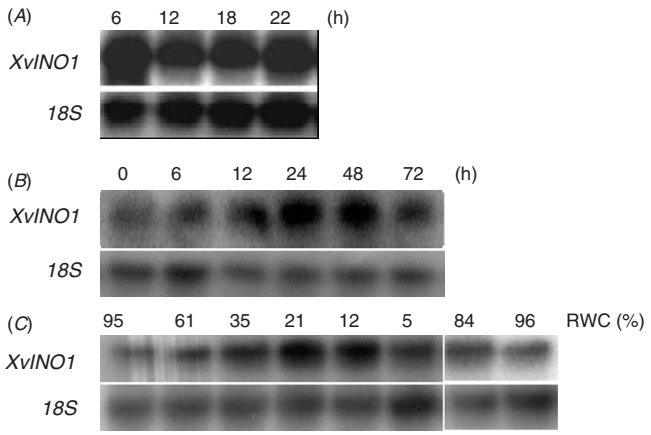

Fig. 5. Northern blot analysis of *XvINO1* expression. (*A*) Day/night cycle; (*B*) salt stress (100 mM); (*C*) dehydration/rehydration. The upper panel is the result of the hybridisation of the *XvINO1* probe and the lower panel is the hybridisation of the same blot with a 18S RNA probe for control purposes.

such as *A. thaliana* and *Z. mays* (Ishitani *et al*. 1996), whereas in halophytic plant species such as *M. crystallinum*, diurnal fluctuation in an *INO1*-like transcript has been reported where it is believed to play a role in the ultimate formation of D-pinitol, considered to be the principle osmoregulator in that species (Ishitani *et al*. 1996; Nelson *et al*. 1998). Since Ino plays such a central role in metabolism related to many different biochemical pathways associated with normal plant growth and development (see Introduction; reviewed in Loewus and Murthy 2000), constitutive expression in *X. viscosa* is not surprising. Excess salinity is not a stress normally associated with the habitats in which *X. viscosa* occurs, but nevertheless, this plant was able to withstand at least 300 mM NaCl (cumulative addition over 3 days) without visible physiological or anatomical damage (Fig. 5*D*). This is in keeping with the extremophille nature of resurrection plants in the *Xerophyta* genus (Mundree *et al*. 2002; Farrant *et al*. 2007).

Importantly, though, both the transcript and the protein levels increased in leaves of *X. viscosa* in response to both salt and desiccation stress and declined again after stress relief in the case of rehydration of desiccated plants (Figs 6 and 7). Since the plants survived these abiotic stresses at the levels imposed here, we propose that this gene (and its downstream products, discussed below) might play a contributory role in the tolerance of *X. viscosa* to at least 300 mM NaCl and desiccation to the air dry state. *XvINO1* transcript levels increased shortly after the application of both stresses (Fig. 6) and preceded increases in the MIPS protein levels (Fig. 7). However, these were generally preceded by substantial increases in levels of soluble sugars in the salt stress treatments (Fig. 8) suggesting that the elevation in carbohydrate levels came about, at least initially, due to fine control regulation at the level of the existing enzymes themselves. We postulate that coarse control at the level of transcription/translation might come into play when stresses are maintained, and that this control declines quite sharply upon cessation the stress.

The substantial increase of the concentrations of Ino, but also of those in the related, downstream carbohydrates, Gol, Raf and Sta in response to salt stress is in accordance with the suggestion that *INO* or, more correctly the MIPS enzymes, play a key regulatory role in carbon partitioning in response to this stress (Ishitani *et al*. 1996; Nelson *et al*. 1998; Wang *et al*. 2003; Taji *et al*. 2004) and abiotic stress is general (Bohnert *et al*. 1995; Cushman 2001; Xiong and Zhu 2002; Munns 2005) and that this might be true also in *X. viscosa*. In contrast, Ino decreased to one third of its original level in response to desiccation, concomitant with large increases in Raf and Sta content (Peters *et al*. 2007). Those authors proposed that this reduction in Ino is due to it being channelled into Gol biosynthesis to provide galactosyl donors for RFO biosynthesis, which in turn protect the subcellular milieu

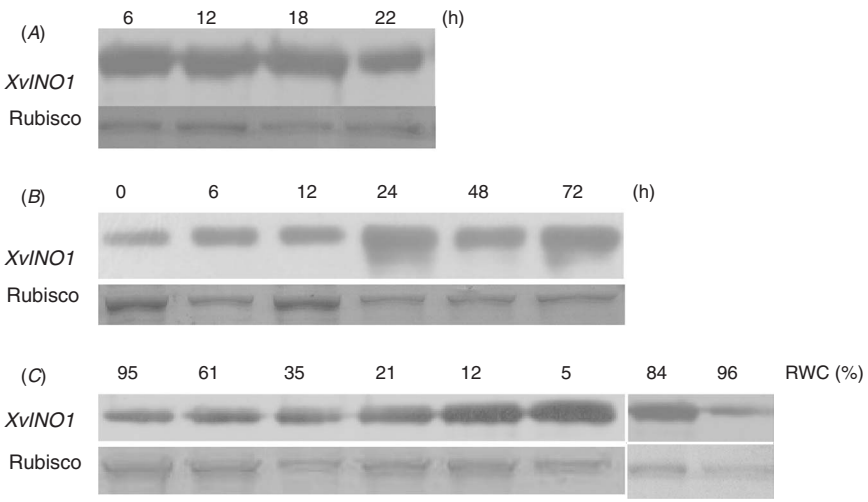

Fig. 6. Western blot analysis of *Xv*MIPS expression. (*A*) Day/night cycle; (*B*) salt stress (100 mM); (*C*) dehydration/rehydration. The upper panel is the result of the hybridisation of the *Xv*MIPS antibody and the lower panel is the hybridisation of the same blot using an antibody to Rubisco.

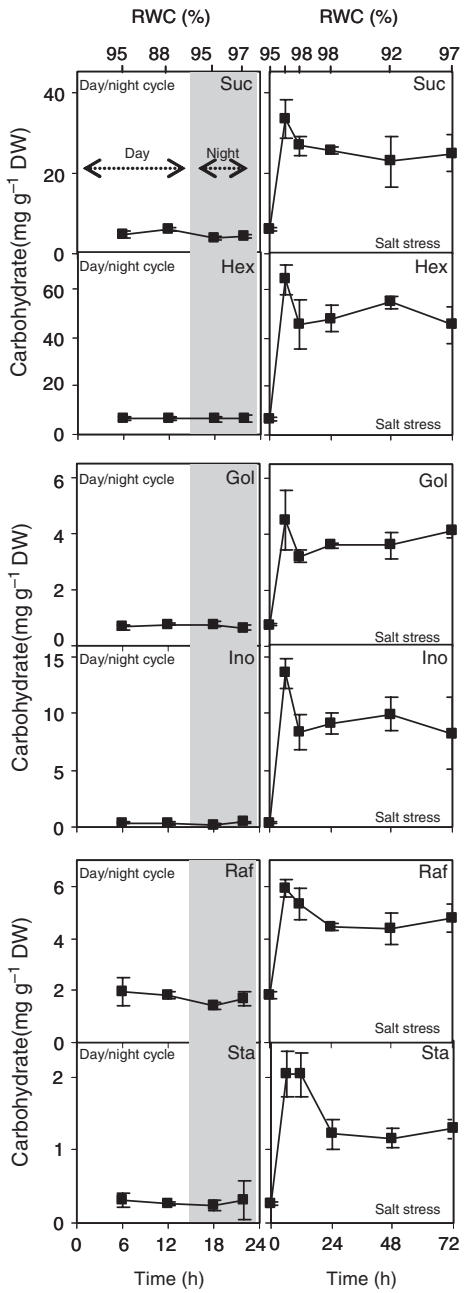

Fig. 7. Changes in the carbohydrate content (mg g^{-1} DW) in leaves of fully hydrated *Xerophyta viscosa* plants during a day/night cycle (left panel) and in salt treated (100 mM) plants (right hand panel). The lower *x*-axis gives the treatment duration on an hourly or daily basis. The upper *x*-axis gives the leaf relative water content (RWC, %) during each treatment. Means of four replicates ± s.d. When no bar is shown, the s.d. is less than the symbol. Suc, sucrose; Hex, hexoses (sum of glucose, fructose and galactose); Gol, galactinol; Ino, inositol; Raf, raffinose; Sta, stachyose.

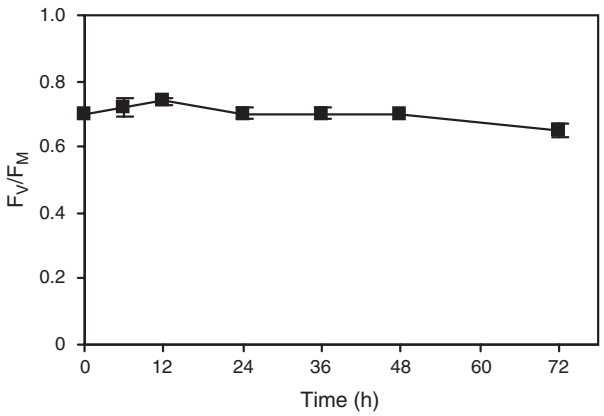

Fig. 8. The effect of salt treatment (100 mM) on PSII activity in leaves of *Xerophyta viscosa*. Means of eight replicates ± s.d. When no bar is shown, the s.d. is less than the symbol.

against effect of water deficit. In the longer term, Ino might be channelled into other metabolic pathways related to recovery and/or repair initiated upon rehydation, including membrane biogenesis and synthesis of cell wall components (Loewus and Murthy 2000).

It has been argued that, at least for desiccation sensitive (mesic) plants, the initial response (minutes to hours) to both salt and water deficit stress is similar, and is geared towards the maintenance of cellular water content of roots and leaves (Cushman 2001; Munns 2002, 2005; Xiong and Zhu 2002). This might conceivably also be true for angiosperm resurrection plants, since they also initially maintain a high water content while protection mechanisms are accumulated, after which water loss occurs relatively rapidly (reviewed in Farrant *et al*. 2007). Under salt stress, maintenance of water content has been reported to be achieved by accumulation of osmolytes, notably polyols and sugars (Bohnert *et al*. 1995; Cushman 2001; Munns 2002, 2005; Xiong and Zhu 2002). We have shown here a very rapid (<6 h) and considerable increase in concentrations of Ino, but also of Hex, Suc and RFOs in leaves of *X. viscosa* (Fig. 8) in response to salt treatment, which we propose contribute to the immediate and sustained maintenance of leaf osmotic potential, water uptake and thus leaf turgor. Interestingly, similar increases (both qualitatively and quantitatively) in carbohydrates do not occur in this time frame in water-deficit stressed *X. viscosa* (Peters *et al*. 2007) albeit that water content is maintained relatively high over the first 7 days (Fig. 5) but instead accumulate when RWC is rapidly declining (Whittaker *et al*. 2001; Peters *et al*. 2007). This is also true of many other resurrection plant species (Bianchi *et al*. 1991; Ghasempour *et al*. 1998; Norwood *et al*. 2000, 2003; Bartels and Salamini 2001; Whittaker *et al*. 2004; Illing *et al*. 2005; Moore *et al*. 2006; Farrant *et al*. 2007) and we propose that their role is to protect against subcellular effects of desiccation rather than for the maintenance of water content as would be the case in desiccation-sensitive (mesic) species.

Since most plants are capable of excluding at best only ~98% of the salt in the soil solution (Munns 2005), we assume that there was some salt accumulation (presumably within vacuoles) within the leaves of *X. viscosa* during the course of this experiment. Thus, the sustained high levels of Ino and

the other carbohydrates measured here could also have served to ameliorate against the vacuolar and cytoplasmic sodium concentration. In addition to facilitating control of osmotic balance, Ino is believed to function in salt tolerance specifically by quenching ROS induced by the effects of salt accumulation in the cytoplasm (Loewus and Murthy 2000; Cushman 2001; Yancey 2005) and organelles such as the chloroplast and mitochondria (RayChaudhuri and Majumder 1996; Nelson *et al*. 1998; Abreu and Aragao 2007) and that this polyol might perform a similar function in *X. viscosa*. The maintenance of PSII activity (Fig. 8) could well have been facilitated by Ino accumulation within the chloroplast, as has been reported for other species such as *Euglena gracilis* Z., *O. sativa* and *Vigna radiata* (L.) wilchzek (Adhikari *et al*. 1987; RayChaudhuri and Majumder 1996; Nelson *et al*. 1998). Hex, disaccharides and particularly Ino and Gol have been shown to be present in higher levels in the salt-tolerant *Thellungiella halphila* O.E. Schulz compared with its sensitive relative *A. thaliana*, and their presence and accumulation in the former is thought to play a role in prevention of sodium induced protein destabilisation and in protein refolding upon alleviation of the stress (Gong *et al*. 2005). Similar roles have been ascribed to Suc and Raf (Bohnert *et al*. 1995; Yancey 2005; Kim *et al*. 2007). Finally, sugars in general are also speculated to serve as an energy source for repair and/or resynthesis of sodium-destabilised macromolecules as salt further accumulates in the cytoplasm of salt-treated plants (Bohnert *et al*. 1995; Balibrea *et al*. 1997; Yancey 2005). The carbohydrates accumulated within leaves of *X. viscosa* may well fulfill some or all these roles in the face of the salt treatment imposed here.

Although specific sugar data is not given here, we have recently reported (Peters *et al*. 2007) that Suc, Raf and Sta accumulate concomitant with the decline in leaf water content and we have proposed above that these sugars play a specific role in subcellular protection against desiccation-associated damage. Suc is accumulated in response to desiccation in all resurrection plants and orthodox seeds tested to date and is thus well established as an important component of the protection system in higher plants (reviewed in Vertucci and Farrant 1995; Berjak 2006; Berjak *et al*. 2007; Farrant *et al*. 2007). Current thinking (Berjak 2006; Berjak *et al*. 2007) is that the most likely role that Suc plays is to form the basis for the formation of a cytoplasmic glassy state, formed at low RWC, that restrains molecular mobility, decreases the occurrence of deleterious chemical reactions, ROS formation and facilitates the protection and stabilisation of membranes, organelles and macromolecules (Burke 1986; Leprince and Walters-Vertucci 1995; Walters 1998; Buitink *et al*. 2002; Berjak 2006; Berjak *et al*. 2007). Suc-based glasses are stabilised and made more effective in the presence of RFOs, notably Raf and Sta (Caffrey *et al*. 1988; Obendorf 1997; Buitink *et al*. 2002; Koster and Bryant 2005) and proteins such as late embryogenesis abundant (LEA) proteins (Wolkers *et al*. 2001; Berjak 2006; Berjak *et al*. 2007). The high levels of both Raf and Sta in dry leaves of *X. viscosa* (Peters *et al*. 2007) and other resurrection plants such as *Myrothamnus flabellifolius* Welw. (Moore *et al*. 2007) and *Eragrostis nindensis* Ficalho & Hiern (Ghasempour *et al*. 1998) might well perform such a stabilising role.

In summary, we cloned and functionally identified a cDNA isolated from the leaves of *X. viscosa* plants encoding a MIPS enzyme involved in Ino synthesis. We have demonstrated that both the corresponding RNA and protein are accumulated during salt and water deficit stress resulting in the accumulation of Ino during salt stress, and of Raf and Sta (RFOs produced downstream from Ino in the cytomatrical pathway of RFO synthesis) during desiccation stress (Peters *et al*. 2007). We have proposed that different roles are played by the carbohydrates produced under these two stresses. Under salt stress, they facilitate maintenance of leaf water content in the short-term, as well as ultimately perhaps protecting against the effects of salt-induced protein denaturation and ROS formation, the latter being of particular importance in chloroplasts. During water deficit stress, the carbohydrates produced do not apparently play a role in the maintenance of water balance (although this might be true in non-resurrection plants), but rather protect the subcellular milieu against desiccation damage, probably by formation of stable cytoplasmic glasses which put a stasis on metabolism and ROS formation. Since *INO* plays a relatively key role in the synthesis of both Ino and RFOs (Obendorf 1997), this is a potentially important candidate for use in production of crops that are more tolerant to abiotic stresses such as drought and salinity. While engineering of such crops is likely to involve many and complex traits, insight gained by characterising roles of individual gene candidates is a starting point towards understanding the bigger picture.

References

Abreu EFM, Aragao FJL (2007) Isolation and characterization of *myo*-inositol-1-phosphate synthase gene from yellow passion fruit (*Passiflora edulis* f. *flavicarpa*) expressed during seed development and environmental stress. *Annals of Botany* **99**, 285–292. doi: 10.1093/aob/mcl256

Adhikari J, Majumder AL, Bhaduri TJ, DasGupta S, Majumder AL (1987) Chloroplast as a locale of L-*myo*-inositol-1-phosphate synthase. *Plant Physiology* **85**, 611–614.

Altschul SF, Gish W, Miller W, Meyers EW, Lipman DJ (1990) Basic local alignment search tool. *Journal of Molecular Biology* **215**, 403–410.

Ashraf M (1994) Breed for salinity tolerance in plants. *Critical Reviews in Plant Sciences* **166**, 3–16.

Ashraf M, Foolad MR (2007) Roles of glycine betaine and proline in improving plant abiotic stress tolerance. *Environmental and Experimental Botany* **59**, 206–216. doi: 10.1016/j.envexpbot.2005.12.006

Avigad G, Dey PM (1997) Carbohydrate metabolism: storage carbohydrate. In 'Plant biochemistry'. (Eds PM Dey, JB Harborne) pp. 143–204. (Academic Press: San Diego)

Balibrea ME, Rus-Alvarez AM, Bolarín MC, Pérez-Alfocea F (1997) Fast changes in soluble carbohydrates and proline contents in tomato seedlings in response to ionic and non-ionic iso-osmotic stresses. *Journal of Plant Physiology* **151**, 221–226.

Bartels D, Salamini F (2001) Desiccation tolerance in the resurrection plant *Craterostigma plantigineum.* a contribution to the study of drought tolerance at the molecular level. *Plant Physiology* **127**, 1346–1353. doi: 10.1104/pp.127.4.1346

Berjak P (2006) Unifying perspectives of some mechanisms basic to desiccation tolerance across life forms. *Seed Science Research* **16**, 1–15. doi: 10.1079/SSR2005236

Berjak P, Farrant JM, Pammenter NW (2007) Seed desiccation-tolerance mechanisms. In 'Plant desiccation tolerance'. (Eds MA Jenks, AJ Wood) pp. 151–192. (Blackwell Publishing: Oxford, UK)

Bernal-Lugo I, Leopold AC (1995) Seed stability during storage: raffinose content and seed glassy state. *Seed Science Research* **5**, 75–80.

Bewley JD, Oliver MJ (1992) Desiccation-tolerance in vegetative plant tissues and seeds: protein synthesis in relation to desiccation and a potential role for protection and repair mechanism. In 'Water and life: a comparative analysis of water relationships at the organismic, cellular and molecular levels'. (Eds GN Somero, CB Osmond, CL Bolis) pp. 141–160. (Springer: Berlin, Heidelberg, New York)

Bianchi G, Murelli C, Bochicchio A, Vazzana C (1991) Changes in low molecular weight substances in *Boea hygroscopica* in response to desiccation and rehydration. *Phytochemistry* **30**, 461–466. doi: 10.1016/0031-9422(91)83705-P

Bohnert HJ, Nelson DE, Jensen RG (1995) Adaptations to environmental stresses. *The Plant Cell* **7**, 1099–1111. doi: 10.2307/3870060

Boyer JS (1982) Plant productivity and environment. *Science* **218**, 443–448. doi: 10.1126/science.218.4571.443

Boyer JS, Westgate ME (2004) Grain yields with limited water. *Journal of Experimental Botany* **55**, 2385–2394. doi: 10.1093/jxb/erh219

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* **72**, 248–254. doi: 10.1016/0003-2697(76)90527-3

Buitink J, Hoekstra FA, Leprince O (2002) Biochemistry and biophysics of tolerance systems. In 'Desiccation and survival in plants. Drying without dying'. (Eds M Black, HW Pritchard) pp. 293–318. (CABI Publishing: Wallingford, Oxon)

Burke MJ (1986) The glassy state and survival of anhydrous biological systems. In 'Membranes, metabolism and dry organisms'. (Ed. AC Leopold) pp. 358–363. (Cornell University Press: Ithaca, New York)

Caffrey M, Fonseca V, Leopold AC (1988) Lipid-sugar interactions. *Plant Physiology* **86**, 754–758.

Chiera JM, Streeter JG, Finer JJ (2006) Ononitol and pinitol production in transgenic soybean containing the *inositol methyl transferase* gene from *Mesembryanthemum crystallinum. Plant Science* **171**, 647–654. doi: 10.1016/j.plantsci.2006.06.006

Chun JA, Jin UH, Lee JW, Yi YB, Hyung NI, *et al*. (2003) Isolation and characterization of a *myo*-inositol-1-phosphate synthase cDNA from developing sesame (*Sesamum indicum* L.) seeds: functional and differential expression, and salt-induced transcription during germination. *Planta* **216**, 874–880.

Cosgrove DJ (1980) 'Inositol phosphates: their chemistry, biochemistry and physiology.' (Elsevier: Amsterdam)

Crowe JH, Crowe LM, Carpenter JF, Widstrom A (1987) Stabilization of dry phospholipid bilayers and proteins by sugars. *The Biochemical Journal* **242**, 1–10.

Cushman JC (2001) Osmoregulation in plants: implications for agriculture. *American Zoologist* **41**, 758–769. doi: 10.1668/0003-1569(2001)041[0758:OIPIFA]2.0.CO;2

Donahue TF, Henry SA (1981) *Myo*-inositol 1-phosphate synthase: characteristics of the enzyme and identification of its structural gene in yeast. *Journal of Biological Chemistry* **256**, 7077–7085.

Farrant JM (2000) Comparison of mechanisms of desiccation tolerance among three angiosperm resurrection plants. *Plant Ecology* **151**, 29–39. doi: 10.1023/A:1026534305831

Farrant JM, Brandt W, Lindsey GG (2007) An overview of mechanisms of desiccation tolerance in selected angiosperm resurrection plants. *Plant Stress Journal* **1**, 72–84.

Flowers TJ (2004) Improving salt stress tolerance. *Journal of Experimental Botany* **55**, 307–319. doi: 10.1093/jxb/erh003

Gaff DF (1971) Desiccation tolerance flowering plants in southern Africa. *Science* **174**, 1033–1034. doi: 10.1126/science.174.4013.1033

Gaff DF (1989). Responses of desiccation tolerant 'resurrection' plants to water deficit. In 'Adaptation of plants to water and high temperature stress'. (Eds KH Kreeb, H Richter, TM Hinckley) pp. 207–230. (Academic Publishing: The Hague, The Netherlands)

Garwe D, Thomson JA, Mundree SG (2003) Molecular characterization of *XvSAP1*, a stress-responsive gene from the resurrection plant *Xerophyta viscosa* Baker. *Journal of Experimental Botany* **54**, 191–201. doi: 10.1093/jxb/54.381.191

Ghasempour HR, Gaff DF, Williams RPW, Gianello RD (1998) Contents of sugars in leaves of drying desiccation tolerant flowering plants, particularly grasses. *Plant Growth Regulation* **24**, 185–191. doi: 10.1023/A:1005927629018

Gong Q, Li P, Ma S, Indu Rupassara S, Bohnert HJ (2005) Salinity stress adaptation competence in the extremophile *Thellungiella halophila* in comparison with its relative *Arabidopsis thaliana. The Plant Journal* **44**, 826–839. doi: 10.1111/j.1365-313X.2005.02587.x

Hasegawa PM, Bressan RA, Zhu J-K, Bohnert HJ (2000) Plant cellular and molecular responses to high salinity. *Annual Review of Plant Physiology and Plant Molecular Biology* **51**, 463–499. doi: 10.1146/annurev.arplant.51.1.463

Illing N, Denby K, Collett H, Shen A, Farrant JM (2005) The signature of seeds in resurrection plants: a molecular and physiological comparison of desiccation tolerance in seeds and vegetative tissues. *Integrative and Comparative Biology* **45**, 771–787. doi: 10.1093/icb/45.5.771

Ingram J, Bartels D (1996) The molecular basis of dehydration tolerance in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**, 377–403. doi: 10.1146/annurev.arplant.47.1.377

Ishitani M, Majumder AL, Bornhouser A, Michalowski CB, Jensen RG, Bohnert HJ (1996) Coordinate transcriptional induction of *myo*-inositol metabolism during environmental stress. *The Plant Journal* **9**, 537–548. doi: 10.1046/j.1365-313X.1996.09040537.x

Iyer R, Mundree SG, Rafudeen S, Thomson JA (2007) XvSAP1, a desiccation tolerance gene with potential in crop improvement. In 'Plant desiccation tolerance'. (Eds MA Jenks, AJ Wood) pp. 283–296. (Blackwell Publishing: Oxford, UK)

Jin S, Chen CCS, Plant AL (2000) Regulation by ABA osmotic-stress induced changes in protein synthesis in tomato roots. *Plant, Cell & Environment* **23**, 51–60. doi: 10.1046/j.1365-3040.2000.00520.x

Keller F, Ludlow MM (1993) Carbohydrate metabolism in drought-stressed leaves of pigeon pea (*Cajanus cajan*). *Journal of Experimental Botany* **44**, 1351–1359. doi: 10.1093/jxb/44.8.1351

Keller F, Pharr DM (1996) Metabolism of carbohydrates in sinks and source: galactosyl-sucrose oligosaccharides. In 'Photoassimilate distribution in plants and crops: source-sink relationships'. (Eds E Zamski, AA Schaffer) pp. 157–183. (Marcel-Dekker: New York)

Kim JK, Bamba T, Harada K, Fukusaki E, Kobayashi A (2007) Time-course metabolic profiling in *Arabidopsis thaliana* cell cultures after salt stress treatment. *Journal of Experimental Botany* **58**, 415–424. doi: 10.1093/jxb/erl216

Klages K, Boldingh H, Smith GS (1999) Accumulation of *myo*-inositol in *Actinidia* seedlings subjected to salt stress. *Annals of Botany* **84**, 521–527. doi: 10.1006/anbo.1999.0946

Kleiger G, Eisenberg D (2002) GXXXG and GXXXA motifs stabilize FAD and NAD(P)-binding Rossmann folds through C-alpha-H.O hydrogen bonds and van der Waals interactions. *Journal of Molecular Biology* **323**, 69–76. doi: 10.1016/S0022-2836(02)00885-9

Koster KL, Bryant G (2005) Dehydration in model membranes and protoplasts: contrasting effects at low, intermediate and high hydrations. In 'Cold hardiness in plants: molecular genetics, cell biology and physiology'. (Eds THH Chen, M Uemura, S Fujikawa) pp. 219–234. (CAB International: Wallingford, UK)

Leopold AC, Sun WQ, Bernard-Lugo I (1994) The glassy state in seed: analysis and function. *Seed Science Research* **4**, 267–274.

Leprince O, Walters-Vertucci C (1995) A calorimetric study of glass transition behaviors in axes of bean with relevance to storage ability. *Plant Physiology* **109**, 1471–1481.

Loewus FA, Loewus MW (1983) *Myo*-inositol: its biosynthesis and metabolism. *Annual Review of Plant Physiology* **34**, 137–161. doi: 10.1146/annurev.pp.34.060183.001033

Loewus FA, Murthy PPN (2000) *Myo*-inositol metabolism in plants. *Plant Science* **150**, 1–19. doi: 10.1016/S0168-9452(99)00150-8

Majee M, Maitra S, Dastidar KG, Pattnaik S, Chatterjee A, Hait NC, Das KP, Majumder AL (2004) A novel salt-tolerant L-*myo*-inositol-1-phosphate synthase from *Posteresia coarctata* (Roxb.) Tateoka, a halophytic wild rice. *Journal of Biological Chemistry* **279**, 28539–28552. doi: 10.1074/jbc.M310138200

Majumder AL, Dattagupta S, Goldwasser P, Donahue TF, Henry SA (1981) The mechanism of interallelic complementation at the *ino-1* locus in yeast: immunological analysis of mutants. *Molecular & General Genetics* **184**, 347–354. doi: 10.1007/BF00352503

Majumder AL, Chatterjee A, Dastidar KG, Majee M (2003) Diversification and evolution of L-*myo*-inositol 1-phosphate synthase. *FEBS Letters* **553**, 3–10. doi: 10.1016/S0014-5793(03)00974-8

Marais S, Thomson JA, Farrant JM, Mundree SG (2004) VATP1XV- a novel stress responsive V-ATPase subunit c homologue isolated from the resurrection plant *Xerophyta viscosa* Baker. *Physiologia Plantarum* **122**, 54–61. doi: 10.1111/j.1399-3054.2004.00389.x

Moore JP, Nguema-Ona E, Chevalier L, Lindsey GG, Brandt WF, Lerouge P, Farrant JM, Driouich A (2006) Response of the leaf cell wall to desiccation in the resurrection plant *Myrothamnus flabellifolius. Plant Physiology* **141**, 651–662. doi: 10.1104/pp.106.077701

Moore JP, Hearshaw M, Ravenscroft N, Lindsey GG, Farrant JM, Brandt WF (2007) Desiccation-induced ultrastructural and biochemical changes in the leaves of the resurrection plants *Myrothamnus flabellifolia. Australian Journal of Botany*, in press.

Mowla SB, Thomson JA, Farrant JM, Mundree SG (2002) A novel stress-inducible antioxidant enzyme identified from the resurrection plant *Xerophyta viscosa* Baker. *Planta* **215**, 716–726. doi: 10.1007/s00425-002-0819-0

Mundree SG, Farrant JM (2000) Some physiological and molecular insights into the mechanisms of desiccation tolerance in the resurrection plant *Xerophyta viscosa* Baker. In 'Plant tolerance to abiotic stresses in agriculture: role of genetic engineering'. (Eds JH Cherry, RD Locy, A Rychter) pp. 201–222. (Kluwer Academic Publishers: The Netherlands)

Mundree SG, Whittaker A, Thomson JA, Farrant JM (2000) An aldose reductase homolog from the resurrection plant *Xerophyta viscosa* Baker. *Planta* **211**, 693–700. doi: 10.1007/s004250000331

Mundree SG, Baker B, Mowla S, Peters S, Marais S, Vander Willigen C, Govender K, Maredza A, Farrant JM, Thomson JA (2002) Physiological and molecular insights into drought tolerance. *African Journal of Biotechnology* **1**, 28–38.

Munns R (2002) Comparative physiology of salt and water stress. *Plant, Cell & Environment* **25**, 239–250. doi: 10.1046/j.0016-8025.2001.00808.x

Munns R (2005) Genes and salt tolerance: bringing them together. *The New Phytologist* **167**, 645–663. doi: 10.1111/j.1469-8137.2005.01487.x

Ndima T, Farrant JM, Thomson JA, Mundree S (2001) Molecular and characterization of *XVT8*, a stress-responsive gene from the resurrection plant *Xerophyta viscosa* baker. *Plant Growth Regulation* **35**, 137–145. doi: 10.1023/A:1014433821730

Nelson DE, Rammesmayer G, Bohnert HJ (1998) Regulation of cell-specific inositol metabolism and transport in plant salinity tolerance. *The Plant Cell* **10**, 753–764. doi: 10.2307/3870662

Nielsen H, Engelbrecht J, Brunak S, von Heijne G (1997) Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Engineering* **10**, 1–6. doi: 10.1093/protein/10.1.1

Norwood M, Truesdale MR, Richter A, Scott P (2000) Photosynthetic carbohydrate metabolism in the resurrection plant *Craterostigma plantigineum. Journal of Experimental Botany* **51**, 159–165. doi: 10.1093/jexbot/51.343.159

Norwood M, Toldi O, Richter A, Scott P (2003) Investigation into the ability of roots of the poikilohydric plant *Craterostrigma plantigineum* to survive dehydration stress. *Journal of Experimental Botany* **54**, 2313–2321. doi: 10.1093/jxb/erg255

Obendorf RL (1997) Oligosaccharides and galactosyl cyclitols in seed desiccation tolerance. *Seed Science Research* **7**, 61–74.

Oliver MJ, Wood AJ, O'Mahony P (1998) "To dryness and beyond" – preparation for the dried state and rehydration in vegetative desiccation-tolerant plants. *Plant Growth Regulation* **24**, 193–201. doi: 10.1023/A:1005863015130

Peterbauer T, Puschenreiter M, Richter A (1998) Metabolism of galactosylononitol in seeds of *Vigna umbellata. Plant & Cell Physiology* **39**, 334–341.

Peterbauer T, Richter A (2001) Biochemistry and physiology of raffinose family oligosaccharides and galactosyl cyclitols in seeds. *Seed Science Research* **11**, 185–197.

Peters SW, Mundree SG, Thomson JA, Farrant JM, Keller F (2007) Protection mechanism in the resurrection plant *Xerophyta viscosa* (Baker): both sucrose and raffinose oligosaccharides (RFOs) accumulate in leaves in response to water deficit. *Journal of Experimental Botany* **58**, 1947–1956. doi: 10.1093/jxb/erm056

Ramanjulu S, Bartels D (2002) Drought- and desiccation-induced modulation of gene expression in plants. *Plant, Cell & Environment* **25**, 141–151. doi: 10.1046/j.0016-8025.2001.00764.x

RayChaudhuri A, Majumder AL (1996) Salinity-induced enhancement of L-*myo*-inositol 1-phosphate synthase in rice (*Oryza sativa* L.). *Plant, Cell & Environment* **19**, 1437–1442. doi: 10.1111/j.1365-3040.1996.tb00023.x

RayChaudhuri A, Hait NC, DasGupta S, Bhaduri TJ, Deb R, Majumder AL (1997) L-*myo*-inositol-1-phosphate synthase from plant source. Characteristics of the chloroplastic and cytosolic enzymes. *Plant Physiology* **115**, 727–736.

Sambrook J, Fritsch EF, Maniatis T (1989) 'Molecular cloning: a laboratory manual.' 2nd edn. (Cold Spring Harbour Laboratory Press: New York)

Schramm G, Bruchhaus I, Roeder T (2000) A simple and reliable 5′-RACE approach. *Nucleic Acids Research* **28**, e96. doi: 10.1093/nar/28.22.e96

Serraj R, Sinclair TR (2002) Osmolyte accumulation: can it really help increase crop yield under drought conditions? *Plant, Cell & Environment* **25**, 333–341. doi: 10.1046/j.1365-3040.2002.00754.x

Serrano R, Mulet JM, Rios G, Marquez JA, de Larrinoa IF, *et al*. (1999) A glimpse of the mechanisms of ion homeostasis during salt stress. *Journal of Experimental Botany* **50**, 1023–1036. doi: 10.1093/jexbot/50.suppl_1.1023

Sherwin HW, Farrant JM (1996) Differences in rehydration of three desiccation-tolerant angiosperm species. *Annals of Botany* **78**, 703–710. doi: 10.1006/anbo.1996.0180

Sherwin HW, Farrant JM (1998) Protection mechanism against excess light in the resurrection plants *Craterostigma wilmsii* and *Xerophyta viscosa. Plant Growth Regulation* **24**, 203–210. doi: 10.1023/A:1005801610891

Sheveleva E, Chmara W, Bohnert HJ, Jensen RG (1997) Increased salt and drought tolerance by D-ononitol production in transgenic *Nicotiana tabacum* L. *Plant Physiology* **115**, 1211–1219.

Smart CC, Fleming AJ (1993) A plant gene with homology to D-*myo*-inositol-3-phosphate synthase is rapidly and spatially up-regulated during an abscissic-acid-induced morphogenic response in *Spirodela polyrrhiza. The Plant Journal* **4**, 279–293. doi: 10.1046/j.1365-313X.1993.04020279.x

Smirnoff N (1998) Plant resistance to environmental stress. *Current Opinion in Biotechnology* **9**, 214–219. doi: 10.1016/S0958-1669(98)80118-3

Sprenger N, Keller F (2000) Allocation of raffinose family oligosaccharides to transport and storage pools in *Ajuga reptans*: the role of two distinct galactinol synthases. *The Plant Journal* **21**, 249–258. doi: 10.1046/j.1365-313x.2000.00671.x

Sun WQ (1997) Glassy state and seed storage stability: the WLF kinetics of seed viability loss at T greater than T-g and the plasticization effect of water on storage stability. *Annals of Botany* **79**, 291–297. doi: 10.1006/anbo.1996.0346

Stein AJ, Geiger JH (2002) The crystal structure and mechanism of 1-L-*myo*-inositol-1-phosphate synthase. *Journal of Biological Chemistry* **277**, 9484–9491. doi: 10.1074/jbc.M109371200

Taji T, Seki M, Satou M, Sakurai T, Kobayashi M, Ishiyama K, Narusaka Y, Narusaka M, Zhu JK, Shinozaki K (2004) Comparative genomics in salt tolerance between *Arabidopsis* and *Arabidopsis*-related halophyte salt cress using *Arabidopsis* micro-array. *Plant Physiology* **135**, 1697–1709. doi: 10.1104/pp.104.039909

Tapernoux-Lüthi EM, Böhm A, Keller F (2004) Cloning, functional expression, and characterization of the raffinose oligosaccharide chain elongation enzyme, galactan: galactan galactosyltransferase, from common bugle leaves. *Plant Physiology* **134**, 1377–1387. doi: 10.1104/pp.103.036210

Valliyodan B, Nguyen HT (2006) Understanding regulatory networks and engineering for enhanced drought tolerance in plant. *Current Opinion in Plant Biology* **9**, 189–195. doi: 10.1016/j.pbi.2006.01.019

Vernon DM, Bohnert HJ (1992) A novel methyl transferase induced by osmotic stress in the facultative halophyte *Mesembryanthemum crystallinum. The EMBO Journal* **11**, 2077–2085.

Vertucci CW, Farrant JM (1995) Acquisition and loss of desiccation tolerance. In 'Seed development and germination'. (Eds J Kigel, G Galilli) pp. 237–271. (Marcel Dekker: New York)

Vicre M, Farrant JM, Gibouin D, Driouich A (2003) Resurrection plants: how to cope with desiccation? *Recent Research Developments in Plant Biology* **3**, 69–93.

Vicre M, Farrant JM, Driouich A (2004) Insights into the mechanisms of desiccation tolerance among resurrection plants. *Plant, Cell & Environment* **27**, 1329–1340. doi: 10.1111/j.1365-3040.2004.01212.x

Walters C (1998) Understanding the mechanisms and kinetics of seed aging. *Seed Science Research* **8**, 223–244.

Walford SA, Thomson JA, Farrant JM, Mundree SG (2004) Isolation and characterisation of a novel dehydration-induced Grp94 homologue from the resurrection plant *Xerophyta viscosa. South African Journal of Botany* **70**, 741–750.

Wang W, Vinocur B, Altman A (2003) Plant responses to drought, salinity and extreme temperatures towards genetic engineering for stress tolerance. *Planta* **218**, 1–14. doi: 10.1007/s00425-003-1105-5

Whittaker A, Bochicchio A, Vazzana C, Lindsey G, Farrant J (2001) Changes in leaf hexokinase activity and metabolite levels in response to drying in the desiccation-tolerant species *Sporobolus stapfianus* and *Xerophyta viscosa. Journal of Experimental Botany* **52**, 961–969. doi: 10.1093/jexbot/52.358.961

Whittaker A, Martinelli T, Bochicchio A, Vazzana C, Farrant J (2004) Comparison of sucrose metabolism during the rehydration of desiccation-tolerant and desiccation-sensitive leaf material of *Sporobolus stapfianus. Physiologia Plantarum* **122**, 11–20. doi: 10.1111/j.1399-3054.2004.00346.x

Wolkers WF, McCready S, Brandt WF, Lindsey GG, Hoekstra FA (2001) Isolation and characterization of a D-7 LEA protein in pollen that stabilizes glasses *in vitro. Biochimica et Biophysica Acta* **1544**, 196–206.

Xiong L, Zhu JK (2002) Molecular and genetic aspects of plant responses to osmotic stress. *Plant, Cell & Environment* **25**, 131–139. doi: 10.1046/j.1365-3040.2002.00782.x

Yancey PH (2005) Organic osmolytes as compatible, metabolic and counteracting cytoprotectants in high osmolarity and other stresses. *Journal of Experimental Biology* **208**, 2819–2830. doi: 10.1242/jeb.01730

Zhu JK (2001) Plant salt tolerance. *Trends in Plant Science* **6**, 66–71. doi: 10.1016/S1360-1385(00)01838-0

Zhu JK (2002) Salt and drought stress signal transduction in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **53**, 247–273.

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