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RESEARCH PAPER

Protection mechanisms in the resurrection plant *Xerophyta viscosa* (Baker): both sucrose and raffinose family oligosaccharides (RFOs) accumulate in leaves in response to water deficit

Shaun Peters[1,2], Sagadevan G. Mundree[1], Jennifer A. Thomson[1], Jill M. Farrant[1] and Felix Keller[2,*]

[1] *University of Cape Town, Department of Molecular and Cell Biology, Private Bag, Rhodes Gift, 7701, Cape Town, South Africa*

[2] *University of Zürich, Institute of Plant Biology, Molecular Plant Physiology, Zollikerstrasse 107, Zürich 8008, Switzerland*

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Abstract

Changes in water-soluble carbohydrates were examined in the leaves of the resurrection plant *Xerophyta viscosa* under conditions of water deficit. Sucrose and raffinose family oligosaccharides (RFOs), particularly raffinose, increased under these conditions, with the highest concentrations evident at 5% relative water content [RWC; 23.5 mg g^{-1} dry weight (DW) and 17.7 mg g^{-1} DW, respectively]. Importantly, these effects were reversible, with concentrations returning to levels comparable with that of the full turgor state 7 d after water deficit conditions were alleviated, providing evidence that both sucrose and RFOs may play a protective role in desiccated leaf tissue of *X. viscosa*. Further, because the sucrose-to-raffinose mass ratio of 1.3:1 observed in the dehydrated state was very low, compared with published data for other resurrection plants (always >5), it is suggested that, in *X. viscosa* leaves, RFOs serve the dual purpose of stress protection and carbon storage. *XvGolS*, a gene encoding a galactinol synthase enzyme responsible for the first catalytic step in RFO biosynthesis, was cloned and functionally expressed. In leaf tissue exposed to water deficit, *XvGolS* transcript levels were shown to increase at 19% RWC. GolS activity *in planta* could not be correlated with RFO accumulation, but a negative correlation was observed between RFO accumulation and *myo*-inositol depletion, during water deficit stress. This correlation was reversed after rehydration, suggesting that during water deficit *myo*-inositol is channelled into RFO synthesis, but during the rehydration process it is channelled to metabolic pathways related to the repair of desiccation-induced damage.

Key words: Desiccation tolerance, galactinol synthase, raffinose family oligosaccharides, resurrection plants.

Introduction

Some 330 angiosperm species have been described to date to belong to a class of plants displaying 'resurrection' capability (Proctor and Pence, 2002). Such plants are typically characterized by their ability to tolerate and survive extremes of vegetative desiccation, subsequently resuming normal cellular metabolism within a short period after water has become available again (Gaff, 1989; Farrant, 2000; Scott, 2000; Vicre *et al*., 2004). Angiosperm resurrection plants all appear to accumulate sucrose (Suc) during drying (Ingram *et al*., 1997; Ghasempour *et al*., 1998; Whittaker *et al*., 1999; Scott, 2000; Norwood *et al*., 2000; Cooper and Farrant, 2002; Zikovic *et al*., 2005). In some of the resurrection species, there are also water deficit-induced increases in the disaccharide, trehalose, and the raffinose family oligosaccharides (RFOs), but never to the same extent as that of Suc (Farrant, 2007).

RFOs have long been suggested to act as anti-stress agents in both generative and vegetative tissues (Koster and Leopold, 1988; Bachmann *et al*., 1994; Brenac *et al*.,

* To whom correspondence should be addressed. E-mail: fkel@botinst.uzh.ch

1997; Taji *et al*., 2002; Pennycooke *et al*., 2003). They are the most widely distributed non-structural carbohydrates in the plant kingdom, occurring in a wide variety of species (Keller and Pharr, 1996; Peterbauer *et al*., 2002). As non-reducing carbohydrates they are good storage compounds, being able to accumulate in large quantities without affecting primary metabolic processes. Research in seeds has revealed strong correlations between accumulation of RFOs, primarily raffinose (Raf), stachyose (Sta), and verbascose (Ver), and desiccation tolerance (Horbowicz and Obendorf, 1994; Hoekstra *et al*., 1997). Orthodox seeds (desiccation tolerant) show high RFO contents, whilst in most recalcitrant seeds (desiccation sensitive) RFOs are absent or detectable only in trace quantities (Lin and Huang, 1994; Sun *et al*., 1994). In orthodox seeds, RFO accumulation has been shown to coincide with the onset of desiccation tolerance during the maturation stage of seed development, and RFOs have thus been suggested to play a protective role against desiccation damage (Horbowicz and Obendorf, 1994; Black *et al*., 1996). In addition, RFO content has been positively correlated with seed longevity during storage (Horbowicz and Obendorf, 1994; Lin and Huang, 1994).

Chemically, RFOs are extensions of Suc to which galactosyl residues are attached via α-1,6 glycosidic linkages, leading to a range of oligosaccharides with degrees of polymerization (DPs) of up to 15. The RFO biosynthetic pathway begins with the synthesis of galactinol [Gol; *O*-α-D-galactopyranosyl-(1→1)-L-*myo*-inositol] from UDP-galactose (UDP-Gal) and *myo*-inositol, catalysed by the enzyme galactinol synthase (GolS; EC 2.4.1.123). Gol itself is found exclusively in plants and serves solely as the galactosyl donor for RFO biosynthesis (Keller and Pharr, 1996; Peterbauer *et al*., 2002). Traditionally, it has been assumed that GolS activity is the key regulatory factor in RFO biosynthesis (Handley *et al*., 1983).

The first RFO member, the trisaccharide Raf, is synthesized by the α-galactosyltransferase, Raf synthase (EC 2.4.1.82), which transfers a galactosyl moiety from Gol to Suc. Subsequent addition of a galactosyl moiety from Gol to Raf by Sta synthase (EC 2.4.1.67) produces Sta (DP 4). Higher DP RFOs are synthesized by the novel chain elongation enzyme, galactan–galactan galactosyl transferase, which operates independently of Gol, utilizing RFOs themselves as galactosyl donors and acceptors (Bachmann *et al*., 1994; Inan Haab and Keller, 2002; Tapernoux-Lüthi *et al*., 2004).

Xerophyta viscosa (family Vellociaceae) is a monocotyledonous poikilochlorophyllic resurrection plant, endemic to southern Africa, that is able to withstand water deficit stress to as low as 5% relative water content (RWC), restoring metabolism to a level comparable with that of the fully turgid state within 72 h after water becomes available (Farrant, 2000). As part of an ongoing project to understand the mechanisms of desiccation tolerance in resurrection plants, so as ultimately to genetically engineer the monocotyledonous crop plant *Zea mays* for abiotic stress tolerance, candidate genes that are differentially expressed in the leaves of the resurrection plant *X. viscosa* during water deficit have been isolated, identified, and characterized. Previous studies have reported: (i) increases in antioxidant enzymes during water deficit (Sherwin and Farrant, 1998); (ii) XvPer1, a novel stress-induced antioxidant (Mowla *et al*., 2002); (iii) a dehydration-induced aldose reductase implicated in sorbitol synthesis (Mundree *et al*., 2000); and (iv) XvSAP1, a protein thought to play a role in membrane stabilization during water deficit (Garwe *et al*., 2003). However, the role of compatible solutes, particularly oligosaccharides such as RFOs, in the desiccation tolerance of *X. viscosa* has not been investigated.

In this work, the successful functional identification of *XvGolS* (GenBank accession no. EF017945), a gene encoding GolS catalysing the first step of RFO biosynthesis, originally identified from a differential screen of a cDNA library constructed from the leaves of *X. viscosa* (Ndima *et al*., 2001) is described. It is also shown that under conditions of water deficit down to 5% RWC, Suc and Raf are the predominant soluble carbohydrates that accumulate in the leaves of *X. viscosa*.

Materials and methods

Unless otherwise stated, experiments were conducted at least twice with a minimum of four biological replicates per experiment.

Plant material

Xerophyta viscosa (Baker) plants were propagated from a sterilized seed stock by *in vitro* culture. Seeds were plated onto MS agar supplemented with 1.5% Suc (w/v) and the plates maintained in a controlled-environment chamber (16 h light, 30 μmol photons m^{-2} s^{-1}, 25 °C, 8 h dark, 60% relative humidity) until the seeds had germinated to plantlets approximately 1.5 cm in height. The plantlets were then transferred into sterile 200 ml bottles containing rooting medium [0.1% (w/v) MS salts, 0.5% (w/v) MS vitamins, 0.8% (w/v) agar, 0.1% (w/v) naphthalene acid, 0.01% (w/v) N^6-benzyl adenine, pH 5.8 KOH] and maintained in the controlled-environment chamber described above. Plantlets were transferred to pots containing equal parts of sterilized peat, potting soil, and vermiculite after approximately 1 year of growth, when they had reached 5 cm in height. The plants were environmentally hardened in a controlled-environment chamber (16 h light, 130 μmol photons m^{-2} s^{-1}, 25 °C, 8 h dark, 60% relative humidity) for 6 months prior to being transferred into glasshouse conditions previously described by Sherwin and Farrant (1996). Plants used for carbohydrate extractions and enzyme activity assays were maintained under greenhouse conditions, previously described by Bachmann *et al.* (1994), at the Institute of Plant Biology, University of Zürich, Switzerland.

Water deficit stress treatment

Water deficit stress was imposed on whole potted plants by withholding irrigation over a period of 20 d, at the end of which the RWC was determined to be 5%. Leaf samples were excised at regular intervals for RNA and carbohydrate extraction, flash-frozen

in liquid nitrogen and stored at −80 °C. Sampling times were determined by visual appraisal of the plant using leaf decoloration and folding as benchmarks, at which times RWC of leaves was determined for the samples. Rehydration was conducted by watering the plants and sampling as described above after 1 week.

RWC was determined at each sampling interval as follows. The initial weight (*Wi*) of each sample was recorded before immersing it in Milli-Q water for 24 h. The weight at full turgor (*Wt*) was recorded and leaf samples subsequently dried at 80 °C for 24 h, and the dry weight (*Wd*) was recorded. The *RWC* was calculated using the formula of Barrs and Weatherley (1962):

$RWC=[(Wi–Wd)/Wd]/\ [(Wt–Wd)/Wd]\times 100$

HPLC-PAD analysis and quantification of carbohydrates

Water-soluble carbohydrates were identified and quantified from plant extracts and enzyme assays by HPLC-PAD (Bachmann *et al*., 1994). A Ca/Na-moderated ion partitioning carbohydrate column was used to separate carbohydrates (Benson BC-100 column, 7.8×300 mm; Benson Polymeric, Reno, NE, USA). It was operated at 90 °C and isocratically eluted with 0.005% (w/v) Ca/Na_2-EDTA at a flow rate of 0.6 ml min^{-1}. 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) operated at 30 °C, and isocratically eluted with 0.6 N NaOH at a flow rate of 0.4 ml min^{-1}.

The BC-100 chromatographic system consisted of a Gynkotek model 480 High Precision Pump, a Gynkotek Gina 50 autosampler, the Chromeleon chromatography software package (version 6.4; Dionex, Olten, Switzerland), and a Jones column temperature controller (Ercatech, Berne, Switzerland). Carbohydrates were detected after post-column addition of NaOH (300 mM, 0.6 ml min^{-1}) using an ESA Coulochem II electrochemical detector (ESA, Cambridge, MA, USA), operated with an ESA 5040 analytical cell. The MA1 column was operated with a BioLC chromatographic system (Dionex). Soluble carbohydrates on both systems were quantified using the Chromeleon software package, against a series of 5 nmol standard sugars. The quantity of standard sugars used corresponds to the linear response range of both chromatographic systems.

Soluble carbohydrate extraction

Ground, freeze-dried *X. viscosa* leaf material (50 mg) was used to extract soluble carbohydrates. Extractions were conducted in 1.5 ml of ethanol [twice for each concentration of 80% and 20% (v/v), respectively] and 1.5 ml of 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* in a bench top centrifuge for 5 min. The supernatants of all extraction steps were pooled and volumes adjusted to 6 ml with dH_2O. Aliquots of 50 µl were desalted and analysed by HPLC-PAD as described.

Desalting of extracts

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

In control experiments, tests were conducted to determine if lipophilic substances (mainly glycosylated secondary plant products and glycolipids) would interfere with the HPLC methods used for carbohydrate quantification. To this end, representative samples of soluble carbohydrate extracts were delipidated by passage through methanol-activated reverse-phase cartridges (C_{18} Sep-Pak classic, 380 mg solid phase; Waters, Rupperswil, Switzerland). The HPLC profiles of non-delipidated and delipidated extracts were identical (data not shown), making a delipification step unnecessary.

Enzyme extractions and GolS activity assays

Plant crude extracts: Freshly harvested *X. viscosa* leaf material (2×8 cm pieces of leaf mid-section) was ground in 500 µl of chilled extraction buffer [50 mM HEPES/KOH pH 7.5, 5 mM $MgCl_2$, 1 mM EDTA, 20 mM dithiothreitol (DTT), 0.1% (v/v) Triton X-100, 1 mM benzamidine, 1 mM phenylmethylsulphonyl fluoride (PMSF), 50 mM Na-ascorbate, 2% (w/v) polyvinylpyrrolidone (PVP)]. Samples were centrifuged at 12 000 *g* (5 min, 4 °C). A 150 µl aliquot of supernatant was desalted by gel filtration at 1400 *g* (2 min, 4 °C) through 5 ml Sephadex G-25 columns (fine, final bed volume of 3 ml). Columns were pre-equilibrated with assay buffer (50 mM HEPES/KOH pH 7.5, 2 mM $MnCl_2$, 10 mM DTT). Pre-equilibration was performed twice with 2 ml of assay buffer. Aliquots (20 µl) of desalted extract were assayed for GolS activity in a final volume of 40 µl of assay buffer containing final concentrations of 50 mM *myo*-inositol and 5 mM UDP-gal, at 30 °C for 20 min. Samples were desalted as described above and analysed by HPLC-PAD.

Escherichia coli *crude extracts*: A single colony (*Escherichia coli* DH5α transformed with pPROEX HTb::*XvGolS*) was inoculated into 5 ml of Luria Broth (LB) with ampicillin (1 µg ml^{-1}), incubated overnight at 37 °C, and used as an inoculum for 100 ml of pre-warmed (37 °C) LB with ampicillin (1 µg ml^{-1}) incubated at 37 °C. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.8 mM when the culture had reached an OD_{590} of 0.8. Subsequently, after 4 h of growth, the cells were collected by centrifugation (Beckmann, 10 000 *g*, 10 min, 4 °C). Cell lysates were prepared under native conditions as described in the QIA*expressionist* user manual (Qiagen). GolS activity was assayed as described above, using 20 µl aliquots of cleared lysate. Negative controls representing *E. coli* (DH5α) that had been transformed with the pPROEX HTb vector were treated in the same manner as described above, desalted, and analysed by HPLC-PAD. Independent experiments were conducted using four individual *E. coli* colonies for inductions, and enzyme activities were measured in triplicate for each experiment.

Enzymatic hydrolysis of Gol: Fractions containing Gol synthesized by the recombinant XvGolS were collected after separation on a BC-100 column, prior to the post-column addition of NaOH. Samples were concentrated in a vacuum concentrator centrifuge and resuspended in 50 µl of McIlvaine buffer (48.5 mM citric acid, 103 mM disodium phosphate, pH 5.0). *Aspergillus niger* α-galactosidase (Megazyme International Ltd, Bray, Wicklow, Ireland) was added to a final concentration of 2 U per 50 µl reaction volume, and incubated at 40 °C for 1.5 h. Samples were desalted and analysed by HPLC-PAD as described, using the Benson BC-100 column.

Northern blot analysis

Total RNA was extracted from stress-treated *X. viscosa* leaves using the TRIZOL LS reagent (Life Technologies, Invitrogen Corporation, Carlsbad, CA, USA), following the manufacturer's

instructions. For RNA gel blot analysis, approximately 10 μg of RNA was electrophoresed in an agarose gel [1.2% (w/v) agarose, 1× TBE−90 mM TRIS-borate, 1 mM EDTA] pre-stained with ethidium bromide, transferred as previously described by Koetsier *et al*. (1993), and UV cross-linked onto nylon filters (Hybond-XL; Amersham Pharmacia Biotech). Filters were incubated in hybridization buffer [0.5 M NaH_2PO4, 0.001 M EDTA, 7% (w/v) SDS, 1% (w/v) bovine serum albumin (BSA)] at 65 °C with a radiolabelled *XvGolS* probe. (A radioactive PCR was conducted on *XvGolS* cDNA using primer A 5′-CTGCTTACCCCTTAGTGGTTGC-3′ and primer B 5′-AGCTTAAGCTGCTTCAAACCAGG-3′ with 50 μCi of [α^{32}P]dCTP.) Filters were washed twice for 10 min in wash buffer [0.1% (w/v) SDS, 0.5× SSC] and autoradiographed at −70 °C onto high-performance autoradiography film (Hyperfilm MP, Amersham Pharmacia Biotech). Subsequently, filters were hybridized as described with a radiolabelled 18s rRNA cDNA.

Results

Suc and RFOs accumulate during water deficit

When a water deficit stress was imposed on *X. viscosa* plants, the classical linear increase in Suc concentration was observed in the leaves (Fig. 1A), increasing nearly 5-fold from 4.8 mg g^{-1} DW to 23.5 mg g^{-1} DW at 5% RWC. Importantly, 7 d after rewatering the plants, the leaf Suc concentrations had decreased to levels comparable with that of the fully turgid state before the application of the stress. The total hexose sugar (Hex) concentration showed a concomitant 3.5-fold decrease to an average of 2.9 mg g^{-1} DW at 5% RWC, but remained at similar levels 7 d after the plants were rewatered (Fig. 1B).

We observed linear increases in concentrations of the RFO members Raf, Sta, and Ver until 50% RWC, after which they remained constant until 5% RWC (Fig. 1E, F, and G, respectively). Importantly, amongst the RFO species, Raf was the most abundant, increasing >3-fold from an average concentration of 5.1 mg g^{-1} DW to 17.7 mg g^{-1} DW at 5% RWC, 1.5-fold higher than Sta and 4-fold higher than Ver at 5% RWC (Fig. 1F and G, respectively). As with the concentrations of Suc, those of the RFO members decreased to levels comparable with those of the fully turgid state 7 d after the stress had been alleviated.

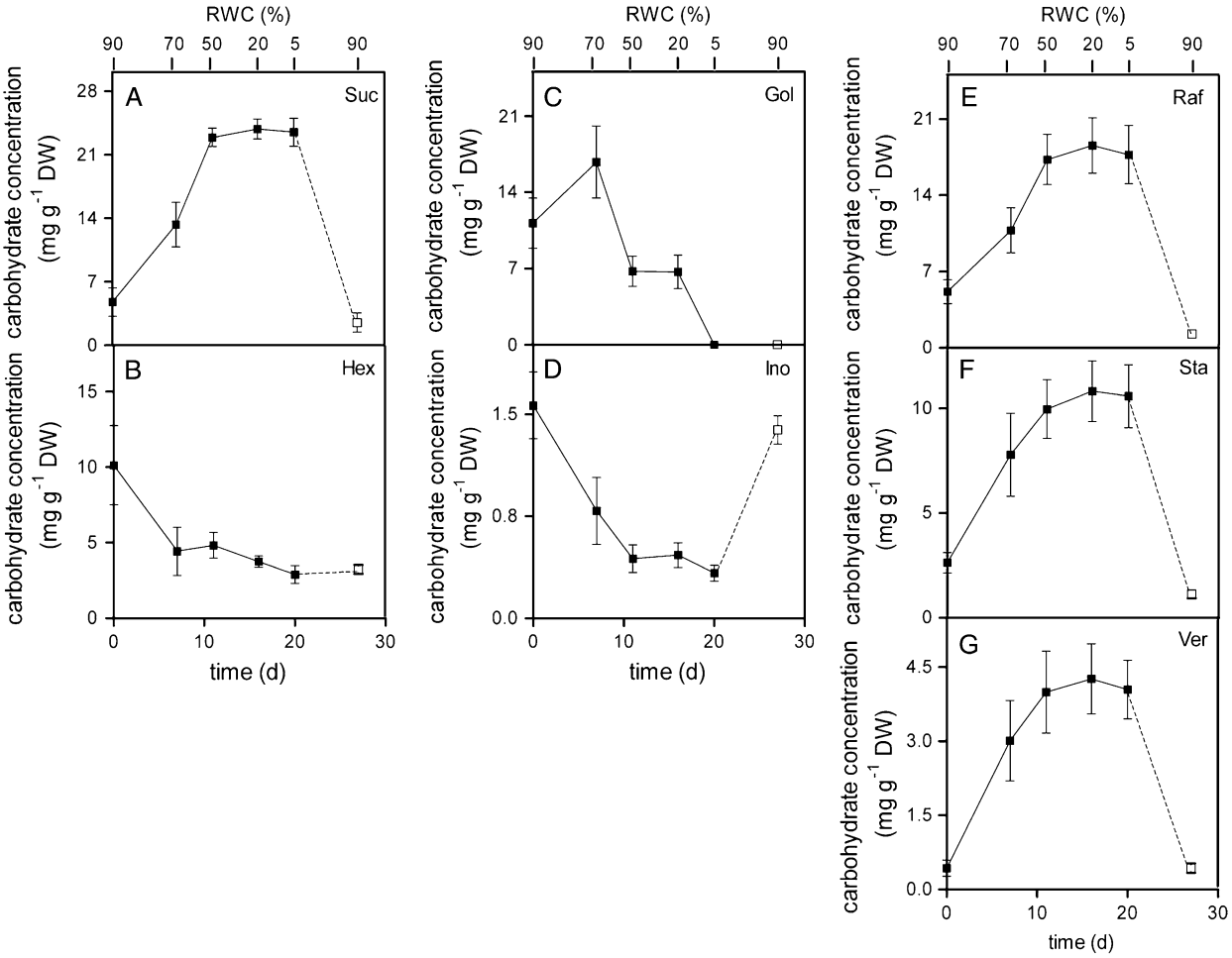

Fig. 1. Changes in the carbohydrate concentrations in the leaves of *X. viscosa* plants subjected to a water deficit stress over a period of 20 d (filled squares). Dashed lines and open squares represent a 7 d period from the point at which water deficit was alleviated by watering the plants. Error bars indicate the standard error between the mean of eight replicates.

GolS activity decreased strongly from 480 μkat g^{-1} DW at 90% RWC to 34 μkat g^{-1} DW at 5% RWC during the water deficit but returned to levels comparable with that of the unstressed state 7 d after rewatering (Fig. 2). This correlated with a decline in leaf Gol concentrations (Fig. 1C), from 11.2 mg g^{-1} DW at 90% RWC to undetectable by HPLC-PAD at 5% RWC; 7 d after rewatering the plants, the leaf Gol concentrations were still undetectable by HPLC-PAD. Interestingly, northern blot analysis indicated that *XvGolS* transcripts only increased at 19% RWC and were maintained once the plants had reached an anhydrobiotic state (7% RWC, Fig. 3). The leaf concentrations of *myo*-inositol decreased 4.6-fold to 0.33 mg g^{-1} DW at 5% RWC (Fig. 5D). After rewatering, the *myo*-inositol concentration was comparable with that observed at full turgor prior to the water deficit, correlating with the changes in RFOs described above.

XvGolS is homologous to known GolSs

An *in silico* analysis of the cDNA sequence, which was subsequently designated *XvGolS*, revealed that it shared high nucleotide homology with *GolS* sequences from various plant species (not shown). This was also evident in high similarities obtained from alignments using the predicted protein sequences of the mature proteins (Fig. 4), with XvGolS sharing high identity to GolS enzymes from *Arabidopsis* (AtGolS I, NP_182240, 81%), *Brassica napus* (BnGolS, AF106954, 80%), *Glycine max* (GmGolS, AY126715, 78%), and *Ajuga reptans* (ArGolSI, AJ237693, 75%). Sprenger and Keller (2000) have reported a putative serine phosphorylation site (Ser263) for a deduced subset of GolS proteins, implying a potential regulatory role for this residue. This residue was cited to be conserved in 14 of 17 full-length sequences analysed (Downie *et al*., 2003). This serine residue was also found to be conserved in XvGolS, along with the characteristic C-terminal penta-peptide APSAA of GolS enzymes (Fig. 4, arrows).

In the absence of a genome sequence for *X. viscosa*, it is uncertain if *XvGolS* is part of a multigene family in this plant. However, given that *A. reptans* has been shown to have at least two active GolS isoforms (Sprenger and Keller, 2000) and *Arabidopsis* at least three (Taji *et al*., 2002), it is believed that XvGolS is most probably part of a small gene family. After sequencing nearly 80% of the full-length cDNAs in a library constructed from RNA isolated from leaves at 60% and 5% RWC, no additional GolS isoforms were identified, suggesting that at least under water deficit, the *XvGolS* described here is the predominant isogene that is up-regulated.

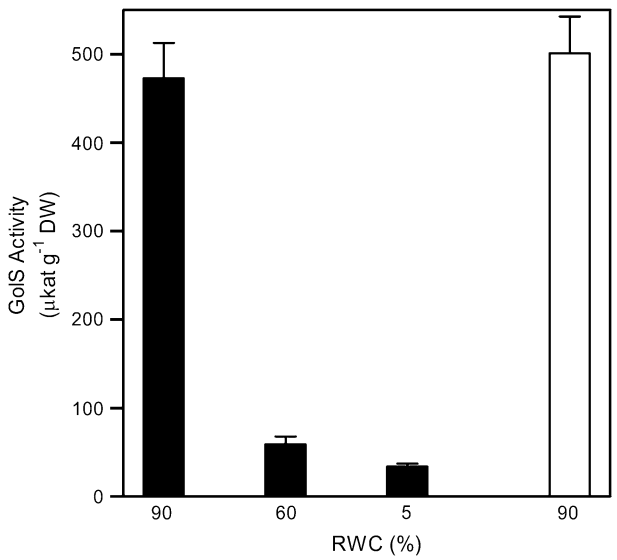

Fig. 2. GolS activity in the leaves of *X. viscosa* plant subjected to a water deficit stress over a period of 20 d. The shaded bar represents GolS activity measured 7 d after alleviation of the stress by watering the plants. Error bars indicate the standard error between the mean of four replicates.

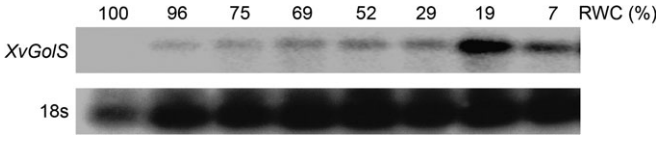

Fig. 3. Northern blot analysis of total RNA isolated from the leaf tissue of plants subjected to a water deficit stress.

Heterologous expression functionally identifies XvGolS

To confirm the identity of *XvGolS*, the cDNA was cloned into the pPROEX HTb expression vector and XvGolS was heterologously expressed in *E. coli*. Crude extracts of induced *E. coli* were able to synthesize Gol from UDP-Gal and *myo*-inositol *in vitro*, contrary to the vector control (Fig. 5). Fractions of Gol synthesized by the recombinant protein were collected after separation on an HPLC column and hydrolysed with an *Aspergillus niger* α-galactosidase specific for the cleavage of the α-1,1 galactosidic linkage in Gol. Both Gol synthesized by recombinant XvGolS and a commercially purchased Gol standard were hydrolysed to *myo*-inositol and Gal in a 1:1 molar ratio (Fig. 6).

Discussion

Suc and RFOs increase during water deficit

Xerophyta viscosa, being a typical resurrection plant, survived desiccation to 5% RWC, recovering to 90% RWC upon rewatering (Fig. 1). It also accumulated Suc during water deficit stress (Fig. 1A), confirming earlier reports on this species (Whittaker *et al*., 2001). The accumulation of Suc is one of the known responses to water deficit stress

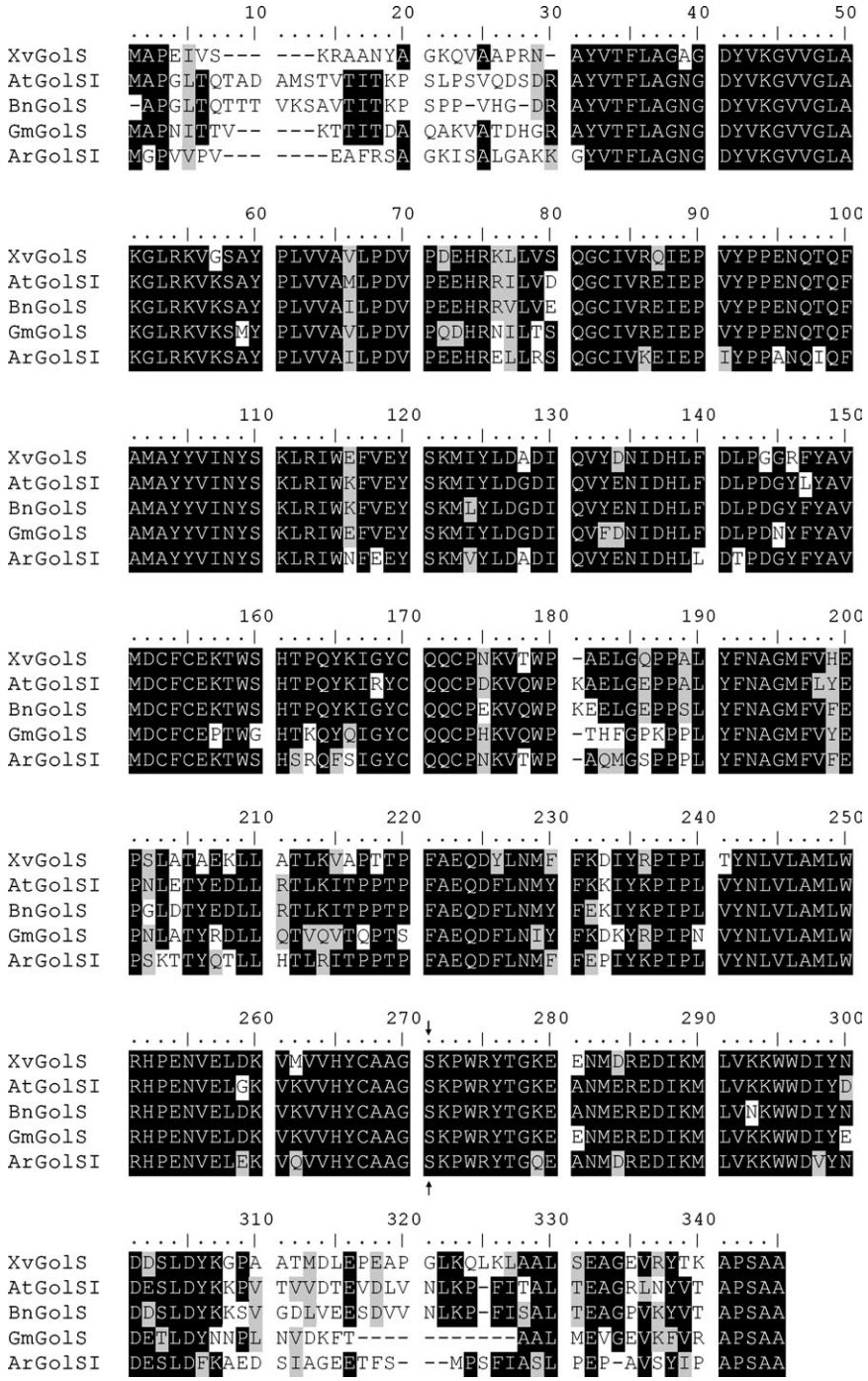

Fig. 4. Amino acid sequence alignment of XvGolS against GolS amino acid sequences from *Arabidopsis* (AtGolS I, NP_182240), *Brassica napus* (BnGolS, AF106954), *Glycine max* (GmGolS, AY126715), and *Ajuga reptans* (ArGolSI, AJ237693). Identical amino acids are shaded in black, whilst similar amino acids are shaded in grey. A conserved serine residue between the sequences is indicated with arrows.

of resurrection angiosperms, such as, for example, *Boea* spp. (Bianchi *et al*., 1991; Ghasempour *et al*., 1998), *Craterostigma* spp. (Bartels and Salamini, 2001; Cooper and Farrant, 2002), *Ramonda* spp. (Müller *et al*., 1997; Ghasempour *et al*., 1998; Zikovic *et al*., 2005), *Xerophyta* spp. (Ghasempour *et al*., 1998; Cooper and Farrant 2002), *Myrothamnus flabellifolius* (Moore *et al*., 2007), and the grasses *Eragrostis nindensis* (Illing *et al*., 2005) and *Sporobulus stapfianus* (Albini *et al*., 1994; Whittaker *et al*., 2001). Norwood *et al*. (2003) state that the amount of Suc accumulation in dehydrated leaf tissue of monocotyledonous poikilohydric plants is between 65 μmol g^{-1} DW and 100 μmol g^{-1} DW, and in dicotyledonous species between 150 μmol g^{-1} DW and 2000 μmol g^{-1}

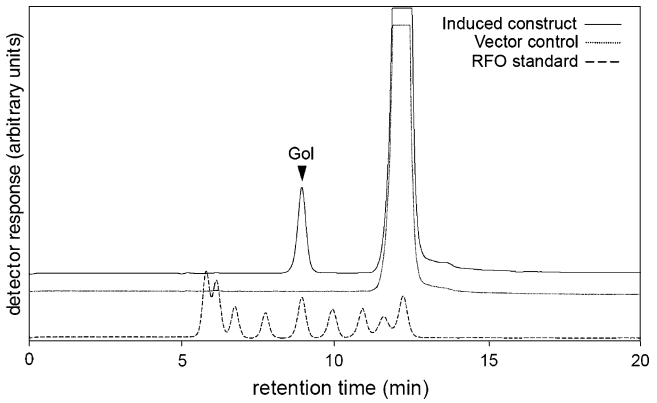

Fig. 5. HPLC-PAD chromatogram representing an *in vitro* Gol synthesis reaction conducted in the presence of UDP-Gal and *myo*-inositol, using crude *E. coli* cell lysates containing recombinant XvGolS. Standard sugars eluted from 5.9 min onwards represent verbascose, stachyose, raffinose, sucrose, galactinol, glucose, galactose, fructose, and *myo*-inositol, respectively.

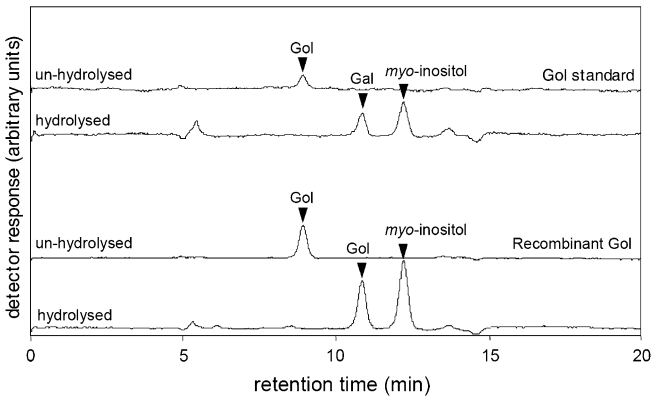

Fig. 6. HPLC-PAD chromatogram representing the enzymatic hydrolysis of Gol synthesized in an *in vitro* reaction using crude *E. coli* cell lysates containing recombinant XvGolS compared with the enzymatic hydrolysis of a commercial Gol standard.

DW. The present HPLC-PAD data indicate that, in *X. viscosa* leaf tissue dehydrated to 5% RWC, Suc accumulates to concentrations that are within this range at an average of 69 μmol g^{-1} DW. It is thus evident that, at least in *X. viscosa* leaf tissue, Suc accumulation must be critical to the protection of leaf cells during anhydrobiosis.

Previously published data indicated that Suc accumulates in *X. viscosa* leaf tissue to approximately 75 mg g^{-1} DW (Whittaker *et al.*, 2001), as opposed to 24 mg g^{-1} DW reported here. Several reasons can be cited for this apparent discrepancy. First, it has been reported that different environmental conditions under which resurrection plants are dried (especially light and temperature levels) can affect the quantity of protectants such as Suc and anthocyanins accumulated (Tuba *et al.*, 1998; Farrant *et al.*, 2003). Secondly, preliminary analyses on plants collected from their natural habitat revealed significant steady-state variations amongst the carbohydrate profiles of individual plants (data not shown), suggesting that innate individual competencies might exist among individuals in wild populations that might be exacerbated by physiological status (e.g. age and environmental history of exposure to abiotic stresses). It was thus chosen to conduct the present study on plants propagated by *in vitro* culture from a seed stock prior to being transferred to soil. This reduced the amount of natural variation, perhaps due to uniformity in plant age and the conditions of environmental hardening under glasshouse conditions. Importantly, in the context of this study, all of the plants used were capable of full recovery from desiccation to the air dry state.

A surprising finding is the pronounced accumulation of RFOs during water deficit stress (Fig. 1E, F, G), the most striking increase being in Raf (Fig. 1E; up to 17.7 mg g^{-1} DW). To our knowledge, this is the first report on such a distinct correlation between water deficit stress and substantial RFO accumulation in the leaves of an angiosperm resurrection plant. Invariably, in the studies cited above, RFO concentrations either decreased (concomitant to Suc increase), remained unchanged, or increased only minimally (up to a maximum of 6.8 mg g^{-1} DW Raf in *Ramonda myconi*; Ghasempour *et al.*, 1998). The reason for this variation in Raf concentration change patterns observed during drying in different resurrection plants is not clear at present. Comparing the sugar concentrations in the leaves of a number of desiccation-tolerant plants, it might be concluded that the Suc-to-Raf mass ratios and not their absolute concentrations are important; the Suc-to-Raf mass ratio was never lower than 5:1 in the dry state (Bianchi *et al.*, 1991; Müller *et al.*, 1997; Ghasempour *et al.*, 1998; Zikovic *et al.*, 2005). Such a high ratio is in clear contrast to the present results obtained from dehydrated *X. viscosa* leaf tissues, where it was only 1.3:1.

One of the experimentally demonstrated functions of Raf is the arrest of Suc crystallization (Caffrey *et al.*, 1988). It has also been observed that Raf is more effective in hydrogen bonding to biomolecules than either Suc or trehalose (Gaffney *et al.*, 1988). This direct hydrogen bonding between sugars and biomolecules has been demonstrated to be imperative in the stabilization of proteins, membranes, and whole cells under conditions of freezing and dehydration (Carpenter and Crowe, 1989; Arakawa *et al.*, 1993; Prestelski *et al.*, 1993). Furthermore, it has been demonstrated in model systems that the combination of Suc and Raf, in ratios of approximately 5.7:1, is optimal for stabilization of glasses that form during desiccation-induced vitrification of the cytoplasm (Koster, 1991; Koster and Bryant, 2005). Given that the present data have demonstrated a typical increase in the leaf Suc concentration during desiccation, a direct role for Raf in preventing its crystallization and/or stabilizing subcellular architecture is probable.

The reasons for the much lower Suc-to-Raf ratio of 1.3:1 found in *X. viscosa* relative to other resurrection species remains a matter for speculation. It is possible that it serves a dual function, primarily for carbon storage and, when needed, for protection against abiotic stresses. Idenficiation of the subcellular location of sugars would facilitate understanding of these putative roles. Storage Raf is speculated to be located in the vacuoles and protection Raf to be distributed evenly in the cytoplasm. To this end, Farrant (2000) and Mundree and Farrant (2000) have shown from ultrastructural studies that the *Xerophyta* spp. prevent cytorrhesis associated with the mechanical stresses of drying, by filling the cytoplasm with vacuoles in which they propose that water is replaced by compatible solutes. Although those authors have not demonstrated what solutes might occur in the vacuoles of *X. viscosa*, Van der Willigen *et al.* (2004) have shown that at least some sugars, along with proline and some protein, accumulate in vacuoles of dry leaves of the resurrection grass *E. nindensis*.

Illing *et al.* (2005) have suggested that the breakdown and mobilization of oligo- and polysaccharides during dehydration might provide the carbon skeletons necessary for Suc synthesis to protect resurrection plants from desiccation-induced damage. The present data demonstrate that in *X. viscosa*, under conditions of water deficit, both Suc and oligosaccharides (i.e. Raf) accumulate, with the carbon skeletons being provided by Hex sugars (via the Hex-P pool), as demonstrated in previous reports (Whittaker *et al*., 2001), and *myo*-inositol (via the Gol pool), respectively. It is proposed that all these sugars contribute toward subcellular protection against desiccation injury, but also provide an energy source to facilitate recovery on rehydration.

Surprisingly, Gol concentrations clearly decreased during the water deficit stress, except for a transient peak evident at 70% RWC (Fig. 1C). Given that increases in Raf starting relatively early in the dehydration process (70% RWC, Fig. 1E) were observed, this would imply that the GolS activity that could be measured is sufficient to synthesize enough Gol for RFO biosynthesis very early in the water deficit stress. Indeed, the Gol concentration in leaves decreased concomitantly with RFO accumulation, with Gol not being detected by HPLC-PAD at 5% RWC nor 7 d after rewatering. The fact that changes in *myo*-inositol that were linked to the accumulation and disappearance of RFOs were observed indicates that *myo*-inositol must be channelled into Gol biosynthesis to provide galactosyl donors for RFO biosynthesis. The absence of Gol 7 d after rewatering, despite a measurable GolS activity comparable with the unstressed state (Fig. 2), could well be due to *myo*-inositol being channelled into other metabolic pathways related to recovery and/or repair mechanisms initiated after the relief of the anhydrobiotic state. The functional roles of *myo*-inositol in plants are known to include the synthesis of cell wall components and membrane biogenesis (Loewus and Murphy, 2000).

RFO mass increases could not be positively correlated either to the accumulation of *XvGolS* transcripts (Fig. 3) or to XvGolS activity during water deficit (Fig. 2). An investigation was conducted into whether the low enzyme activity observed at 5% RWC was due to the presence of an inhibitor, by mixing enzyme extracts from fully turgid leaves with those from dehydrated leaves in a 1:1 ratio (v/v). HPLC-PAD analysis consistently indicated that GolS activity in mixed samples was not lower than 50% of the GolS activity in the extracts from fully turgid leaves (data not shown), precluding the existence of an inhibitor. An inventory of GolS activity and sugar composition in the leaves and seeds of several agronomically important species has previously demonstrated a positive correlation between the accumulation of RFOs and GolS activity (Handley *et al*., 1983). However, there are notable exceptions in recent studies investigating the role of RFOs in stress tolerance (Cunningham *et al*., 2003; Downie *et al*., 2003; Zhao *et al*., 2004), which failed to correlate GolS activity and RFO accumulation in all cases.

XvGolS was functionally expressed in E. coli

When the *XvGolS* cDNA was heterologously expressed as a hexa-His-tagged recombinant protein, in *E. coli*, His-tagged protein could be consistently detected in IPTG-induced cultures by western blots (data not shown). Crude protein extracts from these induced cultures were clearly able to synthesize a compound, from an *in vitro* reaction using *myo*-inositol and UDP-Gal, which eluted with a Gol standard when analysed by HPLC-PAD (Fig. 5). Crude protein extracts from vector control cultures lacked this synthetic capacity. Importantly, when eluates of the compound synthesized by the recombinant XvGolS were collected and hydrolysed with an α-Gal enzyme specific for the α-1,1 linkage between Gal and *myo*-inositol, it was confirmed that these specific components were generated consistently in a 1:1 molar ratio (Fig. 6), unequivocally confirming that *XvGolS* encoded a bona fide GolS enzyme.

In the light of the present observations, it is speculated that an early response to desiccation in *X. viscosa* effects a metabolic switch for the accumulation of sugars (predominantly Suc and Raf) that function in macromolecular and subcellular protection and ultimately cytoplasmic vitrification in the anhydrobiotic state. Once water becomes available again, these protective sugars are broken down and their carbon skeletons used for essential repair functions such as cell wall and membrane biosynthesis. This would explain the absence of Gol in the rehydrated state, despite a high GolS activity and an increase in *myo*-inositol concentration that correlates with a decrease in RFOs after rehydration, suggesting that RFO biosynthesis

is not of key importance to the repair/recovery processes involved during rehydration.

In summary, a cDNA isolated from the leaves of *X. viscosa* plants subjected to water deficit stress was cloned and functionally identified as encoding a GolS enzyme involved in RFO biosynthesis. It has also been demonstrated that Suc and Raf are the predominant soluble carbohydrates to accumulate in the leaves in response to water deficit. Recent experimental evidence has demonstrated that, by genetically manipulating plants to increase RFO levels, they show an enhanced stress tolerance phenotype (Taji *et al*., 2002; Pennycooke *et al*., 2003). These findings strongly suggest a role for RFOs in abiotic stress tolerance. Whether this role, particularly of Raf, in the water deficit tolerance of *X. viscosa* is direct (via interaction with intracellular constituents) or indirect (in the prevention of Suc crystallization) is unclear. It is however clear that the desiccation tolerance observed in *X. viscosa* is part of a multigenic process, due in part to the accumulation of Suc and RFOs.

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