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RESEARCH PAPER

Molecular characterization of *XVSAP1*, a stress-responsive gene from the resurrection plant *Xerophyta viscosa* Baker[1]

Dahlia Garwe, Jennifer A. Thomson and Sagadevan G. Mundree[2]

Molecular and Cell Biology Department, University of Cape Town, Private Bag, Rondebosch, 7701, South Africa

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Abstract

The strategy of 'complementation by functional sufficiency' was used to isolate a cDNA designated *XVSAP1* from a cDNA library constructed from dehydrated *Xerophyta viscosa* Baker leaves. Analysis of the cDNA sequence indicated a highly hydrophobic protein with six transmembrane regions. Southern blot analysis revealed that there are at least two copies of *XVSAP1* in *X. viscosa*. The deduced amino acid sequence showed 49% identity to WCOR413, a low-temperature-regulated protein from wheat. The protein also showed between 25% to 56% identity to WCOR413-like proteins from *Arabidopsis thaliana*. Expression of *XVSAP1* in *Escherichia coli* (*srl*::Tn10) conferred osmotic stress tolerance when the cells were grown in 1 M sorbitol. Analysis of gene expression using semi-quantitative RT-PCR indicated that *XVSAP1* is induced by dehydration, salt stress (100 mM), both low (4 °C) and high temperature (42 °C) and high light treatment (1500 μmol m^{-2} s^{-1}). These results suggest that *XVSAP1* may have a significant role to play in the response of *X. viscosa* to abiotic stresses.

Key words: Cold stress, desiccation stress, heat stress, resurrection plant, salinity stress.

Introduction

Adverse environmental factors such as extremes of temperature and osmotic stress resulting from conditions of high salinity and drought, affect the growth, productivity and distribution of plants (Boyer, 1982). As sessile organisms, plants have evolved a wide variety of mechanisms that enable them to grow and reproduce under hostile environmental conditions. The response to abiotic stresses is mediated through physiological, morphological and metabolic modifications occurring in all plant organs. Expression of genes in response to different environmental stimuli results from a complex signal transduction cascade that commences with perception of the stimulus followed by processing including amplification and integration of the signal. The final step is a response reaction in the form of *de novo* gene expression (Ingram and Bartels, 1996).

Although the general response to abiotic stress is similar in all plants, there is a group of plants known as 'resurrection plants' that have developed mechanisms that enable them to withstand severe water deficit. These plants are unique in their ability to tolerate drying of their vegetative tissues. Resurrection plants can lose over 90% of their water content, survive in their dried state for prolonged periods and then resume active life when water becomes available again (Bartels *et al*., 1990; Gaff, 1971; Sherwin and Farrant, 1996). The molecular basis of desiccation tolerance has been studied in a few species representing different groups: the moss *Tortula ruralis,* the monocotyledonous *Sporobolus stapfianus* (Blomstedt *et al*., 1998; Oliver, 1996; Neale *et al*., 2000) and the dicotyledonous *Craterostigma plantagineum* (Bartels *et al*., 1990). It is thought that two basic mechanisms exist which allow desiccation-tolerant plants to survive water deprivation. The first involves the protection of cellular integrity through inducible and constitutive mechanisms, while the second involves the repair of desiccation or rehydration induced damage. However, both mechanisms are probably employed for desiccation tolerance with different plants utilizing one strategy more than the other (Oliver and Bewley, 1997).

Theories on the mechanisms by which resurrection plants tolerate dehydration have mostly been derived from observations of the cellular processes that occur during drying of

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[2] To whom correspondence should be addressed. Fax: +27 21 689 7573. E-mail: mundree@science.uct.ac.za

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the plant (Ingram and Bartels, 1996). It has been demonstrated that several genes are differentially expressed in response to dehydration (Blomstedt *et al*., 1998; Itturiaga *et al*., 1992; Schneider *et al*., 1993). Both genetic and biochemical studies have established that the phytohormone ABA is crucial in the response to desiccation, salt, and cold (Bray, 1997). Characterization of the ABA inducible genes *Em* from wheat and *rab16A* from rice by expression studies and analysis of protein binding *in vitro*, showed that a *cis*-regulatory ABA-responsive element (ABRE) is important for transcription (Marcotte *et al*., 1989; Mundy *et al*., 1990). The element consists of 8–10 base pairs with the core sequence ACGT. Exogenous application of the plant hormone leads to the expression of most of the dehydration-induced proteins (Bartels *et al*., 1990). ABA also plays an important role in physiological processes such as the closing of guard cells under drought stress and the regulation of several events during late seed development (Zeevaart and Creelman, 1988; McCarty, 1995).

Blomstedt *et al*. (1998) found that a number of genes activated in the early stages of dehydration in resurrection plants are similar to those expressed in the desiccating seed of most plants. The synthesis of globular and extremely hydrophilic proteins known as late embryogenesis abundant (LEA) proteins is one of the well-documented responses to dehydration, salinity and cold stress as is the accumulation of osmolytes. It has been suggested that LEA proteins have a role to play in the maintenance of protein or membrane structure, the sequestration of ions and the binding of water. Osmolytes are thought to function by raising the osmotic potential of the cell and also by stabilizing proteins and membranes when water deficit occurs (Le Rudulier *et al*., 1984; McNeil *et al*., 1999).

Low temperature is one of the major environmental factors limiting plant growth. Freezing temperature induces injuries, particularly to the cellular membrane systems, that result largely from the severe dehydration that occurs upon ice formation within the cells. (Gilmour *et al*., 1988; Thomashow, 2001). Low temperature also affects the normal functioning of integral membrane proteins such as transporters and receptor proteins whose activity is dependent on the fluidity of the membrane (Hazel, 1995). The products of cold-regulated (*cor*) genes such as the *Arabidopsis* COR6.6 and COR78, may protect and help plants to adapt to cold stress (Thomashow, 1999). Studies on cold-regulated gene expression in *Arabidopsis* resulted in the discovery of a DNA regulatory element, the C-repeat (CRT) dehydration responsive element (DRE) which has a conserved core sequence of CCGAC. Transcriptional activators that bind the CRT/DRE designated CBF1, CBF2 and CBF3 or DREB1A, DREB1C and DREB1A, respectively, were subsequently identified. DRE confers responsiveness to low temperature and dehydration (Liu *et al*., 1998; Stockinger *et al*., 1997).

Danyluk *et al*. (1994) identified a low-temperature responsive dehydrin-like gene, *wcor410*, belonging to a family of homologous members, *wcor410, wcor410b* and *wcor410c.* The results from their work suggested that the protein was involved in the cryoprotection of the plasma membrane against freezing or dehydration stress. It was shown that water stress, polyethylene glycol, ABA and, to a lesser extent, salt and wounding also resulted in the up-regulation of members of the *wcor*410 family. Similarly the *wcs120* transcript, which codes for a protein homologous to dehydrins, was found to accumulate in response to cold stress and its promoter was found to be stress-inducible (Oullet *et al*., 1998).

The processes associated with tissue recovery on rehydration in resurrection plants have been less extensively studied with only a few rehydration-associated proteins identified (Oliver *et al*., 1998). Most of the information available is on the fully desiccation-tolerant moss *T. ruralis.* It has been postulated that the moss relies more on the activation of pre-existing repair mechanisms for desiccation tolerance than it does on either pre-established or activated protection systems (Oliver, 1991). In desiccation-tolerant angiosperms recovery is more complex. Studies by Tuba *et al*. (1993) showed that in poikilochlorophyllous plants such as *Xerophyta,* the chloroplasts were extensively altered after a period of desiccation. The rebuilding of chloroplasts and the photosynthetic apparatus occurs on rehydration. However, *Craterostigma wilmsii*, a modified desiccation-tolerant resurrection plant, retains its chlorophyll on drying. Protective mechanisms during dehydration rather than repair on rehydration appear to dominate in modified desiccation tolerant resurrection plants (Sherwin and Farrant, 1996).

X. viscosa is a monocotyledonous resurrection plant that is capable of tolerating severe abiotic stress conditions (Sherwin and Farrant, 1996; Mundree *et al*., 2000). Mundree *et al*. (2000) used an approach called 'complementation by functional sufficiency' to isolate genes from *X. viscosa* that conferred functional sufficiency to osmotically–stressed *E. coli* (*srl*::Tn10). *XVSAP1* was isolated from a cDNA library constructed from dehydrated *X. viscosa* leaves using this strategy. The protein shows 49% identity to a cold-tolerance protein, WCOR413*,* from *Triticum aestivum* (Danyluk and Sarhan, 1996). It also bears close identity (56%) to other uncharacterized proteins from *Arabidopsis* which themselves have 64% identity to WCOR413. In this report the molecular characterization of *XVSAP1* is described.

Materials and methods

Plant material, growth conditions and XVSAP1 *cDNA isolation*

X. viscosa plants were collected from the Buffelskloof Nature Reserve (Mpumalanga Province, South Africa). The plants were

potted and grown under greenhouse conditions as described by Sherwin and Farrant (1996). Experimental plants were watered to ensure full hydration prior to the stress experiments. Relative water content (*RWC*) determination was as described by Sherwin and Farrant (1996). Construction and screening of the cDNA library was as previously described (Mundree *et al*., 2000). A cDNA insert in pBluescriptSK+ (Stratagene, La Jolla, CA) named *XVSAP1* was used for the experiments described in this paper.

XVSAP1 *expression in osmotically stressed* Escherichia coli

XVSAP1 was cloned into the pProEXHT Prokaryotic Expression Vector System (Life Technologies, Inc, USA). *E. coli* (*srl*::Tn10) cells were transformed with the pProEXHT-*XVSAP1* construct and grown in M9 minimal medium supplemented with 1 mM $MgSO_4.7H_2O$, 0.2% glycerol, 0.1% vitamin B, and 100 μg ml^{-1} ampicillin. Cell cultures were induced in duplicate by adding 0.2 mM isopropyl thiogalactopyranoside (IPTG) after the OD_{600} of the cells had reached approximately 0.5. The cells were allowed to grow for a further 2 h before an osmotic stress was imposed by adding 4 M sorbitol to a final concentration of 1 M. The growth of the cells was monitored by taking absorbance readings at 600 nm over a 48 h period. The experiment was repeated three times.

XVSAP1 *sequence analysis*

The nucleotide sequence of *XVSAP1* was determined using the ALFexpress automated DNA sequencer (Pharmacia Biotech AB, Uppsala, Sweden) as described by Mundree *et al*. (2000). The BLAST program of the National Centre for Biotechnology Information (Altschul *et al*., 1990) was used to search databases for sequence similarities. Nucleotide and amino acid sequence comparisons were done using CLUSTAL W (1.5) on the BCM server. The ProfileScan tool on the ISREC bioinformatics server was used to scan *XVSAP1* for conserved motifs. DNAMAN (Version 4.3, Lynnon BioSoft) was used to construct the homology tree.

Southern blot analysis

Genomic DNA from *X. viscosa* was isolated using the plant DNA preparation procedure described by Dellaporta *et al*. (1983) except that in all cases approximately 1 g of leaf tissue was used and DNA was precipitated with isopropanol. Isolated DNA was quantitated spectrophotometrically. 15 μg DNA aliquots were restricted with *Eco*RI, *Xho*I, *Bgl*II, *Hin*dIII and *Eco*RV restriction endonucleases, electrophoresed on 1% agarose gels and blotted onto nylon membranes (Hybond -N, Amersham) by capillary transfer (Sambrook *et al*., 1989). DNA was fixed using a cross-linker (Stratalinker 1800, Stratagene). The complete *XVSAP1* cDNA was labelled with digoxigenin (DIG) using the random primed method according to the manufacturer's instructions (Roche Diagnostics GmbH, Germany). Blots were hybridized with the labelled *XVSAP1* probe for 16 h at 42 °C. The blots were subsequently washed with 2× SSC (sodium citrate buffer), 0.1% SDS at room temperature and stringently with 0.5× SSC**,** 0.1% SDS at 68 °C. The chemiluminescent alkaline substrate disodium 3-(4-methoxyspiro(1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3,3.1.13.7]decan}-4-yl) phenyl phosphate [CSPD (Roche Diagnostics GmbH, Germany)], was used for detection according to the manufacturer's instructions.

Stress induction

X. viscosa plants were dehydrated by withholding water for a period of 2 weeks. Leaves were detached from the plants at 90%, 78%, 63%, 51%, 44%, and 4% *RWC*. Leaf samples were also collected at 4%, 32%, 42%, 85%, and 92% *RWC* on re-hydrating. For the heat treatment, fully hydrated plants were kept in a phytotron at 42 °C (humidity 50–70%, 16/8 h light/dark cycle). The plants were watered regularly to maintain them at full hydration. To determine the effect of cold stress, plants were kept at 4 °C and leaf samples taken every 6 h for 60 h. To test the response of *X. viscosa* to high salinity, the plants were irrigated with 100 mM NaCl daily for 7 d. The high light treatment was carried out by exposing the plants to light at 1500 μmol m^{-2} s^{-1} for 4 d in a phytotron (25 °C, humidity 50–70%). Plants were irrigated with water daily to keep them fully hydrated. To determine if abscisic acid (ABA) had an effect on the expression of *XVSAP1*, *X. viscosa* leaves were sprayed with the phytohormone at a concentration of 100 μM in water once every 24 h. In all cases, leaf samples were taken from the experimental plants just before commencing treatments (time 0). Samples were collected every 24 h thereafter except for the cold treatment, where samples were collected every 6 h. In the case of ABA, samples were taken every 3 h after treatment of the leaves. All leaf samples collected were frozen in liquid nitrogen and stored at –70 °C until further use.

RNA isolation

Total RNA was isolated using the Trizol reagent (Gibco-BRL). *X. viscosa* leaves (200 mg) were ground in liquid nitrogen and homogenized in 0.75 ml of the reagent. Following incubation for 5 min at room temperature, 0.2 ml chloroform was added 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 quantitated spectrophotometrically, separated on a 1.2% agarose formaldehyde gel and stained with ethidium bromide to verify quantitation.

Semi-quantitative RT-PCR

All RNA samples were treated with DNase I (Roche Diagnostics GmbH, Germany) according to the manufacturer's instructions to eliminate DNA contamination. In each case, 2 μg RNA was used for the reverse transcription reaction. The internal control RNA was prepared by deleting a 473 bp (*Nde*I restriction) fragment from *XVSAP1* in pBluescriptSK+ and performing *in vitro* transcription. Two picograms of the truncated clone were used in all the RT reactions except for the ABA RT-PCR where 0.5 pg of the internal standard was used. The reverse transcription reactions were performed using the Omniscript reverse transcriptase kit according to the manufacturer's directions (Qiagen GmbH, Germany). RNase inhibitor was obtained from Roche Diagnostics GmbH, Germany. The cDNA (5 μl) from the RT step was used in 50 μl PCR reactions undiluted. The primer pair (forward primer, 5′-GCACGAGGCA-GATTTGAA TTG-3′; reverse primer, 5′-ATATGGACACGCAT-GACCCA-3′) produced an 829 bp product from *XVSAP1* and a 342 bp product from the truncated clone. Reactions were conducted using a Gene Amp 9700 (Perkin Elmer Applied Biosystems, CA, USA) thermocycler under the following conditions: 95 °C for 2 min followed by 23 cycles of 95 °C for 30 s, 61 °C for 40 s and 72 °C for 45 s and a final extension step for 6 min. The linear portion of the reaction was determined to be between 18 and 25 cycles and 23 cycles were used for all the experiments.

After PCR, the samples were resolved by electrophoresis on a 0.8% agarose gel and stained with ethidium bromide. Gel pictures were obtained using the Gel documentation system GDS 2000 (UVP Ltd, Cambridge, UK).

Results

XVSAP1 *expression in osmotically stressed* E. coli

E. coli (*srl*::Tn10) (Csonka and Clark, 1979) cannot grow on minimal media containing high concentrations of sorbitol. To confirm the osmo-protection function of *XVSAP1*, the cDNA was cloned into a prokaryotic protein

expression vector to yield pPROEXHT-*XVSAP1*. *E. coli* (*srl*::Tn10) cells transformed with this plasmid exhibited significantly better growth in the presence of 1 M sorbitol over a period of 48 h, compared to *E. coli* (*srl*::Tn10) transformed with the vector only, after induction with IPTG (Fig. 1). Although the imposition of osmotic stress by the addition of sorbitol caused an initial decrease in the growth rate of both cultures, 2 h after the stress was imposed there was a steady increase in the growth rate of the experimental cultures.

Sequence analysis of XVSAP1

The nucleotide sequence of *XVSAP1* is 942 bp with an open reading frame of 867 bp (Fig. 2A). The deduced amino acid sequence encodes a basic protein of 264 amino acids with a molecular weight of 29.6 kDa and a predicted p*I* of 9.12. A Prosite motif search revealed that the protein has two prokaryotic membrane lipoprotein lipid attachment sites between amino acid residues 149–159 and 239–249. One possible N-myristoylation site was also found and this is indicated on the *XVSAP1* sequence at position 42–47.

A hydropathic plot [based on the method of Kyte and Doolittle, 1982 (window of 19 amino acid residues)] (Fig. 2B) predicted a protein rich in hydrophobic residues with an average hydrophobicity index of 0.81. The sequence consists of at least six transmembrane helices (Fig. 2A) suggesting that XVSAP1 is likely to be an integral membrane protein. A computer search of protein sequence databanks revealed that XVSAP1 showed 49% identity to WCOR413, a cold-responsive protein isolated from wheat and between 25–56% identity to cold associated proteins identified in *A. thaliana* (Fig. 3). The protein also has 53% identity to a cold associated protein from rice. Results from the BLAST program indicate that the region extending from the lysine residue at position 36 to the phenylalanine residue at 119 (Fig. 2A) bears 12% identity with K^+ potassium transporter family that is conserved across phyla (Quintero and Blatt, 1997). A homology tree based on the amino acid sequences of *XVSAP1* and its homologues (Fig. 3B) indicates that the first three ATCAPs from *Arabidopsis* are the most closely related with over 70% identity. XVSAP1 is closest to these three homologues, The rice cold associated protein (RCAP) and WCOR413 are closely related with nearly 70% identity.

Southern analysis

Southern blot analysis of the *X. viscosa* genomic DNA probed with *XVSAP1* cDNA was carried out to determine the gene copy number. Of the restriction enzymes used, only *Bgl*II has a predicted restriction site within *XVSAP1*. At least seven hybridization bands were detected with this enzyme (Fig. 4). A double-digestion with *Eco*RI and *Xho*I and restriction with *Hin*dIII and *Eco*RV, each resulted in at least four hybridization fragments of varying intensities. These results indicate that there are multiple copies of *XVSAP1* in *X. viscosa*.

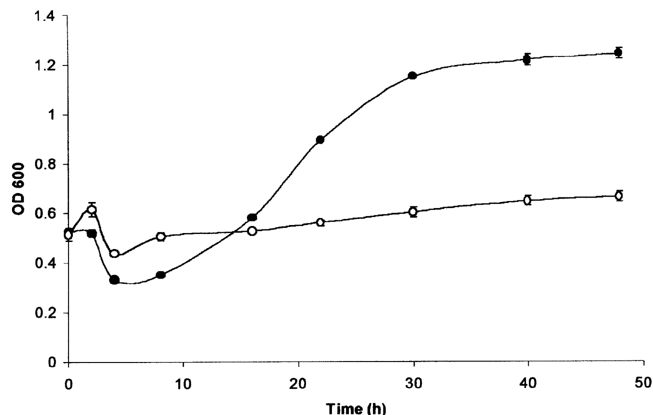

Fig. 1. Growth analysis of *E. coli* (*srl*::Tn10) cells transformed with the prokaryotic protein expression vector pPROEXHT (open circles) and cells transformed with pPROEXHT-*XVSAP1* (closed circles) in minimal media. The expression of XVSAP1 was induced with IPTG at time zero. The cells were allowed to grow for a further 2 h before sorbitol was added to a final concentration of 1 M. Samples were taken at intervals and the absorbance at 600 nm determined. Error bars represent standard deviation based on the average of triplicate samples.

Semi-quantitative RT-PCR

Semi-quantitative RT-PCR was used to compare the relative transcript levels after various stress treatments. Competitor RNA prepared as described in the Materials and methods was used as a control for variations in the RT and PCR reactions. *XVSAP1* was induced by dehydration with the transcript appearing only at 51% and 44% RWC (Fig. 5A). There was no evidence of the transcript at 4% RWC. *XVSAP1* was not detected during rehydration of *X. viscosa* (Fig. 5B). The dehydration–hydration curve (Fig. 5C) for the above treatment revealed that the plant took 12 d to dehydrate to 4% RWC and then completely rehydrated within 4 d after watering. Heat (Fig. 6A), salt (Fig. 6B) and high light (Fig. 6C) resulted in significant induction of *XVSAP1*. The transcripts took 3 d to appear with heat shock and had declined by day 8 of the treatment. Salt shock resulted in the induction of *XVSAP1* expression within 24 h. During the treatment, the transcripts were evident for 7 d, but began to decline on the sixth day of the treatment. The transcripts appeared within 48 h with high light treatment, whereas with the cold treatment (Fig. 6D), the transcripts were evident within the first 24 h. Levels of *XVSAP1* transcription during cold treatment remained fairly steady for the duration of the experiment.

Discussion

The cDNA designated *XVSAP1* was isolated from dehydrated *X. viscosa* leaves using complementation by

A

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1    ATGAGGAACGAGGGTTTTCTGAAAATGAAGACCGACGTTGGAGTCGCCGACGAGGTGATC
1     M  R  N  E  G  F  L  K  M  K  T  D  V  G  V  A  D  E  V  I

61   TCCGGAGATCTCAAGCAGCTTGGTGACGCTGCAAAGCGGCTAGCTAAACATGCGATCAAG
21    S  G  D  L  K  Q  L  G  D  A  A  K  R  L  A  K  H  A  I  K

121  CTCGGCGCCAGCTTCGGGGTTGGCTCTACCATAGTCCAGGCTATTGCTTCGATCGCTGCT
41    L  G  A  S  F  G  V  G  S  T  I  V  Q  A  I  A  S  I  A  A

181  ATCTATTTGTTGATATTGGACCGGACAAACTGGCGTACAAATATCTTGACATCACTTCTA
61    I  Y  L  L  I  L  D  R  T  N  W  R  T  N  I  L  T  S  L  L

241  ATTCCATATGTTTACTTGAGTCTTCCTTCAGTGATATTCAACCTATTCAGGGGCGACCTG
81    I  P  Y  V  Y  L  S  L  P  S  V  I  F  N  L  F  R  G  D  L

301  GGCAGATGGCTTTCATTCATTGGCGTAGTAATGAAGCTCTTCTTCCACCGACACTTCCCA
101   G  R  W  L  S  F  I  G  V  V  M  K  L  F  F  H  R  H  F  P

361  GTTACCTTGGAACTGCTTGTGTCTCTCATTCTCCTGATTGTGGTTTCCCCCACTTTCATT
121   V  T  L  E  L  L  V  S  L  I  L  L  I  V  V  S  P  T  F  I

421  GCCCACACAATCAGAGGCAGTCTCATTGGAGTCTTCATCTTCCTTGTCATCGCCTGCTAC
141   A  H  T  I  R  G  S  L  I  G  V  F  I  F  L  V  I  A  C  Y

481  CTCCTCCAAGAGCACATTAGATCAGCTGGTGGCTTCAAAAACGCGTTCACAAAGAGCAAT
161   L  L  Q  E  H  I  R  S  A  G  G  F  K  N  A  F  T  K  S  N

541  GGGATTTCAAACAGCGTCGGGATCATCATTCTACTGATCCACCCGATCTGGAGCTTGGTG
181   G  I  S  N  S  V  G  I  I  I  L  L  I  H  P  I  W  S  L  V

601  GTGTATTTCCTCTACACGTCTTTGCTGCAACTTCTTGCATACTCTCCTTCCCCTTGTTGT
201   V  Y  F  L  Y  T  S  L  L  Q  L  L  A  Y  S  P  S  P  C  C

661  TGCATATTATACAATAAGTGGTTTAATTTCATGCATGTTTGTAAATGTGTAAGCCTTCAT
221   C  I  L  Y  N  K  W  F  N  F  M  H  V  C  K  C  V  S  L  H

721  ATGTATTCTCAGTCAATTGGGTCATGCGTGTCCATATTTTTCGTGCAGTTTGTATTCATC
241   M  Y  S  Q  S  I  G  S  C  V  S  I  F  F  V  Q  F  V  F  I

781  TATGAAGCTGAATTTTAA
261   Y  E  A  E  F  *
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B

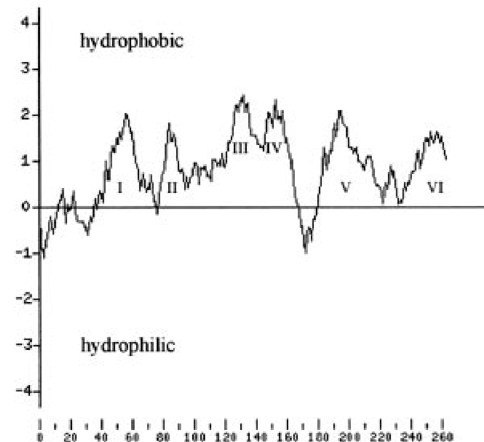

Fig. 2. (A) The nucleotide and deduced amino acid sequences of *XVSAP1*. The start and stop codons are indicated by shading. The *N*-myristoylation site is indicated by broken lines and the prokaryotic membrane lipoprotein lipid attachment sites are underlined by bold lines. The regions of the six transmembrane helices are indicated in bold letters. (B) A hydrophobicity profile of XVSAP1 protein as determined by the method of Kyte and Doolittle (1982) using a window of 19 amino acid residues. The six putative transmembrane domains are indicated by Roman numerals.

functional sufficiency as described by Mundree *et al.* (2000). The *E. coli* (*srl*::Tn10) mutant strain used in this study lacks a specific sorbitol transport system and is unable to catabolize this osmoticum. The cells are

A

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XVSAP1       1 -------MRNEGFLKMKTDVGVADEVISGDLKQLGDAAKRLAKHAIKLGASFGVGSTIVQ
WCOR413      1 ---MAKSFLAMKTGPAAGASEASQALLESDLRELTMAARKLANHAIVLGGGIGFIGTFLQ
ATCAP1       1 -------MGRMDYLAMKTDDVDTVALVNSDMEELKVAAKKLFSDVSKLGG-LGFGVSFLK
ATCAP2       1 ----------MPMKSLRNDHGTLKAMIGSDFNELTIAAKNLATHAFTLTG-LGFGTSVLE
ATCAP3       1 -------MGRGEFLAMKTEEN-AANLINSDMNEFVAAAKKLVKDVGMLGG-VGFGTSVLQ
ATCAP4       1 ---------------------------MENIEYLNEIQAVAGKLIHSYGVPVMITLFLR
RCAP         1 MGKGFMSYLAMKT-DAAGGEAAQAALIDADLQELGVAARKLANHALVLGGGLGFGTTFLK
consensus    1           .    .. .      ... ... .. ....... ... ... ........

XVSAP1      54 AIASIAAIYLLILDRTNWRTN--ILTSLLIPYVYLSLPSVIFNLFRGDLGRWLSFIGVVM
WCOR413     58 WLAFAAAVYLLVLDKTNWKTN--MLTGLLVPYIFFTMPGLLFGFIRGEIGAWIAFVVVVL
ATCAP1      53 FLASFAAIYLLILDRTNWKTK--MLTSLLIPYIFLSLPSVIFNFLSGDVGKWIAFVAVVL
ATCAP2      50 WVASIAAIYLLVLDRTNWKTN--MLTSLLIPYIFFSLPSLIFGIFRGEIGKWIAFVAVVV
ATCAP3      52 WAASIFAIYLLILDRTNWKTK--MLTTLLVPYIFFTLPSVIFQFFSGDFGKWIALIAIIV
ATCAP4      33 WLASIVAVFLMILDQTKWKYSNNIMASLLAPYLFSSLPIVIFQVLRNGVGKWIALLTVIL
RCAP        60 WLAFFAAVYLLILDRTNWKTN--MLTALLVPYIFFTLPGGLFSLLRGEIGKWIAIIAVIL
consensus   61 ..*...*..*..**.*.*...  ....**.**.....*...*.......*.*........

XVSAP1     112 KLFFHRHFPVTLELLVSLILLIVVSPTFIAHTIRGS--LIGVFIFLVIACYLLQEHIRSA
WCOR413    116 RLFFPRHFPDWLELPGSLILLTVVAPAIFADTFRGSWLIIGVGVCLVIGCYLLHEHIKAS
ATCAP1     111 RLFFPKHFPDWLEMPGSLILLLVVSPHFLAHHIRGT--WIGTVISLFIGCYLLQEHIRAS
ATCAP2     108 QLFFPKHAREYLELPVALVLLAVVAPNLIAGTFRDS--WIGLAICLGIGCYLLQEHIRAS
ATCAP3     110 RLFFPKEFPEWLEIPVALILIVVVSPSLIAWTLRES--WVGAVICLVIACYLFHEHIKAS
ATCAP4      93 RLFLPNHFHESLEIPGATILLIVVTPSDIGAIFRDDLRYTGGDVCLLTSFYLINKHTKAC
RCAP       118 RLFFPRHFPDWLELPGAVILLIAVAPNLFASTFRG--DLVGIFICLIIGCYLLQEHIRAS
consensus  121 .**........**......*...*.* ... ..*..  ..*. ..*....**...*....

XVSAP1     170 GGFKNAFTKSNGISNSVGIIILLIHPIWSLVYFLYTSLLQLLAYSPSPCCCILYNKWFNF
WCOR413    176 GGLKEAFQKPNGWSNTIGILLLFIYPVWAVVMWFL-------------------------
ATCAP1     169 GGFRNSFTQPRGVSNTLGIILLLVYPVWALIVRVM-------------------------
ATCAP2     166 GGFRNAFTKANGISNTVGIICLVVFPVWALIF----------------------------
ATCAP3     168 GGFKNSFTQKNGISNTIGIVALLVYPVWTIFFHIF-------------------------
ATCAP4     153 GGIKNSFTQKDKVTYSICLWILFVYPILSSFAALFYL-----------------------
RCAP       176 GGFRNAFRKGNGVSNSIGILLLFIYPVWALVLNFL-------------------------
consensus  181 **....*.. ...........*...*.....  .

XVSAP1     230 MHVCKCVSLHMYSQSIGSCVSIFFVQFVFIYEAEF
WCOR413        -----------------------------------49%
ATCAP1         -----------------------------------56%
ATCAP2         -----------------------------------55%
ATCAP3         -----------------------------------51%
ATCAP4         -----------------------------------38%
RCAP           -----------------------------------53%
consensus  241
```

B

100% 80% 60% 40% 20%

XVSAP1

ATCAP1

ATCAP3

ATCAP2

WCOR413

RCAP

ATCAP4

Fig. 3. (A) Comparison of the deduced amino acid sequence of XVSAP1 with related proteins. The proteins are WCOR413 (GenBank Accession No. T06810), a cold-regulated protein from wheat, cold associated proteins (ATCAP1–4: GenBank Accession Nos T08404, AAD41971, CAB16776, and T02423, respectively) from *Arabidopsis* and an *Oryza sativa* cold-acclimation WCOR413-like protein (RCAP: GenBank Accession No. AAG13395). Only *Arabidopsis* proteins showing the highest homology are shown. Percentages following the sequences indicate the per cent identity to XVSAP1. Identical amino acid residues are indicated by asterisks. Similar amino acids are shown by points. (B) Homology tree for *XVSAP1* and related proteins.

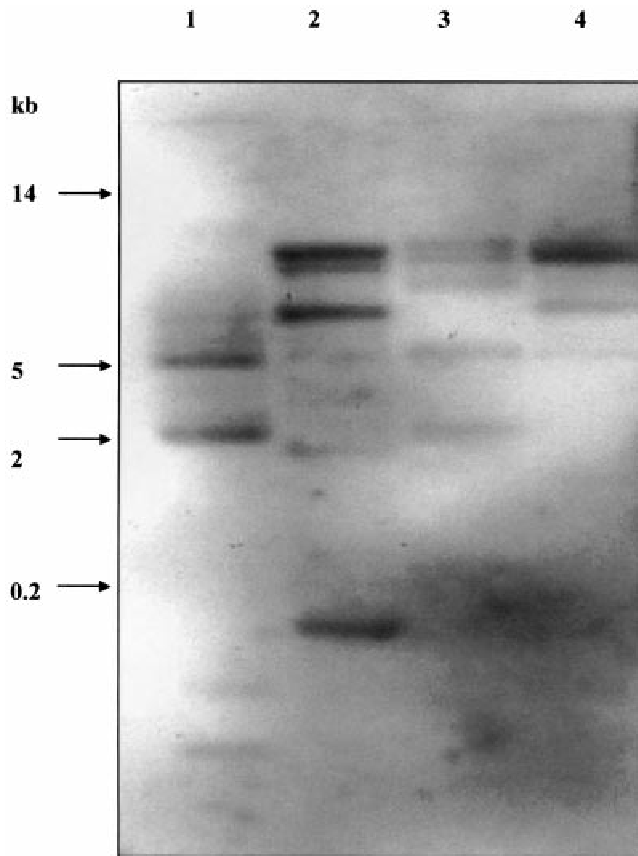

Fig. 4. Southern blot analysis of genomic DNA from *X. viscosa*.15 μg of DNA was cut with *Eco*RI/*Xho*I (lane 1), *Bgl*II (lane 2), *Hin*dIII (lane 3), and *Eco*RV (lane 4), electrophoresed on a 1% agarose gel, transferred to a nylon membrane and probed with DIG-labelled *XVSAP1* cDNA.

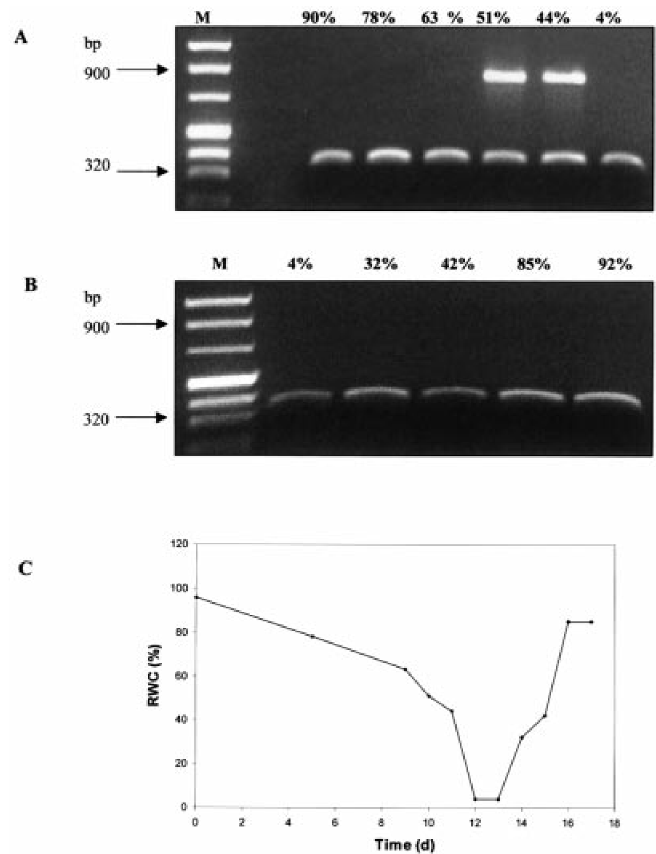

Fig. 5. Expression of the *XVSAP1* transcript during dehydration and rehydration of *X. viscosa* was determined using semi-quantitative RT-PCR. PCR products were visualized on agarose gels stained with ethidium bromide. The native *XVSAP1* product was 830 bp and the competitor 345 bp. M refers to the marker lane. Percentages refer to the relative water content (RWC). (A) *X. viscosa* plants were dehydrated from 90% RWC to 4% RWC by withholding water for a period of 2 weeks. (B) The same *X. viscosa* plants were rehydrated over 5 d by watering. (C) The RWC variation during the dehydration–hydration of *X. viscosa*.

therefore unable to grow in minimal media in which sorbitol is the sole carbon and energy source (Csonka and Clark, 1979). *XVSAP1* cloned in a prokaryotic expression vector was able to rescue *E. coli* (*srl*::Tn10) cells growing in media containing a high concentration of sorbitol confirming this study's hypothesis that the gene is associated with osmotic stress tolerance. Comparable experiments with a construct expressing the related WCOR413 from wheat showed that the protein has similar effects to those of XVSAP1 (data not shown).

The predicted XVSAP1 is a highly hydrophobic protein that is probably anchored in the plasma membrane. XVSAP1 showed significant identity to proteins identified in wheat, rice and *Arabidopsis*. Only the wheat protein, WCOR413, has been partially characterized in this group. The WCOR series of stress-inducible proteins from wheat resembles soluble hydrophilic dehydrins in contrast to XVSAP1, which is predicted to be an integral membrane protein. Data supplied with the protein sequence in the GenBank (Accession number T06810) indicate that the WCOR413 is a cold-regulated protein. The proteins from rice and *Arabidopsis* are classed as cold-associated proteins on the basis of their similarity to WCOR413 at the amino acid level. As is evident from the results of the homology analysis, XVSAP1 is more closely related to the first three ATCAPs from *Arabidopsis* than to WCOR413. The level of identity between XVSAP1 and its homologues suggests that the protein may also be a cold-regulated protein. An analysis of the genomic organization of *XVSAP1* by Southern blotting confirmed that the gene is indeed present in the *X. viscosa* genome. As with the *Arabidopsis* homologues, there is more than one copy of the gene in the nuclear genome suggesting that *XVSAP1* belongs to a small gene family.

An examination of the sequence of XVSAP1 revealed very few clues to the possible functions of the protein in conferring stress tolerance. It is possible that XVSAP1 may be involved in the transport of substances or ions across the plasma membrane as a region stretching from

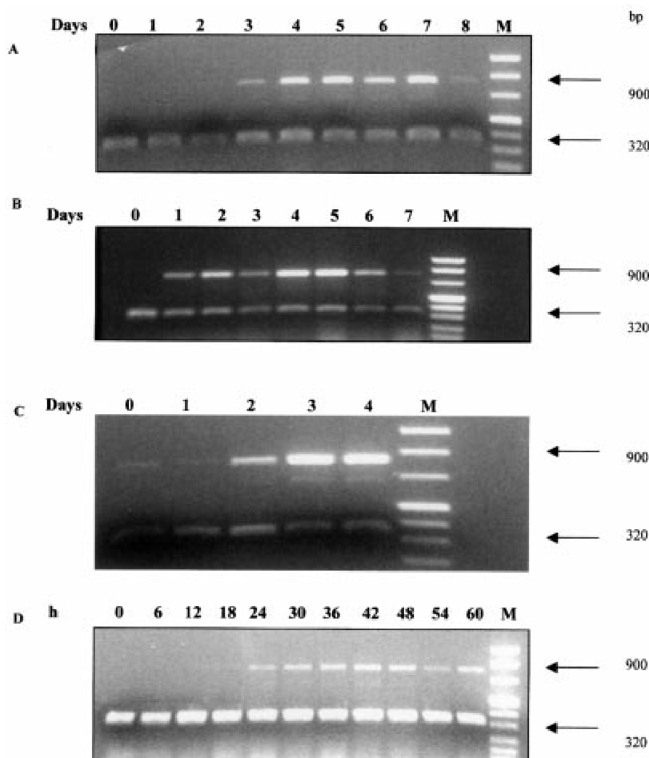

Fig. 6. Induction of *XVSAP1* by heat, NaCl, high light intensity, and low temperature treatments in *X. viscosa* was compared using semi-quantitative RT-PCR. PCR products were visualized on agarose gels stained with ethidium bromide. The native *XVSAP1* product was 830 bp and the competitor 345 bp. M refers to the marker lane. (A) Plants were kept in a 42 °C incubator for 7 d to induce heat stress. (B) Salt shock was induced by irrigating potted plants with 100 mM NaCl. (C) Plants were exposed to high light at 1500 µmol m^{-2} s^{-1} for 4 d in a phytotron (25 °C, humidity 50–70%) for the high light treatment (D) Cold treatment was achieved by keeping plants at 4 °C for 60 h.

amino acid residue 36 to 119 bears 12% identity with a K^+ potassium transporter family. The family is conserved across phyla, having plant (AtKT), yeast (HAK) and bacterial (KUP) sequences as members (Quintero and Blatt, 1997). However, no data are currently available to support this possibility.

It is conceivable that XVSAP1 may be modified for full function as is indicated by the presence of two prokaryotic membrane lipoprotein lipid attachment sites. Modification of proteins by the covalent attachment of lipids appears to be widespread in living systems (Hayashi and Wu, 1990). The presence of these sites supports the concept that XVSAP1 associates closely with the cell membrane. It is interesting to note that the *Arabidopsis* homologues also have prokaryotic membrane lipoprotein lipid attachment sites. XVSAP1 is possibly further processed for full function by the attachment of lipids as is implied by the existence of one putative *N*-myristoylation site. The process of *N*-myristoylation is a cotranslational modification that involves the covalent reaction of myristate and the amino-terminal glycine residue of a growing polypeptide. Proteins so modified have diverse functions and the myristate appears to be critical for mediating protein–protein and/or protein–membrane interactions (Johnson *et al.*, 1994; Ishitani *et al.*, 2000). However, there is currently no evidence that myristoylation is required for the function of XVSAP1 or indeed that it occurs at all.

It has been shown that many genes induced by drought are also induced by salt and cold stresses (Zhu *et al.*, 1997). All these factors result in osmotic stress or water deficit in plant cells. As *XVSAP1* was isolated on the basis of its ability to confer tolerance to osmotically stressed *E. coli* (*srl*::Tn10), it was necessary to establish if *XVSAP1* was involved in the response to these abiotic stresses. An examination of the expression of *XVSAP1* during dehydration and rehydration of *X. viscosa* showed that only dehydration and not rehydration induces the expression of the gene. Interestingly, *XVSAP1* expression was strongly induced at 51% and 44% RWC and not at any other stage. This indicates that *XVSAP1* is not required in the initial stages of dehydration, but is only expressed when dehydration becomes severe and the plant has dried down to approximately 50% RWC. No expression of the gene was evident at 4% RWC. Since XVSAP1 is likely to be an integral membrane protein, one of the roles it could play is the stabilization of membranes during the drying process. As the plant dries further, its metabolic processes decline and eventually stop. This could explain why the expression of *XVSAP1* is not observed at 4% RWC. As no expression of *XVSAP1* was observed during rehydration, this indicates that *XVSAP1* has no role to play during this process. However, the absence of the transcript does not imply absence of the protein product. It has been observed that most of the components required for recovery from desiccation are already present in the plant during dehydration in *X. humilis* (Dace *et al.*, 1998). In *X. viscosa,* XVSAP1 could be one of those components involved in the repair of membrane damage that results from severe water deficit. Studies of expression at the protein level would clarify whether XVSAP1 is involved in just dehydration or whether the protein is also part of the rehydration process.

Heat and high light stresses both strongly induced the accumulation of *XVSAP1* mRNA. In both cases, the transcript only started to accumulate at least 48 h after imposition of the stress. The results obtained suggest that *XVSAP1* is not involved in the initial stages of the response to heat or high light intensity. It is expected that *X. viscosa* would have mechanisms in place to deal with such stresses in the short term as the extremophile grows in environments where it is regularly exposed to high temperature and high light intensity. However, when the duration of these stresses increases, other mechanisms that would have a protective effect come into play. It is known that heat stress affects most cellular processes and causes denatura-

tion of proteins, cellular enzymes, and damage to membranes. The damage is due to the temperature change itself as well as heat-induced oxidative stress (Karim *et al*., 1999; Munro and Pelham, 1985). Survival after heat stress requires an ability to tolerate or repair oxidative damage as well other kinds of heat-induced damage. It is expected that XVSAP1 would have a role to play in the protection of membranes against heat damage.

In a similar manner, high light intensity can result in the formation of reactive oxygen species (ROS). If the free radicals are not quenched, damage in the form of photobleaching and lipid peroxidation occurs (Smirnoff, 1993). *X. viscosa* appears to withstand damage caused by light by a combination of protective and avoidance mechanisms. The poikolochlorophyllous resurrection plant loses its chlorophyll and dismantles its photosynthetic apparatus, while the levels of anthocyanins and antioxidant enzymes increase, affording the plant a degree of protection (Sherwin and Farrant, 1998). As with heat stress, it is proposed that XVSAP1 is involved in the protection of membranes possibly by maintaining structural integrity.

High exogenous salt concentrations cause an imbalance of cellular ions resulting in ion toxicity, osmotic stress and production of ROS (Hasegawa *et al*., 2000). Various molecules including proteins that protect membrane integrity, control ion homeostasis and play a role in ROS scavenging have been reported to attenuate salt stress effects (Hasegawa *et al*., 2000; Ingram and Bartels, 1996; Ishitani *et al.,*1997). Studies in both wheat and barley showed that the induction of genes by salt occurs within 2 h and that many transcripts decrease in abundance within 24 h (Robinson *et al*., 1990). However, in the case of *XVSAP1,* the transcript appeared within 24 h after salt shock and persisted for 7 d. The response *XVSAP1* to salt is again delayed compared to other salt-responsive genes. In addition, it also lasts for a longer period, supporting the earlier theory that the gene is expressed on persistence of a particular abiotic stress.

XVSAP1 has a relatively high identity to cold-responsive WCOR413 and the uncharacterized *Arabidopsis* homologues (Fig. 3A). It was therefore reasonable to consider that the gene could be induced by cold. This proved to be the case. *XVSAP1* was detected within 24 h after the commencement of the treatment. The results correlate well with those obtained with other *COR* genes. Cold-induced mRNAs generally begin to accumulate within a few hours at low temperature and remain at high levels until removal of the stress. The *CBF* genes are induced within 15 min of plants being exposed to low temperatures followed, at about 2 h, by the induction of cold-regulated genes that contain the CRT/DRE regulatory element (Gilmour *et al*., 1998; Thomashow, 1998). It is expected that in the natural habitats of *X. viscosa,* temperatures at night could go well below zero on occasion. *XVSAP1* would therefore form part of the mechanism that assists *X. viscosa* to cope with the stress, particularly since chilling injury is mainly a consequence of destablization of cell membranes.

It has been established that many genes that respond to drought and/or cold stress are also induced by exogenous applications of ABA (Bray, 1997; Chandler and Robertson, 1994). However, in the case of *X. viscosa,* ABA treatment *in planta* failed to induce *XVSAP1*. Moreover, placing of excised leaves in a 100 μM solution of ABA did not have an effect on the expression of *XVSAP1* despite the fact that less competitor RNA was used in the RT-PCR reaction (results not shown). Shinozaki and Yamaguchi-Shinozaki (1997) suggested that there are at least four independent signal pathways that function in the activation of stress-inducible genes. Two of these are ABA-dependent (pathways I and II) and two are ABA-independent (pathways III and IV). The fact that *XVSAP1* was not induced by exogenous applications of ABA suggests that *XVSAP1* responds to environmental stresses through an ABA-independent pathway. It is also possible that the response to ABA is transient and was not detectable under the experimental conditions used.

The data presented here show that *XVSAP1* is a stress-associated gene in *X. viscosa*. The fact that the gene is induced by heat, high salt, cold, and dehydration is not surprising since the gene was isolated on the basis of its response to osmotic stress. It is known that the above abiotic stresses all result in water deficit in the cell. It is predicted that the protein product would play a protective role possibly in stabilizing cell membranes during dehydrative stresses.

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