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An aldose reductase homolog from the resurrection plant *Xerophyta viscosa* Baker

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Abstract. An aldose reductase homologue (*ALDRXV4*) was cloned from the resurrection plant *Xerophyta viscosa* Baker using complementation by functional sufficiency in *Escherichia coli*. A cDNA library constructed from *X. viscosa* leaves dehydrated to 85%, 37% and 5% relative water contents (RWC) was converted into an infective phagemid library. *Escherichia coli* (srl::Tn10) cells transformed with ds-pBluescript phagemids were selected on minimal medium plates supplemented with 1 mM isopropyl β-D-thiogalactopyranoside and 1.25 M sorbitol. Nine cDNA clones that conferred tolerance to the osmotically stressed *E. coli* cells were selected. The phagemid from one clone contained the *ALDRXV4* insert. The *E. coli* cells expressing *ALDRXV4* were capable of tolerating the osmotic stress, whereas control cultures were not. The ALDRXV4 insert contained an open reading frame that can code for 319 amino acids, and the predicted protein had a calculated M_r of 35,667. Amino acid sequence comparisons revealed significant similarity to several aldose reductases, with the highest similarity to aldose reductase proteins from *Hordeum vulgare*, *Bromus inermis* and *Avena fatua*, in the order of 66%, 65% and 65% respectively. Northern blot analysis revealed that *ALDRXV4* was expressed only under dehydration conditions in *X. viscosa* leaves. Western blot analysis detected a protein of 36 kDa under dehydration conditions only. Aldose reductase activity levels in *X. viscosa* leaves increased as the leaf RWC decreased, whereas there was no significant change in aldose reductase activity in *Sporobolus stafianus* as the leaf RWC decreased.

Key words: Aldose reductase – Desiccation stress – Resurrection plant – *Xerophyta*

Introduction

The desiccation-tolerant plant *Xerophyta viscosa* Baker (Family Velloziaceae) belongs to a small group of angiosperms, referred to as "resurrection plants", that are capable of tolerating extremes of desiccation (Gaff 1971; Bewley and Oliver 1992; Vertucci and Farrant 1995; Ingram and Bartels 1996). *Xerophyta viscosa* can be dehydrated to 5% relative water content (RWC) and upon rewatering the desiccated plant rehydrates completely within 80 h, resuming full physiological activities (Sherwin and Farrant 1996). This unique ability of tolerating severe water loss is shared with certain algae and bryophytes (Oliver and Bewley 1997), a few ferns (Reynolds and Bewley 1993), and with specialized structures of higher plants such as seeds and pollen grains (Kermode et al. 1986).

A variety of mechanisms have been identified which enable these extremophiles to survive severe water loss. It has been hypothesized that the mechanisms essential for desiccation tolerance in algae and bryophytes which encompasses repair processes during rehydration, are constitutively present (Oliver 1991; Oliver and Bewley 1997). Resurrection plants, on the other hand, appear to activate the synthesis of specific transcripts and proteins during dehydration (Ingram and Bartels 1996), although it has been proposed that different plants rely on the processes of protection and repair to different extents (Oliver and Bewley 1997).

It is assumed that molecules and compounds synthesized and accumulated during desiccation play an important role in the protection from stress. Such compounds are thought to protect intracellular components such as enzymes, membranes and other macromolecules against damage due to desiccation (Ahmad et al. 1979; Crowe et al. 1984; Oliver and Bewley 1997).

The nucleotide sequence reported will appear in the Genebank Nucleotide Sequence Database under the accession number AF133841

Abbreviations: IPTG = isopropyl β-D-thiogalactopyranoside; RWC = relative water content

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Among the osmoprotective organic solutes which accumulate in higher plants, the most extensively studied compounds include amino acids such as proline (Csonka and Hanson 1991) and quaternary ammonium compounds such as glycine betaine (Hanson and Wyse 1982). Numerous carbohydrates, including sucrose and various reducing sugars (Breins and Larher 1982; Crowe et al. 1984), two classes of polyhydric alcohols (Lewis 1984; Hellebust 1985), the straight-chain alditols (Ahmad et al. 1979; Breins and Laher 1982) and the cyclitols derived from inositol (Ford 1984; Streeter 1985; Loewus 1990) have also been reported extensively. The biochemical and molecular basis for these protective mechanisms is still poorly understood.

In order to isolate genes whose products are functionally important in water-deficit stress, the strategy of "complementation by functional sufficiency" (Mundree 1996) was employed. In this paper we describe the isolation and characterization of one cDNA clone (*ALDRXV4*) that encodes a protein which has significant similarity to aldose reductases, a sub-group of the aldo-keto reductase superfamily (Bohren et al. 1989). The aldo-keto reductases are cytosolic, monomeric oxidoreductases which catalyse the NADPH-dependent reduction of carbonyl metabolites (Bohren et al. 1989).

Materials and methods

Plant material

Xerophyta viscosa Baker plants were collected from the Buffelskloof Nature Reserve (Mpumalanga Province, South Africa). *Xerophyta viscosa* and *Sporobolus stafianus* were grown under glasshouse conditions as described by Sherwin and Farrant (1996). Plant drying and determination of RWC were also described previously (Sherwin and Farrant 1996).

Construction and screening of a cDNA library

Two micrograms of poly(A)$^+$ RNA was extracted from *X. viscosa* leaves that were at 85%, 37% and 5% RWC. The RNA was pooled and used as a template to construct a cDNA library in λ ZAPII (Stratagene, La Jolla, Calif., USA) as described by the manufacturer. The amplified library was converted into an infective, packaged ss-pBluescript phagemid library (Short et al. 1988). A 200-µl aliquot of the λ ZAP cDNA library (average titer = 1.5×10^8 plaque-forming units (pfu) per mL) was added to 200 µl of XL-1-Blue cells (containing 10^{11} cells) (Stratagene) and 1 µl of Exassist helper phage (3×10^{10} pfu/mL). After an incubation period of 15 min at 37 °C, 5 mL of 2× YT medium (1.6% tryptone, 1% yeast extract, 0.5% NaCl) was added and the tube was incubated at 37 °C for 3 h with shaking. The tube was subsequently heated at 70 °C for 20 min and centrifuged at 4,000*g* for 10 min to remove λ phage. The supernatant containing the ds-pBluescript phagemids was used to infect the *E. coli* (K12, pro, leu, lacY, rspLzo, hsdR, hsdM, ara14, galk2, xyl5, srl::Tn10 (Tet^r) recA Δ, supE44, λ^-, F$^-$) mutant strain (Csonka and Clark 1979). Infected bacterial cells were selected on M9 minimal medium plates supplemented with 1.25 M sorbitol, 50 µg/mL ampicillin and 0.1 mg/mL isothiopropylgalactoside (IPTG). Colonies growing on M9 minimal medium plates containing 1.25 M sorbitol were isolated and their respective plasmids purified (Sambrook et al. 1989). SK(+) containing the cDNA insert, referred to as *ALDRXV4*, was used in subsequent experiments.

Sequencing and analysis of DNA

The nucleotide sequence of *ALDRXV4* cDNA clone was determined on both strands using the ALFexpress automated DNA Sequencer AMV3.0 (Pharmacia Biotech AB, Uppsala, Sweden). The sequencing reactions were carried out using the Thermo Sequenase Fluorescent Labelled Primer cycle sequencing kit (Amersham International, Buckinghamshire, UK). The inferred amino acid sequence of ALDRXV4 was used to search for similarities in protein sequence databases using the BLAST network service (Altschul et al. 1990). Amino acid comparisons were done with the CLUSTAL program of DNAMAN (Version 3.0 1997).

Isolation of RNA

Total RNA was isolated from hydrated (100% RWC), and dehydrated (85%, 37% and 5% RWC) leaves of *X. viscosa* using the guanidium thiocyanate-phenol-chloroform method for RNA (Chomczynski and Sacchi 1987). Oligo-dT cellulose affinity chromatogaphy was utilized to isolate poly(A)$^+$ RNA (Sambrook et al. 1989). The RNA samples were quantified spectrophotometrically (Sambrook et al. 1989).

Northern blot analysis

RNA was electrophoresed on 1.2% formaldehyde gels (Chomczynski and Sacchi 1987) in 20 mM Mops pH 7.0, and transferred to nylon membranes (MSI, 0.45 µm) by capillary elution (Chomczynski and Sacchi 1987). Filters were prehybridized at 68 °C for 4 h and hybridized for 18 h at 68 °C in 6× SSC (1× SSC is 150 mM NaCl, 17 mM sodium citrate), 5× Denhardt's solution (Sigma-Aldrich, UK), 0.1% SDS, and 100 µg/mL herring sperm DNA. The *ALDRXV4* insert was labelled with [^{32}P]dCTP by random primer labeling (Boehringer Mannheim, Germany) and used as a probe. Filters were washed at room temperature in 2× SSC, 0.1% SDS for 3 × 5 min, and finally at 68 °C in 0.1× SSC, 0.1% SDS for 2 × 1 h. The membranes were exposed to X-ray film at −79 °C for appropriate durations.

Western blot analysis

Proteins extracted from *E. coli* cells (Barron et al. 1986) and *X. viscosa* leaves (Bartels et al. 1991) were separated on 12% denaturing polyacrylamide gels (Sambrook et al. 1989). Electrophoretic transfer of proteins from SDS-polyacrylamide gels to nitrocellulose membranes was carried out as described by Rybicki and von Wechmar (1982). The membrane was probed with barley aldose reductase antiserum (1:1000 dilution) (Bartels et al. 1991) and detected using goat anti-rabbit antibody conjugated to alkaline phosphatase (Sigma-Aldrich, UK) as described by Sambrook et al. (1989). Molecular weight (MW) was determined by comparison to BioRad low-MW standards.

Measurement of enzymatic activity

The extraction of aldose reductase was carried out according to a modification of the method by Bartels et al. (1991). Extractions were performed at 4 °C. Extracts were prepared by grinding approximately 0.5 g of *X. viscosa* leaf material to a fine powder with liquid nitrogen. An equal amount of polyvinyl polypyrrolidone (PVP) was added to the tissue during grinding. Ice-cold extraction buffer [20 mM potassium phosphate buffer (pH 7.5), 0.5 mM EDTA, 5 mM DTT and 1 mM phenylmethylsulfonylfluoride (PMSF)] was added in a buffer volume to tissue mass ratio

of 30:1. The extracts were homogenised (Kinematica Polytron, model PT-10-35) for 1 min and then centrifuged (Beckman model J2-21) for 10 min at 17,200*g*. Ammonium sulphate was added to the supernatant to attain 40% saturation. The extract was centrifuged for 15 min at 17,200*g*. The supernatant was saturated fully with ammonium sulphate. After centrifugation, the precipitated protein was resuspended in 500 µl extraction buffer and desalted using Sephadex G-25 (particle size 50–150 mm; Pharmacia) columns equilibrated with the extraction buffer. Four replicates for each leaf RWC were obtained. Enzyme activity was expressed on both a dry-mass and a protein basis. Protein concentrations were determined using the method of Bradford (1976), with gamma globulin as a standard.

Escherichia coli (srl::Tn10) cells transformed with *ALDRXV4* cDNA clone were grown in 500-mL flasks containing 100 mL Lauria-Bertani (LB) medium (Sambrook et al. 1989) in the presence and absence of 1 mM IPTG. The flasks were incubated at 37 °C with shaking. Cells were harvested by centrifugation for 10 min at 4000*g* and resuspended in 4 mL extraction buffer [20 mM potassium phosphate buffer (pH 7.5), 0.5 mM EDTA, 5 mM DTT and 1 mM PMSF]. Cells were lysed by rapid freezing in liquid nitrogen followed by homogenization for 1 min. The extracts were centrifuged for 10 min at 17,200*g*. The supernatants were utilized for measurement of aldose reductase activity.

Aldose reductase activities in *X. viscosa* and *S. stapfianus* leaves, and in *E. coli* extracts were measured in a total volume of 1 mL by following the oxidation of NADPH (at 340 nm) using a Beckman Du 650 spectrophotometer. The standard reaction contained 100 mM potassium phosphate buffer (pH 6.9), 50 mM DL-glyceraldehyde and 0.15 mM NADPH as described by Lee and Chen (1993). Activity was initiated by the addition of DL-glyceraldehyde, D-glucose, D-mannose or D-xylose.

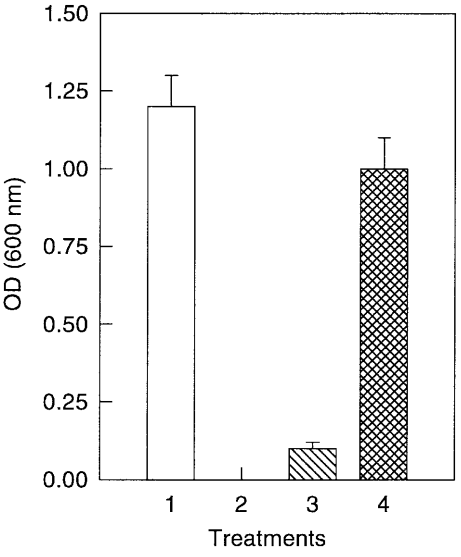

Fig. 1. Growth analysis of *E. coli* (mutant strain srl::Tn10) transformed with SK(+) pBluescript vector grown in M9 minimal medium (*treatment 1*) and in M9 minimal medium supplemented with 1.25 M sorbitol (*treatment 2*). The two further treatments included *E. coli* transformed with *ALDRXV4* [cDNA insert in SK(+)] grown in M9 minimal medium supplemented with 1.25 M sorbitol in the absence (*treatment 3*) and presence (*treatment 4*) of 1 mM IPTG. Cell growth was monitored by determining O.D. readings at 600 nm after 24 hours following inoculation

Results

Isolation of the cDNA encoding ALDRXV4

The strategy of complementation by functional sufficiency used to isolate *ALDRXV4* resulted in the isolation of nine cDNA clones from *X. viscosa*, including *ALDRXV4*. The growth analysis data revealed that the *E. coli* strain srl::Tn10 did not survive in the presence of 1.25 M sorbitol (Fig. 1). *Escherichia coli* (srl::Tn10) containing *ALDRXV4* cDNA clone, exhibited background growth in the absence of IPTG (Fig. 1). However, in the presence of 1 mM IPTG in the growth medium, *E. coli* (srl::Tn10) containing *ALDRXV4* survived in the presence of 1.25 M sorbitol (Fig. 1).

Analysis of ALDRXV4 cDNA

The *ALDRXV4* cDNA insert was 1144 bp long, with an open reading frame of 960 bp (Fig. 2). The first ATG is likely to be the initiation codon. The predicted protein has 319 amino acids and a calculated M_r of 35,667. The protein contained one motif, IPKS, which is highly conserved among aldose reductases (Fig. 2). A computer search for similarities using the BLAST network service revealed that ALDRXV4 has significant similarity to several aldose reductases. The highest similarities were to *Hordeum vulgare*, *Bromus inermis* and *Avena fatua* aldose reductase-like proteins, in the order of 66%, 65% and 65% respectively (Fig. 3). The nucleotide similarities of the 5′ and 3′ untranslated regions between the above clones were considerably lower (approximately 35%).

Northern analysis and drying rates

Northern blot analysis of poly(A)$^+$RNA isolated from hydrated (100% RWC) and dehydrated (85%, 37%, 5% RWC) *X. viscosa* leaves exhibited a single transcript of ≈1.2 kb in leaves that were dehydrated to 85% and 37% RWC only (Fig. 4). The cDNA insert was determined to be a near full-length copy (1144 bp) based on the length of the RNA transcript to which it hybridized. The drying rates of *X. viscosa* and *S. stapfianus* indicated that *X. viscosa* leaves dried to a lower RWC and at a faster rate than those of *S. stapfianus* (Fig. 5).

Western blot analysis

The antibodies raised against a barley aldose reductase cDNA clone by Bartels et al. (1991), were used to analyse the expression of the corresponding protein in *X. viscosa* and *E. coli* (Fig. 6). The antibodies detected a soluble protein of approximately 36 kDa molecular mass in *X. viscosa* leaves (85%, 37% and 5% RWC) and in *E. coli*. The protein was not detected in hydrated (100% RWC) *X. viscosa* leaves (Fig. 6, lane 1). However, the protein in *E. coli* was detected only under induction conditions (IPTG present in the growth meduim) (Fig. 6, lane 6).

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    CGGCACGAGAAGCTACAGAGACGTGAAAGAAGAAGACTCGACTATTTGTTACTTGTAGTAGAAAAAAGATTTTTT  -75

ATG GCG CAT GCA CCG TGT TTT GCT GAT GCG AAG ACA CAG AGC TTC AAG CTC CTC AGC GGG   60
 M  A   H   A   P   C   F   A   D   A   K   T   Q   S   F   K   L   L   S   G   20

CAC TCA ATC CCC GCA GTT GGG CTC GGC ACG TGG AAG TCT GGT GAC AAG GCC GGC AAC GCC  120
 H   S   I   P   A   V   G   L   G   T   W   K   S   G   D   K   A   G   N   A   40

GTA TAC ACT GCC ATC ACT GAG GGA GGA TAC AGG CAC ATT GAT ACC GCA GCA CAA TAT GGA  180
 V   Y   T   A   I   T   E   G   G   Y   R   H   I   D   T   A   A   Q   Y   G   60

GTC CAT GAA GAG GTA GGC AAT GCT CTT CAA TCT GCT TTG AAA GCA GGG ATC AAT AGG AAA  240
 V   H   E   E   V   G   N   A   L   Q   S   A   L   K   A   G   I   N   R   K   80

GCT TTG TTC GTC ACA TCG AAA GTA TGG TGC GAA GAT TTA TCA CCT GAA AGA GTT CGA CCT  300
 A   L   F   V   T   S   K   V   W   C   E   D   L   S   P   E   R   V   R   P  100

GCA TTG AAA AAT ACA CTT GAG GAG CTA CAA CTG GAT TAC CTT GAT CTC TAC CTG ATT CAC  360
 A   L   K   N   T   L   E   E   L   Q   L   D   Y   L   D   L   Y   L   I   H  120

TGG CCT ATC CAC CTT AAA AAG GGC GCA CAC ATG CCT CCT GAG GCT GGT GAG GTG CTA GAA  420
 W   P   I   H   L   K   K   G   A   H   M   P   P   E   A   G   E   V   L   E  140

TTC GAC ATA GGA GGA GTG TGG AGG GAA ATG GAG AAG CTC GTC AAA GTA GGG CTT GTT AGA  480
 F   D   I   G   G   V   W   R   E   M   E   K   L   V   K   V   G   L   V   R  160

GAT ATT GGT ATC TCT AAC TTC ACT GTG AAG AAA CTC GAA AAA CTT CTA AAT TTT GCT GAA  540
 D   I   G   I   S   N   F   T   V   K   K   L   E   K   L   L   N   F   A   E  180

ATA AAG CCC TCG GTG TGC CAG ATG GAG ATG CAC CCG GGT TGG AGA AAG CAC AAG ATG TTT  600
 I   K   P   S   V   C   Q   M   E   M   H   P   G   W   R   K   H   K   M   F  200

GAG ATT TGC AGG AAA TAT GGT ATT CAT ACA ACT GCT TAT TCA CCT CTC GGA TCT TCC GAG  660
 E   I   C   R   K   Y   G   I   H   T   T   A   Y   S   P   L   G   S   S   E  220

CGT GAT CTC CTC AGT GAT CCA ACT GTT TTG AAG ATA GCA AAC AAG CTC AAC AAG AGC CCA  720
 R   D   L   L   S   D   P   T   V   L   K   I   A   N   K   L   N   K   S   P  240

GGT CAA CTT CTG GTG AGA TGG GCT GTT CAA AGA GGA ACT AGT GTC ATC CCA AAA TCG ACC  780
 G   Q   L   L   V   R   W   A   V   Q   R   G   T   S   V   I   P   K   S   T  260

AAC CCG GAG AGG ATA AAG GAG AAC ATC CAG GTC TTC GGG TGG GAG ATT CCT GCA GAG GAT  840
 N   P   E   R   I   K   E   N   I   Q   V   F   G   W   E   I   P   A   E   D  280

TTC CAG ATT TTG AGC AGC CTT AGT GAA CAG AAG AGA GTC TTG GAT GGT GAA GAT CTC TTC  900
 F   Q   I   L   S   S   L   S   E   Q   K   R   V   L   D   G   E   D   L   F  300

GTC AAC AAA ACC CAT GGC CCG TTC AGG AGC GCT GCT GAA CTC TGG GAC GGT GAA GTC TAA  960
 V   N   K   T   H   G   P   F   R   S   A   A   E   L   W   D   G   E   V   X  319

AGTAATTGCAAGGGAAAATACTATCTGGAGAGCCATGCTAGAGACTTTAAATTGTATGCGTATATGTATGTGTGCTTCT 1039
TATAATATGTAGAAAAAAAAAAAAAAAAAA                                                1069
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Fig. 2. Nucleotide and deduced amino acid sequence of *ALDRXV4*. The putative start and stop codons are represented in *bold*. The highly conserved (among aldose reductases) IPKS motif is *underlined*

Aldose reductase activity

In order to link the presence of ALDRXV4 with aldose reductase activity, an enzyme test was conducted on protein extracts from *X. viscosa* and *S. stapfianus* leaves. With a decrease in leaf RWC, there was a significant increase in aldose reductase activity levels in *X. viscosa* (Fig. 7a). The highest mean activity values obtained for *X. viscosa* were in the region from 32% to 15% RWC, and these represented a 6-fold increase in extractable aldose reductase activity in relation to that obtained in leaf samples at 100% RWC . The highest mean activity values obtained for *S. stapfianus* were at 85% RWC, and these represented a 1.5-fold increase in extractable aldose reductase activity in relation to that obtained in leaf samples at 100% RWC (Fig. 7b). Aldose reductase activity was also expressed on the basis of the protein content contained in the 35–100% saturated extract. The specific activity in *X. viscosa* increased from (mean ± SD, $n = 4$) 3.94 ± 0.57 nmol (mg protein)$^{-1}$ min^{-1} (100% RWC) to 14.18 ± 2.58 (32% RWC) and 12.48 ± 2.0 nmol (mg protein)$^{-1}$ min^{-1} (22% RWC). The highest specific activity values obtained for *S. stapfianus* were at 85% RWC, and these again, represented a 1.5-fold increase. The enzymatic activity was dependent on the substrate glyceraldehyde (Table 1). Mannose and xylose were poor substrates while glucose was an efficient substrate (Table 1).

Since the aldose reductase activity is induced during dehydration (Fig. 7a), it was essential to determine whether the induction observed was influenced by either activators or inhibitors which may have co-extracted with the enzyme. Therefore, extracts from the two extremes, i.e. 100% and 22–15% RWC were isolated together and the aldose reductase activity measured (Botha and Small 1987). The aldose reductase activity in the combined leaf tissue extracts was 102 ± 19.4% of that when tissues were extracted separately. The level of aldose reductase activity reported in both hydrated and dehydrated plant material is therefore not attributable to interference by either extractable activators or inhibitors.

Escherichia coli containing the *ALDRXV4* cDNA clone exhibited aldose reductase activity levels of

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ALDRXV4          MAHAPCFADAKTQS-FKLLSGHSIPAVGLGTWKSGDRAGNAVYTAITEGGYRHIDTAAQY  59
HORDEUM          MASAKATMGQGEQDHFVLKSGHAMPAVGLGTWRAGSDTAHSVRTAITEAGYRHVDTAAEY  60
BROMEGRASS       MASAKAMMGQERQDHFVLKSGHAIPAVGLGTWRAGSDTAHSVQTAITEAGYRHVDTAAEY  60
AVENA            MASAKAMG-QGEQDRFVLKSGHAIPAVGLGTWRAGSDTAHSVQTAITEAGYRHVDTAAQY  59
                 ** * .      *. * * ***..********..*. ....* *****.****.****.*

ALDRXV4          GVHEEVGNALQSALKAGINRKALFVTSKVWCEDLSPERVRPALKNTLEELQLDYLDLYLI  119
HORDEUM          GVEKEVGKGLKAAMEAGIDRKDLFVTSKIWCTNLAPERVRPALENTLKDLQLDYIDLYHI  120
BROMEGRASS       GVEKEVGKGLKAAMEAGIDRKDLFVTSKLWCTDLVPDRVRPALEKTLKDLQLDYLDLYLI  120
AVENA            GIEKEVDKGLKAAMEAGIDRKDLFVTSKIWRTNLAPERARPALENTLKDLQLDYIDLYLI  119
                 *...**...*..*..***.** ******.* .* *.*.****..**..*****.*** *

ALDRXV4          HWPIHLKKGAHMPPEAGEVLEFDIGGVWREMEKLVKVGLVRDIGISNFTVKKLEKLLNFA  179
HORDEUM          HWPFRLKDGAHMPPEAGEVLEFDMEGVWKEMENLVKDGLVKDIGVCNYTVTKLNRLLRSA  180
BROMEGRASS       HWPFRLKDGAHKPPEAGEVLEFDMEGVWKEMENLVKDGLVKDIGVCNYTVTKLNRLLQSA  180
AVENA            HWPFRLKDGAHQPPEAGEVLEFDMEGVWKEMEKLVKDGLVKDIDVCNFTVTKLNRLLRSA  179
                 ***..**.*** ***********. ***.***.*** ***.**...*.**.**..**. *

ALDRXV4          EIKPSVCQMEMHPGWRKHKMFEICRKYGIHTTAYSPLGSSERDLLSDPTVLKIANKLNKS  239
HORDEUM          KIPPAVCQMEMHPGWKNDKIFEACKKHGIHVTAYSPLGSSEKNLAHDPVVEKVANKLNKT  240
BROMEGRASS       KIAPAVCQMEMHPGWKNDKILEACKKHGIHATAYSPLCSSEKNLAHDPVVEKVANKLNKT  240
AVENA            NIPPAVCQMEMHPGWKNDKIFEACKKHGIHVTAYSPLGSSEKNLVHDPVVEKVANKLNKT  239
                 .* *.**********...*..* *.*.***.****** ***..*  **.* *.******.

ALDRXV4          PGQLLVRWAVQRGTSVIPKSTNPERIKENIQVFGWEIPAEDFQILSSLSEQKRVLDGEDL  299
HORDEUM          PGQVLIKWALQRGTSVIPKSSKDERIKENIQVFGWEIPEEDFKVLCSIKDEKRVLTGEEL  300
BROMEGRASS       PGQVLIKWALQRGTIVIPKSSKDERIKENIQVFGWEIPEEDFQVLCSIKDEKRVLTGEEL  300
AVENA            PGQVLIKWALQRGTSVIPKSSKDERIKENIQAFGWEIPEDDFQVLCSIKDEKRVLTGEEL  299
                 ***.*..**.**** *****.. ********.****** .**..*.*....**** **.*

ALDRXV4          FVNKTHGPFRSAAELWDGEV  319    100%
HORDEUM          FVNKTHGPYRSAADVWDHEN  320     66%
BROMEGRASS       FVNKTHGPYKSASEVWDNEN  320     65%
AVENA            FVNKTHGPYKSASEVWDHEN  319     65%
                 ********..**...** *
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Fig. 3. Amino acid comparison of ALDRXV4 with related proteins. An asterisk (*) indicates "identity" with the ALDRXV4 sequence while a dot (.) indicates "similarity". Percentages following the sequences indicate the percentage identity to ALDRXV4 as obtained from a computer search using the BLAST network service. The sequences are: *HORDEUM*, an aldose reductase protein from *Hordeum vulgare* (Bartels et al. 1991); *BROMEGRASS*, an aldose reductase-related protein from *Bromus inermis* (Lee and Chen 1993); *AVENA*, an aldose reductase-related protein from *Avena fatua* (Li and Foley 1995)

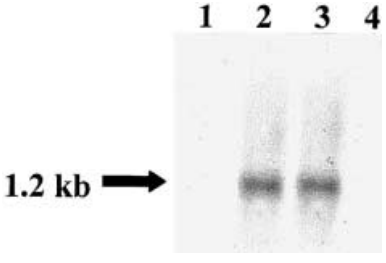

Fig. 4. Northern blot analysis of hydrated (100% RWC, *lane 1*) and dehydrated (85% RWC, *lane 2*; 37% RWC, *lane 3*; 5% RWC, *lane 4*) *X. viscosa* leaves. A 1-μg sample of poly(A)$^+$RNA was fractionated on a 1.2% agarose gel and transferred to a nylon membrane. The blot was probed with ^{32}P-labelled *ALDRXV4* insert. Equivalent amounts of RNA were loaded as determined from Northern analysis using a β-actin probe

(mean ± SD, $n = 4$) 1.94 ± 0.46 nmol ml^{-1} min^{-1} culture in the presence of IPTG in the growth medium. No activity was observed when *E. coli* containing the *ALDRXV4* clone was grown in the absence of IPTG.

Discussion

The strategy of complementing osmotically stressed *E. coli* (srl::Tn10) cells by functional sufficiency (Mundree 1996) provides a novel alternative to cloning function-specific genes. Mundree (1996) utilized this property of λ ZAPII to develop a technique for cloning eukaryotic genes that are functionally important in NaCl stress tolerance. Ten cDNA clones with the ability to independently make *E. coli* tolerant to high salt stress were cloned from tobacco, and another seven clones have been isolated from *A. nummularia* and *L. perezii*, respectively. This strategy was adapted to isolate cDNA clones that conferred tolerance to osmotically-stressed *E. coli* (srl::Tn10) cells.

Like most non-halophilic bacteria, *E. coli* can tolerate and grow in media whose osmolarity corresponds to about 0.7 M NaCl (Gowrishankar 1985). In the face of decreased turgor, the bacteria are known to undergo changes in the cell envelope structure, facilitating the accumulation of compatible solutes so that turgor is

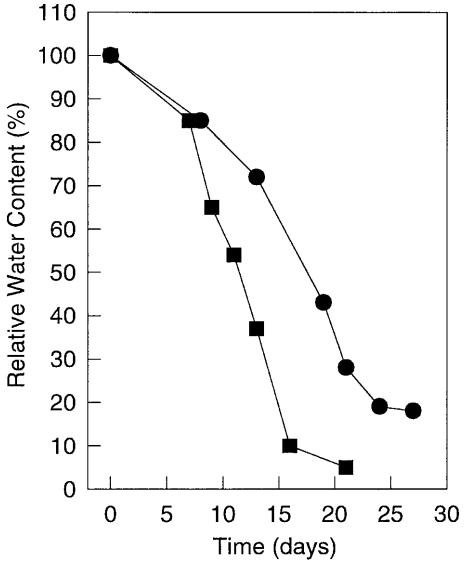

Fig. 5. Drying rates of *X. viscosa* (■) and *S. stapfianus* (●)

restored (McLaggan et al. 1994). However, in the absence of a specific transport system, as evident in the *E. coli* (srl::Tn10) mutant strain, any increase in osmolarity of the growth medium (such as 1.25 M sorbitol) would result in certain death of the cells (Fig. 1). Therefore the ability of *E. coli* cells expressing *ALDRXV4* to tolerate 1.25 M sorbitol in the medium, is indeed significant. The λ ZAPII vector allows for the directional cloning of cDNAs and the efficient rescue of phagemids from this vector (Short et al. 1988). This property of λ ZAPII was utilized to clone eukaryotic genes that are functionally important in osmotic-stress tolerance. Nine cDNA clones with the independent ability to make *E. coli* tolerant to severe osmotic-stress, have been cloned from *X. viscosa*. Although this strategy has been used specifically to isolate genes associated with osmotic stress tolerance, a modification of this scheme has been used to isolate genes that are associated with salinity-stress tolerance (Mundree 1996).

The nucleotide sequence of the *ALDRXV4* cDNA has an open reading frame of 1144 bp (Fig. 2) and shows high levels of similarity to aldose reductases. The inferrred amino acid sequence contains a tetra-amino acid motif IPKS which is highly conserved among NADPH-dependent reductases, and the lysine resudue in this motif is involved in NADPH binding (Bohren et al. 1991). ALDRXV4 shows significant homology to HORDEUM, BROMEGRASS and AVENA aldose reductase-like proteins (Fig. 3). In addition, ALDRXV4 also has significant similarity to aldose and aldehyde reductases of mammals (data not shown) (Tamarev et al. 1984; Kawasaki et al. 1989; Ferraris et al. 1994).

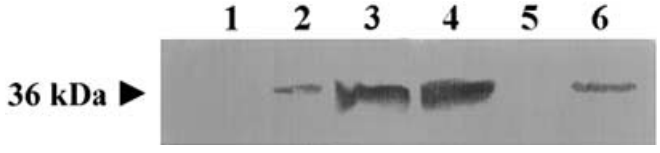

Fig. 6. Western blot analysis of total protein extracts from *X. viscosa* leaves (*lanes 1–4*) and *E. coli* (srl::Tn10 strain) containing the *ALDRXV4* cDNA clone (*lanes 5 and 6*). *Lanes 1–4*, leaves at 100%, 85%, 37% and 5% RWC, respectively. *Lanes 5 and 6*, *E. coli* containing the *ALDRXV4* cDNA clone grown in the absence or presence of 1 mM IPTG, respectively

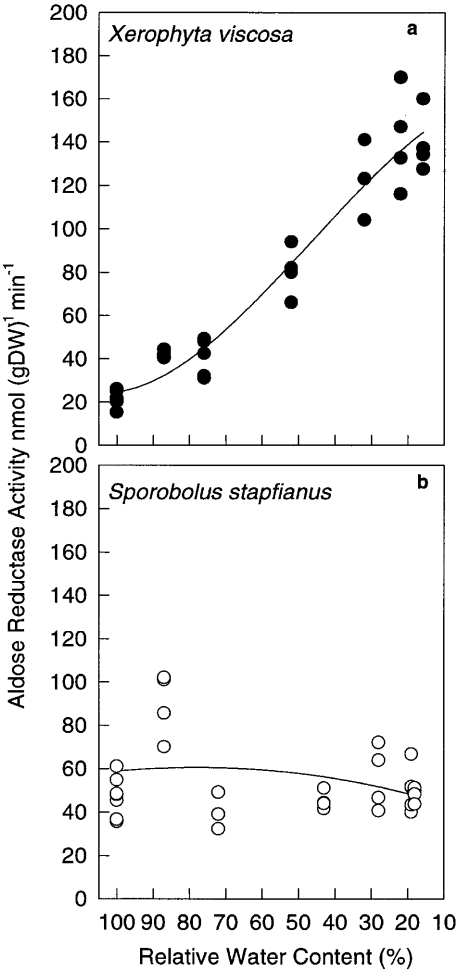

Fig. 7a,b. Aldose reductase activity in *X. viscosa* (**a**) and *S. stapfianus* (**b**) leaves at various relative water contents. Each point on the graph (○ or ●) represents an independent measurement. The line represents a best-fit curve

Table 1. Aldose reductase activity in *X. viscosa* leaves (37% RWC) expressed as percent maximum activity with glyceraldehyde as substrate. Data are means ± SD, $n = 3$

Substrate	Aldose reductase activity (%)
Glyceraldehyde	100
D-Glucose	60.1 ± 2.0
D-Mannose	44.1 ± 2.9
D-Xylose	32.2 ± 3.43

The barley aldose reductase gene was isolated from barley embryos using the strategy of differential screening (Bartels et al. 1991). Antibodies raised against the barley aldose reductase detected a protein of similar molecular weight in dehydrated leaf tissue of the resurrection plant *Craterostigma plantagineum* (Bartels

et al. 1991). Bartels et al. (1991) have provided evidence for the existence of a metabolic pathway in barley embryos that could be involved in the synthesis of osmoprotective molecules such as sorbitol. However, the role of aldose reductases in *C. plantagineum* has not been fully elucidated. The efficiency of glucose as a substrate in the enzyme assay (Table 1), together with the observation of increased levels of sorbitol in dehydrated *X. viscosa* leaves, provides some support for the existence of a metabolic pathway leading to the accumulation of sorbitol in *X. viscosa* leaves. An aldose reductase homolog that is expressed during the desiccation phase of *Avena fatua* embryogenesis has also been isolated using the same strategy (Li and Foley 1995). Li and Foley (1995) have postulated that the *A. fatua* aldose reductase may play an important metabolic role in desiccation tolerance and long-term viability of *A. fatua* seeds. In addition, an aldose reductase homolog to which ALDRXV4 shows 65% similarity, was cloned in the context of freezing-stress tolerance (Lee and Chen 1993).

The fact that ALDRXV4, together with the aldose reductase-homologs to which it shows highest similarity, was isolated from tissues experiencing severe osmotic stress, is noteworthy. As with barley aldose reductase, ALDRXV4 exhibits significant similarity to crystallin, a major structural protein of frog eye lens (Tomarev et al. 1984). This protein also belongs to the aldo-keto reductases (Tomarev et al. 1984). On the basis of the molecular structure of ALDRXV4 it seems likely that ALDRXV4 may play a structural and functional role in desiccation tolerance in *X. viscosa*.

The expression of *ALDRXV4* (Fig. 4) correlates with the occurrence of the protein at 85% and 37% RWC (Fig. 6). Transcripts of *ALDRXV4* were not detected at 5% RWC (Fig. 4) whereas the protein was (Fig. 6). This suggests that the ALDRXV4 protein was stably maintained at 5% RWC. Differences in the aldose reductase activities in dehydrating leaf material (Fig. 7a) correlate with the presence of the aldose reductase protein (Fig. 6). This demonstrates that aldose reductase in *X. viscosa* leaf material, as in desiccated barley embryos and *Craterostigma plantagineum* (Bartels et al. 1991), could in part be modulated by coarse regulation of transcriptional and/or translational factors. The significant increase in aldose reductase activity in dehydrated *X. viscosa* leaves (Fig. 7a) suggests a possible metabolic role of this enzyme in the tolerance of *X. viscosa* to severe dehydration stress. Cross-reactivity between the anti-barley fusion protein pG22-69 polyclonal serum and the *X. viscosa* protein is indicative of antigenic similarity between *X. viscosa* aldose reductase, and aldose reductases of barley embryos and *C. plantagineum* (Bartels et al. 1991). The *X. viscosa* aldose reductase is resolved as a single cross-reacting polypeptide of 36 kDa, which further demonstrates structural similarity between *X. viscosa* aldose reductase and those of the above-mentioned plants species.

The precise manner in which increased levels of aldose reductase may function in desiccated leaves of *X. viscosa* will require more definitive evidence. However, the cloning of *ALDRXV4* using the strategy of complementation by functional sufficiency, and the finding of the reductase activity in *E. coli* suggests that *ALDRXV4* is capable of rescuing *E.coli* from severe osmotic stress.

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