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Abstract

New Mexico agriculture depends upon irrigation to avoid water deficit stress in agricultural crops. Targeted manipulation of plant genomes for enhanced salinity and water deficit tolerance through genetic engineering will require a sophisticated understanding of plant water stress proteins and their genes.

Photosynthetic cyanobacteria, the free-living relatives of chloroplasts, share with higher plants at least one ubiquitous water stress protein, dehydrin. The biochemical function of dehydrin is still unknown. Our goal has been to clone and sequence the cyano-dehydrin gene. Knock-out mutagenesis would then afford an opportunity to study dehydrin function during water stress. Ultimately, these studies should empower rational improvements in water and salt-stress tolerance.

The 40 kilo-Dalton (kD) cyanodehydrin protein was purified and the sequence of 16 N-terminal amino acids was obtained from an immuno-positive 20 kD CNBr peptide. An expression library was constructed in lambda gt11 and screened with the affinity-purified antibodies. Three immuno-positive clones were identified. One of these three contains a 2.2 kb fragment that hybridized with a degenerate oligonucleotide probe based on the amino acid sequence information. Preliminary DNA sequence has identified the coding region for the peptide sequence in a 700 bp *Eco*R1-*Sca*I fragment, thus unambiguously identifying this fragment as containing a portion of the cyanodehydrin gene. Three short regions of similarity to barley dehydrin (*dhn5*) were identified in the derived amino acid sequence for the cyanodehydrin using the computer similarity program, BLAST. One of these includes the sequence IKQKLP, similar to one of the barley *dhn5* K-segments. Southern hybridization with the 700 bp *Eco*R1-*Sca*1 fragment identified a series of unique fragments in *Anabaena* 7120 genomic DNA, suggesting there is only one copy of the dehydrin gene in this organism. The entire 2.2 kb fragment identifies multiple bands in genomic southern blots, suggesting that a segment of DNA downstream of the dehydrin gene itself is part of a small gene family. Preliminary sequence from the repeated region shows similarity in BLAST searches to bacterial insertion sequences. Six genomic clones of 12-16 kb have been isolated using the 2.2 kb fragment to probe a lambda L47.1 library of *Anabaena* 7120 DNA. These will used to determine the sequence of the 5' end of the dehydrin gene which is not present on the 4b insert.

INTRODUCTION

It is now well established that virtually all plant embryos accumulate a related set of proteins during the latter stages of embryogenesis. First observed during cotton embryogenesis, these proteins are named LEA for *l*ate *e*mbryogenesis *a*bundant in order to reflect their developmental period and high concentration (Dure et al. 1981). Some fifteen years later, it now seems likely that all LEA protein families will be represented in all plants. The LEA proteins are presumed to play a role in the desiccation tolerance of seed tissues, and some LEA proteins have been shown to accumulate in other tissues in response to dehydration or exogenous abscisic acid treatments (Skriver and Mundy 1990; Bray et al. 1993). The exact functions of most of the LEA's remains elusive although structural and biophysical studies are beginning to narrow the field of possibilities (Dure 1993).

It is also becoming widely appreciated that proteins with LEA structural signatures occur not just in angiosperms, but are also found in cyanobacteria (Close and Lammers 1993; Curry and Walker-Simmons 1993a), the eukaryotic alga *Chlorella vulgaris* (Joh et al. 1995), in gymnosperms (Close et al. 1993), the desiccation-tolerant fern *Polypodium virginianum* , and moss *Tortula ruralis* (Reynolds and Bewley 1993; Bewley et al. 1993), and finally in a desiccation tolerant liverwort *Exormotheca holstii* (Hellwegge et al. 1994). Thus, it is reasonable to predict that, rather like the heat-shock paradigm, protoplasmic dehydration tolerance will share common mechanisms in all organisms. If this is true, then mechanistic studies in any organism within the shared set are, by definition, relevant to any other organism in that same set.

This introduction begins with an overview of the similarities between plant and cyanobacterial protoplasmic dehydration responses. What follows is a discussion of published reports that homologs from at least 4 plant Lea dehydration protein families can be found in the desiccation-tolerant filamentous cyanobacterium *Anabaena* sp. strain PCC 7120. These four families include the dehydrin group (Close and Lammers 1993), the Em (wheat)/D-19 (cotton) group, the group 3 LEA (D-7 cotton), and the group 3 LEA II family (Curry and Walker-Simmons 1993a). The results section will document the purification, partial amino acid sequence, cloning and partial DNA sequence of the cyanobacterial dehydrin gene from *Anabaena* 7120. Evidence for dehydration-induced and salt-stress-induced expression of cyano-dehydrin protein will also be presented. These results provided the groundwork for the exciting prospect of using the power of prokaryotic genetics to create null mutants, over-expressors and eventually mutants with altered proteins to tease out functional roles for dehydration proteins in *Anabaena* 7120.

PLANT DEHYDRATION/COLD STRESS RESPONSES

Plant survival in nearly all environments necessitates the ability to withstand water-deficit stress resulting from drought, elevated temperature or salinity. Low temperatures also cause dehydration stress by generating a chemical potential gradient leading to the net loss of intracellular water, and also by increasing the resistance of the roots to water movement into the plant as the permeability of endodermal cells decreases below 5 ^{o}C. As might be expected, similarities exist at the cellular level between plant responses to all environmental stresses which result in water deficit. Furthermore, cross-adaptation to multiple stresses commonly occurs after adaptation to a single stress (Levitt, 1958). The various types of adaptive responses known in plants are outlined below.

PHYSIOLOGICAL CHANGES

Plant responses to the water imperative include avoidance of water deficit or tolerance to water stress. The immediate response to water stress is stomatal closure mediated by the hormone abscisic acid (Walton, 1980). Avoidance responses include dormancy during cyclical dry periods, morphological changes in roots or shoots, and even changes in carbon fixation pathways (C3 to CAM) to reduce evaporative water loss and improve water use efficiently (Paleg and Aspinall, 1981; Bohnert et al. 1988). This report focuses entirely on the components of cellular tolerance, therefore a detailed discussion of avoidance mechanisms is omitted here. It should be noted that the protoplasmic adjustments described below can be relevant to the transition periods after the onset of water deficiency in plants capable of avoidance responses.

The mechanisms by which individual cells protect themselves from water-deficit-induced damage are termed protoplasmic adaptations. Protoplasmic tolerance of osmotic and chilling stress in plants has been studied for decades (see review by Steponkus 1980). Major biochemical changes include the accumulation of a variety of compatible solutes, changes in membrane composition, and the synthesis of a recognizably similar group of stress-inducible proteins.

COMPATIBLE SOLUTES: MAINTAINING THE NATIVE STATE

Protoplasmic tolerance is well correlated with the accumulation of compatible solutes in plants and bacteria. Proline and glycine betaine are widespread compatible solutes, but sugars and polyhydric alcohols are also effective (Lee and Timasheff 1981; Gekko and Morikawa, 1981; Csonka, 1989; Paleg et al. 1984). Taken as an entire class of molecules, the compatible solutes seem to share the property of being extensively

excluded from the immediate protein domain. This property forms the basis of the "exclusion theory" of macromolecular stabilization as articulated by Arakawa and Timasheff (1985). The presence of elevated concentrations of compatible solutes results in an increase in the surface free energy of water and hence the surface tension. While this would seem to represent a thermodynamically unfavorable situation with respect to protein solvation, there is an elegant twist to the story. Stabilization of the native protein structure is the net result, because native state globular proteins have a smaller surface area than denatured proteins. Thus, the equilibrium between native and denatured protein should shift in favor of the native state as the surface tension of water increases in the presence of compatible solutes.

During the more severe dehydration that occurs in a freeze-dry cycle, compatible sugars have been postulated to contribute to the stabilization of membranes and proteins through hydrogen bonding between the alcohol groups of the solutes and polar groups on phospholipids and proteins. Evidence supporting the water replacement hypothesis has been summarized (Crowe et al. 1993) and may be particularly important in membrane stabilization.

MEMBRANE ADAPTATIONS: MINIMIZING THE DAMAGE

Changes occurring at cell membranes during desiccation can lead to lysis by at least two mechanisms. For example, in non cold-adapted cells, an episode of freezing leads to loss of water from the cytoplasm due to the decreased chemical potential of water outside the cell. Non-adapted cells shrink due to water loss with the concomitant endocytotic vesiculation. As the extracellular water potential increases during thawing, water moves back into the cytoplasm. Expansion-induced lysis results because the surface area of the plasma membrane cannot increase fast enough to compensate. However, in cold adapted cells, exocytotic extrusion of membranes accompanies contraction, so the total surface area of the membrane does not change. Thus, cold adapted membranes do not experience expansion-induced lysis (Steponkus et al. 1993)

Crowe et al. (1993) report a correlation between free fatty acids and poor survival after drying in yeast cells. Furthermore, cells from the resurrection plant *Selaginella*, which survives desiccation, showed no accumulation of free fatty acids during long periods of storage in the dry state. These authors propose that inhibition of phospholipase A_2 may be occurring in desiccation-tolerant organisms, and implicate dehydrin proteins (see below) in having predicted similarities to proteins known to inhibit PLA_2 (Conricode and Ochs 1989; Bohn et al. 1992). Whether dehydrins can inhibit PLA_2 activity in desiccation-tolerant organisms has yet to be determined.

STRESS INDUCED PROTEINS AND THEIR GENES

This section focuses entirely on the four LEA proteins or their genes for which we have evidence of occurrence in *Anabaena* 7120: the dehydrins, the dehydrin family, the Em (wheat)/D-19 (cotton) group, the group 3 LEA (D-7 cotton), and the group 3 LEA II family.

Dehydrin (D-11; LEA-2) family

All members of the dehydrin family share a consensus 15 amino acid lysine rich motif: EKKGIMDKIKEKLPG also known as the K segment (Close 1995). Indeed, we used affinity purified, anti-K segment antibodies to detect the osmotically regulated cyanobacterial dehydrin analog in filamentous cyanobacteria from the genera *Anabaena, Nostoc* and *Calothrix* (Close and Lammers 1993). Dehydrins have between one and eleven copies of the K segment per monomer and molecular weights range from 9 kDa to 200 kDa for rice *Wsi724* and wheat WCS200, respectively (Takahashi et al. 1994; Oullet et al. 1993). The K segment is predicted to form an amphipathic alpha helix (Dure 1993) and, by analogy with the J domain of the Dna J protein which also forms an amphipathic alpha helix (Szyperski et al. 1994), accounts for the hypothesis that dehydrins may play a chaperone-like role during dehydration stress by associating with exposed hydrophobic surfaces on partially denatured proteins (Close 1995). Evidence that the cyanobacterial dehydrin protein might share such a property derives from our use of hydrophobic interaction chromatography during cyanodehydrin purification (see Results and Discussion).

The majority of dehydrins also share another conserved sequence motif (V/TDEYGNP) near the amino terminus. Known as the Y segment, it has significant amino acid sequence relatedness to a portion of the nucleotide binding site of chaperones of plants and bacteria (Martin et al. 1993). However, we are not aware of any published evidence for nucleotide binding by Y segment-containing dehydrins. Some but not all dehydrins have a serine-rich tract, the S segment, which can become phosphorylated. There is evidence that dehydrins with a phosphorylated S segment may be targeted for translocation to the nucleus (Goday et al. 1994; Godoy et al. 1994). Finally, some but not all dehydrins have a Gly, Thr rich composition which leads to the prediction that a large fraction of these dehydrin proteins will exist in a random coil configuration (Dure 1993). Other dehydrin proteins seem to have replaced the Gly, Thr enrichment for a Lys, Glu enrichment, which drives the secondary structure predictions away from random coil to high levels of alpha helix. In either case, a working model put forth by Close (1995) is that the majority of the each dehydrin protein will be

hydrophilic, forming an envelope of highly ordered water. This is predicted to enhance the exclusion of compatible solutes from macromolecular surfaces, and drive a partially denatured protein back into its native state or inhibit further unfolding.

Using the YSK nomenclature and an amino to carboxy terminal convention, Close (1995) groups 55 different sequences into five dehydrin types:

	# Y segments	# S segments	# K segments	Strongly Induced by
Y_nSK_2	2	1	1-3	dehydration
K_n	0	0	3-9	cold
K_nS	0	1	1-3	cold & dehyd.
SK_n	0	1	2-3	-
Y_2K_n	2	0	1-2	-

Thus, the S segment can occur before or after the K segment, while the Y segment, if present, is always near the N-terminus.

Currently we know very little about the sequence motifs present in the cyanodehydrin protein from *Anabaena* 7120, other than the immunological similarity previously described (Close and Lammers 1993). The cyanobacterial gene has recently been cloned however, and detailed sequence comparisons will soon be available (see below). Using the affinity purified anti-K segment antibodies, we have shown that the cyanodehydrin protein is expressed after dehydration, osmotic shock and salt shock, but is not normally expressed in liquid culture (see below for details).

The Lea-3 (Cotton D7) Family

The LEA 3 or D7 family has been described in wheat (Curry et al. 1991), *Brassica napus* (Harada et al. 1989), carrot (Seffens et al. 1990), cotton (Dure 1993), and *Anabaena* 7210 (Curry and Walker-Simmons 1993a). This group of proteins usually contains many repeats of the short motif: TAQ(E)AAKE(Q)KAXE. The number of tandem repeats of this motif (5 to 13) accounts for the variation in size of the LEA 3 proteins. Like the dehydrin K segment, the repeating motif is predicted to fold into an amphiphilic alpha helix The cotton protein is very abundant in all cell types of cotton embryos. Computer-modeling of the helical interfaces has led Dure (1993) to predict that the LEA 3 proteins will sequester ion pair combinations like K_2HPO_4 as amino acid salts, thereby preventing their precipitation in desiccating tissue.

A LEA 3 homolog was detected in *Anabaena* 7120 by hybridization with a wheat cDNA probe (pMA2005) in Southern blots of restricted genomic DNA. Four very

strongly hybridizing bands were observed in both *Hin*dIII and *Hinc*II digests of cyanobacterial DNA, ranging in size from 1.2 to 3.5 kb. This suggests the possibility that multiple LEA 3-hybridizing loci exist in the *Anabaena* 7120 genome. LEA 3-hybridizing transcripts were identified in RNA isolated from slow-desiccated *Anabaena* cells, but not in RNA from liquid cultures. Furthermore, antibodies directed to the wheat LEA 3 protein identified a 25 kDa protein from dehydrated but not hydrated cyanobacterial cells (Curry and Walker-Simmons 1993a).

Lea-3-(II) Family

Proteins in the Lea-3(II) group share a short 11 amino acid repeat repeated 11 times plus a shared unique 36 amino acid motif at the carboxyl end of the proteins. Examples have been reported in wheat (Curry and Walker-Simmons 1993b), carrot and soybean (Hsing et al. 1992). The 11 residue repeats in the Lea-3-(II) proteins are partially related to the sequence of Lea-3 protein repeats. The significance of the unique sequence at the carboxy terminus is unknown, although it is repeated twice in the wheat protein but only once in the carrot and soybean homologs.

The wheat Lea-3(II) probe (pMA1949) identified single bands of 3.8 and 3.0 kb, respectively, in *Hin*dIII and *Hinc*II digests of genomic DNA from *Anabaena* 7120 (Curry and Walker-Simmons 1993a). No data regarding constitutive or stress-induced expression of the Lea-3(II) hybridizing locus are currently available.

The D19 Family

Proteins in the D-19 family have been found in wheat (Em), maize, carrot, sunflower, barley, rice and *Anabaena* 7120 (Dure 1993; Marcotte and Quatrano 1993; Curry and Walker-Simmons 1993a). All display sequence similarity along the entire length of the proteins. The sequences are high in glycine and charged amino acids, while secondary structure predictions all suggest a high degree of random coil. It has been proposed that the highly hydrophilic and amorphous structural characteristics of the D-19 proteins may result in the retention of large amounts of ordered water within desiccating tissues, potentially preventing total desiccation.

The evidence for a D-19 homolog in *Anabaena* 7120 is based on hybridization of a wheat Em probe (pMA1959) to genomic DNA (Curry and Walker-Simmons 1993a). A single band was observed in both *Hin*dIII and *Hinc*II digests, while signal was significantly weaker than that with the Lea-3 probe. No cyanobacterial expression data at either the RNA or protein levels exists .

CYANOBACTERIAL RESPONSES TO DEHYDRATION AND OSMOTIC STRESSES

TURGOR PRESSURE

The immediate effects of water stress will manifest as a loss in turgor pressure. Turgor pressure is the hydrostatic pressure (P) that balances the difference in osmotic pressure between the external medium (p_e) and the internal cytoplasm (pi) according to the equation $P = p_i - p_e$. Turgor pressure can be measured directly in cyanobacteria that contain gas vesicles (Walsby 1988). Gas vesicles collapse in response to an applied pressure that eventually exceeds the critical pressure of the gas vesicle, causing the cells to sink. Experimentally, the percentage of gas vesicles collapsed is monitored as a function of applied pressure for cells in culture medium and for cells in culture medium containing 0.5 M sucrose. The critical pressure distribution curve for the sucrose solution is similar to that for isolated vesicles because p_e is higher than p_i and the turgor pressure falls to zero. The difference between the two curves gives a measure of turgor pressure which varies within the range of 0.2-0.5 MPa for freshwater cyanobacteria. Sucrose is used to collapse the turgor pressure because rapid uptake of sucrose to balance the internal and external osmotic pressures is not possible in most cyanobacteria.

COMPATIBLE INTRACELLULAR SOLUTES

Reed and Stewart (1988) point out that when the cyanobacteria are divided into the least, intermediate, and most halotolerant forms, a clear correlation emerges with observations of the accumulation of disaccharides, polyol-derivatives, and betaines (least to highest). Furthermore, this is the same order of protection afforded to enzymatic activities measured *in vitro* by these organic osmotica (Warr et al. 1984). Specifically, freshwater cyanobacteria largely utilize disaccharides, particularly sucrose (a few use trehalose). These cyanobacteria constitute the first of the three groups defined by Reed and Stewart (1988) graded from least to most halotolerant. Group 2 strains of intermediate halotolerance (including *Synechocystis* 6803) that can tolerate freshwater or marine environments usually accumulate glucosylglycerol as the organic osmoticum following salt stress (MacKay et al. 1983; Reed et al. 1984; Reed and Stewart 1988). The most halotolerant strains comprising Group 3 usually accumulate glycine betaine or glutamate betaine in response to salinity stress.

Although sucrose accumulation in response to osmotic stress in *Anabaena* 7120 has not been reported in the literature to our knowledge, "all isolates belonging to the

genera *Anabaena* and *Nostoc* which have been screened to date have accumulated sucrose as their sole organic osmolyte" (Reed and Stewart 1988). Regulation of the biosynthetic pathways leading to carbohydrate accumulation in response to osmotic stress is largely unknown for the cyanobacteria. A prediction is that the activity and/or concentration of sucrose synthase (SS) or sucrose phosphate synthase (SPS) will increase after water stress in those strains that accumulate sucrose.

PROTEIN PROFILES

The most relevant studies with respect to this proposal have already been discussed in the previous section on plant dehydration-induced proteins, and will not be repeated here. Dehydration induced proteins have been found in *Nostoc commune* and a discussion of those studies is found in the section on desiccation tolerance that follows immediately. Most studies on cyanobacterial water stress proteins have focused on salt tolerant cyanobacteria.

Changes in protein profiles have been documented in two *Anabaena* strains with different salt tolerance characteristics, the brackish water strain *Anabaena torulosa* and a salt-sensitive strain *Anabaena* sp. strain L-31 (Apte and Bhagwat 1989). Unique changes in two-dimensional protein profiles were observed in the two strains in response to sublethal NaCl concentrations. Salt-induced proteins in strain L-31 were transiently expressed, with synthesis often ceasing between 2 and 6 hours after salt-stress. In contrast, *A. torulosa* salinity-induced proteins were persistent with new synthesis occurring throughout the period of salinity stress. Changes could easily be observed within 10 minutes after salt stress. Molecular cloning of cosmids containing genes differentially regulated by NaCl has been reported using differential cDNA hybridization from *A. torulosa* (Apte and Haselkorn 1990).

Further experiments by the same group explored changes in protein profiles induced by sucrose-mediated osmotic stress (OSPs) in *Anabaena* sp. strain L-31 (Bagwat and Apte 1989). Sucrose-mediated osmotic shock induced the synthesis of the same 15 polypeptides that were observed with salinity stress imposed with NaCl and KCl. Three of these were induced by heat shock as well. 10 mM KNO_3 was subsequently shown to induce the same set of OSPs, and treatment of cultures with 100 mM sucrose and 10 mM KNO_3 conferred significant osmoprotection to *Anabaena* sp. strain L-31 (Iyer et al. 1994). Independent regulation of OSPs and NaCl-induced proteins has also been demonstrated in this strain (Fernandes et al. 1993).

Changes in protein profiles during salinity-stress in the unicellular cyanobacterium *Synechocystis* PCC6803 have been reported as well. Three proteins of 14.2, 21.1 and 52

kDa are transiently induced during salinity stress adaptation (Hagemann et al. 1991). Based on what we have seen in the filamentous strains, we expected this class to include cyano-dehydrin (Close and Lammers 1993). This did not turn out to be the case. Affinity-purified anti-K-segment antibodies revealed no dehydrin-like proteins on Western blots of salinity-stress proteins from *Synechocystis* PCC 6803 (data not shown). Coomassie brilliant blue staining revealed a similar protein pattern to that reported previously (Hagemann et al. 1991). We conclude this strain may not have the cyanodehydrin gene, or that the rapid of response of *Synechocystis* PCC 6803 to salt-stress lowers the level of osmotic stress below the level necessary to induce cyanodehydrin expression. *Synechocystis* is not desiccation tolerant, thus induction by dehydration was not attempted.

DESICCATION TOLERANCE IN *NOSTOC COMMUNE*

The most intensively studied cyanobacterium with respect to water deficit stress is *Nostoc commune* found in terrestrial limestone environments in the tropics, the poles, and temperate regions. For a recent review see Potts (1994). Extremes of water availability subject these communities to repeated cycles of wetting and drying (Whitten et al. 1979). Surprisingly few changes in protein profiles were detected during desiccation of immobilized *N. commune* UTEX 584 cells (Potts 1986), nor did a novel class of proteins appear upon rewetting. However, a cluster of water stress proteins (WSP) with acidic pI and molecular masses between 30 and 40 kDa were identified in field-desiccated, but not liquid-grown laboratory cultures, of *N. commune* collected in Heibei province, China (Scherer and Potts 1989). The difference observed in the two studies may be a reflection of the drying rate, other uncontrolled variables, or genotypic differences between UTEX 584 and field isolates. It is perhaps significant that natural isolates are found imbedded in a water-absorbing extracellular carbohydrate-containing sheath that may effectively slow the rate of water loss *in situ*. Differences in phospholipids and carotenoids have also been noted in comparisons between field-grown *N. commune* isolates and laboratory-grown UTEX 584, which may, or may not, be a result of differential water-stress responses (Potts et al. 1987).

It seems that the extremely desiccation-tolerant cyanobacteria, like the field grown *N. commune* may resemble the desiccation-tolerant moss *Tortula ruralis*, which also shows few changes in protein patterns during desiccation, and which have been shown to constituitively produce WSPs related to dehydrins (Bewley et al. 1993). We showed that another *Nostoc* (Strain MAC) constituitively produced dehydrin, although dehydrin protein also increased after osmotic stress (Close and Lammers 1993).

Anabaena 7120 may be somewhat less desiccation tolerant on an absolute scale than the *Nostoc* strains by virtue of having an inducible dehydrin response, although this has yet to be demonstrated.

An abundant water stress protein with an apparent molecular mass of 39 kDa has been purified from *N. commune* (Scherer and Potts 1989). *In vivo* this protein may exist as high molecular weight complexes. Recent results show that the 39 kDa protein is an isoform related to water-stress proteins (WSPs) of 32 and 37 kDa. These also show homology with carbohydrate-modifying enzymes and copurify with an associated 1,4-β-xylanxylanohydrolase activity (Hill et al. 1994). We are quite certain that the 39 kDa WSP from *Nostoc* is <u>not</u> related to the 40 kDa WSP from *Anabaena* 7120. Partially purified cyanodehydrin did not cross react with antibodies directed against the 39 kDa *Nostoc* WSP (M. Potts, personal communication), nor did the purified 39 kDa *Nostoc* WSP cross react with affinity purified anti-dehydrin K-segment antibodies (T.J. Close, personal communication).

DESICCATION TOLERANCE IN *Anabaena* 7120

Very little is known except for the following observations. Loss of water from agar plates leads to formation of a hard, clear, thin chip but does not seem to affect the visual appearance of the cells macroscopically. Microscopically, the cell borders become much less distinct, but the general shape of the filaments is easily recognized in the dehydrated state. If water is added to filaments on a dry agar chip, the filaments become indistinguishable from completely hydrated filaments within a few seconds. Addition of a 0.5 cm^2 agar chip to fresh liquid media results in the appearance of large numbers growing filaments in the liquid phase within 5 to 10 days demonstrating the viability of some fraction of the cells contained on the agar chip. We have not observed the presence of akinetes (spore-like cells) on these plates or in our 7120 strain under any conditions. Similar observations have also been published (Curry and Walker-Simmons 1993a).

Anabaena AS A MODEL GENETIC SYSTEM

Molecular genetic systems for the filamentous cyanobacteria are getting quite sophisticated. With the exception of mapping by phage transduction, and chromosome mobilization by integrating conjugal plasmids, there is little that one might consider that is not possible (Theil 1994; Elhai and Wolk 1988; Buikema and Haselkorn 1993). Conjugation from *E. coli* hosts works quite well, particulary from those carrying restriction modification genes to protected the transferred DNA from host restriction . The plasmid vectors the have been used include those carrying cyanobacterial plasmid replication origins or plasmids carrying a cyanobacteria DNA fragment to target homologous recombination into the chromosome. A positive selection system for driving double homologous recombination (allele replacement) is also available (Cai and Wolk 1990). A variety of selectable markers are available for selecting exconjugants including streptomycin/spectinomycin, neomycin, and chloramphenicol.

SUMMARY OF RESEARCH PROCEDURES

CONSTRUCTION AND SCREENING OF AN EXPRESSION LIBRARY

We constructed an expression library in the phage vector lambda gt11. Starting with purified *Anabaena* 7120 DNA, random fragments were generated using partial digests with three restriction enzymes that recognize 4 base sites: *Sau*3A (GATC), *Alu*I (AGCT) and *Dde*I (CTNAG). These partial digests were size fractionated on agarose gels yielding fragments between 700 and 1,500 base pairs. The ends of these fragments were treated with T4 DNA polymerase and deoxy-nucleotide triphosphates to eliminate overhanging nucleotides, and treated with the *Eco*R1 methylase in preparation for ligation with oligonucleotide linkers containing the EcoR1 restriction site. We utilized three separate linker preparations of 8, 10 and 12 bp in length in a single ligation. This affords a greater probability of producing translational fusions with the β-galactosidase open reading frame in the gt11 vector, *at every random end* present in the insert population. We did not dephosphorylate the blunt-ended *Anabaena* DNA fragments prior to linker ligation. Therefore, even though we used a large molar excess of linkers over DNA fragments, we can not preclude the possibility that *Anabaena* DNA fragments ligated with each other prior to ligating with linker fragments.

The library was constructed by ligation of the EcoR1-digested *Anabaena* fragments with lambda gt11 arms purchased from Stratagene, followed by *in vitro* packaging, and infection of *Escherichia coli* strain Y1090. A portion of the library was amplified by passage through the Y1090 strain and an aliquot equivalent to 100,000 primary recombinant phage set aside for direct screening. Screening the *Anabaena* 7120 gt11 library with affinity purified anti-dehydrin antibodies was as described (Perball, 1988).

The lambda L47.1 library was a gift from Dr. James Golden. It was constructed from size-selected Sau3A partial digests (12-18 kb) cloned into a unique BamHI site in the vector.

CYANODEHYDRIN PROTEIN PURIFICATION

Cyanodehydrin protein was purified from 17 liter cultures of *Anabaena* 7120 treated with 250 mM sucrose for 16 hours prior to harvest to induce cyanodehydrin protein accumulation (Close and Lammers 1993). Proteins that precipitate between 40 and 60% ammonium sulfate saturation were collected and fractionated by ion-exchange (Mono-Q) and hydrophobic interaction (reversed phase C4) chromatography. Cyanodehydrin was assayed by Western blotting, using affinity purified antibodies specific for dehydrin proteins (Close and Lammers 1993). The immuno-positive fractions were

pooled and size fractionated by agarose gel electrophoresis. Cyanodehydrin was fragmented with cyanogen bromide (CNBr), which cleaves proteins after methionine residues. A 20 kD immuno-positive peptide was isolated by agarose gel electrophoresis and eluted from the gel for amino acid sequence analysis. All protein sequence analysis was done at the University of California, Riverside Biotechnology Instrumentation Facility.

Oligonucleotides were purchase from DNAgnecy, Malvern, Pennsylavania.

SDS-PAGE AND WESTERN BLOTTING

Procedures for cell lysis, protein determinations, SDS-polyacrylamide electrophoresis were as descibed previously (Close and Lammers 1993).

RESULTS AND DISCUSSION

A two-pronged strategy was utilized to clone the *Anabaena* 7120 dehydrin gene. First, affinity-purified antibodies were used to screen an *Anabaena* lambda gt11 expression library for immuno-reactive clones. Second, cyanobacterial dehydrin was purified from osmotically stressed cultures and amino acid sequence determined from an immuno-reactive CNBr peptide. This sequence was used to prove that one of the lambda gt11 clones encoded a portion of the authentic dehydrin protein. The protein purification and sequencing was accomplished primarily in the laboratory of Dr. Timothy Close at the University of California, Riverside, while the remainder of the work described here was completed in the Lammers Laboratory with funding from the New Mexico-Water Resources Research Institute (WRRI) and United States Department of Agriculture Competitive Grants Program. The protein work will be described first, although the sequence information only became available at the end of the WRRI project period.

CYANOBACTERIAL DEHYDRIN PROTEIN PURIFICATION AND PARTIAL PEPTIDE SEQUENCE

The dehydrin protein purification began with sucrose-stressed cultures of *Anabaena* as described in Close and Lammers (1993). Soluble protein was subjected to ammonium sulfate fractionation, ion exchange and hydrophobic interaction chromatography and size fractionation on SDS-PAGE. Cyanobacterial dehydrin protein was followed by Western blots of each fraction. Figure 1 shows the results of a silver-stained gel of partially purified dehydrin. The major protein present in the preparation was dehydrin as demonstrated by its immuno-reactivity (data not shown).

The major band was excised from an agarose prep gel and subjected to sequence analysis at the Biotechnology Instrumentation Facility at the University of California, Riverside. No amino acids were released from the mature protein and a blocked amino terminus was inferred. Purified dehydrin protein from a 17-L osmotically-stressed culture was subjected to cyanogen bromide cleavage and a 20 kDa immuno-reactive peptide was isolated by excision from an agarose prep. The first 16 amino acids from this peptide were determined as follows: VTILWXYLRNNAFKQD, where X denotes an unknown residue. Note this is quite distinct from the peptide sequence used to raise dehydrin-specific antibodies: CTGEKKGIMDKIKEKLPGQH.

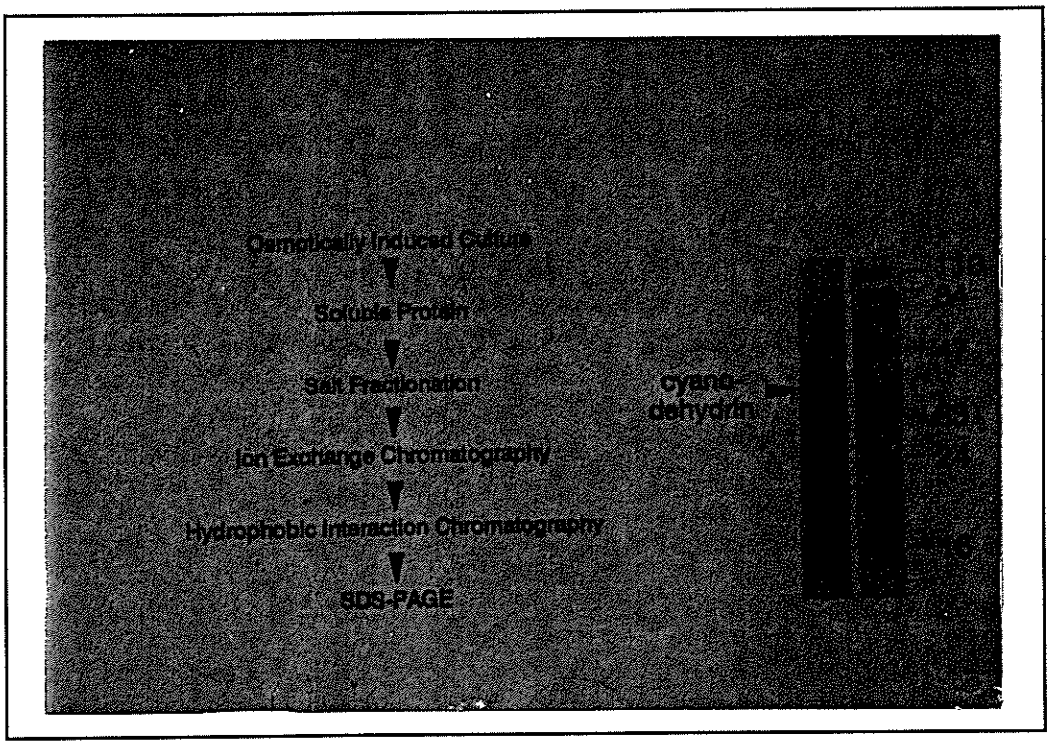

Figure 1. Purification of cyanodehydrin protein. A silver-stained SDS-PAGE gel is shown on the right. The immuno-positive fractions from the hydrophobic interaction chromatographywere loaded on the gel. Molecular weights of the markers in kilo-Daltons are indicated next to each marker.

The majority of the protein work was accomplished by my collaborator, Dr. Timothy Close, who was not an investigator on this grant. Therefore, the details of this work will be published elsewhere than this WRRI Final Technical Report (Close, T.J., R. Fenton, J. Bongianni and P.J. Lammers et al. "Manuscript In Preparation").

ANTIBODY-SCREENING OF AN *Anabaena* 7120 EXPRESSION LIBRARY

A lambda gt11 library was constructed which contained size-fractionated inserts derived from partial digestion of *Anabaena* 7120 genomic DNA using three restriction enzymes with four base recognition sequences. 50,000 primary recombinant plaques were screened with the same affinity-purified antibodies used to identify cyanobacterial dehydrin (Close and Lammers 1993). Three immuno-positive clones were identified and plaque-purified. Phage DNA was isolated from liquid lysates and subcloned into pBluescript. These fragments were designated 4a (390 bp), 4b (2,200 bp) and 1b (1,400 bp). The inserts all appeared to be distinct fragments based on DNA sequence analysis of the ends of each fragment. The 4a and 4b inserts were used to probe Southern blots of all three clones to determine relatedness between the clones. No cross-hybridization was observed with either probe. We concluded that the three clones are derived from separate regions of the *Anabaena* 7120 genome. The sequence information from one end of the 4a clone revealed a short region that resembled the dehydrin consensus as shown below:

```
IPSKCAXXCS  SGTGWPIPTSTADRVKEKLPLIPVPLI        Anabaena
              CTGEKKGIMDKIKEKLPGQH          dehydrin consensus
                   ** **                    * = CONSERVED SUBSTITUTIONS
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However, several lines of evidence contradict the idea that the 4a insert is derived from the authentic cyanobacterial dehydrin gene. Complete nucleotide sequencing of the 4a insert revealed an open reading frame extending over the entire 390 bp insert on the *opposite strand* from the one that encodes the above-mentioned peptide. The larger open reading frame displays a codon utilization bias consistent with other *Anabaena* 7120 genes, while the peptide sequence from the opposite strand does not. The derived sequence from the larger open reading frame does not contain any recognizable similarity with the dehydrin K-segment consensus, nor does it resemble any known protein sequence in Genbank. The 4a insert was used to probe RNA isolated from control and sucrose-stressed *Anabaena* 7120 cultures synthesizing dehydrin protein. A constitutively expressed 1.1 kb mRNA is identified by the 4a probe, and a slight

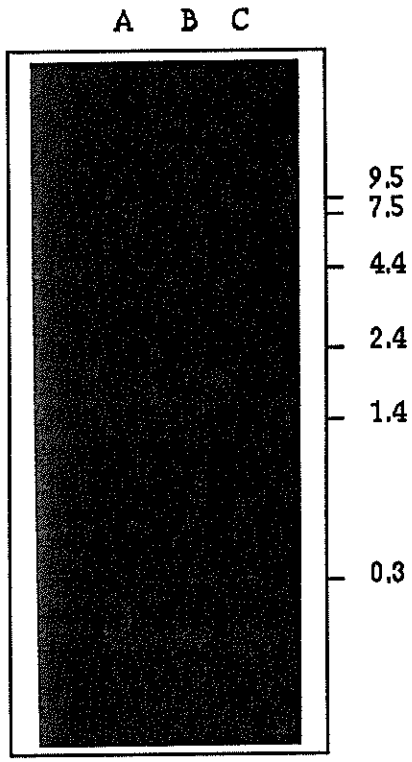


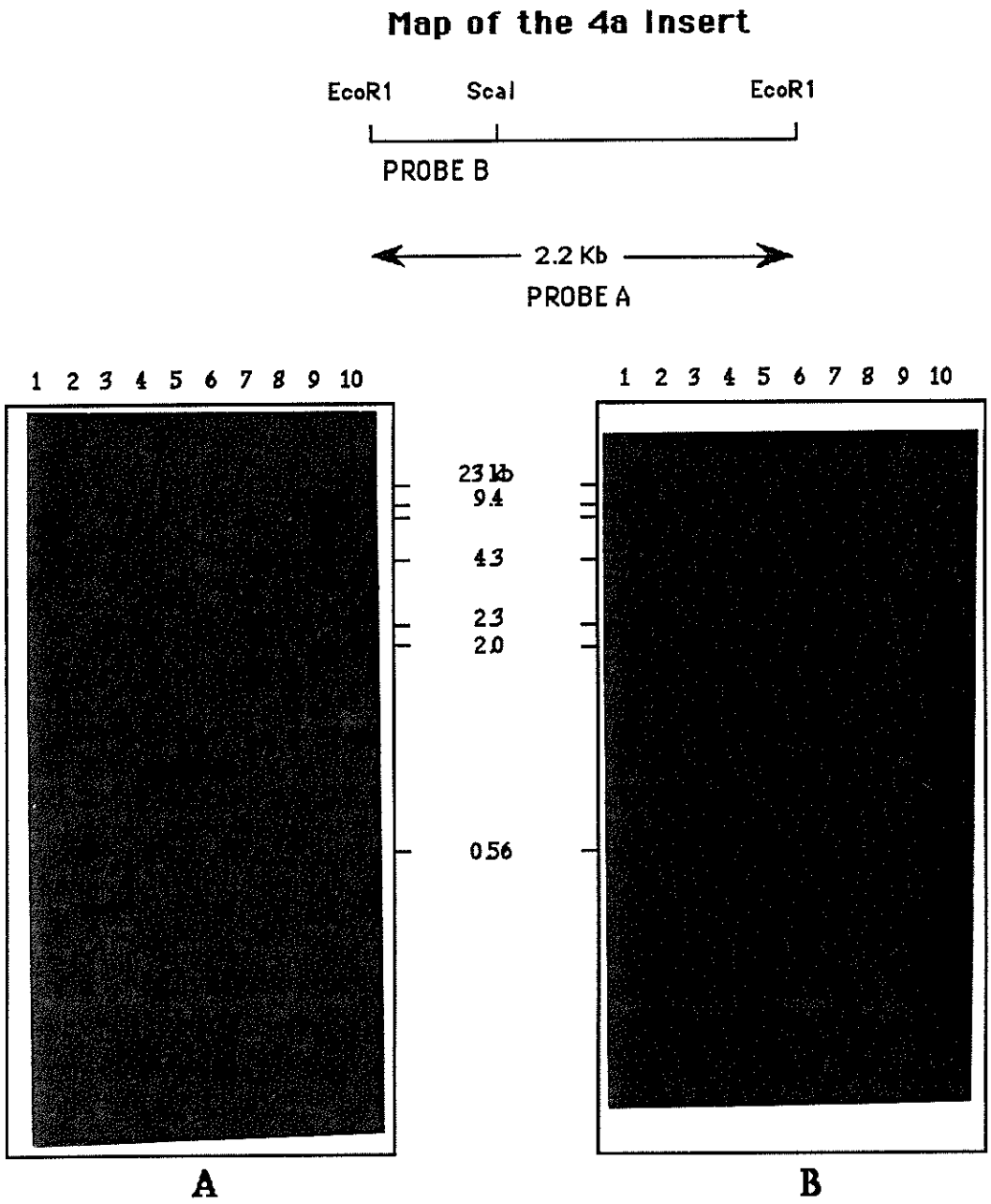
Figure 2. Northern hybridization with the 4a insert probe. RNA transfer blot hybridized with the entire 4a insert (see Appendix 1 for sequence). Samples: RNA was isolated form *Anabaena* 7120 cultures that were unstressed, A; treated with 250 mM sucrose, B; or treated with 500 mM sorbitol, C.

induction of this mRNA is apparent after osmotic stress (Figure 2). However, the signal from unstressed cells is much higher than we would expect based on the absence of dehydrin protein observed without osmotic stress (Close and Lammers 1993). We did not determine the orientation of the 4a clone in the lambda gt11 vector. However, it is likely that the 4a immuno-positive region was derived from the non-coding strand of this unidentified gene.

DEGENERATE OLIGONUCLEOTIDE BASED ON THE PEPTIDE SEQUENCE HYBRIDIZES WITH THE 4B CLONE

A degenerate 21 base oligonucleotide containing all possible coding sequences for the last 7 amino acids from the CNBr fragment was synthesized. This oligonucleotide was end labeled with ^{32}P-ATP for hybridization with the three immuno-positive clones from the lambda gt11 library. This probe identified only the 4b clone, containing a 2.2 kb EcoR1 fragment insert. The 4b clone encodes both an immuno-reactive (dehydrin-like) epitope and part or most of the 20 kDa immuno-reactive CNBr fragment which hybridizes with the oligonucleotide.

A partial restriction map of the 4b insert is shown in Figure 3. The small EcoR1-ScaI fragment hybridizes to the degenerate oligonucleotide based on the immunoreactive peptide sequence. The DNA sequence encoding the peptide maps to the middle of the small EcoR1-ScaI fragment. The entire 4b insert was labeled and hybridized to genomic DNA from *Anabaena* 7120. Figure 3A shows the results of these hybridizations. Note the large number of hybridizing fragments which greatly exceed the size of the probe fragment itself. For example, lane 7 is a HindIII digest in which nine fragments hybridize with the 4b probe ranging in size from 1.6 to 9.4 kb. However, when the small EcoR1-ScaI fragment (600 bp) is used to probe the same blot (after stripping) only a single 2.2 kb HindIII fragment is identified. The most likely interpretation of this data is that the large EcoR1-ScaI fragment contains sequences that are repeated elsewhere in the *Anabaena* 7120 genome. Furthermore, the region containing the dehydrin gene is most like a unique sequence. This will be very helpful for the directed mutagenesis experiments, since only a single gene must be disrupted to create a dehydrin knockout strain.



F

Figure 3. Southern hybridizations with 4b insert and dehydrin subregion. *Anabaena* 7120 DNA restriction digests: lane 1, BspI; lane 2, BspI/DraI; lane 3, BspI/HindIII; lane 4, DraI; lane 5, DraI/HindIII; lane 6, DraI/ScaI; lane 7, HindIII; lane 8, HindIII/ScaI; lane 9, ScaI; lane 10, ScaI/BspI. Panel A was probed with the entire 2.2 kb 4b insert. Panel B was probed with the 700 bp EcoR1-ScaI fragment.

DNA SEQUENCE ANALYSIS OF THE 4b INSERT

A series of unidirectional deletions from the ends of the 4b insert were constructed by the exonuclease III/mung bean nuclease procedure. An 888 bp contiguous sequence was assembled, beginning at one end of the 4b insert, which includes the coding region for the amino acid sequence of the immunoreactive peptide derived from purified cyanodehydrin (Figure 4). This result, together with the immuno-reactivity of the 4b clone proves that the 4b insert encodes the cyanobacterial dehydrin protein. Note that immediately preceding the match is the predicted methionine codon conferring susceptibility to CNBr cleavage. The unidentified residue in the peptide sequence is revealed in the DNA sequence to be a cysteine, which would not survive the chemical cleavage conditions without derivitization, which we did not perform. The 888 bp sequence contains a continuous open reading frame that is still missing both the 5' and 3' ends of the dehydrin gene. The reading frame displays a codon bias consistent with *Anabaena* 7120 codon utilization rates (data not shown; Lammers et al. 1990). The cyanodehydrin sequence responsible for its similarity to the dehydrin consensus motif may be the IKQKLP sequence shown centered about nucleotide #600 in Figure 4. This peptide resembles the K-segment and is aligned with a barley dehydrin sequence (*dhn5*). It also resembles the sequence from the 4a clone, VKEKLP, postulated to be responsible for it's immuno-selection. The possibility that a better match with the K-segment consensus exists nearer to the 3' end of the coding region can't be ruled out at present. The ScaI site used to generate the probes for the Southern blot in Figure 3 is marked in Figure 4.

Features present in many dehydrins that are not found in the amino acid sequence shown in Figure 4 include the presence of repeating Gly-Thr residues. Nor are any direct serine repeats found representing a potential phosphorylation site. However, four serines occur in a 28 residue region beginning at nucleotide residue #60. This region also contains three tyrosines and two threonine residues, all of which could be potential phosphorylation sites. Immediately following this region is a weak Y-segment match: PIYFDP compared with DEYGNP. All of these sequence observations will require biochemical experimentation to verify their significance.

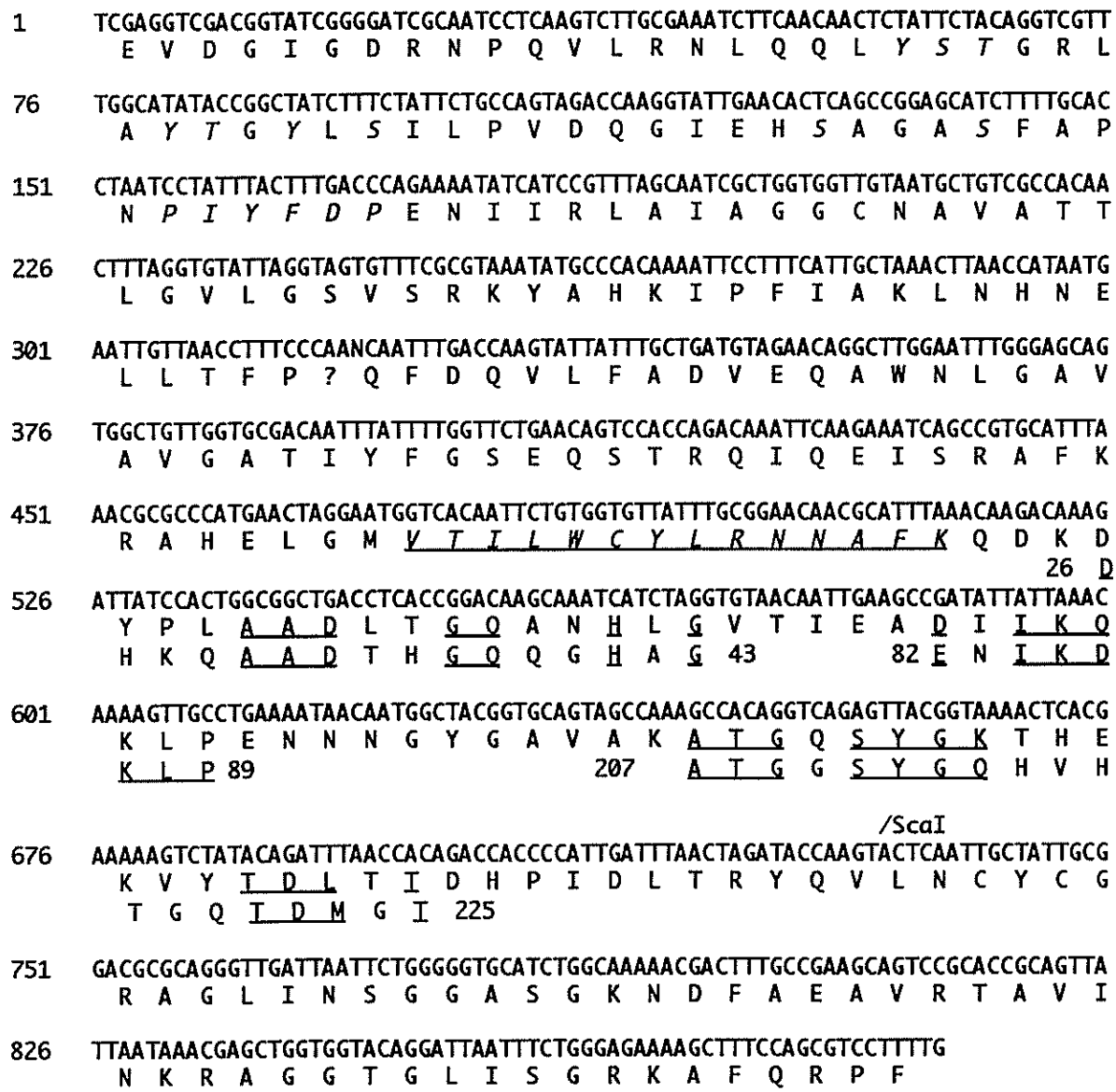


Figure 4. Partial sequence of the 4b clone. The underlined region in italics matches the amino acid sequence from the immunoreactive peptide. Matches to the barley dehydrin 5 (*dhn 5*) amino acid sequence generated by a computer search using the BLAST search program are shown below the derived 4b amino acid sequence. Exact and highly conserved matches with the *dhn 5* sequence are underlined. Numbers near the dhn5 sequence refer to the amino acid number of the *dhn5* sequence. Potential S-segment- and Y-segment-like residues appear in italics.

The 2.2 kb 4b insert was used to probe a lambda L47.1 library containing large inserts derived from Sau3A partial digestions of *Anabaena* 7120 genomic DNA. Six positive clones have been isolated and plaque purified. Subcloning and sequence analysis of one of these clones will be used to determine the sequence of the entire 5' end of dehydrin gene. The selection depends on which one hybridizes with the small EcoR1-Sca1 probe. It is likely that some of the genomic positives from the L47.1 library represent the regions of the genome containing the repetitive sequence present in the large EcoR1-ScaI fragment. The nature of the repetitive sequence is unknown at present (However, see Note Added in Proof, below). The small and large EcoR1-ScaI fragment will be used to probe Northern blots containing RNA from control and osmotically stressed cultures to determine if either or both are induced.

INDUCTION OF *Anabaena* 7120 DEHYDRIN PROTEIN BY DEHYDRATION

One of the unanswered questions about cyanobacterial dehydrin protein is whether dehydration would induce dehydrin accumulation as was shown for osmotic stress. Drought-induced wilting of plant tissues is known to induce dehydrin protein accumulation (Close 1995). Because of the good correlation between any type of osmotic stress and dehydrin accumulation in *Anabaena* 7120, and because this organism is also desiccation-tolerant, we expect that dehydrin will also be induced by dehydration. Positive results would support the hypothesis that cyanobacterial dehydrin protein plays some important role in the cellular response to water deficit stress.

Slow and fast-drying regimes were tested. Cells were harvested from liquid culture by centrifugation and the cell paste placed in sterile petri dishes. For slow drying, the cell paste was left to slow dry on the bench top for three days at which point it had formed a dry crust. Rapid drying was accomplished by placing the cell paste in a vacuum (150 milli-Torr) at room temperature for 30 minutes, at which point the cell paste was crispy-dry. Dehydrin protein was assayed by western analysis of soluble protein as previously described (Close and Lammers 1993). Slow drying on the bench top resulted in significantly higher dehydrin protein levels that did the rapid drying treatment (Figure 4). A single immunoreactive band was observed with both treatments that co-migrates with the immunoreactive protein induced by osmotic stress with 250 mM sucrose treatment.

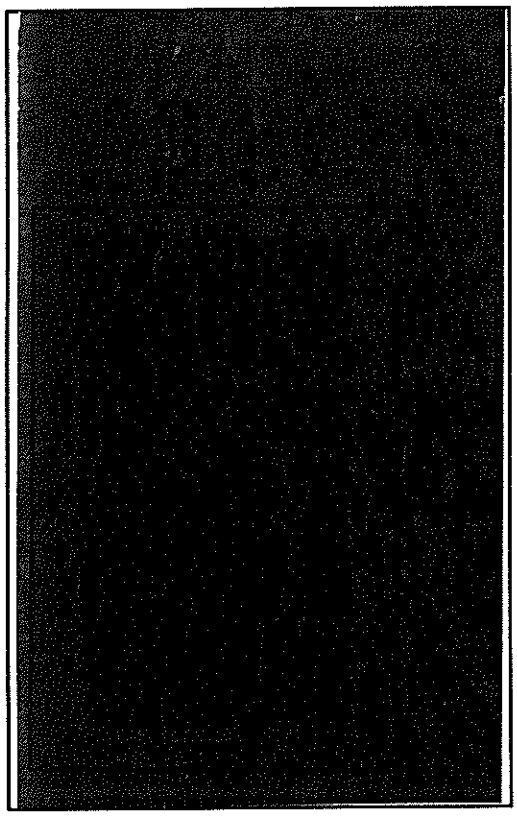


Figure 5. Western blot showing dehydration induced cyanodehydrin protein accumulation in *Anabaena* 7120. Cells from equal volumes of late log phase cultures were harvested and the pellets left to slow dry on the bench top for 3 days or were dried rapidly under vacuum (150 milli-Torr) at room temperature as indicated. Positive control was an osmotically stress culture (250 mM sucrose treatment). Proteins were isolated and subject to SDS-PAGE and western blotting. The blot was probed with affinity-purified anti-dehydrin K-segment antibodies.

It seems likely that dehydrin is a component of a highly conserved dehydration response found in cyanobacteria and photosynthetic eukaryotes. The cloning and characterization of the cyanobacterial dehydrin gene will provide the opportunity to compare the amino acid sequence of the most divergent dehydrins known to date. More importantly, targeted mutagenesis of the dehydrin gene in *Anabaena* 7120 will allow us to apply genetic methods for inference of the unknown function of the dehydrin protein family.

NOTE ADDED IN PROOF

Recent DNA sequence data provide a rationale for the data from the genomic Southern blot probed with the 4b insert shown in Fig. 3. A "BLAST" homology search was initiated with a partial open reading frame from the repeated region of the 4b insert (the 1.5 Kb *Sca*I to *Eco*R1 fragment). The search revealed significant homology to open reading frames from transposable elements of the bacterial insertion sequence class. The highest scoring match is shown below:

```
pir||A32816 hypothetical protein, 33K (insertion sequence IS492) - Pseudomonas
            atlantica >gi|151307 (M24471) protein encoding reversible
            inactivation of extracellular polysaccharide production
            [Pseudomonas atlantica]
            Length = 318

 Score = 190
 Identities = 41/103 (39%), Positives = 60/103 (58%)

Anabaena:        2 DVSKATLDVYIRPIGKALKFANTELEIFNLVEQLKFYDLNLIVLEATGGLETELVIQLQA 61
                   D  K  LD+YIRP       +N E  I   +++++ +    IV+EATG LE   +I
P.atlantica:    14 DTGKFQLDIYIRPHDIYFTVSNDEKGIKEAIKKIQQHSPGRIVIEATGRLEMPFIIACAN 73

Anabaena:       62 AMLPVALINPRQGRNFAKATGKLAKTDTIDVQILAHFGEAMKP 104
                   A LP  + NP   + FA A G+ AKTD +D Q++AH+GEA++P
P.atlantica:    74 ANLPFVVANPIHIKRFAGAIGQRAKTDKLDAQLIAHYGEAIQP 116
```

If an insertion sequence or transposon resides immediately downstream of the dehydrin gene, this would explain why a probe consisting of the entire 4b insert identifies a family of restriction fragments, while the 700 bp probe containing part of the dehydrin gene appears to represent a single copy region.

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Appendix 1.
Raw (Single Stranded) DNA sequence from the ends of the 4a and 1b immuno-positive lambda gt11 inserts.

TextMap of the entire 4a insert sequence.

```
          /EcoR1
          GAATTCCGAGCAAGTGCGCCTNCNGTTGCAGTTCTGGTACTGGTTGGCCTATTCCAACCTCAACAGCAGACAGAG
        1 ----+----|----+----|----+----|----+----|----+----|----+----|----+----|----+----|----+
rf 1      E  F  R  A  S  A  P  ?  V  A  V  L  V  L  V  G  L  F  Q  P  Q  Q  Q  T  E
rf 2       N  S  E  Q  V  R  L  ?  L  Q  F  W  Y  W  L  A  Y  S  N  L  N  S  R  Q  S
rf 3        I  P  S  K  C  A  ?  ?  C  S  S  G  T  G  W  P  I  P  T  S  T  A  D  R  V

                                                                                  /Afl2
          TCAAGGAGAAACTGCCATTGATACCAGTTCCACTGATTTGATTTGCTGTGAGGATTCCTTCGCTTAAGAAGTCGC
       76 ----|----+----|----+----|----+----|----+----|----+----|----+----|----+----|----+----|
rf 1      S  R  R  N  C  H  *  Y  Q  F  H  *  F  D  L  L  *  G  F  L  R  L  R  S  R
rf 2       Q  G  E  T  A  I  D  T  S  S  T  D  L  I  C  C  E  D  S  F  A  *  E  V  A
rf 3        K  E  K  L  P  L  I  P  V  P  L  I  *  F  A  V  R  I  P  S  L  K  K  S  P


          CAGTGTTACTGAAGAATGTTCCTCGTAATTGAGGCAACGTGTATCCTACGATATTCTCATCATCGCCAGGAGTAA
      151 ----+----|----+----|----+----|----+----|----+----|----+----|----+----|----+----|----+
rf 1      Q  C  Y  *  R  M  F  L  V  I  E  A  T  C  I  L  R  Y  S  H  H  R  Q  E  *
rf 2       S  V  T  E  E  C  S  S  *  L  R  Q  R  V  S  Y  D  I  L  I  I  A  R  S  K
rf 3        V  L  L  K  N  V  P  R  N  *  G  N  V  Y  P  T  I  F  S  S  S  P  G  V  K

                                                    /BstX1
          AGTTTCGACCAGCAATGGAGGGATCATCAACCACAAAGTTGGAACCATCCACAAAACTAATGAATGGAGCGGTAG
      226 ----|----+----|----+----|----+----|----+----|----+----|----+----|----+----|----+----|
rf 1      S  F  D  Q  Q  W  R  D  H  Q  P  Q  S  W  N  H  P  Q  N  *  *  M  E  R  *
rf 2       V  S  T  S  N  G  G  I  I  N  H  K  V  G  T  I  H  K  T  N  E  W  S  G  S
rf 3        F  R  P  A  M  E  G  S  S  T  T  K  L  E  P  S  T  K  L  M  N  G  A  V  V

                                                                  /AlwN1
          TTGTGGCTGAGTAGGGTGTTACAGTTGTTCCCAAACCTCTACTCCAGCACCTGTAAGCACTAGATTGATAGTAGA
      301 ----+----|----+----|----+----|----+----|----+----|----+----|----+----|----+----|----+
rf 1      L  W  L  S  R  V  L  Q  L  F  P  N  L  Y  S  S  T  C  K  H  *  I  D  S  R
rf 2       C  G  *  V  G  C  Y  S  C  S  Q  T  S  T  P  A  P  V  S  T  R  L  I  V  D
rf 3        V  A  E  *  G  V  T  V  V  P  K  P  L  L  Q  H  L  *  A  L  D  *  *  *  I

                   /EcoR1
          TATAGAGCGGAATTC
      376 ----|----+----|  390
rf 1      Y  R  A  E  F
rf 2       I  E  R  N
rf 3        *  S  G  I
```

TextMap of "1b.seq", 176 bp.

```
       GATCCCGACATAACCTTAGAAGTATCGAAATCGTTACATAAACATTCACACAAACCACTTGATAAATTTAGCCAA
     1 ----+----|----+----|----+----|----+----|----+----|----+----|----+----|----+
rf 1   D  P  D  I  T  L  E  V  S  K  S  L  H  K  H  S  H  K  P  L  D  K  F  S  Q
rf 2    I  P  T  *  P  *  K  Y  R  N  R  Y  I  N  I  H  T  N  H  L  I  N  L  A  N
rf 3     S  R  H  N  L  R  S  I  E  I  V  T  *  T  F  T  Q  T  T  *  *  I  *  P  M
                                          /Xba1
       TGTAAAAGACTACAGTTTCTCCCCGGTTTAGTTCTAGAGTTACCTTCAGTGAAACATCGGCTGGCGTGTCACGTC
    76 ----|----+----|----+----|----+----|----+----|----+----|----+----|----+----|
rf 1   C  K  R  L  Q  F  L  P  G  L  V  L  E  L  P  S  V  K  H  R  L  A  C  H  V
rf 2    V  K  D  Y  S  F  S  P  V  *  F  *  S  Y  L  Q  *  N  I  G  W  R  V  T  S
rf 3     *  K  T  T  V  S  P  R  F  S  S  R  V  T  F  S  E  T  S  A  G  V  S  R  H

       ATTGAATGAGCATAAATCAATTCAAA
   151 ----+----|----+----|----+- 176
rf 1   I  E  *  A  *  I  N  S
rf 2    L  N  E  H  K  S  I  Q
rf 3     *  M  S  I  N  Q  F  K
```