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NaCl-Regulated Gene Expression

in *Distichlis spicata*

by

Caroline S.M. Furniss

B.Sc Hons. (Dunelm)

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A thesis submitted for the degree of Doctor of Philosophy
in the University of Durham

Department of Biological Sciences
September 1994

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This thesis is entirely the result of my own work. It has not been accepted for any other degree and is not being submitted for any other degree.

Caroline Furniss

September 1994

Abstract

NaCl-induced and -repressed cDNA clones had previously been isolated by differential screening of a cDNA library, prepared from poly(A^+) RNA isolated from *Distichlis spicata* (salt grass) cell cultures grown in the presence of 260 mM NaCl (Zhao, *et al.*, 1989). Eight of these cDNA clones have now been subcloned and/or sequenced and the predicted polypeptides compared with owl sequence data base. Three clones pDZ6.2, pDZVIII 1.2.1 and pDZIX 3.1 encode proline rich proteins, containing an amino acid repeat [PPKKDH(H)Y(Y)]. They have similar amino acid usage to proline-rich cell wall proteins, being rich in P, K, H and Y. The first 20 amino acid residues encode a putative leader sequence, supporting the proposed extracellular role as a cell wall protein. This N-terminal sequence (MPLLVALLLVLAVVAAAGAD) shares some similarity with the leader sequence of a soyabean proline-rich cell wall protein precursor and other extracellular proteins (the conserved residues are underlined). There is an increase in abundance of transcripts hybridising to the inserts from pDZ6.2 and pDZVIII 1.2.1 in response to either 520 mM NaCl or 100 μM ABA, but a decrease in response to 5 mM exogenous proline. It is suggested that the corresponding gene(s) are regulated at the level of either transcription or transcript stability, in response to elevated NaCl, with ABA as a mediator of (or part of) this response. pDZ6.2 and pDZXI 3.1 have identical nucleotide sequences, whilst pDZVIII 1.2.1 differs in three base pairs within the putative open reading frame, suggesting that there may be at least two members of a multi gene family.

A 68 bp GA repeat has been found in the 5' untranslated region of pDZ6.2 and a corresponding transcript identified by northern analysis using this GA sequence as a probe. Such nucleotide repeats can form triplexes (DNA) or hairpin loops (RNA), which is dependent on pH and ionic conditions. Therefore this GA repeat may play a role in the regulation of the gene corresponding to pDZ6.2 at the level of transcription or translation, possibly by attenuation of these processes, either by the formation of triplexes or hairpins, or the binding of a protein to this GA region, at low ionic strength. However initial *in vitro* transcription experiments, to compare the transcriptional activity of pDZ6.2 and pDZVIII 5.1.1 at different ionic strengths, proved inconclusive. An attempt was also made to identify the corresponding genomic region from *D. spicata* by anchored PCR.

A fourth clone pDZ2.8L encodes a histone 2B protein, having 97.9% similarity to a wheat histone 2B. Its transcript abundance decreased in response to either 520 mM NaCl, 5 mM proline or 100 μM ABA. The sequences of the remaining clones either revealed no significant similarity to any known sequences or were assigned as being cloning artefacts.

D. spicata cells accumulate proline within eight hours of exposure to 260 mM NaCl (Heyser, *et al.*, 1989b). An unsuccessful attempt was also made to isolate a pyrroline-5-carboxylate reductase gene homologue from *D. spicata*, by heterologous probing of Southern blots with a soyabean cDNA pProC1 and PCR.

ABBREVIATIONS

°C	degrees Celsius
%	percent
kb	kilo base
bp	base pair
FW	fresh weight
v/v	volume for volume
w/v	weight for volume
h	hour
min	minute
s	seconds
psi	pounds per square inch
rpm	revolutions per minute
DNA	deoxyribonucleic acid
RNA	ribonucleic acid
ATP	adenosine triphosphate
CTP	cytidine triphosphate
GTP	guanosine triphosphate
TTP	thymidine triphosphate
ABA	abscisic acid
cTAB	mixed alkyltrimethyl ammonium bromide
EDTA	ethylenediamine tetra acetic acid (disodium salt)
MES	2-[N-morpholino] ethanesulphonic acid
MOPS	3-[N-morpholino] propanesulphonic acid
PEG	polyethylene glycol
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid
TE	tris EDTA
Tris	tris (hydroxymethyl)methylamine

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Chapter 1

Introduction

1.1 Saline soils

Salinity effects approximately 9 x 10^8 ha of the earth's land surface (Epstein, *et al.*, 1980), including 40 % of all irrigated agricultural land. This includes productive areas, such as the Mediterranean basin, California, mid west USA and southern Asia. The cumulative effects of adding water, containing some dissolved salts, to arid and normal agricultural lands, has led to an accumulation of salt in the soil (Norlyn, 1980). Even water that is recycled by precipitation often contains small amounts of salts and therefore using this for irrigation adds to the problem, despite it being considered 'fresh water' (Aller, 1982). In the region of Sahel, W. Africa, rice growing has been encouraged. Simple irrigation, not accompanied by drainage of the ground, has resulted in the accumulation of some sodium salts and in some cases a salt crust appearing on the surface of the soil, rendering the land unusable (Madeley, 1993). Initial solutions to the problem of salinity have been technological and engineering approaches, such as land reclamation, surface and sub-surface drainage and improved irrigation (Epstein, *et al.*, 1980). However, despite reducing salinity, these solutions have proved expensive. Therefore there is a need to develop salt tolerant plants, since most agriculturally important crops have a comparatively low tolerance to salinity. This would not only enable the continued use of present arable land with increased salt levels, but may also open up the possibility of farming naturally high saline soils, such as coastal regions, that were previously unavailable. In some areas the fresh water supply is also a problem, by improving the salt tolerance of crops, lower quality saline water (including sea water) might be used (Epstein, *et al.*, 1980; Norlyn, 1980). It has been proposed that the development of salt tolerant crops should be based on criteria involving the physiological mechanisms responsible for salt tolerance (Noble and Rogers, 1992). A genetic engineering approach has also been suggested, that involves the overexpression of salt regulated genes (Serrano and Gaxiola, 1994), which may confer tolerance on plants, as well as elucidating critical steps for salt tolerance. However the isolation, identification and characterisation of such genes has so far proved some what difficult, since some salt regulated genes may work collectively and appear useless alone.

1.2 Effects of salinity

Salinity has two effects on plant cells: Firstly Na^+ and Cl^- ions influence the external water activity, which affects the water status of the plant, resulting in loss of turgor, this is osmotic stress; secondly by ion toxicity, the direct effects of ions on physiological and biochemical processes within the cell (Flowers, *et al.*, 1977; Greenway and Munns, 1980).

1.2.1 Osmotic Stress

Under non saline conditions water will enter the cell down a water potential gradient from the surroundings, which have a higher water potential than the cell. This leads to a build up of pressure within the cell, upon the cell wall. This is turgor pressure, which is an essential factor for plant growth. This is based on cell wall loosening, plus a turgor-driven increase in cell volume. However under conditions of high salt, the cell has a higher water potential than the surroundings, therefore water diffuses out of the cell, down the water potential gradient. This results in a reduction of turgor pressure and a decrease in growth through cell expansion. Thus salinity reduces the availability of water from the soil, due to its negative water potential (Salisbury and Ross, 1992).

1.2.2 Ion toxicity

Ion toxicity or 'ion excess', a condition where high internal concentrations cause a reduction in growth, results from increased Na^+, Cl^- and K^+ concentrations within the cytoplasm and cell wall. It also effects metabolism, resulting in a reduction of CO_2 fixation. Most enzymes function in a very narrow range of ionic conditions, 0.1 - 0.2 M K^+, < 50 mM Na^+ and Cl^-, thus 'ion excess' can lead to enzyme toxicity, accounting for the inhibition of photosynthesis (Greenway and Munns, 1980). It has also been proposed that it inhibits membrane function, resulting in a decrease in ion transport and selectivity mechanisms. This may upset mineral nutrition, as the uptake of essential ions such as Ca^{2+}, K^+ and Mg^{2+} is inhibited. Thus an increase in the use of metabolic energy for non growth purposes in the maintenance of tolerance leads to a decrease in growth (Hasegawa, *et al.*, 1986).

1.3 Mechanisms of salt tolerance

The mechanisms adopted by plants to adapt or cope with salt stress can be divided into two categories, accumulation and avoidance (Flowers, *et al.*, 1977; Greenway and Munns,

1980). The first allows cells to cope with ion concentrations, whilst the second allows growing cells to avoid them.

1.3.1 Accumulation

The accumulation adaptation involves the uptake of ions. Na^+ and Cl^- are sequestered into the vacuole, excluding the cytosol as a storage compartment, and minimising enzyme inhibition. This compartmentation of ions is accompanied by the synthesis and accumulation of organic solutes in the cytoplasm, where they are thought to contribute to osmotic potential and protect enzyme activity (Aspinall and Paleg, 1981). This compartmentation results from changes in membrane permeability and ion transport properties, which help the transport of ions against electrochemical gradients (Flowers, *et al.*, 1977; Greenway and Munns, 1980). This and compatible solutes are discussed in more detail in section 1.5. Ion accumulation is thought to have a positive function in osmotic adjustment, maintaining high cell water content in the presence of a low water potential. Increased ion transport also allows the uptake of nutrient ions required for metabolic processes (Flowers, *et al.*, 1977; Greenway and Munns, 1980), including K^+ (Watad, *et al.*, 1991). The accumulation of ions into the vacuole leaves the concentration of ions in the cytoplasm one third to a half of values in the vacuole.

This mechanism of accumulation is mainly adopted by halophytes, but also by some glycophytes adapting to high salt, such as tobacco cells (Binzel, *et al.*, 1987). Some halophytes are able to tolerate concentrations of salts in leaves, equivalent to seawater, through ion accumulation. They are also thought to overcome enzyme inhibition by increasing the substrate concentration (Flowers and Dalmond, 1992). The ice plant *Mesembryanthemum crystallinum* accumulates Na^+ and Cl^-, which increases its ability to absorb water, resulting in little change in the ion concentration (Bohnert, *et al.*, 1988) and causing the leaves to swell.

1.3.2 Avoidance

Avoidance consists of anatomical organisation between the xylem and phloem and allows the actively growing leaf and shoot cells, where sensitivity is high, to avoid high Na^+ and Cl^- concentrations. Ions are taken up by the roots and absorbed by the xylem parenchyma cells, then loaded back into the phloem via the xylem-phloem exchange system. These ions are then transported back to the roots, where they are excluded. Thus a distribution of ion gradients between growing and non-growing parts of the plant is established (Greenway and Munns, 1980; Hasegawa, *et al.*, 1986). This mechanism is mainly adopted by glycophytes and is only effective in low to moderate salinity. Some

halophytes also use an avoidance mechanism, sequestering ions into anatomical structures, such as salt glands, salt bladders and trichomes (Flowers, *et al.*, 1977).

As a result of the different mechanisms adopted by halophytes and glycophytes, the ion distribution within the plants' tissues also differs. In the halophytes 90% of Na^+ is found in the shoots and leaves, due to the preferential transport of Na^+ over K^+ from the roots to the shoots. However glycophytes exclude excessive ions from the leaves, by retaining Na^+ in the vacuoles of the roots (Flowers, *et al.*, 1977).

1.4 *Distichlis spicata*

Distichlis spicata (salt grass), a member of the *Poaceae* family, is a graminoid spike grass found in coastal and inland saline regions of North America (Hansen, *et al.*, 1986). This species shows a very broad distribution with respect to soil salinity, competing effectively in high saline pannes (500-600 mM NaCl), in areas of fresh water seepage (0-170 mM NaCl) and in areas of moderate salinity (250-425 mM NaCl).

The growth of *Distichlis spicata* cell cultures in media with high levels of added NaCl demonstrates that a significant and critical portion of its tolerance functions strictly at a cellular level. This component of NaCl tolerance is therefore not dependent upon tissue organisation or anatomical specialisation, such as the presence of salt glands (Warren and Gould, 1982) and therefore cell suspension cultures of this salt grass provide a model system in which to study aspects of biochemical and molecular mechanisms of NaCl tolerance. Cultured cells have advantages over whole plants, including the fact that they lack the complexity of tissue morphology and developmental regulation. Disadvantages include that fact that the cell cultures are prone to genetic changes when maintained for long periods of time. *D. spicata* does not require prior adaptation to express considerable NaCl tolerance. After growth in the absence of salt for as long as four years, following subsequent inoculation into NaCl-containing media, cells will immediately (within 24 hours) express tolerance, in terms of cell growth (Warren and Gould, 1982). NaCl-stressed cultures of *D. spicata* are characterised by lowered osmotic potential and increased levels of Na^+, protein and proline (Daines and Gould, 1985). Sodium fluxes have been examined using ^{23}Na NMR and the chemical shift reagent dysprosium III triphosphate. A rapid influx of Na^+ can be detected after incubation in media containing 260 mM NaCl (Heyser, *et al.*, 1986). *D. spicata* has 20-60% more protein when exposed to salt than unexposed cells, this correlates with an increase in enzyme synthesis. The specific activities of several enzymes in salt grass have been found to be higher than those of maize and tobacco. This was attributed to an increase in enzyme synthesis and not due to changes in enzyme kinetics (Daines and Gould, 1985). Therefore Daines and Gould (1985) concluded that this increase in enzyme synthesis in *D. spicata* must play a role in salt tolerance.

1.5 Biochemical and genetic adaptations of plants to elevated NaCl

1.5.1 Accumulation and synthesis of compatible solutes

A number of different organic compounds are accumulated in response to salt and osmotic stress, including certain specific carbohydrates, organic acids, amino acids and other nitrogen containing compounds.

1.5.1.1 Organic acids and carbohydrates

Organic acids such as lactate, oxalate and malate accumulate in plants in response to high ionic conditions (Flowers, *et al.*, 1977). Lactate increases in alfalfa roots and nodules to become the most abundant organic acid under salt stress conditions (Fougère, *et al.*, 1991). *Mesembryanthemum crystallinum*, the ice plant, accumulates malate as a result of switching from C_3 metabolism to CAM under salt stress (Höfner, *et al.*, 1987). The exact role these organic acids play remain uncertain, though Fougère *et al.* (1991) propose that lactate may be contributing to osmotic adjustment.

Carbohydrates, such as polyhydric alcohols, have been shown to contribute to the osmotic and ionic balance in a range of organisms, comprising mainly of lower plants and fungi, such as *Asteromyces crutiatus* and *Dendryphiella salina* (Flowers, *et al.*, 1977). Mannitol, arabitol and glycerol constitute major osmotica within these fungi. In yeast (*Saccharomyces cerevisiae*) glycerol is accumulated as a major part of osmoregulation (Brown, 1990). In higher plants, such as alfalfa roots and nodules and *M. crystallinum*, pinitol levels increase significantly and is thought to play a role in salt stress tolerance (Fougère, *et al.*, 1991; Paul and Cockburn, 1989). It can accumulate to >70 % of total soluble carbohydrate in *M. crystallinum*. A cDNA clone *Imt1* has been isolated by screening a subtracted *M. crystallinum* cDNA library, enriched for salt stress-induced transcripts (Vernon and Bohnert, 1992). *Imt1* encodes a 40, 250 MW polypeptide that has significant similarity to several hydroxy methyl transferases. Expression in *E. coli* identified it as myo-inositol *O*-methyl transferase, which catalyses the first step in the biosynthesis of pinitol. *Imt1* mRNA is up regulated in salt stressed leaves, whilst expression in roots initially increases in response to salt stress, but returns to basal levels within a few hours. Therefore regulation is occurring at a transcriptional level or post transcriptionally, through mRNA stability.

Transgenic tobacco plants are able to synthesise and accumulate mannitol, by the introduction of a bacterial gene construct 35s *mltD*, that encodes mannitol-1-phosphate dehydrogenase, which produces a metabolic branch point (Tarczynski, *et al.*, 1992). The transgenic tobacco lines, which don't usually produce mannitol, were able to accumulate up

to 100 mM mannitol. When growth was compared between transgenic and control plants, the mannitol-producing plants had an increased tolerance to high salinity (Tarczynski, *et al.*, 1993). This data and the isolation of the *Imt1* cDNA from *M. crystallinum* provide strong support for the importance of sugar alcohols in establishing tolerance to osmotic stress in higher plants.

1.5.1.2 Amino acids and nitrogen compounds

Amino acids and other nitrogen compounds are accumulated in bacteria, algae and higher plants (Flowers, *et al.*, 1977; Ishitani, *et al.*, 1993b). These include polyamines and diamines, glycine betaine, choline, proline, aspartate and asparagine. They are thought to play a major role in the maintenance of turgor and hydration in salt stressed cells of the afore mentioned organisms. Induction of glycine betaine uptake, following injection of poly(A^+) RNA into *Xenopus* oocytes from renal cells exposed to high extracellular NaCl, has been observed (Brooks Robey, *et al.*, 1991), suggesting that similar responses occur in animals and are regulated transcriptionally or by mRNA stability.

1.5.1.2.1 Polyamines

Putrescine (diamine), spermidine and spermine (polyamines) are accumulated in response to osmotic and salt stress in salt tolerant and salt sensitive cultivars of wheat and rice (Erdei, *et al.*, 1990; Galiba, *et al.*, 1993; Krishnamurthy and Bhagwat, 1989). In wheat, salt stress induced an increase in spermidine and spermine levels, whilst mannitol- or sorbitol-induced osmotic stress caused putrescine and spermidine levels to rise (Erdei, *et al.*, 1990; Galiba, *et al.*, 1993). Thus different stress conditions cause different polyamine/diamine species to accumulate in wheat. In rice, diamines and polyamines are also accumulated in response to salt stress (Krishnamurthy and Bhagwat, 1989). However different rice cultivars accumulated different levels of each. Salt tolerant lines maintained high spermidine and spermine concentrations, whilst putrescine remained unchanged. Salt sensitive lines accumulated excessive amounts of putrescine and low levels of the polyamines. Foliar application of putrescine on salt stressed rice leaves resulted in amelioration of NaCl toxicity (Krishnamurthy, 1991). It inhibited Na^+ and Cl^- uptake and the accumulation of K^+, Ca^{2+}, Mg^{2+}, proline and endogenous putrescine. Putrescine also prevented degradation of chlorophyll and inhibited a decrease in soluble protein, RNA and DNA contents, elevating their concentrations. Krishnamurthy (1991) proposes that putrescine must be heavily involved in salt tolerance of rice, possibly because it can be converted to amino acid synthesis including that of aspartate, glutamate and proline. However ABA-induced proline accumulation was found not to be connected to polyamine levels (Pesci and Reggiani, 1992). ABA lead to a decrease in spermidine and spermine

levels and a slight increase of putrescine, whilst sorbitol (which causes non ionic osmotic stress) lead to an increase in polyamines, but inhibited ABA-induced proline accumulation. However other possible roles for polyamines and diamines have been proposed in regulation of plant growth and "controlling stress related phenomena" (Krishnamurthy and Bhagwat, 1989).

1.5.1.2.2 Glycine betaine

E. coli accumulate glycine betaine as an osmoprotectant and are able to grow on media containing 0.8 M NaCl in its presence and its precursor choline (Le Rudulier, *et al.*, 1984). Glycine betaine is preferentially accumulated above other osmolytes, such as glutamate, K^+ and trehalose, whose levels decrease in the presence of glycine betaine and proline (Cayley, *et al.*, 1992). Accumulation of glycine betaine and proline was found to be accompanied by a large increase (20 - 50 %) in the volume of cytoplasmic water. Thus the molar effects of glycine betaine and proline on water activity and osmotic pressure of the cytoplasm were significantly larger than those of the solutes they replaced (Cayley, *et al.*, 1992). A glycine betaine-binding protein is induced by osmotic stress in *Rhizobium meloliti*, showing its importance in Gram negative bacteria as well (Le Rudulier, 1991). A novel compound 1-methyl-1-piperidino methane sulfonate (MPMS) can block the osmoprotectant activity of choline and proline, but not glycine betaine in *E. coli* (Kunin, *et al.*, 1993). Growth with NaCl was inhibited by MPMS, but restored only with glycine betaine. This is further evidence of the importance of glycine betaine as an osmoprotectant in *E. coli*.

Glycine betaine is accumulated in a number of plant species, including *Atriplex spongiosa*, *Spartina towsendii* (Flowers, *et al.*, 1977), barley, wheat, (Ishitani, *et al.*, 1993a) alfalfa (Pocard, *et al.*, 1991) and spinach (Summers and Weretilnyk, 1993), in response to salt stress. In salt stressed alfalfa plants glycine betaine is translocated to the roots and nodules, where it is thought to maintain the water status and therefore protect nitrogen fixation activity against the high osmolarity (Pocard, *et al.*, 1991). Glycine betaine is synthesised via a two step oxidation of choline, the first step being catalysed by choline mono oxygenase and the second by betaine aldehyde dehydrogenase (BADH). In salt stressed spinach leaves there is an increased diversion of choline to glycine betaine synthesis from phosphatidylcholine synthesis, by an increase in the activity of key enzymes involved in glycine betaine synthesis (Summers and Weretilnyk, 1993). Ishitani *et al.* (1993a) found BADH activity increased in salinization of leaves of glycine betaine accumulating plants. Rice and maize don't accumulate glycine betaine, which in the case of maize may be due to a recessive mutation of choline mono oxygenase that leads to betaine deficiency (Ishitani, *et al.*, 1993a). There is a difference in glycine betaine synthesis between the Gramineae (wheat) and Chenopodiaceae (spinach), since in the Gramineae there is an apparent link between glycine betaine synthesis and membrane lipid biosynthesis

(McDonnell and Wyn Jones, 1988). Rapid accumulation of glycine betaine coincided with membrane development and lipid biosynthesis in wheat and barley. Choline is produced as a biproduct of the breakdown of phosphatidylcholine to diacylglycerol, rather than from hydrolysis of choline phosphate. Thus the Gramineae have adapted one pathway from another, but as a consequence they require phospholipid turnover for glycine betaine and thus don't produce it as rapidly as the Chenopodiaceae in response to salt stress, which may account for their lower salt tolerance.

1.5.1.2.3 Proline

Proline is accumulated in bacteria, algae and higher plants in response to osmotic stress and dehydration (Aspinall and Paleg, 1981; Flowers, *et al.*, 1977), as well as heavy metals (Bassi and Sharma, 1993). Addition of exogenous proline to media of cultures exposed to NaCl, (Pandey and Ganapathy, 1985) water stress (Handa, *et al.*, 1986) and low temperature stress (Duncan and Widholm, 1987) stimulates growth. But other research has shown no correlation between proline accumulation in whole plants and tolerance to water and salt stress (Moftah and Michel, 1987). However in the halophyte *D. spicata*, cell cultures grown continuously in the presence of 200 mM NaCl had elevated levels of proline as compared to those grown in the absence of NaCl (Daines and Gould, 1985). Heyser *et al.* (1989b) observed increases in proline levels when *D. spicata* cells were transferred from 0 mM to 260 mM NaCl containing media, accumulating up to 50 μmol/g FW proline within eight hours. The facultative halophyte *M. crystallinum* accumulates proline in response to 400 mM NaCl, to the extent that in the presence of NaCl it represents 40 % of total amino acids, compared to 5 % in the absence of NaCl (Ostrem, *et al.*, 1987b). These data suggest that proline does have a role in osmoregulation. Like glycine betaine, proline is accumulated in alfalfa nodules to much higher levels than leaves, where its contribution to maintenance of water status protects nitrogen fixation (Irigoyen, *et al.*, 1992). It has also been suggested that it may directly influence protein stability and structure. In the plant *Tamarix jordanis* proline analogues ameliorated the inhibition of Rubisco activity by NaCl, N-methyl-L-proline (MP) being more effective than N-methyl-*trans*-4-hydroxy-L-proline (MHP). Both of these compounds are found at high levels in chloroplasts of *Tamarix* (Solomon, *et al.*, 1994). It is suggested that MP and MHP may be protecting Rubisco by stabilisation of its hydration state and conformation, in the presence of NaCl. MP allowed Rubisco to function well with 100 mM NaCl, since its activity increased in *Tamarix* grown in saline media.

Proline accumulation in plants results from increased *de novo* synthesis from glutamate, whilst in *E. coli* it occurs from uptake via the ProU transport system. *E. coli*, *Saccharomyces cerevisiae*, *D. desulfuricans* and the marine copepod *Tigriopus californicus* all synthesise proline from glutamate, as in figure 1.5.1 (Brandriss and Falvey, 1992;

Burton, 1991a; Burton, 1991b; Csonka, 1983; Fons, *et al.*, 1991; Li and Brandriss, 1992). The initial step is the ATP - dependent phosphorylation of the γ-carboxyl group of glutamate by γ-glutamyl kinase (γ-GK). The resulting γ-glutamyl phosphate is reduced to glutamyl-γ-semialdehyde (GSA) by GSA dehydrogenase, with which γ-GK forms a functional complex. GSA spontaneously cyclizes to Δ^1-pyrroline-5-carboxylate (P5C), which is finally reduced to proline by P5C reductase (P5CR).

It was assumed that plants synthesise proline in a similar way. However although P5CR activity was detected in several different plants, including tobacco (La Rosa, *et al.*, 1991) and *M. nodiflorum* (Treichel, 1986), no γ-GK activity was found. cDNA clones for P5CR were isolated by direct complementation of a *proC* mutation in *E. coli* with a cDNA expression library of soyabean root nodule mRNA. One of the clones, with a 1.2 kb insert, encoded a 28, 586 dalton polypeptide, which had 39% identity to the *E. coli* P5CR (Delauney and Verma, 1990). P5CR cDNAs and genes have since been isolated from pea (Williamson and Slocum, 1992) and *Arabidopsis* (Verbruggen, *et al.*, 1993).

A similar approach was used to isolate cDNAs for γ-GK and GSA dehydrogenase. Direct complementation of *E. coli proA*, *proB*, and *proBA* auxotrophic mutations with a moth bean (*Vigna aconitifolia*) cDNA expression library. A cDNA was isolated that encoded a bifunctional enzyme Δ^1-pyrroline-5-carboxylate synthetase (P5CS) (Hu, *et al.*, 1992) that catalyses the conversion of glutamate to P5C via GSA (figure 1.5.2). The cDNA encoded a 73.2 kD polypeptide that had two enzymatic domains corresponding to the 39 kD *E. coli* γ-GK (proB) (N-terminal) and 43.5 kD *E. coli* GSA dehydrogenase (proA) C-terminal), having 33.3% identity (55.3% similarity) and 35.7% identity (57.9% similarity) respectively. γ-GK and GSA dehydrogenase activities were detected when the P5CS cDNA was expressed in the *E. coli proBA* mutant. There is a 42.4% sequence similarity between the proB and proA *E. coli* proteins. Hu *et al.* (1992) suggest that during evolution duplication of an ancestral gene may have occurred, leading to *proA* and *proB*. They then later developed reductase and kinase activities. In plants the corresponding genes have fused, encoding the bifunctional enzyme. Within each enzymatic domain of P5CS is a leucine zipper, which may facilitate inter- or intramolecular interaction of the protein. Hu *et al.* (1992) suggest these leucine zippers may be another relic of evolution, when the separate enzymes needed to be brought together to form a functional complex, as in *E. coli*. But alternatively they may be acting intramolecularly to maintain the tertiary structure, by allowing the formation of hetero- or homodimer forms of the enzyme.

Plants also synthesise proline from orthinine, by transamination of the δ-amino group by orthinine-δ-aminotransferase (OAT) to give GSA, which is then converted to proline via P5C (figure 1.5.2). A cDNA encoding OAT was isolated by "*trans*"-complementation of an *E. coli proBA* proline auxotroph with orthinine and a moth bean cDNA expression library (Delauney, *et al.*, 1993a). The cDNA encodes a 48.1 kD polypeptide, that when expressed in *E. coli* exists as a monomer, with a high substrate affinity for (K_m) for orthinine. It was

found to have high homology with mammalian and yeast δ-OATs and a family of bacterial and fungal ω-aminotransferases, but no significant similarity with α-aminotransferases. Delauney *et al.* (1993a) suggest this is consistent with the formation of P5C and not P2C, which would be the intermediate product if transamination of the α-amino group of orthinine were to take place. In addition, P2C wouldn't be recognised as a substrate by P5CR in *E.coli* to rescue proline auxotrophy.

P5CR and P5CS are both constitutively expressed in roots and leaves, but their mRNA levels increase when young seedlings are watered with 400 mM and 200 mM NaCl respectively (Delauney and Verma, 1990; Hu, *et al.*, 1992; Verbruggen, *et al.*, 1993; Williamson and Slocum, 1992). This suggests that they are both osmoregulated. In *M. nodiflorum* there is an increase in proline accumulation and P5CR activity with adaptation to NaCl (Treichel, 1986). He suggested that feedback inhibition didn't form part of the regulation of proline synthesis during salt stress, since further proline accumulation wasn't inhibited in the presence of high proline concentrations, but that regulation may occur at the final step, catalysed by P5CR. La Rosa *et al.* (1991) found that there was no increase in P5CR activity in crude extracts from NaCl-adapted tobacco cells compared to non adapted cells. It was concluded that NaCl-dependent regulation of proline biosynthesis doesn't involve a change in the kinetic properties of P5CR and therefore it isn't the rate limiting step. They suggested a point earlier in the pathway, such as the first step. Heyser *et al.* (1989b) found that NaCl-induced proline biosynthesis was inhibited by 5 mM exogenous proline. They attributed this either to feedback inhibition of one of the enzymes involved or by repression of transcription and/or translation of genes encoding those enzymes, and not inhibition of glutamate transport. Other research has suggested that proline accumulation is regulated at the translational level, since it was inhibited by cycloheximide and not actinomycin D (Ketchum, *et al.*, 1991).

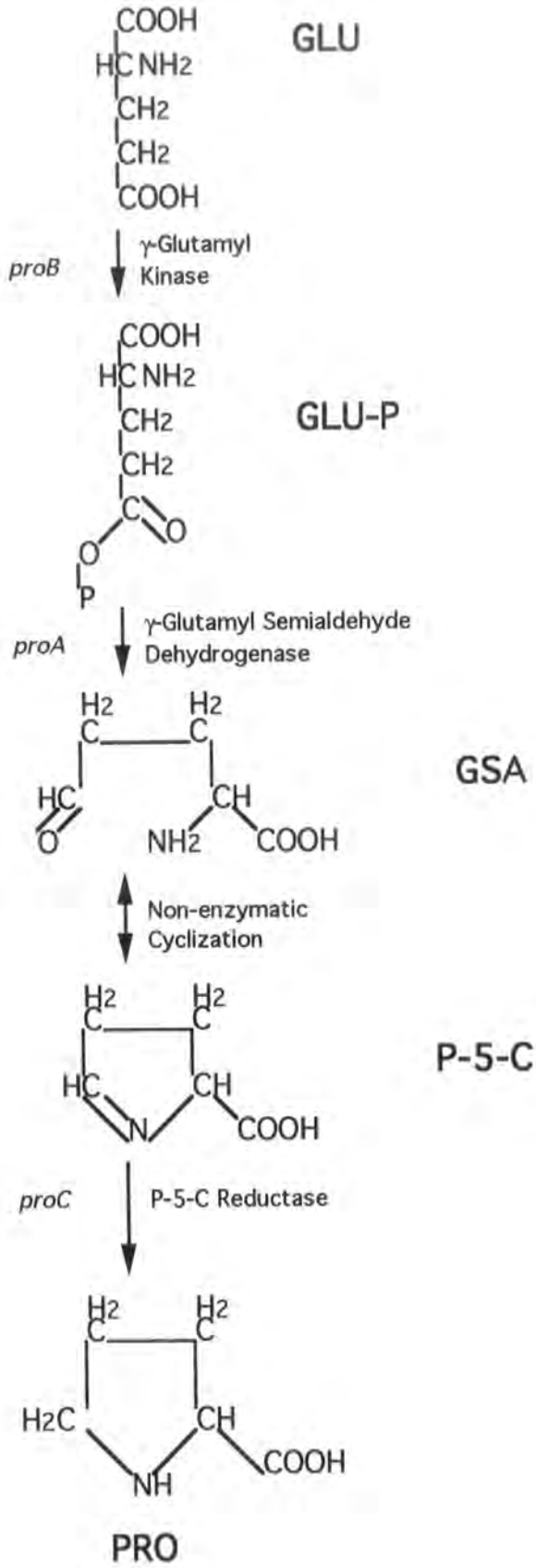
In *E. coli* proline biosynthesis is regulated by allosteric inhibition of γ-GK, the first enzyme in the pathway, by the end product proline (Csonka, 1983; Orser, *et al.*, 1988). A single base pair mutation of the *proB* gene (*proB74)* that results in the overproduction of proline, is 200 times less sensitive to feedback inhibition than the wild type and can also confer osmotolerance (Dandekar and Uratsu, 1988). In the moth bean, P5CS is also allosterically inhibited by proline, but is less sensitive to the end product regulation than the *E. coli* γ-GK, being 50 % inhibited by 6 mM proline as compared to 50 % inhibited by 0.2 mM proline in wild type *E. coli* (Delauney and Verma, 1993b). In the *E. coli* mutant *proB74* allele, the change of an A for G at the nucleotide level, leads to asparagine replacing aspartate at the amino acid level. It is this single base pair change that causes the loss of sensitivity to feedback control. The aspartate residue is conserved in the protein sequence of P5CS and thus a similar mutation may lead to a decrease in feedback inhibition. Delauney *et al.* (1993b) suggest that since the moth bean is a relatively drought tolerant plant, the reduced sensitivity of P5CS to proline inhibition is consistent with the

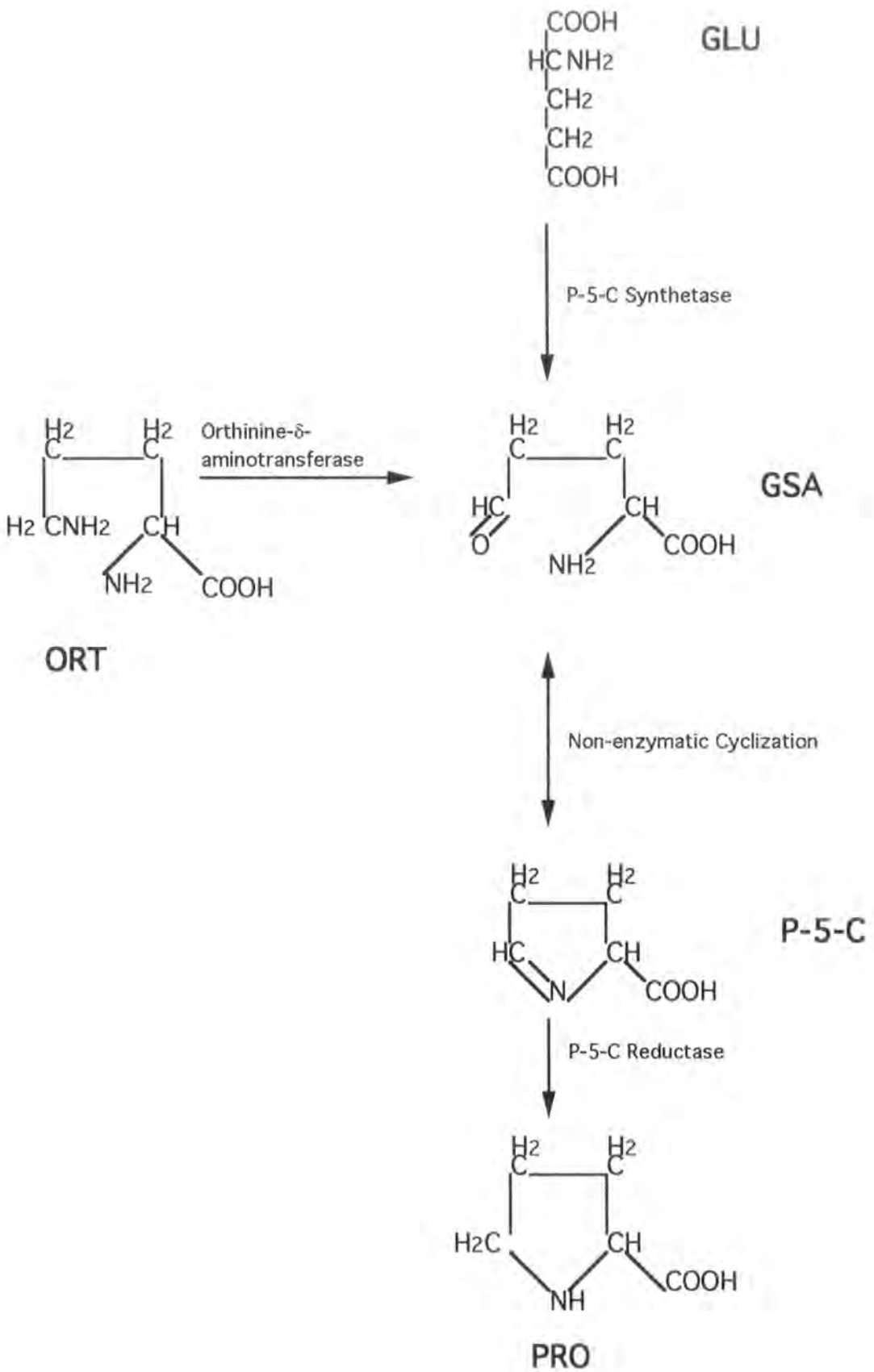
accumulation of proline in salt stressed plants. Therefore in osmotically adapted plant cells P5CS has some how been made less sensitive to feedback inhibition of proline. Blocking proline biosynthesis by preventing P5CS activity, as a means for studying the importance of proline accumulation, may show an increase in osmotic resistance in plants (Delauney and Verma, 1993b). Confirmation that P5CR is not the rate limiting step comes from transgenic tobacco plants overexpressing the genes where there was no significant increase in proline synthesis to reflect the 50 fold increase P5CR activity (Szoke, *et al.*, 1992). Hu *et al.* (1992) suggest that since elevated P5CS may control the flux from glutamate to P5C, it implies that proline synthesis is also transcriptionally controlled, in addition to feed back regulation. Proline biosynthetic enzymes may also be inhibited by to a small extent by NaCl itself. Comparison of metabolism from glutamate to proline between halophytes and nonhalophytes found that enzymes of halophytes were less sensitive to inhibition by NaCl than nonhalophytes (Heyser, *et al.*, 1989a). *D. spicata* was found to have the greatest tolerance, reflecting its greater ability to metabolise glutamate to proline and also its greater salt tolerance. Heyser *et al.* (1989a) conclude that *D. spicata* proline biosynthetic enzymes are less sensitive to inhibition by NaCl than those of other species.

Investigation of the importance of the orthinine versus the glutatmate pathway, by studying transcripts of OAT and P5CS as an indication of gene expression, showed that under normal physiological conditions both pathways are used to synthesise proline (Delauney, *et al.*, 1993a). But when moth bean seedlings are subjected to osmotic stress and nitrogen starvation there is an increase in P5CS mRNA and a decrease in OAT mRNA. In conditions of excess nitrogen the reverse happens, with a decrease in P5CS mRNA levels and an OAT mRNA increase. Delauney *et al.* (1993a) suggest that the glutamate pathway is the primary route for proline synthesis during osmotic stress and N_2 limitation and the orthinine pathway is dominant during high N_2.

In the future Delauney *et al.* (1993b) predict that identification of *cis*-acting elements in the P5CS promoter, that are responsive to drought and salt stress and the corresponding *trans*-acting factors, should help define the mechanisms involved in stress-induced proline synthesis. Whether it is a primary response to a signal transduction pathway or a secondary response, where genes are effected by stress-induced changes in metabolism. Verma *et al.* (Verma, *et al.*, 1992) intend to investigate a possible role for ABA in the regulatory process, since it is involved in other aspects of osmotic stress.

Artificially decreasing the sensitivity of P5CS to end product inhibition, by changing A to G as in the *proB74* mutant, may confer osmotolerance by overproducing proline. If this can be achieved in tobacco or *Arabidopsis*, it could be applied to rice, wheat, soybean and potato (Delauney and Verma, 1993b).


GLU
γ-Glutamyl Kinase
proB
GLU-P
γ-Glutamyl Semialdehyde Dehydrogenase
proA
GSA
Non-enzymatic Cyclization
P-5-C
proC
P-5-C Reductase
PRO


COOH
HC NH2
CH2
CH2
COOH
GLU
P-5-C Synthetase
H2 H2
C C
H2 CNH2 CH
NH2 COOH
Orthinine-δ-
aminotransferase
ORT
H2 H2
C C
HC CH
O NH2 COOH
GSA
Non-enzymatic Cyclization
H2 H2
C C
HC CH
N COOH
P-5-C
P-5-C Reductase
H2 H2
C C
H2C CH
NH COOH
PRO

1.5.2 Changes in membrane transport and permeability during adaptation to elevated NaCl

Compartmentation of ions into the vacuole during adaptation to salinity results from changes in membrane permeability and ion transport mechanisms. It has been proposed that these changes, which contribute to osmotic adjustment of the cell, occur without modifying the ion concentration within the cytoplasm (Flowers, *et al.*, 1977). Maintenance of Na^+ and Cl^- concentration gradients across the plasma membrane and tonoplast is facilitated by H^+-ATPases and changes in K^+/Na^+ selectivity (Flowers, *et al.*, 1977; Greenway and Munns, 1980). The plasma membrane H^+-ATPase is the primary pump of plant cells, driving all the secondary transport systems (Serrano and Gaxiola, 1994 and refs cited within). H^+-ATPase *in vivo* activity increased during osmotic adaptation in carrot, (Reuveni, *et al.*, 1987) tobacco (Watad, *et al.*, 1986) and *in vitro* activity in *Atriplex nummularia* (Braun, *et al.*, 1986). A comparison of H^+-ATPase gene expression in response to NaCl in tobacco (glycophyte) and *A. nummularia* (halophyte), revealed an increase in mRNA levels in both species, there being a greater response in the halophyte than the glycophyte (Niu, *et al.*, 1993). H^+-ATPases have been implicated in both Na^+ extrusion in plant cells in a Na^+/H^+ antiport mechanism (Serrano and Gaxiola, 1994) and K^+ uptake (Watad, *et al.*, 1991). K^+ uptake is enhanced in salt adapted tobacco cells, through cation/H^+ exchange transport or by K^+ entry through specific channels, driven by an electrochemical potential gradient produced by H^+-ATPase. Similarly it is thought that Cl^- efflux down a free energy gradient, established by H^+-ATPase (Niu, *et al.*, 1993). However in all these cases there is no molecular evidence, at either the gene or protein levels, to prove these proposals. Although in the case of Na^+ extrusion, it is thought that plants adopt the Na^+/H^+ antiport mechanism, which is used by the halotolerant alga *Dunialiella salina*, (Katz, *et al.*, 1992) rather than Na^+-pumping ATPases found in animals (Serrano and Gaxiola, 1994). Niu *et al.* (1993) propose that since expression of H^+-ATPase seems to increase in expanded leaves and roots (rather than expanding leaves and stems), it shows that these organs need an increase in H^+-electrochemical gradients for maintenance of the plant's ion homeostasis, for salt adaptation.

Ion transport at the vacuolar membrane occurs via similar mechanisms to those proposed at the plasma membrane, but is better characterised. The accumulation of Na^+ into the vacuole is driven by an Na^+/H^+ antiport mechanism, whose activity increases with salt stress (Serrano and Gaxiola, 1994 and refs. cited within). Specific H^+- transport activity and ATP hydrolysis of a 70 kD subunit of a tobacco tonoplast H^+-ATPase increases during salt adaptation (Reuveni, *et al.*, 1990). A cDNA, encoding this 70 kD subunit has been isolated from tobacco cells and through northern analysis, the corresponding gene showed constitutive expression in adapted and unadapted cell lines (Narasimhan, *et al.*, 1991).

However expression did increase for a short time, after NaCl treatment in NaCl-adapted cell lines.

Further changes in ion transport have also been observed. A cDNA encoding a partial sequence of a putative tobacco endoplasmic reticulum Ca^{2+} ATPase has been isolated from tobacco cells (Perez-Prat, *et al.*, 1992). The abundance of the corresponding mRNA increased two fold with NaCl-shock in deadapted cells and three to four fold in 428 mM NaCl adapted cells. Thus there was an observed correlation between the level of gene expression and the ability of the cells to tolerate NaCl. Perez-Prat *et al.* (1992) proposed that the altered regulation of a Ca^{2+}-ATPase in response to elevated NaCl reflects homeostatic cellular mechanisms that would counteract the disruption of the internal Ca^{2+} concentrations caused by NaCl-induced membrane leakage.

1.5.3 Changes in gene expression during adaptation to elevated NaCl

1.5.3.1 *Distichlis spicata*

Changes in gene expression were studied in *D. spicata* during the first 24 hours after exposure to 260 mM NaCl (Zhao, *et al.*, 1989). Poly (A^+) RNA was isolated and used for *in vitro* translations and for the construction of cDNA libraries. Two dimensional SDS PAGE analysis of the *in vitro* translation products indicated that NaCl stress induced both increases and decreases in a number of different mRNAs, within 24 hours of stress. Transient increases were observed in over 50 polypeptides, suggesting that adjustments to NaCl did not involve large changes in the levels of abundant proteins. Following a search of a cDNA library, in λgt10, for NaCl stress-related transcripts, using a differential screening strategy, a number of clones were isolated. Fifteen clones represented transcripts, whose expression increased in response to salt stress, whilst three represented transcripts, whose expression decreased. However Zhao *et al.* (1989) were unable to find a unique sequence in the library, i.e. one that was not also present in unstressed cells. The kinetics of mRNA accumulation of two of the transcripts whose expression increased, corresponding to clones pDZ6.2 and pDZ2.8, were studied by northern hybridisations. pDZ2.8 increased two fold within four to eight hours of exposure to NaCl and then decreased within 24 hours, it was also abundant in unstressed cells. Subsequent characterisation suggested that this clone may encode a transcript for the protein histone 2B (Zhao, *et al.*, 1989). Transcripts corresponding to pDZ6.2 increased ten fold within 24 hours and remained high in salt adapted cells. Zhao *et al.* (1989) concluded that since proline accumulation occurred within eight hours of exposure to elevated NaCl in *D. spicata*, that it was unlikely that this transcript had a function related to proline biosynthesis.

1.5.3.2 Tobacco (and Osmotin)

Cultured tobacco cells (*Nicotiana tabacum* var. Wisconsin 38) adapted to grow under osmotic stress and PEG-induced water stress synthesise and accumulate a 26 kD protein (Ericson and Alfinito, 1984; Singh, *et al.*, 1985). It was noted that this protein was unique to adapted cells, but non stressed, unadapted cells also incorporated ^{35}S into a protein of a similar size during the midlog phase of culture growth (Singh, *et al.*, 1985). Both these proteins had the same partial proteolysis peptide map and were immunologically cross reactive, but differed in their pI values. Unadapted cells synthesise and accumulate the 26 kD protein during adaptation to elevated NaCl, the start of synthesis corresponding to a period of osmotic adjustment and culture growth (Singh, *et al.*, 1985). Thus Singh *et al.* (1985) concluded that this protein may have a role in adaptation of cultured tobacco cells to osmotic stress and drought stress. The 26 kD protein was designated osmotin (Singh, *et al.*, 1987a). In NaCl-adapted cells it occurs in two forms, an aqueous-soluble form (osmotin I) and a detergent-soluble form (osmotin II) (Singh, *et al.*, 1987a). Both have an identical N terminal amino acid sequence upto residue 22 and are immunologically cross reactive. Osmotin II, however is more resistant to proteolysis. Ericson and Alfinito (1984) and Singh *et al.* (1985) also observed changes in the levels of other proteins, showing that tobacco cells undergo specific changes in expression of multiple genes, whilst adapting to elevated NaCl.

Induction of salt or water (PEG-induced) stress causes osmotin to accumulate in dense inclusion bodies within the vacuole at levels of upto 12 % total protein (Singh, *et al.*, 1987a). Osmotin synthesis is responsive to ABA (Singh, *et al.*, 1987b) . ABA accelerates the rate of adaptation of unadapted cells to salt stress, when applied exogenously (La Rosa, *et al.*, 1985). Osmotin is also accumulated within eight hours of exposure to ABA in unadapted cells. The ABA-induced 26 kD protein is immunologically cross-reactive with the NaCl-induced protein, has an equivalent partial proteolysis map and the same pI value of > 8.2. A correlation between the start of osmotin synthesis and an increase in ABA accumulation was observed (La Rosa, *et al.*, 1985). La Rosa *et al.* (1985) suggest that since ABA-induction of osmotin wasn't permanent without the simultaneous presence of NaCl, and exogenous ABA induces accumulation of osmotin in roots as compared to other tissue, that ABA is involved in the regulation of induction of osmotin synthesis and a low water potential is required for the protein to be accumulated (Singh, *et al.*, 1987b; Singh, *et al.*, 1989). However ABA appears to be necessary for osmotin accumulation since when tobacco cells are osmotically shocked, there is no increase in osmotin mRNA or protein (Singh, *et al.*, 1989). This appears to be because shock-treatment doesn't cause an immediate ABA accumulation. This implies that osmotin plays a role in adaptation of tobacco cells to low water potential, since its synthesis corresponds with a period of osmotic adjustment. Two mechanisms of regulation of osmotin expression have been

proposed (Singh, *et al.*, 1989). Firstly ABA increases the synthesis or stabilisation of osmotin mRNA, which remains constant during the growth cycle, even with changes in the ABA levels. Maximum endogenous ABA levels are similar in adapted and unadapted cells when osmotically stressed, but the peak occurs later in the growth cycle (after the onset of logarithmic growth) with unadapted cells. This may account for the lower level of osmotin accumulation in unadapted cells. ABA treatment at the start or before logarithmic growth leads to an increase in transcription rates and higher levels of osmotin mRNA accumulation. But a similar dose later in the growth cycle results in no osmotin message accumulating (Nelson, *et al.*, 1992). This suggests that the gene is less responsive at this stage in the growth cycle. Secondly a post transcriptional control mechanism is induced by the adaptation to low water potentials, allowing the accumulation of the protein (Singh, *et al.*, 1989). This may occur because osmotin mRNA is preferentially translated in a high NaCl environment or post transcriptional processing may give rise to a more stable form of the protein. La Rosa *et al.* (1992) observed that osmotin mRNA abundance doesn't always reflect the amount of osmotin accumulation. This clearly implies a translational or post translational mechanism of regulation.

The temporal and spatial pattern of regulation of osmotin was studied during normal plant development and adaptation to NaCl. The osmotin promoter was fused to the reporter gene GUS and its activity measured in transgenic tobacco (Kononowicz, *et al.*, 1992). A high level of activity was measured in mature pollen grains during anther dehiscence and in the pericarp tissue at the final, desiccating stages, of fruit development. Activity was lost after pollen germination, thus showing the osmotin promoter to be only active in dehydrated pollen. GUS activity was also found in the corolla tissue at the onset of senescence. Osmotin mRNA was undetected in developing and mature seeds (La Rosa, *et al.*, 1992). Adaptation to NaCl highly stimulated the osmotin promoter in epidermal and cortex parenchyma cells in the root elongation zone; in epidermal and xylem parenchyma cells in the stem internodes; and epidermal, mesophyll and xylem parenchyma cells in developed leaves (Kononowicz, *et al.*, 1992). It was concluded that the spatial and temporal pattern of regulation of the osmotin promoter reflected its proposed functions in osmotic stress and pathogen defence. The osmotin gene promoter can also be transcriptionally activated by ABA and ethylene (Nelson, *et al.*, 1992). The sensitivity of the promoter to exogenous ABA decreases with age in roots and shoots in young seedlings. Accumulation of osmotin mRNA, but not protein, occurred in some plant tissues due to treatment with ABA, wounding and tobacco mosaic virus infection. Ethylene caused accumulation of mRNA, and a small amount of protein, in seedlings but was ineffective in cultured cells. Tobacco plants and cultured cells accumulated both osmotin mRNA and protein on exposure to NaCl (La Rosa, *et al.*, 1992). Thus, in summary, osmotin accumulation is developmentally controlled, as well as being induced by a number of signals (La Rosa, *et al.*, 1992).

The osmotin promoter was searched and found to have sequences with similarity to ABRE's (Marcotte Jr., *et al.*, 1989; Mundy, *et al.*, 1990), *as*-1 (Lam, *et al.*, 1989) and *E-8 cis* element sequences. Deletion studies showed two regions of the osmotin promoter which had specific interactions with nuclear factors isolated from cultured tobacco cells and leaves (Raghothama, *et al.*, 1993). The abundance of these binding factors increased in the presence of NaCl, ABA and ethylene. One region (-762 bp to -642 bp) contained a 35 bp sequence (-757 bp to -722) that was protected by nuclear factors from DNAse I digestion. It contained the sequence TGTACTTCTT, which shares 70 % identity with a motif TCATCTTCTT, that is highly conserved in several stress-induced genes (Goldsbrough, *et al.*, 1993). The other region was from -108 bp to +45 bp and was close to the TATA box. Deletion studies on the osmotin promoter fused to GUS revealed three regions that may have a role in regulation. The first was a fragment from -248 upto -108 bp that was required for reporter gene activity. It was able to activate transcription in response to a number of inducers, including ABA, NaCl, ethylene, desiccation and wounding. Thus it was suggested that "*trans*-acting factors induced by a number of different signals may bind to this region or that the interaction of a constitutive binding factor with the *cis*-regulatory element is enhanced by interactions with protein factors produced in response to the primary inducers" (Raghothama, *et al.*, 1993). It is also possible that such a constitutive transcription factor could also be chemically modified in response to environmental stimuli with no requirement of *de novo* synthesis of other proteins, which would allow binding. This fragment also contained the sequence CACTGTG, which is very similar to the ABRE core element (Marcotte Jr., *et al.*, 1989; Mundy, *et al.*, 1990; Raghothama, *et al.*, 1993). However the extra T may mean it can't act as an ABRE. In addition to these facts, no binding of nuclear factors was observed in this region (-241 bp to -235 bp) nor at -508 bp to -502 bp, where another ABRE motif ACGTGGC, similar to that found in the promoter regions of *rab*16 and *rab*28 (Mundy, *et al.*, 1990; Pla, *et al.*, 1993), was found. A second region upto -1052 bp appeared to be acting as an enhancer-element. It contained one of the two regions (-762 bp to-642 bp) that bound nuclear factors, the *EN*-1 element (-757 bp to -722 bp) that caused maximal expression of the reporter gene. The third region that was identified with a possible role in regulation was located upstream of -1052 bp and contained negative elements. *as*-1 element core sequences were found throughout the promoter region. This activation sequence-1 is a *cis* element found in the cauliflower mosaic virus 35s promoter and confers preferential expression of the heterologous promoter in roots (Lam, *et al.*, 1989). Under osmotic stress there is a high degree of osmotin promoter activity in roots (Kononowicz, *et al.*, 1992). Thus it appears that osmotin transcription may be regulated by a system that is responsive to ABA, NaCl and physical factors, but that it probably does so via a different mechanism to that of other ABA responsive genes.

A peptide immunologically related to osmotin has been detected in cells of the halophyte *Atriplex nummularia* (Casas, *et al.*, 1992), which is constitutively expressed in unadapted

and adapted cells. Two cDNA clones encoding osmotin like proteins have been isolated. pA8 mRNA is constitutively accumulated in unadapted and adapted cells and mRNA levels increase with exogenous ABA treatment. However mRNA is not accumulated with osmotic shock, implying that this clone may be unresponsive to NaCl. By contrast pA9 mRNA is induced by elevated levels of NaCl and increases in cells as a function of NaCl adaptation. It responds to osmotic shock, but not to ABA. This suggests that in this halophyte the two forms of these osmotin-like genes are performing different roles, one (pA8) in an adaptive response and one (pA9) in the initial osmotic shock (Casas, *et al.*, 1992). NaCl-adapted tobacco cells grown in the absence of NaCl retain the ability to readapt more rapidly to osmotic stress than cells never exposed to NaCl before. They also accumulate osmotin constitutively, even in the absence of NaCl (Singh, *et al.*, 1989). Cells adapted to 428 mM NaCl contain 4 to 30 fold more osmotin than unadapted cells. Osmotin levels drop to those intermediate between those seen in adapted and unadapted cells grown in the presence of NaCl when NaCl-adapted cells are transferred to media containing no NaCl (La Rosa, *et al.*, 1989). This ability to accumulate osmotin in the absence of NaCl suggests that the osmotin gene or genes involved in its regulation are altered (though probably not irreversibly) during adaptation (Singh, *et al.*, 1989). Thus cells with stable genetic change affecting osmotin accumulation are selected for during prolonged exposure to high NaCl. This alteration in gene expression leads to salt tolerance (King, *et al.*, 1988).

Osmotin is homologous to a 24 kD NaCl-induced protein from tomato, NP24 (King, *et al.*, 1988), thaumatin (Singh, *et al.*, 1987b), a tobacco maize virus-induced pathogenesis-related protein (Cornelissen, *et al.*, 1986), a maize α-amylase/trypsin inhibitor (Richardson, *et al.*, 1987), a thaumatin-like cDNA from mercuric chloride-treated maize (Frendo, *et al.*, 1992), an ABA responsive cDNA from *Solanum commersonii* expressed during freezing tolerance (Zhu, *et al.*, 1993) and a cDNA clone from tomato planta macho viroid-infected tomato (Nitschmann and Packer, 1992). Thus osmotin is related to a number of genes, which are expressed in response to a variety of environmental stresses and pathogen attack. This suggests a large family of genes that may be involved in a general stress response. Osmotin's temporal and spatial patterns of expression also imply an "antibiotic or insecticidal function", accumulating in roots, epidermal and adjacent layers in the stem and in flowers (Kononowicz, *et al.*, 1992). Osmotin accumulation is also induced by ethylene, which is characteristic of other pathogenesis-related (PR) proteins (La Rosa, *et al.*, 1992). Casas *et al.* (1992) suggest that the different forms of osmotin may be performing separate functions, in response to osmotic stress and pathogen attack.

An osmotin/thaumatin-like protein AP24 was identified from tobacco infected with the tobacco mosaic virus and tomato with *Phytophthora infestans* (Woloshuk, *et al.*, 1991). Both proteins caused lysis of sporangia and growth inhibition of *P. infestans*, either by interacting with the plasma membrane, leading to its disruption and cell lysis, or by having cell wall hydrolysis activity. Further evidence of osmotin having pathogen-defence activity

has been shown by Liu *et al.* (1994). Transgenic potato, constitutively overexpressing the osmotin gene showed delayed development of disease symptoms after leaves were infected with spore suspensions of *Phytophthora infestans*. However tobacco showed no change in the development of disease when infected with *Phytophthora parasitica* var. *nicotianae*, even though purified osmotin showed antifungal activity against different pathogenic fungi during *in vitro* assays (Reuveni, *et al.*, 1993). It is possible that overexpressing one gene alone can't defend the plant against pathogen attack, requiring other factors (Liu, *et al.*, 1994). It may also be that not all PR proteins are involved directly with defence against pathogens. Transgenic tobacco overexpressing PR genes had no resistance against viral infection (Linthorst, *et al.*, 1989).

Pathogenesis-related proteins are soluble proteins induced by the hypersensitive reaction of host plants infected by pathogens, which can also be induced by a number of other types of stress and chemical factors, including salicylic acid and ethylene. PR proteins have been separated into five groups, roughly according to size or function (Ohashi and Ohshima, 1992). Group 2 PR proteins have β-1,3-glucanase activity and group 3 chitinase activity. Group 5 includes osmotin and other (acidic) proteins that are similar to thaumatin. Basic and acidic PR proteins respond differently to plant growth regulators such as ethylene, cytokinins and auxin, and also display different patterns of expression. This may reflect their different functions and localisation (Ohashi and Ohshima, 1992).

Osmotin exhibits behaviour that can be related to its proposed roles in osmotic stress and pathogen attack, such as being regulated by both ABA and ethylene. La Rosa *et al.* (1989) propose that a general stress response is being activated by osmotic stress, and that "this response may have evolved to defend plants against pathogenic organisms when plants are weakened due to environmental stresses".

1.5.3.3 Barley

Barley roots synthesise two 26 kD polypeptides at an increased level when grown in the presence of 200 mM NaCl (Hurkman and Tanaka, 1987). These proteins have pI values of 6.3 and 6.5, which are more acidic than osmotin, to which they are not immunologically related. Synthesis of these polypeptides can be increased in germinated plants grown in NaCl for 6d and also in 5d-old seedlings treated with NaCl for 24h. Synthesis decreases on returning plants to control media, showing that these polypeptides may play a role in adaptation to NaCl. However these polypeptides are not effected by desiccation, PEG, mannitol or heat shock (Hurkman, *et al.*, 1989). Endogenous ABA has no effect upon their synthesis, but exogenous treatment results in the accumulation of the pI 6.5 polypeptide and a 25.5 kD polypeptide with a pI of 6.1, that is immunologically related to the pI 6.3 polypeptide (Hurkman, *et al.*, 1991). Ramagopal (Ramagopal, 1987b) also identified NaCl-induced polypeptides in barley roots corresponding to the two proteins isolated by Hurkman

et al. (1987). He also observed changes in the mRNA populations, concluding that transcriptional and post transcriptional mechanisms were involved in the regulation of gene expression in barley during salt stress (Ramagopal, 1987a).

Characterisation of the two proteins (pI 6.3 and 6.5) showed them to be isoforms (Hurkman, *et al.*, 1991). Both had significant similarity to the N-terminal amino acid sequence of germin, a protein that increases in germinating wheat seeds (Grzelczak and Lane, 1984). Thus they were designated Gs1 (pI 6.3) and Gs2 (pI 6.5) (salt-responsive germin-like polypeptide 1 and 2). Like other germins, Gs1 and Gs2 are resistant to proteases and are glycosylated (Hurkman, *et al.*, 1991). Gs1 and Gs2 are expressed in roots and coleoptiles, but not leaves. Their expression increases in roots and decreases in coleoptiles in the presence of NaCl. Gs1 and Gs2 are mostly found in the soluble cell fraction, but are also in the microsomal and cell wall fractions (Hurkman, *et al.*, 1991). However germins are mainly located within the cell wall and antibodies to germin didn't react with Gs1 or Gs2, whilst neither antibodies to Gs1 nor Gs2 cross-reacted with germin. Germin and Gs 1 and 2 are both expressed in roots, but in different regions. Germin is found in the distal regions and Gs1 and Gs2 in the more mature parts, but not the tip. Despite these differences, isolation of the cDNA clones encoding the germin-like proteins showed the nucleotide sequences to be 98.5 % and 94.8 % similar to the wheat germins gf-2.8 and gf-3.8 respectively (Hurkman, *et al.*, 1994).

A 125 kD oligomer was identified, which consisted of 26 kD subunits that reacted with antibodies to Gs1 and Gs2. Activity blots revealed this 125 kD root protein to have oxalate oxidase activity (Hurkman, *et al.*, 1994). Heating the samples resulted in a loss of activity, as the oligomer denatured into its subunits, which corresponded to the 26 kD proteins with pI values of 6.3 and 6.5. Wheat germin has also been shown to exhibit strong oxalate oxidase activity (Lane, *et al.*, 1993) and antisera against wheat germin was shown to react with barley oxalate oxidase. Thus the germin-like proteins, induced by salt stress in barley, form an oligomer, that has a similar functional activity as the germins from wheat. However these two proteins have different subcellular and tissue location and are also regulated differently, the barley germin-like proteins being induced in response to elevated NaCl and wheat germins, developmentally during germination. Lane *et al.* (1993) proposed a physiological role for germin, due to the correlation between water uptake and germin synthesis during (post) germinative growth of wheat embryos. They suggest that the oxalate oxidase activity of germin may promote water uptake. If this is the case then the germin-like proteins induced in salt stressed barley roots, may promote water uptake from the cell's surroundings to counteract the changes in water potential and to maintain cell turgor pressure, resulting from high NaCl concentrations.

1.5.3.4 Wheat

Amphiploids have been used to study the gene expression of wheat in response to NaCl. Many (18) mRNAs were shown to increase and decrease in the roots of an amphiploid of Chinese spring wheat (salt sensitive) and *Elybrigia elongata* (salt tolerant relative of cultivated wheat) (Gulick and Dvorak, 1987). However none of these changes were unique to the amphiploid, several were observed in the parent plants during adaptation to NaCl. Many more changes were observed in the Chinese spring wheat, compared to the amphiploid and the authors proposed that some genes effected by salinity, due to metabolic changes, may not be directly involved in the mechanism of salt tolerance.

mRNAs were also induced by salt in a *Lophopyrum elongatum* x *Triticum aestivum* amphiploid (Gulick and Dvorák, 1992). cDNAs were isolated, that corresponded to eleven genes, whose expression increased two hours after exposure to 250 mM NaCl, reaching a peak after six hours. However some of these returned to the basal level of expression within 24 hours some within three to seven days. As a result they were designated early salt stress induced (ESIs). One cDNA was found to share homology with *rab* and *dhn*, genes from multi gene families, regulated by ABA. No possible function has been assigned to the other clones.

1.5.3.5 Rice

Several different salt-induced polypeptides have been observed in rice (*Oryza sativa* L.). Ten induced proteins were isolated from rice endosperm in response to 0.5 M NaCl (Limas, *et al.*, 1990). Two were found to have homology with immunoglobulin-binding proteins and one was similar to a α-globulin, in terms of amino acid composition and molecular weight. Four were proposed as being protein synthesis inhibitors, though the exact mechanism has yet to be determined. The authors make no proposals as to how these proteins could contribute to salt tolerant mechanisms in rice. A cDNA *Sal*T has been isolated from a rice cDNA library (Claes, *et al.*, 1990), it corresponds to one of eight proteins, whose expression increased in response to salt stress. Expression of *Sal*T gene was observed in sheaths and roots in response to salt, but not in leaf lamina. The authors propose that this organ-specific response correlates with the pattern of Na^+ accumulation during salt stress and so the *Sal*T protein may be involved in salt stress-induced ion transport.

Proteins with molecular weights of 26 and 27 kD have been shown to increase in expression in salt adapted rice cells (Shirata and Takagishi, 1990). The author draws comparisons with the 26 kD proteins from tobacco (Ericson and Alfinito, 1984) and barley (Hurkman and Tanaka, 1987), but gives no sequence similarity nor proposes a function for these rice proteins.

1.5.3.6 Alfalfa

Changes in the polypeptide population have been observed in both salt tolerant (HG2-N1) and salt sensitive (HG2) cell lines of alfalfa (*Medicago sativa* L.) in the presence of 171 mM NaCl (Winicov, *et al.*, 1989). However the changes seen in HG2-N1 were different to those in HG2 and many of the polypeptides detected in HG2-N1 were expressed constitutively in the presence and absence of NaCl. Differences were also found between adaptation and salt shock, in the polypeptides that were expressed. Winicov *et al.* (1989) proposed that different mechanisms may exist for adapting to high salt and for overcoming NaCl shock. Little is known about the function of these polypeptides, however after screening of an alfalfa cDNA library, a cDNA was isolated, that showed selective expression in salt tolerant lines (Winicov, *et al.*, 1989). The cDNA *Alfin-1*, was found to encode a polypeptide with two amino acid sequences, characteristic of nucleic acid binding proteins, with a role in gene regulation (Winicov, 1993). Putative zinc finger domains were also found in *Alfin-1*, proteins with such domains normally belong to a family of Zn^{2+}-binding transcription factors. Winicov (1993) therefore proposed a possible role of regulation of gene expression in salt tolerant alfalfa cells.

Large increases in photosynthetic-related mRNAs have also been observed in response to salt stress in alfalfa cells (Winicov and Button, 1991; Winicov and Seeman, 1990). HG2-N1 cell lines were found to have a much higher chloroplast DNA copy number, up to 50% compared to HG2 (Winicov and Button, 1991). This was consistent with an increased expression of the plastid-encoded gene *psbA* in HG2-N1 cells in the absence of NaCl, which increased further when exposed to NaCl. In addition, other plastid-encoded photosynthetic genes *psbD, psaB, atpB* and *rbcL* increased in expression in the presence of NaCl, as well as nuclear encoded genes *pCab4, pCab1* and *rbcS* (Winicov and Button, 1991; Winicov and Seeman, 1990). A corresponding increase in enzyme activity of Rubisco was also observed and *rbcL* and *rbcS* could be induced by NaCl in light and dark grown cells (Winicov and Seeman, 1990). There was also an eleven fold higher level of chlorophyll in HG2-N1 cells compared to HG2, in the absence of NaCl, which increased a further two fold when exposed to 1% NaCl (Winicov and Button, 1991). Winicov *et al.* (1990, 1991) propose that the increase in photosynthetic-related mRNA and protein and an increased photosynthetic capacity, may play a role in salt tolerance in alfalfa. An increased photosynthetic capacity could be providing an additional energy source for the compartmentation of ions or carbon skeletons for the synthesis of compatible solutes.

1.5.3.7 *Mesembryanthemum crystallinum*

The facultative halophyte *Mesembryanthemum crystallinum* (the common ice plant), found in arid desert regions, adapts to salt stress, by switching from the C_3 pathway to Crassulacean acid metabolism (CAM) (Winter and von Willert, 1972). CAM plants open their stomata at night and fix CO_2, when evaporative water loss is minimal (Ting, 1985). Phospho*enol*pyruvate carboxylase (PEPCase) catalyses the conversion of phospho*enol*pruvate (PEP) to oxaloacetate during nocturnal CO_2 uptake, as the initial step in the CAM pathway. Oxaloacetate is then converted to malate during the day when the stomata are closed. CAM plants use the accumulated malate as the primary carbon source, it is decarboxylated by $NADP^+$- dependent malic enzyme (NADP-ME) to give pyruvate and CO_2. The pyruvate is phosphorylated to PEP by pyruvate orthophosphate dikinase (PPDK), which can either enter the gluconeogenic pathway, or be used as the substrate for PEPCase. The CO_2 is refixed by ribulose-1,5-bisphosphate carboxylase (Rubisco) to enter the reductive pentosephosphate pathway (Ting, 1985).

The metabolic switch from the C3 pathway to CAM is characterised by the synthesis of the CAM enzymes and a change in expression of the corresponding genes and several other genes. These include *Imt1*, which encodes myo-inositol *O*-methyltransferase, involved in pinitol synthesis (Vernon and Bohnert, 1992); *Gpd1*, which encodes glyceraldehyde-3-phosphate dehydrogenase (Ostrem, *et al.*, 1990); and *B5* ,which encodes a putative thiol protease (Vernon, *et al.*, 1993), which are all up regulated by salt stress. Several fold decreases in mRNA levels have been observed for *rbcs* and *cab*, which encode the small subunit of Rubisco and the chlorophyll a/b binding protein (CAB) respectively (DeRocher, *et al.*, 1987; Michalowski, 1989b). Other genes, such as that which encodes Ferrodoxin-$NADP^+$-reductase (FNR) remain almost unchanged, mRNA levels increase slightly whilst FNR protein levels are unaffected (Michalowski, 1989b).

Salt stress also causes an increased uptake of Na^+ and Cl^- ions, which can facilitate the uptake of water from the soil, by lowering the water potential of plant cells (Demmig and Winter, 1986). This enables halophytes to survive high salinity (Bohnert, *et al.*, 1988). Accumulation of proline (Ostrem, *et al.*, 1987b) and pinitol (Paul and Cockburn, 1989) occurs in the cytoplasm where they act as compatible solutes.

CAM can be induced in *M. crystallinum* by irrigation with 500 mM NaCl, as well as by drought stress and PEG, and is associated with a 40 fold increase in the activity of PEPCase (Höfner, *et al.*, 1987). This linearly correlates with an increase in the total mass of PEPCase protein, as a result of *de novo* synthesis. The salinity-induced increase in levels of PEPCase protein in leaf and shoot tissue occurs via a stress-induced increase in steady state levels of translatable mRNA (Ostrem, *et al.*, 1987a). PPDK and NAD-ME protein activities also increase in the ice plant when exposed to salt or osmotic stress (Schmitt, *et al.*, 1988), which are accompanied by an increase in steady state mRNA, as a result of higher levels of

transcription. CAM doesn't fully establish itself in the ice plant for at least ten days after the onset of stress, although mRNAs for PEPCase and other CAM enzymes increase within two to three days and continue to do so for at least five days (Michalowski, *et al.*, 1989a).

Differential expression of two isogenes of PEPCase has been observed (Cushman, *et al.*, 1989). *Ppc1* and *Ppc2* share 76.4% identity at the nucleotide level and 83% at the amino acid level. The mRNA levels of each differed in salt stressed plants. *Ppc1* transcripts increased 30 fold in leaves within five days, whilst there was only a slight decrease in *Ppc2* transcripts over the same period. NADP-ME transcripts showed a similar pattern of expression, being induced in leaves, but in roots the levels remained unchanged (Cushman, 1992a). PEPCase isoforms have also been isolated from C_3 metabolising leaves, with pI's of 5.2, 5.5, 5.6 and 5.9 and are derived from two genes (Slocombe, *et al.*, 1993). The C3 isoform pI 5.9 is very similar to the CAM isoform and can also be induced by salt stress. Slocombe *et al.* (1993) propose that the CAM isoform of PEPCase may have evolved from this C_3 form of the enzyme.

In cell suspension cultures of *M. crystallinum*, exposed to elevated levels of NaCl, there is almost no change in PEPCase protein, mRNA and activity (Thomas, *et al.*, 1992a). Although Treichel (Treichel, *et al.*, 1988) did observe an increase in PEPCase activity and protein, but less than those seen in whole plants. Thomas *et al.* (1992a) also observed no increase in the activity of other enzymes. They concluded that the switch to CAM may depend on tissue-dependent or photoautotrophy-dependent mechanisms.

The metabolic switch to CAM is environmentally controlled and reversible following the removal of the stress (Vernon, *et al.*, 1988). The ability to switch is also developmentally determined. Three to five day old *M. crystallinum* seedlings show little induction of PEPCase mRNA under stress (Cushman, *et al.*, 1990; Ostrem, *et al.*, 1987a). At six weeks there is strong induction of PEPCase activity, by nine weeks the plant is able to accumulate PEPCase, even in the absence of stress. At this time, transcriptional activation of *Ppc1* is no longer observed, despite an increase in both Ppc1 mRNA and protein (Cushman, *et al.*, 1990). Thus it has been suggested that a developmental programme regulates PEPCase transcription and mRNA stability, which appears to work in conjunction with the plant's natural environment (Cushman, *et al.*, 1990).

Many of the genes expressed in response to NaCl are regulated at the level of transcription (Vernon, *et al.*, 1993). These include *Imt1*, *B5*, and *Ppc1*. But they may also be controlled post transcriptionally, such as by mRNA stability. Investigation of transcriptional mechanisms, by characterisation of the 5' flanking region of *Ppc1*, revealed a transcription start 332/333 bp from the ATG start (Cushman and Bohnert, 1992b). Nuclear DNA-binding factors were found to interact with this region. One factor PCAT-1 binds to the *Ppc1* promoter at two A/T-rich sites, -128 bp to -158 bp and -187 bp to -205 bp, which share a consensus motif AARTAAC(T/A)A(G/T)TTTY. PCAT-1 is more abundant in nuclear extracts from salt stressed leaf tissue. Additional binding activities that interact

with the PCAT-1 binding sites have been detected, which decrease or increase in abundance or binding affinity with salt stress. Another nuclear factor PCAT-2 has been identified, which may have a role in repressing *Ppc1*, since the PCAT-2 complex was only observed in root or unstressed leaf tissue. This is consistent with low *Ppc1* expression (Cushman and Bohnert, 1992b). However a fusion of the *Ppc1* 5' flanking region and GUS, that was constitutively expressed, when introduced into mature tobacco, showed no stress-induced expression, infact it was slightly repressed (Cushman, *et al.*, 1993). The authors concluded that the tobacco was able to recognise the *cis*-acting elements, resulting in the constitutive expression, but that stress-induced expression probably required other factors, not present in tobacco, that may be consistent with developmental control.

Plant growth regulators, such as ABA and cytokinins, have been implicated in regulating the metabolic switch to CAM in *M. crystallinum.* ABA and cytokinins can induce CAM enzymes and other salt induced genes such as *Imt1* and *B5* (Chu, *et al.*, 1990; Thomas, *et al.*, 1992b; Vernon, *et al.*, 1993), though cytokinins are more effective than ABA in terms of eliciting proline and PEPCase accumulation (Thomas, *et al.*, 1992b). It has been proposed that cytokinins regulate PEPCase gene expression by inhibiting its salt induced transcription (Schmitt and Piepenbrock, 1992a), however this is inconsistent with the previous data, that shows that cytokinins promote *M. crystallinum* salt responses (Thomas, *et al.*, 1992b). They concluded that cytokinins and NaCl are independent initiators of a pathway that leads to an increase in PEPCase gene expression and that salt-induced accumulation of proline and PEPCase is coincident with , but not attributable to, the increase in endogenous ABA and zeatin concentrations. Thus environmental stress and PGR's are distinct signals, that lead to overlapping responses (Thomas and Bohnert, 1993). Light and temperature have been shown to moderate the induction of PEPCase (a CAM marker) by NaCl or ABA (McElwain, et al., 1992). Thus gene expression in *M. crystallinum* is effected by a combination of several environmental stimuli. This is consistent with the hypothesis that molecular responses to stress in *M. crystallinum* are triggered by multiple signals (Vernon, *et al.*, 1993) and that parallel response pathways and gene-regulatory mechanisms exist, which allow specific genes or subsets of genes to be up regulated in response to a range of environmental conditions.

1.5.3.8 Abscisic acid regulated gene expression

Abscisic acid is involved in the regulation of seed development. ABA accumulates during the late stages of embryogenesis concomitantly with an increase in fresh weight. It is implicated in the maintenance of embryogenesis and the prevention of precocious germination. When seeds begin to desiccate and maturation has been reached, the levels of ABA decrease (Goldberg, *et al.*, 1989). As ABA levels rise there is an accumulation of embryo-specific mRNAs and proteins, such as Lea (late embryogenesis abundant) mRNAs.

Lea proteins have been well characterised from cotton (Baker, *et al.*, 1988). They can be separated into six groups according to their protein sequences, D-7, D-11, D-19, D-29, D-34 and D-113. Five are hydrophilic proteins, four have no cysteine or tryptophan residues and four have domains that give rise to amphiphilic helical structures. Other *lea* and *lea*-like proteins and/or genes have been isolated from a number of different plants, including *Brassica napus* (Harada, *et al.*, 1989), barley (Hong, *et al.*, 1988), rice (Mundy and Chua, 1988), tomato (Cohen and Bray, 1990), maize (Gómez, *et al.*, 1988; Vilardell, *et al.*, 1990) and wheat (Litts, *et al.*, 1987; Williamson, et al., 1985). Desiccation of plant seeds is characterised by the accumulation of these Lea proteins and mRNAs , but treatment with exogenous ABA can induce their expression at earlier and later stages of seed development (Dure III, *et al.*, 1989). These embryo-specific proteins and mRNAs can also be induced in non-embryonic, vegetative tissue on exposure to ABA and/or environmental stress.

Drought stressed leaves of tomato (*Lycopersicon esculentum*) show many changes in their polypeptide population (Bray, 1988). Three cDNAs pLe25, pLe16 and pLe4 have different expression patterns (Cohen and Bray, 1990). All were first isolated from wilted or ABA-treated tomato leaves (Cohen and Bray, 1990), but corresponding mRNA was also found to accumulate in developing seeds (Cohen, *et al.*, 1991). pLe25 was more greatly expressed in seeds than vegetative tissue and the predicted protein sequence was found to have 50% identity with the cotton Lea D-113 (Cohen and Bray, 1992). pLe4 and pLe16 mRNAs were accumulated to higher levels in drought-stressed vegetative than developing seeds (Cohen, *et al.*, 1991; Plant, *et al.*, 1991). pLe16 mRNA could also be induced in leaf by PEG-induced water deficit, salt, cold and heat stress (Plant, *et al.*, 1991). In *B. napus* the Lea-like mRNA can accumulate in embryos at all times of seed development with ABA treatment, but is also present at high levels in water stressed seedlings (Harada, *et al.*, 1989). The *rab*-16 homologues (A, B, C and D) are all induced by ABA and osmotic stress in vegetative tissue, but *rab*-16-D is not expressed in embryos like the other three (Yamaguchi-Shinozaki, *et al.*, 1990). In wheat the E_m polypeptide is very abundant in dry embryos. The expression of its gene can be induced early in developing embryos by ABA and also by osmotic stress in the absence of an increase in endogenous ABA levels. E_m mRNA also accumulates in osmotically stressed germinating seeds. Precocious germination, which is inhibited by ABA in developing embryos, is also prevented by osmotic stress (Morris, *et al.*, 1990). But under ABA treatment alone the expression of E_m is inefficient in vegetative tissue, requiring high concentrations and long exposures to ABA. An ABA-inducible mRNA that accumulates in epidermal cells during late embryogenesis can also be induced by water stress and wounding in leaves (Gómez, *et al.*, 1988). However there are additional *lea*-like genes which are only expressed in vegetative tissue in response to ABA or environmental stress and not in embryonic tissue, such as *TAS*14 from tomato (Godoy, *et al.*, 1990) and the dehydrins from barley and maize seedlings (Close, *et al.*, 1989). Thus there appears to be a group of genes that are regulated by increased levels

of endogenous ABA, whether it be during embryogenesis or due to environmental stresses. ABA is accumulated during drought (Bray, 1988), salt (Henson, 1984) and low temperature stress (Daie and Campbell, 1981).

In tomato an ABA-deficient mutant *flacca* (*Lycopersicon esculentum* Mill c.v. Ailsa Craig) accumulates 50% less ABA than the wild type during drought stress. Wild type tomato (*L. esculentum*) produces drought stress-induced polypeptides which in *flacca* are either not detected or are synthesised to a lower level. However exogenous ABA treatment on *flacca* leaves results in the accumulation of these polypeptides, thus showing that these particular polypeptides require ABA for their expression (Bray, 1988). Three sets of polypeptides were identified in drought stressed tomato leaves according to their rate of expression. One set increased rapidly, another showed a slow, transient increase and a third decreased. On rehydration there was an exact reverse in their expression pattern. ABA levels increased with drought stress and showed an equivalent decrease on rehydration. Both these changes correlated with those observed in gene expression (Bray, 1990). A similar correlation was observed in maize leaves, on dehydration maximum mRNA levels of an ABA induced embryo gene are accumulated as maximum ABA levels are reached (Gómez, *et al.*, 1988). Bray concludes that these ABA-regulated genes may not all respond in the same way. Some may require more than just elevated ABA levels for accumulation of their mRNAs and proteins, such as additional signals or controls, since different patterns of expression by these genes were observed (Bray, 1991). Further evidence for there being more than one response pathway comes from work performed by Finkelstein *et al.* (1990). ABA responses were studied in three *Arabidopsis* ABA-insensitive mutants. Two, *abi*1 and *abi*2, showed decreased sensitivity to ABA for inhibition of seedling growth, induction of proline accumulation and alterations of protein synthesis during vegetative growth, but there was no effect on their seed storage reserves. The third mutant *abi*3 had wild type sensitivity for induction of proline accumulation, with a slight decrease in sensitivity for inhibition of growth and protein synthesis. However the major effects of this mutant were on seed development, it could only accumulate two thirds of wild type storage protein levels and one third of eicosenoic acid (the major fatty acid component) (Finkelstien and Somerville, 1990). It was concluded that there was more than one pathway involved in the response of plants to ABA, but that they probably worked in parallel.

It has been suggested that the induction of gene expression by osmotic or drought stress is consistent with the role that plants are using ABA-regulated gene products, that confer the desiccation tolerance of cereal embryos, for the "programmed desiccation that occurs during maturation" (Morris, *et al.*, 1990). However this may only be a general role for these genes and may be in addition to the specific changes in gene expression that occur when a plant is subjected to a particular environmental stress. The expression of the four ABA-regulated *le* genes (*le*4, *le*16, *le*20 and *le*25) from tomato was studied in *L. pennelli* (a drought-resistant relative of tomato). All four genes were found in the genome of *L.*

pennelli and their expression induced by water deficit (Kahn, *et al.*, 1993). However the accumulation of the *le* mRNA occurred after much longer periods of drought in *L. pennelli* than tomato, and the endogenous ABA levels that increased in *L. pennelli* with water deficit, did so to much lower levels. In tomato and the F_1 of the two species, all four genes were induced by exogenous ABA. Only three, *le*16, *le*20 and *le*25 responded to similar treatment in *L. pennelli*. Thus it was concluded that although these genes performed similar functions in the two genotypes, they couldn't be attributed to being those responsible for the greater drought-resistance exhibited by *L. pennelli*. Therefore this particular set of genes may also be playing a role as that proposed by Morris *et al.* (1990) for the E_m gene and other ABA-regulated embryogenesis genes, as mentioned earlier. However it may be that under adverse conditions, such as environmental stresses that cause a change in turgor pressure, these normally dormant genes are switched on to enable the plant to survive the immediate "shock", and there are other genes that provide the specific tolerance, expressed simultaneously or later in the plant's response. ABA has been shown to accumulate with a decrease in turgor pressure (Henson, 1984) and is likely to play a part in the signal transduction pathway that results in the expression of embryonic/stress genes.

Other types of genes that aren't involved in embryogenesis nor have some similarities to these genes, are also regulated by ABA. Two such groups are the pathogen-related or osmotin-like genes, and these have been discussed earlier. ABA is able to induce chilling tolerance in chilling sensitive maize suspension cultures (Xin and Li, 1993b) and a set of polypeptides, that may play a role in chilling tolerance (Xin and Li, 1993a). It also regulates the expression of a low temperature-induced gene *blt*4 in barley (Hughes, *et al.*, 1992) . ABA-deficient mutants of Arabidopsis were unable to acclimate to frost, but this was reversed on the application of exogenous ABA (Heino, *et al.*, 1990). Therefore it appears that ABA has a role in low temperature acclimation in plants.

cDNAs corresponding to genes whose mRNAs accumulate in the early stages of salt stress have been isolated from wheat (Galvez, *et al.*, 1993). These ESIs (Early Salt Stress-Induced) showed a biphasic response in roots of wheat exposed to 250 mM NaCl. The initial accumulation occurred within the first few hours of shock and couldn't be induced by non-saline osmoticum, suggesting that these cDNAs encode proteins that are specific for osmotic shock. The second phase involved a constant or gradual accumulation of mRNA levels. It was suggested that this biphasic response could be regulated by ABA, because it is synthesised in roots on exposure to elevated levels of NaCl. Therefore ABA could be the signal that causes the expression of these ESIs. ABA can elicit the salt-induced gene *sal*T in rice (Claes, *et al.*, 1990), which only accumulates in sheaths and roots of mature plants and seedlings. Thus ABA may also be responsible for regulating the expression of a number of genes that are induced by environmental stresses.

ABA can induce the faculative halophyte *Mesembryanthemum crystallinum* to switch from C_3 metabolism to CAM (Chu, *et al.*, 1990). When activities of the CAM-specific

enzymes PEP carboxylase (PEPC) and NADP-malic enzyme (NADP-ME) were measured in the presence of ABA, NaCl and PEG, ABA caused equivalent increases in the activities of the enzymes as those by NaCl and PEG. The ABA effect was dependent upon temperature and light intensity, being more effective in the presence of high intensity light in inducing CAM (McElwain, *et al.*, 1992). Although ABA levels increase eight to ten fold in salt stressed leaves of *M. crystallinum*, inhibition of this accumulation didn't effect the normal salt-induced responses (for example the induction of PEPcase and proline accumulation) (Thomas, *et al.*, 1992b). Thus since ABA is not necessary for PEPcase and proline accumulation, then the observed increase in ABA after NaCl treatment is likely not to be the primary salt stress response (Thomas, *et al.*, 1992b). Further studies showed that ABA, when compared to NaCl and cytokinins, was not as efficient in inducing the accumulation of PEPcase, proline, pinitol and an osmotin like protein (Thomas and Bohnert, 1993). Therefore it appears that in this facultative halophyte, ABA is playing some other kind of role in the salt response, rather than one that is directly involved in the switch from expression of enzymes required for C_3 to those required for CAM. ABA can induce the accumulation of proline in barley leaf fragments (Pesci and Reggiani, 1992), which can be enhanced by light (Pesci, 1992). This enhancement is mimicked by glucose (Pesci, 1993). Therefore ABA has the ability to regulate other factors involved in osmotic stress, not just the embryonic proteins, eliciting entire responses as demonstrated in *M. crystallinum*.

The regulation of some of the embryogenesis ABA-responsive genes, has been proposed to be at the transcriptional level. The expression of E_m was first thought to be controlled at the transcriptional level or mRNA or protein stability (Williamson, *et al.*, 1985). However later experiments showed that in the presence of a transcriptional inhibitor (α-amantin) there was still mRNA accumulation with ABA. Thus it was concluded that post-transcriptional regulation was probably taking place (Williamson and Quatrano, 1988). Mundy and Chua (1988) proposed that preformed nuclear and/or cytosolic factors were involved in the regulation of *rab*16 (formally designated *rab*21), as no protein synthesis was required for accumulation of *rab*16 mRNA. Consensus sequences have been found in the 5' regulatory regions of both these genes (Marcotte Jr., *et al.*, 1989; Mundy, *et al.*, 1990) and designated ABREs (ABA responsive elements). Marcotte *et al.* (1989) proposed that a fragment (+6 bp to +86 bp) from the 5' untranslated region may have a role in quantitatively increasing the ABA response, by stabilising or increasing the efficiency of translation of mRNA, which is consistent with the proposed post transcriptional regulation (Williamson and Quatrano, 1988). However, in addition transient assays with the E_m gene identified a 260 bp (-168 bp to +92 bp) fragment responsible for a 15 to 20 fold increase in GUS expression (the fragment was ligated to the β-glucoronidase gene) in the presence of ABA (Marcotte Jr., et al., 1989). Further studies identified a 50 bp (-152 bp to -103 bp) within that region that when fused in either orientation to the 5' end of the cauliflower mosaic virus

35S promoter, caused a three fold increase in activity with ABA. The reduced response to ABA, as compared to the 260 bp fragment, can be attributed to this 50 bp region not containing the 81 bp (+6bp to +86 bp) fragment. Two sequences were found within the 50 bp fragment, Em1a at -149 bp (cACGTggcgC) and Em2 at -125 bp (CGAGCAG). Another element Em1b was found at - 94 bp (Marcotte Jr., *et al.*, 1989). Em2 is similar to sequences found in the triticin gene from wheat. Em1a and 1b are similar to motif I found in the 5' regulatory region of *rab*16 (Mundy, *et al.*, 1990),which has the sequence RTACGTGGR (R = purine). Another GC-rich element, motif II was found upstream of *rab*16, it has the sequence CGSCGCGCT (S = G/C). This motif occurs in rab16 where it is contained within genes as part of sequences that have similarity to the mammalian transcription factor SP1 binding site (Mundy, *et al.*, 1990). The number of copies of this motif differs between the homologues of this gene. Two copies are found in *rab*16-A and only one in *rab*16-B, *rab*16-C and *rab*16-D. A *cis*-acting regulatory element CCACGTGG has been shown to be involved in the ABA and water-stress responses of a maize gene *rab*28 (Pla, *et al.*, 1993). Regulatory nuclear factors were shown to have binding affinity for this sequence and so it was proposed that this ABRE sequence may be involved in the control of ABA action during embryo development and in other tissues under osmotic stress.

A gene for a DNA binding protein EmBP-1 has been isolated from wheat (Guiltinan, *et al.*, 1990), that interacts with the 8 bp ABRE, CACGTGGC (Em1a). A 2 bp mutation in this sequence (C<u>C</u>CG<u>G</u>GGC) inhibited binding and caused a reduction in the ability of the sequence to confer ABA-responsiveness on the CaMV 35S promoter. Mundy *et al.* (1990) also found that protein factors bind at motifs I and II from gel retardation and DNAse 1 experiments. Since similar sequences have been identified in the *lea* gene family, there may be other factors that play the same role as EmBP-1 in the transcription of these genes. They may form a conserved family of protein factors, able to recognise sequences in the regulatory regions of a number of genes involved in different responses (Guiltinan, *et al.*, 1990). However ABREs are not found in every ABA-regulated gene, therefore the non-*lea*-like genes, that are also regulated by ABA, may be subject to different control mechanisms.

1.6 Aims

The aims of this project were to investigate aspects of the molecular basis of salt tolerance in *Distichlis spicata*. This initially involved the characterisation of eight cDNA clones, which were either NaCl-induced or repressed. These were previously isolated by differential screening of a cDNA library from poly (A^+) RNA, prepared from cells of *D. spicata*, grown in the presence of 260 mM NaCl (Zhao, *et al.*, 1989). It was hoped that the sequencing of these clones would lead to the identification of genes, which may have a role in salt tolerance and their function be investigated further. It was also considered important to invesitagte the regulation of these genes by the identification and characterisation of regulatory elements.

ABA has been implicated in regulation of the salt stress response in plants. It is accumulated in the salt stressed plant cells and has been shown to cause the induction of NaCl-regulated genes or enhance their expression. Therefore the molecular mechanisms of induction of these NaCl-responsive sequences were studied to investigate a possible regulatory role for ABA, similar to that seen other plants. This was initially approached by northern analysis of abundance of mRNAs corresponding to the inserts of the clones, in response to NaCl and/or ABA.

Proline is accumulated in cells of *D. spicata* within eight hours of exposure to 260 mM NaCl and this NaCl-induced proline biosynthesis is inhibited in the presence of exogenous proline (Heyser, *et al.*, 1989a). Therefore northern analyses were carried out to see if there was a similar decrease in transcript abundance corresponding to the cDNA clones, in response to exogenous proline, that would indicate a possible function in proline biosynthesis or its regulation. Proline is synthesised in plants from glutamate via pyrroline-5-carboxylate , but this has yet to be established in *D. spicata*. Therefore an attempt was made to isolate a *D. spicata* homologue of the gene that encodes pyrroline-5-carboxylate reductase, the enzyme that catalyses the final step in proline synthesis from glutamate. This was carried out by heterologous probing of Southern blots, of genomic DNA, using a soyabean cDNA clone pProCI (Delauney and Verma, 1990) and using this clone and the amino acid sequences from yeast and *E. coli*, to design primers for PCR.

Chapter 2

Materials and Methods

2.1 Materials

2.1.1 *Distichlis spicata* cell cultures and growth media

Cell suspension cultures of *Distichlis spicata* were obtained from J.W. Heyser (Los Alamos National Laboratory, Los Alamos, New Mexico 87545, USA). Cultures (20 ml) were maintained in the dark, in 100 ml baffled Duran flasks, in a modified Linsmaier and Skoog media (Heyser, *et al.*, 1986; Linsmaier and Skoog, 1965; Warren and Gould, 1982). Cultures were grown on a gyratory incubator (Infors-AG) at 120 rpm and 30 °C. Cells were transferred to fresh media every seven days; 10 ml of culture were added to 10 ml of media. Growth was measured using the visual scale of Hooker and Nabors (Hooker and Narbors, 1977). Callus cultures were maintained on solid media (containing 0.4% 'Phytagel') and transferred every fourteen days. All of the data were obtained from cultures initiated from callus and then taken through one transfer to fresh media.

2.1.2 *E. coli* strains and growth media

The *E. coli* (K12) strains used were 'Sure': e14-(*mcrA*), D(*mcrBC-hsdRMS-mrr*)*171*, *endA1*, *supE44*, *thi-1*, l-*gyrA96*, *relA1*, l*ac*, *recB*, *recJ*, *sbcC*, *umuC*::Tn5 (*kan*^{r}), *uvrC*, [F', *proAB*, *lac*^{q}Z M15, Tn10, (*tet*^{r})]; XL1-Blue: F'::Tn10. (*tet*^{r}), *proA*$^+$*B*$^+$, *lac*^{q}, D(*lacZ*), M15/*recA1*, *endA1*, *gyrA96* (*Nal*^{r}), *thi-1*, *hsdR17*(r_k-m_k+), *supE44*, *relA1*, *lac* ; JM109: F' *tra*D36, *lac*^{q}D(*lac*Z)M15, *pro*AB/*rec*AI, *end*A1, *gyrA96*(*Nal*^{r}), *thi-1*, *hsdR17*(r_k-m_k+), *supE44*, e14-(*mcr*A-), *rel*A1, D(*lac-pro*AB). Sure and XL1-Blue were obtained from Strategene Ltd, Cambridge, UK and JM109 from Promega Ltd, Enterprise Rd, Southampton, UK. *E. coli* strains were grown in Luria-Bertani(LB) medium (Sambrook, et al., 1989).

2.1.3 Plasmids

The following commercially supplied plasmids were used: pBluescript KS, pGEM3z and pGEMT. pBluescript KS was obtained from Strategene Ltd, Cambridge, UK, and pGEM3z and pGEMT from Promega Ltd, Enterprise Rd., Southampton, UK. Other plasmids used during the course of this research were pProC1 ((Delauney and Verma, 1990); an 1100 bp soyabean P5CR proline cDNA clone, in pBluescript™) was obtained with great thanks from Dr D.P.S. Verma; pDZ2.8L, pDZ2.8S, pDZ2.11.5, pDZ6.2, pDZVIII1.2.1, pDZVIII5.1.1, pDZVIII6.1.6, and pDZXI3.1 ((Zhao, et al., 1989); all cDNA clones in pBluescript from a *Distichlis spicata* λgt10 cDNA library) were obtained with much appreciation from Dr J.W. Heyser, Los Alamos National Laboratory, New Mexico, USA and Dr. H.J. Bohnert, Departments of Biochemistry and Molecular Biology, University of Arizona, Tucson, USA.

2.1.4 Chemicals, reagents and other consumables

General laboratory chemicals were obtained from Sigma Chemical Co., Poole, Dorset and BDH (NELS), Newton Aycliffe, CO. Durham. Other chemicals and reagents are as below:
Restriction enzymes, DNA modification enzymes, Taq Polymerase and Deoxynucleotide triphosphates; Boehringer Mannheim UK, Lewes, Sussex, UK.
Radiochemicals and hybridisation membranes ('Hybond N'); Amersham International Ltd, Bucks., UK.
3MM chromatography paper and glass fibre filter discs (GF/F, 2.5cm): Whatman Ltd, Maidstone, Kent, UK.
Electrophoresis grade agarose: GIBCO BRL Ltd, Paisley, Scotland.
Bacto-Agar; Difco, Detroit, Michigan, USA.
Yeast Extract, Trypticase peptone (tryptone); Becton Dickinson, F-38240, Maylan, France.
Fuji RX X-ray film; Fuji Photo Film, Co. Ltd, Japan.
Phenol (redistilled); International Biotechnologies Inc., Newhaven, Connecticut, USA.
IPTG, Xgal; Northumbria Biologicals Ltd, Cramlington, Co. Durham, UK.
Scintillation fluid (Ecoscint A); National Diagnostics, Mannville, New Jersey, USA.
MEGAscript™ *in vitro* translation kit; Ambion c/o AMS Biotechnology UK Ltd, 5, Thorney Leys Park, Witney, Oxon., OX8 7GE, UK.
Silica fines ('Finebind'); Amersham International Ltd, Bucks., UK.
Other commercially supplied consumables and equipment are acknowledged at the first reference to use.

The water used in growth media and for work with DNA and RNA was double-deionised (MilliQ - 17-18 MΩ cm^{-1} resistivity; Millipore, Watford, UK). Water used for RNA manipulations was further treated, to denature RNAses, by the addition of diethylpyrocarbonate (0.1%v/v), incubation at 25 °C for 16 h, followed by autoclaving (15 psi, 20 min).

2.2 Buffers

2.2.1 Buffers used in DNA and RNA manipulation

Restriction enzyme and DNA modification enzyme reaction buffers were supplied by the manufacturers with the enzymes. Those buffers not described in individual protocols were as specified by Sambrook *et al.*(Sambrook, *et al.*, 1989).

2.2.2 NaI Solution

NaI (90.8 g) and Na_2SO_3 (1.5 g) were added to 100 ml distilled water (final volume is greater than 100 ml). The solution was filter sterilised and a further 0.5 g Na_2SO_3 added (the final solution should be saturated). The resulting solution was stored in the dark at 4 °C.

2.3 Methods

2.3.1 General molecular biology methods

Those methods not described in detail in this section were performed as specified by (Sambrook, *et al.*, 1989).

2.3.1.1 Preparation of genomic DNA from *D. spicata*

(modified from (Murray and Thompson, 1980))

Fresh callus culture, (3 to 4 g) frozen in liquid nitrogen, was ground to a fine powder in liquid nitrogen with a mortar and pestle. 2.5 ml 2X cTAB buffer / g fresh weight (100 mM Tris-HCl pH 8.0, 2% (w/v) cTAB, 700 mM NaCl, 20 mM EDTA pH 8.0, 1% (v/v) β-mercaptoethanol, 1% (w/v) Sodium bisulphite) were added to the powder and ground again after the mixture had thawed. When it was ground to a fine powder, it was transferred to a 50 ml polypropylene tube and incubated at 65 °C for 30 to 35 min, being mixed occasionally. After incubation the tube was allowed to cool to room temperature and then an equal volume of chloroform/octanol (24:1 v/v) added, mixed by inversion for 5 min and centrifuged 900 x g, 10 min (MSE Centaur). The upper aqueous layer was removed to a clean tube, and an equal volume of isopropanol added to precipitate DNA. The tube was rocked gently until the DNA appeared. Using a glass pasteur pipette, the DNA was spooled out and, transferred to a 1.5 ml Eppendorf tube containing 76% Ethanol/0.2 M sodium acetate, where it was incubated for 20 min. After this the DNA was transferred to another tube containing 76% Ethanol/10 mM ammonium acetate and left for 2 min. Finally the DNA was transferred to a tube containing 0.3 - 1.0 ml TE (pressing against the side of the old tube to remove the ethanol), and placed at 4 °C overnight to ensure total resuspension.

In cases where greater purity was required CsCl and ethidium bromide (10 mg/ml stock) were added at a concentration of 0.94 g/g solution and 30 ml/g solution respectively and the solution transferred to 1/2 x 2 inch quick seal centrifuge tubes, which were heat sealed. The tubes were centrifuged at 230,000 x g for 16 h using a Sorval OTD65B Ultracentrifuge. DNA was recovered as in 2.3.1.4.

2.3.1.2 Exposure of *D. spicata* cells to NaCl, proline and ABA

Cell cultures of *D. spicata* were exposed to different treatments three days after their first transfer, following initiation from callus cultures. NaCl was added to final concentrations of 260, 520, and 780 mM; L-Proline to a final concentration of 5 mM and ABA to 100 μM. All solutions of these compounds were prepared in culture media. Equivalent volumes of media were added to other cultures as zero controls. Cells were exposed to treatments for 24 hours, after which they were harvested by collection through filter paper supported on a Buchner funnel and frozen immediately in liquid nitrogen. Cells were stored in polypropylene tubes at -80 °C.

2.3.1.3 Preparation of RNA from *D. spicata*

(modified from (Logemann, *et al.*, 1987).)

Frozen suspension culture cells of *D spicata* were ground to a fine powder in liquid nitrogen with a mortar and pestle, that had been ethanol flamed and pre-cooled with liquid nitrogen, and left to thaw. On thawing, 2 ml freshly prepared guanidine extraction buffer [5 X MES/EDTA (100 mM MES, 100 mM EDTA, pH 7.0), 8 M Guanidine hydrochloride, 50 mM β-mercaptoethanol] were added to the powder and ground thoroughly. 1 ml each of chloroform:isoamyl alcohol (24:1 v/v) and phenol (TE saturated) was added and ground quickly to mix. The mixture was transferred to a 15 ml corex centrifuge tube, mixed using a vortex-mixer for 10 seconds and then centrifuged at 3200 x g for 30 min (Beckman J2-21, JS13.1 rotor) at 15 °C. The upper aqueous layer was collected, extracted again with phenol and chloroform/isoamylalcohol, and centrifuged at 3200 x g for 10 min at 15 °C. Total nucleic acids were precipitated by adding 0.4 ml acetic acid and 3 ml -20 °C 100% ethanol to the aqueous layer and incubated at -80 °C and -20 °C for 30 min each. The nucleic acids were pelleted by centrifugation, at 9000 x g for 30 min and washed with 70% ethanol (in DEPC-treated water). The pellet was air-dried over sterile tissue paper for 15 min, resuspended in 400 µl sterile DEPC-treated MilliQ water and incubated on ice for 10 min. The nucleic acid solution was transferred to a 1.5 ml Eppendorf tube, and the corex tube washed out with a further 100 µl MilliQ water to remove any remaining nucleic acids and added to the Eppendorf. Debris was removed by centrifuging in a microfuge 11,600 x g, 5 min at 4 °C. 470 µl supernatant were removed to a fresh Eppendorf tube and 117.5 µl 10 M LiCl (final concentration 2.5 M) added slowly, gently swirling to prevent precipitation of the LiCl. The tube was mixed by gentle inversion and incubated on ice, in a covered box at 4 °C, overnight to precipitate RNA. RNA was pelleted by centrifugation, 11,600 x g, 15 min at 4 °C and washed with 200 µl 3 M sodium acetate (pH 5.2) and 200 µl 70% ethanol. The pellet was dried under vacuum for 2 min, resuspended in 100 µl DEPC MilliQ water and incubated on ice for 30 min. Debris was removed by centrifugation 11,600 x g, 5 min, the supernatant was transferred to a clean Eppendorf tube and 2 µl removed to make a 1/250 dilution for estimation of RNA concentration.

2.3.1.4 Plasmid mini-preps from *E. coli* by alkaline lysis

(modified from (Birboim and Doly, 1979))

Escherichia coli cultures (5 ml) were grown in LB broth for 16 h at 37 °C with appropriate antibiotic selection (dependent on the plasmid concerned). An aliquot (1.5 ml) was removed to an Eppendorf microfuge tube and the cells harvested by centrifugation 11,600 x g, 1 min. The supernatant was then removed. The cells were resuspended in 100 µl of an ice cold solution of 50 mM glucose, 10 mM EDTA, 25 mM Tris-HCl (pH 8.0). After a 5 min incubation at room temperature, 200 µl of a solution of 0.2 N NaOH, 1 % (w/v) SDS were added and the contents of the tube were mixed by inversion and incubated on ice for 10 min. Ice cold potassium acetate solution (150 µl) (5 M potassium acetate 60ml, glacial acetic acid 11.5 ml, H_2O 28.5 ml) was then added and the tube was incubated on ice for a further 10 min. After centrifugation at 11, 600 x g for 5 min, the supernatant was removed to a fresh tube, RNAse A was added to a final concentration of 20 mg/ml and the tube was incubated at 37 °C for 30 min. Phenol/chloroform (500 µl) (1 : 1) was then added, the contents mixed, centrifuged at high speed and the aqueous phase transferred to a fresh tube. Two and a half volumes of 100% ethanol were added and the tube was incubated at -80 °C for 10 min. Plasmid DNA was rescued by centrifugation at 11, 600 x g for 5 min. The pellet was then washed in ice cold 70% ethanol and dried briefly under vacuum. The plasmid DNA was resuspended in 16 µl of water. A 4 µl aliquot of 4 M NaCl was added, followed by 20 µl of 13% polyethylene glycol, and the tube was incubated on ice for 30 min. The purified plasmid DNA was recovered by centrifugation, washed using 70% ethanol, dried under vacuum and resuspended in 20 µl of water.

2.3.1.5 Plasmid maxi-preps from *E. coli*

Large-scale preparations of plasmid DNA from *E. coli* were performed essentially as a scale up of steps 1 to 10 of the mini-prep protocol as given in section 2.3.1.4. Larger cultures (500 ml) were used and the cells were collected by centrifugation at 1000 x g; Beckman J2-21 centrifuge. Further centrifugation steps were performed using 30 ml glass corex tubes at 12, 000 x g using a Beckman JA-20 rotor. When greater purity of plasmid DNA was required, the following procedure was included: The dried DNA pellet was resuspended in 8 ml of TE buffer (pH 8.0) and 8.6 g of CsCl was added followed by 0.45 ml of a 10 mg/ml solution of ethidium bromide. The solution was placed in two 1/2 x 2 inch quick-seal centrifuge tubes which were then heat sealed. The tubes were then centrifuged at 230,000 x g for 16 h using a Sorval OTD65B ultracentrifuge. The nucleic acid bands were visualised under UV illumination and the supercoiled plasmid band removed using a 10 ml syringe. The ethidium bromide was removed by extraction with

isoamyl alcohol saturated with CsCl, and salts removed by dialysis against TE buffer (pH 8.0). The plasmid DNA was precipitated using 2.5 volumes of 100 % ethanol at -20 °C.

2.3.1.6 Growth and titration of R408 helper phage

The R408 phage stock (Strategene Ltd) was diluted 100 fold with Bbroth (per litre: 10 g tryptone, 8 g NaCl, 10 ml 20% (w/v) glucose). Five further 1/100 X dilutions were made of the diluted phage stock and 0.1 ml aliquots of each added to 0.2 ml XL1-Blue *E. coli* cells [10 ml culture in TYP broth (per litre: 16 g tryptone, 16 g yeast extract, 5 g NaCl, and 2.5 g K_2HPO_4), inoculated with a 1.0 ml aliquot of a 5 ml overnight culture grown in LB broth, supplemented with 5 µg/ml tetracycline]. These were incubated at 37 °C for 60 min, after which they were left to stand at room temperature for 5 min. A 4 ml aliquot of B top agar (as for B broth, with 6 g agar /l) at 45 °C was added and the cell/phage mixture was poured onto B plates (as for B broth with 15 g agar /l), which were incubated at 37 °C overnight. A single plaque was picked and used to inoculate 50 ml TYP broth, which was incubated at 37 °C for 8 - 12 hours. The cells were pelleted by centrifugation at 12,000 x g for 15 min, the supernatant collected and centrifuged for a further 15 min. The supernatant was transferred to a sterile polyprolylene tube and heated at 55 °C for 30 min to kill any remaining bacterial cells. The phage were stored at 4 °C.

The supernatant was titred by making a number of 1/100 X serial dilutions in B broth, which were treated and plated out as before. The number of plaques were counted on each plate and the number of plaque forming units/ml, calculated.

2.3.1.7 Preparation of single stranded DNA

XL1-Blue *E. coli* cells, containing the plasmid pBluescript (KS+ or KS-) vector, were grown at 37 °C overnight in LB broth supplemented with the appropriate antibiotic. An aliquot (3 ml) of 2x TY (per litre: 16 g tryptone, 10 g yeast extract, 10 g NaCl, pH 7.2 - 7.4) was inoculated with 300 µl of the overnight culture (approximately 7.5 x 10^8 cells). Cells were grown at 37 °C in a sterile 50 ml conical flask. The helper phage was added to the culture during log phase (OD_{600} = 0.3 using a Titertek Multiskan® MCC plate reader) at multiplicity of infection of 200:1 (phage:bacterial cells) and incubated at 37 °C for 8 hours. Phage and bacteria were transferred to 1.5 ml Eppendorf tubes, heat shocked at 55 °C for 15 min, centrifuged at 11,600 x g for 2 min and the supernatant stored at 4 °C overnight. Tubes were centrifuged again as before and the supernatant transferred to a fresh tube. A 3.5 M ammonium acetate (pH 7.5) : 20% PEG solution (1/4 volume) was added, mixed by inversion and incubated at room temperature for 15 min. The tubes were centrifuged at 11,600 x g for 20 min and the pellet resuspended in 200 µl TE buffer. Contents were extracted once with phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v) and once with

chloroform/isoamyl alcohol (24:1v/v). DNA was precipitated by the addition of 150 µl of 7.5 M ammonium acetate (pH 7.5) and 600 µl of -20 °C 100 % ethanol followed by incubation on ice for 15 min. DNA was pelleted by centrifugation at 11,600 x g for 20 min at 4 °C, washed with 80% ethanol and dried under vacuum. Single stranded DNA was resuspended in 10 µl double de-ionised water and stored at -20 °C.

2.3.1.8 Preparation and transformation of high efficiency competent *E. coli* cells

The method to prepare high efficiency competent cells was essentially as described by (Alexander, *et al.*, 1984).

Cultures (5 ml) of *Escherichia coli* cells were grown in 2 XL overnight at 30 °C. An aliquot (1 ml) was used to inoculate 100 ml 2 XL prewarmed to 30 °C in a 500 ml Erlenmeyer. This was cultured (with shaking) at 30 °C until the OD_{600} = approximately 0.2, then sterile 2 M $MgCl_2$ was added to a final concentration of 20 mM. Growth was allowed to continue until OD_{600} = 0.5 (0.45-0.55). The cells were then incubated in ice-water for 2 h. Aliquots (40-50 ml) were spun down in sterile blue-capped Falcon tubes at 900 x g for 5 min in a bench-top centrifuge (MSE Centaur) and the supernatant was aspirated. The resulting pellets were resuspended gently in one half of the original volume of ice-cold Ca/Mg medium, 100 mM $CaCl_2$, 70 mM $MnCl_2$, 40 mM NaAc, pH 5.5. This solution was prepared fresh and filter sterilised (the pH of a stock NaAc solution (pH 7), was adjusted down after the addition of the Ca^{2+} and Mn^{2+} salts). The cells were then incubated on ice for 1 h and collected by centrifugation at 900 x g in the bench top centrifuge for 5 min. The resulting pellet was resuspended (very gently) in 1/20 the original culture volume of Ca^{2+}/Mn^{2+} solution containing 15% (v/v) glycerol. Aliquots (0.2 ml) of cells were collected in 1.5 ml Eppendorfs, frozen in liquid nitrogen and stored at -80 °C until required.

The cells were thawed on ice and transformed immediately by the addition of DNA in 100 µl TE and incubation on ice for 30 min. The transformation sample was subjected to heat shock (37 °C for 5 min), diluted to 4 ml with 2 XL broth (pre-warmed to 37 °C) and grown with shaking for 1.5 h at 37 °C. The transformed cells were then plated out onto LB agar containing the desired selective agent.

2.3.1.9 Preparation and transformation of all other competent *E. coli* cells

Method from (Hanahan, 1983)

Cultures of (5 ml) of *Escherichia coli* cells were grown in LB medium overnight at 37 °C and used to inoculate 500 ml LB. This was incubated further at 37 °C until the culture reached an OD_{600} of 0.3-0.35. Cells were chilled on ice for 5 min and pelleted at 900 x g (MSE Centaur) for 7 min in 50 ml polypropylene tubes. The pellet was resuspended in 2/5 volume of TFb1 solution (3O mM KAc, 100 mM RbCl, 10 mM $CaCl_2$, 50 mM $MnCl_2$, 15% v/v glycerol pH 5.8 with acetic acid). Resuspended cells were incubated on ice for 5 min before centrifugation as described earlier. The pellet was resuspended in 1/25 volume of TFb2 solution (10 mM MOPS, 75 mM $CaCl_2$, 10 mM RbCl, 15% v/v glycerol, pH 6.5 with 10 M KOH),and incubated on ice for 15 min. 110 ml aliquots were measured into prechilled 1.5 ml Eppendorf tubes, frozen in liquid nitrogen and stored at -80 °C.

Competent cells were thawed and then incubated on ice for 10 min. Plasmid DNA was added (up to 2/5 volume of cells, no more than 55 ng/110 ml cells) and the tube was incubated on ice for 15 - 45 min. The contents were heat shocked for 90 seconds, and incubated on ice for a further 2 - 3 min. 800 ml of LB broth were added and incubated for 1 hour at 37 °C, with occasional shaking. The transformants were plated on selective LB agar.

2.3.1.10 Agarose gel electrophoresis of DNA

Agarose gel electrophoresis was performed as described by (Sambrook, *et al.*, 1989).

Generally 0.8% (w/v) agarose gels were used, although higher concentrations up to a maximum of 2 % were also used, as specified for each experiment, where small fragments (to a minimum of 100 bp.) were to be separated. All gels were cast using Tris-borate buffer (89 mM Tris-borate, 2 mM EDTA). DNA was loaded into the wells of the gel after addition of loading dyes (0.25% w/v each of bromophenol blue and xylene cyanol, 40% w/v sucrose).

2.3.1.11 Isolation of restriction fragments from agarose gels

Gel slices containing fragments to be isolated were cut from agarose gels using a clean scalpel blade. The DNA was purified from the gel block by binding to silica fines as described below.

The required band was excised and placed in an Eppendorf tube. An aliquot (1 ml) of NaI solution was added and the tube was incubated 60 °C for 10 min. An aliquot (5 µl) of silica fines was then added, followed by incubation at room temperature for 10 min. The fines were collected by centrifugation at 11, 600 x g for 15 seconds, followed by the

aspiration the supernatant. The pellet of fines was washed in 1 ml 70% ethanol (70% ethanol: 30% TE buffer) and centrifuged for 15 seconds in a microcentrifuge. The ethanol was then aspirated and the fines were resuspended in 50 ml TE buffer. The DNA was then eluted off by incubation at 37 °C for 10 min. The fines were collected by centrifugation for at 11, 600 x g for 15 seconds and the supernatant containing the DNA was used in ligations or used to make radioactive probes.

2.3.1.12 Cloning of DNA fragments

General methods employed for cloning of DNA fragments (restriction and ligation) were as described by (Sambrook, *et al.*, 1989).

2.3.1.13 Radioactive labelling of DNA

Double stranded DNA was radioactively labelled with ^{32}P by random priming using [α-^{32}P]dCTP with Klenow polymerase, as described by (Feinberg and Vogelstein, 1983). Following the labelling reaction, unincorporated radioactivity was separated from the DNA fragments by Sephadex G-50 gel permeation chromatography using a glass pasteur pipette as a column.

2.3.1.14 Formaldehyde-agarose gel electrophoresis of RNA

Formaldehyde-agarose gels were prepared and run as described by (Sambrook, *et al.*, 1989). Ribosomal RNA bands from *D. spicata* and *Synechococcus* sp. were used as size markers for these gels. Samples of 15 µg of RNA were run per lane.

2.3.1.15 Southern and northern blotting

DNA and RNA was transferred to nylon hybridisation membranes essentially as described by (Sambrook, *et al.*, 1989). DNA was denatured prior to transfer by soaking agarose gels in an excess of denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 1 h, followed by neutralisation of the gel by soaking in an excess of neutralisation solution (1.5 M NaCl, 0.5 M Tris; pH 7.5) for 1 h. Gels were blotted for 16 h using 10X SSC, after which time complete transfer of nucleic acids had occurred. Filters were UV irradiated for 5 min to crosslink the nucleic acids and then placed under vacuum at 80 °C for 1 h prior to hybridisation of radioactive probes.

2.3.1.16 Hybridisation of radioactive DNA probes to filter-immobilised nucleic acids

All hybridisation reactions were carried in hybridisation tubes (Techne). Hybridisation of probe to northern blots was carried out at 42 °C, and to Southern blots at 65 °C. In both cases, filters were pre-hybridised for 1 h prior to the addition of probe. Solutions used for both prehybridisation and hybridisation were as described by (Sambrook, *et al.*, 1989). Hybridisations were carried out for 16 h. Filters were washed using 3 changes of 1X SSC, 0.1 % (w/v) SDS over a period of 1 h, after which the radioactive filter was placed on 3MM paper, orientated by the addition of several spots of radioactive ink, and exposed to X-ray film. Film cartridges were maintained at -80 °C for the required exposure time. After film development, the filter could be washed to a greater stringency, or completely stripped of radioactivity by incubation at 90 °C in 0.1 % (w/v) SDS which allowed the filter to be re-probed as desired.

2.3.1.17 Quantification of nucleic acids

Nucleic acid concentrations were estimated by measurement of absorbance at 260 nm using a Beckman DU7500 spectrophotometer. It was taken that an OD_{260} =1.0 was equivalent to 50 µg/ml DNA; 40 µg/ml RNA and 20 µg/ml oligonucleotide primers. The 260/280 OD ratio was taken as a measurement of purity, ideally being 1.8 - 2.2.

2.3.1.18 DNA sequencing

Direct sequencing of plasmid clones (double and single stranded DNA) was performed by the dideoxy-sequencing method of (Sanger, *et al.*, 1977), using dye-linked universal M13 primers. Sequences were analysed using an Applied Biosystems 373A sequencer.

2.3.1.19 Synthesis of DNA oligonucleotides

Oligonucleotides were synthesised using an Applied Biosystems 381A DNA synthesiser operated with a standard synthesis programme. After cleavage and deprotection, the oligonucleotides were dried under vacuum, twice resuspended in water and vacuum dried. Oligonucleotides were stored at -20 °C, either dry or as an aqueous solutions and were used without further purification.

2.3.1.20 *in vitro* transcription of pDZ6.2 and pDZVIII5.1.1

in vitro transcription reactions were performed using a MEGAscript™ T3 kit (Ambion). Template DNA was prepared by linearising the plasmids pDZ6.2 and pDZVIII5.1.1 by digestion with *Xba*I (cleaves 3' of the predicted transcript of the insert). Digestion reactions were terminated by the addition of 1/20 volume of 0.5 M EDTA, and the DNA precipitated by adding 1/10 volume 3 M sodium acetate, 2 volumes of 100% ethanol and chilling at -20 °C for 15 min. DNA was pelleted by centrifugation at 11,600 x g for 15 min, the supernatant removed by double aspiration and the pellet resuspended in double de-ionised water to a final concentration of 0.5 mg/ml. 20 µl transcription reactions were set up as follows: 7.5 mM each of ATP, CTP, TTP, GTP; 1 µg DNA, 1 µl [^{32}P] UTP; enzyme mix and buffer as in manufacturer's instructions. Each reaction was incubated at 37 °C in an oven for 4 hours. Percentage incorporation of [^{32}P] UTP into transcripts was assessed by TCA precipitation.

2.3.1.21 Determination of yield of transcription products by TCA precipitation

A 1 µl aliquot was removed from a reaction and thoroughly mixed with 400 µl of 1 mg/ml herring sperm DNA (RNAse-free). An aliquot (100 µl) of the diluted RNA sample was transferred to a 3 ml glass tube and 2 ml 10% (v/v) TCA added. The tube was mixed briefly using a vortex mixer and incubated on ice for 5 min. The precipitated RNA was collected by vacuum filtration through a glass fibre filter (prewetted with 10% TCA). The filter was rinsed successively with ice cold 10% TCA and 100% ethanol. Filters were added to scintillation fluid (Ecoscint) and counted (2000CA Tri Carb Liquid Scintillation Analyzer). An aliquot (200 µl) of the diluted RNA sample were added directly to scintillation fluid and counted, this represented total counts. The ratio of the two values represented the percentage incorporation of the radiolabelled nucleotide into transcription products. Three 1 µl aliquots were removed from each reaction, so that a mean percentage incorporation could be calculated.

2.1.3.22 Use of the polymerase chain reaction (PCR) for *in vitro* amplification of DNA

PCR reactions were carried out essentially as described by (Saiki, *et al.*, 1988). Reaction conditions were as follows: 5U Taq Polymerase; 10 µl 10 x reaction buffer; 50 µM each of dATP, dTTP, dGTP and dCTP; 1 µM each primer in a total reaction volume of 100 µl. Taq Polymerase and reaction buffer were from Boerhinger Mannheim. The amount of DNA added, varied with each experiment, and was added last. Contents were mixed, overlaid with mineral oil and reactions were carried out on a Techne PHC-3 Thermal Cycler. Details of temperatures and time settings used to control denaturing, annealing and extension, and the number of amplification cycles used, are stated for individual reactions.

2.3.1.22.1 Design of primers and conditions for the amplification of a P5CR gene homologue from *D. spicata*

PCR primers were designed, based upon the comparison of the amino acid sequences of pProC1 (soyabean cDNA clone) and the yeast P5CR gene. Inosine - containing PCR primers were synthesised corresponding to two peptide regions shown in figure 2.3.1. These peptides were selected due to their high similarity within the yeast and soyabean sequences and to maximise the proportion of the coding region of the gene homologue that would be amplified. Inosines were included at the positions where greater than two fold degeneracy occurred. The sequences of the primers are shown in section 3.1.2.

PCR was carried out as described in section 2.3.1.22, using the primers described above, and template DNA from *D.spicata*. The PCR contained primers at a concentration of 1 µM and 0.5 µg of template DNA. Reactions were subject to 29 cycles of the following series of temperatures and times: denaturation at 94 °C for 1.5 min, annealing at 48 °C for 1.5 min and elongation at 72 °C for 3 min; and one cycle (cycle 30) with the same denaturation and annealing conditions, but elongation at 72 °C for 5 min.

2.3.1.22.2 Design of primers and conditions for the amplification of GA repeat sequence from pDZ6.2

A 68 bp GA repeat was found in the 5' untranslated region of pDZ6.2, as explained in sections 3.2.4.1 and 3.5.1

The 3' PCR primer was designed from the nucleotide sequence of pDZ6.2 as in figure 2.3.2, represented as primer 3. The primer sequence is shown in section 3.5.1. This region was selected for its proximity to the GA repeat sequence and because it wasn't part of the putative open reading frame of pDZ6.2. The M13 reverse primer was used as the 5' primer.

The PCR was carried out as described in section 2.3.1.22 using the above primers and pDZ6.2 as the DNA template. The PCR contained each primer at a concentration of 1 μM and 0.5 μg of template DNA. Reactions were subject to 29 cycles of the following series of temperatures and times: denaturation at 94 °C for 1.5 min, annealing at 45 °C for 1.5 min and elongation at 72 ° C for 3 min; a 30^{th} cycle included elongation at 72 °C for 5 min (the other steps being the same).

2.3.1.22.3 APCR (Anchored PCR) strategy for the amplification of a genomic clone of pDZ6.2

The APCR strategy entailed ligating a genomic fragment onto a vector, to produce a known primer binding site at the end of the genomic fragment. This would allow subsequent amplification using "generic" primers to the vector in conjunction with three specific primers designed from pDZ6.2.

A sample (6 μg) of *D. spicata* genomic DNA was digested with *Alu*I, a four base pair cutter that doesn't cleave within the predicted coding region of pDZ6.2. This approach was taken in order to produce genomic fragments of 300 to 400 bp, containing part of the pDZ6.2 gene, including the GA repeat region. 7 μg of pBlusescript KS were digested with *Sma* I and ligated to 315 ng of the *Alu*I cut genomic DNA, a 3 : 1 molar ratio of vector to insert. After ligation 0.35 mg aliquots of the ligation mixture were used in the first APCR.

The 3' primers were designed from the nucleotide sequence of pDZ6.2 as in figure 2.3.2. Each region was chosen for its position relative to the GA repeat region, and in relation to each other, being 100 bp apart. The GC : AT ratio was also taken into consideration, looking for equal numbers of each. For primers **1** and **2** the ratio was 10 : 10. Primer **3** was that used in the amplification of the GA region in section 2.3.1.16.2. The sequences of the primers are shown in section 3.5.4.

The ligation population used as the template in the APCR contained diverse *Alu*I fragments ligated to pBluescript KS. The M13 and T7 primers would hybridise to all of these ligation products. However, only fragments containing the pDZ6.2 gene should amplify exponentially when the ligation mix was used as a template for APCR, due to the

specifically designed *D. spicata* primers. Due to the size of the genome of *D. spicata*, as compared to species used before (Robinson, *et al.*, 1990), it was considered that further specificity would be required. The nested set of primers were designed 100 bp apart, the furthest 3' primer being used first and the other two used in succession. In conjunction with the M13 and T7 primers, the smaller region being amplified in each successive reaction, should increase the specificity, such that enough product would be amplified to be detected.

2.3.1.22.4 APCR amplification conditions

APCRs were performed using conditions described in section 2.1.3.22 with some modifications. The template DNA used in the first APCR (1) was 0.35 µg of the ligation mix described in 2.1.3.16.3; the second and third successive APCRs (2 & 3) used 5 µl of the previous reaction as template DNA. 10 µg of each *D .spicata* specific primer and 0.5 µg of the M13 universal forward primer and T7 primer. Primer 1 and M13 forward primer were used in APCR 1; Primer 2 and T7 primer in APCR 2; Primer 3 and T7 in APCR 3. Reactions were subject to 29 cycles of the following series of temperatures and times: denaturing at 94 °C for 1.5 min; annealing at 55 °C for 2 min and elongation at 72 °C for 3 min. A further cycle was used with the same denaturing and annealing conditions, but elongation at 72 °C of 5 min.

2.3.2 Computer analysis of nucleotide and protein sequences

2.3.2.1 Comparison of sequences with nucleic acid and protein databases

Nucleic acid sequences were manipulated using the University of Wisconsin Genetics Computer Group (UWGCG) Sequence Analysis Software Package, (Devereux, *et al.*, 1984), mounted on the SEQNET VAX 3600 at SERC, Daresbury, U.K and then compare against the GenBank and EMBL databases using the Fasta programme (Pearson and Lipman, 1988). Open reading frames (ORF's) in the cDNA sequences were identified using DNA Strider, translated into their protein sequence and compared against the Owl database using the SEQNET programme Sweep.

2.3.2.2 Alignment of nucleotide and protein sequences

Gap alignments of nucleotide and protein sequences were performed using the UWGCG programme GAP, which uses the algorithm of Needleman and Wunsch (Needleman and Wunsch, 1970). Multiple alignments were carried out using the SEQNET programme Clustal V (Higgins and Sharp, 1988).

Figure 2.3.1

Gap alignment between yeast (1-286 aa) and soyabean (1-274 aa) P5CR sequences.

Percent Similarity: 58.015 Percent Identity: 33.588

```
                .         .         .         .         .
 Yeast   1 ........MTYTLAILGCGVMGQALLSAIYNAPKAADETAAAFYPSKIIT 42
                 .|||:::|.| |:::.: .         :.  .:.: ||:| |
  Soya   1 MEIFPIPAESYTLGFIGAGKMAESIAR........GAVRSGVLPPSRIRT 42
                .         .         .         .         .
Yeast   43 CNH.DEPSAQQVTDLVETFDESPQGIKVESTYGHQVSAVEEASVVLLGTK 91
           . | : :. .....:. |. .| :::             | |..||:|:.|
 Soya   43 AVHFNLARRGAFESFGVTVLPSNDDV.............VRESDVVVLSVK 80
                .         .         .         .         .
Yeast   92 PFLAEEVLNGVKSVIGG.KLLISLAAGWTIDQLSQYTST..VCRVMTNTP 138
           | |..:|:. :.:::.  |||:|:||| .:.:| ::.:.  ..|||.|||
 Soya   81 PQLVKDVVSKLTPLLTKHKLLVSVAAGTKLKDLQEWAGNDRFIRVMPNTP 130
                .         .         .         .         .
Yeast  139 AKYGYGCAVVSYSADVSKEQKPLVNELISQVGKYVELPEKNMDAATALVG 188
           |  | :..|:|.::....|:  ::.:|:: :||. . .|| :|| |:| |
 Soya  131 AAVGQAASVMSLGGSATEEDGNIIAQLFGSIGKIWKAEEKYFDAITGLSG 180
                .         .         .         .         .
Yeast  189 SGPAFVLLMLESLMESGLKLGIPLQESLECAMKVLEGTVKMVEKSGAHPS 238
           ||||:|.| :|.| ::|:  |:| : ||. | ..: |...||...| ||:
 Soya  181 SGPAYVYLAIEALADGGVAAGLPRDLSLSLASQTVLGAASMVSQTGKHPG 230
                .         .         .         .
Yeast  239 VLKHQVCTPGGTTIAGLCVMEEKGVKSGIINGVEEAARVASQLGQKKK 286
           ||.:|..||||||.|:  :|: |.::.::|:| .||: ..:|:
 Soya  231 QLKDDVTSPGGTTITGIHELENGGFRGTLMNAVVAAAKRSRELS.... 274
```

Figure 2.3.2

CAACAAAATCTCGCCGAGTACTGAAA**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG**

3

AGAGAGAGAGAGAGAGAGAGACGAGAGCGC**GAGCGTAGCCGACAT**TGCGAACGCGGCCTGGTG**ATG**CCGCTTC

2

TGGTGGCTCTACTTCTCGTCCTAGCCGTGGTCGCCGCCGCCGGTGCCGACCAC**TACAAACCCAAGGACCCCTA**

CCACCCGCCCAAAAAACACTACTACCCACCGAAGAAGGATCACTACTACCCACCCAAGAAAGACCATCACTAC

1

TACCCGC**CCAAGAAGGATCACCACTAT**GAGCCACCGAAGAAGCACCATGACCCGCCTAAGAAGCACCCCTACC

CGCCTAAGAAGGACCATGAGCACTGGCCGGGCAAGCCTGGCTAC**TGA**GCTGCATTCATAAGCTAAGCTGCTGG

ACCATACCACAGTATCTTTCAATAAGAGACATGCATATGCAGCCATGCATGCGATTACTACTTGTGTTGTTTA

GCTATTGGATTTATGTGTGTCGTGCTTTGTTATGGGTCCTGCTAGCTTCTTATCCCATGTGTCTGCTACCTCA

ACTTGTACTGTGTGATTACCTACCTTATG**AATAAA**ATTCATTTGTGATTGTGCTATCTAAAAAAAAAAAAAAG

GTGGCTCCCTACTCCGGCCATATGCCACTCAATATTCGGTAAACCCCCTGCGGTCTTAACATGGGATTACGAA

TTTCTCCGCATGAGGCTCAAGAATCTGTACACTGCGAAATGTGGTTATGCAAGATCTAGATTTCGGACCACCT

ATTTTTATGTGCCATTTGTTGTTTGTTCAGTTTGTTATTTTGGTAATTAGCATGTGTTGGCTGACTACTGGGC

ACTGGCCTGCTGGCCAGAAAACTGAAGGGTGGCCGGTTCAACTGACCGTCAGTTATTGGAGGGTAGATGTTTT

AGAAGTGTTTGGATGTGTAGAAATCGTGCTTTAGTGCTTTACTGAGGATGTGCATTGGATATGACTGTTCAGA

ACCAGCGGCTGGCCGAAAACTCGCGCTAGATGGACCTGAAATTGTGCTTTACCAAGTAATTTTAGGTTCACTC

AAAGTTAAATCTTTTTAACTTTTAAAAAAAAG

Chapter 3

Results

3.1 An attempt to isolate a P5CR gene homologue from *Distichlis spicata*

3.1.1 Heterologous probing of Southern blots

The first approach to attempt to identify and hence isolate a P5CR gene homologue was to use the ^{32}P radiolabelled pProCI clone from soyabean (Delauney and Verma, 1990) to probe Southern blots of *D. spicata* genomic DNA. *D. spicata* genomic DNA was digested with *Eco*RI, *Xba*I, *Hind*III, *Sal*I and *Bam*HI and the products electrophoresed on a 0.7 % agarose gel. Samples were blotted on to Hybond N and then hybridised with the labelled pProCI. After exposure to X-ray film no bands were observed (results not shown). As no suitable controls (i.e. soyabean DNA) had been included (due to unavailability) it was difficult to distinguish whether this was due to incorrect hybridisation conditions or the absence of the *D. spicata* homologue. This procedure was repeated several times, lowering the stringency of the hybridisation conditions, by decreasing the hybridisation temperature from 65 °C to 55 °C, to optimise the binding of this heterologous probe. No hybridisation occurred between the soyabean pProCI and *D. spicata* DNA, thus a different approach was necessary to attempt to isolate a P5CR gene homologue.

3.1.2 PCR

Primers were designed by comparison of known P5CR protein sequences from yeast, *E. coli* and Soya bean (see section 2.3.1.22.1). The sequences are shown below:

5' primer: 5' **GTI ATG CCI AAT/C ACI CCI GC** 3'

3' primer: 5' **AT IGT IGT ICC ICC IGG** 3'

The estimated product size was 357 bp (assuming the absence of introns in the homologus region of the *D. spicata* genome). Initially the reactions were performed with an annealing temperature (T_m) of 53 °C, this was calculated as shown below:

$$T_m = [4 (G+C) + 2(A+T)] - 3$$

Figure 3.1.1 Electrophoresis of reaction products from PCR to amplify a P5CR gene homologue from *D. spicata*. An aliquot of 50 µl (of 100 µl reaction) of each reaction was run on a 1.2 % agarose gel. Lane 1 - *D. spicata* genomic DNA; lane 2 - pProCI; lane 3 - yeast (*Saccharomyces cereviseae*) genomic DNA (60 cycles); lane 4 - yeast genomic DNA (30 cycles). The predicted band size (357 bp) is marked, as compared with the λ/*PstI* ladder.

Thus for the 5' primer T_m = [4(10) + 2(11)] - 3 = 59 °C

3' primer T_m = [4(8) + 2(9)] - 3 = 47 °C

Average T_m = 53 °C

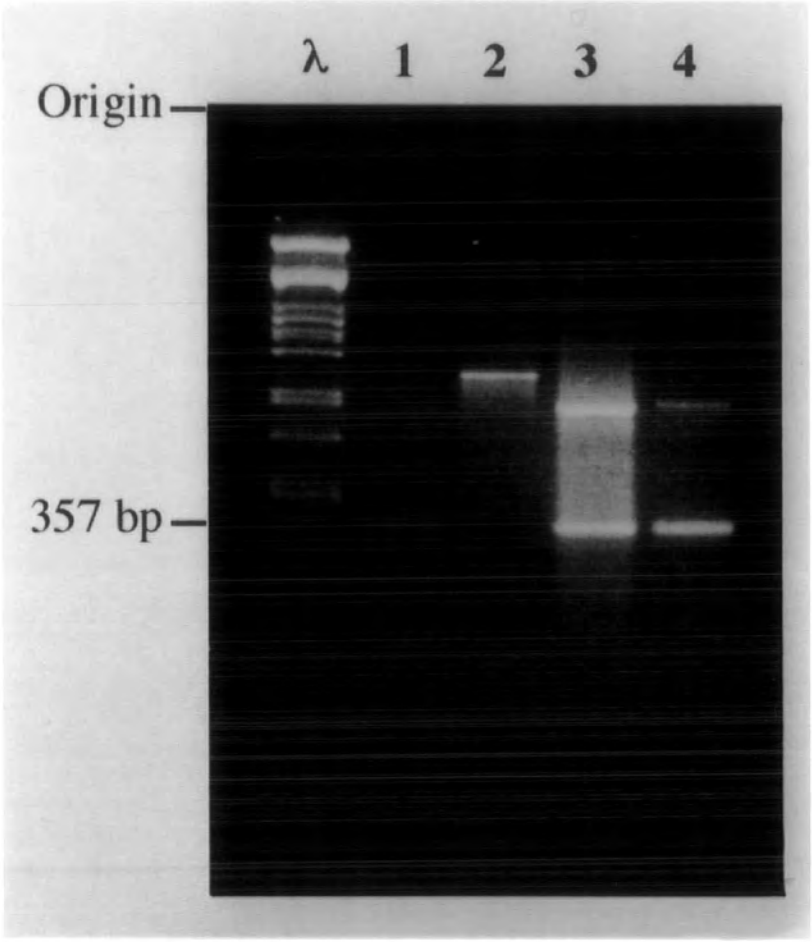
Control reactions were also performed, using yeast genomic DNA and pProCI. Products from the reactions were analysed by electrophoresis on 1 % agarose gels.

At first no bands of the predicted size were obtained from any of the reactions, so the annealing temperature was lowered to 48 °C. Figure 3.1.1 shows a photograph of PCR products. There is a very bright band in the yeast lane of the predicted size, approximately 357 bp. This was confirmed to be the P5CR gene by hybridisation with ^{32}P-labelled pProCI (results not shown). This also confirmed that the other bands present were not the desired product or at least not sufficiently similar in sequence to pProCI to cross-hybridise. The corresponding region from pProCI was not amplified. The faint bands visible in lane 1 were attributed as being contaminants due to the yeast DNA.

A magnesium chloride titration was carried out to see whether an increase in the $MgCl_2$ concentration would improve the reaction conditions enough to allow amplification of a P5CR homologue from *D. spicata* and pProCI control. Reactions were performed with the following $MgCl_2$ concentrations; 1.5, 2.5, 3.5, 4.5, 5.5, 6.5, 7.5, 8.5, and 9.5 mM (1.5 mM being the concentration present in the Taq polymerase buffer). No products of the appropriate size were obtained from either the *D. spicata* or pProCI reactions. The amount of yeast product decreased with increasing $MgCl_2$ concentration (results not shown).

3.1.3 Heterologous probing with ^{32}P-labelled yeast PCR product

The final approach was to use the yeast PCR product to probe Southern blots of *D. spicata* genomic DNA. No signal was obtained, even after exposure to X-ray film for one month. Low stringency hybridisation conditions were used (a hybridisation temperature of 55 °C and using 3X SSC) to maximise the binding of this heterologous probe (results not shown).


λ
1
2
3
4
Origin
357 bp

3.2 Sequencing the *D. spicata* NaCl-responsive cDNA clones

3.2.1 cDNA clones

Eight cDNA clones were obtained from J.W. Heyser and H. J. Bohnert. These had been isolated following a differential screen of a *Distichlis spicata* cDNA library in λgt10 and cloned into the *Eco*RI site of pBluescript™ KS (Zhao, *et al.*, 1989). Table 3.2.1 shows the information first available before the clones were sequenced; this was supplied with the clones and is given in Zhao's paper (Zhao, *et al.*, 1989).

3.2.2 Size estimation of cDNA clones

To determine the sizes of the cDNA inserts in the clones, plasmid DNA of each was digested with *Eco*RI and the products analysed by electrophoresis on a 1% agarose gel. Figure 3.2.1 shows the electrophoresed products (pDZVIII6.1.6 is not visible on the photograph, but was visible to the eye). Sizes were calculated by comparison with the known band sizes of the λ/*Pst*I ladder, using a semi log plot. Table 3.2 2 shows the estimated sizes of the inserts released from the eight cDNA clones, the sizes range from 100 bp (pDZVIII 6.1.6) to 1512 bp (pDZVIII5.1.1).

3.2.3 Restriction mapping the cDNA clones

Restriction maps of the larger clones, namely pDZVIII 5.1.1 (1512 bp), pDZ2.11.5 (1280 bp) and pDZ6.2 (1127 bp) were made for sub cloning purposes.

pDZ2.11.5 released three bands (sizes 187 bp, 405 bp and 688 bp) when digested with *Xba*I and *Sal*I. Figure 3.2 2 shows these bands electrophoresed on a 1% agarose gel. These were excised from the gel, the DNA extracted using silica fines and ligated into appropriately digested pBluescript™ KS. These sub clones were used for the sequencing of pDZ2.11.5.

Restriction maps of pDZVIII 5.1.1 and pDZ6.2 were not made via restriction, however, during the sequencing process restriction sites were identified using the DNA Strider™ programme (Marck, 1986) and these sites were subsequently used for sub cloning.

Table 3.2.1 Information known about cDNA clones

Clone	Vector	Response to elevated NaCl	Other Information
pDZVIII5.1.1	pBluescript KS	UP	-----
pDZVIII1.2.1	pBluescript KS	UP	-----
pDZV1116.1.6	pBluescript KS	UP	-----
pDZIX3.1	pBluescript KS	UP	-----
pDZ2.11.5	pBluescript KS	DOWN	-----
pDZ2.8L	pBluescript KS+	UP	Similarity to Histone 2B (No Sequence Data)
pDZ2.8S	pBluescript KS+	-----	Subclone of pDZ2.8L Unknown piece of cDNA
pDZ6.2	pBluescript KS+	UP	-----

All cDNAs cloned into the *EcoR*I site of pBluescript.

Figure 3.2.1 Electrophoresis of cDNA clones after digestion with *Eco*RI. A 1 μg aliquot of each cDNA clone was digested for 2 hours 37 °C with *Eco*RI and the products electrophoresed on a 1 % agarose gel. Lane 1 - pDZ6.2; lane 2 - pDZVIII 5.1.1; lane 3 - pDZ2.8L; lane 4 - pDZ2.11.5; lane 5 - pDZIX 3.1; lane 6 - pDZVIII 6.1.6; lane 7 - pDZVIII 1.2.1; lane 8 - pDZ2.8S. The sizes of the inserts released from each clone are marked as base pairs. Sizes were estimated by comparison with the λ/*Pst*I ladder, using a semi log plot.

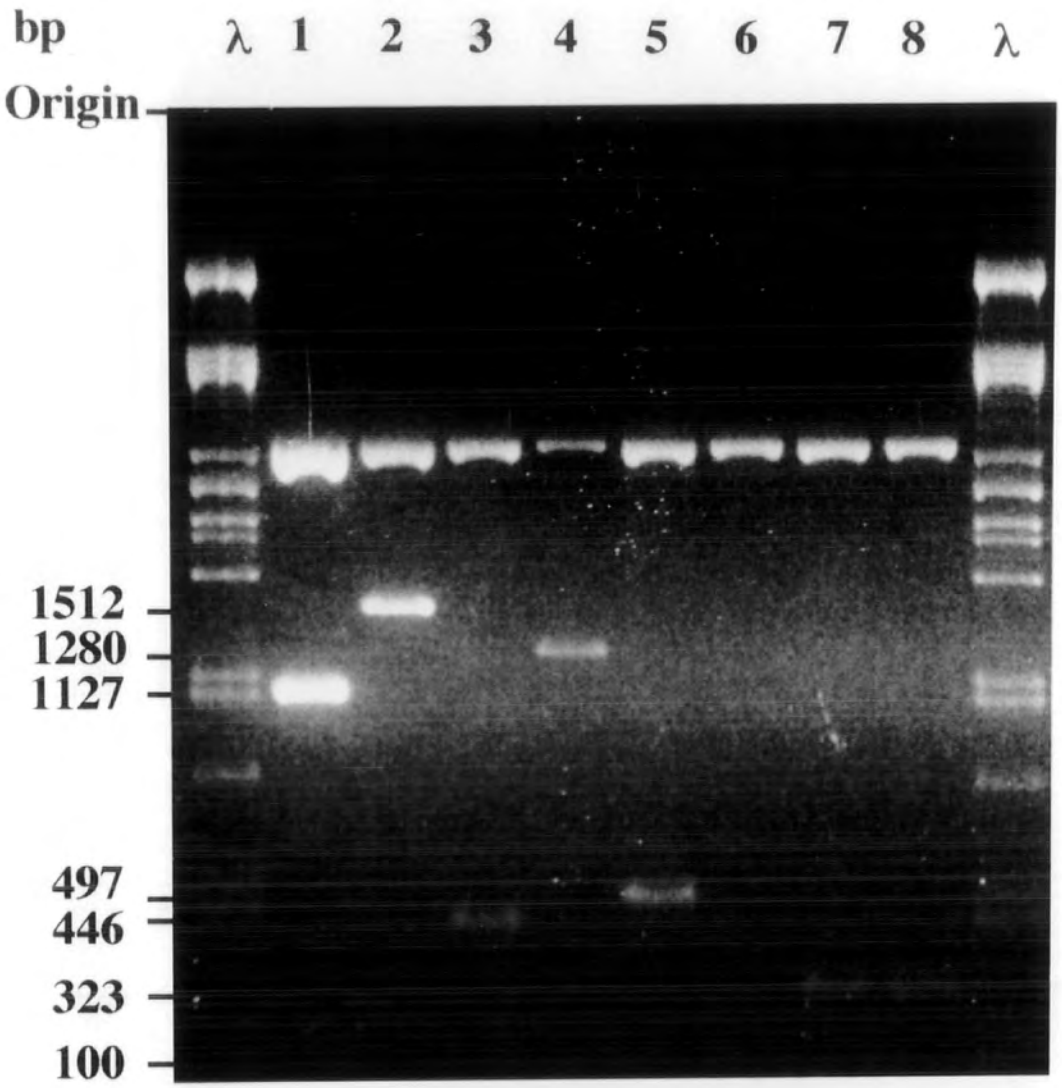
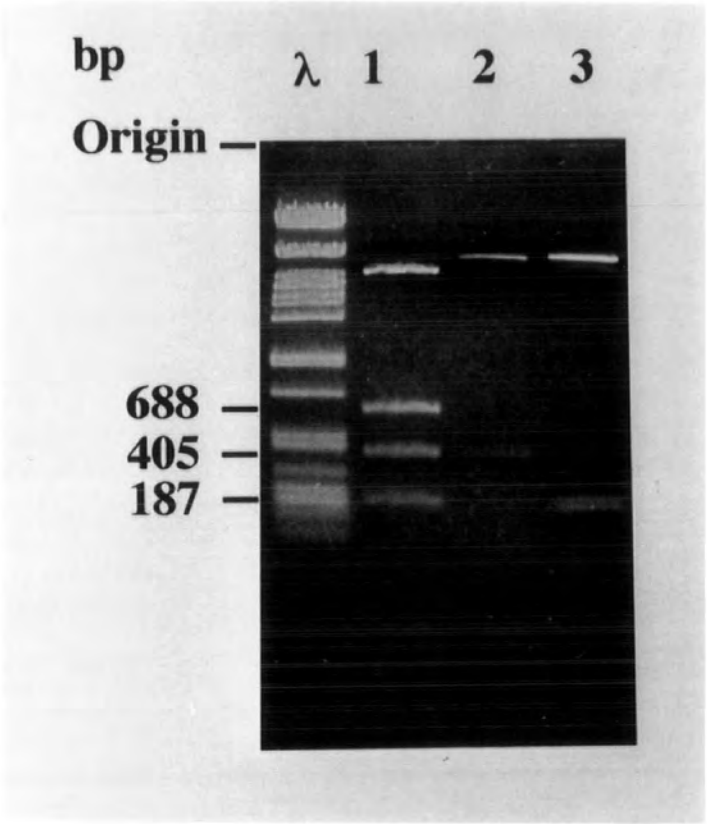

bp
λ 1 2 3 4 5 6 7 8 λ
Origin
1512
1280
1127
497
446
323
100

Table 3.2.2 Size estimation of cDNA clones

Sizes are shown as base pairs and were estimated by comparison with known band sizes of λ DNA digested with *Pst*I using a semi log plot

cDNA Clone	Size (bp)
pDZVIII5.1.1	1512
pDZVIII1.2.1	323
pDZVIII6.1.6	100
pDZIX3.1	497
pDZ2.11.5	1280
pDZ2.8L	446
pDZ2.8S	320
pDZ6.2	1127


bp
λ
1
2
3
Origin
688
405
187

3.2.4 Sequencing and analysis of the clones

Sequencing of the clones and their sub clones was performed using both double stranded and single stranded DNA as templates. Single stranded DNA was isolated by coinfection of pBluescript™ KS with a helper phage R408 and double stranded DNA by a modified alkaline lysis method. Figures 3.2.3. to 3.2.10 show the sequencing strategy used for each clone. Sequences were initially edited and analysed using the DNA Strider™ programme. Figures 3.2.11 to 3.2.18 show the nucleotide sequence of each clone. Features such as possible stop and start codons, poly $(A)^+$ tails , polyadenylation sites and other potentially interesting sequences are highlighted.

3.2.4.1 pDZ6.2

The nucleotide sequence of pDZ6.2 shows a 68 bp GA repeat sequence. This region was sequenced several times to ensure that this sequence was not an artefact of the sequencing reaction. On occasion only 34 bp were observed, the exact half length sequence. Since no other repeat length sequences were observed it was thought that this may not be due to random slippage of the Taq DNA polymerase, but perhaps due to a change in the conformation of the DNA , such as a hairpin loop, that might effectively cause the DNA available to the enzyme to exactly halve in length. A sample of plasmid DNA of pDZ6.2 was sequenced using the SEQUENASE™ system and the chemistry performed by the manufacturers ABI to definitely confirm the length of the repeat sequence. This system is less sensitive to errors due to repeat sequences. This is discussed further in the next chapter. Only one inframe start codon was found at 137 bp and a possible polyadenylation signal AATAAA at 615 bp, 29 bp upstream of the poly $(A)^+$ tail.

Two potential poly $(A)^+$ tails have been marked; it seems that the first one is the one most likely to be used, as it is consistent with the data obtained from the clones pDZIX 3.1 and pDZVIII 1.2.1 (see following two sections). The 471 bp of sequence downstream of this is proposed to be an extra piece of DNA having attached to the cDNA insert during library construction.

3.2.4.2 pDZIX 3.1

On comparison of the nucleotide sequences of pDZ6.2 and pDZIX 3.1, they were found to be identical. Figure 3.2.19 shows where the two sequences overlap in a multiple alignment. pDZIX 3.1 is missing 163 bp of the 5' sequence, this includes the region that encodes the first nine amino acid residues of the peptide sequence. It is also missing the 471 bp of 3' sequence.

3.2.4.3 pDZVIII 1.2.1

The nucleotide sequence of pDZVIII 1.2.1 is also very similar to that of pDZ6.2 and pDZIX 3.1. Figure 3.2.19 shows a multiple alignment of the nucleotide sequences of the three clones. Within the overlap region, there are three differences; the first a cytosine substituted with a thymidine gives rise to a change in the peptide sequence, a histidine being replaced with a tyrosine. The second two are in the third nucleotide position of the codon such that the peptide sequence is conserved. In the sequence contained within the 3' untranslated region prior to the poly (A)+ tail, there are 17 differences. These observations suggest that the different clones may contain inserts corresponding to different members of a multi-gene family.

3.2.4.4 pDZ2.8L

No unusual features were observed in this sequence. The probable stop codon is highlighted, but the clone appears to be too short to include the start codon.

3.2.4.5 pDZ2.8S

This sequence contained no possible start codon, but the probable stop codon is marked. A possible polyadenylation site is marked, though it does vary from the usual AATAAA found in some of the other clones.

3.2.4.6 pDZVIII 6.1.6

This is the shortest of the clones. A possible, but shortened, polyadenylation site is marked. A putative stop codon has also been marked.

3.2.4.7 pDZVIII 5.1.1 and pDZ2.11.5

No poly $(A)^+$ tail, or polyadenylation site were present in either of these sequences.

Figure 3.2.3

pDZ2.11.5 1280 bp

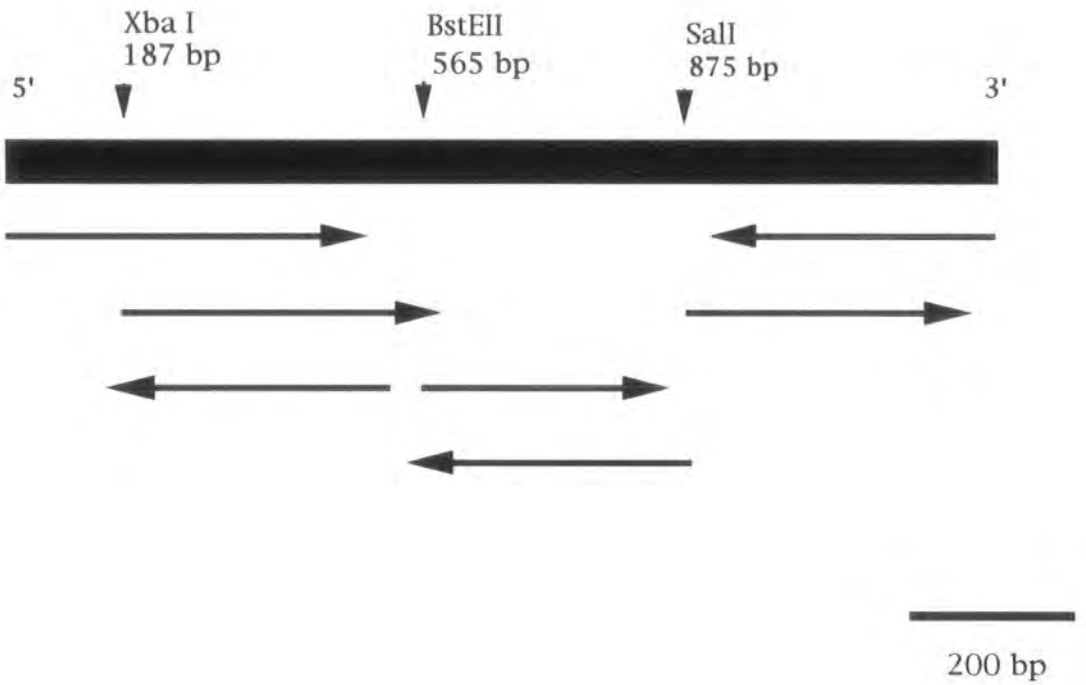


Figure 3.2.4

pDZ2.8L 446 bp

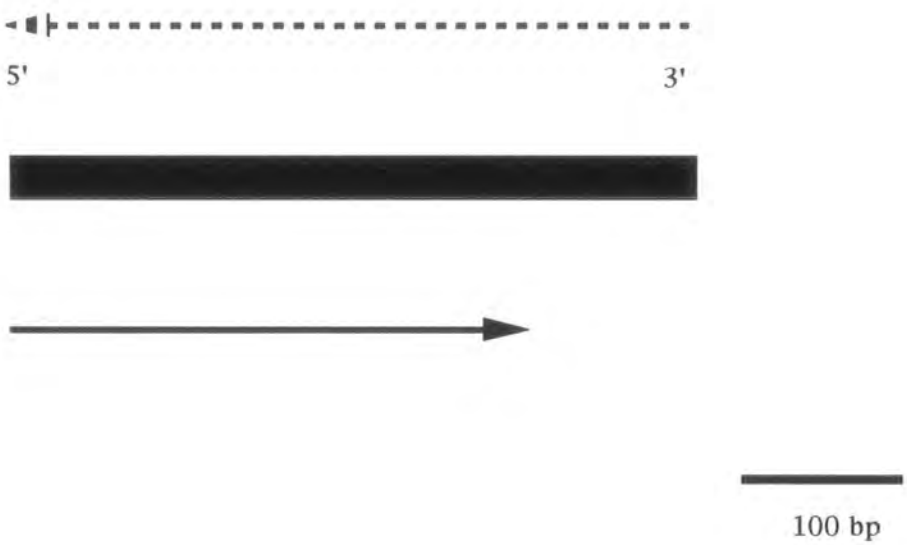


Figure 3.2.5

pDZ2.8S 320 bp

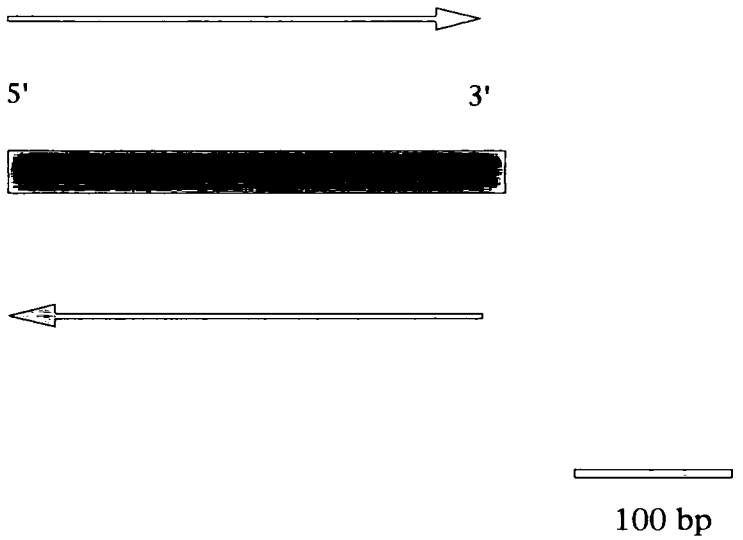


Figure 3.2.6

pDZ6.2 1127 bp

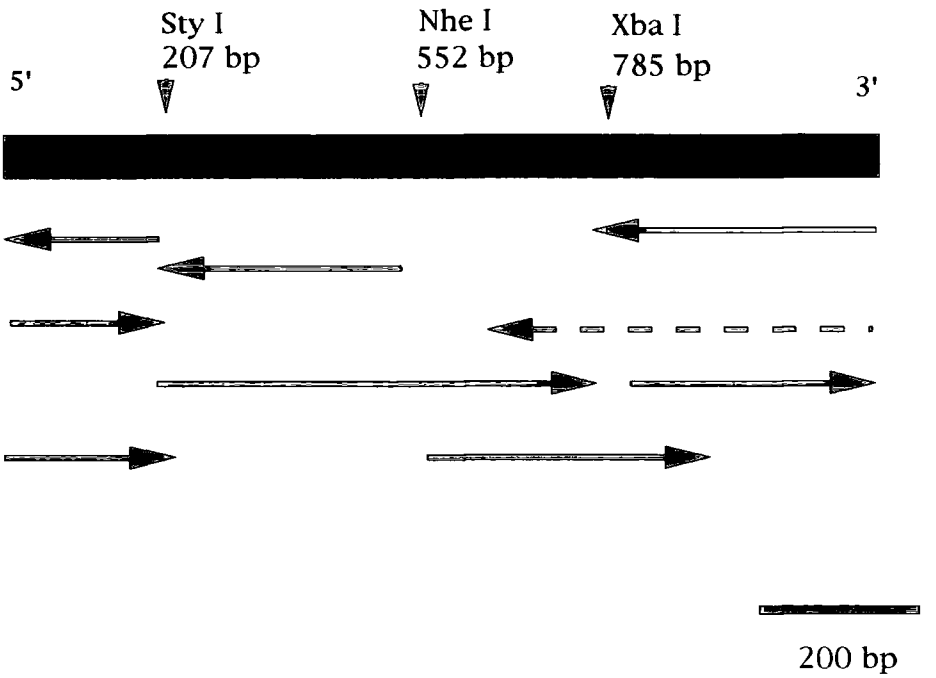


Figure 3.2.7

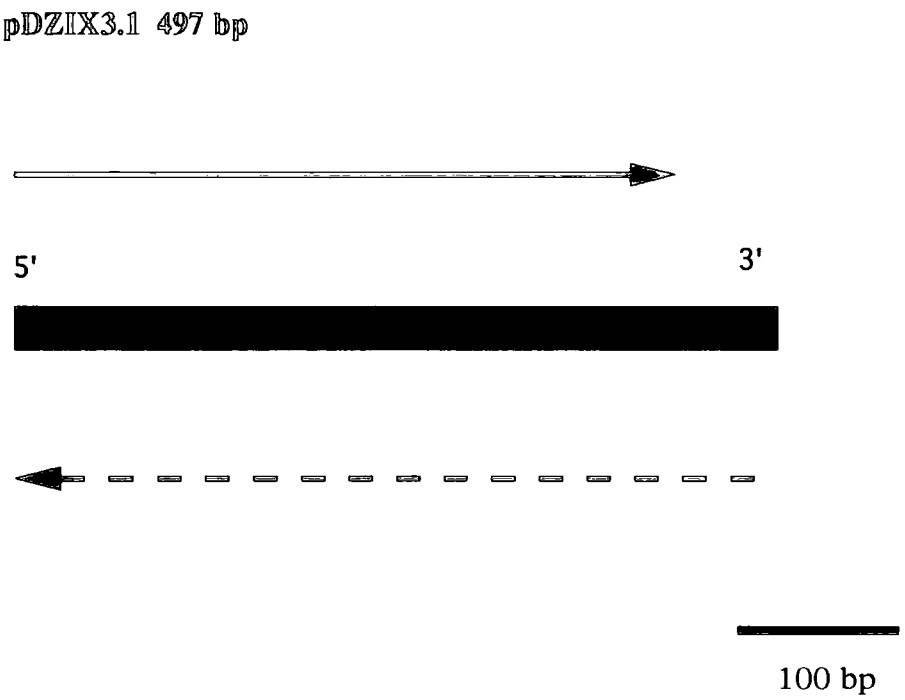


Figure 3.2.8

pDZVIII1.2.1 322 bp

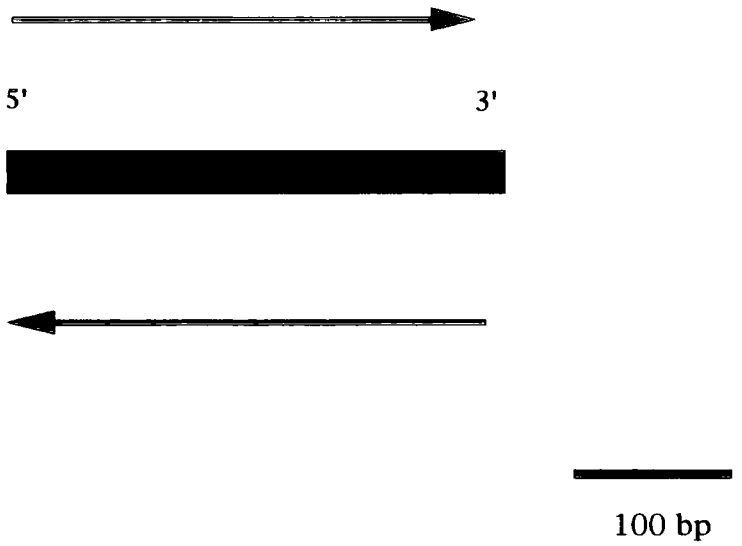


Figure 3.2.9

pDZVIII5.1.1 1512 bp

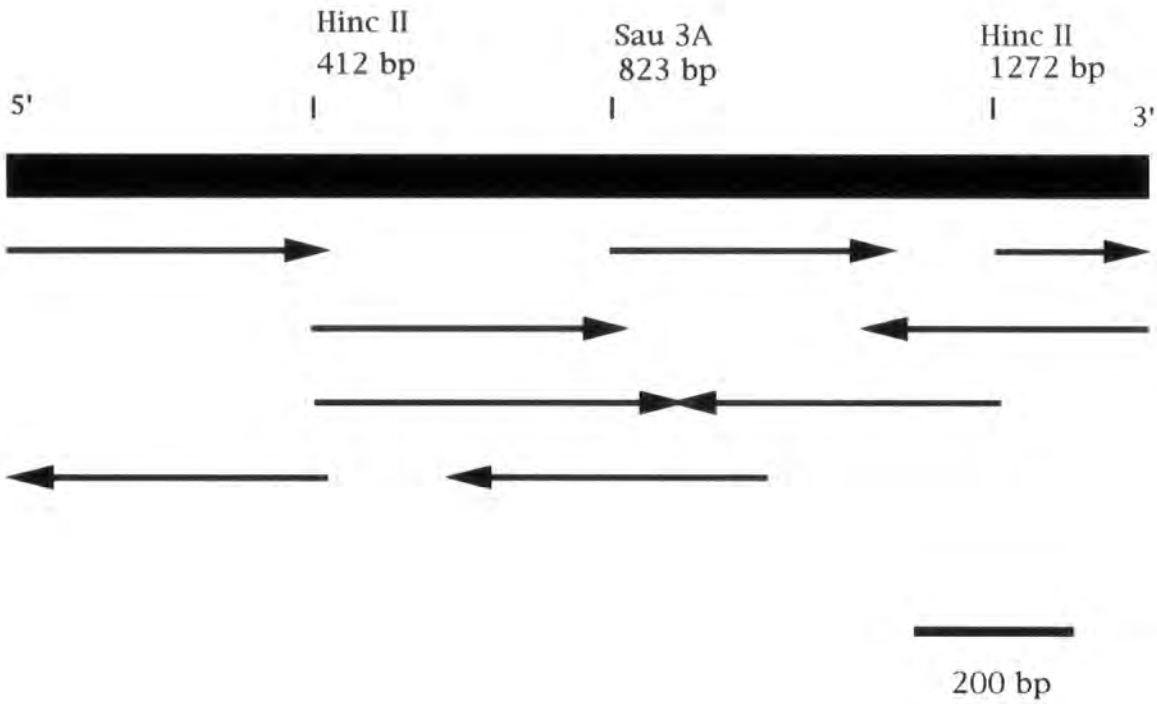


Figure 3.2.10

pDZVIII6.1.6 100 bp

Figure 3.2.11

Nucleotide sequence of pDZ2.11.5

CACGAAATACATATTTCCACAGCCCCCTTGAGCATTTCCCACTCTTGATTGCAAAGATTTG
AGAAGAAAAAGAGAGTTGGGTTGAGGGATTGAGAGATTGAAGAAAAGAGAGCAAAATTCAT
TCTTGAGCACTTGAGTTCATAGCAAGAGATCTTTGTGGCATTTGTTACTCTTGAGGATTCA
TCCTCTAGACGACAAGGTGTTGCCCGGTGAGCTCCCAAGTTTGTGGTGAGTCACGGGAAGT
TTGTGAAGGTTTCGATTCACCTCCGCAAGGAAAGGAATCAAGAAAAGGAAACCGAGGAAAA
GGGTTGAAAGAGACCCGGCTCAAGTGTGACCGAGCCCCTCAACGGAGACGTAGGACTCTTG
AGGAGTCCGAACTCCGGGAAACAAATCTCCGTGTCTCTTCTCTTGGTTTGCTCATACTTTT
ATTTGCATATTTATTTTGAGCAAATCTTTGTGATCTTTTTTTTTTTTTTTAAAGACCACTTC
GCAGCAATTAAACAAAGTCTCTTAGGGAAGCAGCAAGTGAATAGTCATAGTAGTCAACTAG
AGTAATAGGTTAAGAGGTCACCAGCTGCCACTCAGTTTAACCCTGTAGTGACTCCCAAAAT
AATTACAACCATTTCCAGGACACATAAAAACAGCATTGCAAGGTAGTAGGGGCTTGGTGCT
CCTCTACTCGAAGGCCCACTGGTTCTCCTGCGGATCTCCTCTTCCTCCTCAGGGGTGAAGT
CATTCTTGATGTTGAAGGTCTTGCGGATCTCCTCAGGCGTCTTGCCCTTGATCATGTCAGC
AACAGTCTGGCAGGTAAGGTCCAGCAGCCCCTTAATGTTGAGGTAGTTTGCAGCCAGGATG
AGATCGAAGAGGGTGGCCTGGTCGACCTTAACGAAGCGGCGTCCCAATTCTTAAGGTCCTC
GCCGGAGCCTGCAGTGGTGGAGGCGGAGTGTTGTTGGCGCCGGCGTCGTCGGAGCCAGCCT
TGGCGGCGGCCGCGGCGTGGACGTGCTTGTTGCAGTACTCGATGACCTTGGAGAGGATCTT
GGAGTTGACGTTGGGGAGCGGGATGCCGTTGTCGGCGCAGTCGTCCTCGATCATGTGGCGG
ATGGTCTGCGACTCCATCGCCACCGCCTCCTCCACCTCGAACTCCTCGCCGTCGGAGCTCT
TGAGCGTGATCATCTTCTTATCGCCCTCGGCCGCCATGGAGGTAGGTGGATTGGGATCGGT
CGCCTGGGAGGAGGACGAAACCTAGCTCGGGCTCGGCGCACGAGATTGGCGAGGGCGGAG

Figure 3.2.12

Nucleotide sequence of pDZ2.8L

CAAGAAGGCCAAGAAGACCGTCGAGACCTACAAGATCTACATCTTCAAGGTGCTCAAGCAGGTGC
ACCCGGACATCGGGATCTCCTCCAAGGCCATGTCCATCATGAACTCCTTCATCAACGACATCTTC
GAGAAGCTGGCGCAGGAGGCCGCCCGCCTCGCCCGCTACAACAAGAAGCCCACTATCACGTCCCG
GGAGATCCAGACCTCGGTGCGCCTCGTCCTCCCCGGCGAGCTCGCCAAGCACGCCGTCTCTGAGG
GCACCAAGGCCGTCACCAAGTTCACCTCTTCT**TAG**ATTGGTCGTGCTGGTGTGGCGTGGTGTTTA
CTCTAGTTGTTTGCTTCGACATTTAGGTAGGTTAGGTTTGTGGTGTGGAACAATGTAGAATGTAT
TATGCATGATT**AATAAA**TCTTATCTTGGCCGAGCTCGAAT**AAAAAAAAAAAAAAAAA**

Figure 3.2.13

Nucleotide sequence of pDZ2.8S

CGCACATTGACTTCTCACTGACCAGCCCGTTCGGTGGGGGCCCCCCAGGTAGGGTGAAGAGAAAG
AACCAGAAGAAGGCAAGTAGTGGTGGCGGTGACGGCGGTGATGAAGACGAGGAG**TGA**AGAACAAA
CCAGTAGCCGTTACCTTTTGTGTGCTATGAGTACTTAGCTGGGTGGACTGATACTCAGCCCCGTT
CTGTATGGTGGTGTCATCGGTTTTCCTCGTGACAGGATGGTTACCGCTTTATCTCGGGTTCTTAA
GCTTAGCTATGTATGGAGCTGTTT**AATTTTA**TGGTTTTGACTGCTCTGTGCC**AAAAAAAAA**

Figure 3.2.14

Nucleotide sequence of pDZ6.2

CAACAAAATCTCGCCGAGTACTGAAA**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG**
AGAGAGAGAGAGAGAGAGAGAGAGAGACGAGAGCGCGAGCGTAGCCGACATTGCGAACGCGGC
CTGGTG**ATG**CCGCTTCTGGTGGCTCTACTTCTCGTCCTAGCCGTGGTCGCCGCCGCCGGTGCCGA
CCACTACAAACCCAAGGACCCCTACCACCCGCCCAAAAAACACTACTACCCACCGAAGAAGGATC
ACTACTACCCACCCAAGAAAGACCATCACTACTACCCGCCCAAGAAGGATCACCACTATGAGCCA
CCGAAGAAGCACCATGACCCGCCTAAGAAGCACCCCTACCCGCCTAAGAAGGACCATGAGCACTG
GCCGGGCAAGCCTGGCTAC**TGA**GCTGCATTCATAAGCTAAGCTGCTGGACCATACCACAGTATCT
TTCAATAAGAGACATGCATATGCAGCCATGCATGCGATTACTACTTGTGTTGTTTAGCTATTGGA
TTTATGTGTGTCGTGCTTTGTTATGGGTCCTGCTAGCTTCTTATCCCATGTGTCTGCTACCTCAA
CTTGTACTGTGTGATTACCTACCTTATG**AATAAA**ATTCATTTGTGATTGTGCTATCT**AAAAAAAA**
AAAAAAGGTGGCTCCCTACTCCGGCCATATGCCACTCAATATTCGGTAAACCCCCTGCGGTCTTA
ACATGGGATTACGAATTTCTCCGCATGAGGCTCAAGAATCTGTACACTGCGAAATGTGGTTATGC
AAGATCTAGATTTCGGACCACCTATTTTTATGTGCCATTTGTTGTTTGTTCAGTTTGTTATTTTG
GTAATTAGCATGTGTTGGCTGACTACTGGGCACTGGCCTGCTGGCCAGAAAACTGAAGGGTGGCC
GGTTCAACTGACCGTCAGTTATTGGAGGGTAGATGTTTTAGAAGTGTTTGGATGTGTAGAAATCG
TGCTTTAGTGCTTTACTGAGGATGTGCATTGGATATGACTGTTCAGAACCAGCGGCTGGCCGAAA
ACTCGCGCTAGATGGACCTGAAATTGTGCTTTACCAAGTAATTTTAGGTTCACTCAAAGTTAAAT
CTTTTTAACTTTT**AAAAAAAA**G

Figure 3.2.15

Nucleotide sequence of pDZIX 3.1

CGTCCTAGCCGTGGTCGCCGCCGCCGGTGCCGACCACTACAAACCCAAGGACCCCTACCACCCGC
CCAAAAAACACTACTACCCACCGAAGAAGGATCACTACTACCCACCCAAGAAAGACCATCACTAC
TACCCGCCCAAGAAGGATCACCACTATGAGCCACCGAAGAAGCACCATGACCCGCCTAAGAAGCA
CCCCTACCCGCCTAAGAAGGACCATGAGCACTGGCCGGGCAAGCCTGGCTAC**TGA**GCTGCATTCA
TAAGCTAAGCTGCTGGACCATACCACAGTATCTTTCAATAAGAGACATGCATATGCAGCCATGCA
TGCGATTACTACTTGTGTTGTTTAGCTATTGGATTTATGTGTGTCGTGCTTTGTTATGGGTCCTG
CTAGCTTCTTATCCCATGTGTCTGCTACCTCAACTTGTACTGTGTGATTACCTACCTTATG**AATA**
AAATTCATTTGTGATTGTGCTATCT**AAAAAAAAAAAAAAAAA**

Figure 3.2.16

Nucleotide sequence of pDZVIII 1.2.1

CGAAGAAGCACTATGACCCGCCTAAGAAGCACCCCTACCCGCCCAAGAAGGACCATGAGCACTGG
CCCGGCCAATCCTGGCTAC**TGA**GCTGCACTCATAAGATAAGCTGCTGGACCATACCACGTATCTT
CCAATAAGAGACATGCATATGCAGCCATGCATGCGATTGCTACTTGTGTTGTTTAGCTATTGGAC
TTGTGTGTGTTGTGCTTTGTTATGGGTCCTTCTTGTGATCACCTACCTTATG**AATAAA**ATTTATT
CGTGATTGCGC**AAAAAAAAAAAAA**

Figure 3.2.17

Nucleotide sequence of pDZVIII 5.1.1

ACTGTCGGCGAAGGTAAGTTGATGACTCATGATGAACCCTGTTCTATGGCTCCAGATGACAAACA
TGATCTCATATCAGGGACTTGTTCGCACCTTCTTAGTACTTCCCCCAATAATTGGCTGGTGTTTA
TGCAAAATGGTCAAGAAGTGGTAATTGATAGCGGTAAAAGCGTTAGCTCACACTGACAATGGCTC
CAGCTTTTAAGGATGAAGGGGAACTAACCGACATGACAGATATTACAGGCAATCTGACGGTCCTG
GTGGAGGCAAAATGAACAATGTAAAAATTACTGATTGCCGGAAGTGCCTTTTTTTGCCATGTCAG
CGCAAGCCGCTGATAGAGTATCAATTGACGTTAAGGTGACTCTGGAAGCTGCAGTTTGTACTCCA
ATACTAAGCAACGGCGGAGTTGTTAACTTCGGTAGTCATTCAGTCAATAGACTTTCAACGCAGCA
CTACACACAGATTGGAACACGTAATATTAATATGACTATAACTTGCGAATCCGCTACCGGCATTG
CGATTACCGCTCGTGATACACGAATGGATAGTATGACCACCGGAAAAGATAGTGGGGGTCAGAGT
GGTGTAAAATATACCTTAAATGGCGGAGGTTATATTAGCCAGACAACGCGCTTATTCGGCTTAGG
GAAAACAAAAGATAATAAAAATATCGGTAGTTATGCCGTGTTAATTGATAGCAACAACATTAGTG
CCAGCAATGGTAGCCAGACGTTAGCTGTATCAATAGCAGGTGCAGATGCCGTCATAACAGGGCAA
AAAAGAGCATGGCAAACGCTCACCGCTTATCCGCTTGCAGTCGATCAAAGCTATTATTACACCTT
TGTAAAACCAGGTGAAACAACCCCAACCTGTAACGAACGCCATTATACCGCTTCAGGTGAGCGCA
TCTATCGCGAATGATTTAGGCGGTTCAGAAAAAATCGAACTTGATGGGAAAGCTGTGATAAGCGT
AGTCTACTTGTGATAACAATGCCGGACAGGAGTACTATCAGGCATTCGTTTTATACGATCGGTTA
ATGTTTCAGGAAAGCTCTTCGCATATCTTGGGGAAAGTAAATGTAATACGCTGTTCTTAATTGTT
CAGTGCTCACCCCCCAGAGTATAGCTCATTCCATTATTCAACTTAGCTCCATATAAAGGGACAGA
TAATGGAATCGCTCTCAGAAGGAACCACAGCAGGCTACCAGGAAATCCACGACGGTATTATTCAT
CTGGTCGATAGCGCCCGGACGGAAACGGTACGTAGCGTTAACGCGTTAATGACCGCGACGTACCA
GGAAATTGGCCGACGAATTGTCGAATTTGAACAAGGTGGCGAGGCCAGGGCTGCGTATGGTGCGC
AGCTAATCAAGCGACTATCAAAGGATTTATGTCTAAGGTATAAGCGTGGGTTCTCTGCAAAAAAC
TTACGCCAAATGAGGCTTTTTTACCTCTTTTTTCAACATGTTGAAATTCACCAGACAATGTCTGG
CGAATTAACACCATTGG

Figure 3.2.18

Nucleotide sequence of pDZVIII 6.1.6

CCTCATGTGGCCATGTGGTAAGCACATCGCTTGTATGCATCTTATTGTTGAGAGCGAGTGTTGTC
GCTC**AATA**CTACAGAAATCGTTCCTAC**AAAAAAA**G

Figure 3.2.19

CLUSTAL V multiple sequence alignment

```
pDZIX 3.1       ------------------------------------------------------------
pDZ6.2          CAACAAAATCTCGCCGAGTACTGAAAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
pDZVIII 1.2.1   ------------------------------------------------------------


pDZIX 3.1       ------------------------------------------------------------
pDZ6.2          GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGACGAGAGCGCGAGCGTAGCCGACATTG
pDZVIII 1.2.1   ------------------------------------------------------------


pDZIX 3.1       ------------------------------------------CGTCCTAGCCGTGGTCGC
pDZ6.2          CGAACGCGGCCTGGTGATGCCGCTTCTGGTGGCTCTACTTCTCGTCCTAGCCGTGGTCGC
pDZVIII 1.2.1   ------------------------------------------------------------


pDZIX 3.1       CGCCGCCGGTGCCGACCACTACAAACCCAAGGACCCCTACCACCCGCCCAAAAAACACTA
pDZ6.2          CGCCGCCGGTGCCGACCACTACAAACCCAAGGACCCCTACCACCCGCCCAAAAAACACTA
pDZVIII 1.2.1   ------------------------------------------------------------


pDZIX 3.1       CTACCCACCGAAGAAGGATCACTACTACCCACCCAAGAAAGACCATCACTACTACCCGCC
pDZ6.2          CTACCCACCGAAGAAGGATCACTACTACCCACCCAAGAAAGACCATCACTACTACCCGCC
pDZVIII 1.2.1   ------------------------------------------------------------


pDZIX 3.1       CAAGAAGGATCACCACTATGAGCCACCGAAGAAGCACCATGACCCGCCTAAGAAGCACCC
pDZ6.2          CAAGAAGGATCACCACTATGAGCCACCGAAGAAGCACCATGACCCGCCTAAGAAGCACCC
pDZVIII 1.2.1   ---------------------------CGAAGAAGCACTATGACCCGCCTAAGAAGCACCC
                                                  *

pDZIX 3.1       CTACCCGCCTAAGAAGGACCATGAGCACTGGCCGGGCAAGCCTGGCTACTGAGCTGCATT
pDZ6.2          CTACCCGCCTAAGAAGGACCATGAGCACTGGCCGGGCAAGCCTGGCTACTGAGCTGCATT
pDZVIII 1.2.1   CTACCCGCCCAAGAAGGACCATGAGCACTGGCCCGGCAAGCCTGGCTACTGAGCTGCACT


pDZIX 3.1       CATAAGCTAAGCTGCTGGACCATACCACAGTATCTTTCAATAAGAGACATGCATATGCAG
pDZ6.2          CATAAGCTAAGCTGCTGGACCATACCACAGTATCTTTCAATAAGAGACATGCATATGCAG
pDZVIII 1.2.1   CATAAGATAAGCTGCTGGACCATACCAC-GTATCTTCCAATAAGAGACATGCATATGCAG


pDZIX 3.1       CCATGCATGCGATTACTACTTGTGTTGTTTAGCTATTGGATTTATGTGTGTCGTGCTTTG
pDZ6.2          CCATGCATGCGATTACTACTTGTGTTGTTTAGCTATTGGATTTATGTGTGTCGTGCTTTG
pDZVIII 1.2.1   CCATGCATGCGATTACTACTTGTGTTGTTTAGCTATTGGATTTGTGTGTGTTGTGCTTTG


pDZIX 3.1       TTATGGGTCCTGCTAGCTTCTTATCCCATGTGTCTGCTACCTCAACTTGTACTGTGTGAT
pDZ6.2          TTATGGGTCCTGCTAGCTTCTTATCCCATGTGTCTGCTACCTCAACTTGTACTGTGTGAT
pDZVIII 1.2.1   TTATGGGTCCTTCTAGCTTCTTATCCCATGTGTCTGCTACCTCAACTTGTATTGTGTGAT


pDZIX 3.1       TACCTACCTTATGAATAAAATTCATTTGTGATTGTGCTATCTAAAAAAAAAAAAAAAAA-
pDZ6.2          TACCTACCTTATGAATAAAATTCATTTGTGATTGTGCTATCTAAAAAAAAAAAAAAGGTG
pDZVIII 1.2.1   CACCTACCTTATGAATAAAATTTATTCGTGATTGCGC------AAAAAAAAAAAA----
```

```
pDZIX 3.1       ------------------------------------------------------------
pDZ6.2          GCTCCCTACTCCGGCCATATGCCACTCAATATTCGGTAAACCCCCTGCGGTCTTAACATG
pDZVIII 1.2.1   ------------------------------------------------------------

pDZIX 3.1       ------------------------------------------------------------
pDZ6.2          GGATTACGAATTTCTCCGCATGAGGCTCAAGAATCTGTACACTGCGAAATGTGGTTATGC
pDZVIII 1.2.1   ------------------------------------------------------------

pDZIX 3.1       ------------------------------------------------------------
pDZ6.2          AAGATCTAGATTTCGGACCACCTATTTTTATGTGCCATTTGTTGTTTGTTCAGTTTGTTA
pDZVIII 1.2.1   ------------------------------------------------------------

pDZIX 3.1       ------------------------------------------------------------
pDZ6.2          TTTTGGTAATTAGCATGTGTTGGCTGACTACTGGGCACTGGCCTGCTGGCCAGAAAACTG
pDZVIII 1.2.1   ------------------------------------------------------------

pDZIX 3.1       ------------------------------------------------------------
pDZ6.2          AAGGGTGGCCGGTTCAACTGACCGTCAGTTATTGGAGGGTAGATGTTTTAGAAGTGTTTG
pDZVIII 1.2.1   ------------------------------------------------------------

pDZIX 3.1       ------------------------------------------------------------
pDZ6.2          GATGTGTAGAAATCGTGCTTTAGTGCTTTACTGAGGATGTGCATTGGATATGACTGTTCA
pDZVIII 1.2.1   ------------------------------------------------------------

pDZIX 3.1       ------------------------------------------------------------
pDZ6.2          GAACCAGCGGCTGGCCGAAAACTCGCGCTAGATGGACCTGAAATTGTGCTTTACCAAGTA
pDZVIII 1.2.1   ------------------------------------------------------------

pDZIX 3.1       ------------------------------------------------
pDZ6.2          ATTTTAGGTTCACTCAAAGTTAAATCTTTTTAACTTTTAAAAAAAAG
pDZVIII 1.2.1   ------------------------------------------------
```

3.2.5 Database searches to predicted products of possible open reading frames

Figures 3.2.20 to 3.2.27 show the six phase open reading frame (ORF) map for each clone, as predicted by the DNA Strider™ programme. All the possible ORF's were translated into their protein sequences and compared against the Owl database for sequence similarity with known proteins. Nucleotide sequences were compared with the EMBL and GenBank databases using the Fasta programme. Other long stretches of sequence, that contained no predicted start codon were also analysed, to take account of possible sequencing errors. A 40% identity for whole sequence alignment and chunk alignments was taken as a cut off point. Any scores below this level for any of the possible open reading frames were ignored.

3.2.5.1 p DZVIII 5.1.1, pDZ2.11.5 and pDZVIII 6.1.6.

No significant similarities with any known protein or nucleotide sequence were found for pDZVIII 5.1.1, pDZ2.11.5 and pDZVIII 6.1.6. In the case of pDZVIII 6.1.6, it is very likely that since it is only 100 bp in length, it represents untranslated 3' sequence. However for the other two clones it is possible the inserts could encode previously unknown proteins or just artefacts of cloning. To determine which is the case, the data from northern analysis and Southern blots will have to be taken into consideration.

3.2.5.2 pDZ2.8S

pDZ2.8S showed significant similarity to 40s ribosomal proteins from a number of species, including yeast, rat and a slime mould (*Dictyostelium discoideum*). ORF **a** had a highest score of 69.2% identity and 87% similarity with the 40s ribosomal protein from rat (*Rattus norvegicus*). Figure 3.2.28 shows a gap alignment with this protein sequence. The similarity is with the C-terminal end of the ribosomal protein. There are very few differences between the two peptide sequences, but where changes do occur the amino acid residues have similar properties, such as a phenylalanine (2.8S) for a tyrosine (rat), both having an aromatic ring, and an aspartate for a glutamate. Figure 3.2.29 shows a multiple alignment between the top three scoring matches from the database search. There is a very high level of conservation between the different sequences, most divergence occurring over the last few residues. pDZ2.8S appears to be more closely related to the sequences from rat and yeast than the trypanosome, which itself differs from the other three in its sequence.

3.2.5.3 pDZ2.8L

pDZ2.8L showed significant similarity to histone 2B proteins from a large number of species, as first reported by Zhao (Zhao, *et al.*, 1989). ORF **b** had a highest score of 96.9% identity and 97.9% similarity with the histone 2B from wheat. Figure 3.2.30 shows a gap alignment between this protein and the peptide sequence of pDZ2.8L. There is a very high level of conservancy, as reflected by the percentage similarity score. There are only three differences between pDZ2.8L and this region of the wheat histone protein; two of the amino acid changes are between residues with similar properties, a serine for a threonine and an arginine for a lysine; the third change is a glutamine for glycine. Figure 3.2.31 shows a multiple alignment between pDZ2.8L and histone 2B proteins from a number of species. The sequence of pDZ2.8L aligns with the C-terminal end of the histone proteins, it also matches with a region of very high conservation between them all. Again, any amino acid residue changes that do occur are between those with similar properties.

3.2.5.4 pDZ6.2, pDZIX 3.1 and pDZVIII 1.2.1

pDZ6.2, pDZIX 3.1 and pDZVIII 1.2.1 translate into proline rich proteins, having a characteristic repeat of PPKKDH(H)YY throughout the ORFs. Figure 3.2.32 shows the peptide sequence of pDZ6.2, the repeats are underlined and numbered and it can be seen how they differ by one residue. Repeat 6, however is truncated and occurs twice. During the database searches, the best matches of ORF's **c**, **d** and **e** (respectively) were with extensins, hydroxyproline glycoproteins and other cell wall proteins, all being proline rich. Although the percentage identity scores were relatively low, being on average 30 - 40 %, it was the repeat sequences and amino acid usage that led to the prediction that these clones could encode cell wall proteins. The first 20 amino acid residues of pDZ6.2 found a match with the leader sequence of a proline-rich cell wall protein precursor from soyabean (*Glycine max.*). This putative leader sequence is also shown in figure 3.2.32

pDZVIII 1.2.1 differs from pDZ6.2 and pDZIX 3.1 in its peptide sequence. Amino acid residue 68, a histidine, in the sequence of pDZ6.2 is replaced with a tyrosine in pDZVIII 1.2.1, two amino acids with very different properties. This change can be seen in figure 3.2.19, where an asterisk marks the place where the change in nucleotide sequence gives rise to the change in peptide sequence. It would obviously require the full polypeptide sequence to see whether there are other differences within the sequences of the two potential ORF's.

Figure 3.2.20

Predicted ORF map of pDZ2.11.5

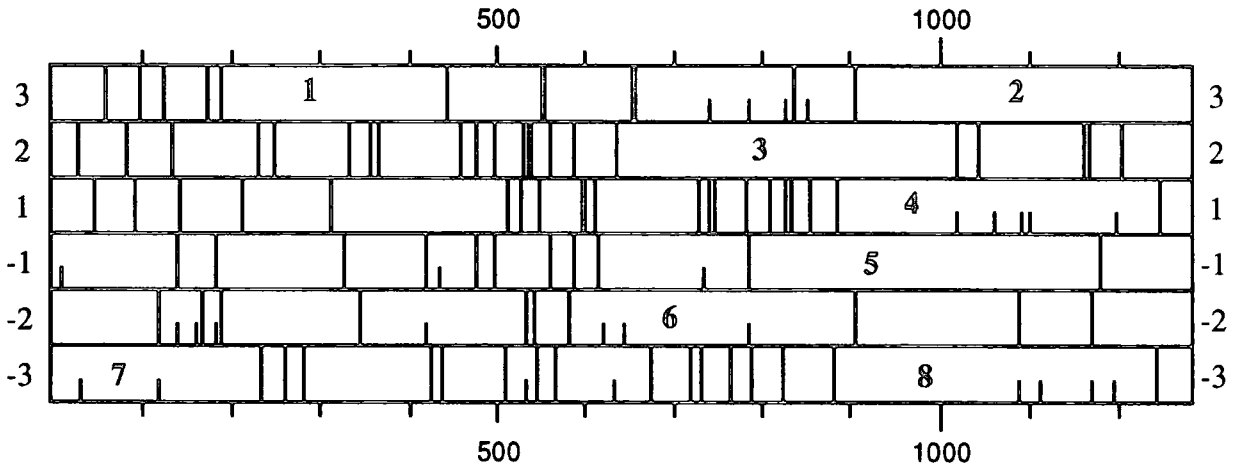


Figure 3.2.21

Predicted ORF map of pDZ2.8L

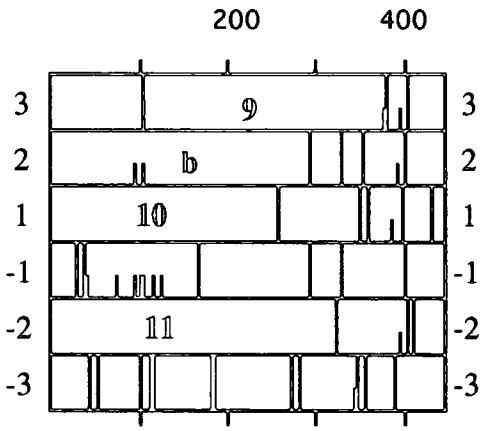


Figure 3.2.22

Predicted ORF map of pDZ2.8S

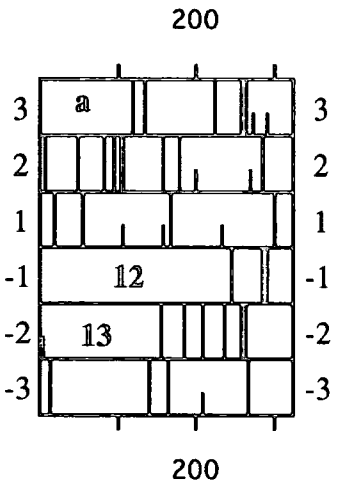


Figure 3.2.23

Predicted ORF map of pDZ6.2

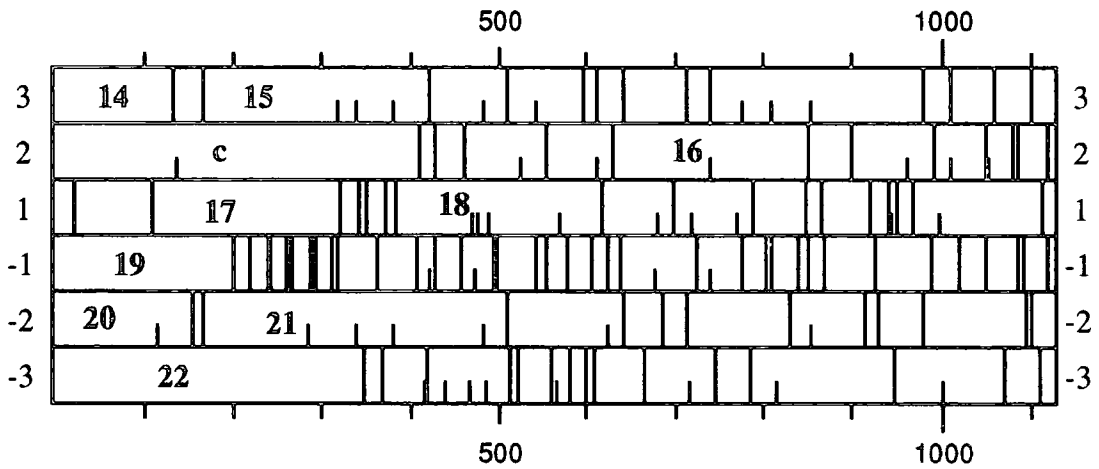


Figure 3.2.24

Predicted ORF map of pDZIX 3.1

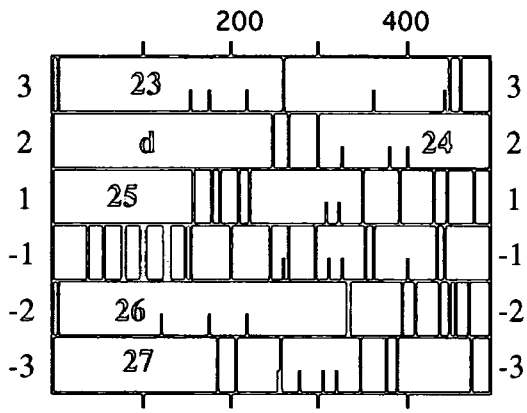


Figure 3.2.25

Predicted ORF map of pDZVIII 1.2.1

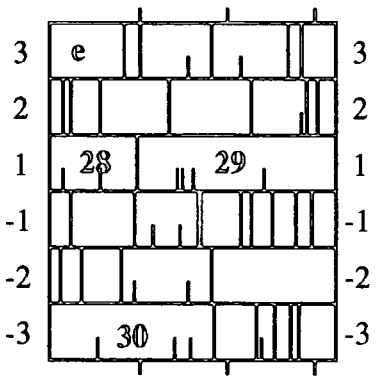


Figure 3.2.26

Predicted ORF map of pDZVIII 5.1.1

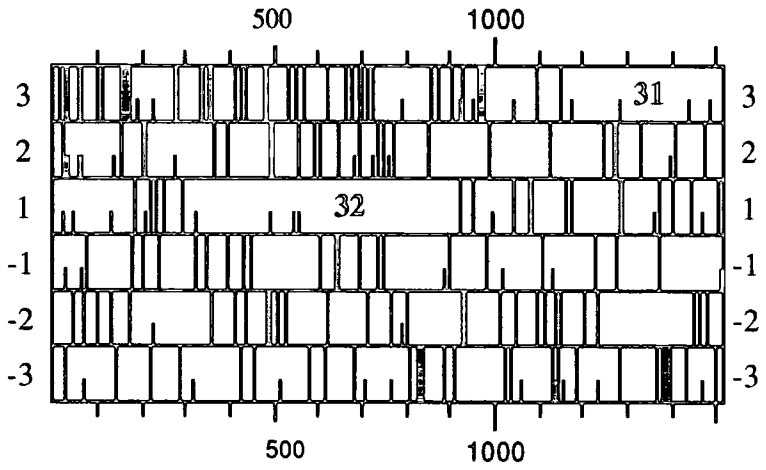


Figure 3.2.27

Predicted ORF map of pDZVIII6.1.6

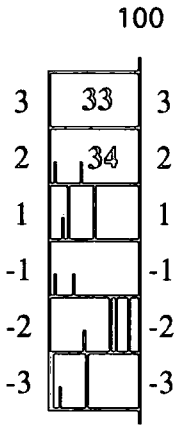


Figure 3.2.28

```
Percent Similarity: 87.179   Percent Identity: 69.231

2.8S x rs9_rat.swiss

                                    .
                .          .          .          .
2.8S 1  .....HIDFSLTSPFGGGPPGRVKRKNQKKASSGGGDGGDEDEE 39
              |||||| ||:|||.||||||||.||: :|:|.|:||:|:
Rat 151 LDSQKHIDFSLLSPYGGGRPGRVKRKNAKKGQGGAGAGDDEEED 194
```

Figure 3.2.29

```
RS11_YEAST    PRAPRTY--SKTYSTPKRPYESSRLDAELKLAGEFGLKNKKEIYRISFQLSKIRRAARDL
RS9_RAT       MPVARSWVCRKTYVTPRRPFEKSRLDQELKLIGEYGLRNKREVWRVKFTLAKIRKAAREL
2.8S          ------------------------------------------------------------
YALU_TRYBB    MRNYNNF--NRVWKAPRRPFEKERLDREMKLCGQYGLRCKREIWRVNMTLSKMRRTARLL

RS11_YEAST    LTRDEKDPKRLFEGNALIRRLVRVGVLSEDKKKLDYVLALKVEDFLERRLQTQVYKLGLA
RS9_RAT       LTLDEKDPRRLFEGNALLRRLVRIGVLDEGKMKLDYILGLKIEDFLERRLQTQVFKLGLA
2.8S          ------------------------------------------------------------
YALU_TRYBB    LTLPENHPRRLLEGSAIMRRCHGYGFLDEDKDKLDYVLSLTVPDILERRLQTVVFKHGLA

RS11_YEAST    KSVHHARVLITQRHIAVGKQIVNIPSFMVRLDSEKHIDFAPTSPFGGARPGRVARRNAAR
RS9_RAT       KSIHHARVLIRQRHIRVLKQVVNIPSFIVRLDSQKHIDFSLLSPYGGGRPGRVKRKNAKK
2.8S          ----------------------------------HIDFSLTSPFGGGPPGRVKRKNQKK
YALU_TRYBB    KSVHHSRVLIQQRHIAVAKQIVTIPSFIVRVSSEHHIAFADASPFGNGRPGRVKRVKRNA
                                                 ** *   ** *   **** *

RS11_YEAST    KAEASGEAADEADEADEE
RS9_RAT       GQGGAGAGDDE----EED
2.8S          ASSGGGDGGDE----DEE
YALU_TRYBB    AKKGSGGGDDD------E
                   *   *       .
```

Figure 3.2.30

Percent Similarity: 97.938 Percent Identity: 96.907

28lhpb x h2b1_wheat.swiss

```
                .         .         .         .         .
 2.8L    1      KKAKKTVETYKIYIFKVLKQVHPDIGISSKAMSIMNSFINDIFEK 45
                |||||.|||||||||||||||||||||||||||||||||||||||
Wheat   51 DKKGKKKAKKSVETYKIYIFKVLKQVHPDIGISSKAMSIMNSFINDIFEK 100
                .         .         .         .         .
 2.8L   46 LAQEAARLARYNKKPTITSREIQTSVRLVLPGELAKHAVSEGTKAVTKFT 95
           ||.|||:|||||||||||||||||||||||||||||||||||||||||||
Wheat  101 LAGEAAKLARYNKKPTITSREIQTSVRLVLPGELAKHAVSEGTKAVTKFT 150

 2.8L   96 SS 97
           ||
Wheat  151 SS 152
```

Figure 3.2.31

```
28LHP        ------------------------------------------------------------KK
H2B1_MOUSE   --------------------------------PEPAKSAPAPKK-GSKKAVTK--AQKKD
H2B1_WHEAT   MAPKAEKKPAAKKPAEEEPAAEKAEKTPAGKKPKAEKRLPAGKSAAKEGGDK---KGKKK
H2B1_YEAST   --------------------------------SAKAEKKPASKAPAEKKPAAKKTSTSTD
H2B2_HUMAN   --------------------------------PDPAKSAPAPKK-GSKKAVTK--AQKKD
H2B2_WHEAT   ---------PXKKPAAENKVEKAAEKTPAGKKPKAEKRLPAGKTASKEAGGEGKTRGRKK
H2B2_XENLA   --------------------------------PEPAKSAPAPKK-GSKKAVTK--TPKKD
H2B3_VOLCA   MAPK-KEEKPASQ-AAEAPEVKAEAKPKAVKAPKKKEKKAPAKKVAKEPSAGGEDGDKKS
H2B4_VOLCA   MAPKTKEEKPASE-AVEP---KAEAKPKAEKAPKKKEKKAPAKKSAKEPAAGDAAEGDKK

28LHP        ---AKKT-VETYKIYIFKVLKQVHPDIGISSKAMSIMNSFINDIFEKLAQEAARLARYNK
H2B1_MOUSE   GKKRKRSRKESYSVYVYKVLKQVHPDTGISSKAMGIMNSFVNDIFERIASEASRLAHYNK
H2B1_WHEAT   ---AKKS-VETYKIYIFKVLKQVHPDIGISSKAMSIMNSFINDIFEKLAGEAAKLARYNK
H2B1_YEAST   GKKRSKARKETYSSYIYKVLKQTHPDTGISQKSMSILNSFVNDIFERIATEASKLAAYNK
H2B2_HUMAN   GKERKRSRKESYSIYVYKVLKQVHPDTGISSKAMGIMNSFVNDIFERIAGEASRLAHYNK
H2B2_WHEAT   GSKAKKG-VETYKIYIFKVLKQVHPDIGISSKAMSIMNSFINDIFEKLAGESAKLARYNK
H2B2_XENLA   GKKRRKSRKESYAIYVYKVMKQVHPDTGISSKAMGIMNSFVNDIFERIAGEASRLAHYNK
H2B3_VOLCA   KKKAKVAKSETYKLYIYKVLKQVHPDTGISSKAMSIMNSFINDIFEKVATEASKLSRYNK
H2B4_VOLCA   KKKAKVAKSETYKLYIYKVLKQVHPDTGISSKAMSIMNSFINDIFEKVATEASKLSRYNK
                      *.*  *..**.** *** *** *.*.*.***.*****..* *...*. ***

28LHP        KPTITSREIQTSVRLVLPGELAKHAVSEGTKAVTKFTSS---
H2B1_MOUSE   RSTITSREIQTAVRLLLPGELAKHAVSEGTKAVTKYTSSK--
H2B1_WHEAT   KPTITSREIQTSVRLVLPGELAKHAVSEGTKAVTKFTSS---
H2B1_YEAST   KSTISAREIQTAVRLILPGELAKHAVSEGTRAVTKYSSSTQA
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H2B2_WHEAT   KPTITSREIQTSVRLVLPGELAKHAVSEGTKAVTKFTSS---
H2B2_XENLA   RSTITSREIQTAVRLLLPGELAKHAVSEGTKAVTKYTSAK--
H2B3_VOLCA   KPTVTSREIQTAVRLVLPGELAKHAVSEGTKAVTKFTSA---
H2B4_VOLCA   KPTVTSREIQTAVRLVLPGELAKHAVSEGTKAVTKFTSA---
             ..*...*****.***.**************.****..*.
```

Figure 3.2.32

CAACAAAATCTCGCCGAGTACTGAAA**GAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAG**

AGAGAGAGACGAGAGCGCGAGCGTAGCCGACATTGCGAACGCGGCCTGGTG**ATG**CCGCTTCTGGTGGCTCTACTTCTCGTCCTAG

M P L L V A L L L V L

0

CCGTGGTCGCCGCCGCCGGTGCCGACCACTACAAACCCAAGGACCCCTACCACCCGCCCAAAAAACACTACTACCCACCGAAGAA

A V V A A A G A D H Y K P K D P Y H P P K K H Y Y P P K K

1 2

GGATCACTACTACCCACCCAAGAAAGACCATCACTACTACCCGCCCAAGAAGGATCACCACTATGAGCCACCGAAGAAGCACCAT

D H Y Y P P K K D H H Y Y P P K K D H H Y E P P K K H H

3 4 5

GACCCGCCTAAGAAGCACCCCTACCCGCCTAAGAAGGACCATGAGCACTGGCCGGGCAAGCCTGGCTAC**TGA**GCTGCATTCATAA

D P P K K H P Y P P K K D H E H W P G K P G Y OPA.

6 6

GCTAAGCTGCTGGACCATACCACAGTATCTTTCAATAAGAGACATGCATATGCAGCCATGCATGCGATTACTACTTGTGTTGTTT

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GTGATTACCTACCTTATG**AATAAA**ATTCATTTGTGATTGTGCTATCT**AAAAAAAAAAAAAA**GGTGGCTCCCTACTCCGGCCATAT

GCCACTCAATATTCGGTAAACCCCCTGCGGTCTTAACATGGGATTACGAATTTCTCCGCATGAGGCTCAAGAATCTGTACACTGC

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GAACCAGCGGCTGGCCGAAAACTCGCGCTAGATGGACCTGAAATTGTGCTTTACCAAGTAATTTTAGGTTCACTCAAAGTTAAAT

CTTTTTAACTTTTAAAAAAAAG

3.3 Southern analysis of cDNA clones

Five 10 µg aliquots of *D. spicata* genomic DNA were digested with *Eco*RI, *Sal*I, *Hind*III, *Bam*HI and *Xba*I and the products analysed by electrophoresis on a 0.7 % agarose gel. Samples were then blotted onto Hybond N and hybridised at 65 °C with randomly primed [^{32}P]-labelled inserts of the cDNA clones.

pDZIX 3.1 was not used as a probed due to its 100 % similarity to pDZ6.2.

3.3.1 pDZVIII 5.1.1, pDZ2.11.5, pDZ2.8S, pDZVIII 1.2.1 and pDZVIII 6.1.6

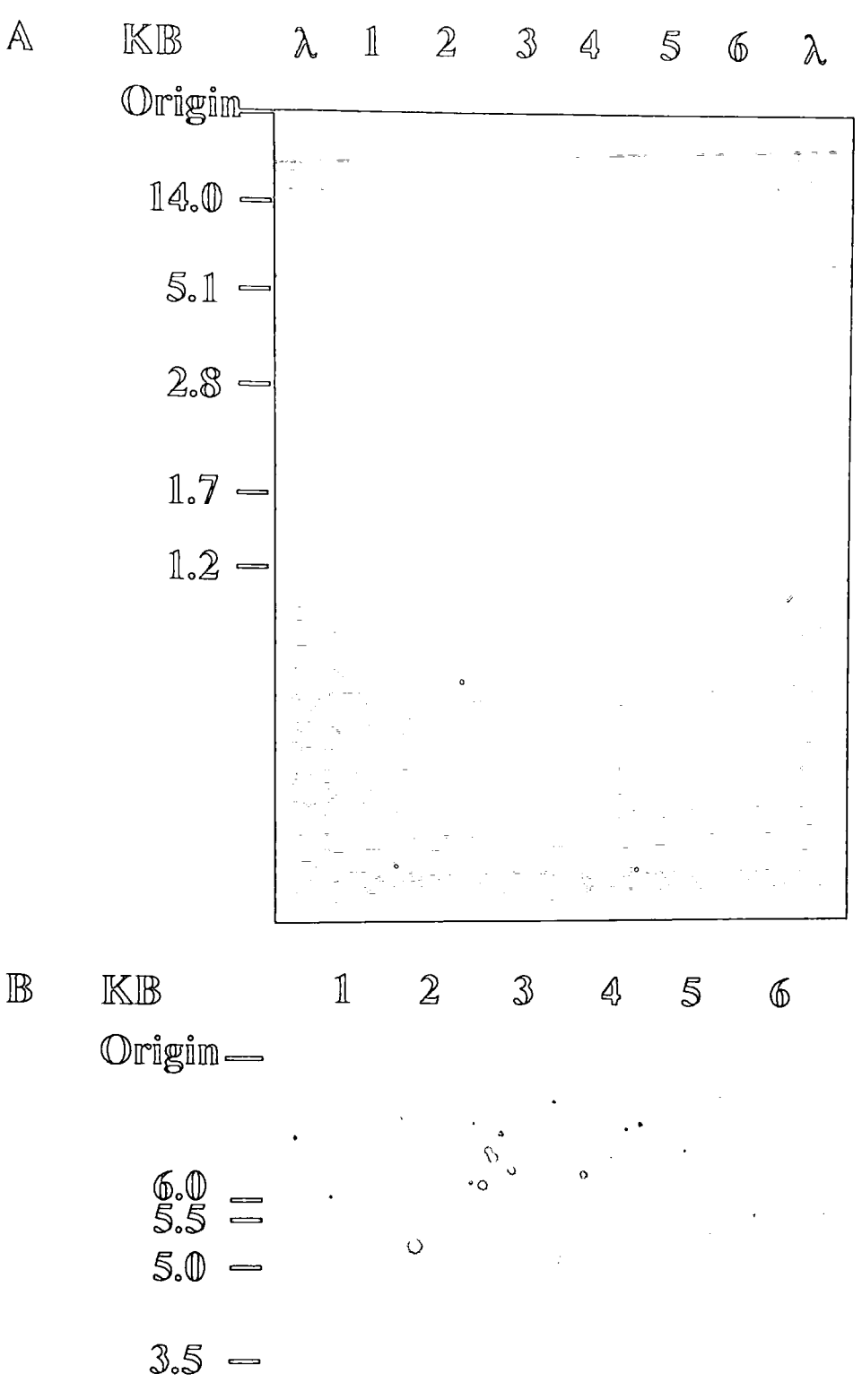
No signals were obtained after hybridisation of any of these cDNA clones with Southern blotted *D. spicata* genomic DNA. Blots were exposed to X-Ray film for over a month to ensure no signal, however weak, was missed. In the case of the smaller clones, pDZVIII 1.2.1, pDZVIII 6.1.6 and pDZ2.8S this may be due to their size, resulting in a weak probe, and thus no measurable signal. The larger inserts in the clones, pDZVIII 5.1.1 and pDZ2.11.5 however, may well have resulted from cloning artefacts , since no signal was obtained.

3.3.2 pDZ2.8L

Figure 3.3.1 shows the gel photograph (panel A) and the corresponding autoradiograph of the probed blot (panel B). The approximate band sizes in panel B are marked; *Eco*R I gave a 3.5 KB band, *Hind*III a 5.0 Kb band, *Bam*H I 6.0 Kb, and *Xba*I a 5.5 Kb band. *Sal*I gave no significant band.

3.3.3 pDZ6.2

Figure 3.3.2 shows the gel photograph (panel A) and the autoradiograph of the probed blot (panel B). The approximate band sizes in panel B are marked; *Eco*RI gave a 5.0 Kb band, *Hind*III a 6.0 Kb band, *Bam*H I a 10.0 Kb band and *Xba*I a 9.0 Kb band. The 11.0 Kb band marked for *Sal*I is likely to be due to uncut DNA and not a restriction fragment. The other bands appear to be consistent with the bands visible on the gel. Therefore it seems more likely that no specific hybridisation has taken place.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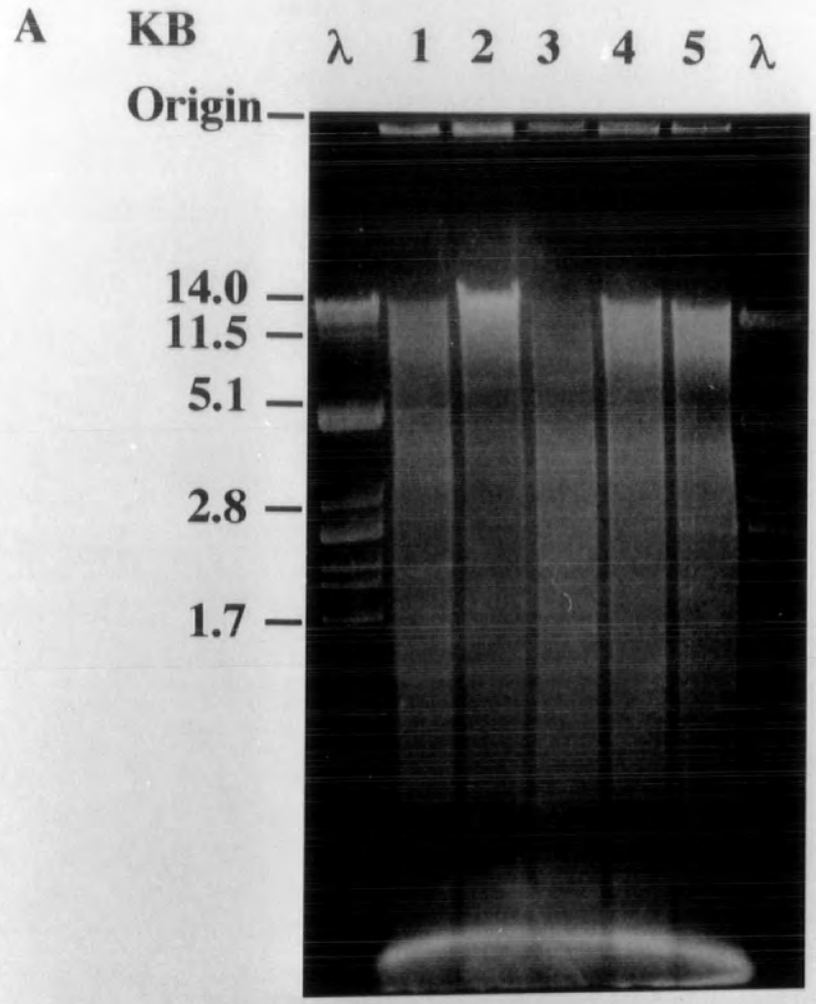
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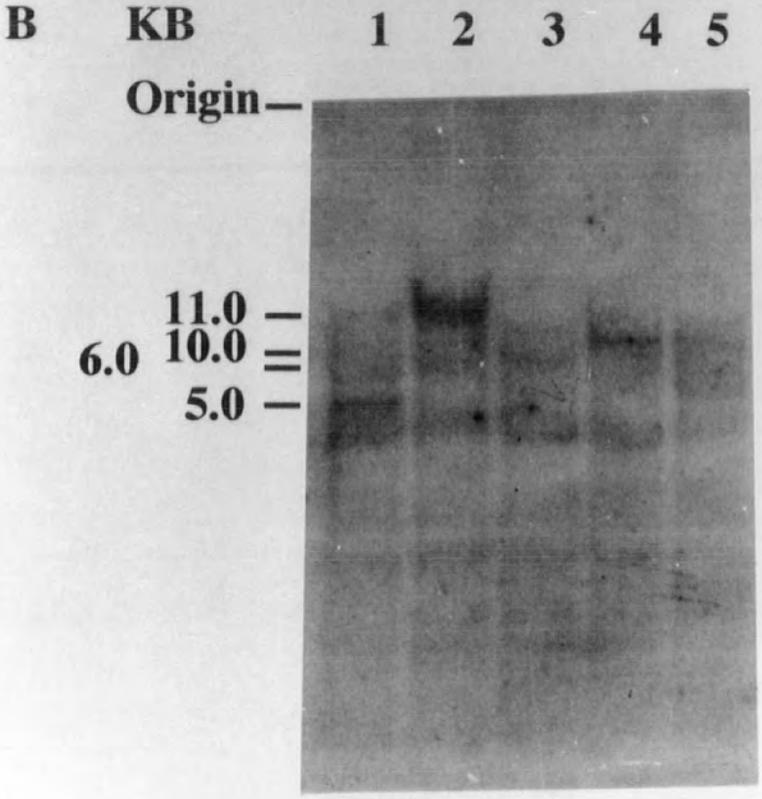
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3.4 Northern analysis of transcript abundance corresponding to the cDNA clones in response to exogenous NaCl, ABA and L-Proline

As pDZIX 3.1 was found to be identical in sequence to pDZ6.2, it was not used as a probe for northern analysis. pDZVIII 5.1.1, pDZ2.11.5, pDZ 2.8S, and pDZVIII6.1.6 gave no measurable signal when hybridised with blots in initial experiments and so were not studied any further. Therefore pDZVIII 1.2.1, pDZ2.8L and pDZ6.2 were the only cDNA clones used as probes used in the experiments described below.

3.4.1 Selection of concentrations of chemical treatments

RNA was extracted from frozen cells (as harvested in section 2.3.1.3), 15 μg electrophoresed on 1.4% formaldehyde agarose gels and blotted onto Hybond N.

Suspension cultures of *D. spicata* were originally exposed to 260 mM NaCl, the level used by Zhao (Zhao, *et al.*, 1989). However when the corresponding blots were probed with ^{32}P labelled pDZ6.2 there was no increase in transcript abundance with 260 mM NaCl as compared to 0 mM samples. Therefore a range of NaCl concentrations was used to find at what level transcript abundance was induced. Cells were exposed to 0, 260, 520 and 780 mM NaCl. Figure 3.4.1 shows the results of hybridising the blot with pDZ6.2. Panel B represents the autoradiographs and shows an increase in transcript abundance at 520 mM NaCl as compared to the 0 mM sample, which is followed by a decrease in the 780 mM sample. Therefore it was decided to use 520 mM NaCl in all further experiments, and 24 hours as the length of the exposure for all treatments. Cells were also exposed to 0 and 5 mM L-proline and 0 and 100 μM ABA. All treatments were done in triplicate.

3.4.2 NaCl

Figure 3.4.2 shows the results following probing with the insert from pDZ6.2, and shows a small increase in transcript abundance in response to 520 mM NaCl in the first two examples, but isn't reproducible in the third. Other larger bands are also visible in some of the lanes, and these appear to correspond to the ribosomal RNA bands when compared to the original gel.

Figure 3.4.3 shows the results following probing with the insert from pDZ2.8L, and in this case there is a decrease in transcript abundance in the presence of 520 mM NaCl, as shown in the first two examples, however this is is not at all clear in the third.

Figure 3.4.4 shows the results after using the insert from pDZVIII 1.2.1, as a probe and there is a slight increase in transcript abundance in response to 520 mM NaCl, as shown in examples one and three, however the second example is too overexposed to draw a definite conclusion. Again larger bands are visible running at the same place as ribosomal bands.

3.4.3 L-Proline

Figure 3.4.5 shows a small decrease in abundance of transcripts corresponding to the insert in pDZ6.2 in the presence of 5 mM proline, though the second replicate is somewhat inconclusive due to its over exposure. Figure 3.4.6 shows a decrease for pDZ2.8L in the first example, but is unclear in the second and third. Figure 3.4.7 also shows a slight decrease in transcript abundance following probing with the insert from pDZVIII 1.2.1 in the first and third examples, the second example is inconclusive due to its overexposure.

3.4.4 ABA

Figure 3.4.8 shows a clear increase of abundance of transcripts corresponding to the insert in pDZ6.2 in response to 100 μM ABA, as does Figure 3.4.10 for pDZVIII 1.2.1. However there is a decrease in transcript abundance of pDZ2.8L in the presence of 100 μM ABA in Figure 3.4.9, which in the third case, is not very clear at all.

3.4.5 NaCl and L-Proline

The effect of L-proline on transcript abundance in the presence of NaCl was studied. Cells were exposed to 520 mM NaCl, or to 5 mM proline and 520 mM NaCl. Northern blots of the resulting gels were hybridised with the three cDNA clones. Size markers were run along side these particular samples in order to estimate the transcript sizes. These are shown in panel A of figures 3.4.12 and 3.4.13.

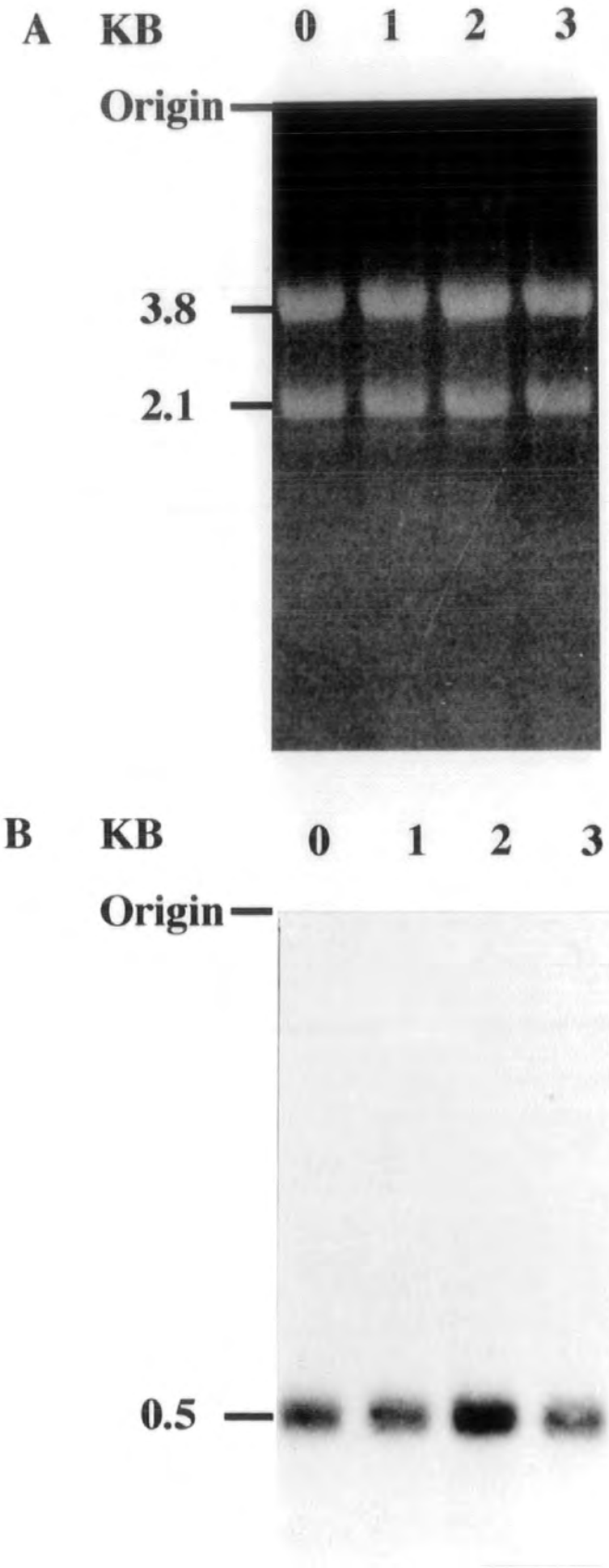
Figure 3.4.11 shows a decrease in transcript abundance following probing with the insert from pDZ6.2 in the presence of NaCl and proline as compared to NaCl alone. A similar decrease in abundance of transcripts corresponding to the insert from pDZVIII1.2.1 is also observed in figure 3.4.13 However in both cases this decrease is only slight and is not reproducible in all the examples. Figure 3.4.12 shows very inconsistent results following probing with the insert from pDZ2.8L, the third replicate showing a decrease in transcript abundance, the second an increase and the first no change at all.

Transcript sizes were estimated by comparison with the markers, using semi log plots. The transcript corresponding to the insert from pDZ6.2 was found to have an estimated size of 0.5 Kbases; that for pDZ2.8L 0.6 Kbases and pDZVIII 1.2.1 0.7 Kbases.

3.4.6 NaCl and ABA

The effect of ABA on transcript abundance in the presence of NaCl was studied. Cells were exposed to 520 mM NaCl, or to 100 μM ABA and 520 mM NaCl Blots were then hybridised with the cDNA clones. Figure 3.4.14 shows an increase in transcript abundance

of transcripts corresponding to inserts from pDZ6.2 in the presence of both NaCl and ABA for the first two replicates, however the third replicate is too over exposed to draw a definite conclusion, but there is a possible increase. Figure 3.4.16 shows an increase in transcript abundance for transcripts corresponding to the insert from pDZVIII 1.2.1 in the first two examples. Figure 3.4.15 shows a decrease in abundance transcripts corresponding to the insert from pDZ2.8L in the presence of NaCl and ABA as compared to NaCl alone.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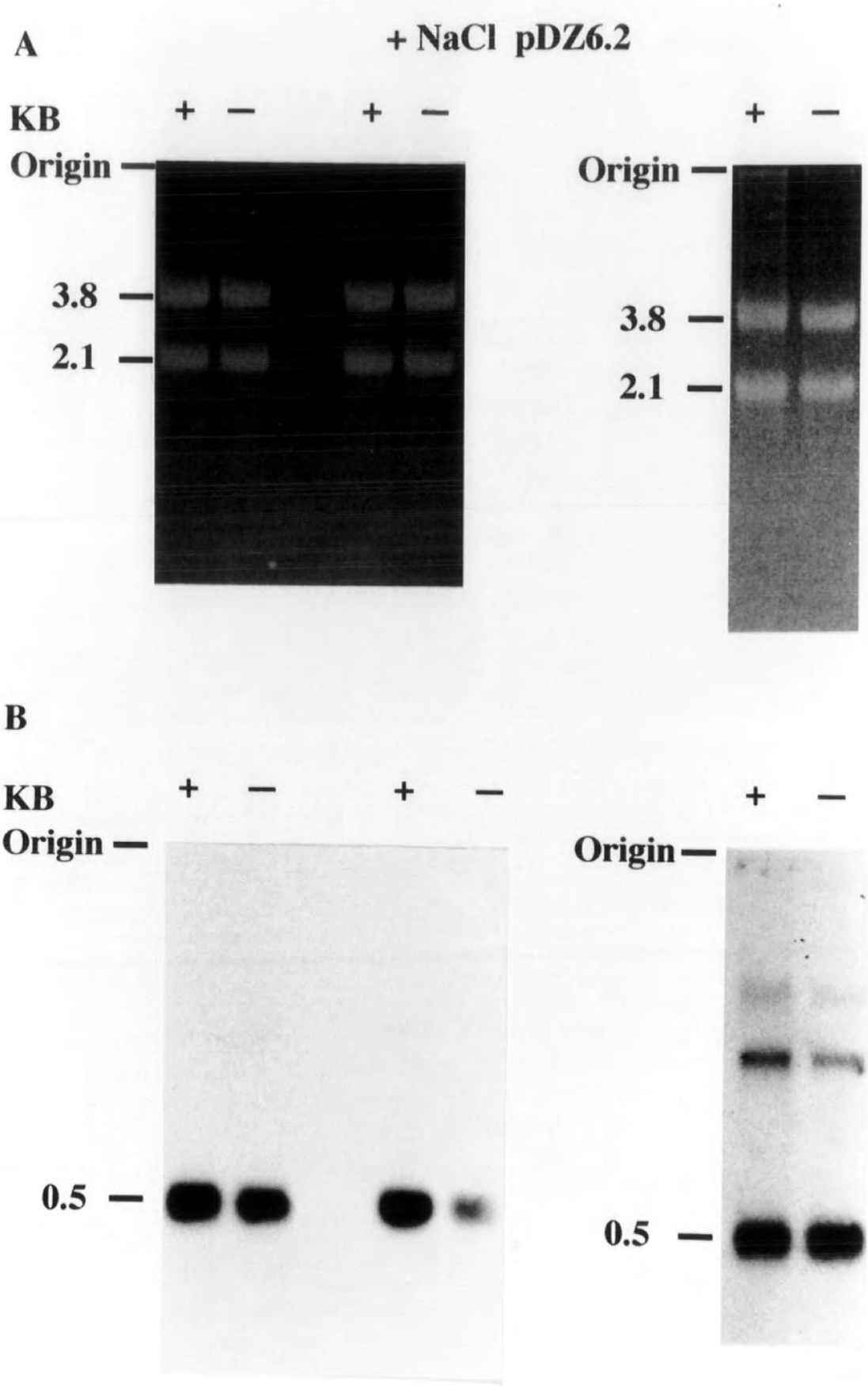
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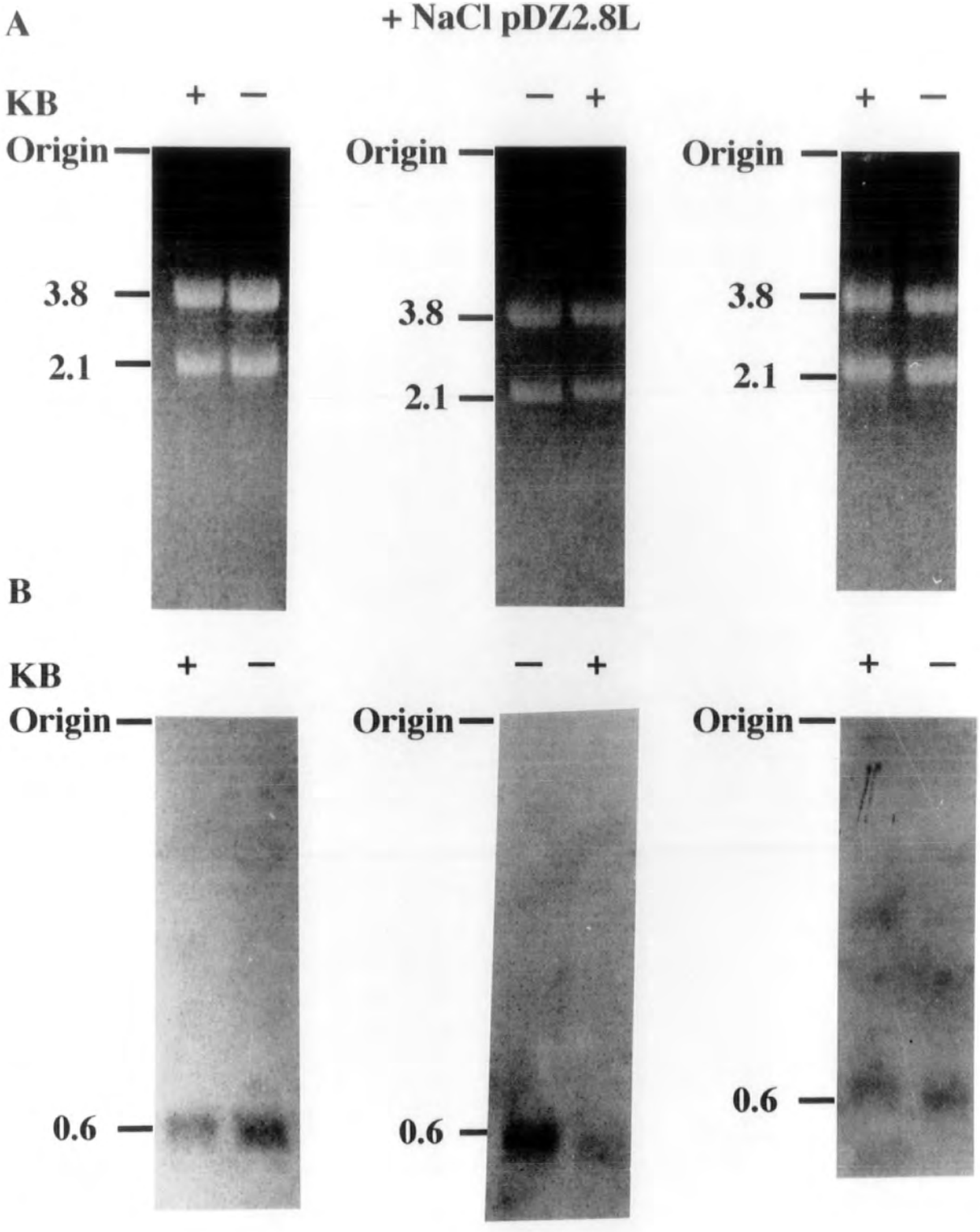
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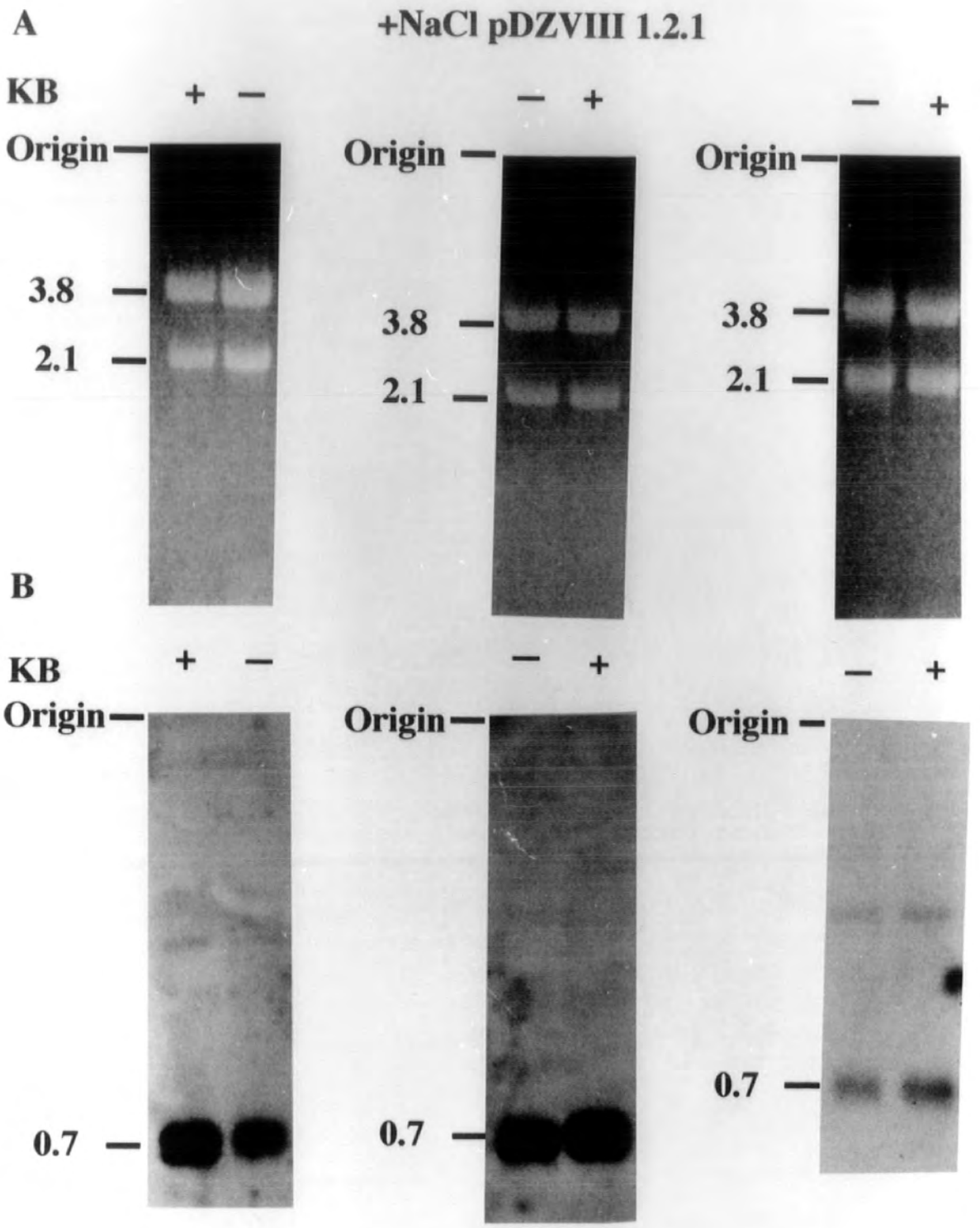
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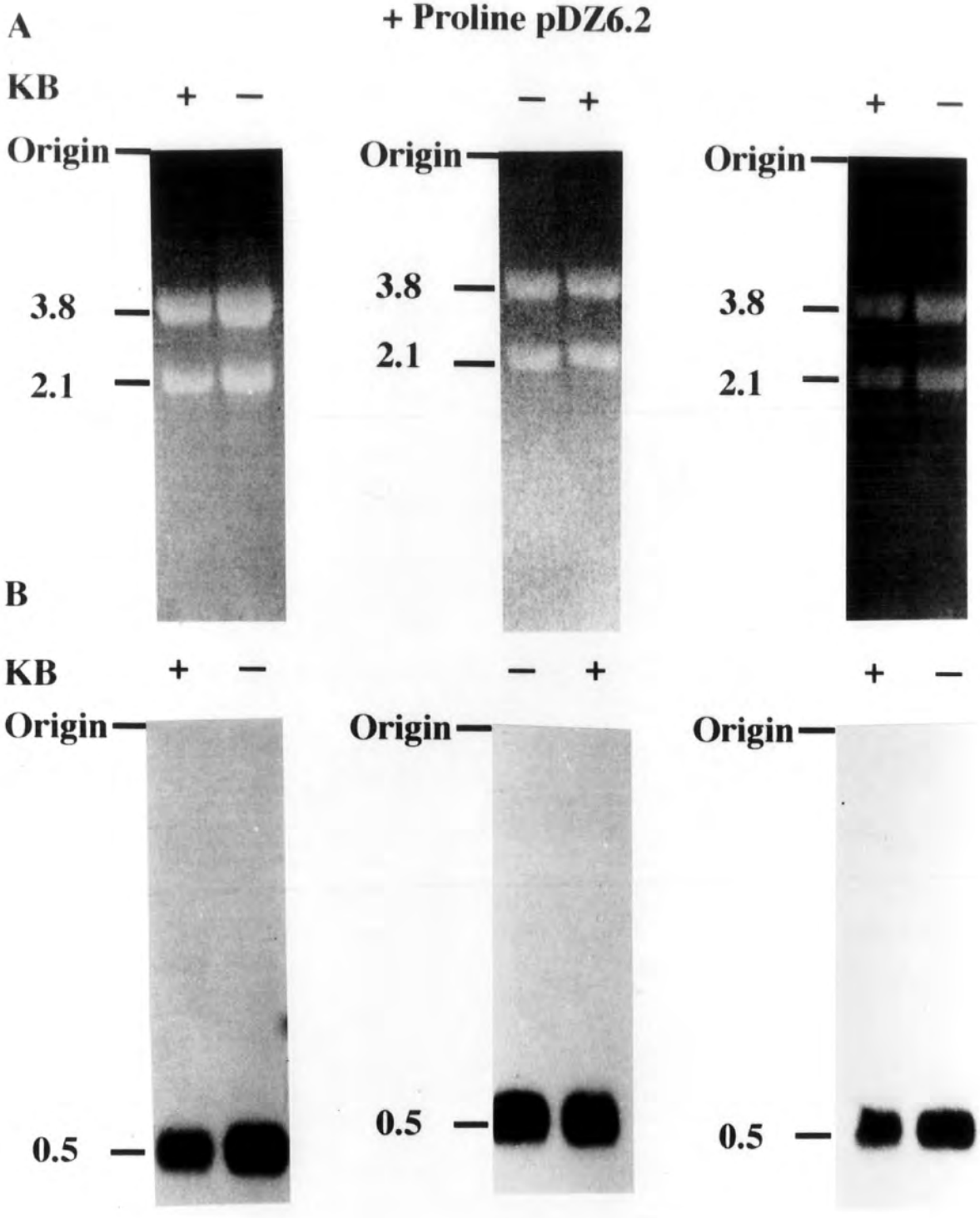
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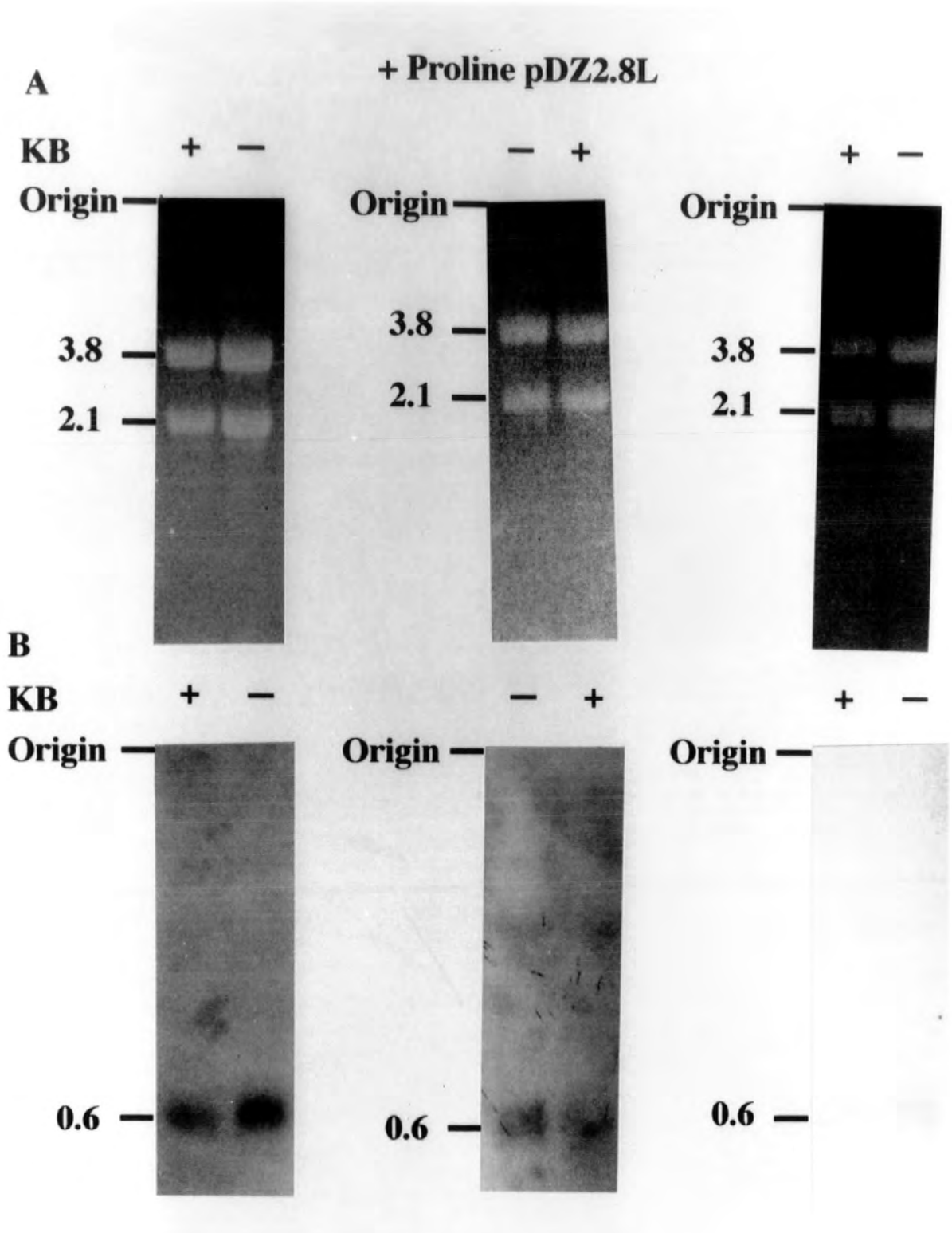
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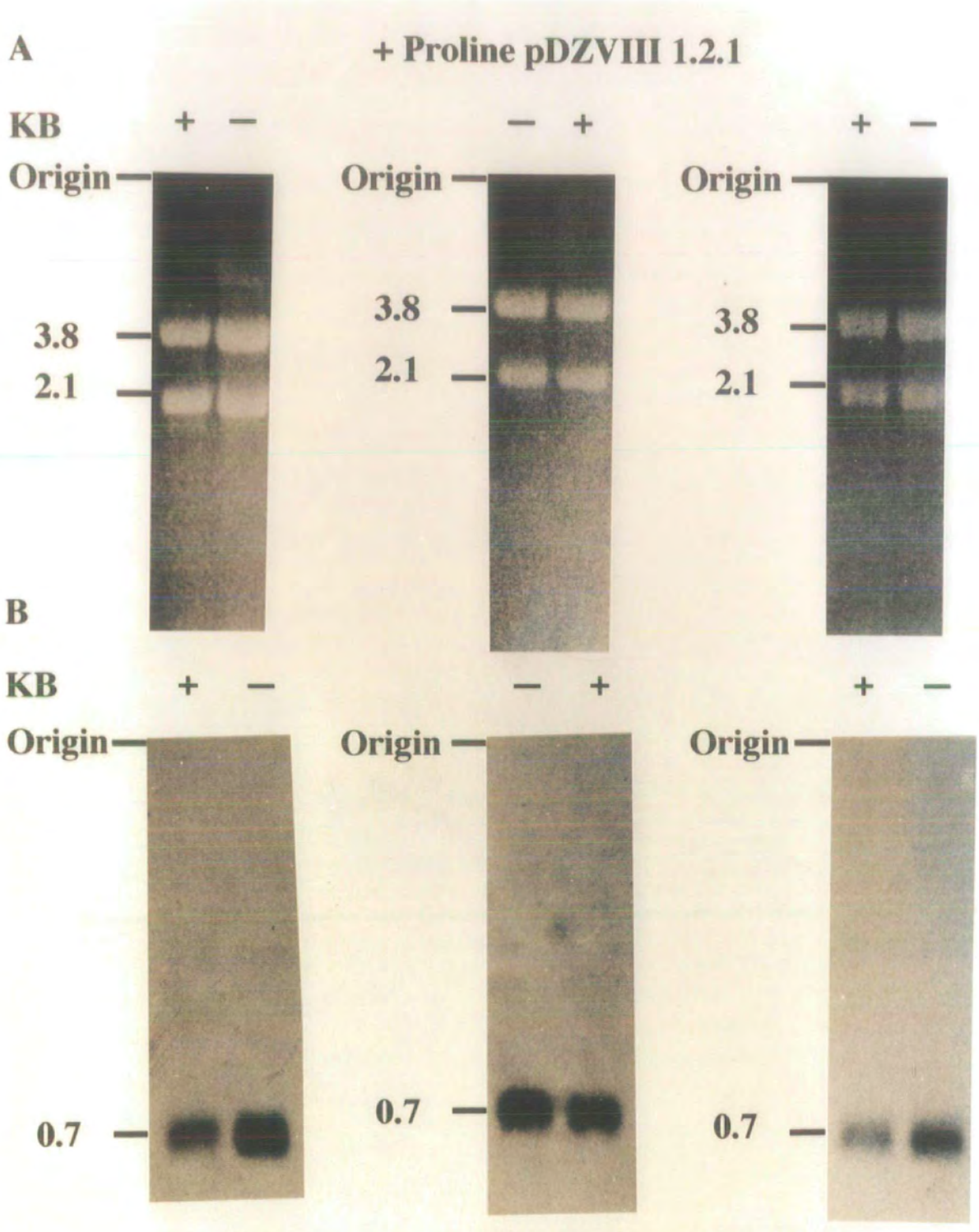
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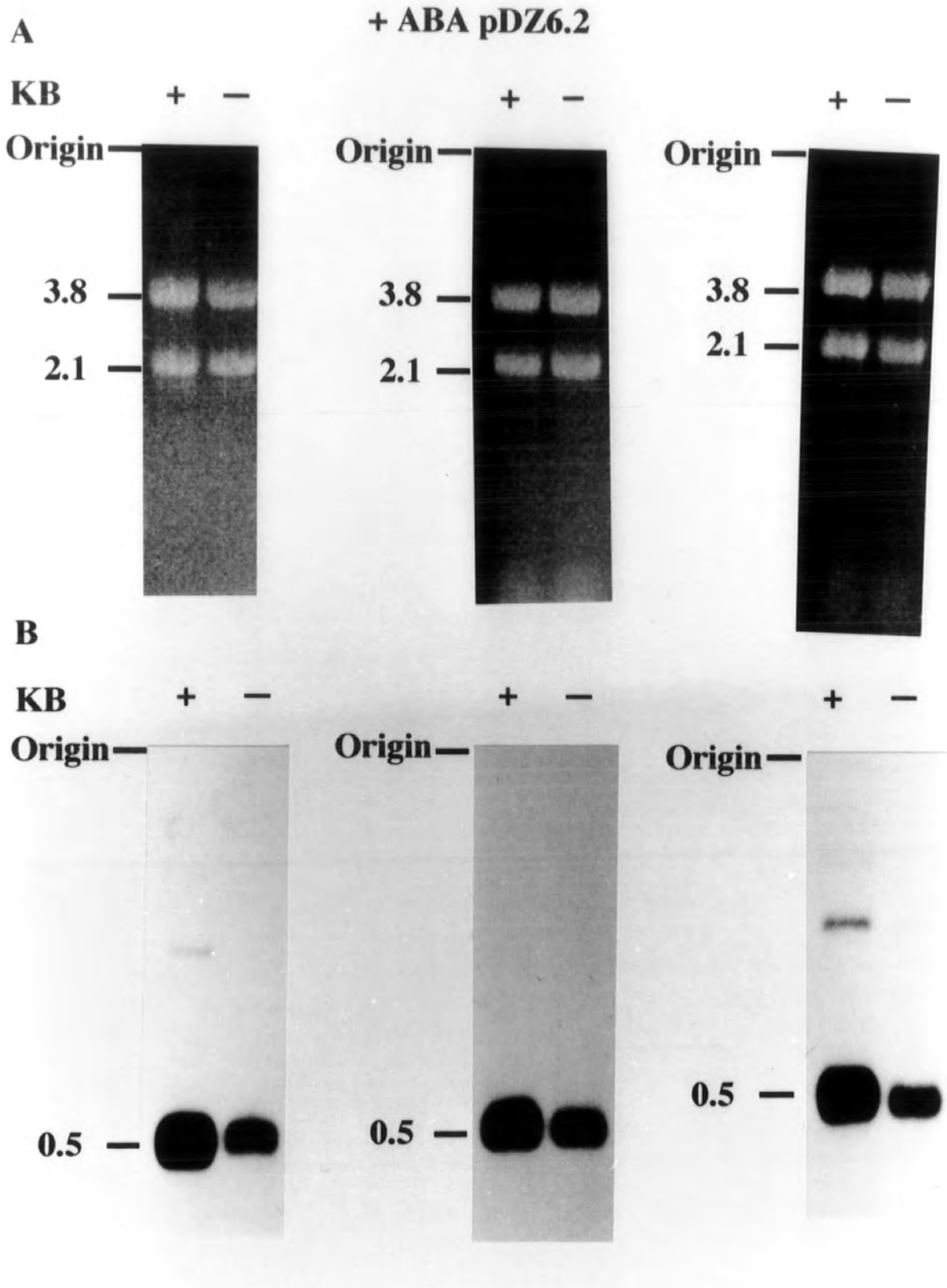
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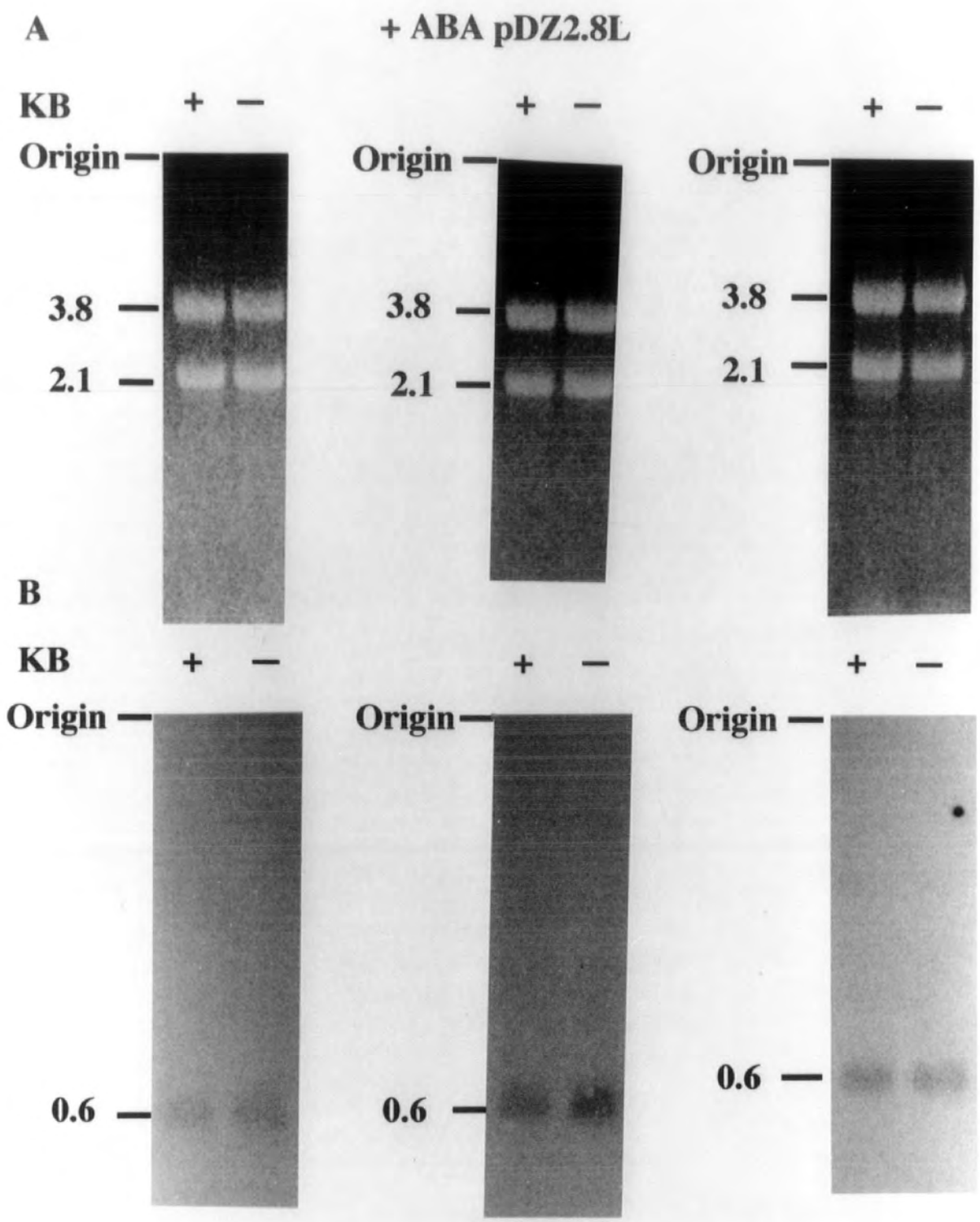
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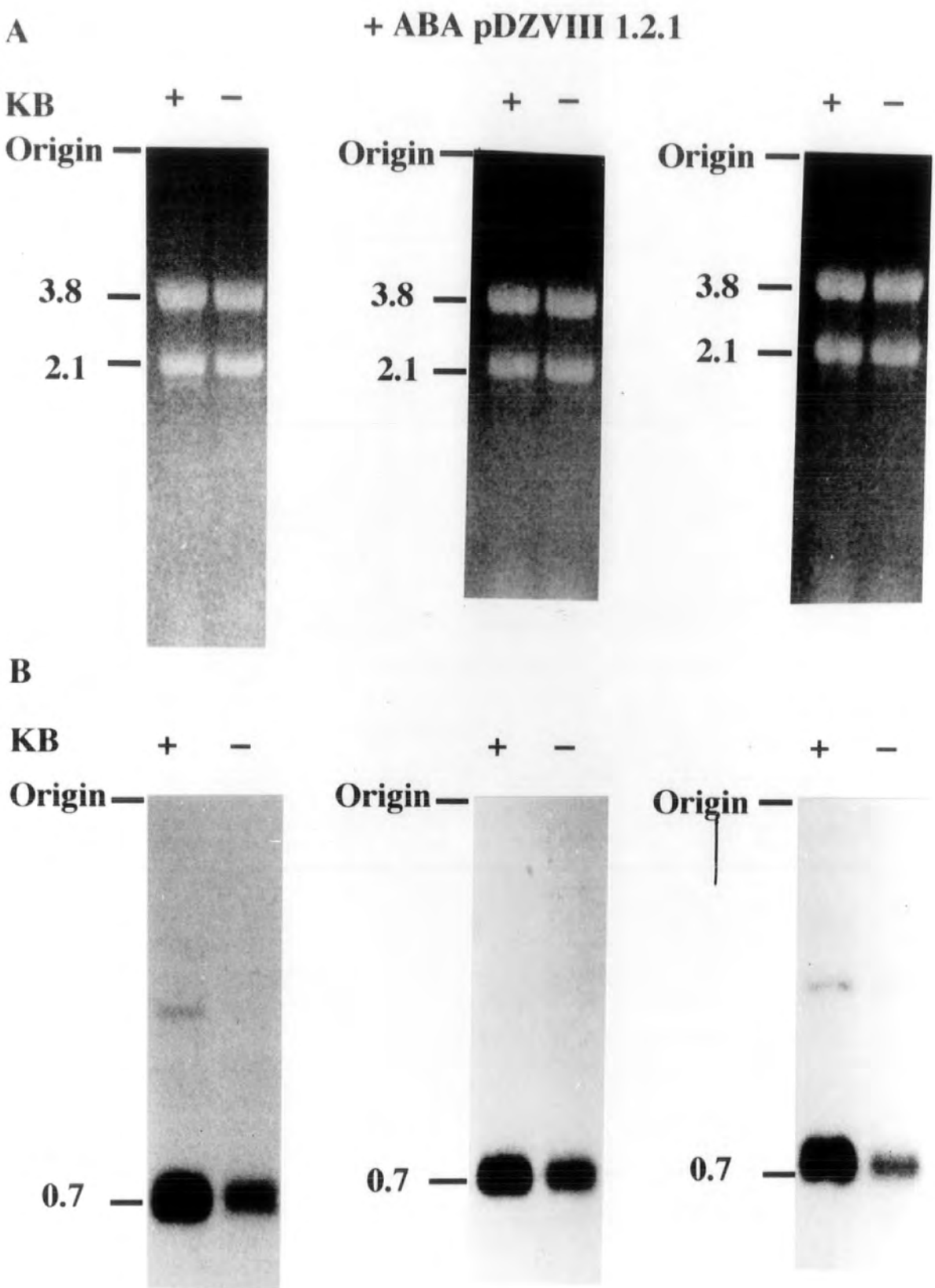
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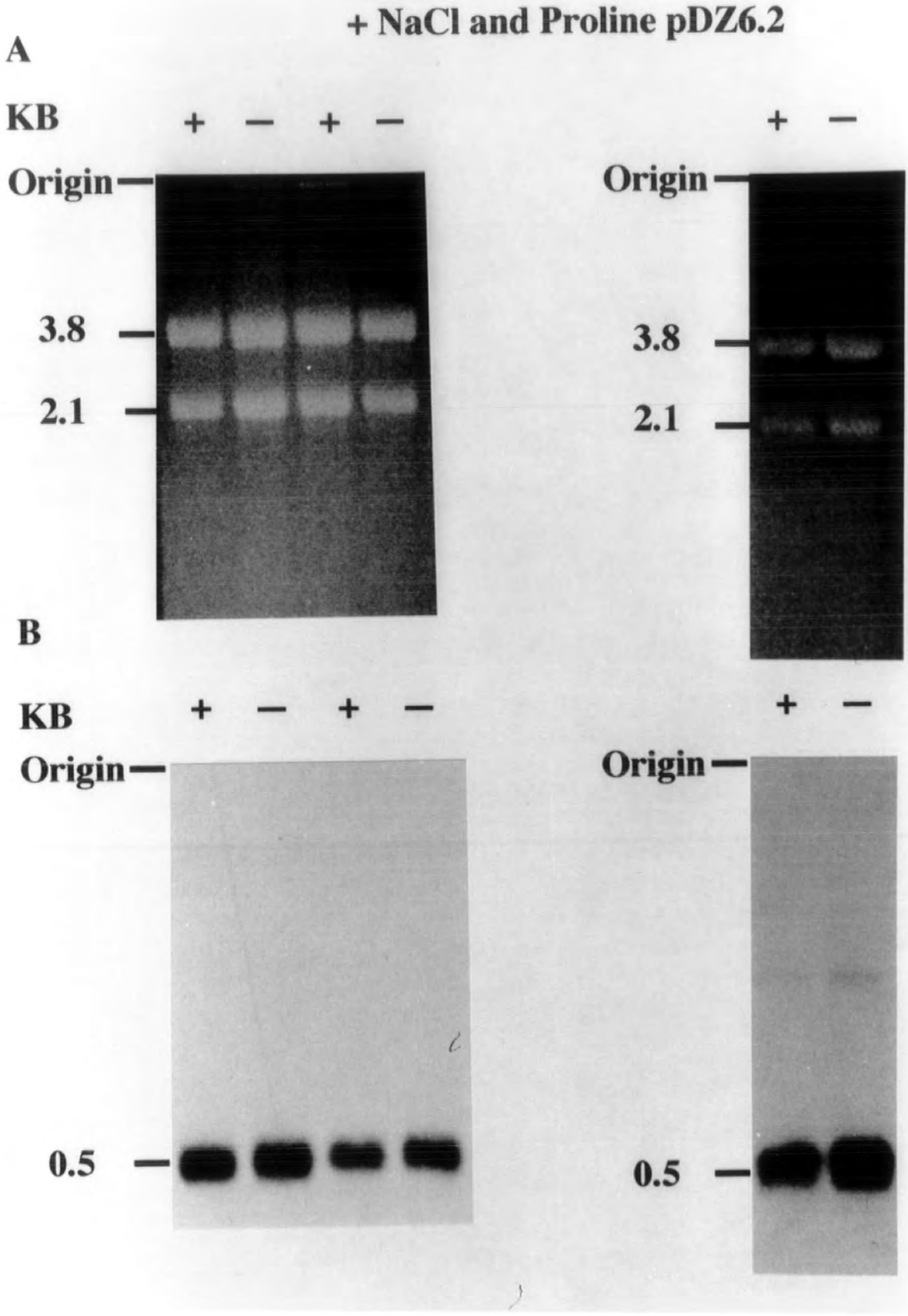
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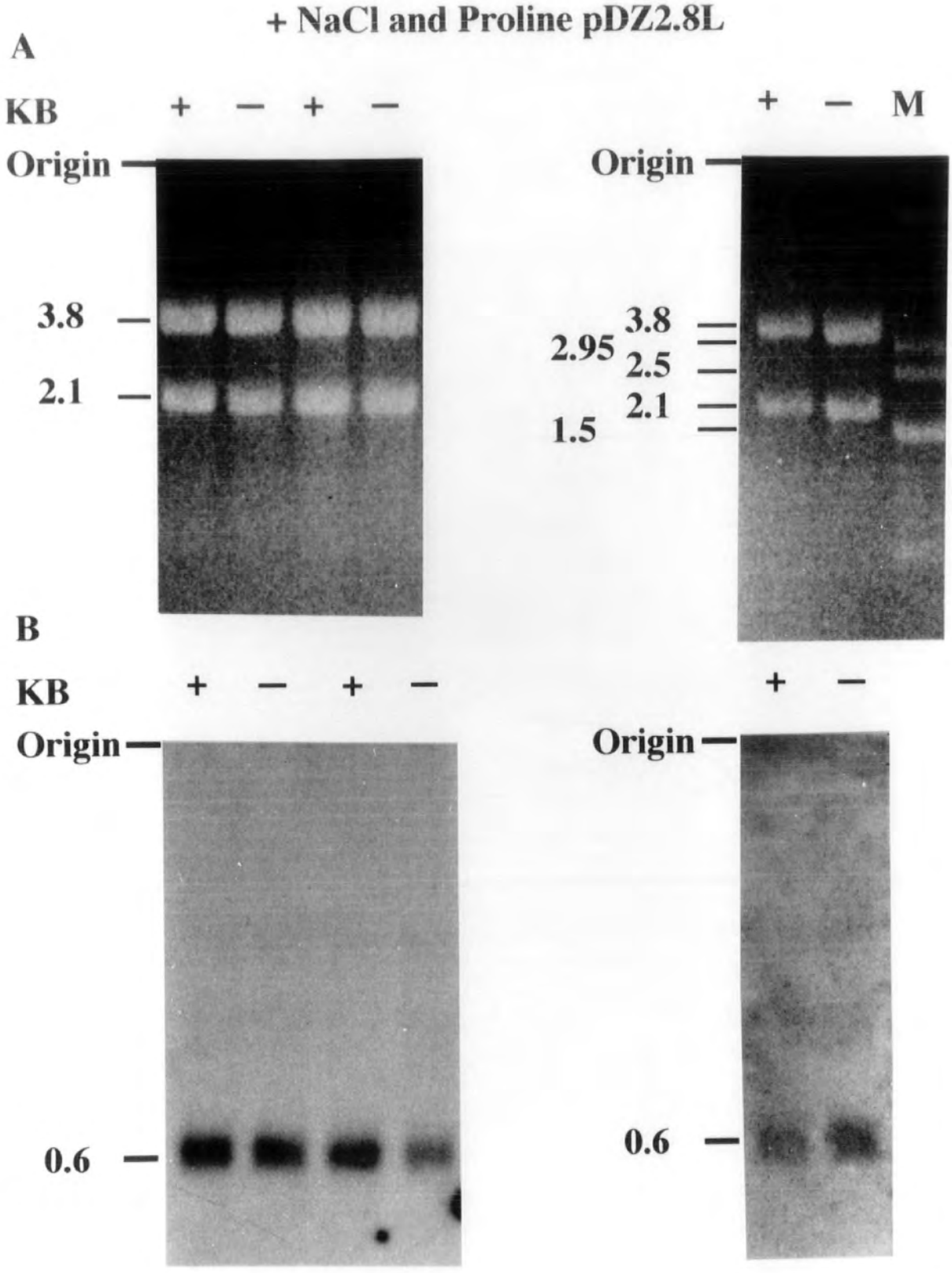
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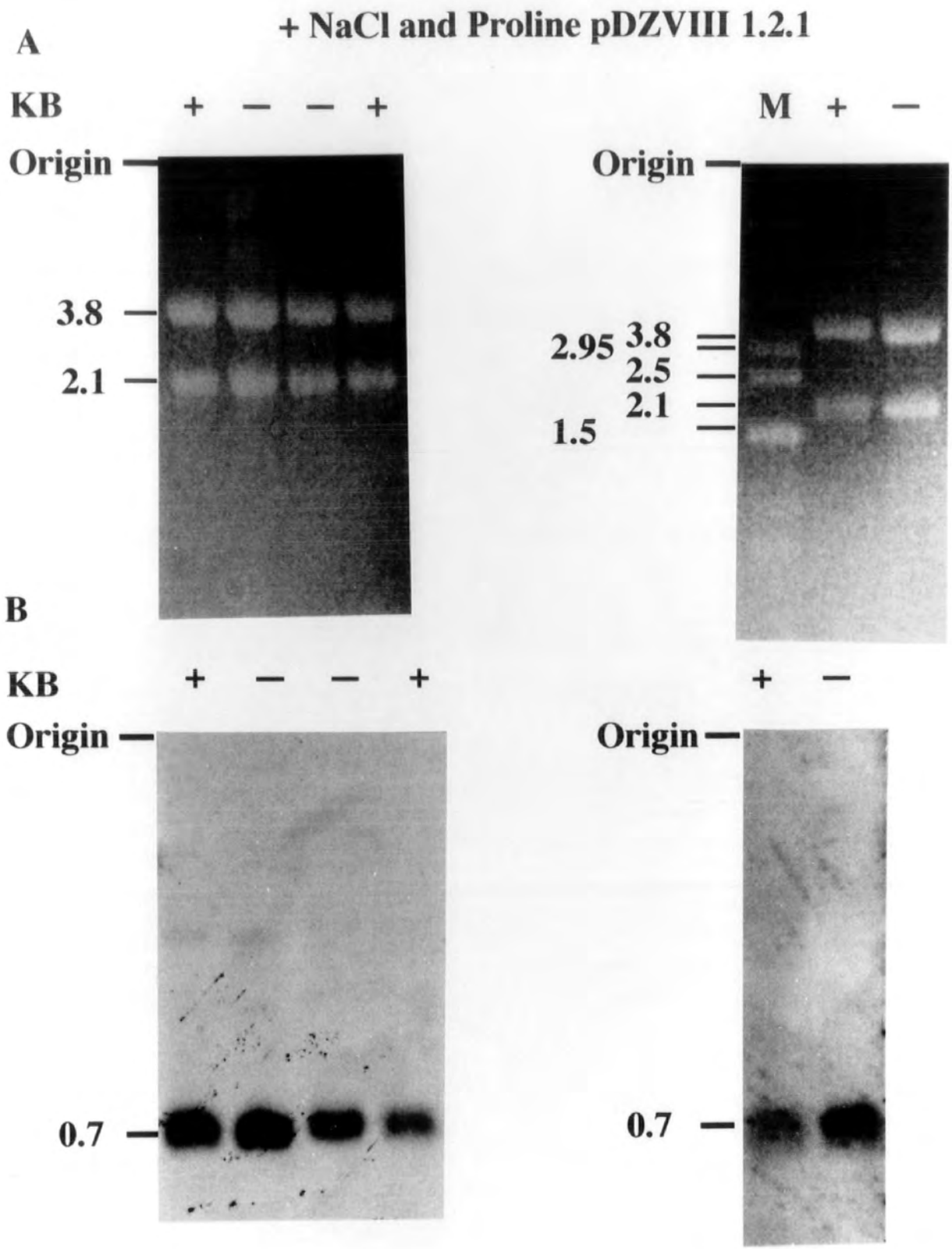
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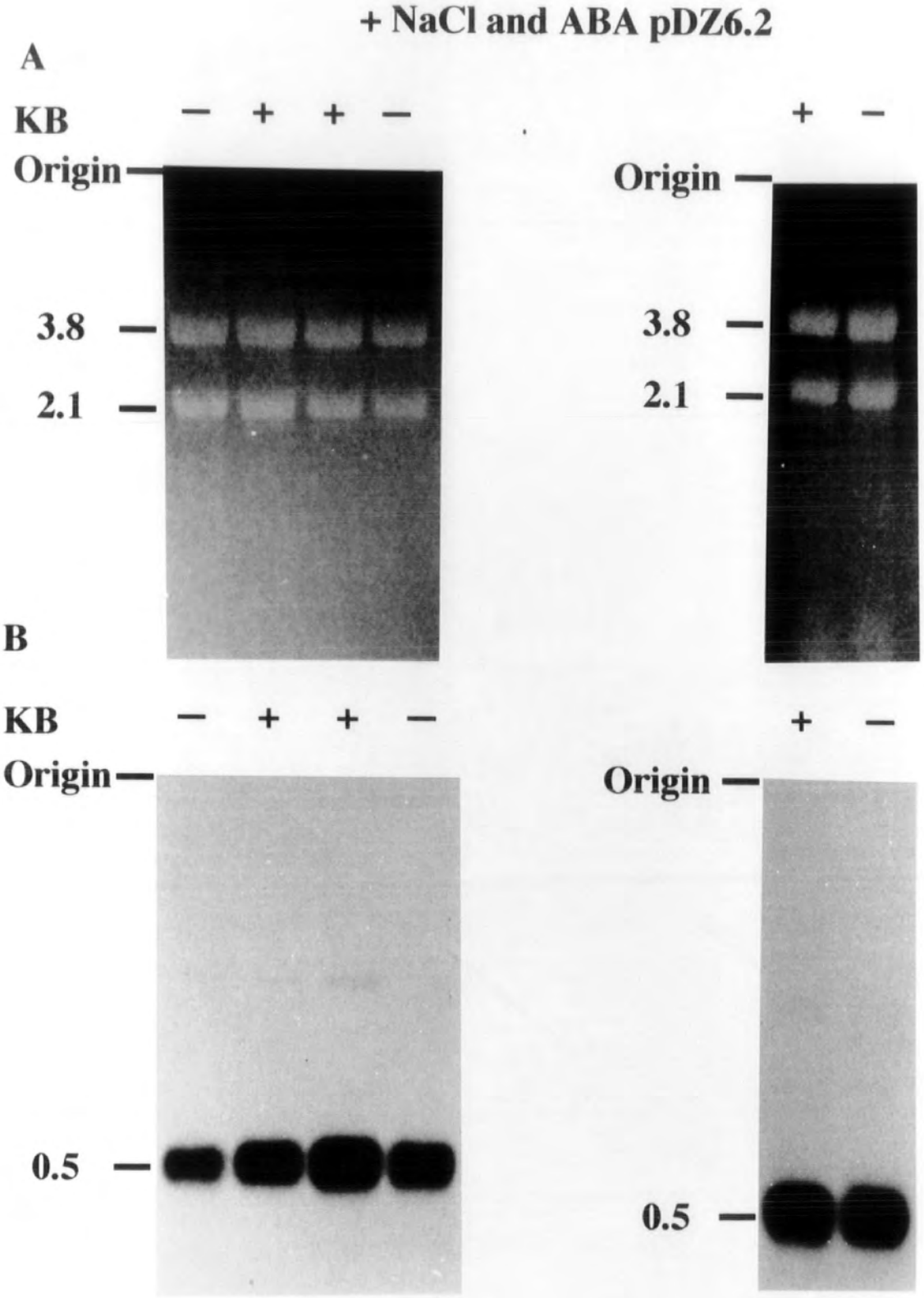
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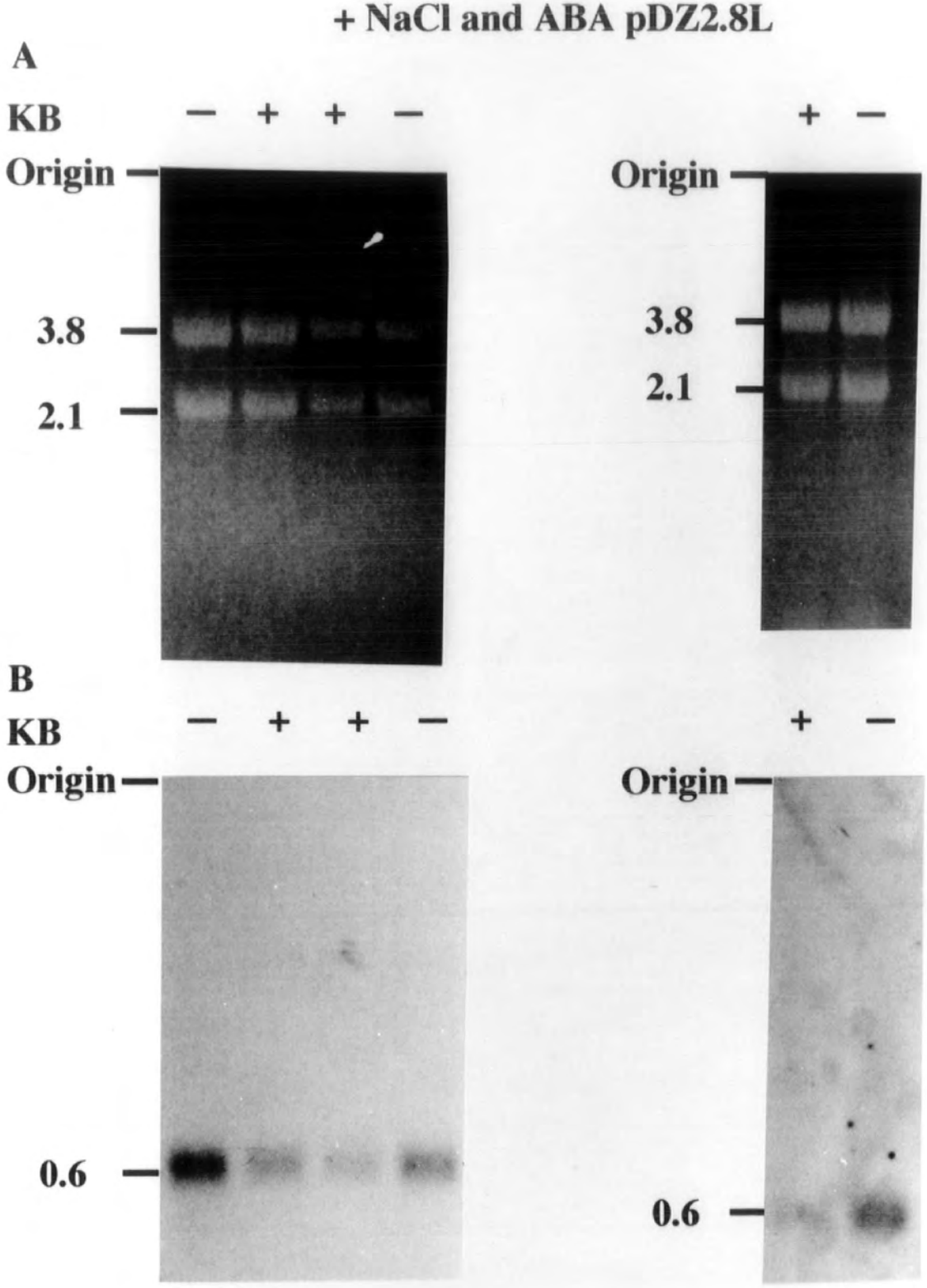
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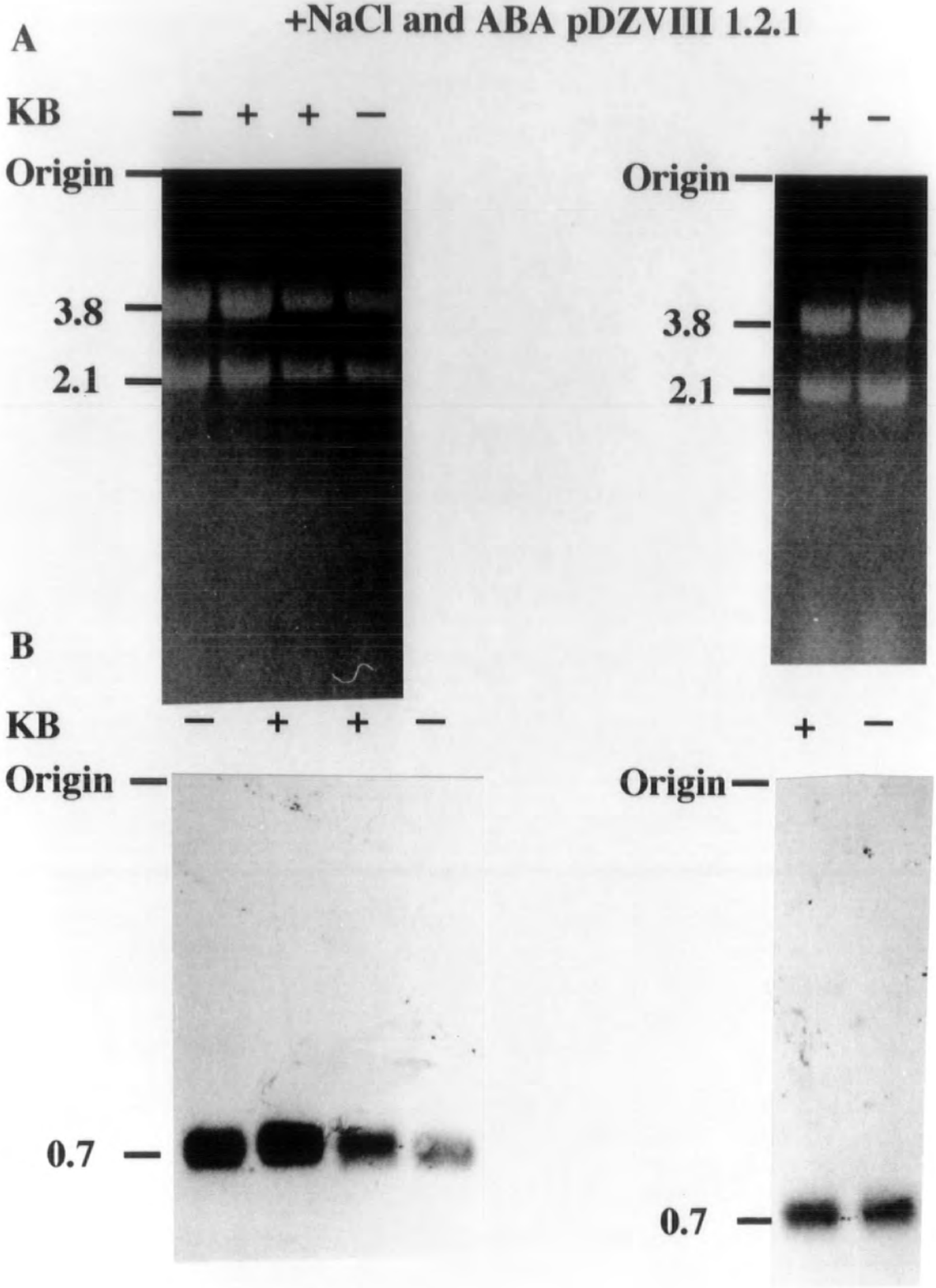
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0.7
+ –
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3.5 Experiments to investigate whether or not a GA repeat occurs in the *D. spicata* genome and to investigate its possible role in gene regulation

3.5.1 Amplification of GA repeat region from pDZ6.2 using PCR

The primers used were as described in section 2.3.1.22.2; the universal M13 reverse primer was used as the 5' primer and the 3' primer as shown below:

5' ATG TCG GCT ACG CTC 3'

The annealing temperature (T_m) was calculated as follows:

$$Tm = [4(G+C) + 2(A+T)]-3$$

$$\therefore\ Tm = [4(9) + 2(6)] - 3$$

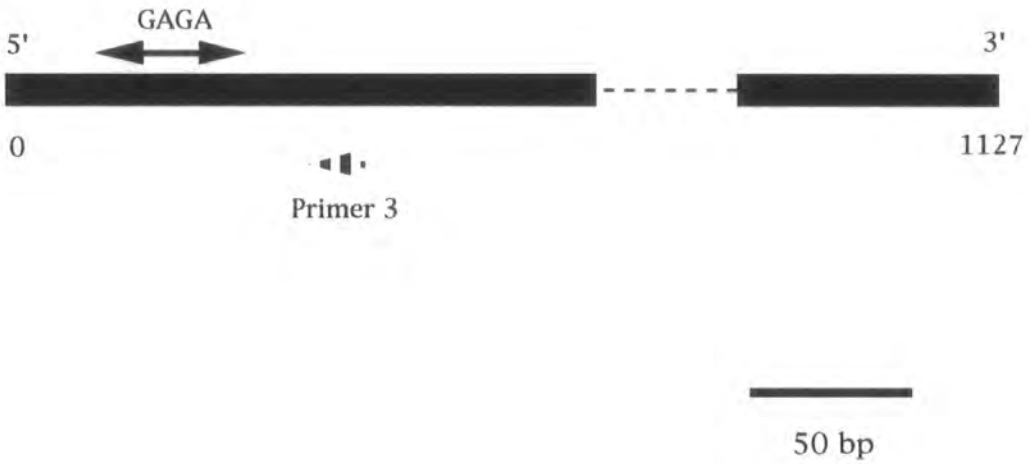
$$= 45\ ^\circ C$$

Figure 3.5.1 is a schematic diagram of pDZ6.2 and shows the position of the primer binding sites relative to the region to be amplified. The predicted product size is 235 bp.

The PCR was performed as described in section 2.3.1.22.2. The products were separated by electrophoresis on a 2% agarose gel, as in figure 3.5.2. There are two products at 201 bp and 235 bp. Both bands were excised from the gel, and the DNA purified by silica fines. Then each was cloned separately into the pGEMT vector to create the plasmids pNRCF0204 (201 bp product) and pCFGA12 (235 bp product). pNRCF0204 was cloned by Prof. N.J. Robinson. JM109 *E. coli* cells were transformed (to antibiotic resistance) with plasmids as in the manufacturer's instructions.

When the plasmids were sequenced they were found to be identical except for the length of the GA repeat. pCFGA12 (235 bp) contained the full length repeat of 68 bp, whereas pNRCF0204 (201 bp) had only half the sequence at 34 bp. This accounts for the 34 bp difference in size between the two PCR products.

Figure 3.5.1




1
2
Origin
235 bp
201 bp

3.5.2 Experiment to show whether or not the GA repeat region is part of the pDZ6.2 transcript

RNA (from cells exposed to 0 and 520 mM NaCl) was electrophoresed in duplicate sets on a 1.4 % formaldehyde agarose gel and each set blotted separately onto Hybond N. One blot was probed with ^{32}P-labelled pDZ6.2 and the other with pNRCF0204, containing the GA repeat. Figure 3.5.3 shows the results after both blots were exposed to the same X-ray film for 72 h. Both sets of bands are running at the same place, implying that they are the same transcript. The band intensities given by each probe differ greatly, pDZ6.2 band being very over exposed in comparison to pNRCF0204, which is only just visible. This is probably due to the difference in their sizes the inserts being 1127 bp (pDZ6.2) compared to 201 bp (pNRCF0204). It can also be noted that for pNRCF0204 there is an apparent increase in transcript abundance in the presence of 520 mM NaCl, which is not seen with pDZ6.2, probably due to over exposure of the X-ray film.

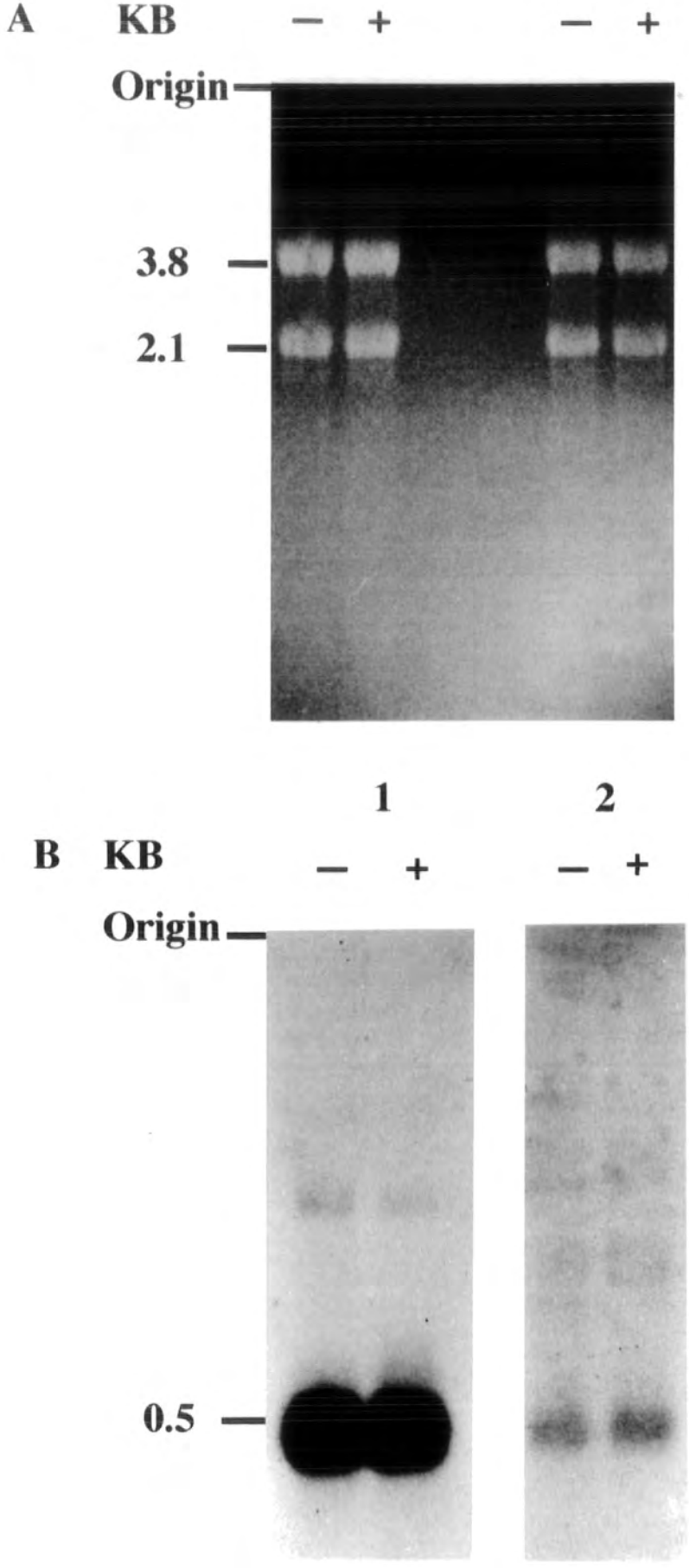
In conclusion there is a corresponding transcript containing the GA repeat region that gives a signal when probed with the GA region alone and the entire clone, from which it comes from.

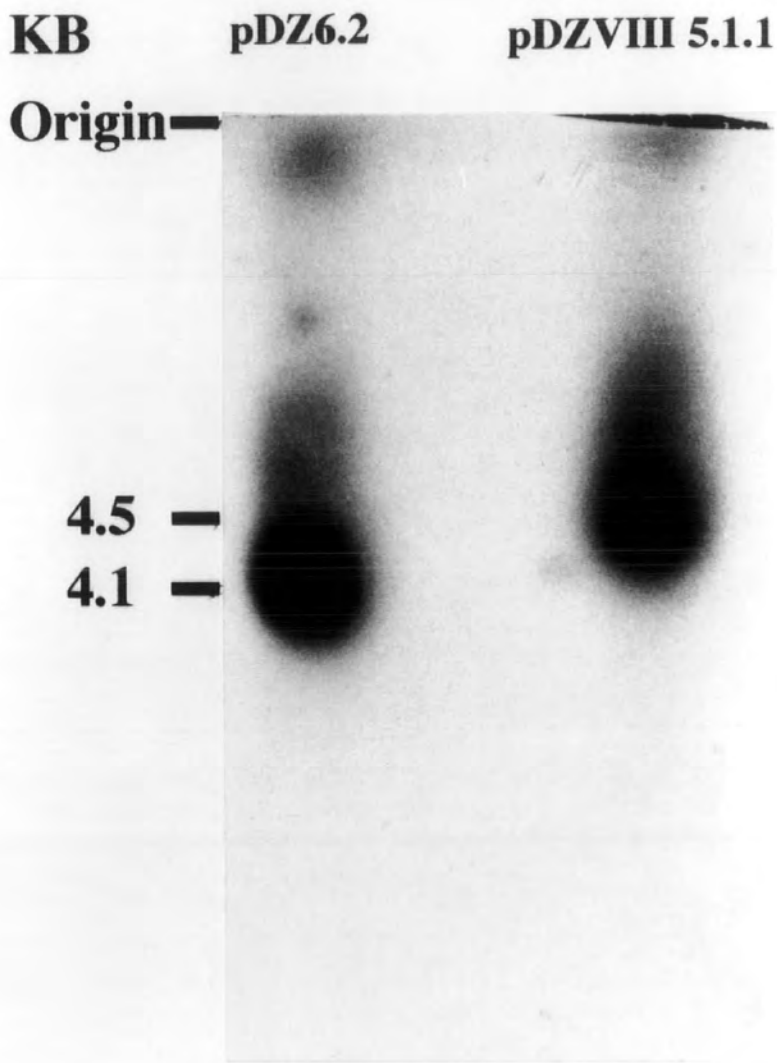
3.5.3 Experiment to show whether the GA repeat region plays a role in transcriptional regulation

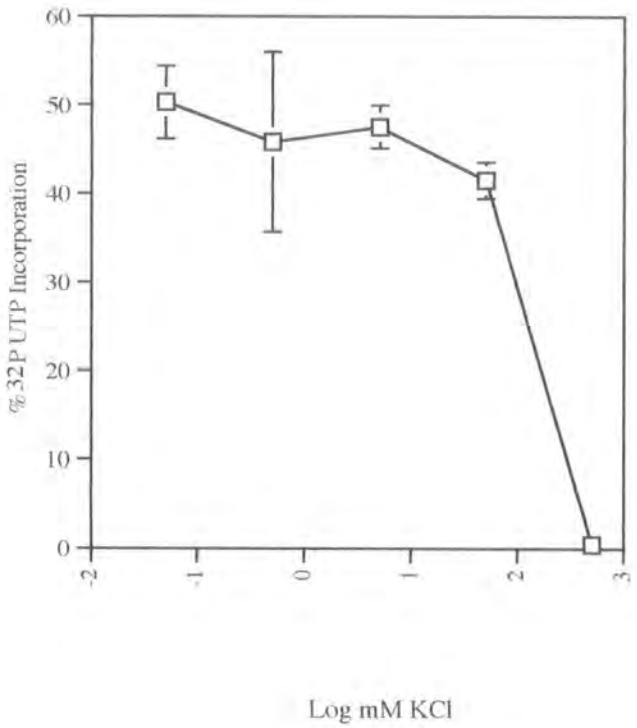
in vitro transcription reactions were performed using a MEGAscript™ T3 kit. pDZVIII 5.1.1 was used as the GA negative control. An initial experiment was carried out to ensure that the ^{32}P UTP was being incorporated into the correct products. Two reactions were set up, one with pDZ6.2 as the template and the other with pDZVIII 5.1.1. After a four hour incubation 12 µl of each reaction was removed and loaded onto a 1.4 % formaldehyde agarose gel. After electrophoresis the gel was frozen and exposed for one hour to X-ray film. Figure 3.5.4 shows the results. The two signals represent the transcriptional products, and relative to each other, the sizes are consistent with those observed before. This shows that bulk of the radiolabel is being incorporated into the specific transcripts and thus in other experiments, when measuring percentage incorporation, all of the precipitable radiolabel is attributed to incorporation into these transcripts.

Reactions were set up, that included a range of KCl concentrations, 0, 5 µM, 50 µM, 500 µM, 50 mM and 500 mM KCl, with pDZVIII 5.1.1 as the template DNA. The incorporated label was TCA precipitated and percentage incorporation measured. Results were plotted as a semi log graph, % incorporation versus Log KCl concentration, as in figure 3.5.5. The percentage incorporation is reasonably stable between 0 and 50 mM KCl, but there is a rapid drop after 50 mM, implying inhibition of the T3 RNA polymerase at approximately 200 - 300 mM KCl.

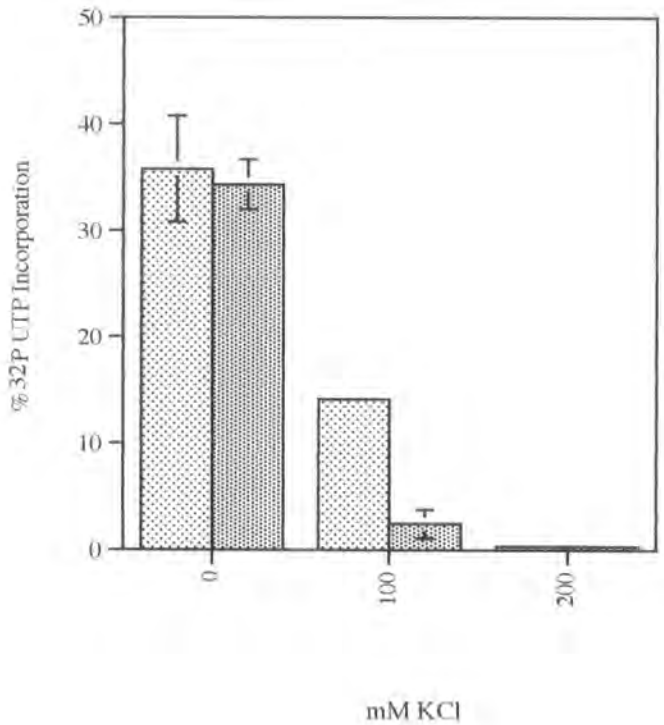
The first experiment to be performed, compared the percentage incorporation into the transcriptional products of pDZVIII 5.1.1 and pDZ6.2 at 0, 100 and 200 mM KCl. The results were plotted as a bar chart as shown in figure 3.5.6. Complete inhibition of activity occurred at 200 mM KCl for both plasmids, but when comparing the incorporation levels at 0 and 100 mM KCl there was a much greater decrease between 0 and 100 mM KCl for pDZVIII 5.1.1 than pDZ6.2. There was a 93 % inhibition of percentage incorporation for pDZVIII 5.1.1 as compared to a 60 % decrease for pDZ6.2. Similar experiments were carried out, comparing 0 and 100 mM KCl. The results obtained were extremely variable and are shown in figures 3.5.7 and 3.5.8. In 3.5.7 there is a much higher percentage incorporation for pDZVIII 5.1.1 at 0 mM KCl as compared to pDZ6.2, but there is a severe reduction at 100 mM KCl a 21% reduction compared to a 14 % increase for pDZ6.2 (29.5 % incorporation at 0 mM KCl to 34.4 % at 100 mM). In figure 3.5.8, the general levels of incorporation are much lower than those obtained in all other reactions. This time there is little (if no) change for pDZ6.2 between 0 and 100 mM KCl, the percentage incorporation staying at 17 %. For pDZVIII 5.1.1 there is a slight reduction from 19.1 % (0 mM) to 14.1 % (100 mM).


A
KB
–
+
–
+
Origin
3.8
2.1
1
2
B
KB
–
+
–
+
Origin
0.5

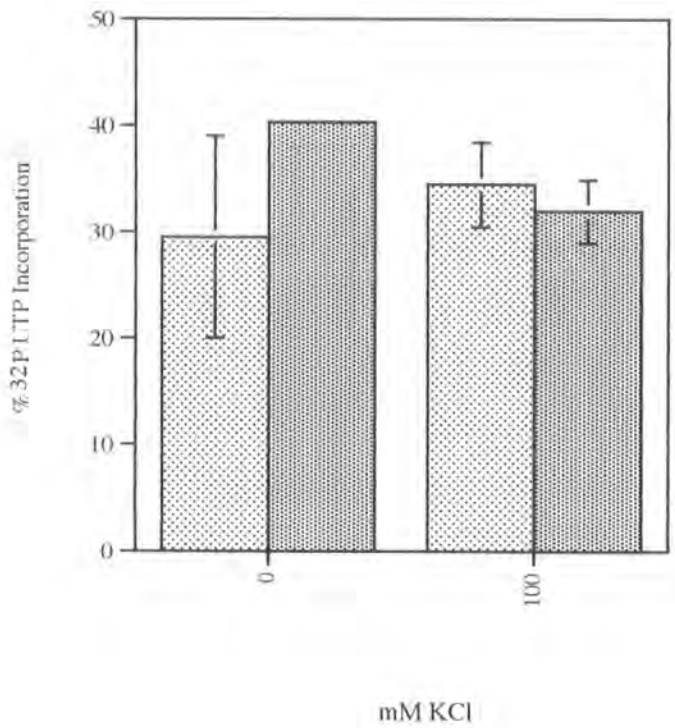

KB
pDZ6.2
pDZVIII 5.1.1
Origin
4.5
4.1


60
50
40
30
20
10
0
% 32P UTP Incorporation
-2
-1
0
1
2
3
Log mM KCl

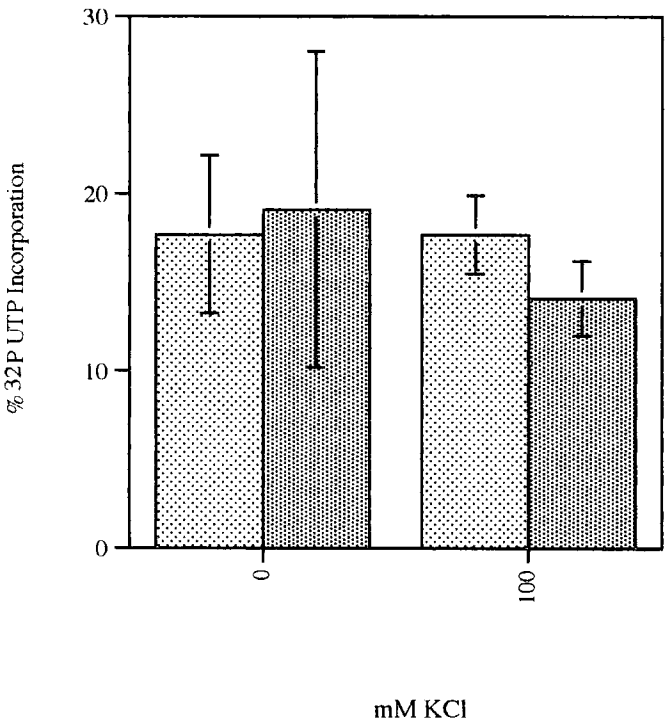
Effect of KCl on *in vitro* transcription
of plasmids pDZ6.2 and pDZVIII 5.1.1



Effect of KCl on *in vitro* transcription
of plasmids pDZ6.2 and pDZVIII 5.1.1



Effect of KCl on *in vitro* transcription
of plasmids pDZ6.2 and pDZVIII 5.1.1



3.5.4 Attempt to amplify a genomic fragment corresponding to the GA-repeat region of pDZ6.2

Experiments were carried out to investigate whether or not the GA-repeat region is in the genome of *D. spicata*, by using anchored PCR.

The 3' primers used in the reactions are shown below, the 5' primers were the universal M13 forward and T7 primers.

Primer 1 5' ATA GTG GTG ATC CTT CTT GG 3'

Primer 2 5' TA GGG GTC CTT GGG TTT GTA 3'

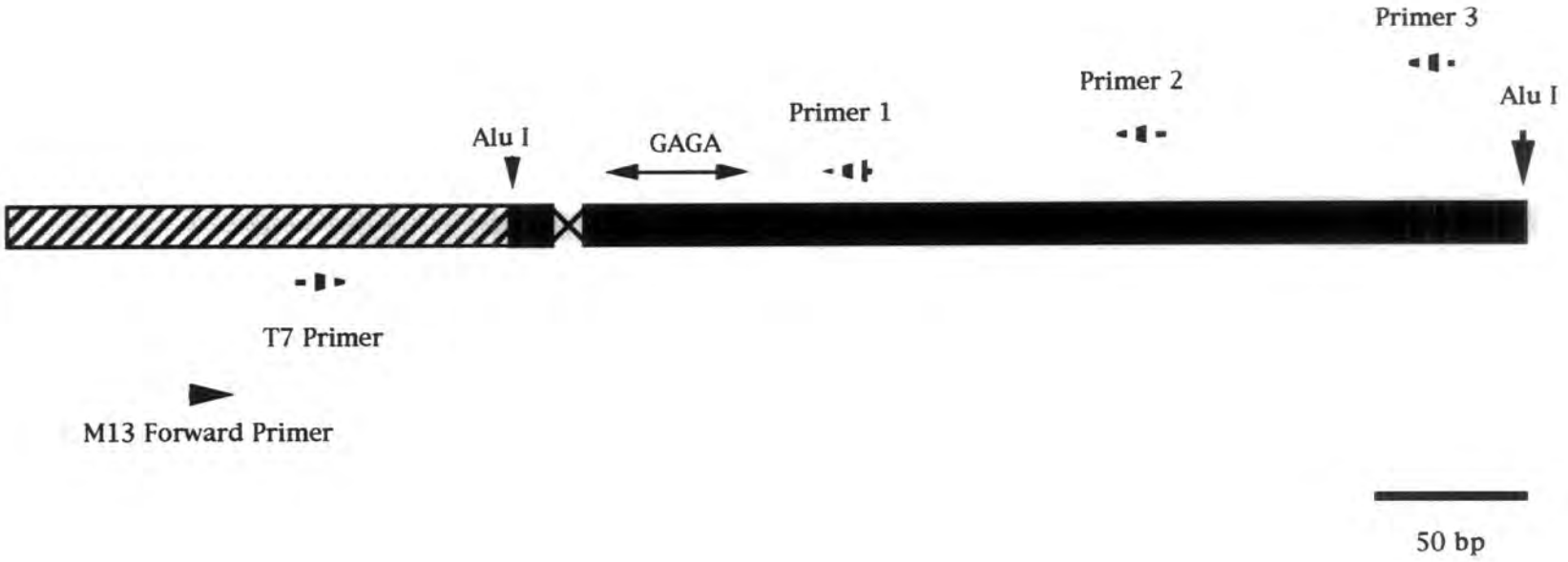
Primer 3 5' ATG TCG GCT ACG CTC 3'

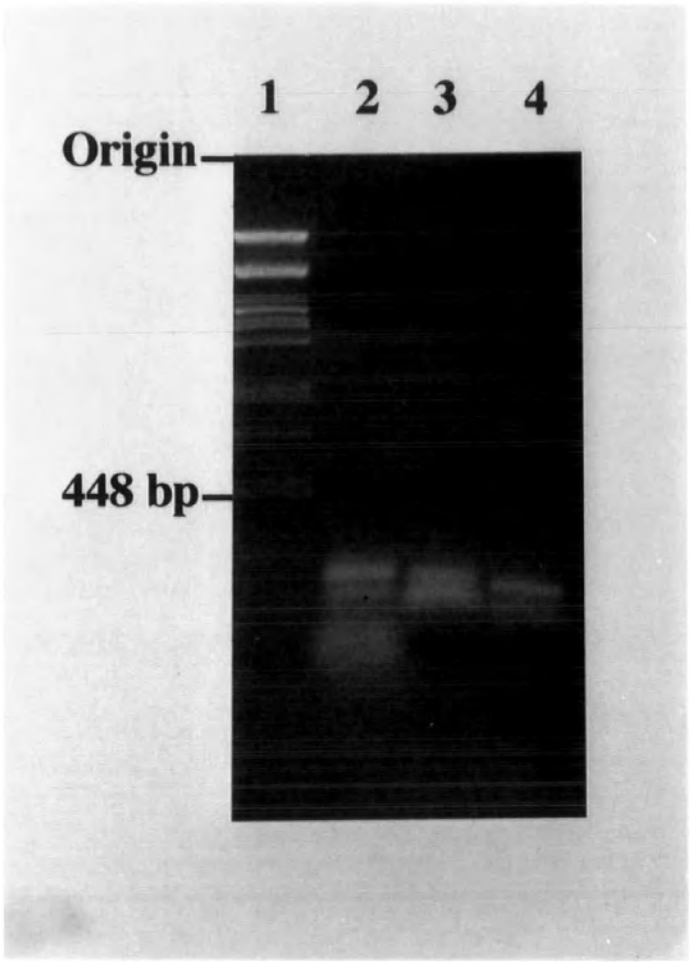
Figure 3.5.9 is a schematic diagram that shows the position of the primer binding sites on the genomic fragment and the strategy behind the experiment. The PCR's were performed in succession and the products from each reaction electrophoresed on a 1.2 % agarose gel. These results are shown in figure 3.5.10, and there are bands present in all three lanes. The smallest of these are most probably the primers, but the others could be the product. However none of them appear to be of the predicted sizes; reaction 1 (primer **1** and M13 forward) would give a product of 417 bp, reaction 2 (primer **2** and T7) would give 291 bp; reaction 3 (primer **3** and T7) would give a 189 bp product. (These sizes are calculated on the assumption there are no introns in the genomic clone of pDZ6.2). Lane 1 doesn't contain a 417 bp band, but there could be a 291 bp band in lane 2, and likewise a 189 bp band in lane 3. It was considered possible that the percentage of the gel wasn't high enough to clearly define these bands, but on repeating it with a higher percentage gel, no better resolution was obtained.

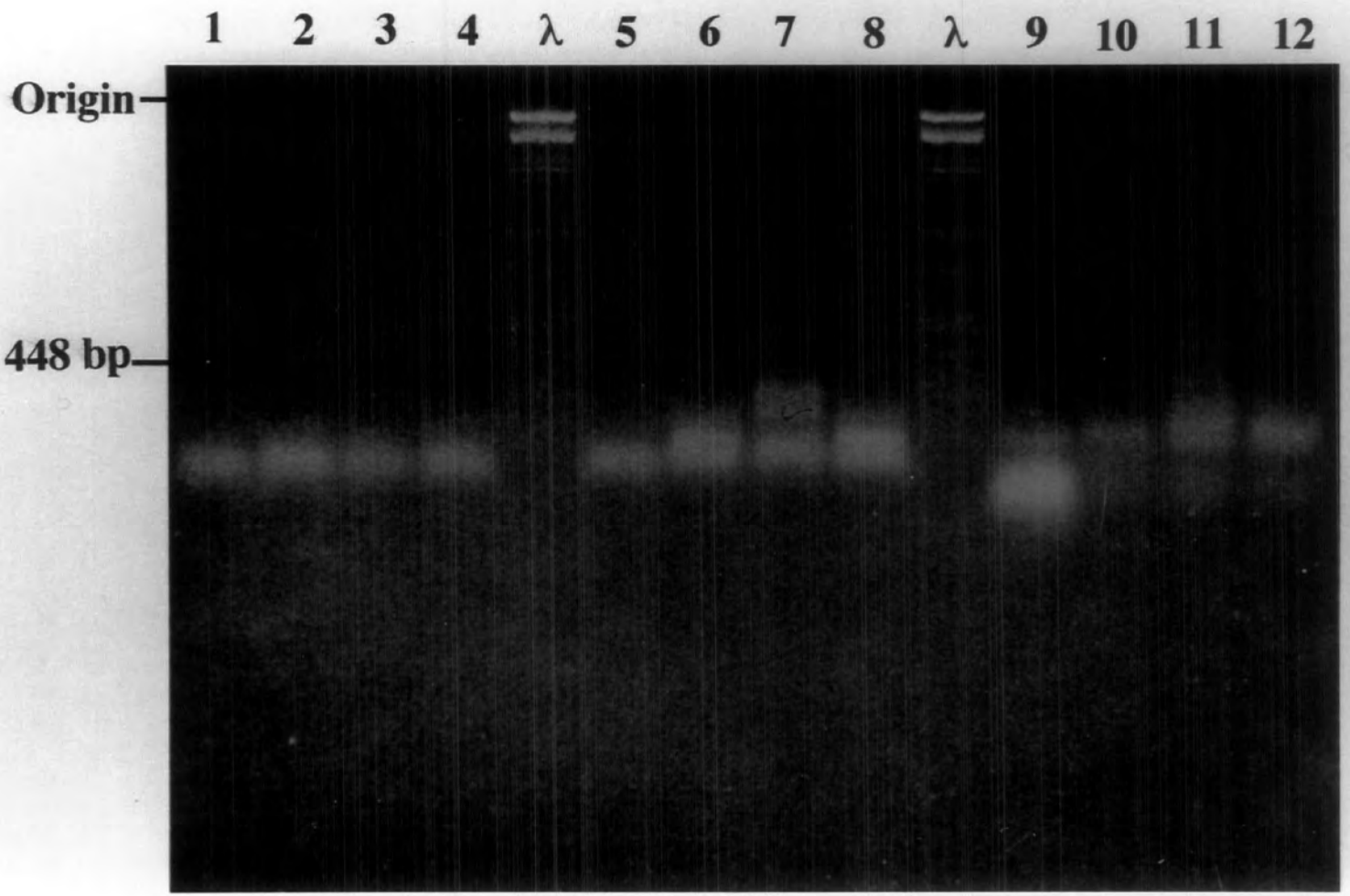
The reactions were repeated, but this time including additional control reactions, genomic DNA alone, vector alone and H_2O. These would define which bands produced were non specific and which, if any, were the desired product. Figure 3.5.11 shows the electrophoresed products. After the first reaction (lanes 1 - 4) there appear to be no products at all from either the controls or the ligation, only the primers are visible. Bands appear after the second reaction, but mostly in the genomic DNA and vector control lanes (lanes 6 and 7). There is no band of the predicted size (291 bp) in the ligation lane (lane 5). After reaction 3 there are more bands visible in the vector lane (lane 11) of larger sizes, but there are still no bands of the predicted size in the ligation lane (lane 9). There are also bands visible in the H_2O lanes that can't be attributed to primers, which leads to the conclusion that there must have been a certain amount of contamination.

Since it was possible that the desired product wasn't visible to the naked eye, a Southern blot was taken of the gel and probed with the ^{32}P labelled insert from pDZ6.2. No clear bands or signal were visible on X-ray film exposed to the hybridised blot for over a month. (Results not shown). The anchored PCR and blot were repeated several times, but no signal was detected. Therefore it appears that this anchored PCR strategy was unable to amplify the desired region of the genomic clone of pDZ6.2, and has not identified whether or not the GA repeat region occurs in the *D. spicata* genome.

Figure 3.5.9


Primer 3
Primer 2
Alu I
Primer 1
Alu I
GAGA
T7 Primer
M13 Forward Primer
50 bp


1
2
3
4
Origin
448 bp


1 2 3 4 λ 5 6 7 8 λ 9 10 11 12
Origin
448 bp

Chapter 4

Discussion

In this chapter the following topics will be discussed; the sequence similarities of the putative proteins encoded by the inserts of cDNA clones and their possible roles in salt tolerance of *D. spicata*; the response of transcripts corresponding to inserts of pDZ6.2, pDZVIII 1.2.1 and pDZ2.8L to NaCl, proline and ABA and the significance of this for salt tolerance mechanisms. Literature describing GArepeat sequences; the presence of a 68 bp GA repeat in the 5' untranslated region of pDZ6.2 and its possible role in gene regulation at a transcriptional or translation level; the failure to identify a P5CR gene homologue from *D. spicata* by heterologous probing of Southern blots or PCR.

4.1 Attempt to isolate of a P5CR gene homologue from *D. spicata*

Proline is accumulated in cells of *D. spicata* within eight hours of exposure to elevated levels of NaCl (Heyser, et al., 1989b). A similar response is seen in *E. coli* (Csonka, 1989). Proline is synthesised from glutamate in *E. coli*, as in figure 1.5.1, as it is in yeast (*Saccharomyces cerevisieae*) (Brandriss and Falvey, 1992). Plants also synthesise proline from glutamate (figure 1.5.2) and via orthinine (Delauney, *et al.*, 1993a). In both these pathways the final step from pyrroline-5-carboxylate (P5C) to proline is catalysed by pyrroline-5-carboxylate reductase (P5CR). P5CR, its activity, or its gene have been isolated from a number of plant species, including, soyabean (Delauney and Verma, 1990), tobacco (La Rosa, *et al.*, 1991), pea (Rayapati, *et al.*, 1989; Williamson and Slocum, 1992), *Mesembryanthemum nodiflorum* (Treichel, 1986) and *Arabidopsis thaliana* (Verbruggen, *et al.*, 1993).

pProCI is the cDNA clone of P5CR isolated from a soyabean cDNA expression library (Delauney and Verma, 1990). This was used as a heterologous probe of Southern blots of *D. spicata* genomic DNA, to isolate a gene homologue. No signal was obtained, even after lowering the stringency of the hybridisation conditions, by lowering the temperature. If there is such a gene present in the *D. spicata* genome, then it must be sufficiently different to pProCI such that no hybridisation was able to take place.

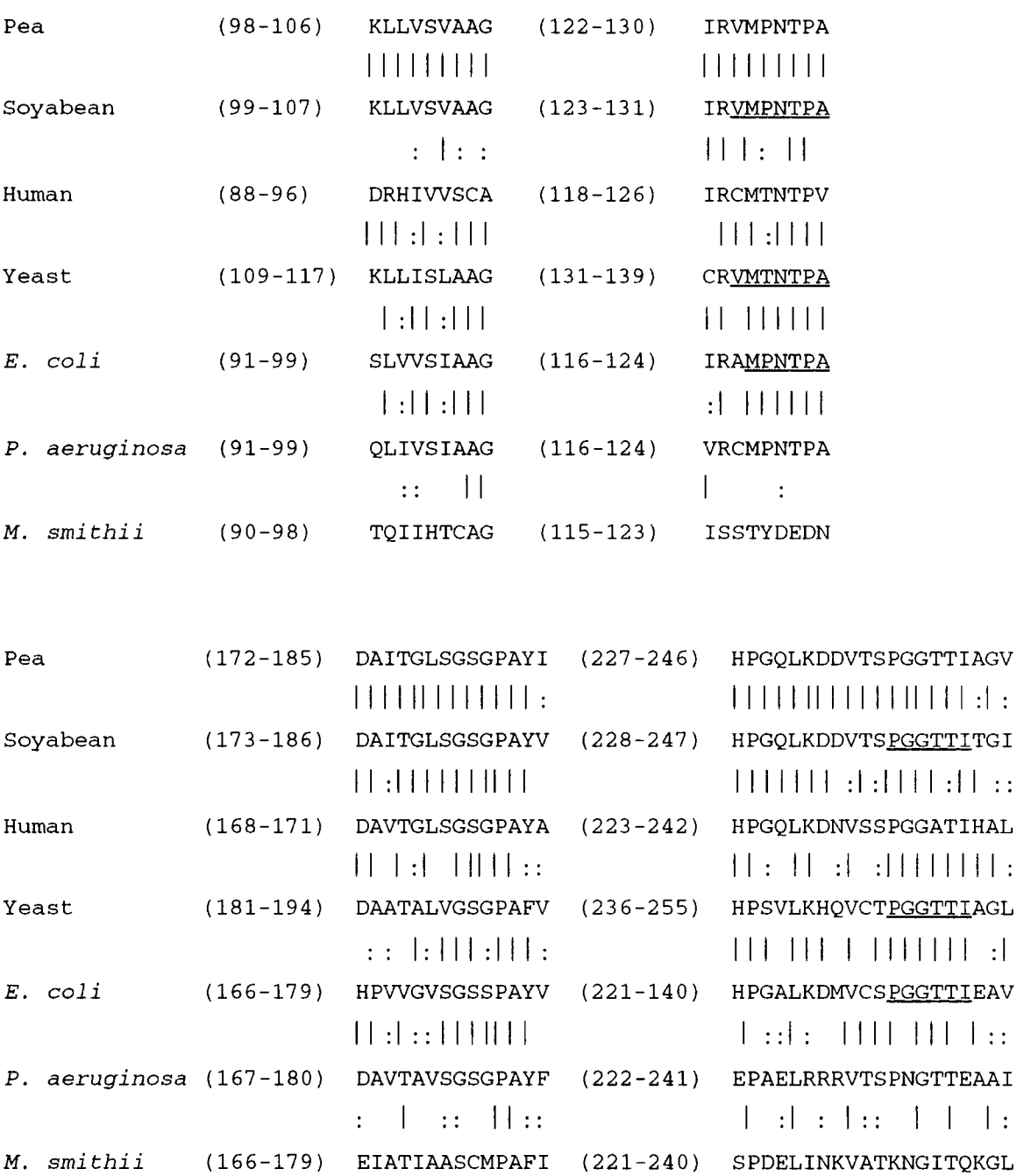
A similar conclusion can be reached to explain the failure of the PCR to identify a P5CR gene homologue. The primers were designed by the comparison of three known P5CR amino acid sequences from *E. coli, S. cerevisieae* and pProCI. There is a certain level of

conservation between the three sequences, and it was the regions of highest similarity that were used as the primers. Unfortunately this resulted in both primers having a high number of inosines, due to the high degree of degeneracy. The primers were only specific enough to produce a P5CR product from *S. cereviseae*, and not from the pProCI template. It is therefore not surprising that no product was obtained from *D. spicata*, when nothing was obtained from pProCI and it might be expected that a P5CR gene from *D. spicata* would have a greater similarity to pProCI, than to *S. cerevisieae* P5CR.

Most organisms appear to use both the orthinine and glutamate pathways for synthesising proline (Adams and Frank, 1980). Both these pathways require P5CR for the last step. Therefore it seems unlikely that *D. spicata* doesn't have this enzyme, although it has never been isolated, or its activity measured, in this particular plant. However, proline biosynthesis has been followed by ^{13}C-NMR analysis from L-[5-^{13}C] to L-[5-^{13}C]-proline and detected ^{13}C enrichment of carbon -5- of proline (Heyser, *et al.*, 1989a). Therefore this demonstrates that proline is synthesised from glutamate in *D. spicata*. Figure 4.1.1 shows conserved sequences among eukaryotic and prokaryotic P5CRs, as compared with the pea P5CR amino acid sequence. The underlined sequences represent those used in the design of the primers, and shows that they lie within regions with the highest level of conservation. The pea and soyabean sequences shown are virtually identical, showing a high level of conservation between these two legumes. However, there are many differences between all the species, even amongst these supposedly conserved areas. For there to be this amount of difference at the amino acid level, means even greater difference at the nucleotide level. The failure of the yeast PCR product to reveal a cognate, when used to probe a Southern blot of *D. spicata* genomic DNA, shows that there is a low level of conservation at the nucleotide level between these two organisms. Therefore it is likely that *D. spicata*, being a monocotyledonous species, has a homologue of this gene that is sufficiently different at the nucleotide level from the other plant homologues and yeast P5CR, such that neither of these two approaches succeeded. The nucleotide sequences of the characterised genes were not available before the experiments were performed. Comparison of these, rather than the amino acid sequences (as was done), may have led to the design of better primers.

Unfortunately the methods used to isolate a gene homologue required a certain level of conservation between the known genes and *D. spicata*. It now appears that complementation of *E. coli* mutants is more likely to succeed in isolating a P5CR gene homologue from *D. spicata*.

Figure 4.1.1


Pea (98-106) KLLVSVAAG (122-130) IRVMPNTPA
Soyabean (99-107) KLLVSVAAG (123-131) IRVMPNTPA
Human (88-96) DRHIVVSCA (118-126) IRCMTNTPV
Yeast (109-117) KLLISLAAG (131-139) CRVMTNTPA
E. coli (91-99) SLVVSIAAG (116-124) IRAMPNTPA
P. aeruginosa (91-99) QLIVSIAAG (116-124) VRCMPNTPA
M. smithii (90-98) TQIIHTCAG (115-123) ISSTYDEDN
Pea (172-185) DAITGLSGSGPAYI (227-246) HPGQLKDDVTSPGGTTIAGV
Soyabean (173-186) DAITGLSGSGPAYV (228-247) HPGQLKDDVTSPGGTTITGI
Human (168-171) DAVTGLSGSGPAYA (223-242) HPGQLKDNVSSPGGATIHAL
Yeast (181-194) DAATALVGSGPAFV (236-255) HPSVLKHQVCTPGGTTIAGL
E. coli (166-179) HPVVGVSGSSPAYV (221-140) HPGALKDMVCSPGGTTIEAV
P. aeruginosa (167-180) DAVTAVSGSGPAYF (222-241) EPAELRRRVTSPNGTTEAAI
M. smithii (166-179) EIATIAASCMPAFI (221-240) SPDELINKVATKNGITQKGL

4.2 Sequence analysis of NaCl-responsive cDNA clones

4.2.1 pDZVIII 5.1.1, pDZVIII 6.1.6 and pDZ2.11.5

None of these clones (or their predicted products) showed any significant similarity to any known protein or gene. Neither did they produce any signal when used to probe Southern and northern blots. In the case of pDZVIII 6.1.6 this may be due to its size, 100 bp. It is possible that the insert represents 3' untranslated sequence and therefore wouldn't show significant sequence similarity with known peptides. However a search for DNA alignments may reveal homology with the 3' untranslated regions of other genes. Its small size may also account for the lack of signals from the Southern and northern analyses.

pDZVIII 5.1.1 and pDZ2.11.5 are the two largest clones, 1512 and 1280 bp respectively. There were no matches with anything in the databases. It could be argued that they are both genes that have never been sequenced before, but no signals were obtained from either northern or Southern blots. In addition to this, neither of these clones contained poly $(A)^+$ tails in their sequences, which is inconsistent with the cDNA synthesis process. Therefore it seems more likely that both of these clones have arisen as artefacts of cloning.

4.2.2 pDZ2.8S

pDZ2.8S showed significant similarity to a 40s ribosomal protein. It is possible this is a NaCl-regulated gene and has a role in NaCl tolerance, but since it gave no signal from probing northern and Southern blots, it seems possible that it is also an artefact of cloning. There is also no evidence in the literature to support such a role. This particular clone arose as a subclone of pDZ2.8L, it was an unknown piece of cDNA attached downstream of that clone, which strengthens the conclusion that it is an artefact and does not correspond to a NaCl-regulated gene.

4.2.3 pDZ2.8L

pDZ2.8L showed significant similarity to histone 2B proteins from a number of different species, the highest being to one from wheat (*Triticum aestivum* L.). This is consistent with Zhao's work, where he initially described the possible product of this clone (Zhao, *et al.*, 1989).

Histone proteins are part of the chromatin structure, the histone 2B being a core protein found inside the coil of the DNA. The N-terminal regions of histones are basic due to lysine residues. Acetylation of these lysine residues reduces basicity by charge

neutralisation, creating histone variants that differ in acetylation and hydrophobicity. Post-synthetic acetylation of histones is associated with chromatin activation for gene transcription in eukaryotes. Differential histone acetylation has been observed in cells of alfalfa (*Medicago sativa*) following growth in NaCl (Waterborg, *et al.*, 1989). Salt-induced conformational transitions in chromatin, which appear to be due to loosening or unfolding of the chromatin structure, are observed in and just above the physiological ionic strength. Intracellular salt and organic solute concentrations may also influence the magnitude and balance of electrostatic and hydrophobic interactions within cellular chromatin. Changes in histone acetylation, induced by salt stress, may therefore signal a change in transcriptional activity at the chromatin level. The presence of histone variants in conditions of high intracellular NaCl, may indicate that adaptive changes in chromatin function are required in salt tolerant cells, growing in the presence of salt. In some cases, altered chromatin function can lead to the death of cells exposed to NaCl (Waterborg, *et al.*, 1989). Functions of histone variants are still unclear, but may constitute a reservoir of histone forms which allow functional chromatin to be maintained in different nuclear environments (Waterborg, *et al.*, 1989). pDZ2.8L may therefore have a protective role in preventing diastic changes to the chromatin structure and functions. There is also the possibility that the induction of a histone 2B protein is merely due to an initial effect of NaCl on the cell cycle, following the early stages of exposure. Zhao found from his northern analyses that pDZ2.8L showed an increases in transcript abundance within 2 - 4 hours of exposure to NaCl, but decreased again within 24 hours (Zhao, *et al.*, 1989). It could be proposed that such an early induction supports the latter argument.

Evidence for a possible role of histone proteins in NaCl tolerance can be found in *E. coli*. Higgins et al. (1990b) have suggested that a histone-like protein (H-NS) plays a role in regulation of gene expression in response to environmental factors in *E. coli* cells (Higgins, *et al.*, 1990b; Owen-Hughes, *et al.*, 1992). The *osm*Z locus is implicated in the control of DNA topology and gene expression, in response to environmental factors (Higgins, *et al.*, 1990a). DNA supercoiling is altered in *osm*Z mutations, which effect many genes scattered throughout the chromosome, including the expression of the osmotically regulated supercoiling-sensitive *pro*U promoter, which shows a decrease in these mutations. The product of the *osm*Z locus is the histone-like protein H-NS (formally designated H1) (Fougère, *et al.*, 1991), one of the most abundant proteins associated with the bacterial nucleoid. Purified H-NS binds non-specifically to DNA and can affect the transcription of a number of promoters *in vitro*. Higgins *et al.* (1990b) also suggest that the gene encoding H-NS (H1) may have a role as a general "silencer" of transcription.

It was found that H-NS interacts with a curved DNA element, located downstream of the *pro*U promoter, and that this was necessary for normal expression (Owen-Hughes, *et al.*, 1992). It was suggested that H-NS-dependent changes in DNA topology may play a role in osmoregulation of *pro*U expression. It was found that not all the curved elements were

functionally equivalent. There was another curved element upstream of the *pro*U promoter, which H-NS binds to, but which plays no role in regulation. Therefore they concluded that other factors must be involved. This conclusion was derived from the facts that *hns* mutations altered plasmid linkage number and the *pro*U promoter was sensitive to perturbations of DNA supercoiling. It was proposed that the H-NS interaction at curved sequences plays a role in both the osmotic control of plasmid linkage number and the osmoregulation of *pro*U expression. This was claimed to be the first evidence of a direct link between environmentally induced changes in DNA topology and changes in gene expression (Owen-Hughes, *et al.*, 1992). Further, they propose that H-NS functions as a "scaffold" to hold the DNA in an appropriate conformation, providing a framework upon which other processes, such as transcription, can be superimposed. In the absence of H-NS there is more flexibility, which allows RNA polymerase to interact productively at the *pro*U promoter. In the presence of H-NS the promoter is maintained in a rigid conformation such that the -35 and -10 regions are not aligned properly to interact with RNA polymerase effectively. Transcription would therefore require a topological change in the DNA template. The H-NS "scaffold" could also constrain local topological changes, for example those caused by a response to osmotic shock, that directly influence DNA conformation or prevent the activity of an enzyme, such as DNA gyrase. This "scaffold" may not require all promoters to behave in the same way. It could provide a framework for sequence-specific regulators or constrain the topological changes caused by other environmental signals such as temperature and anaerobicity (Owen-Hughes, *et al.*, 1992). Owen-Hughes *et al.* (1992) propose that H-NS is providing "topological constraints necessary for transcriptional control at specific promoters and a framework, around which environmentally-induced changes in DNA topology and promoter activity can take affect".

Histone proteins from eukaryotes may play a similar role. Changes in the chromatin structure could allow changes in DNA topology, such that genes become available for transcription, whilst others are made unavailable. Certainly changes in histone variants and the ratios of the different histones, induced by NaCl, could allow for such rearrangements of DNA topology. It is possible that ions such as K^+ could also affect the histone population directly, as they are accumulated within cells in response to elevated NaCl (Watad, *et al.*, 1991).

4.2.4 pDZ6.2, pDZIX 3.1 and pDZVIII 1.2.1

The two clones pDZ6.2 and pDZIX 3.1 were found to have identical nucleotide sequences, except that pDZIX 3.1 was truncated at the 5' end, not having all the putative ORF and the 5' untranslated region. It was also missing 471 bp of sequence downstream of the poly $(A)^+$ tail in comparison to pDZ6.2. This piece of sequence is most likely to be a cloning artefact and represent an extra piece of cDNA that became attached to pDZ6.2

during the cloning process. It showed no similarity to any known protein or gene in the database, although it does have its own poly (A)+ tail. Thus it seems that pDZIX 3.1 and pDZ6.2 are clones corresponding to the same gene.

pDZVIII 1.2.1 was found to be almost identical in sequence to pDZ6.2. Only one of the changes in the nucleotide sequence led to a change in the polypeptide sequence. This resulted in a histidine being replaced for a tyrosine, thereby changing a basic residue for an aromatic one that also contains a reactive hydroxyl group. This change could result in the protein having a slightly different structure, or be indicative of tissue specific expression. All the other changes led to conservation in the sequence within the coding region; all these differences being in the wobble position. There are also differences within the 3' untranslated region. There may well be more differences between the two clones, since pDZVIII 1.2.1 is truncated, missing a lot of the putative coding region and the 5' untranslated sequences.

The predicted products of these three clones showed significant similarity to proline-rich cell wall proteins, including extensins and hydroxyproline rich glycoproteins (HPRGP). The predicted amino acid sequence contains repeat sequences of PPKKDH(H)Y(Y). Proline-rich proteins (PRPs) are abundant components of plant cell walls, which include the extensins and HPRGPs. In dicotyledons, extensins are characterised by the pentapeptide repeat SPPPP or SOOOO (where O represents hydroxyproline). In monocotyledonous plants there are much larger repeat sequences rich in O, T, S and P. In the mature proteins the majority of the proline residues are hydroxylated, and most of these and the serine residues are glycosylated. Other PRPs which have been isolated include ones from soya bean (Bradley, *et al.*, 1992; Suzuki, *et al.*, 1993), *Phaseolus vulgaris* (bean) (Sheng, *et al.*, 1991), *Brassica napus* L. (Coupe, et al., 1993), tomato (Salts, et al., 1991) and maize (Josè-Estanyol, *et al.*, 1992) . These proteins are characterised by tandem repeats of the derived sequence P-P-Λ-Ψ-K, which are often modified to P-O-Λ-Ψ-K in the mature protein (Kleis-San Francisco and Tierney, 1990) and where Λ and Ψ can represent tyrosine, histidine, glutamate or valine (Showalter, 1993).

PRPs are basic proteins and may interact covalently with pectins within the cell wall matrix. The high tyrosine content of PRPs also raises the possibility of isodityrosine crosslinks between PRP molecules and/or between PRPs and GRPs (glycine rich proteins) or extensins (Showalter, 1993). In addition PRPs appear to become covalently attached to components within the cell wall in response to physical damage and elicitor treatment (Bradley, *et al.*, 1992; Kleis-San Francisco and Tierney, 1990). The composition of the plant cell wall can be markedly altered by environmental stimuli, notably in response to biological stress, such as osmotic stress, which causes a decrease in the cell's turgor pressure. Exposure to abiotic and biotic stresses further increases the structural and compositional variation. When soyabean and bean cells are treated with a fungal elicitor or glutathione, it leads to a rapid insolubilization of pre-existing hydroxyproline rich structural

proteins in the cell wall. This involves H_2O_2-mediated crosslinking, which results in a toughening of the cell wall, either in defence or as part of development (Bradley, *et al.*, 1992). In tobacco cells, adapted to growth in high NaCl concentrations, there is a drastically altered growth physiology, that leads to a slower cell expansion and fully expanded cells with volumes one fifth to one eighth of unadapted cells (Iraki, *et al.*, 1989c). There is also a decrease in the proportion of crystalline cellulose in the primary cell wall and a reduction of hydroxyproline in the insoluble protein content to 10 % of that of unadapted cells. It was proposed that the cellulosic-extensin framework is the primary determinant of absolute cell wall tensile strength, but complete formation is sacrificed to divert carbon to substances needed for osmotic adjustment (Iraki, *et al.*, 1989c). There is also a 50 % reduction in cell wall mass of unadapted tobacco cells exposed to 428 mM NaCl and pectin from adapted cells is enriched with rhamnose (Iraki, *et al.*, 1989a). In addition, adaptation of tobacco cells to saline or water stress results in the inhibition of both the hydrolysis of hemicellulosic xyloglucan and the release of uronic acid-rich material into the culture medium (Iraki, *et al.*, 1989b).

The predicted protein sequences of pDZ6.2 and pDZVIII 1.2.1 contain repeats based on the amino acid sequence PPKKDH(H)Y(Y). Whether the proline residues are infact hydroxyprolines can only be determined by analysis of the protein itself, but is possible that some are hydroxylated in the mature protein. The clones are sufficiently different from the extensins, not containing the penetapeptide repeat SPPPP (as in dicots) or the tyrosine and threonine rich repeats (as in monocots), to conclude that they probably don't have this function. The repeats found in the two clones vary significantly from the usual repeat sequences found in other PRPs from plants, in that they don't contain the P-P-Λ-Ψ-K (Λ, Ψ= Y, H, V, D) repeat. However the amino acid usage is more comparable. In pDZ6.2 21.9% of amino acids used are proline, 14.3% histidine, 17.6% lysine and 12.1% tyrosine; this represents 65.9% of the total amino acid usage. This compares to 26.3% proline, 11.3% histidine and 13.1% valine in the bean PRP (Sheng, *et al.*, 1991), and 35.7% proline, 9.3% isoleucine, 8.9% valine and 7.8% lysine in the tomato PRP (Salts, *et al.*, 1991). Table 4.2.1 shows PRPs, their abundant amino acids and amino acid motifs. It is reasonable to hypothesise that the product encoded by pDZ6.2 (and VIII 1.2.1 and pDZIX3.1) may function as a cell wall protein, but this cannot be 'presumed' true.

The first 20 amino acid residues (MPLLVALLLLVLAVVAAAG↓A↓DH) of the putative protein of pDZ6.2 share homology with the leader sequence of a proline-rich cell wall protein precursor from soyabean and other extracellular proteins from a number of different organisms. The residues similar to those found in other leader sequences are shown in bold, those underlined are conserved with respect to the soyabean protein. Therefore it is suggested that this hydrophobic N-terminal sequence represents a possible leader sequence for the putative pDZ6.2 protein. Two possible cleavage sites are represented by arrows, as predicted by von Heijne's (-3, -1) rule (von Heijne, 1984). It also conforms to the pattern of

predicted by von Heijne's (-3, -1) rule (von Heijne, 1984). It also conforms to the pattern of other leader sequences, in that the region has a hydrophobic core and a polar C-terminal part (von Heijne, 1984). The presence of this possible leader sequence is consistent with the proposed function of the pDZ6.2 protein as a cell wall protein, as it increases the likelyhood that this protein could be targeted to the endoplasmic reticulum and ultimately exported. Figure 4.2.1 is a diagrammatic representation of pDZ6.2 and the predicted protein sequence, showing the position of the putative leader sequence and the peptide repeats.

The production of different proline-rich proteins is regulated by a number of different factors, including fungal elicitors, wounding, development, light, red light, ethylene and cell culturing (Showalter, 1993). Synthesis of other cell wall proteins, such as GRPs, are regulated by water stress, ABA, mercuric chloride, wounding and development in monocots. Salicylic acid, viral infection, drought stress, wounding and development regulate their synthesis in dicots (Showalter, 1993). This suggests a role in the defence response of plants for these cell wall proteins. pDZ6.2 and pDZVIII 1.2.1 are regulated by NaCl, ABA and proline (as will be discussed later). Therefore it is possible that elevated levels of NaCl are having a similar effect on the cell wall composition of *D. spicata* cells as wounding and fungal elicitors have on soyabean and bean cell walls, triggering a "defence" response.

Some proline rich proteins and extensins are members of multi- or small gene families; e.g. maize extensin (Hood, *et al.*, 1993), or PRP from *Brassica napus* L. (Coupe, *et al.*, 1993). However others such as PvPRP1 from bean (Sheng, *et al.*, 1991) and maize (Sheng, *et al.*, 1991) are from a single copy gene. The overlying trend is for PRPs and extensins from dicotyledons to be encoded by a multi-gene family and monocotyledons by single copy genes (with the exception of the extensin from maize (Gaxiola, *et al.*, 1992)). However it appears that pDZ6.2 and pDZVIII 1.2.1 are members of a multi gene family. The protein sequences are very similar, only differing in one amino acid residue, though there may be more since pDZVIII 1.2.1 is truncated. There are more differences between the nucleotide sequences within the coding regions, most leading to conservation of the encoded amino acid sequence, as well as many in the 3' untranslated sequence. In addition to this the estimated transcript sizes (from the northern analysis) for the two clones were quite different, pDZ6.2 a size of 0.5 Kbases and pDZVIII 1.2.1 0.7 Kbases. As stated these are only approximate sizes, and in fact may be much closer in size, the predicted size of pDZ6.2 from the cDNA sequence is nearer to 650 Kbases. However this could be further evidence of a multi-gene family. The sequence pDZIX 3.1 is 100 % identical to pDZ6.2. Unfortunately no hybridisation has been detected to Southern blots for either pDZ6.2 or pDZVIII 1.2.1 to confirm this observation.

Some proline rich proteins show tissue specific expression within an organism; e.g. SbPRP1 and SbPRP2 in soyabean (Suzuki, *et al.*, 1993). Therefore in the future, studies

need to be carried out to look at the temporal and spatial expression patterns of these two cDNA clones to determine whether they show tissue-specific expression within the whole plant. However the sequences of the transcripts are so similar, that they are likely to cross-react with one another. Therefore the most likely way to distinguish between them will be via reporter gene fusions to the different 5' flanking regions. Further studies also need to be performed to fully understand their regulation. The full length gene of both clones will need to be isolated to identify all the differences in the nucleotide sequences and to identify any further members of this putative family.

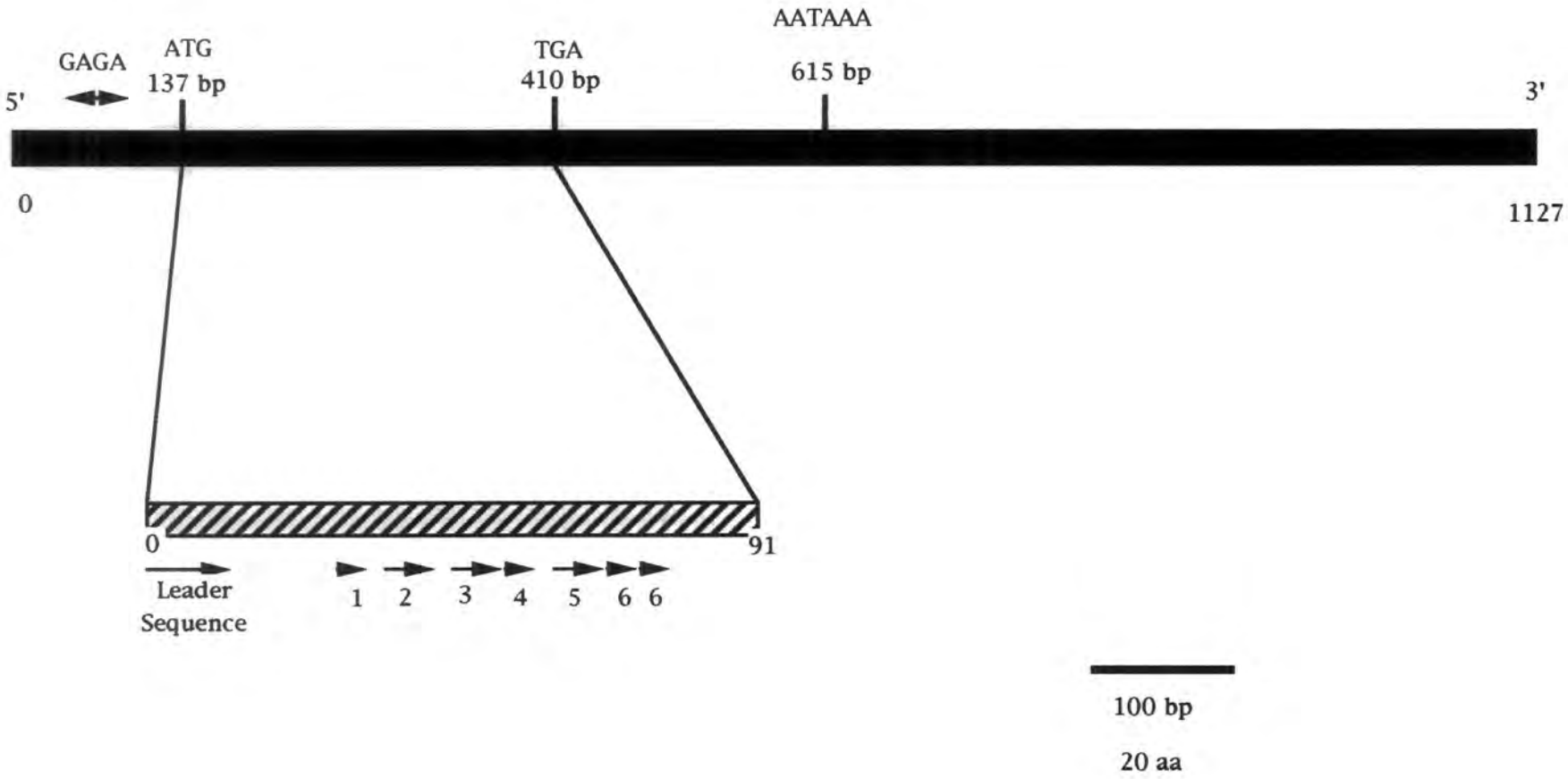
Table 4.2.1

pDZ6.2 Predicted Amino Acid Sequence:

MPLLVALLLV LAVVAAAGAD HYKPKDPYHP PKKHYYPPKK DHYYPPKKDH

HYYPPKKDHH YEPPKKHHDP PKKHPYPPKK DHEHWPGKPGY

PROTEIN	Abundant AAs	AA Motifs
Extensins (dicot)	O,S,K,Y,V,H	SOOOOSOSSSSSYYYK SOOOK SOOOOOTOVYK SOOOOOVYKYK
"Extensins" (monocot)	O,T,S,P,K	TPKPTPPTYTOSOKPO ATPKPP
"Extensins" (Chlamydomonas)	O,S	PPP and PP SP PXP and PXXP
"Extensins" (Volvox)	O,S	$S(O)_{3-7}$
PRPs (soya bean)	O,P,V,Y,K	PPVYK and PPEVK
(nodulins)	(O),P,E,Y,H,K	PPHEK and PPPEYK
(*Phaseolus vulgaris*)	P,V,H,K	PVHPPVKPPV
(*Brassica napus*)	P,S,K,D,N,T,V	PSPKPKPDPS PKPNPKPKPTPTPSPV
(Maize)	P,Y,V,T,R,S	PPYVPP TPRPS
(Tomato)	P,P,H,V,K,S,T,,I	PPHVKPPSTPK PPIV
pDZ6.2 (Salt Grass)	P,D,Y,H,K,	PPKKDH(H)YY


AATAAA
GAGA
ATG
137 bp
TGA
410 bp
615 bp
5'
3'
0
1127
0
91
Leader
Sequence
1 2 3 4 5 6 6
100 bp
20 aa

4.3 Examination of the expression of the different sequences

4.3.1 pDZVIII 1.2.1 and pDZ6.2

pDZVIII 1.2.1 and pDZ 6.2 both showed the same response to exposure to 520 mM NaCl, 5 mM proline and 100 μM ABA. Although the estimated transcript sizes were different for the two clones, pDZVIII 1.2.1 0.7 Kbases and pDZ6.2 0.5 Kbases, it appears that the inserts from these clones were hybridising to the same transcript, due to there being a high degree of sequence similarity between them.

4.3.1.1 NaCl

Initial experiments with pDZ6.2 showed no induction in response to 8 and 24 hour exposures to 260 mM NaCl. This was inconsistent with Zhao's data, which showed a continuous increase over 24 hours, by a factor of at least 10 (Zhao, *et al.*, 1989). This may be due to the different cell culturing conditions used. Zhao used cells that were grown on a five day transfer cycle, whilst cells used in the experiments described here were on a seven day transfer cycle, which may have resulted in them being more dilute or dividing more infrequently and being less metabolically active. pDZ6.2 showed an increase in transcript abundance in the presence of 520 mM NaCl, when cells were exposed to a range of NaCl concentrations, where 260 mM gave no increase and 780 mM showed a decrease relative to the levels shown in response to 520 mM. However the increase observed in figure 3.4.2 was only small and not entirely reproducible across the three examples. The reasons why a putative proline cell wall protein should be regulated by NaCl, have already been discussed. It is possible that changes in turgor pressure due to changes in NaCl could require changes in cell wall elastic properties and hence expression of different cell wall proteins, which reflect the observed changes in the polysaccharide population (Iraki, *et al.*, 1989a; Iraki, *et al.*, 1989b; Iraki, *et al.*, 1989c).

4.3.1.2 Proline

There was an indication that pDZVIII 1.2.1 and pDZ6.2 showed a small decrease in transcript abundance in response to exogenous 5 mM proline, since these results were not reproducible in all examples. Proline is accumulated in *D. spicata* cells within eight hours of exposure to elevated levels of NaCl (Heyser, *et al.*, 1989b). This NaCl-induced endogenous proline biosynthesis is inhibited in the presence of 5 mM exogenous proline (Heyser, *et al.*, 1989b), therefore the expression of any genes involved in the NaCl-induced accumulation of proline would not be switched on to the same extent. This is consistent with the observed decrease in pDZ6.2 and pDZVIII 1.2.1 transcript abundance in some

examples in the presence of NaCl and proline. However no sequence similarity was found between the inserts of either of these clones and genes involved in proline biosynthesis. A 54 % inhibition of cell growth of *D. spicata* suspension cultures by 2 mM proline has been observed in the absence of NaCl, and a 22 % inhibition by 10 mM proline in the presence of 260 mM NaCl (Rodriguez and Heyser, 1988), showing the inhibitory concentration of proline to be less for cells grown in the presence of NaCl than those grown its absence. At 58.5 mM proline all growth was inhibited in both cell lines. The uptake and metabolism of 10 mM L[1-^{13}C]-proline was followed in these cell lines by ^{13}C NMR and ninhydrin analyses of suspension cultures. It was found that only proline accumulated to high levels and not any of its metabolites, suggesting that it was proline inhibiting growth. $^{13}C_1$ in proline was either retained or disappeared, no metabolite of ^{13}C-proline was detected by ^{13}C NMR, therefore confirming the accumulation of proline. The observed decrease in transcript abundance of transcripts corresponding to the inserts from pDZVIII 1.2.1 and pDZ6.2 could be as a result of inhibition of cell growth by proline. However in the presence of 520 mM NaCl there was still a decrease in transcript abundance of pDZ6.2 and pDZVIII 1.2.1 in response to 5 mM proline. Since proline plays a role in NaCl tolerance in *D. spicata* cells, it is possible that by supplying the cell with exogenous proline in the presence of NaCl, that the normal detrimental effects of high NaCl concentrations are reduced such that other genes involved in NaCl tolerance are not induced to the same degree. There is still a decrease in transcript abundance corresponding to both these clones in response to proline alone. This could be an inhibition of cell growth as mentioned before, or it could be that proline is still able to switch on the signal pathway that reduces their expression, even in the absence of NaCl.

4.3.1.3 ABA

There was a marked increase in abundance of transcripts hybridising to the inserts from pDZVIII 1.2.1 and pDZ6.2 on exposure to 100 μM ABA, in the presence and absence of 520 mM NaCl. There were enhanced levels of transcripts in the presence of NaCl and ABA, compared to NaCl alone, suggesting that ABA has a greater effect than NaCl in the regulation of the corresponding gene(s). It also suggests that ABA may be regulating expression independently from NaCl, via a different signal transduction pathway. ABA induces a number of proteins in response to environmental factors, in tobacco (Singh, *et al.*, 1987a), tomato (Godoy, *et al.*, 1990), rice (Mundy and Chua, 1988) and maize (Gómez, *et al.*, 1988). ABA-induced proteins have a variety of functions, including maintenance of dormancy within seeds, storage proteins, lectins and enzyme inhibitors (Mundy and Chua, 1988). Exogenous application of ABA accelerates adaptation of tobacco (Singh, *et al.*, 1989) and rice (Kavi-Kishor, 1989) to high NaCl and induces biochemical changes similar to those induced by NaCl, such as the accumulation of osmotin in tobacco (Singh, *et al.*,

1987a). ABA seems to have a role in adaptation to elevated NaCl, as it enhances the rate of adaptation and increases tolerance in tobacco (La Rosa, *et al.*, 1985). Correlations between gene expression changes induced by ABA during freezing tolerance suggest that ABA has a role in cold acclimation (Godoy, *et al.*, 1990). ABA may therefore have a general role in the adaptation of plants to different environmental factors.

Consequently it seems that pDZVIII 1.2.1 and pDZ6.2, which encode putative proline rich cell wall proteins are under regulation by ABA, such that their expression is enhanced in the presence of NaCl and ABA. This may be consistent with a NaCl-induced increase in ABA, since endogenous levels of ABA increase dramatically under NaCl and water stress in other plant cells (Wright and Hiron, 1969). Future experiments could be performed to find out whether such increases occur in cells of *D. spicata* on exposure to elevated NaCl. This might show that supplying the cell with exogenous ABA is only mimicking a natural effect, such that ABA is accumulated in order to regulate NaCl-induced gene expression.

In the future, as well as looking at endogenous ABA levels in response to NaCl, it would also be of interest to identify any ABA responsive elements and corresponding *cis*- or *trans*-acting factors, such as those identified in wheat (Guiltinan, *et al.*, 1990; Marcotte Jr., *et al.*, 1989), rice (Mundy, *et al.*, 1990), maize (Pla, *et al.*, 1993) and tobacco (Raghothama, *et al.*, 1993). The isolation of the corresponding genomic sequence will be a necessary prerequisite for the search of such *cis*-acting elements.

All of the above data suggests that the corresponding genes for pDZ6.2 and pDZVIII 1.2.1 are regulated transcriptionally, or post transcriptionally by mRNA stability. *in vitro* nuclear run-on experiments would identify which mechanism does control their expression.

4.3.2 pDZ2.8L

4.3.2.1 NaCl

There was an indication from the results that pDZ2.8L showed a decrease in transcript abundance on exposure to 520 mM NaCl for 24 hours. This appears to be inconsistent with Zhao's data, but in fact he observed an increase from 4 - 8 hours after exposure to NaCl, followed by a decrease after 24 hours (Zhao, *et al.*, 1989). Thus the decrease observed at 24 hours is consistent with Zhao. Therefore it would be necessary to study the transcript abundance of sequences hybridising with the insert from pDZ2.8L at several time points, including 4 and 8 hours, to ensure that pDZ2.8L does show an increase in transcript abundance, consistent with Zhao's data. pDZ2.8L encodes a protein with significant similarity to a histone 2B protein and its role in NaCl tolerance has already been discussed. However, the early response to elevated NaCl could be as a result of the cell cycle being shocked and so arrested at different stages. Thus experiments could be carried out, where synchronous cultures are produced by treatment with colchicine which should leave the

cells at the same phase of the cell cycle, and the transcripts of interest could then be examined at different stages of the cell cycle in a series of northern blots.

4.3.2.2 Proline

There was an indication that pDZ2.8L transcripts decreased in abundance in response to a 24 hour exposure to 5 mM proline. No significant change was observed when both NaCl and proline were present. It is possible that in the presence of proline alone, an inhibition of cell growth is occurring, as mentioned previously (Rodriguez and Heyser, 1988), and this inhibition is reduced in the presence of NaCl. Rodriguez noted a 54 % inhibition of growth by 2 mM proline, and 22% in the presence of 10 mM proline and 260 mM NaCl. So it is possible that a similar effect is occurring here, since there was no decrease in transcript abundance in the presence of NaCl and proline, which is consistent with the observations of Rodriguez and Heyser (1988). However in view of the exposure time used in the experiments it would interesting to examine the effects of proline in the presence and absence of NaCl at shorter exposure times.

pDZ2.8L encodes a putative histone 2B protein, therefore some constitutive expression might be predicted. It seems more likely that proline has no regulatory role in the expression of this clone, and that the observed decrease in transcript abundance is as a result of inhibition of cell growth. But it is difficult to draw conclusions, when there are no time points with which to compare with Zhao's data.

4.3.2.3 ABA

pDZ2.8L showed a possible decrease in transcript abundance in response to a 24 hour exposure to 100 μM ABA and also with 520 mM NaCl and 100 μM ABA. As mentioned previously ABA has a regulatory role in the expression of stress-induced genes, often enhancing the effect of that stress response. Therefore, since 520 mM NaCl gives a decrease in the expression of this clone, it seems unsurprising that a similar response is observed with ABA. Once again it is necessary to observe the effects at shorter exposure times, so as to compare with Zhao's previous data. Since ABA is a known regulator of stress-induced gene expression, it might also confirm that this clone, a possible histone 2B protein, is playing a role in NaCl tolerance and hasn't just arisen as a result of the cell being arrested at different phases of the cell cycle.

As for pDZVIII 1.2.1 and pDZ6.2, the corresponding gene for pDZ2.8L is either regulated at the level of transcription or post transcriptionally through mRNA stability and experiments, as suggested before, would identify which.

4.4 The identification of a GA repeat sequence in the 5' untranslated region of pDZ6.2 and its possible significance

4.4.1 Is the GA repeat an artefact of cloning?

A 34 bp dinucleotide GA repeat (therefore 68 bp in total) was found in the 5' untranslated region of pDZ6.2, from 27 bp to 94 bp. There was concern that a sequence located close to the 5' end of the insert could have arisen as a cloning or cDNA synthesis artefact. Figure 4.4.1 shows three sequences that contain dinucleotide repeats that are thought to have arisen as artefacts. They are being shown with the permission of H. Hooper (sequences 4.13 and 1.30) and L. Gatehouse (sequence p256C7). Sequences 4.13 and 1.3 were cloned from a *Brassica napus* embryo cDNA λZapII library. The GA repeat in 4.13 appears at the 3' end of the clone and could therefore have occurred as a result of the cDNA synthetic process, where the 3' primer contains a GA repeat. The repeat in sequence 1.30, although it occurs at the 5' end, is followed by a poly T region. This infers that it could be in the 3' untranslated region as TC repeat. p256C7 was isolated from a *Manduca sexta* λZapII cDNA library and contains an 80 bp CT repeat at its 5' end. A possible ATG has been highlighted, and although the repeat occurs upstream of this, it appears to be an artefact, as it bears considerable similarity to a linker region from λZapII. In 1.30 and p256C7 the sequence starts with the repeat.

pDZ6.2 was cloned from a *D. spicata* cDNA λgt10 library. By contrast to the clones illustrated in figure 4.4.1, the GA repeat in pDZ6.2 starts at 27 bp and the sequence upstream of this has no similarity with any known nucleotide sequence, including λ vectors. Therefore it seems unlikely that it could have arisen as an error during the cDNA synthesis or during cloning. Furthermore a GA repeat has been observed in the 5' untranslated region of a phosphatase 2A cDNA clone from an *Arabidopsis thaliana* λgt10 cDNA library (Casamayor, *et al.*, 1994). The 44 bp poly (GA) tract was found in the cDNA PP2A-3, and also occurred in the corresponding genomic region when it was sequenced, showing it not to be an artefact. A much shorter 14 bp GA repeat in another isoform of the gene the cDNA PP2A-4 was also found (Casamayor, *et al.*, 1994). They propose that such a repeat could have a role as a transcriptional regulator, and it may control the expression of PP2A-3, despite not occurring in the transcriptional regulatory region. GA repeats have also been located downstream of the transcription start in several salt stress-induced genes in *Mesembryanthemum crystallinum*, (Bohnert, 1994) and in the 5' untranslated region of a drought stress-induced gene from tomato (Terry, 1994). An 8 bp dinucleotide CT repeat occurs in the 5' untranslated region of a cDNA from *Phaseolus vulgaris* that encodes a proline rich cell wall protein (Sheng, *et al.*, 1991). The authors only noted its existence and made no mention of any function or purpose. It is interesting to note the occurrence of such

a repeat in a similar position of a cDNA clone that encodes a protein with a similar function to that proposed for pDZ6.2.

Further evidence has been obtained to suggest that this GA repeat may not be artefactual. The results obtained from the northern blot, comparing RNA probed with pDZ6.2 (whole clone) and pNRCF0204 (GA repeat region) show the two sets of bands to be running at exactly the same point on the gel. The bands from pNRCF0204 had a much less intense signal than those from pDZ6.2. This can probably be explained by the difference in sizes, in the case of pNRCF0204 only approximately 100 bp of sequence would have been hybridising to the RNA as compared to 656 bp of pDZ6.2 (excluding the 471 bp of putative junk cDNA from its 3' end). This indicates the existence of transcripts containing the GA repeat.

The APCR strategy was adopted to show the presence of a corresponding repeat within the *D. spicata* genome. However it was unsuccessful in amplifying the GA region found within pDZ6.2. From information obtained after the experiments were performed, it seems that the primer design and usage may not have been optimal and may have contributed to the lack of success in this approach to amplify the genomic region. All of the specific primers were designed with an approximately equal A/T : G/C ratio. However it now appears that nearest-neighbour thermodynamics have a considerable effect on the efficiency of the primers to bind to the DNA template. This means the distribution of the bases within the oligonucleotide is important; guanine and cytosine bases must be neighboured by themselves to give a more efficient binding capacity (De Bellis, *et al.*, 1992). Of the primers used in the APCR's, the M13 forward primer and primers **2** and **3** are of the right sequence to give efficient binding, whilst the T7 primer and primer **1** are not (Bartley, 1994; De Bellis, *et al.*, 1992), . When considering the pairs of primers used in each reaction, an efficient binding primer has been paired with an inefficient one (primer **1** with M13, primer **2** with T7 and primer **3** with T7). Thus optimum conditions were not used, and this is probably the main contributor to the failure of this method. If the experiments were to be repeated it might be better to either use the M13 forward primer in all reactions or the KS primer instead of T7. The reason for using more than one pBlusecript™ primer was to allow a greater decrease in the size of the predicted products to complement the decrease in size that the *D. spicata* specific primers would have given. It is always possible that the strategy did work, but not to a high enough degree to be able to detect the product, even by Southern analysis. This strategy was designed to amplify a 189 bp product from a genome size of approximately 10^9 Kb and perhaps not even increasing the number of cycles in each reaction (as was done) or using the primary amplification mix as a template would make any difference in detection of the product. The strategy was designed to select this particular region over all other DNA sequences in the genome and obviously needs modifying. It was only ever designed as a quick method for identifying the genomic homologue of the 5' untranslated region of pDZ6.2. Of course it may be that such a region

doesn't exist within the genome of *D. spicata*, despite the evidence from the northern analysis, which identified a transcript corresponding to the GA region. The only definite way in which to isolate the genomic clone is to make and probe a *D. spicata* genomic library.

4.4.2 Triplex DNA, its functions and possible significance of the GA repeat in pDZ6.2

$d(GA \cdot CT)_n$ sequences are fairly abundant in eukaryotic genomes including rodents, primates (Manor, *et al.*, 1988) and plants (Casamayor, *et al.*, 1994; Sheng, *et al.*, 1991). They have been shown to adopt non-B DNA structures such as intramolecular triplexes (H-DNA). Only sequences that contain all purine in one strand and pyrimidines in the other are suitable for forming triplex DNA. There are two types of triplexes, differing in base pairing, alignment and third strand polarity. The first are pyrimidine-purine-pyrimidine (Y.RY) triplexes and require a low pH, which enables the protonation of cytosine residues. In this case thymine and protonated cytosine residues in the third strand participate in Hoogsteen base pair formations with adenine and guanine residues in the purine strand, to form isomorphous T.AT and C^+.GC triads (Radhakrishnan, *et al.*, 1993). In these triplexes the Hoogsteen pyrimidine third strand and Watson-Crick purine strand are parallel to each other. The second type are purine-purine-pyrimidine (R.RY) triplexes (H'-DNA) and support three base-pairing alignments with Watson-Crick and Hoogsteen purine strands adopting an antiparallel orientation relative to each other. Besides the third strand guanine residues recognising other guanines and cytosines in G.C base pairing, third strand adenine or thymine residues have been shown to recognise adenines in the A.A/T base pairs to give T.AT and A.AT triplexes (Radhakrishnan, *et al.*, 1993). These triplexes occur at a more neutral pH (Martínez-Balbás and Azorín, 1993). Figure 4.4.2 shows an example of Y.RY triplexes and how the structures form. Intramolecular triplexes contain several regions that are single stranded. These regions include a single stranded loop located in the centre of the strand that folds back into the major groove of the duplex, half of the purine and pyrimidine strands and the junction regions. This single stranded loop shows hypersensitivity to the reagent OsO_4, which reacts with single stranded DNA (Wells, *et al.*, 1988).

Martínez-Balbás *et al.* (1993) propose that R.RY H'-DNA triplex forms from $d(GA \cdot CT)_n$ sequences in the presence of Zn^{2+}. They suggest that Zn^{2+} have a primary role in the formation of H'-DNA, causing local denaturation, so that a 'bubble' appears in the DNA before the triplex is formed. They also observed that Zn^{2+} were required for stabilisation of the structure, but a further increase of the Zn^{2+} concentration caused a change from H' triplex DNA to a H' hairpin. Thus they concluded that at higher concentrations, the Zn^{2+} cause destabilization of the altered DNA conformation through direct interaction with the bases (Martínez-Balbás and Azorín, 1993). It was also observed that ionic strength effected the formation of H'-DNA. In low ionic conditions (10 mM NaCl) H'-DNA formed, when

similar experiments were performed at 50 mM NaCl there was no H'-DNA formation. However this was dependent on the presence of Zn^{2+} and a bacterial negative superhelical density of $-\sigma = 0.05$ (Martínez-Balbás and Azorín, 1993). Dolinnaya *et al.* (1993) have observed an effect of ionic strength on the transition of a $d(A^{+}\text{-}G)_{10}$ oligonuleotide to an intramolecular helix. They found the structure was stabilised by ionic bonds between the positively charged adenine residues and distal negatively charged phosphate groups. The formation of the helix was accompanied by the co-operative uptake of nine protons, corresponding to the nine adenines that can form ionic bonds with all the available phosphates. On increasing the ionic strength, there was no formation of the helix. However, a decrease in ionic strength led to an increase in the transition of the oligonucleotide to the helix structure (Dolinnaya, *et al.*, 1993). It has been observed that Na^{+} ions preferentially bond with the backbone phosphate groups of DNA (Kurkela, *et al.*, 1988). Thus if the surrounding NaCl concentration is decreased, it allows these phosphates to form bonds with the adenine residues of this oligonucleotide, so stabilising the helix. Dolinnaya *et al.* (1993) also propose that if such a single stranded oligonucleotide can form secondary structures, then there maybe a possibility that RNA could behave in a similar way .

Several functions have been proposed for these structures from these dinucleotide purine-pyrimidine repeats. They include roles in DNA replication (Brinton, *et al.*, 1991; Sridhara Rao, *et al.*, 1988) , DNA polymerisation (Baran, *et al.*, 1991) and recombination (Bernués, *et al.*, 1991). In *E. coli* there is a single stranded DNA binding protein (SSB), that has been shown to bind to plasmids containing features associated with triplex DNA formation (Klysik and Shimizu, 1993). Klysik *et al.* (1993) observed that SSB caused a site-preferential increase in OsO_4 reactivity. This is apparently in accord with a previous hypothesis, that proposed SSB removes secondary folds from DNA. SSB appears to be able to remove triplexes as a result of relaxation of superhelical turns (Klysik and Shimizu, 1993). During DNA polymerisation the phenomenon of 'replication arrest' takes place. This has been observed near the centre of oligopurine and oligopyrimidine templates, due to the folding process that results in triple stranded DNA formation. 'Replication arrest' was prevented when SSB was included in the reaction mixture, as it removed the folded back structures (Baran, *et al.*, 1991). Thus it appears that these triplex structures can interrupt processes such as DNA polymerisation (as above) and replication, where pausing at the replication fork was observed when d(GA.CT) sequences were incorporated into a virus genome (Sridhara Rao, *et al.*, 1988) or plasmids (Brinton, *et al.*, 1991). Other groups have proposed a role in regulation (Morgan and Wells, 1968; Palecek, 1991). Morgan *et al.* (1968) noted that the addition of a third strand to a duplex, to generate a triplex, gave a transcriptionally inert template, as compared to the active duplex. This suggests a possible role in regulation of transcription by these triplex structures.

The 68 bp GA repeat found in pDZ6.2 could form non B-DNA structures, given the appropriate conditions. During sequencing reactions and PCR, half length repeats (of 34 bp) were sometimes observed. No other different length repeats were observed during these processes, which infers that the differences didn't arise due to random slippage of the Taq DNA Polymerase. Therefore this suggests that the DNA is sometimes forming some sort of structure, such as triplex or hairpin DNA, so that only half of the sequence is available to the enzyme during sequencing reactions and PCR, which were performed at pH 8.0. Since the average pH of the cytosol within a plant cell lies within a range of 7 - 7.5, a neutral value, it is likely that this repeat region could form R.RY triplexes, rather than Y.RY, which form at a lower more acidic pH (Wells, *et al.*, 1988).

Although the GA repeat does not lie within the regulatory region of this gene, there is a possibility that it could still play some role in transcriptional regulation. Under conditions of low ionic strength, when *D. spicata* cells are not exposed to elevated levels of NaCl, triplex DNA could form and attenuate transcription by yielding the DNA template inert, as suggested by Morgan *et al.*(1968). If, like other sequences, the GA repeat is sensitive to ionic strength, then at higher concentrations, such as when the cells are NaCl stressed, there may be a relaxation of the triplex DNA, which in turn could lead to any inhibitory effects being cancelled out and the transcription of the gene proceeding. There is also the possibility that it could regulate translation in a similar way, by inhibiting the ribosomal activity. This would be dependent on the messenger RNA being able to adopt secondary structures, such as hairpin loops, as suggested by Dolinnaya *et al.* (1993).

In *Drosophila* there is a protein, the GAGA factor, that binds to GA repeat sequences (Biggin and Tjian, 1988) whose gene and cDNA has been isolated (Soeller, *et al.*, 1993). The GAGA factor is a multi-transcriptional activator which binds to $(GA/CT)_n$ sites in a host of promoters. Genetic studies have shown that the function of GAGA, at least at the heat shock loci, is probably one of promoting chromatin rearrangement (Lu, *et al.*, 1993). In *Drosophila* cells, activation of heat shock genes is a multi step process. In these cells the transcription complex exists in a 'poised' state, with the RNA polymerase II already in place and engaged. Release of the polymerase and start of transcription requires the binding of heat shock factor (HSF) proteins, whose binding is in turn conditional on the binding of the GAGA factor (Lis and Wu, 1993). Tsukiyama *et al.* (1994) investigated the effect of the GAGA factor on the *hsp*70 locus of *Drosophila* in reconstituted chromatin. In the absence of the GAGA factor, it was found that the assembly system placed a nucleosome overlapping two of the four GA/CT sequences in the promoter. A nuclease hypersensitive site was formed in this locus on the addition of partially purified chromatin GAGA factor, before, during and after completion of chromatin assembly and this seemed to help position nucleosomes in the surrounding regions. Specific antibodies were used to confirm the requirement for the GAGA factor. Nucleosome disruption was inhibited, but not completely eliminated, with the addition of histone H1 (absent in Drosophila embryo

extract) (Tsukiyama, *et al.*, 1994). Lu *et al.* (1993) predicted that HSF and GAGA would have separate roles in gene regulation , HSF setting up chromatin structure, and GAGA "exploiting it". Tsukiyama et al. (1994) also observed that the modifications in chromatin structure were facilitated by ATP. If the ATP-hydrolysing enzyme apyrase was added after the chromatin was assembled, the GAGA factor was found to be ineffective. A depletion of ATP had the same effect. Effectiveness of the GAGA factor was restored by addition of ATP to depleted systems, but the addition of non-hydrolysable ATP analogues did not (Tsukiyama, *et al.*, 1994). van Holde (1994) proposed that this may be the first time that an exogenous energy source has been shown to be involved in chromatin disruption. There may be another unknown factor involved since the GAGA factor doesn't appear to bind ATP, nor possess any ATPase activity (van Holde, 1994). For transcription to occur the chromatin structure surrounding the gene and its flanking region must be changed. The most important step may be allowing the transcription factors to bind, removing the nucleosomes that protect the promoter region. Felsenfeld (Felsenfeld, 1992) proposed that special proteins, such as the GAGA factor are required for this chromatin rearrangement. van Holde (1994) suggests that this may be a way for normally inactive genes, in a particular cell type, to suddenly become expressible in response to an external stimulus (van Holde, 1994). This suggests a different role in the regulation of transcription (or translation) for such repeat sequences. It is possible that the GA repeat found in pDZ6.2 is recognised by a binding factor, whose binding results in attenuation of transcription or inhibition of transcriptional initiation. This GA factor could either recognise triplex DNA or just the GA repeat alone and thus there are possibilities of this binding being NaCl-dependent or -independent. Similar mechanisms could be involved in the regulation of translation through hairpin loop formation. In addition to this, the GA repeat could be the binding site for a protein that causes changes in chromatin structure and thus regulates gene expression as the heat shock genes are in *Drosophila.* Although this particular sequence doesn't lie within the 5' regulatory region (as they do in *Drosophila*), there may be other sequences further upstream in the genomic clone, as yet unidentified that may have this function. Once the genomic clone has been isolated, the identification of any *trans*-acting factors that bind to the GA repeat would help to investigate further the mechanism by which this repeat may regulate gene expression.

The main contributor to changes in ionic strength is not likely to be Na^+ ions, due to their detrimental effects within the cell. In *D. spicata* Na^+ are sequestered into the vacuole, when cells are exposed to elevated NaCl levels (Heyser, *et al.*, 1986). Changes in ionic strength are more likely to be caused by intracellular K^+ concentrations. In NaCl-adapted tobacco cells, there is an enhanced net K^+ uptake (Watad, *et al.*, 1991), such that in the presence of 160 mM NaCl, K^+ uptake in NaCl-adapted cells was 3.5 fold greater than unadapted cells. In yeast cells overexpressing a yeast gene *HAL*1, Na^+ toxicity is counteracted by the accumulation of K^+ (Gaxiola, *et al.*, 1992). In *E. coli* there is a turgor-

induced accumulation of K^+ ions, to the extent that the intracellular K^+ concentration increases by 200 - 400 mM (Higgins, 1987). Thus in this case K^+ is almost certain to be the primary contributor to the change in ionic strength within the cell, since in *E. coli* Na^+ ions are maintained at a constant low level, even at high extracellular concentrations (Higgins, 1987). Higgins (1987) also proposed that ionic strength (and K^+ ions) may regulate gene expression in two ways. Firstly by influencing the conformation of regulatory proteins and their binding to the gene promoter. In the case of *pro*U (on which he bases his proposals) the ionic strength may upset the equilibrium between two forms of the protein, that regulates its transcription, between monomer and dimer. Betaine (which is accumulated within *E. coli* in response to elevated external NaCl concentrations) has also been shown to facilitate the multimerization of proteins under what would normally be denaturing conditions. Secondly by altering the RNA polymerase-promoter interactions (no regulatory proteins being involved). He also suggests that the changes in ionic strength may be of sufficient magnitude to disrupt B-DNA conformation and lead to the formation of open or closed complexes at the promoter.

4.4.3 Possible roles for the GA repeat in pDZ6.2

Listed below are some of the formally possible ways that the GA repeat could exert a regulatory role:

1) DNA/Transcriptional level

a) Formation of triplex DNA causes attenuation of transcription, under high ionic conditions the collapse of the triplex structure allows transcription to proceed.

b) Triplex DNA is recognised by a protein binding factor, whose binding causes attenuation of transcription, under high ionic conditions the collapse of the triplex causes the release of the factor and transcription to proceed.

c) Triplex DNA is recognised by a binding factor whose binding inhibits transcriptional initiation, under high ionic conditions this factor is released allowing transcription to initiate.

d) Transcription is regulated by a GA binding factor, which is itself modified in the presence of NaCl to either allow or prevent binding.

2) RNA/Translational level

a) Formation of a RNA hairpin loop results in translational attenuation, under high ionic conditions the collapse of the structure allows translation to proceed.

b) A hairpin is recognised by a binding protein, whose binding causes translational attenuation, under high ionic conditions the factor is released, allowing translation to proceed.

c) Translation is mediated by a GA-binding protein, but one that only binds at high salt, conferring mRNA stability.

To test the hypothesis of the possible role of this GA repeat in the regulation of transcription of pDZ6.2, *in vitro* transcription reactions were performed. These reactions were carried out at different KCl concentrations, comparing the transcription rates of two plasmids, pDZ6.2 (containing the GA repeat) and pDZVIII 5.1.1 as a non GA control. If the GA repeat sequence was regulating transcription by adopting triplex or hairpin DNA, then at low ionic strength (0 mM KCl) it would be predicted that a higher rate of transcription would be observed with pDZVIII 5.1.1 (- GA) than pDZ6.2 (+ GA). At this concentration the secondary structures would be able to form in pDZ6.2, so inhibiting transcription. At higher ionic strength, the inhibition of transcription should cease, so allowing pDZ6.2 to be transcribed at the same rate as pDZVIII 5.1.1 However the results obtained from these *in vitro* transcription reactions were inconclusive. Although the results didn't prove the hypothesis, they neither disproved it. It could be argued that using a viral RNA polymerase with a plasmid containing cDNA of plant origin isn't an accurate comparison to the plant RNA polymerase activities. However if a viral RNA polymerase is inhibited by such structures, then it is likely the equivalent plant enzyme will behave in a similar way. Furthermore there is evidence that these GA repeats limit amplification of integrated polyoma virus DNA (Baran, *et al.*, 1987) and cause pausing of DNA replication, when inserted into the simian virus 40 genome (Sridhara Rao, *et al.*, 1988), thus showing that other DNA processes in viruses are affected themselves by such sequences. Another potential argument that could be put forward, is that this experiment isn't a true representation of the plant cell, since there is no chromatin present that could inhibit direct contact between ions and DNA. However these were the initial experiments to investigate the effect of ionic strength on transcription rates of this piece of DNA. In the event of more positive results, *in vivo* experiments would have been performed to investigate how the presence of chromatin and other factors might effect direct contact between ions and DNA in some way. Transient assays, involving reporter gene constructs, would show any *in vivo* role of this region by monitoring expression at different ionic strengths and comparing constructs with and without the GA region. If these experiments were to be repeated, a - GA control (pDZ6.2 with the GA region removed) would make a better negative control.

This would give more comparable results and a better indication of the differences in RNA stability and transcription rates.

Of course there is always the possibility that this region doesn't play a role in regulation of transcription or translation and is an artefact of cloning or cDNA synthesis. However if all the possible ways in which this repeat could perform regulatory role in transcription are considered, then only the first mechanism (1a) could produce results. All the other options listed, involve protein binding factors, which would have not been present in the reactions. This would account for the inconclusive results and suggests that the GA repeat could regulate transcription by one of these other mechanisms. However it could be involved in translation through hairpin loops of RNA as suggested by Dolinnaya *et al.* (1993) or by conferring messenger RNA stability, which would also explain the results. To investigate this possible role in translational regulation, *in vitro* translation experiments could be performed using a wheat lysate system, by comparing the expression levels of two plasmids (+ and - GA) and pDZ6.2 at increasing KCl concentrations. A steady or increased level of expression of pDZ6.2 and the + GA plasmid (as compared to the - GA) with increasing ionic strength would show that GA region was conferring some sort of regulation on this process. Such experiments would produce a positive result if 2a were the regulatory mechanism used, although a wheat lysate system, which is a fairly crude protein extract, may include protein factors similar to those GA factors that may be involved in gene regulation in *D. spicata*. This would possibly lead to an increase in the translational activity of pDZ6.2 under high ionic conditions, if one of the other options (2b-c) were the regulatory mechanism, although the wheat system may contain GA factors not responsive to NaCl.

There is another possibility that the triplex or hairpin structure formed by the GA region may not directly regulate transcription or translation. It may be that it prevents the binding of a protein that does regulate one of these processes. Bhasker *et al.* (1993) (working on iron responsive elements) propose that regulation of translation by such structures in this way depends on two factors; how close the putative regulatory structure lies to the 5' end of the transcript, (they suggest within 20 bp) and secondly the Gibb's free energy of the structure's sequence, they suggest it must be less than - 50 Kcal/mol (Bhasker, *et al.*, 1993) to be involved.

There is no evidence that these structures do form from the GA repeat region in pDZ6.2. Thus there is a need to perform studies, such as those described by Martínez-Balbás *et al.* (1993) and Dolinnaya *et al.* (1993). Treatment with OsO_4 and other reagents that target single stranded DNA would show the presence of such structures, as they would react with the single stranded "fourth" strand not involved in the triplex (see figure 4.4.2).

Figure 4.4.1

4.13

ATTCG*CT*GA*CGT*TC**GTGAGAGAGTGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
GAGAGAGAGAGAGCGAGAGAGAGAGAGAACTAGT

1.30

GGCACGAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGCNC
T*G*C*C*TTT*C*AT**CA**T*T*TCTANCCCC***C*CT*GTGTTTTTTTTTTTTTTTT
TT*AAAAA**C*T*TCTTTT**CCCCCT**GCGGTAT**TCTCTT**CACAA****CA*A**
*TTT****CACA**TTTTTTTTGGGCACACACCCCATTT*TGTAG*GTTT*CA*A*ATA*CC
ACACACA**TTTTTCCCCG*TG*TATACAC*CTCT*G*G*TTTT**TTT*AACCCCTTTTT*
T***TCCCCCG*GT****TTTTT*TT*AGGG*A*ACAAATT*T**TTT*GG*G**TTT*TTA
AAA*A*TCT*GGAA*TGGTG*CG*GG**TT*T*G*TAA***C*TTC*A**TAT*AT**A*AT
TTT*TT*CCC*CT*TTC*CCTT*TT*T*CTGTTTTT*****CCCTTTTTTT*ATAG*TTT**
GG**G***T*TAA***TTGNAAA*A*AAAAT***T*CC**GGGG***T*TT***CGTT****
AT*T*G*C*TTTTTTG**TC

p256C7

TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
TCTCTCTCTCTCTCTCTCCTCGTGCCGAATTCGGCACGAGCATTTGTGTTCTTAATCACA**AT**
GAATTTATTATATTTCCTTTCGTTTCTGGGCTGTATTACTCTCTGCTTGAGTGCCGGTTTGT
ACAAACCTCCTAACAACATAGAATCTGAGAACGAAGTTTACACCGGAAATATTTGCTTCTTG
CCATTGGAAGTTGGGGTATGCCGAGCTCTGTTCTTTAGGTACGGATACGATCCAGCGATAAA
GGCATGCAAGGAATTCATGTACGGCGGTTGCCAAGGGAACGCTAACAATTTCAAGACTTTAG
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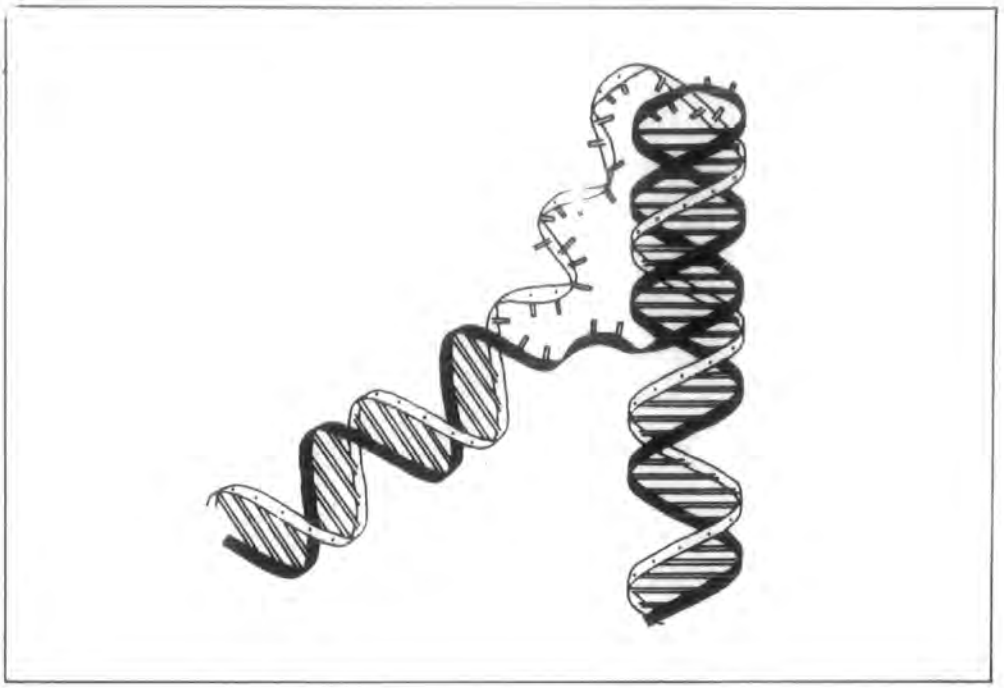
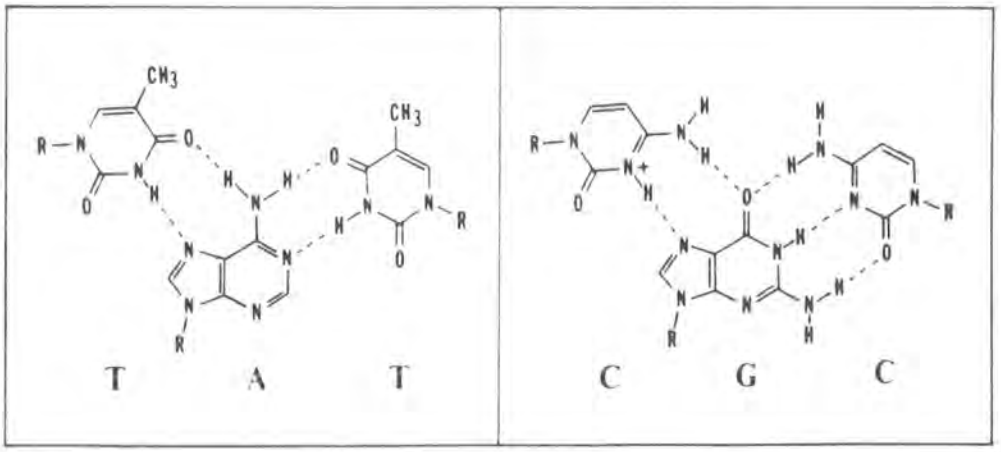
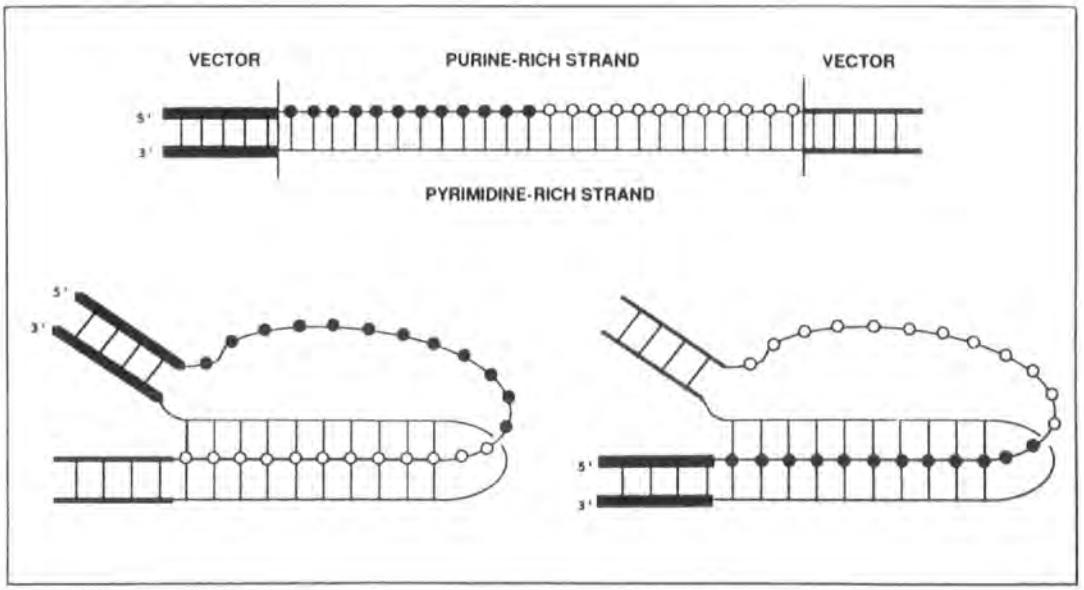
ATATAACACTTCAATAAAAAGTTATAAATAAAAAAAAAAAAAAAAAAA




T A T
C G C


VECTOR
PURINE-RICH STRAND
VECTOR
5'
3'
PYRIMIDINE-RICH STRAND
5'
3'
5'
3'

4.5 Future Studies

Heterologous probing of Southern blots of *D. spicata* genomic DNA with a soyabean cDNA failed to isolate a homologue of the P5CR gene, as did PCR. Complementation of *E. coli proC* mutants with a *D. spicata* cDNA expression library could therefore be used, as done by Delauney *et al.* (1990).

The construction of a *D. spicata* genomic library would enable the isolation of the corresponding genomic clones of the inserts of pDZ2.8L, pDZ6.2 and pDZVIII 1.2.1. The 5' regulatory regions of each could then be searched for any consensus sequences that may represent those responsible for NaCl regulation. Reporter gene studies of these elements fused to GUS may reveal those that play a major role in NaCl-induced gene regulation. DNAse I protection assays would identify *trans*-acting nuclear binding factors that may bind to such *cis*-acting elements.

Further studies on the abundance of transcripts corresponding to the insert of pDZ2.8L, need to be carried out at different time intervals after exposure to NaCl, ABA and proline. This would determine whether these transcripts do increase under such conditions, in accordance with Zhao's data, which showed an increase in transcript abundance four to eight hours after exposure to 260 mM NaCl (Zhao, *et al.*, 1989). In addition transcript abundance should also be examined at different stages of the cycle, to ensure that any changes in transcript abundance are due to the effects of NaCl stress and not because the cells are being arrested at different stages of the cell cycle. Synchronous cultures could be obtained by treatment with colchicine, which would leave cells at the same point in the cell cycle and then washed thoroughly, prior to examining the transcripts of interest. Such experiments would determine whether or not a histone 2B protein really does play a role in salt tolerance and is not just an artefact.

pDZ6.2 and pDZVIII 1.2.1 appear to be members of a multigene family, since they encode similar proline rich proteins, but differ in their nucleotide sequences. Screening a genomic library would identify other members of this family. In addition expression studies in the whole plant may determine tissue-specific expression of the different members of the gene family. To differentiate between the different genes, reporter gene fusions with the 5' flanking regions would have to be used, such as GUS, LUX and CAT, since the genes appear to be so similar, their transcripts would be likely to crossreact with one another. pDZ6.2 and pDZVIII 1.2.1 both encode proline rich proteins, to determine whether any (or all) of these proline residues are actually hydroxyprolines, the peptide should be isolated and sequenced. Reverse genetics could be used to investigate the function of these putative proline-rich cell wall proteins in NaCl tolerance. If genes were overexpressed in tobacco grown at different NaCl concentrations, would they exhibit increased NaCl tolerance? Likewise if expression of these genes was blocked in *D. spicata* cells, through antisense hybridisation, would they show decreased NaCl tolerance? An

antibody could be raised by expressing the genes in *E. coli*, which could be used to identify the localisation of the protein within the plant.

pDZ6.2 and pDZVIII 1.2.1 showed an increase in transcript abundance to ABA. Therefore their 5' flanking regions should be searched for ABA responsive elements and corresponding *trans*-acting factors, similar to those found in other ABA-regulated genes. Endogenous ABA levels should also be examined to determine if they increase upon exposure to NaCl and therefore show whether ABA could play a role in the regulation NaCl-induced genes.

The northern analyses of transcript abundance suggested that the genes corresponding to pDZ2.8L, pDZ6.2 and pDZVIII 1.2.1 are regulated transcriptionally or post transcriptionally by mRNA stability. *in vitro* nuclear run on experiments could be used to determine which mechanism is involved, or alternatively the use of transcription inhibitors , such as actinomycin-D or α-amantin.

A genomic clone of pDZ6.2 would conclusively show whether the 68 bp GA repeat, found in the cDNA, does exist at the genomic level. The initial *in vitro* transcription experiments to show whether this repeat does have a regulatory function, proved inconclusive. Therefore they should be repeated, including a -GA control (pDZ6.2 with GA repeat removed). This would give a better comparison for RNA stability and transcription rates. DNAse I protection assays may identify any factors that bind to either triplex DNA or the GA repeat alone and further identify the possible mechanism by which this region may be performing a regulatory role. The GA repeat may be regulating at the translational level (rather than transcription). To investigate this, *in vitro* translation experiments could be carried out, using a wheat lysate system, to compare the expression of two plasmids, with and without GA repeats, at different ionic strengths. A steady or increased level of expression may indicate that the GA repeat, through the formation of DNA hairpin loops, is conferring some sort of regulation on this process and thus gene expression. To prove that this GA repeat region can form non B-form DNA structures and that this may be ionically determined, treatment with OsO_4 and other reagents that target single stranded DNA, at different ionic strengths, would show the presence of such structures, by reacting with the "fourth" strand not involved in the triplex.

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