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**Studies of a Cold-Induced Gene Encoding a
Hybrid Proline-Rich Protein in *Brassica napus***

A thesis submitted to the University of Glasgow for the degree of
Doctor of Philosophy

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September, 1995

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Contents

Section	Title	Page
	Acknowledgements	i
	Contents	ii
	Abbreviations	ix
	Summary	1
Chapter 1	Introduction	3
1.1	Abiotic stress	3
1.2	Low temperature stress	4
1.2.1	Definition of low temperature stress	4
1.2.2	Classification of stresses	5
1.3	Freezing low temperature-induced injury	6
1.3.1	Intracellular freezing	6
1.3.2	Extracellular freezing	7
1.3.2.1	Freeze-induced dehydration	8
1.3.2.1.1	Dehydration-induced membrane injury	9
1.4	Genetics of freezing tolerance	11
1.5	Cold acclimation	11
1.5.1	Dehydration- and ABA-induced cold acclimation	13
1.5.2	Kinetics of cold acclimation	13
1.6	Biochemical changes	14
1.6.1	Membrane composition	15
1.6.2	Carbohydrate content	18
1.6.3	Protein metabolism	19
1.6.4	Isozyme composition	21
1.6.5	Structural proteins	22
1.6.6	Ribosomal RNA	23

1.6.7	Cryoprotectants	24
1.7	Gene expression	26
1.7.1	Cloned genes	27
1.7.1.1	Temporal expression of cold-induced genes	28
1.7.1.2	Spatial expression of cold-induced genes	30
1.7.1.3	Regulation of cold-induced genes in response to other stimuli	31
1.7.2	Structure and putative functions of cold-induced genes	32
1.8	Signal transduction in low temperature responses	35
1.8.1	Abscisic acid	36
1.8.2	Involvement of calcium and protein phosphorylation	38
1.8.3	Cold specific <i>cis</i> -elements	40
1.9	Project aims	42
Chapter 2:	Materials and Methods	46
2.1	Materials	46
2.1.1	Chemicals	46
2.1.2	DNA modifying enzymes	46
2.1.3	Radiochemicals	46
2.2	General preparatory procedures	46
2.2.1	pH measurement	46
2.2.2	Autoclaving	47
2.2.3	Filter sterilisation	47
2.2.4	Glassware	47
2.2.5	Solutions and equipment for RNA work	47
2.3	Growth media	47
2.3.1	Liquid media	48
2.3.2	Solid media	48
2.3.3	Antibiotics, IPTG, and X-Gal	49
2.4	Bacterial strains and cloning vectors	49

2.5	Plant material	50
2.5.1	Growth of plants	50
2.5.2	Treatment of plants	51
2.6	Isolation of RNA from plant tissue	52
2.6.1	Total RNA	52
2.6.2	Isolation of poly(A) ⁺ RNA	53
2.7	The amplification and purification of plasmid DNA	54
2.7.1	Preparation of competent cells	54
2.7.2	Transformation of competent cells	54
2.7.3	Preparation of plasmid DNA	55
2.7.3.1	Small-scale preparation of plasmid DNA	55
2.7.3.2	Small-scale preparation of DNA for sequencing template	56
2.7.3.3	Large-scale plasmid DNA preparation	57
2.8	Nucleic acid precipitation	58
2.9	Quantification of DNA and RNA	58
2.10	Agarose gel electrophoresis	59
2.10.1	Denaturing electrophoresis of RNA	59
2.10.2	Alkaline electrophoresis of DNA	60
2.10.3	Electrophoresis of DNA	60
2.10.4	Marker DNA	61
2.11	Isolation of DNA fragments from agarose gels	61
2.11.1	GeneClean [®]	61
2.11.2	Magic [™] DNA purification system	62
2.12	Digestion of DNA with restriction endonucleases	62
2.13	Ligation of DNA inserts into plasmid vectors	63
2.14	Blotting of nucleic acids	63
2.14.1	Northern blotting	63
2.14.2	Southern blotting	64
2.15	Radio-labelling of DNA	64

2.15.1	Preparation of the DNA to be labelled	64
2.15.2	Random priming of dsDNA	65
2.15.3	Labelling of dsDNA by nick-translation	65
2.15.4	Separation of labelled DNA from un-incorporated radio-nucleotides using spin columns	66
2.15.5	Measurement of the incorporation of radionucleotides into DNA probes	66
2.16	Hybridisation analysis	67
2.16.1	Hybridisation analysis of northern blots	67
2.16.2	Washing of northern blots	67
2.16.3	Hybridisation analysis of Southern blots	68
2.16.4	Washing of Southern Blots	68
2.16.5	Autoradiography	69
2.16.6	Stripping filters of bound radioactive probes and blocking agents	69
2.16.7	Staining of filter-bound RNA/DNA with Methylene Blue	69
2.17	Construction of a cDNA library	70
2.17.1	cDNA synthesis	70
2.17.2	Blunting the cDNA termini	71
2.17.3	Ligation and subsequent kinasing of <i>EcoR</i> 1 adaptors	72
2.17.4	<i>Xho</i> I digestion and size fractionation	72
2.17.5	cDNA precipitation and ligation into the Uni-ZAP™ XR Vector	73
2.17.6	Packaging, Plating, titering, and amplification of the cDNA library	73
2.18	Screening libraries	75
2.18.1	Preparation of plating bacteria	75
2.18.2	Plating bacteriophage	75
2.18.3	Plaque lifts	76
2.18.4	Selection of bacteriophage plaques	77
2.18.5	Preparation of plate lysate stocks	78
2.19	PCR	78

2.19.1	DNA preparation	78
2.19.2	PCR reaction	79
2.20	<i>in vivo</i> excision from the Uni-ZAP™ XR bacteriophage	79
2.21	Large-scale preparation of DNA from genomic clones	80
2.22	Dideoxy DNA sequencing	82
2.22.1	Annealing of template and primer	82
2.22.2	Labelling and termination reactions	83
2.22.3	Sequencing gel electrophoresis	83
2.23	Generation of clones for sequencing by directed deletions	84
2.23.1	Linearisation of plasmid and protection of the priming site	84
2.23.2	Digestion with <i>Exo III</i>	85
2.23.3	Creation of blunt ends	85
2.23.4	Ligation and characterisation of deletions	85
2.24	Transformation of <i>Arabidopsis thaliana</i>	86
2.24.1	Surface-sterilisation of seed	86
2.24.2	Growth of plants	87
2.24.3	Transformation of roots	87
2.24.4	Analysis of progeny from transformed plants	88
2.24.5	Histochemical localisation of β -glucuronidase	89
Chapter 3:	Characterisation of the cDNAs LF5B1 and BnPRP	90
3.1	Introduction	90
3.2	Sequencing of LF5B1	90
3.3	Analysis of the LF5B1 sequence	91
3.4	Identification of the sequence hybridising to the cold-induced transcripts.	91
3.5	Construction of a cDNA library	92
3.5.1	Isolation of poly(A) ⁺ RNA	93
3.5.2	cDNA production, cloning, and amplification	94
3.6	Screening of the cDNA library	95

3.7	Sequencing of the cDNA clone 6	96
3.8	Sequence analysis of the cDNA clone 6	97
3.9	Comparison of LF5B1 with BnPRP	97
3.10	Discussion	98
Chapter 4:	Isolation and characterisation of the genomic clone λBnPRP	103
4.1	Introduction	103
4.2	Screening of a <i>Brassica napus</i> genomic library	103
4.3	Isolation of genomic clones	105
4.4	Restriction enzyme and Southern analysis of the isolated genomic clones	105
4.5	Restriction enzyme mapping and subcloning of λ BnPRP genomic clone 2	107
4.6	Sequencing of λ BnPRP genomic clone 2	109
4.7	Analysis of the sequence of <i>BnPRP</i>	111
4.8	Comparison of BnPRP with other plant hybrid proline-rich proteins	113
4.9	Discussion	115
Chapter 5:	Analysis of the expression of <i>BnPRP</i>	121
5.1	Introduction	121
5.2	Cold-induced expression of <i>BnPRP</i>	121
5.2.1	Temporal expression of <i>BnPRP</i>	121
5.2.2	Spatial expression of <i>BnPRP</i>	122
5.3	Expression of <i>BnPRP</i> in response to dehydration	122
5.4	Expression of <i>BnPRP</i> in response to exogenous ABA	123
5.5	Expression of <i>BnPRP</i> in response to heat-shock	124
5.6	Expression of <i>BnPRP</i> in response to wounding	124
5.7	Expression of a <i>BnPRP</i> cognate in <i>Arabidopsis thaliana</i> in response to low temperatures	125

5.8	Discussion	126
Chapter 6	Analysis of the putative <i>BnPRP</i> promoter	133
6.1	Introduction	133
6.2	Isolation of the putative promoter	133
6.3	Analysis of the <i>BnPRP</i> upstream sequence	134
6.4	Transformation of <i>Arabidopsis thaliana</i> with promoter-GUS constructs.	136
6.4.1	Production of the promoter-GUS construct	136
6.4.2	Transformation of <i>Agrobacterium tumefaciens</i>	137
6.4.3	Transformation of <i>Arabidopsis thaliana</i>	138
6.4.4	Analysis of the transformants	138
6.4.5	GUS expression driven by the <i>BnPRP</i> putative promoter	139
6.6	Discussion	140
Chapter 7:	General Discussion	147
7.1	Isolation of <i>BnPRP</i>	147
7.2	The <i>BnPRP</i> gene family	147
7.3	Expression of <i>BnPRP</i>	148
7.4	Putative Function of BNPRP	148
7.5	Future work	152
References		

Abbreviations

Abbreviations not described in 'Instructions to Authors', The Biochemical Journal (1992) 281: 1-19 are listed below, with their definitions.

cpm	counts per minute
CsCl	caesium chloride
dsDNA	Double stranded DNA
EtBr	ethidium bromide
GUS	β -glucuronidase
IPTG	isopropylthiogalactoside
OAc	acetate
OD	optical density
pH	hydrogen ion concentration, $-\log_{10}$ of
PRP	Proline-rich protein
psi	pounds per square inch
PVP	polyvinyl pyrrolidine
ssDNA	Single stranded DNA
TEMED	N,N,N',N'-tetramethylethylene diamine
(v/v)	(volume/volume)
(w/v)	(weight/volume)
X-Gal	5-bromo-4-chloro-3-indolyl β -D-galactopyranoside
X-Gluc	5-bromo-4-chloro-3-indolyl glucuronide

Summary

Studies were undertaken to examine molecular events that occur during cold acclimation. This is a process whereby a plant increases its tolerance to low temperatures. The LF5B1 cDNA from *Brassica napus* had previously been isolated (Pallas, 1992). The cDNA was sequenced, which revealed that it was a composite cDNA, apparently composed of at least three separate cDNAs. The cDNA had been shown to hybridise to cold-induced transcripts, but it was not known which part of the cDNA was hybridising. The restriction enzyme *Msp* I cut the cDNA into five fragments. Using each of these fragments to probe northern blots allowed identification of the portion of the cDNA that was hybridising to the cold-induced transcripts.

One of the *Msp* I fragments that hybridised to cold-induced transcripts was used to screen both a "cold-induced" leaf cDNA library and a *Brassica napus* genomic library. Ten cDNAs were isolated, none of which represented a full length cDNA. The cold-induced transcripts had been estimated to be approximately 1.5 kb (Pallas, 1992) whereas the longest cDNA isolated was less than 1 kb. The largest cDNA was sequenced and found to be similar to LF5B1, though not identical. The sequence revealed that the encoded protein was a hybrid proline-rich protein; the N-terminal part was very rich in the amino acid proline while the C-terminal part contained regions that are typical of membrane spanning domains. This cDNA was named BnPRP, this stood for *Brassica napus* proline-rich protein. Seven genomic clones were also isolated and partially characterised and one of these clones was subcloned and sequenced. The sequence in the corresponding region was identical to the cDNA BnPRP and the clone contained all of the putative coding sequence of *BnPRP*. This allowed the entire putative protein, BnPRP to be analysed. In addition to the information derived from the cDNA it revealed a putative signal peptide at the N-terminus and three distinct

domains within the proline-rich region. The lack of identity of the LF5B1 and BnPRP cDNAs indicates the presence of a gene family. This had been previously suggested based on data from Southern blots of *Brassica napus* DNA probed with the LF5B1 cDNA (Pallas, 1992). The possible function of the BnPRP protein in relation to cold-tolerance is discussed.

Preliminary expression studies showed that LF5B1 hybridised to cold-induced transcripts and no expression in response to heat-shock was detected (Pallas, 1992). Further investigations into the expression of *BnPRP* were undertaken. Transcripts were detectable within eight hours of transfer to 4°C and increased up to 24 hours of cold treatment. Upon removal of the cold stimulus the transcript level rapidly fell; after two hours of deacclimation only very low levels were detectable and no transcripts were detected after eight hours deacclimation. The transcripts were found to be highly abundant in leaf tissue, lower levels were detected in stem tissue and no expression was detected in the root tissue; in all tissues the transcript was only detected after cold treatment. Dehydration stress caused no detectable accumulation of the transcript and no increase in transcript abundance was detected in response to abscisic acid (ABA), heat-shock or wounding. A control probe from a dehydration- and ABA-inducible gene was used to show that dehydration had occurred and that ABA had entered the tissue.

The genomic clone contained several kb 5' of the putative coding region and 1.35 kb of this DNA was isolated from the genomic clone by PCR. This was subcloned upstream of the β -glucuronidase gene (*GUS*) in the PBI101.1 vector to make a promoter-GUS construct. This vector was used to transform *Arabidopsis thaliana* plants. Three lines of transformants were analysed for GUS activity but no activity was detected in plants maintained at control temperatures (22°C) or cold treated (4°C).

Chapter 1: Introduction

1.1 Abiotic stress

Abiotic stress can be defined as any environmental factor that is potentially unfavourable for living organisms (Levitt, 1980). Plants are exposed to a number of abiotic stresses, including low temperatures, high temperatures, dehydration, flooding, and high salt. The intrinsic abilities to withstand the stresses that plants are subjected to vary widely between plant species, though they usually reflect the environment that the plant is found in. In addition plants have evolved mechanisms which enable them to acclimate to a variety of environmental stresses (Levitt, 1980; Lange *et al.*, 1981). Acclimation is a process by which the plant is able to acquire an increased tolerance to a stress after exposure to a moderate level of the stress. The degree to which plants can acclimate to any given stress again varies widely (Levitt, 1980).

"The responses of plants to the severities of their environment have occupied the attention of man long before the beginning of the science of biology" (Levitt, 1941). Plants, in particular crop species, have been selected since the beginnings of agriculture for resistance to environmental stresses, enabling the plants to survive and still produce valuable yields. Despite plants' abilities to endure and adapt to prevalent stresses large areas of the earth's land are too cold, hot, dry, wet, or salty to allow cultivation (Weiser, 1970). An understanding of the nature of the stresses and the mechanisms which allow plants to withstand the stresses, defining individual components, will eventually allow further improvements to the stress resistance of crop plants, allowing previously uncultivable land to be cultivated.

1.2 Low temperature stress

Various aspects of low temperature stress have been studied. The main aims of these studies have been to determine the cause of low temperature-induced damage and to elucidate the mechanisms which allow plants to endure and adapt to low temperatures. The importance of low temperatures in determining the distribution of plants has not been overlooked; low temperature along with drought stress have been acknowledged to be the two environmental factors that have the greatest effect in limiting the distribution of plants (Levitt, 1956; Woodward, 1988).

1.2.1 Definition of low temperature stress

Abiotic stress is a major reason for the disparity between potential and actual yield (Boyer, 1982), low temperature can play an important role in the reduction of actual yield and can cause injury and death if the temperatures are below a plant's adaptive range. Defining what constitutes a low temperature, or a low temperature which is stressful, is not straight forward and depends on the plants in question. The same temperature, i.e. 15°C, can cause heat stress in some temperate plants and low temperature stress in other plants that are native to the tropics (see Howarth and Ougham, 1993). Plants have been divided into different classes to indicate the degree of low temperature they can tolerate; chilling sensitive, freezing sensitive, and freezing tolerant are commonly used (Larcher, 1981). Plants that have the ability to be freezing tolerant have also been termed hardy, while those that have not, tender (Levitt, 1980). The divisions between the categories are ill defined. Plants also have the ability to cross these boundaries as they acclimate and deacclimate. Alternative categories have been suggested. Howarth and Ougham (1993) suggested defining plants according to temperature ranges that elicit specific physiological responses, such as optimal or

above, suboptimal but non-damaging, damaging but non-lethal, and lethal. Plants' abilities to move between these boundaries complicates this system.

Definitions in any system are further complicated as many plants' responses to low temperatures can vary depending on the developmental stage. Plants are particularly vulnerable to low temperature stress when they are undergoing rapid growth. For this reason plants are sensitive as seedlings, during spring and summer growth and during floral development (Levitt, 1980). Even plants with high levels of freezing tolerance, for example conifers, can be severely injured by a light summer frost (Levitt, 1980). Conversely plants which do not have high levels of freezing tolerance can be extremely freezing tolerant during the seed stage; seeds can commonly survive temperatures below -100°C (Levitt, 1980).

Classifying what constitutes low temperature stress is also complicated by the requirement of many plants to vernalize. This is a process in which the plants require a period of low temperature, which would normally be stressful, to trigger flowering. Many species also require light stimulation (Salisbury and Ross, 1985; Metzger, 1988).

1.2.2 Classification of stresses

Chilling low temperature stress is used to describe temperatures above 0°C while freezing low temperature stress describes temperatures below 0°C . The injuries caused by low temperatures have similarly been divided into chilling injury and freezing injury. Chilling injury is taken to be caused by temperatures above 0°C , freezing injury below 0°C (Levitt, 1980). This division is again somewhat arbitrary as the water within the plant may well not freeze at higher subzero temperatures.

Many plants that grow in temperate climates are subjected to subzero temperatures at some point of their life cycle resulting in freezing low temperature stress. The remainder of this section will discuss the direct and indirect effects of freezing low temperature stress, the sites of freezing low temperature stress and injury within the plant, the ability of plants to withstand freezing low temperature stress, and the biochemical and genetic alterations that occur during cold acclimation.

1.3 Freezing low temperature-induced injury

Freezing injury can potentially occur in all plants and can manifest itself in several ways. Primary direct injury is caused by intracellular freezing, secondary injury can be caused by extracellular freeze-induced dehydration and other freeze-induced stresses. In virtually all cases ice crystals are the agents of direct and indirect injury.

1.3.1 Intracellular freezing

Ice crystals can form within the cell by two means. If temperature cooling rates are sufficiently rapid ice crystal nucleation within the cell can occur (see Levitt, 1980). Spontaneous nucleation does not generally occur unless the plant is cooled to at least -10°C (Mazur, 1977). The rate of cooling required for internal ice crystals to form has been estimated to be between 3 to $16^{\circ}\text{C}\cdot\text{min}^{-1}$ and $3^{\circ}\text{C}\cdot\text{h}^{-1}$ the first estimate was from studies of protoplasts suspended in liquid medium while the second was from studies of intact plants (Steponkus *et al.*, 1983; Steffen *et al.*, 1989). As environmental cooling rates are usually less than $1^{\circ}\text{C}\cdot\text{h}^{-1}$ ice crystallisation within the cell is not a common cause of injury (Steffen *et al.*, 1989). An exception to this is sun scald where the sun on a cold winter's day can raise the temperature above freezing, when the sun is

suddenly hidden, i.e. behind a cloud, the temperature can drop rapidly, leading to intracellular freezing (Weiser, 1970). The second way that ice crystals can enter the cell is by penetration of the plasma membrane by extracellular ice crystals (Mazur, 1969; Steponkus *et al.*, 1983). Intracellular freezing by either means is considered to be lethal to the cell (Burke *et al.*, 1976; Levitt, 1980). Exceptions to this have been demonstrated experimentally. Ultra-rapid freezing in liquid nitrogen, followed by transfer to warm (25-30°C) aqueous solution did not kill onion epidermis cells that would normally have been killed by temperatures as high as -10°C (see Levitt, 1980). This was explained by the water becoming vitrified rather than crystalline; vitrified water displays a glassy state and ice crystals do not form. The water may have been pseudovitrified, containing small, submicroscopic ice crystals. The experiments did demonstrate that the onion epidermis cells were not damaged directly by the low temperatures, instead relatively large ice crystal growth, and the resulting cellular disruption caused cellular injury. In nature, the temperatures and the rate of cooling/warming occurring would lead to cell death if intracellular crystals formed. Even if the ice crystals were initially small and not damaging to the cell, the relatively long time that the plant tissue would be exposed to the freezing temperature would lead to ice crystal growth and subsequent injury (Levitt, 1980).

1.3.2 Extracellular freezing

The apoplastic solution has a lower solute concentration than the cellular solution (Wenkert, 1980), and consequently has a lower freezing point depression. Ice crystal formation has been shown to occur in the large xylem vessels (Kitaura, 1967), this is expected as their sap is very dilute and probably has the highest freezing point of any of the plant's water. In addition the cells are large which does not favour undercooling of the liquid. Subsequent freezing of extracellular water is relatively rapid, spreading from the nucleation points in the vessels to all parts of the plant. The

internal surface of the cell wall and external surfaces of the plants have also been found to be nucleation sites for ice crystal growth (Jeffree *et al.*, 1987; Pearce and Ashworth, 1992). The plasma membrane acts as an effective barrier to ice growth and does not allow the external ice to initiate the nucleation of ice crystals within the cell (Steponkus, 1984). Ice crystals form throughout the intercellular spaces at the expense of water vapour in the air and water associated with the cell wall.

The growth of intercellular ice crystals has been proposed to cause several indirect stresses. The most prevalent is freeze-induced dehydration (Section 1.3.2.1). Others include ice pressure, air expulsion and evaporative freeze-dehydration. Little experimental evidence exists to support the importance of any of these secondary stresses except for freeze-induced dehydration and the associated injurious effects.

1.3.2.1 Freeze-induced dehydration

Prior to extracellular freezing the cell must be at temperature and vapour pressure equilibrium with the cell wall and intercellular spaces. As ice crystallisation begins the extracellular vapour pressure drops rapidly. Consequently cell water will have a higher vapour pressure. The cell contents are able to undercool, the vapour pressure of the cell water rises as the temperature falls below the freezing point of the cell contents and this leads to a gradient, with water leaving the cell. The extracellular ice crystals will grow as more water leaves the cell (Levitt, 1980). If the temperature continues to drop, water will continue to leave the cell until the cell sap is sufficiently concentrated to be in equilibrium with the extracellular spaces.

Two indirect stresses are imposed on the cell by freeze-induced dehydration; one of these is an increased concentration of the cell sap. Removal of the water leads to the increase in sap concentration and is referred to as the "solution effect". The

increased concentration of salts in the protoplasm was thought to cause injury, though no direct evidence supports this; also no correlation exists between low temperature tolerance and salt content (see Levitt, 1956; Levitt, 1980). The second stress is cell collapse, caused by the reduction in cell volume. If the reduction is sufficient the cell wall collapses (Pearce, 1988; Pearce and Ashworth, 1992). This phenomenon is not universal, red osier dogwood cells displayed protoplasmic contraction rather than cell collapse (Ristic and Ashworth, 1994), the degree of damage to the contracted cells varied seasonally.

1.3.2.1.1 Dehydration-induced membrane injury

Evidence has accumulated to indicate that the plasma membrane, and to a lesser extent other cellular membranes are the primary sites of freezing injury. "Expansion-induced lysis" is one form of injury. It has been well characterised in rye protoplasts (Gordon-Kamm and Steponkus, 1984 a, b; Dowgert and Steponkus, 1984; Dowgert *et al.*, 1987; Webb *et al.*, 1994). The injury occurred during the freeze-thaw cycle. Protoplasts contracted as the temperature was reduced to -5°C ; significant contraction occurred at this temperature and the excess plasma membrane formed endocytotic vesicles. When the temperature of the protoplasts was increased rehydration occurred, the endocytotic vesicles could not be reincorporated into the plasma membrane, which led to an osmotic pressure that caused the protoplasts to burst. The rye protoplasts experienced expansion-induced lysis upon rewarming after incubation at between -2.5 to -6°C (Uemura and Steponkus, 1989). The same expansion-induced lysis was detected in oat protoplasts, though it occurred over the higher temperature range of -2 to -4°C (Webb *et al.*, 1994). This correlated with the relative freezing tolerance of the two species, rye is more freezing tolerant. Whether these events occur *in vivo* is not known. Experiments on rye mesophyll cells found that multilamellar vesicles formed upon lethal freezing (Singh *et al.*, 1987). These multilamellar vesicles were also

formed when the cells were subjected to severe dehydration, which supports the hypothesis that damaging effects are caused by cellular dehydration. The same response to dehydration was seen when using cultured *Brassica napus* cells.

Other forms of membrane damage have been reported. When protoplasts are cooled to more severe low temperatures a loss of osmotic responsiveness has been observed (Steponkus and Lynch, 1987; Webb *et al.*, 1994). This loss of osmotic responsiveness is associated with changes in the structure of the plasma membrane. Singh *et al.* (1987) suggested that there was a continuum of plasma membrane injury during freezing, which eventually led to cell death. Examining rye mesophyll cells, the multilamellar vesicles that formed became increasingly tightly packed. Eventually loss of osmotic responsiveness occurred, though no phase transitions in the plasma membrane were detected. This was in contrast to the experiments on rye protoplasts that concluded that there were two distinct phases in injury, the contraction of the plasma membrane which could lead to expansion-induced lysis, and, at lower temperatures, phase transitions, causing loss of osmotic responsiveness (Gordon-Kamm and Steponkus, 1984b). The differences could be due to differential behaviour of isolated protoplasts and whole cells. The cell wall has been shown to play an important role in rye, alfalfa, and *Brassica napus* cells during osmotically-induced plasmolysis. Strands of plasma membrane forming between the cell wall and the plasmolysed protoplast (Johnson-Flanagan and Singh, 1986). Upon rehydration expansion-induced lysis occurred if dehydration was severe enough, similar to the effects of freeze-induced dehydration.

Compositional differences in plasma membranes of different plant species have been associated with different levels of resistance to freeze-induced injury (Uemura and Steponkus, 1994). Experiments by Steponkus *et al.* (1988) showed that increasing monounsaturated and diunsaturated phosphatidylcholine molecules increased the

freezing tolerance of the protoplasts, supporting the hypothesis that the composition of the membranes plays an important role in resistance to freeze-induced injury.

1.4 Genetics of freezing tolerance

Plants are genetically programmed to have tolerance to low temperatures. The level of tolerance varies widely between species (Levitt, 1980). The integrated developmental changes in freezing tolerance are also under genetic control (Weiser, 1970). Crosses between parents with different tolerance levels typically yield progeny with a range of intermediate values, this behaviour is characteristic of a quantitatively inherited trait (Parodi *et al.*, 1983; Norell *et al.*, 1986). Wheat is the most intensively studied species and most of the studies have largely agreed with the inheritance being a quantitative trait. However, though some studies indicated partial dominance of freezing sensitivity, while others found freezing tolerance to show some dominance (see Thomashow, 1990). Studies on oats (Jenkins, 1969) and barley (Rhode and Pulham, 1960) also found the trait to be quantitatively inherited. Again, the recessive/dominant nature of the trait in these studies varied, which led to the conclusion that different genes had effects under different degrees of freeze stress and at different times in the tolerance process.

1.5 Cold acclimation

Many temperate perennials, annuals and biennials that grow in regions of the world that are subject to subzero temperatures have the ability to alter their freezing tolerance when they are exposed to low temperatures. This process is termed cold acclimation. The ability of plants to cold acclimate varies widely. The woody perennials birch and dogwood demonstrate the potential of cold acclimation to increase

a plant's freezing tolerance. Both birch and dogwood are killed at around -10°C prior to acclimation, but fully cold acclimated they can survive extreme low temperatures and can grow in regions frequently exposed to temperatures of -40°C to -50°C (Levitt, 1980). Cereals also show high levels of cold acclimation. Non-acclimated wheat and rye are killed at -5 to -10°C , whereas fully cold acclimated wheat can survive -15 to -20°C while rye can survive -25 to -30°C . Other plants show much lower levels of resistance. *Arabidopsis thaliana* can survive temperatures of around -3°C when non-acclimated, increasing to between -8 and -10°C after full cold acclimation (Gilmour *et al.*, 1988). Some plants are "resistance stable", their freezing tolerance does not vary much over the year. This is more prevalent amongst lower plants including algae, mosses and liverworts than higher plants (Levitt, 1980).

A plant's ability to cold acclimate is dependent on two factors. One is the ability of the plant to function biochemically at the lower temperatures, the second involves the plant's increased ability to prevent freeze-induced injury. The plant must perceive the environment in order to "know" when to acclimate. Woody perennials use photoperiodic cues to detect when the active growing season is coming to an end. This leads to the onset of cold acclimation (Van Huystee *et al.*, 1967), which is further enhanced by exposure to low non-injurious temperatures (Weiser, 1970). Herbaceous plants are not affected by photoperiodic cues, but rely totally on exposure to low temperatures in order to cold acclimate (Fennel and Li, 1985). Herbaceous plants deacclimate in response to increased temperatures (Fennel and Li, 1985; Boothe *et al.*, 1995), whereas woody perennials deacclimate both in response to photoperiod and elevated temperatures (Levitt, 1980).

1.5.1 Dehydration- and ABA-induced cold acclimation

Exposure to low temperatures is not absolutely necessary for cold acclimation. Dehydration and ABA have both been shown to induce cold acclimation in a number of species. The link between drought stress and low temperature stress is severe desiccation; in low temperature stress freeze-induced dehydration causes desiccation of the plant tissue. Siminovitch and Cloutier (1982) demonstrated that desiccation treatment led to rye plants becoming acclimated to low temperatures and drought. The application of ABA has also been shown to cause cold acclimation in the absence of low temperatures in several species including bromegrass (Reaney *et al.*, 1989), potato (Chen *et al.*, 1983) and *Arabidopsis thaliana* (Lång *et al.*, 1989). Induction of cold acclimation in response to exogenous ABA has also been seen in suspension cultures, in several species, including bromegrass, alfalfa (Reaney and Gusta, 1987), *Brassica napus* (Johnson-Flanagan and Singh, 1987), and potato (Chen and Gusta, 1983). The accumulation of ABA has been observed under desiccation stress (see Walton, 1980) and in response to low temperatures, Chen *et al.* (1983) measured an increase in ABA following exposure to low temperature in the leaves of potato and a similar increase has been measured in the above-ground tissues of wheat (Taylor *et al.*, 1990).

1.5.2 Kinetics of cold acclimation

Plants show varying response times for cold acclimation, leading to increased cold tolerance. The increased low temperature tolerance of a plant is usually measured by calculating the LT₅₀ of the plants after increasing periods of cold acclimation. This is the temperature which results in 50% of the plants being lethally injured. Gilmour *et al.* (1988) tested the abilities of two *Arabidopsis thaliana* races, Landsberg erecta and Columbia, to acclimate. Both races followed a similar pattern of induction. Grown at a

constant temperature of 24°C both races had a LT₅₀ of around -3.5°C. This increased relatively rapidly to approximately -6.6°C after 24 hours at 4°C and after 12 days of acclimation at 4°C the LT₅₀ was -9.3 to -9.4°C. Acclimation for a further 13 days at 4°C did not increase the tolerance of Columbia (Landsberg erecta was not acclimated past 12 days). Similar results were obtained with two varieties of winter oat; acclimation was detected after 48 hours, and full acclimation occurred after 12 days (see Howarth and Ougham, 1993). Other plants require longer periods of cold acclimation to achieve maximum cold tolerance. Ivy (*Hedera helix*) acclimated progressively over a period of six weeks in order to obtain the highest levels of tolerance (Steponkus and Lanphear, 1968). Species which require photoperiodic cues to initiate cold acclimation also take longer to acclimate fully (Van Huystee, 1967).

Deacclimation is the loss of the cold tolerance acquired during cold acclimation. Deacclimation is generally a rapid process, though as with the time scale of acclimation there is variation. Spinach and ivy which acquired increased cold tolerance over one and six weeks respectively, both had an initial LT₅₀ of -7°C. After acclimation the LT₅₀ increased to -17 and -21°C respectively. Both deacclimate, losing virtually all their increased cold tolerance after one week (Fennel and Li, 1985; Steponkus and Lanphear, 1968). *Brassica napus* plants acclimated for 42 days retained a high level of the acquired cold tolerance after one week of deacclimation. Prior to acclimation the plant could withstand -2°C, acclimated for 42 days the LT₅₀ was -16°C and after one week deacclimation the LT₅₀ was still -10°C (Boothe *et al.*, 1995).

1.6 Biochemical changes

Studies of the biochemical changes that occur during cold acclimation have encompassed many factors. The plasma membrane and changes that occur in its composition have been studied in depth, as this is the proposed site of freeze-induced

injury. Other studies have looked at changes in the protein content and profile, carbohydrate content, isozyme composition and accumulation of compounds that act as cryoprotectants.

1.6.1 Membrane composition

As discussed in Section 1.3.2.1.1 the plasma membrane has been implicated as a major site of cold-induced injury. It is therefore not surprising that a number of changes have been detected in the composition of the plasma membrane during cold acclimation. The first studies focused on measuring the fatty acid unsaturation of lipids, since the increase in unsaturation was proposed to alter membrane fluidity (Raison *et al.*, 1971; Lyons, 1973). Membrane fluidity was thought to stabilise the membrane structure, allowing it to function at low temperatures. Steponkus (1984) demonstrated that injury was due primarily to freeze-induced dehydration stress, and was not primarily dependent upon membrane fluidity but on the lipid composition which influences the lyotropic phase behaviour of cellular membranes. Lynch and Steponkus (1987) found large differences in the lipid composition of winter rye after cold acclimation. During cold acclimation the abundance of phospholipids and free sterols increased while cerebrosides, sterylglucosides, and acylated sterylglucosides all decreased in abundance. The increase in phospholipids was caused primarily by an increase in diunsaturated species of phospholipid. No lipid species were unique to cold acclimated or non-cold acclimated tissue, only the proportions of the different lipids varied. In addition to differences in the initial composition of membranes from plants with different non-acclimated levels of freezing tolerance (e.g. the more resistant winter rye has higher levels of phospholipids and lower levels of cerebrosides than spring oat), the changes that occur during cold acclimation also vary. In both rye and oat the levels of unsaturated phospholipids increased, though the increase was greater in rye. A large decrease in the level of cerebrosides occurred in rye, while no significant

decrease was observed in oat (Uemura and Steponkus, 1994). This is associated with the increased level of cold acclimation that occurs in rye.

The changes in the lipid composition are associated with the increased tolerance of the plasma membrane to freeze-induced dehydration. Dowgert and Steponkus (1984) showed that protoplasts isolated from non-acclimated plants formed endocytotic vesicles that were not reincorporated into the plasma membrane upon cellular rehydration, leading to expansion-induced lysis. Protoplasts isolated from cold acclimated plants formed exocytotic vesicles upon freeze-induced dehydration, these were reincorporated as the protoplasts were rehydrated, reducing expansion-induced lysis (Gordon-Kamm and Steponkus, 1984a). Similar changes in behaviour were seen when using rye and *Brassica napus* suspension cultures (Singh *et al.*, 1987); cold acclimated cells formed no vesicles while non-cold acclimated cells formed multilamellar vesicles. The non-cold acclimated cells formed membrane strands that could not be reincorporated into the plasma membrane upon rehydration in response to osmotic stress. The membrane strands were more abundant when cold acclimated cells were used and they could reincorporate into the plasma membrane, preventing expansion-induced lysis. The lipid composition of the rye and *Brassica napus* plasma membrane was not measured so no associations could be made between behavioural changes and the lipid content of the plasma membrane. Gordon-Kamm and Steponkus (1984b) observed that phase transitions occurred with less frequency in the protoplasts from cold acclimated plants.

Direct evidence for the importance of the plasma membrane lipid composition in cold acclimation was obtained in experiments where the lipid composition was altered artificially. Steponkus *et al.* (1988) increased the amount of phospholipids in protoplasts isolated from plants that were not cold acclimated. When these protoplasts were subjected to mild freeze-induced dehydration, exocytotic rather than endocytotic vesicles formed, mimicking the effects of cold acclimation. Similarly, artificial

enrichment of protoplasts isolated from non-cold acclimated rye protoplasts with diunsaturated species of phospholipids led to a decrease in phase transitions in response to severe freeze-induced dehydration (Sugawara and Steponkus, 1990).

Further evidence for the direct involvement of the plasma membrane lipids in low temperature tolerance has been provided by studies using transgenic plants. Murata *et al.* (1992) demonstrated that the manipulation of fatty acid saturation had an effect on cold tolerance in plants. cDNAs encoding glycerol-3-phosphate acyltransferase, an enzyme that has been shown to be important in determining the level of phosphatidylglycerol saturation, were obtained from the chilling sensitive squash plant and the chilling resistant *Arabidopsis thaliana*. Both cDNAs were transformed into tobacco plants. The squash cDNA caused an increase in phosphatidylglycerol saturation, whereas the *Arabidopsis* cDNA had the opposite effect. The tobacco transformed with the *Arabidopsis* cDNA had an increased tolerance to low temperatures relative to wild type plants, but the tobacco with the squash cDNA had an increased sensitivity to low temperatures. A similar correlation between the level of saturation and resistance to low temperatures was seen by Wolter *et al.* (1992). The transformation of *Arabidopsis thaliana* with glycerol-3-phosphate acyltransferase from *E. coli* caused an increase in lipid saturation and an associated increase in low temperature sensitivity. The increased saturation observed by Wolter *et al.* (1992) was confined largely to the plastids, implying that plastid membranes in addition to the plasma membrane play a role in determining the level of freezing tolerance. An increase in cold tolerance was also seen in tobacco when it was transformed with the ω -3 fatty acid desaturase from *Arabidopsis thaliana* which caused an increase in fatty acid unsaturation (Kodama *et al.*, 1994). Mutants of *Arabidopsis thaliana* have been isolated that have decreased amounts of unsaturated fatty acids. Miquel *et al.* (1993) characterised an *Arabidopsis thaliana* mutant deficient for the *fad2* gene which was deficient in the production of polyunsaturated fatty acids. The *fad2* mutants were killed by long-term exposure to low-non-freezing temperatures, although previously they had

moderate levels of freezing tolerance. The *fad5* and *fad6* mutants which are deficient in unsaturated chloroplast lipids displayed abnormal plastid development at low temperatures (Hugly and Somerville, 1992).

As well as changes in the lipid content of plasma membranes during cold acclimation changes in the membrane protein profile occur. In cold acclimated rye plasma membranes, Uemura and Yoshida (1984) reported that 20 proteins changed in abundance during acclimation, 11 increased while nine declined. In addition, 26 proteins that were unique to acclimated proteins appeared. A 20 kDa protein increased in abundance during the cold acclimation of a *Brassica napus* suspension culture (Johnson-Flanagan and Singh, 1987) and this polypeptide was associated with a membrane fraction that was enriched in endoplasmic reticulum. It was proposed that the 20 kDa protein would be incorporated into the plasma membrane. This hypothesis was based on the observation that plasma membrane renewal during the development of cold tolerance is mediated by the fusion of proliferating endoplasmic reticulum vesicles with the plasma membrane (Niki and Sakai, 1981).

1.6.2 Carbohydrate content

The increased accumulation of carbohydrates in response to low temperatures has been known for a long time. Sucrose is the most frequently found carbohydrate in freezing tolerant plants, its levels have been shown to increase ten fold in response to low temperatures (Salerno and Pontis, 1989). Whether the accumulation of carbohydrates is a specific response, leading to increased low temperature tolerance, or is a by-product of other metabolic events that occur in the cell in response to low temperatures is not clear. Levitt (1980) suggested that carbohydrate may play a direct role in increased low temperature tolerance by decreasing the osmotic potential of the cell contents, therefore reducing the freeze-induced dehydration. It was also proposed

that it could have an effect by depressing the freezing point of the cell contents, reducing the probability of intracellular freezing. The importance of increased levels of carbohydrate was not supported by Pollock *et al.* (1988) who calculated that the accumulated carbohydrate in seedlings of *Lolium perenne* could only account for a freezing point depression of 0.5°C in a plant that was able to increase freezing tolerance by 6°C. The conclusion that carbohydrate accumulation plays only a small part in increased freezing tolerance is further supported by a poor correlation between accumulated carbohydrate and freezing tolerance in a number of forage grasses (Suzuki, 1989).

1.6.3 Protein metabolism

A large number of studies have investigated a number of aspects of protein metabolism. An increase in soluble protein content (percent of dry weight) during cold acclimation was first recorded by Siminovitch and Briggs (1949) studying the bark cells of black locus. Numerous studies followed this and found that an increase in protein was a common occurrence, although no direct link between increased protein content and increased cold tolerance has been made. There are exceptions where no increase in protein content in response to low temperatures has been detected (Guy and Haskell, 1987), undermining the requirement for a general increase in protein content in cold acclimation.

More recent studies have concentrated on detecting specific changes in protein profiles using two dimensional gel analysis techniques rather than general changes in content. Guy (1990) summarised the information that was present at the time. Studies had been done on 18 species and some of these, such as wheat and *Brassica napus*, had been studied by several independent researchers. The species studied ranged from highly freezing tolerant, including wheat and barley to rice which is chilling sensitive.

Alterations in the protein profile were found to be relatively subtle with the vast majority of proteins being the same in cold acclimated and non-cold acclimated plants. The number of new proteins that were detected was generally less than ten. The subtle nature of the response also applied to the number of proteins that were present in both cold acclimated and non-cold acclimated tissue but varied in relative abundance between the two states. None of the cold-induced or repressed proteins detected were common between any number of species, suggesting that the response to low temperatures is not highly conserved. However, a group of high molecular weight proteins (150-200 kDa) have been observed in a number of species including spinach (Guy *et al.*, 1985; Guy and Haskell, 1987), *Arabidopsis thaliana* (Gilmour *et al.*, 1988), bromegrass (Robertson *et al.*, 1988), and wheat (Perras and Sarhan, 1989; Abromeit *et al.*, 1992). The related size of these proteins has led to the proposal that they are important components in the cold acclimation process. This is supported to some degree by the level of expression of some of these proteins being higher in varieties with greater freezing tolerance (Perras and Sarhan, 1989; Abromeit *et al.*, 1992). The role, if any, of the proteins that increase in abundance is further confused by their abundance varying between different tissues. In wheat a total of eight newly synthesised proteins were detected (Perras and Sarhan, 1989), but only the high molecular weight species (200 kDa) was induced concomitantly in the root, leaf and crown tissue; two were specific to the leaf tissue, two to the root tissue, and three to the crown. In addition, the pattern of up- and down-regulated proteins varied between the tissue types. These differences may reflect different needs of the different tissue types in the cold acclimation process; alternatively it may imply that these proteins are not fundamental to the process.

Sparse information exists on the identity and function of any of the proteins that increase in abundance or are synthesised *de novo* in response to low temperatures and detected by two dimensional gels. An exception to this is the identification of some of the proteins that increase in abundance in spinach as being related to the heat-shock

proteins (Neven *et al.*, 1992). The protein profiles that occur in response to low temperatures are not similar to the profile that is obtained after heat-shock, where the synthesis of the majority of proteins that are normally present is repressed, while a group of highly conserved heat-shock proteins become highly abundant (see Howarth and Ougham, 1993). However, some relationship seems to exist between the two stresses with regards to the induced protein profiles. Neven *et al.* (1992) reported that several 79 kDa proteins which were present at 20°C in spinach leaves increased in abundance upon exposure to low temperatures. The identification was based on sequence analysis and immunological cross-reactivity to antibodies raised against a member of the 70 kDa heat-shock family. The 79 kDa proteins are not typical heat-shock proteins as they are present in tissue that has not been heat-shocked and are not highly responsive to heat-shock.

1.6.4 Isozyme composition

In addition to the appearance of a small number of new proteins, and the up- and down-regulation of others, the presence of different isoforms has been observed. These observations were not surprising when considering the effects that temperature can have on protein structures; small changes in temperature commonly affect the K_m values and activation energies of enzymes (Thomashow, 1990). Roberts (1969) proposed that the different isozymes may play a role in the cold acclimation process by increasing the protein's tolerance to the higher salt concentrations present in the dehydrated cytosol.

Evidence has been collected to support the idea that the low temperature-induced isoforms are better suited to functioning at low temperature, thereby reducing the low temperature stress imposed on the plant. Huner and Macdowall (1976, 1979) characterised ribulose biphosphate carboxylase/oxygenase (Rubisco) from cold

acclimated and non-cold acclimated winter rye plants. The enzyme from the cold acclimated plants had twice the specific activity compared to the enzyme from non-cold acclimated plants. It was also shown that the enzyme from the cold acclimated plants had a lower K_m at 5°C while the enzyme from non-acclimated plants had a lower K_m at 25°C, the cross over point was estimated to be 10°C. Guy and Carter (1984) detected a cold-induced glutathione reductase isozyme with 66% higher activity than the enzyme isolated from non-cold acclimated tissue, this was correlated with the appearance of two electrophoretic variants that were unique to cold acclimated tissue.

1.6.5 Structural proteins

A number of proteins have been observed to increase during cold acclimation that have structural functions. These are involved in the cell wall and the cytoskeleton. The cell wall surrounds plant cells and has been shown to change in mechanical behaviour in response to a number of stresses. Salt (Bolanos and Longstreth, 1984) and drought stress (Ike and Thurtell, 1981) both altered the cell wall elasticity and the change was considered to be an important part of the stress acclimation process. Changes in the plant cell wall during cold acclimation have been reported. Cell walls have been shown to thicken during cold acclimation (Huner *et al.*, 1981). Weiser *et al.* (1990) measured changes in the cell wall content of pea during cold acclimation. The arabinosyl content increased 100%, glycosyl and cellulose increased by 20%, and the hydroxyproline content increased by 80%. Arabinose and hydroxyproline are both constituents of the extensin class of cell wall structural proteins extensin. Anderson *et al.*, (1983) illustrated that freeze-induced water loss is slowed as the bulk mechanical properties of the tissue increase. Other functions related to the altered cell wall composition may be important to the plant's survival. The presence of a cell wall altered the behaviour of rye plasma membrane (Singh *et al.*, 1987) and constituents in

the acclimated cell wall may be important in preventing dehydration-induced membrane damage.

Isoforms of alpha and beta tubulin have been shown to alter in response to cold acclimating temperatures in rye (Kerr and Carter, 1990b) and beta isoforms have been shown to alter in response to low temperatures in spinach (see Chu *et al.*, 1993) and *Arabidopsis thaliana* (Chu *et al.*, 1993). The behaviour of the microtubules also changes. Kerr and Carter (1990a) observed that microtubules were more easily depolymerised in cold acclimated plant cells than in non-acclimated cells. Treatment of the plant tissue with taxol, a compound that stabilises microtubules, led to a decrease in freezing tolerance, indicating that the composition of the microtubules plays an important role in freezing tolerance.

1.6.6 Ribosomal RNA

RNA has been proposed to play an important role in cold acclimation. Changes occur in the messenger and ribosomal RNA content of plants; mRNA changes are discussed in Section 1.7. The synthesis of ribosomal RNA (rRNA) has been shown to increase in response to low temperatures (Páldi and Dévay, 1983). In wheat the increase in rRNA synthesis was shown to be variety-specific. Increases in winter varieties were 3 to 1.5 fold, but in contrast no increase in rRNA synthesis could be detected in spring varieties with low freezing tolerance upon exposure to low temperatures (Páldi and Dévay, 1983). It was concluded that the higher rates of synthesis in the winter varieties were primarily due to the higher cistron number in winter wheats, since the activities of the rRNA cistrons varied as a function of temperature. Sarhan and Chevrier (1985) reported an increase in RNA polymerase I in response to low temperatures, contributing to the increased rRNA levels. Again this was more pronounced in winter varieties. The increases in rRNA have been associated

with polysomes that have been shown to accumulate to higher levels in cold acclimated wheat seedlings than seedling that were not cold acclimated (Perras and Sarhan, 1990). Laroche and Hopkins (1987) found that the protein content of the ribosomes of rye also varied between cold acclimated and non-cold acclimated plants. A 140 kDa ribosomal protein was only detected in cold acclimated tissues. Both the increases in rRNA and polysomes are associated with the increase in protein content in response to low temperatures (Section 1.6.3).

1.6.7 Cryoprotectants

A large number of compounds have been isolated from plants and have been shown to have a cryoprotectant activity (see Guy, 1990). These include carbohydrates, polyol, sorbitol, glycinebetaine, proline and polyamines. All of the compounds act as compatible solutes (Yancey *et al.*, 1982). Compatible solutes have been postulated to act as cryoprotectants by ordering the water around proteins during freeze-induced dehydration (Alberdi and Corcuera, 1991). Spraying alfalfa with the cryoprotectant glycinebetaine was shown to confer increased levels of membrane integrity in response to freezing temperatures (Zhao *et al.*, 1992), suggesting another possible site of action for cryoprotectants. As with carbohydrate accumulation, the actual role and importance of the other cryoprotectants is not clear (Alberdi and Corcuera, 1991). Recent experiments with near isogenic lines of barley, differing only in a single gene for spring growth habit, showed a good correlation between the levels of accumulated glycinebetaine and the freezing tolerance of the lines, suggesting a role for glycinebetaine in cold tolerance (Kishtani *et al.*, 1994). Still no direct involvement of glycinebetaine in increased cold tolerance was demonstrated.

Proteins have been isolated with cryoprotective properties. Griffith *et al.*, (1992) isolated antifreeze proteins from the apoplast of rye plants. Endogenous

antifreeze proteins had first been proposed to be in plants by Kurkela and Frank (1990). This was based on the cloning of *Kin1* which had sequence similarity to a flounder antifreeze protein. KIN1 was proposed to be in the cytosol, and would presumably prevent intracellular ice formation if it actually functioned as an antifreeze protein. The rye antifreeze protein is proposed to modulate the formation of extracellular ice. Proteins with antifreeze properties have been isolated from intercellular spaces of insects and these have been shown to modify the formation of ice in the extracellular spaces (see Griffith *et al.*, 1992).

Attempts have been made to create transgenic plants that express proteins that order the formation of ice crystals. The proteins that have been expressed fall into two classes, antifreeze proteins that prevent the nucleation of ice crystals, and ice nucleation proteins that aid the formation of ice crystals. Antifreeze proteins have been isolated and characterised from marine animals that live in ice-laden sea water at subzero temperatures (see DeVries, 1983). One that has been structurally defined is an alanine-rich antifreeze protein isolated from flounder (Yang *et al.*, 1988). Cutler *et al.* (1989) found that vacuum infiltration of this protein into the intercellular spaces of *Arabidopsis thaliana* and *Brassica napus* tissue caused a decrease in the temperature at which ice crystal formation was first observed; the most significant decrease of 1.8°C was in the *Brassica napus* tissue. Attempts have been made to constitutively produce the protein in transgenic plants. Kenward *et al.* (1993) produced transgenic plants that accumulated the flounder antifreeze protein, but this only occurred at low temperatures, suggesting the post transcriptional mechanisms are involved in the protein's regulation. No antifreeze activity was reported. A second approach to the regulation of ice crystal formation in plant tissue was to express a bacterial ice nucleation gene in plants (Baertlein *et al.*, 1992). Controlled ice formation in the intercellular spaces is potentially beneficial to plants as it prevents deep supercooling. Spontaneous ice formation in supercooled solutions leads to rapid ice crystal growth. This is too fast to allow ordered efflux of cellular water and leads to increased cellular damage (Mazur,

1977). Baertlein *et al.* (1992) transformed tobacco and potato with an ice crystal nucleation protein from *Pseudomonas syringe*. This caused ice crystals to form at temperatures higher than in untransformed plants, demonstrating that nucleation proteins can function in plants. No increase in the freezing tolerance of the plants was reported.

1.7 Gene expression

Environmental stresses have been shown to elicit specific gene expression responses. The involvement of gene expression in cold acclimation was first proposed by Weiser (1970). The first observed changes in gene expression were by Guy *et al.* (1985) who observed differences in the protein profiles from *in vitro* translation experiments, using RNA from spinach plants that had been exposed to low temperatures and RNA from plants grown continually at non-acclimating temperatures. Similar changes in the *in vitro* translated protein profiles have since been observed in several other species including *Brassica napus* (Meza-Basso *et al.*, 1986), *Arabidopsis thaliana* (Gilmour *et al.*, 1988; Kurkela *et al.*, 1988), alfalfa (Mohapatra *et al.*, 1987), potato (Tseng and Li, 1990), barley (Hughes and Pearce, 1988; Cattivelli and Bartels, 1989) and wheat (Danyluk *et al.*, 1990; Danyluk and Sarhan, 1990). These studies associated the changes in gene expression with increased levels of cold tolerance, though no direct link could be demonstrated. However, the changes in mRNA populations did lead to attempts, largely by differential screening, to isolate cold-induced genes.

1.7.1 Cloned genes

Since the differential expression of genes in response to low temperatures was first reported, a large number of cDNAs/genes have been cloned from a number of species. The cDNAs/genes cloned from dicots include HH29 (*cor6.6*), HH28 (*cor78*), HH67 (*cor15*), and HH7.2 (*cor47*) (Hajela *et al.*, 1990), *lti30* (Welin *et al.*, 1994), *lti140* (Nordin *et al.*, 1991), *rab18* (Lång and Palva, 1992), *Ccr1* (Carpenter *et al.*, 1994), *RCII* (Jarillo *et al.*, 1994), and *Adh* (Dolferus *et al.*, 1994) from *Arabidopsis thaliana*; *BN115* (Weretilnyk *et al.*, 1993), *BN28*, (Orr *et al.*, 1992), and *BnC24A* (Sáez-Vasquez *et al.*, 1993) from *Brassica napus*; *SM2358*, *SM2201* (*cas15*), *SM784* (*cas18*) (Mohapatra *et al.*, 1989), *cas17* (Wolfraim and Dhindsa, 1993), *msaciA* (Laberge *et al.*, 1993), and *msaCIC* (Castonguay *et al.*, 1994) from alfalfa; *cap85* (Neven *et al.*, 1993) and *HSC70* (Anderson *et al.*, 1994) from spinach; and *A13* (Zhu, B *et al.*, 1993) from potato. A number of cold-induced cDNAs/genes have also been isolated from monocots; these include *Wcs120* (Houde *et al.*, 1992), *Wcs19* (Chauvin *et al.*, 1993), and *cor39* (Guo *et al.*, 1992) from wheat; *rlt1412* (Zhang *et al.*, 1993) from rye; and *blt4* (Dunn *et al.*, 1991), *blt14* (Dunn *et al.*, 1990), *blt63* (Dunn *et al.*, 1993) and *blt101* (Goddard *et al.*, 1993), pAO29, pAO86, pAF93, pT59, and pV60 (Cattivelli and Bartels, 1990) from barley.

A number of these genes have been found to be in small gene families. These include *Kin1* (*cor6.6*) and *Kin2* (Kurkela and Frank, 1990; Kurkela and Borg-Frank, 1992), *cor15a* and *cor15b* (Lin and Thomashow, 1992a; Wilhelm and Thomashow, 1993), *cor78*, *lti78*, *lti65*, *rd29A*, and *rd29B* (Nordin *et al.*, 1993; Yamaguchi-Shinozaki and Shinozaki, 1994), *RCII* and *RCI2* (Jarillo *et al.*, 1994), *BN115*, *BN19*, and *BN26* (Weretilnyk *et al.*, 1993), *rlt1412* and *rlt1421* (Zhang *et al.*, 1993), and *blt4.1*, *blt4.2*, *blt4.6*, and *blt4.9* (Dunn *et al.*, 1991; White, A.J *et al.*, 1994).

The cDNAs/genes that have been isolated have been characterised to varying degrees, although most have been examined with respect to the DNA sequence and the primary amino acid sequence of the translated open reading frame and the expression of the gene in response to low temperatures. Other aspects that have been examined include the expression of the genes in response to other stimuli and in different tissue types, the regulation of promoters isolated from cold-induced genes, and the biochemical action of proteins purified from *in vitro* translations of isolated cDNAs.

1.7.1.1 Temporal expression of cold-induced genes

The induction of cold-induced mRNAs has been observed in several studies (Section 1.7). Most of the changes were detected using RNA from plants that had been exposed to low temperature for more than 24 hours. Danyluk *et al.* (1990) found that in wheat there were two distinct classes of cold-induced translatable mRNAs, of 71 translatable mRNAs that increased or were induced 18 were expressed transiently. These reached their peak levels within 24 hours and declined after this point; the remaining 53 mRNAs also increased or were induced rapidly but their levels remained relatively constant for the four week duration of the low temperature treatment:

None of the cloned genes have displayed the transient expression, all are expressed for the duration of the low temperature treatment. There is considerable variation in the rate of induction and subsequently decline upon removal of the low temperature stimulus for the different cold-induced transcripts, and also in the relative abundance of the specific mRNAs. Guo *et al.* (1992) found that the *cor39* transcript from wheat accumulated rapidly and was detectable after two hours of low temperature treatment; the transcript was present at maximal levels after ten hours of low temperature treatment and the levels remained constant over the two week duration of the treatment. Upon removal of the low temperature stimulus (deacclimation) the

decline in transcript levels was equally rapid; after two hours the transcript levels had fallen and no transcripts were detectable after four hours. The *cor39* transcripts accumulated to relatively high levels, as estimated from the signal of the northern blot. Relatively high transcript levels occur with most of the genes examined, although these judgements are again based largely on the strength of signal from the northern blots. This is not universally true, however, since *RC11* and *RC12* transcripts (Jarillo *et al.*, 1994) accumulate only to very low levels. *RC11* and *RC12* transcripts are also at the other end of the temporal expression spectrum. Both the transcripts accumulated only after three days exposure to low temperature; at this point the levels rose relatively rapidly, although transcripts increased in abundance further after seven days. The decline in *RC11* transcripts was relatively rapid and none could be detected after one day deacclimation, *RC12* was considerably slower; only a small decline occurred after three days and transcripts had declined to basal levels after seven days deacclimation. Between these extremes observed with *cor39* and *RC11* and *RC12* lie a range of temporal expression patterns.

Limited work has been done to examine the increases in the proteins that correspond to the cold-induced transcripts. Boothe *et al.* (1995) examined the increase in protein relative to mRNA accumulation for BN28. Whereas the mRNA level had reached near maximal levels after 24 hours of low temperature treatment the protein was not detected on immunoblots until after eight days, and did not reach maximum levels until after 14 days cold acclimation. The deacclimation was similarly delayed; the transcripts were not detectable after 12 hours while the protein abundance had not declined after two days deacclimation. A lag period between transcript and protein accumulation was also observed for members of the 70 kDa heat-shock family (Neven *et al.*, 1992). Similar studies on the CAP85 protein from spinach (Guy *et al.*, 1992; Neven *et al.*, 1993) showed that the lag in protein accumulation was not as great. Increases in both transcript levels and protein were seen after 24 hours; the protein

levels remained elevated over the basal level for five days after deacclimation, although the mRNA levels had returned to near basal levels after 24 hours.

1.7.1.2 Spatial expression of cold-induced genes

Differences have been detected in the tissue specific expression of several of the cold-induced genes that have been characterised. Cattivelli and Bartels (1990) isolated five cold-induced cDNAs, three of which (pAO29, pAO86, and pAF93) are expressed in roots, shoots, and leaves, though the levels of expression vary between tissue types. The pT59 clone was expressed only in the shoots and leaves, while pV60 was only detected in the shoots. Similarly *blt4* from barley and *BN115* from *Brassica napus* were only detected in the leaf and shoot tissue (Hughes *et al.*, 1992; Weretilnyk *et al.*, 1993). Members of the same gene family also display differential tissue specific expression. Zhang *et al.* (1993) found that *rht1412* was expressed predominantly in rye root tissue while *rht1421* was mainly expressed in leaf tissue; both of these are cognates of the barley *blt14* gene. Other genes such as *RC11* and *RC12* (Jarillo *et al.*, 1994) showed no tissue specific expression. Detailed examination of tissue specific gene expression has been possible using transgenic plants with promoter-GUS constructs. The *cor78* promoter produced GUS expression at a relatively similar level in all tissues except the anthers, ovaries, stigmas and styles in transgenic *Arabidopsis thaliana* (Horvath *et al.*, 1993). The *cor15a* promoter was also analysed in transgenic *Arabidopsis thaliana*. GUS expression was induced by low temperatures in all tissues except the root; however, constitutive expression was detected in the anthers (Baker *et al.*, 1994).

1.7.1.3 Regulation of cold-induced genes in response to other stimuli

As discussed in Section 1.5.1 cold acclimation can be induced by exposure of plants to drought stress and also application of ABA. It is therefore not surprising that a number of the cold-induced genes that have been isolated are induced by drought stress and ABA. These include *cor6.6*, *cor15*, *cor47*, and *cor78* (Hajela *et al.*, 1990), *lti30* (Welin *et al.*, 1994), *lti140* (Nordin *et al.*, 1991) and *rab18* (Lång and Palva, 1992) from *Arabidopsis thaliana*. Other genes that are induced by both of these stimuli include *MsaciA* from alfalfa (Laberge *et al.*, 1993), *A13* from potato (Zhu, B *et al.*, 1993), members of the *blt4* family from barley (Dunn *et al.*, 1991; White, A.J. *et al.*, 1994), and *cor39* from wheat (Guo *et al.*, 1992). The degree to which the different genes and even different members of gene families are induced by the different stimuli varies. For example *blt4.1* is induced to comparable levels by both low temperatures and drought while *blt4.9* is only drought-induced to approximately 15% of the level that it is cold-induced (White, A.J. *et al.*, 1994). The *Kin1* and *Kin2* transcripts (related to *cor6.6*) are induced to similar levels by both low temperatures and ABA, though only *Kin2* is strongly induced by drought (Kurkela and Borg-Franck, 1992). A more extreme example of differential regulation of members in gene families are the genes *rd29A* and *rd29B* (related to *cor78*) (Yamaguchi-Shinozaki and Shinozaki, 1994), *rd29A* is induced by low temperatures, dehydration, and salinity stress; *rd29B* is induced by drought and salinity stress and shows no increase in response to low temperatures. Moreover, it is of interest that the effects of ABA and dehydration stress appear to vary between species. Of particular interesting are *cor6.6* and *cor15* from *Arabidopsis thaliana* (Hajela *et al.*, 1990) and their cognate genes from *Brassica napus*, *BN28* (Orr *et al.*, 1992) and *BN115* (Weretilnyk *et al.*, 1993). Both of the genes from *Arabidopsis thaliana* are induced by low temperatures, dehydration and ABA, whereas the genes from *Brassica napus* are induced only by low temperatures.

1.7.2 Structure and putative functions of cold-induced genes

The functions and/or importance of cold-induced genes have been estimated in a number of ways. The simplest way of predicting if a particular gene is important in conferring increased resistance to low temperatures is correlating the expression of a gene with increased low temperature tolerance. This is not particularly helpful as the changes in transcript levels may not reflect the changes in the protein levels. Time courses relating to the proteins rather than the gene transcripts would be useful when trying to establish a link between the accumulation of a protein and the acquisition of the cold acclimated state. The *Brassica napus* BN28 was shown to decline to basal levels before the acquired low temperature tolerance had been lost, implying that the BN28 protein did not play a fundamental role in cold acclimation (Boothe *et al.*, 1995).

Some of the genes isolated have been proposed to be important for cold acclimation on the basis that the levels of transcript accumulation are higher in cultivars that obtain higher levels of low temperature tolerance. In alfalfa the accumulation of transcripts hybridising to the cDNAs SM784 (cas18), SM2201 (cas15), and SM2358 were correlated with the freezing tolerance of four alfalfa cultivars (Mohapatra *et al.*, 1989). Similar correlations have been found for other genes. Transcripts of the wheat gene *wcs120* (Houde *et al.*, 1992) were expressed to similar levels in three cultivars with different levels of freezing tolerance. In one cultivar the transcripts declined to almost basal levels after 36 days exposure to low temperatures while in the more freezing tolerant cultivars the transcript levels remained relatively constant. Moreover, Zhang *et al.* (1993) found that the transcript levels of *rlt1412* and *rlt1421* were higher in the Puma cultivar than in the less freezing tolerant Rhayader. Further evidence that genes may be important in cold acclimation is their occurrence in a range of species. The implication is the greatest when cognate genes are present in species that show a large evolutionary divergence. The only gene that has been reported to be represented

in both monocot and dicot plants is *cor39* from wheat (Guo *et al.*, 1992), which is related to *lii30* from *Arabidopsis thaliana* (Welin *et al.*, 1994) and *cap85* from spinach (Neven *et al.*, 1993).

Most of the cloned cold-induced genes have been sequenced. For some of the genes, such as *blt14* (Dunn *et al.*, 1990), *rlt1412*, and *rlt1421* (Zhang *et al.*, 1993), no sequence homology to any other sequences was found and no function could be based on the protein sequence. Some insight has been gained into the function of other genes from the sequence information. In order to withstand freezing temperatures a plant must be able to tolerate high levels of cellular dehydration (Section 1.3.2.1). It is therefore interesting that a number of genes encode proteins that have been identified as belonging to the DHN/LEA/RAB family of proteins, which have been proposed to enable the cell to survive severe dehydration. These include COR47 (Lin *et al.*, 1990), LTI30 (Welin *et al.*, 1994), RAB18 (Lång and Palva, 1992), CAS17 (Wolfrain *et al.*, 1993), CAP85 (Neven *et al.*, 1993), WCS120 (Houde *et al.*, 1992), and COR39 (GUO *et al.*, 1992). These proteins are related by stretches of highly conserved amino acids, including a stretch of seven to nine serine residues in the central region of the protein. The C-terminal region contains a lysine-rich repeat that is present in at least two copies. The proteins have the unusual property of remaining soluble upon boiling. The putative role of these proteins in protecting the cell from dehydration is based on their abundance in water stressed plants and highly dehydrated seeds, so far no definite function has been demonstrated for these proteins (Dure *et al.*, 1989).

A second group of genes/proteins that have been found to increase in response to low temperatures, that are associated with another stress response and have been well characterised, are the heat-shock proteins. Neven *et al.* (1992) reported that transcripts of the 70 kDa heat-shock family increased upon exposure to low temperatures; however, a less pronounced increase in levels of the corresponding proteins was observed. The *HSC70* gene which is related to the 70 kDa heat-shock

proteins also shows induction in response to low temperatures (Anderson *et al.*, 1994). Similarly, *hsp90*, a member of the 90 kDa heat-shock family was shown to increase in response to low temperatures (Krishna *et al.*, 1995). The heat-shock proteins are known to have a role in protein transport, folding, assembly, and disassembly (see Gething and Sambrook, 1992). The increased levels in response to low temperatures may play a role in metabolic adjustment and allow otherwise cold-labile proteins to fold correctly.

A family of barley cold-induced genes encode proteins BLT4.1, BLT4.2, BLT4.6 and BLT4.9 that have sequence similarity with lipid transfer proteins (Hughes *et al.*, 1992; White, A.J. *et al.*, 1994). These proteins are characterised by their ability to transfer phospholipids between donor and acceptor membranes *in vitro* although their function *in vivo* has not been demonstrated. As discussed in Sections 1.3.2.1.1 and 1.6.1 the membrane composition has been shown to play an important role in the freezing tolerance of plants, so if these proteins function in the same manner *in vivo* they may play an important role in cold acclimation.

Further examples where the sequence has suggested a function include the *RCI1* and *RCI2* genes from *Arabidopsis thaliana* (Jarillo *et al.*, 1994). These display high sequence similarity to 14-3-3 proteins, which are a family of putative kinase regulators and may play a role in signal transduction during cold acclimation. CCR1 and CCR2 are two glycine-rich proteins (GRPs) from *Arabidopsis thaliana* (Carpenter *et al.*, 1994). GRPs are known to associate with the cell wall, although CCR1 and CCR2 have no signal peptide and are therefore presumed to be cytosolic. Other cytosolic GRPs and the CCRs contain two domains, an N-terminal RNA-binding domain and a glycine-rich carboxyl domain. The function of these RNA-binding proteins is unclear, but they may bind to specific or nonspecific RNAs, and may play roles in mRNA stability, splicing, and translational initiation/termination. Dunn *et al.* (1993) isolated a cold-induced cDNA BLT63 from barley. This encoded a protein

synthesis elongation factor 1 α (EF-1 α). This is a highly conserved protein involved in the translation of mRNAs. The increase in a component of the cell's translational system is consistent with the increase in cellular protein that is often seen in cold treated plants.

As discussed in Section 1.6.5 changes in cell wall composition have been observed during cold acclimation and may be important in the acquisition of increased cold tolerance. Weiser *et al.* (1990) reported that the changes seen in pea cell wall composition were associated with increases in the levels of transcripts that hybridised to an extensin probe. In addition a cDNA encoding a hybrid proline-rich protein has been isolated from alfalfa (Castonguay *et al.*, 1994). This is proposed to be located in the cell wall and has a high proline content which is characteristic of some cell wall proteins. (see Josè and Puigdomènech, 1993).

To date the only direct evidence that any of the cold-induced genes encode proteins that play a role in increasing the cell's tolerance to low temperatures comes from studies on COR15. Lin and Thomashow (1992b) measured the effect of the purified COR15 (from *in vitro* translations) on protecting the cold-labile enzyme lactate dehydrogenase (LDH) against freezing inactivation. COR15 was found to be 10^6 times more effective than sucrose and 10^2 - 10^3 times more effective than other proteins including BSA in protecting LDH activity. However, it is not known if the protein has the same potent activity *in vivo*.

1.8 Signal transduction in low temperature responses

As discussed in Section 1.7.1 numerous genes that are induced by low temperatures have been isolated and characterised. Despite the volume of research little is known about the mechanisms of cold perception and signal transduction. The

experiments that have been done to date have concentrated on the roles of ABA and calcium in signal transduction. Preliminary work has been done to identify *cis*-elements that confer responsiveness to low temperatures. These are likely to interact with the transcription factor targets of the signal transduction pathways.

1.8.1 Abscisic acid

As discussed in Section 1.5.1 ABA has been implicated in the signal transduction pathway(s) of responses to cold, the application of ABA mimicking the effect of low temperatures, leading to an increase in cold tolerance. The induction of several of the cold-induced genes (Section 1.7.1.3) by the application of ABA supports the involvement of ABA in the signal transduction pathway. Nordin *et al.* (1991) investigated the role of ABA and low temperatures in the signal transduction pathway using *Arabidopsis thaliana* mutant plants. The *lti140* transcript, in wild type *Arabidopsis thaliana*, was found to increase in abundance after application of ABA, exposure to low temperatures and dehydration. The accumulation of the transcript did not appear to be tissue specific. However, the requirement for an increase in endogenous ABA to trigger the low temperature accumulation of *lti140* transcripts was not necessary; the transcript levels were not affected when using the *aba-1* mutant, which is deficient in ABA biosynthesis. In agreement with this, when wild type plants were treated with the ABA biosynthesis inhibitor fluridone no decrease in transcript accumulation in response to low temperatures was seen. Normal induction of *lti140* in response to low temperatures was also seen in the ABA insensitive mutant *abi-1*. Application of ABA to the *aba-1* mutant induced the normal levels of expression at non-inducing temperatures. These results suggested that the *lti140* gene can be regulated by two separate pathways, one requiring increases in ABA, the other requiring low temperature. However, when both of the stimuli were applied simultaneously no additional increase in the transcript accumulation was seen, which suggested that the

two pathways converge at some point before the final steps of the signal transduction pathways. The induction of *lil140* in response to dehydration was slightly different. The *abi-1* mutant displayed reduced accumulation of the *lil140* transcript suggesting that ABA does play a role in the dehydration signal transduction pathway, though reasonable levels of expression are still seen. The presence of separate signal transduction pathways is supported by a set of experiments similar to those with *lil140* (Gilmour and Thomashow, 1991). The expression of three *cor* genes, *cor47*, *cor78*, and *cor6.6*, in response to low temperatures was not affected in the *aba-1* and *abi-1*, *abi-2*, and *abi-3* mutants. Regulation of the three genes by the application of ABA was severely reduced in the *abi-1* mutant suggesting that this mutant is deficient in a key element of the ABA signal transduction pathway for the *cor* genes, as well as the *lil140* gene. Despite the separate signal transduction pathways for the cold-induction of genes, ABA does seem to play a role in the acquisition of freezing tolerance, at least in *Arabidopsis thaliana* (Heino *et al.*, 1990; Gilmour and Thomashow, 1991). Both studies found that the *abi* mutations had no detectable effects on the cold acclimation of the plants, *aba* mutant however prevented the plants from becoming fully cold acclimated. The two studies disagreed on the level of cold acclimation that the *aba* mutants could obtain, Heino *et al.* (1990) reported that the *aba* mutant completely lost the ability to cold acclimate while Gilmour and Thomashow, (1991) using the most severe *aba* mutant, *aba-4* found that it could cold acclimate, though only to a low level. It was hypothesised that the *aba* mutant's impaired cold acclimation was caused by an increased susceptibility to dehydration.

Monroy *et al.* (1993) found that ABA did not mimic the effects of low temperature treatment in alfalfa (see Section 1.8.2). These studies were done using several of the cDNAs/genes cloned from alfalfa which are not induced by ABA (Monroy *et al.*, 1993; Wolfrain *et al.*, 1993a + 1993b; Castonguay *et al.*, 1994). This implies that ABA is not important in the signal transduction of cold-induced genes in alfalfa. However, ABA does have the potential to play some role in cold signal

transduction in alfalfa, since the cold-induced *MsaciA* transcript is also induced by application of ABA (Laberge *et al.*, 1993). This suggests that the signal transduction pathways may show considerable variation between species. Application of ABA does lead to cold acclimation in alfalfa, although the level obtained is approximately half the levels induced by low temperatures (Mohapatra *et al.*, 1988). These experiments support the conclusions of Nordin *et al.* (1991) and Gilmour and Thomashow (1991) that freezing tolerance can be induced through separate signal transduction pathways.

1.8.2 Involvement of calcium and protein phosphorylation

Calcium has been shown to function as a second messenger in a wide range of organisms. Knight *et al.* (1991) demonstrated that cytosolic calcium levels increased transiently in response to exposure to low temperatures (5°C). Levels also increased in response to other stimuli, including touch and fungal elicitors. The influx of calcium into plant cells in an alfalfa suspension culture was measured at a number of temperatures (Monroy *et al.*, 1995). A decline in temperature between 25 and 15°C had little effect on the influx, the influx increased at temperatures below this and at 4°C the rate of influx was 15 times the rate seen at 25°C (Monroy *et al.*, 1995). Components of a calcium signal transduction chain have been characterised in plants including calmodulin, calmodulin-dependent enzymes and calcium-dependent protein kinases (see Roberts and Harmon, 1992). A direct role for calcium in the signal transduction of low temperatures was provided by Monroy *et al.*, (1993, 1995) studying *cas* (cold acclimation-specific) transcripts. Monroy *et al.* (1993) studied the acclimation of a suspension culture of alfalfa. When the acclimation was carried out in the presence of the antagonist of calmodulin and calcium-dependent protein kinases N-(6-amino-hexyl)-5-chloro-1-naphthalene-sulfonamide hydrochloride, or the calcium channel

blocker La^{3+} , several changes were observed. Firstly, the development of freezing tolerance was lost, and the accumulation of the transcripts from cold acclimation specific genes *Acs784 (cas18)*, *Acs2201 (cas15)*, and *Acs2358* (Mohapatra *et al.*, 1989) substantially decreased. In a further study the use of calcium channel blockers prevented the observed influx of calcium at low temperatures and prevented the accumulation of the *cas15* and *cas18* transcripts (Monroy *et al.*, 1995). The effects of low temperatures on the influx of calcium and the expression of the *cas* genes could be mimicked by the use of a calcium ionophore or a calcium channel agonist, again implying the importance of calcium in the signal transduction pathways. The levels of calcium in the cell are tightly regulated and the increase in calcium in response to low temperatures occurred within seconds. Levels had returned to basal after three minutes (Knight *et al.*, 1991; Gilroy *et al.*, 1993). This transitory influx of calcium was sufficient to stimulate *cas* gene expression and the requirement for calcium in cold acclimation was also shown to be transient (Monroy *et al.*, 1995). However, the influx of calcium alone was not sufficient to cause a sustained response, because the level of the *cas* transcripts began to fall within hours unless the cold stimulus remained, suggesting that a second signal transduction pathway played a role in sustaining the response.

Further studies with the alfalfa cell culture showed that low temperature caused a relative increase in phosphorylation of several proteins and a relative decrease in phosphorylation of several others. The application of cycloheximide to the culture at concentrations that inhibited 90% of protein synthesis did not inhibit the cold-induced changes in protein phosphorylation, suggesting that the phosphorylations occur on proteins that already exist (Monroy *et al.*, 1993). The involvement of phosphorylation in the signal transduction chain was further supported by the observed differential regulation of calcium-dependent protein kinase transcripts in response to low temperatures (Monroy *et al.*, 1995). One of the transcripts, *MSCK1* increased eight fold after only three hours at 4°C , while another *MSCK2* decreased in response to low

temperatures. The significance of increased protein kinase transcripts is not clear as Monroy *et al.* (1993) previously demonstrated that high levels of protein synthesis were not required for the detected alterations in the phosphorylated protein profile. Holappa and Walker-Simmons (1995) reported that transcripts of the putative protein kinase, *PKABA1*, increased in response to low temperatures and dehydration. Other cDNAs have been isolated that have been putatively identified as protein kinase regulators. Jarillo *et al.* (1994) identified two cDNAs, *RCI1* and *RCI2*, the were expressed at low levels in response to low temperatures. Both the cDNAs had high levels of sequence similarity with a family of proteins known as 14-3-3 proteins, which are believed to be involved in the regulation of multifunctional protein kinases.

1.8.3 Cold specific *cis*-elements

As discussed in Section 1.7.1 numerous cold-induced genes have been characterised. Only a small number of promoter regions of these genes have been analysed in detail. These have involved examining the regulation of the promoter regions connected to reporter genes in transgenic plants and transient expression systems. Several promoter regions examined in this manner are from *Arabidopsis thaliana* including *cor15a* (Baker *et al.*, 1994), *cor78* (Horvath *et al.*, 1993), *Adh* (Dolferus *et al.*, 1994), and *rd29A* (Yamaguchi-Shinozaki and Shinozaki, 1994). *BN115*, a *cor15* cognate from *Brassica napus* (White, T.C *et al.*, 1994) has also been analysed using transgenic plants. All of the promoter sequences analysed, with the exception of *BN115* were responsive to dehydration and combinations of ABA, salt and hypoxia stress. Stimuli other than low temperature are known to affect the expression of *BN115*. The regulation of the transgene under the control of *BN115* was very similar to the regulation of the *BN115* transcript, in that an increase in activity was only seen in response to low temperatures (White, T.C *et al.*, 1994). In common to all of the promoters analysed, except the *Adh* promoter, is an element that has been named

the low temperature response element (LTRE). This is based around the pentamer CCGAC, occurring twice in all promoters where it is found. The two elements were contained in a 162 bp fragment in the *rd29A* promoter. These were shown to function in either orientation, though expression was not as high in the reverse orientation. Expression was also detected when the upstream LTRE motif was deleted. Mutation of the one remaining LTRE motif to CAACC lead to a complete loss of promoter activity (Yamaguchi-Shinozaki and Shinozaki, 1994). The presence of the LTRE motif is not an absolute requirement for low temperature responsiveness of a promoter. The *Adh* promoter does not contain this sequence, and neither do the barley *blt4.2* and *blt4.9* genes where the sequences of the putative promoter elements have been analysed. The barley genes do contain motifs that are similar to the LTRE; these include CCGTC from *blt4.2* and CCGAA and CCCAC from *blt4.9* (White, A.J. *et al.*, 1994). This suggests that other, as yet uncharacterised low temperature response elements exist. As yet little work has been done to characterise the nuclear DNA-binding proteins that interact with cold-inducible promoters. Gel retardation studies with the 162 bp fragment from the *rd29A* gene showed that a nuclear factor bound to the fragment (Yamaguchi-Shinozaki and Shinozaki, 1994). The nuclear factor was present in untreated and salt-treated plants; cold-treated plants were not examined. The retardation was also observed with a synthetic oligonucleotide which contained the LTRE suggesting that this sequence was binding a nuclear factor (Yamaguchi-Shinozaki and Shinozaki, 1994).

Deletion/mutation studies on the *Adh* promoter found that one region of the promoter was vital for the response to dehydration, ABA, and anoxia. It was proposed that this region may bind a general transcription factor. Other regions of the promoter appeared to be important in response to each of the stresses. This led to the proposal that different DNA-binding factors may be concerned with the responses to different stresses and that different signal transduction pathways are involved (Dolferus *et al.*, 1994). This hypothesis was supported by the analysis of the *rd29A* promoter. An element named DR1 which contained the LTRE pentamer was necessary for low

temperature and dehydration responsiveness, although the ABA signal transduction pathway did not act upon this motif (Yamaguchi-Shinozaki and Shinozaki, 1994). Analysis of the *cor15a* promoter suggests that signals produced in response to low temperatures do not act as systemic regulatory molecules and the signal transduction pathway(s) act in a cell autonomous manner. The evidence for this was that no promoter-GUS activity was observed in transgenic tissues maintained at 22°C whereas cold-induction of GUS activity was seen in parts of the same plant exposed to 4°C (Baker *et al.*, 1994).

1.9 Project aims

The cDNA LF5 had been isolated from *Brassica napus* cv. cobra (Pallas, 1992). The cDNA was identified by differential screening of a cDNA library in an attempt to isolate genes involved in floral morphogenesis. The cDNA library was made from poly(A)⁺ RNA that was isolated from the floral apices and constructed in the vector λ NM1149. Tissue was collected from the developmental stage where only sepal primordia were present. The tissue was collected from field-grown plants, which provided large amounts of tissue. The cDNA library was differentially screened using first strand cDNA probes made from poly(A)⁺ RNA from two tissue types. The 'plus' probe was from floral apices, the 'minus' probe was from vegetative tissue. Ten cDNAs were isolated and studied further. Transcripts hybridising to one of the cDNAs, LF5, were shown to be up-regulated in response to low temperatures. The up-regulation was detected in both leaf tissue and flower buds, but no expression was detected in root tissue. No expression was detected in any tissues when the plants were maintained at 22 °C. The floral apices were taken from field-grown plants between November and May and thus the plants were frequently exposed to low temperatures. The high levels of the *LF5* transcript in the tissue collected from the field (and used as

the 'plus' probe), as opposed to the undetectable in levels in vegetative tissue (used as the 'minus' probe), explain why the cDNA was isolated by differential screening.

The transcripts that the LF5 probe hybridised to were estimated to be approximately 1.5 kb in size, whereas the pLF5 cDNA was approximately 4 kb. This discrepancy in size suggests that the LF5 cDNA contained sequence that is unrelated to the sequence that hybridises to the cold-induced transcripts. In fact several of the clones isolated from this library were larger than the transcripts that they hybridised to, suggesting that the library contained a high proportion of chimaeric cDNAs. The LF5 cDNA was digested with *Bam*HI, which cut the cDNA insert into four fragments, and each of these was subcloned into pBluescript[®] SK (-) (Pallas, 1992). Only a 1 kb fragment hybridised to cold-induced transcripts and this subclone was named LF5B1. The LF5B1 cDNA was partially sequenced (200 bp from each end), but this did not reveal any putative open reading frames. One region of interest was a stretch of 38 adenine residues, located internally. Such a region is usually associated with the 3' end of a mRNA transcript and its presence internally suggests that the LF5B1 contains cloning artefacts.

At the time the project began few cold-induced cDNAs had been cloned. Thus, it was of interest to characterise the cDNA and this was the aim of the project.

In characterising LF5B1 several aspects would be investigated. The priority would be to sequence the pLF5B1 clone, which would determine if any of the sequence within the chimaeric cDNA was related to any of the cold-induced cDNAs already characterised. It would also be the first step towards defining which part of the cDNA (or if all the cDNA subclone LF5B1) was hybridising to the cold-induced transcripts. It was expected that LF5B1 would contain more than one unrelated cDNA. Therefore it was likely to prove necessary to isolate another cDNA that contained no cloning artefacts in order to define precisely the sequence corresponding to the cold-induced

transcripts. As the library used to isolate LF5B1 appeared to contain a high proportion of cDNAs with cloning artefacts it would be preferable to make another cDNA library, and isolate a clone from this.

A preliminary study of the expression of the *LF5* transcripts had been undertaken (Pallas, 1992). This had shown that the transcripts were induced rapidly, accumulating to high levels after 12 hours of low temperature treatment. The fall in transcript levels on returning plants to normal temperatures was rapid; transcripts were undetectable levels after eight hours. Preliminary experiments had failed to detect transcript accumulation in response to wounding and the application of ABA. The expression studies would have to be repeated once the part of the cDNA hybridising to the cold-induced transcripts had been identified and isolated, or a second cDNA with no artefacts was isolated. Suitable controls would have to be incorporated to validate any negative results.

A second desirable way to examine the expression of the putative cold-induced gene would be to isolate a genomic clone corresponding to LF5B1. It is likely that the region 5' of the open reading frame would contain the *cis*-elements necessary to regulate the gene's expression. This region could be used to create a promoter-GUS construct that could be used to transform plants, enabling the activity of the promoter to be examined.

Cold-acclimation has been studied in a number of plant systems. *Brassica napus* is a suitable species to study the response for several reasons. It is a crop species that is limited in its distribution by low temperatures, which are also responsible for plant losses in severe winters (Thompson and Hughes, 1986). It has the ability to cold acclimate, winter varieties injured at -2°C can acclimate to survive -16°C (Boothe *et al.*, 1995). In addition, several previous studies had utilised *Brassica napus*, making it one of the model species to study cold acclimation (Meza-Basso *et al.*, 1986; Johnson-

Flanagan and Singh, 1986; Johnson-Flanagan and Singh, 1987; Singh *et al.*, 1987; Johnson-Flanagan *et al.*, 1991).

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals

The chemicals were obtained from BDH (AnalaR[®] grade; Poole, Dorset) unless otherwise indicated.

2.1.2 DNA modifying enzymes

All restriction enzymes were purchased from Gibco-BRL, Paisley, Strathclyde together with their reaction buffers, which were provided at a 10X concentration.

2.1.3 Radiochemicals

The radiochemicals used in this study were supplied by Amersham International plc. (Amersham, UK.).

2.2 General preparatory procedures

2.2.1 pH measurement

The pH of solutions other than phenol was measured using a Corning pH meter 220 and combination electrode (Corning Incorporation, New York, USA). The pH of phenol-containing solutions was measured using Whatman Narrow Range pH paper (pH 6.0 - pH 8.0) (Whatman, Maidstone, UK).

2.2.2 Autoclaving

Equipment and solutions were sterilised at 15 psi for 20 min. Small batches were sterilised in a Prestige High Dome pressure cooker, with larger batches being done in a Laboratory Thermal Equipment Autoclave 225E.

2.2.3 Filter sterilisation

Heat-labile solutions were sterilised by passing them through a Corning syringe filter (pore diameter 0.2 μm) into a sterile receptacle.

2.2.4 Glassware

Glassware was sterilised by baking in an oven at 180°C for at least 12 h.

2.2.5 Solutions and equipment for RNA work

Solutions for RNA work were treated with 0.1% (v/v) diethyl pyrocarbonate (DEPC; Sigma Chemical Co., Dorset, UK) overnight and then autoclaved. Solutions containing Tris-Cl were made with DEPC-treated distilled water. Sterilised glassware and plasticware was used throughout.

2.3 Growth media

All media were sterilised by autoclaving at 15 psi for 20 min.

2.3.1 Liquid media

L-broth (1% bacto-tryptone (w/v) (Difco Laboratories, Michigan, USA), 1% NaCl (w/v), 0.5% yeast extract (w/v) (Difco), pH 7.2) was generally used for culture of *E. coli*, supplemented with the appropriate antibiotic. For preparation of competent cells *E. coli* XL1-Blue strain (Stratagene, Cambridge, UK) was cultured in ϕ -broth (2% bacto-tryptone (w/v) (Difco), 0.5% yeast extract (w/v) (Difco), 0.4% MgSO₄ (w/v), 10 mM KCl). For preparation of plasmid DNA for sequencing, *E. coli* XL1-Blue was cultured in TYN (1% bacto-tryptone (w/v) (Difco), 1% yeast extract (w/v) (Difco), 0.09 M NaCl). For preparation of SURE[®] (Stratagene) and XL1-Blue plating cells L-broth was used, supplemented with 0.2% maltose (w/v) and 10 mM MgSO₄. For DL491 plating cells NYCYM media (1% NZ amine (w/v) (Sigma), 0.1% casamino acids (w/v) (Sigma), 0.5% yeast extract (w/v) (Difco), 0.5% NaCl (w/v), 10 mM MgSO₄) supplemented with 0.2% maltose (w/v) was used.

2.3.2 Solid media

For plating out *E. coli* and bottom agar L-agar was prepared by adding 1.5% agar (w/v) (Difco) to L-Broth prior to sterilisation. Top agarose was prepared by adding agarose to an appropriate concentration (0.5-1%) (w/v) and 10 mM MgSO₄ to L-broth or NZYCM media. *Agrobacterium tumefaciens* was grown on AB medium which was prepared by combining solution I (20X, 60 g·l⁻¹ K₂HPO₄, 20 g·l⁻¹ NaH₂PO₄) and solution II (20X, 20 g·l⁻¹ NH₄Cl, 6 g·l⁻¹ MgSO₄, 3 g·l⁻¹ KCl, 3 g·l⁻¹ CaCl₂, 50 mg·l⁻¹ FeSO₄) to a final concentration of 1X, and adding 0.5% glucose and 1.5% Oxoid agar.

2.3.3 Antibiotics, IPTG, and X-Gal

All antibiotics used were supplied by Sigma Chemical Co. except vancomycin which was supplied by Eli Lilly and Co. (Basingstoke, UK). Ampicillin, 25 mg·ml⁻¹ in H₂O, and kanamycin, 10 mg·ml⁻¹ in H₂O and added to a final concentration of 50 µg·ml⁻¹. Tetracycline, 12.5 mg·ml⁻¹ in 50% EtOH, was used at a final concentration of 12.5 µg·ml⁻¹. Rifampicin, 20 mg·ml⁻¹ in MeOH, was used at a final concentration of 100 µg·ml⁻¹. All were filter-sterilised before adding to sterile media which had been allowed to cool to 50°C.

Isopropyl thiogalactoside (IPTG; Life Technologies, Gaithersburg, USA) was made as a 1 mM stock in H₂O and used at a final concentration of 0.1 mM. X-Gal (5-bromo-4-chloro-3-indolyl β-D-galactoside; Life Technologies) was made as a 20 mg·ml⁻¹ stock in dimethylformamide and used at a final concentration of 50 µg·ml⁻¹.

2.4 Bacterial strains and cloning vectors

Plasmids were maintained and amplified in *E. coli* XL1-Blue (Bullock *et al.*, 1987) (Stratagene). *E. coli* SURE[®] (Greener, 1990) (Stratagene) was used as the recipient strain for the cDNA library, *E. coli* XL1-Blue was used for subsequent amplification and plating of the cDNA library and of any isolated clones. *E. coli* SOLR[™] (Greener, 1990) (Stratagene) was used for *in vivo* excision of cDNA clones. *E. coli* DL491 (supplied by Dr N.A. Urwin, Division of Biochemistry and Molecular Biology, University of Glasgow) was used for plating of the genomic library and amplification of any isolated clones.

The strain used for plant transformation was *Agrobacterium tumefaciens* C58^{C1} Rif^R containing pGV2260 (supplied by Dr J.A. Jackson, Division of Biochemistry and

Molecular Biology, University of Glasgow). This strain was grown at 28°C to maintain the plasmid. The helper plasmid pRK2013 was maintained in *E. coli* DH5 α .

The Bluescript[®] II SK (-) phagemid (Short *et al.*, 1988) (Stratagene) was used to maintain, amplify and subclone DNA for restriction digest analysis, sequencing and the production of deletions. The cDNA library was constructed in the bacteriophage vector Uni-ZAP[™] XR (Huse and Hansen, 1988) (Stratagene). This allowed cDNA clones to be *in vivo* excised. The genomic library was provided in EMBL3 (Frischauf *et al.*, 1983) (Clontech, California, USA) and provided by Dr A. Greenland (Zeneca seeds, Bracknell, Berkshire). The PBI101.1 cloning vector (promoterless, β -glucuronidase (GUS) reporter gene and *nos* 3' terminator; Jefferson *et al.*, 1987) was used to construct the promoter-GUS fusion, the PBI121 vector (CaMV-35S promoter, GUS reporter gene, and *nos* 3' terminator; Jefferson *et al.*, 1987) was used as a positive control for histochemical localisation of GUS (supplied by Dr J.A. Jackson, Division of Biochemistry and Molecular Biology, University of Glasgow).

2.5 Plant material

2.5.1 Growth of plants

Brassica napus L. cv 'Cobra' seeds (winter oilseed rape) were obtained from Booker Seeds, Sleaford, Lincolnshire. *Arabidopsis thaliana* Landsberg erecta wild type was obtained from the Nottingham *Arabidopsis* Stock Centre (University of Nottingham). Seeds were stored at 4°C and germinated on ICI potting compost. Plants were grown in continuous light (150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; Osram 45W 'warm white' fluorescent tubes) at a temperature of 22°C.

2.5.2 Treatment of plants

Cold-induction took place at 4-6°C in continuous light (150 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$; Osram 45W 'warm white' fluorescent tubes). Plants were heat-shocked by placing whole plants into a 40°C incubator. The plants were first placed into a polythene bag to limit the dehydration of the plants during heat-shock. This had the effect of reducing the amount of light to 75 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$.

Dehydration was induced by withholding water from day 15 post-germination. Tissue was harvested between 21 and 28 days post-germination. The degree of dehydration (relative water content, RWC) was estimated as described in Guo *et al.* (1992). Two leaves of similar size and developmental stage were cut, at the base of the petiole, from the same plant. One leaf was harvested immediately into liquid nitrogen and used for RNA extraction. The second leaf was weighed to determine its fresh-matter weight (W_f), the petiole was then placed in distilled water at 4°C in the dark for 12 h and then weighed to determine the weight at water saturation (W_s). The dry-matter weight (W_d) of the leaf was measured after drying the leaf at 80°C for 12 h. The RWC content was determined by $(W_s - W_f / W_s - W_d) \times 100$.

For ABA treatment regularly watered plants were sprayed to run-off with 10^{-4} and 10^{-5} M ABA (Sigma, mixed isomers, 50:50). The ABA was dissolved in a minimal volume of MeOH and diluted to the appropriate concentration in H₂O. Control plants were sprayed with H₂O containing an equal amount of MeOH as the ABA solutions.

Wounding was inflicted by cutting leaf tissue into pieces approximately 25 mm² sections. The sections were placed on wetted 3MM filter paper (Whatman) in a Petri dish and incubated in constant light at 22°C. Control leaf sections, after wounding,

were placed at 4°C for 24 h to ensure that the tissue was not wounded to the point that the cold-induced transcripts could not increase in abundance.

2.6 Isolation of RNA from plant tissue

2.6.1 Total RNA

This method is based on the method of Chomczynski and Sacchi (1987) with modifications based on Shirras and Northcote (1984).

Harvested tissue was frozen immediately in liquid nitrogen and stored at -70°C until required. Root and stem material was cut into small pieces before freezing to aid subsequent grinding. The tissue (approx. 1 g) was ground in a mortar and pestle under liquid nitrogen.

After powdering the liquid nitrogen was allowed to evaporate off and the powder was then transferred to a 15 ml Corex[®] tube containing 5 ml solution D (4 M guanidine thiocyanate (Sigma); 25 mM sodium citrate (pH 7.0); 0.5% sarkosyl (w/v) (Sigma); 0.1 M 2-mercaptoethanol) and mixed. 0.5 ml 3M NaOAC (pH 4.0), 5 ml of phenol (pH 7.6), and 1 ml chloroform:*iso*-amyl alcohol (v/v, 23:1) were added. After mixing and centrifugation for 5 min at 2,000g at 4°C the aqueous phase was recovered, with care being taken to avoid debris at the interface, and transferred to a clean 15 ml Corex[®] tube, containing 4 ml phenol (pH7.6) and 1 ml chloroform:*iso*-amyl alcohol (v/v, 23:1). The sample was mixed and centrifuged as above, the upper aqueous phase was extracted once more with phenol: chloroform: *iso*-amyl alcohol (v/v/v, 23:23:1), and then once with an equal volume of chloroform.

In a clean 15 ml Corex[®] tube the RNA was precipitated by the addition of 1 volume *iso*-propanol, the precipitation was allowed to proceed for at least 2 h at -20°C.

The RNA was pelleted by centrifugation for 15 min at 3,000g at 4°C. The pellet was washed with 1 ml of 3 M NaOAc (pH 5.5) and repelleted, the pellet was then washed in 1 ml 80% EtOH, allowed to dry and dissolved in DEPC-treated H₂O.

The purity of the preparation was estimated by measuring the absorbance at 260 nm and 280 nm. A pure preparation was taken as having an A₂₆₀/A₂₈₀ ratio of 1.8-2.0. The integrity of the RNA was examined on a 2% (w/v) agarose mini-gel. All RNA preparations were stored at -80°C.

2.6.2 Isolation of poly(A)⁺ RNA

Poly(A)⁺ RNA isolated was undertaken according to the method of Sambrook *et al.* (1989), as were all experimental procedures unless otherwise stated.

Oligo(dT) cellulose type 7 (Pharmacia, Milton Keynes, UK.) was suspended in loading buffer (0.5 M NaCl, 10 mM Tris-Cl (pH 7.5), 0.1% SDS (w/v)) and the suspension poured into a disposable plastic column to give a column volume of approximately 1 ml. The column was washed with 1 volume H₂O, 1 volume 0.1 M NaOH, 5 mM EDTA, and again with H₂O until the pH of the column effluent was less than 8.0. The column was then re-equilibrated with loading buffer. Total RNA was heated to 65°C for 5 min and chilled on ice. An equal volume of two-fold concentrated loading buffer was added to the RNA which was then loaded on the column. The column effluent containing unbound RNA was heated to 65°C, chilled on ice and re-applied to the column. The column was washed with loading buffer until the A₂₆₀ of the column effluent reached zero. Poly(A)⁺ RNA was eluted from the column into a siliconised 15 ml Corex[®] tube with elution buffer (10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.05% SDS (w/v)) at 65°C. The RNA was precipitated for 2 h with 0.1 volume 3 M NaOAc (pH 5.2) and 2.5 volumes EtOH at -20°C. After pelleting the poly(A)⁺

RNA, the pellet was washed with 70% EtOH and dried. The RNA was resuspended in DEPC-treated H₂O and stored at -80°C.

2.7 The amplification and purification of plasmid DNA

2.7.1 Preparation of competent cells

E. coli were grown up overnight in 3 ml of ϕ -broth at 37°C. 20 ml of fresh broth was inoculated with 1 ml of the overnight culture and was shaken at 37°C for 100 min. The culture was transferred to sterile 15 ml centrifuge tubes. The cells were then pelleted by centrifugation for 5 min at 2000g at 4°C and the supernatant decanted. The cells were then resuspended in a total of 10 ml of ice-cold 50 mM CaCl₂ and left on ice for 20 min. The suspension of cells was then centrifuged as before and the pellet resuspended in a total of 0.6 ml of 50 mM CaCl₂. These cells could then either be stored at -80°C after flash freezing in liquid nitrogen or left on ice until used.

2.7.2 Transformation of competent cells

The competent cells prepared as in Section 2.7.1 were divided into 0.2 ml aliquots in 1.5 ml Eppendorf® tubes. One 1.5 ml Eppendorf® tube was used per transformation. Approximately 200 ng of plasmid DNA in a volume of 5-10 μ l was added to the cells and the suspension was mixed by gentle shaking prior to leaving on ice for 15-20 min. After this period the cells were heat-shocked at 37°C for 1 min and then returned to ice for a further 2 min. Then 0.6 ml of ϕ -broth was added to the cells and the cells were shaken at 37°C for 45 min to allow expression of the antibiotic resistance encoded on the plasmid. 100 μ l serial 1 in 10 dilutions of the cell suspension were plated out on agar plates with the appropriate antibiotic. The plates were allowed to dry and then placed at 37°C overnight. Controls carried out were transformed cells

plated on a plate containing no antibiotic and competent cells which had been treated as described except that no DNA was added, plated on antibiotic-containing plates.

2.7.3 Preparation of plasmid DNA

The methods used were based on that of Birnboim and Doly (1979).

2.7.3.1 Small-scale preparation of plasmid DNA

A single bacterial colony was used to inoculate 3 ml of TYN medium which contained suitable antibiotic(s) in a 7 ml bijou tube. The cultures were incubated overnight at 37°C with vigorous shaking.

The next day the cultures were centrifuged at 10,000g, at 4°C for 5 min. The liquid medium was aspirated off. The pellets were resuspended in 200 µl of ice-cold GTE (50 mM glucose, 25 mM Tris-Cl (pH 8.0), and 10 mM EDTA, 100 µg·ml⁻¹ lysosome (Sigma, 48,000 U·mg⁻¹ solid), and vortexed to aid the resuspension process. Next, 400 µl of freshly prepared 0.2 M NaOH and 0.1% SDS was added to each of the resuspended pellets and mixed by gentle agitation. The lysed bacterial suspensions were stored on ice for several min and afterwards transferred into 1.5 ml Eppendorf® tubes. To each suspension 300 µl of ice-cold 3 M KOAc pH 4.8 was added. The Eppendorf® tubes were stored on ice for a few min before they were centrifuged at 10,000g for 15 min at 4°C. Next, 0.9 ml of the supernatant was removed to a fresh 2 ml microfuge tube and precipitated by the addition of 1 ml EtOH followed by immediate centrifugation at 10,000g for 10 min at 4°C. The supernatant was aspirated from the pellet, which was then washed in 200 µl 70% EtOH. The pellet was left to air dry for 10 min. DNA was resuspended in 50 µl of H₂O, which contained 500 ng of DNAase-free RNAase (Sigma, Type I-A).

2.7.3.2 Small-scale preparation of DNA for sequencing template

This method used the Magic™ minipreps DNA purification system (Promega, Madison, USA) which was used in accordance with the manufacturers instructions.

A single bacterial colony was picked with a sterile loop and used to inoculate 2.0 ml of TYN supplemented with the appropriate antibiotic in a 7 ml bijou tube. The culture was grown overnight with shaking at 37°C. One ml of the culture was transferred into a 1.5 ml Eppendorf® tube and centrifuged at 10,000g for 5 min. The supernatant was discarded and the bacterial pellet was resuspended by vortexing in 200 µl of cell resuspension solution (50 mM Tris-Cl (pH 7.5), 10 mM EDTA, 100 µg·ml⁻¹ RNAase A (Sigma, Type I-A). Once fully suspended 200 µl of cell lysis solution (0.2 M NaOH, 1% SDS) was added and mixed by gentle inversion, 200 µl of neutralisation solution (1.32 M KOAc (pH 4.8)) was then added and mixed by inversion. The Eppendorf® tube was centrifuged at 10,000g for 5 min. The supernatant was removed to a clean 2.0 ml centrifuge tube where 1 ml of Magic™ minipreps DNA purification resin was added and mixed by inversion. The resin/DNA mix was transferred to a 3 ml disposable syringe which was attached to a minicolumn, the plunger was then inserted slowly, pushing the slurry into the minicolumn. The syringe was detached from the minicolumn and the plunger removed. The syringe barrel was reattached to the minicolumn and 2 ml of column wash solution (100 mM NaCl, 10 mM Tris-Cl (pH 7.5), 2.5 mM EDTA) was added, the plunger re-inserted and the wash solution gently pushed through the minicolumn. The minicolumn was removed from the syringe and transferred to a 1.5 ml Eppendorf® tube and was centrifuged at 10,000g for 20 s in a microfuge to dry the resin, the minicolumn was transferred to a clean 1.5 ml Eppendorf® tube, 50 µl of sterile H₂O was added, and after 1 min centrifuged at 10,000g for 20 s in a microfuge to elute the plasmid DNA. The minicolumn was discarded.

2.7.3.3 Large-scale plasmid DNA preparation

A single colony of bacteria containing the plasmid was picked off an agar plate containing the appropriate antibiotic and used to inoculate 3 ml of TYN also containing the appropriate antibiotic. This was shaken at 37°C overnight. The following day the culture was placed at 4°C until required. One ml of the culture was used to inoculate 1 l of TYN which was shaken at 37°C for approximately 18 h. The culture was decanted into sterile screw top 250 ml centrifuge bottles and centrifuged at 10,000g for 10 min at 4°C to pellet the cells. The supernatant was decanted off and the cells were resuspended in 20 ml of GET (50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA). 200 mg of lysozyme (48,000 U.mg⁻¹ solid; Sigma) was dissolved in 2 ml of GET and this was added to the resuspended cells and they were incubated at room temperature for 10 min. Then 40 ml of 0.2 M NaOH, 1% SDS (w/v) was added, the suspension mixed gently, and then left on ice for 10 min. After this incubation 20 ml of 3 M KOAc (pH 4.8) was added and the suspension was mixed by inverting prior to being returned to ice for a further 30 min. The mixture was then centrifuged at 2,000g for 10 min at 4°C as above. The supernatant was poured into another 250 ml centrifuge bottle through 1 layer of muslin and the pellet and precipitate discarded. 50 ml of *iso*-propanol was added to the filtrate, the phases mixed and left at room temperature for 5 min. The DNA and RNA was then pelleted by centrifugation at 5,000g for 10 min at 4°C. The supernatant was discarded and the centrifuge bottle inverted to allow the pellet to dry. The dried pellet was dissolved in 7.1 ml of TE (10 mM Tris-Cl (pH 8.0), 1 mM EDTA) and transferred to an ultracentrifuge tube containing 6.72 g of CsCl. The tube was inverted and 425 µl of 10 mg·ml⁻¹ EtBr was added and the tube left at room temperature for 30 min to allow all the CsCl to dissolve. The tube was then centrifuged at 18,000g for 48 h at room temperature. After centrifugation the plasmid band was removed with a sterile Pasteur pipette into a 15 ml Corex[®] tube. An equal volume of CsCl-saturated *iso*-amyl alcohol was added and the phases mixed. The phases were allowed to separate and the upper phase was removed. This *iso*-amyl alcohol extraction

was repeated until the aqueous phase contained no pink colour. The aqueous phase was then dialysed against a 1000X volume of TE (pH 8.0) in dialysis tubing (Sigma) for 2 h at room temperature and then overnight against fresh TE (pH 8.0) at 4°C. The DNA solution was then stored in aliquots at -20°C.

2.8 Nucleic acid precipitation

Nucleic acids were precipitated by the addition of NaOAc (pH 5.2) to a concentration of 0.3 M, followed by the addition of two volumes of cold 100% EtOH. DNA precipitation was aided by chilling the samples at -20°C for at least 20 min.

In precipitations where *iso*-propanol was used, addition of 0.6-1 volumes of the alcohol was sufficient to precipitate the nucleic acids. In these cases, samples were centrifuged immediately to recover the nucleic acids.

Nucleic acid pellets were washed in 70% EtOH, briefly centrifuged and air dried and finally resuspended in an appropriate volume of H₂O.

2.9 Quantification of DNA and RNA

An aliquot of the nucleic acid solution to be quantified (usually 5-10 µl) was made up to 1 ml with DEPC-treated H₂O. The absorbance of the nucleic acid containing solution was measured between 220 nm and 300 nm against a DEPC-treated distilled H₂O blank. An absorbance at 260 nm (A₂₆₀) of 1 was taken to indicate the following concentrations :

Form of nucleic acid	Concentration ($\mu\text{g}\cdot\text{ml}^{-1}$)
Double stranded DNA	50
Single stranded DNA and RNA.	40
Oligonucleotides.	20

2.10 Agarose gel electrophoresis

All agarose (*ultra PURE*TM) was supplied by Life Technologies.

2.10.1 Denaturing electrophoresis of RNA

This method was used for gels that were to be northern blotted for northern hybridisation analysis. The appropriate amount of agarose (1.3-1.5 g) was added to 10 ml of 10X MOPS buffer (0.2 M MOPS (Sigma), 0.05 M NaOAc, 0.01 M EDTA pH 7.0) and 73 ml of DEPC-treated distilled water. The agarose suspension was then heated in a microwave oven until all the agarose had dissolved. Once the agarose solution had cooled to 60°C 17 ml of formaldehyde (37% v/v; Sigma) was added and the gel was mixed by swirling prior to pouring into the electrophoresis apparatus. After a period of 30 min to allow the gel to set the gel was just covered with 1X MOPS running buffer. The RNA (0.125-20 μg) was prepared in a solution of 50% (v/v) formamide (Fluka Biochemicals, Gillingham, UK), 1X MOPS, 5.92% formaldehyde (v/v) to a volume not greater than 50 μl . This solution was heated to 65°C for 15 min and then placed on ice where a one-tenth volume of running buffer (50% glycerol (v/v), 1 mM EDTA (pH 8.0), 0.25% bromophenol blue (w/v), 0.25% xylene cyanol FF (w/v)) and 2 μl of 2.5 $\text{mg}\cdot\text{ml}^{-1}$ EtBr were added. The sample was then loaded onto the gel with a pipette and the gel was run at 10 to 100 mA until the bromophenol blue had migrated two thirds of the distance down the gel. The gel was then washed in

DEPC-treated H₂O for 15 min and then visualised under UV light (Spectroline[®], Model TC-312A transilluminator).

2.10.2 Alkaline electrophoresis of DNA

One percent alkaline gels were used to separate the first and second strand cDNA synthesis. Seventy two ml of H₂O were added to 0.8 g of agarose and heated in a microwave oven until all the agarose had dissolved, the solution was allowed to cool to 55°C before adding 8 ml of 10X Alkaline Buffer (0.9 M NaOH, 0.04 M EDTA). The gel solution was then poured into the electrophoresis apparatus and allowed to set for 30 min. After setting the gel was covered in 1X Alkaline Buffer. Samples were loaded using an equal volume of 2X loading buffer (20% v/v glycerol, 0.025 M NaOH, 0.25% bromophenol blue (w/v), 0.25% xylene cyanol FF (w/v)). The gel was run at 100 mA until the bromophenol blue had migrated three-quarters the distance of the gel. The gel was dried down in a slab drier for 2 h and then autoradiographed as described in Section 2.16.5.

2.10.3 Electrophoresis of DNA

The appropriate concentration of agarose (0.5-2.0% w/v) was added to the volume of 1X TBE (0.09 M Tris-borate, 2 mM EDTA) or 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA) required for the gel being cast. The agarose suspension was heated in a microwave oven until all the agarose had dissolved. The agarose solution was then allowed to cool to around 60°C at which point 10 mg·ml⁻¹ EtBr was added to a final concentration of 1 µg·ml⁻¹. The gel solution was then poured into the electrophoresis apparatus and allowed to set for 30 min. Enough 1X TBE/TAE running buffer was then added to just submerge the gel. The samples to be loaded were mixed with one-tenth volume of loading buffer (50% glycerol (v/v), 1 mM EDTA (pH 8.0), 0.25% bromophenol blue (w/v), 0.25% xylene cyanol FF (w/v)). The gels

were run at 10 to 100 mA until the bromophenol blue had migrated two-thirds of the way down the gel. The gel was then washed in H₂O for 15 min and then visualised under UV light.

2.10.4 Marker DNA

Marker DNA was run on agarose gels to size the DNA that was being analysed. The 1 kb ladder (Gibco-BRL) was most frequently used, this contained marker bands ranging in size between 75 bp and 12 kb (see Figure 3.3). For the analysis of the cDNA synthesis λ DNA digested with *Hind* III was used, this was labelled by nick-translation to enable visualisation

2.11 Isolation of DNA fragments from agarose gels

2.11.1 GeneClean[®]

The GeneClean[®] kit was supplied by BIO 101 Inc. (La Jolla, USA) and was used according to the manufactures instructions.

The DNA of interest was run on a TAE gel to separate the DNA of interest from any other contaminating DNA. The DNA band required was identified by illumination of the gel with long-wave UV light and excised with a clean scalpel blade. The amount of agarose was estimated by weighing (1 g = 1 ml) and 2.5 volumes of 6 M NaI (supplied with kit) added. The agarose was dissolved by incubation at 55°C for 10 min. The required amount of Glassmilk[®] (5 μ l for up to 5 μ g DNA) was added, the contents mixed and allowed to stand at room temperature and after pelleting of the glass beads at 10,000g for 1 min the supernatant was removed. The pellet was washed three times with 0.5 ml ice-cold NEW wash (1X NEW salts, 50% EtOH). The DNA was eluted by resuspension of the pellet in 30 μ l TE (pH 8.0) and incubation at 55°C for 3

min. After pelleting the supernatant was transferred to a clean Eppendorf[®] tube and the pellet extracted once more with 20 μ l of 1 x TE. This procedure yielded 50-60% recovery of DNA.

2.11.2 Magic[™] DNA purification system

The Magic[™] DNA purification system was provided by Promega.

The DNA of interest was isolated from an agarose gel as described in 2.11.1. One ml of Magic[™] DNA purification resin was added to 300 μ l of agarose (300 mg), and the agarose was dissolved by heating at 65°C for 10-15 min. The resin/DNA mix was allowed to cool to room temperature and then transferred to a 3 ml disposable syringe which was attached to a minicolumn. The plunger was then inserted, slowly pushing the slurry into the minicolumn. The syringe was detached from the minicolumn and the plunger removed. The syringe barrel was reattached to the minicolumn and 2 ml of column wash solution (80% *iso*-propanol) was added, the plunger re-inserted and the wash solution gently pushed through the minicolumn. The minicolumn was removed from the syringe and transferred to a 1.5 ml Eppendorf[®] tube and was centrifuged at 10,000g for 20 s to dry the resin. The minicolumn was transferred to a clean 1.5 ml Eppendorf[®] tube, 50 μ l of sterile water were added, and after 1 min centrifuged at 10,000g for 20 s in a microfuge to elute the DNA. The minicolumn was discarded. This procedure yielded 75-90% recovery.

2.12 Digestion of DNA with restriction endonucleases

The DNA to be restricted was prepared in a solution of 1X the appropriate buffer and 1-20 units of restriction enzyme were added ensuring that its concentration didn't exceed 10% (v/v). The reactions were incubated at 37°C for 2 to 24 h and the

reactions were monitored where sufficient DNA was available by running an aliquot of the reaction on an agarose gel against uncut DNA.

2.13 Ligation of DNA inserts into plasmid vectors.

Ligations were carried out in 1.5 ml Eppendorf® tubes. A three fold excess of DNA insert over vector was used in each ligation. The DNA insert was added to 1 µl vector (10 ng·µl⁻¹), 2 µl of 10X Ligation Buffer (660 mM Tris-Cl (pH 7.6), 100 mM MgCl₂, 100 mM DTT, 3 mM rATP, 10 mM spermidine), 2 µl of 0.1 M DTT, 1 µl of T4 DNA ligase (1 U·µl⁻¹) and sterile H₂O to 20 µl.

Complementary-ended ligations were incubated at 15°C overnight or at room temperature for 4 h. Routine control reactions of only vector DNA incubated with and without ligase and only ligase in 1X Ligase Buffer were incorporated into every ligation experiment.

2.14 Blotting of nucleic acids

2.14.1 Northern blotting

A denaturing agarose gel was run to separate the RNA as described in Section 2.10.1. A support was prepared onto which was placed a wick of Whatman 3 MM paper which was then soaked with 20X SSC (3 M NaCl, 0.3 mM trisodium citrate). The wick was also dipped into a reservoir of 10X SSC. The gel was placed on top of the wick with the well side of the gel touching it, ensuring that there were no air bubbles between the wick and the gel. A piece of nylon membrane (Hybond-N, Amersham) was cut to the same size as the gel and placed on top of the gel, again ensuring that there were no air bubbles between the nylon and the gel. Two pieces of Whatman 3 MM paper cut to the size of the gel were then soaked in 2X SSC and placed

on top of the nylon membrane. The area of the wick within a few mm of the gel was covered with cling film to prevent a short circuit and a large quantity of paper towels was then placed on top of the 3 MM paper. A glass plate was placed on top of the stack of towels and a 250 g weight placed on top. The blot was left overnight and then disassembled. The filter was placed RNA side down on a UV transilluminator for 90 s to fix the RNA. At this point the transfer of the RNA could be assessed as the RNA on the filter is still stained with EtBr.

2.14.2 Southern blotting

DNA was separated on agarose gels of differing percentages as described in Section 2.10.3. Prior to blotting the DNA was depurinated by soaking in 3 volumes of 0.25 M HCl for 15 min. It was then denatured by soaking in 3 volumes of denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 45 min, neutralised by soaking in neutralising solution (1 M Tris-Cl (pH 8.0), 1.5 M NaCl) for 45 min. The gel was rinsed in 2X SSC before blotting. The gel was then blotted and fixed as described above. The DNA could not be visualised as the EtBr was washed out during the depurination, denaturation, and neutralisation.

2.15 Radio-labelling of DNA

2.15.1 Preparation of the DNA to be labelled

The DNA fragment to be labelled was cut out of its host plasmid using the appropriate restriction enzyme(s) and purified as described in 2.11.1 or 2.11.2. The amount of insert DNA was estimated by running an aliquot of the probe on an agarose gel as described in Section 2.10.3 and comparing it to known amounts of DNA loaded onto the same gel.

2.15.2 Random priming of dsDNA

DNA was labelled with [α - 32 P] dCTP using the Megaprime DNA labelling kit supplied by Amersham International.

Approximately 25 ng of DNA prepared as above was made up to a volume of 10 μ l and 5 μ l of primer solution (random nona-nucleotides) was added. The solutions were mixed in a screw top 1.5 ml Eppendorf[®] tube and then heated to 95°C for 5 min. The tube was put on ice and 10 μ l of buffer solution (dATP, dGTP, dTTP), 50 μ Ci [α - 32 P]dCTP (3000 Ci·mmol⁻¹), 2 μ l of enzyme solution (2 units of DNA polymerase 1 'Klenow' fragment) and 18 μ l of distilled water were added. The tube was incubated at 37°C for 60 min. After this time the reaction was stopped by the addition of 5 μ l of 0.5 M EDTA and a further 45 μ l of H₂O.

2.15.3 Labelling of dsDNA by nick-translation

DNA was labelled with [α - 32 P]dCTP (Amersham) using the nick-translation system supplied by Gibco-BRL.

Approximately 250 ng of DNA was placed in a 1.5 ml Eppendorf[®] tube (sitting on ice). Five μ l of solution A2 (0.2 mM dATP, 0.2 mM dGTP, 0.2 mM dTTP), 25 μ Ci [α - 32 P]dCTP (3000 Ci·mmol⁻¹) and H₂O were added to make a final volume of 45 μ l. 5 μ l of solution C (0.4 U· μ l⁻¹ DNA polymerase I, 40 pg· μ l⁻¹ DNAase I, 50 mM Tris-Cl (pH 7.5), 5 mM MgOAc, 1 mM 2-mercaptoethanol, 0.1 mM PMSF, 50% (v/v) glycerol, 100 μ l·ml⁻¹ BSA) were added and mixed. The reaction was incubated at 15°C for 90 min. The reaction was stopped by the addition of 5 μ l of stop solution (300 mM EDTA (pH 8.0)).

2.15.4 Separation of labelled DNA from un-incorporated radio-nucleotides using spin columns

A sterile 1 ml syringe barrel was plugged with siliconised glass wool and filled with Sephadex TESN. The Sephadex TESN was made by autoclaving Sephadex G 50 medium (Pharmacia, Milton-Keynes, UK) in a 20 fold volume of TESN (10 mM Tris-Cl (pH 7.5), 1 mM EDTA, 0.1 M NaCl, 0.05% (w/v) SDS). The column was centrifuged in a 15 ml Corex[®] tube at 700g for 4 min at room temperature. This process was repeated until the column volume was 1 ml. Once this column volume was achieved 100 µl of TESN was loaded onto the top of the column and the column centrifuged as before. This wash with TESN was repeated once more and then the solution of labelled DNA and un-incorporated nucleotides was loaded onto the column and a clean screw-top Eppendorf[®] tube was placed under the column to collect the elute from the column. The column was then centrifuged as before and the DNA solution in the Eppendorf[®] tube was stored at -20°C until used. The column was then disposed of.

2.15.5 Measurement of the incorporation of radionucleotides into DNA probes

Two µl of the probe solution to be measured was spotted one cm above one end of a piece of Whatman DE 81 paper. This end of the paper was then suspended into 0.3 M ammonium formate solution (pH 8.0), ensuring that the liquid level didn't cover the area where the probe was spotted. The ammonium formate solution was allowed to migrate up the piece of paper. When the liquid front had travelled approximately 5 cm past the origin the paper was removed from the liquid, wrapped in cling film and autoradiographed for 10 min at room temperature. The paper was cut in half so that the spot at the origin, which represents the incorporated label, was separated from the smear, which represents the unincorporated label. The two halves of the paper were

then placed in 4 ml of Ecoscint A (National Diagnostics, Georgia, USA) in a scintillation vial and radioactivity was measured in a scintillation counter. The percentage incorporation could then be worked out as: (the number of cpm at the origin/the number of cpm in the smear plus the number of cpm at the origin) multiplied by 100. Specific activities of labelled probes (i.e. cpm incorporated / μg of DNA) could be calculated given that the amount of radiolabelled deoxy-nucleotide, template DNA and percentage incorporation of the radiolabel were known. A typical random priming or nick-translation reaction with an incorporation of 60% would yield a probe with a specific activity of 1.7×10^9 cpm $\cdot\mu\text{g}^{-1}$.

2.16 Hybridisation analysis

2.16.1 Hybridisation analysis of northern blots

Nylon filters, prepared as in Section 2.14.1, were pre-hybridised for 6 h in a shaking water bath. Just enough pre-hybridisation solution was used (5X SSC, 50% formamide (v/v), 5X Denhardt's solution (0.1% Ficoll 400 (w/v), 0.1% PVP 360 (w/v), 0.1% BSA (w/v) (all Sigma), 100-200 μg denatured, sonicated salmon sperm DNA (Sigma), 0.1% SDS (w/v)) to cover the filter(s) in the bottom of a tupperware box. The probe was denatured by heating to 95°C for 2 min, cooled on ice and then added to the pre-hybridisation solution. One to 2 million cpm of labelled probe was added per ml of hybridisation solution. Hybridisation was for 16 h. The pre-hybridisation and hybridisation were carried out at 42°C unless otherwise stated.

2.16.2 Washing of northern blots

After hybridisation the filters were washed three times, once in each of wash solutions 1 (2X SSC, 0.1% SDS (w/v)), 2 (0.5X SSC, 0.1% SDS (w/v)), and 3 (0.1X SSC, 0.1% SDS (w/v)); 300 ml was used for every wash. Each wash was for

20 min at 60°C unless otherwise stated. Filters were rinsed in 2X SSC before autoradiographing.

2.16.3 Hybridisation analysis of Southern blots

Nylon filters, prepared as in Section 2.14.2, were pre-hybridised for 6 h in a shaking water bath. Just enough pre-hybridisation solution (4X SET, 10X Denhardt's, 0.1% SDS (w/v), 0.1% sodium pyrophosphate (w/v), 100 $\mu\text{g}\cdot\text{ml}^{-1}$ denatured, sonicated salmon sperm DNA (Sigma) was used to cover the filter(s) in the bottom of a tupperware box.. The 4X SET was prepared by dilution of 20X SET (3.0 M NaCl, 20 mM EDTA, 0.4 M Tris-Cl (pH 8.0)). The probes were denatured by heating to 95°C for 2 min, cooled on ice and then added to the pre-hybridisation solution. One to 2 million cpm of labelled probe was added per ml of hybridisation solution. The hybridisation was in the same solution as pre-hybridisation, for 16 h. All filters were prehybridised and hybridised at 68°C unless otherwise stated.

2.16.4 Washing of Southern blots

After hybridisation the hybridisation solution was discarded. The filter was covered in 300 ml of wash solution 1 (3X SET, 0.1% SDS (w/v), 0.1% sodium pyrophosphate (w/v)) and incubated with shaking for 20 min. This was repeated twice. The washing was repeated twice with wash solution 2 (1X SET, 0.1% SDS (w/v), 0.1% sodium pyrophosphate (w/v)) and once with wash solution 3 (0.1X SET, 0.1% SDS (w/v), 0.1% sodium pyrophosphate (w/v)). All filters were washed at 68°C unless otherwise stated. Filters were rinsed in 2X SSC before autoradiography.

2.16.5 Autoradiography

Filters to be autoradiographed were wrapped in cling film and exposed to Fuji X-ray film type RX in a film cassette with intensifying screen at -80°C for the appropriate length of time.

2.16.6 Stripping filters of bound radioactive probes and blocking agents

After hybridisation and autoradiography the bound probe and blocking agents were removed by boiling a solution of 0.1% (w/v) SDS and pouring the boiling solution over the filter. The solution was then allowed to cool to room temperature, and the process was repeated a further two times. The filter was then wrapped in cling film and autoradiographed overnight to check that the bound probe had been removed. The presence of RNA on the filter after stripping could be detected as described below.

2.16.7 Staining of filter-bound RNA/DNA with methylene blue

As described by Herrin and Schmidt (1988)

The filter containing the bound RNA/DNA was placed in 0.04% methylene blue (w/v), 0.5 M NaOAc (pH 5.2) for 5 to 10 min. The filter was then washed in DEPC-treated, H_2O until the RNA bands appeared as blue bands on a white background. The stain could be removed by washing the filter in 20% (v/v) acetic acid until the bands were no longer visible.

2.17 Construction of a cDNA library

The ZAP-cDNA[®] synthesis kit (Stratagene) was used to construct the cDNA library according to the manufacturer's instructions.

2.17.1 cDNA synthesis

For the first strand synthesis 5 μl of 10X first strand buffer (500 mM Tris-Cl (pH 8.3), 750 mM KCl), 5 μl 0.1 M DTT, 3 μl 10 mM first strand methyl nucleotide mixture (10 mM dATP, dGTP, dTTP; 5 mM 5-Me-dCTP), 2 μl linker-primer (1.4 $\mu\text{g}\cdot\mu\text{l}^{-1}$), 24 μl DEPC-treated H₂O, 1 μl RNAase block (40 U) were added in order to an Eppendorf[®] tube and mixed thoroughly. 5 μl (5 μg) of poly(A)⁺ RNA isolated as in Section 2.6.2 was heated to 65°C for 5 min, chilled on ice and added to the reaction. The primer and template were allowed to anneal at room temperature for 10 min. Finally 2.5 μl of M-MuLr reverse transcriptase was added and the tube was gently vortexed. 5 μl of this reaction was added to a separate Eppendorf[®] tube containing 0.5 μl of [α -³²]dATP (800 Ci·mmol⁻¹) (Amersham) as a control. Both reactions were incubated at 37°C for 1 h. The reactions were then removed and placed on ice, the control reaction was frozen at -20°C for later analysis.

To the 45 μl non-radioactive first strand synthesis, while on ice, 40 μl 10X second strand buffer (500 mM Tris-Cl (pH 7.5), 100 mM MgSO₄, 500 $\mu\text{g}\cdot\text{ml}^{-1}$ BSA), 15 μl 0.1 M DTT, 6 μl 10 mM second strand nucleotide mix (10 mM dATP, dGTP, dTTP; 26 mM dCTP), 280.6 μl sterile H₂O, 2 μl [α -³²]dATP (800 Ci·mmol⁻¹) (Amersham) were added. The tube was briefly vortexed prior to the addition of 4.5 μl RNAase (1 U· μl^{-1}) and 6.9 μl DNA polymerase I (14.5 U· μl^{-1}). After mixing and spinning the contents to the bottom of the tube the reaction was incubated for 2 1/2 h at 16°C. The tube was then transferred to ice.

400 μ l of phenol (pH 7.6): chloroform (v/v, 1:1) were added to the reaction and vortexed. The contents were centrifuged at 10,000g for 2 min and the upper aqueous phase was transferred to a clean Eppendorf[®] tube. An equal volume of chloroform was added and vortexed. The Eppendorf[®] tube was recentrifuged, and the upper aqueous phase was transferred to a clean Eppendorf[®] tube. The reaction was precipitated by adding 33.3 μ l 3M NaOAc and 867 μ l 100% EtOH, followed by gently vortexing and incubating at -20°C for 16 h. The precipitate was pelleted by centrifuging at 10,000g for 60 min at 4°C. The supernatant was gently aspirated off, and the pellet was gently washed (not vortexed) with 1 ml of 80% EtOH. The precipitate was recentrifuged, the EtOH was aspirated off, and the pellet allowed to dry. The precipitated cDNA was resuspended in 43.5 μ l H₂O and 4.5 μ l of the resuspended cDNA was removed for alkaline gel analysis. Alkaline gel electrophoresis was carried out as described in Section 2.10.2.

2.17.2 Blunting the cDNA termini

To the remaining 39 μ l from the cDNA synthesis 5 μ l of 10X T4 DNA polymerase buffer (300 mM Tris-acetate (pH 8.0), 700 mM KOAc, 100 mM MgOAc, 5 mM DDT, 1 mg·ml⁻¹ BSA), 2.5 μ l dNTP mix (2.5 mM), 3 μ l T4 DNA polymerase (3.2 U· μ l) were added, while the tube was on ice. The reaction was incubated at 37°C for 30 min and returned to ice. 50 μ l of sterile H₂O was added to bring the final volume to 100 μ l. An equal volume of phenol (pH 7.6): chloroform (v/v, 1:1) was added and vortexed. The contents were centrifuged at 10,000g for 2 min and the upper aqueous phase was transferred to a clean Eppendorf[®] tube. An equal volume of chloroform was added and vortexed. The Eppendorf[®] tube was recentrifuged, and the upper aqueous phase was transferred to a clean Eppendorf[®] tube. The reaction was precipitated by adding 7 μ l of 3 M NaOAc and 226 μ l of 100% EtOH and incubated on ice for 1 h. The cDNA was pelleted by centrifugation for 1 h at 10,000g. The pellet was washed in 300 μ l of 80% EtOH, recentrifuged and the pellet allowed to dry.

2.17.3 Ligation and subsequent kinasing of *EcoR* I adaptors

The dried cDNA pellet was resuspended in 7 μ l of *EcoR* I adaptors, mixed and spun down to the bottom of the Eppendorf[®] tube. One μ l of 10X ligase buffer (500 mM Tris-Cl (pH 7.5), 70 mM MgCl₂, 10 mM DTT), 1 μ l rATP (10 mM), and 1 μ l T4 DNA ligase (4 U· μ l⁻¹) were added and mixed. The reaction was incubated at 8°C for 48 h. The ligase was then heat inactivated by placing it in a water bath at 70°C for 30 min.

After the ligase was heat inactivated the adaptors were kinased by adding 1 μ l 10X ligase buffer, 2 μ l rATP (10 mM), 6 μ l sterile H₂O, and 1 μ l T4 polynucleotide kinase (10 U· μ l⁻¹), the reaction was incubated at 37°C for 30 min. The T4 polynucleotide kinase was then heat inactivated by incubation at 70°C for 30 min.

2.17.4 *Xho* I digestion and size fractionation

Twenty eight μ l of *Xho* I buffer supplement and 3 μ l of *Xho* I were added to the kinased cDNA and incubated at 37°C for 2 h. Five μ l of 10X STE (100 mM Tris-Cl (pH 7.5), 2% SDS (w/v), 1% 2-mercaptoethanol (v/v), 1 M NaCl) were added to the reaction in preparation to pass it through a sephacryl[®] S-400 column (Pharmacia).

The column was prepared by blocking a 1 ml syringe with a small cotton plug. Using a Pasteur pipette the syringe was filled with Sephacryl[®] S-400. The syringe was then placed in a 15 ml Falcon tube and centrifuged for 2 min at 600g. The syringe was filled to 1 cm from the top and recentrifuged and the column was rinsed twice with 300 μ l of 1X STE. The cDNA was loaded onto the column and centrifuged for 2 min at 600g, the eluent was removed and placed into a clean Eppendorf[®] tube. 60 μ l of 1X STE was loaded onto the column and recentrifuged and the eluent was pooled to that from the first spin. The column was discarded.

The cDNA was extracted twice with an equal volume of phenol:chloroform (v/v). After centrifugation for 2 min at 10,000g the aqueous phase was transferred to a clean Eppendorf® tube and extracted once with an equal volume of chloroform.

2.17.5 cDNA precipitation and ligation into the Uni-ZAP™ XR Vector

Two volumes of 100% EtOH were added to the cDNA and the precipitation was allowed to proceed for 16 h at -20°C. The cDNA was pelleted by centrifugation for 1 h at 10,000g, the pellet was gently washed in 200 µl of 80% EtOH, the EtOH was removed and the pellet was allowed to dry before resuspending the cDNA in 10 µl of H₂O.

The cDNA was quantified by spotting 0.5 µl of the cDNA on a plate, made with 0.8% agarose (TAE) containing 1 µg·ml⁻¹ EtBr along with DNA of known concentrations, ranging from 10 ng·ml⁻¹ to 200 ng·ml⁻¹.

One hundred ng of cDNA was used in the ligation reaction. This was added in an Eppendorf® tube to 0.5 µl 10X ligation buffer, 0.5 µl 10 mM rATP, 1.0 µl Uni-ZAP™ XR vector (1 µg·µl⁻¹), and H₂O to a volume of 4.5 µl. After mixing 0.5 µl of T4 DNA ligase (4 U·µl⁻¹) were added and the ligation was allowed to proceed for 3 days at 4°C. A control ligation containing test insert was also carried out.

2.17.6 Packaging, plating, titering, and amplification of the cDNA library

The library was packaged using the Gigapack® II packaging extract (Stratagene). This is *mcrA*, *mcrB* and *mrr*-, and therefore does not destroy hemimethylated DNA, leading to higher titer libraries. An appropriate number of extracts

were removed from the -70°C freezer, DNA was added to freeze/thaw extracts as soon as they began to thaw and placed on ice. 15 µl of sonicated extract was added and mixed, this was incubated on ice for 2 h. 500 µl of SM phage buffer (50 mM Tris-Cl (pH 7.5), 100 mM NaCl, 10 mM MgSO₄ and 2% gelatin (v/v)) were added, then 20 µl of chloroform were added and the reaction was mixed. Debris was allowed to settle, the supernatant was removed and stored at 4°C.

One µl of the final packaged reaction was mixed with 200 µl of SURE[®] cells with an OD₆₀₀ of 0.5. SURE[®] cells were used as it is a *recA*-, *mcrA*-, *mcrCB*-, and *mrr*- *E. coli* strain and does not destroy hemi-methylated DNA. The phage and bacteria were incubated for 15 min at 37°C and then mixed with 3 ml of top agarose (48°C), 15 µl of 0.5 M IPTG, and 50 µl X-Gal (250 mg·ml⁻¹) and immediately poured onto L-agar bottom plates. The plates were inverted and placed at 39°C for 12 h. Packaged phage were titered by plating out a series of phage dilutions ranging from 10²-10⁷. The following equation was used to calculate the titer:

$$\begin{aligned} & \text{Number of plaques} \times \text{dilution} \times \text{volume of packaging extract per ml basis} \\ & = \text{pfus ml}^{-1} \text{ original packaging extract} \end{aligned}$$

The library was amplified immediately. 50 ml of L-broth supplemented with 0.2% maltose and 10 mM MgSO₄ were inoculated with a single SURE[®] colony and grown overnight at 30°C with constant shaking. The cells were transferred into 50 ml Falcon tubes, pelleted by centrifugation for 10 min at 500g and resuspended in 10 mM MgSO₄, this was diluted to an OD₆₀₀ of 0.5, using 10 mM MgSO₄. Aliquots of the packaging reaction containing approximately 50,000 pfu were mixed in 15 ml Falcon tubes with 600 µl of the diluted SURE[®] cells (20 amplifications gave 1,000,000 pfu for subsequent screening). After 15 min at 37°C 8 ml of top agarose (48°C) was added and mixed. This was spread immediately on bottom agar (150 mm plates). The plates were inverted and incubated at 39°C until the plaques had a diameter of 1-2 mm. The

plates were then overlain with 8 ml of SM phage buffer and stored overnight at 4°C with gentle rocking. The SM phage buffer from all the plates was pooled into 50 ml Falcon tubes. The plates were rinsed with an additional 2 ml of SM phage buffer, chloroform was added to 5% (v/v) and the cell debris was removed by centrifugation for 10 min at 2,000g. The supernatant was transferred to a clean 50 ml Falcon tube, chloroform was added to 0.3% (v/v) and storage was at 4°C.

2.18 Screening libraries

The same protocols were used to screen both the cDNA and genomic libraries.

2.18.1 Preparation of plating bacteria

Different strains of *E. coli* were used for plating of the cDNA and genomic libraries. SURE[®] was used for the cDNA library, DL491 was used for the genomic library. A single bacterial colony was picked and used to inoculate 50 ml of L-broth/NZYCM supplemented with 0.2% maltose (w/v) and 10 mM MgSO₄. This was grown overnight at 30°C with constant shaking. The cells were transferred into 50 ml Falcon tubes, pelleted by centrifugation for 10 min at 500g and resuspended in 10 mM MgSO₄. The suspension was diluted to an OD₆₀₀ of 0.5 using ice-cold 10 mM MgSO₄. This cell suspension was kept stored at 4°C and used within three weeks.

2.18.2 Plating bacteriophage

A 200 µl aliquot of plating bacteria, as prepared in Section 2.18.1, was dispensed into a sterile 15 ml Falcon tube, to which 50 µl of an appropriate dilution of bacteriophage was added. After the test tubes had been mixed, they were incubated at 37°C for 15 min. After this incubation, 3 ml of top agarose (48°C) was added to each

tube and was briefly mixed and immediately poured onto a dry pre-warmed 90 mm Petri dish which contained 20 ml of hardened L-agar supplemented with 0.1 M MgSO₄ and 0.2% maltose (L-agarose was used to plate the cDNA library, NZYCM was used to plate the genomic library). The plate was swirled gently to allow the formation of an even top agarose layer and then left to stand on a flat surface.

The plates were left to stand for 5 min at room temperature to allow the top agar to harden. Then, all the Petri dishes were inverted and incubated at 37°C for 12-16 h.

2.18.3 Plaque lifts

The method used was based on that of Benton & Davis (1977).

Plates were prepared as outlined in Section 2.18.2. Plates with a diameter of 150 mm were used in primary screens of a library and 90 mm diameter plates were used for all subsequent rounds of screening. In the primary screens 3×10^4 pfu were used. A much lower number, approximately 20-500 pfu, were used for subsequent rounds.

Plates were chilled at 4°C for 30-60 min to allow the top layer of agar to harden before plaque lifts were attempted. Meanwhile, the appropriate number of nylon membranes (Hybond-N, Amersham) were labelled individually and marked asymmetrically in three locations with a permanent marker.

A filter was laid carefully onto each plate from the centre outward, so that it made direct contact with the plaques. The position of the reference points on the filter was recorded onto the bottom of the plate and after 30-60 s the first filter was peeled off the plate with a pair of blunt-ended forceps. Duplicate lifts were made, the second filter was left on the plate for a duration of 2 min.

After its removal from the plate, each filter was placed, DNA side up, onto two sheets of Whatman 3MM paper soaked in denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 4 min. Then, the filters were carefully drained of excess solution and transferred to two sheets of 3MM paper soaked in neutralisation solution (0.5 M Tris-Cl (pH 7.5), 1.5 M NaCl) for a further 4 min. Finally, the filters were rinsed for 5 min in 2X SSC and left, DNA side up, on Whatmann 3MM paper to dry. DNA was immobilised onto the filter by baking at 80°C for 2 h.

2.18.4 Selection of bacteriophage plaques

Hybridisation analysis of filters and washing was the same as described for Southern blots in Section 2.16.3. In cases where the ratio of the background to specific signal was high, filters were washed again in 0.1X SSC, 0.1% SDS (w/v) at 65°C for 20 min. Filters were wrapped in clingfilm and autoradiographed at -80°C.

The pattern of dots produced on the film, by hybridisation of plaque DNA with a probe, was aligned with the corresponding position on the plate with the help of the reference marks on the filter and plate. Plaques of interest were removed from initial rounds of screening with the wide end of a Pasteur pipette. The agar/agarose plug was transferred to a 1.5 ml Eppendorf® tube which contained 1 ml of SM phage buffer and 50 µl of chloroform, vortexed briefly and left at room temperature for 1 h to allow the phage to diffuse out into the solution. The phage suspension was titered as described in Section 2.17.6. The process of screening was repeated with a lower plaque density per plate until plaque purity was attained. This typically took three to four rounds of screening.

2.18.5 Preparation of plate lysate stocks

An inoculum of 10^5 pfu was used to produce confluent lysis of a bacterial lawn grown on a 90 mm diameter plate. Phage were eluted out of the agarose by the addition of 5 ml of SM phage buffer to each plate, these were left, gently shaking at 4°C overnight. The bacteriophage suspension was recovered into a sterile 10 ml polypropylene tube and each plate was rinsed with a further 1 ml of SM phage buffer. A volume of 5% (v/v) chloroform was added to each suspension, vortexed and incubated at room temperature for 15 min. Bacterial cell debris was removed from the suspension by centrifugation at 200g for 5 min. Then the supernatant was transferred to a fresh tube, chloroform was added to 0.3% (v/v) and the lysate was titered. Phage lysates were stored at 4°C.

2.19 PCR

2.19.1 DNA preparation

A PCR based method was used to estimate the sizes of cDNA clones from the cDNA library (isolated as described in Section 2.18) prior to *in vivo* excision. Purified phage were plated at 20-100 pfu on a 90 mm plate, an individual plaque was picked from the plate using a Pasteur pipette and placed into 50 µl of H₂O. This was placed in boiling H₂O for 5 min to melt the agarose, releasing the phage and denaturing the phage coats. This was then placed into a PCR reaction.

PCR on the promoter fragment from *BnPRP* used plasmid DNA prepared as described in Section 2.7.3.2.

2.19.2 PCR reaction

For PCR based sizing of the cDNA inserts the reagents were added to a 0.5 ml centrifuge tube in the following order; 10 μ l of 10X PCR Buffer (670 mM Tris-Cl (pH 8.8), 67 mM MgCl₂, 1.7 mg·ml⁻¹ BSA and 166 mM (NH₄)₂SO₄), 16 μ l of 10X dNTPs (20 mM with respect to each: dATP, dCTP, dGTP and dTTP), 1 μ l of 25 pmol· μ l⁻¹ T3 and T7 primer, H₂O to 100 μ l and 0.5 μ l of *Taq* Polymerase (Promega, 2.5 U· μ l⁻¹) were used. The same reagents were used for the PCR of the promoter except that *Pfu* polymerase was used (Stratagene) in place of *Taq* polymerase. This used a different 10X buffer (200 mM Tris-Cl (pH 8.2), 100 mM (NH₄)₂SO₄, 20 mM MgCl₂, 1000 μ g·ml⁻¹ BSA, 1% Triton X-100 (v/v)) All the reagents were mixed thoroughly, layered with two drops of mineral oil (Sigma) to reduce evaporation and the tubes were placed in a thermal cycler (Perkin-Elmer 2400, Perkin-Elmer, Cetus, USA).

The thermal cycler was programmed to denature the sample for 5 min at 94°C and then to complete 25 cycles of 94°C for 1 min (denaturation), 50°C for 1 min (primer annealing) and 72°C for 2 min (extension). At the end of the last cycle the sample was heated at 72°C for a further 10 min to ensure full extension of the product and then cooled down to 4°C.

2.20 *in vivo* excision from the Uni-ZAP™XR bacteriophage

Phage lysates of purified cDNA clones were isolated as described in Section 2.18.5. In a 50 ml Falcon tube 200 μ l of XL1-Blue cells grown to an OD of 600 in L-Broth were combined with 100 μ l phage lysate (containing > 1 X 10⁵ phage particles) and 1 μ l ExAssist™ helper phage (Stratagene) (containing > 1 X 10⁶ pfu·ml⁻¹) and mixed. The mixture was incubated for 15 min at 37°C. 3 ml of 2 X YT

media (1.6% bacto-tryptone (Difco) (w/v), 1% yeast extract (Difco)(w/v), 0.18 M NaCl) was added and incubated for 2 h at 37°C with constant shaking. The tube was then heated for 20 min at 70°C, and centrifuged for 15 min at 4,000g. The supernatant which contains the Bluescript® II SK (-) phagemid packaged as a filamentous phage particle was decanted into 2 clean 1.5 ml Eppendorf® tubes and was stored at 4°C.

Two hundred µl of SOLR® cells (Stratagene) were grown to an OD of 600 in L-Broth were placed in two 1.5 ml Eppendorf® tubes, 1 µl of phage stock isolated as above was added to 1 tube, 50 µl of the same stock was added to the second tube, the tubes were incubated for 15 min at 37°C. 100 µl from each of the reactions was plated onto a L-agar plate (supplemented with ampicillin, 50 µg.ml⁻¹). Single colonies were isolated and restreaked on a fresh plate, pBluescript® II SK (-) was amplified and isolated for analysis as described in sections 2.7.3.1 and 2.7.3.3. The SOLR® strain was used as it is non-suppressing and therefore does not allow the replication of the ExAssist™ helper phage which contains an amber mutation, removing the possibility of co-infection.

2.21 Large-scale preparation of DNA from genomic clones

A culture of DL491 cells was grown in 100 ml of NZYCM supplemented with 10 mM MgSO₄ and 0.2% maltose (w/v) at 30°C with vigorous shaking overnight. The next day, 4 ml of 1 M MgSO₄, 6 ml of the overnight bacterial culture and 1 x 10⁹ pfu of the bacteriophage clone of interest (prepared as in Section 2.18.5) were added to 400 ml of NZYCM in a 2 l Erlenmeyer flask. The phage were allowed to adsorb to the cells for 15 min at 37°C without shaking. Then, the culture was left to grow at 37°C, shaking at 250 rpm until lysis occurred. This usually took about 5 h and was characterised by the clearing of the culture and the appearance of white 'threads' within the culture.

Once lysis had occurred, 4 ml of chloroform was added and the flask was shaken as before for a further 20 min. Digestion of bacterial nucleic acids was achieved by the addition 16 g of NaCl, $1 \mu\text{g}\cdot\text{ml}^{-1}$ of DNAase (Type VIII) and $1 \mu\text{g}\cdot\text{ml}^{-1}$ of RNAase (Type I-A) (both Sigma) to the culture and the flask was swirled gently to aid salt dissolution. The culture was left for 1 h at room temperature and then centrifuged in 400 ml sterile bottles at 16,000g for 15 min at 4°C to bring down cellular debris. The supernatant was poured into a fresh flask containing 10% (w/v) polyethylene glycol 8000 (PEG 8000), swirled very gently until all the PEG 8000 had dissolved and then left to stand at 4°C overnight to precipitate the bacteriophage.

The next day, the bacteriophage were harvested by centrifugation at 16,000g for 15 min at 4°C. The supernatant was discarded, and the precipitate was resuspended gently in 7 ml of SM phage buffer. The tubes were washed with a further 7 ml of phage buffer and both washes were combined in a sterile 50 ml Falcon tube. An equal volume of chloroform was added to the bacteriophage suspension and the contents of the tube were emulsified by repetitive gentle inversion. Next, the suspension was incubated on ice for 30 min to allow the PEG to precipitate out of solution. After a 10 min centrifugation at 200g the upper aqueous phase was transferred to a fresh tube and $0.75 \text{ g}\cdot\text{ml}^{-1}$ CsCl was added. Once the CsCl had dissolved, the solution was transferred into an 18.5 ml polyallomer ultracentrifuge tube.

The bacteriophage were banded by ultracentrifugation at 18,000g, at 10°C overnight. The next day the single iridescent blue band produced was harvested with the use of a 23 gauge needle and a 5 ml syringe. The band was best visualised in a dark room with a single white light source illuminating the band in the ultracentrifuge tube.

Dialysis tubing (Sigma) was boiled in 10 mM MgCl₂ for 15 min and then left to cool down to room temperature before it was used for dialysis. The banded

bacteriophage solution was poured into dialysis tubing and clipped at either end to contain the solution. The CsCl was removed from the bacteriophage solution by dialysis against a 100 fold volume of dialysis buffer (10 mM NaCl, 50 mM Tris-Cl (pH 8.0) and 10 mM MgCl₂) at 4°C overnight.

The next day, the dialysed solution was transferred from the dialysis bag to a sterile tube. To this was added EDTA to a final concentration of 20 mM, 50 µg·ml⁻¹ of proteinase K and SDS to a final percentage of 0.5% (w/v). The contents of the tube were mixed and then incubated at 65°C for 1 h. This process removed the protein coat from the bacteriophage DNA. After this incubation, the solution was extracted twice with phenol (pH 7.8), once with chloroform and then finally precipitated with 2.5 volumes of EtOH. The resulting DNA pellet was washed with 70% EtOH, air dried and resuspended in 500 µl H₂O and then stored at -20°C until required.

2.22 Dideoxy DNA sequencing

DNA was sequenced using the Sequenase[®] Version 2.0 enzyme and kit obtained from United States Biochemicals (Ohio, USA). Double-stranded template DNA was prepared as in Section 2.7.3.2.

2.22.1 Annealing of template and primer

The mini-prep template DNA (3-5 µg) was denatured in 0.2 M NaOH, 0.2 mM EDTA for 15 min at 37°C. Denatured DNA was precipitated by addition of 0.1 volume 2 M NH₄OAc (pH 4.0) and 2 volumes 100% EtOH and incubated for 10 min on ice, centrifuged at 10,000g for 5 min, washed in 200 µl of 80% EtOH, and recentrifuged. The washed DNA pellet was resuspended in 7 µl H₂O, 2 µl reaction buffer (0.2 M Tris-Cl (pH 7.5), 0.1 M MgCl₂, 0.25 M NaCl, supplied with kit) and 1 µl (2.5 ng)

primer. Annealing took place at 37°C for 20 min after which time the tube was stored on ice for up to 2 h.

2.22.2 Labelling and termination reactions

To the annealed template-primer was added: 1.0 µl 0.1 M DTT, 2.0 µl diluted labelling mix (usually dGTP mix; 1.5 µM dGTP, 1.5 µM dCTP, 1.5 µM dTTP), 0.5 µl (5 µCi) [α -³⁵S]dATP (1000 Ci·mmol⁻¹), 2.0 µl diluted Sequenase[®] 2.0. The contents were mixed and incubated for 2-5 min at room temperature. Four microfuge tubes were labelled A, C, G and T. Into each tube was placed 2.5 µl of the appropriate termination mix and the tubes prewarmed for 1 min at 37°C. On completion of the labelling reaction, 3.5 µl of the reaction mix was placed in each of the four tubes. Termination reactions proceeded for 3-5 min at 37°C after which time 4 µl of stop solution (50% formamide (v/v)) was added to the reactions. When DNA with regions of high secondary structure was sequenced the temperature of the termination reaction was increased to 50°C. The tubes were stored at -20°C prior to electrophoresis. Samples were denatured by heating to 75-80°C for 2 min before loading on the gel.

2.22.3 Sequencing gel electrophoresis

Gels of dimension 20 cm x 40 cm x 0.4 mm were prepared using 50 ml of de-gassed linear sequencing mix (6% acrylamide:bisacrylamide (w/v, 38:2), 7 M urea, 1 x TBE), to which was added 300 µl 10% ammonium persulphate (w/v) and 50 µl TEMED (Sigma). Aluminium plates were placed either side of the gel to ensure even heat distribution during electrophoresis. The gels were pre-run for 20 min at a constant power of 40-45 W before loading the samples. Gels were run for periods of 2 and 5 h at a constant power of 30-35 W for reading the maximum number of nucleotides. After electrophoresis the gel plates were removed from the sequencing apparatus and separated. The gel was fixed in 10% acetic acid (v/v), 10% MeOH (v/v) for at least 15

min. The gel was transferred onto Whatman 3MM paper and dried on a slab gel-drier at 80°C for 1 h. Gels were autoradiographed for 16-48 h without intensifying screens at room temperature.

2.23 Generation of clones for sequencing by directed deletions

The methods used were based on those from Murphy and Ward (1989). The Stratagene *Exo* III deletion kit was used according to the manufacturer's instructions. All enzymes and buffers except those for digestion of the DNA were provided.

2.23.1 Linearisation of plasmid and protection of the priming site

The amount of starting material varied according to the length of DNA which was to undergo exonuclease deletion. As a rough rule of thumb, 5 µg of DNA which had been purified on a CsCl gradient as described in Section 2.7.3.3 was used for every time point to be taken.

The digestion to produce a protective 3' overhang using *Sac* I or *Kpn* I was carried out for 3-4 h at 37°C. After verification of complete digestion, on a 1.2% agarose gel, the sample was cleaned using a Magic™ DNA clean-up kit (Promega), as described in Section 2.11.2, with the exception that the DNA was mixed directly with the resin and did not have to be heated. Then the sample underwent its second digestion for 3-4 h with the restriction enzymes *Xho* I or *Eco*R I, which would produce a 5' overhang. Control digestions were done to ensure the restriction enzymes had enough time to digest the DNA.

2.23.2 Digestion with *Exo* III

The linearised sample was treated with *Exo* III and samples were removed at appropriate intervals, with the consideration that the activity of *Exo* III decreased as the length of incubation increased. Also, *Exo* III can be controlled by the temperature at which the reaction is conducted. The rate that base pairs are deleted min^{-1} is 400 at 37°C, 375 at 34°C, 230 at 30°C, and 125 at 23°C. The whole reaction was started in a 1.5 ml Eppendorf® tube, with aliquots being removed at pre-determined time intervals. The reaction consisted of the following components per time point: 5 µg of double digested DNA, 12.5 µl of 2X *Exo* III Buffer (100 mM Tris-Cl (pH 8.0), 10 mM MgCl_2 , 20 µg·ml⁻¹ of tRNA) 2.5 µl of fresh 100 mM 2-mercaptoethanol and 20 units of *Exo* III in a total volume of 25 µl. Aliquots of 25 µl were removed to pre-cooled microfuge tubes on dry ice, which contained 20 µl of 10X mung bean buffer (300 mM NaOAc (pH 5.0), 500 mM NaCl, 10 mM ZnCl_2 and 50% glycerol (v/v)) diluted in 155 µl of H_2O . Once all the aliquots had been removed, the tubes were incubated at 65°C for 15 min to denature the enzyme and then transferred to ice.

2.23.3 Creation of blunt ends

Blunt ends were generated with the use of mung bean nuclease. To each sample 15 units of mung bean nuclease diluted from a concentrated stock into 1 x mung bean dilution buffer (10 mM NaOAc (pH 5.0), 0.1 mM ZnOAc, 1 mM cysteine, 0.1% Triton-X-100 (v/v) and 50% glycerol (v/v)) was added and then the reaction was incubated at 30°C for 30 min.

2.23.4 Ligation and characterisation of deletions

Fourteen µl of each sample was run out on an 1.2% TBE agarose gel, along with the following two controls: linearised vector which contained a full-length insert

and linearised vector without insert. These controls helped ascertain the rate of deletion of the insert.

Samples from appropriate time points were ligated overnight at 15°C. The ligation reaction consisted of the following additions: 3 µl of DNA sample, 2 µl of 10X Ligation Buffer (500 mM Tris-Cl (pH 7.5), 100 mM MgCl₂, 10 mM spermidine, 10 mM rATP and 1 mg·ml⁻¹ BSA), 2 µl of 100 mM DTT and 8 units of T4 DNA ligase in a total reaction volume of 20 µl.

Transformations were carried out using 3 µl of the ligation reaction in 200 µl of competent *E. coli* XL1-Blue as described in Section 2.7.2. The transformations were plated onto L-broth medium plates supplemented with antibiotic. Transformed colonies were picked at random and screened for insert size.

Plasmid DNA was isolated from transformations as described in Section 2.7.3.1. 50 ng of the DNA was placed into a PCR reaction as described in Section 2.19. Again the T3 and T7 primers were used as these flank the multicloning site and can be used to accurately size the deletion. The products were run on a 1.2% agarose gel along with the PCR products from the full length insert and the pBluescript® II SK (-) with no insert as size markers.

2.24 Transformation of *Arabidopsis thaliana*

2.24.1 Surface-sterilisation of seed

Seeds were placed on Whatman no. 1 filter paper (9 cm diameter) which was then folded into quarters and folded once more to form a packet. The packet was secured with a plastic-coated paper-clip and labelled in pencil. The packet was immersed in 70% EtOH in a Magenta pot for 2 min and allowed to drain before

transferring to a Magenta pot containing 10% sodium hypochlorite (1.4% (v/v) available chlorine), 0.02% Triton X-100 (v/v) (Sigma) for 15 min. From this point manipulations were carried out in a sterile flow hood. The packet was placed in a Magenta pot containing H₂O for 5 min to remove any bleach. This was repeated 5 times. The packet of seed was dried on a Magenta jar lid in the flow hood for at least 3 h. The seeds were stored in a Petri dish sealed with Micropore[®] tape (3M Health Care, Loughborough, UK).

2.24.2 Growth of plants

Surface-sterilised seed was sown onto Petri dishes containing germination medium (GM; 1X Murashige and Skoog salt mixture (Flow Labs, Irvine, Scotland, UK), 2% sucrose (w/v), 100 mg·l⁻¹ inositol, 1 mg·l⁻¹ thiamine, 0.5 mg·l⁻¹ pyridoxine, 0.5 mg·l⁻¹ nicotinic acid, 0.5 g·l⁻¹ MES (pH 5.7), 0.8% agar). The seeds were grown in continuous white light for approximately three weeks and roots harvested before the plants flowered.

2.24.3 Transformation of roots

The transformation procedure used is based on that of Valvekens *et al.* (1988) with modifications suggested by Dean *et al.* (1991). All manipulations were carried out in a flow hood.

Roots were cut off plants, pulled apart, then roots were placed on solid callus inducing medium (CIM; 1 x Gamborg's B5 medium (Sigma), 2% glucose (w/v), 0.5 g·l⁻¹ MES (pH 5.7), 0.5 mg·l⁻¹ 2,4-dichlorophenoxyacetic acid, 0.05 mg·l⁻¹ kinetin) ensuring that all roots were in contact with the medium. The plates were incubated for 3 days in low white light (15 μmol·m⁻²·s⁻¹). The roots were stacked in an empty Petri dish and cut into 0.5 cm explants. The explants were placed in a sieve and washed in

20 ml liquid CIM in a petri dish. 500 µl of an overnight culture of *A. tumefaciens* was added and the explants left to co-cultivate for 2 min. The sieve was drained and excess medium removed by blotting the root explants on sterile Whatman no. 1 filter paper. The root explants were transferred in small clumps to a Petri dish containing solid CIM. The plates were incubated in a growth room at 24°C for 2 days to allow co-cultivation of the *A. tumefaciens*. After incubation, the root explants were washed in 20-25 ml of liquid shoot-inducing medium (SIM; 1 x Gamborg's B5 medium, 2% glucose (w/v), 0.5 g·l⁻¹ MES (pH 5.7), 5 mg·l⁻¹ N6-(2-isopentenyl)adenine, 0.15 mg·l⁻¹ indole-3-acetic acid). The root explants were blotted on sterile filter paper and transferred to solid SIM plus 750 mg·l⁻¹ vancomycin, 50 mg·l⁻¹ kanamycin (SIM V750 K50). Root explants which had not been co-cultivated with *A. tumefaciens* were placed on control plates of SIM plus 750 mg·l⁻¹ vancomycin (SIM V750) (positive control) and SIM V750 K50 (negative control). The plates were placed in continuous white light to allow callus formation. After approximately 10 days callus can be seen on the positive controls and callus appears on the non-control plates after about three weeks. The explants were transferred to fresh SIM V750 K50 plates every 10 days until shoots appeared. The shoots were excised, placed on GM plus 500 mg·l⁻¹ vancomycin and allowed to flower. On formation of flower buds plants were transferred to individual Magenta pots containing GM with the lids loosely attached with Micropore[®] tape (3M Health Care) to allow the anthers to dehisce. Seeds were harvested when the pods were a yellowish-brown colour.

2.24.4 Analysis of progeny from transformed plants

Seed collected from transgenic plants was surface-sterilised and plated out on GM plus 50 µg·ml⁻¹ kanamycin. Wild-type seed was included as a control. The plates were placed in continuous white light. Seedlings were examined after expansion of the cotyledons for expression of kanamycin resistance. Kanamycin-sensitive seedlings are bleached and do not develop leaves. The segregation ratio (kanamycin resistant:

kanamycin sensitive) was calculated. Plants were transferred to compost on formation of flower buds and seed collected.

2.24.5 Histochemical localisation of β -glucuronidase

Arabidopsis thaliana transformed with the construct PBI121 (35S CaMV promoter, β -glucuronidase (GUS) reporter gene and nos 3' terminator; Jefferson *et al.*, 1987) was used as a positive control. The histochemical assay for GUS expression was performed according to Scott *et al.* (1988).

The tissue was incubated in 300 μ l of 1 mg·ml⁻¹ 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) in 50 mM sodium phosphate (pH 7.0) for 8-24 h at 37°C. The tissue was then cleared in 70% EtOH.

Chapter 3: Characterisation of the cDNAs LF5B1 and BnPRP

3.1 Introduction

The cDNA LF5B1 (see Section 1.9) had been partially sequenced using the T3 and T7 primers, which flank the multicloning site of pBluescript[®] II SK (-) (Table 3.1a). The sequence generated using the T7 primer revealed a region containing 38 consecutive adenine residues. This region of polyadenylic acid is characteristic of the poly(A) tail found at the 3' end of most eukaryotic mRNAs (Lewin, 1987). In LF5B1 it was located internally, within the cDNA. This suggested that the LF5B1 cDNA was a compilation of two or more cDNAs. Hence the first objective of experiments described in this section was to characterise LF5B1 and identify sequences corresponding to the cold-induced transcripts. Subsequently a further cDNA was isolated and characterised.

3.2 Sequencing of LF5B1

The first step in characterising the cDNA LF5B1 was to sequence the entire clone. The cDNA had been sequenced from the 5' and 3' ends using the T3 and T7 sequencing primers. A set of three deletions created using *Exo* III nuclease and mung bean nuclease were used to sequence the remainder of the cDNA. The digestion proceeded from the T7 side of the multicloning region. The restriction enzyme *Eco*R I created a 5' overhang that allowed the *Exo* III nuclease to digest the cDNA. *Kpn* I created a 3' overhang that protected the plasmid DNA from *Exo* III digestion. The sizes of the deletions were estimated by analysis of PCR products generated across the multicloning region using the T3 and T7 primers. Three deletions were selected (Table

Table 3.1 Primers and deletions used to sequence the cDNAs LF5B1 and BnPRP

The cDNAs LF5B1 and BnPRP were sequenced using various synthetic oligonucleotide primers in combination with a series of deletions.

a shows the T3 and T7 primers that bind to the regions of pBluescript[®] II SK (-) that flank the multicloning site, along with synthetic oligonucleotide primers made to regions of known sequence in the BnPRP clone. The region the primer was made to is indicated.

b shows the deletions of the plasmids LF5B1 and BnPRP and the first base detected when sequencing the deletion with the oligonucleotide primer indicated.

The numbers are based on the sequences shown in Figs. 3.2 and 3.9.

a

Primer	Sequence (5' - 3')	Template	Template binding position
T3	AATTAACCCTCACTAAAGGG	Bluescript	792 - 772
T7	GTAATACGACTCACTATAGGGC	Bluescript	624 - 646
GF6	ACACCACCGGTTCGTAACG	BnPRP	139 - 157
PR1	CTCACACACGCAAATTAACC	BnPRP	735 - 716
GR1	GTGTCGATTGGGCACGTT	BnPRP	396 - 378

b

Deletion	Template	First nucleotide sequenced	Primer used for sequencing
8	LF5B1	141	T3
11	LF5B1	289	T3
5	LF5B1	591	T3
10	BnPRP	377	T3
17	BnPRP	603	T3

3.1b), the deletions were sequenced using the T3 primer. Fig. 3.1a schematically represents the sequencing of LF5B1.

3.3 Analysis of the LF5B1 sequence

The full sequence is shown in Fig. 3.2. Translation of the sequence revealed only one substantial uninterrupted reading frame. This is not a true open reading frame as it does not start with a methionine residue. The translated amino acid sequence is shown below the corresponding nucleotide sequence in Fig. 3.2. This is 138 residues long, coded for by the nucleotides 191 to 603. The sequence 5' of this sequence, and 3' of the poly(A) region show no clear relationship to the sequence encoding the reading frame. This implies that LF5B1, is composed of at least three unrelated regions of sequence.

A putative polyadenylation signal (AATAAA) is encoded by nucleotides 722 to 727 . This is 52 bp upstream of the first adenine of the poly(A) tail contained within the clone. This is further away than is normally found in plant genes (Joshi, 1987b).

No firm conclusions can be drawn regarding which part of the LF5B1 cDNA hybridises to the cold-induced transcripts without further investigation.

3.4 Identification of the sequence hybridising to the cold-induced transcripts

The uninterrupted reading frame was the most likely part of the sequence to hybridise to the cold-induced transcripts but other parts of the sequence could not be ruled out. It was possible that the non-coding region of the cold-induced transcripts

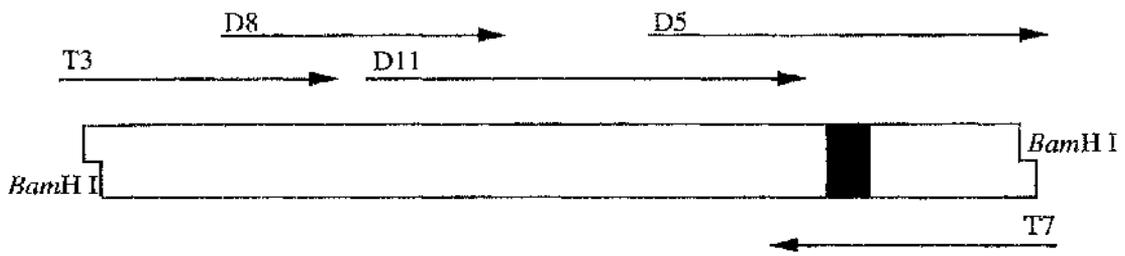
Figure 3.1 Schematic representation of the sequencing of LF5B1 and the digestion of LF5B1 with *Msp* I

The LF5B1 cDNA is represented by the rectangles. The restriction enzymes bordering the inserts are shown.

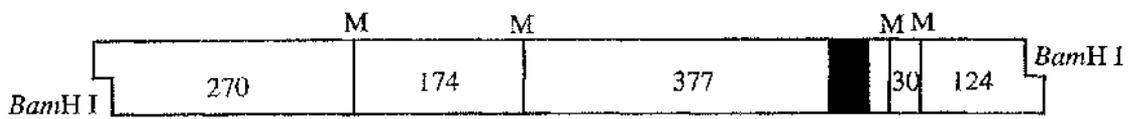
a The lines correspond to the sequence information obtained from the oligonucleotide primer or deletion, the name of the oligonucleotide primer or deletion is shown above the line. The arrow shows the direction that the sequencing reaction proceeded in. Information on the oligonucleotide primers and deletions is shown in Table 3.1.

b The positions that *Msp* I cuts LF5B1 are indicated by M. The sizes of the resulting fragments are indicated.

a



b



0 100 200 300
nucleotides / base pairs

 Poly(A)

Figure 3.2 Nucleotide sequence of the cDNA LF5B1

The numbers at the end of each line correspond to the nucleotide at the end of each line. Non-determined nucleotides are indicated by N. The amino acids of the longest open reading frame are shown under the corresponding nucleotide sequence. Stop codons are indicated by an asterisks. A putative polyadenylation signal (AATAAA) present at position 722-727 is underlined. The sequence was determined using the T3 and T7 primers, together with three deletions created using *Exo III* nuclease and mungbean nuclease.

The *Bam*H I sites, GGATCC, and the *Msp* I sites, CCGG are indicated by underlining.

GGATCCAACCTATCCCTCCTCGCTTTCTTAAGATTCTCGGTCGTTTTCTCTTTGGCTCTA 60
GCTTTCTGAGCCTTGCTTAGACCTGGAAACCACTTCCTTTCTGTCTCATCAGGTAAGTAG 120
AATCTCGCGGGGATGAGCTCTACCATTGTATCGAAAACTCGGAATGCTGATGAACCATT 180
GACTTAATAGTATCCAATCCAGAATCAACAACCTTTCTCCTTTTTCTTTGCCATCGGTACG 240
* Y P I Q N Q Q L S P F S L P S V R
ACGATGGTGCCTAATACCTCCAACACCACCGGTCGTAACGCCACCGACACCAACCCCTCC 300
R W C V I P P T P P V V T P P T P T P P
TGTCGTAAACACCACCAACACCAACTCCACCTAAGCCAGAAAACATGCCCAATCGACACGTT 360
V V T P P T P T P P K P E T C P I D T L
GAAGCTAGGCGCTTGTGTAGACGTTCTTTGGAGGTTTGATTACATCGGGCTTGGTGGAG 420
K L G A C V D V L G G L I H I G L G G S
TAGCGCCAAGAAAGAGTGTGTTCGGTTTTTGGGAGGCTTAGTTGACTTAGACCGCAGCTGT 480
S A K K E C C P V K G G K V D L D A A V
TTGTCTATGTACCACCATTAAGCCAAACTTCTCAACCTCGACCTTATTATCCCCATTGC 540
C L C T T I K A K L L N L D L I I P I A
TCTTGAGCTTCTTATCGACTGTGGAAAGACTCCGCCACCTGGCTTCAATGTCCCTCTTAA 600
L E L L I D C G K T P P P G F N V P L N
TGAATGAAGTGTCTCTCTATCGTGTTTTTCTTTCTTTCTTTCAAGAATTTGTTAGTTGA 660
E *
TAATCTCTTGGGTGAGGTTAATTTGCGTGTGTGGGACCTTTAATCACTTGTTTTGTGTA 720
CAATAAAAGGCTATGTAAAAACGGTATAATTATCTTTCAACAAAAACAAGTTTATATCTCA 780
AAACCGCGTGGAAAATGGANNNGN 840
GCCGAGTAACCGGAGAAGCGNCAACCCAAAAGAGGATCGTGCTCGCATCACTATCGGCAA 900
TGGTCGCAGAGTCAGTTACGTTCCGATTGATCTCACAAAGACCAGAATGCAGCTCCATG 960
GTTATGCTCGGATCC 975

was represented in LF5B1. To examine this, suitable restriction enzymes that would cut the cDNA into several pieces were identified. Each restriction fragment could be used to probe a northern blot containing RNA from cold treated plants and plants grown at control temperatures. The LF5B1 sequence was mapped for suitable restriction enzymes using the UWGCG programme MAP (Devereux *et al.*, 1984). The enzyme *Msp*I was found to be most suitable, cutting the cDNA at positions 270, 444, 821, and 851. This produced five fragments of 270 bp, 174 bp, 377 bp, 30 bp, and 124 bp (Fig. 3.1b). The four largest fragments were isolated from an agarose gel (Fig. 3.3), radioactively labelled, and used to probe a northern blot containing RNA from cold treated and control plants. The 377 bp and 174 bp fragments gave a positive signal, hybridising to transcripts only found in cold treated plants (Fig. 3.4). These fragments contain the region encoding the 138 amino acid uninterrupted reading frame.

The sequence of the cDNA could not be defined by the boundaries of the 174 bp fragment and the poly(A) tail as artefacts could be present in the 174 bp fragment and between the poly(A) tail and the uninterrupted reading frame, part of the uninterrupted reading frame could also be a cloning artefact. To unambiguously deduce the sequence of the cDNA it was decided to isolate another cDNA using the 174 bp fragment of LF5B1 as a probe. This would hopefully be free of cloning artefacts.

3.5 Construction of a cDNA library

The LF5B1 cDNA, along with other cDNAs from the same library had been shown to be chimaeras (Pallas, 1992). It was decided to construct a new cDNA library in order to attempt to isolate a full length clone containing no artefacts. Another reason for constructing a new library was that the library used to isolate LF5B1 was not specifically from cold treated plants, and the cold-induced transcript that hybridises to LF5B1 may not be present at maximal levels. The cDNA library was constructed in the

Figure 3.3 Agarose gel showing the restriction pattern of the LF5B1 insert, cut with *Msp* I.

The LF5B1 insert was excised from pBluescript[®] II SK (-) with *Bam*H 1. The fragment was gel purified using GeneClean[®] and digested with *Msp* I. The products were separated on a 1.5% agarose gel.

- Lane 1 + 5 kb ladder.
- Lane 2 LF5B1 digested with *Bam*H 1.
- Lane 3 Gcl purified LF5B1 insert.
- Lane 4 Purified LF5B1 insert cut with *Msp* I. The 5 fragments are arrowed.

The figures on the left refer to the size of the fragments, in bp, of the kb ladder. The numbers on the right refer to the sizes, in bp of the DNA fragments from LF5B1 after digestion with *Msp* I.

1 2 3 4 5

12,200 →

3,050 →

2,030 →

1,600 →

1,000 →

517 →

506 →

390 →

340 →

290 →

220 →

200 →

150 →

130 →

75 →

← 377

← 270

← 174

← 124

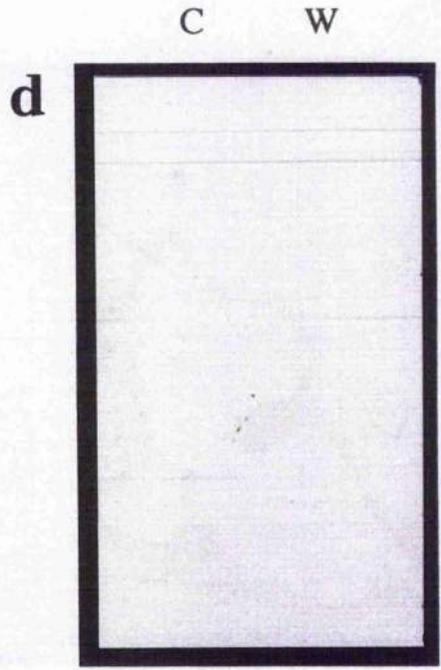
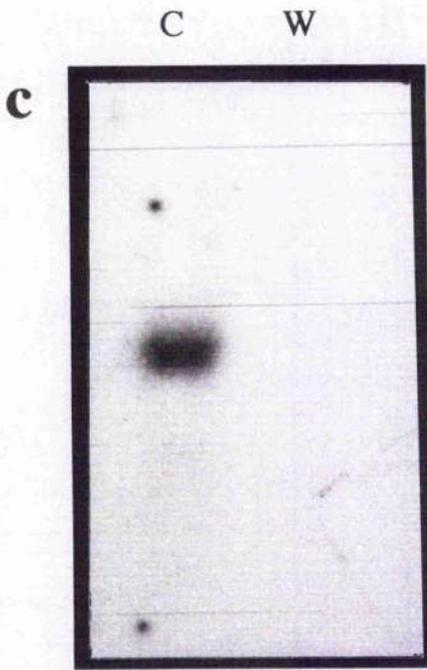
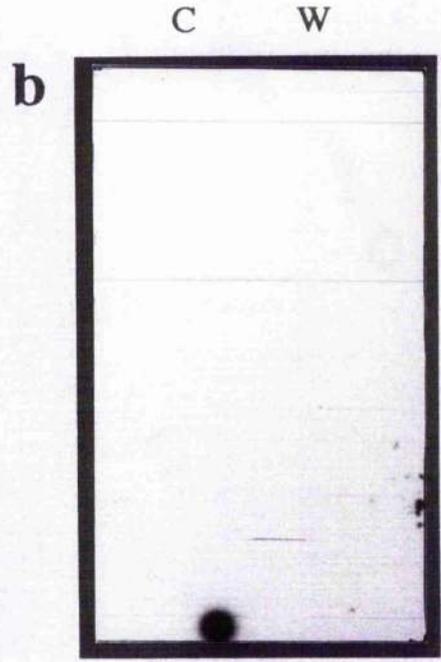
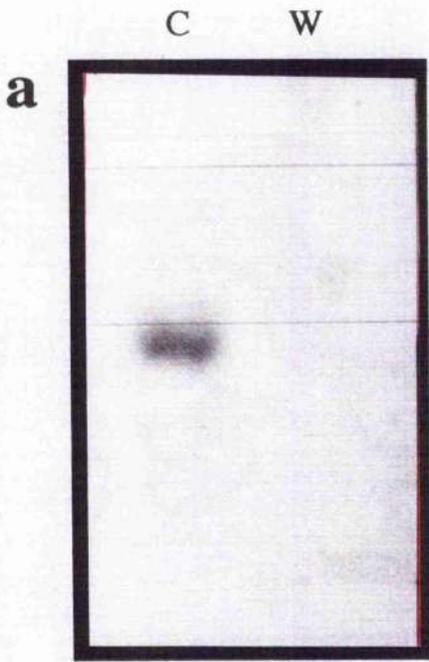
← 30



Figure 3.4 Northern blots of RNA isolated from control and cold treated *B.napus* plants probed with fragments of LF5B1, generated with *Msp* I

15 µg samples of total leaf RNA from plants cold treated for 24 h (C) and control plants grown at 22°C (W) were run on a denaturing agarose gel. Replicate blots were made and each was hybridised with one of the *Msp* I generated fragments from LF5B1 labelled to similar specific activity (see Section 2.16.1). **a** Probed with the 377 bp fragment. **b** Probed with the 270 bp fragment. **c** probed with the 174 bp fragment. **d** probed with the 124 bp fragment.

The blots were autoradiographed for 16 hours.



Uni-ZAP™ XR vector using the ZAP-cDNA® synthesis kit (Stratagene) from poly(A)⁺ RNA isolated from cold treated plants. The Uni-ZAP™ XR vector allowed the inserts to be directionally cloned and *in vivo* excision of the cDNA from the lambda clone.

3.5.1 Isolation of poly(A)⁺ RNA

The RNA was isolated from leaf tissue of plants that had been cold treated for 24 hours. This treatment is sufficient to induce maximal levels of *LF5B1* transcript (Pallas, 1992). The poly(A)⁺ RNA was separated from the total RNA using oligo(dT) cellulose. The isolated poly(A)⁺ RNA was analysed before the cDNA library was constructed. Aliquots of poly(A)⁺ RNA were separated on an agarose gel, samples of total leaf RNA from cold treated plants and control plants grown at 22°C were run on the gel as controls. RNA fractions that were washed off the oligo(dT) cellulose columns before the poly(A)⁺ RNA was eluted were also analysed to ensure that minimal amounts of the message of interest were being lost. The northern blot (Fig. 3.5) of total and poly(A)⁺ RNA, probed with the 174 bp fragment of *LF5B1* showed that the cold-induced transcripts were enriched in the poly(A)⁺ RNA fraction and present at relatively high abundance. It also showed that the transcripts were generally not degraded. A signal was detected in the RNA isolated from plants grown continually at 22°C, although at a much lower level than in cold-treated plants. This will be discussed further in Chapter 5.

The RNA appeared to be of sufficient quality to proceed with the library construction. Further checks often carried out on the RNA, including testing the ability of the RNA to produce high molecular weight products in an *in vitro* translation system (Sambrook *et al.*, 1989), were not considered necessary as we were interested specifically in the *LF5B1* transcripts which we had detected in the poly(A)⁺ RNA.

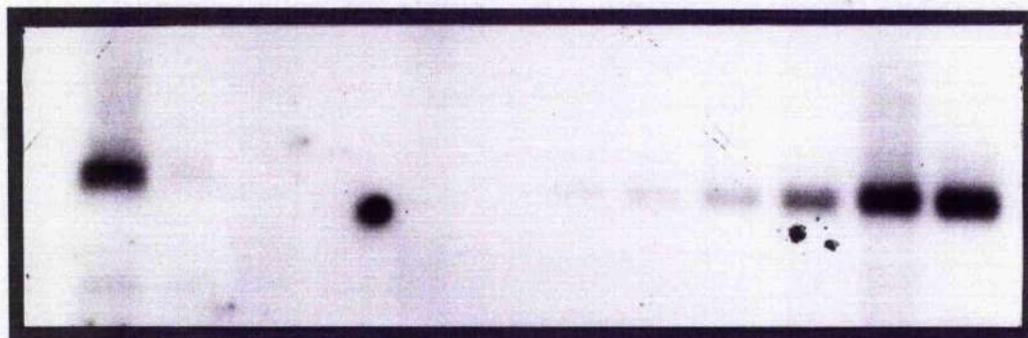
Figure 3.5 A northern blot detecting cold-induced transcripts in the poly(A)⁺ RNA population

Poly(A)⁺ RNA was isolated both from plants that were cold treated (4°C) and plants grown at 22°C. A northern blot of total and poly(A)⁺ RNA was probed with the 174 bp fragment generated by digestion of LF5B1 with *MspI*. RNA fractions retrieved from the washes of the oligo(dT) column were monitored to ensure minimal amounts of the cold-induced transcripts were being lost during the washes.

The blots were autoradiographed for 16 hours.

- Lane 1 15 µg total RNA from cold treated plants
- Lane 2 15 µg total RNA from plants grown at 22°C
- Lane 3 15 µg RNA from cold treated plants from the first wash of the oligo(dT) cellulose column
- Lane 4 15 µg RNA from plants grown at 22°C from the first wash of the oligo(dT) cellulose column
- Lane 5 15 µg RNA from cold treated plants from the second wash of the oligo(dT) cellulose column
- Lane 6 15 µg RNA from plants grown at 22°C from the second wash of the oligo(dT) cellulose column
- Lane 7 0.15 µg poly(A)⁺ RNA from plants grown at 22°C
- Lane 8 0.45 µg poly(A)⁺ RNA from plants grown at 22°C
- Lane 9 1.5 µg poly(A)⁺ RNA from plants grown at 22°C
- Lane 10 0.15 µg poly(A)⁺ RNA from cold treated plants
- Lane 11 0.45 µg poly(A)⁺ RNA from cold treated plants
- Lane 12 1.5 µg poly(A)⁺ RNA from cold treated plants

1 2 3 4 5 6 7 8 9 10 11 12



3.5.2 cDNA production, cloning, and amplification

The cold-induced transcripts were in the poly(A)⁺ RNA fraction. The cDNA library was constructed using 5 µg of poly(A)⁺ RNA. [α -³²P]dATP was added to the first and second strand synthesis reactions which allowed the efficiency of each stage and the length of the first and second strand to be monitored (Fig. 3.6). The first strand synthesis was 24% efficient, the second strand synthesis 20% efficient; these values fall between the values expected (15-30%) (Sambrook *et al.*, 1989). The length of the cDNA synthesised ranged between 400 and 4,000 bp, with the majority of the products sized between 500 and 1,500 bp. This is in the expected range, although the size of 4,000 bp as the largest products from the first and second strand synthesis is slightly low, values of 5,000 bp are usually being observed (Sambrook *et al.*, 1989). This was not considered to be a large enough discrepancy to necessitate the first and second strand synthesis being repeated.

After the first and second strand synthesis and addition of the primers, the cDNA was size fractionated using Sephacryl[®] S-400 spin columns to remove any cDNAs under 400 bp. Before ligation into the vector the cDNA was precipitated and quantified. A total of approximately 700 ng of cDNA was produced, 100 ng was ligated with the vector arms. The cDNA was cloned directionally into the vector Uni-ZAP[™] XR. The cDNA was packaged using the Gigapack[®] II Gold packaging extract (Stratagene). A control positive packaging using wild type lambda DNA (c1857 Sam7) was used to monitor the efficiency of the packaging extract. This produced 370 plaques when 0.2 µg of the DNA was packaged and 10 µl of the 10⁻⁴ dilution was plated out. This is close to the 400 plaques predicted in the manufactures instructions, illustrating that the packaging extracts were working well. The primary library was plated out in the presence of IPTG and X-Gal which allowed blue/white colour selection. The white recombinant clones were approximately 80 fold above the background blue plaques. This was within the limits of 10 to 100 fold above

Figure 3.6 First and second strand cDNA synthesis

Aliquots from the first and second strand synthesis reactions were separated on a 1% alkaline agarose gel, which was subsequently dried and autoradiographed overnight.

Lane 1 + 4 End labelled λ DNA/*Hind* III marker
Lane 2 First strand synthesis
Lane 3 Second strand synthesis

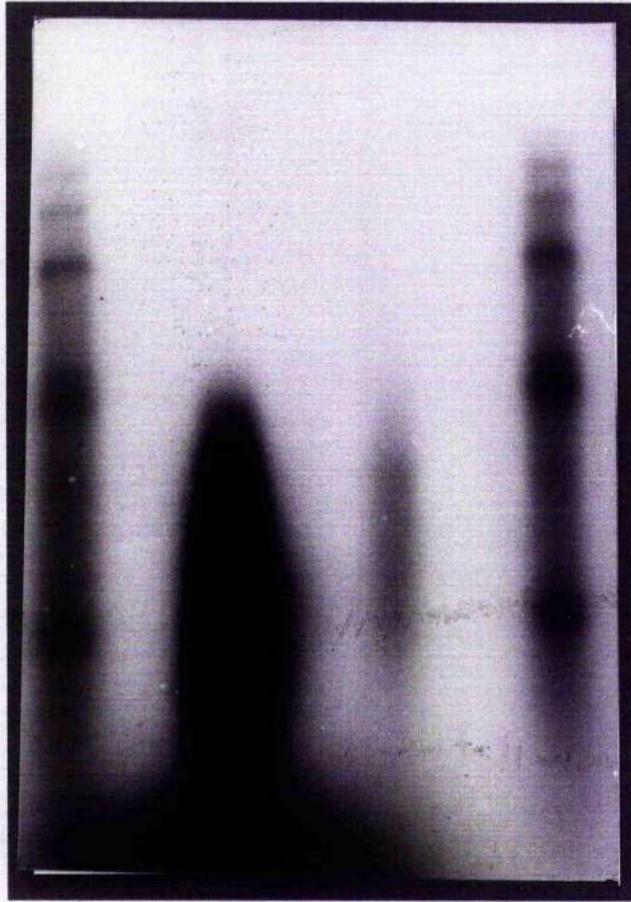
The size markers, indicated in bp, were end labelled fragments of Lambda DNA produced by digestion with *Hind* III.

1

2

3

4



← 9,416

← 6,557

← 4,361

← 2,322

← 2,027

← 564

background expected (Stratagene). The primary library was unstable and to counter this the library was amplified once. After amplification the titer of the library was calculated to be 0.5×10^9 pfu·ml⁻¹. Ten clones were picked at random and placed in a PCR reaction, using the T3 and T7 primers to amplify any insert the clones contained. The products were analysed on an agarose gel (results not shown). The insert sizes varied from 500 bp to 2 kb, which illustrated that the library contained clones sufficiently large to contain a full length LF5B1 clone.

3.6 Screening of the cDNA library

The library was screened using the 174 bp fragment generated by digesting the cDNA LF5B1 with *Msp*I. This fragment was chosen as it was from within the uninterrupted reading frame of LF5B1 and it hybridises to the cold-induced transcripts. The northern blots (Fig. 3.5) show that the transcripts hybridising to the 174 bp fragment of LF5B1 were represented at a moderate level in the poly(A)⁺ RNA population. It was estimated that the screening of 500,000 clones should produce several positive clones. Approximately 500,000 clones were screened, 50,000 were grown on each of 10 large (150 mm) plates and duplicate plaque lifts were taken from these plates. Hybridisation to the 174 bp fragment produced 5-10 putative positives from each plate. Ten of the putative positives were plaque purified. The insert was then sized using PCR. A plaque was picked and placed directly into a PCR reaction. The T7 and T3 primers were used to amplify the multicloning region and any inserts contained within it. The amplified inserts were analysed on an agarose gel, Southern blotted and probed with the 174 bp fragment of LF5B1 to confirm that they were positive clones (Fig. 3.7). The size of the PCR products is 130 bp more than the size of the corresponding insert as 130 bp of the multicloning region is also amplified by the T3 and T7 primers. The largest of the inserts, cloned (clone 6) was approximately 1 kb. This is not a full length cDNA, it is approximately 400 bp shorter than the 1.5 kb

Figure 3.7 Southern blot analysis of inserts from putative positive cDNA clones

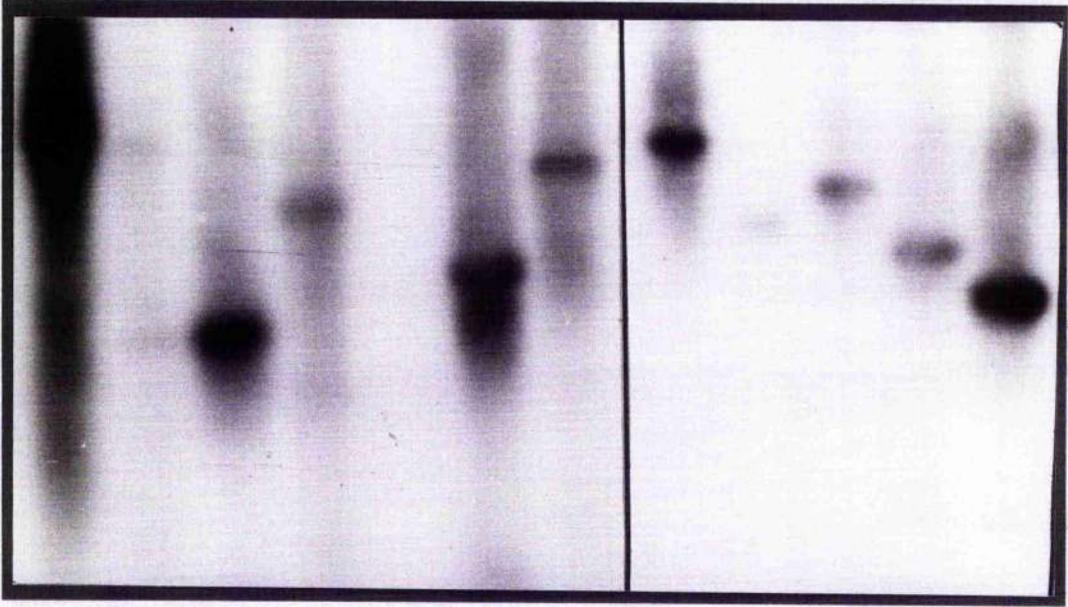
The inserts from putative positive clones were amplified by PCR, using the primers T3 and T7 which flank the multicloning region of the Uni-ZAP™ XR vector. The pBluescript® II SK (-) vector with the LF5B1 insert and with no insert were amplified as controls. The PCR products were separated on 1.5% agarose gels. The gels were Southern blotted and probed with the 174 bp fragment, generated by digestion of LF5B1 with *MspI*.

The blots were autoradiographed for 16 hours.

Lane 1	Amplified LF5B1 insert in pBluescript® II SK (-)
Lane 2	Amplified multicloning region of pBluescript® II SK (-)
Lane 3	Amplified insert of putative clone 1
Lane 4	Amplified insert of putative clone 2
Lane 5	Amplified insert of putative clone 3
Lane 6	Amplified insert of putative clone 4
Lane 7	Amplified insert of putative clone 5
Lane 8	Amplified insert of putative clone 6
Lane 9	Amplified insert of putative clone 7
Lane 10	Amplified insert of putative clone 8
Lane 11	Amplified insert of putative clone 9
Lane 12	Amplified insert of putative clone 10

The figures indicate the size range of the bands and are in bp; they were calculated from the 1 kb ladder DNA that was also on the agarose gel.

1 2 3 4 5 6 7 8 9 10 11 12



mRNA that the 174 bp fragment hybridises to. It was decided not to screen any further cDNAs to try to find a full length cDNA but subsequently to isolate a corresponding genomic clone.

This cDNA should establish that we have isolated a cDNA that is induced in response to cold temperatures, showing which sequences from LF5B1 were hybridising to the cold-induced transcripts. A genomic clone should enable us to examine the 5' region of the gene, which will include the missing 5' region from the cDNA.

3.7 Sequencing of the cDNA clone 6

The cDNA was cloned into the *Xho* I and *Eco*R I sites of the Bluescript[®] II SK (-) phagemid. This allowed sequencing of part of the cDNA using the T3 and T7 primers (Table 3.1a). The cDNA also contained a *Sal* I site at position 557 to 562. This allowed the two fragments to be subcloned into pBluescript[®] II SK (-) allowing additional amounts of the cDNA to be sequenced with the T3 and T7 primers. To sequence the cDNA on both strands synthetic oligonucleotides were made to portions of known sequence. One of these oligonucleotides (PR1) enabled the cDNA to be sequenced across the *Sal* I site; this showed that there was only one *Sal* I site in this region and that a small fragment of the cDNA had not been lost during subcloning (sequencing using deletion 10 also showed this). On one strand it was not possible to use oligonucleotide primers as the DNA contained repeated sequence motifs at the 3' end and primers to this region annealed to the template at more than one position. Two deletions were produced using *Exo* III nuclease and mung bean nuclease which allowed this region to be sequenced. The deletions were produced in an *Eco*R I to *Xho* I direction. An *Eco*R I digest left 5' overhanging ends allowing *Exo* III to digest the clone. The plasmid was protected by digestion with *Sac* I which produced a 3'

overhanging end. The deletions' sizes were again estimated using PCR across the multicloning region. The deletions are described in Table 3.1b, the sequencing of the cDNA is represented schematically in Fig. 3.8.

3.8 Sequence analysis of the cDNA clone 6

The cDNA was sequenced using the Sequenase[®] II enzyme. The sequence is shown in Fig. 3.9. The cDNA was 842 bp long (excluding the primers used in its construction and the poly(A) tail). As was expected from the size of the cDNA compared to the size of the transcripts that it hybridises to, the cDNA was truncated. The sequence when translated revealed an unbroken reading frame of 212 amino acids. This again was not a true open reading frame as it does not start with a methionine residue. The cDNA was named BnPRP, this is based on the name of the plant, *Brassica napus*, that it was isolated from and the fact that part of the translated sequence is rich in proline, such proteins are called proline-rich proteins. The amino acid sequence will be analysed further in Chapter 4.

Three prime of the putative coding sequence there are 205 bp of untranslated sequence. A consensus poly(A) signal AATAAA (Joshi, 1987b) is present 27 bp upstream of the first adenine of the poly(A) tail.

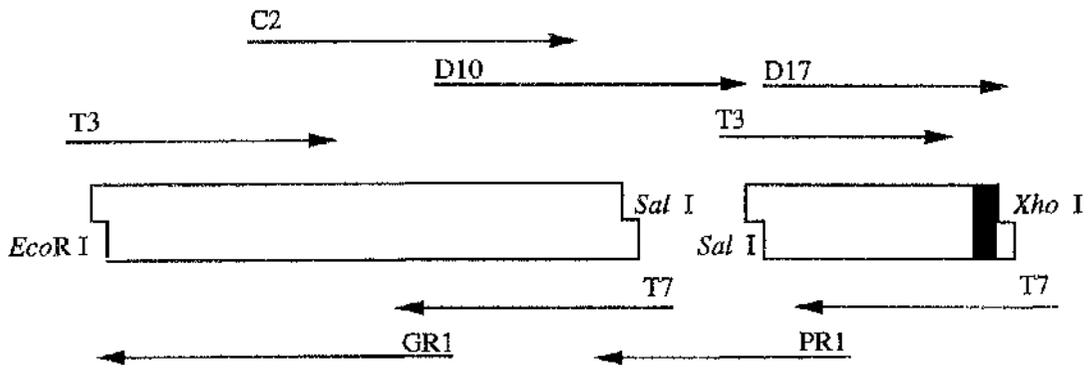
3.9 Comparison of LF5B1 with BnPRP

Fig. 3.10 shows alignments of the nucleotide sequences of LF5B1 and BnPRP. The sequences are broken down into translated (Fig. 3.10a) and untranslated regions (Fig. 3.10b). The putative amino acid sequences from the putative coding regions are compared in Fig. 3.11. The LF5B1 and BnPRP cDNAs are not identical. The cDNA

Figure 3.8 Schematic representation of the sequencing of the cDNA clone 6

The cDNA is represented by the rectangles. The restriction enzymes bordering the cDNA fragments are shown, the internal *Sal* I site in cDNA is also shown as this was used in the sequencing strategy.

The lines correspond to the sequence information obtained from the oligonucleotide primer or deletion. The name of the oligonucleotide primer or deletion is shown above the line. The arrow shows the direction that the sequencing reaction proceeded. Information on the oligonucleotide primers and deletions is shown in Table 3.1.



0 100 200 300
 nucleotides / base pairs

 poly(A)

Figure 3.9 Sequence of cDNA BnPRP

The nucleotide sequence of the cDNA BnPRP and deduced amino acid sequence of BnPRP are shown. The first 13 nucleotides (AATTCGGCACGAG) representing the primer used in the cDNA synthesis are not shown in the sequence. The numbers refer to the nucleotide and amino acids at the end of each line. * indicates the termination codon (TGA), the putative polyadenylation site (AATAAA) is indicated by underlining. The poly(A) tail of the cDNA is indicated by (A)_n.

CAAGCCACCAACAGTGTGCCACCGCCAACACCAACACCAACCCACCTGTGTGTAACGCC	60
K P P T V C P P P T P T P T P P V V T P	20
ACCAACACCACCAACACCAACTCCACCAGTCGTTACGCCACCAACACCAGCCCCACCTGT	120
C T P P T P T P P V V T P P T P A P P V	40
TGTAACGCCACCAACACCAACACCACCGGTCGTAACGCCACCGACACCAACCCCTCCTGT	180
V T P P T P T P P V V T P P T P T P P V	60
CGTAACACCTCCAACACCACCGGTCGTAACGCCACCGACACCAACTCCTCCTGTGTAAC	240
V T P P T P P V V T P P T P T P P V V T	80
ACCACCAACACCACCGGTCGTAACGCCACCGACACCAACCCCTCCGTGTAACACCTCC	300
P P T P P V V T P P T P T P P V V T P P	100
AACACCACCGGTCGTAACGCCACCGACACCAACCCCTCCTGTGTAACACCACCAACACC	360
T P P V V T P P T P T P P V V T P P T P	120
AACTCCACCTAAGCCAGAAACGTGCCCAATCGACACGTTGAAGCTAGGCGCTTGTGTAGA	420
T P P K P E T C P I D T L K L G A C V D	140
CGTTCTTGGAGGTTTAATTCACATCGGGCTTGGTGGAAAGTAGCGCCAAGAAAGAGTGTG	480
V L G G L I H I G L G G S S A K K E C C	160
TCCGGTMTTGGGAGGCTTAGTTGACTTAGACGCAGCTGTTGCTATGTACCACCATCAA	540
P V L G G L V D L D A A V C L C T T I K	180
AGCCAAACTTCTCATCGTCGACCTTATTATCCCCATGCTCTTGAGCTTCTTATCGACTG	600
A K L L I V D L I I P I A L E L L I D C	200
TGGAAAGACTCCGCCACCTGGCTTCAAATGTCCCTCTTGATGAATGAAGTGTCTCTAT	660
G K T P P P G F K C P S *	212
CGTGTMTTCTTTCTTTCTTTCAAGAATTTGTTAGTTTGATAATCTCTTGGGTGAGGTTA	720
ATTTGCGTGTGTGAGACCTTTAATCACTTGTMTTGTGTFACAATAAAAGGCTATGTTTGG	780
GTTTGTGATGTAATTCTAAGAAGAAGAAATAAAAAATGTAAAACGGTATAAATATCTT	840
TC (A)n	842

Figure 3.10 Comparison of the LF5B1 and BnPRP nucleotide sequences

The nucleotide sequences of LF5B1 and BnPRP shown in Figs. 3.2 and 3.9 respectively were aligned using the UWGCG programme GAP. This also calculates the percentage identity between the sequences which is shown above the sequence. The figures at the beginning and end of the lines correspond to the nucleotide number designated in Figs. 3.2 and 3.9. Identical nucleotides are represented by a vertical line, gaps introduced into the sequence are represented by a dot in the sequence.

a Shows the alignment between the nucleotide sequences of the putative coding regions of LF5B1 and BnPRP. The BnPRP sequence is the upper sequence.

b Shows the alignment between the nucleotide sequences of the putative untranslated regions of LF5B1 and BnPRP. The BnPRP sequence is the upper sequence. The last six nucleotides of the reading frame of LF5B1 are included in the untranslated alignment due to the similarity with the first six nucleotides of the BnPRP untranslated sequence.

Figure 3.11 Comparison of the putative amino acid sequences of LF5B1 and
BNPRP

The putative reading frames deduced from the nucleotide sequences of LF5B1 and BnPRP were translated using the UWGCG programme MAP (Figs. 3.2 and 3.9).

These sequences were aligned using the UWGCG programme GAP. This also calculated the degree of sequence identity and sequence similarity.

The upper sequence represents BNPRP and the lower sequence represents LF5B1. The numbers correspond to the amino acids at the beginning and the end of each line, designated according to Figs. 3.2 and 3.9. A vertical line represents sequence identity, a double dot represents a highly conserved substitution, a single dot represents a conserved substitution.

Sequence identity = 94.8% Sequence similarity = 95.7%

Gaps inserted = 0

97	VIPPTPPVVTPPTTPPPVVTPTPTPPKPE _{TC} PID _{TL} KL _{GAC} VD _{VL} GGLI	146
	.	
21	VIPPTPPVVTPPTTPPPVVTPTPTPPKPE _{TC} PID _{TL} KL _{GAC} VD _{VL} GGLI	70
	.	
147	HIGLGGSSAKKECCPVLGGLVDLDAAVCLCTT _{IK} AKLLI _{VD} LI _{IPI} ALEL	196
	:	
71	HIGLGGSSAKKECCPVLGGLVDLDAAVCLCTT _{IK} AKLLN _{LD} LI _{IPI} ALEL	120
197	LIDCGKTPPPGFKCPS	212
	. .	
121	LIDCGKTPPPGFNVPLNE	138
	. .	

BnPRP has sequence similarity to the 174 bp and 377 bp *Msp* I fragments of LF5B1. This is consistent with the data from the northern blots (Fig. 3.4). In Figures 3.10 and 3.11 the sequence of LF5B1 is shown from the point that it starts to match the sequence of BnPRP. The sequences were shown to be extremely similar. The coding regions were 98.27% identical in nucleotide sequence. The 174 bp fragment of LF5B1 used to isolate BnPRP was 98.6% identical with the corresponding sequence in BnPRP. Three of the six substitutions cause changes in the putative amino acid sequence. The two translated proteins LF5B1 and BnPRP are 94.8% identical, 95.7% similar. The single nucleotide deletion in the LF5B1 sequence is manifested in the amino acid alignment, three of the last four residues are non-identical and the translated sequence from LF5B1 is two residues longer than the sequence from BnPRP.

The untranslated sequences were also non-identical, but the two sequences are 98.8% identical between the nucleotides present in both untranslated regions. Along with two mismatches an interesting feature is the extra 45 bp present in BnPRP. Furthermore, the LF5B1 sequence has an additional 22 bp at the 3' end of the sequence.

3.10 Discussion

The sequencing and northern blot analysis revealed that the cDNA LF5B1 was a chimaeric cDNA. It appeared to be made-up of at least three distinct pieces of DNA. The only sequences that hybridised to the cold-induced transcript were located internally within the clone, flanked by DNA that was not related. This suggested that no other sequences related to the cold-induced transcript would be contained within the original, larger cDNA LF5 that LF5B1 was derived from. These findings were consistent with previous data showing that none of the other DNA fragments generated

from the cDNA LF5 by digestion with *Bam*H I hybridised to cold-induced transcripts (Pallas, 1992).

By hybridisation to a *Msp* I fragment derived from LF5B1 several cDNAs were isolated from a 'cold-induced' cDNA library constructed in the UNI-ZAP™ XR vector. None of these represented a full length cDNA, the longest was 842 bp long. Judging from the transcript size it was approximately 500 bp short of being full length. It is not clear why we were unable to isolate a full length clone. One possibility is that the first and second strand synthesis did not produce sufficiently long products. This would be more plausible if the desired cDNA had been longer. The first and second strand synthesis did produce products greater than 1.5 kb. Clones of 2 kb (the size was estimated by PCR) were randomly isolated from the library (results not shown) which also suggested that sufficiently long clones were present in the library. Another possible reason for not obtaining a full length clone is the low efficiency that DNA polymerase transcribed the DNA *in vitro*. This is evident from the sequencing of the *BnPRP* genomic clone in Chapter 4. In the region of genomic clone with repeated DNA sequence the DNA polymerase was prone to pausing at several sites, this was visualised as bands in all four lanes on the sequencing gel. Very low amounts of sequence products could be extended beyond these pause sites. If the DNA polymerase used during the second strand synthesis also paused at several sites it would prevent many extensions that were initiated 5' of the pausing sites proceeding past this region, which would lead to a very low abundance of full length *BnPRP* clones in the library.

The LF5B1 and *BnPRP* clones contained 198 and 205 bp untranslated regions respectively. These untranslated sequences were analysed, looking for commonly found sequence motifs. Both the LF5B1 and *BnPRP* clones contain the hexanucleotide AATAAA. This is an almost ubiquitous signal for polyadenylation in animal and viral pre-mRNAs (Proudfoot and Brownlee, 1976) and has been shown to act as the polyadenylation sequence in some plant genes (Dhaese *et al.*, 1983; Joshi, 1987b). It

may well act as the polyadenylation signal for the two transcripts. The positioning of the AAUAAA in relation to the poly(A) tail varies considerably between the two transcripts. In the *LF5B1* transcript it is 52 nt from the poly(A) tail while in the *BnPRP* transcript it is 27 nt from the poly(A) tail. The positioning of the AATAAA motif in plant genes is usually between 9 and 27 nt from the point where the poly(A) tail is added to the transcript (Joshi, 1987b). The larger distance present in *LF5B1* could be caused by miss identifying the end of the transcript. It has been established that this cDNA is chimaeric, and it is not known whether the poly(A) tail is part of this cDNA or a part of one of the other pieces of DNA in the clone. It is possible that the AAUAAA motif is not the poly(A) signal for one or both of the transcripts. It has been shown that this motif is less conserved in plant mRNA than animal and virus mRNA (Joshi, 1987b), with 61% of the plant genes examined varying in at least one position from the consensus sequence. The majority of these (90%) only vary in one position. In the *LF5B1* transcript the AACAAA sequence 17 nt upstream of the poly(A) tail or the AACAAAG sequence 11 nt upstream of the poly(A) tail both vary from the consensus sequence by only one and two nucleotides respectively and could act as polyadenylation signals. It is possible that one or both of the transcripts are polyadenylated at more than one site, using different polyadenylation signals; this has been observed in a number of plant genes (Dean *et al.*, 1986; Montoliu *et al.*, 1990). The *LF5B1* and *BnPRP* transcripts both have untranslated regions that are rich in adenine and uracil (69 and 70% respectively). This is a feature commonly observed in transcript's untranslated regions (Joshi, 1987b).

Comparisons of the sequences revealed that the corresponding regions of *LF5B1* and *BnPRP* were not identical, but contained several substitutions and an insertion. This applied at the nucleotide and amino acid levels. The sequences were obviously very closely related, displaying over 98% and 94% identity at the nucleotide and amino acid levels respectively. The most likely explanation is that they represent two members of a gene family. Southern blots of *Brassica napus* DNA digested with

several different restriction enzymes probed with the LF5B1 cDNA insert showed the presence of 4-6 hybridising fragments in the genome (Pallas, 1992). These may represent a small gene family encoding several highly related genes. However, some of the bands observed by Pallas (1992) may be hybridising to the other sequences in LF5B1, that do not hybridise to the cold-induced transcript. The Southern blot was not repeated with the 174 bp fragment from LF5B1 or the BnPRP cDNA. The presence of the two highly similar cDNAs indicates that there are at least two members in the gene family. Gene families have been reported for both cold-induced genes (Dunn *et al.*, 1991; Dunn *et al.*, 1993; Wilhelm and Thomashow, 1993; Jarillo *et al.*, 1994; Kurkela and Borg-Franck, 1992; Zhang *et al.*, 1993) and genes encoding proline-rich proteins (reviewed in Josè and Puigdomènech, 1993).

The putative untranslated sequence is as conserved between the two cDNAs as the putative coding sequences, except for the regions of additional sequence present in both LF5B1 and BnPRP. This is surprising as the untranslated regions of related genes usually show much greater divergence. The conservative forces are not usually as great on the sequences of non-coding regions because changes do not result in changes in the amino acid sequence. Where two members of a cold-induced gene family have been cloned the 3' untranslated sequence shows greater divergence than the putative coding regions (Kurkela and Borg-Franck, 1992; Jarillo *et al.*, 1994). The lack of divergence in the untranslated region has been observed in other genes; for example, four members of the extensin gene family isolated from *Brassica napus* show greater than 99% homology in the untranslated region (Evans *et al.*, 1990). This is proposed to be due to the relatively recent duplication of the genes, and may be the reason that the untranslated regions of LF5B1 and BnPRP do not show greater divergence.

The two regions of additional 3' untranslated sequence, one of 45 bp in BnPRP and one of 22 bp in LF5B1, are particularly interesting. The significance, if any, of

these additional sequences is not understood. The sequence was investigated to see if the additional sequence in the BnPRP cDNA could be an unprocessed intron. No GT-AG motifs that characterise intron boundaries (Brown, 1986) were present at the beginning and the end (GT and AG respectively) of the additional sequence, indicating that it was an integral part of the *BnPRP* transcripts. This also applies to the additional sequence in LF5B1.

Both of the sequences (nucleotide and amino acid) were placed into searches of the EMBL and GenBank data bases. The results of these are the same as for the *BnPRP* genomic clone and are discussed in Chapter 4.

Chapter 4: Isolation and characterisation of the genomic clone λ BnPRP

4.1 Introduction

If a full length genomic clone could be isolated the entire ORF, and hence the full length putative protein could be identified and analysed. In addition, if a sufficiently large genomic clone was identified it may contain the gene's promoter. The isolation of the promoter may allow the identification of regulatory sequence motifs through functional studies and comparison with other cold regulated promoters. It may also be possible to identify tissue specific regulatory motifs. In addition, by making a promoter-GUS fusion the regulation of the gene could be looked at in more detail. A *Brassica napus* genomic library was therefore screened at the same time as the 'cold-induced' cDNA library.

4.2 Screening of a *Brassica napus* genomic library

A genomic library constructed in the EMBL3 bacteriophage λ vector, purchased from Clontech and provided by Dr A. Greenland (Zeneca seeds, Bracknell, UK) was used to screen for genomic clones. The library was constructed by ligating genomic DNA from *B. napus* cv. Bridger into the arms of the EMBL3 vector. The high molecular weight *B. napus* DNA was partially digested with the restriction enzyme *Sau3A* I to produce smaller overlapping fragments. The ends on the DNA fragments were compatible with the ends of the vector arms after the stuffer fragment had been removed by complete digestion with the restriction enzyme *BamH* I. The DNA was ligated into the vector arms and packaged. The library contained 2.5×10^6

independent recombinant clones. The inserts in the clones varied between 8 and 31 kb, the average length of the inserts was 15 kb.

The library was screened using as a probe the 174 bp fragment generated by the digestion of the cDNA LF5B1 with *Msp* I. A total of approximately 1,000,000 clones were screened. This was more than the necessary 3.48×10^5 to 3.79×10^5 recombinants required to represent 99% of all DNA sequences in the genome (Clarke and Carbon, 1976). This is the number of clones usually screened in order to maximise the chances of isolating the sequence of interest (Sambrook *et al.*, 1989). More clones than necessary were screened in an attempt to isolate more than one clone and to increase the chances of isolating a full length clone together with the promoter sequence. It also allowed for any under estimation of the genome size.

The number of clones that would represent 99% of all DNA sequences was estimated by the equation:

$$N = \frac{\ln(1 - P)}{\ln(1 - f)}$$

where N is the necessary number of recombinants, P is the desired probability (i.e. 99%), and f is the proportion of the genome in a single recombinant (Clarke and Carbon, 1976). The average size of the inserts in the recombinants was 15 kb. The approximate genome size of *B. napus* is 1,129 to 1,235 Mbp (1C) (Arumuganathan K. and Earle E.D., 1991).

$$N = \frac{\ln(1-0.99)}{\ln\left(1 - \left[\frac{1. \times 10^4}{1.129 \text{ to } 1.235 \times 10^9}\right]\right)} = 3.48 \text{ to } 3.78 \times 10^5$$

4.3 Isolation of genomic clones

Plaque lifts were taken from 20 large (150 mm) plates containing approximately 50,000 clones each. Approximately 130 putative positives were identified from the primary screen. Of the 130 putative positives, plaques from 20 were isolated, and plated out at a lower density than in the primary screen (approximately 200 plaques on 70 mm plates), giving mainly individual plaques. These were rescreened using the 174 bp fragment of LF5B1 generated by *Msp* I. Individual plaques that were identified as positives were picked off the plate. A tertiary screen on the plaques from the secondary screen was carried out to ensure the plaques contained only one recombinant; any that did not were screened repeatedly until they all contained only one recombinant. Of the 20 putative positives that were plaque purified seven proved to be true positives, repeatedly hybridising with the probe.

4.4 Restriction enzyme and Southern analysis of the isolated genomic clones

The seven clones that had been plaque purified were analysed by restriction digestion together with Southern blotting. Phage from the seven clones were amplified from individual plaques and DNA was isolated. The cloned DNA in the EMBL3 vector is flanked by *EcoR* I and *Sal* I restriction enzyme sites. These enzymes were used to digest the cloned DNA. These enzymes removed the vector arms in all the digests, this made the analysis of the resulting DNA fragments less complicated. *BamH* I digests were also used in the analysis of the clones. In constructing the genomic library genomic DNA was digested with *Sau3A* I, the genomic DNA was then ligated into the vector arms using the *BamH* I site on the arms. The join between a *BamH* I site and *Sau3A* I sites reproduces a *BamH* I site only 25% of the time, making the *BamH* I digests harder to interpret. The DNA of all of the clones was restricted in single digests

with *EcoR* I, *Sal* I or *BamH* I; and in double digests with *EcoR* I and *Sal* I, *EcoR* I and *BamH* I, *Sal* I and *BamH* I. The DNA was also digested with *Msp* I to see if a 174 bp fragment was produced; this would suggest that the DNA was highly related to the LF5B1 DNA.

The single digests were separated on 1.2% and 0.5% agarose gels which were used to analyse small (< 3 kb) and large (> 3 kb) fragments respectively. Both gels for each digest were Southern blotted. The double digests were separated on 1.2% agarose gels before Southern blotting. The Southern blots were probed using the 174 bp fragment of the cDNA LF5B1 generated by digestion with *Msp* I. The Southern blots from the 1.2% agarose gels revealed no small fragments, (<2 kb), and were not very informative as the DNA fragments were not separated sufficiently (results not shown). The intensity of the signal was high for the five clones, 2, 3, 4, 6, and 7; the intensity of signal from the two clones, 1 and 5 was substantially lower. This implied that clones 2, 3, 4, 6, and 7 contained DNA identical or very similar to the DNA in the 174 bp probe and that clones 1 and 5 contained DNA which was not identical to the 174 bp probe DNA.

The most informative blots were of the *Sal* I digest (Fig. 4.1a), the *EcoR* I digest (Fig. 4.1b), and the *Sal* I together with *EcoR* I digest (Fig. 4.2a). The digests using *BamH* I were not very informative as the DNA fragments were too large to separate (with the exception of clone 5 which produced a 9 kb hybridising fragment (Fig. 4.1c). Also when using *BamH* I it was not known if one of the vector arms was attached to any of the hybridising fragments. The *Sal* I digest (Fig. 4.1a) produced a fragment of approximately 10 kb that hybridised to the probe, in clones 1, 2, 3, 4, and 7; the fragments that hybridise to the probe in clones 5 and 6 are slightly larger, approximately 15 kb and 12 kb respectively (the bands appear progressively smaller due to the gel running at an angle). The fragment hybridising from clone 5 produced a very weak signal, a larger signal was detected when the blot was washed at a lower

Figure 4.1 Southern blots of phage DNA (single digests)

Seven purified phage that gave positive signals with the 174 bp probe derived from the cDNA LF5B1 were Southern blotted. One μg of DNA from each phage was digested with **a** *Sal* I, **b** *Eco*R I, **c** *Bam*H I, and **d** *Msp* I.

Prior to Southern blotting the digested DNA was separated on 0.5% TBE agarose gels, run at 10 volts for 8-10 hours. The Southern blots were probed with the 174 bp probe.

The blots **a**, **b**, and **c** were autoradiographed for 16 hours, blot **d** was autoradiographed for 8 hours.

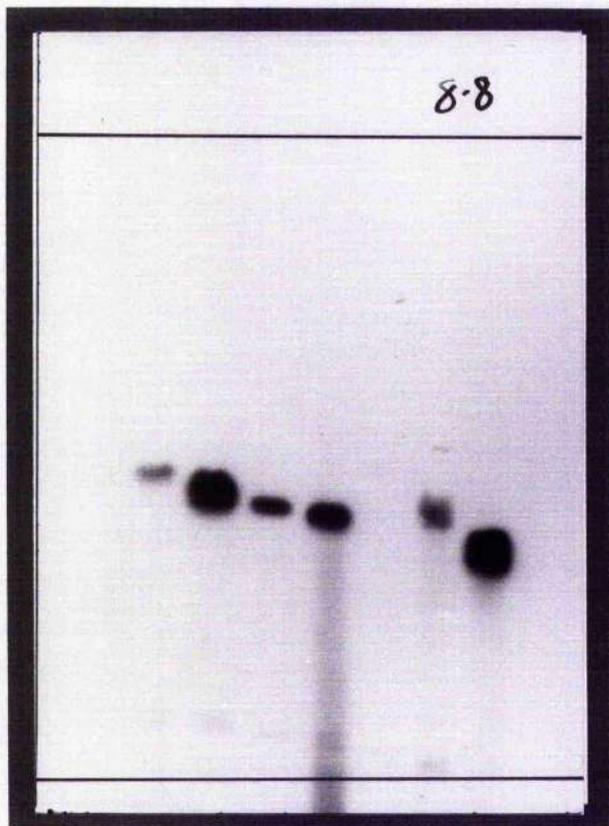
- Lane 1 One μg of DNA isolated from phage 1
- Lane 2 One μg of DNA isolated from phage 2
- Lane 3 One μg of DNA isolated from phage 3
- Lane 4 One μg of DNA isolated from phage 4
- Lane 5 One μg of DNA isolated from phage 5
- Lane 6 One μg of DNA isolated from phage 6
- Lane 7 One μg of DNA isolated from phage 7

- C 200 ng of the 174 bp probe after gel purification (Fig. 4.1d only)

The figures refer to the size of the fragments (bp), which were calculated by measuring the distance the fragments migrated in relation to the 1 kb ladder marker.

1 2 3 4 5 6 7

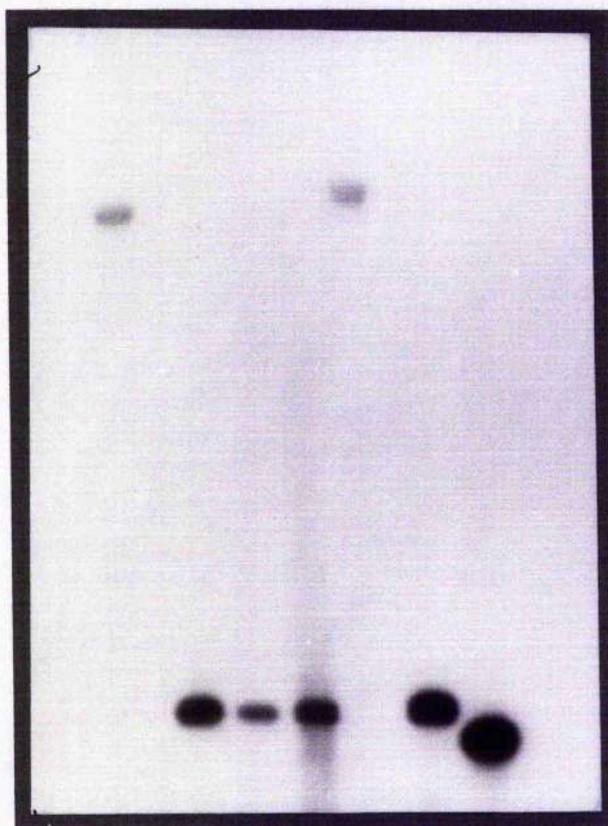
a



← 15,000
← 12,000
← 10,000

1 2 3 4 5 6 7

b

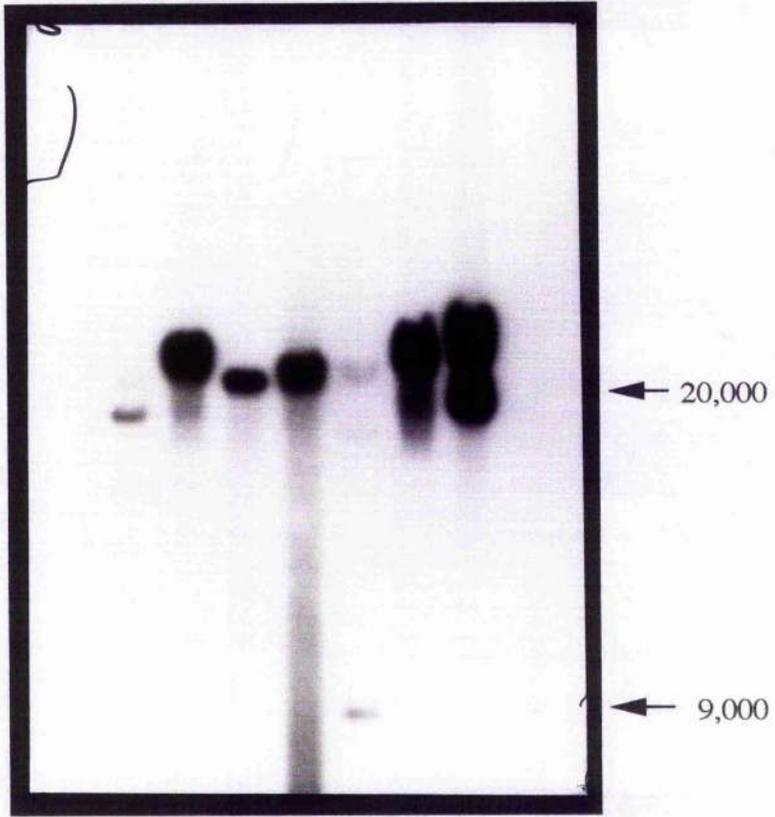


← 20,000

← 3,000
← 2,800

1 2 3 4 5 6 7

c



C 1 2 3 4 5 6 7 C

d

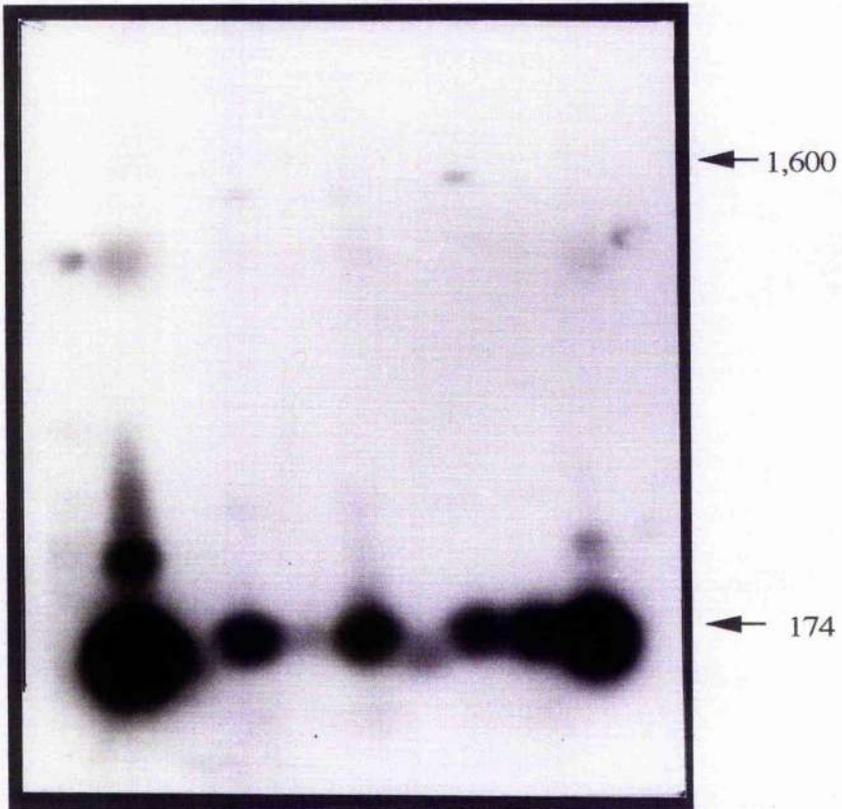


Figure 4.2 Southern blots of phage DNA (double digests)

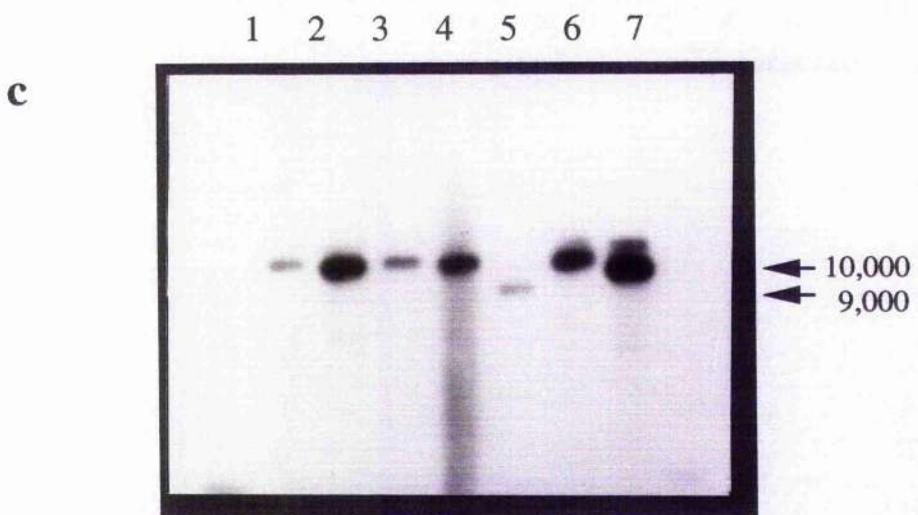
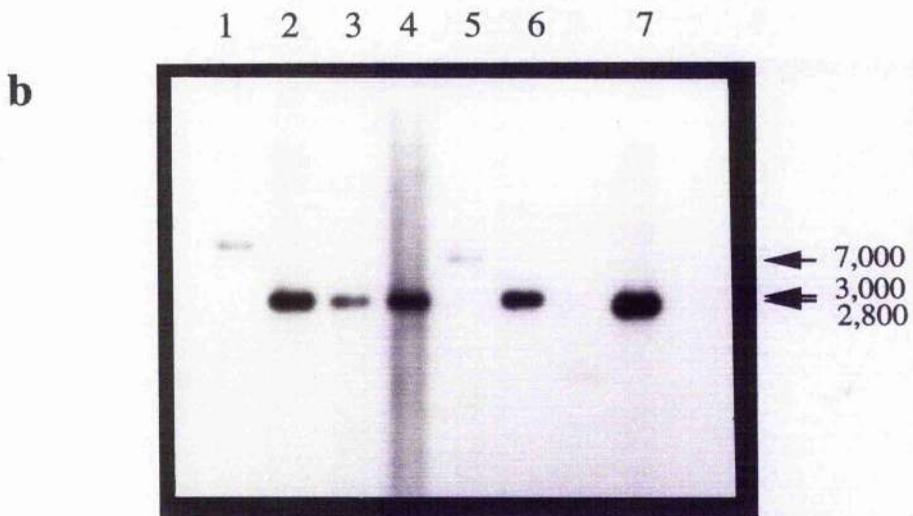
One μg of DNA isolated from each of the seven purified phage that gave positive signals with the 174 bp probe derived from the LF5B1 cDNA was digested with **a** *Sal* I and *Eco*R I, **b** *Eco*R I and *Bam*H I **c** *Bam*H I and *Sal* I.

Prior to Southern blotting the digested DNA was separated on 1.2% TBE agarose gels, run at 10 volts for 8-10 hours. The Southern blots were probed with the 174 bp probe.

The blots were autoradiographed for 16 hours.

- Lane 1 One μg of DNA isolated from phage 1
- Lane 2 One μg of DNA isolated from phage 2
- Lane 3 One μg of DNA isolated from phage 3
- Lane 4 One μg of DNA isolated from phage 4
- Lane 5 One μg of DNA isolated from phage 5
- Lane 6 One μg of DNA isolated from phage 6
- Lane 7 One μg of DNA isolated from phage 7

The figures refer to the size of the bands (bp); these were calculated by measuring the distance the fragments migrated in relation to the 1 kb ladder marker.



stringency (50°C rather than 65°C) (results not shown). The *EcoR* I digests (Fig. 4.1b) produced bands of approximately 3 kb from clones 2, 3, 4, and 6; the hybridising fragment from clone 7 is slightly smaller, approximately 2.8 kb; the hybridising fragments from clones 1 and 5 are greater than 20 kb, indicating that the inserts contain no *EcoR* I sites. The *Sal* I together with *EcoR* I digest (Fig. 4.2a) produced hybridising fragments of 2.4 kb from clones 2, 3, 4, and 6; clones 1 and 5 produced hybridising fragments of approximately 10 kb; clone 7 contains a 2.8 kb fragment, which is the same size of fragment that hybridised from the *EcoR* I digest. The double digests using *BamH* I (Figures 4.2b and 4.2c) produced fragments apparently the same size as the *EcoR* I and *Sal* I single digests with clones 2, 3, 4, 6, and 7. This suggested that there are no *BamH* I sites in the hybridising DNA fragments from these clones. In the *BamH* I together with *EcoR* I digest (Fig. 4.2b) of clones 1 and 5 the 7 kb fragment shows the presence of an internal *BamH* I site since the *EcoR* I single digest produced fragments greater than 20 kb. The 15 kb fragment from clone 5 produced with *Sal* I (Fig. 4.1a) is reduced to a 9 kb fragment (which is the same as when *BamH* I is used on its own (Fig. 4.1c)) in the *BamH* I and *Sal* I digest (Fig. 4.2c), supporting the presence of internal *BamH* I sites in both clones 1 and 5. The DNA digested with *Msp* I produced hybridising fragments of approximately 174 bp from all of the clones (Fig. 4.1d). Clone 3 as well as clones 1 and 5 gives a weak signal, which is due to less DNA being loaded onto the gel rather than weak homology to the probe.

4.5 Restriction enzyme mapping and subcloning of λ BnPRP genomic clone 2

Genomic clone 2 was chosen for further analysis. This was based on the strong signal obtained from the Southern blots indicating that the clone was identical or very similar to the probe, at least along the length of the probe. This clone also

appeared to be related to the clones 3, 4, and 6. Restriction mapping of the clone would show the position of the hybridising fragment within the clone. If this fragment was flanked by DNA on both sides it would make it suitable for further analysis, as it would be likely that it contained the whole coding region and the promoter sequence.

DNA from clone 2 was cut with the restriction enzymes *Sal* I, *Eco*R I, and in a double digest with both of the enzymes, the products of the digest were analysed on a 0.8% agarose gel (Fig. 4.3). The sizes of the large DNA fragments (> 4 kb) could not be sized accurately as the separation of large fragments is limited.

The EMBL3 vector arms can be seen in all three lanes, the highest band corresponds to the 20 kb left arm, the band at 8.8 kb corresponds to the right arm. The size of the right arm appears to be larger than 8.8 kb judging from the marker DNA (10-11 kb). All the sizes are estimated using the marker DNA, and not too much importance is attached to the sizes of the larger fragments. The *Sal* I digest produced a fragment corresponding to the right arm that appeared more intense and wider than the *Eco*R I and *Eco*R I with *Sal* I double digest. This is due to it being a doublet, one band is the vector arm, the other is a *Sal* I fragment, this fragment hybridises to the probe (Fig. 4.1a). Two other bands are present, one approximately 15 kb, the other 4 kb. The largest band from the *Eco*R I digest co-migrated with the 20 kb left arm. Four other bands were produced, a 3 kb band, which hybridises to the probe in the Southern blot (Fig. 4.1b), and bands of 1.4 kb, 1 kb, and 0.8 kb. In the double digest the 10 kb fragment from the *Sal* I digest has been reduced to an 8 kb and a 2.4 kb fragment (again illustrating the inaccuracy of the sizing of larger fragments). The probe hybridised to the 2.4 kb fragment from this digest (Fig. 4.2a) so this must originate from the 10 kb *Sal* I fragment. The *Eco*R I 3 kb fragment had been cut to produce the 2.4 kb fragment that hybridised to the probe, the 0.6 kb fragment was also produced from this cleavage. The 4 kb *Sal* I fragment had been cut to produce four fragments of 1.4 kb, 1 kb, 0.8 kb, and 0.6 kb.

Figure 4.3 Restriction digests analysis of genomic clone 2

Two μg of DNA isolated from genomic clone 2 was digested with *Sal* I, *Eco*R I, and *Eco*R I together with *Sal* I.

The digested DNA was separated on a 0.9% TBE agarose gel.

Lane 1+ 5 1 kb ladder DNA marker

Lane 2 2 μg of DNA digested with *Sal* I

Lane 3 2 μg of DNA digested with *Eco*R I

Lane 4 2 μg of DNA digested with *Sal* I + *Eco*R I

The figures refer to the size of the bands (bp); these were calculated by measuring the distance the fragments of clone 2 migrated in relation to the 1 kb ladder marker.

1 2 3 4 5



← 10,000
← 8,000

← 4,000
← 3,000
← 2,400

← 1,400

← 1,000
← 800
← 600

DNA from clone 2 was subcloned into the Bluescript II SK (-) phagemid. The DNA of the genomic clone and pBluescript II SK (-) was digested with the restriction enzymes *EcoR* I and *Sal* I. After purification of the DNA from the restriction digests the genomic DNA was ligated into the plasmid. After transformation of *E. coli* and antibiotic and β -galactosidase selection, DNA was isolated from cultures of *E. coli* and the subclones analysed by digesting with *EcoR* I and *Sal* I, followed by agarose gel electrophoresis (Fig. 4.4). The 8 kb fragment is in subclone F, the 2.4 kb fragment is present in subclone G (and possibly E). The 1.4 kb fragment is present in subclone D along with the 1 kb fragment, and a previously undetected 0.1 kb fragment, this 0.1 kb fragment is also present in subclone A. The 0.8 kb and 0.6 kb fragments are both contained in subclone C, subclone B also contained the 0.6 kb fragment. The multiple fragments that appear in two of the clones are due to incomplete digestion of the genomic DNA. The large 15 kb fragment was not subcloned; this was not possible as the fragment has only *Sal* I sites. The information from the subcloning, together with the results from the Southern blots allowed a restriction map to be drawn (Fig. 4.5). The multiple fragments in clones C and D enabled these *EcoR* I fragments to be ordered to some degree as the 0.8 kb and 0.6 kb fragments must be adjacent within the clone. The same applies to the 1.4 kb, 1 kb, and 0.1 kb fragments. The positions of the restriction sites within these multiple subcloned fragments can be inferred as the fragments were cloned using *EcoR* I and *Sal* I sites, and the 0.6 kb and 0.1 kb fragments were cloned as individual fragments and therefore must have *EcoR* I and *Sal* I sites. The fragments were not excised from the clones with multiple inserts using *Sal* I alone.

4.6 Sequencing of λ BnPRP genomic clone 2

Each of the subcloned genomic fragments produced by digestion with *EcoR* I and *Sal* I were isolated from the Bluescript II SK (-) phagemid. Each of these

Figure 4.4 Analysis of fragments of genomic clone 2, subcloned into pBluescript® II SK(-)

DNA was isolated from seven independent *E. coli* colonies transformed with fragments of DNA from genomic clone 2 digested with *Sal* I and *EcoR* I and ligated into pBluescript® II SK(-).

Three µg of the DNA was digested with the restriction endonucleases *EcoR* I and *Sal* I, and separated on a 1.2% TBE agarose gel.

Lane 1 + 9	1 kb ladder DNA marker
Lane 2	Three µg of digested DNA isolated from colony A
Lane 3	Three µg of digested DNA isolated from colony B
Lane 4	Three µg of digested DNA isolated from colony C
Lane 5	Three µg of digested DNA isolated from colony D
Lane 6	Three µg of digested DNA isolated from colony E
Lane 7	Three µg of digested DNA isolated from colony F
Lane 8	Three µg of digested DNA isolated from colony G

The figures refer to the size of the bands (bp); these were calculated by measuring the distance the fragments of clone 2 migrated in relation to the 1 kb ladder marker.

1 2 3 4 5 6 7 8 9



← 8,000

← 2,400

← 1,400

← 1,000

← 800

← 600

← 100

Figure 4.5 Restriction map of genomic clone 2

The data from Figs. 4.1, 4.2, 4.3, and 4.4 was analysed to construct the genomic restriction map. The figures for the fragments' sizes that are shown in kb are only approximate, particularly for the larger fragments. The orientation of the clone within the EMBL3 vector is not known. The orientation of the 1 and 1.4 kb fragments could not be determined, this is indicated by the double arrowed line.

The positions of the *EcoR* I and *Sal* I restriction enzyme sites are indicated by E and S respectively.

fragments were used to probe northern blots containing 15 µg of total RNA from plants cold treated for 24 hours and plants grown constantly at 22°C. As expected the 2.4 kb fragment hybridised with cold-induced transcripts of the same size as hybridise with the LF5B1 cDNA. Of the other fragments only the 0.6 kb fragment hybridised to the cold-induced transcripts (results not shown), indicating that it contained part of the coding or untranslated sequence of *BnPRP*. Unless large introns were present in the gene these two subcloned fragments should contain all the transcribed sequence of the gene, and may contain part of the promoter sequence. The two subclones C and G, which contained the 2.4 kb and 0.6 kb fragments, were sequenced.

The two subclones were sequenced using a combination of synthetic oligonucleotide primers and deletions. The primers were made to known regions of the DNA that had been sequenced with other primers or deletions. The primers, their sequence and the position that they hybridise to the subclone are shown in Table 4.1 (the subclone G is numbered from the *EcoR* I to *Sal* I restriction enzyme site; subclone C is numbered from the *Sal* I to *EcoR* I restriction enzyme site). The deletions were created using the enzymes *Exo* III and mung bean nuclease. Plasmid G was digested with *Sac* I and *EcoR* I for deletions in the *EcoR* I to *Sal* I direction, and *Kpn* I and *Xho* I for deletions in the *Sal* I to *EcoR* I direction. Each set of enzymes leaves one 3' overhang which is resistant to *Exo* III digestion, and therefore protects the Bluescript II SK (-) phagemid. The other enzymes leave 5' overhangs which are vulnerable to *Exo* III digestion, and allow deletions to proceed into the subcloned fragment. The deletions used are listed in Table 4.1b, together with the first nucleotide that was sequenced using the deletion, the primer used to sequence the deletion, and the direction of the deletion.

Fig. 4.6 is a schematic representation of the primers and deletions used, showing the regions of DNA that were sequenced with each primer and deletion. Apart from a 126 bp stretch of DNA in plasmid G between positions 1665 and 1792, all of

Table 4.1 Description of the primers and deletions used to sequence the 2.4 kb and 0.6 kb subcloned fragments from genomic clone 2.

The 2.4 kb and 0.6 kb fragments from genomic clone 2 were subcloned and sequenced using a combination of synthetic oligonucleotide primers and deletions.

The 2.4 kb fragment (subclone G) is numbered from the *EcoR* I to *Sal* I site, the 0.6 kb fragment (subclone C) is numbered from the *Sal* I to *EcoR* I site.

a A list of the synthetic oligonucleotide primers used, showing the region of DNA the primer anneals to. The T3, T7, and SK primers that bind to the regions of pBluescript II SK (-) DNA that flank the multicloning site along with primers made to known regions of *BnPRP* are shown.

b A list of the deletions of the 2.4 kb subcloned fragment (subclone G), indicating the primer that was used to sequence the deletion and the first nucleotide sequenced using the deletion.

a

Primer	Sequence (5' - 3')	Template	Template binding position
T3	AATTAACCCTCACTAAAGGG	pBluescript	792 - 772
T7	GTAATACGACTCACTATAGGGC	pBluescript	624 - 646
SK	CGCTCTAGAACTAGTGGATC	pBluescript	749 - 729
GF1	GTTTATGTTGCTGAGTGC	G	244 - 261
GF2	TTTCTTGGTCACCTTTCG	G	504 - 522
GF3	ACTTTTCTAAGTACAGTG	G	801 - 819
GF4	CGACTACAAAGATACGCC	G	1,116 - 1,134
GF5	CTCATTCTAAGAGATGGG	G	1,341 - 1,359
GF6	ACACCACCGGTCGTAACG	G	1,984 - 2,002
GR1	GTGTCGATTGGGCACGTT	G	2,241 - 2,223
GR2	CTCTTAGAATGAGGGTTTG	G	1,354 - 1,335
GR3	CTTTGTAGTCGTAGATTC	G	1,127 - 1,109
GR4	AACGACAAGTCTGAGAAGG	G	889 - 870
GR5	GCTTGGACATGCTCAGCC	G	644 - 626
GR6	CTTATTAAATTTGAGAAGC	G	382 - 363
GR7	CGTATTAGTAAAGAATATAC	G	241 - 221
PF1	TTAATTTGCGTGTGTGAG	P	155 - 173
PR1	TATAGTGTACACGCAGTC	P	420 - 403
PR2	CTCACACACGCAAATTAACC	P	173 - 153

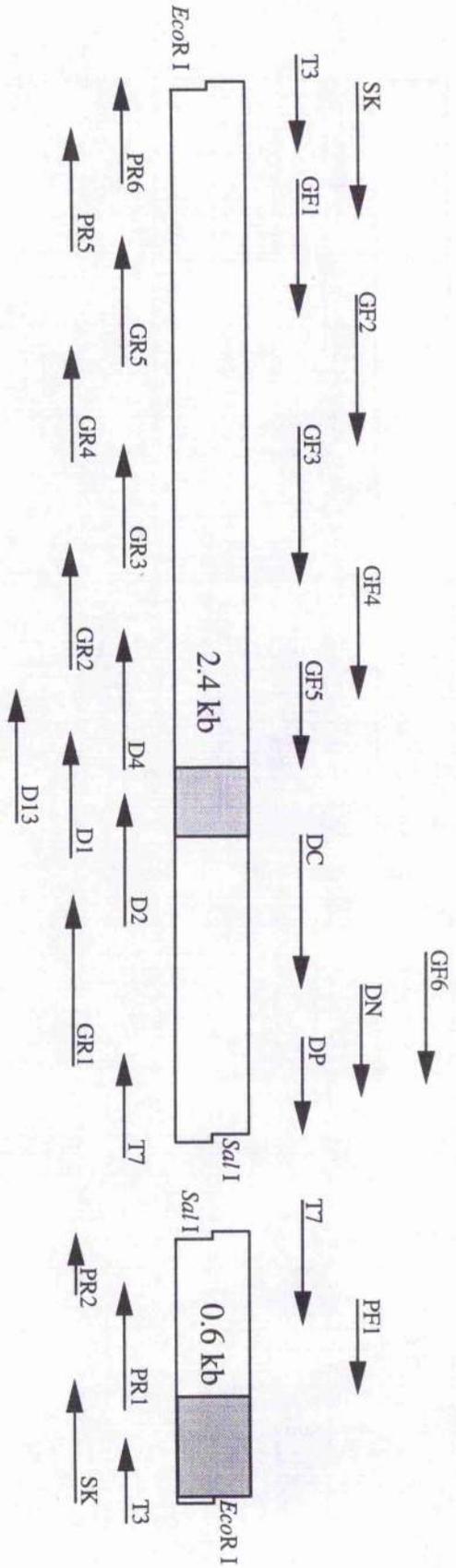
b

Deletion	First sequenced nucleotide	Primer used for sequencing
C	1,792	T3
N	1,949	T3
P	2,185	T3
2	1,925	T7
1	1,767	T7
13	1,681	T7
4	1,602	T7

Figure 4.6 Schematic representation of the sequencing of subclones C and G

The subclone G (2,407 bp fragment) and subclone C (607 bp fragment) are represented by rectangles. The restriction sites used to subclone the fragments into pBluescript II SK (-) are shown.

The lines above and below the subclones correspond to the sequence information obtained from the oligonucleotide primer or deletion, the name of the oligonucleotide primer or deletion is above the line. The arrow shows the direction of the sequencing reaction for each primer/deletion. Information on the oligonucleotide primers and deletions is in Table 4.1



0 100 200 300 400 500
 nucleotides / base pairs

 DNA sequenced on one strand

the 2.4 kb subclone was sequenced on both strands. Primers could not be synthesised to this region due to the repetitive nature of the DNA sequence in this region (Fig. 4.7), this would cause any primer made to this region to prime from multiple points, so the primer would not yield any useful sequence information. The sequence could not be determined by using a long gel run, using the GF5 primer as the sequencing reactions were prone to severe pausing in this region. The pausing could not be removed by elevating the sequencing extension temperature to 50°C (normally 37°C). This region of the sequence was in the putative open reading frame of *BnPRP*, so accurate sequence information was important. Numerous *EcoR* I to *Sal* I deletions were looked at in order to find one that would enable this stretch of DNA to be sequenced, but none was found. However, four overlapping *Sal* I to *EcoR* I deletions covering this region of DNA were sequenced. This allowed confidence in the fidelity of sequence obtained in this region, although sequence of both strands would have been more desirable. A region of subclone C was also sequenced on only one strand between positions 410 and 607. This was not considered important as this region was shown to be downstream of the putative polyadenylation site and therefore not a transcribed part of the gene.

4.7 Analysis of the sequence of *BnPRP*

The sequence from the 2.4 kb (2,407 bp) and 0.6 kb (607 bp) subcloned genomic fragments was analysed. Plasmid G contained 1,354 bp of sequence 5' of the ATG of an open reading frame (ORF). This sequence will be discussed in Chapter 6. The ORF is contained in plasmid G and C, it is 1,128 bp long, encoding a putative protein BNPRP of 376 amino acids (Fig. 4.7) with an estimated molecular weight of 38,673 Da.

Figure 4.7 Sequence of the *BnPRP*

The nucleotide sequence of *BnPRP*'s open reading frame is shown with the deduced amino acid sequence below. The numbers refer to the nucleotide/amino acid at the end of each line. The putative cleavage site of the signal peptide is between amino acid 27 and 28, indicated by the double headed arrow. The first nucleotide of the partial cDNA *BnPRP* is indicated by an arrow at nucleotide 492, a putative polyadenylation signal is indicated by underlining, * indicates the termination codon (TGA), the position of the poly(A) tail (deduced from alignment with the cDNA) is indicated by (An). The nucleotide sequence after the putative polyadenylation site is shown in lower case.

ATGGGGTCTCACACACAAAACCTCTCTTTTCTTATCCTCCTTCTCCTAGGCTTCCTTGCT 60
 M G S H T Q N L S F L I L L L L G F L A 20
 GTCTCATTTCGCTTGGGAATGTAGCCCTCCATAAACCATCTCCGAGACCCCACAAACCGCCT 120
 V S F A C E C→S P P K P S P R P H K P P 40
 AAACATCCCGTCAAACCCGCTAAACCACCAGCAGCCAAACCACAAAACCACCGGCAGTA 180
 K H P V K P P K P P A A K P P K P P A V 60
 AAACCTCCTAAACCCCCACAAAACCTCCACACCCTTAAACCACACCCCCACCTTAAACCT 240
 K P P K P P T K P P T L K P H P H P K P 80
 CCCACCCTTAGACCACATCCACACCCTAAGCCCCCTACCAAACCTCATCCCATCCCAAAA 300
 P T V R P H P H P K P P T K P H P I P K 100
 CCGCCACAAATTAACCACCACCCTCCACACCCAAACCGCCACAAAACCACCCACCGTT 360
 P P T I K P P P S T P K P P T K P P T V 120
 AAACCACCTCCAATCCACCCCTAAGCCGCCACCAAACCACCCACCGTCAAACCACCACCC 420
 K P P P S T P K P P T K P P T V K P P P 140
 TCCACCCCAAAAACCGCCACCAAGCCACCCACCGTCAAACCACCTCCCGTCCACCCCTAAA 480
 S T P K P P T K P P T V K P P P S T P K 160
 CCACCTACCCACAAGCCACCAACAGTGTGCCACCCGCCAACACCAACACCAACCCACCT 540
 P P T H K P P T V C P P P T P T P T P P 180
 GTTGTAAAGCCACCAACACCACCAACACCAACTCCACCAGTCGTTACGCCACCAACACCA 600
 V V T P P T P P T P T P P V V T P P T P 200
 GCCCCACCTGTTGTAACGCCACCAACACCAACACCACCGGTCGTAACGCCACCGACACCA 660
 A P P V V T P P T P T P P V V T P P T P 220
 ACCCTCCTGTGTAACACCTCCAACACCACCGGTCGTAACGCCACCGACACCAACTCCT 720
 T P P V V T P P T P P V V T P P T P T P 240
 CCTGTGTAACACCACCAACACCACCGGTCGTAACGCCACCGACACCAACCCCTCCTGTG 780
 P V V T P P T P P V V T P P T P T P P V 260
 GTAACACCTCCAACACCACCGGTCGTAACGCCACCGACACCAACCCCTCCTGTGTAACA 840
 V T P P T P P V V T P P T P T P P P V V T 280
 CCACCAACACCAACTCCACCTAAGCCAGAAACGTGCCCAATCGACACGTTGAAGCTAGGC 900
 P P T P T P P K P E T C P I D T L K L G 300
 GCTTGTGTAGACGTTCTTGGAGGTTTAATTCACATCGGGCTTGGTGGAAAGTAGCGCCAAG 960
 A C V D V L G G L I H I G L G G S S A K 320
 AAAGAGTGTGTCGGGTTTGGGAGGCTTAGTTGACTTAGACGCAGCTGTTTGTCTATGT 1020
 K E C C P V L G G L V D L D A A V C L C 340
 ACCACCATCAAAGCCAAACTTCTCATCGTCGACCTTATTAATCCCATTTGCTCTTGAGCTT 1080
 T T I K A K L L I V D L I I P I A L E L 360
 CTTATCGACTGTGGAAAGACTCCGCCACCTGGCTTCAAATGTCCTCTTGTGATGAATGAAG 1140
 L I D C G K T P P P G F K C P S * 376
 TGTCCTCTATCGTGTTTTTCTTTCTTTCTTTCAAGAATTTGTTAGTTTGATAATCTCTT 1200
 GGGTGAGGTTAATTTGCGTGTGTGAGACCTTAAATCACTTGTTTTGTGTACAATAAAAG 1260
 GCTATGTTTGGCTTTTGTGTGATGTAATTTCTAAGAAGAAGAAATAAAAAATGTAACCGT 1320
 ATAAATATCTTC (An) acaaaaaacaagttaatatttcatatctgtgtaacgcttatttt 1376

gTTTTTTtAtgtacatatatacgtacgtagtcacttGtggagtagcagaaaTgtggattt 1436
tAtagccatgcaaattgcactgacgcacatgtgatatgcgcocccatagcgtggctactg 1496
TgtactgaccaaatacatgtgtataaatatagttacatacaagtatatatatgctaacaC 1556
atataTcataactttctccaaaaattgatagctaagatagctcttaacaaaattatgtctca 1616
gtcacacagatgattacgacatttgatttGtggattaagaatC 1660

Analysis of the putative amino acid sequence confirmed that BNPRP was a hybrid proline-rich protein. This had been shown from translation of the BnPRP cDNA. The full length genomic sequence revealed several other features. The BNPRP protein is modular, composed of 5 distinct domains. This is illustrated in a hydropathy plot (Fig. 4.8a). A Kyte and Doolittle plot was used, with a nine residue window (Kyte and Doolittle, 1982). Each region of BNPRP has a distinct amino acid composition (Fig. 4.9).

Domain one shows the physio-chemical properties attributed to signal peptides (Von Heijne, 1988). It is 27 amino acids long, having a positively charged amino terminal, a central hydrophobic region, and a polar carboxyl-terminal with a cleavage site (CEC-SP) that conforms to the rules for defining cleavage sites (Von Heijne, 1988). After removal of the signal peptide the BNPRP protein has an estimated molecular mass of 35,762 Da.

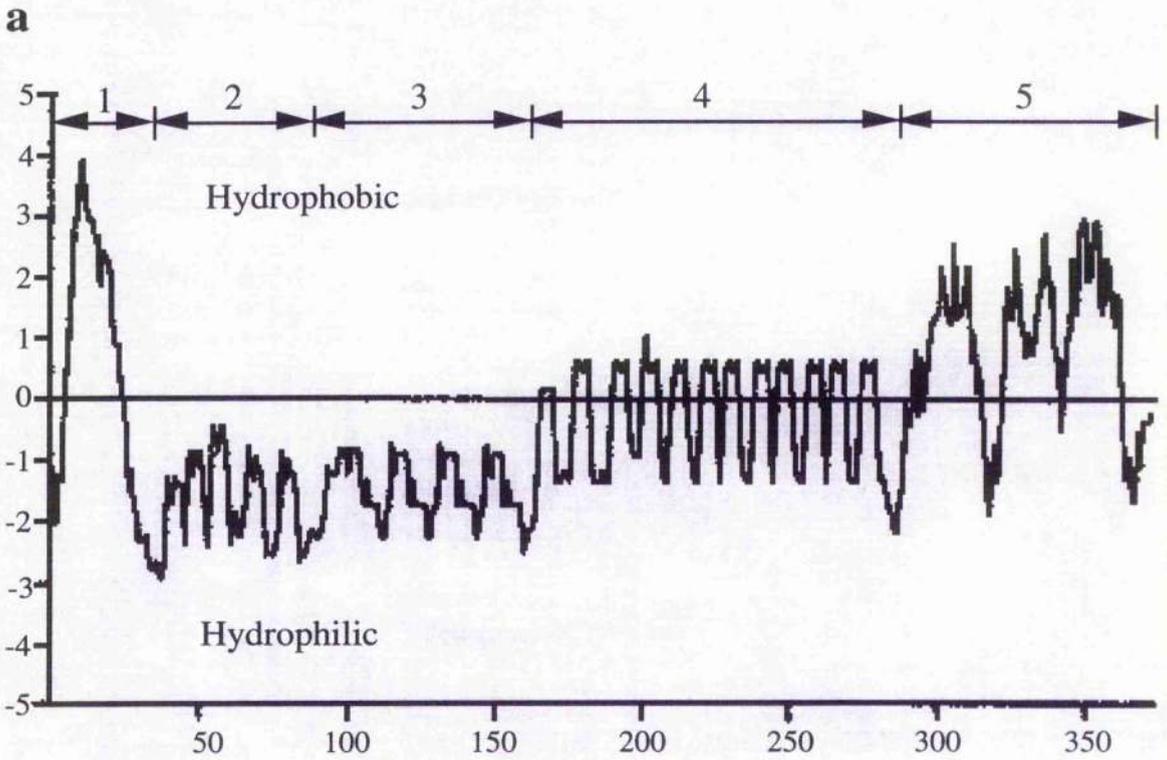
The three central domains span residues 28 to 287. They are all rich in proline (50%), threonine(19.2%), valine (11.2%), and lysine (10.4%). The amino acid motifs within each domain vary and are repeated to varying degrees, the length of the individual repeats also varies. Domain three shows the most ordered repeats (Fig. 4.10).

The carboxyl domain stretches from residue 288 to 376. It contains three putative membrane spanning segments. These segments consist of stretches of approximately 20 relatively apolar, hydrophobic residues (particularly leucine, isoleucine and glycine), which would span the membrane, followed typically by polar, charged, hydrophilic residues (particularly lysine) which would protrude into the cytoplasm or cell wall space. This is represented schematically in Fig. 4.11. The carboxyl domain also has a relatively high content of cysteine and proline.

Figure 4.8 Analysis of the BNPRP putative amino acid sequence

The putative protein BNPRP was analysed using a hydropathy plot (a). The hydrophobicity/hydrophilicity of each residue is measured and an average value for each successive set of nine residues determined and the value plotted. The values on the y-axis refer to the average hydrophobicity/hydrophilicity (hydrophobic values are positive, hydrophilic values are negative). The x-axis refers to the amino acid number, as shown in Fig. 4.7. The double arrowed lines above the plot define the five domains.

b The main features of each of the five domains are indicated.



b

Domain	Features
1	Characteristic properties of a signal peptide.
2	Repeats ranging between 7 and 11 residues. Core motif KPPK/T.
3	Highly regular repeats of 16 residues, KPPT(I/V)KPPPSTPKPPT.
4	Repeats ranging between 8 and 13 residues. Core motif PPVVT PPT.
5	Contains 3 putative membrane spanning regions.

Figure 4.9 The amino acid composition of BNPRP

The amino acid composition of the three regions of BNPRP were calculated. The putative protein was broken down into the putative signal peptide, the proline-rich region and the carboxyl region. The amino acids that these regions cover are indicated in the brackets. The amino acids not present after removal of the putative signal peptide are also indicated.

Domain 1 (signal peptide):	M=1	N=1	E=1
	G=2	L=7	C=2
(1-27)	S=3	F=3	
	H=1	I=1	
	T=1	A=2	
	Q=1	V=1	Total=27

Domains 2-4 (proline-rich):	P=130 (50%)	S=6	C=1
	T=50 (19.2%)	A=4	
	V=29 (11.2%)	R=2	
(28-287)	K=27 (10.4%)	I=2	
	H=8	L=1	Total=260

Domain 5 (putative : trans-membrane)	L=15	A=6	H=1
	G=10	V=6	F=1
	I=9	D=6	
(288-376)	K=8	T=5	
	P=8	E=3	
	C=8	S=3	Total=89

Total=376

Amino acids not present after : M, N, Q, W, Y
removal of the signal peptide

Figure 4.10 The repeated motifs of BNPRP

Domains 2, 3 and 4 of BNPRP all contain repeated sequence motifs. The full amino acid sequences of domains 2, 3 and 4 are shown, they have been broken down into the repeated motifs. The core sequence of each of the repeats is underlined, the numbers refer to the length of each repeat.

Domain 2:	<u>KPPKHPV</u>	7
	<u>KPPKPPAA</u>	8
	<u>KPPKPPAV</u>	8
	<u>KPPKPPT</u>	7
	<u>KPPTLKPHPHP</u>	11
	<u>KPPTVRPHPHP</u>	11
	<u>KPPTKPHPIPK</u>	11

Domain 3:	<u>KPPTIKPPPSTPKPPT</u>	16
	<u>KPPTVKPPPSTPKPPT</u>	16
	<u>KPPTVKPPPSTPKPPT</u>	16
	<u>KPPTVKPPPSTPKPPT</u>	16
	<u>HKPPTVCPPT</u>	11

Domain 4:	<u>PTPTPPVVTPTPT</u>	13
	<u>PTPTPPVVTPTPT</u>	13
	<u>PPVVTPTPT</u>	10
	<u>PPVVTPTPT</u>	10
	<u>PPVVTPTPT</u>	8
	<u>PPVVTPTPT</u>	10
	<u>PPVVTPTPT</u>	8
	<u>PPVVTPTPT</u>	10
	<u>PPVVTPTPT</u>	8
	<u>PPVVTPTPT</u>	10
	<u>PPVVTPTPTPT</u>	12

Figure 4.11 Schematic representation of the putative membrane spanning segments in domain 5

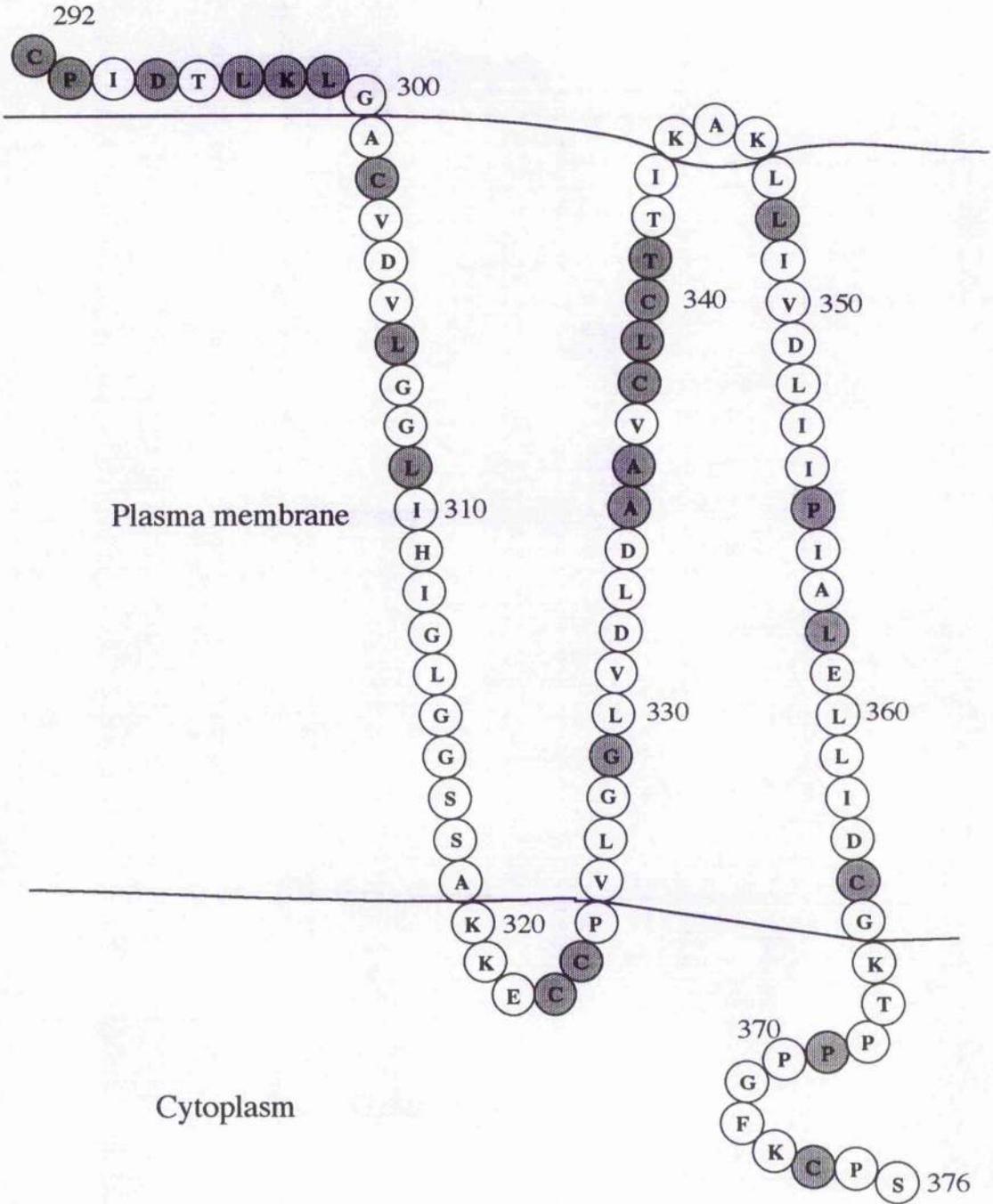
The putative membrane spanning segments of domain 5 were identified by the hydropathy plot (Fig. 4.8a).

The membrane spanning segments contained regions that were hydrophobic (averaged over a nine residue window) over at least 18 residues. The membrane - cell wall/cytoplasm boundaries are defined by the highly hydrophilic lysine residue in four of the six boundaries, the other two boundaries are inserted at likely positions in view of the length of the membrane spanning segment and the relative hydrophobicity / hydrophilicity of the residues in that area. In all cases the exact positions of the boundaries are only approximate as no precise rules exist.

The numbers refer to the amino acid number of BNPRP shown in Fig. 4.7.

The residues that are conserved in a number of other plant proteins (Fig. 4.12) are shaded.

Cell Wall



BNPRP after removal of the putative signal peptide contains only one aromatic amino acid, phenylalanine, which is present only once in the sequence; there are no tryptophan or tyrosine residues. Methionine, asparagine and glutamine residues are also absent from the sequence.

Alignment with the cDNA shows an untranslated region of 205 bp assuming that transcripts from this gene would be polyadenylated at the same position as the cDNA. A consensus polyadenylation signal (AATAAA) (Joshi, 1987b) is present 27 bp upstream of the position of the putative poly(A) tail. The sequence of the cDNA BNPRP where it overlaps with the genomic clone is identical. The continuity of sequence between the cDNA and the genomic clone across the *Sal* I restriction enzyme site is strong evidence that none of the genomic clone had been lost during the subcloning, which could have occurred if two *Sal* I sites had been present in this region of sequence of the genomic clone. The unbroken ORF indicates that the gene contains no introns, and this is supported by the cDNA aligning without the insertion of gaps.

4.8 Comparison of BNPRP with other plant hybrid Proline-rich proteins

The DNA sequence of *BnPRP* and the putative protein BNPRP were entered into a data base search against both DNA and protein sequences. The HGMP computers (Cambridge, UK.) were used for the searching the GenBank and EMBL databases using the BLAST algorithm (Altschul *et al.*, 1990). The DNA sequences were converted into putative protein sequence and analysed using the UWGCG programme PILEUP (Deveraux *et al.*, 1984). The putative protein BNPRP shares a high degree of sequence similarity with several putative plant proteins. The similarity is highest in the carboxyl domain (Fig. 4.12). All are hybrid proline-rich proteins,

Figure 4.12 Comparison of the BNPRP carboxyl domain amino acid sequence with other plant proteins

The nucleotide sequence of *BnPRP* was used to search the EMBL and GenBank databases. The sequences that were identified and found to be highly similar were translated and compared at the amino acid level.

a Alignment of the sequences using the UWGCG programme PILEUP. Dots indicate identical amino acids, highly conserved amino acids are indicated by underlining, dashes indicate where gaps have been introduced into the amino acid sequence. The * symbol above the alignment indicates where the amino acid is conserved in all the proteins, _ indicates highly conservative substitutions in all the proteins, ° indicates a conservative substitution in all proteins. Each substitution's degree of conservation in relation to BNPRP was determined using the BESTFIT alignments.

b Table showing the identity/similarity of the above proteins to BNPRP in the C-terminal domain as determined by the UWGCG programme BESTFIT, the number of gaps inserted to achieve the BESTFIT alignment are indicated.

The accession numbers of *TPRP-F1*, *ADR11-1*, *HyPRP*, *DC2.15*, *SAC51*, *ZRP3*, and *MSACIC* are X57076, S58480, S44189, X15436, X71618, Z12103, and L22305 respectively.

a

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      ** * *** * _ * _ * _ * _ * _ * _ * _ * _ * _ * _ * _ * _ *
BNPRP  KPETCPEDTLKLGACVDVLGGJLHIGLGGSSAKKFCPCPVLGGLVDLD
TPRP-F1 AQP.....A.....I.....QT...L.....
ADR11-1 AQA.....L.....V.....DPV.-NQ.....Q...EVE
HYPRP  AVR.....N.....S.....LVI.QE-.RSK...LVQ.VA...
DC2.15 SAGK..R.A...V.A...-N.V.NVVI..PPTLP..SL.E...N.E
SAC51  ATAK..R.A...V.AN..S..IN.T.-.KPPV.P..TLIK..A..E
ZRP3   SHGR....A...KV.AK...-VYV..POY---EQ...L.E.....
MSACIC TSQK..T.....V.A...-VNVIV.-.P.SSK..TLIQ..A...

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      ** **** _ _ _ * _ _ * _ _ _ * _ _ * _ _ *
BNPRP  AAVCLCTTIKAKLLIVDLIPIALELLI-DCGKTTPPPGFKCPS      376
TPRP-F1 ..I.....RL...NINI..L...QV..D...Y..KD.....T      346
ADR11-1 .....L.L...NLNIYV..L..Q..V-T...S...YT.SL      151
HYPRP  ..L.....E.E..NINIYL...N...-T...HA.S..Q..PLYD      301
DC2.15 .....A...NI.GKN.NI...S.VLNN...QV.N..E.T      137
SAC51  ..A...AL..NI.GIN.N...S.S..LNV.S.KV...Q.      147
ZRP3   ..L....A...NV.GIH.NV..LS.NEILNN..RIC.ED.T..N      129
MSACIC .....A...NI.GIN.NV..T.S..ISA.E.SI.N..Q.S      166

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b

Gene / cDNA	Percent identity	Percent similarity	Gaps inserted
TPRP-F1	77.0	89.7	2
ADR11-1	66.3	84.9	1
HYPRP	62.1	80.5	1
SAC51	52.9	70.6	1
DC2.15	52.9	70.1	2
ZPR3	50.6	71.8	3
MSACIC	54.7	69.8	3

containing two distinct domains, one rich in the amino acid proline, and the other carboxyl domain, rich in hydrophobic residues.

The genes identified are from a number of plants, and expressed in response to diverse developmental and environmental stimuli. *TPRP-F1* is expressed predominantly in young tomato fruit (Salts *et al.*, 1991). *ADR11-1*, isolated from soybean, is down regulated in response to auxin in seedling hypocotyls (Datta *et al.*, 1993). *HyPRP* accumulates in immature maize zygotic embryos (Josè-Estanyol *et al.*, 1992). *DC2.15* is expressed in carrot suspension cultures during the initiation of somatic embryogenesis (Aleith *et al.*, 1990). *ZRP3* accumulates in developing cortical cells, within maize roots (John *et al.*, 1992). *SAC51* accumulates in the dehiscence zone during seed pod formation in oilseed rape (Coupe *et al.*, 1993). *MsaCIC* is expressed in response to low temperatures and wounding in the apices of alfalfa (Castonguay *et al.*, 1994).

A low level of similarity exists in the proline-rich domains. These vary widely in length, BNPRP is the longest with 260 residues, ZRP3 is the shortest with only 22 residues. Similarity is due mainly to the high levels of proline. Some of the motifs are shared with those found in BNPRP, two of the proteins, TPRP-F1 and MSACIC share proline triplets. These are unusual in higher plants, the prolines in most proteins appear in doublets as occurs in proline-rich proteins (PRPs), or fours as occurs in the extensins (Showalter, 1993). The PPVV motif appears in TPRP-F1, ADR11-1, and SAC51.

The carboxyl terminal is very conserved between all the proteins. The majority of residues (75%) are conserved to some extent. The cysteine residues are conserved at all positions, suggesting that they play an important role in the mature protein. The hypothesis that the putative membrane-spanning regions in the carboxyl domain of BNPRP are membrane spanning domains is supported by their sequence similarity with

other membrane spanning segments. The highest sequence similarity (88% similarity, 56% identity, over 18 amino acids) is between the first putative membrane spanning region of BNPRP and the first membrane spanning region of human B cell integral membrane receptor protein CD20 (Einfeld *et al.*, 1988). This was determined by the UWGCG programme BESTFIT (Devereux *et al.*, 1984).

4.9 Discussion

Seven genomic clones were isolated from a genomic library and analysed by restriction digestion and Southern blotting. The strong hybridisation of the probe to the clones indicated that five of the clones were homologous or very similar to the probe, whereas two appeared to be less related. The five clones 2, 3, 4, 6 and 7 that were highly related to the probe appeared very similar to each other. This was based on the Southern blotting analysis.

One of the clones, clone 2 was subcloned, restriction mapped and sequenced. The sequence that contained the ORF spanned two of the subclones, G and C. These contained 3,014 bp of DNA. The sequence of the genomic clone and the cDNA BnPRP were identical over the length of the cDNA. This implied that the gene *BnPRP* would be regulated by low temperatures. This is supported by the observation that both these subcloned fragments hybridised to cold-induced transcripts (data not shown). The cDNA and genomic clone were isolated concurrently, using the same probe that was derived from the LF5B1 cDNA. Neither the cDNA nor the genomic clone are identical to the corresponding sequence of LF5B1. As several genomic clones were isolated it is possible that one of the other clones that was not characterised further was homologous to the relevant sequence in LF5B1. Cloning related genes was possible as Southern blots had indicated the presence of a gene family related to *LF5B1* (Pallas, 1992).

The position of the poly(A) tail was predicted from alignment with of the BnPRP cDNA. As was discussed in Chapter 3, plant transcripts are often polyadenylated at multiple sites. Alternative polyadenylation sites 5' of the one that is present have been discussed in Chapter 3. In addition three other putative polyadenylation sequences aacaaa (1334-1339), tataaa (1518-1523), and aacaaa (1600-1606) which vary from the consensus polyadenylated sequence by only one nucleotide could also act as the polyadenylation signal.

The ORF was 1,128 bp long, encoding a putative protein 376 amino acids long. The putative protein was modular, composed of five distinct domains. The amino terminal domain one has the characteristic features of a signal peptide. The putative signal peptide is 27 amino acids long. Signal peptides can vary in length between 15 and 30 residues. No high sequence similarity has been found between signal peptides (Von Heijne, 1988), though they do all contain three distinct regions, which are called the n-, h- and c-regions. The amino terminal n-region varies in length (one to 20 residues) and amino acid composition but always carries a positive charge (Von Heijne, 1984). In BNPRP the n-region covers the first seven to nine residues, the positive charge is provided by the histidine residue. The h-region of signal peptides can vary between seven and 16 residues, hydrophobic apolar residues (isoleucine, leucine, methionine, valine, tryptophan, and phenylalanine) are abundant (Von Heijne, 1985). The h-region is characterised by 11 centrally located apolar residues in BNPRP. The border between the h- and c-regions is characteristically between the sixth and fifth residues from the cleavage site (-6 and -5 positions) and is often signalled by a proline, glycine, glutamine, serine, or charged residue (Von Heijne, 1985). The serine residue at the -6 position in BNPRP fits this requirement. All naturally occurring cleavage sites conform to the -3, -1 rule; the residues in the -1 and -3 positions must be small and uncharged, the residue at the -2 position is usually large, bulky and charged (Von Heijne, 1983; Perlman and Halvorson, 1983). In BNPRP the cysteine residues at the -1 and -3 positions fit the criteria of being small and uncharged, the glutamic acid

residue at the -2 position is large and negatively charged. The features present in the first 27 amino acids of BNPRP are strong evidence that it represents a signal peptide, and the conformity with the -3, -1 rule allows the end of the signal peptide to be defined with a high degree of confidence. Signal peptides have been shown to direct proteins into the endoplasmic reticulum. The proteins then pass to the golgi apparatus where specific signals are recognised, these signals direct proteins to lysosomes, vacuoles, or back to the endoplasmic reticulum (Johnson *et al.*, 1987; Munro and Pelham, 1987; Pelham *et al.*, 1988). Removal of the signals results in secretion of the protein, this suggests that proteins are secreted by a default pathway. None of the signals which have been shown to lead to retention of proteins, such as the ER retention signals KDEL and HDEL (see Vitale *et al.*, 1993) are present in BNPRP.

Within domains 2 to 4 the amino acids are arranged into repeating units (Fig. 4.10). Each domain has a distinctive core motif. The motifs present do not correspond to any of the common proline-rich protein/extensin motifs previously identified (Josè and Puigdomènech, 1993; Kieliszewski and Lamport, 1994). The PPP and PPVV submotifs are found in the proteins that are highly similar in the carboxyl domain. Both the amino acid composition and the repeated nature of the motifs are characteristic of proline-rich proteins, found in the cell wall (Showalter, 1993; Josè and Puigdomènech, 1993; Kieliszewski and Lamport, 1994). The domains are rich in several amino acids (Fig. 4.9). Proline accounts for 50% of the residues in domains 2 to 4, threonine, valine and lysine are also abundant residues representing 19.2%, 11.2%, and 10.4% of all the residues present in these domains. The high proline content is the feature that defines the proline-rich proteins. The high threonine content is characteristic of proline-rich proteins from monocots (Kieliszewski *et al.*, 1990; Stiefel *et al.*, 1988, 1990), the significance, if any of the threonine is not understood. Lysine and valine are both common in PRPs and extensins, the lysine residues in these proteins have been proposed to interact ionically with cell wall components such as pectic polysaccharides (Miller and Fry, 1992; Smith *et al.*, 1984) and may be functionally important. The

proline-rich domains contain no tyrosine, this is commonly found in PRPs and extensins, and is believed to have a role in forming isodityrosine linkages (Cooper and Varner, 1984). The absence of tyrosine limits the potential cross-linking between BNPRP and other cell wall components. The proline-rich domains have no aspartic acid or glutamic acid, the absence of these residues is commonly seen in PRPs and extensins (Cassab and Varner, 1988).

The carboxyl domain of BNPRP contains three putative membrane spanning domains (Fig. 4.11). These putatively act as membrane integration signals which would prevent the protein from being fully secreted. Membrane integration signals (stop transfer signals, STs) are typically apolar regions of approximately 20 residues (which show little sequence conservation), usually followed by a couple of positively charged residues on the carboxyl side (Von Heijne, 1981). The lysine residues at positions 320 and 321 follow a hydrophobic region; this is the first putative ST signal. Two other putative trans-membrane regions are present, the lysine residues at positions 344 and 346 follow a second hydrophobic putative trans-membrane region, the second polar region is predicted to project into the cell wall space. The third hydrophobic putative trans-membrane region follows these lysine residues, another lysine residue at position 366 predicts the point at which this trans-membrane region would be in the cytoplasmic space.

The predicted mass of the BNPRP protein is 38,673 Da. After the removal of the putative signal peptide the predicted mass is 35,762 Da. The mass of the protein *in vivo* cannot be accurately estimated because extensive post-translational modification may occur and is common, for example, in dicot extensins. The post-translational modifications detected in monocot threonine-rich extensins and PRPs are less extensive (Kieliszewski and Lamport, 1994). The rules for post-translational modification of extensins and PRPs are not clear, though some are beginning to be elucidated. Hydroxylation of proline residues seems to be sequence rather than conformation

dependent (Kieliszewski *et al.*, 1990; Sticher *et al.*, 1992). The proline in the proline-valine dipeptide is invariably hydroxylated (Kieliszewski *et al.*, 1990). This dipeptide occurs in each of the 11 repeats in domain 4 of BNPRP. Other proline residues in BNPRP are likely to be hydroxylated. The serine residues of dicot PRPs are almost always glycosylated with a single galactose (Lampert, 1977), in BNPRP a single serine residue occurs in each of the 16 residue repeats of domain 3. Arabinosylation of hydroxyproline residues increases with hydroxyproline contiguity (Kieliszewski *et al.*, 1992). In BNPRP the proline residues occur predominantly in doublets, and if both proline residues from any doublet were hydroxylated then glycosylation of the second residue would be predicted. The triplets of proline that occur in the five repeats of domain 3 are candidates for more extensive glycosylation, if the residues are hydroxylated. The size of the carbohydrate attached varies from single sugars (arabinose and galactose) to small arabino-oligosaccharides (Lampert, 1977). The rules determining which carbohydrate is added are not clear although arabino-oligosaccharides occur more frequently on contiguous hydroxyproline residues (Kieliszewski and Lampert, 1994).

The sequence similarity with the other hybrid PRPs over their carboxyl domains is striking (Fig. 4.12). The high sequence similarities exist between both monocots and dicots, suggesting that strong evolutionary pressures exist on the carboxyl domain of the protein, which implies that the primary structure is important in its function. From the sequence data it can be assumed that the proline-rich domains are inserted in the cell wall, directed by the signal peptide before it is cleaved. The carboxyl domain presumably inserts into the plasma membrane, acting as a stop transfer signal. The high sequence similarity is primarily due to the carboxyl domain being composed largely of hydrophobic residues, substitutions in this region are largely conserved. A number of residues are conserved in all of the proteins, some of these may be through chance, though some are likely to be important in the function of the proteins. The cysteine residues are likely candidates to play an important role; these have the ability to

form disulphide bridges and are structurally important in a number of proteins (Stryer, 1988). All eight cysteine residues are conserved in the carboxyl domain. The putative positions of these residues (Fig. 4.11) are both inside and outside of the membrane, and they have the potential to interact with other cysteine residues in the carboxyl domains or other proteins, which may also be embedded in the membrane. The length of the carboxyl domains is also highly conserved between the proteins. Gaps are inserted in the PILEUP, but most of these cover only one residue. Alternatively, the putative membrane spanning domains may not be recognised *in vivo* and the whole protein could be secreted. If this occurs the cysteine residues could be important, potentially interacting with other proteins in the cell wall.

Chapter 5: Analysis of the expression of *BnPRP*

5.1 Introduction

The cDNA LF5B1 had been used to probe several northern blots (Pallas, 1992). These had revealed that the transcripts it hybridises to are induced by exposure of the plant to low temperature. Preliminary studies had indicated that the transcript was not induced by heat-shock or wounding. Further experiments were therefore undertaken to examine the expression of *BnPRP* in relation to these stimuli. The response to the related stress of dehydration and the effect of applying ABA, a phytohormone associated with dehydration, and also shown to be involved in some instances in the activation of cold-induced genes, was also examined.

5.2 Cold-induced expression of *BnPRP*

5.2.1 Temporal expression of *BnPRP*

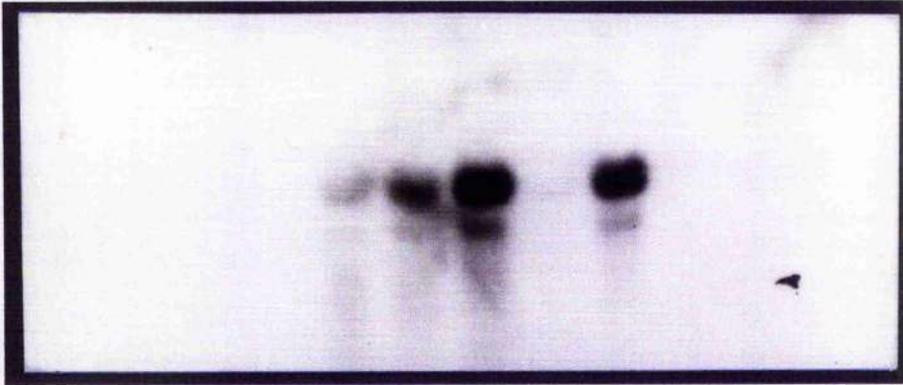
To examine the expression of *BnPRP* in response to low temperature, *Brassica napus* plants were grown for three weeks at 22°C and then transferred to 4°C. An increase of the levels of the *BnPRP* transcript in leaf tissue could be detected on northern blots after eight hours of cold treatment (4°C). The transcript levels continued to increase for up to 24 hours (Fig. 5.1). This level remains reasonably constant for the time the plant is cold treated, up to 14 days (results not shown). A time course of 11 days had been carried out for LF5B1 and the results were very similar (Pallas, 1992).

Figure 5.1 Temporal expression of *BnPRP*

Twenty-one day old *Brassica napus* plants were grown at 22°C were cold treated (4°C) for the times indicated. After 24 hours treatment the plants were returned to the normal temperature, samples were taken at intervals to evaluate how long the transcripts remained. Total RNA was isolated from plants leaves, 15 µg was separated on a 1.5% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with the *BnPRP* cDNA and after washing the blot was autoradiographed for 20 hours.

- Lane 1 Plants grown at 22°C.
- Lane 2 Plants treated at 4°C for one hour.
- Lane 3 Plants treated at 4°C for two hours.
- Lane 4 Plants treated at 4°C for four hours.
- Lane 5 Plants treated at 4°C for eight hours.
- Lane 6 Plants treated at 4°C for 12 hours.
- Lane 7 Plants treated at 4°C for 24 hours.
- Lane 8 Blank.
- Lane 9 Plants treated at 4°C for 24 hours and returned to 22°C for one hour.
- Lane 10 Plants treated at 4°C for 24 hours and returned to 22°C for two hours.
- Lane 11 Plants treated at 4°C for 24 hours and returned to 22°C for eight hours.

1 2 3 4 5 6 7 8 9 10 11



After 24 hours of cold treatment the plants were returned to normal growing conditions (22°C) and the transcript abundance was measured. The transcript levels declined rapidly (Fig. 5.1). Little change was detected after one hour, by two hours the transcripts were barely detectable, and no transcripts could be detected after eight hours at 22°C. The transcripts appeared to be contained in one main hybridising band, though a weaker band was also visible immediately below the main band. The position of this fainter band corresponds with one of the ribosomal RNA bands, and it is believed that this signal is non-specific. The second fainter band is also seen in some of the subsequent blots.

5.2.2 Spatial expression of *BnPRP*

The *BnPRP* transcript levels were measured in different parts of the plant. Leaf, stem and root tissue were examined from plants cold treated for 24 hours and plants grown continually at 22°C. In cold-treated plants the transcripts were detected in the leaf tissue and to a lesser extent in the stem tissue, no expression was detected in the root tissue (Fig. 5.2). The transcripts were barely detectable in tissue from control plants.

5.3 Expression of *BnPRP* in response to dehydration

No increase in the *BnPRP* transcript level was detected in response to dehydration (Fig. 5.3a). The relative water content was reduced to as low as 65 %, The estimates for the relative water content of the leaves are not very accurate as they were not calculated using leaves that the RNA was isolated from. Instead, leaves of the same age on the same plants were used. The leaves that gave the 65 % value appeared wilted and were obviously dehydrated to some extent. A dehydration response was

Figure 5.2 Spatial expression of *BnPRP*

Twenty-one day old *Brassica napus* plants grown at 22°C were used. One set of plants was cold treated for 24 hours before the tissue was harvested, one set of control plants was kept at 22°C. Total RNA was isolated from leaves, stems and roots. Fifteen µg of RNA was separated on a 1.5% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with the BnPRP cDNA and after washing the blot was autoradiographed for 20 hours.

- Lane 1 First emerging leaf of plants after 24 hours at 4°C.
- Lane 2 Second emerging leaf of plants after 24 hours at 4°C.
- Lane 3 Third emerging leaf of plants after 24 hours at 4°C.
- Lane 4 Stem tissue of plants after 24 hours at 4°C.
- Lane 5 Root tissue of plants after 24 hours at 4°C.
- Lane 6 First emerging leaf of plants treated continually at 22°C.
- Lane 7 Second emerging leaf of plants treated continually at 22°C.
- Lane 8 Third emerging leaf of plants treated continually at 22°C.
- Lane 9 Stem tissue of plants treated continually at 22°C.
- Lane 10 Root tissue of plants treated continually at 22°C.

1 2 3 4 5 6 7 8 9 10

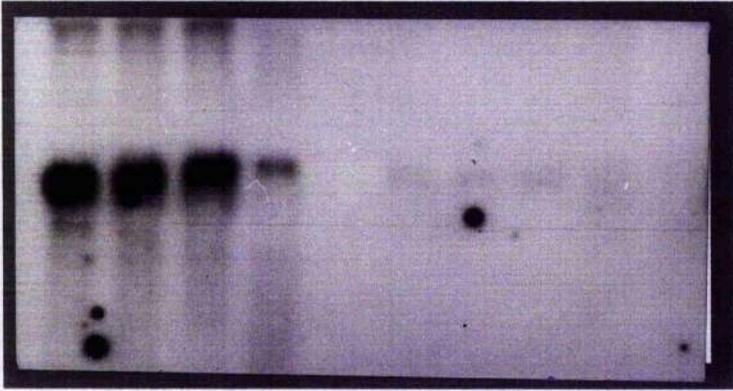


Figure 5.3 Dehydration-induced expression of *BnPRP*

Brassica napus plants were subjected to varying degrees of water stress. Water was withheld from plants from 15 days post germination. Leaves were harvested between 21 days and 28 days post germination. At each point that leaves were harvested the dehydration state of an equivalent leaf (RWC) was monitored. Total RNA was isolated from leaf tissue. The RNA was separated on a 1.5% denaturing agarose gel and blotted onto a nylon membrane.

The northern blot was probed using standard conditions with **a)** the cDNA *BnPRP* and **b)** the cDNA *BnD22*. Blots **a** and **b** were washed using standard conditions. The blots were autoradiographed for 20 hours.

Lane 1 Leaf tissue from plants treated at 4°C for 24 hours.

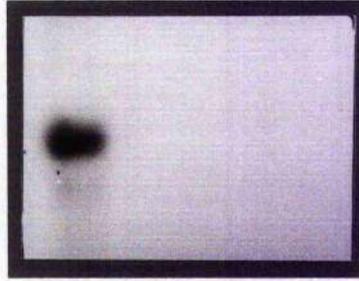
Lane 2 Leaf tissue with RWC of 95%.

Lane 3 Leaf tissue with RWC of 84%.

Lane 4 Leaf tissue with RWC of 65%.

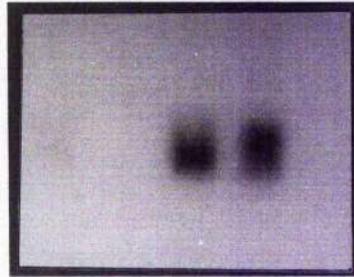
a

1 2 3 4



b

1 2 3 4



detected in the leaves with calculated RWC of 84% and 65%. This was detected by probing the northern blots with the BnD22 probe (Fig. 5.3b). This is a cDNA isolated from *Brassica napus*, which codes for a protein related to the Kunitz inhibitor family and has been shown to be induced in response to dehydration (Downing *et al.*, 1992). This is good evidence that the leaves that the RNA was extracted from were dehydrated to the degree that gene expression had been affected, validating the negative result with BnPRP.

5.4 Expression of *BnPRP* in response to exogenous ABA

No increases in the level of *BnPRP* transcripts could be detected after the application of 10^{-4} M ABA to the leaves of plants (Fig. 5.4a). Again the BnD22 cDNA was used to probe the northern blot. The induction of *BnD22* transcripts with the application of ABA was detected demonstrating that the ABA is penetrating the cell wall, entering the cell, and acting on gene expression. The message can be detected after two hours of application and was present at similar levels 24 hours after application (Fig. 5.4b). The *BnD22* transcripts could also be detected on the northern blot from plants treated with 10^{-5} M ABA. The leaf tissue from plants 24 hours after spraying with 10^{-5} M ABA did show a slight signal when probed with BnPRP, but the level is very low and comparable to the background signal that is sometimes detected from control plants. Moreover, if this was a real induction it is not clear why no signal was seen with a higher ABA concentration.

Figure 5.4 ABA-induced expression of *BnPRP*

Total RNA was isolated from leaves of 21 day old plants grown at 22°C that had previously been sprayed to run-off with either 10⁻⁴ M or 10⁻⁵ M ABA.. Fifteen µg of the RNA was separated on a 1.5% denaturing agarose gel and blotted onto a nylon membrane.

The northern blot was probed with **a)** the cDNA *BnPRP* and **b)** the cDNA *BnD22*.

Blots **a** and **b** were washed using standard conditions. The blot was autoradiographed for 20 hours.

Lane 1 Leaf tissue from plants treated at 4°C for 24 hours.

Lane 2 Leaf tissue sprayed with 10⁻⁴ M ABA after zero time.

Lane 3 Leaf tissue two hours after spraying with 10⁻⁴ M ABA.

Lane 4 Leaf tissue 24 hours after spraying with 10⁻⁴ M ABA.

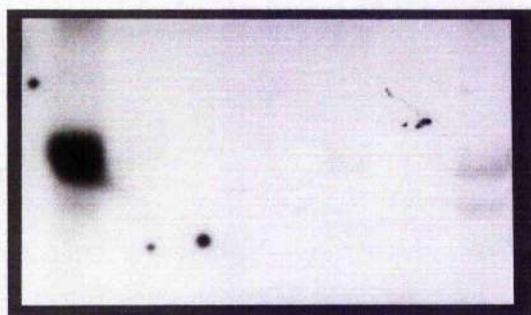
Lane 5 Leaf tissue 24 hours after spraying with no ABA in the solution.

Lane 6 Leaf tissue two hours after spraying with 10⁻⁵ M ABA.

Lane 7 Leaf tissue 24 hours after spraying with 10⁻⁵ M ABA.

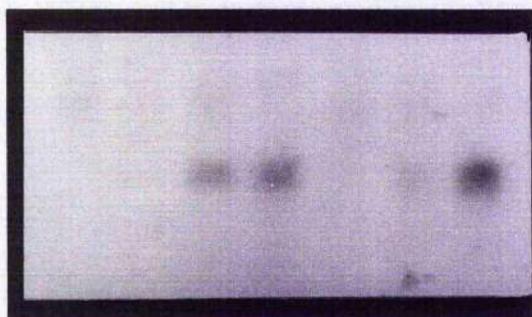
a

1 2 3 4 5 6 7



b

1 2 3 4 5 6 7



5.5 Expression of *BnPRP* in response to heat-shock

No expression of *BnPRP* was detected when the plants were heat-shocked at 40°C for up to eight hours (Fig. 5.5). During the heat-shock treatment the plants were placed in to plastic bags, to help prevent water loss during the treatment. Longer treatments could not be given as the leaves after eight to ten hours of heat-shock appeared wilted and failed to regain turgor after the treatment. No control probe was used as the plants were obviously undergoing heat-shock.

5.6 Expression of *BnPRP* in response to wounding

Leaf tissue was cut into small pieces, approximately 25 mm², and incubated at the normal growth conditions. The wounded tissue was placed on damp filter paper and placed inside a Petri dish to prevent dehydration of the tissue. The wounded tissue showed no detectable increase in *BnPRP* transcripts from one to 48 hours post-wounding (Fig. 5.6). A control experiment was carried out to ensure that the wounded tissue was still capable of activating gene transcription, transcript stabilisation, or a combination of both. Wounded tissue was incubated in the experimental conditions for between 24 hours to five days and then cold treated for 24 hours.

A low level of *BnPRP* transcripts was detected 12 hours post-wounding with no cold treatment. The levels are minimal when compared to the level detected after 24 hours wounding followed by 24 hours of cold treatment and are comparable with the levels occasionally detected in untreated plants. The *BnPRP* transcript levels were lower in leaves which received 48 hours wounding followed by 24 hours of cold treatment, and were not detectable after 5 days wounding followed by 24 hours of cold treatment, which indicated that the leaf tissue had severely deteriorated after five days of wounding.

Figure 5.5 Heat-induced expression of *BnPRP*

Total RNA was isolated from leaves of 21 day old plants grown at 22°C that had been heat-shocked at 40°C for the times indicated. Fifteen µg of the RNA was separated on a 1.5% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed with the BnPRP cDNA and after washing the blot was autoradiographed for 20 hours.

Lane 1 Leaf tissue from plants treated at 4°C for 24 hours.

Lane 2 Leaf tissue from plants grown continually at 22°C.

Lane 3 Leaf tissue from plants heat-shocked for one hour.

Lane 4 Leaf tissue from plants heat-shocked for two hours.

Lane 5 Leaf tissue from plants heat-shocked for four hours.

Lane 6 Leaf tissue from plants heat-shocked for eight hours.

1 2 3 4 5 6

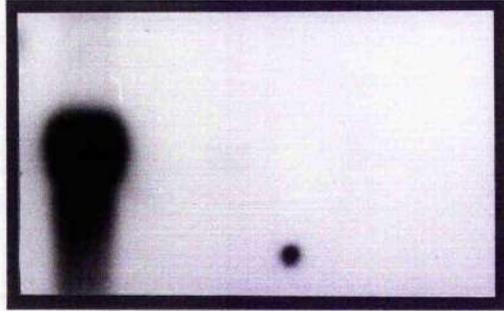
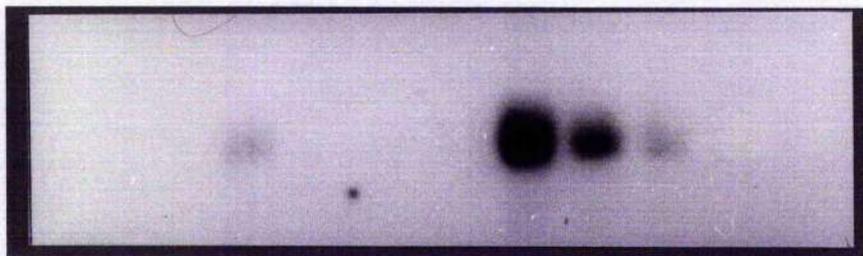


Figure 5.6 Effect of wounding on expression of *BnPRP*

Total RNA was isolated from 21 day old plants grown at 22°C after the leaf tissue had been wounded. As a control, leaf tissue was wounded and then cold treated at 4°C for 24 hours to ensure the leaf tissue was still capable of responding to stimuli. Fifteen µg of the RNA was separated on a 1.5% denaturing agarose gel and blotted onto a nylon membrane. The northern blot was probed using the cDNA *BnPRP*. Standard hybridisation and washing conditions were used. The blot was autoradiographed for 20 hours.

- Lane 1 Leaf tissue from plants wounded for zero hours.
- Lane 2 Leaf tissue from plants wounded for six hours.
- Lane 3 Leaf tissue from plants wounded for 12 hours.
- Lane 4 Leaf tissue from plants wounded for 24 hours.
- Lane 5 Leaf tissue from plants wounded for 48 hours.
- Lane 6 Leaf tissue from plants wounded for five days.
- Lane 7 Leaf tissue from plants wounded for zero hours and cold treated for 24 hours.
- Lane 8 Leaf tissue from plants wounded for 24 hours and then cold treated for 24 hours.
- Lane 9 Leaf tissue from plants wounded for 48 hours and then cold treated for 24 hours.
- Lane 10 Leaf tissue from plants wounded for five days and then cold treated for 24 hours.

1 2 3 4 5 6 7 8 9 10



5.7 Expression of a *BnPRP* cognate in *Arabidopsis thaliana* in response to low temperatures

It was important to see if *Arabidopsis thaliana* contained a cognate of *BnPRP* that responded in a cold-induced manner as it was intended to transform *Arabidopsis thaliana* with a promoter-GUS construct to further investigate the expression of *BnPRP* (Chapter 6). *Arabidopsis thaliana* plants were cold treated at 4°C and leaves were harvested at the same time points used to investigate the cold-induced expression in *Brassica napus*. The northern blot was probed with the *BnPRP* cDNA, but the hybridisation and washing conditions were less stringent than used in all the other northern blots. RNA isolated from the leaf tissue of cold treated and non-cold treated *Brassica napus* plants was included on the blot as controls (Fig. 5.7). The RNA isolated from the cold treated *Brassica napus* plants hybridised as expected with the probe and the RNA from the *Brassica napus* plants treated continually at 22°C also produced a relatively large, though lower signal, which was substantially reduced when the blot was washed using the normal conditions (result not shown). Transcripts that hybridised with the *BnPRP* cDNA probe were detected in *Arabidopsis thaliana*. Unlike in *Brassica napus*, the transcripts were present before any cold treatment. A detectable increase in hybridising transcripts was detectable with cold treatment, and this was particularly noticeable after 12 and 24 hours of cold treatment. The levels appeared to be reasonably constant between 12 and 24 hours of cold treatment suggesting that the increase in transcript abundance had reached a plateau. Experiments using longer time courses were not carried out. No change in transcript abundance was detectable one hour after returning the plants to the control temperatures. A decline was detectable after eight hours, although the transcript level appears to be higher than the basal level. Virtually no transcripts in *Arabidopsis thaliana* were detectable after the blot was washed at high stringency (result not shown).

Figure 5.7 Cold induction of *BnPRP* in *Arabidopsis thaliana*

Total RNA was extracted from leaf tissue of 21 day old *Brassica napus* and *Arabidopsis thaliana* after varying intervals of treatment at 4°C. Fifteen µg of the RNA was separated on a 1.5% denaturing agarose gel and blotted onto a nylon membrane.

The northern blot was probed using the cDNA *BnPRP*, the blot was hybridised at 35°C and washed twice in 2X SSC, 0.1% SDS and once in 1X SSC, 0.1% SDS at 48°C.

The blot was autoradiographed for 20 hours.

- Lane 1 Leaf tissue from *Brassica napus* plants cold treated for 24 hours.
- Lane 2 Leaf tissue from *Brassica napus* plants grown continually at 22°C.
- Lane 3 Leaf tissue from *Arabidopsis thaliana* plants cold treated for zero hours.
- Lane 4 Leaf tissue from *Arabidopsis thaliana* plants cold treated for one hour.
- Lane 5 Leaf tissue from *Arabidopsis thaliana* plants cold treated for two hours.
- Lane 6 Leaf tissue from *Arabidopsis thaliana* plants cold treated for four hours.
- Lane 7 Leaf tissue from *Arabidopsis thaliana* plants cold treated for eight hours.
- Lane 8 Leaf tissue from *Arabidopsis thaliana* plants cold treated for 12 hours.
- Lane 9 Leaf tissue from *Arabidopsis thaliana* plants cold treated for 24 hours.
- Lane 10 Leaf tissue from *Arabidopsis thaliana* plants cold treated for 24 hours returned to 22°C for one hour.
- Lane 11 Leaf tissue from *Arabidopsis thaliana* plants cold treated for 24 hours returned to 22°C for eight hours.

1 2 3 4 5 6 7 8 9 10 11



5.8 Discussion

The *BnPRP* transcript appears to be induced specifically by low temperatures. However, it is possible that stresses we have not investigated may affect the expression of *BnPRP*. We have also not investigated any developmental aspects of the expression.

In most of the experiments no basal level of *BnPRP* transcripts was detected at control temperatures (22°C). Occasionally a low level was detected and this was most noticeable when the northern blot was hybridised and washed at lower stringency than normal (Fig. 5.7). It is likely that the transcript hybridising to the BnPRP probe in RNA from untreated *Brassica napus* leaf tissue is from a gene in the same gene family as *BnPRP* which appears to be regulated in a different manner. This interpretation is supported by the signal with the BnPRP cDNA probe diminishing from the control plants but not the cold treated plants after the blot was washed at high stringency (data not shown). The presence of a gene family which *BnPRP* is a member of is supported by the sequencing of the related cDNA LF5B1 (Chapter 3) and a Southern blot of *Brassica napus* DNA using the cDNA LF5B1 as a probe (Pallas, 1992). The second transcript that the BnPRP cDNA probe hybridised to may also increase in response to cold treatment, but it is not possible to tell as differences in the transcript sizes, if any size differences exist, are indistinguishable on the northern blot. Other cold-induced gene families have members that are differentially regulated. *Kin2* from *Arabidopsis thaliana* is expressed at a basal level while the closely related *Kin1* requires six hours of cold treatment before any transcripts can be detected. Both *Kin1* and *Kin2* increase in abundance in response to cold treatment (Kurkela and Borg-Franck, 1992). Similarly *rit1421*, a cold-induced gene from rye is expressed in the mature leaf tissue of plants grown at 20°C day/15°C night. The related *rit1412* transcripts are not detected unless the plants are grown at 6°C day/2°C nights (Zhang *et al.*, 1993). The transcripts that

BnPRP cDNA hybridises to may also display different regulation between different tissues and in different cultivars.

There is only one size of transcript detected on the northern blots. A second faint band below the main one is sometimes detected and since this corresponds to the position of one of the ribosomal RNA bands it is thought that it is non-specific hybridisation. This is supported by the observation that on the blot of poly(A)⁺ RNA (Fig. 3.5), no second band is detected. The size of the transcripts that BnPRP cDNA hybridises to was not calculated accurately, though the transcripts are approximately the same size as the transcripts that LF5B1 hybridised to. The transcripts are of the size expected from the sequence of λ BnPRP.

The relatively rapid induction of *BnPRP* transcripts in response to low temperature, and the rapid decline in transcript abundance upon removal of the cold stimulus is comparable to a number of other cold-induced genes. The length of cold treatment required to produce a detectable increase in transcript abundance and subsequent maximal levels does vary considerably between genes. Similarly the time that the transcripts remain after the cold stimulus is removed varies widely. For example, the transcripts of *cor39* from wheat is detected after two hours of cold treatment, the transcript level does not increase significantly between ten hours and two weeks of cold treatment, and after removal of the stimulus the transcript is not detectable after four hours (Guo *et al.*, 1992). Transcripts of *Kin1* from *Arabidopsis thaliana* are detected after six hours of cold treatment, the transcript levels are relatively constant between one and seven days of cold treatment and are present at moderate levels 12 hours after the plants are returned to control temperatures (Kurkela and Franck, 1990). Other genes show an extended accumulation period, for example transcripts that hybridise to the cDNAs SM784 (*cas18*), SM2201 (*cas15*), and SM2358 from alfalfa, and *RC11* and *RC12* from *Arabidopsis thaliana* all take approximately seven days to accumulate to the maximal levels (Mohapatra *et al.*, 1989; Jarillo *et al.*,

1994). No information on the rate that the transcript levels fall after removal of the cold stimuli is available for the alfalfa transcripts. The *Arabidopsis thaliana* *RC11* transcript is not detectable after one day deacclimation whereas the *RC12* transcript is still relatively abundant three days after removal of the cold stimulus, but not detectable seven days after removal from the cold stimulus (Jarillo *et al.*, 1994).

The analysis of the transcript abundance revealed that the transcript that BnPRP cDNA hybridises to is very unstable at 22°C. The level of control of the cold-induced transcript is not known but it is likely that the transcripts are unstable at 22°C otherwise the levels of transcript would not diminish as rapidly. The instability may be inherent in the transcripts. This has been observed with the *phyA* transcripts from oats (Seeley *et al.*, 1992). The observed increase in transcript levels may be caused by increased promoter activity. However, the increase in transcript level could also be caused by stabilisation of the transcript in response to the low temperature stimulus. The *E17* transcript increases six fold in tomato fruit in response to ethylene with no increase in transcription rate (Lincoln and Fischer, 1988). A combination of both transcriptional and post transcriptional mechanisms is possible and this has been observed in the *Fed-1* transcript of pea (Gallo-Meagher *et al.*, 1992; Dickey *et al.*, 1992).

Elements have been identified in the untranslated regions of genes that have a direct effect on transcript stability. Animal transcripts with a high AU level and multiple repeats of AUUUA in the untranslated regions have been shown to be unstable (Shaw and Kamen, 1986; Caput *et al.*, 1986). The AUUUA sequence has been shown to destabilise the β -glucuronidase transcript when inserted into the 3' untranslated region (Ohme-Takagi *et al.*, 1993). The AUUUA motif has also been found in unstable plant genes, in tobacco the *par* transcript contains three of the motifs (Takahashi *et al.*, 1989). The unstable *AUX22* transcript from soybean contains six similar AUUAA sequences (Ainley *et al.*, 1988) but the involvement of these sequences in the instability of these transcripts has not been demonstrated. One element from

plant genes that has been directly linked to transcript instability is the DST element. It was discovered in the small auxin-up-regulated (SAUR) transcripts from soybean. The element is located in the untranslated region, between 40 and 100 bp downstream of the stop codon (Newman *et al.*, 1993). A synthetic dimer of the DST element destabilised the β -globin reporter transcript when it was inserted into the untranslated region (Newman *et al.*, 1993).

The AUUUA and DST elements are not present in the *BnPRP* or *LF5B1* untranslated regions. However, the transcripts that the *BnPRP* cDNA probe hybridises to may not be from only the *BnPRP* gene, but could include related members of the gene family. The *BnPRP* transcript may be relatively stable and not strongly cold-induced, and the cold-induced transcripts could come from another related gene. If this is true the untranslated region would not be expected to contain any instability elements. The *LF5B1* may represent a cold-induced transcript, though this has not been proved. Again the transcripts that pPL5B1 recognises could be encoded by another member of the gene family. Alternatively, if the *LF5B1* and *BnPRP* transcripts are cold-induced and unstable at control temperatures (22°C) the untranslated region could contain unidentified instability elements. Or the transcript stability could be controlled by other mechanisms such as using different polyadenylation sites, which has shown to have a large effect on the stability of some transcripts (An *et al.*, 1989; Ingelbrecht *et al.*, 1989). Other as yet uncharacterised instability mechanisms may play a role.

The *BnPRP* transcripts were only detected in the leaf and stem tissue of cold-treated plants. Similar examples of tissue specific expression of cold-induced genes have been reported. For example Cattivelli and Bartels (1990) isolated five cold-induced cDNAs, three of these pAO29, pAO86, and pAF93 were found in all tissues, pT59 hybridised only to transcripts from cold-treated leaf and shoot tissue, pV60 was only detected in cold-treated shoots.

The majority of cold-induced genes are induced by drought and the application of ABA. However, some other genes show no response to dehydration and the application of ABA. These include *Acs784* (*SM784/cas18*), *Acs2201* (*SM2201/cas15*), and *Acs2358* (*SM2358*) (Mohapatra *et al.*, 1989), *msaCIC* from alfalfa (Castonguay *et al.*, 1994), and *Wcs120* from wheat (Horde *et al.*, 1992). Others are stimulated by dehydration and/or ABA to some degree, although the extent to which the transcript is induced varies. For some, such as *cor47* (*HH7.2*), *HH28*, *cor6.6* (*HH29*), *cor15* (*HH67*) from *Arabidopsis thaliana* (Hajela *et al.*, 1990) and *lti78* from *Arabidopsis thaliana* (Nordin *et al.*, 1993) induction by ABA and dehydration is to a lesser degree than induction by low temperatures. Other genes such as *lti65* are induced to a greater extent by ABA and dehydration than by low temperatures. Other cold-induced genes, for example *Kin1* (Kurkela and Borg-Franck, 1992) are induced by ABA and not dehydration, whereas others like *BN115* (Weretilnyk *et al.*, 1993) are induced by dehydration and not ABA application. The use of the control probe BnD22 which hybridised to *BnD22* transcripts from dehydrated and ABA treated plants validates the lack of *BnPRP* expression in response to these stimuli.

The lack of expression in response to heat-shock is not unusual for cold-induced genes, very few of the genes appear to be general temperature-shock genes. Nevertheless a few cold-induced transcripts do increase abundance in response to heat-shock. The C14 gene from tomato (Schaffer *et al.*, 1990) accumulates in the fruit after eight hours of heat-shock at 40°C. The C14 clone has sequence homology to several proteases (Schaffer *et al.*, 1988). The only other transcripts that have been shown to accumulate in response to cold treatment and heat-shock are heat-shock genes. The *hsp90* transcript accumulates rapidly at both 5°C and 42°C, changes are also detectable at the protein level (Krishna and Oeda, 1995). Proteins that increase in response to both high and low temperatures have also been identified. Several 79 kDa proteins accumulate at 5°C in spinach and these are cross reactive to the 79 kDa heat-shock

proteins; mRNA levels were also shown to increase (Neven *et al.*, 1992). Heat-shock proteins of 140 kDa and 85 kDa have been detected in the leaves of cold treated plants, the 85 kDa heat-shock protein also increased in response to dehydration, ABA, and wounding (Kazuoka *et al.*, 1992).

The cold-induced, proline-rich gene transcript *msaCIC* was found to accumulate in response to wounding to levels comparable to cold-induced levels (Castonguay *et al.*, 1994). No significant response of *BnPRP* to wounding was found, although a control probe was not used to ensure the tissue was undergoing a wound response. This was seen as unnecessary as the leaf tissue was clearly injured, and was shown to be capable of responding to the low temperature stimulus and therefore should have been able to respond to the wound-induced stimulus. The majority of cold-induced genes identified have not been studied in response to wounding and no wound-induction of a gene that is also cold-induced apart from *msaCIC* has been reported.

Apart from *msaCIC*, none of the other cDNAs that display high sequence similarity to *BnPRP* have been reported to be cold-induced. These are induced by a diverse set of stimuli, but none have been reported to respond to any abiotic stress. Other proline-rich proteins have been reported to be cold-induced. These were identified by using an extensin genomic clone (pDC5A1) to probe northern blots containing RNA from cold treated pea seedlings (Weiser *et al.*, 1990). None of the cloned proline-rich proteins have been reported to be cold-induced (Josè and Puigdomènech, 1993).

A transcript that hybridises to the *BnPRP* cDNA probe was detectable in *Arabidopsis thaliana* leaf tissue. The presence of cognates has been reported for other cold-induced genes from *Brassica napus* and *Arabidopsis thaliana*. The *cor6.6* and *cor15a* genes from *Arabidopsis thaliana* (Hajela *et al.*, 1990; Lin and Thomashow, 1992a) are homologous to *BN28* and *BN115* from *Brassica napus* (Orr *et al.*, 1992;

Weretilnyk *et al.*, 1993). The regulation of the *Arabidopsis thaliana* *BnPRP* cognate seems to be different to *BnPRP* in *Brassica napus*. Cold-induction was detectable though the hybridising transcripts were also present in plants that had undergone no cold treatment. This could be due to a genuine difference in the regulation of the genes in the two plants or it could be caused by detecting two or more different transcripts in *Arabidopsis thaliana*. One or more of the genes detected could be expressed in a basal manner and may or may not increase in response to cold treatment. The other gene(s) may be expressed only in response to low temperatures. Southern blots of *Arabidopsis thaliana* DNA probed with LF5B1 revealed that two to four copies of a homologous gene were present in *Arabidopsis thaliana*. (Pallas, 1992). These different genes may well be differentially regulated. This appears to occur in *Brassica napus*, as transcripts that are present with no cold treatment were detected when northern blots were washed at low stringency. As discussed previously, examples of differential regulation have been reported in other gene families.

Chapter 6: Analysis of the putative *BnPRP* promoter

6.1 Introduction

The genomic *BnPRP* clone contained sequence upstream of the putative open reading frame which would be likely to include the gene's promoter. The 2.4 kb genomic *EcoR* I-*Sal* I fragment contained 1.35 kb of DNA 5' of the putative open reading frame. A further eight kb was present in the adjoining fragment generated by digestion with *EcoR* I and *Sal* I (Fig. 4.5). It is likely that the 1.35 kb of DNA immediately upstream of the putative open reading frame of *BnPRP* contains sufficient information to direct expression of *BnPRP*, though this depends upon the location of the transcription start site. Isolation of this fragment of DNA would allow a promoter-GUS construct to be produced and transformation of this into a host plant would allow the expression of the gene to be further investigated. The putative promoter region was also analysed to see if any characterised sequence motifs were present.

6.2 Isolation of the putative promoter

Analysis of the putative promoter sequence from the 2.4 kb genomic fragment using the UWGCG programme MAP revealed no suitable restriction enzyme sites that would enable subcloning of the putative promoter sequence. Instead PCR was used to isolate the putative promoter (Fig. 6.1a). A primer named P1 was made (5' GAA GAT CTA GAC TCT TAG AAT GAG GGT TTG GGT TG 3'). Twenty four nucleotides of P1 corresponded to the sequence complementary and immediately 5' of the open reading frame. An extra 11 nucleotides were added onto the 5' end of the primer incorporating two restriction enzyme sites (*Xba* I and *Bgl* II) to ease subsequent

Figure 6.1 Isolation of the putative promoter sequence

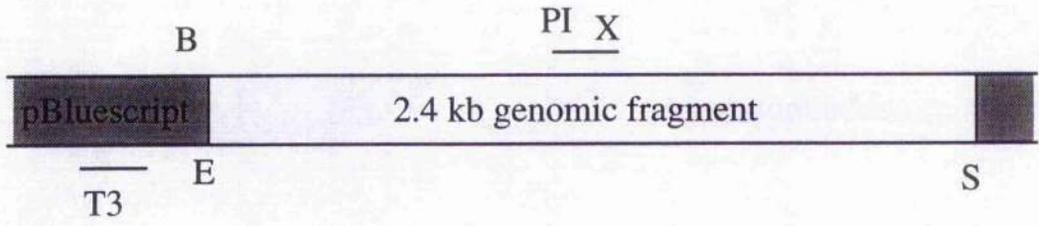
The putative promoter was isolated using PCR from the 2.4 kb genomic clone (plasmid G).

a The T3 and PI primers (described in Table 4.1 and Section 6.2) were used to amplify the putative promoter region in a PCR reaction. The diagram shows the 2.4 kb genomic fragment within pBluescript[®] II SK (-) (represented by the shaded areas). The restriction sites used to clone the fragment and subsequently used to clone the PCR product into pBluescript[®] II SK (-) and PBI101.1 are indicated.

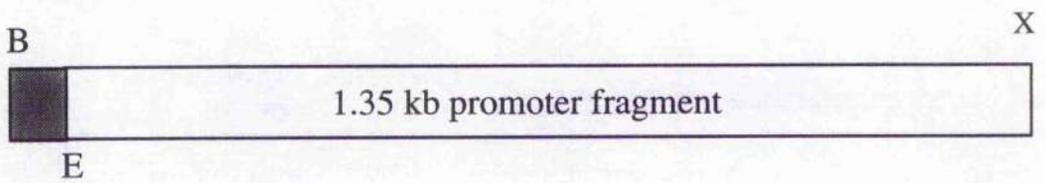
b The PCR product was isolated and separated from target plasmid DNA and unincorporated primers. After cutting the PCR product with the restriction enzymes *Xba* I and *Bam*H I, it was cloned into the same sites of pBluescript[®] II SK (-) for amplification and sequencing. The diagram shows the insert.

B = *Bam*H I, E = *Eco*R I, S = *Sal* I, X = *Xba* I

a



b



Cloned into pBluescript

cloning of the amplified fragment. The second primer used to amplify the putative promoter was the T3 primer (5' AAT TAA CCC TCA CTA AAG GG 3'). This primed from the proximal end of the multicloning site in pBluescript® II SK (-). The primers were included in a PCR reaction with the plasmid G as the template. The PCR produced a product of 1,448 bp, 1,354 bp of putative promoter together with 11 bp of the PI primer containing the restriction sites, and 83 bp of pBluescript® II SK (-) (Fig. 6.1b). The PCR product was gel purified, and subsequently digested with the restriction enzymes *Xba* I and *Bam*H I, and ligated into the same sites in pBluescript® II SK (-).

The *Pfu* DNA polymerase was used to amplify the putative promoter. This was used in preference to *Taq* DNA polymerase as the *Pfu* has a 12 fold higher fidelity (Stratagene product catalogue, 1994). The higher fidelity is important when amplifying the putative promoter region as small changes in the sequence may have a large effect on the promoter's activity in transgenic plants. The isolated promoter was sequenced to ensure no errors had been incorporated during the PCR. The primers that were used when the promoter region was initially sequenced as a part of plasmid G (Fig. 4.6) were again used. The sequence is shown in Fig. 6.2, sequence motifs that are discussed in Section 6.3 are indicated. Sequencing revealed that no changes had been incorporated during the amplification. The putative promoter region was easy to manipulate once subcloned into pBluescript® II SK (-).

6.3 Analysis of the *BnPRP* upstream sequence

The putative *BnPRP* 5' non-coding sequence was analysed for sequence motifs characteristic of all plant promoters and those specific to cold-induced promoters. Other features that may be important in the regulation of the putative promoter were identified. The putative *cis*-elements of most interest are shown in Fig. 6.2.

Figure 6.2 Sequence of the putative *BnPRP* promoter

The primers used to sequence the promoter are shown in Fig. 4.7. In addition the T3 and T7 primers were used respectively to sequence the 3' and 5' regions of the isolated putative promoter after it was cloned into pBluescript® II SK (-).

The numbers refer to the nucleotides at the end of each line, with reference to the first nucleotide of the putative open reading frame. The *cis*-elements that potentially play a role in the regulation of the promoter are indicated. The shaded boxes with no annotation are all G box related with the ACGT core motif. Two putative LTREs are shown along with a sequence element that is similar to the D motif from *cor15a*. Two 25 bp direct repeats are heavily underlined along with putative CAAT and TATA boxes. A putative transcription initiation site (-48 to -54) is also underlined; the A nucleotide is heavily underlined, indicating the putative point of transcript initiation. The first two residues of BNPRP (M + G) are shown to indicate the putative translation start point.

GAATTCTGAA ATTCATTCTC ATGGTCA**CAC** **GTG**ACTACAA AATAATGTTG -1305
 TAATGTTTCA CAATTAATAT ATAAAAAAGA TTATTGGCAT GCTTCACGAA -1255
 GCTAATTTTT GACCAAATAT**T** **GCA**AATATAA ACTATGGAAA TTTCTTAGGA -1205
 ACATACTAGT AATGAAAATC ATTGGAGATC GTECTATCGT TATTCGTTAC -1155
 TGATTCTTT**T** **GCA**AAAATAA ACATATAAGA AATGATTATG CTAGTTTATG -1105
 TTGCTGAGTG CTACAGTTTA TGGGTCTGAC CGGTGACCAT TCATAAAACA -1055
 AGAGGAACAA CAGAAAAAGA ATATACCGGA AAAAAATAGC AAGAATGAAA -1005
 GTGAATGGTT ATTGCTTCTC AAATTTAATA AGGAATAATT TTATTTTTTA -955
 ATTTTCTACA AAAAAAAGA ACGAAAAAGA ATGAGATAGA ATTATTATTC -905
 CTTGTGAATG GTGATTTTTT ATAGGA**ACGT** TAGAGAA**TGC** **A**TTATTCCC -855
 ATCATTCTT GGTACCTTT CGTAAAATAG TGTGTTTTT CTAATTAAGC -805
 ATTTTCATGT TTTTGTTTTT CTAACATTT TCATGTTGTT TGTTTTCTA -755
TACATTTCA TGTTACGGTA TATATAGGCT GAGCATGTCC AAGCTATTCA -705
 AAATTAACCG AATCGTGGAA CAGATCCAGT AACTAGCTAA TTCATAG**TTC** -655
 D motif
TATTAAAATTT GAGGTTAAGT ATATAAAAGA AAAGAAAAA CAAAGAAATG -605
 AATAGTAACA AACTAGAGA GAGAACAGAA AGGAATAGAG ATTTTGCCGA -555
 AACTTTTCTA AGTACAGTGT CTGAGTATTT TTTTGGTAAT CATGTTAAAT -505
 CAGTGTCTGA GTTCTTCAA CCTTCTCAGA CTTGTCGTTT GTAATTAATC -455
 LIRE-1
 ATTATC**TGGC** **CGAT**TAAAAA TGATTA**ACGT** ATTTGATTTT GTGTTTTCCG -405
 AATGCTTTCA CACTAACCAA TTACATTTTC ACAAATTTA CAAATAGGTT -355
 LIRE-2
 CGTAGAGC**AT** **GCAGCC**ATGT GAT**GAGCAG**A AAAGTGAACA ATTCTTTAGC -305
 AGTTTACTTA TATCA**ACGT**A AATAGCGAAT GTTATTCCTA AATTTCCCTG -255
 AAAAGATAAG AATCTACGAC TACAAAGATA CGCCAATTTA GGAAAGTATG -205
 ACAAACCCTT TAATTTGTT TTGGAACATA GTGACCTTAT ACCTGCTTAA -155
 TAAAATCTAA TATT**CCAATC** ATCTAATCAC AGTTTGGTGC TTACACTATT -105
 AAAAAATAAC AAATATCGGC GAATACTTTT TCTATAAAAG GGTGCACACT -55
ATCATATACC TACTTACTAA GTCGTTTACA CAACCCAAAC CCTCATTCTA -5
 AGAGATGGGG

A number of *cis*-sequences have been found to be important for the translation and transcription of genes. In order to successfully translate a messenger RNA eukaryotic ribosomes recognise a sequence motif known as the Kozak sequence. The 40S ribosome is proposed to scan from the 5' cap site, stopping at the first AUG codon in a favourable context (Kozak, 1989). The consensus sequence in plants is TAAACAATGGCT (Joshi, 1987a). *BnPRP* has the sequence TAAGAGATGGGG. We do not know the point of transcription initiation. Plants have the weakly conserved transcript initiation sequence CTCATCA (Joshi, 1987a) and a sequence similar to this (ATCATAT) is present in *BnPRP* at -54 to -48. Two putative TATA boxes are present between nucleotides -1 and -300; the first is at -50 to -47, the second between -72 and -69. A putative CAAT box is also present in the *BnPRP* putative promoter, located between -139 and -136.

As well as putative TATA and CAAT boxes *BnPRP* also contains several putative USEs (upstream elements) related to the G-box (CACGTG), containing the ACCG core motif. This motif is present in several regulatory elements (Armstrong *et al.*, 1992). These were positioned in the forward orientation between -1327 and -1322, -878 and -875, -428 and -425, and between -289 and -286. They also occur in the reverse orientation between -1235 and -1232, -1145 and -1142, and between -867 and -864. A G-box like element (CACGAG) is present in the reverse orientation between -331 to -326.

One element labelled LTRE-1 (low temperature response element-1) in Fig. 6.2 is similar to the TGGCCGAC element (LTRE-1/2) identified in *cor15a* and *BN115* (Baker *et al.*, 1994; White, T.C., *et al.*, 1994), except there is a one bp difference in *BnPRP*. The difference occurs in the LTRE core element (CCGAC). The core LTRE element is present once in the reverse orientation, located within the motif LTRE-2 between -343 and -339.

Two imperfect 25 bp direct repeats are present at -792 to -768 and -765 to -741, these differ by only one bp. A single copy of a motif that was found to be repeated in the promoter of *cor15a* is present between -657 and -647. The motif, TTCTATTACAA, from *cor15a* (Baker *et al.*, 1994) was the D motif; the related repeat in *BnPRP* varies by one bp.

6.4 Transformation of *Arabidopsis thaliana* with promoter-GUS constructs.

6.4.1 Production of the promoter-GUS construct

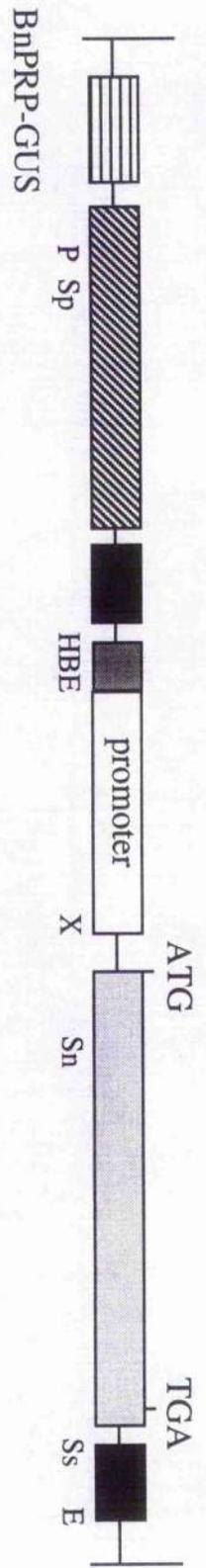
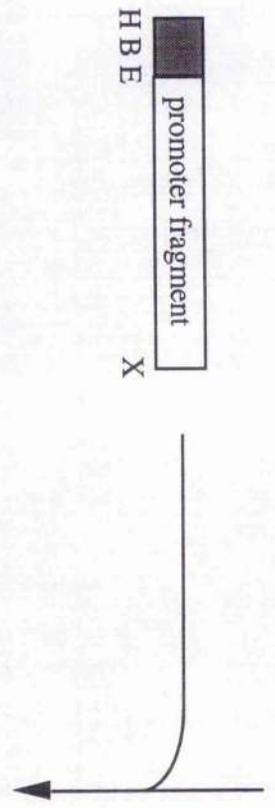
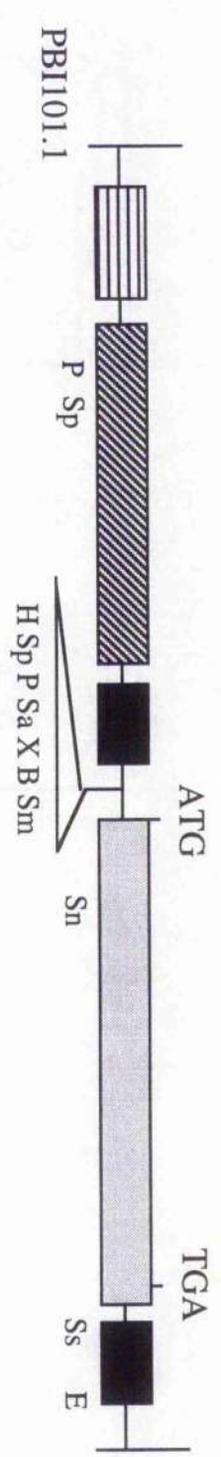
The isolated promoter fragment was cloned into the multicloning site of PBI101.1 a 'promoterless' GUS cassette in the *Agrobacterium* binary vector pBIN19 (Fig. 6.3) (Jefferson *et al.*, 1987). It contains a promoterless gene encoding GUS. The multicloning site lies 5' to the *GUS* coding region. Sequences from the 3' non-coding region of the nopaline synthase gene which include a polyadenylation site lies 3' of the *GUS* coding region. PBI101.1 also contains the *npt-II* coding sequence transcribed under the control of the nopaline synthase promoter in plants. The gene product confers resistance to kanamycin in transformed plant cells and facilitates selection of transformants. The pBIN19 vector contains a second *npt-II* gene which is active in, and confers kanamycin resistance to bacteria.

The putative promoter fragment was excised from pBluescript® II SK (-) with the restriction enzymes *Xba* I and *Hind* III and directionally cloned into the multicloning site of PBI101.1. The -1 nucleotide (with respect to the translation start site) of the *BnPRP* gene lies 30 nucleotides 5' of the adenine of the ATG translation initiation site of the β -glucuronidase protein. The excised fragment contained 46 bp from pBluescript® II SK (-) 5' of the putative promoter and therefore 1,384 bp 5' of

Figure 6.3 Construction of the plasmid BnPRP-GUS

The putative promoter fragment was excised from pBluescript[®] II SK (-) with the restriction enzymes *Xba* I and *Hind* III. After isolation from pBluescript[®] II SK (-) it was cloned into the corresponding restriction sites in PBI101.1. The different boxes are not drawn to scale.

B = *Bam*HI, E = *Eco*R I, H = *Hind* III, P = *Pst* I, Sa = *Sal* I, Sm = *Sma* I, Sn = *Sna*B I, Sp = *Sph* I, Ss = *Sst* I, X = *Xba* I



-  *nos* promoter
-  *npt*-II coding sequence
-  *nos* poly(A) signal
-  pBluescript fragment
-  β -glucuronidase coding sequence

the ATG site of the β -glucuronidase (Fig. 6.3). The promoter-GUS fusion was examined by restriction analysis to ensure that only one copy of the putative promoter was in place. The plasmid was digested with *Bam*H I and *Hind* III. The *Bam*H I site is situated 3' of the *Xba* I site in the PBI101.1 multicloning site; these enzymes excised the entire subcloned fragment, which was approximately 1.4 kb long (results not shown) and therefore could only contain one copy of the putative promoter. The structure of the construct was also examined by sequencing. A primer that primed from the *GUS* coding region (5' GAT TTC ACG GGT TGG GGT TTC T 3') was used to sequence across *Xba* I restriction site; this revealed the expected sequence. The construct was named pBnPRP-GUS.

6.4.2 Transformation of *Agrobacterium tumefaciens*

The pBnPRP-GUS construct was transferred from *E. coli* XL1-Blue to *Agrobacterium tumefaciens* strain C58^{C1} by triparental mating together with *E. coli* DH5 α containing the helper plasmid pRK2013. Two separate triparental matings were done. A streak was taken from each of two plates containing the three strains. These were restreaked to give two sets of individual colonies of transformed *A. tumefaciens*. A single colony was picked from each of the independent triparental matings. Plasmid DNA was prepared from each of these colonies and analysed to ensure that no major rearrangements had occurred during triparental mating. In order to analyse the pBnPRP-GUS DNA the plasmid was retransformed into *E. coli* XL1-Blue to allow easier manipulation of the DNA. The DNA from each of the transformations was digested with *Dde* I and *Sau*3A I and the resulting DNA fragments were analysed on an agarose gel (Fig. 6.4). Both the enzymes are frequent cutters and give a characteristic profile. The profiles of both the isolates appear to be identical; this is good evidence that no major rearrangements occurred during the triparental mating as it is unlikely that the same rearrangements would occur in two independent triparental matings.

Figure 6.4 Analysis of the pBnPRP-GUS construct after triparental mating

The BnPRP-GUS construct was transferred into *Agrobacterium tumefaciens* by triparental mating. To ensure that no large rearrangements/deletions occurred during the triparental mating two of the isolates were analysed. The BnPRP-GUS plasmid was isolated from the *Agrobacterium* host and used to transform *E.coli* (XL1-Blue) to aid manipulation of the DNA. BnPRP-GUS plasmid DNA was isolated from the recipient *E.coli* and digested with two restriction enzymes, *Dde* I and *Sau* 3AI. These enzymes cut frequently within the plasmid, producing a characteristic profile.

The products of the restriction digest were separated on a 1.5% agarose gel. The numbers down the right indicate the size of selected markers from the kb ladder, the sizes are in bp.

- Lane 1 Isolate A digested with *Dde* I.
- Lane 2 Isolate B digested with *Dde* I.
- Lane 3 Isolate A digested with *Sau* 3AI.
- Lane 4 Isolate B digested with *Sau* 3AI.

1 2 3 4 5 6



← 4,000

← 500

← 75

6.4.3 Transformation of *Arabidopsis thaliana*

The isolate A of *A. tumefaciens* containing pBnPRP-GUS was used to transform root explants of *Arabidopsis thaliana* ecotype Landsberg erecta were transformed using the method of Valvekens *et al.* (1988). Nine putative primary transformants were obtained, this was based on their kanamycin resistance. Transformed plants containing pBnPRP-GUS were selfed and the seedlings (T1 generation) analysed. Seed was collected from individual T1 plants that had not been grown with kanamycin in the media, these plants had self-fertilised and this formed the T2 generation.

6.4.4 Analysis of the transformants

Seed from the T1 and T2 generations was grown on germination medium plus 50 µg/ml kanamycin. Table 6.1 details the phenotype of the T1 and T2 generations of transformants with respect to kanamycin resistance. Of the nine transformants obtained five of the plants gave some progeny which were kanamycin resistant, while four of the plants gave no kanamycin resistant phenotypes. The progeny from the transformants 2, 3, and 8 showed no kanamycin resistance and no seed from transformant 6 germinated. Seed collected from the transformants 1, 4, and 7 grown with no selection was chosen for further analysis. These plants were chosen as their progeny showed good germination rates and segregation ratios of around 3:1 (kan^R:kan^S) in the T1 generation. This suggested that single transformation events had occurred in the transformants, although not enough progeny were examined to be confident that this was true. Seed was collected from four selfed T1 plants from each of the selected transformants and this T2 generation was subjected to kanamycin selection (Table 6.1). Some of the individuals from the T2 generation did not show any segregation, progeny from 1², 1⁴, 4² and 4³ were all sensitive to kanamycin; progeny from 1³, 4⁴, and 7⁴

Table 6.1 Analysis of progeny of pBnPRP-GUS transformants

Primary transformants were selfed and the T1 seed was collected. These seeds were plated out on germination medium supplemented with kanamycin. The number of kanamycin resistant (Kan^R ; green leaves developed) and kanamycin sensitive (Kan^S ; cotyledons bleached, leaves did not develop) were counted and the $Kan^R:Kan^S$ ratio calculated. T1 seed from transformants 1, 4, and 7 were grown with no selection, seed (T2) was collected of four individual plants from each transgenic line. The $Kan^R:Kan^S$ analysis was repeated with the T2 seed. Ratios are shown only when segregation was observed.

Plant	Kan ^R	Kan ^S	Kan ^R :Kan ^S	Comments
T1 generation				
1	7	2	3.5	
2	0	3		Low germination
3	0	3		Low germination
4	16	7	2.29	
5	2	2	1	
6	0	0		None germinated
7	7	2	3.5	
8	0	22		
9	1	0		Low germination

T2 generation

1 ¹	16	7	2.29
1 ²	0	23	
1 ³	22	0	
1 ⁴	0	18	
4 ¹	39	5	7.8:1
4 ²	0	29	
4 ³	0	35	
4 ⁴	36	0	
7 ¹	36	8	4.5
7 ²	25	7	3.5
7 ³	18	15	1.2
7 ⁴	43	0	

were all kanamycin resistant. Progeny from all other plants all showed some segregation, the values varied from 7.8:1 to 1:1 (kan^R:kan^S).

6.4.5 GUS expression driven by the *BnPRP* putative promoter

As controls, wild type *Arabidopsis thaliana* were examined for any background GUS activity in the plants. *Arabidopsis thaliana* transformed with the plasmid PBI121.1 (Jefferson *et al.*, 1987) was used as a positive control for X-Gluc staining (supplied by Dr. J.A. Jackson, Dept. Division of Biochemistry and Molecular Biology, University of Glasgow). The PBI121.1 construct contains the β -glucuronidase gene cloned into the binary plasmid vector pBIN 19 behind an 800 bp fragment of the CaMV 35S promoter.

The transformants that were homozygous for kanamycin resistance (1³, 4⁴, and 7⁴) were analysed for β -glucuronidase activity in the T₂ generation. Seedlings grown on germination medium containing kanamycin for ten days post-germination were cold treated for 48 hours and then analysed, control seedlings that had not been cold treated were also analysed. No significant GUS activity could be detected in either normal or cold-treated plants using X-Gluc staining. Seedlings that had been germinated on germination medium containing kanamycin were transferred to potting compost after ten days and grown for a further 11 days. The cold treatment and analysis was repeated, again using control and cold treated plants. Again no GUS activity could be detected using X-Gluc staining. Unfortunately time constraints prevented the expression studies being repeated at different developmental stages or under different environmental stresses. In all experiments GUS activity was detected in the 35S CaMV-GUS positive controls, demonstrating that the assay was working.

6.5 Discussion

The *BnPRP* putative promoter was PCR-amplified without any changes in the nucleotide sequence from the genomic subclone G. This was used along with the PBI101.1 vector to produce the pBnPRP-GUS construct. The pBnPRP-GUS construct was transformed into *Arabidopsis thaliana* plants. Not all of the primary transformants were in fact transformed. The false transformants could have originated from cells close to a part of the explant that had been transformed, thus conferring some degree of kanamycin resistance to the cells around it. Enough genuine transformants were produced to assess the expression of the pBnPRP-GUS construct. Three lines of transformants were chosen to examine the expression. All three of these lines showed some degree of segregation in the T1 generation, good germination rates and appeared to adopt a normal growth habit. The plants from the T2 generation that appeared to be homozygous for the pBnPRP-GUS construct were used in the analysis. The homozygosity was not confirmed by analysis of further generations.

No reporter gene activity was detected in the three lines of plants that were examined. There are a number of reasons for the lack of detectable GUS activity. It is possible that not all the regulatory elements required to direct cold-induced expression of the *GUS* gene were present in the pBnPRP-GUS construct. This seems unlikely as 1,354 bp of the putative promoter was placed into the construct; considerably shorter promoter elements have been sufficient to drive cold-induced expression of reporter genes. The nucleotides -305 to +78, -384 to +53, -417 to +17, and -802 to +100 from the *cor15a*, *Adh*, *rd29A*, and *BNI15* promoters respectively have all driven high levels of GUS expression in transformed plants (Baker *et al.*, 1994; Dolferus *et al.*, 1994, Yamaguchi-Shinozaki and Shinozaki, 1994; White, T.C. *et al.*, 1994). It is possible that a repressor element is present in the *BnPRP* putative promoter which negates any cold-induced activity in the construct. Repressor elements have been reported in the promoters of cold-induced genes. The most powerful element that has been identified

is in the *Arabidopsis thaliana Adh* promoter; deletion of the nucleotides -575 to -510 results in approximately a four fold increase in expression of the *GUS* gene (Dolferus *et al.*, 1994). Such an element may exist in *BnPRP* although it would be unlikely to account for the complete lack of detectable GUS expression and more likely lead to a reduction in expression.

As discussed previously *BnPRP* is probably a member of a gene family and members of gene families have been shown to have differential responses to the same stimulus. It is possible that *BnPRP* is not cold-induced, but highly related to a gene that is cold-induced. Several examples of cold-induced gene families and the differential regulation of members of these families have been discussed, including the *bltLTPs*, *Kin1* and *Kin2*, and *rd29A* and *rd29B* (White, A.J. *et al.*, 1994; Kurkela and Franck, 1990; Kurkela and Borg Franck, 1992; Yamaguchi-Shinozaki and Shinozaki, 1994). Some of the gene families show different degrees of cold-induction within the family. For example, *Kin2* is induced predominantly by drought whereas *Kin1* is induced predominantly by low temperatures (Kurkela and Borg-Franck, 1992). In more extreme cases one member of the family shows no cold-induction. The *rd29A* gene is cold-induced whereas the *rd29B* gene shows no detectable cold-induction (Yamaguchi-Shinozaki and Shinozaki, 1994).

The putative promoter of *BnPRP* may function as a cold-induced promoter *in vivo*. The altered sequence environment of the PBI101.1 cassette may not be conducive to the promoter's activity; for example the increased length of the leader sequence may have a deleterious effect on expression, though no obvious mechanism exists to explain such an occurrence. Alternatively there may be regulatory elements that are not present in the construct but, as discussed above it is unlikely that these would reside 5' of the 1,354 bp included in the construct. There are regulatory elements that have been found 3' of the TATA box. These include the first intron of the maize *Adh1* gene that is required for high levels of expression (Luehrsen and Walbot,

1991) and the coding sequence of the ferredoxin I gene plays a role in gene expression (Elliot *et al.*, 1989). It is unlikely that such an element would play a role in the expression of *BnPRP* as such elements are rare. Also these auxiliary elements may play a role in mRNA stability rather than promoter activity.

As well as being in a heterologous sequence environment in the PBI101.1 vector, the vector has been transformed into a different species. The transformation of *Brassica napus* would have taken too long to perform given the time limitations of the project. *Arabidopsis thaliana* was chosen because of its rapid regeneration time (Dean *et al.*, 1991). It is a suitable host to examine genes from *Brassica napus* because it is a closely related species. The DRE (drought responsive element) from *Arabidopsis thaliana* which also directs cold-induction retained activity in response to dehydration in transgenic tobacco; the effects of low temperature were not reported (Yamaguchi-Shinozaki and Shinozaki, 1994). The cold-induced expression of a *BnPRP* cognate in *Arabidopsis thaliana* (Fig. 5.7) suggested it would be a suitable host to examine the regulation of the gene. However, differential activation of the *BnPRP* promoter may occur between the two species, such differences have even been observed in different cultivars of the same species. The *blt4.2* gene from the Igri cultivar (White, A.J. *et al.*, 1994) is induced by low temperatures but a version of this gene *ltp4*, cloned from the spring cultivar Bomi, which is 98% identical in the transcribed region and presumed to be a different allele of the same gene shows no cold-induction (Molina and Garcia-Olmedo, 1993). The Bomi cultivar does not cold-acclimate to the same extent as the winter Igri cultivar, which may be reflected in the accumulation of the *blt4.2* transcripts in response to low temperatures. *Arabidopsis thaliana* does not acclimate to the same degree as *Brassica napus* and this could explain the difference in *BnPRP* promoter activity. The northern blot data identified increased expression of a *BnPRP* cognate in response to low temperatures. However, a basal level of expression was also detected, so cognates may be present in *Arabidopsis thaliana* which are not induced by low temperatures. A final possible explanation for the lack of GUS expression is the

potential incorporation of the pBnPRP-GUS into regions of the genome that are not active. Such positional effects are well documented, for example in *rbcs* promoter fusions (Jones *et al.*, 1985). It is possible that pBnPRP could have inserted into 'silent' regions in all three of the transgenic lines.

Despite the lack of detectable GUS expression in the transgenic lines tested the putative promoter did contain putative regulatory elements. The *cis*-elements that a promoter requires to be functional can be split into two classes. The first includes the transcription and translation initiation sequences ; these are present in all messenger RNAs that are transcribed by RNA polymerase II. The Kozak sequence in *BnPRP* differs from the consensus sequence, but this is not necessarily a problem as a large degree of variation has been observed in plant genes around the ATG motif; no strict rules can be applied when identifying a translational initiation sequence (Joshi, 1987a). Similarly the transcriptional initiation point cannot be defined on the basis of sequence despite the reasonable match with the consensus sequence. Primer extension or S1 mapping would be the only way to determine accurately the point of transcript initiation. Of the two putative TATA boxes present, the second one between -72 and -69 is more likely to be functional; the sequence surrounding the TATA conforms reasonably well to the consensus sequence TCACTATATATAG. The first TATA box is probably too close to the translation initiation site to allow for the usual 25-30 bp between the TATA box and transcription initiation site and the 5' untranslated sequence which is usually between 40 and 80 bp long (Joshi, 1987a). As with the other sequence elements discussed, the TATA box cannot be defined accurately by sequence alone. Not all the TATA boxes studied conform to the TATA motif (Joshi, 1987a). One base change in this motif would allow numerous sites within the *BnPRP* putative promoter to function as the TATA box. The CAAT box is a well defined upstream element. In animal genes the activity of some enhancer elements has been shown to be dependent on the CAAT box (Dierks *et al.*, 1983; Bienz and Pelham, 1986). Strong evidence exists that the CAAT box also performs a role in enhancing the effect of

upstream enhancers in plant systems (Fang *et al.*, 1989). The putative CAAT box in the *BnPRP* putative promoter is 63 bp upstream of the putative TATA box, which is in a range that is often found in promoters.

The second class of *cis*-elements act as enhancers, increasing/initiating the expression of genes in response to developmental and environmental cues. Some of these elements are common to genes with disparate expression patterns. The best defined of these is the G-box, and in particular the ACGT core motif that is part of the G-box (CACGTG). This has been shown to be important in the expression of light-regulated genes (Block *et al.*, 1990; Armstrong *et al.*, 1992), genes regulated by ABA (Marcotte *et al.*, 1989; Guiltinan *et al.*, 1990; Mundy *et al.*, 1990; Oeda *et al.*, 1991), and it has been suggested to be important in the regulation of cell cycle-dependent genes (Tabata *et al.*, 1991) and in plant pathogen response elements (Lam *et al.*, 1989; Bouchez *et al.*, 1989). The majority of the elements occur in the ACGT 5' to 3' orientation, although some do exist in reverse orientation, for example the hex1 from the wheat histone promoter (Tabata *et al.*, 1991). Furthermore, a 75 bp fragment of the ABA-induced wheat *Em* gene which contains an ACGT element, was equally effective at directing gene expression in an expression cassette in either orientation (Guiltinan *et al.*, 1990). Several ACGT motifs are present in the putative promoter sequence of *BnPRP*. The sequence context that they are in does not conform to the motifs identified in other genes such as Box II (5'-CCACGTGGCC-3') and III (5'-GTACGTGG-3') from the parsley chalcone synthase promoter (Armstrong *et al.*, 1992) and the *Em* gene ABA-response element (5'-CACGTGGC-3') (Guiltinan *et al.*, 1990). *BnPRP* is not regulated by ABA or to our knowledge by light, so it is not surprising that the same motifs as found in ABA and light regulated genes are not present. The sequences surrounding the ACGT motifs are in most cases ill-defined (Straub *et al.*, 1994) and the motifs in *BnPRP* may be common to other cold-induced promoters, not yet characterised.

Several cold-induced genes have been examined in an attempt to define *cis*-elements that are specific to cold-induction. *BnPRP* contains several sequences of interest. It only contains one putative low temperature response element (LTRE), and this is in the reverse orientation. The LTREs of *rd29A* were able to function in either orientation with respect to low temperature induction (Yamaguchi-Shinozaki and Shinozaki, 1994). A second element that is closely related to the LTRE motif, particularly to the LTREs from *BN115* and *cor15a* (White, T.C. *et al.*, 1994; Baker *et al.*, 1994) is present in the *BnPRP* putative promoter. LTREs have not been identified in all cold-induced genes; the *blt4.2* and *blt4.9* genes from barley contained only imperfect repeats of the core motif (White, A.J. *et al.*, 1994). This implies that either the imperfect core motif can still function as a LTRE or that other, as yet undefined LTREs exist.

The direct repeats that occur in the *BnPRP* putative promoter are potentially important for the expression of the gene. Direct repeats have been observed in other cold-induced gene promoters. The *rd29A* promoter contains two direct repeats (Yamaguchi-Shinozaki and Shinozaki, 1994), at least one which maybe necessary for cold-induced expression. However, the LTRE core motif is located within the repeats and it is likely that it is this rather than the larger repeats that is necessary for expression. The deletion of two direct repeats of 31 bp and 22 bp from the *BN115* promoter had no effect on promoter activity (White, T.C. *et al.*, 1994). Long direct repeats of the *Brassica rapa* acyl carrier protein gene have also been shown to be non-essential for its expression (Scherer *et al.*, 1992). Other smaller repeats were detected in the *cor15a* promoter (Baker *et al.*, 1994), although some of these again contained the core LTRE motif. A sequence element similar to one of these motifs that does not contain the LTRE (motif D) is present in the *BnPRP* putative promoter. No functional importance has been attached to this motif.

In summary, the putative BnPRP promoter appears to have several sequence elements which could be involved in initiating transcription (e.g. TATA box) and which are similar to elements that are concerned with cold-induction in other genes. These observations suggest that the *BnPRP* putative promoter should be functional and its inability to drive GUS expression in transgenic *Arabidopsis thaliana*, even in a non-cold-induced manner, is surprising. Further experiments, using different length promoter sequences and different host species, should help explain these findings.

Chapter 7: General Discussion

7.1 Isolation of *BnPRP*

The cDNA LF5B1 was sequenced, which revealed that it was a chimaeric cDNA composed of at least three independent cDNAs. Part of the cDNA that encoded a hybrid proline-rich protein was identified as hybridising to cold-induced transcripts. A fragment of the cDNA LF5B1 from the hybrid proline-rich region was used to probe a cDNA library constructed using RNA isolated from cold treated plants. The longest clone isolated was sequenced and found to be highly similar but not identical to LF5B1; the new cDNA was named *BnPRP*. Unfortunately this cDNA was not a full length clone. A genomic clone λ *BnPRP* was isolated, again using a fragment of LF5B1 as a probe. This allowed the sequence from the 5' coding region of the gene to be determined, along with 1.35 kb of the sequence upstream of the coding region.

7.2 The *BnPRP* gene family

Two separate lines of evidence indicated that *BnPRP* is a member of a gene family. Firstly, the full length LF5B1 cDNA hybridised to several bands on a Southern blot of *Brassica napus* genomic DNA, four to six members were predicted to be in the gene family (Pallas, 1992). A Southern blot of *Arabidopsis thaliana* DNA also revealed hybridising bands, indicating that two to three genes with complementary sequences were in the genome. Secondly, sequencing of the cDNA LF5B1 and subsequent isolation and sequencing of the cDNA *BnPRP* showed that the two cDNAs were non-identical, but highly similar. The Southern blot analysis was not repeated using *BnPRP* as a probe so it is not possible to predict accurately the number of genes in the family. Some of the bands detected using the cDNA LF5B1 may have resulted from

hybridisation to parts of the cDNA not related to the sequence that hybridises to cold-induced transcripts.

7.3 Expression of *BnPRP*

The expression of *BnPRP* has been investigated using two separate approaches. Northern blots using both cDNAs LF5B1 and *BnPRP* as probes showed that the corresponding transcript levels increased relatively rapidly in response to low temperatures. The transcript accumulation was mainly in the leaf tissue. Transcript accumulation was not induced by dehydration, ABA, heat-shock, or wounding. Unfortunately it was not possible to corroborate these results using transgenic plants containing the p*BnPRP*-GUS construct. The lack of expression in transgenic plants does not necessarily negate the data obtained from the northern blots. As discussed in Chapter 6, there are a number of possible explanations that could explain the lack of transgenic activity including the possible presence of silencing elements in the selected promoter region and the heterologous genetic background that existed in *Arabidopsis thaliana*. The possibility was also discussed that *BnPRP* may not itself be strongly expressed but may be sufficiently similar in sequence to a cold-induced member of the gene family for it to recognise the corresponding transcripts on a northern blot.

7.4 Putative Function of *BNPRP*

The putative function of the *BNPRP* protein has not been addressed experimentally. However, the characterisation of the gene's sequence and expression allow us to speculate as to the potential function(s) of the protein *in vivo*. Our studies have shown that the gene is expressed in response to low temperatures. It has not been demonstrated unequivocally that *BnPRP* itself is cold-induced; if it is not then a closely

related member of the same gene family is cold-induced, and presumably would play a similar role in the plant.

The sequence analysis of *BnPRP* revealed that the putative BNPRP protein was a hybrid proline-rich protein. Several hybrid proline-rich proteins have been identified. A function for some of the proteins has been conjectured. One of the main storage proteins from maize, γ -zein has a proline-rich domain followed by a domain rich in cysteine (Prat *et al.*, 1987). The cysteine-rich domain of γ -zein has homology to a large family of proteins that have been proposed to function as storage proteins or hydrolase inhibitors (Henrissat *et al.*, 1988; Kreis and Shewry, 1989). It is unlikely that BNPRP functions in this manner, although the expression has not been studied in the developing seeds of the plant, the presence of high levels of the *BnPRP* transcript in leaf tissue after exposure to low temperatures would not indicate a storage role. This is supported by the amino acid composition of BNPRP which is low in amino acids such as glutamine that have been proposed to play an important role in nitrogen storage. In addition the putative signal peptide and the proline-rich domain indicate that the mature protein BNPRP would be secreted into the cell wall. It has been shown that some proline-rich proteins are located in the cell wall (Ye *et al.*, 1991). A soybean hydrophobic protein has some sequence similarity to BNPRP, and this protein has been shown to be secreted (Odani *et al.*, 1987). Whether BNPRP is situated in the cell wall or not has not been demonstrated experimentally.

As discussed previously, several cDNAs which have very similar sequence to the BNPRP C-terminal domain have been identified. *In situ* hybridisation studies of the *HyPRP* and hydroxy-rich glyco protein (*HRGP*) maize genes showed that they are expressed in different cells in the developing embryos (Josè-Estanyol *et al.*, 1992). This indicated that there are cells types in the embryo that differ in wall composition, and the investigators postulated that *HYPRP* could modify the structure of the wall, protecting the cell during later development. The SAC51 putative protein has been

proposed to play a role in seed dehiscence, but this is based on northern blot analysis, and no precise function has been proposed (Coupe *et al.*, 1993). *In situ* hybridisation showed that the maize *ZRP3* transcript, which has high sequence similarity to *BnPRP*, was expressed specifically in the cells of the cortical ground meristem (John *et al.*, 1992). The inner cortical cells are adjacent to the procambium and may play a role in the transport of molecules to or from the vascular tissue. It has also been proposed that the *ZRP3* polypeptide may play a role in the differentiation of the inner cortical cells (John *et al.*, 1992). The MSACIC protein is the only other proline-rich protein that has been reported to be cold-induced; the transcript levels accumulated to higher levels in alfalfa cultivars that display higher levels of cold tolerance, implying that the protein is important in conferring cold tolerance (Castonguay *et al.*, 1994). This correlates with the observation that the accumulation of some of the cold regulated translation products are related to freezing tolerance in alfalfa (Castonguay *et al.*, 1993).

As discussed in Chapter 4, the BNPRP contains three putative membrane spanning domains, although it is not known whether these act as such *in vivo*. If they do it would lead to the proline-rich domain protruding into the cell wall with the C-terminal domain embedded in the membrane. The highly conserved cysteine residues are presumably functionally important. This is based on their high conservation and upon their ability to form disulphide bridges which can be important in the structure of the protein and the way the protein interacts with other proteins. The putative arrangement of the C-terminal domain is depicted in Fig. 4.11. This predicts that some of the cysteine residues are located within the plasma membrane while others are on the cell wall or cytoplasmic side of the membrane. This would enable the cysteine residues to interact intramolecularly, or intermolecularly with proteins on both sides of, and within the plasma membrane.

If the BNPRP protein was situated at the cell wall-plasma membrane boundary it could potentially act as a cell wall-membrane linker protein. HRGP proteins have

been postulated to perform this function in onion epidermis cells (Pont-Lezica *et al.*, 1993). This hypothesis was based on proteins that were recognised by antibodies to an HRGP being concentrated at points in the plasma membrane where Hechtian strands were seen upon plasmolysis. The proteins in this study were termed Hechtian wall-to-membrane linkers. Some evidence suggested that as well as attaching the cell walls to the plasma membrane these Hechtian wall-to-membrane linkers also connected to the cytoskeleton. The location of the protein and its possible ability to cause Hechtian strands upon dehydration does not itself reveal the protein's function, though it does enable some hypotheses to be suggested. Structurally, the protein could help to maintain cell polarity by anchoring the plasma membrane and cytoskeleton against slippage, minimise disruption of the cell structure upon cellular dehydration and subsequent rehydration or transfer stresses that are exerted on the cell wall to the plasma membrane. Akashi *et al.* (1990) reported that extensin was capable of stabilising cortical microtubules in tobacco protoplasts that were otherwise cold-labile, supporting the hypothesis that HRGPs can function as linker proteins and that these can affect the cellular response to low temperatures. In this case an intermediate transmembrane protein would presumably be required to couple the extensin and the cytoskeleton. BNPRP presumably would not require an intermediate transmembrane protein if it functioned in a comparable manner.

An increase in membrane components that were immunologically related to human fibronectins (involved in cell to cell binding in mammalian systems) has been observed in response to salt stress in tobacco cells (Zhu, J.-K. *et al.*, 1993). The increase correlated with tight zones of adhesion forming between the plasma membrane and the cell wall. Salt, like freezing imposes a dehydrative stress upon the plant. BNPRP could act in a similar manner, maintaining adhesion of the cell wall to the plasma membrane during the cellular dehydration that can occur in response to freezing low temperatures. However, BNPRP could have functions other than structural, it could play a role in stress detection. It is likely that stress is detected at least in part by

stretch activated ion channels in the plasma membrane (Falke *et al.*, 1988; Ingber *et al.*, 1991). It is possible that by maintaining links between the cell wall and plasma membrane during plasmolysis, BNPRP would create a stress on the plasma membrane leading to the activation of ion channels. This in turn would signal that the cell was becoming dehydrated.

We have no evidence that the putative membrane integration signals function in BNPRP and the entire protein may be secreted into the cell wall (assuming that the putative signal peptide is functional). Freeze-induced water loss is slowed down as the bulk of the tissue increases (Anderson *et al.*, 1983), and cell wall thickening during cold acclimation is consistent with this observation (Huner *et al.*, 1981). BNPRP could contribute to the increased cell wall mass. The potential cross links that could occur in the wall may also increase the cell wall strength, which could prevent cell wall cavitation/collapse that has been reported in response to freeze-induced dehydration (Weiser and Wallner, 1988).

In summary, although the above hypotheses regarding the function of BNPRP are entirely speculative, they do provide plausible functional models and provide a basis for further experimentation.

7.5 Future work

Several interesting aspects arose from this study that merit further work. The presence of a gene family, containing at least two members has been shown. Only one of the genes, *BnPRP*, has been fully analysed at the sequence level. In order to further characterise the different members of the gene family cloning of cDNAs and genes related to *BnPRP* is necessary. This should be a relatively simple task; as described in Chapter 3, ten cDNA clones were isolated from the 'cold-induced' cDNA library using

a fragment of the cDNA LF5B1 as a probe. Only cDNA 6 was sequenced and used to probe northern blots. Sequencing of the 3' ends of the remaining nine cDNAs would probably reveal some of the other members of the gene family. Unfortunately, as discussed in Chapter 3, none of the cDNAs represent full length clones (assuming that all the members of the gene family are of similar length). The most likely explanation of this problem is that the sequence in the 5' region of the gene causes the DNA polymerase to pause, terminating its progression in the majority of cases, which in turn leads to the low number of full length cDNAs represented in the 'cold-induced' library. One technique that may enable us to isolate full length cDNAs representing different members of the family is to use a 5' region of the *BnPRP* gene to reprobe the 'cold-induced' cDNA library. Alternatively we could characterise the genomic clones isolated as described in Chapter 4. Seven clones were isolated and three to four of these seemed to be closely related to *BnPRP* on the basis of the size and intensity of the bands identified on Southern blots. These may represent different members of the gene family. The two genomic clones that did not appear to be very closely related to *BnPRP* may also be members of the gene family. A genomic Southern blot using *BnPRP* as a probe would allow us to estimate the total number of members within the family.

If we could identify different members of the gene family this would enable us to carry out more precise gene expression studies. The cDNA BNPRP was used to probe northern blots. Although it was assumed that this hybridised to a single species of transcript, it may have hybridised to other related transcripts, leading to incorrect information regarding the expression of *BnPRP*. By characterising the other members of the gene family it would be possible to differentiate between the expression patterns of the different family members. Two different approaches would enable us to do this. Identifying regions within the genes that differed in sequence would allow gene-specific oligonucleotides to be produced which could be used to probe northern blots. A similar approach was used to differentiate between the expression of the three

different members of the *rbcS* gene family in *Phaseolus vulgaris* (Sawbridge *et al.*, 1994). Alternatively, if we cloned the genes rather than the cDNAs we could make more promoter-GUS constructs that would allow expression to be studied if the constructs were functional *in vivo*. A different approach to studying the expression of the promoter-GUS constructs may help; transforming *Brassica napus* rather than *Arabidopsis thaliana* may result in the constructs being functional. With the exception of low temperatures we do not know what regulates the other members of the gene family, the use of transgenic plants would ease the process of examining the effect of different environmental and developmental stimuli. If we could produce plants with transgene activity (cold-induced or otherwise) it would create the possibility of studying the promoter(s) in more detail and possibly defining *cis*-acting elements that are functionally important. Transient expression could be useful for this purpose. A transient expression system utilising particle bombardment has previously been used successfully to examine promoter deletions of a cold-induced gene in *Brassica napus* (White, T.C. *et al.* 1994). Such a system would speed-up the analysis of any constructs.

The function of the protein is of interest. To understand what it does it is important to know exactly where the protein is expressed. This could be done by *in situ* hybridisation which would locate the cell types and the location in the cell. However, it would be preferential to study the protein rather than the gene transcripts. The protein may be regulated in a manner we did not expect as we are assuming the protein's accumulation in response to low temperature will correlate with the increase in transcript accumulation. One way to study the protein levels and cellular location would be to raise an antibody against the purified protein. It may be possible to produce sufficient amounts of protein by cloning the *BnPRP* coding sequence into an expression vector which would potentially produce large amounts of the protein in an *E. coli* host. As well as allowing the protein levels to be monitored in response to low temperatures, an antibody would enable the location of the protein to be examined. The

sequence data predicts that the proline-rich region of the protein will be in the cell wall and the C-terminal domain will be in the plasma membrane. Antibodies have been used to successfully locate other proline-rich proteins in the cell wall (Ye *et al.*, 1991). An alternative to tissue prints would be to fractionate the cell into cell wall, membrane and cytoplasmic fractions and measure the levels of the protein in each of the fractions.

Determining the location of the protein would not actually demonstrate its function. Two approaches are possible to address the question. Down-regulation of the gene by antisense RNA and co-suppression has been used to successfully knock out genes, for example the polygalacturonase gene has been successfully down-regulated in tomato fruit using antisense constructs (Smith *et al.*, 1990). This has not been done with proline-rich proteins. The main problem with proline-rich proteins has been predicting what phenotype the down-regulation of the gene would lead to. Another approach would be the constitutive expression of BNPRP in a transgenic plant. Again it is necessary to hypothesise the function of the protein in order to examine the effect of over expression. We could test if BNPRP acts as a Hechtian wall-to membrane linker by over expressing it and investigating if this increased the number of Hechtian strands seen upon dehydration.

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